Genomic Structure of MUNC18-1 Protein, Which Is Involved in Docking and Fusion of Synaptic Vesicles in Brain*

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MUNC18-1 (n-Sec1) is a brain-specific protein and is known to play a role in neurotransmitter release by mediating docking and fusion of synaptic vesicles to presynaptic membranes. The protein is also implicated in the cellular excretion process of hormones and other biological substances in other mammalian tissues and yeasts. We have studied the structure of mouse munc18-1 gene by sequencing the genomic munc18-1 gene and its 5′-flanking region. munc18-1 gene comprises 19 exons whose size ranges from 50 base pairs (2nd exon) to 1676 base pairs (19th exon) with a total gene size of approximately 56 kilobases. In the 5′-flanking region, there are several transcription factor binding sites such as for HSF2, Lyf-1, and Sp1 but no TATA or CAAT sequences. munc18-1 gene was mapped on mouse chromosome 2 between two anchor markers D2Mit152 and D2Mit242. Transfection experiments employing these and upstream sequences suggest the presence of a sequence(s) that negatively regulates the expression of munc18-1 gene.

MUNC18-1 (n-Sec1) protein, the mammalian counterpart of the Unc-18 protein originally discovered in Caenorhabditis elegans (1), is a brain-specific protein with a molecular mass of 67 kDa, which associates with synaptic membranes through interaction with syntaxin, one of the synaptic membrane proteins (2–4). MUNC18-1, by forming a complex with syntaxin, is believed to play a role in neurotransmitter release through mediating docking and fusion of synaptic vesicles to presynaptic membranes. Upon docking of the vesicles to the membrane, however, MUNC18-1 protein dissociates from the complex. Phenotypes of mutants (unc-18) defective for the protein in C. elegans and its homolog (rop) in Drosophila melanogaster include, in addition to impairment of neurotransmitter release at synapses, uncoordinated movement (unc-18), retardation of postembryonic development (unc-18, rop), abnormal accumulation of acetylcholine (unc-18), and others (5–8). The knock-out mutation of munc18-1 gene in mouse was lethal (9).

Although munc18-1 gene is highly expressed in brain, it is also expressed in pancreatic endocrine cells (10) along with other genes responsible for fusion of synaptic vesicles, suggesting that the role played by MUNC18-1 protein is not limited to neurotransmitter release but includes the excretion of biologically active substances such as hormones. In fact, mutants in yeast defective in sec1, a counterpart of munc18-1 in yeast, are known to have an impaired secretion pathway, which is involved in trafficking of secretory vesicles (11). In this paper we report the organization of mouse munc18-1 gene as well as characterization of the 5′-regulatory sequence of the gene.

EXPERIMENTAL PROCEDURES

Construction of Mouse Genomic Library and Screening for Genomic munc18-1 DNA—A mouse genomic DNA library was constructed in Escherichia coli LE392 after ligating partial Sau3AI DNA digests of mouse genomic DNA (strain 129/Sv) at the BamHI site of a phage vector (EMBL3). For screening clones carrying munc18-1 gene, phage plaques were transferred to Hybond-N filters and (after alkaline treatment) hybridized with two [α-32P]dCTP-labeled rat munc18-1 cDNA probes (one covering nucleotides 1–1363 and the other nucleotides 1291–3488), which were isolated in this laboratory. The hybridization was performed overnight at 65 °C in a hybridization buffer that contained 500 mM Na2HPO4 (pH 7.2), 7% SDS, and 1 mM EDTA (12). After hybridization, the filters were washed with 0.1 M SSC and exposed to X-ray films (Kodak XAR-5). Among approximately 6 × 108 phage plaques examined, 19 clones were reactive to the munc18-1 cDNA probes.

DNA Sequencing—DNA of the 19 phage clones was digested with SacI and their restriction fragment patterns indicated that 4 clones (M-7:18kb, M-9:18kb, M-16:17kb, and M-1/1:20kb) could represent the whole DNA sequences covered by the 19 clones. Because preliminary experiments by hybridization of restriction fragments of the 4 clones described above with munc18-1 cDNA suggested the presence of a considerably large number of exons in the gene, we adopted the following sequencing strategy for the munc18-1 gene. Plasmid (pBluescript SK−) subclones were obtained from each of the 4 clones after SacI digestion, and hybridization experiments indicated that all of the SacI subclones carried a DNA insert with at least one exon for munc18-1 gene. The DNA sequence at the termini of the subclones was sequenced by the dideoxy chain termination method (13) using a sequencing kit (BcaBEST Dideoxy Sequencing Kit, Takara Shuzo Co., Ltd.) employing [α-32P]dCTP. After sequencing the terminal, the DNA was digested by the dideoxy chain termination method (13) using a sequencing kit (BcaBEST Dideoxy Sequencing Kit, Takara Shuzo Co., Ltd.) employing [α-32P]dCTP. After sequencing the terminal, the DNA was digested with appropriate restriction enzymes and subjected to a series of further plasmid subcloning and sequencing. We sequenced the DNA (total of over 40 kb) from the 4 clones. All the sequence of the munc18-1 cDNA was included in the determined sequence, thus having identified 19 exons. Sequencing one of the DNA strands gave corroboratory information in most cases, but when any ambiguity arose we sequenced the other strand of the same DNA or DNA clones obtained from other restriction fragments.

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The nucleotide sequence data reported in this paper will appear in the DDBJ/GenBank™/EMBL nucleotide sequence data bases with the following accession numbers: AB010151 (the sequence of the 5′-flanking region of munc18-1 gene and exon 1), AB012581 (the sequence in the first intron used for PCR primers to determine the size of the intron), AB012582 (the sequence between exon 8 and 9), AB012583 (the sequence between exon 8 and 9), AB012584 (the sequence between exon 10 and 13), AB012585 (the sequence of exon 14), AB012586 (the sequence between exon 15 and 17), AB012587 (the sequence of exon 18), AB012588 (the sequence of exon 19), and AB012697 (the sequence of downstream of exon 19).

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† The abbreviations used are: kb, kilobase(s); PCR, polymerase chain reaction; bp, base pair(s); PIPES, 1,4-piperazinediethanesulfonic acid; RACE, rapid amplification of cDNA ends; EUCIB, European Collaborative Interspecific Backcross; DMEM, Dulbecco’s modified Eagle’s medium; CAT, chloramphenicol acetyltransferase.

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The genomic fragments covered all potential introns except for a part of the intron between the first and second exons. The missing sequence of the intron was recovered by PCR using whole genomic mouse DNA as a template using primers (EL sense primer, 5'-GTGACGACACTTCTGGCCCTTAAATC-3'; A7 primer, 5'-CCTCCACTGCCCTTCTCCATCAC-3') and a kit (Expand Long Template PCR System, Boehringer Mannheim). After having confirmed the authenticity of the product by sequencing, the size of the intron was calculated by gel electrophoresis. S1 Nuclease Analysis—S1 nuclease analysis was performed as described (14). A single-stranded DNA probe for S1 nuclease mapping was prepared as follows. A 200-bp Smal genomic DNA fragment (see Fig. 2), which covers the potential transcription initiation site suggested from the 5'-terminal sequence of munc18-1 cDNA, was cloned at the EcoRI site of pBluescript SK+, excised from the vector (double digestion by PstI/EcoRI), treated with alkaline phosphatase, heat-denatured, and separated by polyacrylamide (6%)/urea (7 M) gel electrophoresis. A smaller single-stranded DNA band corresponding to the 186-bp antisense strand was recovered from the gel. The DNA was end-labeled with γ-[32P]ATP using T4 polynucleotide kinase and (after purification) incubated with total mouse brain RNA (10 μg) in a buffer (20 mM PIPES (pH 6.4), 1 mM EDTA, 400 mM NaCl, and 80% (v/v) formamide) for 10 min at 85 °C and then overnight at 30 °C. 200 μl of S1 nuclease analysis buffer (50 mM sodium acetate, pH 4.5, 280 mM NaCl, 4.5 mM ZnSO4, 20 μg/ml salmon sperm DNA) was then added with S1 nuclease (200 units) and incubated for 30 min at 37 °C. The reaction products were precipitated with ethanol, subjected to polyacrylamide (6%)/urea (7 M) gel electrophoresis, and autoradiographed.

3'-RACE—3'-RACE was performed using a kit (3'-RACE System, Life Technologies, Inc.). In essence, the first cDNA strand was synthesized from total mouse brain RNA employing a 3'-poly(A) tailing reaction. The first cDNA strand was then reversely transcribed using a gene-specific deoxyoligonucleotides (5'-CTGATATGATATCCCAGC-3') corresponding to nucleotides 2110 to 2126 of rat cDNA (diameter) dish. The cells continued to be incubated at 37 °C with gentle agitation (2 min at 5 and 24 h). After 48 h of incubation after transfection, the cells were collected by a scraper, washed with phosphate-buffered saline, resuspended in 100 μl of 250 mM Tris-HCl (pH 8.0), and subjected to repeated freezing and thawing (three times). After centrifugation (15,000 rpm for 5 min at 4 °C), the supernatant fractions were subjected to chloromphenicol acetyltransferase (CAT) and β-galactosidase assays. For L cells, essentially the same protocol was employed except that ES medium and Lipofectamine (Life Technologies, Inc.) solution (12 μl in 100 μl of ES medium) were used instead of DMEM and LipofectAMINE, respectively.

CAT and β-Galactosidase Assays—The assays were performed as described (14) with minor modifications. For CAT assay, cell extracts (100 μg of protein) in 80 μl of 250 mM Tris-HCl (pH 8.0) were heat-treated for 10 min at 65 °C, and supernatant fractions after centrifugation (5 min at 4 °C) were incubated with 10 μl of 10 mM acetyl-CoA and 20 μl of [14C]chloramphenicol (ICN, 0.2 μCi, specific activity 2.22 GBq/mmol) for 2 h at 37 °C and further incubated overnight at 37 °C after addition of 10 μl of fresh acetyl-CoA (10 mM). The reaction mixture was vigorously mixed with 200 μl of ethyl acetate, and the ethyl acetate fraction was subjected to TLC (CHCl3:CH3OH, 95:5) after concentration. The TLC plates were exposed to x-ray films (Kodak XAR-5), and the CAT activity (expressed as percent conversion) of each sample was quantitated by an image analyzer (Fuji, BAS2000). To correct transfection efficiency, β-galactosidase activities derived from co-transfected pCMVβ in the same sample were also assayed by incubating 2 μg of cell extracts in a reaction mixture (200 μl), which contained 7.5 mM Na2HPO4/NaNH2PO4 (pH 7.0), 75 mM NaCl, 0.75 mM MgCl2, 0.075% NaN3, 0.075% bovine serum albumin, and 0.225 mM 4-methylumbelliferol β-D-galactoside for 1 h at 37 °C. The reaction was terminated by addition of 600 μl of 0.1 M glycine-NaOH (pH 10.3), and fluorescence at 450 nm (excitation at 360 nm) was measured.

FIG. 1. Organization of mouse munc18-1 gene. The organization of munc 18-1 gene as revealed by sequence analysis of the genomic mouse munc18-1 DNA is diagrammatically presented. The numbers corresponding to those of the exons are placed as they are arranged in the gene. The width of the bars under exon numbers do not reflect the actual size of exons except for exon 1 and exon 19. Under the gene organization map, we show how munc18-1 mRNA is derived from the exons. The relative size of each exon calculated from sequence data as well as their possible functions (coding or non-coding) are also shown.

RESULTS

Organization of munc18-1 Gene—The organization of mouse munc18-1 gene was determined based upon information obtained from genomic DNA sequences. We first screened for genomic munc18-1 DNA fragments from a library constructed from partial restriction enzyme (Sau3AI) digests of mouse genomic DNA. By employing two rat munc18-1 cDNA sequences as probes (see “Experimental Procedures”), we isolated 19 clones that were reactive to the probes. Restriction fragment analysis of the clones revealed that each of the clones carried at least a part of the munc18-1 cDNA sequences, as a whole they covered the total munc18-1 cDNA sequence. We selected 4 clones by which the entire munc18-1 cDNA sequence could be covered and sequenced the DNA (total, over 40 kb). As all of the sequence of the munc18-1 cDNA was included in the determined sequence, we identified 19 exons for munc18-1 gene. As for the introns, the genomic fragments covered all potential introns except for a part of the intron between the first and second exons. The missing part of the intron was recovered by
Fig. 2. Nucleotide sequence of the 5'-flanking region of the munc18-1 gene. Position +1 denotes the putative major transcription start site (cap site). The sequence underlined was used for S1 nuclease analysis to determine the cap site. The first codon (+199/+201) is shown in bold, and relevant restriction sites are boxed. Sequences with possible biological significance (HSF2, Lyf-1, c-Rel, MZF1, and Sp1 (see text)) are indicated in the shaded boxes. The first exon/intron boundary located at +235/+236 is also shown.

PCR using whole genomic DNA as a template, and the size was calculated to be approximately 15 kb.

Thus, munc18-1 gene comprises 19 exons, whose sizes range from 50 bp (2nd exon) to 1676 bp (19th exon) with the total gene size of 56 kb. The organization of mouse munc18-1 gene is shown in Fig. 1. The precise locations of the exon/intron boundaries were primarily determined from observed sequence discrepancies between genomic DNA and cDNA. For cases in which the locations could not be determined from sequence discrepancy alone, positions of gt and ag-splicing specific dinucleotides (15) in the introns were taken into consideration. The positions for transcription initiation site and polyadenylation site were determined by S1 nuclease analysis and 3'-RACE, respectively, as described below.

Determination of Transcriptional Initiation Site—The precise location of the transcription initiation site of munc18-1 gene was determined by S1 nuclease analysis (14). Conventional primer extension was first tried, but despite a series of experiments performed under various conditions, the extension was not sufficient to determine the site, probably because of the presence of significantly GC-rich sequences (82% between +1 and +150 nucleotides) just downstream of the putative transcription initiation site. For S1 nuclease analysis, an antisense single-stranded DNA was prepared from one of the genomic DNA fragments, which was thought to cover the potential transcription initiation site and further extend up to 65 bp upstream of the site (underlined in Fig. 2). The labeled antisense single-stranded DNA (see “Experimental Procedures”) was first hybridized with total RNA from mouse brain, followed by S1 nuclease digestion. After electrophoresis, two closely mobilizing bands at positions 116 and 117 bp were detected (Fig. 3). The protected sequences contained the consensus sequence for the CAP site (CA and pyrimidine-rich sequence) without the consensus sequence for splicing. The 116-bp band was apparently produced by overdigestion of S1 nuclease (16), and we concluded that the transcription initiation site of mouse munc18-1 gene is located 117 bp upstream of the terminus of the antisense single-stranded DNA (nucleotide 1 shown in Fig. 2). The identification of the transcription initiation site by S1 nuclease analysis confirmed that exon 1, designated from the sequence analysis, is in fact the first exon of mouse munc18-1 gene.

Determination of the Polyadenylation Site—The size of exon 19 along with the polyadenylation site of munc18-1 gene was determined by 3'-RACE and subsequent sequencing of the product. cDNA primed at the polyadenylation site was synthesized from rat brain RNA and then subjected to PCR using a primer specific to the munc18-1 gene sequence. The PCR product was sequenced, and the size of exon 19 (1676 bp) and the polyadenylation site were determined (see Table I).
shown in Fig. 2. Characteristics of the sequence will be discussed below (see “Discussion”).

Chromosomal Location of munc18-1 Gene—We attempted to map munc18-1 gene on mouse chromosomes by interspecific (M. spretus and C57BL/6) backcross analysis. An informative restriction fragment length polymorphism was obtained following digestion with TaqI, yielding a 3.2-kb hybridizing band in M. spretus and a 2.8-kb band in C57BL/6. We concluded that munc18-1 gene is located on mouse chromosome 2 between two anchor markers D2Mit6 and D2Mit242. The order and distance (±1 S.E.) are: Cen–D2Mit6–(15.4 ± 4.5 centimorgans)–munc18-1–(10.5 ± 4.1 centimorgans)–D2Mit242. Haplotype analysis between these two anchor markers revealed that one recombinant between D2Mit152 and munc18-1 existed and the order is: Cen–D2Mit6–D2Mit152–munc18-1–D2Mit242. According to the Mouse Genome database (Mouse Genome Informatics Project, The Jackson Laboratory, Bar Harbor, ME; URL: http://www.informatics.jax.org. April, 1998), D2Mit152 and D2Mit242 are mapped at 21 and 29 centimorgans from the centromere, respectively. Because this region is syntenic to human chromosome 9q33-q34, the human homologue of munc18-1 is likely mapped to human chromosome 9q33-q34.

Expression of Promoter-Reporter Constructs in PC12D and L Cells—To examine whether sequences essential for the expression of munc18-1 gene, in addition to munc18-1 promoter, are present upstream of the gene, we constructed a series of composite constructs, in which 5′-upstream sequences as far as 2391 bp upstream of munc18-1 gene were placed at the proximal position of a reporter CAT gene. Rat PC12D cells, as well as mouse L cells, were transfected with the constructs, and CAT activities in extracts of the transfected cells were assayed. PC12D cells, a cell line established from rat adrenal pheochromocytoma, differentiate to form neuron-like cells in vitro after the addition of nerve growth factor to medium (17) and have been used extensively as a model for neuronal differentiation. Results of the transfection experiments are shown in Fig. 4, A and B. As seen in the figure, CAT activity in PC12D cells gradually increased to a maximum of approximately 2-fold of control, as the upstream sequence was truncated from 23911 to 2292 bp, suggesting the presence of a motif or sequence in that region, which negatively affects expression of the gene. As also shown in Fig. 4 (A and B), the CAT activities in mouse L cells after transfection were much lower than those observed in PC12D cells, and the activities were relatively constant regardless of all the constructs examined. This suggests that the sequence(s) necessary for expression of munc18-1 gene or regulation of expression is not playing an active role in mouse L cells.

When the upstream sequence was further truncated, CAT activity was decreased drastically in both PC12D and L cells with a significant drop in activities in the constructs truncated beyond 195 bp, indicating that the munc18-1 promoter is located downstream of nucleotide –195.

DISCUSSION

We have studied the structure of mouse munc18-1 gene, whose product (MUNC18-1) is implicated in neurotransmitter release and the cellular excretion process in mammalian cells and yeasts. The gene consists of 19 exons with a total gene size of approximately 56 kb. There are several interesting characteristics in the sequence of the 5′-flanking region. First, the immediate upstream sequence of the gene from the cap site is characterized by significantly high GC content, as we observed 78% GC content between nucleotides –1 and –150. Also, there
is no apparent TATA or CAAT box in this region. These may be unique features of some genes expressed in neural tissues. On the other hand, we were able to identify several specific sequences known as binding sites for transcription factors. For example, motifs for HSF2 (18), Lyf-1 (19), c-Rel (20), MZF1 (21), and Sp1 (22) are located at 2287 to 2278, 2262 to 2254, 2201 to 2192, 2162 to 2155, and 2175 to 2168, respectively (see Fig. 4), suggesting that transcription factors that bind to these motifs are involved in the expression of munc18-1 gene. Besides these, we examined whether the 5'-flanking sequence (8.9 kb), the introns, and the sequence downstream of the polyadenylation site (1.5 kb) include possible coding sequences or sequences with any biological significance by searching for homologous sequences in DDBJ/EBI/GenBank data bases. No sequences that are homologous to them were hit in the data bases. Furthermore, Northern hybridization against mouse brain RNA using blocks of the sequences from the 5'-flanking region (6.3 and 2.6 kb) as probes failed to give any signals. These results suggested that any genes are localized in the immediate vicinity of munc18-1 gene.

In transfection experiments using rat PC12D cells and composite DNA constructs, in which upstream sequences of munc18-1 gene were placed at the proximal position of a reporter CAT gene, we obtained evidence for the presence of a motif or sequence that negatively affects expression of the gene. Furthermore, because observed CAT activities were constant and much lower among all the constructs examined in mouse L cells than those observed in rat PC12D cells, the sequence(s) necessary for expression of munc18-1 gene or regulating of expression in PC12D cells are inactive. At present, it is not clear how the enhancer (silencer)-like motif or sequence in this upstream region functions in neural and other cells in which munc18-1 gene is expressed, but the presence of a similar silencer-like sequence has been reported in other genes, including those for neuron-restrictive silencer factors (23).

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