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Sex-Specific DNA in Reptiles With Temperature Sex Determination

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ABSTRACT Banded krait minor ("Bkm") satellite DNA, originating in the W-chromosome of the snake *Bungarus fasciatus*, has been found in the genome of diverse eukaryotic species including fruit fly, quail, and horse. Concentrations of Bkm have been found in the presumptive W-chromosome of snakes with isomorphic sex chromosomes and in the male-determining region of the Y-chromosome in mouse and man. We therefore asked whether Bkm-related DNA might be present in quantitative excess in DNA from males or females in two related species of sea turtle, *Chelonia mydas*, in which sex is determined by the temperature of the incubating egg, and *Lepidochelys kempi*, in which the critical sex-determining temperature has recently been described. Filter hybridization with the Bkm 2(8) probe revealed male-specific fragments in both species; female-specific fragments were also revealed in *C. mydas*. Sex-specific DNA sequences in temperature-sex-determined species such as Kemp's ridley and the green turtle were unexpected, but could be explained if there were an underlying genetic mode of sex determination in these animals, or alternatively, if temperature-influenced sex determination involved structural modifications in DNA adjacent to, or directly concerned with, the sex-determining genes. If these results are confirmed across a broader sample of sea turtles, the techniques described in this paper might be used routinely to identify gender in the young of these endangered animals, in which male and female are grossly indistinguishable.

Two types of sex determination have been described in the vertebrates. The first, genotypic sex determination (GSD), is usually associated with occurrence of the heteromorphic sex chromosomes. The second, environmental sex determination (ESD), is found in certain fish, amphibians, and reptiles—in general, in species lacking identifiable sex chromosomes (reviewed in Bull, '80). In sea turtles such as the olive ridley (*Lepidochelys olivacea*), the green turtle (*Chelonia mydas*), and the loggerhead (*Caretta caretta*), sex is determined by the temperature of the incubating egg. Females are produced at higher temperatures (30–35°C), and males, at lower temperatures (20–27°C). For each species there is a threshold or pivotal temperature at which males and females are produced (Raynaud and Pieau, '85). The type of sex determination has not been ascertained in Kemp's ridley sea turtle (*Lepidochelys kempi*), but early indications are that this too is a temperature-sex-determined species (Shaver et al., 1988).

In order to evaluate the molecular mechanisms underlying sex determination in Kemp's ridley, we screened the DNA of this turtle for Bkm sequences, which are associated with sex chromatin in certain reptiles with identifiable sex chromosomes (Jones and Singh, '81). DNA of the green turtle (*C. mydas*), a known ESD species (Morreale et al., '82), was evaluated for comparison.

Bkm is a minor satellite DNA isolated from the W-chromosome of the banded krait (*Bungarus fasciatus*). The highly repetitive GATA sequences that characterize Bkm have been found in a broad range of eukaryotic species including fruit fly and quail (Singh et al., '81) and horse (Kent et al., '88). Although Bkm occurs throughout the genome in most species, concentrations of Bkm have been located on the W- or Y-chromosomes in many cases. In the mouse, for example, Bkm repeats are closely linked to male-determining sequences (Singh and Jones, '82). Our results indicate that certain Bkm-related sequences may also be sex-linked in the sea turtles, *L. kempi* and *C. mydas*.

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MATERIALS AND METHODS

Fifteen males and 15 females of *L. kempi* and five males and five females of *C. mydas* were studied. With the exception of four Kemp's ridley turtles raised at the National Marine Fisheries Service Laboratory in Galveston, the animals came from diverse locations including the Florida Coast, Grand Cayman Islands, and the Gulf of Mexico. They ranged in age from 2 to 10 years. Gender was verified by laparoscopy or at necropsy.

DNA from red and white blood cells was isolated in SDS lysis buffer and extracted with phenol and chloroform essentially according to standard procedures (Maniatis et al., '82). The DNA was digested with restriction enzymes obtained from New England Biolabs or Betheseda Research Laboratories (BRL); digestion was performed in accordance with the manufacturer's specifications.

Electrophoresis was carried out in horizontal agarose gels (0.75–1.0%) in TAE buffer at pH 8.0. From 2 to 10 μg DNA was placed in each lane; the quantity of DNA was consistent for each experiment. DNA was transferred to nitrocellulose filters according to Southern ('75). Hind III digests of phage lambda DNA were used as markers. Bkm DNA is described in Singh et al. ('84). It consists of repeats of the tetranucleotide GATA, originally isolated by isopycnic density centrifugation from DNA of females of the elapid snake, *Bungarus fasciatus*.

The Bkm 2(8) probe that we used (CS314) consists of a sequence recovered after screening of a genomic library of *Drosophila melanogaster* (Singh et al., '84). Probes were labeled with biotin by nick translation according to the manufacturer's specifications (BRL). The probes were hybridized to the filters in 4× SSCP at 65°C overnight. After hybridization, the filters were washed in 2× or 3× SSCP with 0.1% SDS at 65°C. They were developed by use of streptavidin-alkaline-phosphatase conjugate with nitro-blue-tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

RESULTS

DNA's from red or white blood cells, or both, from the 15 males and 15 females of *L. kempi* and the five males and five females of *C. mydas* were restricted with each of the endonucleases indicated below and challenged with the Bkm 2(8) probe. (No differences were noted for DNA from white or red blood cells; turtle erythrocytes are nucleated.) The gender of the blood donors was known in the first eight samples (three males and five females of *L. kempi*) whereas the remaining 32 samples were coded.

In DNA from *L. kempi* and *C. mydas*, the Bkm 2(8) probe revealed a "fingerprint" pattern comprising some 20–25 fragments in each digest. On Southern blotting, two categories of fragments were generated: 1) fragments that appeared to be stable in most or all of the turtles surveyed, and 2) polymorphic fragments in which the number of repeats of the core satellite appeared to differ in samples from different animals, as in the fingerprinting studies by Jeffreys et al. ('86). [Similar patterns have been described with Bkm in the human (Singh and Jones, '86).]

We screened the patterns generated by 20 restriction enzymes. Several enzymes revealed male-specific fragments in DNA from *L. kempi* (Table 1). An example of the banding pattern obtained with one of the enzymes, BstN I, is given in Figure 1. A 16.6 kb fragment was generated after digestion of male DNA with BstNI (lanes 2, 3, 6). This was observed in 13 of 15 males of *L. kempi*. In two males, the band was slightly displaced. Yet bands of this size were not seen in any of the female digests.

In subsequent tests, we screened DNA from the related species, *C. mydas*. In general, samples of DNA from *C. mydas* displayed fewer of the polymorphic fragments than were displayed in samples from *L. kempi*, although many of the stable fragments in DNA from *C. mydas* were similar to those in DNA from *L. kempi* (Table 2, Fig. 2). Digestion of *C. mydas* DNA with BstNI also produced a male-specific fragment of 16.6 kb. In one

TABLE 1. Sex specific Bkm fragments in L. kempi

Enzyme	Restriction sites and sites of methylation sensitivity[1]	Fragments (kb)[4] Male	Female
BstN I	A CC·TGG[2] + C	16.6	—
MboI	·GATC	6.3	—
Dpn I	GA·TC[3] o +	—	—
Sau3AI	·GATC	6.3	—

[1] · = point of cleavage by restriction enzyme;
+ = cleavage blocked by methylation of nucleotide;
o = cleavage unaffected by methylation of nucleotide.
[2]Cleavage unaffected by methylation in this sequence.
[3]Only methylated adenine is cut by Dpn I.
[4]Variation in fragment size was noted; see text and Figure 1.

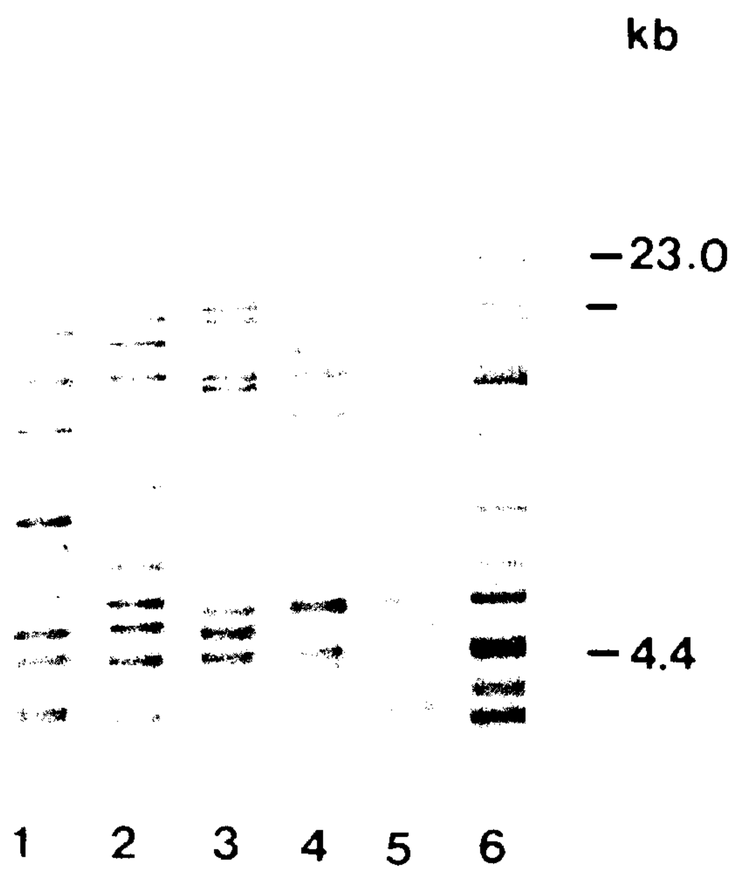

Fig. 1. BstNI digest of *L. kempi* DNA challenged with Bkm probe. Samples from individual males were placed in **lanes 2, 3,** and **6** and samples from individual females in lanes **1, 4,** and **5.** Note the 16.6 kb male-specific fragment (marker); see text and Figure 2.

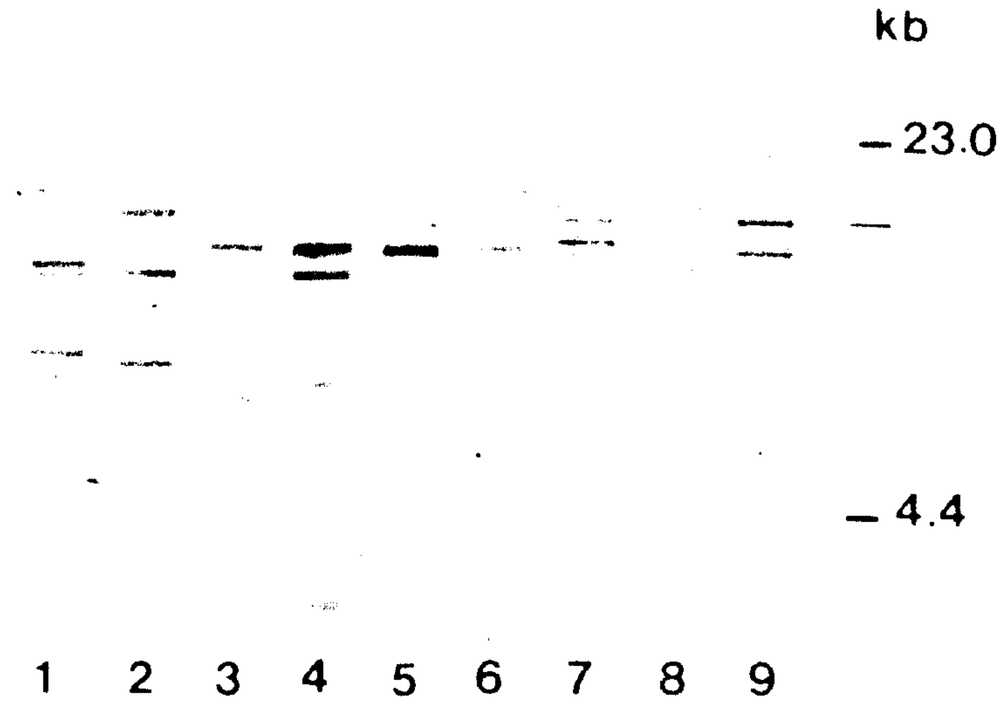

Fig. 2. BstNI digest of *C. mydas* DNA challenged with Bkm probe. Samples from individual males were placed in **lanes 2, 3, 5, 7, 9** and samples from individual females in **lanes 1, 4, 6, 8.** Note 16.6 kb male-specific fragment in lanes 2, 3, 7, and 9, and related fragment in lane 5. See text and compare with Figure 1.

of five males of *C. mydas,* the band was deflected (Fig. 2, lane 5). No bands of this size were observed in DNA from the females.

To determine whether differential methylation could account for appearance of the sex-specific patterns, we used enzymes having the same recognition site, but differing in their ability to cleave methylated DNA. An example of the results obtained is depicted in Figure 3. MboI and Sau3AI each generated a 6.3 kb band in DNA from *C. mydas* (see Table 2, Fig. 3, and Discussion).

In other tests, MboI digests of DNA from each of the five females of *C. mydas* were placed in lanes 1–5 in a gel, and samples of DNA from each of the five males were placed in lanes 6–10. Hybridization with Bkm 2(8) revealed male-specific fragments at 6.3 kb (Fig. 4, lanes 6, 7, 9, 10). A slightly larger male-specific fragment was identified in lane 8. (The BstNI fragment from this male is depicted in Fig. 2, lane 5.) Female-specific MboI fragments were identified at 7.5 kb (see Discussion).

TABLE 2. Male-specific Bkm fragments in C. mydas

Enzyme	Restriction sites and sites of methylation sensitivity[1]	Fragments (kb)[2] Male	Female
BstNI	A CC·TGG	16.6	—
MboI	+ C ·GATC	6.3	7.5
Sau3AI	o + ·GATC	6.3	7.5

[1] · = point of cleavage by restriction enzyme;
+ = cleavage blocked by methylation of nucleotide;
o = cleavage unaffected by methylation of nucleotide.
[2] Variation in fragment size was noted; see text.

DISCUSSION

Appearance of sex-specific Bkm fragments in *L. kempi* and *C. mydas* could be explained as follows: 1) temperature-influenced sex determination (TSD) (Raynaud and Pieau, '85; Shaver et al.,

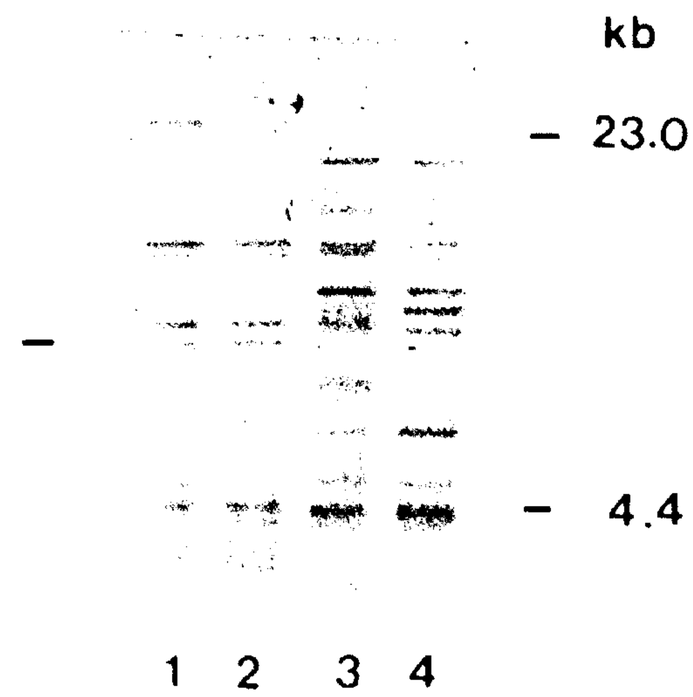

Fig. 3. Hybridization of C. mydas DNA with Bkm after disgestion with MboI and Sau3AI. **Lanes 1** and **3:** Sau3AI digest. **Lanes 2** and **4:** MboI digest. Samples from a single male were placed in lanes 1 and 2 and samples from a single female in lanes 3 and 4. Note 6.3 kb male-specific fragment. Similar results were obtained in five male-female pairs.

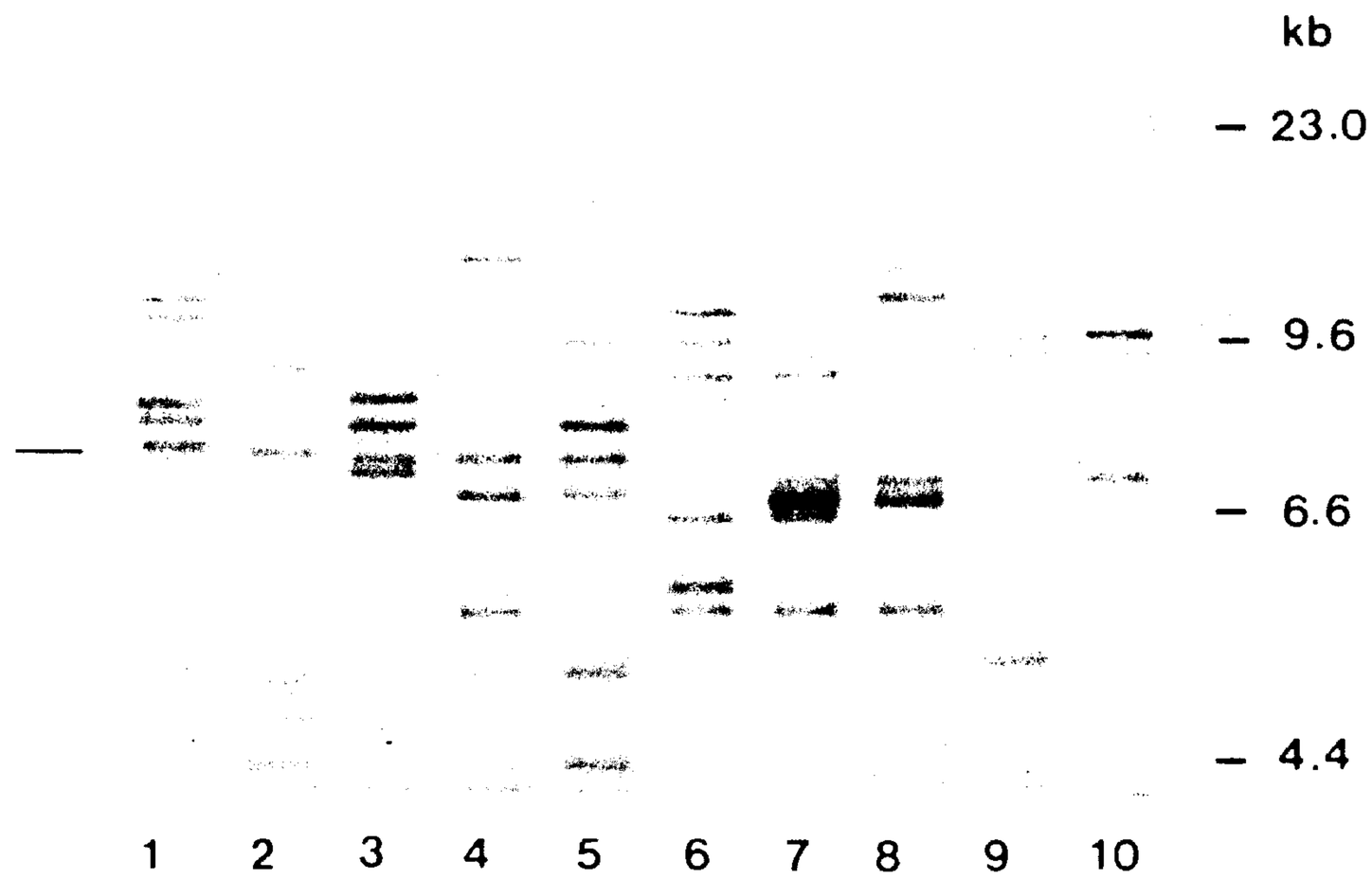

Fig. 4. MboI digest of *C. mydas* DNA challenged with Bkm probe. Samples from individual females were placed in **lanes 1–5** and samples from individual males in **lanes 6–10.** Note female-specific bands at approximately 7.5 kb (left marker) and male-specific bands at approximately 6.3 kb. See text.

'88) may not play a significant role in these species; 2) TSD involves changes such as methylation, or structural changes in chromatin that modify the susceptibility of DNA to restriction endonucleases; or 3) TSD involves modification of DNA in regions involved with or adjacent to sex-determining sequences. (A fourth possibility—fortuitous sex-specific banding due to random polymorphism—deserves mention, although this would seem improbable given that 40 animals were screened and given the number of enzymes used.)

As for the question of methylation, we used three enzymes having the same recognition sequences (GATC) but differing with respect to their ability to cut methylated sites: MboI, DpnI, and Sau3AI. Of these, MboI is blocked only by adenine methylation (this occurs rarely in eukaryotes), DpnI cuts the GATC recognition sequence only when the adenine residue is methylated, and Sau3AI is blocked by methylation of the cytosine residue. BstNI is not affected by methylation. Thus methylation would seem to play no significant role in the appearance of the sex-specific fragments described here.

Unlike most other satellite DNA's, phylogenetically conservative Bkm repeats are transcribed and may contain open reading frames (Singh et al., '84). Moreover, transcription of Bkm appears to occur at certain stages of development. According to a model proposed by Davidson and Britton ('79), certain regions of the genome yield RNA's that control the expression of structural genes—in sea urchins, for example. These regions contain clusters of repetitive sequences that may exhibit transposon-like qualities (Liebermann et al., '83). Insertion of a movable Bkm sequence could effect a rearrangement that would allow the expression or repression of a gene product involved in differentiation of the gonad (see discussion in Standora and Spotila, '85). Alternatively, occurrence of sex-specific fragments could be explained by amplification of the Bkm core repeat during the temperature-sensitive period. This might be expected in embryos destined to become males, as a mechanism to regulate or control gene expression.

Singh et al. ('81) suggested that the sex of mammals could be determined by the "structural alteration of certain DNA sequences" and proposed a general model to account for the detection of male-specific Bkm-related fragments in the mouse. This involved the sex-specific excision of Bkm-related introns adjacent to the sex-determining genes. According to that model, structural modifications could occur in male and/or female DNA; and splicing events in the male would be under the control of the Y-chromosome. A similar model could account for the occurrence of male-specific bands in the sea turtle (Fig. 5); in

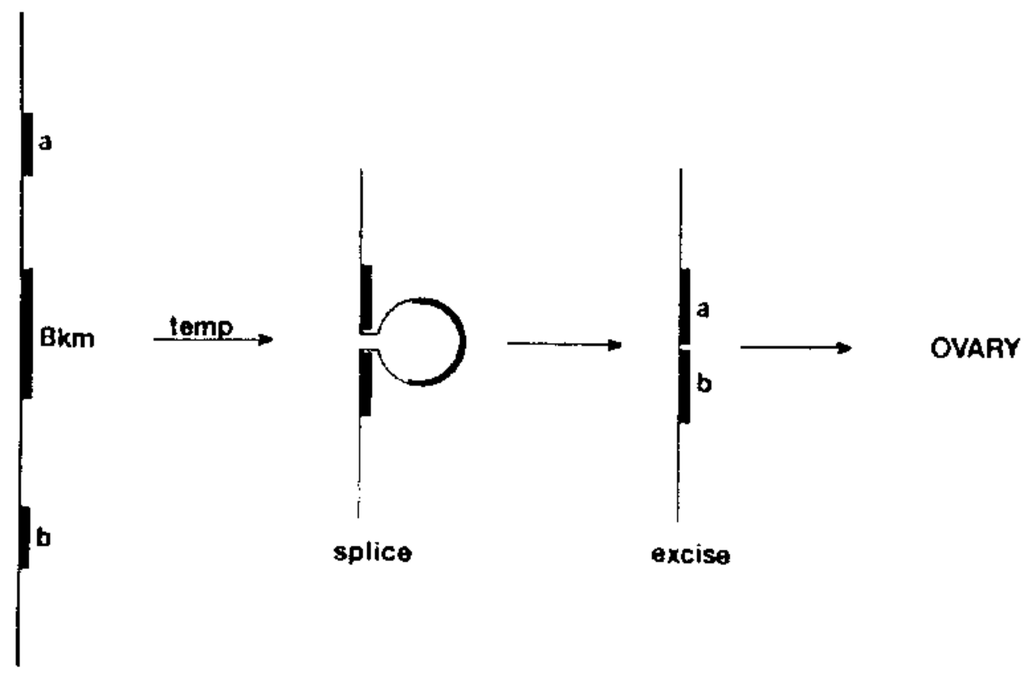

Fig. 5. Temperature-regulated deletion of DNA sequences in sea turtles. According to this model, "a" and "b" are regions of the genome separated by a sequence containing certain Bkm repeats. At temperatures of 27°C, "a" and "b" are involved in testicular development; at temperatures of 30°C, a region containing the Bkm repeats is looped out (by temperature-dependent enzymes?) and excised. As a result, "a" and "b" are juxtaposed and their functions, altered. Modified after the scheme of Singh et al. ('81) in which splicing and concomitant alteration of Bkm restriction patterns were placed under control of the sex chromosomes.

this case, splicing would be under control of the environment.

It might be supposed that ESD is superimposed on a weak genetic element, because males and females both are produced at threshold temperatures in these species. Yet sex chromosomes have been identified neither in *L. kempi* nor in *C. mydas*, and if there were an underlying genetic component overridden by temperature, specific Bkm-related fragments would be expected in all males and females, or in some of the members of each sex—e.g., in all "ZW" or "XY" turtles, of which half would be "sex reversed" by temperature (Zaborski et al., '82). In the Atlantic silverside (*Menidia menidia*), individual broods exhibit different responses to temperature. This indicates that genetic and environmental factors coexist in this species; evidently the genetic component predominates in the more northern populations (Conover and Heins, '87).

Occurrence of male-specific Bkm fragments in peripheral blood could indicate a constitutive, temperature-regulated mode of Bkm expression. This allows the prediction that similar fragments will be found in all of the tissues of the developing *L. kempi* embryo and that those fragments will be deleted, or simply not generated, in the corresponding tissues of the opposite sex during the temperature-sensitive period of development. Alternatively, the male-specific bands described here represent male heterogamety, and Kemp's ridley and the green turtle are actually XX/XY species, like the human and mouse. GSD, and in particular, male heterogamety, are known to occur in reptiles, but it is not easy to accept the notion of male heterogamety in a species such as *C. mydas* in which temperature-regulated sex determination has been documented.

In certain animals, we noted a slight deflection in the position of the sex-specific band. This could be attributed to allelic variation; bands of the same kb were not observed in the opposite sex, in any case. But hypervariability would be expected within the minisatellite regions, especially if the core sequence serves as a recombination signal, as suggested by Jeffreys et al. ('86). As another point, variations in electrophoretic mobility could result from curvature within migrating fragments due to presence of AT-rich DNA (Radic et al., '87).

It may be worth pointing out that Kemp's is an endangered species in which gender cannot easily be identified. Availability of sex-specific markers thus will enable routine identification of sex, at any stage after the temperature-sensitive period. The results and methods described here ought to be confirmed in larger samples, however, before their acceptance and regular application.

We have not found sex-specific Bkm fragments in *Alligator mississipiensis*, and our preliminary study has so far revealed no such fragments in freshwater turtles. So it will be informative to learn whether similar bands occur among any of the other temperature-sex-determined species.

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