DNA Binding by the Male and Female Doublesex Proteins of *Drosophila melanogaster*

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Yolk protein genes are regulated by doublesex male protein (DSXM) in males and doublesex female protein (DSXF) in females. Both proteins bind to the same DNA sites from which DSXM represses and DSXF activates transcription. The proteins are identical through 397 NH2-terminal amino acids that include doublesex protein; DSXM, male doublesex protein; bp, base pair(s).

The proteins differ because the male-specific DSXM and female-specific DSXF proteins encode by the doublesex (dsx) gene have opposite effects that together yield essentially all or no female-specific transcript. DSXM represses and DSXF activates transcription from the same binding sites in Yp DNA. This regulation is synergistic because a single binding site yields 5-fold activation by DSXF and 4-fold repression by DSXM, whereas multiple sites in Yp DNA yield very large DSX-dependent effects (1–4). Second, DSX has a function quite different from sex specificity. It regulates tissue specificity of Yp transcription (3). This regulation also is synergistic, but synergism is between DSXF and a protein not encoded by the dsx gene. Two overlapping binding sites occur in Yp DNA: dsxA, which binds DSX protein; and bzip1, which binds an unidentified bZIP protein related to C/EBP. When the bZIP protein binds bzip1, transcription is activated in a few somatic tissues of the ovary and in no other adult tissues. When DSX binds to dsxA, no transcriptional activation is observed in any adult tissue. However, when both bZIP and DSXF bind, transcription is strongly activated in fat bodies, the normal tissue of Yp transcription, and in no other tissue, including ovarian tissues.

To provide a base for understanding the mechanisms of these several transcriptional functions, we have been investigating the physical properties of DSX proteins. Previous investigations show that DSXM and DSXF are identical over their first 397 amino acids, which include a DNA binding domain (5, 6). The proteins differ because the dsx transcript is spliced differently in males and females. This alternative splicing produces only one difference between the two proteins, a 152-amino acid carboxyl terminus in DSXM and a completely different 30-amino acid carboxyl terminus in DSXF. DSX proteins have two protein oligomerization domains: one co-localized with the DNA binding domain, and the other localized at the splice boundary and requiring both sex-specific and sex-nonspecific sequences for the oligomerization activity (7). Both proteins have highly extended shapes, forming dimers at low protein concentrations and higher order oligomers at higher protein concentrations (8). Although the equilibria between protein oligomers and DNA are complex, the fundamental DNA binding species of each protein is a dimer (8).

In this paper we investigate the DNA binding properties of purified DSXM and DSXF dimers. We find that DSXM and DSXF affinities for short DNA oligomers are indistinguishable whether those oligomers contain a specific tight binding site or nonspecific sites. The association and dissociation rates as well as the dependence on monovalent and divalent cations are also indistinguishable between the male and female proteins. These results indicate that the region common to the two proteins is the predominant determinant when dimers bind to individual DNA sites. We infer that the sex-specific oligomerization domain is likely to play roles in binding cooperativity when the proteins bind to multiple DNA sites or when DSX proteins interact with different regulatory proteins, for example, the tissue specifying interaction with the bZIP protein mentioned above. In this investigation we also observed that the dimer-DNA complex dissociation has a first order dependence upon free DNA concentration. We infer that direct transfer between DNAs occurs when DSX proteins are searching for specific sites in the many short open DNA regions of chromatin.

Analysis of yolk protein (Yp) gene regulation has shown that the doublesex proteins (DSXM and DSXF) of *Drosophila melanogaster* have several functions in transcriptional regulation. First, the male-specific DSXM and female-specific DSXF proteins encoded by the doublesex (dsx) gene have opposite effects that together yield essentially all or no female-specific transcript. This alternative splicing produces only one difference between the two proteins, a 152-amino acid carboxyl terminus in DSXM and a completely different 30-amino acid carboxyl terminus in DSXF. DSX proteins have two protein oligomerization domains: one co-localized with the DNA binding domain, and the other localized at the splice boundary and requiring both sex-specific and sex-nonspecific sequences for the oligomerization activity (7). Both proteins have highly extended shapes, forming dimers at low protein concentrations and higher order oligomers at higher protein concentrations (8). Although the equilibria between protein oligomers and DNA are complex, the fundamental DNA binding species of each protein is a dimer (8).

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**MATERIALS AND METHODS**

**DNAs**—The dsxA binding site (shown in bold face type) of the Yp1 gene was made by annealing DNA oligonucleotides 5’-TCGACAACATCTAAATGTGCAATCGGAC-3’ and 5’-TCGAGGCTAGGCTGATTGACCATATGATTGG-3’. Both the synthetic oligonucleotides and annealed product were purified by polyacrylamide gel electrophoresis (9). Binding competition studies assayed by gel mobility shifts demonstrated that the annealed product and the natural dsxA site bound DSX protein with the same affinity. The nonspecific DNA (upper strand sequence: 5’-GGTGCCAGTTGGATCTTAAATTACC-3’) was prepared in the same way. DNA concentrations were determined by absorption at 260 nm (1.0 OD = 50 μg/ml). For DNA binding assays, DNAs were 32P-labeled using radioactive precursors and either T4 polynucleotide kinase or Klenow fragment (9). Quick spin columns (Boehringer Mannheim Corp.) were used to remove unincorporated nucleotides under conditions recommended by the manufacturer. The specific radioactivity of DNA was determined by PhosphorImager (Molecular Dynamics, Inc.) after gel electrophoresis. Poly(dC)·poly(dC) was from Boehringer Mannheim.

**Proteins**—DSX<sub>3</sub> and DSX<sub>2</sub> proteins were purified from baculovirus-infected Sf9 cells and stored at −70 °C (8). At least 70% of the DSX protein molecules in these preparations were active when measured by the mobility shift binding assay under stoichiometric conditions. Throughout this paper the molarity of the DSX protein was calculated using the molecular weight of the dimer and the fraction of active protein.

Equilibrium Binding Assays—Unless otherwise specified, equilibrium DNA binding by the DSX protein was at room temperature in a final volume of 20 μl of DNA binding buffer (25 mM Hepes (pH 7.6), 0.1 M NaCl, 1 mM dithiothreitol, 10% glycerol, 0.1 mM EDTA, and 100 μg/ml bovine serum albumin), 32P-labeled DNA, and freshly diluted DSX proteins. After a 1-h incubation (gel binding assays showed that equilibrium was reached by 1 h when dsxA was at 0.1 nM and the DSX protein was at either 0.1 nM or 1.0 nM), samples were loaded at 300 V onto mobility shift assay gels. 16 × 16-cm slabs of 4% polyacrylamide gel (29.1, acrylamide/ bisacrylamide) containing 0.5 × TBE (90 mM Tris·HCl (pH 8.3), 90 mM boric acid, 2 mM EDTA) (9). To obtain predominantly dimers in these reactions it was necessary to adjust the protein concentration with care because the dissociation constants for dimer-DNA complexes in these reactions it was necessary to adjust the protein concentration. The time required for the sample to enter the gel, approximated, was 4.5 min, was added to the dissociation time.

Association Rate Determination—DSX<sub>M</sub> (0.22 nM) or DSX<sub>2</sub> (0.16 nM) was incubated with 0.025 nM 32P-labeled dsxA in a DNA binding buffer (200 μl) at room temperature. At the indicated times unlabeled dsxA DNA was added to 20-μl aliquots of the reaction to yield a final concentration of 12 nM dsxA to stop the association reaction. Aliquots were mixed, incubated for 1 min at room temperature, and assayed by mobility shifts. A linear regression method was used to determine dissociation rates from a minimum of 6 data points on a plot of ln([P]/[D])/t versus t. The time required for the sample to enter the gel, approximated, was 4.5 min, was added to the dissociation time.

\[
[PD] = [PD]_{0}e^{-k_{d}t}
\]

where the dissociation rate constant \(k_{d} = 8.6 \times 10^{-4} \text{s}^{-1}\) is from Fig. 4A. Data were plotted according to a standard second order rate equation (10).

**RESULTS**

**Equilibrium Binding to Specific and Nonspecific DNAs by Dimers of DSXM and DSXF**—Purified DSXM and DSXF are predominantly dimeric at low concentrations (0.2–2 nM) and tetrameric at higher concentrations (≥20 nM) (8). Although cross-linking studies have shown that both dimers and tetramers bind DNA, assays of equilibrium binding showed that tetramers dissociate into DNA-bound dimers as the DNA concentration is increased, presumably as a second DNA binds to the DSX tetramer (8).

To move the study of DSX binding to a more quantitative level we measured equilibrium binding between DSX protein dimers and DNA using a protein concentration that produced free dimers, dimer-DNA complexes, and no other detectable form of DSX protein. Purified protein was incubated with different concentrations of a radiolabeled DNA (24 bp) that contains dsxA, a specific binding site for DSX proteins. After binding equilibrium was reached (see “Materials and Methods”), the bound and free DNAs were separated by mobility shift assays and quantitated. The apparent equilibrium binding constants were determined by a Scatchard analysis of the data showing that DSXM<sup>2</sup> and DSXF<sup>2</sup> have very similar specific DNA binding constants.

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and the DSX F concentration was varied as indicated (molarity expressed in dimers). The reactions were incubated for 1 h at room temperature before mobility shift assays were done. for binding by DSX the equilibrium between DSX dimers and DNA sites than for dsxA. Since the two selectivity tests give different results, the equilibrium between DSX dimers and tetramers because half of the DNA was bound.

The MgCl2 titration curves showed that the protein/DNA interaction is strongest at 2 mM and declines gradually at higher MgCl2 concentrations for both proteins (Fig. 3B). This contrasts dramatically with other DNA-binding proteins and particularly with the well studied binding between 
repres- sor and its operator, which changes more than 100-fold be-
tween 3 and 10 mM (13). In summary, the results indicate the following: that ionic interactions between dsxA and the male and female proteins are indistinguishable, that the divalent magnesium cation is a surprisingly weak competitor for binding to DNA, and that approximately two monovalent ions are displaced upon binding from the DNA.

DSX Protein-DNA Dissociation Rate Depends on the Concentration of Specific and Nonspecific DNA—The rate of DSXF dissociation from dsxA was measured as the disappearance of preformed DSXF-[32P]-labeled dsxA complex after a large excess of unlabeled dsxA was added. Under these conditions almost all DSXF subsequently released from the complex binds to unlabeled DNA rather than labeled DNA. We measured the dissociation rate at various concentrations of excess unlabeled dsxA DNA to verify that the unlabeled DNA acts only as a binding sink. The dissociation rate, however, increased linearly with the concentration of unlabeled dsxA (Fig. 4A), indicating that

**Fig. 2. Selectivity of DSX binding to specific and nonspecific DNA.** A, DSXF binding to [32P]-labeled specific (dsxA, 24 bp; filled triangles) and nonspecific (25 bp; open triangles) oligomer DNA. DNAs were at 50 nM, and the DSXF concentration was varied as indicated (molarity expressed in dimers). The reactions were incubated for 1 h at 37 °C before mobility shift assays were done. B, binding competition between specific DNA (dsxA; S) and nonspecific homopolymer DNA (poly(dI-dC); NS). DSXF (open circles; 0.18 nM) or DSXN (filled circles; 0.23 nM) was incubated with 0.12 nM [32P]-labeled dsxA DNA and the indicated concentrations of poly(dI-dC)poly(dI-dC) (molarity of nonspecific sites is expressed in nucleotides because each nucleotide with an average length 1-kilobase polymer represents a different site).

In the second selectivity test, one not involving complications of the equilibrium between DSX dimers and tetramers because protein concentration was so low, the concentrations of protein and radiolabeled dsxA were kept constant and the concentration of a nonspecific poly(dI-dC)poly(dI-dC) competitor was varied (Fig. 2B). The \( K_{app} \) for a nonspecific poly(dI-dC) site was \( 1.2 \times 10^{-6} \) fold less than for the dsxA site and 100-fold less than for the 25-bp nonspecific DNA. Thus, both selectivity tests show DSXF has a substantially lower affinity for nonspecific DNA sites than for dsxA. Since the two selectivity tests give different results, the equilibrium between DSXF dimers and tetramers may have an effect on the DNA binding equilibrium although the unusual structure of the copolymer may be a more likely explanation. Fig. 2B also shows that both DSXN and DSXF bind poly(dI-dC)poly(dI-dC) with the same affinity. Based on the apparent affinities for specific and nonspecific DNAs, we infer that DSXN and DSXF bind DNA in very similar, perhaps identical, manners.

**Salt Effects on DSX Protein Interaction with DNA**—To further investigate the DNA binding characteristics of these proteins, the effects of NaCl and MgCl2 were examined (12). DSX proteins were incubated with [32P]-labeled dsxA at different salt concentrations. The NaCl titration curves for binding by DSXM and DSXF in the absence of MgCl2 were indistinguishable, having maxima at 100 mM and decreasing sharply at higher and lower salt concentrations (Fig. 3A). The apparent number of monovalent ions displaced upon protein binding was calculated from the slope of \( -\log K_{app} \) versus \( -\log [\text{NaCl}] \) in the range 0.1–0.5 M NaCl. B, MgCl2 effect on binding. 1 nM DSXF (open circles) or DSXN (filled circles) was incubated with 0.23 nM [32P]-labeled dsxA DNA at 0.1 M NaCl and the indicated concentrations of MgCl2. The results are plotted as a percent of the concentration of protein-DNA complexes at 2 mM MgCl2.
Therefore the reaction pathways can be described as follows, specific or nonspecific ligand through a ternary intermediate. The other two are by direct transfer mechanisms in which the DSX protein transfers from one specific ligand to either a specific or nonspecific sites in the nonspecific DNA duplex (see above).

To interpret these observations, we made the following assumptions. First, the few nonspecific sites in the specific dsxA-competitor, panel A, and a 25-bp nonspecific competitor (see under "Materials and Methods"), panel B, are shown. In panel B the dissociation rates were measured after adding unlabeled dsxA DNA (12 nM; thereby increasing $k_{o,1}$ for all data points by $0.86 \times 10^{-3}$ s$^{-1}$, see panel A) and the indicated quantity of nonspecific DNA sites. The concentration of nonspecific sites is expressed as the molarity of the 25 nonspecific sites/nonspecific DNA helix.

unlabeled DNA not only acts as a binding sink, but also participates in the dissociation mechanism (15). We also observed that the dissociation rate was linearly dependent on the concentration of nonspecific DNA, although with a $10^4$-fold lower slope (Fig. 4B). This slope difference corresponds to the $10^4$-fold lower affinity of DSX$^+$ for the nonspecific competition observed above.

To interpret these observations, we made the following assumptions. First, the few nonspecific sites in the specific dsxA-containing duplex can be ignored because their affinity is so much lower than dsxA. Second, there are approximately 25 nonspecific sites in the nonspecific DNA duplex (see above). Third, the protein-DNA complex has three pathways for dissociation. One pathway is an intrinsic dissociation that is independent of the concentration of added excess unlabeled DNA. The other two are by direct transfer mechanisms in which the DSX protein transfers from one specific ligand to either a specific or nonspecific ligand through a ternary intermediate. Therefore the reaction pathways can be described as follows,

$$P_2 + D^* \underset{k_0}{\overset{k_{o,1}}{\rightleftharpoons}} P_2D^* \overset{k_2[D]}{\longrightarrow} P_2D + D^* \overset{k_4[N]}{\longrightarrow} P_2N + D^*$$

REACTION 1

where $P_2$ is the DSX protein dimer, $D^*$ is the $^{32}$P-labeled specific DNA, and D and N are the unlabeled specific and nonspecific DNAs, respectively, used to quench reassociation with radiolabeled DNA. In this reaction model, $k_0$ is the intrinsic dissociation rate constant and $k_1$ and $k_2$ are the intermolecular transfer rate constants. The rate equation for this is: $k_{off} = k_0 + k_1[D] + k_2[N]$, where both [D] and [N] are considered constants because they are in at least 50-fold molar excess over the protein.

To apply this equation to the data in Fig. 4, we obtained values for $k_0$ ($5.1 \times 10^{-4}$ s$^{-1}$), $k_1$ ($2.4 \times 10^3$ M$^{-1}$ s$^{-1}$), and $k_2$ ($2.4 \times 10^1$ M$^{-1}$ s$^{-1}$). The difference in the first order dissociation rate constants for the specific and nonspecific interactions corresponds well to the difference between the apparent equilibrium constants for specific and nonspecific DNAs, indicating that the dissociation rate is likely to be the only difference between specific and nonspecific binding. We also measured dissociation rates of the DSXM dimer-dsxA DNA, obtaining results that were indistinguishable from those of DSXF (data not shown), a further indication that the DNA binding interactions of the two proteins are indistinguishable under these conditions.

**Association Rates**—The rates of DSXM and DSXF binding to DNA were determined from the initial association with dsxA as measured by the mobility shift assay (Fig. 5). Identical association rate constants of $4.6 \times 10^6$ M$^{-1}$ s$^{-1}$ were calculated for each protein using an equation derived from a simple bimolecular reaction model (10). This association rate appears to be two orders of magnitude slower than the diffusion limit (16–18). Note that the short DNA ligand used in this experiment does not permit rate acceleration by sliding and intersegment transfer mechanisms within the DNA molecule (16). The equilibrium dissociation constant of 0.11 nM calculated from this association rate constant and the intrinsic dissociation rate constant $5.1 \times 10^{-3}$ s$^{-1}$ (Fig. 4) agrees well with the apparent equilibrium dissociation constants of 0.19 nM and 0.18 nM (Fig. 1).

**DISCUSSION**

We have described DNA binding properties of purified DSXM$^+$ and DSXF$^+$ protein dimers. The results indicate that the full-length proteins are indistinguishable in binding to dsxA, a specific DNA regulatory site. The indistinguishable properties we investigated are the apparent equilibrium constants for dsxA, the influence of monovalent and divalent cations on these equilibria, and the DNA association and dissociation rate constants, as well as the first order dependence of those dissociation rate constants on the concentration of specific and nonspecific DNAs. DSXM$^+$ and DSXF$^+$ binding to nonspecific DNAs is also indistinguishable and is at least $1.5 \times 10^4$-fold weaker...
than specific binding. These results for specific and nonspecific DNA binding are consistent with previous reports that both male and female proteins bind the dsxA site in vivo and regulate Yp genes from that site in a sex-specific manner. The results are also consistent with the localization of the DNA binding domain to amino acids 39–104, a region identical between the two full-length DSX proteins.

Sex-specific Regulation by DSX Proteins—The DNA binding results reported in this paper suggest that the sex-specific regulatory functions of male and female proteins are unrelated to DNA binding. Thus, repression by DSX<sup>X</sup> and activation by DSX<sup>Y</sup> appear unrelated to binding to dsxA and that binding may only serve to localize the proteins to a useful place in the Yp gene. This speculation is strengthened by the observation that transcriptional regulators commonly have independently operating domains, one for DNA binding and one for transcriptional activation. It is further strengthened by the observation that the amino-terminal DNA binding domain of the DSX proteins are well separated from their sex-specific carboxyl termini when traced along their amino acid backbones. This separation also is likely to occur in the three-dimensional structures of the proteins because they are highly asymmetric. However, weighing against this possibility of functional separation is the observation that the sex-specific regions contribute substantially to dimerization of the DSX proteins. For this reason, the sex-specific regions may well have more subtle effects on DNA binding not dealt with in the current study. For example, there may be thermodynamic linkage between dimerization and DNA binding.

Implication of the First Order Dependence of Dissociation Rates on DNA Concentration—Dissociation rates of DSX protein-DNA complexes were first order with respect to the concentration of free DNA. This indicates that DSX proteins can transfer directly from one DNA site to another site through a ternary transition state. The dsxA and nonspecific DNAs were both found to facilitate direct transfer, although in keeping with its 10<sup>4</sup>-fold lower affinity, nonspecific DNA had a correspondingly lower rate effect.

The dissociation rate measurements suggest a mechanism by which DSX proteins search for their binding sites in vivo and may also indicate why multiple dsx binding sites are found in the Yp gene, the only regulatory region so far where DSX proteins have been demonstrated to function. Like all other transcriptional regulatory proteins of higher organisms, DSX proteins must find their specific binding sites within the very large mass of DNA in chromatin. This general searching problem has been divided into several theoretical steps: loose binding and hopping in a local, solvent-exposed DNA helical region; binding to a nonspecific DNA site within that region; and then sliding along the exposed helix until a specific site is encountered, transferring to another exposed DNA region or dissociating into solution (16, 20). More compact and chromatized DNA, like that usually found in eukaryotic chromosomes in contrast to prokaryotic chromatin, is likely to make the transfer between exposed DNA regions kinetically more important when proteins search for a specific binding site than sliding within the short lengths of exposed DNA ligands (16). For this reason, the transfer rates between helices where sliding is suppressed experimentally by using short DNA, as in the experiments we report here, are likely to be a useful property to measure for eukaryotic transcriptional regulatory proteins. The direct transfer mechanism also has been proposed for other eukaryotic transcription factors, for example, the HeLa upstream stimulatory factor and 5 S gene-specific RNA polymerase III transcription factor A (21, 22).

Since the DSX proteins can transfer from a specific site to a nonspecific site, the transfer rate may need to be suppressed if DNA binding is to lead to regulatory effects. This might be accomplished by cooperative protein-protein interaction between proteins bound in the same DNA region thereby increasing the stability of the bound complex. If there were only one dsx binding site, the bound protein could dissociate from that site through DNA-facilitated direct transfer, resulting in a failure to regulate transcription. Several lines of evidence suggest that such cooperative localization may apply to DSX protein in the Yp protein. First, DSX proteins have an ability to form oligomers higher than a dimer, suggesting the possibility of cooperativity between several DNA-bound DSX molecules (8). Second, when two dsxA sites are separated by a few helical turns, two sites show some binding cooperativity in vitro. Such cooperative effects of multiple binding sites on protein-DNA complex dissociation have been observed in the study of the Ultrabithorax homeodomain protein of D. melanogaster (23). Third, the multiple dsx binding sites present in the Yp regulatory region are known to act synergistically when regulating transcription in vivo (3). Finally, strong synergistic activation has been observed in vivo between dsxA and two other non-DSX DNA binding sites in a 34-bp mini-enhancer (3). This indicates that DSX proteins not only cooperatively bind to multiple dsx sites in Yp genes but also interact cooperatively with non-DSX proteins.

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