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Differential CpG island methylation of murine adenine nucleotide translocase genes

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Abstract

Adenine nucleotide translocase (Ant) mediates the exchange of ADP and ATP across the inner mitochondrial membrane in eukaryotes. Mice possess three distinct but highly homologous Ant isoforms, encoded by independent genes, whose transcription depends upon tissue type. *Ant1* is expressed selectively in heart and skeletal muscle, *Ant2* is ubiquitously expressed in most tissues but lower in skeletal muscle and testis, while *Ant4* is exclusively expressed in the testis. Of interest, each of these *Ant* genes contains CpG islands in their proximal promoter regions. We investigated the methylation status of the three *Ant* genes in various tissues with active and inactive transcription. In contrast to the *Ant4* gene in which CpG island methylation is essential for gene repression, the CpG islands of *Ant1* and *Ant2* are hypomethylated regardless of the gene expression status throughout the tissues of male mice. Despite the tissue specific expression profile of *Ant1*, CpG methylation is unlikely involved in the regulation of the gene. Consistent with these findings, addition of a CpG-demethylating agent, 5-aza-2'-deoxycytidine, to fibroblasts increased the expression of *Ant4* but not *Ant1* or *Ant2* genes. This study provides insight regarding the differential regulation of *Ant* isoforms in mammals, whereby both the *Ant1* and *Ant2* genes are capable of expression, but the *Ant4* gene is completely repressed throughout somatic tissues. To the best of our knowledge, this is a first example to clearly demonstrate a differential usage of CpG island methylation within a family of genes.

Keywords

Adenine nucleotide translocase; ATP; gene expression; transcription; CpG island; DNA methylation

Introduction

Adenine nucleotide translocase (Ant), also called ADP/ATP carrier (Aac) is expressed on the inner mitochondrial membrane and facilitates the exchange of ADP and ATP between the mitochondria and the cytosol, and thus plays an essential role for energy metabolism in eukaryotes [1-3]. Under aerobic conditions, ATP produced within the mitochondria through oxidative phosphorylation is exported to the cytosol through Ant to support cellular activities. In exchange, ADP is imported through Ant to provide a substrate for the conversion of ADP to ATP by ATP synthase. In contrast, under anaerobic conditions, glycolytic ATP is imported into mitochondria through Ant. This retro-translocation of ATP is considered necessary for the

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energy-dependent processes of mitochondria and is critical for maintaining the mitochondrial membrane potential and preventing apoptosis mediated by mitochondrial membrane permeabilization [4,5].

Of interest, all eukaryotic species investigated so far, from single cellular yeast to multicellular organisms, have multiple *Ant* genes. For example, *Saccharomyces cerevisiae* has *Aac1*, 2 and 3, among which *Aac1* and *Aac3* are expressed almost exclusively in aerobic and anaerobic conditions, respectively [6,7]. Thus, the different *Ants* are likely utilized in order to efficiently cope with varying external oxygen and nutrient conditions in single cellular organisms. Mice have three *Ant* genes (*Ant1*, 2 and 4), and their expression differs depending on the tissue [8-11]. *Ant1* (also called *Aac1*, *Slc25a4*) is predominantly expressed in heart and skeletal muscle, *Ant2* (*Aac2*, *Slc25a5*) is present in most tissues with its lowest expression in skeletal muscle and testis, while *Ant4* (*Aac4*, *Slc25a31*) is expressed exclusively in testicular germ cells [8-10]. It is speculated that different *Ants*, each with unique kinetic properties, may have developed to adapt to the specific energy requirements in particular tissues including heart, skeletal muscle and male germ cells in multicellular organisms [8,10,11]. Disruption of *Ant1* leads to mitochondrial myopathy, cardiomyopathy, and severe exercise intolerance in mice [12], which confirms the specific role of the gene in heart and skeletal muscle. *Ant4* disruption leads to meiotic arrest of male germ cells and male infertility but no apparent abnormalities in somatic tissue or female reproductive organs [10], also confirming the specific role of *Ant4* in testis. It should be noted here that non-rodent mammals including human, cow and dog have an additional *Ant* gene, *Ant3*, which is encoded at the pseudoautosomal region of X chromosome and is ubiquitously expressed [8,13,14,15].

The *Ant* genes are made up of distinct promoter sequences [16], supporting the view that their expression is under different regulatory controls. However, mechanisms underlying tissue specific expression of the *Ant* genes are not fully understood. The human *ANT1* gene promoter possesses the OXBOX element which is also seen at the ATP Syn β promoter [17,18]. This element acts as a positive regulatory element in muscle cells [19]; hence, the OXBOX might account for the high *ANT1* expression in heart and skeletal muscle [8]. However, the other report indicates that the mouse *Ant1* gene promoter lacks the OXBOX element, questioning the role of the element in tissue specific expression [20]. *Ant2* expression is ubiquitous but growth-dependent [8,21,22]. The GRBOX (glycolysis regulated box) motif, Sp1-binding elements, and NF1 binding site in the *Ant2* gene promoter are proposed to regulate *Ant2* expression in a cell cycle-dependent manner and also depending on cellular glycolysis status [4,23-25]. *Ant4* expression is exclusively detected in male germ cells and is particularly high during meiosis [10]. The *Ant4* gene promoter has an E2F6-binding element, which is essential for repression of the *Ant4* in somatic tissue [26].

In addition, the *Ant4* gene has a CpG island which spans its proximal promoter region to exon 1 [9,27]. The CpG island of the *Ant4* gene is hypomethylated in testis where *Ant4* is highly expressed, whereas it is hypermethylated in somatic tissue where *Ant4* expression is undetectable [9,27]. Moreover, the disruption of DNA methyltransferase or the addition of 5-aza-2'-deoxycytidine resulted in a loss of *Ant4* repression in somatic cells [9]. These indicate that DNA methylation is essential for *Ant4* repression in somatic tissues. Since *Ant1* and *Ant2* genes also have CpG islands, here we investigated whether CpG island methylation is involved in the tissue-specific gene expression of other *Ants*.

Materials and methods

CpG island analysis

The promoter regions of *Ant1*, *Ant2*, and *Ant4* were analyzed for the presence of CpG islands using the MethPrimer program (<http://www.urogene.org/methprimer/index1.html>). A CpG

island is defined as a region of DNA greater than 200 bp, with a GC content above 0.5 and an observed/expected CpG ratio above 0.6 [28]. Briefly, sequences containing 500 bp upstream of the transcription start site and 212 bp downstream, were entered and searched by MethPrimer for CpG islands in *Ant1*, *Ant2*, and *Ant4*. The predictions were then further confirmed by sequence analysis of these regions.

Bisulfite sequencing and combined bisulfite restriction analysis

DNA was extracted from various tissues of adult C57BL/6 mice (~8 weeks) using the DNA Wizard Genomic DNA purification Kit (Promega, Madison, WI). All mice have been maintained under standard specific-pathogen-free (SPF) conditions, and the procedures performed on the mice were reviewed and approved by the University of Florida Institutional Animal Care and Use Committee (IACUC). Testes were decapsulated, and seminiferous tubules were collected for analysis. Bisulfite reactions were performed using the EZ DNA Methylation Kit (Zymo Research, Orange, CA). Up to 2 µg of genomic DNA was used for conversion with the bisulfite reagent. Approximately 80 ng of bisulfite-converted DNA was used as template for each polymerase chain reaction (PCR) analysis. PCR was performed in 25 µl reaction mixtures containing 2 µl of bisulfite converted DNA (50-100ng), 1 µM of primers, 0.625 U of HotStar Taq (Qiagen, Maryland, USA), 200 µM deoxy-nucleotide triphosphates and 0.25x Q-solution. Primers used for combined bisulfite restriction analysis (COBRA) were *Ant1*: 5'-GGAAGGGGTGGAAGTTTG and 5'-CTAATCCCCCATACTAAAAACC; *Ant2*: 5'-GGTTTGATTAGGTGTTAAGGGTAAG and 5'-ACATCTATCATATTAAAAACAAAAA; *Ant4*: 5'-GTAGTATTTGGTTAGAGTGTGTTTTTTGG and 5'-ACACTAAAAAAACTAAAAAACC (40 cycles). Following PCR amplification, fragments were purified using QIAquick PCR purification kit (Qiagen) and eluted with a final volume of 35 µl. Digestion of PCR purified fragments was then carried out with HhaI as follows: 35 µl eluted PCR purified fragments, 4.5 µl of NEB buffer 4 (New England Biolabs, Beverly, MA), 1.5 µl of HhaI enzyme (New England Biolabs), 0.25 µl of Bovine serum albumin (New England Biolabs), and 3.75 µl water. Digestion was carried out overnight and products were subsequently subjected to electrophoresis at 100V for 30 min. on 2% agarose gels. Primers used for bisulfite sequencing were *Ant1*: 5'-TGTTTAGGGATTAGTTTAGTTAATG and 5'-CTAATCCCCCATACTAAAAACC; *Ant2*: 5'-GGTTTGATTAGGTGTTAAGGGTAAG and 5'-ACATCTATCATATTAAAAACAAAAA; *Ant4*: 5'-TTGTTGTGTATTGATTGAGTATG and 5'-ACACTAAAAAAACTAAAAAACC. PCR fragments were cloned into pCRII-TOPO cloning vector (Invitrogen, Carlsbad, CA), and individual clones were sequenced.

Real-time RT-PCR

We isolated total RNA from various tissues using the RNAqueous kit (Ambion, Austin, TX). cDNA was synthesized using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA) using random primers. Real-time PCR reactions were performed using the TaqMan Gene Expression Assay (Applied Biosystems) according to manufacturer's instructions. Each 20 µL reaction consisted of 10 µL of TaqMan Universal PCR Master Mix, No AmpErase-UNG; 1 µL of TaqMan Gene Expression Assay Mix, for β -actin (VIC-labeled), *Ant4* (FAM-labeled), *Ant2* (FAM-labeled), or *Ant1* (FAM-labeled); and 9 µL of cDNA (25 ng). Reactions (40 cycles of 95°C, 15 sec and 60°C, 1 min) were performed using Applied Biosystems 7900HT Fast Real-Time PCR instrument. Gene expression analysis was performed using the comparative cycle threshold (Ct) method using β -actin for normalization. The relative transcript levels was calculated using the formula, $2^{-(\Delta Ct)}$.

Cell culture

NIH3T3 cells (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's Modified Eagle Medium (Invitrogen) containing 10% Fetal Bovine Serum. Freshly prepared 5-aza-2'-deoxycytidine (5-aza-dC; Sigma), and/or trichostatin A (TSA; Wako) were added to culture media at the indicated final concentrations.

Results

Identification of CpG islands at the promoter regions of mouse *Ant1*, *Ant2*, and *Ant4* genes

In order to determine whether *Ant1* and *Ant2* are regulated by DNA methylation similarly to *Ant4*, we initially investigated the proximal promoter regions of *Ant1*, *Ant2*, and *Ant4* for the presence of CpG rich areas known as CpG islands. CpG islands are short stretches of DNA in which the frequency of the CpG sequence is higher than in other regions. The accepted definition of a CpG island is a region of DNA greater than 200 bp, with a GC content above 0.5 and an observed/expected CpG ratio above 0.6 [28]. Figure 1 illustrates murine *Ant1*, *Ant2* and *Ant4* genes regions from 500 bp upstream of the predicted transcription initiation site to 212 bp downstream from the transcription initiation site extending into exon 1. Based on the definition, analysis using MethPrimer program revealed that like *Ant4*, *Ant1* and *Ant2* also contained discernable CpG islands between their proximal promoter regions and exon1.

Analysis of *Ant1*, *Ant2*, and *Ant4* transcript levels in various tissues

Transcript levels of *Ant1*, *Ant2*, and *Ant4* in various mouse tissues were then examined using real-time RT-PCR analysis of RNA isolated from male mouse testis, kidney, heart, skeletal muscle, and tail, as well as RNA isolated from female tail. Each tissue was chosen carefully after searching the published data regarding the expression levels of *Ant1*, 2, and 4 in various tissues [8,9,12]. The goal of the expression analysis was to determine the highest and lowest expressing tissues for each *Ant* gene. Within the tissues analyzed, *Ant1* was expressed highly in heart and skeletal muscle but very low to undetectable expression levels in kidney, liver, tail and testis; *Ant2* expression was rather ubiquitously expressed in the tissues examined but lower in skeletal muscle and testis. *Ant4* was expressed highly in testis and had no to very low expression in other tissues (Fig. 2).

Methylation analysis of CpG islands of *Ant1*, *Ant2*, and *Ant4* genes in various tissues

To investigate the methylation status of *Ant1*, *Ant2*, and *Ant4* genes in various mouse tissues we initially utilized combined bisulfite restriction analysis (COBRA). Using the restriction enzyme *HhaI* which digests DNA protected from bisulfite conversion by methylation at the sequence GCGC, we were able to quickly determine the methylation status of the promoter regions of *Ant1*, *Ant2*, and *Ant4* in various tissues. *HhaI* digestion of *Ant1*, *Ant2*, and *Ant4* promoter loci revealed an absence of methylation at the *Ant1* and *Ant2* promoter regions in all male tissues analyzed. *Ant4* gene exhibited hypomethylation in testis but hypermethylation in somatic tissues as described previously (Fig. 3) [9]. Interestingly, the *Ant2* gene, which is located on the X chromosome, shows a partial methylation pattern in female tissue when analyzed by COBRA. In order to further confirm the methylation status of *Ant1*, *Ant2*, and *Ant4* genes, we performed bisulfite sequencing analysis. Bisulfite sequencing analysis of the CpG islands was carried out on selected tissues for *Ant1*, *Ant2*, and *Ant4*. For each of *Ant1*, *Ant2*, and *Ant4* we chose one tissue in which transcript levels were high and also one in which little to no transcript was detectable. This allowed us to determine if there was any differential methylation status present between tissues in which expression of these *Ants* were present or absent to very low. Bisulfite sequencing analysis of the *Ant1* CpG island was performed using heart and liver which express *Ant1* at high and very low levels, respectively. For *Ant2* analysis, we utilized kidney as the highly expressing tissue and skeletal muscle as the low expressing

tissue. We also analyzed female tail to confirm our COBRA data for the partial methylation pattern present. For *Ant4*, we utilized testis as the tissue expressing the gene and kidney as the tissue with little or no detectable expression. Bisulfite sequencing analysis confirmed our COBRA data that there is no significant increase in methylation at *Ant1* and *Ant2* CpG islands in all the male tissues examined regardless of gene expression status (Fig 4). Female tail showed hypermethylation of the *Ant2* CpG island among approximately half samples examined. In contrast to *Ant1* and *Ant2*, the *Ant4* gene demonstrated a distinct methylation profile between tissues with high expression and those without. The *Ant4* CpG island was hypomethylated in testis but hypermethylated in kidney, which was consistent with our COBRA data.

5-aza-2'-deoxycytidine increased the expression of *Ant4* but not *Ant1* or *Ant2* gene in fibroblasts

To further investigate the differential role of CpG methylation in *Ant1*, *Ant2*, and *Ant4* genes, we examined the effects of DNA demethylation on gene expression. NIH3T3 mouse fibroblast cells were treated with a DNA-demethylating agent, 5-aza-2'-deoxycytidine (5-aza-dC) [29], a histone deacetylase (HDAC) inhibitor, trichostatin A (TSA) [30], or both for 48 hours. As shown in Fig. 5, *Ant4* expression was derepressed by 5-aza-dC but not TSA, indicating that DNA methylation, but not histone deacetylation is the primary repression mechanism for the gene. In contrast, *Ant1* or *Ant2* gene expression was not affected by either 5-aza-dC or TSA, supporting the idea that neither the *Ant1* nor the *Ant2* gene is likely epigenetically controlled.

Discussion

Our data demonstrate that all mouse *Ant* genes: *Ant1*, *Ant2*, *Ant4*, similarly possess CpG islands spanning their proximal promoter regions to first exons. However, their methylation status was dissimilar between the family of genes. *Ant1* and *Ant2* promoter loci showed no differential methylation pattern throughout tissues regardless of their gene expression status (Table 1). This is in contrast to the *Ant4* gene which is hypermethylated in tissues lacking expression. Consistent with these findings, addition of a CpG-demethylating agent, 5-aza-deoxycytidine, to fibroblasts increased gene expression of *Ant4* but not *Ant1* or *Ant2*. The present data indicate that, unlike *Ant4*, DNA methylation does not likely play a role in tissue specific expression of *Ant1* and *Ant2*. To the best of our knowledge, this is a first example to clearly demonstrate a differential usage of CpG island methylation within a family of genes.

The state of CpG methylation is believed to regulate chromatin structure and the accessibility of transcriptional machinery to gene promoters [31]. High CpG content is enriched at the proximal promoter regions of genes, and in particular most of the “housekeeping” genes have CpG islands at their promoters [32]. In contrast, genes associated with more specific functions and those expressed in tissue-specific manner typically have low CpG content in their promoters [32]. In this context, in contrast to the previous data that *Ant(s)* are expressed in a tissue-dependent manner, *Ant1* and *Ant2* genes may be more categorized with the “housekeeping” side as are most other mitochondrial protein-encoding genes which have high CpG content at the promoters [32]. Although tissue-selective expression is seen to a degree particularly with *Ant1*, it may not be necessary to completely repress the gene in other tissues. In eukaryotic evolution, it is thought that different *Ants* have developed to cope with differential external oxygen and nutrient conditions [33]. Human *ANT1* gene contains the OXBOX/REBOX element while both human *ANT2* and mouse *Ant2* contain the GRBOX element in their promoters [17-20], indicating that the genes are likely still under the control of external oxygen and nutrient conditions. Thus, tissue selective *Ant1* and *Ant2* expression may not be absolute but more flexible in each tissue. Leaving the gene capable of expression may be important for the whole body to cope with various external conditions. The present data are also consistent with the notion that the X-linked *Ant2* gene is methylated in one allele in female

tissues due to somatic X chromosome inactivation (see review, for example, [34]). This methylation, due to X chromosome inactivation, is unlikely to be regulating expression of *Ant2*.

In contrast to *Ant1* and *Ant2*, the *Ant4* gene appears to follow the DNA methylation pattern of germ cell-specific genes. As seen in many other germline genes [35,36], the *Ant4* CpG island becomes methylated during early embryonic development [9] and is completely hypermethylated in somatic tissues. The reason for complete repression of *Ant4* in somatic tissue is currently unknown. Human ANT4 was shown to have very unique affinity for and kinetics of ADP/ATP exchange when compared to somatic ANT4s [11], which may render *Ant4* expression detrimental to somatic tissues. Alternatively, the *Ant4* gene may be under the control of a yet-unrevealed universal gene repression mechanism common to germline genes in soma, and repression of the *Ant4* gene itself may not be critical in somatic tissues. In conclusion, the present study provided us with further insights regarding differential regulation of *Ant* isoforms in mammals, which would help increase the understanding of how the genes developed throughout evolution and currently function in mammals.

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Figure 1. CpG islands of murine *Ant1*, *Ant2* and *Ant4* genes

The promoter regions along with exon 1 (-500 to +212) of *Ant1*, *Ant2*, and *Ant4* were analyzed for the presence of CpG islands using the MethPrimer program. A CpG island is defined as a region of DNA greater than 200 bp, with a GC content above 0.5 and an observed/expected CpG ratio above 0.6.

Figure 2. Tissue specific expression of murine *Ant1*, *Ant2* and *Ant4* genes

RNA expression of *Ant1*, *Ant2*, and *Ant4* genes in various tissues of adult C57BL/6 mice (~8 weeks) were analyzed using real-time RT-PCR. Relative expression levels of *Ant* genes to β -actin using the comparative Ct method are shown. Sk M: Skeletal Muscle, M: Male, F: Female

Figure 3. CpG methylation of murine *Ant1*, *Ant2* and *Ant4* genes (COBRA)

CpG methylation status of *Ant1*, *Ant2*, and *Ant4* gene promoters in various tissues was analyzed using combined bisulfite restriction analysis (COBRA) as described in Materials and Methods. "U" represents unmethylated DNA fragments, while "M" represents methylated DNA fragments.

Figure 4. CpG methylation of murine *Ant1*, *Ant2* and *Ant4* genes (Bisulfite Sequencing)
CpG methylation status of (A) *Ant1*, (B) *Ant2*, and (C) *Ant4* gene promoters in various tissues was analyzed using bisulfite sequencing analysis as described in Materials and Methods. % Methylation (% Met) for each CpG site investigated is demonstrated in the bar graphs. Actual CpG methylation status of the region in eight sequenced clones is also shown to the right of the graph. The open circles represents unmethylated CpG residues, while the closed circles represents methylated CpG residues.

Figure 5. The effects of 5-aza-2'-deoxycytidine and trichostatin A on *Ant1*, *Ant2* and *Ant4* gene expression

NIH3T3 mouse fibroblasts were treated with 5-aza-2'-deoxycytidine (5-aza-dC, 5 μ M), trichostatin A (TSA, 200 nM) or both for 48 hrs. RNA expression of *Ant1*, *Ant2*, and *Ant4* genes was analyzed using real-time RT-PCR as described above in Fig. 2. Relative expression levels of *Ant* genes compared to β -actin using the comparative Ct method are shown. The mean values for the *Ant4* transcript are: No treatment (1.21×10^{-3}), 5-aza-dC (9.71), TSA (1.53×10^{-3}) and 5-aza-dC+TSA (1.89).

Table 1

	Ant1		Ant2		Ant4	
	Met	Exp	Met	Exp	Met	Exp
Testes (M)	○	-	○	±	○	++
Kidney (M)	○	-	○	++	●	-
Heart (M)	○	++	○	+	●	-
Sk M(M)	○	+	○	±	●	-
Liver (M)	○	-	○	+	●	-
Tail (F)	○	-	●	+	●	-
Tail (M)	○	-	○	+	●	-

Met - Methylation Status
○ - unmethylated
● - methylated
● - mixture

Exp - Expression level
++ high
+ intermediate
± low
- very low to absent