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Tissue-specific transcription pattern of the adenine nucleotide translocase isoforms in humans

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Abstract Three adenine nucleotide translocase isoforms (ANT1, ANT2 and ANT3) are coded by different genes. The relative amounts of the three ANT isoform mRNAs were determined in detail in various human tissues. ANT isoforms were co-expressed in all tested tissues revealing tissue-specific transcription patterns. The highest ANT1 mRNA proportions were found in terminally differentiated tissues like skeletal muscle, heart and brain, whereas ANT2 was mainly expressed in tissues capable of proliferation and regeneration as in the kidneys, spleen, liver, fibroblasts and lymphocytes. The ANT3 mRNA proportion was not prominently expressed in any of the tissues tested. In conclusion, tissue-specific expression of ANT isoforms is strongly related to the state of cellular differentiation.

Key words: Adenine nucleotide translocator; Isoform; Tissue differentiation

1. Introduction

Adenine nucleotide translocase (ANT) is an integral protein of the inner mitochondrial membrane and consists of two identical 32 kDa subunits with a single binding site for ATP and ADP that alternatively faces the matrix (**m** conformation) or the cytosolic side of the membrane (**c** conformation) [1]. Dependent on the conformational change of ANT, mitochondrial ATP is exchanged for cytosolic ADP at a 1:1 ratio. Since ANT is the only mitochondrial translocase for nucleotides, it is the most important link between energy-producing and energy-consuming processes.

Immunological investigations pointed for the first time to the existence of ANT isoforms, since antibodies against ANT protein isolated from heart muscle tissue showed only a partial cross-reactivity to ANT protein from the kidneys and liver [2,3]. Molecular biological investigations confirmed the presence of at least three different human genes for ANT, designated *ANT1*, *ANT2* and *ANT3* [4,5]. The *ANT1* gene is located on chromosome 4, wherease *ANT2* is found on the X chromosome [6,7]. The locus of the *ANT3* gene is still unknown. *ANT* genes have similar structural features consisting of 4 exons of identical length and a 77–79% homology of the nucleotide sequence. However, their respective 5′ flanking regions are distinct and show different promotor elements [4,8,9]. These different regulatory structures suggest various regulating mechanisms for the three isoforms in relation to their development and/or cellular metabolism.

Thus far, the exact proportions of the three human ANT isoform transcripts have not been defined. For this reason, we determined the relative amount of the ANT isoform transcription to get an accurate picture of the tissue-specific ANT isoform expression patterns in different human tissues.

2. Materials and methods

2.1. Materials

Skeletal muscle ($n = 8$), left cardiac ventricle, brain, kidney, spleen and liver specimens ($n = 18$ of each) were investigated for tissue-specific ANT isoform expression. Human autopsies were performed within 24 h after death. Furthermore, myocardial specimens (intraventricular septum, left and right ventricle) obtained from patients undergoing heart transplantation due to coronary heart disease ($n = 25$) were immediately snap-frozen in liquid nitrogen after removal. In the same way, specimens from atrial auricles were obtained from patients undergoing valvular surgery ($n = 12$). All tissues were stored at −80°C until analysis.

Human fibroblasts were cultured in MEM medium supplemented with 10% fetal calf serum at 37°C and 5% CO_2. Cells were harvested in the log phase of growth.

Lymphocytes were isolated from blood of 11 persons by Ficoll centrifugation according to the manufacturer's instructions (Pharmacia Biotech, Uppsala, Sweden).

2.2. Determination of ANT isoform-specific mRNA proportions

Total RNA was extracted using the LiCl method [10] and was reverse-transcribed into cDNA (Gibco BRC, Eggenstein, Germany). Amplification of ANT-specific cDNA was carried out in a PCR reaction mixture containing 50 mM Tris-HCl, 50 mM KCl, 0.001% gelatine, 1.5 mM $MgCl_2$, 0.4 μM of each primer (5′: 5′-CTG CTG CTG CAG GTC CAG CA-3′ and 3′: 5′-CCT GAC TGC ATC ATC AT-3′) and 0.25 U of Taq polymerase (Cetus Perkin Elmer, Weiterstadt, Germany). The denaturation, annealing and extension conditions for the total ANT cDNA amplification were 2 min for each period of cycle at 94°C, 57°C and 72°C, respectively. Thirty-five amplification cycles were performed with a thermal cycler (Biometra, Göttingen, Germany). Contamination and non-specificity of reaction were ruled out by control reactions not including cDNA as a template. The primers used are located in the coding regions of the ANT isoform cDNAs (Fig. 1). They are complementary to regions sharing nearly identical sequences within the isoform cDNAs. Thus, the amplification resulted in a mixture containing ANT1, ANT2 and ANT3 PCR products, each of them 629 bp long (Fig. 2A), with identical end sequences but different ANT isoform-specific intermediate nucleotide sequences. Therefore, ANT1, ANT2 and ANT3 isoform cDNA was amplified in relation to ANT isoform mRNA distribution in the orginal tissue.

Next, the proportions of ANT isoform-specific sequences in this mixture were determined by a special dot-blot hybridization procedure. Aliquots of the PCR product — containing the mixture of amplified ANT1, ANT2 and ANT3 isoform cDNA — were denaturated in 1 vol% 0.5 M NaOH, 1 M NaCl and spotted in triplicate onto three nylon membranes (Amersham, Germany). Additionally, an ANT1 isoform-specific cDNA standard (constructed as described below) was spotted in a dilution of 8–40 fmol onto the first of these nylon membranes; an ANT2 isoform-specific cDNA standard was applied in the same dilution onto the second membrane, and the third nylon membrane obtained the ANT3 isoform-specific cDNA standard. The

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membranes were air-dried for 1 h. Prehybridization was performed in gold hybridization buffer (Amersham, Germany) for 1 h at 42°C. The membranes were then hybridized according to the blotted ANT isoform-specific standards with corresponding ^{32}P-radiolabeled ANT isoform-specific oligonucleotides (for the first membrane ANT1: 5′-GGC AAG GGC GCC GCC CAG CG-3′, for the second membrane ANT2: 5′-GCT GGA GCT GAA AGG GAA TT-3′ and for the third membrane ANT3: 5′-ATC GTG GAC TGC ATT GTC CG-3′). After hybridization at 42°C overnight, the membranes were briefly washed in 6×SSPE, 0.1% Triton X-100 at room temperature and for 10 min at 42°C. This was followed by a final wash step at high-stringency conditions in 3 M tetramethylammonium chloride (TMACl: Sigma, Deisenhofen, Germany), 0.1% Triton X-100 at 62°C for 30 min. Each dot was cut out of the membrane and measured in β-counter. Standard curves were calculated using known molarities of the ANT isoform-specific standards. The measured radioactivity of the samples was compared to the corresponding standard curves, and the proportions of the ANT1, ANT2 and ANT3 isoforms were calculated.

2.3. Preparation of ANT isoform standards

Isoform-specific cDNA standards were amplified by PCR using heart-specific cDNA as template and isoform-specific primer pairs shown in Table 1. The annealing temperatures for ANT1 amplification was 60°C for ANT2 55°C and for ANT3 70°C. The PCR reaction mixture was the same as described above. Specificity of standards and oligonucleotides used for hybridization were confirmed by Southern blotting (Fig. 2B) and restriction analysis (data not shown). Each of the obtained PCR products was cloned into the TA pCR2.1 cloning vector (Fa. Invitrogen NV, Leek) according to the manufacturer's instructions. Cloned inserts were reamplified by PCR using the primers previously mentioned and the respective ANT isoform-specific vector as template. Thereafter, PCR products were purified from primers, protein and salt using QIAquick PCR purification spins (QIAGEN, Hilden, Germany). The eluated ANT isoform-specific PCR products were measured spectrometrically and diluted to 40 fmol solutions.

2.4. Southern blot

The PCR products were subjected to electrophoresis on a 2% agarose gel in 50 mM Tris-borate, 1 mM EDTA. After denaturation in 0.5 M NaOH, 1.5 M NaCl for 30 min, the PCR products were capillarly transferred onto a nylon membrane (N^+ Hybond, Fa. Amersham, Braunschweig, Germany) in 0.4 M NaOH overnight. The membrane was dried and hybridized with ANT-specific oligonucleotides as described for the dot-blot procedure.

3. Results

The relative amount of ANT isoform-specific mRNA was determined in human tissue from different sources. In order to prove the accuracy of the method used and guarantee that the results were not falsified by RNA degradation, the ANT isoform distribution of left ventricular tissue from explanted hearts immediately snap-frozen in liquid nitrogen after removal was compared to the isoform pattern of left ventricular autopsy material. As shown in Fig. 3, the ANT isoform patterns of both groups were identical. Thus, the ANT proportions were not distorted by possible RNA degradation in autopsy tissue. Additionally, although PCR analyses were performed with different amounts of cDNA, various numbers of PCR cycles and annealing temperatures, the PCR products obtained differed only in their cDNA amount but not in the ANT isoform proportions (data not shown).

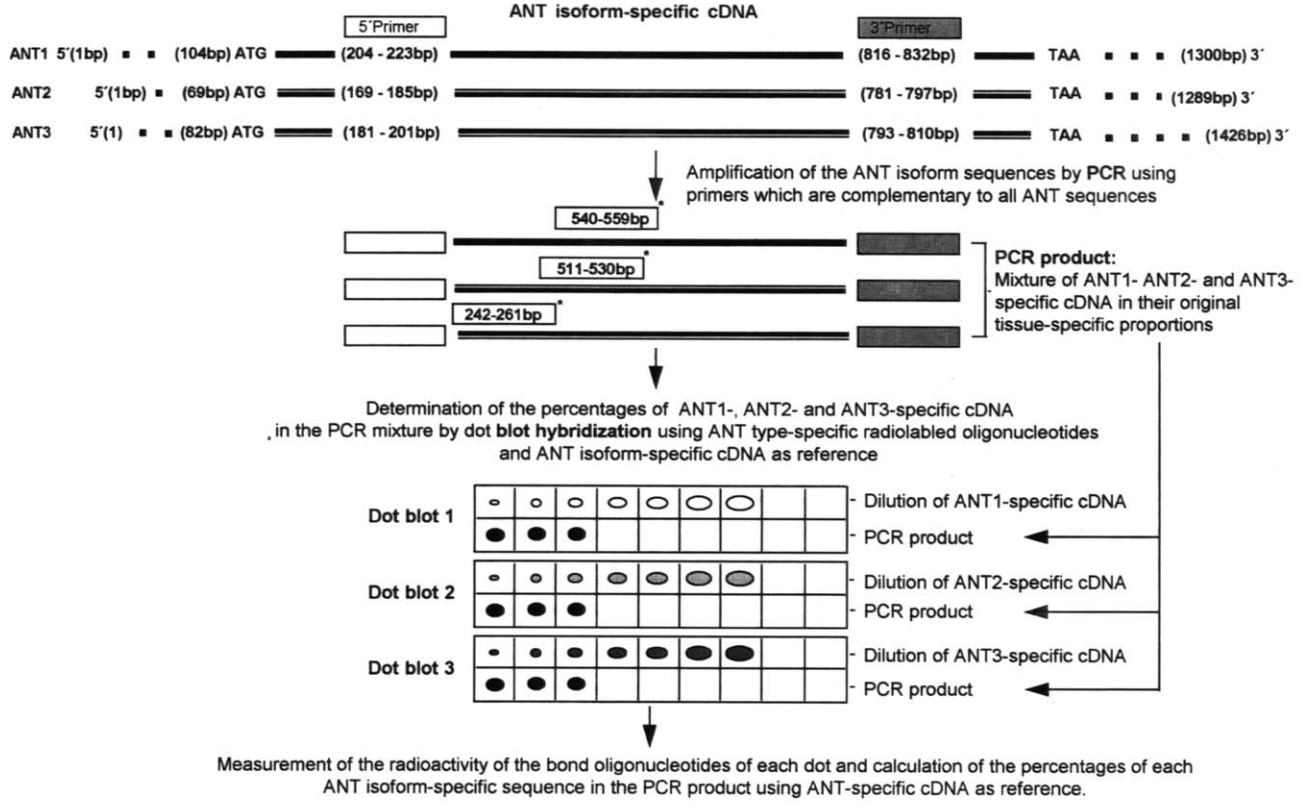

Fig. 1. All ANT isoform-specific sequences were coamplified in a single PCR reaction using only one primer pair that hybridizes to homologous sequences of the three ANT isoforms. The amplification resulted in a mixture of ANT1-, ANT2- and ANT3-specific sequences in relation to the ANT isoform mRNA distribution of the original tissue. The proportions of amplified isoform-specific cDNA in the PCR mixture were finally determined by dot-blot hybridization with isoform-specific radiolabeled (*) oligonucleotides and ANT isoform-specific cDNAs as standards. Regions of primer hybridization are marked. Radioactivity of the samples was measured and compared to the corresponding standard. The proportion of each isoform was calculated.

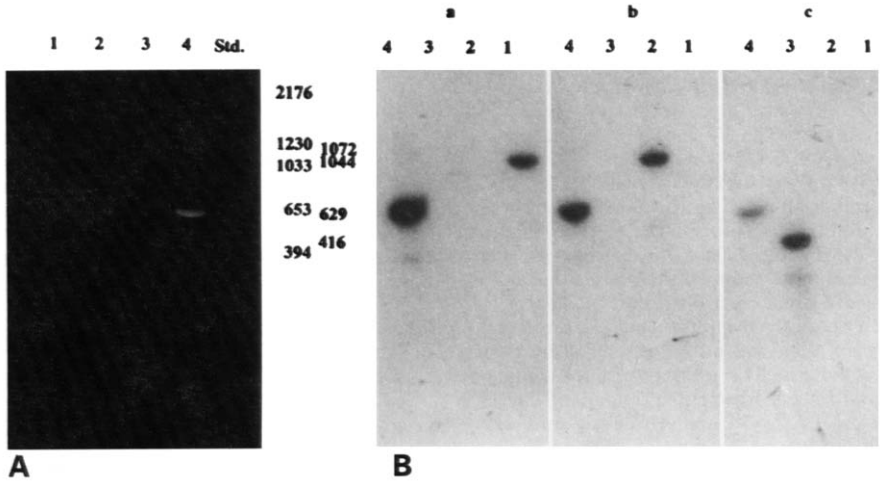

Fig. 2. A: PCR was carried out with ANT isoform-specific primer pairs to amplify ANT1-, ANT2- and ANT3-specific cDNA standards. The PCR products were subjected to electrophoresis on an agarose gel containing ethidium bromide. The PCR product of ANT1 is 1072 bp (lane 1), ANT2 is 1044 bp (lane 2) and ANT3 is 416 bp (lane 3). Lane 4 shows the PCR product of total ANT cDNA from heart with a length of 629 bp amplified with a primer pair which recognizes homologous sequences of ANT1, ANT2 and ANT3. B: The PCR products were blotted onto three nylon membranes by Southern blotting. Each blot was hybridized with radiolabeled oligonucleotides specific for (a) ANT1, (b) ANT2 and (c) ANT3. All oligonucleotides hybridized to the total ANT PCR product (lane 4) and to each of their specific PCR products. No cross-reactions were seen.

The tissues analyzed could be separated into two groups differing in their ability to regenerate and grade of differentiation. The first group was made up of tissues like skeletal muscles, heart and brain which are terminally differentiated and incapable of regeneration. Kidneys, spleen, liver, proliferating cultured fibroblasts and lymphocytes which keep their capacity of regeneration by mitotic division were grouped in a second collective. The ANT isoform transcription pattern differed significantly in these groups. The ANT isoform transcription pattern of terminally differentiated tissues is characterized by a predominant ANT1 expression. Here, the proportion of ANT1 transcription ranges from 56 ± 6 to $69 \pm 6\%$ (Table 2). Hereby, the percentages of ANT1 transcription in heart and skeletal muscle tissue indicate no marked variations. However, in comparison to muscle tissue, the proportion of ANT1 mRNA in the brain is significantly lower. In contrast to the first group, the proportion of ANT1 transcription of tissues capable of regeneration varies between $14 \pm 1\%$ and $29 \pm 6\%$, whereby proliferating fibroblasts indicate the lowest proportion of ANT1 transcription.

However, ANT 2 is the prevalent isoform of tissues qualified for mitotic division. ANT2 mRNA proportions varied between $56 \pm 9\%$ and $77 \pm 8\%$ in these organs. In contrast, the ANT2 mRNA proportion of terminally differentiated tissues amounts only to a fourth of the total ANT transcription.

ANT3 expression is not prominent in any of the tissue tested. The percentages of ANT3 transcription ranges from an average of $7 \pm 3\%$ in the heart and lymphocytes to $29 \pm 3\%$ in fibroblasts.

Beside the description of the organ-specific ANT isoform transcription pattern, the ANT isoform distribution of various regions of the heart like left and right ventricle, septum and auricle were compared (Fig. 4). The different heart regions indicate a common ANT isoform transcription pattern without any variation.

4. Discussion

Immunological studies of ANT protein isolated from the heart, kidneys and liver provided evidence of organ-specific determinants and the existence of at least three ANT isoforms [2,3]. Furthermore, data from molecular biological studies employing the Northern blot technique provided a rough evaluation of the ANT isoform transcription level in various human tissues [6,11,12]. However, up to this date, there was no information about the exact proportions of ANT isoform transcripts in various mammalian tissues. Therefore, we determined the percentages of each isoform in a variety of human organs by employing the PCR technique combined with a dot-blot hybridization procedure. This technique is applied to de-

Table 1
Primer used for the amplification of ANT1-, ANT2- and ANT3-specific cDNA

Primer	Primer sequence	cDNA region (bp)
ANT1		
Forward primer	5′-CTG AGA GCG TCG AGC TGT CA-3′	83– 102
Reverse primer	5′-CTC AAT GAA GCA TCT CTT C-3′	1136–1154
ANT2		
Forward primer	5′-CCG CAG CGC CGT AGT CAA A-3′	1– 19
Reverse primer	5′-AGT CTG TCA AGA ATG CTC AA-3′	1025–1044
ANT3		
Forward primer	5′-CCG TTC TCC GGC TGT CCT CC-3′	39–58
Reverse primer	5′-GCC GGC CGC ACC GCC GGA GGC-3′	434– 454

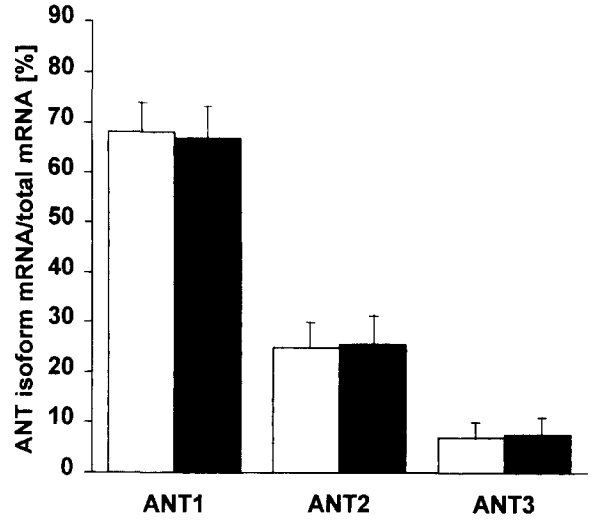

Fig. 3. ANT isoform mRNA proportions of left ventricular tissue obtained from autopsies ($n = 18$; open bars) and explanted hearts ($n = 25$; filled bars). Data are shown as mean ± SD.

termine ANT isoform mRNA proportions of total ANT transcripts in very small tissue biopsies (3 mg wet weight).

Differences regarding the expression of ANT isoforms obviously depend on the state of cellular differention. *ANT1* gene expression predominantly occurs in skeletal muscles, heart and brain, and thus, in cells that are terminally differentiated and incapable of mitotic division. In these tissues, the ANT2 and ANT3 transcription is of minor significance. These findings correspond to the data of Stepien et al. [11], who showed that ANT2 is the prevalent isoform in myoblasts during cell proliferation and in the early stage of differentiation, whereas ANT1 is poorly expressed. However, a dramatic increase in ANT1 transcription took place at the final differentiation stage, while the ANT2 mRNA level decreases.

In contrast to a predominant ANT1 expression in terminally differentiated tissues, a prevailing ANT2 expression occurs in tissues that are able to regeneration by mitotic division. *ANT2* is a growth-regulated gene known to be highly expressed in a variety of proliferating cells [13–16]. In addition to these data, we report here a prevalence of ANT2 expression in proliferating fibroblasts with an isoform proportion pattern similar to that observed in the kidneys, liver, spleen and lymphocytes.

None of the tissue investigated demonstrated any predominance of ANT3 expression. *ANT3* is assumed to be a housekeeping gene which is influenced to a lesser extent by the state of cellular development [4]. This notion is supported by the observation that ANT3 expression is not altered during muscle cell differentiation [11], as shown for ANT1 and ANT2 transcription. Furthermore, there was no significant change of ANT3 transcription during the cultivation of HeLa cells when the cell culture reached the late log phase, as it was observed for ANT2 [12].

The reported data explain the findings of Schultheiss et al. [2,3] and Rojo et al. [17]. Both authors showed a particular cross-reactivity of antibodies against ANT protein from heart with ANT protein isolated from the kidneys and liver. These antibodies are mainly reactive against ANT1, which is the predominant isoform of the heart. Since the proportion of ANT1 is low in kidney and liver, they react only to a small part of ANT protein isolated from these tissues that consists mainly of ANT2 protein. Additionally, Rojo et al. found similar antigenic properties between ANT protein from the heart and brain corresponding to the ANT isoform mRNA pattern shown by our data.

The physiological significance of tissue-specific ANT isoforms is probably additionally related to different functional properties of the isoform proteins. Since the human ANT isoform proteins have not yet been isolated, detailed data about the kinetic characteristics of the individual isoforms are still lacking. However, Klingenberg found a lower ANT transport rate in mammalian heart mitochondria compared to liver mitochondria [18]. Since we measured a predominant ANT1 expression in the heart and a prevalent ANT2 expression in the liver, it may be assumed that the ANT1 isoform has a lower transport capacity than ANT2. This hypothesis is supported by a kinetic analysis of the ADP/ATP-carrier (ACC1-3) of yeast. Here, the nucleotide transport rate of ACC1 protein is approximately 40% of the ACC2 nucleotide transport rate [19,20]. In the mammalian heart, an elevated ANT protein density that are four times higher than in the liver [18,21] may compensate the lower ANT1 isoform transport rate. Further analyses of ANT isoform kinetics still have to be carried out.

In summary, we present the tissue-specific transcription pattern of the ANT isoforms ANT1, ANT2 and ANT3 in various human tissues. Since ANT3 is always subordinately expressed. Thus, alterations in the ANT isoform mRNA pattern mainly depend on the differential transcription of ANT1 and ANT2. We conclude from our data that the distinct expression of the ANT isoforms is strongly related to the state of cellular differentiation, with a predominance of ANT1 in terminally differentiated tissues, and a prevalent ANT2 expression in tissues capable of regeneration and proliferation. We

Table 2
ANT isoform mRNA proportions in various human tissues

	Tissue	ANT1 (%)	ANT2 (%)	ANT3 (%)
Terminally differentiated tissues: No mitotic division	Skeletal muscles	69 ± 6	20 ± 5	11 ± 3
	Heart	68 ± 5	25 ± 5	7 ± 3
	Brain	56 ± 6	27 ± 6	17 ± 5
	Kidney	29 ± 6	56 ± 9	15 ± 7
Tissues with the ability to regenerate by mitotic division	Spleen	23 ± 3	56 ± 9	21 ± 6
	Liver	15 ± 6	63 ± 11	22 ± 10
	Fibroblast	14 ± 1	57 ± 3	29 ± 3
	Lymphocyte	16 ± 5	77 ± 5	7 ± 4

Data are shown as mean ± SD.

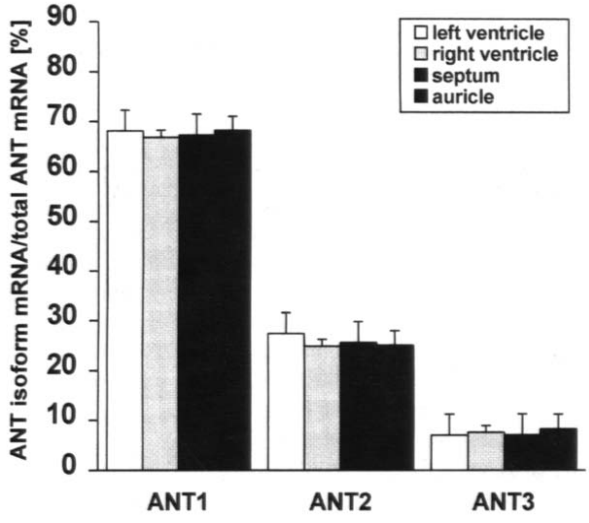

Fig. 4. ANT isoform mRNA proportions of samples from left and right ventricle, septum ($n = 25$ of each) and from auricle ($n = 12$). Data are shown as mean ± SD.

postulate that ANT isoform expression may be related to cell cycle control.

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