A novel gene suppressed in the ventricle of carnitine-deficient juvenile visceral steatosis mice

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Abstract In order to clarify the pathogenesis and pathophysiology of cardiac hypertrophy in carnitine-deficient juvenile visceral steatosis (JVS) mice, we performed mRNA differential display analysis with total RNA extracted from the ventricles of control and JVS mice at 14 days of age. We identified four up-regulated genes, two known and two unknown, and a novel down-regulated gene. Northern blot analysis with a novel cDNA probe derived from the down-regulated gene fragment 8A2 revealed three mRNA species of 1.1-, 1.3-, and 2.6-kb. The 1.1- and 1.3-kb mRNA species were found only in the heart, and the 2.6-kb species was found in the heart, kidney and brain, but not in skeletal muscle or liver. The 1.1- and 1.3-kb species were down-regulated in the ventricles of JVS mice, but not in the auricles, and increased to the control level with carnitine treatment. We isolated cDNA clones from ventricle RNA, termed CDV-1 (carnitine deficiency-associated gene expressed in ventricle) and from brain RNA, termed CDV-1R (CDV-1-related gene) by 5′- and 3′-RACE analyses. The entire nucleotide sequence except the 5′-terminal 64 bp of CDV-1 cDNA was completely identical to the 992 bp sequence from the 3′-end of CDV-1R cDNA. The CDV-1 cDNA contained an open reading frame predicting a peptide of 107 amino acids, which composed the C-terminal portion of CDV-1R peptide consisting of 414 amino acids.

Key words: Carnitine deficiency; Cardiac hypertrophy; Differential display; Gene expression; Juvenile visceral steatosis mouse

1. Introduction

Juvenile visceral steatosis (JVS) mice were described in 1988 by Koizumi et al. [1] as a novel animal model with a Reye-like syndrome suffering from fatty liver, hyperammonemia, hypoglycemia and growth retardation. We have reported the suppressed expression of the urea cycle enzyme genes in the liver of JVS mice to be a mechanism of hyperammonemia [2–6]. In addition, the most important finding in JVS mice study was that the mice were deficient in carnitine [7] and that all the symptoms disappeared after carnitine treatment [8]. The mice were defective in renal carnitine transport [9], and the *jvs* gene causing systemic carnitine deficiency was mapped to chromosome 11 [10]. As in the human counterpart, we found typical and geniune cardiac hypertrophy in JVS mice recognizable from 10 days of age and showed that the gene expression pattern in the hypertrophied JVS heart differed from that in pressure-loaded models [11,12].

To further characterize and define the pathogenesis and pathophysiology of the hypertrophy in JVS mice at the molecular level, we applied mRNA differential display analysis. Uenaka et al. [13] carried out a similar analysis on JVS mice and reported that carnitine palmitoyltransferase I (CPT-I) gene was expressed three to six times higher in the heart of JVS mice than in control. They reported that CPT-I gene was repressed to the normal level when the mice were treated with carnitine. In the present study, we report a novel gene, expressed as three species of mRNA, two of which were down-regulated in the ventricles of JVS mice.

2. Materials and methods

2.1. Animals

We used homozygous mutant (jvs/jvs) mice, with heterozygous (+/jvs) mice as the control. Heterozygous mice show no clinical manifestations and no difference in heart weight from homozygous control mice (+/+) [11,12]. The homozygous mutant and heterozygous control mice were produced as previously described [8,11,12]. In the therapeutic experiment, 5 μmol of L-carnitine were administered i.p. to JVS mice twice a day from 5 to 14 days after birth.

2.2. RNA preparation

Mice at 14 days were used, when JVS mice show significant increase in heart weight [12]. Tissues were taken from the mice under pentobarbital anesthesia (50 mg/kg of body weight). The heart was dissected transversely at the bottom of the auricles. The lower part was used as ventricular tissue. Total RNA was extracted as described by Chomczynski and Sacchi [14] and treated with DNase I using MessageClean kit (GenHunter).

2.3. Differential display

Differential display analysis was performed essentially as described previously [15,16] with the following modifications [17]. Three kinds of 3′-anchored oligo-$(dT)_{15}$ primers ($GT_{15}VG$, $GT_{15}VA$ or $GT_{15}VC$ in which V represented A, C, or G) were used for reverse transcription from total RNA and as the 3′-polymerase chain reaction (PCR) primer. The 5′-PCR primers were 20 kinds of arbitrary primer (Operon Technologies), each composed of 10 mer. The first-strand cDNA was amplified by PCR in 10 μl of final volume containing 0.1 unit of *Taq* polymerase, 1 nmol of dNTP (25 μM each), 4 pmol of 5′-PCR primer, 2.5 pmol of 3′-PCR primer, 1 μCi of [α-^{33}P]dCTP, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.3 under the following conditions; 94°C (3 min), 40°C (5 min), and 72°C (5 min) for the second-strand cDNA synthesis, followed by 30 cycles of 95°C (15 s), 40°C (2 min) and 72°C (1 min) for the amplification, and 72°C (5 min) for the additional final extention. As described previously [16], the labelled PCR products were separated by electrophoresis using 6% polyacryl-

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Abbreviations: JVS, juvenile visceral steatosis

The nucleotide sequences reported in this paper have been registered to the EMBL/GenBank/DDBJ data bank with accession numbers Y10945 (CDV-1R) and Y10496 (CDV-1).

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amide gels. The interesting DNA bands were re-amplified with the same primers as used in the initial reaction, subcloned into pCR II vector using the TA cloning kit (Invitrogen), sequenced with *ΔTaq* fluorescent dye-primer cycle sequencing kit or Thermo sequenase core sequencing kit (both from Amersham) and Texas red-labelled primers, and run on the Hitachi model SQ-5500 DNA auto-sequencer. We confirmed the cloned cDNA fragments to be derived from differentially expressed ones by Southern blot analysis of the originally amplified PCR products using the cloned fragments as a probe.

2.4. Northern blot analysis

Total RNA was analyzed by Northern blotting as previously described [8,12]. The cloned cDNA fragment 8A2 derived from the interesting band, which was down-regulated in the ventricle of JVS mice, was labelled with the 3′-anchored oligo-$(dT)_{15}$ primer, $GT_{15}VA$, in the presence of [α-^{32}P]dCTP and Klenow fragment of DNA polymerase, and used as a probe. The probe for β-actin used as an internal control was generated by labelling a genomic fragment of human β-actin with [α-^{32}P]dCTP and random prime DNA-labelling kit (Amersham).

2.5. cDNA cloning

To obtain the upstream nucleotide sequence of the novel gene transcript containing the sequence of cDNA fragment 8A2, the 5′-RACE analysis was carried out using the 5′-RACE System (Gibco BRL). In the first step, a first-strand cDNA was synthesized with total RNA prepared from ventricle and brain of control mice using fragment 8A2-specific primer, 5′-CGCAGGCTTTATTTACTTTG-3′, and SuperScript II. The first-strand cDNA was purified by using a GlassMAX spin cartridge, and then an anchor sequence was added to the 3′ end of the cDNA using terminal transferase and dCTP. In the second step, PCR amplification was performed using TaKaRa LA PCR kit (Takara Shuzo), a nested fragment 8A2-specific primer, 5′-GCAATCACATCACATCACAA-3′, and an anchor primer provided with the 5′-RACE System. The downstream nucleotide sequence of the novel gene transcript was confirmed by using the 3′-RACE System (Gibco BRL) and fragment 8A2-specific primer, 5′-CCAAATTAGAGCAGGAAGTG-3′. The 5′- and 3′-RACE products were subcloned into pCR II vector using the TA cloning kit and sequenced by using the Thermo sequenase core sequencing kit and the Hitachi SQ-5500 DNA auto-sequencer as described above.

3. Results and discussion

3.1. Differential display analysis of RNAs expressed in ventricles of control and JVS mice at 14 days of age identified a novel down-regulated gene in JVS mice

To identify differentially expressed genes in the hypertrophied ventricles of JVS mice, we compared mRNA expression

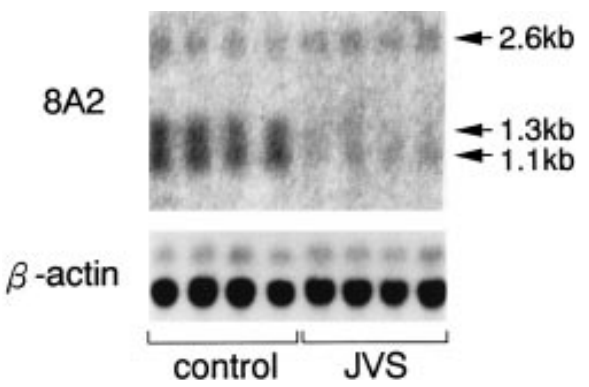

Fig. 1. Two down-regulated and a constitutive mRNA species in the ventricle of JVS mice were detected by Northern blot analysis with cDNA fragment 8A2. Total RNA (15 μg/lane) isolated from the ventricles of control (+/jvs) and JVS (jvs/jvs) mice at 14 days of age was analyzed by Northern blot hybridization with probes, 8A2 and β-actin, as described in Section 2.

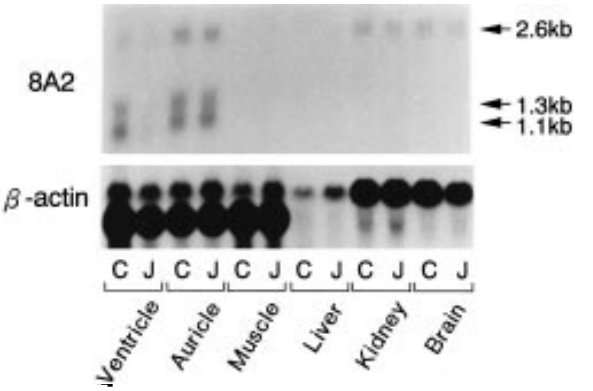

Fig. 2. Tissue distribution of mRNA species detected by cDNA fragment 8A2 in control and JVS mice. Total RNA (15 μg per lane) isolated from ventricle, auricle, thigh muscle, liver, kidney, and whole brain of control (C) and JVS (J) mice at 14 days of age was analyzed by Northern blot hybridization.

patterns in ventricles of control and JVS mice at 14 days of age by differential display analysis. By extracting differentially expressed cDNA fragments from the polyacrylamide gels, cloning and sequencing them with TA cloning system, and confirming the differential expression by Southern blotting with cloned cDNA fragment as probes, we found that four cDNA species were up-regulated to a higher amount and one was down-regulated to a much lower amount in the ventricles of JVS mice. Of the up-regulated cDNA species, two were found to be derived from known genes: ribosomal protein L3, reported to play a role in mRNA binding to the ribosome [18], and fibronectin, reported to be highly expressed in pressure-loaded model [19]. The other two were not identified as known genes with high sequence homology. We confirmed the up-regulated expression of these two novel genes in the ventricles of JVS mice by Northern blot analysis (data not shown), and are now cloning them.

On the other hand, the sequence obtained from the down-regulated cDNA fragment named as 8A2 (306 bp), containing a typical polyadenylation signal, AATAAA [20] at the near 3′-end, showed no significant homology with any published genes. By Northern blot analysis using the cDNA fragment 8A2 as a probe, we found that three mRNA species, two intense bands at 1.1- and 1.3-kb, and a faint band at 2.6-kb, were expressed in the ventricles of control mice and that the 1.1- and 1.3-kb species were much lower in the ventricles of JVS mice but that there was no difference in the 2.6-kb species (Fig. 1).

3.2. Unique tissue distribution of the three mRNA species derived from the novel gene and the unique suppression pattern in carnitine-deficient JVS mice

As shown in Fig. 2, all these three mRNA species were intensely expressed in the auricles of both control and JVS mice. There was no difference in intensity of these three bands between control and JVS mice. In the ventricles, however, the 1.1- and 1.3-kb species were much lower in JVS mice than in control, although there was no difference in intensity of the 2.6-kb species between control and JVS mice (Figs. 1 and 2). The 2.6-kb species was detected in the kidney and brain, but not in skeletal muscle and liver, and there was no difference between control and JVS mice. These results suggest that the 1.1- and 1.3-kb mRNA species were expressed specifically in

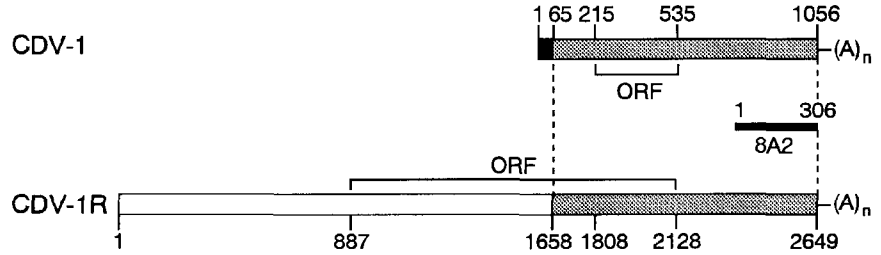

Fig. 3. Diagrammatic representation of nucleotide structure of CDV-1 (carnitine deficiency-associated gene expressed in ventricle) and CDV-1R (CDV-1 related gene) cDNAs cloned with a down-regulated cDNA fragment 8A2 detected by differential display analysis from ventricles. The numbers above and below the column or bar denote nucleotide numbers of each cDNA clone. A down-regulated cDNA fragment 8A2 first cloned from differential display analysis is shown by a bar. Shaded columns indicate completely identical nucleotide sequences between CDV-1 and CDV-1R. The nucleotide sequence of the closed column in CDV-1 differed totally from that of the open column in CDV-1R. Open reading frame is shown as ORF.

heart and suppressed specifically in ventricles of carnitine-deficient JVS mice. When JVS mice were treated with 5 μmol of carnitine twice a day from 5 to 14 days of age for 10 days, the 1.1- and 1.3-kb mRNA species were increased to the level of control mice (data not shown).

3.3. *Cloning and sequencing of the novel cDNAs from heart and brain*

To identify the three mRNA species derived from the novel gene corresponding to the fragment 8A2, we cloned cDNAs by 5′- and 3′-RACE methods using total RNA prepared from the heart and brain of control mice. A full-length cDNA was isolated from the heart. We term this cDNA a carnitine deficiency-associated gene expressed in ventricle (CDV-1). The construction is shown in Fig. 3. We could not obtain any other clones with a sequence different from the CDV-1 cDNA. At present we have no information about the structural differences between 1.1- and 1.3-kb mRNA species. As shown in Fig. 3, we have constructed cDNA isolated from the brain, which we have termed a CDV-1-related gene (CDV-1R). Although both full-length nucleotide sequences were analyzed by searching for homologies with the EMBL/GenBank/DDBJ data bases using FASTA, no significant similarities with any published genes were found. However, the two cDNAs identified in the present study showed part identity with four EST clones, three from human and one from mouse, T80249 (85.4% in 65-366 of CDV-1 and 87.4% in 1573–1953 of CDV-1R), N44901 (84.8% in 234–548 of CDV-1 and 1827–2141 of CDV-1R), N98663 (82.3% in 369–548 of CDV-1 and 1962–2141 of CDV-1R) and AA170087 (99.5% in 514–702 of CDV-1 and 2107–2295 of CDV-1R), respectively.

The entire nucleotide sequence from 65 to 1056 (992 bp) except for the 5′-end of CDV-1 cDNA was completely identical to the 3′-end nucleotide sequence from 1658 to 2649 of CDV-1R cDNA. The 5′-end sequence from 1 to 64 of CDV-1 cDNA was totally different from the corresponding sequence from 1 to 1657 of CDV-1R cDNA. An open reading frame (ORF) in each CDV-1 and CDV-1R cDNA was predicted according to the method of Fickett [21] as shown in Fig. 3. The ORF of CDV-1 started from ATG at 215 and ended at 535 followed by a stop codon, TGA, and in the case of CDV-1R, from ATG start codon at 887 to a last codon at 2128 followed by a TGA stop codon. As shown in Figs. 3 and 4, the peptide sequence deduced from CDV-1 cDNA sequence composed the C-terminal one-fourth of the peptide sequence deduced from CDV-1R cDNA. Interestingly, the nucleotide sequence from 65 to 214 in CDV-1 cDNA was the 5′-noncoding region, but that from 1658 to 1807 of CDV-1R cDNA was involved in the coding region, which is located in the middle one-eighth portion of the CDV-1R peptide. Both CDV-1 and CDV-1R cDNAs contained a 521-nucleotide 3′-untranslated region with a typical polyadenylation signal,

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CDV-1R    1 MKRLEEEIKFNSYMVTEKFPKELESKKKELHFLQKVVSEPAMGHSDLLEL  50

         51 ETKVNEVNTEINQLIEKKMMRNEPIEGKLSLYRQQASIISRKKEAKAEEL 100

        101 QETKEKLASLEREVLVKTNQTREFDGTEVLKGDEFKRYVSKLRSKSTVFK 150

        151 KKHQIIAEFKAEFGLLQRTEELLKQRQETIQHQLRTIEEKKGISGYSYTQ 200

        201 EELERVSALKSEVDEMKGRTLDDMSEMVKKLNSLVSEKKSALAPVIKELR 250

        251 QLRQKCQELTQECDEKKAQYDSCAAGLESNRSKLEQEVRGLREECLQEES 300

CDV-1     1         MIKNLEVELRRATDEMKAYVSSDQQEKRKAIREQYTKNITEQE  43
CDV-1R  301 KYHYTNCMIKNLEVELRRATDEMKAYVSSDQQEKRKAIREQYTKNITEQE 350

CDV-1    44 NLGKKLREKQKAVRESHGPNMKQAKMWRDLEQLMECKKQCFLKQQSPASI  93
CDV-1R  351 NLGKKLREKQKAVRESHGPNMKQAKMWRDLEQLMECKKQCFLKQQSPASI 400

CDV-1    94 GQVIQEGGEDRLVL                                     107
CDV-1R  401 GQVIQEGGEDRLVL                                     414
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Fig. 4. Amino-acid sequences deduced from CDV-1 and CDV-1R cDNAs. Predicted amino-acid sequences are shown by single-letter code. The numbers of amino-acid positions relative to initiating Met are shown. The shaded sequences of CDV-1 and CDV-1R are completely identical.

AATAAA [20], 32–37 bases upstream of the poly(A) tail. These results together with the unique expression pattern of CDV-1 and CDV-1R among tissues in control and JVS mice (Fig. 2) strongly suggest that they share exon(s), but that their expression is independently regulated by different promoters.

3.4. Amino-acid sequence of CDV-1 and CDV-1R peptides

The peptides deduced from CDV-1 and CDV-1R genes (Fig. 4) showed calculated molecular masses of 12 578 and 48 538 Da, respectively. Both peptides were hydrophilic and basic, containing high numbers of Lys, Glu, Gln and Leu. A search of protein data bases using BLAST and FASTA indicated that both CDV-1 and CDV-1R peptides were in a limited region slightly homologous (up to 20%) to proteins having α-helical coiled-coil structure, such as NUF1, which is a potential component of the yeast nucleoskeleton [22], *Caenorhabditis elegans* myosin heavy chain [23] and *Schistosoma mansoni* paramyosin [24]. The peptide sequences contain so-called heptads, having hydrophobic amino acids at 'a' and 'd' positions and hydrophilic amino acids at other positions. This suggests that the peptides may have a coiled-coil structure. It is interesting and important to find out the biological function and pathological implications of the novel peptides, especially of CDV-1, in carnitine deficiency.

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