The complete nucleotide sequence of the gene encoding the nontoxic component of *Clostridium botulinum* type E progenitor toxin

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We have analysed the genes borne on a 6.0 kb *Hind*III fragment cloned from the chromosome of *Clostridium botulinum* type E strain Mashike. This fragment, cloned within plasmid pU9EMH, contains part of the structural gene for botulinum toxin type E neurotoxin as well as the entire structural gene for a nontoxic component of botulinum type E progenitor neurotoxin gene, *ent-120*. *ent-120* is transcribed in the same direction as the neurotoxin gene and consists of one open reading frame encoding 1162 amino acid residues. Western blotting with anti-nontoxic component sera demonstrates that *ent-120* encodes a protein of 120 kDa which forms part of the nontoxic component. *ent-120* is homologous to an analogous gene found in botulinum type C strains (69.3% identity at the nucleotide level and 56.1% at the amino acid level). Two stretches of amino acids at the N-terminus of the *ent-120* protein are highly homologous to amino acid sequences within the type E neurotoxin. The stop codon of the *ent-120* gene is situated 27 nucleotides upstream from the start codon of the neurotoxin gene.

Introduction

Botulinum toxins, produced by *Clostridium botulinum*, are extremely potent neurotoxins which inhibit the release of acetylcholine at the neuromuscular junction. They are classified into seven groups (A to G), based on the antigenicity of the toxin. The 7S neurotoxin is synthesized as a single polypeptide chain of molecular mass 150 kDa, which undergoes cleavage to form a di-chain molecule linked through a disulphide bond. The heavy chain (100 kDa) correlates with the binding of toxin to peripheral synapses, and the light chain is associated with the intracellular activity of blocking acetylcholine release (Kimura *et al.*, 1992). These toxins are produced as progenitor toxins of large molecular sizes: of 12S (M toxin), 16S (L toxin) and 19S (LL toxin) in culture supernatants. Three different molecular forms have been demonstrated in botulinum type A toxin (Sugii & Sakaguchi, 1975).

L and M toxins are recognized in botulinum type C and D toxins (Ohishi & Sakaguchi, 1980). Type E toxin

is exclusively composed of M toxin (Kitamura *et al.*, 1968). L and LL toxins show haemagglutinin activity, but M toxin does not (Ohishi & Sakaguchi, 1980). Molecular dissociation of these progenitor toxins into toxic and nontoxic components occurs in alkaline conditions (Ohishi & Sakaguchi, 1980). The nontoxic component of L or LL toxin is formed by conjugation of the nontoxic component of M toxin with the haemagglutinin. It has been suggested that the nontoxic components are necessary to maintain oral toxicity or to cause food poisoning because they prevent the 7S neurotoxin from degradation by gastric juices at low pH (Ohishi *et al.*, 1977; Ohishi & Sakaguchi, 1980). The molecular composition of the nontoxic fractions is not clear, but they appear to contain haemagglutinin and other macromolecules.

We have previously cloned and determined the complete nucleotide sequences of the structural genes for the haemagglutinin subcomponent (HA-33) and a nontoxic-nonHA component (nontoxic component of M toxin) of *C. botulinum* type C progenitor toxin (Tsuzuki *et al.*, 1990, 1992). In other types of *C. botulinum*, the molecular constitution of the nontoxic component is less clear.

Type E progenitor toxin (M toxin) consists of a nontoxic component and the neurotoxin (Kitamura *et al.*, 1968). The nontoxic component contains no haem-

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agglutinin activity. In this paper, we describe the cloning of the gene encoding the nontoxic component of M toxin from chromosomal DNA of *C. botulinum* type E strain Mashike.

Methods

Recombinant plasmids for DNA sequencing and Western blotting analysis. The recombinant plasmid pU9EMH contains a 6.0 kb *Hind*III fragment encoding the entire light chain and the N-terminal portion of the heavy chain of botulinum type E neurotoxin (Fujii *et al.*, 1992). pU9EMH DNA was digested with *Xba*I and *Kpn*I, then deleted by using a Takara deletion kit containing exonuclease III, Mungbean nuclease and Klenow enzyme (Takara Shuzo Co.). *Escherichia coli* MV 1184 cells transformed with the deleted pU9EMH were plated on TY agar plates (0.8%, w/v, tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar, 50 µg ampicillin ml⁻¹). Plasmids containing deletions were prepared by alkaline lysis of transformed *E. coli* MV 1184, and DNA fragments with appropriate sizes were selected by agarose gel electrophoresis.

Western blotting and DNA sequencing. Cultures of *E. coli* MV 1184 transformed with deletion mutants were grown in liquid medium and lysed by sonication; the protein products were separated by SDS-PAGE, and Western blot analysis was carried out as described previously (Fujii *et al.*, 1991, 1992; Tsuzuki *et al.*, 1990). Anti-type E nontoxic component rabbit serum (7S-NT), which reacts with the nontoxic component of botulinum type E progenitor toxin (Yokosawa *et al.*, 1986), but not with neurotoxin, and the monoclonal antibody (EL161-38) specific for type E neurotoxin were used in Western blot analyses. To reduce background, a 1:1000-diluted antiserum was treated with about 1 mg ml⁻¹ of *E. coli* MV 1184 extracts for 30 min before adding to the filter.

The deletion mutants of pU9EMH described above were used to determine the DNA sequence of the nontoxic component gene of botulinum type E progenitor toxin. DNA sequences were determined by the dideoxy chain-termination method using [α -³⁵S]dATP (NEN Products) and a T7 DNA sequencing kit (Pharmacia LKB Biotechnology), in accordance with the manufacturer's instructions (Fujii *et al.*, 1990, 1991).

Results

Western blotting analysis of gene products

The proteins expressed from *E. coli* MV 1184 containing pU9EMH were analysed by SDS-PAGE and Western blotting. In addition to the 100 kDa protein fragment of type E neurotoxin identified with monoclonal antibody EL161-38 (Fujii *et al.*, 1992), pU9EMH also produced a protein band of approximately 120 kDa which reacted with anti-7S-NT rabbit serum recognizing the nontoxic component of type E progenitor toxin (M toxin) (Fig. 1*a*). The 120 kDa protein did not react with monoclonal antibody EL161-38 recognizing type E neurotoxin. The gene encoding the 120 kDa protein was designated as *ent-120*.

A series of plasmids containing various deletions of the 6.0 kb DNA fragment was prepared. The gene products from these deletion mutants were analysed by

Western blotting using 7S-NT serum. Three plasmids containing a DNA fragment larger than 3.7 kb (pU9EH-D16, pU9EH-D0 and pU9EMH) produced the 120 kDa protein. Lower molecular mass proteins were produced by plasmids containing inserts smaller than 3.7 kb (pU9EH-D23, pU9EH-D29 and pU9EH-D12) (Fig. 1*b*). These results suggested that the entire *ent-120* gene was located within the 3.7 kb fragment contained in pU9EH-D16. Furthermore, deletion of about 500 bp from the 5'-terminus of the 6.0 kb fragment (pU9EH-RD1) resulted in no expression of the 120 kDa protein, suggesting that in this construct the promoter region has been deleted. Therefore, we concluded that the genes for *ent-120* and type E neurotoxin were probably transcribed in the same direction (Fig. 1*b*). In contrast to the plasmids containing deletions of the toxin gene, pU9EH-RD1 still produced the 100 kDa toxin polypeptide. Thus although the putative promoter for *ent-120* has been deleted, the promoter region necessary for the expression of the 100 kDa toxin gene in *E. coli* is still present.

Nucleotide and deduced amino acid sequences of *ent-120* gene

The complete DNA sequence of the *ent-120* gene was determined and is shown in Fig. 2. One open reading frame was composed of 1162 amino acid residues (3486 nucleotides), initiating at nucleotide position 163 with an ATG codon and terminating at position 3649 with a TAA stop codon. The molecular mass calculated from the deduced amino acid sequence was 136849.3 Da, a value slightly higher than that estimated from the blotting analysis described above. The N-terminal amino acid sequence of the *ent-120* gene was very similar (22 out of 30 amino acid residues identical) to that of the botulinum type E nontoxic component reported by Somers & DasGupta (1991). The amino acid residues which differ are marked with stars in Fig. 2. The high degree of identity between the amino acid sequence of the nontoxic component and the deduced sequence of *ent-120* strongly suggests that *ent-120* encodes the nontoxic component of botulinum type E progenitor toxin.

In the 5'-untranslated region of the *ent-120* gene, sequences homologous to the Shine-Dalgarno and to the -10 regions of typical *E. coli* promoters were found 16 bp and 107 bp upstream from the translation initiation start codon respectively (Fig. 2). There was no similarity in the promoter region between the botulinum type E and type C nontoxic component genes. However, sequences resembling the promoters of the *C. botulinum* type A and *Clostridium tetani* neurotoxin genes were detectable in those regions of both the type E and type C nontoxic component genes (Fig. 2). These sequences may

Fig. 1. Characterization of proteins produced by recombinant plasmids. (a) Extracts of *E. coli* MV 1184 transformed with pU9EMH were analysed by Western blotting with anti-neurotoxin monoclonal antibody (EL161-38) (lane 1) and anti-nontoxic component antiserum (7S-NT) (lane 2). (b) The recombinant plasmid pU9EMH was deleted with exonuclease III and transformed into *E. coli* MV 1184, and then gene products were analysed by Western blotting with monoclonal antibody EL161-38 and antiserum 7S-NT. The arrows indicate the orientations of the nontoxic component (*ent-120*) and neurotoxin genes. E, *EcoRI*; H, *HindIII*.

act as promoters in *C. botulinum* strains. Furthermore, the -35 region (TTTACA) of the lactose operon gene was also found near the putative -35 regions of both nontoxic component genes (indicated with a box in Fig. 2). Surprisingly, as shown in Fig. 2, the *C. botulinum ent-120* gene was separated from the type E neurotoxin gene by only 27 nucleotides, although no promoter-like sequence was found in this region. A similar result was found with the type C neurotoxin and nontoxic component genes (Tsuzuki *et al.*, 1992).

Comparative studies on amino acid sequences between botulinum type E and type C nontoxic component

Sequence comparison of type E and type C nontoxic components reveals approximately 69.3% identity at the nucleotide level and 56.1% at the amino acid level. Amino acid sequences homologous of those of type C are underlined in Fig. 2. This is an interesting result in the light of the low homology at the amino acid level between the neurotoxins of *C. botulinum* types E and C (Poulet *et al.*, 1992; Whelan *et al.*, 1992b). This suggests that the nontoxic components are composed of highly conserved amino acid sequences, in contrast to the botulinum neurotoxins. The two nontoxic components

have similar hydrophobicity patterns (data not shown). We reported previously that the N-terminal amino acid sequences of the type C nontoxic component and type C neurotoxin showed high homology (Tsuzuki *et al.*, 1992). Similar homology in the N-terminal amino acid sequences is also found between the type E nontoxic component and botulinum toxins types A, B, C, D and E, and tetanus neurotoxins (Fig. 3). Nevertheless, there is no immunological cross-reaction between type E nontoxic component and other neurotoxins (data not shown).

Discussion

Type E botulinum neurotoxin is produced as a progenitor toxin (M toxin) in culture supernatants (Kitamura *et al.*, 1968). The progenitor toxin dissociates into the neurotoxin and the nontoxic component in alkaline conditions (Kitamura *et al.*, 1968; Sugii & Sakaguchi, 1975). In this paper, we report the nucleotide sequence of the structural gene for the type E nontoxic component, and the possibility of polycistronic transcription of the botulinum type E nontoxic component and neurotoxin genes. The botulinum type E nontoxic component gene has one open reading frame (3486 nucleotides) coding for

1	AAG CTT TCG TGA TTC CTT AGC TTT <u>TGA ATT</u> AGC AAA TAA <u>TTT TAC AGA</u>	48
49	TAT AAT <u>AAA TGA</u> TAT TGT AAA TTG GAA TAA TTT AAA AAT TTC AGA GGT	96
97	TAC AAA TAT TAT TTT AAA TGT TGG ATT TTG TAT ACA GGA AAT ATG AAT	144
145	TAA <u>AGA GGG</u> TGA AAA TTT <u>ATG AAA ATA AAT GGT AAT TTA AAT ATT GAT</u>	192
1	SD Met Lys Ile Asn Gly Asn <u>Leu Asn Ile</u> Asp	10
193	TCT CCT GTA GAT AAT AAG AAT GTA GCA ATT GTT AGA AGT AGA AAT CAG	240
11	<u>Ser Pro Val Asp Asn Lys*Asn Val Ala Ile Val Arg Ser Arg*Asn*Gln*</u>	26
241	<u>ATG TTT TTT AAA GCA TTT CAA GTG GCT CCC AAT ATA TGG ATA GTC CCA</u>	288
27	<u>Met*Phe*Phe*Lys*Ala Phe Gln Val Ala Pro Asn Ile Trp Ile Val Pro</u>	42
289	GAA AGA TAT TAT GGA GAA TCA TTA AAG ATA AAT GAA GAT CAA AAA TTT	336
43	<u>Glu Arg Tyr Tyr Gly Glu Ser Leu Lys Ile Asn Glu Asp Gln Lys Phe</u>	58
337	GAT GGT GGA ATT TAT GAT TCT AAT TTT CTT TCA ACA AAT AAT GAA AAG	384
59	<u>Asp Gly Gly Ile Tyr Asp Ser Asn Phe Leu Ser Thr Asn Asn Glu Lys</u>	74
385	GAT GAC TTT TTG CAA GCA ACA ATC AAG TTA TTA CAA AGA ATA AAT AAC	432
75	<u>Asp Asp Phe Leu Gln Ala Thr Ile Lys Leu Leu Gln Arg Ile Asn Asn</u>	90
433	AAT GTT GTA GGT GCA AAG TTA TTA TCT TTA ATT TCT ACA GCT ATT CCT	480
91	<u>Asn Val Val Gly Ala Lys Leu Leu Ser Leu Ile Ser Thr Ala Ile Pro</u>	106
481	TTT CCT TAT GAA AAT AAT ACT GAA GAT TAT AGA CAG ACT AAC TAC CTT	528
107	<u>Phe Pro Tyr Glu Asn Asn Thr Glu Asp Tyr Arg Gln Thr Asn Tyr Leu</u>	122
529	AGT TCT AAG AAT AAT GAA CAT TAT TAT ACA GCT AAC TTA GTT ATT TTT	576
123	<u>Ser Ser Lys Asn Asn Glu His Tyr Tyr Thr Ala Asn Leu Val Ile Phe</u>	138
577	GGA CCA GGA TCA AAT ATA ATA AAA AAT AAT GTT ATT TAT TAT AAA AAA	624
139	<u>Gly Pro Gly Ser Asn Ile Ile Lys Asn Asn Val Ile Tyr Tyr Lys Lys</u>	154
625	GAA TAT GCA GAA AGT GGA ATG GGA ACC ATG TTA GAA ATA TGG TTT CAA	672
155	<u>Glu Tyr Ala Glu Ser Gly Met Gly Thr Met Leu Glu Ile Trp Phe Gln</u>	170
673	CCA TTT TTA ACA CAT AAA TAT GAT GAA TTC TAT GTT GAT CCA GCT TTA	720
171	<u>Pro Phe Leu Thr His Lys Tyr Asp Glu Phe Tyr Val Asp Pro Ala Leu</u>	186
721	GAG TTA ATA AAA TGT TTA ATA AAA TCT CTT TAT TAT TTA TAT GGA ATA	768
187	<u>Glu Leu Ile Lys Cys Leu Ile Lys Ser Leu Tyr Tyr Leu Tyr Gly Ile</u>	202
769	AAA CCT AAT GAT AAT TTA AAT ATT CCA TAT AGA TTA AGA AAT GAG TTT	816
203	<u>Lys Pro Asn Asp Asn Leu Asn Ile Pro Tyr Arg Leu Arg Asn Glu Phe</u>	218
817	AAT AGT TTA GAA TAT TCA GAG TTA AAT ATG ATT GAT TTT TTA ATA TCA	864
219	<u>Asn Ser Leu Glu Tyr Ser Glu Leu Asn Met Ile Asp Phe Leu Ile Ser</u>	234
865	GGA GGA ATT GAT TAT AAA CTT TTA AAT ACT AAT CCT TAT TGG TTT ATA	912
235	<u>Gly Gly Ile Asp Tyr Lys Leu Leu Asn Thr Asn Pro Tyr Trp Phe Ile</u>	250
913	GAT AAG TAT TTT ATT GAT ACT TCG AAA AAT TTT GAA AAA TAT AAA AAT	960
251	<u>Asp Lys Tyr Phe Ile Asp Thr Ser Lys Asn Phe Glu Lys Tyr Lys Asn</u>	266
961	GAT TAT GAA ATA AAA ATT AAA AAT AAT AAT TAT ATT GCT AAT AGT ATT	1008
267	<u>Asp Tyr Glu Ile Lys Ile Lys Asn Asn Asn Tyr Ile Ala Asn Ser Ile</u>	282
1009	AAA TTA TAT TTA GAA CAA AAG TTT AAG ATT AAT GTA AAA GAT ATA TGG	1056
283	<u>Lys Leu Tyr Leu Glu Gln Lys Phe Lys Ile Asn Val Lys Asp Ile Trp</u>	298
1057	GAA TTA AAT TTA AGT TAT TTT TCT AAA GAA TTT CAA ATC ATG ATG CCA	1104
299	<u>Glu Leu Asn Leu Ser Tyr Phe Ser Lys Glu Phe Gln Ile Met Met Pro</u>	314
1105	GAA AGA TAC AAT AAT GCA CTT AAT CAT TAT TAC AGA AAA GAA TTT TAT	1152
315	<u>Glu Arg Tyr Asn Asn Ala Leu Asn His Tyr Tyr Arg Lys Glu Phe Tyr</u>	330
1153	GTA ATA GAT TAT TTT AAA AAT TAC AAT ATA AAT GGT TTT AAA AAT GGT	1200
331	<u>Val Ile Asp Tyr Phe Lys Asn Tyr Asn Ile Asn Gly Phe Lys Asn Gly</u>	346
1201	CAA ATT AAA ACA AAA TTA CCT TTA TCA AAA TAT AAC AAA GAG ATT ATA	1248
347	<u>Gln Ile Lys Thr Lys Leu Pro Leu Ser Lys Tyr Asn Lys Glu Ile Ile</u>	362
1249	AAT AAG CCT GAA TTA ATA GTT AAC TTG ATA AAT CAA AAT AAT ACT GTA	1296
363	<u>Asn Lys Pro Glu Leu Ile Val Asn Leu Ile Asn Gln Asn Asn Thr Val</u>	378
1297	TTG ATG AAA AGT AAT ATT TAT GGA GAT GGA TTA AAA GGG AAT GTG GAT	1344
379	<u>Leu Met Lys Ser Asn Ile Tyr Gly Asp Gly Leu Lys Gly Asn Val Asp</u>	394

Fig. 2. (For legend see page 84.)

1345	AAT TTC TAT TCT AAT TAT ATA ATT CCC TAT AAT CTA AAT TAT GAA CAT	1392
395	Asn Phe Tyr Ser Asn Tyr Ile Ile Pro Tyr Asn Leu Asn Tyr Glu His	410
1393	TCT ATT AAT TAT TTT TAT TTA GAT AAT GTA AAT ATC GAA GAA ATA GAA	1440
411	Ser Ile Asn Tyr Phe Tyr Leu Asp Asn Val Asn Ile Glu Glu Ile Glu	426
1441	AAA ATT CCT CCT ATT AAT GAT GAA GAT ATA TAT CCT TAT AGA AAA AAT	1488
427	Lys Ile Pro Pro Ile Asn Asp Glu Asp Ile Tyr Pro Tyr Arg Lys Asn	442
1489	GCT GAT ACA TTT ATA CCA GTA TAT AAT ATT ACA AAA GCT AAG GAA ATT	1536
443	Ala Asp Thr Phe Ile Pro Val Tyr Asn Ile Thr Lys Ala Lys Glu Ile	458
1537	AAT ACT ACC ACA CCA TTA CCA GTA AAT TAT TTA CAG GCT CAA ATG ATA	1584
459	Asn Thr Thr Thr Pro Leu Pro Val Asn Tyr Leu Gln Ala Gln Met Ile	474
1585	GAT AGT AAT GAT ATT AAC TTA TCC TCA GAT TTT CTA AAA GTA ATT TCT	1632
475	Asp Ser Asn Asp Ile Asn Leu Ser Ser Asp Phe Leu Lys Val Ile Ser	490
1633	TCT AAG GGA TCT TTA GTA TAT TCG TTT TTA AAT AAT ACA ATG GAT TAT	1680
491	Ser Lys Gly Ser Leu Val Tyr Ser Phe Leu Asn Asn Thr Met Asp Tyr	506
1681	TTA GAG TTT ATA AAA TAC GAT AAA CCC ATT GAT ACA GAT AAA AAA TAT	1728
507	Leu Glu Phe Ile Lys Tyr Asp Lys Pro Ile Asp Thr Asp Lys Lys Tyr	522
1729	TAT AAG TGG TTA AAA GCA ATT TTT AGA AAT TAC TCT CTT GAT ATA ACA	1776
523	Tyr Lys Trp Leu Lys Ala Ile Phe Arg Asn Tyr Ser Leu Asp Ile Thr	538
1777	GAA ACT CAA GAA ATT AGT AAT CAA TTT GGA GAT ACT AAG ATA ATA CCA	1824
539	Glu Thr Gln Glu Ile Ser Asn Gln Phe Gly Asp Thr Lys Ile Ile Pro	554
1825	TGG ATT GGT AGA GCA TTA AAT ATT CTA AAT ACA AAT AAT TCA TTT GTG	1872
555	Trp Ile Gly Arg Ala Leu Asn Ile Leu Asn Thr Asn Asn Ser Phe Val	570
1873	GAG GAA TTT AAA AAC TTA GGA CCA ATT TCT CTT ATT AAT AAA AAA GAA	1920
571	Glu Glu Phe Lys Asn Leu Gly Pro Ile Ser Leu Ile Asn Lys Lys Glu	586
1921	AAT ATA ACT ATT CCT AAA ATA AAA ATT GAT GAA ATA CCT AGT AGT ATG	1968
587	Asn Ile Thr Ile Pro Lys Ile Lys Ile Asp Glu Ile Pro Ser Ser Met	602
1969	TTG AAT TTT TCA TTT AAA GAT TTA AGT GAA AAT TTA TTT AAT ATA TAT	2016
603	Leu Asn Phe Ser Phe Lys Asp Leu Ser Glu Asn Leu Phe Asn Ile Tyr	618
2017	TGT AAA AAT AAT TTT TAT CTA AAA AAA ATT TAC TAT AAT TTT TTA GAT	2064
619	Cys Lys Asn Asn Phe Tyr Leu Lys Lys Ile Tyr Tyr Asn Phe Leu Asp	634
2065	CAA TGG TGG ACA CAA TAT TAT AGT CAA TAT TTT GAT CTA ATT TGT ATG	2112
635	Gln Trp Trp Thr Gln Tyr Tyr Ser Gln Tyr Phe Asp Leu Ile Cys Met	650
2113	GCT AGT AAA TCA GTA TTA GCT CAA GAA AAG TTA ATA AAA AAA CTA ATA	2160
651	Ala Ser Lys Ser Val Leu Ala Gln Glu Lys Leu Ile Lys Lys Leu Ile	666
2161	CAA AAA CAA TTA AGG TAT TTA ATG GAA AAT TCT AAT ATA TCC TCT ACT	2208
667	Gln Lys Gln Leu Arg Tyr Leu Met Glu Asn Ser Asn Ile Ser Ser Thr	682
2209	AAT TTA ATA TTG ATA AAC TTA ACA ACC ACA AAT ACA TTA AGA GAT ATT	2256
683	Asn Leu Ile Leu Ile Asn Leu Thr Thr Thr Asn Thr Leu Arg Asp Ile	698
2257	TCA AAT CAA TCA CAA ATA GCA ATA AAT AAT ATA GAT AAA TTT TTT AAT	2304
699	Ser Asn Gln Ser Gln Ile Ala Ile Asn Asn Ile Asp Lys Phe Phe Asn	714
2305	AAT GCT GCT ATG TGT GTT TTT GAA AAC AAT ATT TAT CCT AAA TTC ACT	2352
715	Asn Ala Ala Met Cys Val Phe Glu Asn Asn Ile Tyr Pro Lys Phe Thr	730
2353	TCT TTT ATG GAA CAA TGT ATT AAA AAT ATA AAT AAA AGC ACC AAA GAG	2400
731	Ser Phe Met Glu Gln Cys Ile Lys Asn Ile Asn Lys Ser Thr Lys Glu	746
2401	TTT ATA CTA AAA TGT ACT AAT ATT AAT GAA ACT GAA AAA TCA CAC TTG	2448
747	Phe Ile Leu Lys Cys Thr Asn Ile Asn Glu Thr Glu Lys Ser His Leu	762
2449	ATT ATG CAA AAT AGT TTT AGT AAT TTA GAT TTT GAT TTT TTA GAT ATT	2496
763	Ile Met Gln Asn Ser Phe Ser Asn Leu Asp Phe Asp Phe Leu Asp Ile	778
2497	CAA AAT ATG AAG AAC CTA TTT AAT TTA TAT ACA GAA CTA CTT ATA AAA	2544
779	Gln Asn Met Lys Asn Leu Phe Asn Leu Tyr Thr Glu Leu Leu Ile Lys	794
2545	GAA CAA ACC TCA CCC TAT GAA TTA TCA TTA TAT GCT TTT CAA GAA CAA	2592
795	Glu Gln Thr Ser Pro Tyr Glu Leu Ser Leu Tyr Ala Phe Gln Glu Gln	810
2593	GAT AAC AAT GTT ATT GGA GAT ACA TCC GGT AAA AAT ACA TTA GTA GAA	2640
811	Asp Asn Asn Val Ile Gly Asp Thr Ser Gly Lys Asn Thr Leu Val Glu	826

Fig. 2 cont. (For legend see page 84.)

2641	TAC CCT AAA GAT ATA GGA TTA GTT TAT GGA ATA AAT AAT AAT GCA ATA	2688
827	Tyr Pro Lys Asp Ile Gly <u>Leu Val Tyr Gly</u> Ile Asn Asn Asn Ala Ile	842
2689	CAT TTA ACT GGG GCT AAT CAA AAT ATA AAG TTT ACC AAC GAT TAT TTT	2736
843	His Leu Thr Gly Ala Asn Gln Asn <u>Ile Lys Phe</u> Thr Asn Asp Tyr Phe	858
2737	GAA AAT GGA TTA ACC AAT AAC TTT TCA ATT TAT TTT TGG TTG AGA AAT	2784
859	Glu <u>Asn Gly Leu Thr Asn Asn Phe</u> Ser Ile Tyr <u>Phe Trp Leu Arg</u> Asn	874
2785	TTA AAG CAA AAT ACT ATT AAA TCT AAG TTA ATA GGT AGT AAA GAA GAT	2832
875	Leu Lys Gln Asn Thr Ile Lys Ser Lys <u>Leu Ile Gly</u> Ser Lys Glu Asp	890
2833	AAT TGT GGT TGG GAA ATT TAT TTT GAA AAT GAT GGA TTA GTT TTT AAT	2880
891	<u>Asn Cys Gly Trp Glu Ile Tyr Phe</u> Glu Asn Asp Gly Leu Val Phe Asn	906
2881	ATA ATA GAT TCT AAT GGA AAT GAA AAA AAT ATT TAT TTA TCT AAT ATT	2928
907	<u>Ile Ile Asp Ser Asn Gly Asn</u> Glu Lys Asn Ile <u>Tyr Leu Ser Asn Ile</u>	922
2929	TCT AAT AAG AGT TGG CAT TAT ATA GTA ATA TCT ATA AAT CGT TTG AAA	2976
923	Ser Asn Lys Ser Trp His <u>Tyr Ile</u> Val <u>Ile Ser</u> Ile Asn <u>Arg Leu Lys</u>	938
2977	GAT CAA TTA CTA ATA TTT ATT GAT AAT ATA CTT GTT GCA AAT GAA GAT	3024
939	<u>Asp Gln</u> Leu Leu Ile Phe Ile Asp Asn Ile Leu <u>Val Ala Asn</u> Glu Asp	954
3025	ATT AAA GAA ATT TTA AAT ATT TAT TCA AGT GAT ATA ATT TCA TTA TTA	3072
955	Ile Lys <u>Glu Ile Leu</u> Asn <u>Ile Tyr Ser</u> Ser Asp <u>Ile Ile Ser Leu</u> Leu	970
3073	AGT GAT AAT AAT AAT GTC TAT ATT GAA GGA TTA TCT GTT TTA AAT AAA	3120
971	Ser Asp Asn Asn Asn Val Tyr Ile Glu Gly <u>Leu Ser Val Leu</u> Asn Lys	986
3121	ACT ATT AAT AGT AAT GAA ATT TTA ACT GAT TAT TTT AGT GAT TTA AAC	3168
987	Thr Ile Asn Ser Asn Glu Ile Leu Thr Asp Tyr Phe Ser Asp Leu Asn	1002
3169	AAT TCA TAT ATA AGA AAT TTT GAT GAA GAA ATA TTA CAA TAT AAT AGA	3216
1003	Asn <u>Ser Tyr Ile Arg</u> Asn Phe Asp Glu Glu Ile <u>Leu Gln Tyr Asn</u> Arg	1018
3217	ACA TAT GAA TTG TTT AAT TAT GTA TTT CCA GAA ATC GCT ATA AAT AAA	3264
1019	Thr <u>Tyr Glu Leu</u> Phe <u>Asn Tyr Val Phe Pro Glu</u> Ile Ala Ile Asn Lys	1031
3265	ATT GAG CAA AAT AAT AAT ATA TAC TTA TCA ATT AAC AAT GAA AAT AAT	3312
1035	Ile Glu Gln Asn <u>Asn Asn</u> Ile <u>Tyr Leu Ser</u> Ile Asn Asn Glu Asn Asn	1050
3313	TTA AAT TTT AAA CCT CTA AAA TTT AAA TTA TTA AAT ACT AAT CCA AAC	3360
1051	Leu Asn Phe Lys Pro Leu <u>Lys Phe Lys Leu</u> Leu Asn Thr Asn Pro Asn	1066
3361	AAA CAA TAT GTT CAA AAA TGG GAT GAG GTA ATA TTT TCT GTA TTA GAT	3408
1067	Lys Gln Tyr <u>Val Gln Lys Trp Asp</u> Glu Val Ile Phe Ser Val Leu Asp	1082
3409	GGT ACA GAA AAA TAT TTA GAT ATA TCT ACT ACT AAT AAT AGA ATT CAA	3456
1083	Gly Thr Glu Lys Tyr Leu Asp Ile Ser Thr Thr <u>Asn Asn Arg Ile Gln</u>	1098
3457	CTA GTA GAT AAT AAA AAT AAT GCA CAG ATT TTT ATT ATT AAT AAT GAT	3504
1099	<u>Leu Val</u> Asp Asn Lys Asn <u>Asn Ala</u> Gln Ile Phe Ile Ile Asn Asn Asp	1114
3505	ATA TTT ATC TCT AAC TGT TTA ACT TTA ACT TAT AAC AAT GTA AAT GTA	3552
1115	Ile Phe Ile Ser Asn Cys Leu Thr Leu Thr <u>Tyr Asn</u> Asn Val Asn Val	1130
3553	TAT TTG TCT ATA AAA AAT CAA GAT TAC AAT TGG GTT ATA TGT GAT CTT	3600
1131	Tyr <u>Leu Ser</u> Ile Lys Asn Gln <u>Asp Tyr Asn Trp</u> Val <u>Ile Cys</u> Asp Leu	1146
3601	AAT CAT GAT ATA CCA AAA AAG TCA TAT CTA TGG ATA TTA AAA AAT ATA	3648
1147	Asn His Asp Ile Pro Lys Lys Ser Tyr <u>Leu Trp Ile Leu</u> Lys Asn Ile	1162
3649	TAA ATT TAA AAT TAG GAG ATG CTG TAT	
***	SD	
		ATG CCA AAA ATT A
		Met Pro Lys Ile
		ORF
		Toxin gene

Fig. 2. Nucleotide and deduced amino acid sequences of botulinum type E nontoxic component gene. The putative Shine–Dalgarno sequence and the sequence homologous to –10 region of typical *E. coli* promoter are indicated by SD and TATA, respectively. The sequences identical to those of the botulinum type C nontoxic component (Tsuzuki *et al.*, 1992) are underlined. The amino acid sequence of the N-terminal region of type E nontoxic component reported by Somers & DasGupta (1991) is indicated by double underlining. Other features marked on the sequence are discussed in the text.

Progenitor toxin	Position	Amino acid sequence
Nontoxic component		
Type E	30–44	K A F O V A P N I W I V P E R
Type C	31–45	K A F K V A P N I W V A P E R
Neurotoxin		
Type A	34–48	K A F K I H N K I W V I P E R
Type C	35–49	K A F R I T G N I W V I P D R
Type D	35–49	K A F M I T O N I W V I P E R
Type E	31–45	K S F N I M K N I W I I P E R
Tetanus	30–44	K A F K I T D R I W I V P E R
Type B	35–49	K A F K I T D R I W I I P E R
Nontoxic component		
Type E	64–98	D S N F L S T N N E K D D F L O A T I K L L O R I N N N V V G A K L L
Type C	65–99	D S N F L S Q D S E R E N F L O A I I I L L K R I N N T I S G K Q L L
Neurotoxin		
Type A	74–108	D S T Y L S T D N E K D N Y L K G V T K L F E R I Y S T D L G R M L L
Type C	73–107	D P N Y L S T D S D K D T F L K E I I K L F K R I N S R E I G E E L I
Type D	73–107	D P S Y L S T D E Q K D T F L K G I I K L F K R I N E R D I G K K L I
Type E	70–104	D P N Y L Q S D E E K D R F L K I V T K I F N R I N N N L S G G I L L
Tetanus	74–108	D P N Y L R T D S D K D R F L Q T M V K L F N R I K N N V A G E A L L
Type B	75–109	D P D Y L N T N D K K N I F L O T M I K L F N R I K S K P L G E K L L
Nontoxic component		
Type E	102–113	S T A I P F P Y E N N T
Type C	103–114	S T A I P F P Y G Y I G
Neurotoxin		
Type C	111–122	S T D I P F P G N N N T

Fig. 3. Homology of deduced amino acid sequence among botulinum nontoxic components and neurotoxins. The sequence of type E nontoxic component is compared with those of botulinum type C nontoxic component (Tsuzuki *et al.*, 1992), and botulinum type A (Binz *et al.*, 1990a; Thompson *et al.*, 1990), type B (Whelan *et al.*, 1992a), type C (Kimura *et al.*, 1990), type D (Binz *et al.*, 1990b), type E (Fujii *et al.*, 1992; Poulet *et al.*, 1992; Whelan *et al.*, 1992b) and tetanus (Eisel *et al.*, 1986; Fairweather & Lyness, 1986) neurotoxins.

1162 amino acid residues. The type E nontoxic component is shorter than type C nontoxic component (1196 amino acid residues). Although the entire light chains of type E and type C neurotoxins are produced in *E. coli* MV 1184 cells harbouring recombinant plasmids of pU9EMH and pCL8, respectively (Kimura *et al.*, 1990; Fujii *et al.*, 1992), the mechanism of expression of the nontoxic component from these plasmids may be different. The type E nontoxic component is expressed in *E. coli* cells transformed with pU9EMH, but that of type C (pCL8) is never found (Tsuzuki *et al.*, 1992). It is possible therefore that there are some differences in the activity of the promoter regions between type E and type C nontoxic component genes, as recognized by *E. coli* RNA polymerase. Comparative studies revealed that there is very little similarity in these promoter regions. It is still unclear which nucleotide sequences are essential for the expression of genes in *C. botulinum*. For the expression of type E progenitor toxin in *E. coli* cells, the neurotoxin and the nontoxic component are produced or transcribed independently because the recombinant

plasmid pU9EH-RD1 (Fig. 1), which lacks 500 bp of the putative promoter region of *ent-120*, produces a 100 kDa N-terminal fragment of the type E neurotoxin. This is also confirmed by our previous finding that the 33 kDa fragment of the type E neurotoxin is expressed from a plasmid containing a 1 kb *EcoRI* 5'-region fragment of the type E neurotoxin gene (Fujii *et al.*, 1990). A similar result is obtained from the plasmid containing a 2.8 kb *HindIII* 5'-region fragment (1.9 kb of 5'-noncoding region and 0.9 kb of 5'-coding region) of the type C neurotoxin gene (unpublished data). However, it is clear that there is only 27 bp between the stop codon of the type E nontoxic component gene and the start codon of the type E neurotoxin gene. Therefore, it is likely that some nucleotide sequence in the nontoxic component gene may work as a promoter for the neurotoxin gene in *E. coli*. Such a promoter region has been proposed by Fujii *et al.* (1990) and Whelan *et al.* (1992b): the '–10' region located between nucleotides 3535 and 3540. However, such a sequence may not function as a promoter in *C. botulinum*. We suggest that the nontoxic

component and neurotoxin genes are transcribed by a polycistronic mRNA species initiated from a promoter located in the 5'-untranslated region of the nontoxic component gene in *C. botulinum*. These results obtained from the nontoxic components of types E and C contrast with the work of Binz *et al.* (1990a), who showed that the transcription of botulinum type A neurotoxin initiates from just upstream of the start codon of the toxin gene. Thus the transcription system may vary in each type of *C. botulinum*. Primer extension and Northern blot experiments using mRNA from *C. botulinum* type C and E strains will be necessary to locate the transcriptional start sites and the size of mRNA from the neurotoxin and *ent-120* genes.

Similarity between the type E and type C nontoxic components indicates that the nontoxic component may be highly conserved in *C. botulinum*, and it may play an important role. The N-terminal regions of both the nontoxic component and neurotoxins of type C showed high homology (Tsuzuki *et al.*, 1992). In the type E progenitor toxin, highly homologous regions are detectable between the nontoxic component and neurotoxin (Fig. 3). As the nontoxic component is closely associated with oral toxicity (Ohishi *et al.*, 1977; Ohishi & Sakaguchi, 1980), this component might act to protect neurotoxin from protease.

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