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Genomic structure and promoter analysis of the mouse neutral ceramidase gene☆,☆☆

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Abstract

We report here the molecular cloning of the mouse neutral ceramidase gene and its promoter analysis. The gene, composed of 27 exons ranging in size from 40 to 292 bp, spans more than 70 kb. Analysis of the 5′-flanking region of the ceramidase genes revealed that the first exon of the gene of mouse liver was exactly the same as that of mouse kidney and Swiss 3T3 fibroblasts but completely different from that of mouse brain. The putative promoter regions of liver and brain ceramidase genes contained several well-characterized promoter elements such as GATA-2, C/EBP, and HNF3β but lacked TATA and CAAT boxes, a typical feature of a housekeeping gene, although the expression is regulated in a tissue-specific manner. Interestingly, a GC box was exclusively found in the putative promoter of mouse liver whereas potential AP1 and AP4 binding sites were present in that of mouse brain. By a luciferase reporter gene assay, it was shown that the GC-rich region, which exists just upstream of the first exon, conferred the promoter activity in Swiss 3T3 cells.

Keywords: Ceramide; Ceramidase; Sphingolipid; Luciferase assay; Sphingosine; Promoter; Genomic structure; Housekeeping gene; GC-rich region; Swiss 3T3

Ceramidase (CDase; *N*-acylsphingosine amidohydrolase, EC 3.5.1.23) is an enzyme that catalyzes the hydrolysis of the *N*-acyl linkage of ceramide (Cer) to generate fatty acid and sphingosine [1]. CDases are classified into three families by their catalytic pH optima and primary structure. Lysosomal acid CDase was purified from human urine [2] and the cDNA cloned from human [3] as well as mouse [4]. The genomic structure of the acid CDase was also characterized [4,5]. CDases which show an optimum pH in the neutral to alkaline range have been purified from bacteria [6], mouse liver [7], and rat brain [8], and the genes/cDNAs encoding the enzyme were cloned from bacteria [9], Drosophila [10], mouse [11], rat [12], and human [13]. Enzymes showing an extremely alkaline pH optima were cloned from yeast [14,15] and human [16].

Over the past decade, Cer and its metabolites, sphingosine and sphingosine-1-phosphate, have emerged as a new class of lipid biomodulators for various cell functions such as cell growth, differentiation, and apoptosis [17]. Sphingosine is not produced from de novo synthesis [18], and thus, CDase is considered to be crucial for producing sphingosine and possibly sphingosine-1-phosphate. That is, the cellular contents of Cer/sphingosine/sphingosine-1-phosphate should be regulated, at least in part, by the expression and activity of CDases.

Interestingly, we found that the 5′-UTR exon of the neutral CDase of mouse liver is somewhat different from that of mouse brain, although both ORFs are exactly the same [11]. Furthermore, it was revealed that the mRNA expression of the neutral CDase was much greater in mouse liver than mouse brain, suggesting that

☆ *Abbreviations:* BAC, bacterial artificial chromosome; Cer, ceramide; CDase, ceramidase; CMV, cytomegalovirus; DIG, digoxigenin; DMEM, Dulbecco's minimal essential medium; FBS, fetal bovine serum; PCR, polymerase chain reaction; 5′-RACE, 5′-rapid amplification of the cDNA end; UTR, untranslated region.

☆☆ The nucleotide sequences reported in this paper have been submitted to the GenBank/EBI Data Bank with Accession Nos. AB093137 and AB093138.

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the expression of the CDase gene was regulated in a tissue-specific manner. To elucidate how the neutral CDase gene expression is regulated, we characterized the genomic organization of the gene and analyzed its promoter function using the luciferase gene reporter assay.

Materials and methods

Materials. All restriction endonucleases were obtained from Nippon Gene (Toyama, Japan). pGL3-Basic vector and Luciferase assay kit were from Promega (WI, USA). All other reagents were of analytical grade.

DNA manipulation. DNA manipulation was carried out according to the method of Sambrook et al. [19]. The bacterial artificial chromosome (BAC) clone DNA was prepared using a Qiagen Large-Construct Kit (Qiagen, CA, USA). The nucleotide sequence was determined in both directions using a Bigdye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, CA, USA). An Applied Biosystems model 377A DNA Sequencer was used for the analysis.

Isolation and characterization of the neutral CDase gene. Three clones containing the neutral CDase gene in the pBeloBAC11 vector were isolated from a 129-mouse genomic library by PCR (Genome Systems, MI, USA). PCR was performed using a sense primer (GSS1: 5′-AACATGACAGATGTGAGCGTCCAG-3′, corresponding to nucleotides 1944–1967) and antisense primer (GSA1: 5′-TAATATTGA GGCCCGAAACTCCAT-3′, corresponding to nucleotides 2038–2061). The isolated genomic clones were designated pBCD-1, pBCD-2, and pBCD-3. These clones were digested with *Eco*RI, *Bam*HI, *Nco*I, or *Hin*dIII, and then subjected to Southern blotting using various portions of mouse brain and liver neutral CDase cDNA as probes. A DIG DNA Labeling Kit (Roche, Mannheim, Germany) was used to prepare the probe according to manufacturer's instructions. pBCD-1 was partially digested by *Sau*3AI. Three- to 10-kb fragments were ligated into *Bam*HI-digested pUC118 (Takara, Shiga, Japan) and these constructs were transfected into *Escherichia coli* JM109. Clones containing exons were isolated by colony hybridization using various portions of mouse brain and liver neutral CDase cDNA as probes.

PCR amplification of the 5′-cDNA end (5′-RACE). Total RNA was isolated from mouse brain, liver, kidney, and Swiss 3T3 fibroblasts with Sepasol RNA 1 (nacalai tesque, Kyoto, Japan). Poly(A) RNA was isolated from 250 μg of total RNA using the Oligotex-dT30 mRNA Purification Kit (Takara). The 5′-UTR of mouse neutral CDase cDNA was cloned by the RACE method using a SMART RACE cDNA Amplification Kit (Clontech, CA, USA). According to manufacturer's instructions, 1.0 μg poly(A) RNA was reverse transcribed with SuperScript II (Invitrogen, Groningen, The Netherlands) using random primers. First strand cDNA was used as the template for the first PCR amplification with Universal Primer Mix (5′-CTAATA CGACTCACTATAGGGCAAGCAGTGGTAACAACGCAGAG T-3′ and 5′-CTAATACGACTCACTATAGGGC-3′) and the gene-specific antisense primer GSP1 (5′-AATCCACCAGTTTCAAGAC-CAGCAT-3′, corresponding to mouse liver neutral CDase cDNA nucleotides 1494–1518 [11]). The resulting PCR products were diluted 50-fold with sterile water and amplified using Nested Universal Primer 5′-AAGCAGTGGTAACAACGCAGAGT-3′ and the gene-specific antisense primer GSP2 (5′-CCTGTGCAATCCGCTCTC-3′ corresponding to mouse liver neutral CDase cDNA nucleotides 974–991 [11]). The final PCR products were subcloned into the pGEM T-easy vector (Promega) and then sequenced.

Luciferase assay. A series of neutral CDase promoter and luciferase gene chimeras with progressively smaller promoter regions were prepared by site-directed PCR amplification using promoter primers (LPF1-LPF6) and a promoter reverse primer (LPR1). *Xho*I and *Hin*dIII sites were incorporated in the 5′ ends of the LPF primers and LPR1 primer, respectively. The sequences of the primers used were as follows:

LPF1: 5′-CATCTCGAGAGGGATCATGTCCTATTTGATA-3′
LPF2: 5′-ATCCTCGAGTCACCTAATGTACATCATTTTC-3′
LPF3: 5′-GACCTCGAGCAATGTACATAATCCCTGAC-3′
LPF4: 5′-GGCCTCGAGCCTCTGGAGGAGGAGGGAG-3′
LPF5: 5′-CGCCTCGAGCTGCGCCACTTCTCTCTCC-3′
LPF6: 5′-GAGCTCGAGCTGCTGGAGACCGGAGACC-3′
LPR1: 5′-CGGAAGCTTAGAGATGGAAACAGAAAGTAA-3′

PCRs were performed using the S-204 fragment as a template. The standard conditions were 98 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s, for 30 cycles. The DNA fragments generated were digested with *Xho*I and *Hin*dIII, and cloned into the pGL3-Basic vector using the

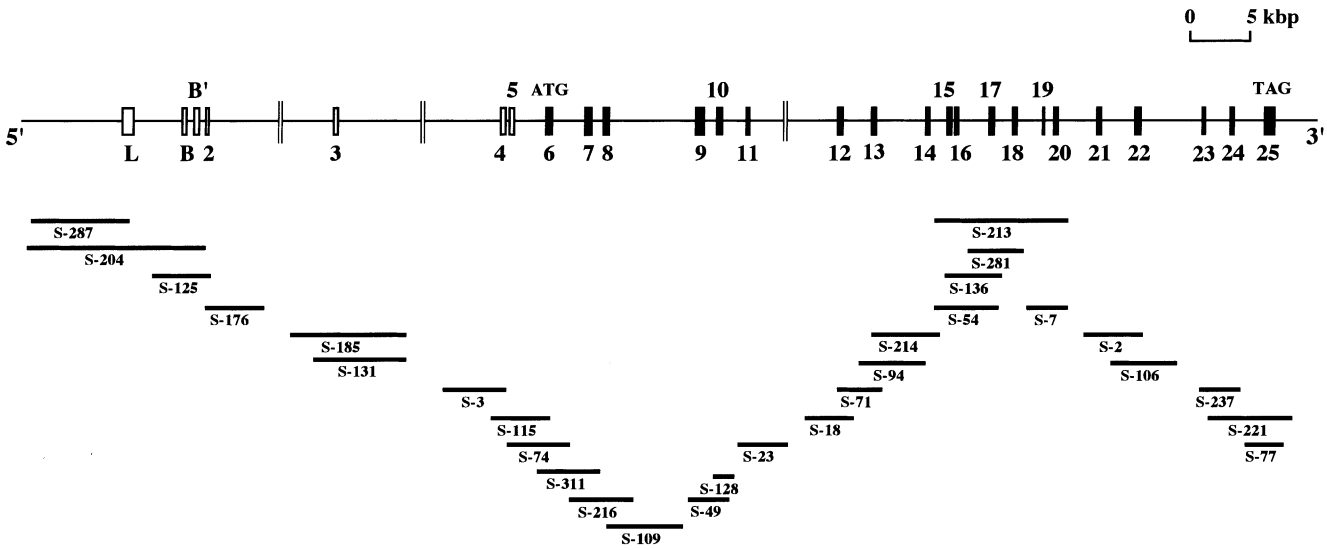

Fig. 1. Genomic structure of the mouse neutral CDase gene. Exons are numbered and depicted as boxes. Solid boxes correspond to the ORF of the neutral CDase gene. Open boxes indicate the noncoding regions at the 5′ end. The positions of the *Sau*3AI clones used for analyses are indicated as solid bars with appropriate clone numbers. L and B represent the first exon of liver-type and brain-type CDases, respectively. Details are described in Results and discussion.

Table 1
Exon/Intron boundaries of the mouse neutral ceramidase gene

Exon number	Position in the cDNA	Size (bp)	Sequences at exon–intron junction	Exon number	Position in the cDNA	Size (bp)	Sequences at exon–intron junction
L	−725 to −434	292	CCTGC-TAAAG**gt**atc	12	822–942	121	aac**ag**CTGGT-GACAG**gt**gag
(B)	(−440 to −319)	(122)	(CCTGC-TTTGG**gt**acg)	13	943–1068	126	ggc**ag**GGACC-GTGGG**gt**agg
(B′)	(−495 to −319)	(176)	(CTCTA-TGAAG**gt**aca)	14	1069–1155	87	ccc**ag**CCTAG-CCAAG**gt**agg
2	−433 to −356	78	tgc**ag**GTCTG-GCCAT**gt**aag	15	1156–1260	105	tct**ag**GAGCT-ACACA**gt**gag
(2)	(−318 to −241)	(78)	(tgc**ag**GTCTG-GCCAT**gt**aag)	16	1261–1342	82	ttc**ag**GTGAA-ACAGG**gt**gag
3	−355 to −244	112	ggg**ag**GGAAT-TCCAG**gt**aac	17	1343–1458	116	aac**ag**GAACT-GAGAG**gt**aag
4	−243 to −125	119	ttc**ag**TAGAT-GTCAG**gt**aag	18	1459–1553	95	ctc**ag**CTGAC-TTAAC**gt**aag
(4)	(−240 to −125)	(116)	(ag**tag**ATTTG-GTCAG**gt**aag)	19	1554–1593	40	ttc**ag**AACCA-AAAAA**gt**aag
5	−124 to −3	122	tct**ag**GGTTG-GACAG**gt**aag	20	1594–1689	96	cct**ag**GAATT-ACCAG**gt**aaa
6	−2 to 127	129	aga**ag**AAATG-CAAAG**gt**aag	21	1690–1785	96	tgc**ag**GCTCA-CTACG**gt**aat
7	128 to 288	161	ttt**ag**ATTCA-ATTTG**gt**agg	22	1786–1932	147	ttc**ag**GACAC-GAGTG**gt**aag
8	289 to 438	150	ttt**ag**ATGGG-TGGAG**gt**gag	23	1933–1992	60	tcc**ag**GAGA-ACCAG**gt**gag
9	439 to 615	177	tca**ag**GTCCT-TGAAG**gt**agg	24	1993–2081	89	ttc**ag**ACCCA-ACGAG**gt**aaa
10	616 to 743	128	ttc**ag**AGCAT-GCAAG**gt**aag	25	2082–2332	251	aac**ag**GTTTT-TTCACacaaa
11	744 to 821	78	gat**ag**GTATT-ATCAG**gt**att				

The +1 indicates the first base of the initiation Met of the neutral CDase. Uppercase letters represent exon sequences, and lowercase letters indicate intron sequences. Boldface letters represent the intron sequences adjoining the splice junctions. Parentheses indicate brain-type exons.

same restriction sites. Swiss 3T3 fibroblasts (4×10^4 cells at 30% confluency) were transfected with each construct (200 ng) and pCMV SPORT β-gal (40 ng, Invitrogen) in the presence of Plus reagent (2 μl) and Lipofectamine reagent (2 μl) (Invitrogen) in 500 μl DMEM in 12-well plates. After incubation at 37 °C for 5 h, the medium was replaced with 1 ml DMEM (Sigma, St. Louis, MO, USA) supplemented with 10% FBS. Twenty-four hours after transfection, cells were precipitated by centrifugation (800*g* for 5 min) and lysed in 200 μl lysis buffer (Promega). The luciferase activity in the cell lysates was determined using a Luciferase Assay System (Promega) and normalized with reference to β-galactosidase activity evaluated using a Chlorophenolred-β-D-galactopyranoside (Roche).

Results and discussion

Genomic organization of the neutral CDase gene

By PCR screening of a mouse 129Sv/J ES genomic library maintained in BAC vectors, three clones were isolated and designated pBCD-1, pBCD-2, and pBCD-3. Southern blot analysis using various portions of the mouse neutral CDase cDNA as probes suggested that pBCD-1 contains the entire neutral CDase gene. pBCD-1 was then partially digested with *Sau*3AI and the *Sau*3AI-fragments were cloned into the *Bam*HI-digested pUC118 vector. To select the clones containing exons, *Sau*3AI-clones were subjected to colony hybridization using various portions of the DIG-labeled CDase cDNA as probes. The exon–intron boundaries of the neutral CDase gene were determined by either sequencing the BAC inserts directly or sequencing the subcloned fragments. The structure of the neutral CDase gene along with the clones used for analyses is shown in Fig. 1. The CDase gene contains 27 exons, ranging from 40 to 292 bp, which are interrupted by 26 introns. The size of the introns was determined by DNA sequencing of *Sau*3AI clones or PCR. All exon–intron boundaries were found to follow the gt/ag rule that introns begin with GT and end with AG, and were in agreement with the consensus sequences for the splicing donor and acceptor sites [20]. All coding exons were covered by the *Sau*3AI clones obtained

Fig. 2. Genomic organization of 5′-UTR region of the neutral CDase gene. Black and open boxes represent exons for the coding region and 5′-untranslated region (UTR), respectively.

(Fig. 1). On the other hand, it was not possible to determine the size of three introns which are indicated as break bars in Fig. 1, because no overlap sequences were obtained for them by genomic PCR. In conclusion, the exact size of the full genomic region of the CDase is unknown at present but predicted to be longer than 70 kb (Fig. 1, see also Table 1).

To investigate the genomic organization of the 5′-UTR of the CDase gene, mRNAs from mouse liver, brain, kidney, and Swiss 3T3 cells were reverse-transcribed and then subjected to 5′-RACE. As a result, it was found that the first exon of liver (exon L) was exactly the same as that of kidney and Swiss 3T3 cells but completely different from that of brain

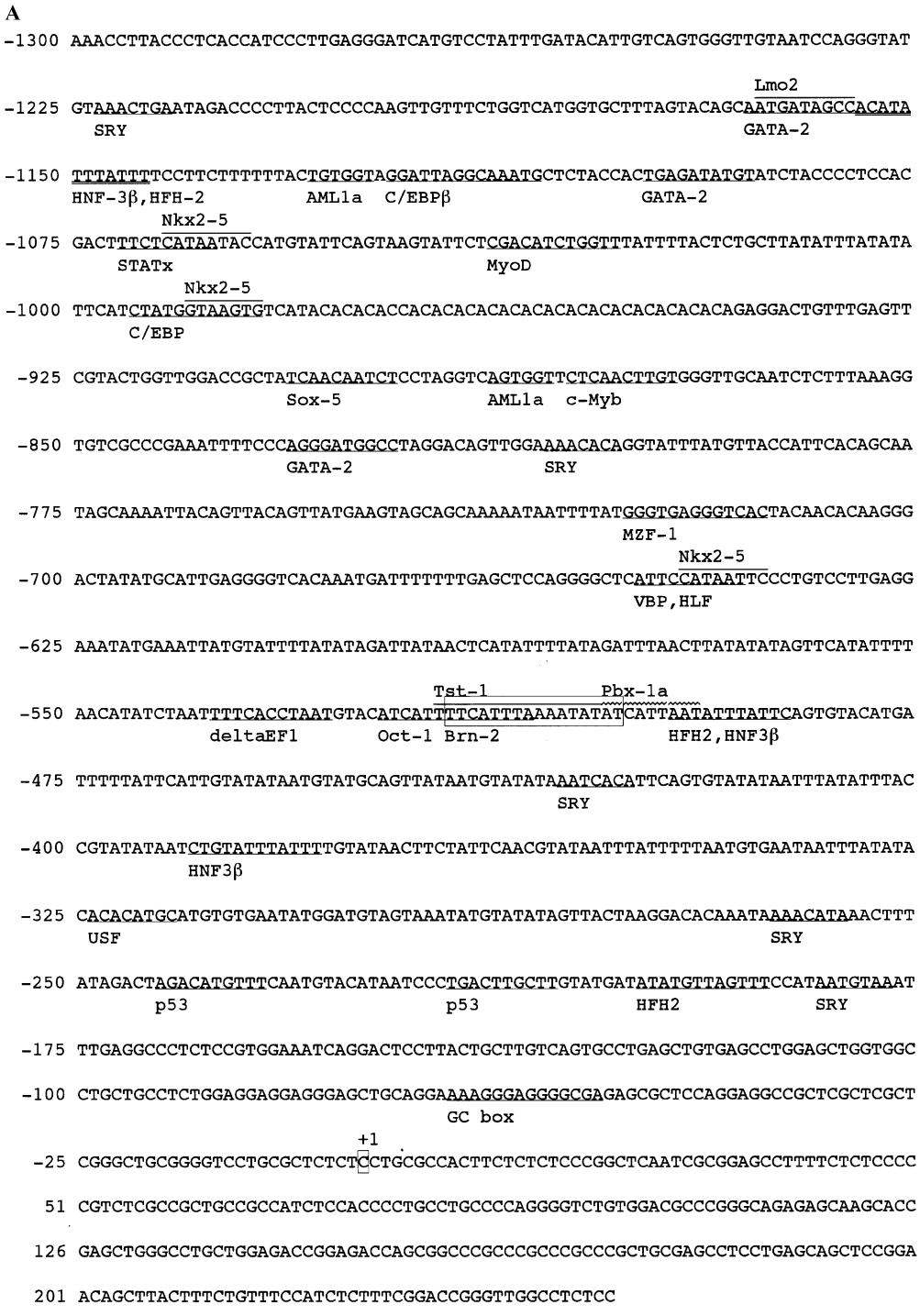

Fig. 3. Nucleotide sequence of the 5′ flanking region and the consensus sequences of transcription factor-binding elements of liver-type (A) and brain-type (B) CDases. Nucleotide sequences upstream of exon L (A) and exon B (B) of the mouse neutral CDase gene were analyzed for potential transcription factor-binding sites, which are underlined. The +1 indicates the putative transcriptional start site.

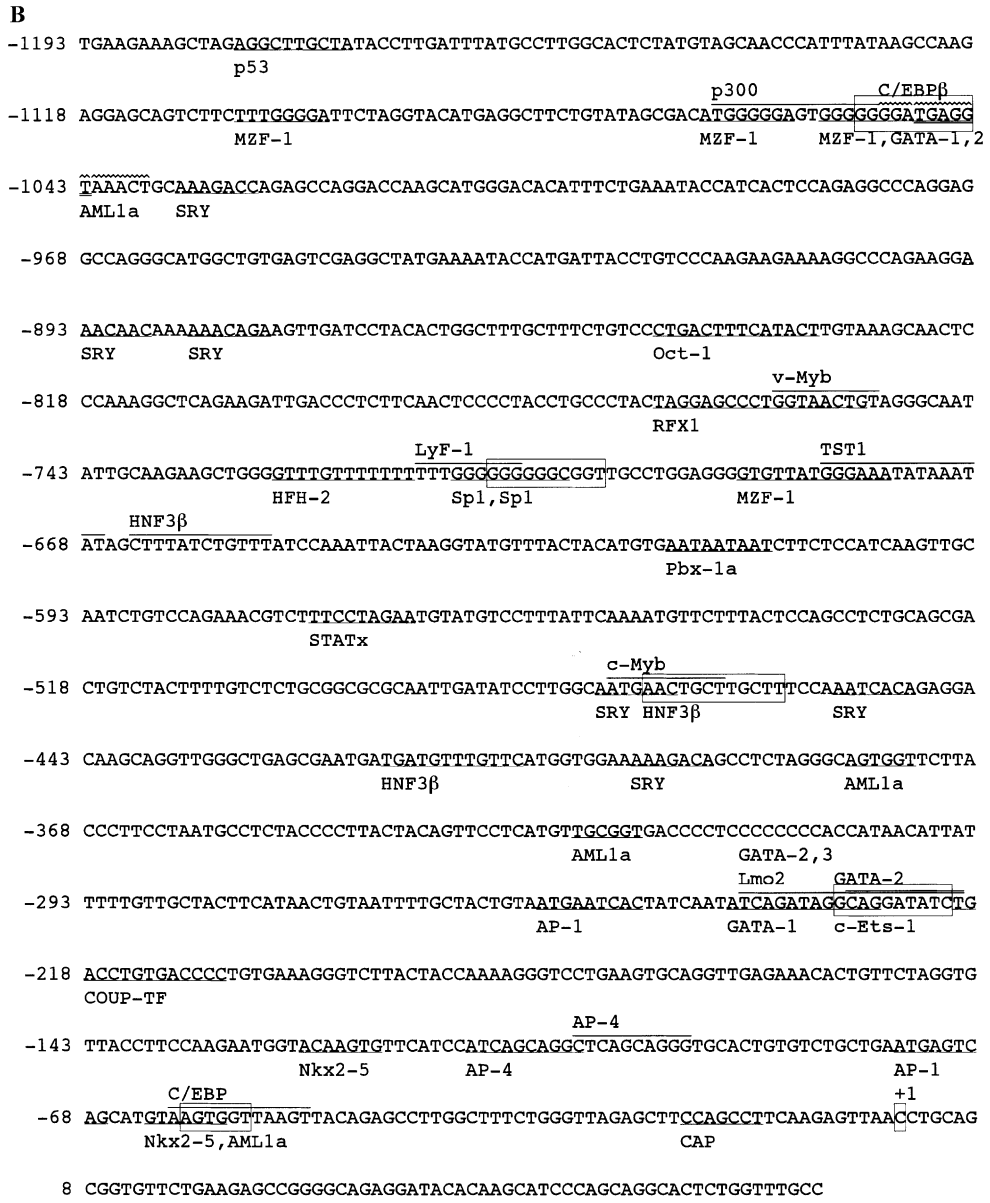

Fig. 3. (*continued*)

(Fig. 2). In the mouse brain two types of first exons, B and B′, were found for the gene. To determine the frequency of B and B′ exons, we analyzed 10 cDNA clones of mouse brain, seven of which contained exon B and the rest exon B′ (data not shown). It is noted that exon 3 was found only in the clones from liver and brain but not from kidney or Swiss 3T3 cells (Fig. 2).

The promoter region of the neutral CDase

The fact that exon L was completely different from exon B or B′ suggests that the CDase is regulated by a tissue-specific promoter. We thus sequenced the 5′-flanking region (~1.3 kbp) of the mouse neutral CDase clones from liver (pAPLCD) and brain (pCMVBCD). Consequently, it was revealed that both 5′-flanking regions lacked canonical TATA or CAAT boxes, although both regions contained several well-characterized promoter elements such as GATA-2, C/EBP, and HNF3β. Interestingly, a GC-rich region and a CAP site [21] were found just upstream of the first exon in mouse liver and brain, respectively. These features are typical for the promoter region of many housekeeping genes [22]. Computer analysis of these putative promoter regions also disclosed that GC box and potential AP1 and AP4 binding sites were exclusively present in the putative promoter region of liver and brain, respectively. Figs. 3A and B show potential regulatory elements in both putative promoters.

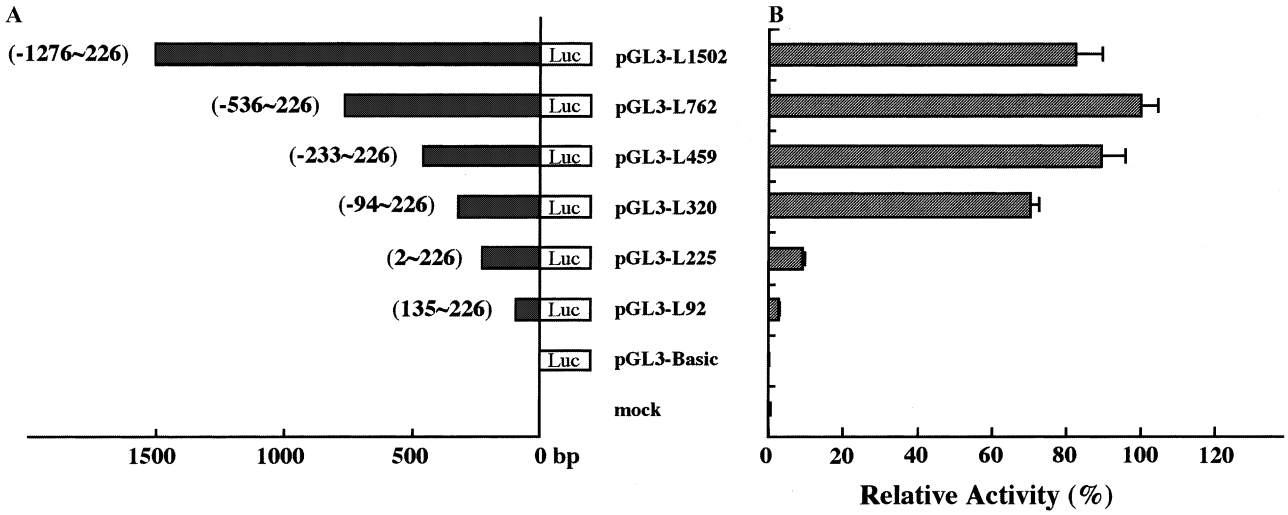

Fig. 4. Deletion analysis of the mouse neutral CDase promoter. Luciferase expression minigenes containing sequentially smaller segments of the neutral CDase chimeric promoter constructs were cotransfected into Swiss 3T3 cells with a plasmid (pCMVSPORT β-gal) containing the CMV promoter-driven β-galactosidase gene. Following liposome-mediated transfection, the cells were incubated at 37 °C for 24 h in DMEM containing FBS. Luciferase (Luc) and β-galactosidase (Gal) activities were measured. The Luc/Gal ratio was determined and the mean (±SD) for triplicate transfections was calculated. Values are expressed as the percent activity relative to that observed in pGL3-L762 (100%). pGL3-Basic is a negative control construct (vector alone) and mock indicates cells subjected to a mock transfection without the construct. Details are described in Materials and methods.

The functional promoter activity of the liver CDase was analyzed using the luciferase gene reporter system. Transfection of the plasmid containing the entire putative promoter region (1.5 kb) into the mouse fibroblast Swiss 3T3 cells led to a significant increase in luciferase activity (Fig. 4, pGL3-L1502), indicating that the region can actually function as a promoter for the neutral CDase. To obtain more information, a series of truncated forms of the promoter sequence were prepared by site-directed PCR amplification, inserted into the reporter plasmids and subjected to the gene reporter assay. As shown in Fig. 4, deletion of the −1276 to −95 region did not affect the promoter activity while deletion of the −94 to 1 region dramatically decreased the activity (Fig. 4, pGL3-L225). It is thus likely that essential sequences for enhancing the neutral CDase transcription are located in the region between −94 and 1 where the GC-rich region is to be found. It is also noted that the upstream region (−95 to −536) had no significant contribution for the regulation of CDase expression in Swiss 3T3 cells (Fig. 4). In contrast to the liver-type promoter, no promoter activity was detected in the putative promoter region of the brain CDase (upstream region of exon B) when Swiss 3T3 cells were used as the host. Further study is necessary to elucidate the brain-specific promoter activity using brain-derived cell lines.

The genomic sequence encoding the acid CDase, consisting of 14 exons separated by 13 introns, was cloned from mouse and a promoter assay was performed [4]. The putative promoter sequence of the acid CDase of mouse brain contains possible binding sites for transcription factors such as Sp1, GATA-1, AP1, and CACC boxes. The acid CDase gene seems to possess a housekeeping promoter, since the putative promoter region is GC-rich and does not contain TATA or CAAT boxes. Northern blot analysis and enzyme assay of mouse tissues revealed that the highest levels of activity and mRNA expression of acid CDase were found in kidney and brain [4] while those of neutral CDase were detected in liver and kidney [11]. The expression of neutral CDase mRNA and the enzyme activity in mouse brain were approximately one tenth that of mouse liver [7,11]. These results indicate that the expression of both enzymes was regulated in a tissue-specific manner possibly by alternative promoter utilization, even though both enzymes were coded on typical housekeeping genes. The tissue-specific expression of some glycosyltransferases was also found to be due to the alternative promoter utilization [23–25].

The data obtained here will provide some insight into the regulation of neutral CDases and facilitate the creation of gene knockout or knock-in mutant mice.

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