Sex-Specific and Non-Sex-Specific Oligomerization Domains in Both of the *doublesex* Transcription Factors from *Drosophila melanogaster*

WENQIAN AN, SAYEON CHO, HARUHIKO ISHII, AND PIETER C. WENSINK*

Department of Biochemistry and The Rosenstiel Center, Brandeis University, Waltham, Massachusetts 02254-9110

Received 24 January 1996/Returned for modification 22 February 1996/Accepted 14 March 1996

The *doublesex* gene of *Drosophila melanogaster* encodes the alternatively spliced, sex-specific transcription factors DSX^M and DSX^F. These factors regulate male- and female-specific transcription of many genes. For example, female-specific transcription of the *yolk protein 1* gene is regulated by DSX^M repression in males and DSX^F activation in females. In this study we used in vitro interaction assays and the in vivo yeast two-hybrid method to identify and examine oligomerization domains of the DSX proteins. A 66-amino-acid segment common to both proteins (amino acids 39 to 104) contains a sequence-specific DNA binding domain and an oligomerization domain (OD1). The OD1 domain oligomerizes up to at least a pentamer, but only dimers bound to a palindromic regulatory site in the *yolk protein 1* gene are detected. Both subunits of the OD1 dimer are in contact with DNA. Another segment of each protein (amino acids 350 to 412 for DSX^F and 350 to 427 for DSX^M) contains a second oligomerization domain (OD2^F and OD2^M, respectively). The OD2 domains have both sex-specific and non-sex-specific sequences which are necessary for oligomerization. On the basis of sequence analysis, we predict that OD2 oligomerizes through coiled-coil interactions. We speculate that the common function of OD1 and OD2 is to oligomerize the full-length proteins, whereas their specialized functions are to form a dimeric DNA binding unit and a sex-specific transcriptional activation or repression unit.

Sex differentiation in *Drosophila melanogaster* is directed by genes that form perhaps the best understood regulatory pathway among all multicellular organisms (3, 4, 21, 27). The *doublesex* (*dsx*) gene lies at the end of this pathway and is regulated by alternative RNA splicing to synthesize either of two transcription factors, the male-specific DSX^M or the female-specific DSX^F. These two DSX proteins link the pathway to target genes specifying the sex-specific structures of the body.

An examination of one target gene, the female-specific *yolk protein 1* (*Yp1*) gene, demonstrated that DSX^F activates transcription from a DNA binding site in females, whereas DSX^M represses transcription from the same site in males (1, 2). Like the female-specific *Yp1* gene, male-specific genes are regulated by the same two DSX proteins, but the necessarily different regulatory activities of the DSX proteins at male-specific genes are unknown.

The mechanisms responsible for the opposing regulatory effects of DSX^M and DSX^F at the *Yp1* gene must be due to differences between the DSX proteins themselves rather than to differences between other female- and male-specific proteins. This conclusion follows from the observation that DSX^M represses and DSX^F activates from a *Yp1* enhancer regardless of whether the flies are otherwise genetically male or female (8).

The differences between the two DSX proteins are localized to a small region of their primary structures. These proteins share a 397-amino-acid N terminus and differ only in the 30- and 152-amino-acid C-terminal extensions of DSX^F and DSX^M, respectively (5). As might be expected from the fact that both proteins regulate from the same *Yp1* DNA site, the DNA binding domain is well separated from the sex-specific C

terminus along the primary sequence backbone and, most likely, in the native proteins as well (7, 10).

We have been investigating the DNA binding and other physical properties of DSX proteins to understand the mechanism of their transcriptional regulation (7). Both of the full-length proteins form dimers and tetramers in the absence of DNA. These oligomers bind with high levels of specificity to a palindromic target regulatory site in *Yp1* DNA, but curiously, upon binding additional DNA molecules the equilibrium shifts dramatically toward dimers. Each dimer appears to have a single DNA binding site, suggesting that occupying the second site in a tetramer reduces affinity between the dimers. These DNA binding and oligomerization properties of the male and female proteins are indistinguishable.

Here we identify oligomerization domains in DSX^F and DSX^M by in vivo two-hybrid interaction assays and in vitro physical studies. We have found that each protein has two oligomerization domains: one is sex independent, and the other is sex specific. We speculate that the common function of the two domains is to oligomerize the full-length proteins, whereas their specialized functions are to form a dimeric DNA binding unit and a sex-specific transcriptional activation or repression unit.

MATERIALS AND METHODS

DNA constructs. All junction sequences in the following constructs were confirmed by sequencing. The LexA and B42 fusions were constructed as follows (25). The pT7-7:DSX^F:mod and pT7-7:DSX^M:mod plasmids, modified as previously described (7), were digested with *Nde*I at the DSX^F initiation codon, filled in with the Klenow fragment of *Escherichia coli* DNA polymerase I, and then ligated to an *Eco*RI linker (catalog no. 1004; New England Biolabs). Similarly, a *Sal*I linker was inserted at the *Cl*aI site in the polylinker downstream of each gene. The *Eco*RI-*Sal*I fragment encoding the full-length protein was cloned into the *Eco*RI-*Sal*I sites in pEG202 (14) to create in-frame LexA fusions DSX^F-LexA and DSX^M-LexA. The same *Eco*RI-*Sal*I fragments were inserted into the *Eco*RI-*Xho*I sites in pJG4-5 (14) to create DSX^F-B42 and DSX^M-B42 fusions.

Truncated DSX structures were generated by PCR using 5' primers containing an *Eco*RI site and a 3' primer with stop codon and *Sal*I site and then insertion of the restriction-endonuclease-digested fragments into the *Eco*RI-*Sal*I sites of

* Corresponding author. Mailing address: Rosenstiel Center, Brandeis University, 415 South St., Waltham, MA 02254-9110. Phone: (617) 736-2421. Fax: (617) 736-2405. Electronic mail address: wensink@hydra.rose.brandeis.edu.

pEG202 and *EcoRI-XhoI* sites of pJG4-5. MB3 is a human immunodeficiency virus Rev protein mutated by replacing three arginines with three glycines (20). The MB3 constructs were generous gifts from Françoise Stutz (Brandeis University).

Glutathione *S*-transferase (GST)-OD2^F was made by cloning a PCR-generated DSX^F fragment (amino acids 350 to 412) into the *EcoRI-SalI* sites of vector pGEX-4T1 (Pharmacia).

By PCR and various subcloning methods (25), we constructed pBlueBac-DSX(39-104) to express in insect cells the DSX segment from amino acids 39 to 104 tagged with hemagglutinin and six histidines at the N terminus. The resulting sequence was inserted into the pBlueBac (Invitrogen, Inc.) *NheI* site: GCTAGC (*NheI* site) ATG (initiation codon) TACCCATACGACGTCCAGACTA CGCG (hemagglutinin tag) CGC (Arg) CACCACCACCACCACCAT (6 His) ATG (Met) GAATTC (GluPhe; *EcoRI* site) AGG (DSX amino acid 39). . . CAC (DSX amino acid 104) CTCGAG (LeuGlu; *XhoI* site) TAA (stop codon) CTA ACTAGC (*SpeI-NheI* site fusion). The expression of this plasmid in insect cells produces DSX(39-104) with an extra 20 amino acids at the N terminus and 2 amino acids at the C terminus.

Yeast transformation and the mating tests. Yeast transformations were done by the lithium acetate (LiOAc) method (16, 18). Mating tests were done as previously described (13).

After mating, protein interaction was assayed by growth on minimal dropout plates containing glucose or galactose and raffinose but lacking the appropriate components (13). β -Galactosidase activity was assayed by color on minimal dropout plates containing X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside). Both the growth and staining intensity of colonies were monitored over a 4-day period at 30°C.

Retention of DSX^F by GST-OD2^F-Sepharose beads. GST and the GST-female oligomerization domain 2 (OD2^F) fusion were purified from *E. coli* (19). Two micrograms of either protein bound to glutathione-Sepharose beads was incubated with 200 ng of purified full-length DSX^F at 22°C for 24 h in 110 μ l of binding buffer (0.5% Nonidet P-40, 20 mM Tris [pH 8], 100 mM NaCl, 1 mM EDTA, 0.5 mg of bovine serum albumin per ml). The beads were washed three times with 0.4 ml of binding buffer. The samples were boiled in 1 $\times$ reducing sodium dodecyl sulfate (SDS) loading buffer and fractionated by SDS-12% polyacrylamide gel electrophoresis (PAGE). Western blotting (immunoblotting) was done by using polyclonal anti-DSX antibody and standard protocols.

Cell culture and production of recombinant baculovirus bearing the gene for DSX(39-104). Sf9 cells were grown at 27°C in TNM-FH medium supplemented with 10% (vol/vol) fetal bovine serum (26). Monolayer cultures of Sf9 cells were cotransfected with pBlueBac-DSX(39-104) and *Autographa californica* nuclear polyhedrosis virus DNA. Recombinant viruses were isolated by successive rounds of plaque purification. The presence of DSX(39-104) in the recombinant baculovirus DNA was confirmed by PCR.

Purification of the recombinant protein. Sf9 cells (2×10^8) were infected with recombinant virus at a multiplicity of infection of 10 in a spinner flask. Infected cells were grown for 3 days before harvest. After cells were harvested by low-speed centrifugation, the cell pellet was washed with 1 $\times$ phosphate-buffered saline. Cells were separated into cytoplasmic and nuclear fractions as previously described (7). To the soluble nuclear extract was added 80% ammonium sulfate in a buffer containing 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.6), 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 M NaCl, 10 μ M ZnCl₂, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 5 μ g of aprotinin per ml, 1 μ g of leupeptin per ml, 1 μ g of antipain per ml, and 1 μ g of pepstatin A per ml to give a final concentration of 50%. The precipitated protein pellet was resuspended in a buffer containing 25 mM HEPES (pH 7.6), 0.1 mM EDTA, 1 mM dithiothreitol, 0.25 M NaCl, and 20% glycerol. The ammonium sulfate fraction was applied to the specific DNA affinity column, and bound protein was eluted as described previously (7). The eluted fraction was dialyzed against the DNA binding buffer (25 mM HEPES [pH 7.6], 0.1 mM EDTA, 1 mM dithiothreitol, 0.1 M NaCl, 10% glycerol) and stored in aliquots at -70°C. The DSX(39-104) protein was judged to be at least 50% pure by SDS-PAGE analysis.

Mobility shift assay. The mobility shift assay was performed as described previously (7) except that 0.5 μ g of nonspecific poly(dI-dC) · poly(dI-dC) was added to the reaction mixture. The *dsx4* site from the *Yp1* gene was the specific binding DNA, and an oligonucleotide of the same length derived from the OE1 enhancer of *Yp2* was the nonspecific DNA competitor (7).

Protein-protein and protein-DNA cross-linking. One hundred nanograms of partially purified DSX(39-104) was cross-linked by incubation with the indicated concentrations of glutaraldehyde in 20 μ l for 30 min at room temperature and then separated by SDS-15% PAGE followed by Western blotting using rat serum against DSX^M and peroxidase-conjugated anti-rat antibody (7). Double cross-linking experiments were performed as previously described (7). A single-stranded DNA oligomer containing DSX protein binding site 5'-TCGACA CAACTACAATGTTGCAATCAGCTAGCC-3' (*dsx4* sequence in boldface type) was hybridized to a 14-nucleotide primer (5'-GGCTAGCTGATTGC-3') and labeled with bromodeoxyuridine and [α -³²P]dCTP. DNA binding reactions were performed in DNA binding buffer containing 1 μ g of poly(dI-dC) · poly(dI-dC) and 5 ng of DSX protein in 20 μ l for 1 h at room temperature. After the reaction, glutaraldehyde was added and incubation continued for 30 min at room temperature. After electrophoresis on nondenaturing polyacrylamide gels, gels were placed on a Fotodyne 305-nm UV transilluminator and illuminated for 30 min.

After autoradiography, shifted bands were excised, eluted, denatured, and analyzed by SDS-15% PAGE and subsequent autoradiography.

RESULTS

Full-length male and female proteins interact with themselves and each other in the yeast two-hybrid system. The yeast two-hybrid system was used to identify protein-protein interactions likely to be responsible for the oligomerization of DSX proteins (12, 17). We first tested whether such interactions could be detected. Full-length DSX^F and DSX^M were separately fused to the LexA DNA binding domain and the B42 transcriptional activation domain. Pairwise interactions were then tested by the stringent mating version of the two-hybrid assay (13).

As shown in Fig. 1, the phenotypes of diploid products from mating between haploids expressing the two DSX^F fusion proteins indicate that full-length DSX^F interacts with itself in this assay. A *LEU2* reporter gene whose activation depends on interaction between the two proteins allowed vigorous growth on selective media (Fig. 1A). A *lacZ* reporter gene with the same interaction dependence also produced strong signals (Fig. 1B). The DSX^F specificity of the interaction was demonstrated by several observations. First, neither fusion protein alone activated either of the reporter genes (data not shown). Second, the DSX^F-B42 protein was necessary because the induction of its gene was required for the expression of both reporter genes (data not shown). Third, a different protein, MB3, fused to the LexA or B42 protein gave no positive signals when tested with any of the DSX^F fusions (Fig. 1).

Identical results were obtained with full-length DSX^M, indicating that it also contains an interaction domain (Fig. 1). Furthermore, DSX^M and DSX^F interact with each other, suggesting that the interaction domains are in sequences shared by these two proteins.

An oligomerization domain of 63 amino acids includes both sex-specific and non-sex-specific sequences. To localize interaction domains within DSX^F, a panel of truncated DSX^F proteins was fused to the B42 activation domain (Fig. 2, rows 1 through 12) and tested for interaction with full-length DSX^F and DSX^M fused to the LexA DNA binding domain (Fig. 2, columns 1 and 2). A single 63-amino-acid segment (amino acids 350 to 412) of DSX^F appears to be responsible for all DSX^F interaction with full-length male and female proteins. Deleting upstream and downstream sequences did not diminish the signal, and large segments of DSX^F that lie outside amino acids 350 to 412 gave no interaction signal. In addition, it appears that most of this DSX^F segment is necessary for interaction because removing 17 amino acids from the amino terminus (structure 367-412) or 15 from the carboxy terminus (structure 305-397) eliminated detectable interaction with full-length DSX^M and DSX^F. We call the 350-412 segment of DSX^F OD2^F because another oligomerization domain (OD1^F) was found later.

The OD2^F domain oligomerizes with itself. When OD2^F rather than full-length protein was tested against the DSX^F truncation panel, signals occurred with OD2^F alone and only when OD2^F was present in the other truncations (Fig. 2, column DSX^F 350-412). This indicates that OD2^F is necessary and sufficient for OD2^F-directed oligomerization. Furthermore, other segments added to OD2^F do not increase the signal, suggesting that OD2^F does not have substantial interaction with other DSX^F segments (11).

Figure 2 also shows that DSX^M has a male-specific oligomerization domain (OD2^M) at approximately the same position as that of OD2^F in the female protein. First, full-length DSX^F and

FIG. 1. Specific interaction between full-length DSX proteins in a yeast two-hybrid system. Photographs of plates assaying for leucine-independent growth (A) and β -galactosidase activity, as identified by X-Gal staining (blue color detected as black here) (B). Both plates were replicas of the same master plate, which had streaks with cells expressing the indicated fusion proteins.

DSX^M gave identical signals with the DSX^F truncation series, indicating that both proteins interact predominantly with the same OD2^F target in DSX^F (Fig. 2, columns 1 and 2). Second, the amino-terminal 48 amino acids (350 to 397) of OD2^F are identical to those in DSX^M and include the 17 amino acids (350 to 366) that are necessary for interaction with both DSX^M and DSX^F. Third, DSX^M terminated at amino acid 427 gave an interaction signal indistinguishable from that of the full-length female protein, indicating that no additional sex-specific portion of OD2^M is necessary. Fourth, the sex-specific carboxy-terminal 15 amino acids (398 to 412) of OD2^F are necessary for interaction with both of the full-length proteins, leading us to conclude that at least some of the male-specific sequence in OD2^M is also necessary.

We conclude that male and female OD2 domains contain common and sex-specific regions that are necessary for oligomerization. Although these domains require sex-specific amino acid sequences, the two-hybrid assay detects no difference between OD2^F and OD2^M interactions.

OD2^F interacts with purified DSX^F in vitro. OD2^F was overexpressed in *E. coli* as a GST fusion, purified, and then immobilized on Sepharose beads. Full-length DSX^F purified from a baculovirus expression system was retained by this immobilized fusion protein but not by immobilized GST (Fig. 3). This demonstrates that OD2^F interaction with DSX^F is not an artifact of the protein fusions used in the two-hybrid system.

A second and independent oligomerization domain within the segment from amino acids 39 to 104. The yeast two-hybrid system detected only one protein oligomerization domain in each DSX protein. However, other observations suggested the presence of a second oligomerization domain in each protein. A 66-amino-acid segment (amino acids 39 to 104) within the shared sequence contains a DNA binding domain that is sufficient to bind a target regulatory site in the *Yp1* gene (10). Since protein dimerization appears to be necessary for tight binding to the DNA site, this segment of DSX is also likely to promote its own protein-protein interaction (2, 7). To explore this possibility, this segment was expressed with an epitope tag in baculovirus-infected cells and partially purified, and its oligomerization and DNA binding properties were examined. The epitope tag contained a six-histidine tag previously shown to have no observable effect on the oligomerization of full-length and truncated p53 proteins (28).

To investigate the oligomerization of this segment in the absence of DNA, we performed protein cross-linking experiments (Fig. 4A). Incubation with 0.01% glutaraldehyde produced protein bands with SDS-PAGE mobilities that ranged from monomers to pentamers. In contrast, incubations without glutaraldehyde produced only the monomer band. Adding an equal concentration of a heterologous protein (carbonic anhydrase) before the cross-linking reaction yielded no heterologous cross-links, indicating that cross-links occurred within molecular complexes rather than between them (data not shown). We conclude that there is another domain, OD1, located within a non-sex-specific segment of the sequence that also contains a previously identified DNA binding domain. Furthermore, at least five monomers can be linked together by OD1.

The DSX(39-104) segment binds DNA as a dimer. The partially purified DSX(39-104) segment bound specifically to a 33-bp DNA oligomer containing a single target regulatory site (Fig. 5). Only one shifted band was detected in these mobility shift assays, suggesting that there is a predominant protein oligomer bound to DNA under these conditions. To determine the number of monomers in the shifted complex, we first cross-linked protein to protein within the complex and then cross-linked protein to radiolabeled DNA. By varying the extent of protein cross-linking, this procedure allows the total number of monomers in a complex bound to a single DNA molecule to be detected after electrophoresis on a denaturing gel (7). Without protein cross-linking, most DNA molecules were cross-linked to monomers and a very small number were cross-linked to two protein monomers (Fig. 4B, lane 1). This indicates that at least two protein monomers can be in contact with a single palindromic DNA site. As the glutaraldehyde concentration was increased and protein-DNA cross-linking remained constant, the ratio of dimer to monomer cross-linked to DNA increased. At 10 times the glutaraldehyde concentration that produced cross-linked pentamers in the absence of DNA, no protein oligomers higher than a dimer were detected (Fig. 4B, lane 3).

		DSX ^M		DSX ^F							MB3	
		1-549	1-427	51-427	305-427	350-427	350-412	39-104				
DSX ^F
1-427	+	+	+	++	++	++	-	-	-	-	-
105-427	+	+	+	ND	ND	ND	ND	-	-	-	-
305-427	+	+	++	++	++	++	ND	-	-	-	-
350-427	+	+	ND	++	++	++	-	-	-	-	-
350-412 *	+	+	ND	++	++	++	-	-	-	-	-
367-412	-	-	-	ND	ND	+/-	ND	-	-	-	-
380-412	-	-	-	-	-	-	ND	-	-	-	-
105-412	+	+	++	++	++	++	ND	-	-	-	-
105-385	-	-	-/+	ND	ND	ND	ND	-	-	-	-
305-397	-	-	-	-	-	-	-	-	-	-	-
1-304	-	-	-	-	-	-	ND	-	-	-	-
39-104	-	-	-	-	-	-	-	-	-	-	-
DSX ^M
1-549	+	+	+	ND	ND	ND	ND	-	-	-	-
1-427	+	+	++	ND	ND	ND	ND	-	-	-	-
MB3		-	-	-	-	-	-	-	-	-	-	-

FIG. 2. Summary of two-hybrid interactions between full-length and truncated DSX proteins. Scale drawings of the protein structures are shown on the left, with OD2^F indicated by an asterisk. Numbers indicate the termini of structures. Full-length DSX^F is 427 amino acids in length, and full-length DSX^M is 549 amino acids in length. An open box indicates a male-specific sequence, a black box indicates a female-specific sequence, and a gray box indicates a non-sex-specific sequence. +, positive interaction; -, no interaction; ND, not determined. The order of interaction strength is as follows: ++ > + > +/- > -/+ > -, as reflected by assays of leucine-independent growth and β -galactosidase activity. Column headings indicate LexA fusions. Row headings indicate B42 fusions.

These results indicate that the species bound to DNA is a dimer that has both subunits in contact with DNA.

DISCUSSION

The results described here identify two oligomerization domains in each of the DSX proteins. One of these domains, OD1, is within a 66-amino-acid segment that also contains a DNA binding domain. This segment is identical in the male and female proteins and is located near the N terminus. The second oligomerization domain, OD2, is hundreds of amino acids downstream of OD1 and includes both non-sex-specific and sex-specific sequences. This domain is a continuous sequence of approximately 65 amino acids in length, 48 amino acids of N-proximal non-sex-specific sequence followed by a C-proximal sex-specific sequence of either 15 amino acids in OD2^F or less than 30 amino acids in OD2^M (Fig. 6A). Our

deletion studies showed that both the sex-specific and non-sex-specific regions are necessary for interactions between the OD2 domains. Therefore, each full-length protein has two domains that can independently cause oligomerization. One domain colocalizes with a DNA binding function and is the same in both proteins, and the other is well separated from the first and is sex specific.

FIG. 3. OD2^F binds DSX^F in vitro. Western blot assay of DSX protein after SDS-PAGE. Lanes 1 through 3, full-length DSX^F (D^F) bound by glutathione-Sepharose beads, by those beads linked to GST, and by those beads linked to GST-OD2^F fusion protein, respectively; lane 4, same as lane 3 except that no DSX^F (-) was present in the binding reaction mixture; lane 5, purified DSX^F, an electrophoretic migration standard. The position of DSX^F is indicated on the right.

FIG. 4. Oligomeric forms of DSX(39-104) in the absence or presence of DNA. (A) Protein cross-linking in the absence of DNA. This segment was cross-linked with 0 and 0.01% glutaraldehyde (GA) and then assayed by Western blotting after SDS-PAGE. The positions of molecular mass standards (in kiladaltons) and oligomeric species are shown on the left and right, respectively. (B) Protein-protein and DNA-protein cross-linking in the presence of ³²P-labeled *dsx4* DNA. Protein-DNA complexes were incubated with the indicated concentrations of GA, fractionated by electrophoresis on nondenaturing gels, illuminated with UV light, extracted, and then analyzed by SDS-PAGE and subsequent autoradiography. The numbers on the left indicate the mobilities of protein molecular mass standards (in kilodaltons), and those on the right indicate the numbers of monomers bound to DNA. Because complexes were not treated with nuclease, mobility is related to the sum of protein and DNA sizes.

FIG. 5. Binding between segment DSX(39-104) and a specific DNA site. An autoradiograph of an electrophoretic mobility shift assay is shown. The baculovirus-expressed segment was incubated with 0.1 ng of ³²P-labeled *dsx4* binding site, a high-affinity site from the *Yp1* gene. Lanes 1 through 4, samples with 0, 0.5, 2, and 4 ng of protein in the binding reaction mixture, respectively; lanes 5 and 6, the same as lane 4 except that the binding reaction mixtures had 400-fold excesses of nonradioactive nonspecific (nonspec.) and *dsx4* DNA, respectively. +, presence; -, absence; spec., specific; comp., competitor.

The OD1 domain. A segment of DSX protein has previously been identified as a region which, when joined to either substantial upstream or downstream DSX regions, can bind specifically to a DNA regulatory site (10). In this study we have shown that this DSX segment is sufficient to bind to this site. We have also found that this segment has a protein oligomerization function. We refer to the two underlying structures as the DNA binding domain and OD1. The functions of these two domains are likely to be interrelated because we have shown that this segment binds DNA as a dimer, strongly suggesting that the structure recognizing DNA is dimeric and that the oligomerization energy provided by OD1 is important for tight and specific binding to DNA (6).

In the absence of DNA, this DSX segment can oligomerize into at least a pentamer, whereas the predominant form bound to DNA is a dimer. Although the equilibria and rates of oligomerization and DNA binding remain to be examined, it appears that DNA binding has a significant and negative effect on oligomerization. The shift from a higher-order polymer to dimer as DNA concentration increases is reminiscent of an effect observed with the full-length DSX proteins (7). DNA binds to full-length dimers and tetramers, but increasing the DNA concentration dramatically shifts the equilibrium toward the dimer complex with DNA. Presumably, the higher DNA concentration increases occupancy of the second DNA binding site in a tetramer and leads to its dissociation into dimers. The similar behavior of the OD1-containing segment suggests that

FIG. 6. Structure of DSX proteins. (A) Locations of functional domains in the DSX protein sequence. The locations of male (OD2^M) and female (OD2^F) oligomerization domains and of the region which contains both the sex-independent oligomerization domain (OD1) and the DNA binding domain are shown by horizontal lines above the sequence. The sex-independent sequence is represented by a gray box, and the female- and male-specific sequences are represented by black and open boxes, respectively. (B) The sequences of the sex-specific oligomerization domains. The regions predicted to be alpha helical (24) are boxed, and the amino termini of the sex-specific sequences are marked by arrows. (C) A wheel diagram of a proposed OD2^F alpha-helix that includes all three predicted alpha-helical segments from panel B and the intervening sequences. The male-specific sequence at the carboxy terminus of the third predicted helix in OD2^M is shown immediately counterclockwise to the female spokes of the wheel. The hydrophobic amino acids in the proposed coiled-coil interaction surface are underlined, and all negatively (-) and positively (+) charged amino acids in this helical region are shown.

tetramerization of the full-length DSX protein critically depends on direct interaction between two pairs of OD1 domains. Further, the observed inhibition of tetramerization of the full-length protein is unlikely to be caused by an indirect mechanism, such as conformational changes within the full-length protein, but is more likely to be an effect localized to this segment, perhaps involving steric hindrance or electrostatic repulsion between DNAs bound to each dimer.

Similar DNA effects on protein oligomerization have been found in other DNA-binding proteins. For example, Sp1 and p53 form high-molecular-weight protein-DNA complexes but dissociate into lower-molecular-weight protein-DNA complexes as the DNA concentration is increased (9, 22). In these two examples, disruption is most likely caused by electrostatic repulsion between bound DNAs. In contrast, the tetrameric Lac repressor also has two DNA binding sites but does not dissociate into dimers when both DNA binding sites are occupied. This most likely results from the strong tetramerization forces and well separated DNA binding sites in the Lac repressor (15, 23).

The OD2 domains. Coiled-coil interactions are likely to be the basis for OD2-OD2 oligomerization. Secondary structure predictions from the primary sequence indicate that the OD2 domains are mostly alpha helical (Fig. 6B). Three helices of 11, 6, and either 22 (for OD2^F) or 25 (for OD2^M) amino acids are separated by short gaps of 4 and 9 amino acids (24). If the three helices and the gaps of OD2^F are represented together as a single helix in an alpha-helical axial projection, a remarkably amphipathic distribution is observed, consistent with an extended coiled-coil interaction (Fig. 6C). The male domain has an essentially identical amphipathic distribution, although the male structure allows seven rather than six successive pairs of hydrophobic interactions at positions a and d (Fig. 6C).

Two oligomerization domains in a single protein. The observation that there are two independent oligomerization domains in DSX transcription factors leads us to speculate that the domains have both common and specialized functions. We suppose that the common function is to bring the full-length polypeptides together as dimers or tetramers. Since the two domains are well separated in the primary sequence and the proteins also have highly asymmetric shapes (7), we suggest that the two domains are at opposite ends of the protein, holding it together at those two points. We speculate that at one end of this protein the specialized function of OD1 oligomerization is to form the dimeric DNA binding unit. This OD1-OD1 interaction may be weak, as suggested by its lack of detection in the two-hybrid assay (Fig. 2, row 12). The specialized function of OD2 at the other end of the protein is likely to be related to the sex specificity of DSX interaction with the transcriptional machinery or to DNA binding cooperativity when the proteins bind naturally occurring multiple regulatory sites in DNA. For example, it may form a dimeric sex-specific regulatory structure. This regulatory structure in DSX^M would repress transcription of the *Yp1* gene, and in DSX^F it would activate.

ACKNOWLEDGMENTS

W. An and S. Cho made approximately equal contributions to this research.

This research was supported by Public Health Service grant GM46237 from the Institute of General Medical Sciences.

We thank Roger Brent (Harvard Medical School) for the necessary strains and vector plasmids used in the two-hybrid system and Zushi Huang, Françoise Stutz, Jie Tang, Xiaohua Wu, and Xiaochun Zhun (Brandeis University) for the gifts of constructs used in our two-hybrid tests and for technical advice.

REFERENCES

- An, W., and P. C. Wensink. 1995. Integrating sex- and tissue-specific regulation within a single *Drosophila* enhancer. *Genes Dev.* **9**:256–266.
- An, W., and P. C. Wensink. 1995. Three protein binding sites form an enhancer that regulates sex- and fat body-specific transcription of *Drosophila* *yolk protein* genes. *EMBO J.* **14**:101–110.
- Belote, J. 1992. Sex determination in *Drosophila melanogaster*: from the X:A ratio to *doublesex*. *Semin. Dev. Biol.* **3**:319–330.
- Burtis, K. C. 1993. The regulation of sex determination and sexually dimorphic differentiation in *Drosophila*. *Curr. Opin. Cell Biol.* **5**:1006–1014.
- Burtis, K. C., and B. S. Baker. 1989. *Drosophila doublesex* gene controls somatic sexual differentiation by producing alternatively spliced mRNAs encoding related sex-specific polypeptides. *Cell* **56**:997–1010.
- Chen, J., and K. S. Matthews. 1994. Subunit dissociation affects DNA binding in a dimeric lac repressor produced by C-terminal deletion. *Biochemistry* **33**:8728–8735.
- Cho, S., and P. C. Wensink. 1996. Purification and physical properties of the male and female *doublesex* proteins. *Proc. Natl. Acad. Sci. USA* **93**: 2043–2047.
- Coschigano, K. T., and P. C. Wensink. 1993. Sex-specific transcriptional regulation by the male and female *doublesex* proteins of *Drosophila*. *Genes Dev.* **7**:42–54.
- Delphin, C., P. Cahen, J. J. Lawrence, and J. Baudier. 1994. Characterization of baculovirus recombinant wild-type p53. Dimerization of p53 is required for high-affinity DNA binding and cysteine oxidation inhibits p53 DNA binding. *Eur. J. Biochem.* **223**:683–692.
- Erdman, S. E., and K. C. Burtis. 1993. The *Drosophila* *doublesex* proteins share a novel zinc finger related DNA binding domain. *EMBO J.* **12**:527–535.
- Estojak, J., R. Brent, and E. A. Golemis. 1995. Correlation of two-hybrid affinity data with in vitro measurements. *Mol. Cell. Biol.* **15**:5820–5829.
- Fields, S., and O. Song. 1989. A novel genetic system to detect protein-protein interactions. *Nature (London)* **340**:245–246.
- Finley, R. L. J., and R. Brent. 1994. Interaction mating reveals binary and ternary connections between *Drosophila* cell cycle regulators. *Proc. Natl. Acad. Sci. USA* **91**:12980–12984.
- Finley, R. L. J., and R. Brent. 1995. Interaction trap cloning with yeast, p. 169–203. In D. M. Glover and B. D. Hames (ed.), *DNA cloning 2: a practical approach*, 2nd ed. Oxford University Press, Oxford.
- Flickert, R., and B. Müller-Hill. 1992. How lac repressor finds *lac* operator in vitro. *J. Mol. Biol.* **226**:59–68.
- Gietz, D., A. St. Jean, R. A. Woods, and R. H. Schiestl. 1992. Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res.* **20**:1425.
- Gyuris, J., E. Golemis, H. Chertkov, and R. Brent. 1993. Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2. *Cell* **75**:791–803.
- Ito, H., Y. Fukuda, K. Murata, and A. Kimura. 1983. Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* **153**:163–168.
- Kaelin, W. G., D. C. Pallas, J. A. DeCaprio, F. J. Kaye, and D. M. Livingston. 1991. Identification of cellular proteins that can interact specifically with the T/E1A-binding region of the retinoblastoma gene product. *Cell* **64**:521–532.
- McDonald, D., T. J. Hope, and T. G. Parslow. 1992. Posttranscriptional regulation by the human immunodeficiency virus type 1 Rev and human T-cell leukemia virus type 1 Rex proteins through a heterologous RNA binding site. *J. Virol.* **66**:7232–7238.
- McKeown, M. 1992. Sex differentiation: the role of alternative splicing. *Curr. Opin. Genet. Dev.* **2**:299–303.
- Pascal, E., and R. Tjian. 1991. Different activation domains of Sp1 govern formation of multimers and mediate transcriptional synergism. *Genes Dev.* **5**:1646–1656.
- Pilz, I., K. Goral, O. Kratky, R. P. Bray, N. G. Wade-Jardetzky, and O. Jardetzky. 1980. Small-angle X-ray studies of the quaternary structure of the lac repressor from *Escherichia coli*. *Biochemistry* **19**:4087–4090.
- Rost, B., and C. Sander. 1993. Prediction of protein structure at better than 70% accuracy. *J. Mol. Biol.* **232**:584–599.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Summers, M. D., and G. E. Smith. 1987. *A manual of methods for baculovirus vectors and insect cell culture procedures*. Texas A&M University, College Station.
- Taylor, B. J. 1992. Differentiation of a male-specific muscle in *Drosophila melanogaster* does not require the sex-determining genes *doublesex* or *intersex*. *Genetics* **132**:179–191.
- Wang, Y., M. Reed, P. Wang, J. E. Stenger, G. Mayr, M. E. Anderson, J. F. Schwedes, and P. Tegtmeyer. 1993. p53 domains: identification and characterization of two autonomous DNA-binding regions. *Genes Dev.* **7**:2575–2586.