Biochemical and Biophysical Research Communications xxx (2009) xxx–xxx

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Fluorescence *in situ* hybridization and chromosomal organization of the sirtuin 4 gene (Sirt4) in the mouse

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ARTICLE INFO

Article history:
Received 5 March 2009
Available online xxxx

Keywords:
Sirtuins
Sirt4
Histones
Chromatin
Histone deacetylase
Chromosomes
Genes
Structural

ABSTRACT

The sirtuins (SIRT1–7), also being referred to as class III HDACs, exert NAD-dependent deacetylase and/or ADP-ribosyltransferase activities in various cellular compartments including the cell nucleus, the cytoplasm and the mitochondria. The sirtuins play a central role in epigenetic gene silencing, DNA repair and recombination, cell-cycle, microtubule organization, and in the regulation of aging. SIRT4 is a mitochondrial protein that lacks deacetylase activities but efficiently works as an ADP-ribosyltransferase. We have isolated and characterized the murine *Sirt4* genomic sequence, which spans a region of 12 kb and which has one single genomic locus. Determination of the exon–intron splice junctions established that SIRT4 is encoded by 6 exons. The 1648 bp murine *Sirt4* transcript encodes a 418 aa protein with a predictive molecular weight of 47.3 kDa. Fluorescence *in situ* hybridization analysis identified a single genomic locus for murine *Sirt4* gene on chromosome 5F and is neighbored by the *PLA2G1B* and *PXN* genes.

Mammalian histone deacetylases (HDACs) catalyze the removal of acetyl groups from the ε-amino group of lysine residues and are divided into four distinct subclasses, which comprise three classes of non-sirtuin HDACs (class I, II and IV HDACs) and the sirtuins (class III HDACs), which received their name based on their homology with the yeast Sir2 protein [1]. The main distinguishing feature, that discriminates the sirtuins from other HDACs, is their exceptional enzymatic mechanism. While class I, II and IV HDACs are Zn^{2+}-dependent hydrolases, the sirtuins exhibit a unique NAD-dependent deacetylase activity and as shown for a few sirtuins, a secondary ADP-ribosyltransferase activity [2,3]. To date, seven human and murine sirtuins have been identified (SIRT1–SIRT7), which share a common Sir2 catalytic domain [4–7]. This deacetylase domain is being flanked by highly variable N-terminal and C-terminal extensions, suggesting the acquisition of new functions through evolution, which may be required for the regulation of their subcellular localization and/or catalytic activity [7,8]. While SIRT1 may be found both, in cell nucleus and in the cytoplasm, SIRT2 has been reported to be exclusively cytoplasmic. SIRT3, SIRT4 and SIRT5 localize to the mitochondria [9,10], while SIRT6 and SIRT7 are solely found in the cell nucleus [11].

SIRT4 is a mitochondrial matrix protein that becomes cleaved at amino acid position 28 subsequent to its import into mitochondria [12]. Unlike the other HDACs, SIRT4 does not show evidence of a deacetylase activity; it does however exhibit strong enzymatic activity as an ADP-ribosyltransferase. Insulin secretion in pancreatic β cells for instance, is being regulated by SIRT4, which ADP-ribosylates and downregulates glutamate dehydrogenase (GDH), which converts glutamate to α-ketoglutarate in β cells during calorie-sufficient conditions, thereby preventing glutamine from subsequently inducing the secretion of insulin [9]. Calorie restriction on the other hand, goes along with increased GDH activity, which upregulates insulin and it has therefore been suggested that calorie restriction does in fact downregulate SIRT4 ADP-ribosyltransferase activity, which appears unexpected at first sight in view of the fact that sirtuin activity is otherwise being increased in the context of calorie restriction [9], but which may also be explained by a lower NAD/NADH ratio during calorie restriction, which may downregulate SIRT4 activity and thus reduce ADP-ribosylation of GDH [9,13]. SIRT4 knockout mice are viable and fertile, and pancreatic islet cells isolated from such mice secrete increased levels of insulin, which further supports the role of SIRT4 in the downregulation of insulin secretion through repression of GDH activity [9,11]. GDH, acetyl-CoA synthetase 2 (AceCS2), IDE (insulin degrading enzyme), ANT2 (ATP/ADP translocase 2) and ANT3 (ATP/ADP translocase 3) are the only SIRT4 targets that have been reported so far,

Abbreviations: HDAC, Histone Deacetylase; HAT, Histone Acetyltransferase.

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0006-291X/$ - see front matter © 2009 Published by Elsevier Inc.
doi:10.1016/j.bbrc.2009.03.092

additional targets of SIRT4 remain still to be identified [9,12]. It has been suggested that SIRT4 may protect from diabetes and aging. Therefore, the loss of SIRT4 could on the other hand contribute to the pathogenesis of diabetes and cancer [5,14]. In the study presented herein, we report the chromosomal localization and genomic organization of the murine Sirt4 gene.

Materials and methods

Identification of the murine Sirt4 cDNA

Homology searches of the EST database at NCBI (National Center for Biotechnology Information) with the human SIRT4 protein sequence (GenPept XP_993153) yielded four murine mRNA sequences of variable length, of which one was then used for the identification of the murine *Sirt4* genomic clone.

Identification of BAC genomic clone RP23-189B10

The Sirt4 cDNA (GenBank clone XM_988059), which contained the full-length murine *Sirt4* cDNA was used to identify the murine *Sirt4* genomic clone from a murine BAC genomic library (RZPD, Berlin, Germany) after *in silico* screening. We found BAC clone RP23-189B10 to contain an approximately 120 kb insert in the vector pBACe3.6, which included the murine *Sirt4* genomic sequence. BAC genomic DNA was prepared according to published protocols [15] and the murine *Sirt4* insert was confirmed by cycle sequencing [16].

Instrumental methods

Dye terminator cycle sequencing was carried out using the ABI PRISM™ BigDye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq™ DNA polymerase (Perkin Elmer, Branchburg, NJ) and analyzed on an ABI PRISM 310 Genetic Analyzer which utilizes the four-color sequencing chemistry.

PCR methods

The murine Sirt4 gene was partially sequenced by primer walking on both strands using a direct sequencing strategy [16]. Sequencing reactions were carried out using 0.6 μg cDNA and 20–30 mer oligonucleotide primers (Thermo Electron, Dreieich, Germany). Sequencing reactions were set up in a volume of 20 μl containing 10 pmol of the sequencing primer, 4 μl BigDye (Perkin Elmer, Norwalk, CT), DNA as indicated and ddH_2O, which was added up to a final total volume of 20 μl. The thermal cycling profile for the sequencing of the cDNA-clones was published earlier [16].

Chromosomal localization by fluorescence in situ hybridization (FISH)

Cell culture and preparation of chromosomes. Standard chromosome preparations were used from a mouse embryonic fibroblast cell line.

Slide preparation. For the removal of excess of cytoplasm, slides were treated with pepsin (0.5 mg/ml in 0.01 M HCl, pH 2.0) at 37 °C for 40 min. Slides where then washed in 1xPBS for 10 min at room temperature, which was then followed by a series of ethanol washes (70%, 90% and 100%) and air drying. BAC genomic clone RP23-189B10, which was shown to contain the murine Sirt4 gene, was used as a probe.

Probe labeling. BAC DNA was labeled by standard nick translation. Digoxigenin (Roche Diagnostics) was used as labeled dUTP at a concentration of 40 μM. Probe length was assessed on a 1% agarose gel. The probe showed the optimal average length of approximately 300 bp after nick translation.

Hybridization and probe detection. About 50 ng DNA were pooled together with 2 μg cot-1 in 10 μl hybridization buffer (50% formamide, 2xSSC, 10% dextransulfate). The DNA was applied to chromosomes fixed on a slide, mounted with a cover slip and sealed with rubber cement. Probe DNA and chromosomes were denatured together at 72 °C for 3 min. Hybridization was then carried out over night at 37 °C in a wet chamber. The cover slip was then carefully removed and the slides were washed in 2xSSC for 8 min and subsequently incubated at 70 °C in 0.4xSSC/0.1% Tween for 1 min. After equilibration in 4xSSC/0.1% Tween for 5 min the rhodamine coupled antibody was added in a dilution of 1:400. Incubation was for 45 min at 37 °C. The slide was then washed twice in 4xSSC/0.1% Tween for 10 min at 45 °C followed by staining in DAPI (4′,6-Diamidino-2-phenylindole) for 10 min. For microscopy, the slide was mounted in antifade solution (Vectashield).

Microscopy. In situ hybridization signals were analyzed on a Zeiss Axioplan II microscope. Each image plain (blue and orange) was recorded separately with a b/w CCD camera. Chromosomes and FISH signals were then displayed in false colors and images were merged on a computer. Camera control, image capture and merging were done with SmartCapture X software (Digital Scientific, Cambridge, UK).

Sequence analysis and computer database searches

DNA sequence analysis was carried out on the HUSAR (Heidelberg Unix Sequence Analysis Resources) server hosted by the Biocomputing Service Group at the German Cancer Research Center (DKFZ, Heidelberg) and the UniGene and LocusLink programs at the National Center for Biotechnology Information (NCBI). For sequence comparisons the BLAST algorithm of the GenBank and EMBL databases [17] was used. Protein similarity analyses were calculated with the BLAST algorithm at NCBI [18]. Protein motifs were identified online with the PROSITE program at the ExPASy proteomics server of the Swiss Institute of Bioinformatics (SIB) and double-checked with the MotifFinder program hosted by the GenomeNet server at Institute for Chemical Research, Kyoto University (Japan), but still remain to be confirmed experimentally. Potential transcription factor binding sites were identified with the TRANSFAC program (GenomeNet Computation Service as above). Repetitive elements were identified on the Repeat Masker Server at the University of Washington and CpG elements were found with the CPG software hosted by the European Bioinformatics Institute (EMBL outstation) (Figs. 1 and 2).

Phylogenetic analyses

Phylogenetic trees were constructed from the known SIRT4 protein sequences, which were obtained from a protein sequence similarity search with the murine SIRT4 protein sequence using the BLAST 2.0 program at NCBI database (Non-redundant GenBank CDS: translations + PDB + SwissProt + SPupdate + PIR) and compared to the yeast SIR2 protein (GenPept P06700). Progressive multiple sequence alignments were performed with the CLUSTAL W Multiple Alignment Program Version 1.7 [19]. Trees were calculated and drawn with the PATH (phylogenetic analysis task in HUSAR) software, which estimates and realizes phylogenies by executing the three main phylogenetic methods: distance, parsimony and maximum likelihood and which is hosted by the HUSAR (Heidelberg Unix Sequence Analysis Resources) server from the Biocomputing Service Group at the German Cancer Research Center (DKFZ, Heidelberg) (Fig. 4).

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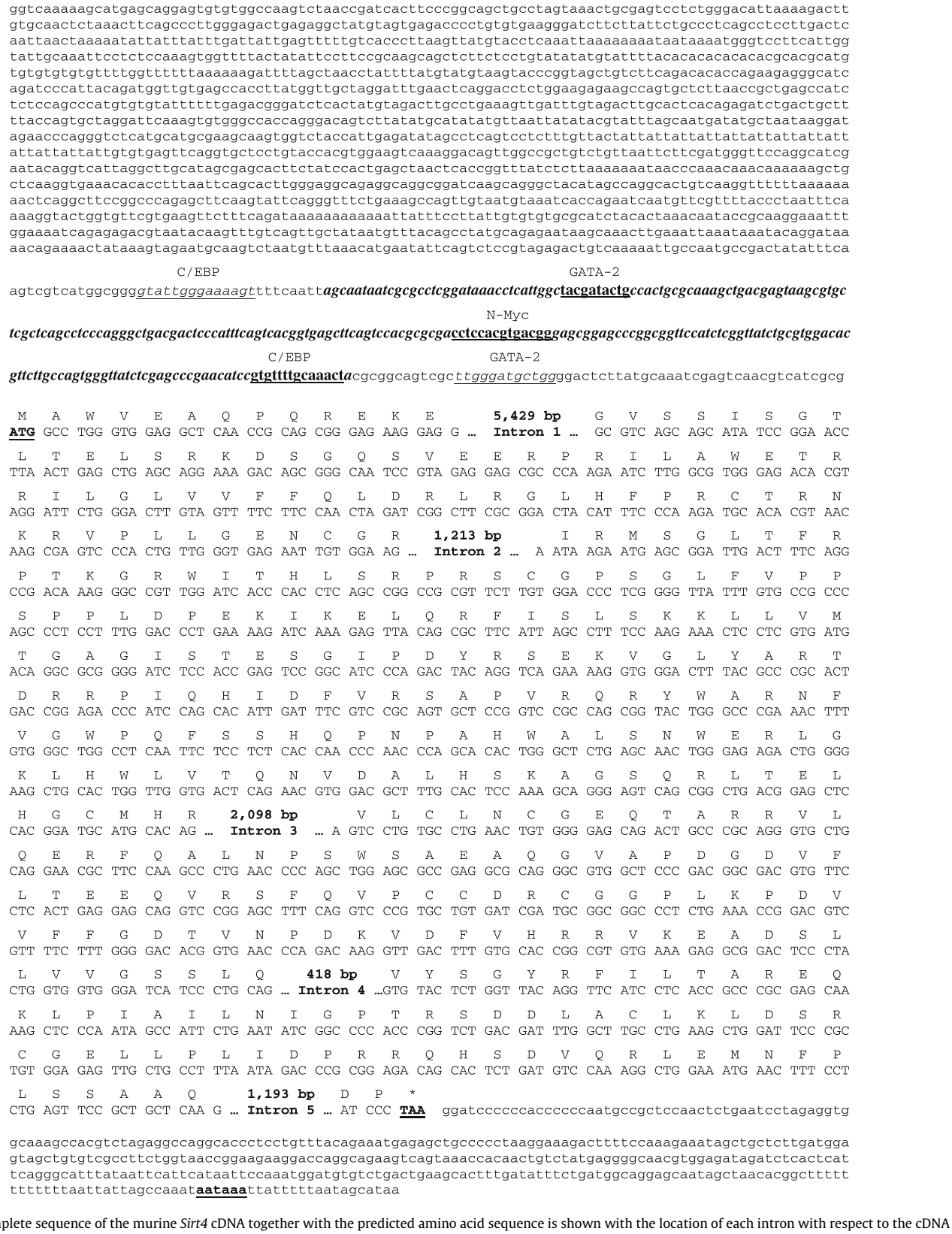

Fig. 1. The complete sequence of the murine *Sirt4* cDNA together with the predicted amino acid sequence is shown with the location of each intron with respect to the cDNA sequence. The 1648 bp murine *Sirt4* mRNA has an open reading frame of 1254 bp, which yields a 418 aa protein and an untranslated 3′ flanking region, which is 367 bp long (distance from the translational termination codon to the polyadenylation signal). About 1.9 kb of the 5′ upstream promoter region are indicated 5′ upstream of the translational start codon. Putative transcription factor binding sites are underlined. A CpG island that measures 247 bp in length (pos. −58 to −305), is shown in fat lower case italics. The translational start (ATG) and stop codons (TAA) are marked fat and underlined, the polyadenylation signal (aataaa) is also shown in fat lowercase and underlined letters.

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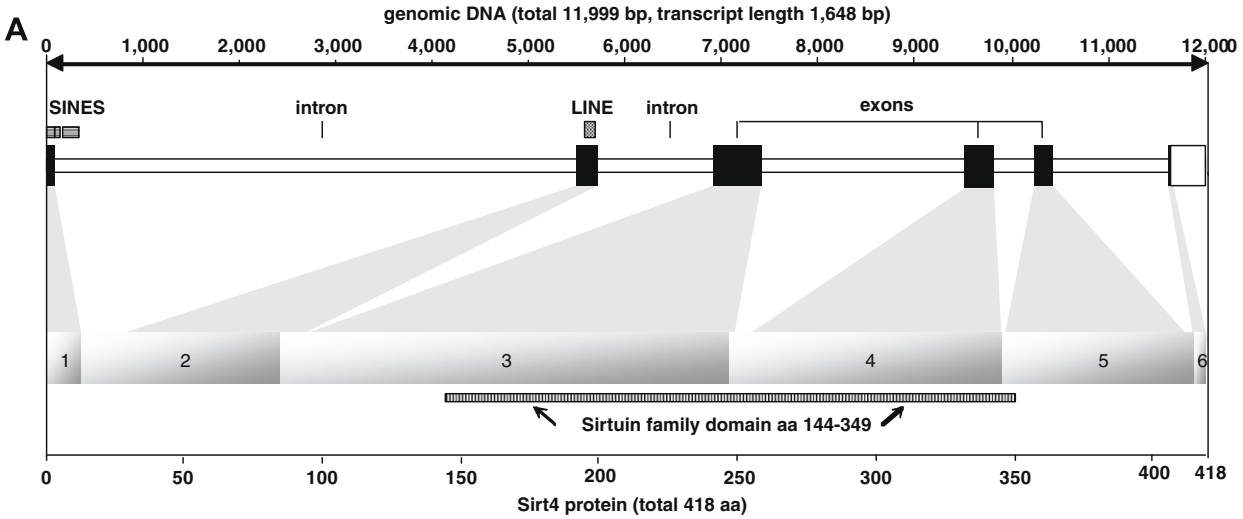

B

Exon no.	Exon size	5´-Splice donor	Intron no.	Intron size	3´-Splice acceptor
1	40	GAGAAGGAGG**gt**aagccagaag	1	5429	tcattgcccc**ag**GCGTCAGCAG
2	208	ATTGTGGAAG**gt**aagtgttctg	2	1213	ctttctctcc**ag**AATAAGAATG
3	495	GCATGCACAG**gt**gcagaagtct	3	2098	atttgtcttt**ag**AGTCCTGTGC
4	295	ATCCCTGCAG**gt**acctgactct	4	418	ttgcctgggc**ag**GTGTACTCTG
5	211	GCTGCTCAAG**gt**aaagagaaag	5	1193	atttcttttc**ag**ATCCC**TAA**GG
6	8 (399)				

Fig. 2. (A) Genomic organization of the murine *Sirt4* gene. Repetitive sequences (SINEs and LINEs) are indicated. The sirtuin catalytic domain overlaps the SIRT1 protein region that is encoded by exons 3 and 4. (B) Exon/Intron splice junctions of the murine *Sirt4* gene: exon sequences are given in uppercase and intron sequences in lowercase letters. Exon/Intron sizes are indicated.

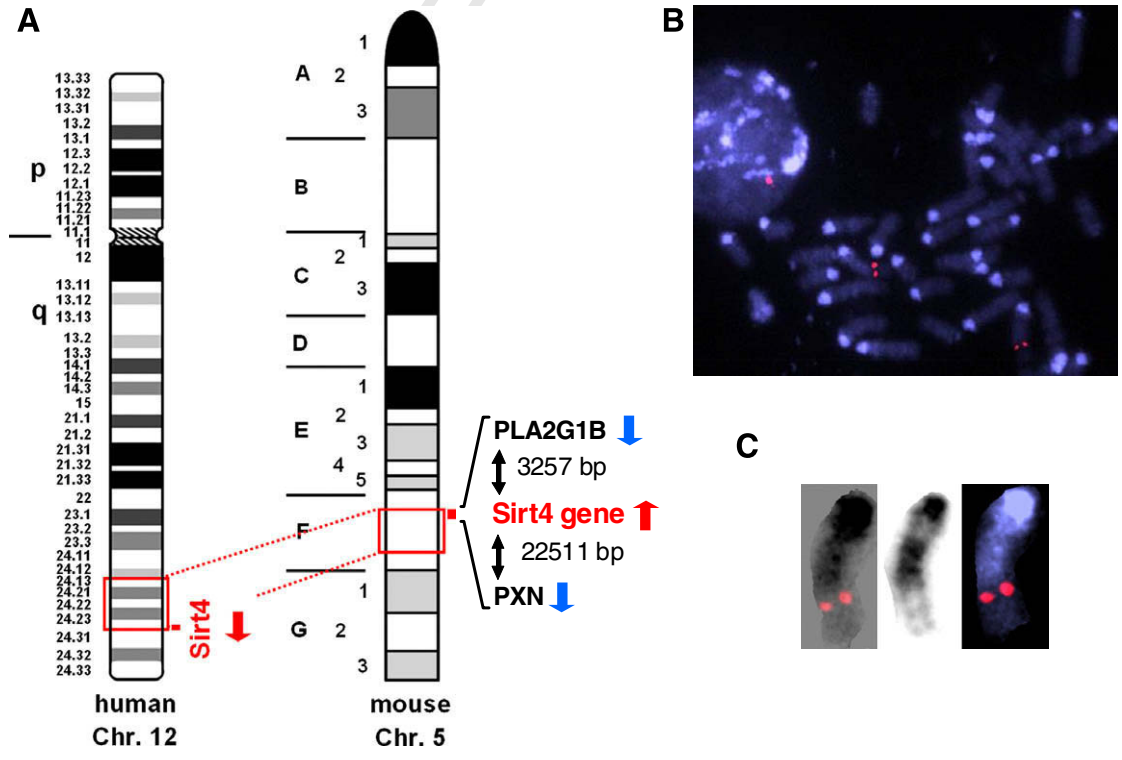

Fig. 3. Chromosomal mapping of the murine *Sirt4* gene to mouse chromosome 5F. (A) mouse and human chromosomal idiograms showing *Sirt4* within autosomal gene synteny groups (mouse *Sirt4*: Chr. 5F; human *Sirt4*: Chr. 12q24.23). Mouse *Sirt4* is located in close neighborhood to the PLA2G1B and PXN genes. (B/C) Lower right panel: Fluorescence *in situ* hybridization of *RP23-189B10* to mouse chromosome 5F.

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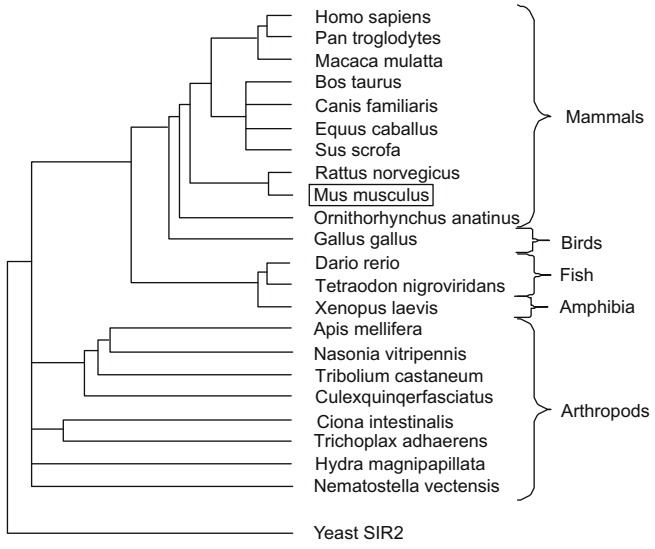

Fig. 4. Consensus evolutionary tree on the basis of an alignment of SIRT4 from different species together with their common ancestor protein, yeast SIR2.

Results

Identification of cDNAs encoding murine Sirt4

Homology searches of the EST database at NCBI (National Center for Biotechnology Information) with the murine SIRT4 protein sequence (GenPept XP_993153), yielded 4 murine mRNA sequences of variable length (GenBank clones XM_988059: 1648 bp; AK016400: 1639 bp; BC022653: 1460 bp and XM_485674: 1900 bp), of which mRNA XM_485674 was predicted to yield a 502 aa protein, which has however never been detected in the literature that is available so far. Sequence XM_988059, which has been reported to yield a 418 aa protein [12], was the correct full-length murine Sirt4 mRNA and was therefore used for the identification of the murine Sirt4 genomic clone. The authenticity of its insert was confirmed by DNA cycle sequencing. Sequences flanking the 5′ and 3′ ends of the Sirt4 open reading frame were identified from the *Sirt4* murine genomic clone BAC *RP23-189B10*. Characterization of the 5′ flanking genomic region, which precedes the *Sirt4* open reading frame, revealed a number of putative optimal transcription factor binding sites for C/EBP and GATA in addition to a small 247 bp CpG island (pos. −58 to −305). However, their biological relevance remains still to be investigated experimentally. The 1648 bp murine Sirt4 transcript has an open reading frame of 1254 bp, which encodes a 418 aa protein with a predictive molecular weight of 47.3 kDa and an isoelectric point of 9.53. Fluorescence *in situ* hybridization analysis localized the murine *Sirt4* gene to chromosome 5F. The murine *Sirt4* gene is neighbored by the *PLA2G1B* and *PXN* genes. Translational stop codons in all reading frames precede the murine *Sirt4* open reading frame. The 3′ flanking region was shown to contain the eukaryotic polyadenylation consensus signal AATAAA 367 bp downstream of the termination of translation signal TAA (Fig. 1).

Identification and characterization of the murine Sirt4 genomic locus

The murine Sirt4 genomic clone was obtained from an arrayed mouse BAC genomic library (RZPD, Berlin, Germany) after *in silico* screening with the murine *Sirt4* cDNA (GenBank clone XM_988059), which was shown to contain the full-length murine *Sirt4* cDNA sequence. BAC clone *RP23-189B10* was identified to contain inserts with an average size of approximately 120 kb in the 11.6 kb vector pBACe3.6, which included the murine *Sirt4* genomic sequence. BAC genomic DNA was prepared according to published protocols [15] and the *Sirt4* insert was confirmed by cycle sequencing [16]. Genomic sequence comparison analyses with the BLAST algorithm helped us to identify mouse chromosome 5 genomic contig GenBank NT_078458, which is part of the largely finished reference sequence (C57BL/6 J) that contains small amounts of WGS and HTGS Draft sequence and was assembled by NCBI in consultation with the Mouse Genome Sequencing Consortium. We have used this sequence for the determination of Sirt4 introns and exon/intron boundaries (Fig. 2). The murine *Sirt4* gene spans a region of 11,999 bp (Fig. 2). Determination of the exon-intron splice junctions established that SIRT4 is encoded by 6 exons ranging in size from 40 bp (exon 1) to 495 bp (exon 3). Particularly within intron 1, we identified an accumulation of interspersed repetitive elements, SINEs (short interspersed nuclear elements) and within exon 2 a significant LINE (long interspersed nuclear element) (Fig. 2) [20]. We were not able to identify any STS or RH markers within the *Sirt4* genomic sequence or its close neighborhood. The sirtuin catalytic domain, which is highly conserved in all members of mammalian sirtuins that have been described so far as well as in their Sir2 yeast ancestor protein, is found between amino acid residues 144–349, i.e. within exons 3 and 4 of the protein (Fig. 2).

Murine Sirt4 is a single-copy gene

Our fluorescence in situ hybridization studies identified *Sirt4* on mouse chromosome 5F. These analyses identified one single site of hybridization of *Sirt4* on murine metaphase chromosomes and its specific localization on chromosome 5F (Fig. 3). *Sirt4* is therefore located within autosomal gene synteny groups in mouse and man (Fig. 3).

Sirt4 expression analyses

In silico expression profile analyses have been carried out with the UniGene EST profile viewer, which is hosted by the NCBI homepage and which suggested the strongest expression of murine *Sirt4* in the heart, followed by sympathetic ganglia, the spinal cord, the fertilized ovum, the thymus, testis, connective tissue and the lung on the basis of an analysis of EST counts.

Phylogenetic analyses and pairwise sequence comparisons

We have screened the non-redundant protein sequence (nr) database (NCBI) with the mouse SIRT4 protein sequence and identified SIRT4 in different species. Phylogenetic trees were constructed from the following SIRT4 protein sequences: Apis mellifera (GenPept XP_623654), Bos taurus (GenPept NP_001069253), Canis familiaris (GenPept XP_851150), Ciona intestinalis (GenPept XP_002125010), Culex quinquefasciatus (GenPept XP_001843271), Dario rerio (GenPept NP_001005988), Equus caballus (GenPept XP_001488758), Gallus gallus (GenPept XP_415273), Homo sapiens (GenPept NP_036372), Hydra magnipapillata (GenPept XP_002163018), Macaca mulatta (GenPept XP_001115744), Mus musculus (GenPept XP_993153), Nasonia vitripennis (GenPept XP_001162415), Nematostella vectensis (GenPept XP_001627929), Ornithorhynchus anatinus (GenPept XP_001517990), Pan troglodytes (GenPept XP_001160214), Rattus norvegicus (GenPept NP_001100617), Sus scrofa (GenPept NP_001107742), Tetraodon nigroviridans (GenPept CAG06926), Tribolium castaneum (GenPept XP_972967), Trichoplax adhaerens (GenPept XP_002114969), Xenopus laevis (GenPept NP_001084634) and compared to the yeast SIR2 protein (GenPept P06700). Progressive multiple sequence alignments were performed with the CLUSTAL W Multiple Alignment

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Program Version 1.7 [19]. Trees were calculated and drawn with the PATH (phylogenetic analysis task in HUSAR) software, which estimates and realizes phylogenies by executing the three main phylogenetic methods: distance, parsimony and maximum likelihood and which is hosted by the HUSAR (Heidelberg Unix Sequence Analysis Resources) server from the Biocomputing Service Group at the German Cancer Research Center (DKFZ, Heidelberg) (Fig. 4).

Discussion

The sirtuins, also referred to as the class III NAD^+-dependent histone deacetylases (HDACs), comprise seven homologs (SIRT1-7) of the yeast Sir2 protein. They are known to catalyze NAD^+-dependent histone deacetylation and to produce nicotinamide and 2′-*O*-acetyl-ADP-ribose. The conservation of the sirtuins throughout evolution and their extraordinary dependence upon NAD^+ consumption to regulate an increasing list of cellular processes implies that this class of HDACs may connect transcriptional control with energy homeostasis [21]. Even though the sirtuins were initially characterized primarily as histone deacetylases controlling transcription, they increasingly turn out to be also key elements in the regulation of metabolic processes [22]. Sirtuin-mediated control of metabolism takes place in the most different tissues and involves numerous biological pathways: while in white adipose tissue SIRT1 decreases PPARγ activity and mobilizes free fatty acids [23], it represses UCP2 (Uncoupling protein 2) and thus increases glucose-stimulated insulin secretion in pancreatic β cells [24,25]. In the liver, SIRT1 controls gluconeogenesis via deacetylation and activation of PPARGC1A (peroxisome proliferator-activated receptor-gamma, coactivator 1, synonymous: PGC-1α), which regulates oxidative phosphorylation and energy homeostasis in mitochondria [26].

The mammalian Sir2 homolog SIRT4 is a mitochondrial protein, which unlike other sirtuins and despite having a conserved sirtuin domain, lacks a deacetylase activity, but instead functions as an ADP-ribosyltransferase. ADP-ribosyltransferases transfer an ADP-ribosyl group from NAD^+ onto specific target proteins, an activity that is sensitive to inhibition by nicotinamide. In the mitochondria of pancreatic β cells SIRT4 directly interacts and represses the enzyme glutamate dehydrogenase (GDH) via ADP-ribosylation and thus inhibits the secretion of insulin in response to glucose and amino acids [9]. The finding that SIRT4 is involved in the regulation of insulin secretion in pancreatic β cells in response to glucose and amino acids is in accordance with the concept that sirtuins regulate the mitochondrial energy supply.

In the present study, we report the cloning, characterization and mapping of murine *Sirt4* on the genomic level. *Sirt4* is a single-copy gene in the mouse that spans a region of approximately 12 kb. The 1648 bp murine Sirt4 transcript encodes a 418 aa protein with a predictive molecular weight of 47.3 kDa and an isoelectric point of 9.53. Fluorescence *in situ* hybridization analysis identified a single genomic locus for murine *Sirt4* gene on mouse chromosome 5F.

Even though the knowledge on sirtuins is rapidly increasing, for most of the sirtuins a lot needs to be done in terms of characterization of their enzymatic activity, identification of substrates and regulatory functions. With respect to the translation of biological functions that have been described for SIRT4 into the pathogenesis of disease, it has been suggested that SIRT4 may protect from diabetes and aging. Therefore, a loss of SIRT4 could on the other hand expedite the pathogenesis of diabetes and cancer.

Acknowledgment

This work was supported by grants from the Deutsche José Carreras Leukämie-Stiftung e. V. (DJCLS R 05/11).

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