Cooperative DNA-binding and sequence-recognition mechanism of *aristaless* and *clawless*

Ken-ichi Miyazono¹, Yuehua Zhi¹,
Yuriko Takamura¹, Koji Nagata¹,
Kaoru Saigo², Tetsuya Kojima³
and Masaru Tanokura^{1,*}

¹Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, University of Tokyo, Bunkyo-ku, Tokyo, Japan, ²Department of Biophysics and Biochemistry, Graduate School of Science, University of Tokyo, Bunkyo-ku, Tokyo, Japan and ³Department of Integrated Biosciences, Graduate School of Frontier Sciences, University of Tokyo, Kashiwa-city, Chiba, Japan

To achieve accurate gene regulation, some homeodomain proteins bind cooperatively to DNA to increase those site specificities. We report a ternary complex structure containing two homeodomain proteins, *aristaless* (Al) and *clawless* (Cll), bound to DNA. Our results show that the extended conserved sequences of the Cll homeodomain are indispensable to cooperative DNA binding. In the Al–Cll–DNA complex structure, the residues in the extended regions are used not only for the intermolecular contacts between the two homeodomain proteins but also for the sequence-recognition mechanism of DNA by direct interactions. The residues in the extended N-terminal arm lie within the minor groove of DNA to form direct interactions with bases, whereas the extended conserved region of the C-terminus of the homeodomain interacts with Al to stabilize and localize the third α helix of the Cll homeodomain. This structure suggests a novel mode for the cooperativity of homeodomain proteins.

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Introduction

Homeodomain proteins, which make up one of the most widespread superfamilies of eukaryotic DNA-binding proteins, regulate the transcription of various kinds of genes that are indispensable to development (Gehring *et al*, 1994a, b). The homeodomain consists of 60 highly conserved amino acids and forms an N-terminal arm structure and the following three α helices. The homeodomain reads target DNA sequences using its N-terminal arm and the third helix from the minor and major groove of DNA, respectively (Qian *et al*, 1989; Wolberger *et al*, 1991; Klemm *et al*, 1994). Because of the high level of conservation of both the

amino-acid sequences and three-dimensional structures, homeodomain recognition sequences are highly similar to one another (Laughon, 1991). Although these high degrees of similarity would seem to conflict with the fact that homeodomain proteins regulate the expression of a large number of development-related genes with high specificity, homeodomain proteins resolve this paradox by cooperative binding with cofactors, including the other homeodomain proteins (White, 1994; Mann and Chan, 1996).

Some homeodomain proteins bind cooperatively to specific DNA sequences. MATA1 and MAT α 2 are well-characterized homeodomain proteins from *Saccharomyces cerevisiae* that bind cooperatively to the sites upstream of haploid-specific genes and repress the expression of those genes (Goutte and Johnson, 1993). Although the DNA-binding affinity and specificity of MATA1 in the absence of MAT α 2 are very low, the heterodimer of MATA1 and MAT α 2 can regulate a number of genes with high specificity (Goutte and Johnson, 1993; Phillips *et al*, 1994). In the ternary complex structure of MATA1–MAT α 2–DNA, a conserved C-terminal tail region of MAT α 2 forms a short α helix and interacts with a hydrophobic patch between helix α 1 and helix α 2 of the MATA1 homeodomain (Li *et al*, 1995). This protein–protein interaction is indispensable to the cooperative binding of these homeodomain proteins to minimize the overall energy cost of cooperative DNA binding (Ke and Wolberger, 2003). The other examples are cooperative binding of Hox homeodomain proteins and three amino-acid loop extension (TALE) family homeodomains (Chang *et al*, 1995; Mann and Chan, 1996; Neuteboom and Murre, 1997). The TALE family homeodomain, which includes Pbx1 (as well as Pbx2 and Pbx3) from human and Exd from *Drosophila*, possesses a TALE region between helix α 1 and helix α 2 that is involved in their interaction with Hox proteins (Bertolino *et al*, 1995). Cooperative DNA binding of the Hox protein with the TALE family homeodomain is dependent on a conserved YPWM motif located N-terminal to the Hox homeodomain (Knoepfler and Kamps, 1995). The three-dimensional structures of this type of complex (HoxB1–Pbx1–DNA (Piper *et al*, 1999), HoxA9–Pbx1–DNA (LaRonde-LeBlanc and Wolberger, 2003), Ubx–Exd–DNA (Passner *et al*, 1999), and Scr–Exd–DNA (Joshi *et al*, 2007)) revealed that the YPWM motif conserved in the region N-terminal to the Hox homeodomain interacts directly with the hydrophobic pocket formed around the TALE region. Interaction of the YPWM motif with the Pbx1 or Exd homeodomain can increase the DNA-binding affinity of these homeodomains by stabilizing the DNA recognition helix α 3 of Pbx/Exd. Furthermore, the sequence-specific structure of DNA is critical to the accurate sequence recognition of this type of homeodomain.

In this paper, we focused on the cooperativity of two homeodomain proteins, *aristaless* (Al) and *clawless* (Cll), from *Drosophila melanogaster*. These homeodomain proteins have an important function in *Drosophila* leg development. In the distalmost region, homeobox genes, *al*, *Lim1*, and *ccl*

*Corresponding author. Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan.
Tel.: +81 3 5841 5165; Fax: +81 3 5841 8023;
E-mail: amtanok@mail.ecc.u-tokyo.ac.jp

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(also known as *C15*), are expressed to specify the region, whereas a pair of *Bar* homeobox genes are expressed in its immediate neighbour (distal tarsus) (Campbell, 2005; Kojima *et al*, 2005). For the accurate differentiation of these regions, *Bar* expression is repressed by the cooperative mechanism of Al and Cll in the distalmost region. An earlier study showed that these two homeodomain proteins bind cooperatively to a specific DNA sequence, which has a consensus sequence of 5'-(T/C)TAATTAA(T/A)(T/A)G-3' and is included in the *Bar* enhancer element (Kojima *et al*, 2005). Both Al and Cll have several homologues in humans that are known to be involved in various aspects of embryogenesis, such as spleen, leg, craniofacial and neuronal development, and that are also known to cause T-cell acute lymphoblastic leukaemia, intestinal dysplasia, and neural tube defects (Hatano *et al*, 1991, 1997; Kennedy *et al*, 1991; Roberts *et al*, 1994, 1995; Zhao *et al*, 1994; Salvati *et al*, 1995; Shirasawa *et al*, 1997; Qu *et al*, 1999; Beverdam *et al*, 2001; Kanzler and Dear, 2001; Cheng *et al*, 2004; Kuijper *et al*, 2005). Among them, *Cart1* (an Al homologue; sharing 75% sequence identity with Al in its homeodomain) and *Hox11L1* (a Cll homologue; sharing 85% sequence identity with Cll in its homeodomain) (Dear *et al*, 1993; Zhao *et al*, 1994) have already been examined for their cooperative DNA-binding activity. The *Cart1*–*Hox11L1* complex, as well as the Al–*Hox11L1* and *Cart1*–Cll complexes, was shown to bind specifically to the same sequences as the Al–Cll complex, indicating that this cooperative DNA-binding activity is a conserved feature of Al- and Cll-type homeodomain proteins (Kojima *et al*, 2005).

Al belongs to a paired class of homeodomain proteins that can bind cooperatively as homo- or heterodimers to a palindromic DNA sequence (Wilson *et al*, 1993). When the amino-acid sequence of Al and its homologous proteins were compared, only the homeodomain and OAR domain, which is thought to repress the DNA-binding affinity of Al, were highly conserved (Figure 1A) (Brouwer *et al*, 2003). On the other hand, the conserved region of Cll was extended for 14 residues at the N-terminus and 8 residues at the C-terminus of its homeodomain (Figure 1B). The cooperative DNA-binding mechanism of these homeodomain proteins, particularly the function of the extended conserved region of the Cll homeodomain, has not been clarified.

To elucidate the structural basis of the cooperative DNA-binding mechanism of Al and Cll, we first determined the minimal region of Al and that of Cll that are indispensable to the cooperative DNA-binding mechanisms of these proteins. We then tried to determine the homeodomain structures of Al, Cll, *CART1*, and *Hox11L1*, a binary complex structure of Al–DNA, and a ternary complex structure of Al–Cll–DNA to analyse the structural basis of the cooperativity of these proteins. In this study, we determined four structures by X-ray crystallography: that of the Al homeodomain, that of the *Hox11L1* homeodomain, that of the binary complex of Al–DNA, and that of the ternary complex of Al–Cll–DNA. Our results show a novel ternary complex structure formation of homeodomain proteins that is distinguished from pre-existing structures.

Results

Cooperative DNA-binding regions of Al and Cll

To identify the minimal regions of Al and Cll where each homeodomain protein binds cooperatively to DNA, we over-

expressed constructs of Al and Cll (Al–HD (–5–63 in Figure 1A), Cll–N3C3 (N3–C3 in Figure 1B), Cll–N3C2 (N3–C2), Cll–N2C3 (N2–C2), Cll–N2C2 (N2–C2), Cll–N1C2 (N1–C2), and Cll–N2C1 (N2–C1)) and analysed their cooperative DNA-binding ability by EMSA (see Supplementary data). The EMSA results are shown in Figure 2. First, we confirmed the cooperative DNA binding of Al–HD and Cll–N3C3 constructed in this report. The Al homeodomain bound the target DNA by itself, whereas the Cll homeodomain bound the DNA weakly. In addition, Al–HD and Cll–N3C3 bound the DNA cooperatively in a sequence-specific manner (Figure 2A and B). Second, we shortened the Cll construct step-by-step from its N-terminus and C-terminus and analysed the cooperative DNA-binding ability of the resulting constructs. When the N2–N1 region of the Cll was deleted, Cll completely lost its cooperative DNA-binding ability with Al. This region is also indispensable to the cooperative DNA binding of human homologue of Cll, *Hox11L1* (Supplementary Figure S1). When the C1–C2 region of the Cll was deleted, the cooperative DNA-binding ability of Cll was somewhat reduced (Figure 2C). Taken together, these results indicate that the cooperative-binding mechanism of Al and Cll requires the Al–HD and Cll homeodomain with its extended N-terminal and C-terminal conserved regions (Figure 2D and E).

The structure of the Al–Cll–DNA complex

The crystal structures of the Al homeodomain, the *Hox11L1* homeodomain, the binary complex of Al–DNA, and the ternary complex of Al–Cll–DNA were determined at 1.00, 1.54, 2.25, and 2.70 Å resolutions, respectively (Figure 3; Supplementary data). The structural model of the ternary complex contains residues 2–58 of Al, –15–63 of Cll, and 17 bp of double-stranded DNA. In the ternary complex structure, the Al and Cll homeodomains bind DNA with a head-to-tail orientation, which is also observed in the structure of Hox–Pbx/Exd–DNA complexes (Passner *et al*, 1999; Piper *et al*, 1999; LaRonde-LeBlanc and Wolberger, 2003; Joshi *et al*, 2007): the C-terminal region of the Cll homeodomain lies adjacent to the N-terminal region of the Al homeodomain. The Al and Cll homeodomains observed in the ternary complex form a typical homeodomain structure consisting of an N-terminal arm structure followed by three α helices and bind to DNA using the N-terminal arm and the third α helix, in the manner of previously determined homeodomain–DNA complex structures. The third α helices of the Al and Cll homeodomains exist in the major groove of DNA in tandem, and each N-terminal arm of the homeodomain follows the minor groove of DNA. In addition to these protein–DNA interactions, Cll also binds to DNA using its conserved residues located in the region N-terminal to the homeodomain. In this study, we presented this region as an extended N-terminal arm. The extended N-terminal arm is well ordered in both structures of the Al–Cll–DNA ternary complexes (chains ABCD and EFGH) in the asymmetric unit and contacts the minor groove of DNA as the N-terminal arm of the homeodomain, though the N-terminal arm of the homeodomain is frequently disordered in the structures of homeodomain–DNA complexes (Wolberger *et al*, 1991; Billeter *et al*, 1993; Hirsch and Aggarwal, 1995; Li *et al*, 1995; Tucker-Kellogg *et al*, 1997; Fraenkel and Pabo, 1998; Fraenkel *et al*, 1998; Passner *et al*, 1999; Piper *et al*, 1999;

Figure 1 Sequences of Al, Cll, and the recognition site. (A) Alignment of the homeodomain sequences of Al and its homologues from other organisms (*A. gambiae*, *A. mellifera*, human (Cart1), *Gallus gallus*, and *Danio rerio*). The secondary structure assignment of Al is shown by helices (α helix). The numbering follows the convention for the 60 residues of the homeodomain. (B) Alignment of the homeodomain sequences of Cll and its homologues from other organisms (*A. gambiae*, *A. mellifera*, human (Hox11L1 and Hox11L2), *G. gallus*, and *D. rerio*). Secondary structure assignments of Cll are shown by helices (α helix) and TT (β turn). (C) Sequence of a dsDNA used for the structure determination of the Al-Cll-DNA complex. The recognition DNA sequence of Al and Cll is shown in bold. The bases are numbered from 1 to 17.

Hovde *et al*, 2001). The contact surface area between the DNA and the extended N-terminal arm is 960 Å², which is 37% of the total contact surface area between Cll-N2C3 and the DNA.

The extended N-terminal arm of Cll forms a unique conformation (Figure 3). It lies in the minor groove and wraps around the DNA. The four residues in the extended N-terminal arm (Tyr-8, Gln-7, Asn-6, and Arg-5) are folded into a classical type I β turn and form a hydrogen bond between Tyr-8 and Arg-5. In this region, the side chains of Tyr-8 and Arg-5 in the β turn and His-10 are inserted into the minor groove of DNA.

The structure of the homeodomain region of each protein was not modified by the formation of the ternary complex. The r.m.s.d. of the Al homeodomain before and after the ternary complex formation was 0.4 Å in the 51 aligned α positions (Supplementary Figure S3A). In the structure of the Al homeodomain, the length of the third α helix is extended by 1 residue in the ternary complex. The r.m.s.d. between the Cll homeodomain in the ternary complex and the Hox11L1 homeodomain was 0.4 Å in the aligned 53 α atom positions (Supplementary Figure S3B). In the structure of the Cll homeodomain, the length of the third α helix is longer than that of the Hox11L1 homeodomain by one residue.

DNA sequence-recognition mechanism of Al and Cll

Al and Cll are thought to regulate *Bar* expression by directly binding to sequences in the *Bar* enhancer region, which are fit to the 11 bp consensus sequences, including 5'-T₄T₅A₆A₇T₈T₉A₁₀A₁₁T₁₂T₁₃G₁₄-3' (Kojima *et al*, 2005). The sequence-recognition mechanisms by Al and Cll observed in this study are summarized in Figure 4A. The Al homeodomain forms a set of conserved DNA contacts that have been observed in other homeodomain-DNA complex structures (Figure 4B). Al recognizes the 5'-T₄T₅A₆A₇T₈N₉A₁₀-3' sequence in the ternary complex. The first three bases of the sequence (5'-T₄T₅A₆-3') are recognized by the N-terminal arm residues, Arg3 and Arg5, from the minor groove of DNA. The Arg3 forms a hydrogen bond with the O2 atom of Thy12' (the counterpart of Ade6; see Figure 1C), whereas Arg5 forms a pair of hydrogen bonds with the O2 atom of Thy5 and the N3 atom of Ade14'. In the major groove, the last four bases (5'-A₆A₇T₈N₉A₁₀-3') are recognized by the residues in the third α helix of the Al homeodomain, especially by the invariant bidentate hydrogen bonding between the highly conserved Asn51 residue and the adenine base (Ade7). In addition, Al recognizes three bases—Ade6, Thy8, and Thy8'—from the major groove of DNA using Asn51, Val47, and Gln50, respectively, by hydrogen bonding and/or van der Waals contacts.

Figure 2 EMSA analysis. (A) Sequence-specific DNA binding of AI-HD and CII-N3C3. In all, 10 pmol of AI-HD, 20 pmol of CII-N3C3, and 2 pmol of FITC-labelled dsDNA were used for each binding reaction. (B) Cooperative DNA binding of AI-HD and CII-N3C3. The following AI and CII constructs were added to each binding reaction—lane 1: no protein; lanes 2, 3, and 4: 1.25, 2.50, and 5.00 pmol of AI-HD, respectively; lanes 5, 6, and 7: 1.25, 2.50, and 5.00 pmol of AI-HD, respectively, and 260 pmol of CII-N3C3; lanes 8, 9, and 10: 16.3, 32.6, 65.2 pmol of CII-N3C3, respectively; lanes 11, 12, and 13: 16.3, 32.6, 65.2 pmol of CII-N3C3, respectively, and 100 pmol of AI-HD. Each binding reaction contains 2 pmol of FITC-labelled dsDNA. (C) Cooperative DNA-binding affinities of CII constructs (CII-N3C3, CII-N3C2, CII-N2C3, CII-N2C2, CII-N1C2, and CII-N2C1) with AI-HD. In all, 10 pmol of AI-HD, 20 pmol of CII constructs, and 2 pmol of FITC-labelled dsDNA were used for each binding reaction. (D) Binding affinities (K_D s) of the indicated proteins. (E) Summary of the EMSA analysis. The N2-C2 region is required for the cooperative DNA binding with AI-HD.

CII recognizes the 5'-T₈T₉A₁₀A₁₁T₁₂T₁₃G₁₄-3' sequence, which overlaps with the recognition sequence of AI. The base-recognition mechanism of the CII homeodomain is also similar to that of the typical homeodomain (Figure 4C). Arg5 in the N-terminal arm is inserted into the minor groove of the DNA and forms two hydrogen bonds with two bases (Ade10' and Thy9) to recognize these sequences. The three residues (Thr47, Gln50, and Asn51) of the third α helix interact directly with bases from the major groove of the DNA to recognize the 5'-A₁₀A₁₁T₁₂N₁₃G₁₄-3' sequence. In addition to these typical homeodomain-DNA interactions, the extended N-terminal arm of CII interacts directly with the last three

bases of the recognition sequence (5'-T₁₂T₁₃G₁₄-3') to improve the sequence specificity and DNA-binding affinity of CII (Figure 4D). The three residues, His-10, Tyr-8, and Arg-5, lie within the minor groove of DNA and interact directly with the bases and the sugar groups of the recognition sequence. His-10 is inserted into the minor groove of DNA and forms a direct hydrogen bond with the N3 atom of Gua14 at a distance of 3.03 Å. The guanidium nitrogen atom of Arg-5 lies deeper within the minor groove of DNA to form a hydrogen bond with the N3 atom of Ade6' at a distance of 2.89 Å. In addition, the guanidium nitrogen of Arg-5 also lies next to the O2 atoms of Thy13 and Ade5' at distances of 3.42

Figure 3 Structure of the ternary complex of Al-Cll-DNA. Overall structure of Al-Cll-DNA. Al and Cll are coloured cyan and red, respectively. Secondary structure assignments are labelled on the ribbon model. The side chains of His-10, Tyr-8, and Arg-5 of Cll are shown by a stick model.

and 3.73 Å, respectively, and may be involved in the recognition of these bases. On the other hand, Tyr-8 forms van der Waals contacts with the sugar groups of Thy7' and Thy8', though the residues are not involved in the sequence-recognition mechanisms through direct interaction with the bases.

Characteristic DNA structure modification in the Al-Cll-DNA complex

In the ternary complex structure of Al-Cll-DNA, the DNA structure was partially modified from the typical B-form structure by the binding of two homeodomain proteins. The most characteristic feature is that the minor groove of DNA is largely expanded around the region to which the extended N-terminal arm of Cll binds (Figure 5A and B). The average minor groove width of the 5'-T₉A₁₀A₁₁T₁₂T₁₃-3' region is 7.7 Å. In general, AT-rich regions often lead to narrow minor grooves due to negative propeller twisting (Crothers and Shakked, 1999). As expected, the average value for the propeller twist in this region is -12.2°. Although the propeller twisting is negative, the minor groove is expanded by three inter-base pair steps (T₉pA₁₀, T₁₂pT₁₃, and T₁₃pG₁₄ steps) with large roll values (12.0°, 11.4°, and 4.9°, respectively). The large positive roll value is expected to lead to a wide minor groove.

The expanded minor groove is stabilized by the residues of the Al homeodomain (Figure 5C). The phosphate group of Thy8' is tightly stabilized by the hydrogen-bonding network between the phosphate group and Tyr25, Arg53, and Arg57 of Al. The analysis of the temperature factor of the ternary complex structure shows that this region possesses the lowest *B* factor in the complex (Supplementary Figure S4).

The minor groove width in this region is also expanded in the structure of the Al-DNA complex (Figure 5A and B). In the binary complex, residues of the Al homeodomain form several hydrogen bonds with the phosphate group of DNA in this region (Figure 5D). The oxygen atom of Gln46 of Al forms a hydrogen bond with the phosphate group of Cyt2' (the counterpart of Gua13; see Supplementary Figure S2D) at a distance of 2.92 Å. In addition, the side chains of Tyr25, Arg53, and Arg57 of Al form a strict hydrogen-bonding network with the phosphate group of Thy4' at distances of 2.54, 2.81, and 2.48 Å, respectively. The side chain of Arg57 also

forms a hydrogen bond with the phosphate group of Ade5' at a distance of 2.63 Å.

Intermolecular interactions between Al and Cll on target DNA

The intermolecular interactions between Al and Cll are focused on the extended conserved region of the Cll homeodomain (Figure 6A). In the Al-Cll-DNA complex, two intermolecular interaction sites (sites 1 and 2) are observed. Site 1 consists of intermolecular interactions between the Al homeodomain and the extended N-terminal arm of Cll. In this site, Arg-14 of Cll forms a salt bridge with Glu32 of Al at a distance of 2.69 Å (Figure 6B). In addition, Ile-12 of Cll has hydrophobic interactions with Val28 and Phe29 of Al. Gln-7 of Cll also has a hydrophobic interaction with Tyr25 of Al. On the other hand, site 2 consists of intermolecular interactions around the C-terminal residues of the Cll homeodomain. In this site, Glu62 of Cll forms a salt bridge with Arg44 of Al at a distance of 3.60 Å (Figure 6C). Glu62 may also form a water-mediated hydrogen-bonding network with Thr41 of Al, though the coordinates of the water molecules could not be determined due to the low-resolution data.

Mutation analysis of the extended conserved region of the Cll homeodomain

To elucidate the importance of the extended conserved region of the Cll homeodomain, we overexpressed alanine mutants and analysed the cooperative DNA-binding affinities with Al-HD by EMSA. When we mutated the residues in the extended N-terminal arm, the His-10Ala, Tyr-8Ala, and Arg-5Ala mutants lost the cooperative DNA-binding affinity with Al-HD, though the other mutants bound to the target DNA cooperatively with Al-HD (Figure 7A). These three residues undergo characteristic interactions with DNA and are involved in the DNA's recognition mechanism. The intermolecular interactions of the residues in the extended N-terminal arm of Cll (His-10, Tyr-8, and Arg-5) are indispensable to the cooperative DNA binding of Al and Cll. When we mutated the Glu62, which forms a salt bridge with the Al homeodomain, the cooperative-binding affinity of Al and Cll was reduced (Figure 7B and C). The *K_D* value of the cooperative DNA binding of the Cll-N2C3 Glu62Ala mutant is nearly identical to that of Cll-N2C1, which is a deletion mutant of the extended conserved residues in the C-terminal region of the Cll homeodomain. The salt bridge in the extended conserved region of the C-terminus of the Cll homeodomain is also important for the cooperative DNA binding of Al and Cll.

Discussion

DNA structural characteristics that are indispensable to the cooperative DNA binding of Al and Cll

DNA structure is known to affect the specific sequence-recognition mechanism of homeodomain proteins. In the HoxA9-Pbx1-DNA structure, a series of contacts mediated by the HoxA9 N-terminal arm residues Pro7 and Tyr8, and a first helix residue Thr13 compress the minor groove of DNA and allow an additional sequence-recognition mechanism by Gln51 of HoxA9 (LaRonde-LeBlanc and Wolberger, 2003). On the other hand, one of the Hox proteins, Scr, recognizes the sequence-specific structure of DNA (Joshi *et al*, 2007). The DNA structure is slightly modified depending on its sequence.

Figure 4 Protein–DNA contacts. (A) Base recognition mode of Al and Cll. Residues from Al and Cll are coloured blue and red, respectively. Bases recognized by Al and Cll are coloured cyan and red, respectively. Hydrogen bonds are indicated with arrows, and van der Waals contacts are shown as lines terminating in a circle. Residues interacting with DNA from a minor groove are underlined. (B) DNA sequence-recognition mechanism of the Al homeodomain. Residues involved in the sequence-recognition mechanisms are shown by a stick model. Hydrogen bonds are shown by blue dotted lines. Residues from Al are labelled in blue and bases are labelled in black. (C) DNA sequence-recognition mechanism of the Cll homeodomain. Residues from Cll are labelled in red. (D) DNA sequence-recognition mechanism of the extended N-terminal arm of Cll.

Consecutive ApA, TpT, or ApT base pair steps are known to result in a narrow minor groove due to negative propeller twisting that is stabilized by inter-base pair interactions in the major groove (Crothers and Shakked, 1999). In the ternary complex structure of Scr–Exd–DNA (Joshi *et al*, 2007), His-12 and Arg3 of Scr are inserted into a narrow region of the minor groove and recognize the sequence-specific structure of DNA to serve a paralog-specific function.

Contrary to this general tendency between sequence and structure, the minor groove region of the DNA where the extended N-terminal arm of Cll binds is greatly expanded from a typical B-form DNA, though the DNA sequence of this region consists of ApA, TpT, or ApT base pair steps (5'-T₉A₁₀A₁₁T₁₂T₁₃G₁₄-C'). This characteristic substructure suggests that the structure of the DNA is highly affected by

the protein–DNA contacts. In the structure of the Al–Cll–DNA complex, the DNA kinks at the T₉pA₁₀, T₁₂pT₁₃, and T₁₃pG₁₄ steps to increase the minor groove width. As the expanded minor groove is also observed in the binary complex structure of Al–DNA, the expansion of the minor groove would be induced by the binding of the Al homeodomain to the DNA. In the structure of Al–DNA, Al forms a set of conserved DNA contacts that have been observed in other homeodomain–DNA complex structures to recognize the sequence (Supplementary data). In addition to the general contacts, the residues of Al (Tyr25, Gln46, Arg53, and Arg57) form direct hydrogen bonds with the phosphate group of DNA in the expanded minor groove region. The expansion of the minor groove would be induced by these interactions. The residues expanding the minor groove are highly conserved

Figure 5 Characteristic DNA structure. **(A)** Plots of major and minor groove widths as a function of the DNA sequence. Horizontal grey lines indicate the average values for B-form DNA. Only the sequence of the top strand is shown. **(B)** Superposition of the binary complex of Al-DNA (red), the ternary complex of Al-Cll-DNA (blue), and 17 bp of B-form DNA (green). **(C)** Expanded minor groove of DNA in the Al-Cll-DNA complex. Each residue is shown by a stick and sphere model. Al and Cll are coloured cyan and red, respectively. **(D)** Expanded minor groove of DNA in the Al-DNA complex. Al is coloured cyan.

among the homologous proteins of Al (Figure 1A). The high conservation may suggest the importance of the minor groove expansion mechanism of these proteins.

The residues of the extended N-terminal arm of Cll interact directly with sugar and base groups of the DNA in the expanded minor groove region. These interactions increase the binding affinity and sequence specificity of Cll (Figures 2 and 4). The expansion of the minor groove supports the interactions between the residues in the extended N-terminal arm and DNA from the minor groove side by increasing the contact area of the minor groove side. As the expansion of the minor groove is induced by the Al homeodomain, the interaction from the minor groove at this site would not occur by the binding of Cll solely to the target sequence of the DNA. The observation that Cll, by itself, binds the target sequence with low affinity supports the importance of the intermolecular interaction between the expanded minor groove and the extended N-terminal arm.

Importance of the extended N-terminal arm for cooperative binding

The N-terminal arm of the Cll homeodomain is extended for 14 residues compared with typical homeodomains. In this paper, we showed that this extension of the N-terminal arm is indispensable to the cooperative DNA-binding mechanism of Al and Cll. In the ternary complex structure of Al-Cll-DNA,

the extended N-terminal arm of the Cll forms a typical type I β turn and recognizes the DNA sequence through direct interactions. Although characteristic secondary structure formations, such as a β turn or 3_{10} helix, and sequence-recognition mechanisms using this region have been reported earlier (Passner *et al*, 1999; Piper *et al*, 1999; Slupsky *et al*, 2001; Joshi *et al*, 2007), the reason why this region is important for the cooperativity is different.

In the structures of Hox-Pbx/Exd-DNA, a Hox-specific YPWM motif folds into the 3_{10} helix or type I β turn. But the substructure is used for the intermolecular interactions with Pbx/Exd. The YPWM motif of Hox homeodomains interacts with the hydrophobic pocket formed around the TALE region of the Pbx/Exd homeodomain using an invariant tryptophan residue. On the other hand, the β -turn formation in the extended N-terminal arm of Cll assists the structure and sequence recognition mechanism by the extended N-terminal arm. In the Al-Cll-DNA complex, the first residue of the β turn (Tyr-8) is inserted into the expanded minor groove region and interacts with the sugar group of Thy7' and Thy8' to recognize the expanded minor groove structure. This protein-DNA interaction would be important to recruit the residues around the β turn, such as His-10 and Tyr-8, to the expanded minor groove. These two residues are indispensable to the sequence recognition mechanism of Cll from the minor groove.

Figure 6 Intermolecular interactions between AI and CII. (A) Intermolecular interaction sites observed in the ternary complex structure of AI–CII–DNA. AI and CII are coloured cyan and red, respectively. Interaction sites are indicated by black circles (site 1 and site 2). (B, C) Intermolecular interactions observed in sites 1 and 2. Ion pairs and hydrogen bonds are shown by a blue dotted line. Residues involved in the intermolecular contacts are shown by a stick model. Residue numbers of AI and CII are coloured blue and red, respectively. A grey mesh indicates the $2F_o - F_c$ map contoured at 1.0σ . DNA is shown with a green surface model.

Although protein–DNA interactions from the minor groove side using histidine and arginine residues were also observed in the structure of the Scr–Exd–fkh250 complex (Joshi *et al*, 2007), these residues do not interact directly with the bases but recognize the DNA sequence using the sequence-specific shape of the DNA: the guanidium group of arginine and the imidazole ring of histidine lie in approximately the same plane and are inserted into the narrow region of the minor groove to recognize the shape of the DNA. In contrast, we observed direct interactions between the residues and bases in the structure of AI–CII–DNA. These two residues approach and come into closer proximity to the base groups of DNA than do the residues of the Scr–Exd–DNA complex from the minor groove side and increase the sequence specificity and binding affinity of the cooperative DNA binding of AI and CII by direct interactions. In most cases, the extended conserved regions of homeodomains are used for the intermolecular interactions or the recognition of the sequence-specific structure of the minor groove of the DNA. The direct recognition of the DNA sequence from the minor groove using the extended N-terminal arm is a structural characteristic identified for the first time in this report.

Intermolecular interactions between two homeodomain proteins

Intermolecular interactions between two homeodomain proteins support the cooperative DNA binding (Stark and Johnson, 1994; Chang *et al*, 1995; Li *et al*, 1995;

Figure 7 Mutation analysis. (A) EMSA analysis of the alanine mutants of CII–N2C3. His–10Ala, Tyr–8Ala, and Arg–5Ala lost the ability to bind cooperatively with AI. In all, 10 pmol of AI construct, 20 pmol of CII constructs, and 2 pmol of FITC-labelled dsDNA were used for each binding reaction. (B) Cooperative DNA binding of CII–N2C3 and CII–N2C3 Glu62Ala with AI–HD. The following AI–HD and CII constructs were added to each binding reaction—lane 1: no protein; lanes 2, 3, and 4: 100 pmol of AI–HD and 17.5, 35.0, and 70.0 pmol of CII–N2C3, respectively; lanes 5, 6, and 7: 100 pmol of AI–HD and 17.5, 35.0, and 70.0 pmol of CII–N2C3 Glu62Ala, respectively. Each binding reaction contains 2 pmol of FITC-labelled dsDNA. (C) Binding affinities (K_D s) of the indicated proteins.

Neuteboom and Murre, 1997; Passner *et al*, 1999; Piper *et al*, 1999; LaRonde-LeBlanc and Wolberger, 2003; Joshi *et al*, 2007). In the structure of AI–CII–DNA, two homeodomain proteins interact with each other at two sites (Figure 6). At site 1, the extended N-terminal arm of CII interacts with the $\alpha 1$ – $\alpha 2$ loop and $\alpha 2$ of the AI homeodomain. In the AI–CII–DNA complex, the three residues in the extended N-terminal arm, His–10, Tyr–8, and Arg–5, form remarkable contacts with the minor groove of DNA. These interactions are indispensable to the cooperative DNA binding of AI and CII. The intermolecular interaction between AI and CII around the extended N-terminal arm of CII may help to recruit the three residues to the correct position by the support of AI, which can bind to DNA more tightly than CII, though single mutants of the residues of AI involved in the protein–protein interface did not show any significant decrease of the cooperative DNA-binding ability.

Meanwhile, intermolecular interactions at site 2 seem to stabilize and localize the third α helix of the CII homeodomain. Although the structure of the CII homeodomain in the ternary complex is nearly identical to that of the Hox11L1 homeodomain, the existence of the extended conserved residues of the C-terminal region of the CII homeodomain is important for the maximum cooperative DNA-binding affinity with AI (Figures 2C, D and 7C). The K_D values of the CII–N2C3 Glu62Ala mutant and CII–N2C1 indicate that the

existence of the glutamic acid residue at this position is critical for the maximum cooperative DNA-binding mechanism using the extended conserved residues in the C-terminal region of the Cll homeodomain. In this region, Glu62 of Cll forms a salt bridge with Arg44 of the Al homeodomain at a distance of 3.60 Å. Al seems to attract the third α helix of the Cll homeodomain toward Al and DNA by this interaction, thereby increasing the contact surface area between the third α helix of the Cll homeodomain and the major groove of DNA. This interaction is thought to support the stabilization and localization of the third α helix of the Cll homeodomain to maximize the cooperative-binding affinity of Al and Cll.

Cooperative DNA-binding mechanism of Al and Cll

The extended conserved regions of the Cll homeodomain are indispensable to the cooperative DNA-binding mechanism of Al and Cll. The importance of the extended N-terminal arm is also confirmed in the human homologue of Cll, Hox11L1. The EMSA analysis showed that both of the extended conserved regions are indispensable to the cooperative-binding mechanism of Al and Cll. These extensions are the characteristic features of Cll-type homeodomain proteins. Although Hox11L1, which is a human homologue of Cll, possesses a conserved YPMW motif-like Hox proteins, Cll and its homologues of insecta (*Anopheles gambiae*, *Apis mellifera*, and so on) do not possess this sequence motif. The Cll homeodomain would have evolved in a different way from other homeodomain proteins and obtained a Cll-specific function, that is, the ability to bind DNA cooperatively with Al.

This cooperative mechanism is quite different from those observed in the earlier ternary complex structures of homeodomains and DNA. The most important part of this finding is that the residues in the extended conserved regions of the homeodomain are used in the sequence-recognition mechanism to increase the sequence specificity of the cooperative DNA binding. In general, homeodomain proteins are believed to recognize DNA sequences using their homeodomain or additional DNA-binding domain, and the sequence preferences of the homeodomain regions have been well characterized (Berger *et al*, 2008; Noyes *et al*, 2008). In the homeodomain family, there are subfamilies that possess extended conserved regions around the homeodomain (DuBoule, 1994). Our results suggest that these extended conserved regions may promote the diversity of recognition sequences of homeodomains by the cooperative DNA-binding mechanisms with other homeodomain proteins.

Materials and methods

Protein expression and purification

Gene fragments of Al, Cll, CART1, and Hox11L1 were obtained by PCR from cDNA. Amplified gene fragments were cloned into the T7 polymerase-based protein expression vectors pET26b and pET28a. The expression vectors were transformed into *Escherichia coli* strain Rosetta (DE3) cells for protein expression. Transformants were cultivated at 37°C in LB medium until the optical density at 600 nm reached 0.6. The protein expressions were induced by the addition of IPTG at a final concentration of 1 mM. Cultivations were further continued at 25°C for 16 h to allow the accumulation of expressed proteins. Each construct was purified by cation exchange chromatography using CM Sepharose and Resource S (GE Healthcare) columns and dialysed against 10 mM Tris–HCl (pH 8.0) and 200 mM NaCl.

Protein–DNA-binding assays

The 5'-FITC-labelled 30-mer dsDNA (5'-FITC-AAAGTTTCTTAATTAATGCTAAGCCAAAA-3'; the recognition sequence of Al and Cll is underlined) for the DNA-binding assay was designed as described earlier (Kojima *et al*, 2005). Each protein construct and dsDNA were mixed and incubated in a buffer containing 20 mM Tris–HCl (pH 7.5), 100 mM KCl, 0.25 mg/ml BSA, 0.1% NP-40, and 0.2 mg/ml poly(dI-dC) $\times$ poly(dI-dC) for 60 min at 4°C. Bound and unbound DNAs were separated on a 12% polyacrylamide gel, and fluorescence was measured by an LAS4000 Mini system (Fujifilm, Tokyo, Japan). The protein composition of the binding reactions and the labelled DNA probe used are described in the figure legends. K_{DS} values were measured by at least four independent experiments, each containing at least eight protein concentration data points fit to nonlinear hyperbolic curves using Prism4 software (GraphPad Software Inc., San Diego, CA).

Crystallization and structure determination

All crystallization experiments were performed using the sitting-drop vapour-diffusion method. Crystals of the Al and Hox11L1 homeodomains were obtained in a reservoir solution containing 0.1 M acetate buffer (pH 4.8), 0.1 M CdCl₂, and 30% PEG400; and 0.1 M Tris–HCl (pH 8.6), 0.2 M Li₂SO₄, and 35% PEG4000, respectively.

For co-crystallization of the Al–DNA complex, 14-mer dsDNA (5'-GGGTTTAATTAGGG-3') was mixed with an equimolar amount of Al–HD. The binary complex solution was concentrated to 300 μ M for crystallization. Binary complex crystals were obtained in a reservoir solution containing 0.1 M Tris–HCl (pH 8.5), 0.2 M sodium acetate trihydrate, and 26% PEG4000.

For co-crystallization of the Al–Cll–DNA complex, 17-mer dsDNA (5'-GGCTTAATTAATTGCGG-3') was mixed with an equimolar amount of Al–HD and Cll–N2C3. The ternary complex solution was concentrated to 250 μ M for crystallization. Ternary complex crystals were obtained in a reservoir solution containing 0.2 M sodium malonate (Ph 7.0), 18% PEG3350, and 0.01 M spermidine within 2 weeks.

X-ray diffraction data sets of the Al homeodomain, the Hox11L1 homeodomain, the binary complex of Al–DNA, and the ternary complex of Al–Cll–DNA were collected at beamline AR-NW12 at the Photon Factory (Tsukuba, Japan). The crystal of the Al home-

Table 1 Data collection and refinement statistics of the Al–Cll–DNA complex

<i>Data collection</i>	
Space group	$P2_12_12_1$
<i>Cell dimensions</i>	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	70.93, 85.40, 110.68
Observed reflections	266 303
Unique reflections	18 995
Resolution (Å)	20.0–2.70 (2.77–2.70) ^a
<i>R</i> _{merge} (%)	6.7 (59.2)
<i>I</i> / σ <i>I</i>	27.10 (4.24)
Completeness (%)	99.6 (100)
Redundancy	14.0 (10.1)
<i>Refinement</i>	
<i>R</i> / <i>R</i> _{free} (%)	23.4/28.2
<i>No. of atoms</i>	
Protein	2286
DNA	1382
<i>r.m.s.d.</i>	
Bond length (Å)	0.011
Bond angle (deg)	1.769
<i>Ramachandran plot</i>	
Favoured region (%)	94.5
Allowed region (%)	5.1
Outer region (%)	0.4

^aValues in parentheses are for the highest-resolution shell.

odomain was diffracted to a resolution of 1.00 Å. The crystal of the Al homeodomain belonged to space group $P3_221$ with unit cell dimensions of $a = b = 45.81$ and $c = 48.98$ Å. The crystal of the Hox11L1 homeodomain was diffracted to a resolution of 1.54 Å. The crystal of the Hox11L1 homeodomain belonged to space group C2 with unit cell dimensions of $a = 73.06$, $b = 33.17$, $c = 34.43$ Å, and $\beta = 102.12^\circ$. The crystal of the Al–DNA binary complex was diffracted to a resolution of 2.25 Å. The complex crystal belonged to space group $P4_32_12$ with unit cell dimensions of $a = b = 42.35$ and $c = 162.73$ Å. The crystal of the Al–Cll–DNA ternary complex was diffracted to a resolution of 2.70 Å. The complex crystal belonged to space group $P2_12_12_1$ with unit cell dimensions of $a = 70.9$, $b = 85.4$, and $c = 110.7$ Å. All X-ray diffraction data were integrated and scaled with the program XDS (Kabsch, 1993). Data collection statistics are summarized in Table I and Supplementary Table I.

The structures of Al and Hox11L1 were determined by the molecular replacement method using the program MOLREP (Vagin and Teplyakov, 1997) with the coordinates of the homeodomain structure of Pax (PDB ID: 1FJL) (Wilson *et al*, 1995) and HoxA9 (PDB ID: 1PUF) (LaRonde-LeBlanc and Wolberger, 2003) as templates, respectively. Initial models were refined and manually rebuilt with the programs Refmac5 (Murshudov *et al*, 1997) and XtalView (McRee, 1999).

The binary complex structure of Al–DNA and the ternary complex structure of Al–Cll–DNA were determined by the molecular replacement method using the program MOLREP with the coordinates of Al, Hox11L1, and dsDNA obtained from the HoxA9–Pbx1–DNA structure (LaRonde-LeBlanc and Wolberger, 2003). The initial model was refined and manually rebuilt with the programs Refmac5 and XtalView. The structures were validated by the programs PROCHECK (Laskowski *et al*, 1993) and Rampage (Lovell *et al*, 2003). The refinement statistics are summarized in Table I and Supplementary Table I.

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Computational

The structures of Al, Hox11L1, and the ternary complex were analysed using a set of computer programs: Pymol (<http://pymol.sourceforge.net/>) to depict the structure, Curves + (Lavery *et al*, 2009) to analyse the DNA geometry, Surface in CCP4 (Collaborative Computational Project Number 4, 1994) for calculation of the protein surface area, and Dalilite (Holm and Park, 2000) for the superposition of molecules.

Accession numbers

The coordinates have been deposited in the RCSB Protein Data Bank with the accession codes 3A02 (Al homeodomain), 3A03 (Hox11L1 homeodomain), 3LNQ (Al–DNA), and 3A01 (Al–Cll–DNA).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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