REVIEW ARTICLE

New Y chromosomes and early stages of sex chromosome differentiation: sex determination in *Megaselia*

WALTHER TRAUT*

Institut für Biologie, Zentrum für medizinische Struktur- und Zellbiologie, Universität Lübeck, Ratzeburger Allee 160, D-23538 Lübeck, Germany

Abstract

The phorid fly *Megaselia scalaris* is a laboratory model for the turnover and early differentiation of sex chromosomes. Isolates from the field have an XY sex-determining mechanism with chromosome pair 2 acting as X and Y chromosomes. The sex chromosomes are homomorphic but display early signs of sex chromosome differentiation: a low level of molecular differences between X and Y. The male-determining function (*M*), maps to the distal part of the Y chromosome's short arm. In laboratory cultures, new Y chromosomes with no signs of a molecular differentiation arise at a low rate, probably by transposition of *M* to these chromosomes. Downstream of the primary signal, the homologue of the *Drosophila doublesex* (*dsx*) is part of the sex-determining pathway while *Sex-lethal* (*Sxl*), though structurally conserved, is not.

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Introduction

Sex-chromosome mechanisms are the prevalent type of sex determination and sex allocation in organisms with two sexes, 'gonochoristic' species in animals and 'dioecious' species in plants. A sex-chromosome mechanism acts formally as a single-factor Mendelian backcross, where one sex is always homozygous (called 'homogametic' in sex-chromosome systems) while the other is constantly heterozygous ('heterogametic'). The sex chromosomes are designated X and Y when males are the heterogametic sex and W and Z in female-heterogametic systems. The peculiar mode of transmission is due to the presence of a sex-determining gene or genes in one chromosome of that particular pair of chromosomes. The activity of this gene or genes forms (or contributes to) the primary sex-determination signal which acts in a cascade of genetic switches that finally govern the development of an embryo into a female or male individual.

Sex chromosomes are often conspicous elements in the karyotype and can even be recognized in interphase nuclei as heterochromatic elements. They have originally been discovered as an unequal pair of chromosomes in karyotype analysis (Wilson 1911) and inferred from sex-linked inheritance of phenotypic markers (Bateson and Punnet 1911; Doncaster and Raynor 1906). It is now generally agreed that most sex chromosomes arise from a pair of homologous chromosomes by acquisition of the sex-determining function. All molecular and morphological differentiation of sex chromosomes are evolutionary consequences of that function. Given sufficient time, a non-recombining region forms around the sex-determining locus and spreads further on. Clonal inheritance of the Y-specific region and constant heterozygosity allow and favour differentiation of the sex chromosomes and lead to genetic erosion of the Y chromosome (for a review, see Charlesworth *et al*. 2005).

Sex-chromosome systems are rather stable in some animal groups. In placental mammals, the same linkage group constitutes the X chromosome in all species investigated (Ohno 1967). The Y chromosomes of mammals show traces of the ancient homology to the X: a small collection of genes including the male-determining *Sry* are obviously paralogues of those present on the X. The existence of these genes demonstrates that the Y chromosome—in spite of its present dissimilarity—has been derived from the same original chromosome pair as the X chromosome, and has been functioning as the Y for more than 180 million years (Graves 2006). In

*E-mail: traut@molbio.uni-luebeck.de.

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birds, with the exception of ratites, W and Z chromosomes are conserved and shown to be derived from an ancestral pair of autosomes (Fridolfsson *et al*. 1998). Stability and conservation in evolution is what one would expect from a fundamental biological process that has direct bearing on reproduction and fitness.

However, this is not a general rule. Even in mammals, the Y chromosome and the *Sry* gene can get lost and the sex-determining function can be taken over by some other still unknown gene, as in the Caucasian vole, *Ellobius lutescens* (Vogel *et al*. 1998). There are many animal groups in which primary sex determination is highly variable and sex chromosome mechanisms are not conserved at all. Among vertebrates, this is especially conspicuous in teleost fishes where species with hermaphroditism, gonochorism, environmental sex determination, polygenic sex determination, XY and WZ mechanisms occur (Mank *et al*. 2006). In a single genus, *Oryzias*, the medaka, or even in a single species, *Xiphophorus maculatus*, the platyfish, different sex-chromosome mechanisms coexist (Takehana *et al*. 2007; Tanaka *et al*. 2007; Schultheis *et al*. 2009). Among insects, Diptera are a group with an extraordinary variety of sex-determining mechanisms (Saccone *et al*. 2002) and also intraspecific variation of the sex-chromosome mechanism, as in the midges *Chironomus tentans* and *C. pallidivittatus* (Beermann 1955) and the flies *Musca domestica* (Dübendorfer *et al*. 2002) and *Megaselia scalaris* (Traut 1994).

The scuttle fly, *Megaselia scalaris*, affords a singular opportunity among animals as sex chromosome turnover can be studied in the laboratory. *M. scalaris* has a wide tropical and subtropical distribution and lives in a variety of habitats. The larvae feed preferentially on decaying organic matter but can become facultative predators, parasitoids or even parasites (Disney 2008). In laboratory cultures, they are reared on *Drosophila* medium. In the present paper, I review sex determination and sex chromosome differentiation in *M. scalaris*.

Sex chromosome differentiation

The karyotype of *M. scalaris* consists of three chromosome pairs, two metacentrics, chromosomes 1 and 2, and an acrocentric, chromosome 3 (Ondraschek 1953; Tokunaga 1953; Traut *et al*. 1990). Comparison between homologues is facilitated in mitosis by somatic pairing, a phenomenon also known from other dipterans. But none of the three chromosome pairs reveals itself as a heteromorphic sex chromosome pair under the microscope (figure 1). In mitotic metaphases of *M. scalaris*, X and Y are indistinguishable when stained with conventional chromosome dyes or with DAPI, with C banding, by comparative genomic hybridisation (CGH) or visualised by scanning electron microscopy: the sex chromosomes are 'homomorphic' (Traut *et al*. 1990; Wolf *et al*. 1994; Traut *et al*. 2001). Polytene chromosomes, which usually present much finer cytogenetic details in dipterans, are not sufficiently organized in bands and interbands in this species; they are fuzzy and, hence, inadequate for cytogenetic analysis.

With X-ray-induced translocations, all chromosomes of the wild-type strain 'Wien' have been cytogenetically labelled and identified. Seven translocations were transmitted in the male line only, a characteristic of Y chromosomes. They were reciprocal translocations between two or all three chromosomes, t(1; 2^Y), t(2^Y; 3) or t(1; 2^Y; 3), and identified the Y chromosome as a chromosome 2 (Johnson *et al*. 1988; Traut *et al*. 1990).

Although no X–Y differentiation was seen at the cytogenetic level, sex chromosome differentiation was evident at the molecular level. Several random-single-copy probes detected male-specific bands in Southern hybridization of genomic DNAs (Willhoeft and Traut 1990). Moreover, multicopy probes, retrotransposon probes as well as a simple repeat probe, (GATA)*n* (figure 2), exposed sex-specific hybridization patterns, revealing Y-specific bands (Willhoeft and Traut 1990; Traut and Wollert 1998; Suck and Traut 2000). Another indicator of molecular differentiation between X and Y of the 'Wien' strain were RAPDs (random amplified polymorphic DNAs). For all chromosomes, RAPD markers have been established including common as well as distinguishing markers for X and Y (Traut 1994).

A small, ~1.8 kb, segment from the X chromosome and its homologue from the Y chromosome exhibited some

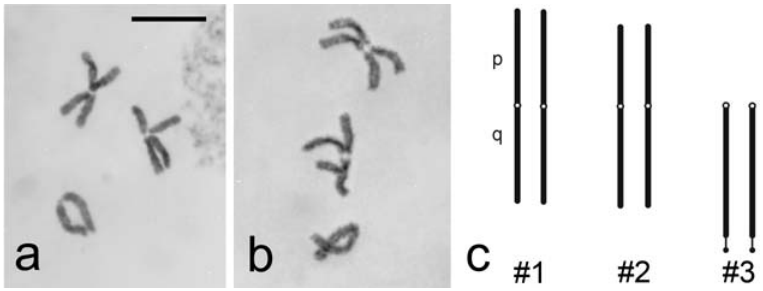

Figure 1. Mitotic chromosomes of *M. scalaris* 'Wien'. (a) female, (b) male chromosomes from neuroblast cells; note that homologues are somatically paired; orcein-stained; bar 10 μm; (c) idiogram derived from measurements of cytogenetically labelled karyotypes (Traut *et al*. 1990).

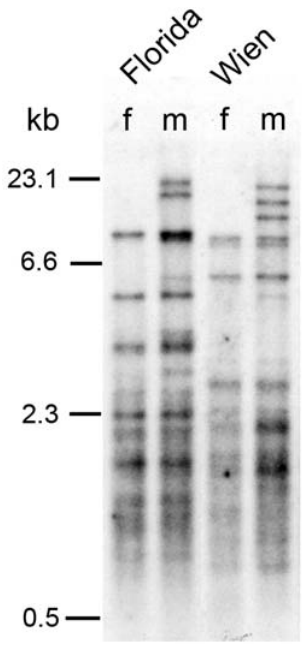

Figure 2. (GATA)*n* microsatellite loci in females (f) and males (m) of the 'Florida' strain and the 'Wien'-derived geT4 strain. Southern hybridization of a (GATA)*n* probe to *Sau3*AI-digested genomic DNAs.

details of the molecular differentiation (Traut and Wollert 1998). The segment was anchored to the start of the *vespid antigen 5* gene but otherwise noncoding. X and Y were 72% identical in this segment. A multitude of single nucleotide exchanges and insertions/deletions of various sizes including a mobile element of 332 bp were detected in the non-coding region. Only a few synonymous changes, however, were found in the coding region, indicating that this Y-chromosomal gene was under negative selection and, therefore, probably still active.

Thus, a low degree of molecular differentiation distinguishes X and Y in *M. scalaris*. This is precisely what we expect from young sex chromosomes. Besides the mainly investigated strain 'Wien', two more wild-type isolates of separate geographic origin, 'Tennessee' and 'Florida', showed a similar degree of molecular sex chromosome differentiation and, hence, had 'young' sex chromosomes too. There is a caveat, however, concerning the origin of differentiation. Some of the differences between X and Y may not have evolved *in situ* but may be the result of an invading Y chromosome that had originally arisen in a distant population.

In contrast to *Drosophila*, crossover does occur in *Megaselia* males, though at a much reduced rate compared to females. Crossover rates of males are 1/6 - <1/2000 those of females, depending on the chromosome region studied (Tokunaga 1955b; Tokunaga and Honji 1956; Springer 1958; Burisch 1963). There is also regular though rare exchange between the sex-determining locus and linked phenotypic markers (Tokunaga 1955b; Springer 1958). The molecular differentiation markers seen in the Y chromosomes, in contrast, appeared to be stably inherited in males. This may mean that the Y chromosome consists of two regions, a Y-specific, which contains the sex-determining locus and all molecular differentiation markers, and a pseudoautosomal region in which exchange with the X chromosome occurs at a low rate. But it is not clear whether the type of Y chromosomes discussed here was the same as used in Tokunaga's (1955b) and Springer's (1958) linkage experiments. They did not distinguish between the original types as found in the field ('young' Y chromosomes) and 'new' ones that arose in laboratory cultures (see next section). 'Young' Y chromosomes have a Y-specific non-recombining segment of unknown size (which may cover the whole chromosome) while in 'new' Y chromosomes no such region has been detected.

Young and even younger Y chromosomes

In the years 1950s and 60s, Tokunaga's and Mainx's groups had isolated several phenotypic markers, which were assigned to three linkage groups (Tokunaga 1955b; Mainx 1964, 1966). When flies trapped in the field were investigated, sex determination was always linked to group I. In laboratory cultures, however, males arose whose sex determination had changed to linkage groups II and III, or even back to group I (Tokunaga 1958; Mainx 1964, 1966). The term 'alternative sex determination' was coined by Mainx (1964) to describe the situation in *Megaselia*. Tokunaga (1955a) and Mainx (1964, 1966) hypothesized from their experiments that the changes were due to translocations of a terminal chromosome segment including a dominant or epistatic male-determining factor *M*.

When work on sex determination in *M. scalaris* was resumed in the 1980s, a wild-type strain from Mainx's cultures had survived and was subsequently called 'Wien'. The three chromosome pairs were labelled cytogenetically with X ray-induced translocations but could not safely be assigned to the formel linkage groups because all previously isolated phenotypic marker strains had been lost. A few phenotypic markers were newly isolated and mapped to the respective chromosomes in crosses with translocations strains (Johnson *et al*. 1988; Traut *et al*. 1994). But only one, *ge* from chromosome 3, was a 'good' marker, i.e. a stably expressed and easily recognisable one. Besides phenotypic markers, RAPD markers were defined for all the chromosomes (Traut 1994).

In the wild-type strain 'Wien' and two new acquisitions, 'Tennessee' and 'Florida', chromosome 2 provided the Y chromosomes. They were the sex chromosomes dubbed 'young' in the preceding section. Sex determination appeared to be rather stable in these strains as (i) all three had Y chromosomes derived from the same source chromosome, i.e. chromosome 2, and (ii) several molecular markers were stably associated with the sex-determining locus *M*. The association with these markers proved that *M* had not moved in or out of this chromosome recently in any of the three strains.

An exceptional X–Y recombinant, 'Except42', was used to close in on the *M* locus in the 'Wien' Y chromosome. *M* was retained together with 5 of the 16 former Y-specific molecular markers in 'Except42' but the remaining ones were lost in the presumed irregular recombination event (Willhoeft and Traut 1995). Assuming an even distribution of the markers in the original Y chromosome, only 1/4 to 1/3 of the old Y chromosome had persisted while the major part had apparently been exchanged against the homologous X chromosomal region. The retained segment from the old Y may be even smaller if—as discussed above—the Y is indeed composed of two regions, a Y-specific and a pseudoautosomal region. By *in situ* hybridization, one of the conserved molecular markers was localized at the distal end of the short arm of chromosome 2. The conserved region including the male-determining locus *M*, therefore, maps to the distal part of chromosome arm 2p.

To test the earlier reports on a turnover of sex chromosomes, a strain was constructed in which *M* and the phenotypic marker *ge* of chromosome 3 were linked by the reciprocal translocation *T4*, a t(2^Y; 3) translocation. Females in this strain were *ge*/*ge*, had grey eyes and a normal karyotype whereas males were *ge*/*ge*$^+$ *T4*, had black wild-type eyes and the chromosome translocation. Exceptional, rarely appearing grey-eyed males were candidates for changes of the sex-determining Y chromosome. The exceptional males were used to start separate lines for genetical, cytogenetical and molecular tests in order to identify the male-determining chromosome. New Y chromosomes appeared with a frequency of 0.06% (Traut and Willhoeft 1990). The figure compares with rates of 0.08%–0.3% in the experiments of Mainx (1966).

In this way, strains have been established with new Y chromosomes that arose in the laboratory. The Y chromosomes were derived from all three chromosomes of the *Megaselia* standard karyotype, including the former X chromosome (table 1). Although the male-determining function had been transferred to the new chromosome, none of the former Y chromosomal molecular markers had been transferred together with it. No difference between females and males was detected with molecular probes in these strains. Considering the rather high rate, the best explanation for the sex chromosome turnover is that a male-determining gene *M* had been transposed to different locations, thus creating new Y chromosomes at each such event (Traut and Willhoeft 1990; Traut 1994) (see Conclusions for more discussion).

Stability of the sex-chromosome system in the field

The rather regular turnover of sex chromosomes in the laboratory provokes the question why the sex-chromosome system is apparently stable in the field. 'Wien', 'Tennessee' and 'Florida' have chromosome 2-Y chromosomes. Mainx (1966) observed a similar phenomenon: only linkage group I-Y chromosomes were found in flies from various regions of the world, Spain, Canary Islands, Malta and Hawai. Presumably, 'linkage group I' and 'chromosome 2' refer to the same chromosome of *M. scalaris*.

Table 1. Y chromosomes in *Megaselia scalaris*.

Derived from chromosome	Strains
Chromosome 1	Except45
Chromosome 2	Wien, Tennessee, Florida
Chromosome 2 (former X chromosome)	Except11, Except46
Chromosome 3	Except1, Except10

Springer (1967) tested the adaptive value of the three types of Y chromosomes experimentally in population cages. In competition experiments, an equilibrium was reached with different Y chromosomes dominating, depending on the experimental conditions. Under conditions of crowding, linkage group III-Y chromosomes were successful while in well-aerated population cages, linkage group I-Y chromosomes were dominant. The latter result may explain their preponderance in the field. Linkage group I supposedly is polymorphic and contains alleles which, when expressed in males, confer competitiveness under most field conditions.

Dosage compensation

In advanced X–Y systems, e.g. in mammals and *Drosophila*, a double dose of X chromosomal genes is present in females but only a single dose in males. To restore a balanced expression between X chromosome and autosomes, dosage compensation mechanisms have evolved in these groups. In *Drosophila*, the expression of X-chromosomal genes is enhanced in males to achieve the same level as in females (for a review, see Gilfillan *et al*. 2004); in mammals, the second X of females is inactivated to attain the same level as in males (for a review, see Payer and Lee 2008).

In *Megaselia*, X-chromosomal genes have active counterparts on the Y chromosome. Heterozygotes of sex-linked recessive alleles are wild type, no matter whether the wild-type allele is in the X or the Y chromosome. This holds for sex chromosomes from each of the three linkage groups (Mainx 1964). Although this does not prove the absence of dosage compensation, it suggests that dosage compensation is not required in *M. scalaris*.

The sex-determining pathway

Somatic sex-determination in *Drosophila* has been the paradigm for insects (for a review, see Cline and Meyer 1996). It is known now that only parts of the *Drosophila* sex-determining pathway are conserved in other insects. Nevertheless, it is the most intimately known case and, hence, all insect systems are studied with reference to it. In *Drosophila*, the primary sex-determining signal is generated by the interaction of a couple of transcription factors. They are encoded by X-linked 'numerator' genes, an autosomal 'denominator'

gene, and maternal genes. The primary decision is passed down in a cascade-like fashion to the genes that express sex-specific somatic functions. The primary signal regulates the expression of *Sxl*, the first step in the cascade. Sxl is a splice regulator which regulates its own splicing in an autofeedback cycle and that of *tra*, the next step of the cascade. Tra is also a splice regulator. It regulates, together with Tra2 and Rbp1, the splicing of the *dsx* transcript. As a consequence, two isoforms of Dsx are generated, the female-specific Dsx^F and the male-specific Dsx^M. They are transcription factors which regulate the expression of genes for sex-specific functions, e.g. those encoding yolk proteins.

An attempt was made to isolate the inferred primary sex-determining *Maleness* (*M*) gene of *Megaselia* from one of the new Y chromosome strains, 'Except1', by representational difference analysis (RDA). In this strain, *M* was the only expected genomic difference between females and males. The attempt failed, although a positive control (a mouse sequence added to the genomic DNA in the same molar concentration as the supposed *M*) was recovered by the procedure (W. Traut, unpublished data). Thus, the nature of *M* is still unknown.

Sxl, the next step in *Drosophila's* sex-determining cascade, is conserved in *Megaselia*. Several splice variants have been identified but none of them was sex-specific. Therefore, it is unlikely that *Sxl* is part of the sex-determining cascade in *Megaselia* (Sievert *et al*. 1997, 2000). *Megaselia* is not different from other non-Drosophilid insects in this respect. A sex-determining function of *Sxl* has only been found in *Drosophila* species. There are two paralogues of the gene in *Drosophila*, however: *Sxl* and *CG3056*. Both are orthologues of the single *Sxl* gene in other insects. But only *Sxl* is spliced sex-specifically in *Drosophila* while *CG3056* is not (Traut *et al*. 2006). The function of *CG3056* is not known yet. In *Megaselia*, hot spots of *Sxl* expression were found in the female and male gonads by *in situ* hybridization (Sievert *et al*. 2000).

The homologue of *tra*, the next step in *Drosophila's* sex-determining cascade, has not yet been isolated in *Megaselia*. The *tra* gene is known to have very low sequence conservation in insects (Pane *et al*. 2002). From the presence of a conserved Tra2/Rbp1/Tra binding site in the *Megaselia dsx*, however, one may deduce that *tra* is present and functioning in the sex-determining cascade (Kuhn *et al*. 2000). The *tra* gene is known to form the cell memory of the sexual status of an individual in flies other than *Drosophila*, e.g. *Anastrepha*, *Bactrocera*, *Ceratitis* and *Musca* (Pane *et al*. 2002; Lagos *et al*. 2007; Ruiz *et al*. 2007; Hediger *et al*. 2010).

The last step in *Drosophila* sex-determining cascade, *dsx*, has a well-conserved orthologue with sex-specific splice products in *Megaselia* (Sievert *et al*. 1997; Kuhn *et al*. 2000). Thus, its sex-determining function also appears to be conserved, like in other insects investigated, e.g. *Apis*, *Bombyx*, *Ceratitis* and *Musca* (Suzuki *et al*. 2001; Saccone *et al*. 2002; Beye 2004; Hediger *et al*. 2004).

In *Drosophila*, *dsx* is known to upregulate the transcription of the yolk protein genes in females (Bownes and Nöthiger 1981). In *Megaselia*, three distinct yolk proteins can be distinguished by Western blotting and detection with antibodies against the *Drosophila* yolk proteins (M. Bownes and W. Traut, unpublished data) but there is no evidence yet for a regulation of yolk protein synthesis by *dsx*-encoded transcription factors.

Conclusion

The most conspicuous feature of the *Megaselia* case is sex chromosome turnover. Since no linked marker accompanies the change and since the rates are similar to those known from transposition events, the best explanation for the mechanism is transposition of a male-determining gene *M*. Yet, there is no direct evidence for it. An alternative, though more speculative interpretation is that (i) female sex determination requires a couple of unlinked genes in double dosage and (ii) insertional inactivation of any of these genes by a transposon creates a male-determining condition in the respective chromosome, i.e. creates a new and non-homologous '*M*' and thereby a new Y chromosome. If this were true, it could explain the failure to extract *M* by RDA. Knowledge of the illusive *M* and the mechanism that causes sex chromosome turnover is highly desirable. This holds not only for *Megaselia*. The primary sex-determining signal is only known from two insect species yet, *Drosophila* and the honeybee (Cline and Meyer 1996; Beye 2004).

In a similar case, the old Y chromosome is being substituted by former autosomes or even the X-chromosome as the male-determining chromosome in many field populations of the housefly, *Musca domestica* (see Dübendorfer *et al*. 2002). In a recent paper, Hediger *et al*. (2010) show that *Mdtra*, the orthologue of *Drosophila tra*, forms an autofeedback cycle which serves as the cell memory of the sexual status in the housefly. The primary male determinant *M* in old and new Y chromosomes is thought to act by disrupting the female cycle of *Mdtra*. Like in *Megaselia*, the nature of *M* is still unknown. Hediger *et al*. (2010) suggest that transposition of the original *M* or generation of new '*M*'s by mutation of unrelated genes may be the basis for the replacement of Y chromosomes.

Whatever the mechanism is: why should a species have a selective advantage from a regularly occurring sex chromosome turnover? One good reason may be the adaptive value of different Y chromosomes under changing environmental conditions (Springer 1967). Recurrent sex chromosome turnover at a low rate will allow rapid adaptation without much fitness sacrifice. Another reason may be the replacement of deteriorating Y chromosomes. In the absence of dosage compensation, loss-of-function mutations may be difficult to tolerate in a Y-specific non-recombining region. The ability to create a new Y chromosome, even from the former X, would overcome this problem.

Megaselia exemplifies the ease with which the primary signal can be changed in evolution while at the same time downstream steps of the sex-determining pathway are conserved. *Megaselia* is a promising model to further elucidate the primary sex-determining signal and the initial steps of sex chromosome differentiation and a unique system to explore the suitability of specific linkage groups as sex chromosomes.

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