Chromosoma (Berl.) 59, 43–62 (1976)

CHROMOSOMA

Satellite DNA and Evolution of Sex Chromosomes

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Abstract. The satellite DNA (satellite III) which is mainly represented in the female of *Elaphe radiata* (Ophidia, Colubridae) has been isolated and its buoyant density has been determined ($\varrho = 1.700$ g cm^{-3}). In situ hybridisation of radioactive complementary RNA of this satellite DNA with the chromosomes of different species has revealed that it is mainly concentrated on the W sex chromosome and its sequences are conserved throughout the sub-order Ophidia. From hybridisation studies these sequences are absent from the primitive family Boidae which represents a primitive state of differentiation of sex chromosomes. Chromosome analysis and C-banding have also revealed the absence of heteromorphism and of an entirely heterochromatic chromosome in the species belonging to the primitive family and their presence in the species of highly evolved families. It is suggested that the origin of satellite DNA (satellite III) in the W chromosome is the first step in differentiation of W from the Z in snakes by generating asynchrony in the DNA replication pattern of Z and W chromosomes and thus conceivably reducing the frequency of crossing-over between them which is the prerequisite of differentiation of sex chromosomes. Presence of similar sex chromosome associated satellite DNA in domestic chicken suggests its existence in a wider range of vertebrates than just the snakes.

Introduction

The presence of constitutive heterochromatin in sex chromosomes is a wide spread phenomenon in vertebrates. In general the Y chromosome in mammals is constitutively heterochromatic and rich in satellite DNA with only a few exceptions, for example in calf and in mouse (Kurnit et al., 1973). In birds, however, invariably the entire W chromosome is positively stained by C-banding methods and is rich in repetitive DNA as has been shown in *Gallus domesticus* (Stefos and Arrighi, 1974). In a snake, the common Indian Krait, C-banding is exclusively localised in the W chromosome (Singh and Ray-Chaudhuri, 1975).

Such a consistent occurrence of constitutive heterochromatin in the W chromosome of snakes and birds and the Y chromosome of mammals perhaps reflects its biological significance rather than its coincidental occurrence. In order to understand the significance of the heterochromatin in sex chromosomes at the molecular level, snakes offer a particularly interesting system. They exhibit various states of differentiation of the Z and W chromosomes, apparently according to the evolutionary status of the families, being homomorphic in primitive families and well differentiated in highly evolved ones (Ohno, 1967; Ray-Chaudhuri et al., 1971; Singh, 1972). Ray-Chaudhuri et al. (1970) have convincingly demonstrated that somatic interphase nuclei of different tissues of heterogametic females of various species of snakes with a differentiated W chromosome possess a distinct darkly stained chromocenter comparable to the characteristic mammalian sex chromatin and have termed it "W chromatin". Ray-Chaudhuri et al. (1971) have further shown that nuclear sexing is possible not only in those species of snakes where the W chromosome is morphologically distinguishable from the Z, but also in those species where it is not so, but shows an asynchrony in the replication pattern. However, in primitive snakes belonging to the family Boidae the sex chromosomes are morphologically indistinguishable and do not show asynchrony in their DNA replication pattern (Ray-Chaudhuri and Singh, 1972). In these species there is no W chromatin in the interphase nuclei and hence no nuclear sexing is possible (Ray-Chaudhuri et al., 1971).

On the basis of such observations it has been suggested (Ray-Chaudhuri et al., 1971) that heterochromatinization rather than establishment of structural changes is the first step in differentiation of the W chromosome in snakes. Due to the previous lack of any direct evidence for the localisation of satellite DNA in the W chromosome in snakes, the heterochromatic nature of the W has been interpreted to mean that all heterochromatic DNA is not highly repetitious (Comings, 1972). However, because heterochromatinization of the W and morphological differentiation and evolution of sex chromosomes in snakes are interlinked we have been encouraged to analyse both male and female DNA separately on isopycnic gradients.

Materials and Methods

1. DNA and Satellite Preparation. Two male and two female adult individuals of the snake *Elaphe radiata* belonging to the family Colubridae were obtained from Thailand. Other snakes utilised in the present study were obtained from Thailand, Africa and India. *Xenopeltis unicolor* was obtained from a London dealer. DNA was isolated from liver, kidney, testis and heart tissues from each individual and processed separately by the method of Marmur (1961) with the inclusion of repeated phenol-chloroform, RNase and Pronase treatments. Satellite DNA was observed in Cs_2SO_4/Ag^+ gradients by the procedure of Jensen and Davidson (1966). The Ag^+ to DNA ratio for optimum separation was 0.20 (Fig. 1). Satellites I, II and III were isolated from the female and purified by successive Cs_2SO_4 and CsCl centrifugations in the M.S.E. 8×40 Ti rotor for 80 h at 32 K r.p.m. at $+25°C$. The buoyant densities were determined in neutral CsCl in the Spinco model E analytical centrifuge at $+25°C$, 44,000 r.p.m. for 20 h using *Micrococcus lysodeikticus* DNA ($\varrho = 1.731$ g cm^{-3}) as density marker. The buoyant densities were, I = 1.681, II = 1.693 and III = 1.700 g cm^{-3} respectively. Satellite IV was not isolated. Care was taken to avoid any contamination of satellite IV, which bands very close to the satellite III in the Ag^+/Cs_2SO_4 gradient (Fig. 1a), by successive Cs_2SO_4 and CsCl gradient centrifugation. Contamination of satellite IV DNA with satellite III, however, can not be ruled out unequivocally.

2. Transcription of Satellite DNA. cRNA of satellitee IIII DNA was prepared by using equimolar amount of ATP (S.A. 29 Ci/mM), UTP (21 Ci/mM), GTP (15 Ci/mM) and CTP (18 Ci/mM), 1–2 μg of satellite DNA and 2 units of *E. coli* DNA dependent RNA polymerase as described by Moar et al. (1975).

3. Filter Hybridisation. Male and female DNAs of various species of snakes and birds were denatured and loaded onto filters (HAWP 0.45 μ 13 mm) according to the procedure of Gillespie and Spiegelman (1965). Each filter contained 0.05 μg of total DNA with 3 μg of *Micrococcus lysodeikticus* DNA as a carrier. Filters containing *M. lysodeikticus* DNA (3 μg/filter) served as controls. Before hybridisation, the filters containing the bound DNA were soaked in 3 × SSC. The hybridisation was carried out for 2.5 h at 60° C (T_{opt}), satellite III cRNA concentrations of 0.03 μg/ml in 3 × SSC. After hybridisation, filters were washed and RNased by the batch method (Birnstiel et al., 1968), dried and counted. The counts hybridised are the average of 3 filters of ♂, ♀ and control expressed in the Table 1 along with the standard error.

4. Determination of T_{opt}. The initial rate of hybrid formation was studied by filter hybridisation (Gillespie and Spiegelman, 1965) in 3 × SSC in cRNA excess (Bishop, 1969; Birnstiel et al., 1972) (Fig. 2). The temperature optimum (T_{opt}) for hybrid formation in 3 × SSC of *Elaphe radiata* ♀ satellite III cRNA-DNA was 60° C.

5. Kinetic Studies. For kinetic studies, the hybridisation medium was brought to optimal temperature, filters introduced and individual ones withdrawn and placed into chilled 6 × SSC at various times. Filters were washed by the batch method (Birnstiel et al., 1968). Controls using the heterologous carrier *Micrococcus lysodeikticus* alone bound less than 0.015% of the input cRNA.

6. Dissociation of RNA-DNA Hybrids. cRNA-satellite DNA hybrids were formed at optimal temperature in 3 × SSC as described above and the hybrids dissociated as described in Figure 14.

7. Chromosome Preparations. Chromosome preparations were made either from monolayer cultures established from lung cells using MEM medium supplemented with 10% fetal calf serum or from short-term leukocyte cultures by the usual air-drying procedure. Cultures were treated with colchicine (0.015 μg/ml) for 4 h, 0.075 M KCl for 8 min and fixed in 1:3 acetic acid alcohol. Slides of air-dried chromosome preparations were stored in slide boxes in cold room and were used when required.

8. C-banding Procedure. For the staining of constitutive heterochromatin the procedure of Sumner (1972) was followed. Chromosomes were treated for 1 h in 0.2 N HCl at room temperature, 4–10 min in 5% aqueous solution of Barium hydroxide octahydrate at 50° C, 1.5 h in 2 × SSC at 60° C and stained for 1.5 h in buffered Giemsa (1 ml Giemsa + 50 ml buffer pH 6.8).

9. In situ Hybridisation. The procedure described by Jones (1973) was followed. Chromosome preparations were treated with 0.2 N HCl for 20 min to denature the DNA, dried through an alcohol series and hybridised with 5 μl cRNA/slide at 60° C (T_{opt}) in 3 × SSC at a cRNA concentration of 1 μg/ml for 3 h (i.e. 30 reaction half lives) (specific activity 1.7×10^7 counts/min/μg). Hybridised slides were RNase treated (20 μg/ml, 30 min at 37° C in 2 × SSC), washed (2 × SSC, 6 h at 4° C), and dried through an alcohol series. Slide preparations were dipped in Ilford K_2 nuclear emulsion diluted 50:50 with distilled water. Slides were exposed for 2–4 months at 4° C, developed in Kodak D19b developer and stained in Giemsa (1 ml Giemsa + 50 ml buffer pH 6.8) for 20 min. Photographs were taken by using Agfapan 25 ASA 35 mm film, Zeiss NF Research microscope with camera attachment.

Results and Discussion

When total DNA of *Elaphe radiata* male and female was centrifuged to equilibrium in neutral CsCl, in the analytical ultra centrifuge, no satellites were observed in either sex. However, when total DNA was centrifuged to equilibrium in Ag^+/Cs_2SO_4 gradients, four major bands were seen in the female and

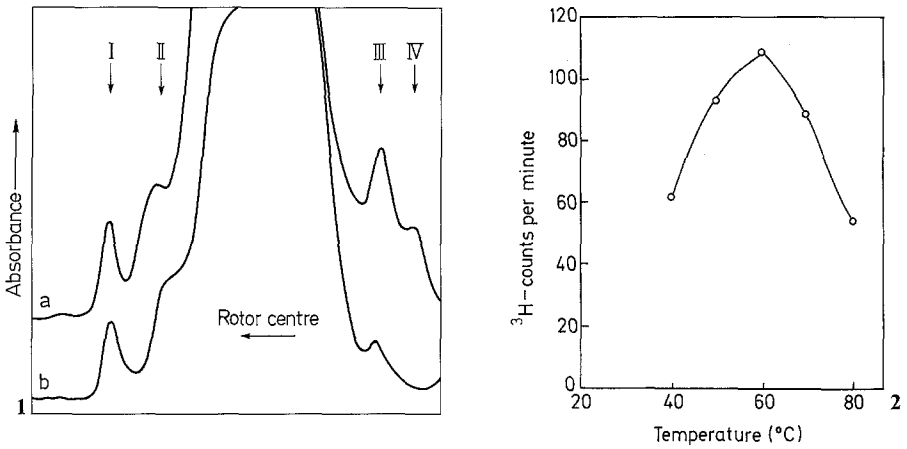

Fig. 1a and b. Analytical equilibrium density gradient centrifugation of total DNA of *Elaphe radiata* male and female in $Ag^+Cs_2SO_4$ gradient. 70 µg of male and female DNA were centrifuged to equilibrium in Cs_2SO_4/Ag^+ gradients at a Ag^+ to DNA-phosphate ratio (R_F) of 0.20 in the Spinco model E analytical centrifuge at 25° C, 44,000 r.p.m. for 20 h. Four major bands were seen in the female **a** and three in the male **b**. Satellites I, II and III were isolated from the female and purified by successive Cs_2SO_4 and CsCl centrifugations. The buoyant densities were, I = 1.681, II = 1.693 and III = 1.700 g · cm^{-3} respectively. Satellite IV was not isolated

Fig. 2. Temperature dependence on the initial rate of cRNA-satellite hybrid formation. *Elaphe radiata* ♀ satellite III cRNA was hybridised to filter discs containing 0.05 µg of *Elaphe radiata* ♀ DNA with 3 µg of *M. lysodeikticus* DNA as carrier (Gillespie and Spiegelman, 1965) at each of the temperatures indicated, in a pre-warmed solution of cRNA in 3 × SSC. The cRNA concentration was 0.006 µg/ml and the time of incubation 40 min. The filters were washed and RNased by the batch method (Birnstiel et al., 1968) dried and counted

three in the male (Fig. 1a–b). Satellite I and II were common to both male and female without any apparent quantitative difference. However, a sex difference was observed in the content of satellite III which appeared as a prominent peak in the female (Fig. 1a) and was poorly represented in the male (Fig. 1b). Satellite IV was found to be restricted to the female and therefore presumably concentrated on the W chromosome. The minute quantity of satellite IV in the genome made it difficult to isolate it from the limited amount of the female DNA which was available to us. Here we are mainly concerned with satellite III.

The optimal rate temperature (Birnstiel et al., 1972) (T_{opt}) for satellite III DNA-cRNA hybridisation was determined to be 60° C in 3 × SSC (Fig. 2).

$Crt^1/_2$[1] can be used to measure the kinetic complexities of RNA species in hybridisation reactions (Birnstiel et al., 1972). Satellite III DNA-cRNA hybridised with a $Crt^1/_2$ of 1.44×10^{-3} moles · sec/l under optimal rate conditions. It therefore appears to be 6 times more complex than human satellite I DNA-cRNA measured under similar conditions (56° C, 4 × SSC) (Jones et al., 1974), having a kinetic complexity of 200 bases (Moar et al., 1975).

[1] $Crt^1/_2$ = Product of initial RNA concentration, in moles/litre and the time taken to reach half saturation (sec)

The difference in satellite III content between males and females seemed likely to be due to its concentration on the W chromosome. However, chromosomes of *Elaphe radiata* were not available to demonstrate this directly. We reasoned, however, that should this be so, conservation of this DNA may have occurred during the evolution of the W chromosome, by analogy with the conservation of the X chromosome in mammals (Ohno, 1967). In this case the chromosomes of related snakes which were available would serve to test this point. Accordingly we cross hybridised *Elaphe radiata* female satellite III cRNA with the chromosomes of *Ptyas mucosus* male and female and *Natrix piscator* female (Family Colubridae), of *Bungarus caeruleus* female and *Bungarus fasciatus* female of the highly evolved family of poisonous snakes (Elapidae) and of *Python reticulatus* female and *Xenopeltis unicolor* female of the primitive family Boidae. With the exception of *Python reticulatus* and *Xenopeltis* unicolor which are included in the present study (Figs. 3 and 4), karyotype analyses of these species have been described earlier (Singh et al., 1968, 1970; Singh, 1972, 1974). More than 100 hybridised metaphase spreads were analysed in each case. In the case of *Natrix piscator, Bungarus caeruleus* and *Bungarus fasciatus*, grains were exclusively concentrated on the W chromosome (Fig. 5a–c). In these species the W chromosome is morphologically differentiated and could unequivocally be identified by C-banding (Fig. 5d–f). Occasionally a few grains were observed scattered on other chromosomes but they were not significantly above the background level and their locations were not consistent. Further exposure for 3 months did not reveal any consistent and significant number of grains on any chromosome other than the W (Fig. 6a). In interphase nuclei grains were generally concentrated in a single region corresponding to the W chromatin body. Occasionally in interphase nuclei the W chromosome remained slightly in extended condition in which a chromosome outline could be seen over which grains were concentrated (Fig. 6b). No grains were observed on any chromosome in the case of *Ptyas mucosus* male (Fig. 7a). However, in the case of *Ptyas mucosus* female (Fig. 8a) there were significant numbers of grains localised mainly on one entire chromosome which is the W chromosome and which could also be identified by C-banding (Fig. 8b). It should be pointed out that unlike the highly differentiated W chromosome of other species, in this species Z and W chromosomes are homomorphic (Singh, 1972) (Fig. 9) and the W chromosome does not show very intense C-banding. It seems to be intermediate in this respect between non-differentiated sex chromosomes which do not stain differentially and highly differentiated sex chromosomes which do. Thus sex chromosomes in this species, as revealed by in situ studies, are differentiated at the molecular level. Because of their otherwise 'primitive' features for example absence of asynchrony in DNA replication or formation of a W chromatin body in interphase nuclei (Ray-Chaudhuri and Singh, 1972) which are the characteristics of the differentiated W chromosome in other species of snakes and birds, these perhaps represent the first step in differentiation of sex chromosomes. This process is indicated to involve a differential concentration of satellite DNA on the otherwise indistinguishable W chromosome. No grains were observed on any chromosome even after 4 months exposure in the case of *Python reticulatus* female (Fig. 7b) and *Xenopeltis unicolor* female

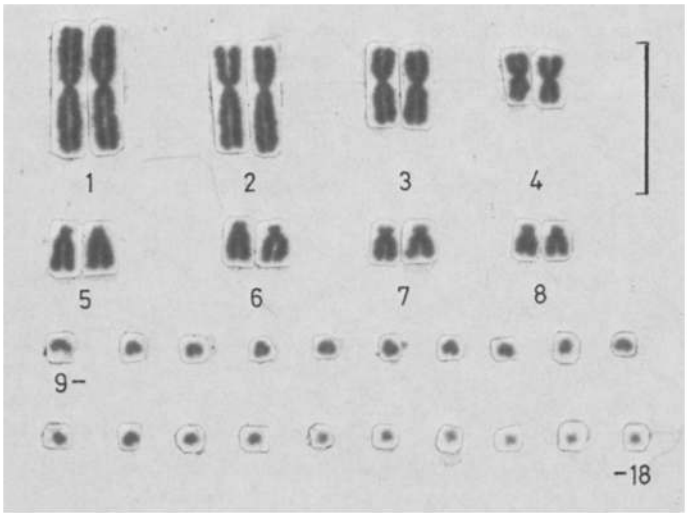

Fig. 3. Female karyotype of *Python reticulatus* (2n=36) from short term leukocyte culture. Sex chromosome heteromorphism is absent

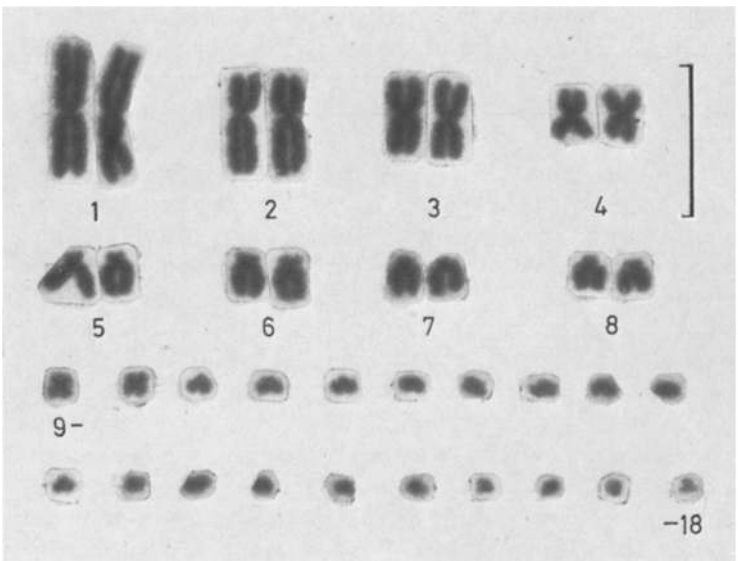

Fig. 4. Female karyotype of *Xenopeltis unicolor* (2n=36) from short term leukocyte culture. No sex-chromosome heteromorphism

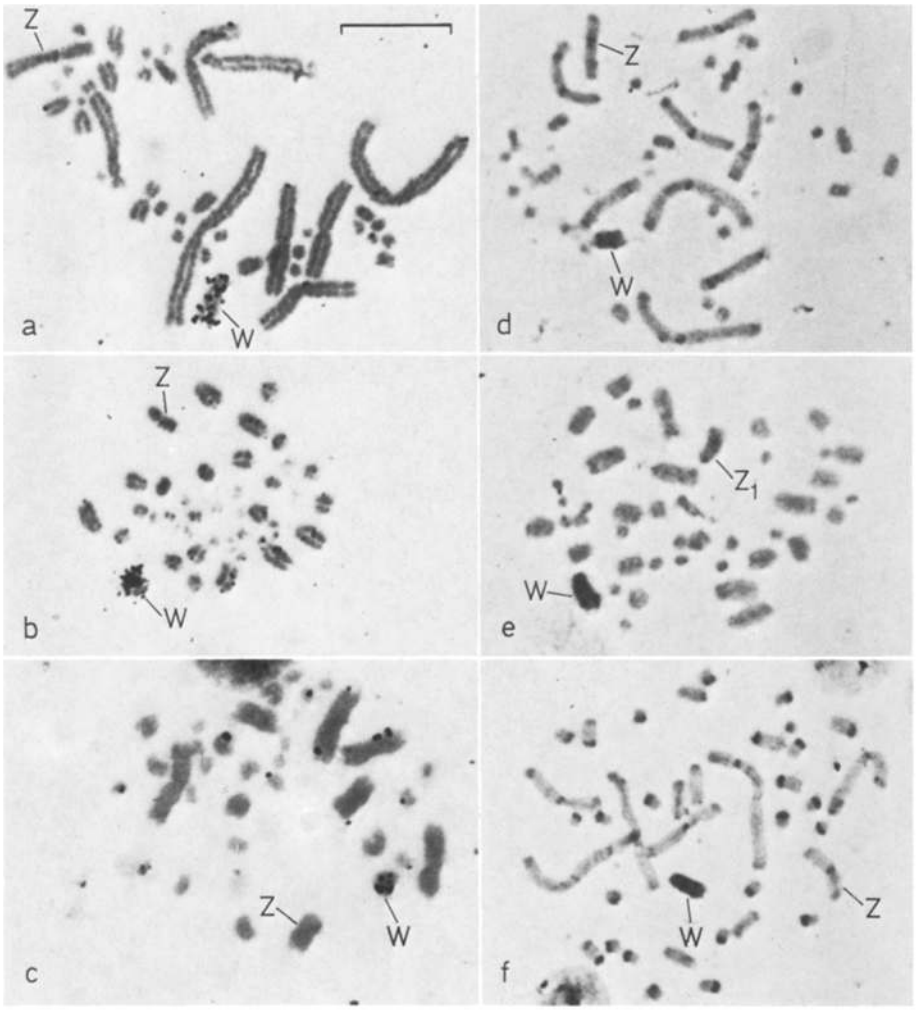

Fig. 5a–f. Cross hybridisation of *Elaphe radiata* ♀ satellite III cRNA with metaphase chromosomes of **a** *Natrix piscator* ♀ (Colubridae), **b** *Bungarus caeruleus* ♀ (Elapidae), **c** *Bungarus fasciatus* ♀ (Elapidae). Chromosome preparations were hybridised after 20 min denaturation in 0.2 N HCl excepting in the case of *Bungarus fasciatus* in which chromosomes were denatured in 0.1 × SSC at 100°C for 5 s. In situ hybridisation was carried out by using 5 µl cRNA/slide at 60°C (T_{opt}) in 3 × SSC at a cRNA concentration of 1 µg/ml for 3 h (i.e. 30 reaction half lives). Slides were exposed for 2 months in the case of **a** and **b** and for 1 month in the case of **c**. Grains are exclusively localised all along the W chromosome. Conditions for in situ hybridisation in other cases were the same unless otherwise indicated. **d–f** Metaphase chromosomes showing C-banding. **d** *Natrix piscator* ♀, **e** *Bungarus caeruleus* ♀, **f** *Bungarus fasciatus* ♀. Note the darkly stained entire W chromosome apart from centromeric C-banding in other chromosomes in the case of **d** and **f**, whereas, in the case of **e** C-banding is exclusively localised in the W chromosome. *Bungarus caeruleus* (**b** and **e**) has a multiple sex chromosome constitution of $Z_1Z_1Z_2Z_2♂/Z_1Z_2W♀$ type; where W is the largest element in the whole complement (Singh et al., 1970). The above metaphase spreads (**b** and **e**) are polymorphic individuals obtained from India in which the large W chromosome having a translocated autosome has undergone fission and subsequent translocation with one of the micro-chromosomes giving rise to a small W chromosome (our unpublished data) (representing the original W chromosome) which could be recognised by C-banding (**e**)

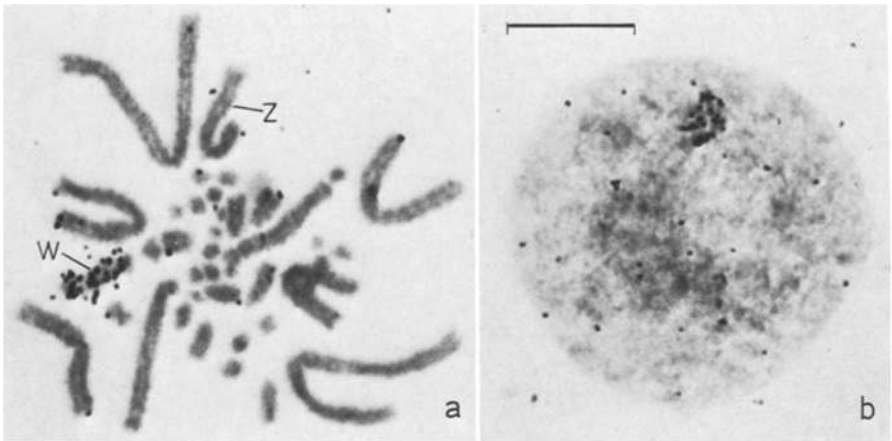

Fig. 6a and b. Cross hybridisation of *Elaphe radiata* ♀ satellite III cRNA with: **a** metaphase chromosomes of *Natrix piscator* ♀. Additional exposure for 3 months did not reveal any significant number of grains on any chromosome other than the W. Grains are highly concentrated on the W chromosome. **b** Interphase nucleus of *Bungarus caeruleus* ♀ showing the position of the W chromosome over which grains are concentrated

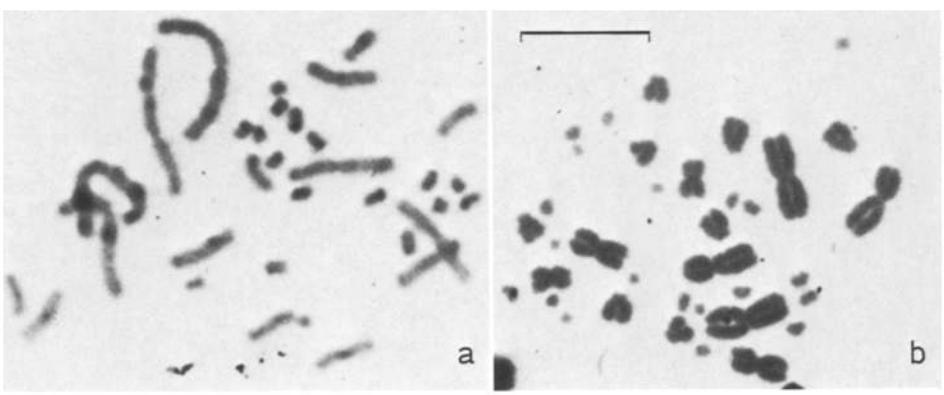

Fig. 7a and b. Cross hybridisation of *Elaphe radiata* ♀ satellite III cRNA with: **a** metaphase chromosomes of *Ptyas mucosus* male (Colubridae). Slides were exposed for 4 months. No grains are detected on any chromosome. **b** Metaphase chromosomes of *Python reticulatus* ♀ after 4 months of exposure. Note the absence of grains on all the chromosomes

(Fig. 10a). Analysis of C-banding in *X. unicolor* ♀ also revealed the absence of any entirely heterochromatic chromosome comparable to the heterochromatic W in other species of snakes and birds (Fig. 10b).

It is clear from these observations that the satellite DNA which is present in females of *E. radiata* is also conserved in other, even distantly related species where it is concentrated upon the W chromosome. It, therefore, appears probable

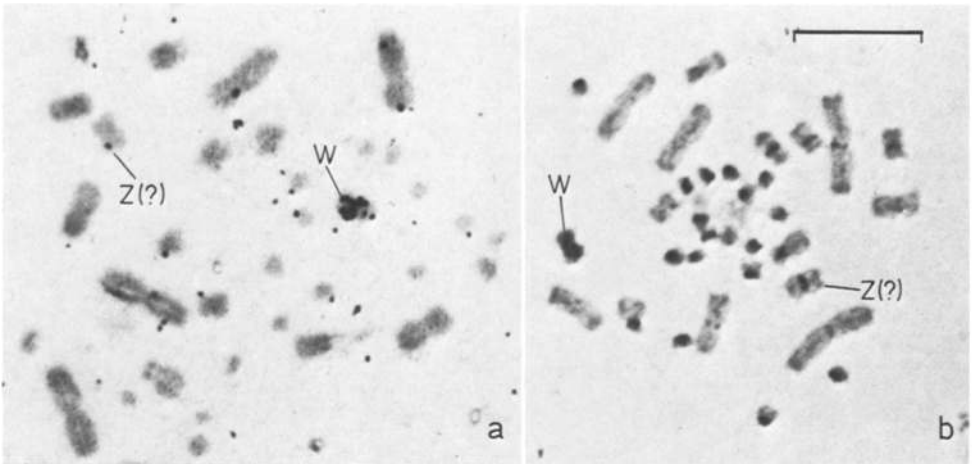

Fig. 8a and b. Cross hybridisation of *E. radiata* ♀ satellite III cRNA with metaphase chromosomes of *Ptyas mucosus* female **a** (Colubridae). Chromosomes were denatured in 0.1 × SSC at 100° C for 5 s. Slides were exposed for 1 month. Grains are mainly concentrated on the entire length of only one chromosome presumably the W. **b** Female metaphase plate of *P. mucosus* from leucocyte culture showing C-banding. Note the differential staining of one of the macrochromosomes apart from centromeric C-banding of other chromosomes. On the basis of its size and morphology this appears to be the same chromosome showing cross in situ hybridisation with the *Elaphe* ♀ satellite III cRNA (see **a**)

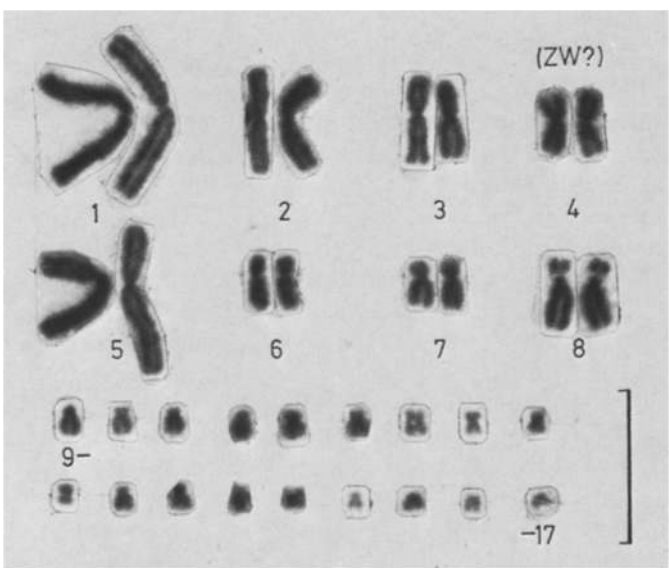

Fig. 9. Female karyotype of *Ptyas mucosus* (2n = 34) from lung culture showing the absence of sex chromosome heteromorphism

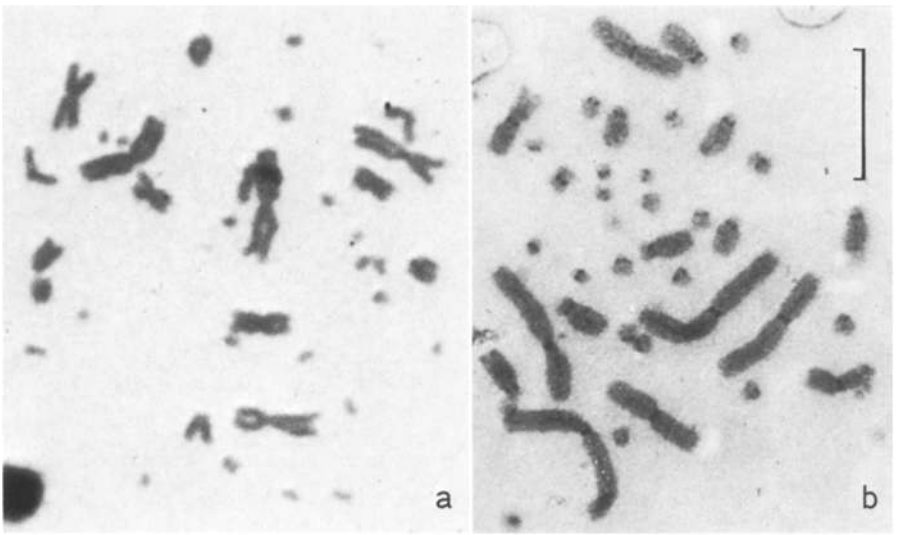

Fig. 10a and b. Cross hybridisation of *E. radiata* ♀ satellite III cRNA with: **a** metaphase chromosomes of *Xenopeltis unicolor* ♀ (Boidae). Slides were exposed for 4 months. No grains are detected on any chromosome. **b** Female metaphase plate of *Xenopeltis unicolor* from short term leucocyte culture showing C-banding. Note the absence of any entirely C-band positive macrochromosome comparable to the W sex chromosome. Centromeric regions of most of the chromosomes are C-band positive

that this reflects its distribution in *E. radiata* also. This is supported by the fact that in *Python reticulatus* and *X. unicolor* without a differentiated sex chromosome pair, related sequences are not evident. Lack of in situ hybridisation in the Z in females or with any chromosome in males, is unexplained.

In order to investigate the apparent content of satellite III DNA as seen from analytical ultracentrifugation in male DNA, male and female total DNA of *E. radiata* were loaded onto millipore filters and hybridised with satellite III cRNA (see Table 1). It is apparent from Table 1 that the total number of counts hybridised per unit of DNA is almost twice as much in the female as in the male. The percentage of *E. radiata* genome complementary to satellite III cRNA was calculated from the saturation values using double reciprocal plots (Birnstiel et al., 1972; Bishop, 1969) (Fig. 11). This showed that *E. radiata* female satellite III DNA comprises 1.56% of the double stranded genome in the female and 0.66% in the male. The haploid DNA content of man and other placental mammals is 3.5×10^{-9} mg, the snake genome is approximately 60% of this value (Ohno, 1967). 1.6% of the double stranded genome of *E. radiata* ♀ therefore represents some 26,000 copies/genome. Approximately 12,000 of which are in the W chromosome.

The same amount of female total DNA (0.05 μg/filter) of *Python reticulatus* and *Xenopeltis unicolor* of the primitive family Boidae, total male and female DNA of *Ptyas mucosus* and *Rhamphiophis oxyrhynchus rostratus* belonging to the same family Colubridae, and total female DNA of *Vipera russelli russelli*

Table 1. Male and female DNA of various species of snakes was denatured and loaded onto filters (HAWP 0.45 μ 13 mm). Each filter contained 0.05 μg of total DNA with 3 μg of *Micrococcus lysodeikticus* DNA as a carrier. Filters containing *M. lysodeikticus* DNA (3 μg/filter) served as controls. The hybridisation was carried out for 2.5 h at 60° C (T_{opt}), satellite III cRNA concentrations of 0.03 μg/ml in 3 × SSC. After hybridisation, filters were washed, RNased, dried and counted. The counts hybridised are the average of 3 filters of ♂, ♀ and control expressed in the table along with the standard error (±)

Species		Family	Counts per min hybridised		
			Sex		Control
			♂	♀	
1	*Python reticulatus*	Boidae	–	97±8	60±15
2	*Xenopeltis unicolor*	Boidae	–	91±1	67±2
3	*Ptyas mucosus*	Colubridae	268±3	559±9	65±1
4	*Elaphe radiata*	Colubridae	350±23	581±54	48±1
5	*Rhamphiophis oxyrhynchus rostratus*	Colubridae	180±26	326±20	67±2
6	*Vipera russelli russelli*	Viperidae	–	318±67	65±1

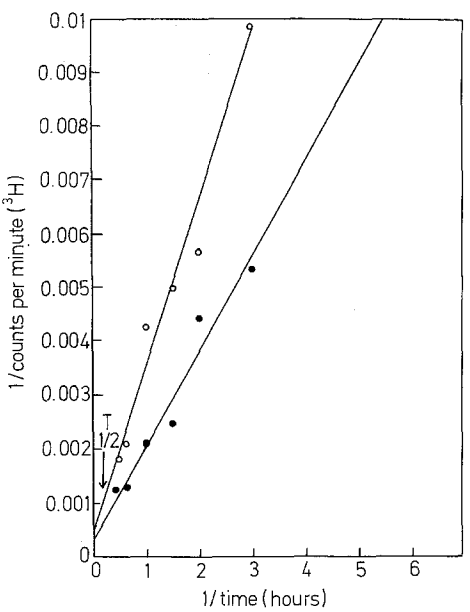

Fig. 11. Kinetics of *Elaphe radiata* ♀ satellite III cRNA-DNA hybridisation using *Elaphe radiata* ♂ and ♀ DNA. Filters contained 0.05 μg *Elaphe radiata* ♂ and ♀ DNA plus 3 μg *micrococcus lysodeikticus* DNA as carrier. The cRNA concentration was 0.03 μg/ml. The specific activity of the cRNA was 1.7×10^7 counts/min/μg. The hybridisation was carried out at T_{opt} (60° C) in 3 × SSC. The results are expressed by the double reciprocal plot method of Bishop (1969). *M. lysodeikticus* filters bound less than 0.015% of the input cRNA. o—o ♂, ●—● ♀

belonging to the highly evolved family of poisonous snakes "Viperidae" was bound to millipore filters and cross hybridised with satellite III cRNA of *E. radiata* female. There were no significant counts above the background level on the female filters of *P. reticulatus* and *X. unicolor* (Table 1). However, twice as many counts were observed on the female filters as on the male of *P. mucosus* and *R. oxyrhynchus rostratus* (Table 1). There were significantly high counts on the female filters of *V. russelli russelli* also (Table 1). These results are in agreement with in situ studies. The high number of hybrid counts obtained on the filters (Table 1) shows the presence of related sequences even in species as distantly related as viper. cRNA of satellite I and II DNA of *E. radiata* however, showed no significant hybridisation over the background with total filter bound DNA of either *P. mucosus* or *Boiga dendrophila* belonging to the same family Colubridae (Tables 2 and 3).

Hybridisation of satellite III cRNA to male DNA in these species with differentiated sex chromosomes but absence of any detectable in situ hybridisation to the Z, even when of equal in size to the W, or to autosomes is a curious aspect of our findings. The level of male DNA-satellite III cRNA hybridisation is approximately one half that seen in female DNA and well above the background represented by female *Python* and *Xenopeltis* DNA. It amounts to 0.66% of the genome and this, by analogy with other satellites in man for example, should enable it to be revealed in situ. In *E. radiata* there is evidence of a small satellite III in male DNA from model E analysis so that failure to find an in situ location for this would be quite exceptional. Unfortunately we do not have chromosomes of this species. In the heterologous species, we have not yet identified the reacting components in terms of satellite DNA so that we have no information concerning their presence or absence in the males. Supposing that they are absent, the hybridisation which we see would be to related non-satellite repeated DNA and this might be widespread. In this event in situ hybridisation would not necessarily reveal anything. A second possibility is that satellite III does not hybridise very efficiently in situ so that difference in general amount between female and male respectively would provide a positive versus a negative result in situ. This seems improbable in view of the very strong hybridisation to the W chromosome, unless one further assumes that their arrangements on the W is a deciding factor. Thirdly, it may be that the results are due to a component in the satellite III fraction which is only present in the W chromosome and which is conserved. As an example of this the ribosomal cistrons of *Xenopus laevis* are conserved but the spacer is not (Brown et al., 1972). Satellite III is indicated to be rather complex kinetically. It is therefore quite conceivable that we are detecting a sequence which is covalently linked and interspersed with satellite III in *E. radiata*. There is some indication of a satellite IV which is rather elusive and apparently only present in *E. radiata* females (Fig. 1). This is therefore presumably a component specific to the W which might be co-extracted with satellite III. Both are indicated to be present on the W so that cross contamination due to their covalent linkage and similar densities cannot be excluded.

The heterologous results on filters may in this case reflect a low hybridisation homology of satellite III with other repeated DNA in both male and female

Table 2. Cross hybridisation of *Elaphe radiata* ♀ satellite I cRNA with the total DNA of different species of snakes bound to millipore filters

Total DNA was denatured and loaded onto filters (HAWP 0.45 μ 13 mm). Each filter contained 0.05 μg of total DNA with 3 μg of *Micrococcus lysodeikticus* DNA as a carrier. Filters containing *M. lysodeikticus* DNA (3 μg/filter) served as controls. The hybridisation was carried out for 2 h at 50° C (T_{opt} for Sat I), satellite I cRNA concentration of 0.016 μg/ml in 3 × SSC. After hybridisation, filters were washed RNased, dried and counted. The counts hybridised are the average of 3 filters

Species	Family	Counts per min hybridized	
			Control
Elaphe radiata	Colubridae	2860	120
Ptyas mucosus	Colubridae	283	
Boiga dendrophila	Colubridae	265	

Table 3. Cross hybridisation of *Elaphe radiata* ♀ satellite II cRNA with the total DNA of *Elaphe radiata* and *Ptyas mucosus* bound to millipore filters

Total DNA was denatured and loaded onto filters (HAWP 0.45 μ 13 mm). Each filter contained 0.05 μg of total DNA with 3 μg of *Micrococcus lysodeikticus* DNA as a carrier. Filters containing *M. lysodeikticus* DNA (3 μg/filter) served as controls. The hybridisation was carried out for 2 h at 62° C (T_{opt} for Sat II) satellite II cRNA concentration of 0.027 μg/ml in 3 × SSC. After hybridisation filters were washed RNased, dried and counted. The counts hybridised are the average of 3 filters

Species	Family	Counts per min hybridized	
			Control
Elaphe radiata	Colubridae	1689	112
Ptyas mucosus	Colubridae	209	

compared with a high homology of an associated sequence which is perhaps functionally conserved on the W chromosome. Because of the possibility that sex determining factors are distributed over the length of sex chromosomes in Drosophila and mammals (Ohno, 1967) this possibility is a rather attractive one and we are investigating it further.

Like snakes, birds also have a ZZ♂/ZW♀ type of sex determining mechanism. In this group of vertebrate the W chromosome is also heterochromatic and can be identified by C-banding. In order to find out whether similar sex chromosome associated satellite DNA does exist in birds also, we isolated DNA from male and female chicken (*Gallus domesticus*) in the same way as in snakes and processed them separately. When male and female DNA were centrifuged to equilibrium in Cs_2SO_4/Ag^+ gradients at a Ag^+ to DNA phosphate ratio (R_F) of 0.25 in the Spinco model E analytical centrifuge, two satellites were observed on the light side of the gradient and one on the heavy side (Fig. 12). There was an indication of difference in the quantity of one satellite (Satellite I) between male and female in favour of the female. This satellite DNA was isolated from female DNA by successive centrifugation in Cs_2SO_4 and CsCl

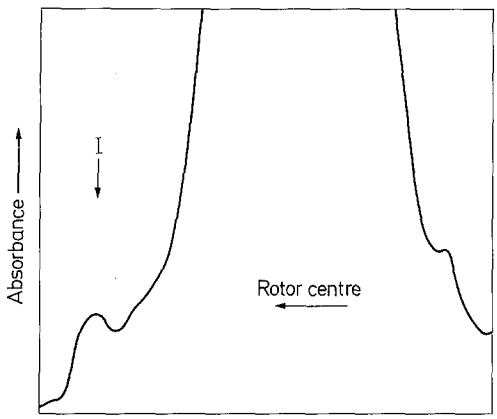

Fig. 12. Analytical equilibrium density gradient centrifugation of total DNA of *Gallus domesticus* female in $Ag^+Cs_2SO_4$. 70 μg of female DNA was centrifuged to equilibrium in Cs_2SO_4/Ag^+ gradients at a Ag^+ to DNA-phosphate ratio (R_F) of 0.25 in the Spinco model E analytical centrifuge at 25° C, 44,000 rpm for 20 h. Total 3 bands, 2 on the light side (one close to the main band) and one on the heavy side of the gradient were seen. Satellite I was isolated from the female and purified by successive Cs_2SO_4 and CsCl centrifugations. The buoyant density was determined to be I = 1.695 g·cm^{-3}

gradients. The buoyant density was determined to be 1.695 g·cm^{-3} in neutral CsCl gradients. cRNA was prepared as described in the material and methods. The optimal rate temperature (T_{opt}) for this satellite I DNA cRNA hybridisation was determined to be 63° C in 3 × SSC (data not shown). In situ hybridisation of female chicken satellite I cRNA to female chicken chromosomes revealed its concentration on the entire length of the W chromosome (Fig. 13a). There were a few grains on 4–6 microchromosomes which would account for the hybridisation to male DNA. In interphase nuclei grains were concentrated in a single region (Fig. 13b).

We have not cross hybridised the cRNA of the female chicken satellite I DNA with any other species of birds. We, therefore, do not know whether its sequences are also conserved in different species of birds.

On the basis of the similarity in the sex determining mechanism of snakes and birds and of the conservation of the sequences of the sex chromosome associated satellite DNA throughout the sub-order Ophidia (species having differentiated Z and W chromosomes), DNA conservation during the evolution of the W chromosome seems probable. Homology in the sequences of sex chromosome associated satellite DNA in snakes and birds was therefore tested. 0.10 μg of male and female DNA of Japanese Quail *Coturnix coturnix japonica* was loaded onto millipore filters and hybridised with the cRNA of *Elaphe radiata* ♀ satellite III at the T_{opt} in 3 × SSC. From Table 4 it can be seen that there are significantly more counts hybridised to the female than to male DNA. The total number of counts however is significantly low compared with snake DNA when allowance is made for the greater amount of bird DNA.

Filters containing total female DNA of various species of snakes and one species of bird hybridised with satellite III cRNA of *Elaphe radiata* female were used for determining the melting profiles of the hybrid in 1 × SSC. The

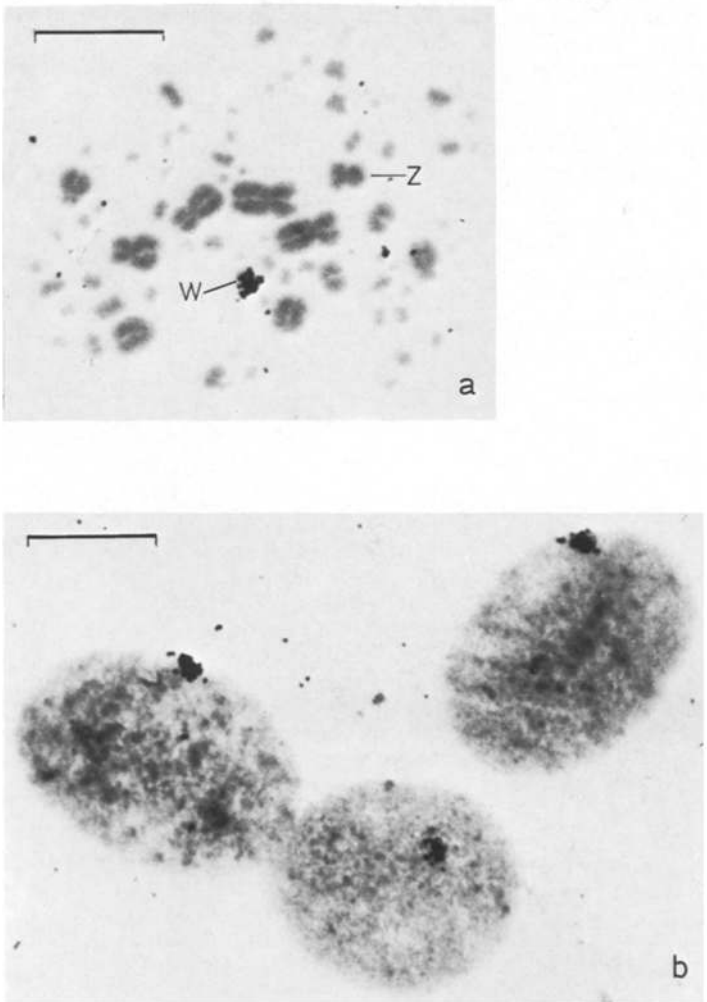

Fig. 13. a In situ hybridisation of *Gallus domesticus* ♀ satellite I cRNA on the metaphase chromosomes of the female of the same species. Chromosome preparations were hybridised after 20 min denaturation in 0.2 N HCl, with 5 µl cRNA/slide at 63°C (T_{opt}) in 3 × SSC at a cRNA concentration of 0.3 µg/ml for 6 h. Slides were exposed for 2 months. Note the high concentration of grains on the W chromosome. Few microchromosomes also have one or two grains. **b** In situ hybridisation of *Gallus domesticus* ♀ satellite I cRNA on the female interphase nuclei of the same species after 2 months exposure showing concentration of grains in a single region

homologous hybrid yielded a sharp monophasic melting curve consistent with a single component. 50% of the homologous as well as heterologous hybrids melted at 70°C suggesting that many of the heterologous hybrid molecules are quite well matched (Fig. 14). However the melting profile at lower temperatures reflects stability differences between homologous and heterologous hybrids consistent with nucleotide divergence (Fig. 14). As expected the less closely related the species the less thermally stable are the hybrids.

Table 4. Male and female DNA of Japanese quail, *Coturnix coturnix japonica* was denatured and loaded onto filters (HAWP 0.45 μ 13 mm). Each filter contained 0.10 μg of total DNA with 3 μg of *Micrococcus lysodeikticus* DNA as a carrier. Filters containing *M. lysodeikticus* DNA (3 μg/filter) served as controls. The hybridisation was carried out for 2.5 h at 60°C (T_{opt}), with the *Elaphe radiata* ♀ satellite III cRNA, at cRNA concentration of 0.03 μg/ml in 3 × SSC. After hybridisation, filters were washed, RNased, dried and counted. Two filters of ♂, ♀ and control were used

Species	Counts per min hybridized			
	Filter No.	Sex		Control
		♂	♀	
Coturnix coturnix japonica (Japanese Quail)	1 –	265	392	93
	2 –	173	346	92

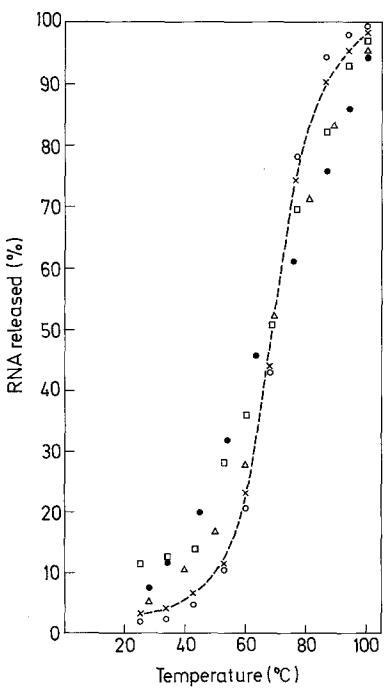

Fig. 14. Melting temperature profiles of RNA-DNA hybrids. 0.15 μg of total female DNA of different species of snakes and 0.3 μg of Japanese quail were hybridised with *Elaphe radiata* ♀ sex-chromosome associated satellite cRNA (satellite III) at optimal temperature (60° C) for 3 h as described in materials and methods. The filters were washed by the batch method and treated with RNase (10 μg RNase/ml) for 20 min in 2 × SSC at room temperature, rinsed with 2 × SSC and vacuum dried. The filters were then incubated in 1 × SSC, 0.1% diethylpyrocarbonate at 20° C for 30 min to remove the nuclease activity, rinsed exhaustively in 1 × SSC and vacuum dried. To determine the temperature at which 50% of the RNA was released from the filter (Tm), the filters were placed in 1 ml of 1 × SSC and heated in temperature increments of approximately 8° C. After 5 min at each temperature, the released RNA was recovered by trichloroacetic acid precipitation (by using Bovine serum albumin 0.1 mg/ml as a carrier), loaded on millipore filter and counted (Birnstiel et al., 1972). × --- × *Elaphe radiata* ♀ satellite III cRNA – autologous DNA hybrid; ○ *E. radiata* ♀ satellite III cRNA – *Ptyas mucosus* ♀ DNA hybrid; □ *E. radiata* ♀ satellite III cRNA – *Vipera russelli russelli* ♀ DNA hybrid; △ *E. radiata* ♀ satellite III cRNA – *Rhamphiophis oxyrhynchus rostratus* ♀ DNA hybrid; ● *E. radiata* ♀ satellite III cRNA – Janpanese Quail (*Coturnix coturnix japonica*) DNA hybrid

In situ hybridisation of *Elaphe radiata* ♀ satellite III cRNA to the chromosomes of female Japanese quail gave a clear indication of hybridisation in the interphase nuclei but there was no convincing hybridisation to any chromosome. This is perhaps because of reduced homology or quantity, or because of the arrangement of the sequences. However, it appears that there is conservation of sex chromosome associated nucleotide sequences and we are investigating this further.

The presence of constitutive heterochromatin on the Y chromosome of mammals including man and W chromosome of birds suggests the possible existence of sex chromosome associated satellite DNA in a wider range of vertebrates than just the snakes. Kunkel et al. (1976) have recently been able to obtain reiterated DNA specific for the human Y chromosome by extensive reassociations between ^{3}H-DNA prepared from men and excess DNA from women. The purified Y-chromosome specific sequences represent between 7% and 11% of the human Y chromosome. In *Elaphe radiata* ♀ satellite III DNA comprises 1.56% of the double stranded genome in the female. The relative length of the W chromosome in this species is not known. We therefore cannot comment what percentage of total DNA of the W chromosome is represented by this satellite DNA in this species.

The distribution of human Y chromosome specific reiterated DNA sequences on the human Y chromosome is not known. However, the pattern of distribution of grains along the entire length of the W chromosome revealed by in situ hybridisation (Figs. 5a–c, 6a, 8a and 13a) indicates that sex chromosome associated satellite DNA is relatively uniformly interspersed among the other sequences which may be present on the W chromosome in snakes and birds. This is unlike the distribution of other satellite sequences which are generally concentrated in a particular region of a chromosome rather than spread along its entire length.

Chromosomal sex determination depends upon the presence of different factors on each of the sex chromosome pair. This is possible only if crossing-over between the two chromosomes (X and Y or Z and W) of the heterogametic sex is suppressed during meiosis. Prevention of crossing-over between the homomorphic Z and W or X and Y is an essential precondition for their gradual differentiation. In a number of species of snakes belonging to the family Colubridae, although the Z and W of the female are still identical in size, a pericentric inversion has occurred in the W. Ohno (1967) suggested that a pericentric inversion in the Y or the W facilitates further differentiation of sex chromosomes. Ray Chaudhuri et al. (1971) however, provided evidence that heterochromatinization of the W chromosome may be the significant process in the differentiation of the W in snakes.

Our findings reveal three very pertinent facts with regard to the evolutional process of sex chromosome differentiation.

1. Sequences of sex chromosome associated satellite DNA are absent along with C-bands, in the species belonging to the primitive family Boidae, which includes the most primitive snakes living today, in which sex chromosomes are in a primitive state of differentiation.

2. Sequences of W chromosome associated satellite DNA are present not only in those species of snakes having morphologically differentiated sex chromosomes but also in those species of highly evolved families in which sex chromosomes are morphologically identical but stain differentially by C-banding.

3. Sequences of W chromosome associated satellite DNA are unusually conserved throughout the sub-order Ophidia and are distributed along the entire length of the W chromosome.

These findings show that origin and distribution of sex chromosome associated satellite DNA in the W chromosome precedes morphological differentiation of sex chromosomes in snakes and suggests that this is a vital aspect of the process. Morphological changes in the W chromosome are thus suggested to be the consequence rather than the cause of the sex chromosome differentiation.

The origin and distribution of sex chromosome associated satellite DNA in an homomorphic W chromosome could bring about asynchrony in DNA replication pattern of the two homologues (Z and W) and thus reduce the frequency of crossing-over between them, which is the prerequisite of sex chromosome differentiation. The homologues may then evolve differently in sequence and in structure.

A possible effect of heterochromatin on chromosome pairing has been suggested by Thomas and Kaltsikes (1974) in Triticale which is the amphiploid between species of tetraploid wheat (*Triticum L.*) and rye (*Secale L.*). In Triticale a number of homologous chromosomes of the rye genome fail to pair at meiosis. Within the rye genome this pairing failure was associated with the presence of large terminal heterochromatic bands. Since these terminal bands of rye chromosome are late replicating, the effect of heterochromatin could arise from an overlap between the processes of chromosome replication and chromosome pairing. Natarajan and Gropp (1971) showed that two species of hedgehogs *Erinaceus europaeus* and *Aethechinus algirius*, possess respectively 3 and 2 pairs of autosomes with large blocks of heterochromatin which pair homologously until the end of pachytene, but separate during diplotene, owing to lack of chiasmata in these regions. These regions are also associated with nucleolus organisation. They suggested that the lack of chiasmata in these regions may be a mechanism to protect vital genes (such as ribosomal cistrons) from crossing-over and for their conservation. These observations support our contention of the function of sex chromosome associated satellite DNA in the prevention of crossing-over between Z and W chromosomes and in protecting associated sex determining factors.

The origin of sex chromosome associated satellite DNA can be explained by the process of saltation invoked by Britten and Kohne (1968). Once this satellite DNA originated on the W chromosome, internal rearrangements during evolution would distribute it along the length of the chromosome under the influence of natural selection favouring its genetic isolation. Such inversions would in themselves also assist in the isolation process (Ohno, 1967).

The fact that the sequences of sex chromosome associated satellite DNA are conserved throughout the sub-order Ophidia while sequences of other satellite DNA have changed even in related species of the same family, strongly suggests that there has been selection pressure in maintaining the base sequences of this particular satellite DNA.

Sex determining factors do not behave in a Mendelian manner like the other sex linked genes. In *Drosophila* the female determining factors are distributed along the entire length of the X, and the female determining factors located at one end of the euchromatic region function in the same way as those located at the other end. It seems that the male determining factors on the mammalian Y are somewhat similar in nature (Ohno, 1967). If these factors have been translocated along with satellite DNA in the manner envisaged here, perhaps they are similarly distributed in snakes and birds also.

Acknowledgements. This work was supported in part by the Cancer Research Campaign and I.C.I. Ltd. L.S. is grateful to the Commonwealth Scholarship Commission of Great Britain for financial assistance.

We gratefully acknowledge not only the efficient and expert technical assistance of Ms. J. Muir and Ms. P. Baillie but also their courageous help in handling the live snakes.

We are thankful to Government of India for giving special permission to get living snakes from that country and to Prof. S.P. Ray-Chaudhuri of Calcutta University, India, for procuring the required species and arranging their shipment to us.

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Received June 15–August 26, 1976 / Accepted by H.C. Macgregor
Ready for press August 27, 1976