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Restriction fragment length polymorphism (RFLP) analysis of bovine nuclear protein genes

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Summary. We have recently cloned both the bovine protamine (Krawetz et al. 1987, DNA 6: 47–57) and high mobility group (HMG-1) cDNAs (Pentecost and Dixon 1984, Bioscience Reports 4: 49–57). They have been used as probes for Restriction Fragment Length Polymorphism analysis of male-female pairs of different species and breeds, within the genus *Bos.* Utilizing this approach we have studied inheritance, chromosomal location and gene copy number of the bovine protamine and HMG-1 genes. This revealed that these nuclear protein genes are highly conserved suggesting that selective pressure has maintained their gene structures during evolution. A polymorphic Taq 1 restriction fragment was identified that was shown to be a heritable marker. These genes are not sex-linked and are present in a single copy for protamine and at least two copies for the HMG-1.

Key words: RFLP (restriction fragment length polymorphism) – Protamine – HMG-1 (high mobility group-1)-Hybridization

Introduction

DNA is packaged tightly within the eucaryotic nucleus into a highly ordered chromatin structure with several levels of organization. In the fundamental unit, the nucleosome, four pairs of histones (H2A, H2B, H3 and H4) form an octameric core particle around which are wound two turns of DNA (Kornberg 1974; McGhee and Felsenfeld 1980). The nucleosomes are spaced by a fifth histone, H1, which lies at the entrance to and exit of the DNA from each nucleosome. H1 also plays a key organizing role in the formation of the next higher order structure, the 30 nm fibre (Thoma et al. 1979). In addition to the histones, a second group of non-histone nuclear proteins, the high mobility group (HMG) are also present at levels ~5–10% of those of the core histones. In many animal species (Bloch 1969), this somatic chromatin organization is totally restructured during the development of the sperm after the synthesis of a new set of basic proteins, the protamines. These progressively replace the somatic histones and generate a new, highly-condensed form of chromatin.

Within the last decade, Restriction Fragment Length Polymorphism (RFLP) analysis has been applied and become an integral part of linkage analysis. This has yielded great insight and provided clinical markers for various genetically based diseases, e.g. hemoglobinopathies (Old and references therein 1986; Orkin and Kazazian and references therein 1984), Huntington's (Gusella et al. 1983), Duchenne muscular dystrophy (Davies et al. 1983), and Familial Alzheimer's disease (St. George-Hyslop et al. 1987). The use of this approach in studying problems outside the human genome has been limited. In the case of the bovine, establishing similar relationships has often relied on more conventional genotypic analysis utilizing isozyme markers (Womack and Moll 1986). Thus far, the method of RFLP analysis has only been sparingly utilized to address the problems of evolution (Ferris et al. 1981; Casanova et al. 1985), even though it shows great potential and provides more detailed information than, for example, C_0t analysis (O'Brien et al. 1985).

Bovine cDNAs corresponding to both protamine (Krawetz et al. 1987) and high mobility group-1 (HMG-1) mRNAs (Pentecost and Dixon 1984) have recently been cloned. We have utilized them as probes for RFLP analysis to study the relationships between the protamine and HMG-1 genes of various bovine

species and breeds. The results of this study are described in the following.

Materials and methods

Chemicals and reagents were purchased from sources previously described (Krawetz et al. 1983). The preparation of random primer probes were as described (Feinberg and Vogelstein 1983). The bull protamine probe was the Sac 1-Hind III fragment (from nucleotide -7 to the poly A tail of the mRNA) from the cDNA clone BPK59 (Krawetz et al. 1987). The bovine HMG-1 probe was the Bst Nl fragment (from nucleotide 3 of the 3′ coding region to 498 of the 3′ untranslated region) of the cDNA clone pBPl (Pentecost and Dixon 1984).

Isolation of high molecular weight genomic DNA

Peripheral blood was drawn into an EDTA vacutainer and white blood cell nuclei were isolated and lysed essentially as described in the lysis solution containing 1 M LiBr (Madisen et al. 1987). Lysis and solubilization was allowed to proceed overnight before extraction of the DNA. The following day, DNA was extracted (Madisen et al. 1987) then redissolved in a minimum volume of 10 m*M* Tris-HCl, pH 7.4 buffer containing 1 mM EDTA.

Restriction fragment analysis

DNA (10 μg) was digested for 16 h with 25 units of either Eco R1 or Taq 1 in the restriction buffers described by Maniatis et al. (1982). Fragments were subsequently resolved on 1% agarose gels then transferred to GeneScreen plus filters and subjected to Southern-hybridization analysis as previously described (Krawetz et al. 1987; Krawetz and Dixon 1984). The filters were initially probed with the protamine probe. Prior to probing with the HMG-1 probe, the bound protamine probe was removed from the filter essentially as described by the manufacturer. Probe removal was varified by autoradiography.

Results

RFLP lineage analysis of bovine species and breeds

As shown in Fig. 1, an *indicus* and *taurus* species and breed lineage map of the genus *Bos*, has been assembled. This was constructed with the use of the data of Becker (1973); Briggs and Briggs (1980); King (1967); Towne and Wentworth (1955) and Zeuner (1963). The breeds shown, were selected from readily available local stocks in order that a varied distribution was represented. From this lineage map, distinct yet related groups can be identified. The most distantly related are those of the species *indicus* and *taurus*, although within *taurus*, three more closely related yet distinct groups are present, i.e. Italian, Other European and Africander cattle. Africander is the most distantly related amongst this group. These groups provided the opportunity to determine whether within a relatively succinct evolutionary period differences could be detected for genes encoding nuclear proteins.

DNA samples were obtained from various *Bos taurus* breeds and subjected to comparative RFLP analysis as described above. As shown in Fig. 2, when

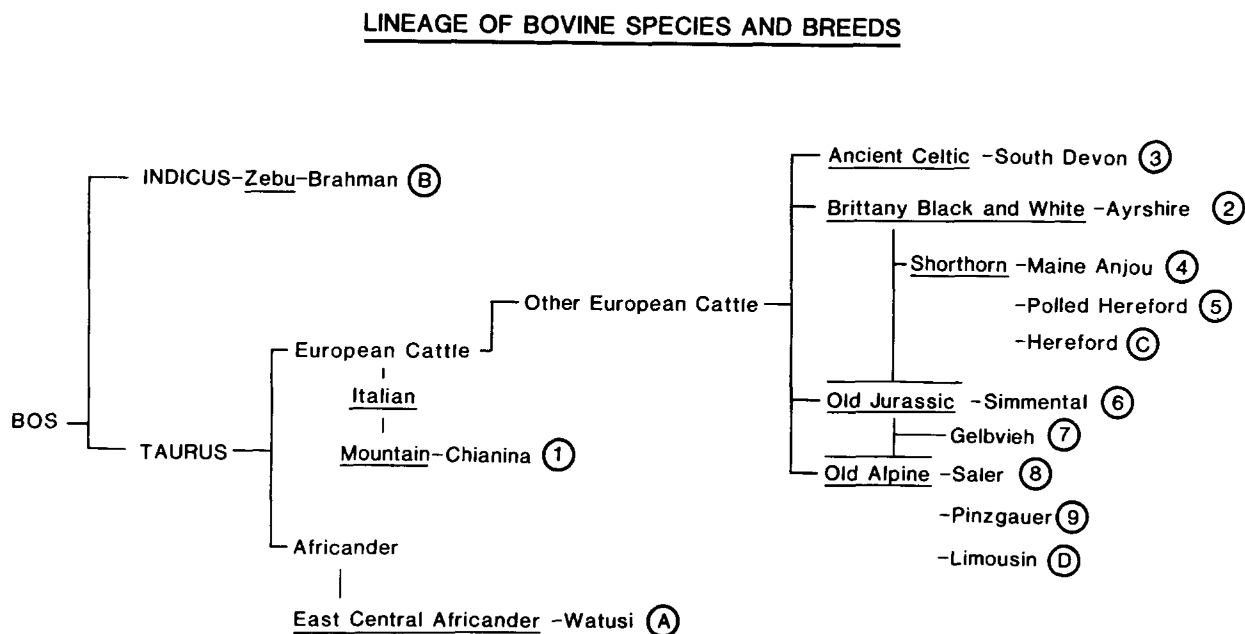

Fig. 1. Lineage of bovine species and breeds. The genus *Bos* with the representative lineage of breeds of the species *indicus* and *taurus* are *outlined.* The relative evolutionary lineage was compiled from the data of Becker (1973), Briggs and Briggs (1980), King (1967), Towne and Wentworth (1955) and Zeuner (1963). The symbols represent the individual breeds

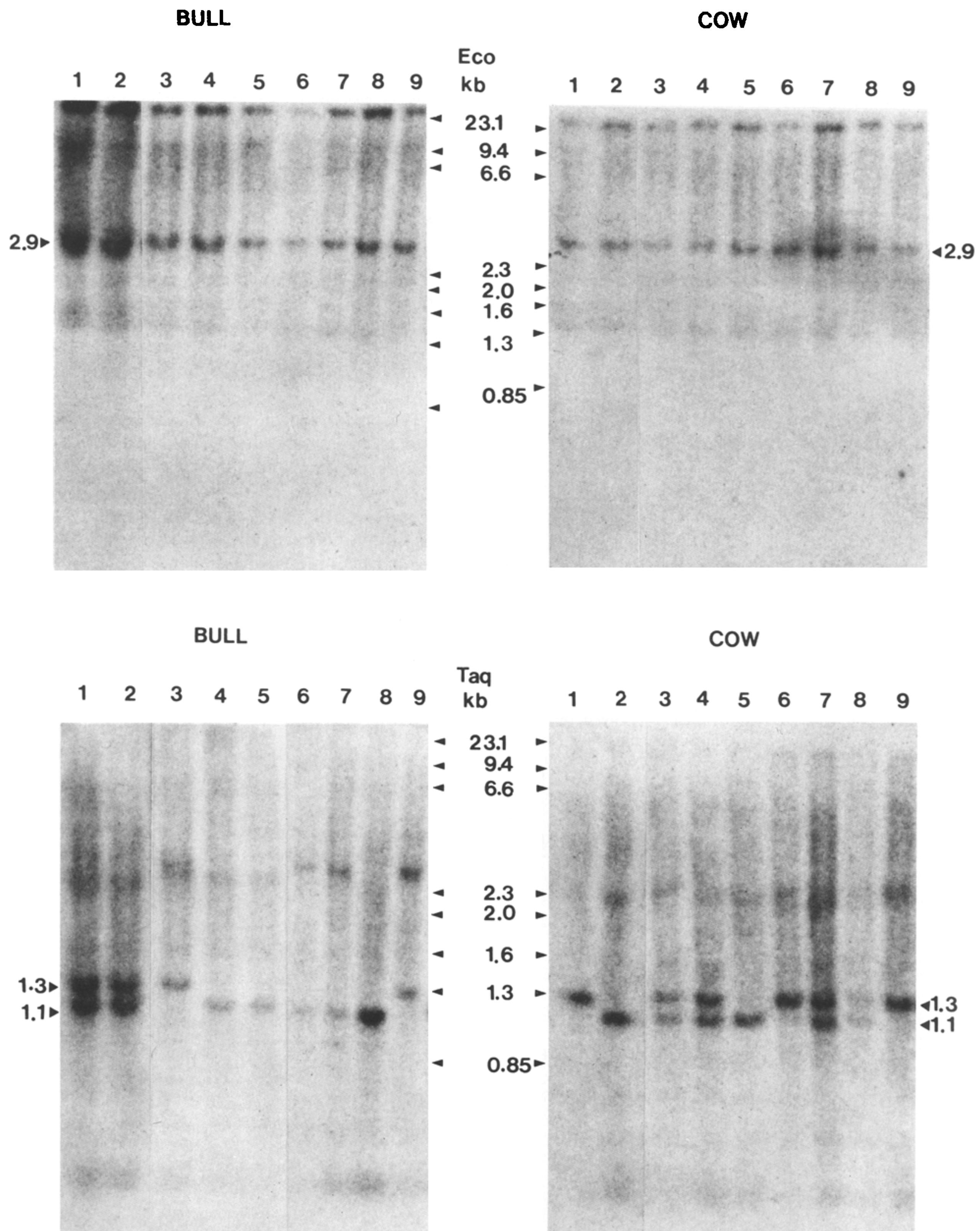

Fig. 2. Southern hybridization analysis of bovine protamine genomic DNA. Bull (*left panels*) and Cow (*right panels*) DNA (10 μg) were digested with either 25 units of Eco R1 (Eco; *upper panels*) or 25 units of Taq 1 (Taq; *lower panels*) for 16 h. The digests were resolved on a 1% agarose gel then electroblotted onto GeneScreen Plus filters. The filters were subsequently hybridized to 10^6 cpm/ml of the protamine probe at 50 °C in the hybridization solution containing 50% formamide. They were subsequently washed, then exposed for six days at −70 °C with a lightening plus intensifying screen. The relative migration of the molecular weight (kb) markers are indicated by the *center arrowheads* and the prominent protamine genomic fragments by the *outer arrowheads. Track 1,* shows the Chianina; *track 2,* Ayrshire; *track 3,* South Devon; *track 4,* Maine Anjou; *track 5,* Polled Hereford; *track 6,* Simmental; *track 7,* Gelbvieh; *track 8,* Saler and *track 9,* Pinzgauer hybridizing fragment(s)

bull or cow Eco R1 digested DNA was probed with the protamine (male gamete nuclear protein) probe a fragment of 2.9 kb in size was detected in the Chianina, Ayrshire, South Devon, Maine Anjou, Polled Hereford, Simmental, Gelbvieh, Saler and Pinzgauer breeds (upper panel). No polymorphism or real difference in intensity of hybridization was observed. This lack of polymorphism was also observed for Sac 1 and other restriction enzyme digested DNAs (S. A. Krawetz et al. 1987; unpublished observations). In order to determine whether a polymorphism could be resolved, DNA was digested with the restriction enzyme Taq 1 (four base recognition sequence) which is known to recognize sites more frequently (Cooper and Schmidtke 1984). As shown in the lower panels, two fragments of 1.3 and 1.1 kb were observed. These results show that individual (upper fragment: Bull, tracks 3, 9; Cow tracks 1, 6, 9; lower fragment: Bull, tracks 4, 5, 6, 7, 8; Cow, tracks 2, 5) or both fragments (Bull, tracks, 1, 2; Cow, tracks 3, 4, 7, 8) can be detected. Since these fragments were not present in all, yet distributed within each breed, this restriction enzyme defines a polymorphic site close to the protamine gene.

The Ayrshire data contains a cow (Cow, Fig. 2, track 2) and bull calf (Bull, Fig. 2, track 2) pair. This data shows that the bull calf is heterozygous for the 1.3 and 1.1 Taq 1 protamine fragments while the cow is homozygous for the 1.1 fragment. This suggests that the sire was either heterozygous, or homozygous for the 1.3 fragment. To address this issue Taq 1 digested sire, cow and bull calf DNA was subjected to Southern blot hybridization analysis with the protamine probe. As shown in Fig. 3 (upper arrow), the sire was heterozygous for the 1.3 fragment. Thus the heterozygotic bull calf inherited the 1.1 fragment (Fig. 3 lower arrow) from the cow and the 1.3 fragment (Fig. 3 upper arrow) from the sire.

When the HMG-1 probe was hybridized to these same filters, a similar simple hybridization pattern was observed. The bull and cow Eco R1 digested DNA resolved two major fragments of ~6.0 and 4.8 kb (Fig. 4, upper panels) when probed with the HMG-1 probe. However, a third fragment larger than 6.0 kb showing varied intensity may also be present (Fig. 4, Bull, track 1 most intense). Its significance has not been assessed. The two clearly resolved major fragments were of similar intensity, present in both bull and cow and showed no polymorphism. However, as in the case of protamine, the restriction enzyme Taq 1 revealed polymorphic sites (bottom panels) contained within the HMG-1 locus. Utilizing the HMG-1 probe, four fragments could be resolved which were ~5.2, 2.8, 2.4, 2.0 kb in size. The 5.2 and 2.0 kb fragments were common to all individuals while the 2.8 and 2.4 were polymorphic. Their distribution is varied within and

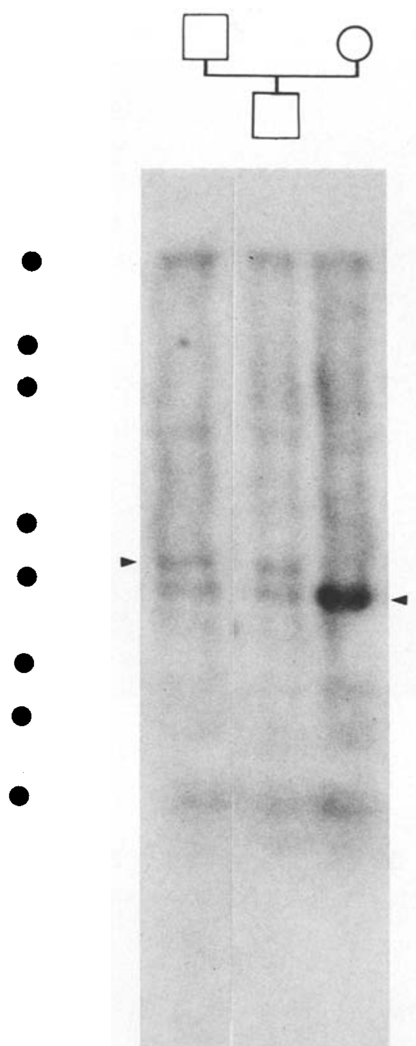

Fig. 3. Inheritance of the bovine protamine fragments in Ayrshire Cattle. Bull sperm (sire; *square, left panel*), cow blood (mother; *circle right panel*) and bull calf blood (off-spring; *square, middle panel*) DNA (10 μg) were digested with 25 units of Taq 1 for 16 h. The digests were resolved on a 1% agarose gel then electroblotted onto a GeneScreen Plus filter. The filters were subsequently hybridized to 10^6 cpm/ml of the protamine probe at 50 °C in the hybridization solution containing 50% formamide. They were subsequently washed then exposed for four days at −70 °C with an intensifiying screen. The relative migration of the molecular weight markers are indicated by the dots. From top to bottom they are 23130, 9416, 6557, 1610, 1310, 854, 672 and 322 bp. The arrowheads (upper 1.3 kb and lower 1.1 kb fragment) indicate the protamine fragment that was inherited from the respective sire and mother

among the individual breeds. In addition to the common 5.2 and 2.0 kb fragments, the additional 2.4 kb fragment was uniquely observed in Bull tracks 1, 4, 9, and Cow track 1, while both the 2.8 and 2.4 kb fragments were observed in Bull tracks 2, 3, 5, 6, 7 and Cow track 9 while the additional 2.8 kb fragment was uniquely observed in Bull track 8 and Cow tracks 2, 3, 4, 5,

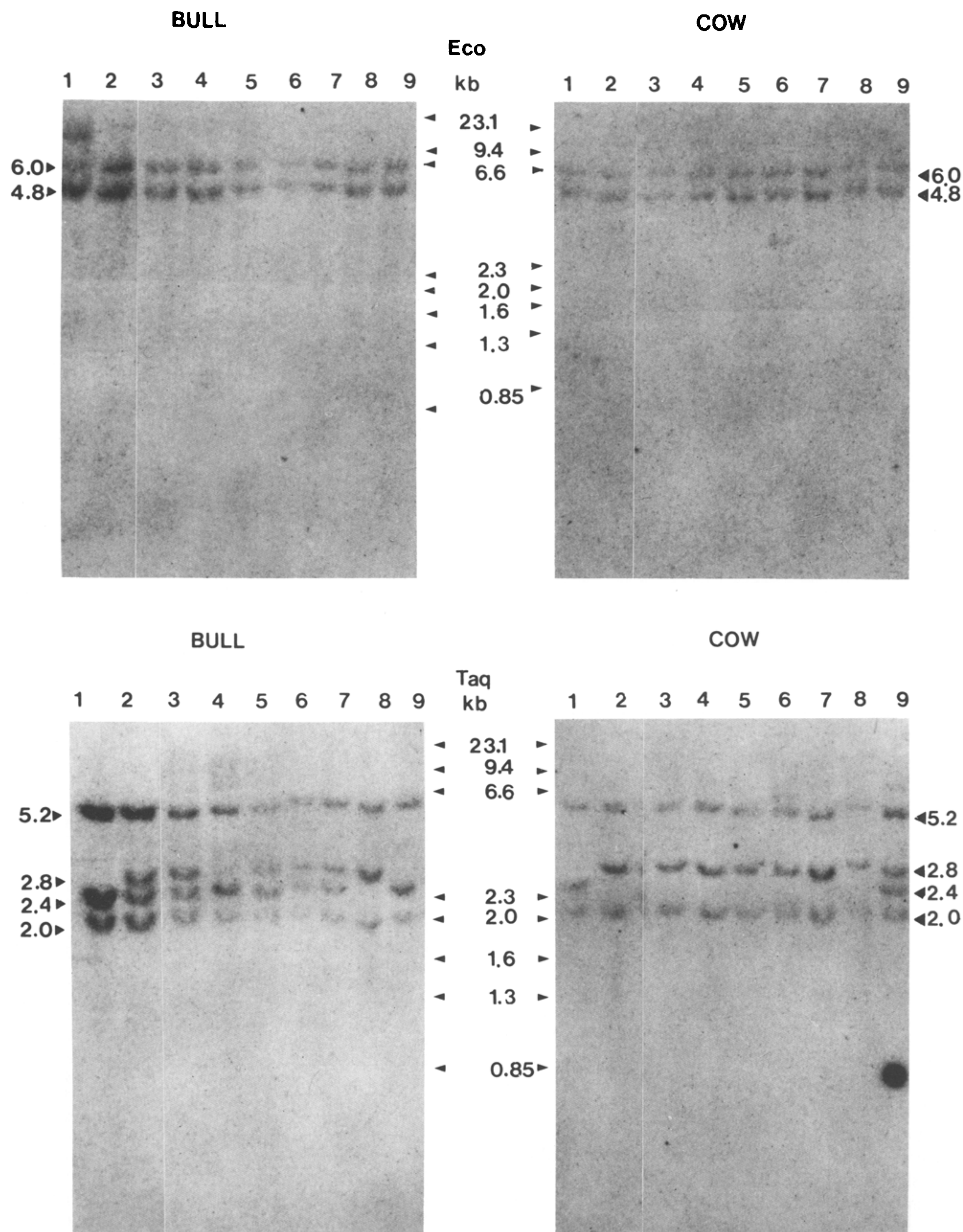

Fig. 4. Southern hybridization analysis of bovine high mobility group-1 genomic DNA. Bull (*left panels*) and Cow (*right panels*) DNA (10 μg) were digested with either 25 units of Eco R1 (Eco; *upper panels*) or 25 units of Taq 1 (Taq; *lower panels*) for 16 h. The digests were resolved on a 1% agarose gel then electroblotted onto GeneScreen Plus filters. The filters were subsequently hybridized to 10^6 cpm/ml of the high mobility group-1 probe at 50 °C in the hybridization solution containing 50% formamide. They were subsequently washed and then exposed for six days at −70 °C with a lightening plus intensifying screen. The relative migration of the molecular weight (kb) markers are indicated by the *center arrowheads* and the prominent high mobility group-1 genomic fragments by the *outer arrowheads. Track 1,* shows the Chianina; *track 2,* Ayrshire; *track 3,* South Devon; *track 4,* Maine Anjou; *track 5,* Polled Hereford; *track 6,* Simmental; *track 7,* Gelbvieh; *track 8,* Saler and *track 9,* Pinzgauer hybridizing fragments

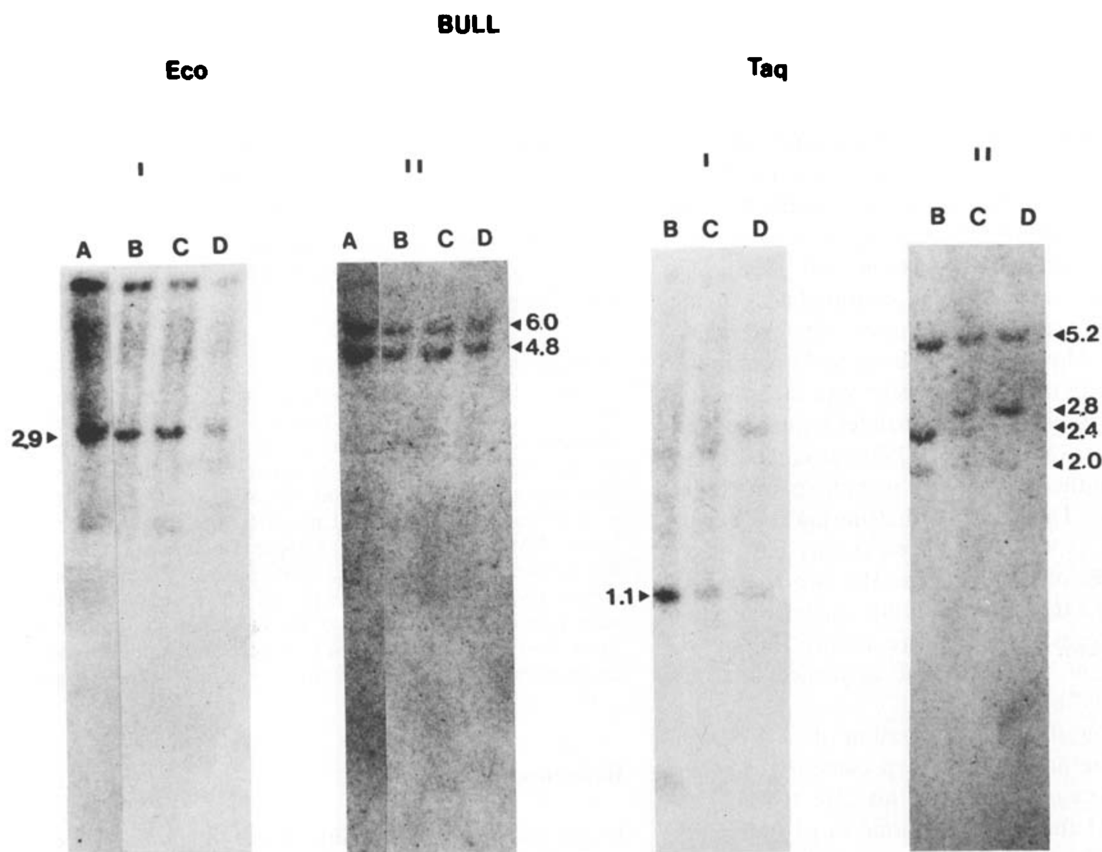

Fig. 5. Comparative Southern hybridization analysis of *Bos indicus* and *Bos taurus* protamine and high mobility group-1 genomic DNA. Bull DNA (10 μg; *track A,* Watusi; *track B,* Brahman; *track C,* Hereford; *track D,* Limousin) was digested with either 25 units of Eco R1 (eco; *left set of panels*) or 25 units of Taq 1 (Taq; *right set of panels*) for 16 h. The digests were resolved on a 1% agarose gel then electroblotted onto GeneScreen Plus filters. The filters were subsequently hybridized to 10^6 cpm/ml of their respective probes (1, protamine and 11, high mobility group-1) at 50 °C in the hybridization solution containing 50% formamide. They were subsequently washed then exposed for six days at −70 °C with a lightening plus intensifying screen. The size (kb) of the corresponding hybridizing gene fragment(s) are indicated by the *arrowheads*

6, 7, 8. It is interesting to note that within each track the relative intensities of the fragments are similar to those fragments observed with the protamine probe (Fig. 2, lower panels).

This data suggests that within the two classes of European cattle the gene segments surrounding both the protamine and HMG-1 genes are similar. However, it did not address the issue of similarity between the species or a more distantly related breed. As shown in Fig. 5, when Brahman (track B; *Bos indicus*) and Watusi (track A; Africander) DNA was subjected to Southern analysis the results were similar to those obtained for the European cattle (Fig. 2, Fig. 4 and Fig. 5, tracks C and D). They show that the *Bos indicus* and the Africander do not possess additional or unique polymorphic Eco R1 or Taq 1 sites. The data supports the view that a high level of sequence conservation amongst the nuclear protein genes was maintained during the relatively short evolutionary period of *Bos indicus* and *Bos taurus.*

Discussion

The application of Southern/RFLP analysis to members of the *Bos* genus provides additional insight into the genetic relationships between various species and breeds. From the data presented above, sex-linkage, gene copy number and inheritance can be simultaneously assessed in one assay system.

Of the breeds examined, both sexes displayed very similar if not identical protamine and HMG-1 restriction fragments. Within each lane, all hybridizing bands yielding the same restriction pattern were of a constant ratio regardless of sex. This suggests that both the protamine and HMG-1 genes are autosomal and not

sex-linked. Developmentally, this is consistent with the view that these gene products do not play a role in sex determination.

The unique protamine Eco R1 fragment and the polymorphic Taq 1 fragments of equal intensity supports the view that the bovine protamine gene is present as a single copy (per haploid genome). This is in marked contrast to the situation in rainbow trout (*Salmo gairdneri*), where there are approximately 15–20 copies per haploid genome (Dixon et al. 1986). It is intriguing that the more complex mammalian genome would have fewer rather than more (less complex) protamine genes. However, the presence of a protamine multigene family in trout is consistent with the isolation of six protamine polypeptides of distinct sequence from a single trout testis (McKay et al. 1986) as compared to the single protamine polypeptide in bull sperm (Mazrimas et al. 1986). This suggests that during the evolution of this gene in salmonid fishes, it may have been subject to a series of duplications. The two fragments produced by Taq 1 digestion from this single copy gene in the bovine suggest that they are allelic. This hypothesis is consistent with genomic sequence analysis (Krawetz et al. 1987 b).

The restriction digest-hybridization data utilizing the HMG-1 probe are similar when compared to that found for the protamine gene. In this case at least two prominent Eco R1 fragments and four Taq 1 fragments were observed (Fig. 4). This suggests that like the trout HMG-T (Pentecost et al. 1985) the bovine HMG-1 is not single copy and that the bovine genome contains at least two copies of the HMG-1 gene per haploid genome.

Similar protamine and HMG-1 fragments were observed in the various breeds and *Bos* species examined even though hybridization conditions were such that only sequences with at least ~82% identity would hybridize (Bonner et al. 1973). These conditions were selected to minimize non-specific hybridization to any possible pseudo- or quasi-related genes. This suggests that within this relatively short evolutionary time span (~1,000,000 years since the divergence of *indicus* and *taurus* and ~5,000 years for European and Africander; Becker 1973; Clutton-Brock 1981; Towne and Wentworth 1955; Zeuner 1963), the pressure to maintain the relative sequence organization of these nuclear protein genes has exceeded their ability to drift. Although this conservation is observed with Eco R1 sites, the Taq 1 polymorphism provided the opportunity to determine sex-linkage and inheritance of these genes, in a manner similar to that utilized in human genetics. As shown above (Fig. 3) the Ayrshire data clearly illustrated Mendelian inheritance. This showed that the bull calf inherited the 1.3 fragment from the sire and the 1.1 fragment from the cow; thus demonstrating the usefulness of the protamine probe to follow this inheritance. A similar heritable relationship was observed for the HMG-1 fragment. The bull calf is heterozygous for the 2.8 and 2.4 kb fragments while the cow is homozygous for the 2.8 fragment. It suggests that the sire must contain the 2.4 fragment in a heterozygous state. The sequence conservation and polymorphism suggests that these probes may be useful genetic markers for parentage analysis and for analyzing and developing breeding strategies for the *Bos* species. This application is presently being explored.

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