Perspectives

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The charms of sex chromosomes in snakes

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1. Introduction

Nearly all vertebrates have morphologically separate male and female sexes, but sex is determined in different ways. In mammals there is male heterogamety XY♂/XX♀, whereas in birds there is female heterogamety ZW♀/ZZ♂. A large number of organisms do not have morphologically distinct sex chromosomes, and yet their sex is genetically determined and the 1:1 sex ratio is maintained. In many species of reptiles, environmental factors such as temperature play an important role in determining the sex ratio (Head *et al.* 1987). For example, in alligators, crocodiles and turtles, during the early stages of embryogenesis, temperature has a determining effect on the sex ratio. In amphibians, the sex ratio is usually determined genetically. Thus, the existence of specialized sex-determining chromosomes poses a range of fascinating and fundamental questions, although few of them have been satisfactorily answered. Why, for example, do some species show morphological differentiation of sex chromosomes but not others? If the sex ratio can be maintained by a set of genes, as in the case of amphibians, then what is the need for specializing one entire chromosome exclusively for this singular sex determination function and maintenance of sex ratio at such an enormous cost that most genes, barring a few specifically involved in sex determination, have been lost from the sex-determining chromosome? And to compensate for this, why did yet another mechanism of dosage compensation have to be invented? What is the mechanism involved? Can we answer these questions?

2. Sex chromosomes in snakes

Snakes offer a unique system representing various states of differentiation of sex chromosomes. Except in snakes belonging to the primitive family Boidae, the W chromosome of all other species, irrespective of whether they are morphologically highly differentiated or not, is late or early (asynchronous) replicating (Ray-Chaudhuri and Singh 1972), deeply stained by C-banding (Singh and Ray-Chaudhuri 1975), and forms a W-chromatin body (Singh 1972; Singh *et al.* 1976). The observation that the entire W chromosome of the poisonous species *Bungarus caeruleus* (common Indian krait), *Bungarus walliwall* and several others was exclusively and entirely deeply stained by the C-banding technique suggested that it may contain W-chromosome-specific repetitive (satellite) DNA, which could possibly be isolated by analytical equilibrium-density-gradient centrifugation. This was based on the established fact that the C-band-positive centromeric region of all the chromosomes in human and mouse contained satellite DNA. As the facilities to carry out this work were not available in India, I went abroad to test the idea that the W chromosome must contain W-specific satellite DNA. Dr KW Jones, at the Institute of Animal Genetics, University of Edinburgh, UK, was among the early pioneers of the technique of *in situ* hybridization, and is internationally well known for his work on satellite DNA in human and chimpanzee (John *et al.* 1969; Jones 1970; Jones *et al.* 1973, 1974). Luckily, I was selected for the Commonwealth Fellowship, which enabled me to join his laboratory in September 1974. Snake tissues preserved in 70% ethanol, which I had carried with me, unfortunately proved to be useless for isolating satellite DNA. I spent several months without any success. Professor SP Ray-Chaudhuri, under whose supervision I had done my PhD, then made untiring efforts to send a consignment of live poisonous and non-poisonous species of snakes to me by British Airways.

Keywords. C-band-positive chromosomes; sex-specific satellite DNA

Figure 1. Banded krait.

3. Sex-chromosome-specific satellite DNA

The live snake samples gave me the break I was looking for – successful isolation of good-quality DNA. By using analytical equilibrium-density-gradient centrifugation of total male and female DNA in $Ag^{+}Cs_2SO_4$ gradient, we observed four satellite peaks, two of which were common to both males and females, and two were female-specific (Singh *et al.* 1976, 1980). *In situ* hybridization of female-specific satellite DNA localized, as predicted, predominantly on the entire W chromosome, providing direct and conclusive evidence of the existence of W-sex-chromosome-specific satellite DNA in snakes. This was the first report of isolation of a chromosome-specific satellite DNA in any species of animal or plant (Singh *et al.* 1976). As it was isolated from the banded krait (*Bungarus fasciatus*, figure 1) we designated it as banded krait minor (Bkm) satellite DNA (Singh *et al.* 1980).

My hunch about the existence of W-sex-chromosome-specific satellite DNA was based on the observation that, in many species of snakes, the W chromosome is exclusively C-band-positive (Ray-Chaudhuri *et al.* 1971; Singh and Ray-Chaudhuri 1975). Although this hunch was correct in one sense, it was wrong in the sense that there were other satellite DNAs present in the snake genome which were shared by both the sexes, although no other chromosome or chromosomal region was C-band-positive. If I had observed C-band-positive regions on other chromosomes as well, perhaps I would not have even attempted to isolate sex-specific satellite DNA. Thus, the simple observation of the exclusive existence of C-banding on the entire W chromosome led me on my obsessive pursuit, which could have been wrong.

Our subsequent studies demonstrated that Bkm sequences are highly conserved in eukaryotes (Singh *et al.* 1981) but are absent in prokaryotes. By using the Bkm probe, we could identify the W chromosome in all the species of snakes belonging to the highly evolved as well as the intermediate groups that possessed the C-band-positive W chromosome. However, we failed to identify the W chromosome in primitive snakes such as python (figure 2) or eryx by using any of the sex-chromosome-specific DNA probes available at that time (Singh *et al.* 1976).

4. Bkm (GATA) repeats in mammals

Having established that Bkm sequences are conserved in snakes and other vertebrates (Singh *et al.* 1980), we asked whether they had a role in the determination of sex. To answer this question, we performed an experiment that no one even in their wildest imagination would have done. We hybridized Bkm sequences both on Southern blot as well as on metaphase chromosomes of male and female as well as sex-reversed (XX_{sxr}) male and carrier (XY_{sxr}) male mice by *in situ* hybridization. The results were astonishing. Southern hybridization revealed a male-specific pattern, irrespective of the presence of the Y chromosome ($XY♂$ and $XX_{sxr}♂$).

Figure 2. The author holding a python.

In situ hybridization localized Bkm sequences in the short arm of the Y chromosome. In the case of sex-reversed (XX_{sxr}) male, it predominantly hybridized at the telomeric region of the long arm of one of the X chromosomes. The carrier male mouse (XY_{sxr}) revealed duplication of the Bkm-positive Y short arm and its translocation to the distant region of the mouse Y long arm. Detailed study of meiosis in the carrier mouse by *in situ* hybridization revealed regular crossing over between one chromatid of the X and one chromatid of the Y chromosome, transferring the duplicated Bkm-positive (Sxr) region of the Y chromosome to one chromatid of the X. This satisfactorily explained how meiotic segregation always produces one-fourth of the progeny with sex reversal (XX_{sxr} male). In one stroke this demolished the earlier conjecture that the sex-reversal gene in mouse could be a dominant autosomal mutation. These findings (Singh and Jones 1982; Singh *et al.* 1984) led to the conclusion that a very small region of the mouse Y chromosome was necessary and sufficient to convert a female mouse into a male. It also led to the demonstration of the existence of a pseudo-autosomal region in the X as well as the Y chromosome, where regular crossing over takes place to ensure their proper segregation. This further led to the detection of a similar phenomenon of sex reversal in humans (Gubbay *et al.* 1990; Koopman *et al.* 1991; Goodfellow and Lovell-Badge 1993). However, when we cloned and sequenced Bkm satellite DNA, it was found to be predominantly made up of tetranucleotide repeat GATA, which does not code for any protein. The fact that the Bkm satellite DNA was highly conserved and was found in all eukaryotes, associated predominantly with sex chromosomes, strongly suggested its functional significance.

5. Evolution and differentiation of sex chromosomes in snakes

In the primitive family of snakes, the sex chromosome pair is homomorphic in both the sexes and the presumptive W chromosome has not undergone heterochromatinization. In more advanced species, although the Z and W chromosomes are homomorphic in both the sexes, the W chromosome in the female has already undergone heterochromatinization. In highly evolved species, the W chromosome has not only undergone heterochromatinization but has also become heteromorphic, and is much smaller than the Z chromosome. The Bkm sequences are interspersed along the length of the W chromosome even in those species in which the Z and W chromosomes are similar in size and morphology (homomorphic) but the W chromosome has undergone heterochromatinization. This suggests that the quantitative evolution of Bkm sequences occurred early on in the evolution of chromosomal sex determination (CSD).

Inactivation of the W chromosome, which prevented both its somatic expression and meiotic exchange, could provide an explanation both for the accumulation of Bkm sequences and for the subsequent rapid morphological evolution of the W chromosome. This raises several fundamental questions: How and when did the functional specialization of the W chromosome originate? How did it progress to morphological differentiation? And why was it skipped in some species? The change at the DNA level, which established a strong genetic control of the sex ratio, might have involved primarily the W chromosome, predisposing it to continue to evolve in some snakes so as to attain the status of a specialized sex determiner.

6. The hijacking hypothesis

It is assumed that in species ancestral to those in which the W chromosome has continued to specialize, the sex determiner assumed control over an adjacent gene or centre involved in chromosomal condensation, which is present in every chromosome. This could have involved structural

alteration(s) such as an inversion in the W chromosome, which brought the sex determiner to the vicinity of the condensation centre. This is substantiated by the fact that in a large number of snake species, the Z chromosome from which the W chromosome has evolved is sub-metacentric, whereas the W is acrocentric (Singh 1972). Once this happened, a spreading effect or read-through from the sex gene was such that the chromosome condensed when it was turned off; the sex determiner thus fortuitously, but effectively, hijacked the entire chromosome (figure 3). This is substantiated by the fact that the W chromosome remains highly condensed and forms a W chromatin body in interphase nuclei of all somatic tissues of the female, but becomes highly decondensed in developing oocytes (Ray-Chaudhuri *et al.* 1971; Ray-Chaudhuri and Singh 1972; Singh and Ray-Chaudhuri 1975). The model postulates that, in some species belonging to the primitive family Boidae, the condensation centre was not located sufficiently close to the sex determiner so as to cause a similar interaction between them. This seems plausible as such an event occurs in one X chromosome in every mammalian female. This inactivated W chromosome had undergone extensive alterations in its DNA, involving amplification of GATA repeats (Jones 1983).

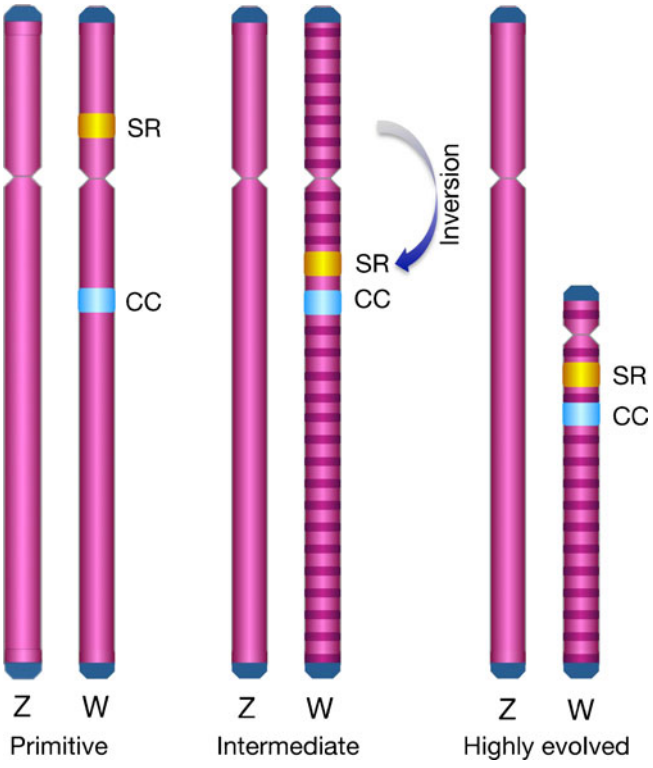

Figure 3. Diagrammatic representation of Z and W chromosomes of different species of snakes representing various states of differentiation of Z and W chromosomes as per their evolutionary status. In *primitive* species, the Z and W chromosomes are homomorphic. In the W chromosome only, the chromosomal condensation region is indicated by 'CC' in the long arm and the sex determining region 'SR' in the short arm. In the *intermediate* stage, although the Z and W chromosomes are homomorphic, the sex determiner 'SR' is now located in the long arm, very close to the condensation centre 'CC' due to inversion. This leads to heterochromatinization, and accumulation of Bkm 'GATA repeats', shown as distinct bands across the length of the W chromosome. Thus, the sex determiner hijacks the W chromosome. In *highly evolved* species, the W chromosome has lost most of the genes homologous to the Z chromosome and has become much smaller in size compared with the Z chromosome.

7. Proposal to test the hijacking hypothesis

It is possible to test the above hypothesis by making cell lines or by doing short-term blood cultures of female snakes representing the primitive, intermediate and highly evolved states of differentiation of sex chromosomes, preparing chromosome spreads, dissecting out the Z and W chromosomes of respective species by laser capture microscopy using a laser dissection microscope, or making a chromosome-specific library and directly sequencing the entire chromosome. This is expected to unravel the mystery of the molecular basis of the origin, evolution and differentiation of sex chromosomes. This is the mystery I would like to unravel by using the snake system again.

Sequencing of the Z chromosome and its homomorphic W chromosome will reveal the extensive homology between the two chromosomes and will, hopefully, identify the region involved in female sex determination. Similarly, sequencing of the W chromosome from the intermediate state of differentiation of the sex chromosome, in which the Z and W chromosomes are homomorphic but differentiated at the molecular level, will reveal sequential changes including the mutation and rearrangement that have taken place in the DNA. The highly differentiated W chromosome DNA, which has become much smaller than the Z during the course of evolution, will reveal the functionlessness and elimination of Z chromosome homologous genes, apart from very high accumulation of GATA repeats. The prediction of the accumulation of transposable elements, which might have aided in the distribution of GATA repeats along the length of the W chromosome and over the entire genome, will be elucidated. Thus, we may be able to determine the molecular mechanisms involved in the origin, evolution and differentiation of sex chromosomes, which are not possible in other groups of animals.

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