Identification, characterization, and complete amino acid sequence of the conjugation-inducing glycoprotein (blepharmone) in the ciliate *Blepharisma japonicum*

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Conjugation in *Blepharisma japonicum* is induced by interaction between complementary mating-types I and II, which excrete blepharmone (gamone 1) and blepharismone (gamone 2), respectively. Gamone 1 transforms type II cells such that they can unite, and gamone 2 similarly transforms type I cells. Moreover, each gamone promotes the production of the other gamone. Gamone 2 has been identified as calcium-3-(2′-formylamino-5′-hydroxybenzoyl) lactate and has been synthesized chemically. Gamone 1 was isolated and characterized as a glycoprotein of 20–30 kDa containing 175 amino acids and 6 sugars. However, the amino acid sequence and arrangement of sugars in this gamone are still unknown. To determine partial amino acid sequences of gamone 1, we established a method of isolation based on the finding that this glycoprotein can be concentrated by a Con A affinity column. Gamone 1 is extremely unstable and loses its biological activity once adsorbed to any of the columns that we tested. By using a Con A affinity column and native PAGE, we detected a 30-kDa protein corresponding to gamone 1 activity and determined the partial amino acid sequences of the four peptides. To isolate gamone 1 cDNA, we isolated mRNA from mating-type I cells stimulated by synthetic gamone 2 and then performed rapid amplification of cDNA ends procedures by using gene-specific primers and cloned cDNA of gamone 1. The cDNA sequence contains an ORF of 305 amino acids and codes a possibly novel protein. We also estimated the arrangement of sugars by comparing the affinity to various lectin columns.

Conjugation in ciliates has been well studied. In conjugation, often old individuals are replaced by new ones with new genotypes. Many ciliates undergo asexual reproduction (binary fission) if provided with sufficient food. When cells are moderately starved, they transform into preconjugants and undergo sexual reproduction (conjugation) if they are sexually mature. The mechanism of induction of conjugation in ciliates has been studied for a long time in *Blepharisma*, *Paramecium*, *Euplotes*, *et al.* (1–4). However, the mechanism has not been well elucidated. In many cases, isolation of conjugation-inducing substances (gamones, mating pheromones, or mating substances) that induce mating-pair formation in conjugation is extremely difficult. In the ciliates, gamone 2 (blepharismone) in *Blepharisma japonicum* was the first identified conjugation-inducing substance (5). Thus far, in only three species, *B. japonicum*, *Euplotes raikovi*, and *Euplotes octocarinatus*, have the conjugation-inducing substances ever been isolated (see ref. 4 for review). In *E. raikovi* and *E. octocarinatus*, the genes of the substances have already been isolated (4, 6, 7). The presumptive receptors have been studied recently (8, 9), but the mechanism of how these substances induce conjugation is still under investigation.

The heterotrich ciliate *B. japonicum* is one of the primitive species of ciliates located in the root of divergence of ciliate phylogeny along with species of karyorelictides. *B. japonicum* retained many primitive features such as the way of macronuclear division (10, 11) and the nuclear changes in conjugation (12). It differs in this respect from *E. raikovi* and *E. octocarinatus*. The mechanism of conjugation in *B. japonicum* may reveal the origin of sexual reproduction in ciliates.

Conjugation of *B. japonicum* is induced by interaction between cells of complementary mating types (I and II; 13, 14). When the moderately starved sexually matured cells are mixed, mating-pair formation occurs after a few hours. The pair formation is induced by gamones secreted by each mating type (Fig. 1). Type I cells excrete gamone 1, which transforms mating-type II cells so that they can unite and at the same time induces them to produce and excrete gamone 2. This gamone in turn transforms type I cells so that they can unite, and it also promotes the production and excretion of gamone 1. Transformed cells can form heterotypic pairs (I–II) or homotypic pairs (I–I, II–II), but only heterotypic pairs complete conjugation.

Gamone 2 was isolated, identified as calcium-3-(2′-formylamino-5′-hydroxybenzoyl) lactate (5), and chemically synthesized (16–19). Gamone 1 (blepharmone) has also been isolated (20) and reported to be a glycoprotein with a molecular mass of about 20 or 30 kDa (by column chromatography and SDS/PAGE, respectively) consisting of 175 amino acids and 6 sugar residues (20, 21). However, this chemical composition was calculated based on the relative amounts of different residues measured with an amino acid analyzer (for amino acids and amino sugars) and gas-liquid chromatograph (for neutral sugars). Tryptophan was not measured for technical reasons. The above chemical composition of gamone 1 therefore is an approximation. Also, no information is available about the sequence of amino acids and the arrangement of sugar residues.

To elucidate the mechanism of induction of conjugation in *B. japonicum*, we characterized gamone 1 more precisely. We established a new method of gamone 1 isolation, and determined the partial amino acid sequences. On the basis of this result, we cloned gamone 1 cDNA and determined the full sequence. We also estimated how the 6 sugar residues are arranged in gamone 1 by comparing its affinity to various lectin columns.

Mating-type systems in ciliates are supposed to have evolved from a primitive binary system to a multiple system by duplication (4). The species *E. raikovi* and *E. octocarinatus*, in which the genes have been isolated, belong to the multiple mating-type system. This is the first case of isolation of a gene for a conjugation-inducing substance in a ciliate with the binary mating-type system.

Abbreviations: CFF, cell-free fluid; RACE, rapid amplification of cDNA ends.

Data deposition footnote: The sequence reported in this paper has been deposited in the DNA Data Bank of Japan (accession no. AB056696).

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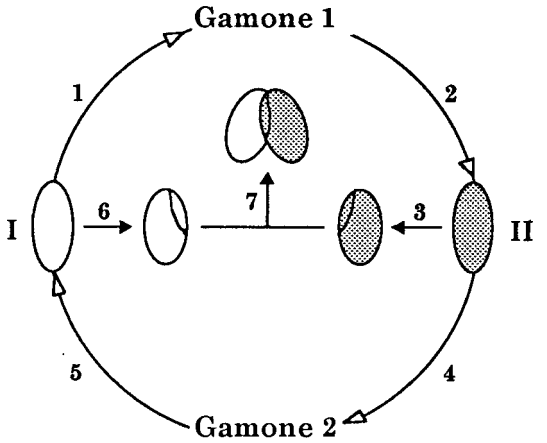

Fig. 1. Interaction of complementary mating-type cells (types I and II) by secreted gamones. Type I cells autonomously excrete gamone 1 (step 1). Gamone 1 specifically reacts with type II cells (step 2) and transforms them so that they can form cell unions (step 3) and at the same time induces the production and excretion of gamone 2 (step 4). Gamone 2 specifically reacts with type I cells (step 5) and transforms them so that they form cell unions (step 6) and enhances the production and excretion of gamone 1. A loop of positive feedback consists of steps 1, 2, 4, and 5. Transformed cells unite in pairs on contact (step 7). Modified from Miyake (15).

Materials and Methods

Cells and Preparation of Cell-Free Fluid (CFF). Mating-type I (strain R1072) cells were cultured in wheat grass powder (Pines, Lawrence, KS) medium (22), concentrated by mild centrifugation, washed with physiological balanced solution, synthetic medium for *Blepharisma* (SMB−), which is SMB III (23) not containing EDTA, and suspended in SMB− containing 320 units/ml synthetic gamone 2 and 0.01% BSA (BSA Fraction V, Sigma) at a density of 5,000–10,000 cells per ml. Gamone 2 was added to stimulate type I cells to promote secretion of gamone 1, and BSA was the protector of gamone 1 (20, 24). After the suspension was kept for 1 day at room temperature, the CFF was obtained by removing cells by mild centrifugation and filtration with a nylon net (5×5 μm). The cells were resuspended as described above, and the process was repeated again. CFF was stored at −85°C and thawed at 4°C before use for gamone 1 purification.

Mating-type II (strain T121) cells, which were used as tester cells for the gamone 1 activity assay, were suspended in SMB− at a density of 1,500–2,000 cells per ml.

Gamone 1 Activity Assay. The activity of gamone 1 was represented by unit and by index of pair formation (0–5). A unit of activity was defined as the smallest amount of gamone activity that could induce at least one face-to-face pair in 750–1,000 cells suspended in 1 ml of SMB−, and a unit was measured by the method described before (23, 24). An index of pair formation was determined on the basis of the ratio of the tester cells forming homotypic pairs and used for screening the peak of gamone 1 activity in many fractions. Then 500 μl of tester cells suspension (1,500–2,000 cells per ml) was added to a 500-μl sample in a well, and the ratio of pairs was judged after 3, 5, and 20 h. The index of pair formation (0–5) indicates no pairs (0), a few pairs (1), about half the cells forming pairs (3), most cells forming pairs (5). 2 and 4 were between 1 and 3, 3 and 5, respectively.

Purification of Gamone 1. We purified gamone 1 protein according to the our established method, which could identify one band corresponding to the activity of gamone 1 by SDS/PAGE (Fig. 2). First, CFF (85 ml, the activity of 6.5×10^4 units/ml) was filtered with a 0.2-μm DISMIC-25cs filter (Toyo Roshi, Tokyo),

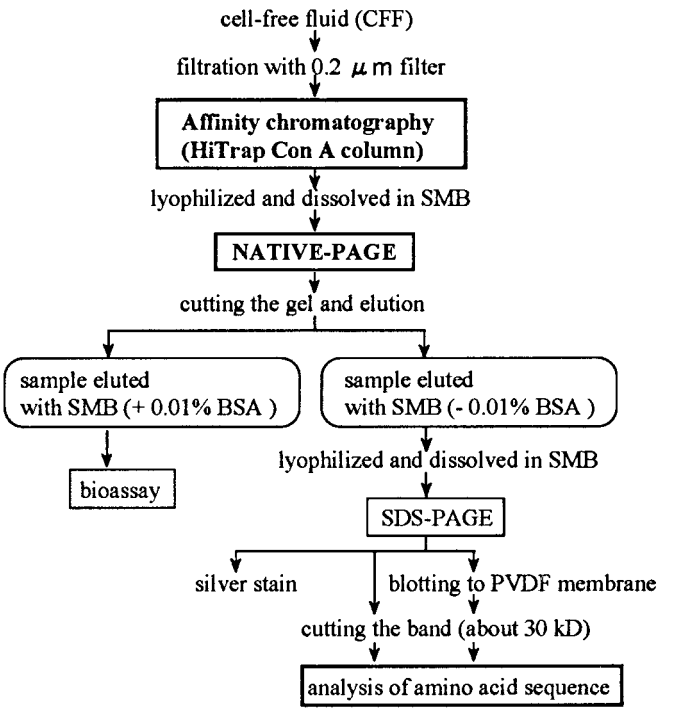

Fig. 2. The method of gamone 1 isolation established in this study.

applied to a HiTrap Con A column (Amersham Pharmacia) with a starting buffer (0.01% BSA in SMB−), and eluted with elution buffer [0.01% BSA and 0.5 M methyl-α-D-mannopyranoside (Sigma) in SMB−] by the HiLoad system (Amersham Pharmacia). The filtered CFF was separated to 61 fractions, and the fractions with high activity of gamone 1 were pooled, lyophilized, and dissolved in 3 ml of SMB−. The concentrated sample was mixed with native PAGE sample buffer [0.1 M Tris·HCl (pH 6.8), 20% glycerol, and bromophenol blue] and fractionated on 7% native polyacrylamide slab gel. The electrophoresed gel was cut, and proteins were extracted from each of the gel pieces according to the method described in the next section.

The extracted samples were measured for gamone 1 activity or lyophilized to concentrate and dissolved in small amounts of SMB− and further separated on 15% SDS/polyacrylamide gels. Proteins were visualized by silver staining.

Protein Extraction from the Electrophoresed Polyacrylamide Gel. All processes were carried out in a cold room (4°C) so as not to lose the activity of gamone 1. The electrophoresed gel, which was not stained, was cut into 5-mm sections from the top of the gel, and each was separated into two parts. They were cut into small pieces and put into separate test tubes. To one was added 2 ml of SMB− with 0.01% BSA for an activity assay, and to the other was added only SMB− for SDS/PAGE. The pieces of gel were crushed with the flat end of a glass rod, and each tube was kept at 4°C for 24 h to allow diffusion of proteins. Then, the sample was filtered with a nylon net (25×25 μm) and then was passed through a 0.2-μm DISMIC-25cs filter to remove the gel pieces completely.

Preparation of the Cells for Total RNA Extraction. *B. japonicum* has red pigments (blepharismin) that are in one of the extrusomes secreted by exocytosis in response to several stimuli. Because the process of extraction of RNA was often hampered by a trace of pigments, we prepared cells from which the pigment had been removed as described below. Mating-type I cell suspension was

prepared as indicated above, and type I cells were stimulated by gamone 2 treatment overnight. The activated type I cells were harvested by mild centrifugation, and the cell pellet was suspended in cold SMB− for 10 min to expel the red pigments. Then, the cells were washed with fresh SMB− and harvested by mild centrifugation.

Cloning of Gamone 1 cDNA. Type I cells that had their pigment removed and had been activated by gamone 2 were suspended in TRIzol reagent (Life Technologies, Grand Island, NY), and total RNA was isolated by the acid guanidinium-phenol-chloroform method. Then poly(A)$^+$ RNA was purified by oligo(dT)$_{30}$-latex beads with an Oligotex-dT30[SUPER] kit (Roche Molecular Biochemicals). Poly(A)$^+$ RNA (1 μg) was incubated with Power script reverse transcriptase (CLONTECH), and cDNA was synthesized with a SMART rapid amplification of cDNA ends (RACE) cDNA amplification kit (CLONTECH) according to the conditions suggested by the supplier. The synthesized cDNA was used as template for RACE PCRs. The gamone 1-specific primers were designed on the basis of the result of partial amino acid sequence analysis of the purified gamone 1 protein and universal primer mix supplied in the kit. After PCR the RACE PCR products were fractionated by electrophoresis in 0.8% agarose gel, purified from the gel, and cloned in pCR2.1-TOPO vector with the TOPO TA cloning kit (Invitrogen).

Estimation of Sugar Arrangement of Gamone 1. Gamone 1 is a glycoprotein containing 6 sugars (glucosamine$_3$, mannose$_3$; ref. 21). To determine the sugar arrangement, CFF of type I cells was applied to several lectin columns (lentil lectin, Con A, and wheat germ agglutinin). HiTrap lentil lectin and Con A columns (Amersham Pharmacia) were used, SMB− with 0.01% BSA was the starting buffer, and SMB− with 0.01% BSA and 0.5 M methyl-α-D-mannopyranoside was used for the elution buffer. In the case of the wheat germ agglutinin column, the elution buffer was SMB− with 0.01% BSA and 0.5 M *N*-acetylglucosamine (Nacalai Tesque, Kyoto). The fractions that were obtained were measured for gamone 1 activity, and the affinity of gamone 1 to the lectin was found. Sugar arrangement of gamone 1 was estimated by whether gamone 1 had an affinity to the examined lectins (25).

Results

Purification of Gamone 1. To determine the partial amino acid sequence of gamone 1, we established a new method of gamone 1 isolation based on the finding that this glycoprotein can be concentrated by using a Con A affinity column. Because gamone 1 is an unstable glycoprotein and loses its biological activity once adsorbed to any of the columns tested before, the introduction of the Con A column greatly facilitated the isolation. We also used native PAGE to remove BSA, which had been added as a protector of gamone 1. This method kept the high biological activity of gamone 1 in all steps, and the gamone 1 band corresponding to the activity could be identified by SDS/PAGE.

CFF of type I cells was applied to the Con A column and separated into 61 fractions. The gamone 1 activity detected in the fractions (43–58, an index of pair formation: 4–5) were pooled, lyophilized, and dissolved in SMB−. The concentrated sample was subjected to native PAGE. A part of the electrophoresed gel that was cut along the length of the gel was silver-stained. It showed that many proteins including BSA were smeared in the lower region, but some bands were detected in the upper region of the gel. The rest of the electrophoresed gel not stained was cut at intervals of 5 mm from the top of the gel, and the proteins were extracted from each gel piece. The extracted samples were evaluated for gamone 1 activity and separated by SDS/PAGE to identify the band of gamone 1. Gamone 1 activity was detected

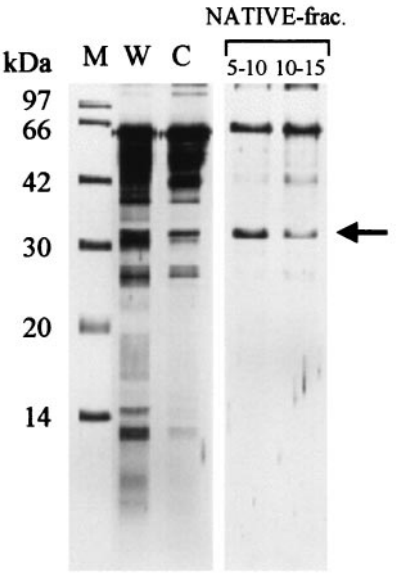

Fig. 3. Purification of gamone 1. CFF containing BSA (whole sample, lane W), the pooled active fraction of Con A column (lane C), and the extracts from gel pieces of native polyacrylamide gel (NATIVE-frac.) were electrophoresed in a 15% polyacrylamide gel and silver-stained. Native fractions that were obtained from gel pieces at the range of 5 to 10 mm (5–10) and 10 to 15 mm (10–15) from the top of the gel are shown here. The band of molecular mass at 30 kDa, corresponding to the gamone 1 activity, is indicated by an arrow. The band of ≈60 kDa is BSA, which was added to protect gamone 1. The protein molecular mass markers are in lane M.

in the range of 0–20 mm from the top. The highest activity was in the sample from the gel piece from 5 to 10 mm, and its activity was $\approx 3.3 \times 10^4$ units. The next highest was from 10 to 15 mm, and its activity was $\approx 8.2 \times 10^3$ units. We applied these samples to SDS/PAGE, and a clear band of slightly larger than 30 kDa was detected by silver staining corresponding to the intensity of the gamone 1 activity in these samples (Fig. 3). Because the molecular mass was consistent with previous reports (20), we concluded that this 30-kDa protein was gamone 1. The band was cut off and analyzed for its amino acid sequences. As a result, partial amino acid sequences of four internal peptides of gamone 1 were determined (Table 1). A band of ≈60 kDa was detected in all the extracted samples (Fig. 3). We considered that it was derived from remaining BSA, because a relatively large amount of BSA was added to keep gamone 1 activity in each step of purification. The recovery of gamone 1 was ≈20%. This method identified the gamone 1 band corresponding to its biological activity in all steps.

Cloning and Sequence Determination of Gamone 1 cDNA. To isolate gamone 1 cDNA, we designed specific primers on the basis of the partial amino acid sequences of the peptides of gamone 1 (Table 1). cDNA was synthesized by using poly(A)$^+$ RNA as a template prepared from the type I cells that had been stimulated by gamone 2. We carried out RACE PCR with various primer combinations, and an ≈500-bp product was amplified by 3′-RACE using the Gm_77/81 primer. The PCR product was purified, cloned, and sequenced by an ABI Prism Big Dye terminator cycle sequencing ready reaction kit. The nucleotide sequence analysis showed that the 3′-RACE product included sequences completely corresponding to the peptides Gm_75 and Gm_57. Thus, the product was regarded as a fragment of gamone 1 cDNA. We designed a gamone 1-specific primer based on the sequence of the fragment and carried out 5′-RACE PCR. An ≈500-bp product was amplified, and its deduced amino acid

Table 1. Partial amino acid sequences of gamone 1 and synthesized oligonucleotides for the isolation of gamone 1 cDNA

Gm_71	Gly-Val-Ser-Asn-Ile-Asn-Tyr-Asn-Arg-Trp-Ile-Ser-Phe-Tyr
	5′-AAY TAY AAY AGA TGG-3′
Gm_77/81	Ser-Met-Arg-Tyr-Asn-Phe-His-Glu-Ala-Leu-Asp-Leu-Val-Ser-Thr
	5′-ATG AGA TAY AAY TTY CAY GAR-3′
Gm_75	Val-Trp-Leu-Gly-Val-Tyr-Val-Ile-Gly-Ala-Tyr
Gm_57	Asp-Thr-Ser-Leu-Trp
	3′-CTR TGD AGD GAV ACC-5′
	3′-CTR TGD AGD AAY ACC-5′

Each amino acid sequence and oligonucleotide was given the name indicated on the left.

sequence included sequences completely corresponding to the peptides Gm_71 and Gm_77/81. Ultimately, we cloned four PCR products, determined those sequences, and connected the sequences at overlapping regions. The sequence of 948 bp was revealed as gamone 1 cDNA, and it coded 305 amino acids that included all sequences of peptides determined by amino acid sequence analysis (Fig. 4). The stop codon of this protein was TAA; TAG and TGA were not found in the reading frame. There has been no indication that the stop codon deviates in *B. japonicum* (26), unlike other ciliates such as *Tetrahymena* (27) and *Paramecium* (28, 29).

Estimation of Sugar Arrangement of Gamone 1. Oligosaccharides attached to glycoproteins are thought to have various important roles, for instance structural roles and information-bearing functions in cell–cell recognition and adhesion. Gamone 1 is a glycoprotein, and its composition of sugars ($glucosamine_3$ and $mannose_3$) was reported previously (21). However, the function of oligosaccharides that are attached to gamone 1 is not elucidated, and it is also unknown how the 6 sugars are arranged in gamone 1.

Protein-linked oligosaccharides can be classified into *O*- and *N*-linked oligosaccharides, which are divided into three major types: high mannose type, hybrid type, and complex type. Because these oligosaccharide types have different affinities to lectins, it is possible to estimate roughly their structure (25). We estimated oligosaccharide attached gamone 1 by examining the affinity of gamone 1 to the lectins (lentil lectin, Con A, and wheat germ agglutinin). It was revealed that gamone 1 had an affinity to Con A, but it had no affinity to lentil lectin or wheat germ agglutinin. This result suggested that the oligosaccharide of gamone 1 is an *N*-linked type without fucose modification in α1,6 linkage to the innermost *N*-acetylglucosamine residue. On the basis of this result and composition, the structure was estimated that one *N*-acetylglucosamine was added to the mannose residue of the end of the common core structure [Manα1–3 (Manα1–6) Manβ1–4 GlcNAcβ1–4 GlcNAc-Asn] of *N*-linked oligosaccharides. Gamone 1 is considered to have one very simple oligosaccharide.

Discussion

We isolated and determined the sequence of gamone 1 cDNA, the conjugation-inducing glycoprotein in *B. japonicum*. In this study we established a method of isolating gamone 1 by using Con A affinity chromatography and native PAGE and identified the gamone 1 band (≈30 kDa) by SDS/PAGE (Fig. 3). We determined the partial amino acid sequences of four peptides (Table 1). On the basis of the sequences, we designed oligonucleotides and performed RACE PCR by using them as gene-specific primers. PCR products were cloned and sequenced, and finally gamone 1 cDNA sequences of 948 bp were determined (Fig. 4). The sequence contains an ORF encoding 305 amino acids, and the deduced protein seems to have a cleavable signal peptide (amino acids 1–16). We also predicted the amino terminus of secreted gamone 1 by the analysis of amino acid sequences of gamone 1 protein (data not shown). As a result, it was concluded that the portion from Gly-34 to Trp-305 constitutes the secreted gamone 1. We assume that gamone 1 mRNA is translated as prepro-gamone 1 of amino acid 305, just like in the case of euplomone, the mating pheromones of *E. raikovi* (6, 8) and *E. octocarinatus* (7), and after cleavage of the secretory signal peptide prepro-gamone 1 is exported into the endoplasmic reticulum as pro-gamone 1 of amino acid 289, and pro-gamone 1 is processed proteolytically before being secreted as mature gamone 1 of amino acid 272. The putative molecular mass of mature gamone 1 deduced from the amino acid sequences was ≈30.7 kDa, and it is consistent with the result of SDS/PAGE.

Amino acid sequence comparisons of *E. raikovi* and *E. octocarinatus* prepro-pheromones showed that prepro domains were relatively conserved between two species (8). We compared the prepro domain of gamone 1 to these pheromones, but we found no significant homology. A homology search for other proteins revealed that gamone 1 was a previously uncharacterized protein.

Previously, the composition of amino acids of gamone 1 was reported to consist of 175 amino acids excluding tryptophan (21), but we showed here that the mature gamone 1 consisted of 272 amino acids including tryptophan. We compared these data about the composition of amino acids. The result suggested that (*i*) they were correlative in the composition, and (*ii*) the unique features of gamone 1, the very high tyrosine content, and the low glutamic acid content were conserved and rather emphasized in our data. We concluded that our data were consistent with the previous studies except for the number of amino acids.

Gamone 1 is the first glycoprotein in conjugation-inducing substances that has ever been isolated in ciliates. It is also a relatively large conjugation-inducing substance (272 amino acids) compared with those in *E. raikovi* (≈40 amino acids) and *E. octocarinatus* (about 80–100 amino acids). The result of deduced amino acid sequences of gamone 1 showed that it had four possible sites that could be linked to *N*-linked oligosaccharide. According to the composition of sugars, one oligosaccharide seems to attach to gamone 1 in one of these sites. In this study, we estimated the structure of oligosaccharide. Whether the oligosaccharide attached to gamone 1 is essential for conjugation-inducing activity remains for further investigation. The function may be examined by using gamone 1 without oligosaccharide produced by enzymatic treatment.

To investigate the signaling pathway of induction of conjugation in *B. japonicum*, here we characterized the ligand molecule (gamone 1). Next, it is necessary to search for the receptor molecule. In *B. japonicum*, by comparing the activity of synthe-

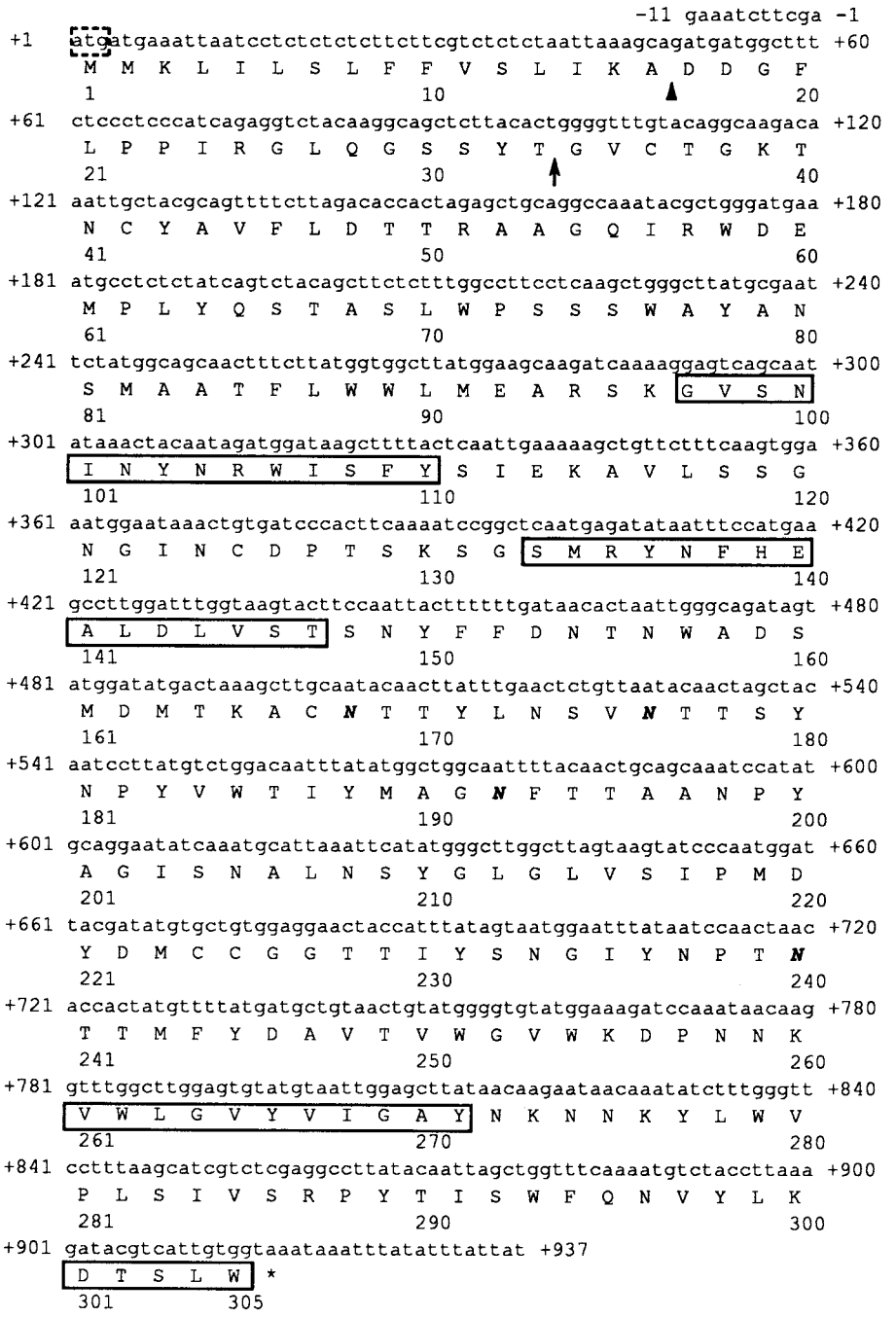

Fig. 4. cDNA sequence and deduced amino acid sequence of gamone 1. The numbering at both ends of the nucleotide sequence indicates nucleotide residues and is shown as + or − relative to the translation initiation codon (a dashed box). The numbering below the amino acid sequence indicates amino acid residues. Solid boxes indicate the amino acid sequences corresponding to the result of amino acid analysis of gamone 1. The stop codon of gamone 1, TAA, is indicated by an asterisk. The arrowhead indicates the presumptive signal peptide cleavage site, and the arrow indicates the putative amino terminus of the secreted gamone 1. The asparagine residues (168, 176, 192, and 240) that are possible attachment sites for *N*-linked oligosaccharides are shown in italics.

sized gamone 2 derivatives, the relative importance of various parts of gamone 2 molecule for gamone activity were examined, and the binding site of gamone 2 to the hypothetical receptor was suggested (18, 30). By using ^{125}I-labeled gamone 2, a relative binding affinity of gamone 2 to type I cells fixed with formaldehyde and glutaraldehyde has been examined (31). Experimental data on the receptor molecule for the mating pheromone (euplomone) in *E. raikovi* have been reported also (9). The *E. raikovi* mating pheromone (euplomone) promotes cells mitotic proliferation (asexual proliferation) and mating-pair formation for conjugation by binding cells in an autocrine-like or paracrine-like manner. The genes encoding these pheromones generate at least two distinct molecules by alternate splicing. One is the soluble pheromone, the other is a pheromone isoform that remains anchored to the cell surface (membrane-bound pheromone isoforms). It has been suggested that these isoforms are effective pheromone binding sites or receptors. In *B. japonicum*, membrane-bound gamone isoforms are unknown. Moreover,

presumptive receptor molecules against gamones are considered to be produced by complementary mating-type cells (1, 4, 23). Identification of receptors of gamones will contribute greatly to elucidate the mechanism of induction of conjugation in *B. japonicum*.

There are three advantages to studying the conjugation in *B. japonicum*. (*i*) *B. japonicum* has a simple binary mating-type system consisting of only two types (types I and II) of cells. Thus the conjugation-inducing substances are of two types (gamones 1 and 2, secreted by type I and II cells, respectively). (*ii*) It is interesting that *B. japonicum* uses very different molecules, gamone 1, a temperature-sensitive glycoprotein, and gamone 2, a tryptophan-derived small molecule, as conjugation-inducing substances. In some species of *Blepharisma* (*B. stoltei, B. americanum, B. musculus, B. tropicum* and *B. japonicum*), gamone 1 seems to be species-specific, whereas gamone 2 is common in all of the five species (32). Gamones of ancestral ciliates are assumed to have been probably small molecules such as blepharismone (gamone 2). *Blepharisma* is suggested to have retained the most primitive type of gamones (4). Mating types in ciliates are classified to the four classes, binary and gamone-excreting, binary and gamone-carrying, multiple and gamone-excreting, and multiple and gamone-carrying. It is supposed that the most primitive of the four classes of mating-type systems is the binary and gamone-excreting system, and the mating-type system in ciliates has evolved from this system to a multiple system (4). *Blepharisma* belongs to the most primitive mating-type classes, the binary and gamone-excreting system. Therefore, *Blepharisma* occupies an important position in discussions of not only induction of conjugation but also the evolution of mating-type systems. (*iii*) There has been no report that the stop codon deviates in *B. japonicum* (26), unlike other ciliates that use specialized stop codons (27–29). Therefore, it should be possible to express *B. japonicum* genes in bacterial cells without any alteration of stop codons.

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