

Binding properties of the human homeodomain protein OTX2 to a DNA target sequence

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Abstract OTX2, a homeodomain protein essential in mouse for the development of structures anterior to rhombomere 3, binds with high affinity to a DNA element (called OTS) present in the human tenascin-C promoter. Here we investigate the binding properties of the full length recombinant human OTX2 and of several deletion mutants to the OTS element. We demonstrate that, upon binding of the protein to its DNA target site, a second molecule of OTX2 is recruited to the complex and that a nearby second binding site is not necessary for this interaction. OTX2 sequences located within a region carboxyl-terminal to the homeodomain are necessary in addition to the homeodomain for binding to DNA. Furthermore, OTX2 dimerization requires the same protein domains necessary for DNA binding.

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1. Introduction

Homeodomain (HD) proteins are transcription factors that regulate many developmental programs in eukaryotes [1,2]. Human OTX2 is a HD protein encoded by a gene related to *orthodenticle*, a *Drosophila* gene implicated in the control of head development [3]. In mouse, *Otx2* transcripts [4] and OTX2 protein [5] have been found in the entire embryonic ectoderm and in the primary endoderm at the pre-streak stage. Later during development, *Otx2* becomes expressed in the anterior portions of the embryo, mainly in the neuroectoderm of the headfold, fated to give rise to fore- and mid-brain [4,5]. Three studies in homozygous null mutant mice clearly demonstrated that *Otx2* plays an essential role in the pathways of primitive streak formation, axial mesendoderm formation and anterior head development [6–8].

The OTX2 HD is very similar to that of the *Drosophila* transcription factor bicoid, characterized by a Lys residue at position 50, corresponding to position nine of the recognition helix [9]. Several studies indicate that Lys-50 confers the DNA binding specificity to bicoid [9–12]. Thus, on the basis of the primary structure of its HD, OTX2 has been hypothesized to share a DNA binding specificity with bicoid [4,9]. Indeed, we previously demonstrated that OTX2 is able to bind with high affinity to a target sequence (OTX2-target sequence, OTS) present in the promoter of the gene encoding the human extracellular matrix protein tenascin-C (TN-C) [13], containing a core element (5'-CCTAATCCT-3') closely resembling the binding site for bicoid present in the hunchback promoter

(5'-TCTAATCCC-3') [10,14]. Moreover, OTX2 has been shown to repress the transcriptional activity of the TN-C gene promoter through the OTS sequence in transiently transfected cells [13]. Recently, the role of OTX2 in regulating the expression of a variety of cell and substratum adhesion molecules, including TN-C, has been underscored [15]. Also the promoter of the interphotoreceptor retinoid binding protein gene contains a sequence very similar to OTS which is bound by OTX2 with a high affinity [16].

Some HD proteins belonging to the paired class (i.e. paired) bind cooperatively as homo- or hetero-dimers to a palindromic DNA site [17,18]. Recently, it has been demonstrated that bicoid binds a target sequence as a monomer and that bicoid-bicoid interactions occur when one molecule is bound to DNA [19]. The similarity between paired, bicoid and OTX2 HDs, allowed us to hypothesize that OTX2 is able to bind to its DNA target sequence as a dimer.

In the present study we investigated in details the binding properties of the full length human recombinant OTX2 and of several OTX2 deletion mutants to the OTS element found in the TN-C gene promoter. Our findings indicate that: (i) OTX2 is able to dimerize on the OTS sequence. (ii) OTX2 dimerization does not require the binding of the second molecule to DNA. (iii) OTX2 sequences located within a region carboxyl-terminal to the HD are necessary in addition to the HD for binding to DNA. (iv) The same protein domains required for DNA binding are required for dimerization.

2. Materials and methods

2.1. Expression and purification of the human OTX2 recombinant protein and its deletion mutants

The human OTX2 protein was produced in the *Baculovirus* system according to the method previously described [13,20]. Several deletion mutants of OTX2 (a schematic representation of OTX2 mutants is in Fig. 4A) were produced in the *Baculovirus* system using the following procedures. Δ 1OTX2, comprising the first 746 bp of the open reading frame of the human OTX2 cDNA (coding for the amino acids 1–249 of the sequence reported in [4]) was generated by PCR and cloned into the *EcoRI*-*BglII* sites of the pAcSG2 *Baculovirus* transfer vector (PharMingen, San Diego, CA, USA). Recombinant *Baculovirus* particles (*Baculogold*/ Δ 1OTX2) were obtained by standard procedures [20] and the protein was purified from nuclear lysates of infected Sf9 cells by ion exchange chromatography using Q and S sepharose fast flow columns (Amersham Pharmacia Biotech, Cologno Monzese, Italy). Δ 3OTX2, Δ 4OTX2 and Δ 5OTX2, encoding the amino acids 1–179, 1–103 and 32–289, respectively, of the human OTX2 protein [4] were generated by PCR and cloned into the *EcoRI*-*BglII* sites of the pAcHLT-A *Baculovirus* transfer vector (PharMingen, San Diego, CA, USA) in frame with the polyhistidine tag. Recombinant *Baculovirus* particles (*Baculogold*/ Δ 3OTX2, *Baculogold*/ Δ 4OTX2 and *Baculogold*/ Δ 5OTX2, respectively) were obtained by standard procedures [20] and proteins were purified from nuclear lysates of infected Sf9 cells utilizing metal affinity chromatography (Talon, Clontech, Palo Alto, CA,

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USA). After elution, the fractions containing the recombinant proteins were dialysed and stored as described [20]. The concentration of the purified recombinant proteins was estimated in SDS-PAGE by densitometric scanning of silver-stained bands corresponding to different amounts of marker proteins. The sequence of PCR-generated inserts was checked prior to their utilization.

2.2. Cells, antibodies and immunofluorescence

Sf9 *Spodoptera frugiperda* cells were cultured as previously described [20]. The rabbit anti-OTX2 antiserum is previously described [5]. For immunofluorescence, cytopspins were prepared and fixed in 50% methanol/50% acetone at -20°C for 5 min. Cells were incubated for 2–16 h at room temperature with the anti-OTX2 polyclonal antiserum (1:1000 dilution) followed by extensive washes and incubation with rhodaminated goat anti-rabbit IgG+IgM for 1 h at room temperature.

2.3. Electrophoresis mobility shift assays

The preparation of nuclear extracts and the electrophoresis mobility shift assays were conducted substantially as reported in Briata et al. [21]. Slices of dried gels corresponding to radioactive bands (bound and free oligonucleotides) were cut out, incubated in Ecolite (ICN Biomedicals, Irvine, CA, USA) and counted by liquid scintillation spectroscopy.

2.4. DNase I footprinting

A 140 bp fragment of the human tenascin-C promoter [22] containing the sequence from -596 to -456 was obtained by PCR. 3' End labelling and purification of the probe were performed as described [23]. The DNA binding reactions and electrophoresis were performed as described [23]. An aliquot of the end-labelled DNA fragment was also subjected to the G+A sequencing reaction [24] and loaded on the same gel for identification of protected sequences.

3. Results

Increasing concentrations of purified recombinant OTX2, obtained with the *Baculovirus* system [5,13], were incubated with a fixed limiting amount of the labelled OTS oligonucleotide [13] and the resulting complex was analyzed by a mobility shift assay. When purified recombinant OTX2 is mixed with OTS at a low protein/oligonucleotide ratio, a single complex

(M) is detected. However, when the ratio is increased, a second slower complex (D) is observed, which becomes more and more predominant as the ratio is further increased until virtually all the available oligonucleotide is shifted to this position (Fig. 1A).

To verify the hypothesis that OTX2 binds to OTS as a dimer, the purified protein was mixed with OTS at a ratio giving only the fast moving complex together with a truncated form of OTX2 ($\Delta 3\text{OTX2}$), lacking the last 109 amino acids but still capable of binding to its target sequence (see details under Section 2 and Fig. 4A,B). $\Delta 3\text{OTX2}$ was added to the mixture at a concentration sufficient to reach the protein/oligonucleotide ratio at which the slower complex would be predominant. As shown in Fig. 1B, a complex migrating to a position intermediate between the D and M complexes was observed, indicating that a complex containing one molecule of OTS and two of OTX2 (one truncated) does indeed form when the protein/oligonucleotide ratio is increased. In order to determine whether OTX2 dimers occurred in solution, gel filtration experiments using a Superdex 200 column (Pharmacia), pre-calibrated with protein markers, was performed. OTX2 is a monomer in solution, migrating at a position corresponding to a calculated molecular mass of approximately 40 kDa (data not shown).

In the OTS sequence the TAATCC core element, known to be bound with a high affinity by OTX2 ($K_d = 0.75 \times 10^{-9}$, [13] and Table 2), is separated by four nucleotides from a lower affinity TTATC sequence ($K_d = 8.2 \times 10^{-9}$, Fig. 3A,C), thus two OTX2 molecules could independently bind to OTS with the observed kinetics. Indeed, a DNase I footprinting analysis indicated that the TN-C promoter region protected by OTX2 spans bases from -535 to -516 containing both the TAATCC and TTATC elements (Fig. 2). To verify the role of the TTATC sequence in the formation of the D complex in mobility shift assays, we repeated the experiments illustrated in Fig. 1A using the oligonucleotide m1OTS (in which the

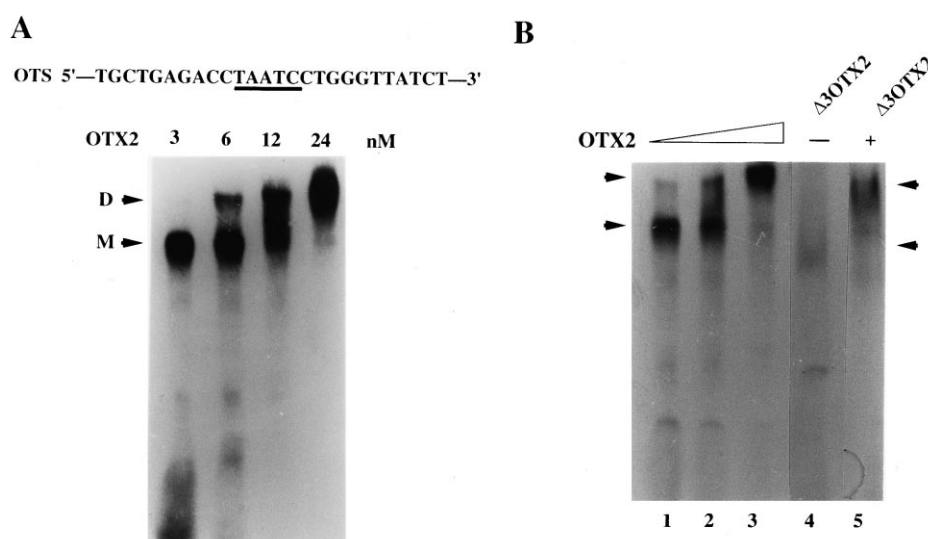


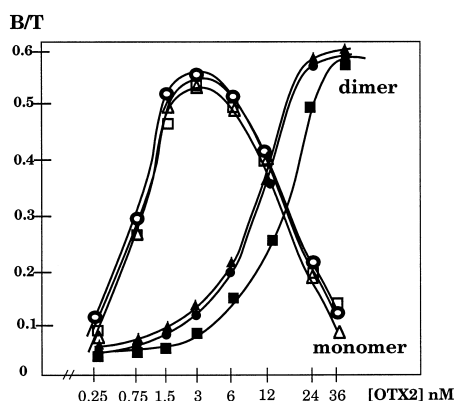
Fig. 1. Recombinant OTX2 binds to OTS as a homodimer. A: Electrophoretic mobility shift assay performed by incubating a fixed limiting amount of the double-stranded labelled OTS oligonucleotide (0.2 nM) with increasing concentrations (3–24 nM) of purified recombinant OTX2, as indicated. Arrows point to the faster migrating complex (M) and the slower migrating complex (D). B: Electrophoresis mobility shift assay performed by mixing 0.2 nM of double-stranded labelled OTS oligonucleotide with increasing concentrations of wild type recombinant OTX2 (lanes 1, 2, 3: 6, 12, 24 nM, respectively), 25 nM $\Delta 3\text{OTX2}$ (lane 4), 6 nM wild type OTX2 plus 25 nM $\Delta 3\text{OTX2}$ (lane 5). The experiments shown in the figure are representative of three other experiments that yielded similar results.

sequence TTATC has been mutated to TCGTC (a sequence to which OTX2 displays only the background affinity shown by HD proteins for any random DNA sequence $K_d = 4 \times 10^{-7}$, unpublished observation)) (Fig. 3A). As expected, the K_d for the monomeric OTX2/DNA complex formation is the same for OTS and m1OTS (Fig. 3B). Surprisingly, despite the absence of a second binding site, the kinetics of the binding of

A

OTS 5'—TGCTGAGACCTAATCCTGGGTATCT—3'
 m1OTS 5'—TGCTGAGACCTAATCCTGGGTCGTCT—3'
 m2OTS 5'—TGCTGAGACCTAATCCTGGGTAATCC—3'
 m3OTS 5'—TGCTGAGACCCGCCGCTGGGTATCT—3'

B



C

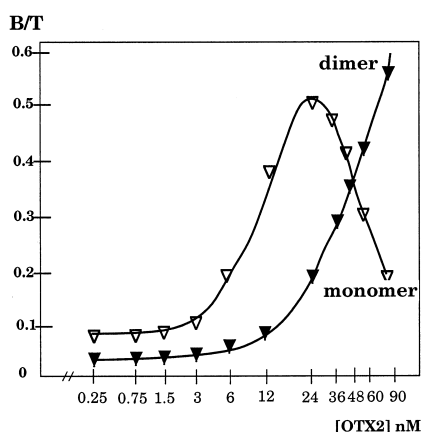


Fig. 2. Saturation analysis of OTX2 binding to either OTS or mutants oligonucleotides. A: Sequences of the oligonucleotides used in the experiments. The OTX2 target element and the mutated elements are underlined. B: Electrophoretic mobility shift assay performed by mixing a fixed limiting amount (0.2 nM) of each double-stranded labelled oligonucleotide (○● OTS, □■ m1OTS, △▲ m2OTS) with the indicated increasing concentrations of recombinant OTX2. C: Electrophoretic mobility shift assay performed by mixing a fixed limiting amount (0.2 nM) of the double-stranded labelled m3OTS oligonucleotide with the indicated increasing concentrations of recombinant OTX2. Gels were dried, autoradiographed, and the bands corresponding to M and D complexes and to the free probe were separately counted. The B/T value corresponds to the ratio between either D or M radioactivity and the total radioactivity input. The figure shows the average of three independent experiments.

G+A 1 2 3

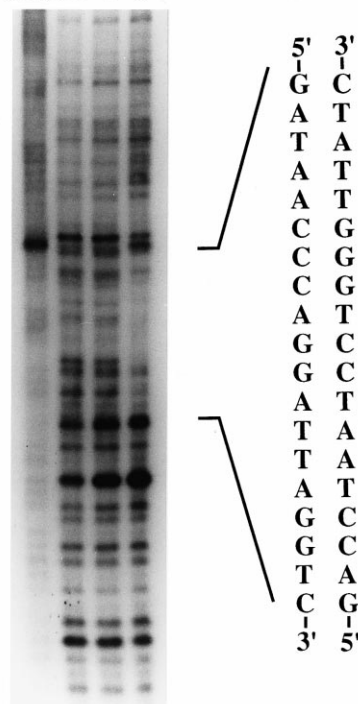


Fig. 3. DNase I footprinting analysis of OTX2 recombinant protein. A 140 bp fragment of the human tenascin-C promoter was end-labelled and reacted with a different amount of recombinant OTX2 (lane 1: no OTX2, lane 2: 10 ng, lane 3: 100 ng) as described under Section 2. The lower strand footprinting is shown. On the left the G+A sequencing reaction is shown.

the second OTX2 to the M complex containing m1OTS ($K_d = 14 \times 10^{-9}$ M) is very similar to that of the dimerization of OTX2 on OTS ($K_d = 8.5 \times 10^{-9}$ M). We also measured the dissociation rates of dimeric OTX2/DNA complexes in mobility shift assays. As shown in Table 1, the dimeric OTX2/OTS complexes are only slightly more stable than the OTX2/m1OTS complexes. To further investigate the importance of the TTATC sequence in the formation of the dimeric complex, we mutated TTATC to TAATC in order to obtain an oligonucleotide containing two high affinity binding sites (m2OTS, Fig. 3A). As shown in Fig. 3B, the association between OTX2 and either OTS or m2OTS proceeds with similar kinetics. Also the dissociation rate between OTX2 and m2OTS is very similar to that between OTX2 and OTS (Table 1) and does not seem to be affected by the presence of a second high affinity binding site. From data presented in Fig. 3B it appears that the binding of the second OTX2 molecule is independent of the presence of a second DNA target site but always occurs when the M complex reaches a given concentration. To confirm this hypothesis, we analyzed in mobility shift assays the interaction between OTX2 and an oligonucleotide containing exclusively the TTATC (m3OTS, Fig. 3A), which would form the M complex at a higher protein/oligo ratio. As shown in Fig. 3C, the affinity of OTX2 for m3OTS is more than one order of magnitude lower ($K_d = 8.2 \times 10^{-9}$ M) in comparison to the affinity of OTX2 for OTS. However, the dimer is formed, even though at high protein/oligonucleotide ratio. The dissociation rate of the dimeric complex OTX2/m3OTS confirms the above described results (Table 1).

Table 1
Dissociation rates of the dimeric OTX2/DNA complexes

Time (min)	0	10	40	60
OTS	100	92	42	14
m1OTS	100	94	27	10
m2OTS	100	96	41	15
m3OTS	100	31	5	3

Electrophoresis mobility shift assays were performed by mixing either OTS or mOTS double-stranded labelled oligonucleotides (0.2 nM) with 24 nM purified OTX2. After a 20 min incubation at room temperature (time 0), a ten-fold excess of either unlabelled oligonucleotides was added and the reaction carried out for the indicated times at 4°C. Gels were dried, autoradiographed and the bands corresponding to the shifted complexes and to the free probes were separately counted. Results are presented as the percentage of B/T values measured at time 0. The table shows the average of three independent experiments

In order to define the binding/dimerization domain(s) of OTX2, we performed the deletion mutant analysis described under Section 2 and schematically illustrated in Fig. 4A. Truncated OTX2 proteins were expressed with the *Baculovirus* system and used in mobility shift experiments. As shown in Fig. 4B, nuclear extracts of Sf9 cells expressing $\Delta 10\text{TX2}$ and $\Delta 30\text{TX2}$, lacking 20 and 109 amino acids, respectively, at the C-terminus and $\Delta 50\text{TX2}$, lacking 31 amino acids at the N-terminus of the protein, form complexes with OTS while $\Delta 40\text{TX2}$ protein, lacking 185 amino acids at the C-terminus, does not. The $\Delta 40\text{TX2}$ deletion mutant is correctly expressed and translocated to the nucleus of Sf9 cells as demonstrated by the staining of Sf9 cells infected with Baculogold/ $\Delta 40\text{TX2}$ with either the anti-OTX2 polyclonal antiserum [5] (Fig. 4C) or the αOTX2 monoclonal [13] (not shown). We also purified the truncated OTX2 proteins to near homogeneity and the K_d

Table 2
DNA binding affinity and dimerization ability of OTX2 deletion mutants

	K_d	Dimerization
OTX2	0.75×10^{-9}	+
$\Delta 10\text{TX2}$	2.1×10^{-9}	+
$\Delta 30\text{TX2}$	4.2×10^{-8}	+
$\Delta 40\text{TX2}$	—	—
$\Delta 50\text{TX2}$	3.4×10^{-9}	+

Electrophoresis mobility shift assay is performed by mixing a fixed limiting amount of the double-stranded labelled OTS oligonucleotide (0.2 nM) with increasing concentrations of recombinant OTX2. Gels were dried, autoradiographed and the bands corresponding to the M complex and to the free probe were separately counted. K_d values were calculated according to Corsetti et al. [31]

of the formation of the monomeric complex between each purified deleted protein and the OTS sequence is presented in Table 2. The affinity of $\Delta 30\text{TX2}$ for OTS is almost 20 times lower in comparison to wild type OTX2. Thus, taking into account that $\Delta 40\text{TX2}$, lacking almost the complete C-terminus, does not bind to OTS, one can conclude that a region C-terminal to the HD is necessary for DNA binding. The data presented in Table 2 also indicate that each deletion mutant which binds to OTS is able to form dimers maintaining the same kinetics displayed by wild type OTX2 (data not shown).

4. Discussion

The major finding of the present work is that the full length recombinant human OTX2 forms a dimer when bound to a DNA target sequence and that this dimerization depends on the interaction of a second molecule with an already DNA bound OTX2 molecule. Some HD proteins interact with DNA in a dimeric form but in a different way. Recombinant paired class HDs have been reported to bind to palindromic DNA sequences composed of two TAAT half sites [18]. Paired HD per se contains sufficient information to cooperatively dimerize on these sites, thus distinguishing the HDs of the paired class from those of other classes which bind as monomers [25]. Not only does OTX2 appear to dimerize on OTS, even if this sequence does not contain any palindromic element, but it binds and dimerizes with similar kinetics on an oligonucleotide containing either a second site for which OTX2 displays a very high affinity (m2OTS) or a second site for which it displays virtually no affinity (m1OTS). From Fig. 3B,C it appears that the only parameter affecting dimerization is the requirement of a pre-constituted OTX2/DNA complex. These findings and the fact that OTX2 is a monomer in solution, suggest that, upon binding to its target site, OTX2 undergoes a conformational change that creates a site on the protein for the binding of a second molecule, which for this interaction does not need to be bound to DNA. On the other hand, the DNase I footprinting data do not imply that OTX2 binds to the low affinity TTATC motif. It is possible that OTX2 binding to the high affinity TAATC site prevents DNase I digestion around the low affinity site by steric hindrance. Our interpretation does not exclude that a second DNA site could be relevant in stabilizing the complex. It is worth noting that the dissociation experiments show a slight (two-fold) decrease in the complex stability when the oligonucleotide contains a second site for which OTX2 dis-

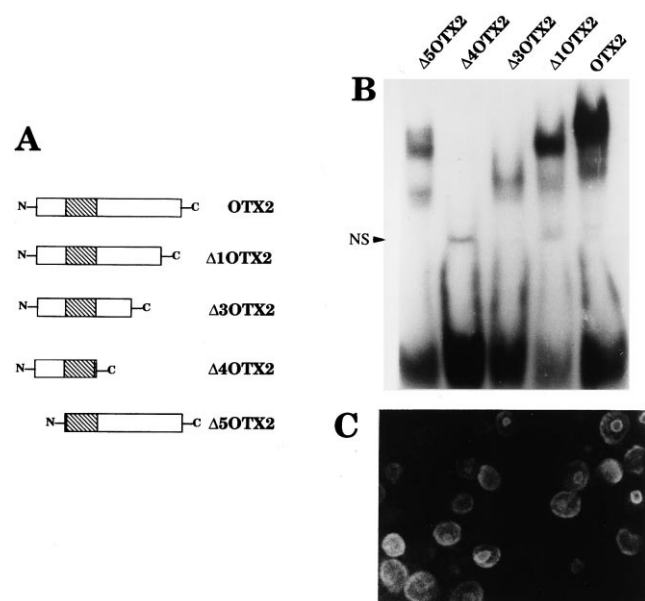


Fig. 4. Deletion mutant analysis of OTX2. A: Schematic representation of OTX2 and OTX2 deletion mutants. Details on the construction of the mutant vectors are reported under Section 2. B: Nuclear extracts of Sf9 cells expressing the recombinant proteins were mixed with double-stranded labelled OTS oligonucleotides and analyzed in mobility shift assays. C: Immunofluorescent staining with the anti-OTX2 antiserum of Sf9 cells infected with Baculogold/ $\Delta 40\text{TX2}$.

plays virtually no affinity (m1OTS). Furthermore, the relevance of a second DNA site could be increased by a different spacing between the two sites that could exist in different promoters.

The evidence that our deletion mutant $\Delta 4\text{OTX2}$, which lacks the C-terminus, is unable to bind OTS underscores the relevance of other OTX2 domains apart from the HD in DNA binding activity. In this respect, the observation is noteworthy that the $\Delta 3\text{OTX2}$, which lacks the C-terminal 109 amino acids, although still able to bind DNA and to dimerize, interacts with OTS with an affinity that is almost 20-fold lower than the affinity displayed by OTX2, thus pointing to a functional relevance of this region that would be interesting to test in vivo. Other authors have demonstrated for HOM-C proteins that sequences C-terminal to the HD in addition to the HDs have significant functions in establishing segment identity in *Drosophila* [26]. The deletion mutant analysis of OTX2 also indicates that the same OTX2 domains (namely the HD and the C-terminal region) required for DNA binding are necessary for dimerization. The large deletion in $\Delta 4\text{OTX2}$ could per se result in protein misfolding and consequent inability to bind DNA. The evidence that $\Delta 4\text{OTX2}$ is correctly expressed and translocated to the nucleus of Sf9 cells where it is recognized by both the anti-OTX2 polyclonal antiserum and the αOTX2 monoclonal antibody, in addition to the finding that also the DNA binding affinity of $\Delta 3\text{OTX2}$ is significantly reduced in comparison to OTX2, makes this possibility unlikely.

Since nearly all HD proteins are believed to bind to short, highly related DNA sequences, the basis for their high specificity of action is not yet understood. This specificity in vivo is probably mediated by transcriptional cofactors. The first example of these protein/protein interactions was provided by the yeast proteins MATA1 and MAT α 2 which interact through a short α helix located C-terminal to the MAT α 2 HD [27]. Recently described examples derive from the products of the *exd/Pbx* genes which have been proposed as Hox cofactors on the basis of both genetic and biochemical evidence [28,29]. Furthermore, a new family of cofactors which confers transcriptional synergism between LIM and one of the Otx HD proteins (P-OTX/Ptx1) has been discovered [30]. It is then tempting to speculate that OTX2 dimerization plays a role in modulating the ability of the protein to interact with yet unknown cofactors.

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