The remarkable ubiquity of DM domain factors as regulators of sexual phenotype: ancestry or aptitude?

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The DM domain is a cysteine-rich DNA-binding motif first recognized in proteins encoded by the Drosophila sex determination gene doublesex (Erdman and Burtis 1993; Zhu et al. 2000). As the name doublesex (dsx) suggests, this gene has functions in both sexes: Its transcripts undergo sex-specific alternative splicing, so that it can encode either a male-specific isoform, DSX M, or a female-specific isoform, DSX F (Baker and Wolfner 1988; Burtis and Baker 1989). These proteins have the same N-terminal DNA-binding domain, but different C termini that confer different regulatory properties on the two forms. The expression of DSX M directs male development, and the expression of DSX F directs female development, throughout most of the somatic tissues of the fruit fly.

More recently, the same domain was discovered in nematodes, in the Caenorhabditis elegans male-abnormal gene mab-3, which has several biological functions similar to those of DSX M (Shen and Hodgkin 1988; Raymond et al. 1998). Male worms mutant for mab-3 synthesize yolk proteins and show defects in male genital development similar to phenotypes seen in male flies that lack DSX M. Underlining these similarities, it was found that ectopic expression of DSX M (but not DSX F) in C. elegans can partly rescue the mab-3 mutant phenotype (Raymond et al. 1998). This was the first example of a possible component shared between sex-determination mechanisms in different phyla, and the DM family was named on the basis of these two genes (dsx/mab-3).

Searches for similar genes in other phyla were rewarded by the discovery of DMRT1 and related genes in vertebrates. DM family members have been increasingly implicated as playing important roles in sexual development, in a variety of different vertebrate and invertebrate species.

mab-23: a new male-specific DM domain gene

In this issue of Genes & Development, Lints and Emmons [2002] report the investigation of another sex-specific member of the DM family in C. elegans, defined by the gene mab-23. There are striking similarities in the function and regulation of mab-23 and mab-3. Both are required for several different elements of male somatic development, but appear to be dispensable for normal development of hermaphrodites, the alternative sexual form in C. elegans. Hermaphrodites (XX) can be regarded essentially as modified females, able to differentiate both oocytes and sperm and therefore capable of self-fertilization. Males (XO) differ extensively from hermaphrodites in anatomy, development, and behavior.

Mutations of mab-23 result in multiple defects in male development. The most conspicuous alterations are seen in the highly differentiated structures of the male tail, particularly in overall tail morphology and in sensory structures called rays, which have changes both in the pattern of neurotransmitter synthesis and in the guidance of their sensory axons. In addition, mutant males show abnormalities in sex-specific musculature and associated behaviors, being unable to achieve a characteristic ventral curling of the tail that occurs during mating. Detailed examination revealed yet further defects in the copulatory spicules and in the junction of genital tract and hindgut. The affected tissues were all observed to express a MAB-23::GFP reporter construct, suggesting that the gene acts cell-autonomously to direct normal male differentiation in multiple different cells. Other components of male development are, however, normal.

There is considerable, although incomplete, overlap between tissues affected by mab-23 and those affected by the other characterized DM domain gene, mab-3. For example, both are required for normal development of the sensory rays and male musculature. However, the two genes have distinct functions within these tissues. Moreover, mab-3 has a major function in the adult male intestine, acting to repress transcription of the yolk protein genes, but mab-23 has no as-yet identified function in the intestinal cells and does not appear to be expressed in them. In terms of sequence, the two genes are clearly related, because the single DM domain of MAB-23 clusters with one of the two DM domains of MAB-3. Therefore, it may be that multiple male-specific functions carried out by a single ancestral gene comparable to dsx...
have become partitioned during evolution between \textit{mab-3} and \textit{mab-23} and perhaps other genes.

The two genes \textit{mab-3} and \textit{mab-23} also display notable similarities in their regulation. All aspects of sexual differentiation in \textit{C. elegans} appear to be under the control of a master regulator gene \textit{tra-1}, which encodes a CI/GLI-related zinc finger protein, TRA-1A [Zarkower and Hodgkin 1992]. Genetic and molecular studies indicate that TRA-1A activity is both necessary and sufficient to dictate female development of all tissues, so that constitutive mutations of \textit{tra-1} transform \textit{XO} animals [normally male] into females, and null mutations transform \textit{XX} animals (normally hermaphrodite) into males [Hodgkin 1987]. TRA-1A is therefore believed to have multiple targets for transcriptional control in the \textit{C. elegans} genome, activating female-specific genes or repressing male-specific genes such as \textit{mab-3}. Studies of the \textit{mab-3} regulatory region have revealed a binding site for TRA-1A, which is essential for the correct regulation of \textit{mab-3} in one sexually differentiated tissue, the intestine [Yi et al. 2000]. A \textit{mab-3} :: \textit{gfp} reporter gene is expressed in the intestinal cells of adult males but not adult hermaphrodites, consistent with the role of \textit{mab-3} in preventing yolk protein synthesis in males. Mutation of the TRA-1A binding site, however, results in expression of the \textit{mab-3} reporter gene in the intestinal cells of both sexes, indicating that \textit{mab-3} transcription in the hermaphrodite intestine is normally directly repressed by TRA-1A. One might therefore expect this simple pattern of control to be repeated elsewhere in the animal, but, in fact, MAB-3 is expressed in a largely non-sex-specific manner in other tissues [Yi et al. 2000]. The same kind of non-sex-specific distribution has now been found for \textit{MAB-23}. So both of these proteins are expressed in multiple tissues in both sexes, despite the fact that they appear to be completely male-specific in action. A possible explanation for their lack of effect in the other sex (female/hermaphrodite) is that their activities are blocked by a female-specific factor such as TRA-1A. Alternatively, the MAB-3 and MAB-23 proteins might have different and less obvious functions in female differentiation. This possibility is unlikely in the case of \textit{mab-23} because the first \textit{mab-23} mutation to be discovered was found in KR314, a wild isolate of \textit{C. elegans} obtained from Vancouver [Hodgkin and Doniach 1997]. KR314 hermaphrodites are fully viable and healthy, but homozygously mutant for \textit{mab-23}, so the occasional spontaneous males that arise in populations of KR314 are severely abnormal. The existence of this natural race provides a strong reason for believing that \textit{mab-23} has no significant function in hermaphrodites. The apparently wasteful unregulated expression of proteins with sex-limited functions is not without precedent: For example, male sea urchins express yolk proteins at high levels, despite the fact that they do not make eggs [Shyu et al. 1986].

At least nine more DM domain genes have been identified in the \textit{C. elegans} genome [Ottolenghi et al. 2002], but it remains to be seen if any have sex-specific roles comparable to \textit{mab-3} and \textit{mab-23}.

**DM domain genes in vertebrates**

A vertebrate member of the DM family was first identified in the form of a human testis-specific gene, \textit{DMRT1} [Raymond et al. 1998]. The chromosomal location of the gene, on the short arm of Chromosome 9, was suggestive of a function in sex determination because hemizygous deletions of this region have been observed to correlate with defective testis formation. However, no unambiguous point mutations of \textit{DMRT1} affecting human sex determination have been found thus far [Raymond et al. 1999b]. The corresponding mouse gene, \textit{Dmrt1}, has been studied in detail and found to be expressed in the early genital ridge, before sexual differentiation [Raymond et al. 1999a]. A \textit{Dmrt1} knockout mouse was then made and was found to show normal female development in \textit{XX} individuals, but \textit{XY} individuals showed multiple defects in testis differentiation [Raymond et al. 2000]. Nevertheless, early events in testis differentiation occurred normally in the knockout male mice, indicating that \textit{Dmrt1} may not play a major early role in mammals, despite its expression pattern.

In other vertebrates, corresponding genes have been identified and found to be similarly expressed at a very early point in genital ridge differentiation [Raymond et al. 1999a; Smith et al. 1999; Guan et al. 2000; Kettlewell et al. 2000; Marchand et al. 2000]. Furthermore, in most cases higher expression is seen in male as opposed to female primordia. In birds, the \textit{Dmrt1} homolog is sex-linked, on the Z chromosome, so there is a higher dosage of the gene in male birds (karyotype ZZ) as compared with females (karyotype ZW). Homologs have also been examined in reptile species with temperature-dependent sex determination, such as turtles and alligators [Smith et al. 1999; Kettlewell et al. 2000]. In representatives of each of these groups, expression of \textit{Dmrt1} was found to be higher in embryos exposed to male-determining temperatures than in embryos exposed to female-determining temperatures. The difference in expression level is the earliest sexual dimorphism so far detected in these species, so there is a circumstantial case for suspecting that these DM genes have a more important male-determining role in nonmammalian vertebrates than they do in mice.

Direct evidence for a DM gene playing a role in primary sex determination has been provided by Matsuda et al. [2002], studying the medaka fish. In this species, males have an \textit{XY} karyotype, and females have an \textit{XX} karyotype. The male-determining region on the Y-chromosome was narrowed to a 250-kb region, within which a Y-specific gene was identified and named \textit{DMY} because it contains a DM domain. The expression pattern of \textit{DMY} is consistent with a role in testis determination, and two natural mutations of \textit{DMY} were identified. Both of these cause sex reversal, so that \textit{XY} fish carrying a mutant \textit{DMY} develop as female, not male. It remains to be seen whether expression of \textit{DMY} in XX fish would be sufficient to cause the reverse transformation, from female into male. The data so far suggest that \textit{DMY} plays a role analogous to \textit{SRY} in mammals.
As in invertebrates, all vertebrate genomes so far examined contain multiple members of the DM domain family (Ottolenghi et al. 2002). Some of these genes are likely to have developmental functions unrelated to sexual development. For example, the zebrafish gene terra and its mouse homolog are both specifically expressed in early somitic mesoderm, and may be required for somitogenesis (Meng et al. 1999).

**Regulation of DM factors**

In the cases for which information is available, the regulation of DM gene expression is conspicuously diverse. In the simplest case, that of medaka DMY, because the gene is located on the male-specific Y-chromosome, its expression is controlled simply by the gene being present in males and absent in females. Along the same lines, the chicken Dmrt1 gene is located on the avian Z-chromosome, and therefore it is present in double dose in male embryos (ZZ) but only single dose in female embryos (ZW). Conceivably, the higher dosage in ZZ embryos is responsible for the higher expression observed by in situ hybridization, and this higher expression might be enough to trigger male development. For this explanation to work, the gene would have to escape sex-chromosome dosage compensation, which is now believed to occur in birds, but this is not inconceivable. On the other hand, the difference in expression level between males and females is more than twofold, thus regulation must entail more than just the difference in gene dosage (Raymond et al. 1999a; Smith et al. 1999).

In both turtles and alligators, the expression levels of Dmrt1 homologs respond to temperature, but the mechanism of this regulation is wholly unknown. Similarly, little is yet known about the transcriptional or posttranscriptional control of mammalian Dmrt1 homologs.

In *Drosophila*, the difference between synthesis of DSXM and DSXF is achieved by alternative splicing (for review, see Cline and Meyer 1996). Default splicing, using the normal cellular machinery, results in the formation of a message encoding DSXM. Modified splicing, which occurs only in females and depends on the presence of the female-specific TRA product, results in a message encoding DSXF. The fact that the default product is DSXM might suggest that the gene is ancestrally male-determining, and that the production of DSXF has been a more recent evolutionary addition. However, observations on a distantly related insect, the silkmoth *Bombyx mori*, reveal a different pattern (Suzuki et al. 2001). In the silkmoth, the *doublesex* homolog undergoes different patterns of splicing in the two sexes, as in *Drosophila*, but both sequence features and in vitro splicing experiments indicate that here the default mode is to produce a female-specific isoform. Thus the situation is reversed, relative to *Drosophila*. It remains to be seen which arrangement is the more common, among other groups of insects. With further comparative data, it may become possible to infer what the ancestral regulation of DSX involved.

In contrast to the situation in insects, the two nematode genes so far studied (mab-3 and mab-23) are not regulated by splicing. One gene, mab-3, is under sex-specific transcriptional control in the intestine, but in other tissues it is expressed in both sexes. The observations on mab-23 suggest that it, too, is expressed in multiple tissues in both sexes, as discussed above.

**Targets of DM factors**

Few targets of regulation by DM domain factors have yet been identified for certain. The most convincing examples are provided by yolk protein genes, in both *Drosophila* and *C. elegans*, which are synthesized in large amounts in adult females of both species and appear to be directly regulated at a transcriptional level by DSX (in flies) or MAB-3 (in worms). The different behavior of DSXM and DSXF is well illustrated by their actions on Yp (yolk protein) genes in *Drosophila*: Both proteins recognize the same sequence at multiple sites in the enhancers of these genes, but DSXM represses transcription, whereas DSXF activates it (Coschigano and Wensink 1993; An and Wensink 1995). In *C. elegans*, the six vit (yolk protein) genes all contain potential upstream MAB-3-binding sites, and mutation of one of the sites in a vit-2 reporter results both in loss of MAB-3 binding in vitro and in expression of the reporter in both sexes (Yi and Zarkower 1999). Therefore, MAB-3 seems to be acting in exactly the same way as DSXM, as a direct repressor of yolk protein gene transcription.

At first sight, this looks like an impressive piece of evolutionary conservation. However, the yolk proteins of flies and worms are not homologous (Wahl 1988; Spieth et al. 1991), nor are the tissues in which they are synthesized (fat body in flies, intestine in worms). Therefore, if the biological similarities represent conservation, then there must have been substitution of one set of target yolk protein genes for a different set, in one or both evolutionary lineages, or else both sets were present and similarly regulated in the common ancestor of nematodes and insects.

Recent studies in *Drosophila* have identified additional possible targets for DSX regulation. An interesting candidate is the gene *branchless* (*bnl*), which encodes a fibroblast growth factor (FGF) protein and is expressed sex-specifically in the male genital disc primordium (Ahmad and Baker 2002). As a result, additional mesodermal cells are recruited to the male disc and differentiate as parts of the male genitalia. Lack of *bnl* expression in the corresponding female primordium is dependent on the presence of DSXF. Furthermore, the upstream region of *bnl* contains multiple DSX-binding sites, suggesting that it is directly repressed by DSXF in females. If so, then the protein acts as a repressor of *bnl* transcription, in contrast to its activating effect on the Yp genes. As with many transcription factors, the properties of both DSXF and DSXM are likely to be context-dependent, making it hard to generalize about their modes of action on different target genes.

Another candidate for regulation by *dsx* is the *bric-a-
brac [bab] gene, which regulates sexually dimorphic pigmentation in the abdomen of Drosophila melanogaster, and may be under direct positive transcriptional control by DSX [Kopp et al. 2000]. Comparison of related Drosophila species indicates that bab is not regulated in a sex-specific manner in some of the species with monomorphic abdominal pigmentation, and in these species it may therefore lack the putative transcriptional input from dxs. If so, then this case shows the ease with which genes can be gained or lost as targets for DSX regulation.

No definite targets for regulation by MAB-3 or MAB-23 in C. elegans have yet been identified, other than the vit genes regulated by MAB-3. However, in C. elegans [and also Drosophila] it is likely that genomic analyses and microarray experiments will soon lead to the identification of new candidates for investigation.

Primordial role or convergence?
The assorted observations of members of this transcription factor family add up to something of a puzzle. In terms of biological function, DM-related genes have been frequently found to be associated with sex-specific aspects of development, and often with male-specific development in particular. Yet in terms of biochemistry, neither the regulation of these genes, nor the targets they act on, show significant conservation, at least in the cross-phylum comparisons so far available. The idea that DM-related genes had a primordial role as the ancestral male-determining factors in the common ancestor of all metazoa is therefore tenable, but only by assuming extensive swapping both of downstream targets and of upstream regulators, during the subsequent evolution of different animal groups. Such a scenario is not entirely unreasonable, given that the processes of both sex determination and sexual differentiation are subject to rapid evolutionary change.

An alternative hypothesis is that the recruitment of DM-domain factors to male-specific functions in development has occurred multiple times independently, as a result of convergent evolution, and that there is no link to a primordial sexual differentiation mechanism. Why then should this have happened so often—why should members of this family turn up so frequently as necessary for male development, when there are dozens of other kinds of transcription factor that might seem equally qualified? Is there even something special about male development that attracts this particular protein family?

At present, there is some danger of ascertainment bias, because new DM factors are now likely to be sought for and scrutinized with a particular emphasis on finding yet more sexual regulators. But even allowing for possible bias of this type, the involvement of these genes in male development in many different kinds of animal is hard to dismiss as accidental.

A similar argument has existed for some time over the role of the Pax6 gene family in eye development (for review, see Pichaud and Desplan 2002). One present view is that the many different kinds of eyes found in different sighted creatures have a common ancestry, and that Pax6 is a primordial vision-determining gene. Alternatively, it may simply be that Pax6 has properties that make it more likely to be recruited to functions in sensory neuron development, thus its repeated involvement in eye development is a consequence of convergent evolution. In support of this alternative, it is conspicuous that in C. elegans, which does not have eyes, the Pax6 homolog is used in the differentiation of other kinds of sensory neuron instead [Chisholm and Horvitz 1995].

In the case of DM factors, irrespective of whether their sex-specific functions result from homology or convergence, it is evident that concentrating on them has been a productive way to discover much about the origin of sexual dimorphism in many different kinds of animal. It will be particularly interesting to see whether DM-related genes are also associated with sexual development in other major animal groups, such as annelids and molluscs.

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