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# Transcription of the adenine nucleotide translocase isoforms in various types of tissues in the rat

A. Dörner *, M. Olesch, S. Giessen, M. Pauschinger, H.-P. Schultheiss

*Department of Cardiology, Benjamin Franklin Klinik, Free University Berlin, Hindenburgdamm 30, 12200 Berlin, Germany*

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**Abstract**

Two different isoforms of the adenine nucleotide translocase (ANT1 and ANT2) have been identified in the rat. In order to obtain enhanced knowledge of the ANT isoform expression, we analyzed the transcription pattern of both isoforms and their mRNA levels in various tissues of the rat using the PCR technique. A predominant ANT1 mRNA percentage was recorded in the skeletal muscle, heart and brain, ranging from 81 to 58%. In contrast to these tissues, the percentages of ANT2 were dominant with a range from 59 to 75% in the kidney, lung, spleen and liver. The level of total ANT mRNA varied markedly in the various organs. Tissues with a dominant ANT1 percentage simultaneously showed a high level of total ANT transcription (24–41 attomol/ng total RNA). In comparison to the latter, tissues with a prevalent ANT2 transcription were shown to have an even lower ANT transcription level (2–5 attomol/ng total RNA). The predominance of the ANT1 expression appeared to be restricted to tissues with an inability to regenerate by means of mitotic division, whereas a prevalent ANT2 transcription is found in cell types able to proliferate. The level of total ANT transcription but not the individual ANT isoform expression depends to a great extent on the energy requirements of the tissue. 

*Keywords:* Adenine nucleotide translocase; Isoform; Gene expression

## 1. Introduction

The adenine nucleotide translocase (ANT) is an integral protein of the inner mitochondrial membrane [1,2]. It enables the transport of ADP and ATP across the impermeable mitochondrial membrane. Consequently, the ANT links the energy consuming reactions in the cytosol to the energy producing processes in the mitochondrion. The ANT consists of two identical subunits of 32 kDa with a single binding site for ATP and ADP, which alternately faces the matrix (m conformation) or the cytosolic side of the membrane (c conformation). One of the ANT subunits is made of 293 amino acids (AS). Three sections of intramembranous α-helices, each of about 100 amino acids, which are combined by loops outside the membrane, build up the tertiary structure of a subunit. The amino acid sequences of these sections are highly homologous to each other. This tertiary structure is common for the ANT proteins in several species. Thus, it is assumed that the current ANT isoform genes descend from a common primordial gene, which originally coded one of the transmembrane structures [3]. The current gene structure was subsequently developed by the process

* Corresponding author. Fax: +49 (30) 84453565;
E-mail: doerner@ukbf.fu-berlin.de

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of gene duplications. The present isoforms were then derived from this forerunner gene.

Two different isoforms (ANT1 and ANT2) were identified in the rat [4]. The homology of their amino acid sequences was shown to be 98%. Moreover, by comparing the sequences of the rat-specific ANT isoforms to the respective isoforms in man [3,5], cattle [3] and mouse [6], high similarities of 94–98% were found among the various species. This important protein complex appeared to be highly sustained in mammals. In order to improve our understanding for the ANT isoform transcription we determined the exact distribution of the isoform transcripts in various tissues of the rat and in addition measured the ANT transcription level using the quantitative PCR technique.

## 2. Methods

### 2.1. Animals

Heart, skeletal muscle, brain, kidney, lung, spleen and liver were taken from eight, 5 month old, male Wistar Kyoto rats. The animals were delivered by the Schönwalde Company, Germany. Specimens were immediately snap-frozen in liquid nitrogen after removal and stored at −80°C awaiting analysis.

### 2.2. Amplification of ANT isoform-specific cDNA

Total RNA was extracted from each tissue using the LiCl method [7] and 250 ng of it was reverse transcribed into cDNA in a reaction mixture (RT) with an end volume of 20 μl containing 4 μl 5×first strand buffer (Gibco, Karlsruhe, Germany), 40 U RNase inhibitor (Boehringer, Mannheim, Germany), 2.5 nmol of each dNTP, 8 ng random primer, 10 μM DTT and 200 U MMLV-Reverse transcriptase (Gibco). Amplification of ANT1- and ANT2-specific cDNA was carried out in a mixture of 50 mM Tris-HCl, 50 mM KCl, 0.001% gelatine, 1.5 mM $MgCl_2$, 0.4 μM of each primer (Table 1), 0.25 units of *Taq* polymerase (Cetus Perkin Elmer, Weiterstadt, Germany) and 10 μl of the RT mixture, containing cDNA and dNTPs, in an end volume of 50 μl. The denaturation, annealing and extension conditions for the amplification were 45 s at 94°C, 45 s at the corresponding annealing temperature (Table 1) and 90 s at 72°C. Thirty-five amplification cycles were performed with a thermal cycler (Biozyme, Oldendorf, Germany). Both isoform-specific cDNAs were simultaneously amplified by PCR using a primer pair, which was complementary to the homologous sequences in the coding regions of the ANT isoforms (Table 1). The amplification resulted in a PCR product (tANT), containing ANT1 and ANT2 sequences, each of them 629 bp long, with identical end sequences but different ANT isoform-specific intermediate nucleotide sequences.

### 2.3. Determination of ANT isoform-specific mRNA proportions

The proportions of ANT1- and ANT2-specific sequences in the tANT product were subsequently de-

Table 1
Primers used for the amplification of ANT-specific sequences

| Isoform | Primer sequences | cDNA region (bp) [4] | Annealing temperature (°C) | Length of the PCR products (bp) |
|---|---|---|---|---|
| ANT1 | | | | |
| Forward primer | GTA GGC AAG AGC AAA AGA G | 21–39 | 60 | 79 |
| Reverse primer | GGA TCT GTG AAC CTG TGA A | 999–981 | | |
| ANT2 | | | | |
| Forward primer | AAC ATG ACA GAT GCC GCT G | 52–70 | 55 | 922 |
| Reverse primer | GGG GAG AAC AAC TAG GAC ATA A | 973–952 | | |
| tANT | | | | |
| Forward primer | CTG CTG CTG CAG GTC CAG CA | ANT1: 175–194; ANT2: 154–173 | 57 | 629 |
| Reverse primer | CCA GAC TGC ATC ATC AT | ANT1: 803–787; ANT2: 782–766 | | |

bp, base pair.

termined by a special dot blot hybridization procedure as previously described in detail by Dörner et al. [8]. This method was adapted to the rat-specific nucleotide sequences. In brief, aliquots of the tANT PCR product were denatured and dropped onto two nylon membranes (Amersham, Germany). Additionally, an ANT1- and ANT2-specific cDNA standard, respectively, was dropped in a dilution of 5–30 fmol onto one of these membranes. The membranes were hybridized with corresponding $^{32}$P-radioactively labeled ANT isoform-specific oligonucleotides (ANT1: 5′-GAT CGT CAT AAG CAG TTC-3′ and ANT2: 5′-GCT GGA GCT GAA AGG GAA-3′). The hybridization and washing conditions of the blots as well as the evaluation of the hybridization were the same as already published.

#### 2.3.1. Preparation of ANT isoform standards

ANT1- and ANT2-specific standard cDNA for the dot blot procedure were produced as described by Dörner et al. [8], using rat-specific primer pairs for the amplification of the ANT isoform-specific nucleotide sequences (Table 1).

### 2.4. Southern blotting

Southern blot analyses were performed as previously published [8].

### 2.5. Determination of the amount of ANT-specific mRNA

The competitive q-PCR was employed for the determination of the total ANT mRNA amount in the various tissues of the rat. An internal mRNA standard (competitor) was used, which was simultaneously reverse transcribed with the target RNA and competitively amplified in the PCR.

#### 2.5.1. Construction of the competitor

The competitor was produced using the PCR MIMIC construction kit of Clontech (Heidelberg, Germany). ANT-specific primer sequences (tANT) were linked to the delivered *Bam*HI-*Eco*RI fragment of *erbB*, an ANT non-homologous, neutral DNA fragment of 574 bp, by means of the PCR technique. PCR was carried out in a reaction mixture as described above with 2 ng DNA fragment and 0.4 μM of each composite primer. Composite primers consisted of ANT-specific nucleotide sequences attached to a nucleotide stretch of the sequence, designed to hybridize on the heterogeneous DNA fragment (forward primer: GCA GGT CCA GCA CAA GTT TCG TGA GCT GAT TG; reverse primer: GCA TCA TCA TTT GAG TCC ATG GGG AGC TTT). The denaturation, annealing and extension conditions for the amplification were 45 s at 94°C, 45 s at 60°C and 90 s at 72°C, respectively. Sixteen amplification cycles were performed with a thermal cycler (Biozyme). The amplified PCR product was diluted 1/100 and 2 μl was put into the second PCR reaction. This PCR was made with the primers used for the simultaneous amplification of both isoforms (tANT) (Table 1) leading to a DNA fragment with an ANT non-homologous internal sequence, 471 bp long and flanked by ANT-specific primer sequences.

This PCR product was inserted into the pCR2.0 vector and was multiplied in *Escherichia coli* INVαF′ according to the manufacturer's instructions (Invitrogene, Leek, The Netherlands).

After plasmid preparation using a standard protocol, 5 μg plasmid was digested with *Bam*HI according to the instructions of the manufacturer (Boehringer). 1 μg digested plasmid was transcribed into cRNA using T7 polymerase (Gibco). The transcription mixture was subjected to a 1% low-melting gel electrophoresis. cRNA was extracted from the gel using the QIAquick gel extraction kit (Qiagen, Hilden, Germany). In order to eliminate contaminating DNA, cRNA was subjected to a DNA digestion. The reaction was performed in a mixture containing 1 μg purified RNA, 1 μl 10×DNAse I buffer, 1 U DNAse I amp. grade (Gibco, Karlsruhe, Germany) ad 10 μl diethyl pyrocarbonate treated $H_2O$ for 15 min at 37°C. The cRNA was purified by phenol/chloroform extraction and quantified spectrophotometrically at 260 nm. The cRNA was stabilized with tRNA in a concentration of 1 μg tRNA to 1 ng RNA competitor RNA. The purity of the competitor was proved by PCR using non-transcribed cRNA as a template and the tANT-specific primer pair. No PCR product was generated (data not shown).

#### 2.5.2. Quantitative PCR

RNA of the different types of tissue was isolated

as described by Auffray et al. [7]. The conditions of the PCR were optimized, especially in view of the cycles used and the amount of isolated total RNA and standard cRNA. In the end the following conditions were used. Equal amounts of total RNA (4 ng) were mixed with increasing amounts of competitor (173, 86.5, 43.3, 21.6, 10.8, 5.4 or 2.7 attomol) in a RT mixture of 20 μl containing 4 μl 5×first strand buffer (Gibco), 40 U RNase inhibitor (Boehringer), 2.5 nmol of each dNTP, 8 ng random primer, 10 μM DTT and 200 U MMLV-Reverse transcriptase (Gibco). Competitive PCRs were carried out in a 25 μl reaction mixture containing 50 mM Tris-HCl, 50 mM KCl, 0.001% gelatine, 1.5 mM $MgCl_2$, 2.5 nmol of each dNTPs, 0.4 μM of each tANT primer, 1 μl RT mixture and 0.25 units of *Taq* polymerase (Cetus Perkin Elmer). Conditions of amplifications were the same as mentioned above. Thirty-five amplification cycles were performed with a thermal cycler (Biometra, Göttingen, Germany). PCR products were subjected to agarose gel electrophoresis and stained with ethidium bromide. Gels were scanned by a computer based video system (Polaroid, Offenbach, Germany). The intensity of the ethidium bromide stained bands of the target and the competitor RNA was evaluated by a computer based image system (NIH image). In order to correct for differences in size of the target (629 bp) and the competitor (471 bp) PCR products, the band densities of the respective competitor were multiplied by the specific factor of 1.33. The target/competitor ratio was plotted against the amount of competitor in a log scale. The linear regression was calculated. The amount of competitor corresponding to a log ratio value of 0 is equivalent to the amount of total ANT-specific RNA in the specimens. The level of ANT transcription was expressed as attomol/ng of total RNA.

## 3. Results

### 3.1. ANT isoform transcription patterns in various tissues of the rat

PCR analyses were performed using ANT1- and ANT2-specific primer pairs and reverse transcribed RNA isolated from various types of tissue of the rat. Both ANT isoforms were shown to be transcribed in all tissues tested (Fig. 1). PCR products generated a clear sign of the predicted size on the ethidium bromide strained agarose gel. PCR using primers, which were complementary to homologous sequences of both isoforms, led to an amplification of total ANT (tANT) containing ANT1 and ANT2 cDNA with a length of 629 bp. The specificity of the PCR reactions was subsequently tested performing Southern blot hybridization. Isoform-specific radioactively labeled oligonucleotides hybridized especially with their corresponding type-specific PCR product as well as with the tANT product, consisting of ANT1- and ANT2-specific nucleotide sequences (data not shown).

The exact percentages of ANT1- and ANT2-specific cDNA contained in the tANT PCR product were subsequently determined by a dot blot hybridization procedure. The binding of ANT1- and ANT2-specific radioactively labeled oligonucleotides to the tANT product was compared to that of ANT isoform-specific DNA standards of known molarity,

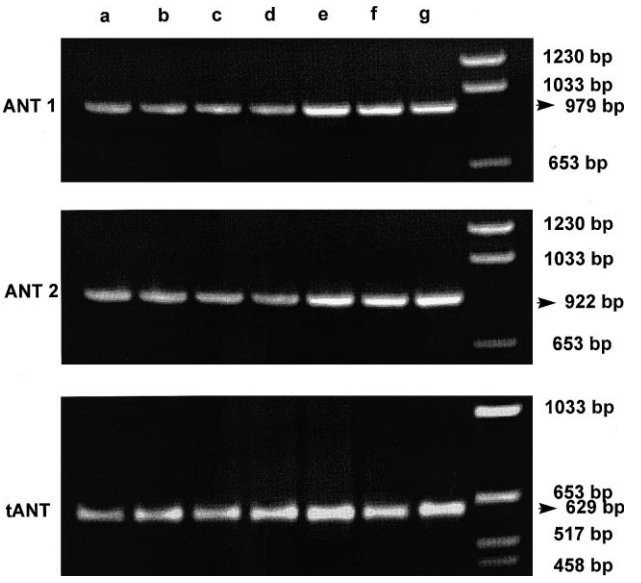



Fig. 1. Transcriptions of the ANT1, ANT2 and total ANT (tANT) were analyzed by qualitative PCR in the liver (a), spleen (b), lung (c), kidney (d), brain (e), skeletal muscle (f) and heart (g). PCRs were performed utilizing ANT1- and ANT2-specific primer pairs as well as a primer pair which recognized homologous sequences in both ANT isoforms (Table 1). PCR products were subjected to agarose gel electrophoresis. The ANT1, ANT2 and tANT PCR products had predicted lengths of 979 bp, 922 bp and 629 bp respectively.

also applied to the dot blot. Using these standard dilutions the content of each ANT isoform-specific cDNA in the tANT product was determined and the percentages of ANT1 and ANT2 were calculated. A predominant proportion of ANT1 transcription was found in skeletal muscle, in heart tissue and in the brain (Table 2). The lung, kidney and spleen had a similar ANT isoform pattern characterized by a dominant ANT2 proportion, ranging from 59 to 65%. The highest proportion of ANT2 was found in the liver.

### 3.2. ANT transcription level in the various tissues of the rat

Competitive PCR was employed in order to quantify the original amount of total ANT (tANT) mRNA in the various tissues of the rat. Quantitative PCR was carried out using an internal RNA standard and the primer pair, leading to a simultaneous amplification of all ANT isoform sequences (tANT) (Fig. 2).

As illustrated in Fig. 3a, the level of total ANT mRNA varied markedly in the various tissues of the rat. The highest value of total ANT transcription was found in the heart tissue, making up 40.9 ± 8.5 attomol/ng total RNA. The level of ANT transcription was determined as being 28.0 ± 5.3 attomol/ng total RNA in the brain and amounted to 23.9 ± 2.1 attomol/ng total RNA in skeletal muscle. The ANT mRNA amount was substantially lower in the kidney, making up 4.6 ± 1.0 attomol/ng total RNA. However, even lower levels were found in the lung, spleen and liver and were quantified to be an average of 2.4 attomol/ng total RNA.

Table 2
Percentages of the ANT isoform mRNAs in various tissues of the rat

| Tissue | ANT1 (%) | ANT2 (%) |
|---|---|---|
| Skeletal muscle | 81 ± 4 | 19 ± 4 |
| Heart | 63 ± 4 | 37 ± 4 |
| Brain | 58 ± 3 | 42 ± 3 |
| Lung | 41 ± 6 | 59 ± 6 |
| Kidney | 35 ± 6 | 65 ± 6 |
| Spleen | 36 ± 7 | 64 ± 7 |
| Liver | 25 ± 9 | 75 ± 9 |

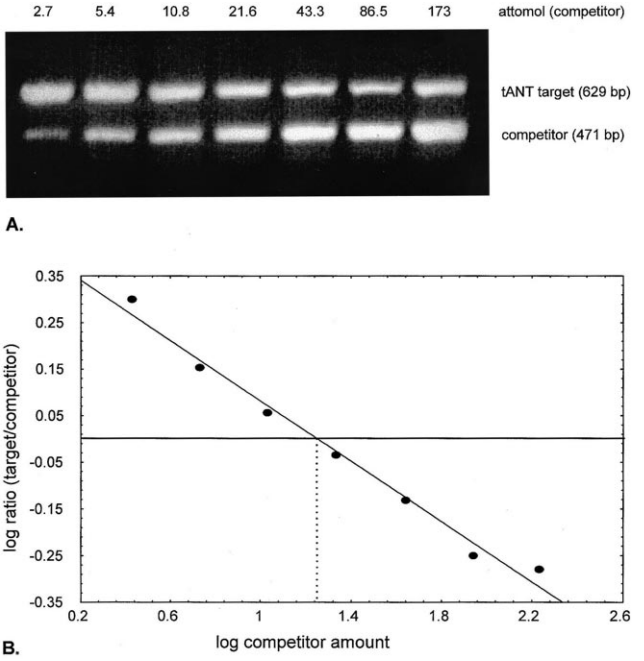
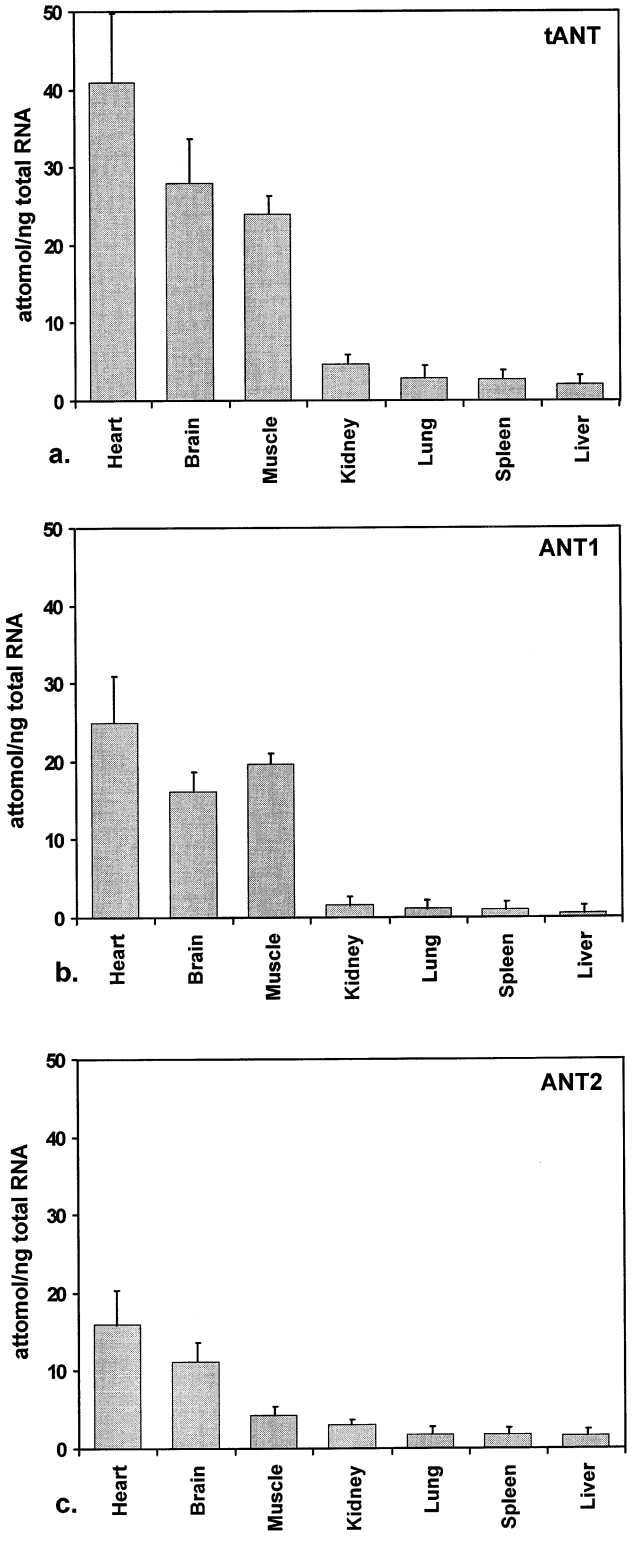



Fig. 2. Total ANT mRNA quantification by competitive PCR. A constant amount of total RNA (4 ng) isolated from kidney was mixed with an increasing amount of competitor cRNA, reverse transcribed and amplified by PCR. (A) The PCR products were subjected to agarose gel electrophoresis, stained and scanned. The PCR products of the ANT target and the competitor had expected lengths of 629 bp and 471 bp, respectively. The band intensities were evaluated using a computer based image system. (B) The ratio of target/competitor products was plotted against the known amount of competitor on a log scale. At the competition equivalence point (log ratio = 0) the original content of the target mRNA corresponds to the initial amount of competitor RNA used.

In order to additionally determine the precise amount of ANT1- and ANT2-specific mRNA in the various types of tissue, the values of total ANT mRNA amount, quantified by q-PCR, were combined with the percentages of the ANT isoforms, obtained from the dot blot hybridization procedure. The highest ANT1 transcription level amounting to 24.9 ± 5.7 attomol/ng total RNA was found in the heart, followed by that determined in skeletal muscle (19.6 ± 1.8 attomol/ng total RNA) (Fig. 3b). The ANT1 mRNA amount in the brain was also significantly higher (16.2 ± 3.0 attomol/ng total RNA) than in tissues which have an ANT isoform transcription pattern with a predominant ANT2 proportion. The amount of ANT1 mRNA made up 1.6 ± 0.3 attomol/ng total RNA and 1.1 ± 0.6 attomol/ng total RNA in




the kidney and the lung, respectively. In the spleen the ANT1 mRNA amount was found to make up $0.9 \pm 0.3$ attomol/ng total RNA. However, the lowest ANT1 transcription level was measured in the liver amounting to only 2% of that determined in the heart ($0.5 \pm 0.4$ attomol/ng total RNA).

A maximum ANT2 transcription level was also recorded in the heart ($16.0 \pm 1.7$ attomol/ng total RNA), followed by that of the brain ($11.8 \pm 2.5$ attomol/ng total RNA) (Fig. 3c). The value of ANT2 mRNA amount was calculated to be $4.3 \pm 1.0$ attomol/ng total RNA in the skeletal muscle. The ANT2-specific mRNA amounted to $3.0 \pm 0.8$ attomol/ng total RNA in the kidney and was even lower in the lung, spleen and liver, making up 1.7 attomol/ng total RNA on an average.

## 4. Discussion

Two different ANT isoforms were identified in the rat, which were shown to be differently expressed in various tissues [4,9]. However, the significance of tissue-specific ANT isoform expression has not been totally understood. In order to find new aspects which improve our knowledge of the ANT isoform expression, we determined in this study the exact percentages of the ANT isoform mRNAs and in addition quantified the level of the isoform transcription in various organs of the rat. The Northern blot technique was previously employed to describe the transcription of the ANT isoform genes. This technique, however, allows only for a rough appraisal of the transcription levels. We therefore developed a competitive PCR for the accurate quantification of the ANT transcription. For this purpose, we used an internal RNA standard and primers, which enabled simultaneous amplification of both ANT sequences in the mRNA original distribution. These primers had already been utilized for the amplification of

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Fig. 3. The content of total ANT (tANT) (a), ANT1 (b) and ANT2 (c) mRNA in various types of tissue in the rat. The level of total ANT transcription was determined by the use of the competitive PCR technique as described in Fig. 2. The ANT1- and ANT2-specific mRNA amounts were calculated by combining the data obtained from the q-PCR with those from the dot blot hybridization procedure.

the three human ANT isoforms and can also be employed for the simultaneous multiplication of ANT isoform sequences from cattle [3] and mouse [6]. We subsequently determined the percentages of each ANT isoform sequences in the PCR product of total ANT by means of a dot blot hybridization procedure. The combination of the q-PCR with the dot blot hybridization procedure allowed additionally the quantification of the ANT1 and ANT2 transcription levels. The described technique is especially recommendable if only small tissue samples are available. The employed method provides an exact picture of the ANT isoform transcription level and pattern in the various types of tissues from the rat.

A predominant proportion of the ANT1 isoform was found in the skeletal muscle, heart and brain, ranging from 81 to 58% of total ANT transcription. However, tissues such as lung, kidney, spleen and liver showed a prevalent percentage of ANT2 transcription. In contrast to the rat, the human organism expresses three different ANT isoforms. Nevertheless, ANT3 was not found to be prominent in any of the tissues tested [8]. Thus, ANT3 seems to be of subordinate significance, particularly since it is missing in several species including the rat [6]. ANT3 aside, the distribution of the ANT1 and ANT2 isoforms in the rat tissue is very similar to that found in man. Highly conserved tissue-specific ANT isoform transcription programs obviously exist in mammals.

A common feature of the lung, kidney, spleen and liver is, besides the predominant ANT2 mRNA percentage, the ability for cell regeneration, distinguishing these tissues from the organs with a predominant ANT1 transcription as shown in heart, skeletal muscle and brain. In contrast to the ANT1 isoform, ANT2 was shown to be a strongly growth-related gene [10,11]. In connection with this, the influence of thyroid hormones on the ANT expression was intensively investigated [9,12,13]. Thyroid hormones play a key role in the regulation of metabolism, growth and development. ANT2 was shown to be sensitive to $T_3$, responding tissue-specifically with an increased transcription. In contrast, the ANT1 transcription was not positively affected by $T_3$. Moreover, it has been found that thyroid hormones influence the binding of regulatory proteins with a negative-regulating element of the ANT1 gene [14], thus possibly inhibiting the transcription in this way. In addition, the developmental dependence of the ANT gene regulation is impressively shown during the development of myoblasts to already determined muscle cells [15]. During the proliferation of the myoblasts a predominant ANT2 transcription was observed, whereas it decreased when the cells entered the differentiation stage. At the beginning of this phase a dramatic increase in the ANT1 mRNA amount was found. Hence, ANT1 appeared to be the isoform of tissues no longer subjected to proliferation, whereas the ANT2 percentage is predominant in tissues able to divide mitotically.

The above does not eliminate a close correlation between the oxygen consumption of the tissue, which is a marker for the degree of oxidative phosphorylation and thus of ATP production, and the level of total ANT expression. Since the ANT is the only carrier for ATP in the mitochondrion, its amount is adapted to the ATP production and to the amount of mitochondria in the cell. Biochemical studies have shown that the content of total ANT in the heart mitochondria, making up 14% of the total mitochondrial protein, was markedly higher than the mitochondrial ANT amount in kidney or liver, amounting to only 6% and 3% of the total mitochondrial protein, respectively [16]. In this study, the ANT mRNA amount in the heart tissue was found to be approx. 9 and 17 times higher than that measured in the kidney and liver, respectively. However, it is necessary to take into consideration that not only the ANT density in the mitochondria differs in the various types of tissue but also the amount of mitochondria itself. Since the amount of mitochondria in the heart is substantially higher than in the kidney and liver, the ANT transcription consequently has to be markedly higher in the myocardium. The correlation between the ANT transcription level and the degree of oxidative phosphorylation is moreover shown by the positive regulation of both isoform genes by oxygen. Recent studies demonstrated that hypoxia caused a decrease in the amount of ANT2 mRNA in human hepatocellular carcinoma cells [17]. In addition, it was shown that the amount of ANT1 in heart mitochondria decreased as a result of ischemia [18]. Furthermore, investigations of the ANT isoform regulation in yeast proved that the isoforms are differently regulated by oxygen. It was shown that ACC1 and ACC2 are positively regulated by the oxy-

gen tension, whereas ACC3 is subjected to a negative control of $O_2$ [19–21]. Thus, both the levels of ANT1 and ANT2 expression are adapted to the energy demand of the cell. This is confirmed by our findings showing that there is not only a high level of ANT1 but also of ANT2 transcription in the heart and muscle tissue.

The correlation between ATP production and ANT expression level in the tissues fits exactly, with the exception of brain tissue. Comparing the oxygen consumption of the various tissues, the energy requirement of the brain is lower than that of the kidney. Therefore, the energy metabolic conditions in the brain would lead us to expect a lower level of ANT transcription than in the kidney. Nevertheless, the level of total ANT transcription in the brain is about 6 times higher than that in the kidney. The observation made concerning the ANT transcription of the brain, an organ with a prevalent ANT1 proportion, led us to the conclusion that (1) the main factor which determines the predominance of the ANT isoform expression in the various tissues is not the energy requirement of the tissue but the ability or inability to proliferate. (2) A predominant ANT1 expression is accompanied by a high level of total ANT transcription.

In summary, ANT isoform transcription is subjected to a regulatory program in which developmental and energy-requiremental aspects are involved.

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