The DSX (Doublesex) transcription factor regulates somatic sexual differentiation in Drosophila. Female and male isoforms (DSX^f and DSX^m) are formed due to sex-specific RNA splicing. DNA recognition, mediated by a shared N-terminal zinc module (the DM domain), is enhanced by a C-terminal dimerization element. Sex-specific extension of this region in DSX^f and DSX^m leads to assembly of distinct transcriptional preinitiation complexes. Here, we describe the structure of the extended C-terminal dimerization domain of DSX^f as determined by multidimensional NMR spectroscopy. The C-terminal dimerization element is well ordered, giving rise to a dense network of interresidue nuclear Overhauser enhancements. The structure contains dimer-related UBA folds similar to those defined by x-ray crystallographic studies of a truncated domain. Whereas the proximal portion of the female tail extends helix 3 of the UBA fold, the distal tail is disordered. The structures of proximal tail disrupt the sex-specific binding of IX (Intersex), an obligatory partner protein and putative transcriptional coactivator; IX–DSX^f interaction is, by contrast, not disrupted by truncation of the distal tail. Mutagenesis of the UBA-like dimer of DSX^f highlights the importance of steric and electrostatic complementarity across the interface. Temperature-sensitive mutations at this interface have been characterized in yeast model systems. One weakens a network of solvated salt bridges, whereas the other perturbs the underlying nonpolar interface. These mutations confer graded gene-regulatory activity in yeast within a physiological temperature range and so may provide novel probes for genetic analysis of a sex-specific transcriptional program in Drosophila development.

 Sexual differentiation in Drosophila melanogaster is regulated by the X:autosome ratio and a sex-specific RNA-splicing pathway (Fig. 1A) (1). A principal target is doublesex (dsx); expression of male- and female-specific transcription factors (DSX^m and DSX^f) in turn directs most aspects of somatic sexual differentiation (2). The DSX isoforms are encoded by mRNAs sharing the first three exons; the C-terminal segment of DSX^f is encoded by exon 4, whereas that of DSX^m is encoded by exons 5 and 6. Male and female isoforms are thus identical for the first 397 residues but differ thereafter (Fig. 1B) (2). DSX^m and DSX^f share two recognized domains, an N-terminal DNA-binding domain (3) and a C-terminal dimerization domain (4, 5). The DNA-binding domain (the DM motif) contains a nonclassical zinc module (6). C-terminal dimerization enhances DNA binding (7) and is mediated by a novel α-helical dimer containing ubiquitin-associated (UBA)^5-like) folds (8).6 Mutations in either the DM domain or dimerization domain have been isolated in association with intersexual phenotypes (3, 4, 9, 10).7 Broad conservation of the DM motif in metazoan proteins related to sexual differentiation suggests that mechanisms of sexual dimorphism are in part universal (11).

In this paper, we describe the solution structure of the C-terminal domain (CTD) of DSX^f and its functional implications. This domain (residues 350–427) mediates both strong dimerization (K_d < 1 nM) and the sex-specific recruitment of an obligatory partner protein, Intersex (IX) (12). Such recruitment has provided a biochemical basis for observations that (i) the intersex gene (ix) interacts genetically with dsx^f but not dsx^m (13, 14) and (ii) mutations in ix cause an intersexual phenotype in XX:AA ix-null flies (15) that is identical to that of XX:AA dxx-null flies. Such corresponding phenotypes make it unlikely that IX interacts with targets other than DSX^f. The ix gene

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6 The abbreviations used are: UBA, ubiquitin-associated domain; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside; CTD, C-terminal domain; IP, immunoprecipitation; dsx^f and dsx^m, specific DNA binding sites for Doublesex in the fat body enhancer; HA, hemagglutinin; NOE, nuclear Overhauser enhancement; NOESY, NOE spectroscopy; r.m.s., root mean square; HSQC, heteronuclear single-quantum coherence; TOCSY, total correlation spectroscopy; Y1H, yeast one-hybrid; Y2H, yeast two-hybrid; AD, activation domain; DBD, DNA binding domain.

7 It is not known whether the structural analogy between the CTDs of the DSX isoforms implies a functional role of the ubiquitination machinery in sex-specific gene regulation or whether this resemblance is incidental (8).

8 Mutations in the DM motif (encoding residues 35–110) affect both DSX^m and DSX^f. Because the sex-specific region begins at residue 398 (Gly in DSX^f and Ala in DSX^m), mutations in exons 4 – 6 affect either DSX^f (exon 4, encoding residues 398 – 427) or DSX^m (exons 5 and 6, encoding residues 398 – 546). XX females homozygous for G398D, for example, are intersexual, whereas males are unaffected, since exon 5 is unchanged (3, 72).
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Bacterial Expression and Isotopic Labeling—Uniformly $^{13}$C- and $^{15}$N-double-labeled domains CTD$^F$-p (residues 350–427) and CTD$^F$-p$_A$ (residues 350–412) were expressed as thrombin-cleavable fusion proteins (8) in modified M9 minimal medium containing 3 g/liter $^{13}$C-glucose, and 1 g/liter $^{15}$NH$_4$Cl as sole carbon and nitrogen sources, respectively. The polypeptides each contained an additional N-terminal dipeptide (GS) derived from the vector. A 50-ml overnight culture was used to inoculate 1 liter of minimal medium; the starting cell density exhibited an absorbance value of 0.15 at 600 nm ($A_{600}$). When aerobic bacterial growth reached an $A_{600}$ value of 0.5 following shaking at 37 °C at 250 rpm, protein overexpression was induced for 6 h by the addition of isopropyl-$\beta$-D-thiogalactopyranoside to a final concentration of 1 mM. Cells were harvested by centrifugation at 6370 × g for 15 min at 4 °C. The cell pellet (from 4 liters of culture) was resuspended in 100 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 250 mM NaCl and 60 μg/ml lysozyme. Cell lysis was affected by French press at least twice. The lysate was centrifuged at 20,100 × g for 45 min, and the supernatant was loaded onto a cobalt resin column equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 250 mM NaCl. The DSX fusion protein was eluted with 150 mM imidazole and cleaved by thrombin. Final purification of the DSX domains was accomplished by gel filtration chromatography using an Amersham Biosciences Superdex-75 column. The protein was >98% pure as assessed by SDS-PAGE; molecular masses were verified by mass spectrometry.

NMR Methods—Protein samples were prepared in nitrogen-purged H$_2$O solution (7% D$_2$O) containing 10 mM $^3$H-Tris-HCl (pH 6.5) and 250 mM NaCl in a 300-μl Shigemi NMR tube; the protein concentration was in each case ∼1.5 mM. One-, two-, and three-dimensional NMR spectra were acquired at 30 °C at 700 MHz using a triple resonance probe and a shielded (x, y, z)-gradient unit; four-dimensional $^{1}$C/$^{13}$C NOE spectra were acquired at 600 MHz. Spectra were in each case processed with the program nmrPipe (22) and analyzed with pipp (23). Three-dimensional NMR triple-resonance spectra (HNCACB, CBCA(CO)NH, C(CO)NH, H(CCO)NH, HCCH-TOCSY, and HNCO experiments) were acquired to enable complete main-chain and side-chain resonance assignments (24–26). Stereo-specific assignments were obtained by analysis of approximate iminoresidue and sequential iminoresidue distances involving NH, C$_2$H, and C$_3$H protons, as derived from three-dimensional $^{15}$N-edited and $^{13}$C-edited NOE spectra with short mixing times (35 ms) to avoid spin diffusion (27). Three NOE spectra (three-dimensional $^{13}$C-separated NOEY- HSQC, three-dimensional $^{15}$N-separated NOEY-HSQC, and four-dimensional $^{13}$C/$^{13}$C-edited NOEY) were used to derive distance restraints. Three-dimensional $^{13}$C-edited (F3)/$^{15}$N, $^{13}$C-filtered (F1) experiments were used to extract intermolec-

encodes a protein of 188 amino acids (12) that is homologous to a component of the mammalian Mediator transcriptional co-activation complex (16). Whereas IX lacks recognizable motifs of DNA binding, its N-terminal region is also homologous to Caenorhabditis elegans SUR-2 (encoded by suppressor of ras) and to the SYT class of human synovial sarcoma translocation proteins; the latter can function as transcriptional co-activators in in vitro assays. Heterologous expression of insect and mammalian homologs of ix in transgenic flies provides evidence that its functional interactions with DSX$^F$ are (at least in part) conserved (17). Although IX is expressed in both female and male flies, it has no known function in males. The molecular basis of the sex-specific recruitment of IX by DSX$^F$ is not well understood but is presumably mediated by the female-specific C-terminal domain (CTD$^F$).

To investigate the structural basis of DSX$^F$ dimerization and IX recruitment, we have determined the structure of CTD$^F$ and explored its function by mutagenesis. Because this domain appears to be refractory to crystallization (18), heteronuclear multidimensional NMR methods were employed to define its ordered substructure and delineate disordered segments. A dense network of interresidue nuclear Overhauser effects (NOEs) was defined by three- and four-dimensional edited NMR spectroscopy; intra- and intermolecular contacts were distinguished by asymmetric isotopic labeling (19–21). The structure of the constituent UBA-like folds and their mode of dimerization in solution are similar to features observed in the crystal structure of a dimeric fragment comprising residues 350–412 (CTD$^F$-p$_A$) (8). The female-specific portion of CTD$^F$-p (residues 398–427) in part extends the C-terminal α-helix of the UBA fold (residues 350–408) but is otherwise disordered (residues 409–427). Deletion analysis and Ala-scanning mutagenesis collectively demonstrate that IX binding is mediated by the proximal helical portion of the female tail. The dimer interface of CTD$^F$ is remarkable for an electrostatic network of salt bridges and charge-stabilized hydrogen bonds braced by underlying nonpolar side chains. Two temperature-sensitive (ts) mutations at this interface have been characterized in yeast model systems. These mutations may provide in vivo probes to enable studies of sex-specific transcriptional regulation in D. melanogaster with application to developmental neurogenetics.

EXPERIMENTAL PROCEDURES

FIGURE 1. Sexual differentiation cascade in D. melanogaster and domain organization of DSX. A, sex is determined by Xautosome ratio, leading to an RNA-splicing cascade. B, organization of DSX isoforms. The shared region spans residues 1–397, containing the DM domain (residues 35–105; gray box) and proximal portion of the dimerization domain (residues 350–397; black box). Sex-specific regions comprise residues 398–427 (red and 398–549 (DSX$^F$; green). CTD$^F$ interacts with co-activator IX. Other targets of the tra factors in the sex-determining hierarchy include fruitless (not shown), whose male-specific isoform directs male courtship behavior (67, 73–75).
ular NOEs between labeled and unlabeled protomers in a solution containing a 1:1.5 ratio of labeled and unlabeled domains (28, 29). Four-dimensional $^{13}C/^{13}C$-edited NOESY was employed to identify additional dimer-related NOEs.

**Structure Calculations and Molecular Modeling**—An ensemble of structures was calculated using a hybrid distance-geometry dynamic simulated annealing method (30, 31) using the National Institutes of Health version of XPLOR (32, 33). Initial structures were calculated by employing only unambiguous NOEs; calculations were iteratively performed on further NOE analysis. Assigned intra- and intermolecular NOEs were classified as strong, medium, weak, and very weak, corresponding to respective interproton distance restraints of 1.8–2.7 Å (1.8–2.9 Å for NOEs involving NH protons), 1.8–3.3 Å (1.8–3.5 Å for NOEs involving NH protons), and 1.8–5.0 Å and 1.8–6.0 Å (34, 35). Restraints on main-chain $\phi$ and $\psi$ torsion angles were generated using TALOS (36), in which minimum error ranges were set to $\pm 20$ ($\phi$) and $\pm 30$ ($\psi$) from the average predicted value (37, 38). The structure of CTD$^F$-p$_A$ was calculated based on 3057 NOE distance restraints, including 418 long range restraints and 275 contacts between protomers across the dimer interface. The structure of CTD$^F$-p was obtained using 2847 NOE restraints. Ramachandran plots were calculated to examine the quality of the final structures using PROCHECK (39). Models were visualized using InsightII and molmol software (40).

**Mutagenesis**—Site-directed substitutions were introduced by PCR-based two-stage overlap extension mutagenesis as described (8). Random mutations were introduced by error-prone PCR, generating a DNA fragment spanning the CTD$^F$ coding region. Protocols for PCR amplification and subcloning have been described (41).

**Yeast Assays**—Dimerization of CTD$^F$ was probed using the Matchmaker GAL4 Y2H system (BD Clontech, Palo Alto, CA). For Y2H library screening, pGBK7-CTD$^F$ wild-type and pGADT7-CTD$^F$ variants were co-transformed into yeast strain Y187 by the lithium acetate/polyethylene glycol method. Interactions were monitored on SD/-Leu/-Trp-selective plates supplemented with 80 μg/ml X-gal. Plates were incubated at 30 °C for 7 days. White colonies were picked and spotted on new selective plates, incubated at 20 °C until new colonies appeared. Plasmid DNA from blue colonies was recovered by PCR and sequencing. Interactions were otherwise characterized using a quantitative β-galactosidase enzymatic assay (see below). Specific DSX$^F$-DNA binding was probed using a MATCHMAKER Y1H system (BD Clontech) in which lacZ is regulated by a 48-bp fragment of the DSX-responsive fbe (fat body enhancer in Drosophila yolk protein genes yp1 and yp2) containing binding sites dsx$^F$ and dsx$^P$ (42). The design and validation of this system have previously been described (41). Expression levels of DSX fusion proteins were verified in each case by Western blot using anti-GAL4 antiserum (Upstate Group, Charlottesville, VA).

**DSX$^F$-IX Interactions**—The sex-specific interaction of DSX$^F$ and IX was probed by two assays developed by Baker and coworkers (12). (i) Initial studies employed a Y2H system in which the bait plasmid expressed a fusion protein containing the specific DNA-binding domain (DBD) of GAL4 linked to DSX$^F$, DSX$^M$, or respective fragments containing C-terminal deletions; the prey plasmid expressed the GAL4 activation domain (AD) linked to IX. In each construct, specific independent DNA binding by the DM domain of the DSX fusion proteins was blocked by the mutation R91Q to avoid toxicity (3). Yeast transformants (strain AH109) were grown at 30 °C on selective plates containing the His3 competitive inhibitor 3-AT. Under these conditions, little or no growth is observed in control studies of the DSX$^M$ fusion proteins. To identify determinants of IX binding, Ala-scanning mutagenesis of CTD$^F$ was conducted in this context. Ala substitutions were tested for effects on interaction with IX by Y2H as described above. (ii) Structure-activity relationships inferred from Y2H studies were verified by co-immunoprecipitation (co-IP) of recombinant tagged IX and DSX$^F$ proteins from Drosophila S2 nuclear extracts (12). Transfected S2 cells were grown at 25 °C, and nuclear extracts were obtained and analyzed at 4 °C. Wild-type or variant DSX isoforms were expressed as V5 epitope-tagged constructs, whereas IX was co-expressed as hemagglutinin (HA)-tagged constructs. Full-length IX, DSX$^M$, DSX$^F$, and DSX$^D$ deletion variant coding sequences were subcloned in frame into the pAc5.1/V5-HisA vector (Invitrogen). To generate HA-tagged pAc5.1-IX expression vector, an HA epitope was added to the N terminus of IX sequence by PCR. Constructs were verified in each case by case DNA sequencing. Cells were transfected using Effectene transfection reagent (Qiagen, Valencia, CA) according to the manufacturer’s protocol. Nuclear extracts were prepared using a nuclear extract kit from Active Motif (Carlsbad, CA). 100-μl nuclear extracts were incubated with 2-μg polyclonal anti-HA antiserum (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) with gentle rocking overnight at 4 °C, followed by incubation with Protein A-agarose beads (Santa Cruz Biotechnology) for an additional 3 h. The beads were collected and gently washed three times with cell lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na$_3$VO$_4$, protease inhibitor mixture (Roche Applied Science) and 1 mM phenylmethanesulfonyl fluoride). Proteins were resolved on a 12% SDS-polyacrylamide gel and detected by immunoblotting with a mouse monoclonal anti-V5 antibody (Invitrogen).

**Enzyme Assays**—Liquid state o-nitrophenyl-β-D-galactose assays were performed according to the vendor’s protocol (BD Clontech). Results (given in Miller’s units) represent the mean ± S.D. of triplicate experiments (43).

**RESULTS**

Our study has three parts. We first undertook the heteronuclear NMR analysis of CTD$^F$-p and its dimeric fragment CTD$^F$-p$_A$. This analysis permits comparison of the solution structure of the extended domain with the crystal structure of the fragment, in turn enabling characterization of the female-specific tail. We next investigated the role of the tail in IX binding by deletion analysis and Ala-scanning mutagenesis. Finally, we employed random- and site-directed mutagenesis to probe the dimer interface. Yeast models were exploited to identify ts mutations at this interface, which may be of future value as genetic probes.
Solution Structure of DSX CTD⁻ —NMR spectra of CTD⁻ exhibit residue-specific spin systems corresponding to the number of residues in a protomer, indicating that the dimer is symmetric; within the dimer, any exchange between asymmetric conformations must be fast on the time scale of NMR chemical shifts. Despite the presence of two prolines (Pro³⁷⁰ and Pro³⁷⁵), minor conformations characteristic of cis-trans isomerization were not observed. ¹H-¹⁵N HSQC “fingerprint” spectra of the ¹⁵N-labeled domains are superimposed in Fig. 2.

The spectrum of CTD⁻ contains additional cross-peaks that are poorly resolved near random coil chemical shifts. Patterns of chemical shifts are otherwise similar in the two spectra, indicating a correspondence of structures within respective UBA folds. Complete sequential assignment was in each case obtained.

Analysis of Secondary Structure — Trends in the secondary chemical shifts of Hα, Cα, and Cβ resonances, together with diagnostic NOE patterns, indicate that CTD⁻ contains three long α-helices (76% of the sequence): Gln³⁵³–Lys³⁶⁶ (α₁), Trp³⁷¹–Asn³⁸³ (α₂), and Ile³⁸⁸–Gln⁴⁰⁸ (α₃). These helices each exhibit large positive secondary Cα shifts, negative secondary Cβ shifts, and strong or medium strength NOEs between amide protons within the helix (Hα(i, i + 1) and Hα(i, i + 3) contacts in Wüthrich format (44). Although such NOE patterns can sometimes be observed in nascent (but not stably folded) helical segments, observation of the accompanying canonical helix-associated ¹³Cα and ¹³Cβ chemical shifts indicates that these elements of secondary structure are well ordered. NMR-defined helical end points in CTD⁻ are in accord with the crystal structure of CTD⁻-D₃ (8).

Long Range and Dimer-related NOEs —Analysis of tertiary structure requires assignment of long range contacts, which are predominantly between α-helices. Unambiguous assignment requires dimer-related NOEs to be distinguished from intramolecular NOEs. The latter are critical for determining the folding of individual protomers, whereas intermolecular NOEs aid in characterizing the dimer interface. The pattern of long range and dimer-related NOEs is in overall accord with the crystal structure; representative three- and four-dimensional NOESY spectra are provided as supplemental material.

Of particular interest are two anomalous NOE cross-peaks involving in one dimension the side-chain HN⁺ resonance of Arg³⁹⁴ at 8.55 ppm and in the other dimension respective resonances at 6.45 and 6.84 ppm (Fig. 3B). By elimination, the latter resonances cannot be due to aromatic, main-chain amide, or side-chain carboxamide protons. These anomalous NOE cross-peaks are asymmetric, indicating that the unassigned resonances at 6.45 and 6.84 ppm are broader than that of Arg³⁹⁴ HN⁺. Four corresponding NOE cross-peaks are likewise observed in the ¹³C-edited NOESY spectrum involving the Arg³⁹⁴ CβH₂ methylene group (Fig. 3A). We hypothesize that these resonances belong to the Arg³⁹⁴ NH₂ side-chain guanidinium moieties, which are seldom observable in proteins due to rapid solvent exchange. The two-dimensional ¹H-¹⁵N HSQC spectrum of CTD⁻-D₃ contains five cross-peaks in the chemical
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shift range characteristic of exchangeable Arg side-chain resonances (Fig. 3C). Four are assigned to Arg HN\_s resonances, whereas the additional cross-peak at 6.84/72.00 ppm is likely to be an Arg NH\_2 guanidinium resonance. We speculate that an electrostatic network, including charge-stabilized hydrogen bonds, protects this resonance from solvent exchange, enabling its observation (see “Discussion”).

**Mobility of C-terminal Tail**—Whereas the initial residues of the female-specific tail of CTD\^F-p (residues 350 – 427) extend \(\alpha\)-helix \(\alpha_5\) of the UBA fold (see above), the remainder of the tail is disordered. Evidence of disorder is provided by four sets of observations. First, resonances in the C-terminal tail exhibit motional narrowing relative to resonances in the UBA folds. Such differences in line widths lead to systematic differences in cross-peak patterns in the three-dimensional HNCACB spectrum (supplemental material). Although, due to line broadening, residues in the UBA fold predominantly exhibit intraresidue cross-peaks with few interresidue \(C_\alpha\) and no \(C_\beta\) cross-peaks, residues in the C-terminal portion of the sex-specific tail (residues 409 – 427) exhibit complete \(C_\alpha\) and \(C_\beta\) connectivities (supplemental material). Second, the pattern of chemical shifts in the C-terminal portion of the tail, unlike those in the UBA fold, is consistent with random coil. Third, the absence of stably folded tail structure is further indicated by an absence of medium and long range NOEs; only sequential and intraresidue NOEs were observed between residues 409 – 427 in three- and four-dimensional \(^{13}\text{C}\)- and \(^{15}\text{N}\)-edited NOESY spectra. Finally, truncation of the tail after residue 412 (i.e. comparison of CTD\^F-p and CTD\^F-p\_A) reveals a correspondence of chemical shifts and NOE patterns, demonstrating that the presence or absence of an intact tail does not modulate the folding of the core domain.

**Solution Structure and Dimer Interface**—The structure of CTD\^F-p was determined based on 2847 distance restraints and 226 dihedral-angular restraints. 40 structures were obtained; the number of restraints per residue in the ordered moiety (residues 352–408) is 46. The average root mean square (r.m.s.) difference between this set of structures and their mean coordinates is 0.25 Å for main-chain atoms and 0.77 Å for all heavy atoms. The best fit superposition of the polypeptide backbone (\(C_\alpha\) trace) is well defined (residues 352–408; Fig. 4A); a ribbon model is shown in Fig. 4B. The proximal portion of the female-specific tail (residues 398–407) contributes to the \(\alpha\)-helical structure of UBA fold, whereas the distal portion (residues 408–427) is largely disordered. The extensive dimer interface is illustrated in Fig. 4C by packing of a ribbon model of one protomer against a space-filling model of the other.

The solution structure of CTD\^F-p\_A was also determined by parallel NMR analysis. The four-dimensional \(^{13}\text{C}/^{15}\text{N}\) edited NOESY spectra of the intact and truncated domains exhibit nearly identical NOE patterns for residues in the hydrophobic core and dimer interface (supplemental material). Due to improved line widths (and hence signal-to-noise ratio) in the fragment, additional NOE distance restraints were obtained to a total of 3057. The corresponding ensemble of 40 structures of CTD\^F-p\_A exhibits a precision of 0.18 Å (main-chain atoms) and 0.65 Å (all heavy atoms). Although these values are slightly lower than those of CTD\^F-p, the two structures are essentially identical.

**Comparison of NMR Structure and Crystal Structure**—For residues 354 – 408, pairwise comparison reveals that the main-chain r.m.s. difference between the CTD\^F-p\_A ensemble and its crystal structure (Protein Data Bank accession code 1ZV1) (8) is 0.79 Å. The NMR ensemble nonetheless exhibits differences in certain detail. In Fig. 5A is shown the best fit superimposition between the crystal structure and the average structure of 40 NMR-derived models. The core regions, including the dimerization interface, are almost identical in their main-chain orientation and pattern of side-chain packing (Fig. 5B); main chain r.m.s. deviation in these regions (residues 361–381 and residues 389–398) is 0.48 Å. Differences between the solution structure and crystal structure are observed involving the N-terminal portion of helix \(\alpha_1\) and the C-terminal portion of \(\alpha_2\). Main-chain r.m.s. deviations for the N-terminal portion of \(\alpha_1\) (residues 354–360) and the C-terminus portion of \(\alpha_3\) (residues 399–408) are 1.14 and 0.86 Å, respectively. Systematic displacement is observed between corresponding side-chain positions (Fig. 5C), in part due to relative “sliding” of the two helices between the NMR-derived and crystal structures. Further structural differences are observed in the solvent-exposed linker region between \(\alpha_2\) and \(\alpha_3\) with main-chain r.m.s. deviation of 0.91 Å. It is possible that these differences are due to effects of crystal packing.

**Mechanism of IX Binding**—The mechanism of DSX\^F-IX binding was investigated by deletion analysis and Ala-scanning mutagenesis. Initial studies employed a Y2H screen developed by Baker and co-workers (12). The prey plasmid expressed the transcriptional activation domain of GAL4 fused to IX, whereas alternative bait plasmids were constructed expressing the DNA-binding domain of GAL4 fused to the DSX isoforms (Fig. 6A). In this system, IX interacts with DSX\^F but not DSX\^M, as indicated by viability on selective plates (top two rows in Fig. 6B). Such sex-specific binding is maintained on truncation of respective tails at residue 412 (DSX\^F-\Delta and DSX\^M-\Delta); bottom two rows in Fig. 6B). These observations suggest that (in the context of otherwise intact fusion proteins) the proximal portion of the female-specific tail is sufficient to direct IX binding, whereas the disordered distal tail is dispensable. Specificity of the Y2H system in the reverse direction was verified through the construction of eight Ala mutations in IX, introduced at Cys and His residues (which are often important for protein function, in some cases via formation of a metal ion-binding site). Whereas four of the variants (H94A, H95A, C123A, and H132A) retained sex-specific binding to DSXF, the other four variants (H102A, C109A, C110A, and H116A) did not bind to either DSX\^F or DSX\^M (data not shown). Control studies of protein expression levels by Western blot indicate that these substitutions do not impair expression of the IX fusion protein.

To extend this analysis to the milieu of a Drosophila cell, the well characterized S2 cell line was employed in a co-IP assay. Co-transfection of plasmids respectively expressing IX (linked to HA as a N-terminal epitope tag) and DSX\^F (linked to V5 as a C-terminal epitope tag) enabled analysis of nuclear extracts.
for assembly of complexes; IP by a polyclonal anti-HA antiserum was followed by immunoblotting with a monoclonal anti-V5 antiserum. V5-tagged variants of DSX are constructed containing successive C-terminal deletions (Fig. 6C). Results of Western blots are shown in Fig. 6D: detection of DSX proteins in IX-specific IPs (top box), direct detection of DSX proteins in the nuclear extracts (middle box), or direct detection of IX in the nuclear extracts (bottom box). As described by Baker and co-workers (12), binding of IX is observed to wild-type DSX (lane 9, top box) but not to DSXM (lane 3, top box). Formation of this female-specific complex in S2 nuclear extracts does not require specific DSXF-DNA binding, since an identical co-IP result is obtained when the inactive R91Q variant of DSXF is co-transfected with IX rather than wild-type DSX (data not shown). As expected, control co-IP reactions conducted in the absence of transfected DSX or DSXM (lane 1) or in the absence of transfected IX (lane 2) yield no detected co-IP signal.

The co-IP assay was employed to test a series of successive C-terminal deletions in DSX (residues 1–420 (Δ421–427), 1–412 (Δ413–427), 1–406 (Δ408–427), 1–397 (Δ398–427), and 1–349 (Δ350–427); Fig. 6C). Deletion of residues 421–427 and 413–427 does not lead to an attenuated co-IP signal (Fig. 6D, lanes 7 and 8, respectively), whereas deletion of residues 408–427 causes a 2-fold reduction in band intensity (lane 6). Further deletion abolishes detection of an IX complex (lanes 4 and 5); deletion of residues 350–427 removes CTD, whereas deletion of residues 398–427 removes the female-specific tail. The control blots in the middle box indicate that these deletion variants are expressed in S2 cells at least as well as full-length DSX.

To determine whether the above deletion fragments retain the ability to homodimerize (and thus present a structured binding surface to IX), we generated corresponding dele-
Figure 5. Structural comparison of NMR structure and crystal structure of CTD\(^{p,\Delta}\). A, best fit superposition of the crystal structure (Protein Data Bank accession code 1ZV1) and the average structure of the 40 NMR-derived models in the ensemble (Fig. 4A). The crystal structure is shown in red, and the mean NMR structure is shown in green. B, comparison of selected residues in major core. Side-chain positions are almost identical. C, side-chain reorganization in NMR structure relative to crystal structure near the N-terminal region of helix \(\alpha_3\) and C-terminal region of \(\alpha_2\). In B and C, the single crystallographic conformation of each side chain is shown in relation to the NMR ensemble. Although the ensemble is well converged, individual side chains differ in precision.

Although truncation of the C-terminal region at residue 412 (\(\Delta 413–427\)) does not affect dimerization, the additional deletions introduced perturbations in reporter gene expression. Truncation of the tail to residue 406 (\(\Delta 407–427\)) leads to >10-fold reduction in \(\beta\)-galactosidase activity; deletion of all female-specific residues (\(\Delta 398–427\)) completely abolished \(\beta\)-galactosidase activity (data not shown). These Y2H data suggest the possibility that the most extensive deletions impair (\(\Delta 407–427\)) or block (\(\Delta 398–427\)) DSXF-IX binding (Fig. 6D) by nonlocal destabilization of the CTD\(^{p}\) binding surface rather than by shaving of specific C-terminal contact residues.

Based on these results, we undertook Ala-scanning and charge reversal mutagenesis of helix \(\alpha_3\) in full-length DSXF to identify individual side chains in contact with IX as probed by the IX-DSXF\(^{p}\) Y2H. The following 10 substitutions were thus introduced: E389R, E390R, R393A, E396R, Q399A, Y400A, N403A, E404A, Y705A, and R407A. Of these substitutions, Y400A and N403A were observed to disrupt binding of IX. Further Y2H studies of CTD\(^{p}\) homodimerization indicated that neither substitution perturbs dimerization, suggesting that global folding of the mutant domains is maintained (data not shown). The Ala substitutions each block DSXF\(^{p}\) binding in the co-IP assays described above (Fig. 6E). These side chains are contiguous, exposed in a groove on each side of the dimer (red in Fig. 6F).

Mutagenesis of the Dimer Interface—Although the interface between UBA folds is predominantly hydrophobic, its periphery contains an extensive network of salt bridges and charge-stabilized hydrogen bonds. One cluster involves the side chains of Asp\(^{383}\), Glu\(^{390}\), Arg\(^{394}\), and their dimer-related mates (Fig. 7B); a second cluster involves Tyr\(^{378}\), Lys\(^{382}\), Glu\(^{397}\), and their dimer-related mates (supplemental material).\(^8\) The importance of these side chains has in part been demonstrated by Ala-scanning mutagenesis (8). The structural environment of Arg\(^{394}\) is of special interest in relation to its protected side-chain NH\(_2\) resonances. The positively charged guanidinium group is surrounded by the negative charges of Asp\(^{383}\) and Glu\(^{390}\) and near the partial negative charge (dipole) of the Asp\(^{383}\) main-chain carbonyl oxygen. The structure suggests that one NH\(_2\) group of Arg\(^{394}\) is engaged in a salt bridge (and possible hydrogen bond) with the side-chain carboxylate of Asp\(^{383}\), whereas the other NH\(_2\) group forms a hydrogen bond with the Asp\(^{383}\) main-chain carbonyl oxygen. A favorable \(i, i + 4\) electrostatic interaction between Arg\(^{394}\) and Glu\(^{390}\) may also contribute to the helical stability of \(\alpha_3\). In addition to this electrostatic network, the methylene side chain of Arg\(^{394}\) packs against the aliphatic side chains of Ile\(^{380}\), Ile\(^{395}\), and Val\(^{379}\); such close packing seals one face of the dimer interface and may limit the flexibility of the terminal guanidinium group, reducing the entropic cost of its engagements in an electrostatic network.

The structural role of Arg\(^{394}\) (Fig. 7, A and B) and its distinctive side-chain NH\(_2\) H NMR resonances motivated further studies by site-directed mutagenesis. Homodimerization of CTD\(^{p,\Delta}\) and variant domains was monitored by an Y2H system in which the bait and prey plasmids contained the same substitutions. Design of this system (originally described by Burtis and co-workers) (4, 8, 9) was validated by characteriza-

\(^8\) Primed residue numbers refer to dimer-related protomer, and primed symbols \(\alpha_3^\prime, \alpha_2^\prime, \text{ and } \alpha_1^\prime\) likewise refer to dimer-related helices.
in reporter gene expression (rather than extent of colony growth on a selective medium) to be measured, enhancing the power of the Y2H assay to compare effects of substitutions.

Y2H screening at 30 °C demonstrates that substitution R394K is well tolerated, whereas substitution by 10 other amino acids (Ala, Gly, Gln, Asn, Glu, Asp, Cys, Thr, Met, or Pro) impairs expression of β-galactosidase by at least 10-fold (Fig. 7C). These data indicate that a positive charge is required at this site but that the particular arrangement of hydrogen bond donors in the guanidinium group of Arg^{394} is not necessary to stabilize the dimer-related electrostatic network. Loss of function by an R394M analog further suggests that maintenance of nonpolar packing by the aliphatic portion of Arg^{394} is not in itself sufficient to stabilize the dimer. Similarly, loss of function is observed on substitutions R394N and R394Q, whose neutral carboxamide functions are nonetheless capable of accepting and donating hydrogen bonds.

Identification of Temperature-sensitive Mutants—Temperature-sensitive (ts) mutants of a protein can provide powerful tools for studying protein function in vivo (see “Discussion”). As a first step toward identifying such mutations in DSX, the Y2H system was utilized to test whether R394A CTD-Dα and other dimerization-defective Ala variants (P370A, P375A, Y378A, I380A, K382A, and I395A) (8) might regain Y2H activity at 20 °C. Bait and prey plasmids contained the same variant coding region, giving rise in each case to homodimers. Of this set of seven variant domains, only R394A scored as a potential ts mutation (Fig. 7E). Although at the higher temperature, expression of β-galactosidase was reduced by 17-fold, at the lower temperature, expression was reduced by only 2-fold. Thus, although dimerization remains partially impaired at 20 °C, its significant restoration implies that the Arg^{394}-associated electrostatic network augments the stability of the dimer rather than providing an essential contribution. By contrast, instability due to removal of
either proline (P370A or P375A), introduction of cavities (Y378A, I380A, or I395A), or loss of a positive charge (K382A) cannot be overcome by lowering the temperature to 20 °C. These results suggest that at most sites Ala substitution represents too severe a perturbation to allow conditional dimerization of CTD-p

Accordingly, to extend our search for candidate ts mutations beyond Ala and without bias, random PCR mutagenesis was employed to create a library of variants. This library was then screened by replica plating (using blue-white X-gal indicator plates) at permissive and nonpermissive temperatures (20 and 30 °C). We thus sought yeast colonies that were white at the higher temperature (indicating loss of Y2H-detectable dimerization) but blue at the lower temperature (indicating restoration of dimerization). This protocol avoided sequencing of background wild-type colonies. Screening of 150 white colonies at 30 °C led to identification of one that was blue at 20 °C; DNA sequencing revealed the substitution V379T. Because such Y2H screening employs one variant gene and one wild-type gene (thus probing temperature-dependent formation of a heterodimer), the V379T allele was recloned into both bait and prey plasmids to investigate the stability of the variant homodimer. At 20 °C, enzyme assays demonstrated that the homodimeric V379T Y2H colony expressed a level of β-galactosidase activity similar to that of a wild-type Y2H colony, consistent with the corresponding native blue colors of their colonies. By contrast, at 30 °C the expression of β-galactosidase in the mutant colony is reduced by 7-fold relative to a wild-type colony (Fig. 7E). Interestingly, Val379 packs against the aliphatic portion of Arg394 (Fig. 7B). Thus, random mutagenesis and structure-based screening have converged to the same region of the molecule.

To characterize further the importance of Val379, six additional substitutions were introduced at this site and tested as variant homodimers by Y2H: Ala, Gly, Glu, Phe, Leu, and Arg. Of these, only V379A and V379T variants exhibit partial activity at 20 or 30 °C. The other substitutions led to 10-fold reduction in β-galactosidase activity (Fig. 7D). These results suggest that a small hydrophobic side chain is required at Val379. Unlike the ts V379T variant, the V379A variant does not confer wild-type expression of β-galactosidase at 20 °C. Substitution of Val by Thr thus imposes a more subtle perturbation than substitution by Ala, presumably due to their effects of side-chain volume at a closely packed interface.

Temperature-dependent Transcriptional Regulation—Residues Val379 and Arg394 are invariant among insect dsx genes,
suggesting their biological importance. To test the gene-regulatory properties of DSX	extsuperscript{F} alleles bearing candidate ts mutations V379T or R394A, we exploited a Y1H system (41) designed to probe specific DSX-DNA recognition. This system employs a model operon regulated by the Drosophila fat body enhancer (fbe) (45). The molecular logic of this system (illustrated in Fig. 8A) probes specific DNA binding but not other aspects of sex-specific transcriptional regulation in Drosophila.

Expression of β-galactosidase in the Y1H system reflects binding of DSX	extsuperscript{F} fusion proteins to specific target sites (dsxA and dsxB in the Drosophila fbe); DSX-regulated expression is appropriately blocked by either intersexual mutations in the DM DNA-binding domain or nucleotide substitutions in the target site that impair such binding in vitro (41). Limiting values of β-galactosidase expression are provided by wild-type DSX	extsuperscript{F} (highest; left-hand side of Fig. 8B) and its inactive R91Q variant (lowest; right-hand side of Fig. 8B). In the Y1H system, a single DSX	extsuperscript{F} fusion protein is expressed, restricting this assay to homodimers.

An intermediate level of expression is observed on impairment of dimerization by the intersexual G398D mutation (Figs. 7E and 8B). This mutation, which leads to complete loss of Y2H-detectable dimerization (4, 8), leaves intact the N-terminal DNA-binding domain of DSX (the DM motif) (6, 11). The isolated DM domain is itself capable of dimer-specific DNA recognition, albeit with lower affinity (6, 10). Deletion of CTDF	extsuperscript{F} leads to a similar intermediate level of expression due to partial binding of the enhancer elements by the DM-containing fusion protein (data not shown). Comparison of Y1H expression levels at 20 and 30 °C demonstrates that at the lower (permissive) temperature, the V379T and R394A fusion proteins direct levels of reporter gene expression similar to that of the wild-type fusion protein, whereas at the higher (nonpermissive) temperature, the mutations impair reporter gene expression to a level similar to that of the G398D control allele at 20 °C (Fig. 8B). Since at this temperature, the G398D dsxA allele in female flies is without biological activity (4), our results suggest that at 30 °C, the variant V379T and R394A dsxA alleles would likewise be inactive in vivo.

**DISCUSSION**

The DSX transcription factors (2) control a major branch of the sex-determining hierarchy of D. melanogaster (1, 42). The dsx gene encodes sex-specific protein isoforms due to sex-specific RNA splicing (2). We have sought to determine the structure of CTDF	extsuperscript{F} as a foundation to elucidate molecular mechanisms of sex-specific gene regulation. The CTDs mediate dimerization and (in the case of DSX	extsuperscript{F}) sex-specific interactions with transcriptional co-activator Intersex (IX). Our overarching goal is to exploit protein structure to enable genetic analysis of development.

The DSX isoforms control most aspects of somatic sexual development and differentiation in flies (1, 46, 47); related proteins function in sex determination in many animals, including C. elegans and humans (11, 48–50). Although DSX	extsuperscript{F} and DSX	extsuperscript{M} have opposing biological functions (13, 51–53), they exhibit similar DNA-binding properties (42, 45, 54). DSX	extsuperscript{F} (but not DSX	extsuperscript{M}) interacts with transcriptional co-activator Intersex (IX), presumably leading to assembly of a female-specific multiprotein-DNA complex at target genes (12, 55). The integration of sex-specific and positional signals during development is achieved, at least in part, by dsx and homeotic genes jointly acting through a common set of cell-cell signaling cascades and transcription factors (53, 56–60).

**NMR Studies of CTDF**—The solution structure of the ordered portion of CTDF	extsuperscript{F}-p is essentially identical to that observed in the crystal structure of CTDF	extsuperscript{F}-pA. This structure has an ellipsoidal shape with head-to-tail orientation; the distal half of the female tail is disordered. Each protomer forms a compact three-helix bundle connected by two well ordered short loops and/or turns, resembling a UBA fold (8). Interhelical angles within this fold are consistent with those of the UBA1 and UBA2 domains in the human homolog of yeast Rad23A (HHR23A) (61, 62). Variation is nonetheless observed in the length of helical segments relative to canonical UBA domains: α	extsubscript{1} comprises residues 352–366 (13 residues), α	extsubscript{2} helix from 371–383 (13 residues), and α	extsubscript{3} from 388–409 (22 residues), thus including female-specific sequences 398–409. It is not known whether the resemblance between CTDF	extsuperscript{F} and the UBA fold is incidental or implies a role for ubiquitination in regulation of sexual differentiation.
Structure and Function of Doublesex

The present study illustrates the complementary utility of NMR and x-ray crystallography. In the crystal structure, CTD\(^{D}\)-p\(_A\) exhibited both a major subunit interface and a less extensive lattice contact involving an unrelated surface. Interpretation of the major contact as the actual site of dimerization in solution was inferred based on buried surface area and supported by mutagenesis in an Y2H system (8). The present NMR analysis of dimer-related NOEs by asymmetric isotope labeling explicitly verifies this interpretation. Further, although the positions of almost all side chains in UBA folds are well defined in the crystal structure of CTD-P\(_A\) (irrespective of whether in the core or on the protein surface), NMR provides a distinction between flexible and well ordered surface side chains. For the latter, the precision of the solution structure, made possible by three-/four-dimensional filtering of NOEs, enables stably maintained side chain interactions to be inferred.

The present NMR analysis has identified key NOEs and secondary chemical shifts that are diagnostic of the distinctive structural features of CTD\(^{D}\). These features are likely to provide a valuable foundation for comparative future studies of variant domains. An example is provided by prominent interprotonic NOEs observed between the Pro\(^{375}\) pyrroldine ring and the \(\gamma_2\) methyl group of Val\(^{402}\), a signature of an unusual kink in helix \(\alpha_2\). The Pro\(^{375}\)-associated kink appears to optimize a helix-helix contact at the dimer interface (8). The exchangeable \(^{1}H\) NMR resonances of Arg\(^{394}\) are also of special interest. Their anomalous prominence (analogous to the NMR features of Arg side chain immobilized at a peptide-RNA interface) (63, 64) suggests that C-NH\(_2\) bond rotation and solvent exchange are hindered. The guanidinium NH\(_2\) groups thus appear to be involved in charge-stabilized hydrogen bonding. Although the identity of such interactions is not well defined in the crystal structure, evidence for such interactions in solution is provided by three-dimensional \(^{15}N\)- and \(^{13}C\)-edited NOESY spectra. Despite these intriguing spectroscopic features, Y2H analysis of dimerization indicates that Arg\(^{394}\) may functionally be replaced at 30 °C by Lys. Thus, whereas a positive charge at this site stabilizes the dimer-specific electrostatic network, the geometric precision in principle made possible by bidentate guanidinium-related hydrogen bonds is not necessary for stability.

Function of the Female-specific Tail—The NMR structure of CTD\(^{D}\)-p\(_A\) demonstrates that the proximal portion of the female-specific tail extends helix \(\alpha_5\) of the UBA motif, whereas the distal portion is disordered. The structure and function of this tail are of central biological importance in relation to the sex-specific regulatory properties of the female isoform. We have investigated the role of the tail in the binding of IX, an obligatory partner protein of DSXM and presumed transcriptional co-activator. In XX:AA flies (chromosomal females), null alleles of either the \(dsx\) or \(ix\) genes give rise to indistinguishable intersexual phenotypes (13, 14). Although IX is expressed in both male and females, its binding to DSXM\(^{F}\) is weak or absent, and null \(ix\) alleles have no phenotype in males (15). Intersex homologs are conserved in mammals as a component of the Mediator co-activator complex (17).

We have utilized two assays of DSXM\(^{F}\)-IX recognition: a Y2H system and co-IP of their complex from nuclear extracts, obtained from transfected Drosophila S2 cells. Originally developed by Baker and co-workers (12), these assays demonstrate specific binding of IX to DSXM\(^{F}\) rather than DSXM\(^{A}\), thus providing a biochemical basis for the classical sex-specific genetic interaction between \(dsx\) and \(ix\) in female flies. The fidelity of the Y2H system was verified herein by its sensitivity to point mutations in either DSXM\(^{F}\) or IX. Surprisingly, the C-terminal portion of the female-specific tail can be deleted without hindering DSXM\(^{F}\)-IX recognition. Ala-scanning mutagenesis of the proximal tail highlights the importance of Tyr\(^{400}\) and Asn\(^{403}\) in the female-specific extension of helix \(\alpha_5\). By contrast, charge reversal mutagenesis in the non-sex-specific portion of this helix has no effect on IX binding. Because Ala scanning can underestimate the size of contact surfaces between proteins (65), however, we anticipate that the DSXM\(^{F}\)-IX complex may contain additional contacts, either in the proximal tail or spanning a non-sex-specific protein surface elsewhere.

Determining the structural mechanism of sex-specific IX recruitment, a model of a genetic switch in a program of sexual differentiation, poses an important future problem. The proximal portion of the male-specific tail (ARVEINRTV; residues 358–406) differs in sequence from that of DSXM\(^{F}\) (sequence GQYVVVEYS). In particular, Tyr\(^{400}\) in DSXM\(^{F}\) is substituted by Val in DSXM\(^{F}\) (in boldface type above). Although Asn\(^{403}\) (underlined) is shared by the two isoforms, it is not known whether its respective structural contexts are similar; to our knowledge, how these male-specific sequences extend the cognate UBA folds in DSXM\(^{F}\) has not been addressed. Because the structure of CTD\(^{M}\) may exhibit nonlocal interactions between the core dimerization domain and its substantial male-specific extension (residues 398–547), the attenuated binding of IX to DSXM\(^{F}\) does not in itself exclude a potential contribution of non-sex-specific residues in DSXM\(^{F}\) to its overall IX-binding surface.

Applications to Molecular Genetics—\(dsx\) is representative of a class of genes that not only specify aspects of the body plan but also influence behavior (66, 67). Genetic studies have shown that \(dsx\) is important for female sexual behavior and has a minor role in male sexual behaviors (14, 68). Studies of female sexual behavior are presently limited by the absence of \(ts\) alleles of \(dsx\). The power and utility of a \(ts\) allele in the sex-determining hierarchy of \(D.\) melagonaster were first demonstrated by Belote and Baker (69) in behavioral studies of XX flies containing one \(tra-2\) allele and deleted for the other allele (XX; \(tra-2^{+}\) \(Df(2R)trix\)). The removal of \(tra-2\) function at the restrictive temperature resulted in a shift in the pattern of sex-specific splicing of \(dsx\) and \(fruitless\) from female to male. Temperature shifts (from 29 to 16 °C) were imposed at different stages of development. These experiments highlighted the importance of a critical time in the middle of the pupal period for development of the male behavioral program.

The above results were confirmed and extended by Nöthiger and co-workers (70) using a heat shock-inducible transgene to

Chromosomal female (XX) flies expressing DSXM\(^{M}\) but not DSXM\(^{F}\), although male in external appearance, do not court wild-type females (68). Chromosomal male \(dsx\) \(^{F}\) flies ectopically expressing DSXM\(^{F}\) (in addition to endogenous DSXM\(^{A}\)) exhibit reduced male courtship behavior, generate feminine sex appeal, are actively courted by other males, and will copulate with males (albeit with increased rejection behavior) (14), indicating that female sexual behavior is dependent on \(dsx\)。“
express the female-specific isoform of TRA (TRA F) in tra -/- XX and XY flies. The two studies disagree, however, with respect to the functional plasticity of the adult central nervous system. A shift of adult XX; tra-2+/Df(2R)trix flies from permissive to restrictive temperatures in the studies of Belote and Baker led in some flies to a change in courtship behavior from female to male after 6–10 days (69). This seemed to imply that operation of the SDH is continuously required to maintain female-specific behaviors in the adult; neural circuitry can be rewired in the adult brain to switch to male courtship behavior. In contrast, Nöthiger and co-workers (70) found that sex-specific behaviors are “hard wired” during the critical period in pupation; heat-shocked flies, when returned to low temperature, never regained male behavior. The reasons for this discrepancy are unclear but may be related to the different genetic backgrounds used.

An important limitation of the above behavioral studies arises from the position of tra and tra-2 genes in the sex-determining hierarchy; shifts in temperature simultaneously affect the sex-specific splicing patterns of dsx, fruitless, and potentially other possible targets of tra/tra-2 regulation. The contributions of individual genes were thus not resolved. In particular, when a temperature shift is implemented to turn on/off dsx F, one is simultaneously doing the opposite to dsx M. A similar difficulty arises with respect to fru M when tra-2 F is employed to study the role of dsx F; whenever one turns off/on dsx F with a temperature shift, one is also turning on/off fru M. We envisage that these limitations may be overcome through the use of a designed ts allele of dsx.

The present study has provided a first step toward the identification and characterization of ts mutations in DSX. A Y2H system probing the dimerization of CTD-pA has been to screen a collection of Ala scanning mutations. Of seven variants tested at 20 and 30 °C, one (R394A) was found to exhibit impaired dimerization at the higher temperature and restored dimerization at the lower temperature. Such restoration implies that a positive charge at position 394 functions to augment the stability of the dimer at higher temperature (see above) but is not integral to the overall motif. This screening strategy was extended by random mutagenesis to identify a second candidate ts mutation (V379T). Interestingly, the two side chains are in contact across the dimer interface. Whereas Val and Thr have similar sizes and shapes, they differ in polarity. Subtle perturbation of the dimer interface by the polar β-OH group of V379T is sufficient to confer its temperature-dependent assembly. Because the dimerization of CTD F contributes to the strength of specific DNA binding (54), these substitutions would in turn be expected to confer temperature-dependent changes in enhancer recognition. This prediction is in accord with Y1H studies of wild-type and variant DSX F fusion proteins in a system designed to recapitulate DSX-DNA recognition in a well-characterized Drosophila enhancer element (41). It would be of future interest to introduce these mutations in the dsx locus of D. melanogaster by homologous recombination (71).

Temperature-dependent function of variant dsx alleles in vivo would provide novel probes of a sex-specific transcriptional network with application to the neurogenetic basis of female courtship behavior.

Conclusion—The present study has extended our prior cryo-
tallographic analysis of a dimeric fragment of DSX F (CTD F-pA) to a C-terminal domain containing the complete female-specific tail (CTD F-p). Because the tail domain was refractory to crystallization, heteronuclear NMR methods were employed to obtain a solution structure. The proximal female-specific sequences contribute to the UBA fold, its dimer interface, and IX-binding surface. The remainder of the tail is disordered in the free domain and, although not required for IX binding, may fold upon binding other factors in a female-specific transcriptional preinitiation complex. The DSX DM domain similarly contains a disordered segment C-terminal to its zinc module; this segment folds on specific DNA binding as a recognition α-helix (6, 10). DSX thus contains a modular organization of folded and unfolded elements. Order-disorder transitions on macromolecular assembly are a general feature of eukaryotic transcriptional regulation.

The structure of DSX not only rationalizes aspects of its biochemical function in sex-specific gene expression but may also enable development of novel genetic reagents. Because of its role at a point of bifurcation in the sex-determining hierarchy of Drosophila, temperature-sensitive alleles of dsx would be of biological interest as probes of its contribution to sex-specific developmental processes, including the specification of female courtship behavior by specific circuitry in the central nervous system (66, 67). Structure-based design of variant alleles in genetic model organisms offers a promising approach toward integrating molecular and systems level understandings of metazoan development.

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