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Cloning and Sequencing of the Gene for a *Tetrahymena* Fimbrin-Like Protein[1]

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***Tetrahymena* F-actin–binding protein, which induces bundling of *Tetrahymena* F-actin, was localized to a division furrow during cytokinesis. We report here the cloning and characterization of the gene and cDNA of a *Tetrahymena* F-actin–binding protein. The cDNA encodes a protein comprising 579 deduced amino acids with a calculated molecular mass of 65.9 kDa. The predicted amino acid sequence shares 37.7, 41.8, and 39% identity with the sequences of yeast fimbrin, *Arabidopsis thaliana* fimbrin, and *Dictyostelium discoideum* plastin, respectively. The *Tetrahymena* F-actin–binding protein also shares two actin-binding domains previously identified in the fimbrin/plastin family, but lacks the EF-hand Ca^{2+}-binding motif, suggesting that this protein is a novel-fimbrin–like protein in *Tetrahymena*. Moreover, we cloned a genomic DNA encoding the *Tetrahymena* fimbrin-like protein and performed Southern and Northern hybridizations. The results indicate that the genomic DNA possesses 9 introns and that both the gene and transcript of *Tetrahymena* fimbrin-like protein are single. Thus, we suggest that *Tetrahymena* fimbrin-like protein localizes to the division furrow and probably cross-links actin filaments in a Ca^{2+}-insensitive manner during cytokinesis.**

Key words: cytokinesis, F-actin bundling, fimbrin/plastin family, RACE, *Tetrahymena*.

Animal cells are divided by cytokinesis through constriction of the contractile ring, which is mainly composed of actin filaments and myosin. The force for constriction is generated through actin–myosin interaction (*1–4*). The formation and disappearance of the contractile ring are considered to be due to the actions of actin-modulating proteins that are present in the division furrow region.

The ciliate *Tetrahymena* is one of the best experimental systems for studying the mechanism of cytokinesis; (i) *Tetrahymena* cells exhibit synchronous division induced by heat treatment (*5*), and (ii) cell-division-arrest mutants (*cda* loci) have been isolated and partially characterized (*6–13*). The contractile ring structure in *Tetrahymena* is like that in animal cells, and is composed of several division-associated structures (*14*). One of these structures is the lateral stripe, which bundles the contractile ring microfilaments (*12*). The lateral stripe may be involved in the contractile ring organization, and one of its components corresponds to a kind of F-actin–binding protein. Therefore, to understand the dynamic properties of the contractile ring, it is important to elucidate the biochemical properties and the biological roles of the F-actin–binding proteins found in the division furrow.

To ascertain the functions of the actin-binding proteins, we had tried to isolate F-actin–binding proteins as contractile ring structure proteins. Recently, we succeeded in the isolation of a 60-kDa F-actin–binding protein from *Tetrahymena pyriformis* using an F-actin affinity column, and determined its biochemical properties and its localization during the cell cycle (*15*). An N-terminal 21–amino acid sequence of one of the fragments released by cyanogen bromide digestion showed homology with an actin binding-site (amino acid residues 260–281) of yeast fimbrin. An antibody prepared against a synthesized 21-mer oligopeptide reacted with a 71-kDa protein in *T. pyriformis* cell extract, suggesting that the 60-kDa protein was a digested product of the 71-kDa protein and this partial digestion occurred during the isolation step. The 60-kDa fragment of the 71-kDa protein bound to *Tetrahymena* F-actin and induced the bundling of *Tetrahymena* F-actin. Indirect immunofluorescence microscopy showed colocalization of the 71-kDa protein and actin in the oral apparatus and the deep fiber bundles in *T. pyriformis*. On the other hand, in *T. thermophila*, the 71-kDa protein was localized in the oral apparatus and the contractile vacuole pores during interphase. At the dividing stage, the 71-kDa protein was localized in the division furrow. Therefore, the 71-kDa protein seems to associate with the actin cytoskeleton and to play an important role in actin filament organization during phagocytosis and cytokinesis in *Tetrahymena*. We tried to isolated the intact 71-kDa protein from *Tetrahymena*, but failed because of proteolytic digestion.

To further understand the molecular structure of the 71-kDa protein, we have cloned and sequenced a full length

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Abbreviations: AUAP, abridged universal amplification primer; GSP, gene specific primer; MP, maximum parsimony; NJ, neighbor joining; RT, reverse transcription; RACE, rapid amplification of cDNA ends; T.t., *Tetrahymena thermophila*; UTR, untranslated region.

cDNA encoding it. We report here that the primary structure of the 71-kDa protein has high sequence homology with that of fimbrin/plastin family proteins. Fimbrin/plastin family proteins have two EF-hand Ca^{2+}-binding motifs and two actin-binding domains, whereas the 71-kDa protein has only two actin-binding domains. These results suggest that the 71-kDa protein is a novel fimbrin/plastin family protein in *Tetrahymena*.

MATERIALS AND METHODS

Cell Culture—Tetrahymena thermophila strain B was axenically cultivated in medium containing 1% proteose peptone, 0.5% yeast extract, and 0.87% dextrose at 26°C until early stationary phase (*16*).

Isolation of Total RNA, Poly(A)$^+$ RNA, and Genomic DNA—Total RNA was isolated from *T. thermophila* using ISOGEN (Wako, Tokyo) and Poly(A)$^+$ RNA was isolated from total RNA using oligotex dT30 super (Takara, Tokyo). Genomic DNA was isolated from macro- and micronuclei of *T. thermophila* prepared by the method of Mita *et al.* (*17*).

Cloning and Sequencing of the 71-kDa Protein cDNA—Based on the 21-amino acid sequence of the 71-kDa protein (Fig. 1) and biased codon usage of *Tetrahymena* (*18*),

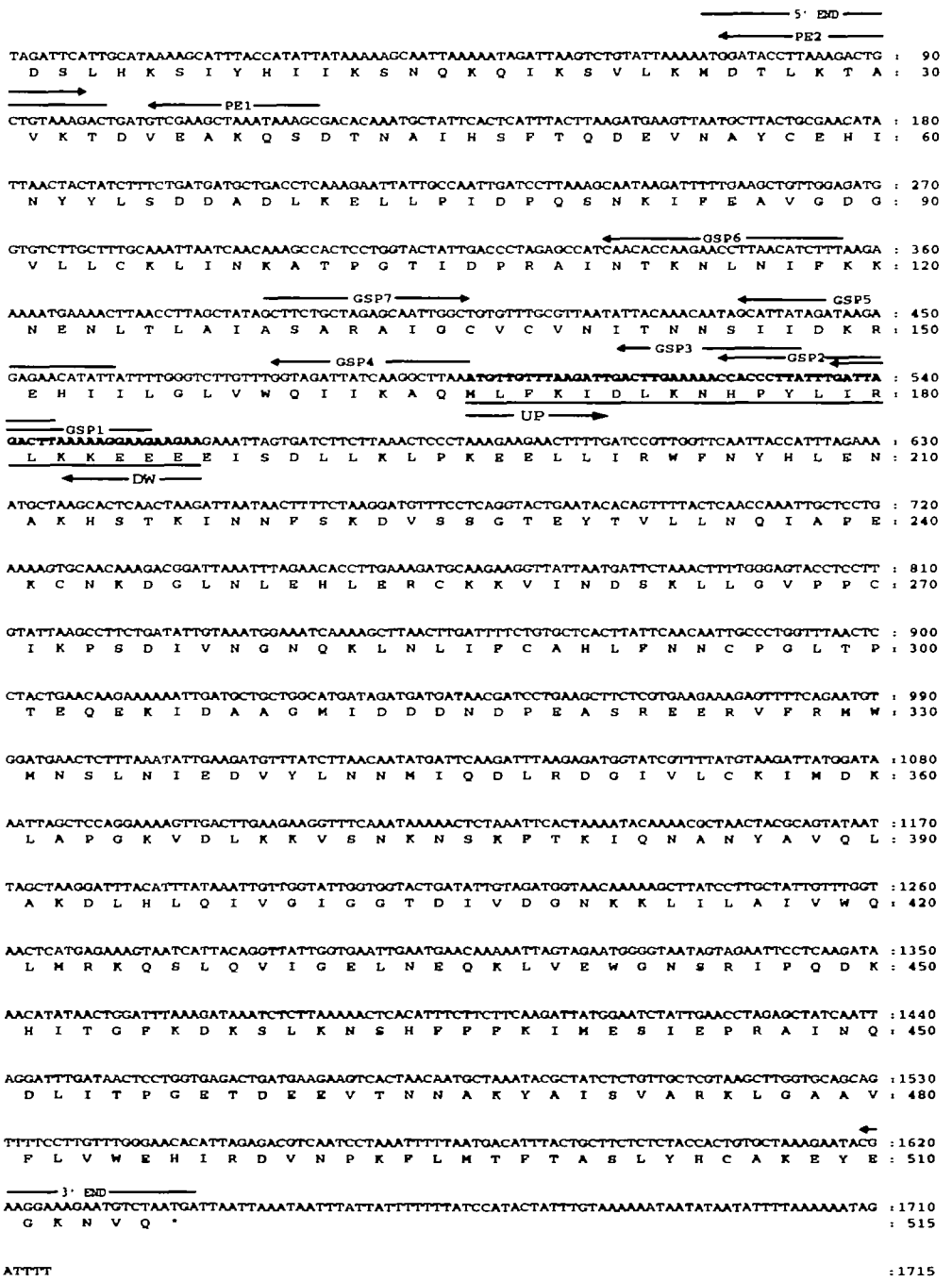

Fig. 1. **The nucleotide and deduced amino acid sequences of a partial cDNA of the 71-kDa protein.** The asterisk (*) denotes the termination codon and the arrows indicate the positions of primers used for 5′ RACE, 3′ RACE, PCR, and primer extension (see "MATERIALS AND METHODS"). The amino-terminal 21-amino acid sequence of the CNBr fragment of the 71-kDa protein is underlined.

two degenerated primers containing *Bam*H1 sites were synthesized. The upstream primer was 5′-GGATTCATGT-TYTTYAARAT-3′ and downstream primer was 5′-GGAT-TCTTCTTCTTRYTTYTT-3′. the Poly(A)$^+$ RNA (1 µg) was reverse transcribed to cDNA with Superscript II reverse transcriptase (Life Technologies, Gaithersburg, MD, USA). PCR was performed for 30 cycles using the two degenerated primers with ExTaq DNA polymerase (Takara, Tokyo) at 94°C for 1 min, at 37°C for 1 min, and at 72°C for 1 min with cDNA as the template. The amplified DNA fragment was subcloned into pBluescript cloning vector and nucleotide sequence was determined by the dideoxy chain termination method (*19*) using an ABI Prism-DNA sequencer 377 (Perkin-Elmer, Foster City, CA, USA). For cloning the 5′ end of the cDNA encoding the 71-kDa protein, 5′ rapid amplification of cDNA ends (5′ RACE) was performed twice using a 5′ RACE amplification kit (Life Technologies, Gaithersburg, MD, USA) (*20, 21*). Poly(A)$^+$ RNA (1 mg) was reverse transcribed to first strand cDNA at 42°C for 1 h with 100 units of Superscript II reverse transcriptase using gene specific primer 1 (GSP1) or GSP4 (Fig. 1). After polyC tailing by terminal deoxy transferase, the first PCR was performed for 30 cycles using anchor primer (5′-GGCCAC-GCGTCGACTAGTACGGGIIGGGIIGGGIIG-3′) and GSP2 or the anchor primer and GSP5 with ExTaq DNA polymerase at 94°C for 1 min, at 59°C for 1 min, and at 72°C for 2 min with first strand cDNA containing the 3′ anchor sequence as the template. A second nested PCR was performed with the first PCR product using AUAP (5′-CGC-CACGCGTCGACTAGTAC-3′) and GSP3 or AUAP and GSP6 as templates. Conditions of the second nested PCR were the same as those of the first PCR. The amplified DNA fragments were subcloned into pCR2.1 vector by the TA cloning method (Invitrogen, San Diego, CA, USA) and nucleotide sequences were determined with an ABI Prism-DNA sequencer 377. For cloning of the 3′ end of the cDNA encoding the 71-kDa protein, 3′ rapid amplification of cDNA ends (3′ RACE) was performed. Poly(A)$^+$ RNA (1 mg) was reverse transcribed to the first strand cDNA at 42°C for 1 h with 200 units of Superscript II reverse transcriptase using 50 ng of 3′ RACE adapter primer (Life Technologies, Gaithersburg, MD, USA). PCR was performed for 30 cycles using GSP7 (Fig. 1) and AUAP with ExTaq DNA polymerase at 94°C for 1 min, at 55°C for 1 min, and at 72°C for 3 min with the first strand cDNA with 5′ adapter sequence as the template. The amplified DNA fragment was subcloned into pCR2.1 vector and its nucleotide sequence was determined.

Primer Extension Experiment—For cloning the 5′ untranslated region of the 71-kDa protein gene, cassette PCR was performed as described by Isegawa *et al.* (*22*). PCR was performed for 30 cycles using cassette primer (5′-GTA-CATATTGTCGTTAGAACGCG-3′) and PE2 (Fig. 1) with ExTaq DNA polymerase at 94°C for 1 min, at 55°C for 1 min, and at 72°C for 3 min with the *Eco*RI cassette (Takara) ligated to genomic DNA digested with *Eco*RI as the template. Primer extension was performed as described by McKnight and Kingsbury (*23*), with the following modifications. The primers, PE1 (5′-GCTTTATTTAGCTTCGAC-3′) and PE2 (5′-GTCTTTACAGCAGTCTTTAAGGTATCC-3′) were labeled with T4 polynucleotide kinase (Wako, Tokyo). Labeled primer (2 × 10^5 cpm) was added to 5 mg poly(A)$^+$ RNA. The mixture was incubated at 70°C for 10 min and allowed to cool on ice. Reverse transcription was carried out with 100 units of Rev-tra Ace (Toyobo, Tokyo) at 50°C, and the primer extension products were subjected to electrophoresis in a 6% sequencing gel.

Analysis of the Putative Amino Acid Sequence and Construction of a Phylogenetic Tree—Alignment of the deduced

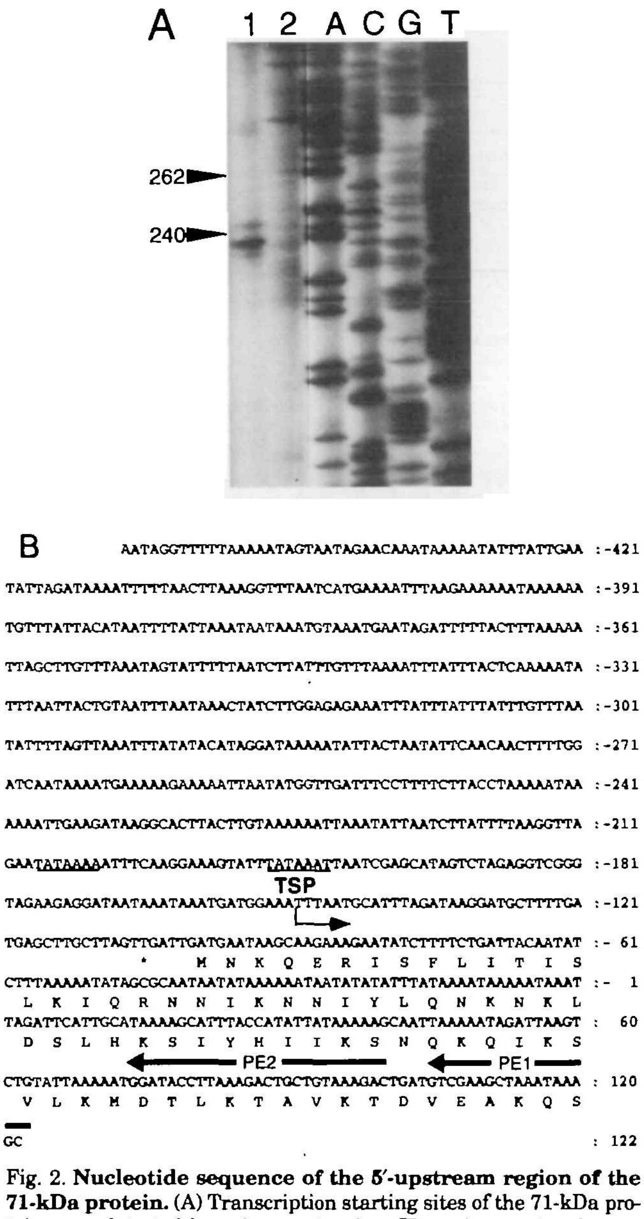

Fig. 2. **Nucleotide sequence of the 5′-upstream region of the 71-kDa protein.** (A) Transcription starting sites of the 71-kDa protein gene detected by primer extension. The primer extension experiment was performed using control RNA (yeast rRNA) with PE1 primer and PE2 primer or *Tetrahymena* poly(A)$^+$ RNA with PE2 primer (lane 1) and PE1 primer (lane 2). Candidate transcription sites are shown with both nucleotide numbers and arrowheads. For reference, sequence ladders (A, C, G, T) using the same ^{32}P-labeled primer as used in the primer extension method are represented in the rightmost 4 columns. (B) The nucleotide sequence of the 5′-upstream region. An arrow indicates the transcription start point (TSP) determined by the primer extension experiment. The asterisk (*) denotes the termination codon. The amino acid sequence is indicated in the coding region. TATA box consensus sequences are underlined.

amino acid sequence of the 71-kDa protein with other characterized members of the fimbrin/plastin family was performed using the GENETYX program Ver. 10 (Genetic Information Processing Software, Software Development, Tokyo), making minor adjustments by eye to optimize alignments. Phylogenetic analysis was carried out using the neighbor-joining distance matrix (NJ) method of Saitou and Nei (*24*) and the maximum persimony (MP) method of Eck and Dayhoff (*25*). A phylogenetic tree was constructed using PROTDIST, NEIGHBER, and PROTPARS in PHYLIP (Phylogeny Inference Package ver3.5c, University of Washington, Seattle; Felsenstein). Bootstrap analysis was carried out by the method of Felsenstein (*26*). The NJ and MP methods were performed on alignment in PHYLIP 100 times with the input order randomized.

Cloning and Sequencing of the 71-kDa Protein Genomic DNA—The 5′ end and 3′ end primers (shown in Fig. 1) for PCR were designed from the nucleotide sequence of 71-kDa protein cDNA. PCR was performed for 30 cycles using these primers with ExTaq DNA polymerase at 94°C for 1

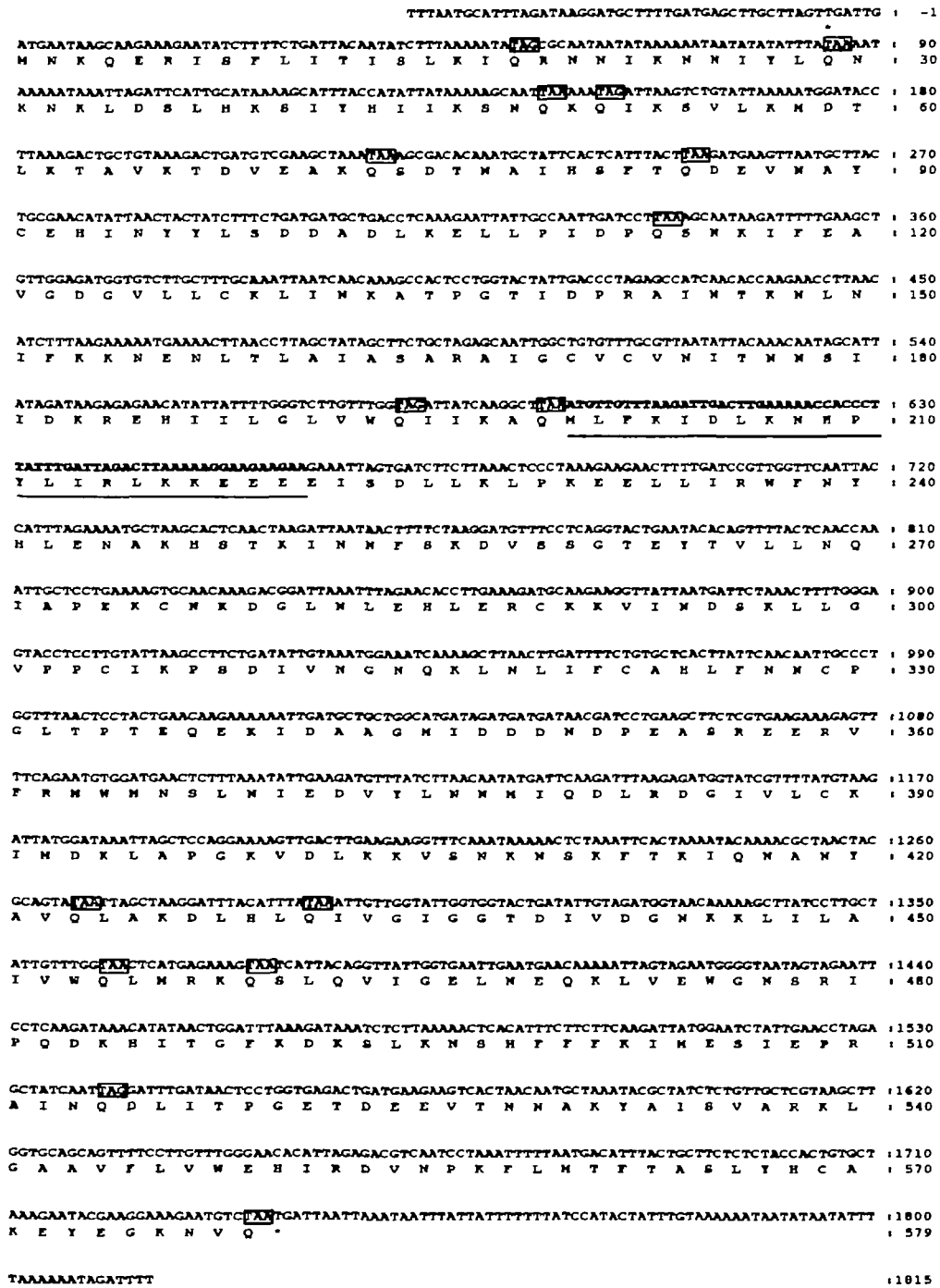

Fig. 3. **The nucleotide and deduced amino acid sequences of the 71-kDa protein cDNA.** The asterisk (*) denotes the termination codon; the amino-terminal 21–amino acid sequence of the CNBr fragment of the 71-kDa protein is underlined. Boxes indicate 11 triplet codon TAAs and 4 TAGs encoding glutamine. The sequence has been deposited in the DDBJ/EMBL/GenBank nucleotide sequence database under the accession number AB024706.

min, at 37°C for 1 min, and at 72°C for 1 min with genomic DNA as the template. The amplified DNA fragment was subcloned into pCR2.1 vector and its nucleotide sequence was determined by the dideoxy chain termination method.

Southern and Northern Hybridization—Genomic DNA (9 μg) digested with *Eco*RI was electrophoresed in a 0.8% agarose gel, transferred to a GeneScreen Plus membrane (NEN Life Sciences Products, Boston, MA, USA), and hybridized with the ^{32}P-labeled genomic DNA (+172 to +2948, 1.0×10^6 cpm/ml) as a probe. Total RNA (10 μg) and Poly(A)$^+$ RNA (2 μg) were electrophoresed in a 0.8% formaldehyde denaturing agarose gel, transferred to a GeneScreen Plus membrane, and hybridized with the ^{32}P-labeled PCR product (5.0×10^5 cpm/ml) using GSP7 and AUAP primers as probes. Southern and Northern hybridizations were carried out under the same conditions as described by Takemasa *et al.* (*27*).

RESULTS

Cloning and Sequencing of the cDNA Encoding the 71-kDa Protein—The probe used to screen the *T. thermophila* cDNA library was prepared by RT-PCR using two oligonucleotide primers (shown in "MATERIALS AND METHODS"). The obtained RT-PCR product (75 bp) was subcloned into pBluescript cloning vector and sequenced (Fig. 1, underline). The deduced amino acid sequence was highly homologous to fimbrin/plastin family proteins of other sources. Using this RT-PCR product as the probe, a λgt10 *T. thermophila* cDNA library was screened, but no positive clones were isolated. Then, 5′ RACE was carried out to clone the 5′

TABLE I. **Amino acid identities of some fimbrin/plastin family proteins.**

VS *Tetrahymena*	Whole	A	B
Yeast fimbrin	37.7%	45.9%	38.6%
Arabidopsis fimbrin	41.8%	45.6%	40.7%
Dictyostelium plastin	39.0%	50.2%	37.1%
Rat T-plastin	38.0%	42.3%	37.7%
Human T-plastin	37.9%	42.3%	37.3%
Chick fimbrin	35.5%	43.1%	35.0%
Drosophila fimbrin	35.4%	41.0%	36.2%

The 422 amino acids of the *Tetrahymena* protein sequence are compared. A and B indicate the two actin-binding domains.

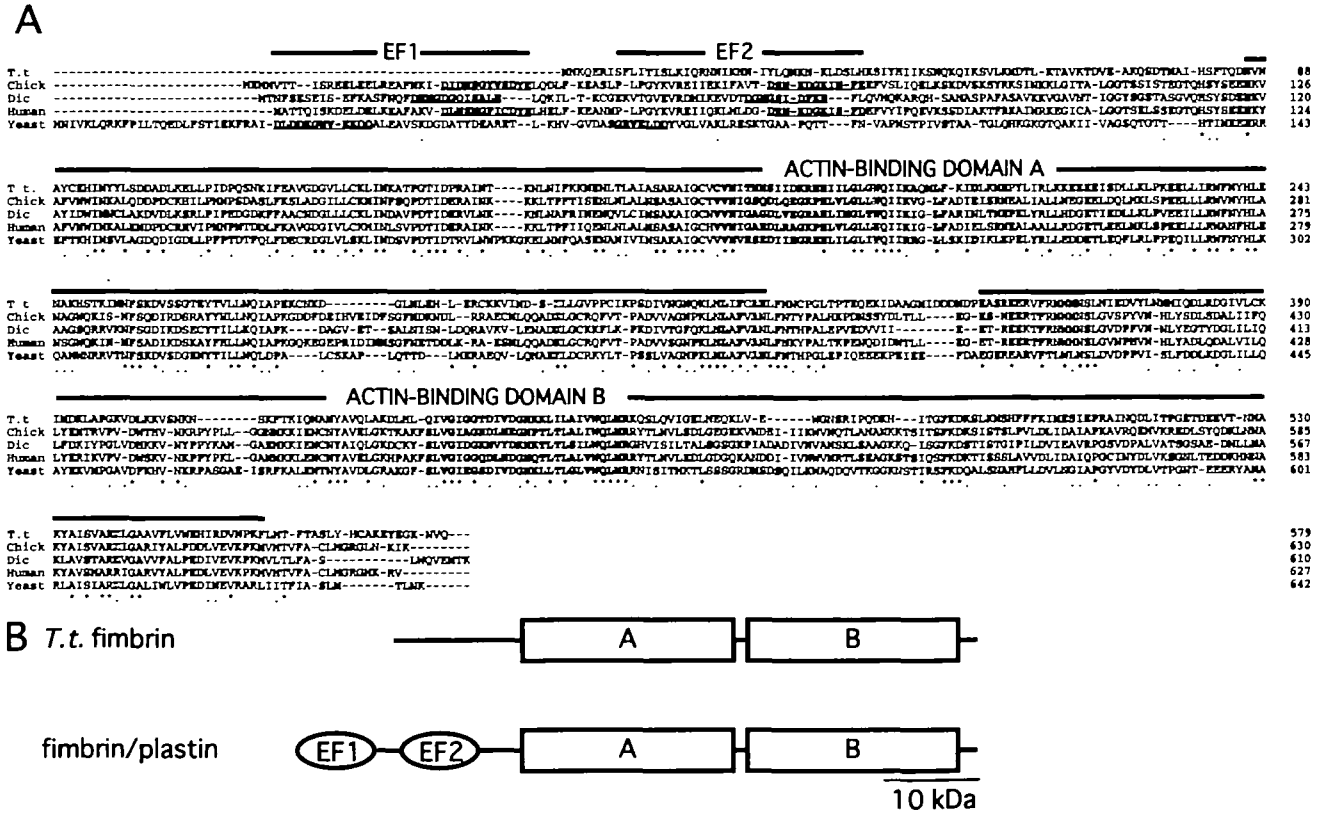

Fig. 4. **Comparison of the cDNA-derived amino acid sequence of the 71-kDa protein with those of the fimbrin/plastin family.** (A) Alignment of the deduced amino acid sequence of *Tetrahymena* fimbrin-like protein (T.t.) with that of chicken brush border fimbrin (Chick; accession number, A37097), *Dictyostelium discoideum* plastin (Dic; P54680), human T-plastin (Human; P13797), and yeast fimbrin (Yeast; P32599). Amino acid sequences are aligned and gaps (-) are introduced to maximize homology. Identical amino acids are marked by asterisks (•), and similar amino acids by dots (•). Lines on top of the sequence indicate two Ca^{2+}-binding motifs (EF1 and EF2) and two actin-binding domains of the fimbrin/plastin family. Bold letters with underlines indicate Ca^{2+}-binding domains in EF1 and EF2. Bold letters in the two actin-binding domains indicate a conserved stretch of 27 amino acids in each of the two actin-binding domains. Outlined letters indicate amino acid residues involved in the interaction between actin and the yeast fimbrin homologue, Sac6p. (B) A diagram of the modular arrangement of domains in the 71-kDa protein and fimbrin/plastin. EF1 and EF2 identify the EF-hand Ca^{2+}-binding motifs. A and B regions indicate the actin-binding domains.

end of the 71-kDa protein cDNA, and 200-bp fragments were obtained by the first 5′ RACE. The first 5′ RACE product contained neither a starting codon nor the 5′ UTR sequence, so a second 5′ RACE was tried with GSP4, GSP5, and GSP6. The second 5′ RACE amplified 356-bp fragments, but the 5′ UTR and a starting codon were not detected in this sequence. In order to clone the 3′ end of the 71-kDa protein cDNA, 3′ RACE was performed and a 1,329-bp fragment was obtained. Those nucleotide sequences were combined by assembling the sequences of 5′ and 3′ RACE products. The combined nucleotide sequence contained 1,715 nucleotides with a partial open reading frame of 1,637-bp, but this open reading frame did not contain an initiation codon or 5′ upstream region (Fig. 1). Therefore, it is necessary to determine the nucleotide sequence of the 5′ upstream region and the transcription starting sites.

Transcription Starting Sites of the 71-kDa Protein Gene—To examine the transcription starting sites of the 71-kDa protein gene, the nucleotide sequence of the 5′ upstream region was determined by cassette PCR (−708 to +101; Fig. 2B), and then the transcription starting site(s) and the position of the putative TATA box(es) were investigated. The transcription starting sites of the 71-kDa protein were determined by primer extension experiments. As shown in Fig. 2A, a 240-bp primer extension product was obtained using PE2 as the primer (+72 to +100). On the other hand, using the PE1 primer (+104 to +122), a 262-bp extension product was obtained. If the number of initiation starting sites is one, the difference in the length between the primer extension product using the PE2 primer and that using the PE1 primer is 22 bp. Therefore, the transcription starting site may be located 140-bp upstream (Fig. 2B). Since a termination codon TGA is located 106-bp upstream, the ATG located 100-bp upstream may be the initiation codon (Fig. 2B). Three TATA box consensus sequences (TATAT/AAT/A) (*28*) were found 107 to 137 bp upstream from the initiation codon ATG (Fig. 2B). The sites of the putative TATA boxes correspond to 67 to 97 bp upstream from the transcription starting site (Fig. 2B).

The 71-kDa protein cDNA contains 1,855 nucleotides with a putative open reading frame of 1,737-bp, a 5′-untranslated stretch of 40-bp, and a 3′-untranslated region of 78 bp (Fig. 3). No poly(A) stretch was found at the end of the nucleotide sequence (Fig. 3). The open reading frame of this isolated cDNA clone contains 579 deduced amino acids with a calculated molecular mass of 65,926 Da. The amino-terminal 21–amino acid sequence of the CNBr fragment of the 71-kDa protein (*15*) corresponds to the sequence of amino acid residues 200–220 in the open reading frame (Fig. 3). The 19 amino acids in the amino-terminal 21–amino acid sequence are identical to residues 200–220. The difference between these two amino acid sequences (L201, E218) may be caused by an error in amino acid sequencing (*15*). Although there are 11 TAA and 4 TAG codons within the open reading frame, codons that are usually recognized as universal stop codons, they have been proved to code for glutamine in *Tetrahymena* (*18*).

Sequence Comparison between the 71-kDa Protein and Fimbrin/Plastin Family—A search of sequence data bases (GenBank and NBRF) showed that the 71-kDa protein shares 36–42% sequence identity with fimbrin/plastin family proteins such as yeast fimbrin (37.7%) (*29*), *Arabidopsis thaliana* fimbrin (41.8%) (*30*), *Dictyostelium discoideum* plastin (39%) (*31*), rat T-plastin (38%), human T-plastin (37.9%) (*32*), chick fimbrin (35.5%) (*33*), and *Drosophila melanogaster* fimbrin (35.4%) (Table I). Fimbrin is an actin-bundling protein identified as a major component of the microvilli in the intestinal brush border (*34–36*), and is distributed widely in nonintestinal cell microvilli and filopodia (*37*). The sequence of chicken fimbrin shows a high degree of homology to human L-plastin (*33*). In addition to L-plastin, two other isoforms, T-plastin and I-plastin, have been identified in human (*38, 39*).

The fimbrin/plastin family proteins consist of four modules: a pair of EF hand motifs in the N-terminal region and two actin-binding domains of about 250 amino acid residues: one in the center (domain A) and the other in the C-terminal region (domain B) (Fig. 4, A and B) (*33*). The two EF hand motifs are closely related to the Ca^{2+}-binding sites of calmodulin. However, the 71-kDa protein does not show

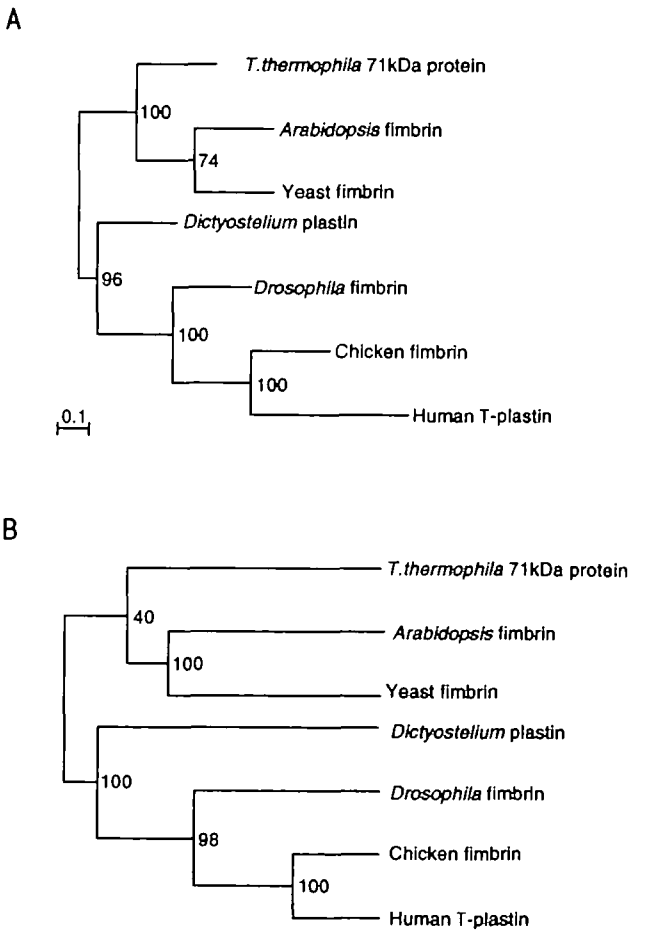

Fig. 5. **Molecular phylogenetic tree analysis of fimbrin/plastin family proteins.** The construction of the phylogenetic tree was based on two actin-binding domains of fimbrin/plastin family proteins by the NJ method (A) and the MP method (B). The number at each branch point represents the bootstrap probability that two lineages join together to form a cluster. The accession numbers of these proteins are: *Tetrahymena* 71-kDa protein, AB024706; *Arabidopsis* fimbrin, U66424; yeast fimbrin, P32599; *Dictyostelium* plastin, P54680; *Drosophila* fimbrin, AF053225; chicken fimbrin, A37097; human T-plastin, A34789.

sequence identity with the EF hand motifs of *Tetrahymena* calmodulin (data not shown). Strikingly, the sequence of the 71-kDa protein is remarkably shorter than the sequences of fimbrin/plastin family proteins because there are no EF-hand motifs in the N-terminal region (Fig. 4, A and B).

The sequences of the actin-binding domains of the fimbrin/plastin family show 22–31% identity to the actin binding domains of α-actinin, β-spectrin, and dystrophin. The actin-binding domains contain a highly conserved motif of 27 amino acids, which is presumed to be essential for F-actin–binding (*40*). The 71-kDa protein consists of two actin-binding domains, domains A and B, which show 41–50 and 32–38% identity, respectively, to the domain sequences of fimbrin/plastin family proteins (Table I). Both domains in the 71-kDa protein also contain the essential 27 amino acid F-actin–binding motif (Fig. 4A).

Molecular Phylogenetic Tree Analysis of the 71-kDa Protein—The phylogenetic tree analysis based on the NJ method suggests that the 71-kDa protein is classified in the fimbrin/plastin family with a bootstrap probability of 100% (Fig. 5). This result, together with the sequence identity, strongly suggests that the 71-kDa protein is a *Tetrahymena* homologue of fimbrin/plastin family proteins. Therefore, we

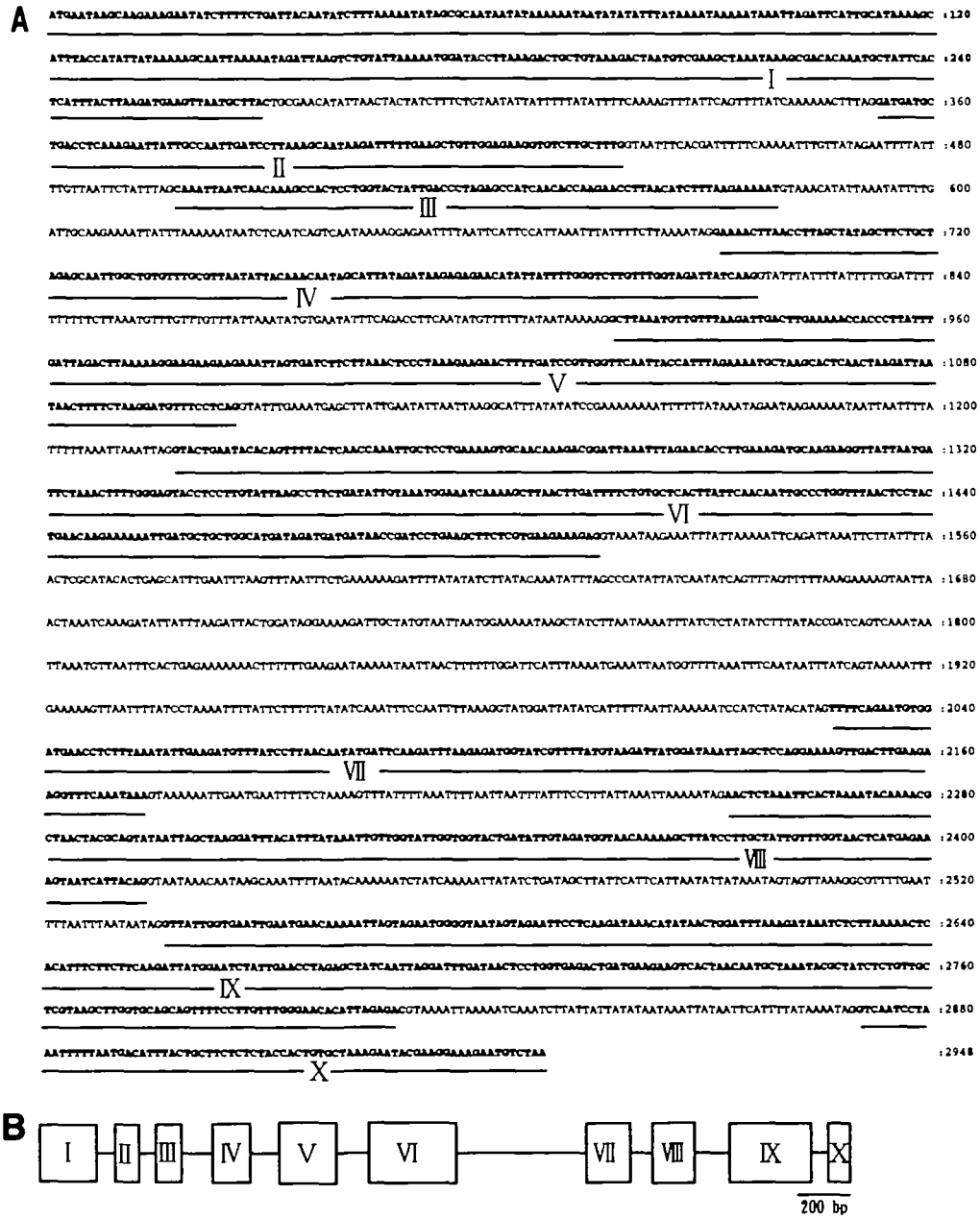

Fig. 6. **The nucleotide sequence of genomic DNA encoding *Tetrahymena* fimbrin-like protein and the structural organization of genomic DNA.** (A) Genomic DNA sequence showing 10 exons in boldface and underlines with Roman numerals and 9 introns in lightface. (B) Schematic illustration of the genomic DNA structure. The boxes with Roman numerals denote the 10 exons and the lines denote the 9 introns.

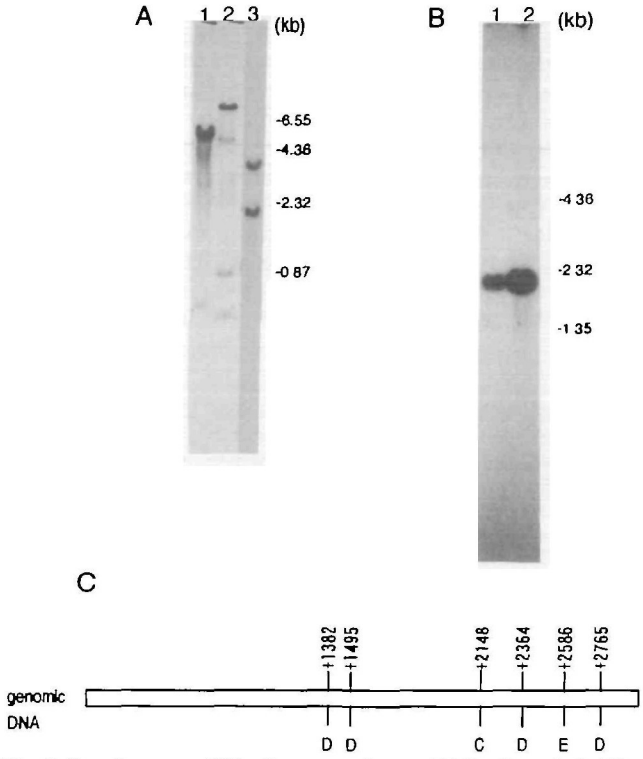

Fig. 7. **Southern and Northern analyses.** (A) Southern hybridization patterns of genomic DNA digested with *Eco*RI (lane 1), *Hin*dIII (lane 2), or *Hin*cII (lane 3) using the genomic DNA (+172 to +2948) encoding *Tetrahymena* fimbrin-like protein as a probe. The kb-markers are represented on the right using *Hin*fI-digested φx174 and *Hin*dIII-digested λDNA. (B) Northern hybridization patterns of poly(A)$^+$ RNA (lane 1) and total RNA (lane 2). The probe was the PCR product obtained using GSP7 and AUAP primers. Molecular mass markers are represented on the right using *Hin*fI-digested φx174 and *Hin*dIII-digested λDNA. (C) For reference, the restriction map of genomic DNA encoding *Tetrahymena* fimbrin-like protein. Symbols for restriction enzymes: E, *Eco*RI; D, *Hin*dIII; C, *Hin*cII. Digestion sites for restriction enzyme are indicated by symbols and nucleotide numbers.

designated the 71-kDa protein as *Tetrahymena* fimbrin-like protein.

Cloning and Sequencing of Genomic DNA Encoding Tetrahymena Fimbrin-Like Protein—Although the calculated molecular mass from *Tetrahymena* fimbrin-like protein cDNA (65,926 Da) was different from the previous estimate of 71 kDa obtained by gel electrophoresis by the method of Laemmli (*15*), the calculated molecular mass corresponds to the molecular mass of 66.7 kDa (data not shown) estimated by the method of Weber and Osborn (*41*). The lack of EF hand motifs suggests the existence of several genes or several transcripts: That is, one encodes a protein without EF hand motifs and the other may encode a protein with EF hand motifs. Therefore, it is necessary to examine the genomic DNA for *Tetrahymena* fimbrin-like protein and to ascertain whether the gene and its transcript are single or not.

To examine the structural organizations of the genomic DNA for *Tetrahymena* fimbrin-like protein, the genomic DNA was cloned by PCR using primers corresponding to the 5′ and 3′ ends of the cDNA with the genomic DNA as a template. The amplified DNA fragment was subcloned into pCR2.1 vector, and sequenced. The sequence of amplified DNA fragment was combined with the genomic DNA sequence between the 5′ end primer and the initiation codon. Comparison of the genomic DNA sequence and *Tetrahymena* fimbrin-like protein cDNA sequences elucidated the structural organization of the genomic DNA encoding *Tetrahymena* fimbrin-like protein and the existence of 9 introns in the genomic DNA (Fig. 6). The ends of the introns are in accordance with the junction consensus GT/AG rule (*42*). The A+T contents of introns in the genomic DNA are between 80 to 89% (Table II). Globally, the introns of the *Tetrahymena* fimbrin-like protein gene have a mean A+T content of 83%. The introns range in size from 54 to 511 bp (Table II), with a mean size of 134 bp. Half of the introns are less than 100 bp. Since *T. thermophila* introns have a mean A+T content of 85% and approximately half of the introns are less than 100 bp (*43*), the introns in genomic DNA encoding *Tetrahymena* fimbrin-like protein are perfect examples of *Tetrahymena* introns.

TABLE II. **Nucleotide numbers and (A+T) contents of introns.**

	bp	(A+T) contents (%)
Intron I	82	85
Intron II	59	82
Intron III	111	83
Intron IV	100	84
Intron V	111	86
Intron VI	511	82
Intron VII	78	90
Intron VIII	122	83
Intron IX	61	82

Southern and Northern Hybridizations—To ascertain how many genes for *Tetrahymena* fimbrin-like protein exist in the macronucleus, Southern blot analysis was performed using the genomic DNA (+172 to +2948) as a probe. Two bands of 4.6 and 0.51 kb in the *Eco*RI-digested DNA, four bands of 7.7, 4.5, 0.87, and 0.4 kb in the *Hin*dIII-digested DNA, and two bands of 3.7 and 2.1 kb in the *Hin*cII-digested DNA were recognized (Fig. 7A), although a 112-bp band in the *Hin*dIII-digested DNA was not detected because of its small size. These results coincide with patterns deduced from the restriction map of the genomic DNA (Fig. 7C). This suggests that *T. thermophila* possesses only a single type of *Tetrahymena* fimbrin-like protein gene in the macronucleus. We then performed Northern hybridization with poly(A)$^+$ RNA and total RNA from *T. thermophila*. The probe used was the PCR product obtained using GSP7 and AUAP primers. As shown in Fig. 7B, a single band of 1.8 kbp hybridized intensely with the probe, suggesting that the transcript of the *Tetrahymena* fimbrin-like protein gene is only a single type. In addition, it suggests that the *Tetrahymena* fimbrin-like protein gene is actively transcribed *in vivo* and contains a full length *Tetrahymena* fimbrin-like protein cDNA.

DISCUSSION

The present study reveals the following: (i) *Tetrahymena* fimbrin-like protein is the *Tetrahymena* counterpart of fimbrin/plastin family proteins; (ii) *Tetrahymena* fimbrin-like protein has two actin-binding domains, but lacks the EF

hand motif; (iii) *Tetrahymena* possesses a single gene, a single transcript, and a single translational product of *Tetrahymena* fimbrin-like protein. These results show that the lack of an EF hand motif in *Tetrahymena* fimbrin-like protein is not due to apparent sequencing or amplification errors in cloning of *Tetrahymena* fimbrin-like protein cDNA by PCR, and that the *Tetrahymena* fimbrin-like protein is a novel type of fimbrin.

The fact that *Tetrahymena* fimbrin-like protein is localized in the division furrow, binds to F-actin, and induces the bundling of actin filaments suggests that it plays an important role in cytokinesis. It consists of two actin-binding domains (A and B) of about 250 amino acid residues each, domain A in the N-terminal region and domain B in the C-terminal region. In yeast, genetic analysis has been used to identify amino acid residues involved in the interaction between actin and the yeast fimbrin homologue, Sac6p (*44*). Analysis of Sac6p mutations, which suppress temperature sensitive mutations in actin, identified nine residues that may be involved in the interaction with actin. All of these residues cluster in the putative actin-binding domains of Sac6p (*44*). Of these nine residues in Sac6p, seven residues (E140, W252, L259, D264, H268, L379, A383) are identical to seven residues (E86, W193, L201, D205, H209, L319, A323) in *Tetrahymena* fimbrin-like protein, according to the alignment shown in Fig. 2. In addition, four other residues in Sac6p (F381, H384, F560, K610) identified as potential actin-binding sites by site-directed mutagenesis (*44*) are also identical to four residues (F321, H324, F489, K539) in *Tetrahymena* fimbrin-like protein. At the amino acid level, therefore, residues that have been demonstrated to be important in actin/fimbrin interactions in yeast are also highly conserved in the *Tetrahymena* counterpart. Of these eleven residues, nine are in domain A, while two are in domain B. In addition, domains A and B in *Tetrahymena* fimbrin-like protein show 41–50 and 32–38% identity to domains A and B of fimbrin/plastin family proteins, respectively (Table I). Our results suggest that the two actin-binding domains in *Tetrahymena* fimbrin-like protein is able to bind F-actin, and that *Tetrahymena* fimbrin-like protein as well as fimbrin enable cross-linking between actin filaments as a monomer. The close spacing of actin filaments in bundles induced by *Tetrahymena* fimbrin-like protein might result from the tandem arrangement of slightly separated actin-binding domains.

Consistent with the presence of two perfect EF hands, the bundling of *D. discoideum* plastin to actin proved to be sensitive to Ca^{2+}, with 92% inhibition at 200 μM Ca^{2+} (*31*). However, the functional role of Ca^{2+} in fimbrin regulation is not understood because preliminary evidence suggests that fimbrin binding to actin is independent of Ca^{2+} (*36*). A novel future of the *Tetrahymena* fimbrin-like protein is the lack of EF hand motifs in the amino-terminal region, suggesting that it cross-links actin filaments in a Ca^{2+}-insensitive manner. The lack of EF hand motifs is not seen in the non-plant fimbrin/plastin family, but *Arabidopsis* fimbrin has a single EF hand-like domain in the amino terminal. Thus, *Tetrahymena* fimbrin-like protein is the first report of a fimbrin-like protein without an EF hand motif.

Each actin-binding domain consists of a duplicated sequence suggesting that the *Tetrahymena* fimbrin-like protein structure may have arisen from a gene duplication event. The tandem arrangement of the actin-binding domains and the lack of EF hand motifs show that *Tetrahymena* fimbrin-like protein has a simple modular arrangement of domains (Fig. 4B). Therefore, *Tetrahymena* fimbrin-like protein is likely to be an ancestor of fimbrin/plastin family proteins.

Because it lacks EF-hand motifs, *Tetrahymena* fimbrin-like protein must cross-link actin filaments in a Ca^{2+}-insensitive manner. Another actin-bundling protein identified as elongation factor-1α is localized in the division furrow in *Tetrahymena* and its actin-bundling activity is regulated by Ca^{2+}/calmodulin (*45*). In *Tetrahymena*, both a Ca^{2+}-independent actin-bundling protein and a Ca^{2+}-dependent actin-bundling protein may regulate the organization of contractile ring microfilaments. Destruction of the *Tetrahymena* fimbrin-like protein gene will clarify whether it plays an important role in cytokinesis.

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