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Mediterranean river buffalo oxytocin-neurophysin I (OXT) gene: structure, promoter analysis and allele detection

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ABSTRACT: Oxytocin (OXT) is a very abundant nonapeptide neurohypophysial hormone implicated in several aspects of reproduction, including social, sexual and maternal behaviour, induction of labour and milk ejection. The nucleotide sequence of the whole OXT-neurophysin I encoding gene (OXT) in Mediterranean river buffalo was determined, plus 993 nucleotides at the 5' flanking region. Buffalo oxytocin gene sequence analysis showed two transitions in the promoter region (C→T in position – 966 and G→A in position – 790) and one transversion G→T at the 170th nucleotide of the second exon, responsible for the Arg^{97}→Leu aa substitution which identifies an allele named OXT B. A PCR-RFLP based method for a rapid identification of carriers of these alleles has been developed.

Key words: River buffalo, Oxytocin-neurophysin I, *OXT*, Nucleotide sequence, Allele, Milk ejection.

INTRODUCTION - Oxytocin (*OXT*) is a very abundant nonapeptide neurohypophysial hormone. In the central nervous system, the *OXT* gene is primarily expressed in magnocellular neurons in the hypothalamic paraventricular nucleus and supraoptic nucleus. Action potentials in these neurosecretory cells trigger the release of *OXT* from their axon terminals in the neurohypophysis (Poulain & Wakerley, 1982). Oxytocin is synthesized as an inactive precursor in the hypothalamus along with its carrier protein neurophysin I. The complex is known as prepro-oxytocin-neurophysin I. Oxytocin's actions are somewhat different. One of the classic roles assigned to *OXT* is milk ejection from the mammary gland. In particular, oxytocin administered prior to milking provides a significant increase in milk production, due to an increase in milk flow rate and a decrease in the machine milking time (Lollivier *et al*., 2002). Other roles, however, characterize oxytocin: it stimulates the uterine smooth muscle contraction during labour; it controls the oestrous cycle length, follicle luteinization in the ovary, and ovarian steroidogenesis; furthermore it is involved in cognition, tolerance, adaptation and complex sexual and maternal behaviour (Gimpl & Fahrenholz, 2001). In

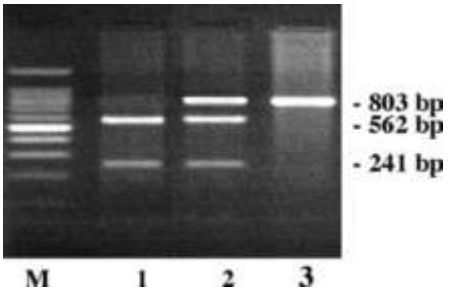
this paper we report the complete nucleotide sequence of the river buffalo oxytocin-neurophysin I encoding gene, promoter analysis and an allele detection at this *locus*.

MATERIAL AND METHODS - *DNA samples, Primers design, PCR conditions and DNA sequencing*: Genomic DNA of 13 Mediterranean river buffaloes reared in the province of Caserta (Italy) was extracted from leukocytes. Primers for amplification and sequencing were designed using the DNA sequence of bovine (EMBL nos. X00502, X58474) and ovine *OXT* gene (X55131) as templates. A typical 50 µl reaction mix comprised: 200 ng of genomic DNA, 3 mM $MgCl_2$, 10 pmol of each primer, dNTPs each at 400 µM, 1.25 U of LA Taq™ DNA Polymerase (Takara BIO INC.), LA PCR Buffer II 1X (Takara). The thermal amplification conditions was as follows: 97 C° for 2 min, 65 C° for 45 sec, 72 C° for 1.5 min, 1 cycle; 94 C° for 45 sec, 65 C° for 45 sec, 72 C° for 1.5 min, 29 cycles; 94 C° for 45 sec, 65 C° for 45 sec, 72 C° for 1.5 min, 30 cycles. *Genotyping of OXT alleles by means of DraII PCR-RFLP*: A 803 bp fragment spanning from the 66th nt of exon 1 to 186th nt of exon 3 was PCR amplified with the following primers: 5'-GGCCTCCTGGCGTTGA-3' (forward) and 5'-GGGGATGATTACA-GAGGGAG-3'(reverse) (EMBL no. AM234538). The amplification protocol and the thermal amplification conditions were as reported in the previous paragraph. Digestion of 17µl of each PCR amplification was accomplished with 10 U of *Dra*II endonuclease (Promega) for 5 h at 37°C following the supplier's direction for buffer condition.

RESULTS AND CONCLUSIONS - By using genomic DNA as template we sequenced the whole oxytocin-neurophysin I gene (*OXT*) plus 993 nucleotides at the 5' flanking region (EMBL nos. AM234538, AM234539). The gene organization is similar to that described by Rupert *et al*. (1984) and by Ivell *et al*. (1990) for the homologous gene in bovine and ovine species. The river buffalo *OXT* gene was found to extend over 912 bp, including 512bp of exonic regions and 400bp of intronic regions. The gene contains only 3 exons, ranging in size from 153bp to 202bp, and 2 introns of 311bp (intron 1) and 89bp (intron 2). The 5'UTR comprises the first 33 nucleotides of the first exon, while the 3'UTR includes the last 104 nucleotides of the 3rd exon. The ORF region codes for the peptide leader and for 106 aa of the "oxytocin-neurophysin I" complex. The first exon (153bp) encodes a translocator signal (19 aminoacid residues encode from nucleotide 34 to 91), the nonapeptide hormone (from nucleotide 92 to 118), the tripeptide processing signal (GKR) (from nucleotide 119 to 127), and the first nine of the 94 residues of neurophysin I; the second exon (202bp) encodes the central part of neurophysin I and the third exon (157bp) encodes the COOH-terminal region of neurophysin I. The methionine initiation codon is located at nucleotides +34/+36 and the translation stop codon TGA is located at nt 54-56 of exon 3. All splice junctions follow the 5' GT/3'AG splice rule. Moreover, the polyadenylation signal and polyadenylation site were identified in the 3'UT region (3rd exon), located between nucleotides +75/+83 and +89 from the terminator codon, respectively. A modified RNA polymerase binding TATA box (TTAAA), is located between nucleotides –24/–29. TGACC and TCACC pentamers, target elements for nuclear hormone receptors repressing activity (Parker et al. 1993), are present in the proximal flanking region of buffalo *OXT* gene. A highly conserved DNA element exists at about 160 nucleotides upstream of the transcriptional initiation site: a "composite hormone response element" (monomeric orphan receptor binding site, CATAACCTTGACC), conserved across species (Gimpl & Fahrenholz, 2001), including the binding sequence sites of the es-

trogen response elements (ERE), COUP-TF sites and steroidogenic factor-1 (SF-1) (Burbach *et al*., 2001). A putative AP-1 site (GTGACAA) is located at nucleotides –622/–628. The Mediterranean river buffalo *OXT* gene comparison with the corresponding bovine (EMBL no. X00502) and ovine (EMBL no X55131) sequences shows a similarity of about 97.7 % and 93.8 % respectively. The comparative analysis of the river buffalo promoter sequences shows two transitions: C → T in position – 966 and G→A in position – 790. While the first mutation does not seem to have any effect upon known possible regulatory sites of the *OXT* gene, the second one seems to create a potential binding site for CCAAT/enhancer binding protein-alpha (C/EBP alpha) (by TRANSFAC® 7.0 database) that is a member of the basic region-leucine zipper (B-ZIP) family of transcription factors. The C/EBP alpha cooperates with other transcription factors, including pituitary-specific homeodomain protein (Pit-1), in the regulation of lactotropes gene transcription in the anterior pituitary (Schaufele *et al.* 2001). Polymorphic sites have not been evidenced at intronic level, instead a non conservative mutation (transvertion G→T) at the 170th nucleotide of the second exon responsible for the Arg^{97}→Leu aa substitution (66th aa residue of nerophysin I) was detected. The presence of guanine at the *OXT* exon 2 might represent the ancestral condition of the gene because it has been found also in other species. For this reason, the allele containing the guanine was named *OXT* A, whereas the changed form was named *OXT* B. According to the sequence analysis of the DNA samples belonging to individuals with informative genotypes, the T in position – 966 and the A in position – 790 are in *cis* with the T at 170th nucleotide of exon 2. The nucleotide transvertion in position 170 of the 2nd exon is responsible also for the change of a restriction site of the *Dra* II endonuclease. Therefore, a PCR-RFLP method for a rapid identification of carriers of these alleles has been developed. A DNA region spanning from 66th nt of the first exon to the 186th nt of the third exon was amplified. By *Dra* II PCR-RFLP, *OXT* A homozygous individuals show a single undigested fragment of 803bp whereas *OXT* B homozygous individuals show two fragments of 562 and 241bp (Figure 1). Genotyping of 13 buffaloes reared in the same breeding unit was carried out, revealing the following genotype distribution: 3 *OXT* A/A, 8 *OXT* A/B and 5 *OXT* B/B. The results obtained from this work represent the first example of genetic polymorphism detected at the *OXT locus* in a mammalian species, thus opening the way to further interesting prospects. It is possible, for instance, to hypothesize that in buffalo the different aa composition of the neurophysin I coded by such alleles or the mutations detected in the promoter region could be responsible for a different gene expression. The main function of neurophysin I, a small (93–95 residues) disulfide-rich protein, appears to be related to the proper targeting, packaging, and storage of *OXT* within the granula before release into the bloodstream.

Figure 1. Genotypes observed after DraII digestion of 803bp fragment obtained by PCR of buffalo genomic DNA. Line 1: OXT B/B; lane 2: OXT A/B; lane 3: OXT A/A; M = Marker (100bp, DNA Ladder).



OXT is found in high concentrations in the neurosecretory granules of the posterior pituitary complexed in a 1:1 ratio with neurophysin I. In such complexes, *OXT*-neurophysin I dimers are the basic functional units (Rose *et al*., 1996). Other researches in this direction are necessary to estimate the frequency of such mutations in larger population samples in order to better understand the association between the observed polymorphisms and the physiological processes controlled by this hormone.

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