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Utility of syntenic relationships of VDAC1 pseudogenes for not only an understanding of the phylogenetic divergence history of rodents, but also ascertaining possible pseudogene candidates as genuine pseudogenes

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ABSTRACT

Rodent and human genomes were screened to identify pseudogenes of the type 1 voltage-dependent anion channel (VDAC1) in mitochondria. In addition to the 16 pseudogenes of rat VDAC1 identified in our recent study, 15 and 13 sequences were identified as pseudogenes of VDAC1 in mouse and human genome, respectively; and 4, 2, and 1 sequences, showing lower similarities with the VDAC1 sequence, were identified as "possible pseudogene candidates" in rat, mouse, and human, respectively. No syntenic combination was observed between rodent and human pseudogenes, but 2 and 1 possible pseudogene candidates of VDAC1 of rat and mouse, respectively, were found to have syntenic counterparts in mouse and rat genome, respectively; and these syntenic counterparts were genuine VDAC1 pseudogenes. Therefore, syntenic combinations of pseudogenes of VDAC1 were useful not only for a better understanding of the phylogenetic divergence history of rodents but also for ascertaining possible pseudogene candidates as genuine pseudogenes.

1. Introduction

Mitochondria have two membrane systems, i.e., the inner and outer mitochondrial membranes. Of these, the inner mitochondrial membrane shows high resistance against the permeation of solutes and ions to enable effective energy conversion, because the electrochemical gradient of H^+ across the inner mitochondrial membrane is used as a driving force for ATP synthesis. By contrast, the outer mitochondrial membrane is known to be highly permeable to various molecules. The voltage-dependent anion channel (VDAC), present in the outer mitochondrial membrane, is responsible for the free permeation of metabolites smaller than 5000 Da across this membrane; and 3 isoforms of VDAC, i.e., VDAC1, VDAC2, and VDAC3, are expressed in mammals [1–4].

Our previous study on the transcripts encoding rat VDAC isoforms indicated the possible existence of an mRNA showing structural similarity with rat VDAC1 mRNA (Ishida et al., unpublished). We assumed this unexpected mRNA would have been formed by transcription of this VDAC1 pseudogene, and so we explored pseudogenes of VDAC1 in the rat genome. As a result, 16 rat genomic segments showed structural similarity with the mRNA of rat VDAC1 [5]. Further characterization revealed that they are processed pseudogenes of VDAC1, and 8 of them are slightly expressed in certain tissues such as brain and testis [5].

The possible presence of pseudogenes of mouse and human VDAC was reported earlier [6–8], but their detailed characterization has not yet been achieved. To obtain a clue as to how and when pseudogenes of VDAC were formed in mammals, comparison of pseudogenes of VDAC among mammalian genomes would seem to be an effective strategy. Thus, in the present study we characterized pseudogenes of rat, mouse, and human VDAC1.

2. Methods

2.1. Identification of candidate pseudogenes of VDAC1

Identification of candidate pseudogenes of VDAC1 was performed as described by Zhang et al. [9,10]. Briefly, at the web page of BLAST in NCBI (http://blast.ncbi.nlm.nih.gov), we first chose a species (rat, mouse or human) whose genome was to be searched. After having entered the accession no. of the amino acid sequence of rat, mouse or human VDAC1 (NP_112643, NP_035824, or NP_003365, respectively), we screened the genome database of the target species "Genome (reference assembly scaffolds)" with the "TBLASTN program (search translated nucleotide database using a protein query)." Algorithm parameters of an expected threshold of "0.0001" and word size of "2" and a filter of "low complexity regions" were employed. We carried out this screening

Abbreviations: VDAC, voltage-dependent anion channel.

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at the end of April 2013. (This statement is necessary because the database is updated frequently.)

2.2. Synteny analysis

Synteny analysis was performed as described previously [11,12].

3. Results

3.1. Identification of "possible pseudogene candidates of rat VDAC1"

In our previous study on the identification of pseudogenes of VDAC1 in the rat genome [5], the database of the rat genome was screened with the BLASTN program, essentially as described in the Methods section; and we obtained 34 BLAST hits. Of these hits, 14 sequences were excluded from being pseudogene candidates of VDAC1, because 3 of the sequences were those of genuine genes encoding 3 rat VDAC isoforms, 9 of them were more similar to VDAC2 or VDAC3 than to VDAC1, and 2 sequences showed poor structural similarity. Finally, the remaining 20 sequences were further inspected with the criterion of "whether these sequences could encode a continuous amino acid sequence of rat VDAC1 longer than 11 residues;" and 4 of them were excluded from the pseudogenes of VDAC1 by this criterion. These results are re-summarized in Table 1. Because the number of sequences eliminated by the final screening stage (4 sequences) was not remarkable, in the present study we handled these 4 sequences as "possible pseudogene candidates of rat VDAC1" in a manner distinguished from the 16 pseudogenes identified in our previous study. As these sequences do not have their specific gene symbols, individual sequences are tentatively referred to as "BLAST hit Rn" in the present manuscript (see Table 2, lines "rat"). When the structural features of these 4 sequences of possible pseudogene candidates of rat VDAC1 were examined, all of the candidates were found to show markedly split structures comprising 5 to 7 segments (see Supplementary Fig. 1 and Table 2, lines "rat"), possibly indicating that these pseudogenes had been massively rearranged after their formation by retrotransposition. In addition, one possible pseudogene candidate of rat VDAC1, referred to as BLAST hit R25, was found to be located at the 5th intron of the type II inositol polyphosphate-4-phosphatase gene, Inpp4b, as shown in Table 2, column "Intron."

3.2. Identification of VDAC1 pseudogenes of mouse and human

In the present study, we further examined the features of mouse and human VDAC1 pseudogenes. When mouse and human genomes were screened with the amino acid sequence of their respective VDAC1, 27 and 26 BLAST hits, respectively, were obtained. Likewise, as in the case of the analysis of rat pseudogenes, these sequences were classified into individual groups as summarized in Table 1; and we identified 15 and 13 pseudogenes of mouse and human VDAC1, respectively. When we loosened the screening conditions for pseudogenes by omitting the use of the criterion "whether these sequences could encode a continuous amino acid sequence of target protein longer than 11 residues," we identified 2 and 1 sequences in the mouse and human genome, respectively, and referred to them as "possible pseudogene candidates of VDAC1." It should be noted that none of the pseudogenes or pseudogene candidates identified in the rodent or human genome retained intron/exon boundaries of genuine genes encoding VDAC1, thus indicating that these sequences had been formed by retrotransposition of mRNA encoding VDAC1. In addition, likewise as in the case of BLAST hit R25 of the rat pseudogene, 3 mouse pseudogenes (Gm16102, BLAST hit M24, and Gm17072) and 6 human pseudogenes (VDAC1P4, LOC100420568, VDAC1P7, VDAC1P11, VDAC1P3, and VDAC1P6) were found in the intron of certain genes (see column "Intron" in Table 2).

3.3. Features of pseudogenes of mouse VDAC1

As for the pseudogenes (and possible pseudogene candidates) of mouse VDAC1, there was no pseudogene precisely or largely retaining the original structure of the entire cDNA (i.e., complete or semi-complete pseudogene, respectively, according to the classification used in our previous study [5]), and all of them showed characteristic structures of having been split into multiple segments, from 3 (Gm7910 and Gm6008) to 12 (Gm2988) pieces, possibly reflecting the occurrence of genome rearrangements after retrotransposition of the mRNA encoding VDAC1 (see Table 2, **lines "mouse"** and Supplementary Fig. 2). Furthermore, pseudogenes and possible pseudogene candidates of mouse VDAC1 showed two additional features (see Supplementary Fig. 2). First, pseudogenes Gm13758 and Gm6008 each had a relatively long (6.93 and 4.11 kbp, respectively) irrelevant DNA sequence between pseudogene segments, also supporting the occurrence of genome rearrangements. The second feature was the presence of twin pseudogenes having a completely identical structure (Gm5379 and Gm16480), and a pseudogene showing high structural similarity with these two (Gm16479). These twins may have been formed from one of them as a common ancestor by gene duplication, because they are closely located on the same chromosome. The rate of locus-specific gene duplication in mammals has been reported to be 10^{-5} to 10^{-6}/gene/generation [13]. However, further discussion on the questions as to when and how these twin pseudogenes were formed is difficult at this stage of our study.

3.4. Features of pseudogenes of human VDAC1

As for the pseudogenes of human VDAC1, of the 13 sequences identified in the present study, 12 of them were already assigned as pseudogenes of VDAC1 with names of VDAC1P1 to VDAC1P12. In agreement with the HUGO Gene Nomenclature Committee (HGNC), theremaining 1 sequence, LOC100420568, has been named VDAC1P13. In the aspect of the structural features of these pseudogenes of human VDAC1 (for details, see Table 2, **lines "human"** and Supplementary Fig. 3), the following two points are worthwhile to note: First, all pseudogenes lacked the 5′ end region of their cDNA and started with nucleotide ~240. The exact reason for this characteristic structure is uncertain, but a possible explanation is as follows: The gene encoding human VDAC1 consists of 9 exons, and the first one is a non-coding exon with a length of 239 bps. Possibly, all of these pseudogenes of human

Table 1
Numbers of DNA segments showing structural similarities with cDNA of VDAC1 in individual species.

		Rat	Mouse	Human
Number of total BLAST hits		34	27	26
BLAST hits indicating	Genuine genes of VDAC1, 2, 3	3	3	3
	Sequences more similar to VDAC2 or 3 than to VDAC1	9	7	8
	Sequences showing poor structural similarity with VDAC1	2	0	1
	Pseudogene of VDAC1	16	15	13
	Possible pseudogene candidates of VDAC1	4	2	1

Table 2
Features of pseudogenes and possible pseudogene candidates of rat, human, and mouse VDAC1.

Animal species	Gene name	Chromosome	Intron	Strand	Split	Length
Rat	BLAST hit R26	2q24		−	5	35.0
	BLAST hit R13[a]	2q45		+	7	55.2
	BLAST hit R33	3q23		−	6	40.4
	BLAST hit R25[b]	19q11	5th intron of Inpp4b	−	6	22.2
Mouse	Vdac1-ps1 (Gm7910)	1B		+	3	79.8
	Vdac1-ps2 (Gm16102)	1C1.1	1st intron of Glup1	+	6	83.4
	Vdac1-ps3 (Gm13360)	2A3		−	5	96.4
	Vdac1-ps4 (Gm13758)	2E1		+	6	87.1
	Vdac1-ps5 (Gm2988[a])Gm2988[a]	3H4		−	12	51.2
	BLAST hit M24	5G2	11th intron of Cyp3a13	−	4	54.0
	Vdac1-ps6 (Gm8459)	6F1		−	5	80.4
	Vdac1-ps7 (Gm6008)	7F1		−	3	72.3
	Vdac1-ps8 (Gm7506)	8A1.1		−	6	87.3
	Vdac1-ps9 (Gm7319)	8A4		+	4	85.3
	Vdac1-ps10 (Gm17072[b])	8C2	5th intron of Inpp4b	+	8	55.1
	Vdac1-ps11 (Gm16479)	XA1.1		+	5	53.4
	Vdac1-ps13 (Gm5379)	XA1.1		−	4	44.5
	Vdac1-ps12 (Gm16480)	XA1.1		−	4	44.5
	Vdac1-ps14 (Gm15132)	XF3		+	5	80.1
	Vdac1-ps15 (Gm13655)	2C3		−	8	39.6
	Vdac1-ps16 (Gm18656[c])	9A4		+	5	19.5
Human	VDAC1P9	1q23		−	6	55.4
	VDAC1P4	1q24-q25	3rd intron of ACBD6	+	3	85.9
	VDAC1P10	1q41		+	3	82.1
	VDAC1P13	2p21	1st intron of KCNG3	−	3	69.6
	VDAC1P7	3p12	3rd intron of ROBO2	−	1	86.1
	VDAC1P8	6q24		+	1	87.8
	VDAC1P11	9q22	2nd intron of ZNF169	−	2	86.3
	VDAC1P5	12q13		−	1	86.4
	VDAC1P12	13q12		−	4	84.9
	VDAC1P2	Xp11		−	1	86.9
	VDAC1P1	Xq21		+	1	86.6
	VDAC1P3	Xq21	2nd intron of PCDH11X	−	1	85.6
	VDAC1P6	Yp11	2nd intron of PCDH11Y	−	1	86.4
	LOC644169	11p15		−	5	83.4

As stated in the text, pseudogenes were identified by several screening steps, and sequences that could encode a continuous amino acid sequence of VDAC1 longer than 11 residues were classified as "pseudogenes of VDAC1", and those that failed to encode a continuous amino acid sequence of VDAC1 longer than 11 residues were classified as "possible pseudogene candidates of VDAC1." As for the rat, 16 sequences were identified as pseudogenes of VDAC1 as reported previously [5], and they are not listed in this table. However, the 4 sequences identified as possible pseudogene candidates of VDAC1 are listed. As for the mouse and human, 15 and 13 sequences, respectively, were identified as pseudogenes of VDAC1; and 2 and 1 sequence, respectively, were identified as possible pseudogene candidates of VDAC1. The broken line in the table distinguishes the pseudogenes (above the line) and possible pseudogene candidates (below the line). The gene names of mouse are shown by two ways, like Vdac1-ps1 (Gm7910). The former starting with Vdac1 is the new gene symbols, certificated by the Mouse Genomic Nomenclature Committee (MGNC), and the latter shown in the parenthesis is old symbols found in the NCBI database.
The column "Chromosome" represents the chromosomal localization of the individual pseudogenes (or possible pseudogene candidates). If the target sequence was found in the inside of the other gene, this information is stated in the column "Intron." The columns of "Split" and "Length" represent numbers of split DNA segments and length of the nucleotide sequence corresponding to the cDNA of VDAC1 relative to the full length of the cDNA, respectively.
The superscripts "a"–"c" represent the 3 combinations of pseudogenes showing syntenic relationship between mouse and rat. The rat counterpart of the mouse pseudogene Gm18656, RGD1562882, is not shown in this table, because it was identified as one of the pseudogenes of rat VDAC1 in our previous study [5].

VDAC1 were formed by retrotransposition of the mRNA starting with the second exon. It is noteworthy that all sequences retained the 5′ end region of the open reading frame (i.e., protein-coding sequence) of the mRNA; and 8 sequences retained the entire region of the open reading frame, but none of them encoded the whole VDAC1 protein due to mutations. Thus, even if they were transcribed, their transcript could not function as an mRNA of the VDAC1 protein. The second point is that, although all of the pseudogenes lacked their 5′ end, half of the identified pseudogenes and the candidate one, i.e., 7 sequences, retained most of the entire structure of the cDNA. Possibly, these "non destructured" pseudogenes had been recently formed.

3.5. Synteny analysis of pseudogenes of rat, mouse, and human VDAC1

The most intriguing question regarding studies on pseudogenes is how and when they are formed during the process of evolution. To obtain possible clues to answer this question, we further conducted synteny analysis of individual pseudogenes. No syntenic combination was observed with pseudogenes of VDAC1 between human and rodent, but 3 combinations of pseudogenes of VDAC1 were found to show synteny between mouse and rat. Apparently, these syntenic pseudogenes would have been formed by retrotransposition of the mRNA encoding VDAC1 before developmental divergence between mouse and rat. These combinations were a) Gm2988 of mouse and BLAST hit R13 of rat, b) Gm17072 of mouse and BLAST hit R25 of rat, and c) Gm18656 of mouse and RGD1562882 of rat [5]. These pseudogenes or possible pseudogene candidates of VDAC1 in Table 2 were highlighted with superscripts "a"–"c", and their chromosomal localizations are depicted in Fig. 1. Interestingly, one member of the individual combinations (BLAST hits R13, R25 of rat and Gm18656 of mouse) were judged as possible pseudogene candidates (i.e., they failed to encode a continuous amino acid sequence of mouse or rat VDAC1 longer than 11 residues), but their counterparts (Gm2988 and Gm17072 of mouse, and RGD1562882 of rat) were judged as genuine pseudogenes (i.e., they could encode a continuous amino acid sequence of mouse or rat VDAC1 longer than 11 residues). Because the presence of a syntenic counterpart as a genuine pseudogene could be strong supporting evidence, we concluded that these 3 possible pseudogene candidates having their syntenic counterpart were genuine pseudogenes. We also compared the structural features of the syntenic combinations of pseudogenes, as shown in Fig. 2 (note that all figures showing the structural features of individual pseudogenes are shown in Supplementary Figs. 1 and 2, and in Fig. 1 in our recent paper [5]; but to make structural

comparison easier, they are re-cited as this independent Fig. 2). The split manner of the nucleotide sequences showing homology with the nucleotide sequences of the cDNA encoding rat or mouse VDAC1 was poorly conserved between the combination of Gm2988 and R13, slightly conserved between that of Gm17072 and R25, and partially conserved between the combination of Gm18656 and RGD1562882. Possibly, the differences in the similarities of the split manner of the nucleotide sequence between the genomes of mouse and rat may reflect the time of the formation of the individual pseudogenes.

4. Discussion

In the present study, we sought to identify the pseudogenes of VDAC1 in rodent and human genomes. By the standard conditions

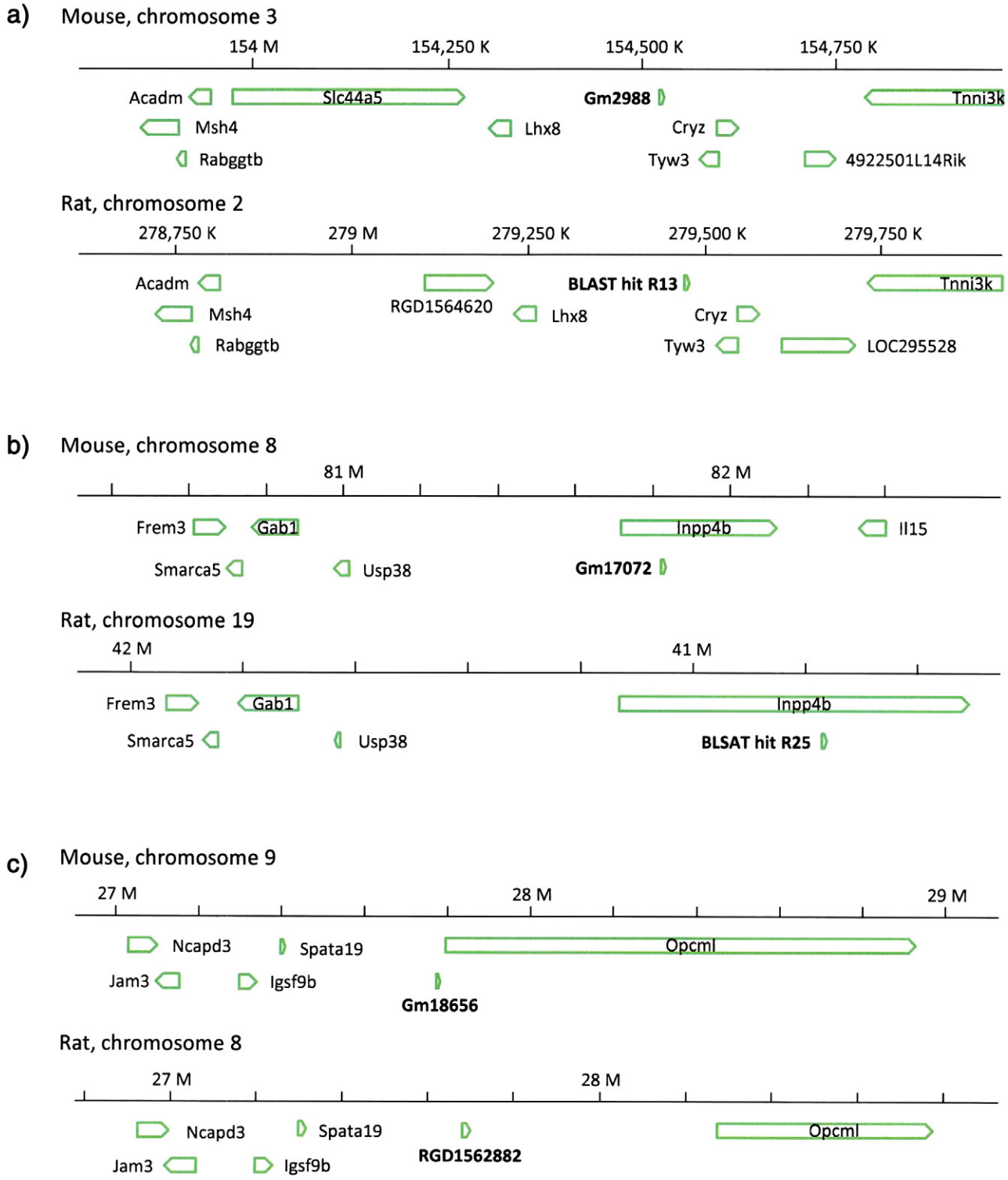

Fig. 1. Comparison of the chromosomal localizations of the 3 pseudogene combinations showing syntenic relationship between mouse and rat. The graphic displays of individual genomic annotations of the 3 syntenic combinations of mouse and rat pseudogenes (or possible pseudogene candidates) of a) Gm2988 and BLAST hit R13, b) Gm17072 and BLAST hit R25, and c) Gm18656 and RGD1562882 were obtained by use of NCBI tools. The nucleotide databases used to make the diagrams were NC_000069, NC_005101, NC_000074, NC_005118, NC_000075, and AC_000076 for Gm2988, BLAST hit R13, Gm17072, BLAST hit R25, Gm18656, and RGD1562882, respectively.

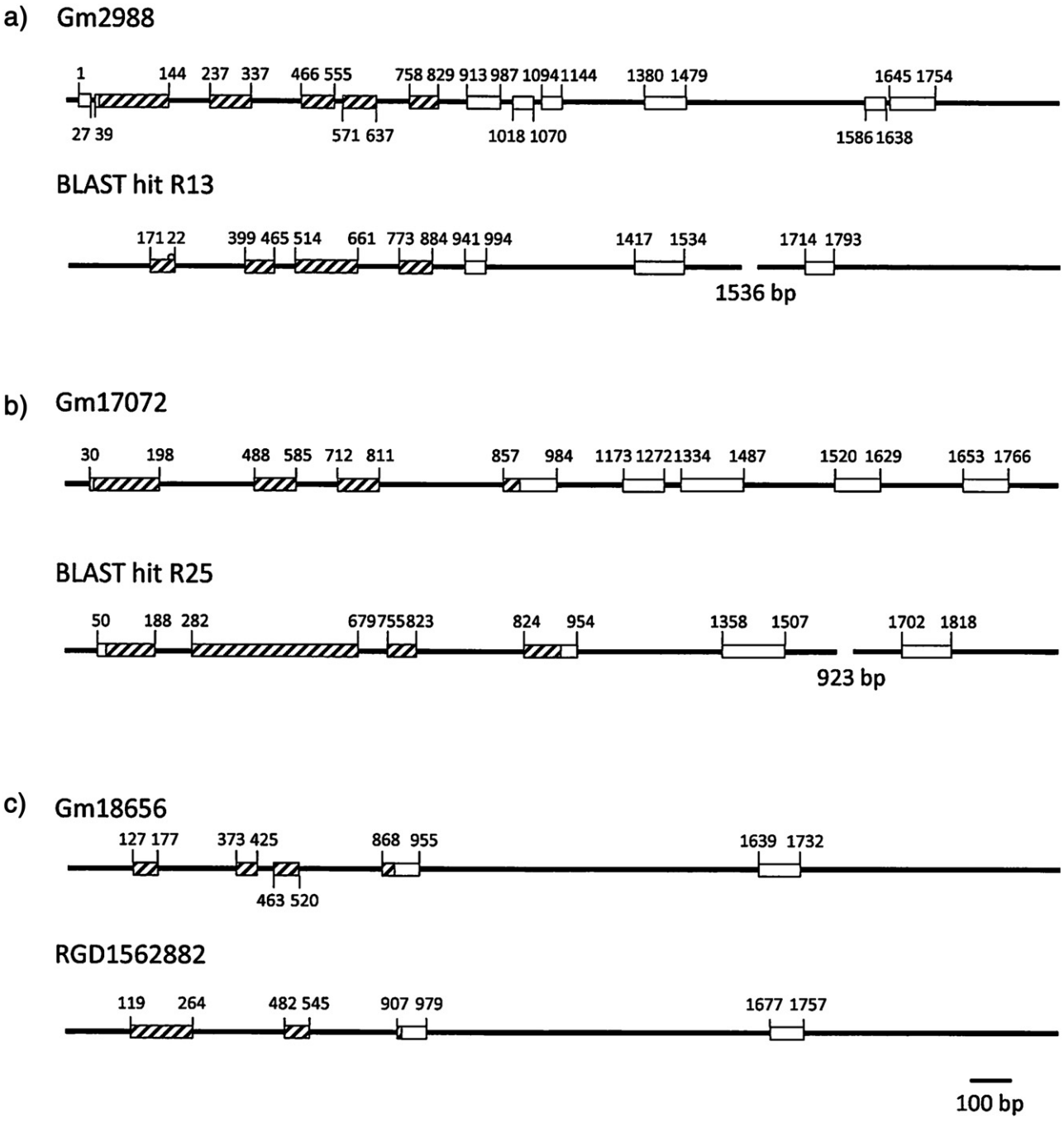

Fig. 2. Comparison of the structural properties of the pseudogenes showing syntenic relationship between mouse and rat. For comparisons of the structural properties of the pseudogenes showing syntenic relationship between mouse and rat, regions in the individual pseudogenes showing structural similarities with the nucleotide sequences of the cDNAs encoding mouse or rat VDAC1 are shown by open boxes; and those not relevant to this cDNA are shown by horizontal lines. The nucleotide regions encoded by individual DNA segments are numbered according to the numbering of nucleotides in the mRNAs of mouse or rat VDAC1 (NM_01169 and NM_03135, respectively). The DNA segments containing the nucleotide regions corresponding to the open reading frame of the cDNA are shown by hatched boxes.

used for the identification of pseudogenes, 15 and 13 pseudogenes of VDAC1 were identified in the mouse and human genome, respectively. By loosening the screening conditions for pseudogenes, we identified 4, 2, and 1 sequences in the rat, mouse and human genome, respectively, as possible pseudogene candidates of VDAC1. As expected, upon loosening the screening conditions, conservation of the original nucleotide sequences became poor. Therefore, it becomes difficult to be convinced that these possible pseudogene candidates of VDAC1 are genuine pseudogenes. Interestingly, however, synteny analysis revealed that two possible pseudogene candidates of rat VDAC1 and one possible pseudogene candidate of mouse VDAC1 had syntenic counterparts in the mouse and rat genome, respectively, indicating that these three possible pseudogene candidates of murine VDAC1 were genuine pseudogenes. Thus, syntenic combinations of pseudogenes of VDAC1 would be useful to ascertain the possible pseudogene candidates as pseudogenes.

In addition to the fact that VDAC plays important roles in the regulation of mitochondrial function and other biological processes in vivo, because multiple VDAC isoforms (paralogs) are present in various organisms, their phylogenetic history has been well studied [14–16]. In our present study, we discovered 3 syntenic pseudogene combinations of VDAC1 between mouse and rat genomes, which synteny was formed

before their phylogenetic divergence. The obtained results are also expected to be useful for a better understanding of the molecular evolution of the VDAC genes.

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