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Genetics

Sex determination: A hypothesis based on noncoding DNA

(Banded krait minor satellite DNA/*Caenorhabditis elegans*/maleness factors/origins of sex)

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ABSTRACT **Certain recent models of sex determination in mammals, *Drosophila melanogaster*, *Caenorhabditis elegans*, and snakes are examined in the light of the hypothesis that the relevant genetic regulatory mechanisms are similar and interrelated. The proposed key element in each of these instances is a noncoding DNA sequence, which serves as a high-affinity binding site for a repressor-like molecule regulating the activity of a major "sex-determining" gene. On this basis it is argued that, in several eukaryotes, (*i*) certain DNA sequences that are sex-determining are noncoding, in the sense that they are not the structural genes of a sex-determining protein; (*ii*) in some species these noncoding sequences are present in one sex and absent in the other, while in others their copy number or accessibility to regulatory molecules is significantly unequal between the two sexes; and (*iii*) this inequality determines whether the embryo develops into a male or a female.**

Sex determination in *Drosophila melanogaster* (1–4) and *Caenorhabditis elegans* (5) appears to be based on interactions among a small number of genes, with the level of activity of one gene being of primary importance. Consequently, it becomes feasible to look upon the choice of sexual phenotype in development as being mediated through a kind of genetic switch that opens up one or the other of two alternative pathways. If so, one is then led to inquire about the nature of the regulatory elements that might constitute such a switch.

If sex determination is based on a small number of critical genes and on an "either/or" mechanism, it is reasonable to expect both single-gene mutations and, occasionally, environmental perturbations to shift sexual development from one pathway to the other. Mutations that transform sex in this manner are known in *D. melanogaster*, in *C. elegans*, and in mammals. In some reptiles sex is determined by the temperature at which egg incubation occurs. The sharp temperature thresholds seen in these experiments (6, 7) and the absence of intersexes or significant egg mortality suggest that not only a small number of genes might be involved but also a high degree of cooperativity in the relevant regulatory processes.

On the basis of a recent model for mammalian sex determination (8, 9) and related results (2), I wish to suggest that certain noncoding DNA sequences could be responsible for the choice of sexual phenotype during development. The term "noncoding" sequence is used in this paper to mean that the DNA sequence is not the structural gene for a sex-determining protein. Rather, its role in sex determination is to bind a repressor-like molecule and, thereby, to regulate indirectly the activity of a "sex-determining" gene. "Repressor" denotes a regulatory molecule that has high affinity for the noncoding sequence(s) but whose primary role is in controlling the activity of a major sex-determining gene.

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THE MODELS

Mammals. There are at least three observations that must be faced by any model of primary sex determination in mammals: (*i*) the failure to map, in over 20 yr of search by the methods of classical genetics, any structural gene with male-determining properties on the Y chromosome of mouse or man (8, 9); (*ii*) the origin of certain XX males from mutant genes transmitted through females (10, 11), which suggests that a Y chromosome is not indispensable for male differentiation; and (*iii*) the occurrence of fertile XY females in *Myopus schisticolor* (12), which demonstrates that the presence of a normal Y chromosome does not guarantee a male phenotype. A genetic regulatory model that takes into account these and other results has been suggested (9) (Fig. 1*a*). The key element in the model is a multicopy, noncoding sequence. This sequence is assumed to be carried on the Y chromosome, and its function is to bind with high affinity a repressor of autosomal origin, which occurs in limiting concentrations. In the absence of the sink provided by the Y chromosome, the repressor would bind to a testis-determining gene (designated *Tdx*) on the X chromosome, for which it has lower affinity than for the Y chromosome-linked (Y-linked) noncoding sequences. As a consequence, transcription of *Tdx* would occur in XY cells but not in XX or X0 cells. The product of the *Tdx* gene (TDX) is assumed to be essential for determinination of the male sex. When TDX synthesis does not occur, as in XX and X0 embryos, development would proceed in the direction of the "constitutive" female sex.

XX mice carrying the "sex-reversed" property (X*Sxr*) develop as males (17). However, mice heterozygous for the X*Sxr* chromosome and the Searle translocation (T16H/X*Sxr*) can be female (18, 19) because the translocation is preferentially active and the *Sxr*-carrying X chromosome is inactive. This secondary sex reversal has been attributed to inactivation of the "male-determining *Sxr* sequences" (19) on the X*Sxr* chromosome. According to the present model (9) (Fig. 1*a*), these mice develop as females because the condensed state of the *Sxr*-carrying X chromosome prevents the *Tdx* repressor from binding to the *Sxr* sequences in spite of their affinity for each other being normally high. The repressor binds instead to *Tdx* on the active X chromosome, a site for which it normally has lower affinity than for *Sxr*. Thus, synthesis of TDX is blocked, and gonadal differentiation reverts to the female pathway. The present model is thus not dependent on a protein synthesized from the Bkm DNA-related *Sxr* sequences. (For other views about the role of Bkm DNA in sex determination, see refs. 13 and 20–23).

Certain recent discussions concerning the mechanism of sex determination in mammals have centered around the "H-Y antigen" (24, 25). One of the models suggested for sex-specific regulation of this antigen involves an X chromo-

Abbreviations: Bkm, banded krait minor satellite; X/A, ratio of X chromosomes to the sets of autosomes; X- and Y-linked, X and Y chromosome-linked.

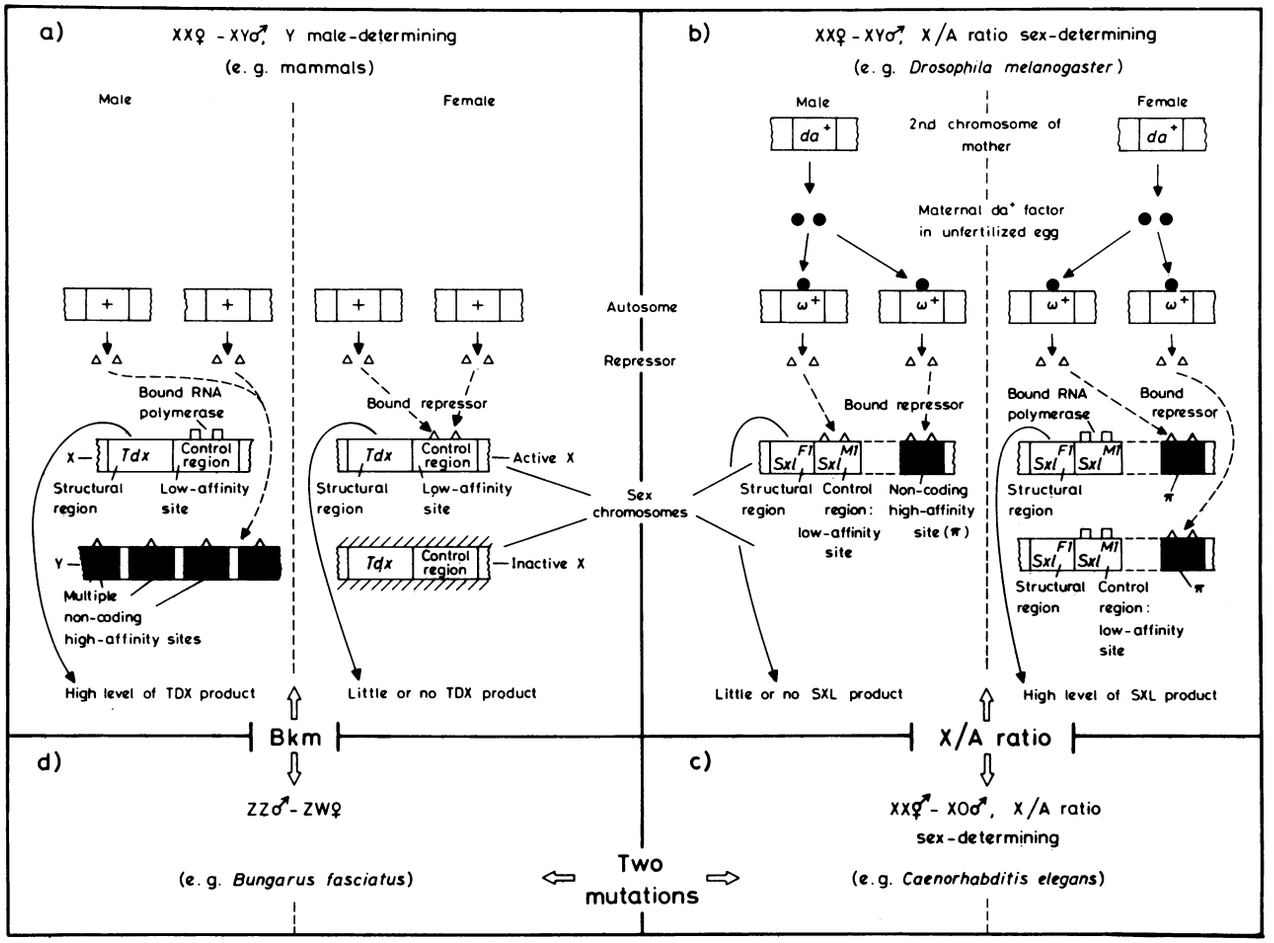

FIG. 1. Mechanistic models of sex determination in mammals (*a*) and *D. melanogaster* (*b*) and their relationship to an XX♀–X0♂ system (e.g., *C. elegans*) (*c*) and a ZZ♂–ZW♀ system (e.g., birds and snakes) (*d*). The solid rectangles on the sex chromosomes represent DNA sequences postulated to be noncoding as defined in the text; their role in sex determination is to bind with high affinity the repressor of a sex-determining gene. The repressor is expected to occur in limiting concentrations in both sexes. The large open arrows connecting *a* with *d*, *b* with *c*, and *c* with *d* indicate, respectively, that the mammalian type of sex determination can be related to that in birds and snakes through Bkm DNA (13), that the *D. melanogaster* system can be related to the *C. elegans* system through the X/A ratio, and that a ZZ♂–ZW♀ system can be derived from an XX♀–X0♂ system with as few as two mutations (14). (*a*) XX♀–XY♂ (Mammals): A model for sex-specific regulation of a postulated testis-determining gene (*Tdx*) on the X chromosome (after ref. 9). A limited quantity of a repressor, synthesized by an autosome, competes with RNA polymerase for *Tdx* and the multiple, high-affinity sites on the Y chromosome. The repressor has higher affinity for the Y-linked, noncoding sequences than for *Tdx*. In males, all available repressor is bound by the Y chromosome, thus permitting RNA polymerase to initiate transcription of *Tdx*. When the Y-linked high-affinity sites are absent, as in XX cells, repressor binds to *Tdx* on the active X and prevents its transcription. (*b*) XX♀–XY♂ (*D. melanogaster*): A model for measurement of the X/A ratio (the number of X chromosomes to the number of sets of autosomes) in *D. melanogaster* (after ref. 2). The Y chromosome plays no role in sex determination; therefore, it is not shown in the diagram. The da^+ factor (●) is produced by the maternal gene da^+ and stored in the egg. After fertilization, this factor binds to a specific autosomal site, ω, in the embryo, resulting in the production of a small quantity of repressor (△). In the male (left half of *b*), there is one X chromosome and, therefore, only one set of low-affinity *Sxl* and high-affinity π sites. The repressor binds to both of these sites. As a result, on average little or no RNA polymerase (□) binds to *Sxl* and little or no Sxl^+ product (SXL) is synthesized. In the female (right half of *b*), there are two X chromosomes but the same quantity of repressor as in the male, and this quantity is just sufficient to bind the high-affinity π sites. RNA polymerase binds to *Sxl* and initiates synthesis of SXL. Females are viable at high levels of SXL and males at low levels. As a result of interactions postulated to occur among *Sxl*, π, and ω, transcription from the *Sxl* site would be proportional to the X/A ratio such that the levels of SXL are low in males, high in females, and intermediate in the intersexes. (*c*) XX♀–X0♂ (*C. elegans*). Wild-type animals are either male or hermaphrodite. However, a single-gene mutation can convert an XX hermaphrodite into an XX female (5). Similarities between the system in *D. melanogaster* and that in *C. elegans* are discussed in the text. As few as two mutations convert the XX♀–X0♂ system of *C. elegans* to a ZZ♂–ZW♀ system (14). (*d*) ZZ♂–ZW♀ (birds and certain snakes, including *Bungarus fasciatus*). Banded krait minor satellite (Bkm) DNA is a repetitious DNA originally isolated from *B. fasciatus*. This DNA is localized almost exclusively in the W chromosome of this snake. Bkm DNA-related sequences occur in the Y chromosome of the mouse. The *Sxr* mutation in mouse provides genetic evidence that these Bkm DNA-related sequences are in some way male-determining (13). In some birds and snakes, sex determination may be based on the Z/A ratio rather than a dominant W chromosome (15).

some-borne repressor locus (25, 26). However, because these models have not been clearly defined (25) and because they do not address the three major observations referred to in the beginning of this section, they are not discussed here. Moreover, serious doubts have been raised about the role of this antigen in primary sex determination (27).

D. melanogaster. Sex in *D. melanogaster* is determined by the ratio of the number of X chromosomes to the number of sets of autosomes (X/A). Genes concerned with measurement of the X/A ratio appear to act early in the pathway that leads to sex determination (1, 3). A model for X/A ratio measurement has been proposed by Gadagkar *et al.* (2) (Fig. 1*b*), and it rests on the genetic analysis of three sex-determination genes, *da*, Sxl^{M1} and Sxl^{F1} (1, 3). An essential element in

the model is a DNA sequence on the X chromosome, referred to as the π site. I consider this site to be noncoding as defined earlier. π is critical to the process of X/A ratio measurement because, although nearly all other relevant loci are expected to be present equally in both sexes, this sequence and the gene *Sxl*, both X chromosome-linked (X-linked), would be present in females in twice the number of copies as in males (Fig. 1*b*). If the high-affinity π and low-affinity *Sxl* sites compete for limiting concentrations of a repressor, it can be shown that under certain plausible binding conditions, transcription from the *Sxl* site would be proportional to the X/A ratio such that the levels of *Sxl* product (SXL) are low in males and high in females. If, as proposed by Cline (1), SXL is an inhibitor of X-chromosome activity, dosage compensation also would occur (2).

C. elegans. Males have two sets of autosomes and a single X chromosome (AAX0), whereas hermaphrodites are AAXX, there being no naturally occurring females. Sex is dependent, as in *D. melanogaster*, on the ratio of the number of X chromosomes to the number of sets of autosomes (28) (Fig. 1*c*). When small X-chromosome duplications are added to a AAAXX complement (phenotype:male), there is an increase in the X/A ratio and a corresponding shift in the sexual phenotype towards hermaphroditism. These results have led to the conclusion that there are on the X chromosome "at least three (and perhaps many more) dose-sensitive sites that act cumulatively in determining sex" (28).

Although the sex chromosome constitution of *D. melanogaster* is XX♀–XY♂, this is in effect XX♀–X0♂ because the Y has no influence on sex determination. Thus, *D. melanogaster* and *C. elegans* can be said to have similar sex-chromosome constitutions. In both species, (*i*) sex determination is based on the X/A ratio; (*ii*) the product of a particular structural gene is deemed necessary for determination of the female sex, and its absence, for that of the male sex (Fig. 1*b*) (2, 5); and (*iii*) the addition of small X-chromosome duplications to a male or intersexual genome shifts the phenotype in the direction of femaleness (28). In *D. melanogaster*, most X-linked genes are dosage-compensated (4). Recent work suggests that in *C. elegans* also X-linked genes are subject to dosage compensation (29). Further evidence of similarity between the two systems comes from the major sex-determination genes. The gene *dpy-26* of *C. elegans*, for example, appears analogous to the *daughterless* gene of *D. melanogaster* (16). In terms of its effect on sex determination, the *tra-1–her-2* complex in *C. elegans* is remarkably similar to the Sxl^{F1}–Sxl^{M1} complex of *D. melanogaster*. However, it should be noted that, in the model of Gadagkar *et al.* (2), the *Sxl* locus is a component of the process that leads to X/A ratio measurement, whereas Hodgkin (5) places *tra-1* subsequent to X/A ratio measurement. Quantitative modeling of the *C. elegans* system might permit clarification of this point as well as recognition of further analogies between the two sex-determination systems. Thus, although the mechanism by which cells of *C. elegans* measure the X/A ratio is not understood, there appears to be sufficient correspondence between *D. melanogaster* and *C. elegans* in this respect to permit extrapolation of the *D. melanogaster* model (Fig. 1*b*) to the question in *C. elegans* (Fig. 1*c*), but with one modification. This modification is necessitated by the fact that, although the X chromosome of *C. elegans* is sex-determining in a karyotypic sense and is the most thoroughly mapped of the six chromosomes, it does not carry any of the seven major sex-determination genes so far detected in this organism (5, 16). All seven of them are on autosomes. The failure to find sex-determining genes on the X chromosome of *C. elegans* is reminiscent of the situation in mammals just discussed.

How does the X chromosome of *C. elegans* determine sex and still remain mutationally silent for such genes? I suggest that there are multiple, sex determining but noncoding sequences on the X chromosome and that these provide high-affinity binding sites for the repressor of a major, autosomal sex-determining gene. This autosomal gene is expected to participate in X/A ratio measurement, and the critical inequality between embryos that develop into males and those that develop into hermaphrodites would be in the copy number of the postulated noncoding sequences in the X chromosome. This copy number difference leads to significantly different consequences in terms of X/A ratio measurement and, therefore, sex determination. This interpretation is consistent with Madl and Herman's conclusion (28) that there are several dose-sensitive sites on the X chromosome of *C. elegans* that act cumulatively in sex determination.

Haplodiploidy. The genetic basis of sex-determination by haplodiploidy has been investigated in several insects, in particular *Habrobracon* (30), the honey bee (31), and the coccids (see ref. 32 for a review). There are no sex chromosomes and, in general, haploid embryos develop into males and diploids develop into females. In honeybees unfertilized eggs develop into males and fertilized eggs develop into females. Among certain coccids, although fertilization is necessary for initiating embryonic development, haploidy is subsequently induced in some embryos by elimination of the father's chromosomes in a large proportion of embryonic cells; such embryos develop into males. In both of these systems, there is no evidence for heterozygosity-dependent mechanisms of sex determination.

The generally accepted interpretation is that, with the possible exception of *Habrobracon* (30), sex determination in haplodiploid systems is the result of the action of genes with additive effects (33). Haploids would have *n* such genes, and diploids, 2*n*. It appears possible to understand the basis of such additive gene action in terms of the models illustrated in Fig. 1. It is necessary, again, to assume that (*i*) a repressor of a structural gene with sex-determining properties occurs in limiting concentrations and in equal amounts in all embryos; (*ii*) the sex-determining gene as well as a number of copies of a noncoding sequence compete for this repressor, and (*iii*) the repressor has greater affinity for the noncoding sequence than for the sex-determining structural gene. Since diploid embryos would be expected to have twice the number of these high-affinity repressor-binding sequences as haploid embryos, a set of plausible binding conditions can be defined under which the product of a sex-determining gene would be synthesized in diploids but not in haploids (unpublished data). To obtain such a result in the coccid system in which inactivation rather than elimination of a haploid set occurs, it is necessary to assume that the high-affinity sequences are inaccessible or unresponsive to the repressor when the chromosomes are in a condensed, inactive state (9) (see below).

EVOLUTIONARY RELATIONSHIPS

As judged from chromosome constitution, sex-determining mechanisms would appear to show wide variation among eukaryotes, sometimes within the same family (33), but it is my contention that this apparent variety of sex-chromosome types probably masks a basic unity in the genetic and molecular basis of sex determination. Such an assumption, while seemingly at variance with the results of karyotype cytology, is consistent with certain recent views that sex may have had a polyphyletic origin among unicellular organisms but not among the Metazoa (34). It has even been argued that sex is monophyletic and that it predates the evolution of eukaryotes (35). In any case, if there is a homology between sex-determining mechanisms among eukaryotes, it should be possible to derive one sex-chromosome system from another with very few mutational steps or to demonstrate otherwise

a close relationship. The following results show that this is possible.

The Bkm Sequence. Bkm DNA was originally isolated as a minor satellite from females of a poisonous Indian snake, the banded krait (13). When radiolabeled Bkm DNA is hybridized to metaphase preparations of this snake, the label is almost exclusively localized to the W chromosome. This DNA also hybridizes to the W chromosome of birds. The mammalian Y chromosome is positive for a Bkm-related sequence. The *Sxr* mouse provides genetic evidence that this Bkm-related sequence is male-inducing in some manner (13). Homologous or homeologous DNA is found in a number of other eukaryotes, from yeasts to man (36). In several eukaryotes this DNA is quantitatively specific to one or the other sex. Among certain snakes, the evolutionary emergence of a morphologically distinct W chromosome is correlated with a 5- to 10-fold increase in Bkm copy number in the genome (36). I have conjectured previously (9) that in mammals, the postulated noncoding sequences responsible for primary sex determination are Bkm-related.

In considering possible roles for Bkm DNA in sex determination, we are confronted with the curious situation that in three of the four types of sex determination illustrated in Fig. 1, none of the chromosomes traditionally considered sex-determining (the X of *C. elegans*, the Y of mammals, and the W of birds) is known to carry structural genes with sex-determining properties (5, 9, 43). In *D. melanogaster*, the X chromosome carries a strongly Bkm-positive region, but this region is away from *Sxl*, the only major sex-determining gene known on the X chromosome, suggesting that Bkm may be noncoding in this species.

In *D. melanogaster* (37) (as in *C. elegans*), addition of small X-chromosome duplications to an intersexual (or male) genome shifts the phenotype towards femaleness, and there is a correlation between the size of the duplication and the extent of femaleness. These results suggest that female-determining genetic elements are distributed at many sites along the X chromosome and that they act cumulatively. I favor the view that these multiple, female-determining elements consist of regulatory rather than structural sequences. If so, ancillary questions arise. Do these female-determining sequences collectively perform the function of the π site in Fig. 1*b*? If Bkm has a role in sex-determination, what relationship is there, if any, between these multiple female-determining elements and the Bkm-positive site? Whether there is a single, localized region of such putative noncoding sequences or whether they are distributed at several points along the X chromosome, the essential requirement of this model is that there should exist two sets of such sequences in the female but only one in the male. X-linkage of these sequences appears to satisfy this requirement.

Different Chromosomal Systems of Sex Determination Are Closely Interrelated. Hodgkin's observation (14) in *C. elegans* (wild type:XX♀–X0♂) that it is possible to derive, by as few as two single-gene mutations, a stock formally equivalent to a ZZ♂–ZW♀ sex-chromosome system demonstrates (*i*) the close phylogenetic relationship between the XX–X0 and ZZ–ZW systems (Fig. 1*d*), (*ii*) that a sex-determination system based on the X/A ratio can change over to another not dependent on this ratio, and (*iii*) that a system based on male heterogamety can readily shift to one based on female heterogamety. Winge, as well as Bellamy (see ref. 38), had earlier observed a changeover from male heterogamety to female heterogamety in certain species of fish. Male heterogamety is characteristic of most populations of *Chironomus tentans* (Diptera) but female heterogamety has been reported in some populations, and crosses between the two types are fertile (39). Additional evidence for the close relationship between XX–XY and ZZ–ZW systems comes from amphibians in which both systems sometimes occur within the same family (40). In certain other amphibians, morphologically distinct sex chromosomes are not recognizable in either sex. In one such species, *Rana clamitans*, sex-linked inheritance of an enzyme marker provides (*i*) evidence for male heterogamety and (*ii*) evidence that one of the chromosomes carries a male-inducing element (41). Therefore, the system in *R. clamitans* can be looked upon as a primitive XX♀–XY♂ system in which a morphologically distinct Y has not emerged.

Among coccids, the XX♀–X0♂ sex-chromosome system is clearly ancestral to the haplodiploid systems because it is found in morphologically primitive coccid families as well as in other, closely related homopterans (42). Since evidence from *C. elegans* (28) suggests that sex determination in XX♀–X0♂ organisms is based on the X/A ratio, it appears likely that in coccids haplodiploidy evolved from organisms in which sex determination was based on the X/A ratio.

DISCUSSION

In terms of this hypothesis, certain noncoding DNA sequences control the male/female switch during development and thus the choice of sexual phenotype in a developing embryo. One of the two sexes does not carry these particular noncoding sequences or carries them in a significantly lower copy number or in a manner inaccessible or unresponsive to the repressor of a sex-determining gene. The system in *C. elegans* seems particularly instructive in this regard (5). All of the major sex-determining genes so far identified in this species are autosomal and, therefore, are expected to be present equally in both sexes. The only apparent inequality between males and hermaphrodites is in the number of X chromosomes, but, significantly, the X chromosome to date has remained mutationally silent for sex determining genes. Genetic methods have not so far permitted detection of a structural gene with sex-determining properties on two other well-studied sex chromosomes—the Y of mammals (8, 9) and the W of *Gallus domesticus* (43). The present hypothesis provides a plausible explanation for this apparent anomaly.

Mutations in Noncoding Sequences. Mutations in the postulated noncoding sequences in sex chromosomes would not be readily detectable if such sequences occurred in multiple copies. The copy number of such sequences relative to the amount of repressor would be expected to have a bearing on the probability with which mutations are expressed. If there are significantly more copies of the noncoding sequences than are necessary to bind all available repressor, mutations would accumulate in them. A mammalian Y chromosome would be expected to cease to be male-determining when its capacity to bind repressor falls below a certain critical level because of accumulated mutations. If several mutations are required to make the Y chromosome ineffective in sex determination, the frequency of such ineffective Y chromosomes in a population would be lower than the frequency of an equivalent but nonfunctional (mutated) single-copy gene. Furthermore, because the most obvious phenotypic consequence of such mutant Y chromosomes would be on the sexual phenotype itself, one would expect to see an immediate loss of fitness and, therefore, a failure to transmit such chromosomes to the next generation. Loss of fitness should become apparent only when mutation reduces the effective copy number of the noncoding sequences to below critical level; that is, the system as it has evolved should be fine-tuned, with a lower as well as an upper limit on copy number. One consequence of these conditions is that it would be difficult to recover and maintain such mutant Y chromosomes in laboratory mammals. For these reasons, it might appear that the mammalian Y chromosome is mutationally silent for sex-determining genes.

Noncoding DNA and Origins of Sex. Using population ge-

netic models, Hickey (44) has shown that noncoding, transposable DNA sequences that induced sexuality in organisms carrying them would spread in populations. On the basis of this result, he has proposed that genes controlling sex may have been selected originally as transposable elements from among selfish DNA (45–47). This hypothesis provides one possible mechanism for the origin of sex, a continuing puzzle in evolutionary theory (48). Another attractive feature of Hickey's hypothesis is that, under conditions that promote outbreeding, a transposing sequence could spread rapidly in populations even if it were noncoding and made a negative or zero contribution to fitness. In other words, selfish, transposable sequences may be an inevitable consequence of "nonphenotypic" selection (46, 47). If one such element becomes linked with sex, or itself induces sex in some manner, it will spread intergenomically and may, through mutations, eventually come under the purview of phenotypic selection (44). Mobile genetic elements are known to determine sex in a few organisms. Examples include the transposon that controls mating type in yeast (49) and the mobile maleness factors in *Megaselia* and certain other insects (50).

Inaccessibility or Unresponsiveness of Noncoding DNA in Condensed, Inactive Chromatin to Repressor-Like Molecules. In terms of the present model, inactivation of a haploid set of chromosomes leads to male determination in mealybugs because the copy number of the regulatory sequences available for binding the repressor becomes halved. It is expected that, concurrently, the effective copy number of structural genes concerned with sex determination also would be halved by chromosome inactivation. However, we consider inactivation of the noncoding regulatory sequences as the more significant event for sex determination. In other words, inactivation of structural genes would not lead, by itself, to sufficient disparity between male and female embryos in respect of a critical sex-determining gene product. This implies that regulatory sequences in condensed chromosomes are inaccessible or unresponsive to repressor-like molecules, although at present there is no evidence from molecular biology that bears on this assumption. However, as noted earlier, certain genotypically male mice (X*Sxr*/T16H) can develop as females because of preferential inactivation of the X*Sxr* chromosome. In order to interpret these results within the framework of the present model (Fig. 1*a*), it is necessary to assume that the X*Sxr* chromosome as well as the normal X chromosome are unresponsive to the repressor of *Tdx* when they are heterochromatic and inactive (9).

Inactivation of one of two homologous chromosomes is known only among coccids and mammals. In coccids such inactivation is clearly associated with determination of the male sex. Therefore, it is reasonable to ask whether the evolution of X-chromosome inactivation in mammals is in some way related to sex determination. This possibility will be examined in another publication.

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