A 1.3 kb Satellite DNA from *Bubalus bubalis* not Conserved Evolutionarily is Transcribed

Sunita Bhatnagar[a,*], Anu Bashamboo[b], Munmun Chattopadhyay[b], Supriya Gangadharan[b], and Sher Ali[b]

[a] Regional Plant Resource Centre, Nayapalli, Bhubaneswar 751015, Orissa, India.
E-mail: sunita_bhatnagar@hotmail.com
[b] National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi 110067, India

* Author for correspondence and reprint requests

Z. Naturforsch. **59 c**, 874–879 (2004); received July 5/August 11, 2004

A 1.3 kb satellite DNA from a size defined genomic library of mammal *Bubalus bubalis* was cloned and sequenced. The clone pSB1 is AT rich with 447 A (33.6%), 262 C (19.7%), 240 G (19.0%) and 383 T (28.8%). There were about 1400 copies of contig in the bubaline genome but it did not uncover allele length variation when used as probe in conjunction with a number of restriction enzymes. The contig pSB1 is not conserved evolutionarily and cross hybridizes only with the Bovideae family. A set of primers from 5′ (nt 422 to 441) and 3′ (nt 962 to 947) deduced from the clone used for PCR amplification with four members of the Bovideae family gave the expected 530 bp band of equal intensity indicating a similar number of copies in all the four species namely *Bos indicus*, *Capra hircus*, *Ovis aries* and *Bubalus bubalis*. Expression studies with pSB1 following slot-blot hybridization with total RNA isolated from ovary, testes, kidney, lung and spleen revealed varying signal intensities in all the tissues with a most prominent signal in spleen but a faint one in ovary. Further sequence analysis revealed the presence of several eukaryotic transcriptional elements such as NF-E1, Poly-A signal, lariat consensus sequences, and CTF/NF1 binding sites. Blast search showed 90% sequence similarity with the reverse transcriptase gene of *Bos taurus* and sequences from nt 283 to 636 within the contig showed highly conserved reverse transcriptase like signatures along with *N*-glycosylation and protein kinase C phosphorylation sites. From the data we conclude that the pSB1 representing satellite DNA is associated with transcribing sequences. The prospect of identifying functional genes linked with the satellite fraction in higher vertebrates is discussed.

Key words: Bubalus bubalis, Satellite DNA, Bovideae

Introduction

The bubaline *Bubalus bubalis* is an important species in Asia, Egypt and Southeast Europe. Owing to its importance in milk and meat industry, attention has been focused to improve the genetic potential of this animal. A number of breeds on the basis of physical and physiological attributes have been reported and few of them are well known for their good milk, disease resistance and lower fodder requirement but genetic information on them is lacking. Absence of such information and indiscriminate slaughter of buffaloes not only reduces the number of elite animals but also obliterates the uncharacterized breeds with possible untapped genetic potential (Mary and Ali, 1997). In earlier attempts, RFLP studies on mitochondrial DNA (Tanaka *et al.*, 1995) and genetic variability at the MHC loci (Wenink *et al.*, 1998) have been conducted. More recent approach of DNA profiling particularly with the use of synthetic oligonucleotide probes specific to minisatellite regions (Ali and Epplen, 1991) has facilitated the analysis of bubaline genome. Other synthetic probes have been attractive candidates for analyzing a number of vertebrate genomes including humans (Epplen, 1988), their use does not reveal the transcriptionally active sequences associated with satellite DNA. Information on the satellite DNA linked with transcriptionally active sequences or a functional gene would be critical in deciphering the biological significance of repetitive DNA in higher eukaryotes in addition to in-depth characterization of this species. Keeping this in mind, a recombinant representing satellite DNA isolated from a size-defined genomic library was characterized and the biological significance of the same was highlighted.

0939–5075/2004/1100–0874 $ 06.00 © 2004 Verlag der Zeitschrift für Naturforschung, Tübingen · http://www.znaturforsch.com · D

Materials and Methods

Samples

Blood samples from five different breeds *i.e.*, Mehsana, Murrah, Surti, Toda and a non-descript breed from Assam were collected and DNA was isolated according to standard protocols (Kunkel *et al.*, 1977).

Construction of size-defined genomic library

Pooled genomic DNA (10 μg) representing 2 μg from each breed of *Bubalus bubalis* was digested with MbOI enzyme following supplier's specifications, resolved on 0.8% agarose gel and fragments in the range of 1–5 kb were excised from the gel. The purified DNA was cloned in pGEM-7Zf(+) after Ali *et al.* (1999). A total of 40 recombinant clones was obtained after initial screening. Insert size was estimated following symmetrical PCR using universal reverse and forward primers. Amplified products were resolved on 1% agarose gel, transferred onto a nylon membrane, UV fixed and hybridized with a random labelled bubaline genomic probe followed by autoradiography using standard protocols (Ali *et al.*, 1999).

Zoo-blot hybridization

Approx. 200 ng of heat-denatured genomic DNA in 2XSSC from different sources such as *Homo sapiens*, *Presbytis entellus*, *Macaca mulatta*, *Macaca radiata*, *Bubalus bubalis*, *Bos indicus*, *Capra hircus*, *Ovis aries*, *Sus scrofa*, *Tammar wallaby*, *Oructolagus cuniculus*, *Rattus norvegicus*, *Heteropneustes fossilis*, *Camelus dromedarirs*, *Gavialis gangeticus*, *Columba livia*, *Naja naja*, *Acheta domesticus* and *E. coli* were used for slot/zoo-blot preparation.

Blot was semi dried and cross-linked with UV using Stratalinker–1800 (Stratagene, San Diego, CA, USA). Zoo-blot hybridization was conducted following standard protocols (Ali *et al.*, 1999).

Sequencing of pSB1 and its analysis

pSB1 was fully sequenced using a sequenase kit (Amersham, version 2.0) employing Sanger's dideoxy chain termination method (Sanger *et al.*, 1977) and the sequence was deposited in GenBank, accession No. Af-119573. Sequence analysis for the presence of short-tandem repeat motifs, restriction sites, ORF, possible coding potential and presence of eukaryotic transcriptional elements was conducted using the Gene Runner Program (Hastings Software, Los Angeles, USA) and DNAsis software (LKB/Pharmacia, Sweden). Homology search was conducted using Blast search (http://www.ncbi.nlm.nih.gov, NIH, Bethesda, Maryland, USA).

PCR amplification for the assessment of organizational variation of pSB1 in related bovids

A set of primers from 5′ (nt 422–441), 20-mer (5′ GTCATCATAAGAAACGCTGG 3′) and 3′ (nt 962–947), 18-mer (3′ TGCTATGGGTCTTG-TGGC 5′), respectively, was deduced from the contig. Symmetrical PCR was conducted with genomic DNA from four different breeds of buffaloes, namely Mehsana, Murrah, Surti, Toda, cattles goat and sheep. The reaction was carried out in a 25 μl volume containing approx. 25 ng of genomic DNA as template, 20 pmol of primer, 0.25 units of Taq DNA polymerase (Bangalore Genei, Bangalore, India), 2.5 mM $MgCl_2$, 200 μM of each dNTP, 50 mM KCl, 20 mM Tris [Tris(hydroxymethyl)-aminomethane]-HCl (pH 8.3) and 0.1% Triton X-100 and an equal volume of mineral oil on a Thermal Cycler (Perkin Elmer, Cetus). The reaction mixture was first heat-denatured at 96 °C for 5 min, and amplified for 35 cycles comprising subsequent steps of denaturation at 96 °C for 45 s, annealing at 58 °C for 45 s and extension of the primer at 72 °C for 45 s. Final extension was carried out at 72 °C for 5 min. 15 μl of the amplified products were resolved on 1–5% agarose gel, stained with ethidium bromide and photographed under UV light.

RNA isolation and hybridization of contig with total RNA slot-blot.

For total RNA isolation, tissue samples of kidney, spleen, testis, ovary and lung were obtained in liquid nitrogen from the local slaughter house and total RNA was isolated using the Tri-X reagent (Molecular Research Center, Cincinnati, Ohio) according to manufacturer's specifications. Approx. 5 μg total RNA were spotted onto the nylon membrane and fixed under UV light for 10 min. The blot was hybridized with (∞P32) dCTP labelled pSB1 clone and autoradiography was carried out after Ali *et al.*, (1999). A β-actin cloned probe was used as control.

Northern blot analysis

Approx. 10 μg of total RNA isolated from the above mentioned tissues were electrophoretically separated on 1% agarose gel following standard procedures and transferred onto a nylon membrane and hybridized with the pSB1 labelled probe.

cDNA synthesis and reverse transcriptase polymerase chain reaction

For the assessment of transcription, RT-PCR was conducted for the first strand of cDNA of total RNA from spleen, lung, testis, ovary and kidney; the Superscript preamplification system (GIBCO-BRL, Bangalore, India) was used according to supplier's specifications. RT-PCR amplicons were resolved on 1.5% agarose gel, transferred, UV fixed, hybridized and autoradiographed. For positive control β-actin primers were used.

Results

Zoo-blot hybridization showed that pSB1 hybridizes with buffalo, cattle, goat and sheep genomic DNA giving rise to almost equal signal intensity indicating that the same is present in equal copy number in these species. However, other non-bovid species failed to show signals even after a 10-fold prolonged exposure indicating that the sequences are not conserved evolutionarily. Following standard approach (Ray *et al.*, 1999), bubaline genome was found to contain approx. 1400 copies of 1.3 kb contig.

The pSB1 contig is AT rich with 447 A (33.56%), 262 C (19.67%), 240 G (19.02%) and 383 T (28.75%). The search for repeat motifs showed presence of 2–10 mers repeats with a majority of 5 mers but none was present consecutively. Further the contig showed 6 ORFs, of which the longest one consisted of 83 amino acids ranging from nt 655 to nt 906, followed by a frame of 72 amino acids from nt 1116 to nt 1331. Unlike these two, another coding stretch consisted of a start codon at nt 1116 but it lacked a stop codon. This may be due to the fact that contig seems to be a part of functional gene. The schematic representation of the pSB1 with respect to major ORFs and reverse transcriptase like signature sequences is given in Fig. 1.

Blast search showed maximum sequence (90%) homology (Fig. 2B) of a part of contig with the reverse transcriptase gene bovine *Bos taurus* and placental lactogen precursor (PL) gene of ovine *Ovis aries*. Further, as shown in Fig. 2A, translation of nucleotides into protein sequence revealed the highly conserved signatures of reverse transcriptase gene (Hattori *et al.*, 1986; Michel and Lang, 1985; Minghetti and Dugaiczyk, 1993).

Primers deduced from the contig were used for genomic amplification of the samples from four buffalo breeds (Fig. 3A), along with related species such as cow, goat and sheep (Fig. 3B). They revealed similar signal intensity suggesting that the copy number in all the species is same. The clone did not uncover allele length variation when used as a probe for DNA typing with a number of restriction enzymes such as Alu1, BamH1, Rsa1 and Bgl1 indicating that this contig is probably uniformly organized in bovid genomes.

The cloned pSB1 probe hybridized with bubaline derived total RNA revealed signals in most of the tissues with varying intensity but the reason for this variation was due to different amounts of RNA slotted on the blot as confirmed by the β-actin probe (Fig. 4A and 4B). The transcriptional status of pSB1 was further confirmed by RT-PCR using cDNA from somatic as well as germline tissues. RT-PCR generated amplicons were hybridized with the pSB1 probe and with the β-actin probe (control) revealed two different bands, one at 520 bp and another at 480 bp (Fig. 4C). This

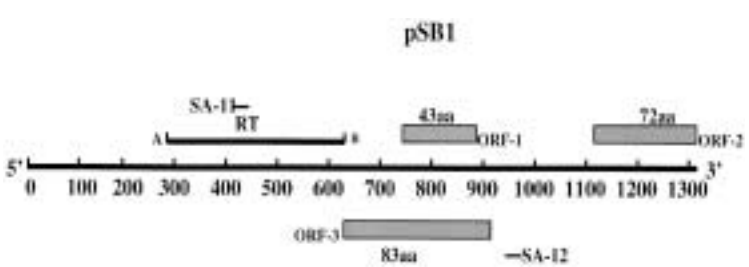

Fig. 1. Schematic representation of pSB1 contig. Note three major open reading frames (ORF-1, 43 amino acids, ORF-2, 72 amino acids, and ORF-3, 83 amino acids). A-B stretch of contig shows homology with reverse transcriptase gene (RT) with its signatures. SA-11 (nt 422–441) and SA-12 (nt 962–947) are the primer sites that were used for PCR and RT-PCR.

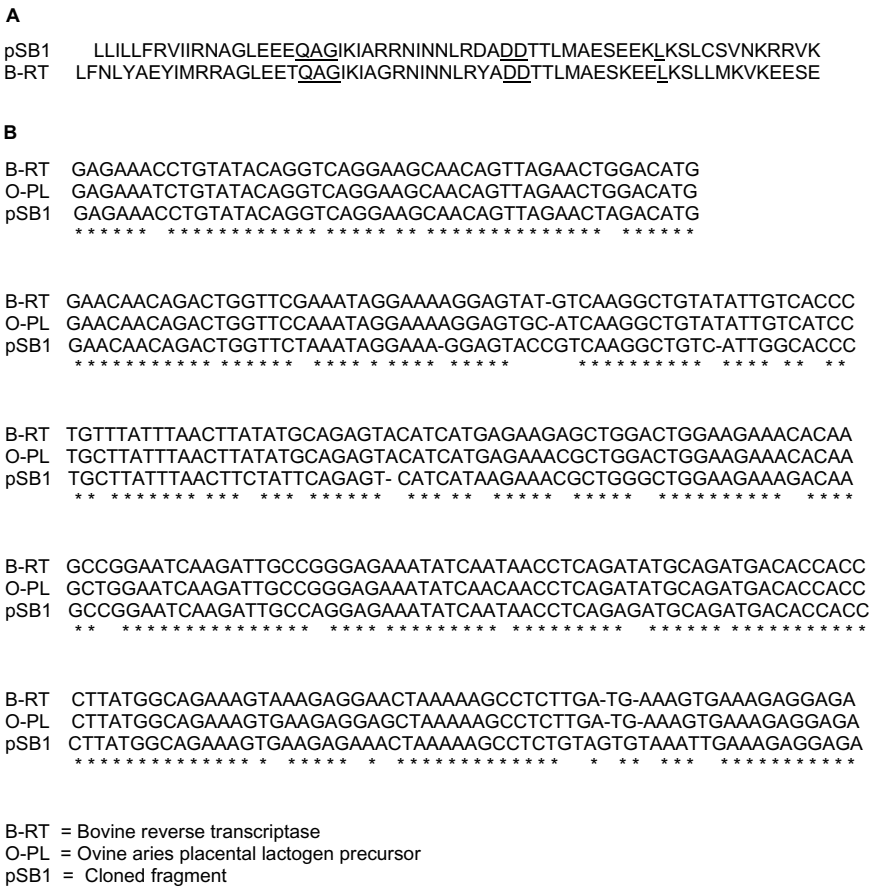

Fig. 2. Blast search of the pSB1 translated sequences amplifications showing protein homology with reverse transcriptase gene of bovine origin. Note the underlined conserved RT gene signatures (A). CLUSTAL W (1.74) multiple sequence alignment showing nucleotide sequence homology of the pSB1 contig with bovine reverse transcriptase and *Ovis aries* placental lactogen precursor genes (B).

observation was in homology with pSB1 contig with the bovine reverse transcriptase and ovine placental lactogen precursor genes. Although less likely the possibility of alternative splicing of the RT gene may not be ruled out. Thus, pSB1 contig shares the part homology with the reverse transcriptase gene of bovine origin and ovine placental lactogen precursor gene *i.e*, the same is conserved in the Bovideae family only and could possibly be used as probe in its identification.

Discussion

The pSB1 contig is rather unique since it is AT rich, which is in contrast with earlier reports where the bubaline genome has been reported to be GC rich (Szemraj *et al.*, 1995). Several eukaryotic transcriptional elements, such as NF-E1, Poly-A signal, lariat consensus sequences, TATA box, translational initiation sequences and CTF/NF1 binding sites were detected in pSB1 contig suggesting it to be a part of coding sequences. The same was evident since it showed signals both in somatic as well as germline tissues in case of total RNA slot-blot,

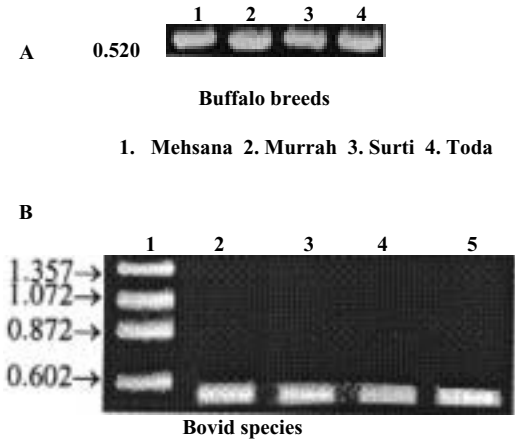

Fig. 3. PCR amplification of genomic DNA of the four breeds of buffaloes (A) and related bovids (B) with the primers SA-11 and SA-12. Note the 520 bp expected amplicons of similar intensity in all the samples seen in ethidium bromide stained gel.

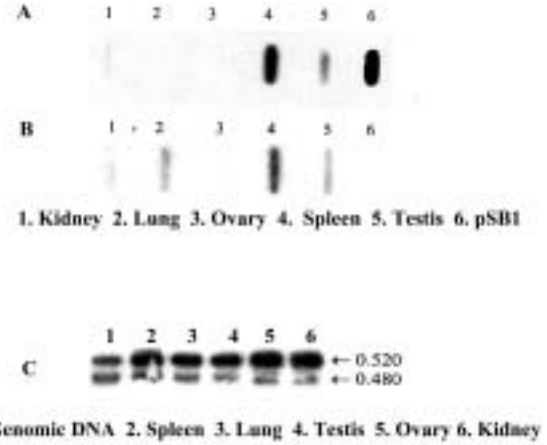

Fig. 4. Slot-blot hybridization of pSB1 contig with bubaline derived total RNA isolated from germline and somatic tissues (A) and hybridization of the same blot with bubaline derived cloned β-actin probe (B). The expression of pSB1 was also confirmed by RT-PCR using cDNA synthesized from the tissues as shown (C). Note the strong signal intensity in all the samples and two bands of about 520 and 480 bp comprising with at least two genes showing high level of sequence homology. Also note that two bands became apparent only after hybridization of the RT-PCR blot and not in ethidium bromide stained gel.

Northern blot hybridization and RT-PCR amplification.

Sequence further showed homology with the RT gene of bovine origin and homology with *Ovis aries* placental lactogen precursor gene which is in accordance with RT-PCR data exhibiting two bands. The contig also revealed distinct RT signatures at several places in addition to the presence of eukaryotic transcriptional elements and ORFs. Thus the precise role of pSB1 contig is still not clear but the data suggest that this is associated with transcribing sequences belonging to a family of genes at least conserved in the bovids and perhaps play some still unidentified regulatory roles in the bubaline and related genome.

Acknowledgements

This work was supported by a grant (no. SP/SO/D-51/93) from the Department of Science and Technology, New Delhi to S. A. Acknowledgement is due to CSIR for the support provided to S. B. in the form of Senior Research Associateship. We thank Shri Khem Singh Negi for technical assistance.

Ali S. and Epplen J. T. (1991), DNA fingerprinting of eukaryote genomes by synthetic oligodeoxyribonucleotide probes. Ind. J. Biochem. Biophys. **28**, 1–9.

Ali S., Azfer M. A., Bashamboo A., Mathur P. K., Malik P. K., Mathur V. B., Raha A. K., and Ansari S. (1999), Characterization of a species specific repetitive DNA from a highly endangered wild animal *Rhinoceros unicornis* and assessment of genetic polymorphism by microsatellite associated sequence amplification (MASA). Gene **228**, 33–42.

Epplen J. T. (1988), On simple repeated GATA/GACA sequences: A critical reappraisal. J. Herid. **79**, 409–417.

Hattori M., Kuhara S., Takenaka O., and Sakaki Y. (1986), L1 family of repetitive DNA sequences in primates may be derived from a sequence encoding a reverse transcriptase-related protein J. Virol. **321**, 625–628.

Kunkel L. M., Smith K. D., Boyer S. H., Borgaonkar D. S., Wachtel S. S., Miller O. J., Breg W. T., Jones H. W., and Rary J. M. (1977), Analysis of human Y chromosome specific reiterated DNA in chromosome variants. Proc. Natl. Acad. Sci USA **74**, 1245–1249.

Mary V. J. and Ali S. (1997), Synthetic DNA-based genetic markers reveal intra and inter species DNA sequence variability in the *Bubalus bubalis* and related genomes DNA. Cell. Biol. **16**, 369–378.

Michel F. and Lang F. B. (1985), Mitochondrial class II introns encode proteins related to the reverse transcriptases of retroviruses. Nature **316**, 641–643.

Minghetti P. P. and Dugaiczyk A. (1993), The emergence of new DNA repeats and the divergence of primates. Proc. Natl. Acad. Sci. USA **20**, 3933–3937.

Ray P., Gangadharan S., Chattopadhyay M., Bashamboo A., Bhatnagar S., Mallik P. K., and Ali S. (1999), A bubaline derived satellite DNA probe uncovers generic affinities of gaur with other bovids. J. Biosciences **24**, 101–104.

Sanger F., Nicklen S., and Coulson A. R. (1977), DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**, 5463–5467.

Szemraj J., Plucienniczak G., Jaworski J., and Plucienniczak A. (1995), Bovine Alu-like sequences mediate transposition of a new site-specific retroelement. Gene **152**, 261–264.

Tanaka K., Yamagata T., Masangkay J. S., Faruque M. O., Vu-Binh D., Salundik, Mansjoer S. S., Kawamot Y., and Namikawa T. (1995), Nucleotide diversity of mitochondrial DNAs between the swamp and the river types of domestic water buffaloes. *Bubalus bubalis*, based on restriction endonuclease cleavage patterns. Biochem. Genet **33**, 137–148.

Wenink P. W., Groven A. F., Roelke-Parker M. E., and Prins H. H. (1998), African buffalo maintain high genetic diversity in the major histocompatibility complex in spite of historically known population bottlenecks Mol. Ecol. **10**, 1315–1322.