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The Doublesex proteins of *Drosophila melanogaster* bind directly to a sex-specific yolk protein gene enhancer

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The *doublesex* (*dsx*) gene of *Drosophila melanogaster* encodes both male-specific and female-specific polypeptides, whose synthesis is regulated by alternative sex-specific splicing of the primary *dsx* transcript. The alternative splicing of the *dsx* mRNA is the last known step in a cascade of regulatory gene interactions that involves both transcriptional and post-transcriptional mechanisms. Genetic studies have shown that the products of the *dsx* locus are required for correct somatic sexual differentiation of both sexes, and have suggested that each *dsx* product functions by repressing expression of terminal differentiation genes specific to the opposite sex. However, these studies have not shown whether the *dsx* gene products function directly to regulate the expression of target genes, or indirectly through another regulatory gene. We report here that the male- and female-specific DSX proteins, expressed in *E.coli*, bind directly and specifically *in vitro* to three DNA sequences located in an enhancer region that regulates female-specific expression of two target genes, the yolk protein genes 1 and 2. This result suggests strongly that *dsx* is a final regulatory gene in the hierarchy of regulatory genes controlling somatic sexual differentiation.

Key words: doublesex/enhancer/repressor/sex determination/yolk protein

Introduction

Genetic and molecular studies have established that differentiation of the fruit fly, *Drosophila melanogaster*, is controlled by numerous regulatory genes, often arranged in hierarchical cascades. The end result of these regulatory hierarchies is to control the expression of the genes that actually define morphogenetic cell properties, first termed 'realisator' genes by Garcia-Bellido (1975) and more commonly described as downstream or target genes. The identities of these target genes and the mechanisms by which their expression is controlled by the final regulatory genes in the hierarchies are largely unknown, leaving a significant gap in our understanding of *Drosophila* differentiation at the molecular level. In this manuscript we report the first evidence of a direct molecular linkage between the regulatory hierarchy controlling somatic sexual differentiation and a specific set of target genes.

Somatic sexual differentiation in *D.melanogaster* is controlled by a well characterized hierarchy of regulatory genes (reviewed in Baker, 1989; Slee and Bownes, 1990; Steinmann-Zwicky *et al.*, 1990). The initial setting of this hierarchy as either male or female involves transcriptional controls (Salz *et al.*, 1989; Erickson and Cline, 1991) while the transmission of sexual identity down the cascade is by the regulation of RNA processing (Boggs *et al.*, 1987; Bell *et al.*, 1988; Nagoshi *et al.*, 1988; Burtis and Baker, 1989; Sosnowski *et al.*, 1989). Genetic experiments suggest that the hierarchy regulates sexual differentiation through negative control (reviewed by Baker and Ridge, 1980; Baker and Belote, 1983; Wolfner, 1988; Steinmann-Zwicky *et al.*, 1990). However, to date there has been no direct evidence regarding the molecular mechanism by which the information transmitted down this hierarchy is passed on to the target genes that mediate the final steps in somatic sexual differentiation.

The *doublesex* (*dsx*) gene appears to be at the bottom of the hierarchy and thus may directly regulate target genes. The primary transcript of the *dsx* gene is alternatively spliced in males and females to yield sex-specific mRNAs which encode male-specific and female-specific polypeptides (Burtis and Baker, 1989). The two *dsx* proteins are identical for the first 397 amino acids (aa), but have unique carboxy termini of 152 aa (male) and 30 aa (female). It has been proposed that the male-specific product of the *dsx* locus, DSX^M, represses the expression of female-specific genes in males and that the DSX^F protein, perhaps in conjunction with the product of the *intersex* gene, represses the expression of male-specific genes in females (Baker and Ridge, 1980; Nöthiger *et al.*, 1987; Burtis and Baker, 1989). This repression could occur through direct binding of DSX proteins to target genes.

Regulation of the yolk protein genes provides an ideal system in which to look for a direct molecular interaction between the DSX proteins and a target gene. The yolk protein genes are expressed sex-specifically in a tissue found in both sexes, the adult fat bodies. Molecular genetic experiments have demonstrated that proper expression of these genes requires the continuous action of the sex determination hierarchy (Belote *et al.*, 1985). Moreover, germline transformation experiments have shown that a 127 bp fat body enhancer (FBE) of the yolk protein genes 1 and 2 (*yp1* and *yp2*) is likely to be the target of sex-specific regulation because this enhancer is sufficient to direct the female-specific transcription characteristic of the *yp* genes in adult fat bodies (Shepherd *et al.*, 1985; Garabedian *et al.*, 1986).

In this manuscript we report that the male- and female-specific protein products (DSX^M and DSX^F) of the *dsx* locus, expressed in *E.coli*, bind specifically to the FBE, demonstrating a direct molecular interaction between the sex determination hierarchy and a target gene. The observation that both the male- and female-specific DSX proteins interact

with the FBE is discussed with respect to prior genetic and molecular results.

Results

Gel mobility shift assays using doublesex proteins overexpressed in E.coli

Coding sequences from male- and female-specific *dsx* cDNAs (Burtis and Baker, 1989) were inserted into the expression vector pT7-7 (Tabor and Richardson, 1985) in order to overexpress the *dsx* polypeptides in *E.coli* under the control of the T7ϕ10 promoter. In the experiments described in this paper, the expression constructs utilized a T7-derived initiation codon, resulting in the production of a fusion protein containing 10 additional amino acids at the amino-terminus as described in Materials and methods. Subsequent experiments with constructs expressing DSX polypeptides without additional amino acids have yielded identical results (data not shown). Soluble extracts were prepared from cells carrying either the pT7-7 vector plasmid alone (control extract), the pT7-7 vector containing a cDNA encoding the DSX^M polypeptide (male extract), or the pT7-7 vector containing a cDNA encoding the DSX^F polypeptide (female extract). Migration of the female and male proteins on denaturing SDS–polyacrylamide gels indicate apparent molecular weights of 52 kDa for the female fusion protein and 67 kDa for the male fusion protein (Figure 1a), somewhat larger than the molecular weights of 45.8 kDa (female) and 58.5 kDa (male) calculated from the amino acid sequence.

Initial evidence for direct interaction between DSX proteins and DNA sequences containing the FBE was obtained by gel mobility shift assays. Aliquots of each extract were incubated with a mixture of four end-labeled restriction fragments; a large fragment derived from vector sequences, and three smaller fragments derived from sequences located between −888 and −161 nt relative to the transcriptional start site of the yolk protein 1 gene. The smallest of these three fragments (extending from −347 to −161) includes sequences (−322 to −196) previously identified a sex-, stage- and tissue-specific enhancer of the *yp* genes (Garabedian *et al.*, 1986). As seen in Figure 1b, incubation with either the DSX^M or DSX^F extracts, but not the control extract, resulted in a substantial reduction in the amount of unbound DNA present in the smallest restriction fragment (containing the FBE), but not in the quantity of unbound DNA in the two adjacent yolk protein gene restriction fragments. Thus, DSX^M and DSX^F are sequence-specific DNA binding proteins *in vitro*. Mobility shift assays using a 4-fold lower concentration of protein and the FBE-containing fragment alone revealed several discrete protein–DNA complexes with differing mobilities (Figure 1c).

Further evidence for the specificity of this binding has been derived from experiments involving the addition of a competitor oligonucleotide (containing sequences from −309 to −285, a binding site identified by subsequent DNase I footprinting analysis; see below) to the binding reaction (Figure 1d). The appearance of shifted complexes in the reactions containing control extract is unaffected by the presence of a 1000-fold molar excess of this competitor, indicating that these are due to non-specific interactions between *E.coli* proteins and labeled DNA. However, the

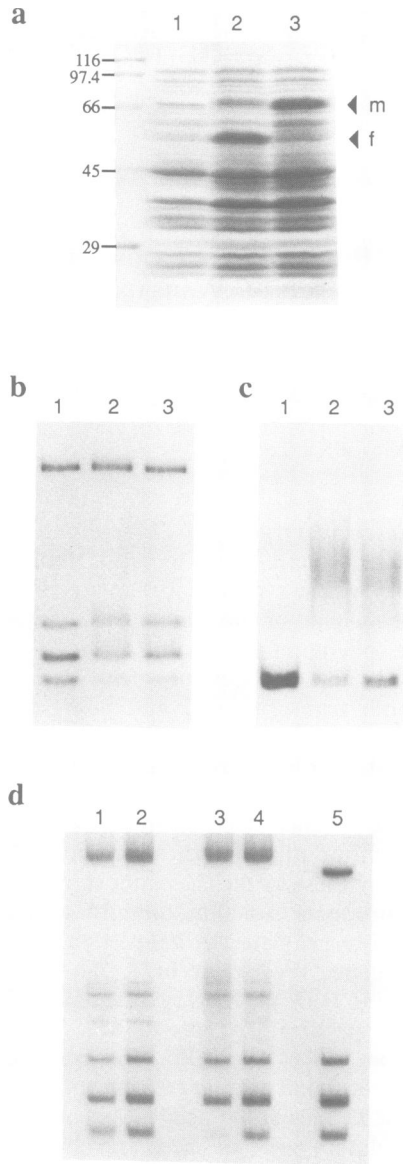

Fig. 1. DSX protein interaction with *yp* DNA. (**a**) Expression of DSX proteins in *E.coli*. Cells containing the pT7-7 vector alone (lane 1), the pT7-7:DSX^F construct (lane 2) or the pT7-7:DSX^M construct (lane 3) were boiled in loading buffer and electrophoresed on a 9% denaturing polyacrylamide gel (Laemmli, 1970). The M_r of protein standards (in thousands) are indicated at the left. Positions of DSX^M (m) and DSX^F (f) are indicated by markers at the right; both migrate more slowly than predicted by amino acid sequence. (**b**) Gel mobility shift assay with several *yp* restriction fragments. The three smallest restriction fragments contain sequences from −888 to −667 (221 bp), −666 to −347 (319 bp) and −346 to −161 (185 bp); all relative to the *yp1* transcription initiation site. The largest fragment (4.4 kb) contains pBR322 vector linked to *yp* sequences (−160 to −89). The smallest band contains the 127 bp FBE (−322 to −196) (Shepherd *et al.*, 1985; Garabedian *et al.*, 1986). 1 μg of pUC18 DNA was added as an additional non-specific competitor. Labeled DNA was incubated with control extract (lane 1), DSX^F extract (lane 2) or DSX^M extract (lane 3). (**c**) Gel mobility shift assay with only the FBE-containing restriction fragment. A 4-fold reduced extract concentration (0.5 μl per reaction) was used. Labeled DNA was incubated with control extract (lane 1), DSX^F extract (lane 2) or DSX^M extract (lane 3). (**d**) Competition by synthetic oligonucleotide. 2.5 nmol of a synthetic competitor oligonucleotide (1000-fold molar excess over the radiolabeled fragment; see text) was added to the reactions shown in lanes 2 and 4. Reactions included either control extract (lanes 1 and 2), DSX^M extract (lanes 3 and 4), or no extract (lane 5).

shift of the FBE-containing fragment by the extract containing DSX^M protein is completely eliminated in the presence of competitor oligonucleotide (Figure 1d, lane 4), indicating that the interaction between DSX^M protein and the FBE-containing fragment is specific.

Several explanations are possible for the heterogeneous mobility of bound complexes seen in these experiments. These include binding of multiple molecules of DSX to one fragment, binding of altered forms of the protein (e.g. proteolytic fragments), and loss of protein from DNA during

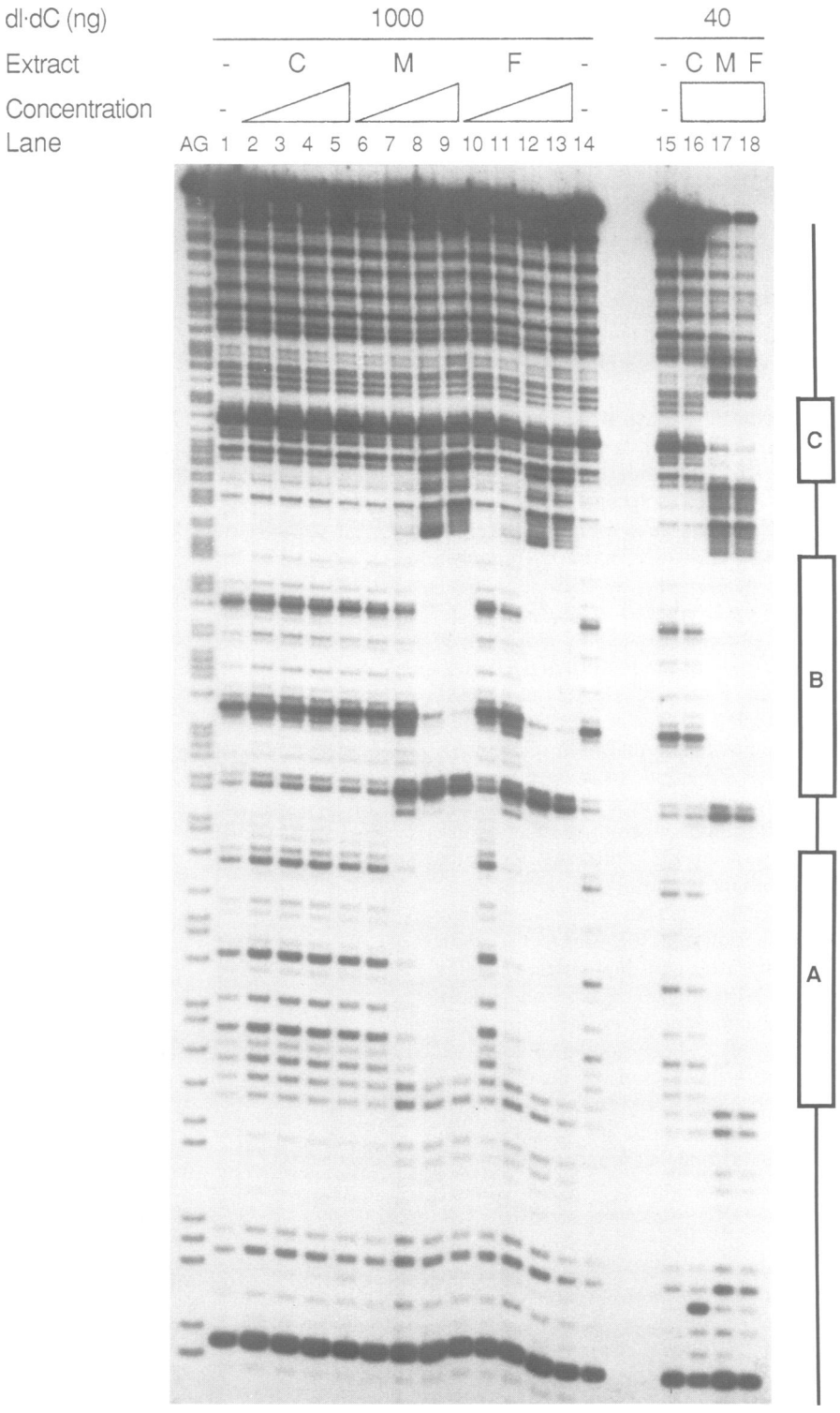

Fig. 2. DNase I footprints of DSX protein interaction with FBE DNA. The indicated extracts (C, control; M, DSX^M; F, DSX^F) diluted 10^4-fold (lanes 2, 6, 10), 10^3-fold (lanes 3, 7, 11) 10^2-fold (lanes 4, 8, 12), or 10-fold (lanes 5, 9, 13, 16–18) were incubated with radiolabeled FBE and analyzed by DNase I footprinting. No extract was added to reactions for lanes 1, 14, 15. The AG lane is an (A+G) chemical degradation sequencing ladder of the FBE fragment (Maxam and Gilbert, 1980). Footprints are indicated to the right and their endpoints and sequences are shown in Figure 4.

the course of electrophoresis. We show below that at least three DSX molecules can bind simultaneously to this DNA fragment (Figure 2, lane 17). Additional experiments involving the addition of competitor oligonucleotide after the formation of bound complexes (K.C.Burtis, unpublished data) have indicated a rapid dissociation rate for the DSX^M protein (essentially complete release after 5 min), suggesting that loss of complex during electrophoresis is also a reasonable possibility.

Localization of DSX binding sites by DNase I footprinting assays

Binding sites were localized within the enhancer region by DNase I footprinting assays. Increasing amounts of protein extract were incubated with a constant amount of radiolabeled enhancer DNA (Figure 2). Control extract gave no footprints at any of the concentrations tested (lanes 2–5, 16). However, extract from cells overproducing DSX^M protein gave three footprints (lanes 6–9, 17). A single footprint appeared at low protein concentration (footprint A, lane 7). A 10-fold increase in protein concentration yielded a second footprint (footprint B, lane 8). A further 100-fold increase in protein (data not shown) or a further 10-fold increase in protein and 25-fold decrease in non-specific competitor DNA (lane 17) yielded footprint C. Extract from cells overproducing DSX^F protein gave footprinting results indistinguishable from those with DSX^M extracts (Figure 2, lanes 10–13, 18).

A further indication that the DNA binding specificities and affinities of the two DSX proteins are very similar is given by the ionic strength sensitivity of the footprints (Record *et al.*, 1981). As shown in Figure 3, DSX^M and DSX^F binding decreased in parallel at all sites as the concentration of KCl was increased. We conclude that both of the DSX proteins present in *E.coli* extracts bind to three sites in the FBE and that each protein binds DNA in a very similar if not identical manner. This similarity strongly suggests that the DNA binding domain of the DSX proteins lies in the amino acid sequence common to the two proteins.

A comparison of the three binding sites revealed a potential 9 bp consensus recognition sequence, CTACAAAGT. Four sequences match this consensus with homologies ranging from seven out of nine to nine out of nine nucleotides (Figure 4). The consensus copies occur at similar positions in the three footprints, four nucleotides from the 5′ ends and five to six nucleotides from the 3′ ends of the footprints (Figure 4), indicating that the consensus sequences are likely to be involved in the binding reaction. Two copies occur in the larger footprint B, indicating that it may be composed of two binding sites.

Discussion

Direct regulation of yolk protein gene expression by dsx

The experiments described above supply compelling evidence that a product of the *dsx* locus is a direct link between the elaborate cascade of regulation controlling somatic sexual differentiation in *Drosophila* and the sex-specific enhancer of a particular target gene. They demonstrate unequivocally that the proteins encoded by the *dsx* gene are sequence-specific DNA binding proteins, and that a high-affinity DSX binding site lies within the 127 bp sequence previously found to be a sex-specific, tissue-specific, and stage-specific enhancer of the yolk protein genes. Previous experiments have suggested that DSX^M negatively regulates expression of the yolk protein genes in male fat bodies (Postlethwait *et al.*, 1980; Bownes and Nöthiger, 1981; Ota *et al.*, 1981; Belote *et al.*, 1985; Burtis and Baker, 1989). We now propose that this repression

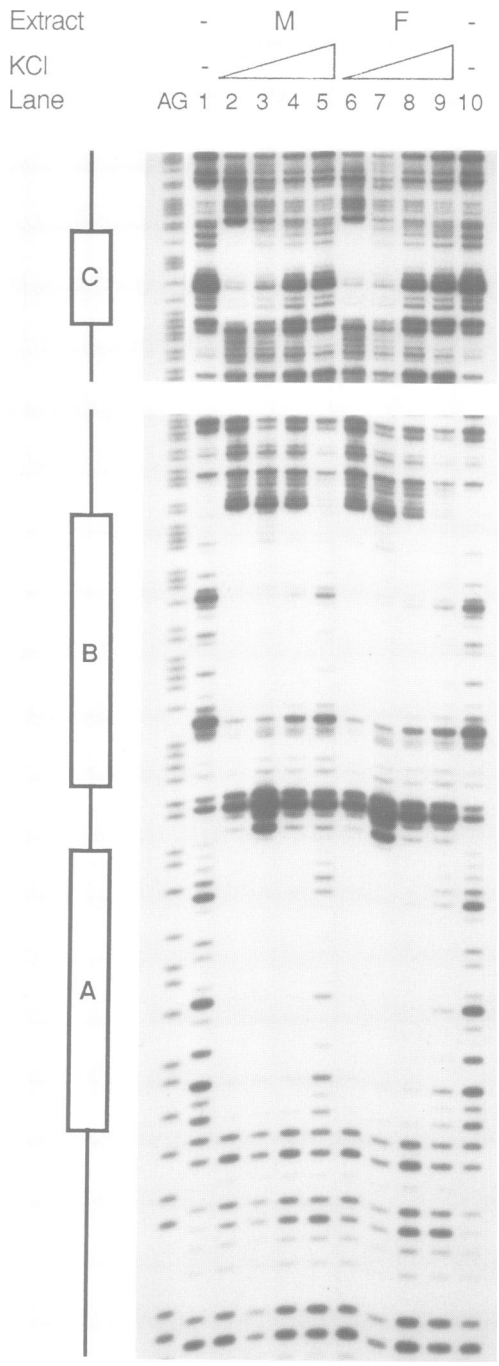

Fig. 3. Ionic strength dependence of DSX footprints. The indicated extract (M, DSX^M; F, DSX^F) was incubated with radiolabeled FBE DNA in the presence of increasing concentrations of KCl and analyzed as in Figure 2. No extract was added to reactions for lanes 1 and 10. The final KCl concentrations in the binding reactions were 50 mM (lanes 1, 2, 6, 10), 100 mM (lanes 3, 7), 200 mM (lanes 4, 8) and 400 mM (lanes 5, 9). Reactions examining footprint C (upper panel) contained 40 ng of the non-specific competitor and 10-fold diluted extract. Reactions examining footprints B and A (lower panel) contained 1000 ng of non-specific competitor and 100-fold diluted extract.

occurs because DSX^M protein binds directly to the FBE in males, interfering with enhancer action and thus repressing the expression of the yolk protein genes. The determination of whether this mechanism is also used in the repression of other female-specific genes in males must await identification of additional target genes.

Repressors have been found with increasing frequency to play an important role in the regulation of eukaryotic gene expression, in many cases through interactions with positive enhancer elements (reviewed by Levine and Manley, 1989; Stenlund and Botchan, 1990). The mechanism of repression may involve direct competition for the binding sites of activator proteins, protein−protein interactions leading to interference with activator function but not binding, or direct interference with the basal transcriptional machinery. Although it remains to be determined which of these mechanisms is involved in the regulation of *yp* expression by DSX, the location of the binding site would favor one of the first two possibilities.

Determination of whether the DSX proteins act directly to regulate the expression of other sex-specific target genes is dependent on the identification and isolation of these genes. The most probable candidates for direct regulation include other genes regulated continuously by the sex determination hierarchy during adult life, such as the genes regulating male courtship behavior (Belote and Baker, 1987), and genes controlling the determination of sex-specific tissues, which genetic evidence suggests are regulated by the hierarchy at various times during larval and pupal development (Belote and Baker, 1982). The regulation of genes expressed in sex-specific tissues of adult flies may be less direct. It has been established in some cases that adult expression of these genes is no longer responsive to the status of the sex determination hierarchy (reviewed by Wolfner, 1988), and that the critical period during which the hierarchy acts to determine the expression of these genes coincides with the period during which the morphological development of the tissue occurs, days before the genes are expressed (Chapman and Wolfner, 1988). It appears that these genes are directly controlled by tissue-specific rather than sex-specific mechanisms and are not direct targets of *dsx* regulation, although the possibility cannot be excluded that a transitory direct interaction with the DSX proteins during the critical period results in a stable expression state (Wolffe and Brown, 1988).

Site-specific binding of DSX^F to the FBE

An unpredicted observation in these experiments is the *in vitro* binding of DSX^F to the FBE, since previous experiments have suggested no role for DSX^F in regulating yolk protein gene expression in females. In considering this unexpected result, it is important to note that DSX^F and DSX^M are identical for the first 397 amino acids, but have unique carboxy termini of 152 aa in DSX^M and 30 aa in DSX^F. It seems most likely that the shared 397 amino acid region mediates the specific binding we observe *in vitro*. Thus the different repression effects observed *in vivo* are likely to be due to the unique carboxy termini. We propose two hypotheses to explain the different *in vivo* activities.

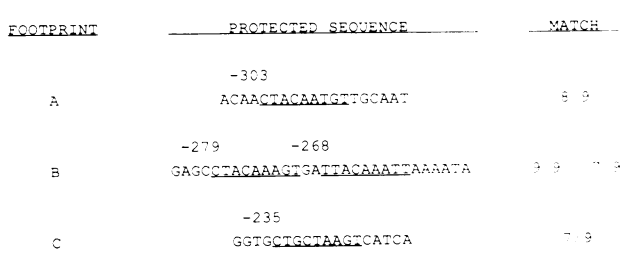

Fig. 4. Footprint sequences. The sequences protected in each footprint are shown. Homologies to the proposed consensus are underlined and their first nucleotides and matches to the consensus are indicated.

In the first hypothesis, the two proteins bind to the same set of sites *in vivo* but have different effects. In this model the unique domain of DSX^M interferes with the function of an activator protein bound to female-specific enhancers and the unique domain of DSX^F interferes with the function of an activator bound to male-specific enhancers. A prediction of this model is that binding of DSX^F *in vivo* to the binding sites in the FBE would not lead to repression of the *yp* genes, which is crucial since both DSX^F and the *yp* genes are expressed in females. It also predicts that if DSX^F were expressed inappropriately in males, it might bind non-productively to the FBE binding sites, preventing DSX^M from binding to the FBE and thus preventing the repression of *yp* expression. Previous experiments have indeed shown that expression of small quantities of DSX^F in male flies, produced from a cDNA copy of the gene introduced into the genome by P-element mediated transformation, results in a significant derepression of *yp*1 mRNA expression (Burtis and Baker, 1989). A precedent for inhibitory domains on repressor proteins has been found with the yeast protein $\alpha 2$, which binds to operator sequences adjacent to a-specific genes. Binding of $\alpha 2$ interferes with the expression of a-specific genes by blocking the activation of these genes mediated by the MCM-1 protein (Keleher *et al.*, 1988). Hall and Johnson (1987) constructed several mutant versions of the *MAT*$\alpha 2$ gene with in-frame deletions. These genes encoded mutant $\alpha 2$ proteins with internal deletions, which still displayed tight binding to operator sequences *in vitro* (and probably *in vivo*), but failed to repress an operator-regulated gene *in vivo*, thus indicating that binding and repression were mediated by different domains of the $\alpha 2$ protein.

An alternative hypothesis is that DSX^F binds to the FBE *in vitro* but not *in vivo*. In this model, the unique domain of DSX^F interacts with another protein or is modified post-translationally, altering its binding specificity and directing it to an alternative set of target genes. One candidate for an interacting protein is the product of the *intersex* gene, which has been shown by genetic studies to be required specifically in females in addition to the *dsx* gene product for proper regulation of somatic sexual differentiation (for review see Baker and Belote, 1983; Wolfner, 1988). It is possible that an interaction between *dsx* and *intersex* gene products may lead to a heterodimer with an altered DNA binding specificity, presumably leading to specific binding of sequences adjacent to male-specific genes. Again, there is a precedent in the mating type genes of *S.cerevisiae*, in which interaction of the *MATa1* gene product (a1 protein) with the $\alpha 2$ protein leads to a heterodimer that no longer recognizes operators adjacent to a-specific genes, but rather binds to operators regulating haploid-specific genes (Goutte and Johnson, 1988).

Materials and methods

Expression of dsx proteins

DSX^M and DSX^F proteins were over-expressed in *E.coli* using the expression plasmid pT7-7 (Tabor and Richardson, 1985). DSX^F or DSX^M coding sequences from cDNAs AC16 and AC32 (Burtis and Baker, 1989)

were inserted into the vector between the *Eco*RI and *Hin*dIII or *Cla*I sites, respectively. The resulting constructs encoded proteins with 10 additional N-terminal amino acids (MARIRSEAGI) fused to full length DSX polypeptides. Expression was induced in 25 ml of cells in mid-log phase using M13 phage mGP1-2 harboring the T7-7 RNA polymerase gene as described (Tabor and Richardson, 1985). Cells were then pelleted and resuspended in 4 ml of buffer Z-100 (100 mM KCl, 25 mM HEPES pH 7.9, 12.5 mM $MgCl_2$, 1 mM DTT, 0.1% NP40, 10% glycerol, 1 mM PMSF) with 2 mM benzamidine, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin added. Cell lysates were prepared using a French press (two passes at 18 000 p.s.i.). Lysates were then centrifuged at 27 000 *g*, 10 min. The supernatant was brought to 40% saturation with ammonium sulfate and precipitate collected by centrifugation (12 000 *g*, 10 min). The pellet was resuspended at 1 to 10 mg/ml in buffer Z-50 (same as buffer Z-100, except 50 mM KCl), and dialysed twice against 100 vol of buffer Z-50 over a 12 h period.

Gel mobility shift assays

Interactions between the DSX proteins and *yp* DNA were assayed by gel mobility shift assays (Fried and Crothers, 1981; Garner and Rezvin, 1981). Standard binding reactions contained (in the order added) 4 μl of 5× buffer Z-50, 2 μg poly[(dI·dC):(dI·dC)], 2 μl (~2 μg total protein) of extract (control, DSX^F, or DSX^M), and 2.5 fmol of each of the ^{32}P-end-labeled restriction fragments, in a total vol of 20 μl. Reactions were incubated at room temperature for 20 min, then electrophoresed in non-denaturing 4% acrylamide gels (30:0.8 ratio acrylamide:bisacrylamide, 25 mM Tris, 190 mM glycine, 1 mM EDTA, pH 8.5).

DNase I footprinting

The DNase I footprinting method (Galas and Schmitz, 1978) as modified by Heberlein *et al.* (1985) was further modified as follows: 25 μl of extract (diluted as indicated from 10 mg/ml), 1 ng (7 fmol) of 5′ ^{32}P-end-labeled FBE (−322 to −196) DNA fragment (label on coding strand), and indicated amount of non-specific competitor (poly[(dI·dC):(dI·dC)]) were incubated in 12.5 mM HEPES (pH 7.6), 0.05 mM EDTA, 6.25 mM $MgCl_2$, 5% glycerol, 0.5 mM DTT, 50 mM KCl, 2% polyvinyl alcohol in a final volume of 50 μl on ice, 26 min. Following DNase I digestion, reactions were stopped by adding 90 μl of 500 mM NaCl, 0.1% SDS, 20 mM EDTA, 12 mg/ml tRNA.

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