acteristics, CPF-G2Im matches well with the typical secondary structures of group II introns reported elsewhere (7, 44).

DISCUSSION

A unique *C. perfringens* strain, CPBC16ML, which showed a larger *cpa* gene by PCR, was isolated from a broiler chicken. Although nucleotide and amino acid sequence differences for the *cpa* gene of *C. perfringens* have been reported (12, 32, 39, 43), there is no previous report of a gene fragment inserted into the *cpa* gene. However, sequence analysis of the *cpa* gene demonstrated that it was not translated as an intact alpha toxin protein because several stop codons existed in the insertion fragment. Nevertheless, strain CPBC16ML showed both PLC and hemolytic activities. Two possibilities may help to explain these results. First, the truncated alpha toxin peptide from the 5′ terminus of *cpa* possesses both PLC and hemolytic activities; second, CPF-G2Im may be deleted from the flanking *cpa* gene by some mechanism, resulting in the ligation of the flanking *cpa* fragments.

It is known that the *C. perfringens* alpha toxin protein comprises two domains: the N-terminal domain of 246 amino acid residues carries the active site for phospholipid hydrolysis, and the C-terminal domain, consisting of the remaining 124 residues, is essential for lethality to mice and for hemolytic and sphingomyelinase activities (16, 30, 31). It has been reported that the C terminus of alpha toxin is devoid of enzymatic and toxic activities; however, a mutant of the alpha toxin which lacked the 121 amino acids from the C terminus retained PLC activity but abolished hemolytic activity (30, 31), suggesting that the interaction of the two domains is required for hemolytic activity (31). Therefore, expression of both the N-terminal and C-terminal domains of the alpha toxin produced by CPBC16ML was deduced.

To verify that the CPF-G2Im fragment was integrated into the *cpa* gene of strain CPBC16ML, Southern hybridization analysis was also performed. The results showed that the fragment is present in the *cpa* gene of CPBC16ML. Moreover, it appeared that the insertion fragment (CPF-G2Im) is also inserted into other parts of the genomic DNA of CPBC16ML, suggesting the presence of a mobile element.

cpa gene expression at the transcriptional level was examined by Northern hybridization. *C. perfringens* strain 13 was used as a control since its whole genome sequence has been determined. The three probes shown in Fig. 2 were designed for the gene expression analysis. When probe 1, consisting of *cpa* and CPF-G2Im, was used, three bands were detected for strain CPBC16ML (Fig. 5A). These bands may suggest that the mRNA of *cpa* was first transcribed together with CPF-G2Im as a primary transcript (about 3.0 kb) and then separated into two fragments (about 1.5 kb and 0.9 kb). Since *C. perfringens* strain 13 has no sequence similarity with CPF-G2Im, the smallest band (about 0.9 kb) detected for CPBC16ML, but not for strain 13, might be derived from CPF-G2Im, which was spliced out of the flanking *cpa* exons after transcription.

Sequence analysis of the RT-PCR product from the *cpa* gene amplified from CPBC16ML confirmed that CPF-G2Im was excised completely from the precursor mRNA. However, RT-PCR using a combination of primers designed for the CPF-G2Im sequence and the *cpa* ORF (capF2/cpaR10 and cpaF8/cpalphatox1-R, respectively) could not amplify any of the cDNAs (Fig. 5B). These results, as well as a very faint 3.0-kb band detected by Northern hybridization, may imply that CPF-G2Im has a very efficient splicing activity.

These findings suggest that CPF-G2Im is likely a mobile element. Although group II introns are easily recognized through an RT encoded by an ORF within the introns (24, 44), the fragment in our study does not contain any known RT sequence. Although an ORF encoding 44 amino acid residues could be predicted for the 3′-end region of CPF-G2Im, the short peptide had no similarity with any known proteins and is not likely a remnant of a known RT. A predicted group II intron RT gene sequence in *C. perfringens* was recently deposited in GenBank under accession number AB236336.1. This was found during complete sequencing of the enterotoxin-encoding pCPF4969 plasmid of *C. perfringens* (26). A BLAST search of CPF-G2Im indicated that two regions in CPF-G2Im (bp 1 to 661 and 715 to 834) show high similarity (95% and 97%, respectively) to two noncoding regions flanking the predicted group II intron RT gene sequence. This suggests that CPF-G2Im may be a group II intron which lacks an ORF encoding an RT and that it is likely a remnant of the plasmid group II intron. The results of secondary structure prediction analysis showed that CPF-G2Im has the typical secondary structure of group II introns. It consists of six helical domains emerging from a central wheel (Fig. 6A) and seems to be a bacterial group II class B2-like intron (7, 44). Several ORF-less introns have been reported among prokaryotes. The first was found in *Methanosarcina acetiborans*, and some introns are inserted into the ORFs of other introns to form nested organizations called twintrons (9). Recently, ORF-less group II introns inserted in a conserved protein-encoding gene have been reported for a *Cyanobacterium* sp. (23). Furthermore, an ORF-less intron has also been found in a putative conjugative plasmid, pAW63, in the *Bacillus cereus* group (46), although functional analysis has not yet been carried out. To our knowledge, the CPF-G2Im sequence detected in the conserved alpha toxin-encoding gene is the first ORF-less group II intron which is inserted into an ORF encoding a functional toxin protein in *C. perfringens*.

It is known that the efficient splicing or reverse splicing (mobility) of group II introns requires proteins to help the intron RNA fold into the catalytically active structure (9, 18, 29). CPF-G2Im was capable of self-splicing, but nevertheless, it does not encode any RT. It is not clear whether the ORF-less CPF-G2Im sequence can move autonomously by using its intrinsic self-splicing activity or whether it requires additional proteins, such as possible RTs, carried by the organism that may act in *trans*. For a better understanding of the expression of the alpha toxin in CPBC16ML, and also in order to exclude the possibility that the PLC and hemolytic activities were produced by another gene(s), the *cpa* gene carrying CPF-G2Im was cloned into a pQE30 vector and transformed into *E. coli* M15 cells. The transformants harboring the intact *cpa* gene showed a PLC with the normal molecular weight, even though the reactivity of the expressed protein against anti-PLC antibody was relatively weak. This demonstrated that the phospholipase C was also produced in *E. coli* cells, despite the presence of CPF-G2Im. It should be noted that gene expression occurred in both *C. perfringens* and *E. coli*, implying that the

splicing of CPF-G2Im may occur autonomously, using its intrinsic self-splicing activity alone, since *E. coli* M15 is derived from *E. coli* K-12, whose complete genome sequence has been determined (GenBank accession number NC_000913) and shows no evidence of group II introns being carried. However, it has been reported that group II introns can undergo splicing with the help of some proteins that function as RNA chaperones (15, 27) and/or other host-encoded protein splicing factors which are not related to intron-encoded proteins (33). Therefore, we cannot exclude the possibility that the splicing of CPF-G2Im may also be promoted by proteins with RNA chaperone activity or by other host-encoded splicing cofactors. It may be a rare occurrence, but the present study provides evidence for the existence of splicing-efficient bacterial group II introns. On the other hand, it remains unclear whether CPF-G2Im has the capacity for being mobile. The results of Southern hybridization with strain CPBC16ML demonstrated that CPF-G2Im is present in other parts of the genome (Fig. 3). Further studies are required to clarify the splicing mechanism and to determine the possibility of horizontal transfer of ORF-less group II introns.

BLAST analysis of CPF-G2Im showed another interesting finding. Four short fragments, ranging from 42 to 86 bp, with 90 to 96% sequence similarity to the Tn*554*-related transposase A (GenBank accession number AE017195) carried on plasmid pBC10987 of *B. cereus* ATCC 10987 were distributed in CPF-G2Im. The presence of transposase fragments within a catalytic intron RNA structure is very rare and unlikely. The possible significance of this finding remains unclear.

To date, the frequency of group II introns in *C. perfringens* remains unclear. Studies on the distribution and localization of group II introns, with or without RT gene sequences, in *C. perfringens* strains are ongoing in our laboratory. These studies may help to clarify not only the phylogenetic relationships among group II introns of bacteria but also the pathogenesis of *C. perfringens*.

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