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FELINE ATRIAL NATRIURETIC PEPTIDE (ANP) HAS A SINGLE COPY GENE
(O peptídeo natriurético atrial felino (PNA) possui uma única cópia gênica)

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RESUMO – O peptídeo natriurético atrial felino (PNA) é um hormônio sintetizado pelo miocárdio cardíaco atrial que, uma vez liberado na circulação, produz diurese, natriurese e vasodilatação periférica. Nós recentemente isolamos, sequenciamos e estabelemos a expressão cardíaca normal do PNA felino. No presente estudo, nós apresentamos o número de cópias gênicas do PNA em gatos. Foram extraídas amostras de DNA genômico do sangue periférico humano e felino, as quais foram purificadas e digeridas com as enzimas de restrição *EcoRI, BamHI* e *HindIII*, com posterior realização da técnica de Southern Blots. Primers para PCR, confeccionados para regiões de alta conservatividade entre espécies, foram utilizados para amplificação, clonagem e sequenciamento de um produto de 900 pares de bases, posteriormente utilizado como sonda de DNA. O gene do PNA felino possui uma única cópia gênica, a exemplo do PNA humano. Devido a sua similaridade, sondas de DNA felino puderam ser utilizadas para a hibridização de DNA genômico tanto do gato como do homem.

Palavras chave: peptídeo natriurético atrial, PNA, gato, gene, copia gênica, coração.

ABSTRACT – Atrial natriuretic peptide (ANP) is a hormone normally synthesized by the cardiac atrial myocardium that once released, produces diuresis, natriuresis and peripheral vasodilation. We have recently isolated, sequenced and assessed the normal cardiac expression of feline ANP. In this study, we report on the number of copies of the feline ANP gene, which could have an effect on future molecular biology studies of ANP gene expression in cat models of cardiovascular disease. Cat and human genomic DNA (gDNA) were extracted for Southern blotting from blood. The purified DNA was digested with the restriction enzymes *EcoRI, BamHI* and *HindIII* followed by agarose gel electrophoresis and blotting onto nytran membranes. A feline ANP cDNA probe of approximately 900 base pairs was used for hybridization of the membranes. The results of Southern blotting showed that both the feline and human genomes contain a single copy of the ANP gene.

Key words: atrial natriuretic peptide, ANP, cat, feline, human, gene copy, heart.

Introduction

Atrial natriuretic peptide (ANP) is a hormone normally synthesized by the cardiac atrial myocardium (CHAPEAU *et al*., 1985; COLBATZKY *et al.*, 1993) and stored in cytoplasmic granules as a prohormone (MIFUNE *et al*., 1992; FORSSMANN *et al.*, 1998; TURK, 2000). Upon release into the circulation it is cleaved to the mature peptide causing, diuresis, natriuresis and peripheral vasodilation (LEVIN *et al.*, 1998; MELO *et al.*, 2000; TURK, 2000). A positive correlation has been established between plasma ANP concentrations and the severity of heart failure in several species with various forms of heart disease including, dilated cardiomyopathy, chronic valvular regurgitation, and other anomalies causing atrial distension (ITOH *et al*., 1988; SUNDSFJORD *et al.*, 1988; LERMAN *et al*., 1993; HAGGSTROM *et al.*, 1994; HALL *et al*., 1994; CLERICO *et al*.,

1996; MUDERS *et al.*, 1997; NUMATA *et al.*, 1998; CHENBURNETT, 1999; FRUHWALD *et al.*, 1999a; FRUHWALD *et al.*, 1999b).

Atrial natriuretic peptide was first described in human beings (DE BOLD *et al.*, 1981) and its gene was subsequently cloned and sequenced few years later (OIKAWA *et al*., 1984). The nucleotide and amino acid sequence of ANP has been established for many species, including rats, rabbits, pigs, horses, cattle, sheep, dogs (KANGAWA *et al*., 1984; NAKAYAMA *et al*., 1984; SEIDMAN *et al*., 1984; OIKAWA *et al*., 1985; VLASUK *et al*., 1986; MAGERT *et al*., 1990; AITKEN *et al*., 1999) and recently determined in our laboratory for domestic cats (BIONDO *et al*., 2002). No studies either on the number of copies or on mutations of the feline ANP gene have been performed to date, however in man it has been shown that a single copy of the ANP gene exists (LEWICKI *et al*., 1986).

Our laboratory is currently evaluating the potential of ANP as a cardiac marker of heart failure in cats. Cats develop a form of hypertrophic cardiomyopathy (HCM) that may make them a good model of heart failure with HCM of man (KITTLESON *et al*., 1999). We are also evaluating the plasma levels of natriuretic peptides in a variety of feline cardiovascular diseases and investigating the potential correlations between the expression ANP gene and feline myocardial diseases as well (BIONDO et al., 2001).

The purpose of this study is to determine the number of copies of the ANP gene within the feline genome, which could be important in future studies concerning genetic manipulation of ANP gene expression in feline myocardial cell cultures and the inheritance patterns of feline HCM.

Material and Methods

Feline and human whole blood samples were obtained and processed within 6 hours after collection. Genomic DNA (gDNA) was extracted from blood using a commercial kit.[a] DNA concentrations were determined in two different dilutions by spectrophotometry at 260 nm, and purity evaluated by the 260/280 nm ratio and gel analysis. Ten mg of each sample was digested for 3 hours at 37 ºC with 1ml of restriction enzymes EcoRI, BamHI and HindIII,[b] 2 ml of respective buffer and water up to 20 ml. The total volume of each sample was loaded onto a 1.5 % commercial agarose gel for electrophoresis. Following separation the DNA was visualized using ultraviolet light and denatured in a solution 0.4 M of NaOH and 0.8 M of NaCl. The gel was then blotted for 16 hours with 20X Standard Saline Citrate (SSC) solution onto a nytran membrane.[c] After blotting the DNA was fixed to the nytran membrane by ultra violet crosslinking.

The cloning and sequencing were done using standard techniques (MANIATIS, 1987). The upstream and downstream consensus sequence primers (5' ACGACGCCAGCATGAGCTCCTTC 3' and 5' CGGAAGCTGTTGCAGCCCAG 3') were designed using known feline ANP gene sequence previously obtained in our laboratory. Polymerase chain reactions (PCR) were performed under standard conditions. The thermocycle profile included an initial denaturing step (3 min at 95 ºC), 35 cycles of denaturing (30s at 95 ºC), annealing (30s at 62 ºC) and extension (45s at 72 ºC), a final extension (7 min at 72 ºC) and a 4 ºC end step. The resulting PCR product was purified using a commercial PCR purification kit.[d] The purified PCR product was ligated into a commercial vector[e] following the manufacturer protocol. Plasmids identified with the properly sized inserts were sent to a commercial DNA sequencing facility.[f] Sequence results were compared to those of feline ANP in our databank and a piece of 900 base pairs was selected as a probe.

Probe labeling and Southern blot analysis

[a] Wizardâ genomic DNA purification kit, cat. 1125, Promega, Madison, WI, USA.
[b] Promega Restriction Enzymesâ, Promega, Madison, WI, USA.
[c] Nytranâ Membranes, Schleicher & Schuell, Inc., Keene, NH, USA.
[d] Wizardâ PCR preps DNA purification kit, cat. A7170, Promega, Madison, WI, USA.
[e] pGEMâ- T Easy Vector System I, cat. A1360, Promega, Madison, WI, USA.
[f] ACGTâ Inc, Northbrook, IL, USA.
[g] Transcendâ Non-Radioactive Translation Detection Systems, Promega, Madison, WI, USA.

were performed using a commercial enhanced chemiluminescent kit following the manufacturer's protocol.[9] Probes were labeled with fluorescein-II-dNTP (FI-dNTP) in a random primed synthesis reaction. After hybridization, anti-fluorescein antibody conjugated to a horseradish peroxidase was added to the blot. Detection of the bound horseradish peroxidase was done using the kit's detection reagents. After the labeling, hybridization and washing the blot was exposed to a Rx hyperfilm for 5 minutes. The film was developed and analyzed.

Results

The digestion of cat and human chromosomal DNA was successfully performed (FIGURE 1), the Southern blot analyses conducted and the results registered (FIGURE 2). The digestions with all three restriction digestion enzymes resulted in single bands. EcoRI and HindIII digestion resulted in products about 5 to 8 kilobase long. The digestion with BamHI resulted in a band of about 1.2 kb, corresponding to the size of the feline ANP gene.

FIGURE 1 – 1.5% AGAROSE GEL SHOWING THE RESTRICTION ENZYME DIGESTION OF CAT AND HUMAN DNA. LANES 1,2 AND 3 ARE HUMAN PRODUCTS FROM DIGESTION WITH EcoRI, BamHI AND HindIII, RESPECTIVELY. LANES 4,5 AND 6 ARE FELINE PRODUCTS FROM DIGESTION WITH EcoRI, BamHI AND HindIII, RESPECTIVELY. THE SIZE OF

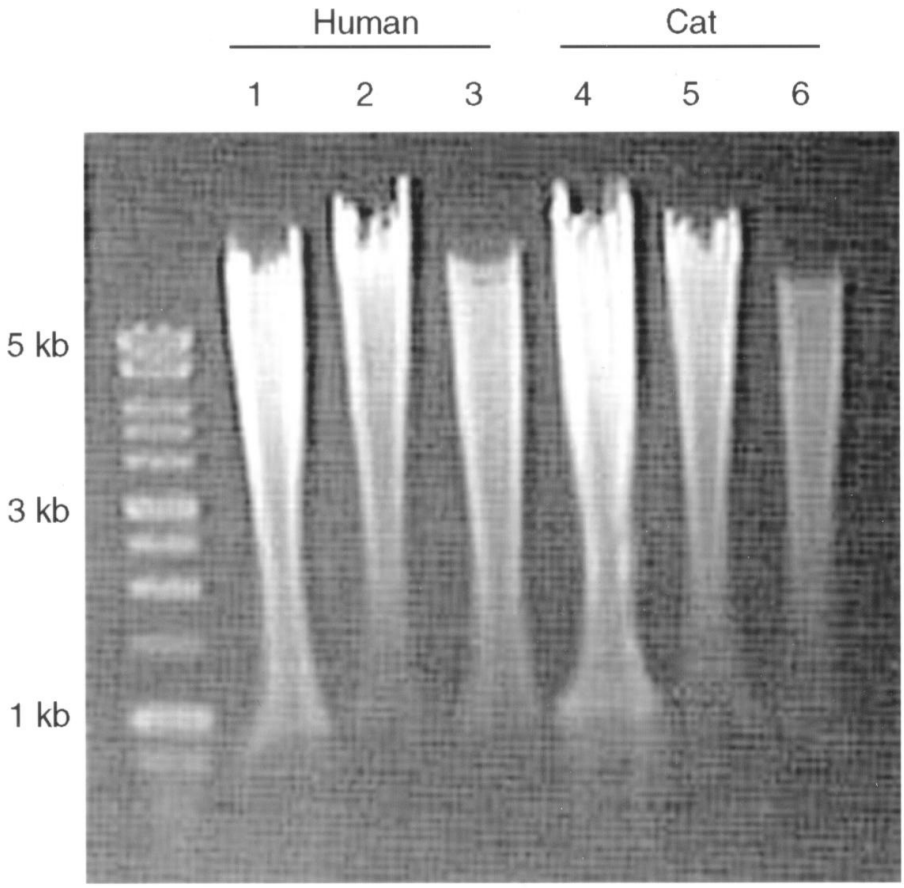
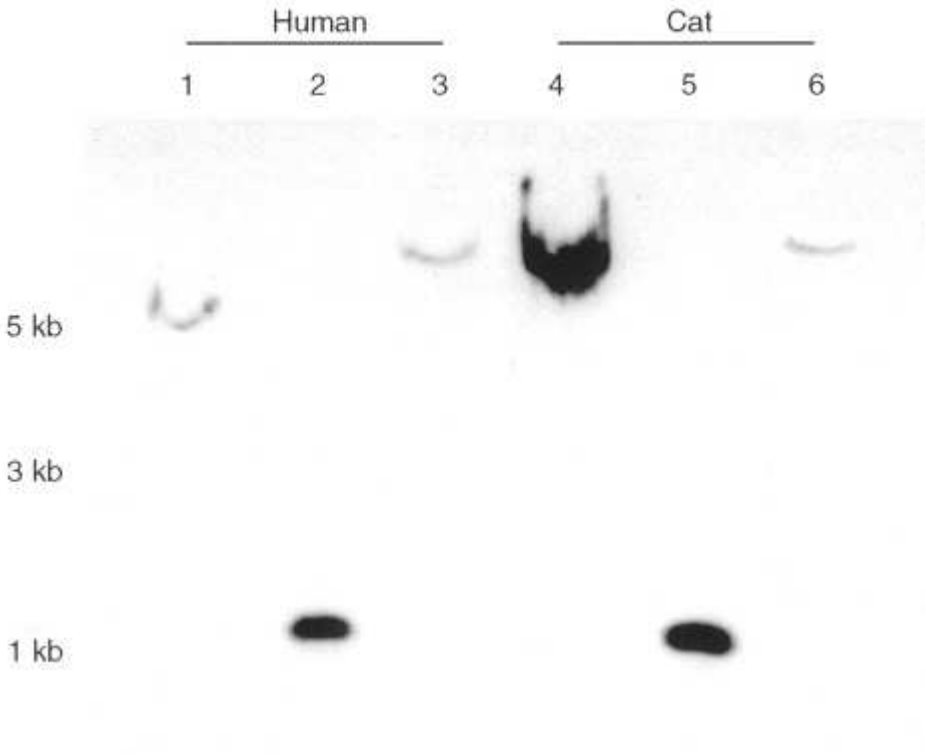


FIGURE 2 – SOUTHERN BLOT ANALYSIS OF CAT AND HUMAN ANP GENES. DIGESTION WAS PERFORMED WITH EcoRI (LANES 1 AND 4), BamHI (LANES 2 AND 5) AND HindIII (LANES 3 AND 6). THE SIZE OF THE PRODUCTS ARE SHOWN IN KILOBASES (Kb). THE SIZE OF THE DIGESTION BY BamHI IS ABOUT THE SIZE OF THE ANP GENE IN BOTH SPECIES.



Discussion and Conclusions

The application of Southern blots analysis has shown that ANP gene is a single copy gene in various species including human and rodents (ZIVIN *et al*., 1984; SEIDMAN *et al*., 1985; LEWICKI *et al*., 1986; TAKEI *et al*., 1997). Chemiluminescence applied to Southern blotting has been referred to as a very sensitive technique for DNA analysis (SHEFFIELD *et al*., 1992). In the present study, the Southern blot analysis of cat and human digested genomic DNA revealed a similar pattern of restriction enzymes digestion. This was anticipated based on the fact that ANP has highly conserved nucleotide sequence among species (BIONDO *et al*., 2002). If the feline ANP gene had more than one copy, digestion with restriction enzyme BamHI would result in either multiple bands or different intensity/size when compared with human ANP gene. Hence, the presence of single bands with similar intensities for both the cat and human ANP genes with digestion by three separate restriction enzymes suggests that the ANP gene exists as a single copy in the feline genome as in human beings. It should also be noted that the cDNA probe used in these experiments for Southern blotting, was effectively used with both feline and human DNA, supporting the notion that the nucleotide sequence of ANP among species is highly conserved.

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