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Archives of Medical Research 32 (2001) 102–107

Archives of Medical Research

ORIGINAL ARTICLE

Trichomonas vaginalis Has Two Fibronectin-Like Iron-Regulated Genes

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Received for publication October 5, 2000; accepted November 27, 2000 (00/146).

Background. *Trichomonas vaginalis*, a protozoan parasite of the human urogenital tract, interacts with fibronectin (FN), a glycoprotein of the extracellular matrix. We, therefore, attempted to identify genes of this eukaryote encoding FN-binding proteins.

Methods. A cDNA clone, C1, representing an incomplete gene was obtained from an expression library based on its FN-binding ability and was characterized. The full-length 378-bp gene encoding a 14.8-kDa protein of 125 amino acids was obtained.

Results. The amino acid sequences revealed homology with the type III-14 repeat of the heparin-binding domain at the carboxyl terminal end of FN. This fibronectin-like protein gene, *flp1*, was single copy in all the *T. vaginalis* isolates examined. Levels of *flp1* transcript were elevated in cells grown under low-iron conditions. Another low-iron-regulated gene, *flp2*, with 70 and 67.5% identity to *flp1* at the nucleotide and amino acid levels, respectively, was recovered from the trichomonad genome. Both *flp1* and *flp2* had consensus Inr promoter-like elements immediately adjacent to the start codon. *flp2* also contained an additional Inr element followed by an ATG 24-bp within the gene.

Conclusions. Unlike *flp2*, the *flp1* gene had AU-rich destabilizing elements in the 3′-untranslated region (UTR).

Key Words: Fibronectin, Genes, Iron, *T. vaginalis*.

Introduction

The protozoan *Trichomonas vaginalis* is the causative agent of the most common, non-viral, sexually transmitted disease (STD), trichomonosis (24). It is estimated that in the U.S. alone, over 5 million women will be infected each year (10). Colonization of the vaginal tract by trichomonads is essential for initiation of infection, and four *T. vaginalis* surface proteins, whose expression is under the control of iron, permit cytoadherence to the terminally differentiated vaginal epithelial cells (VECs) (1,8,9). This protozoan also produces numerous cysteine proteinases that cause contact-dependent cytotoxicity (2,3,5–7) and, when combined with the hormonally regulated exfoliation of the epithelial outer layers, may lead to erosion of the epithelium and exposure of underlying structures. While parasites do not bind immature VECs (parabasal cells), it was recently shown that trichomonads interact in a receptor-mediated fashion to fibronectin (FN) (11).

In this paper, we describe the discovery of two single-copy genes, *flp1* and *flp2*, both of which encode proteins with homology to FN. Functional screening with FN of a cDNA expression library identified one of the genes. It is noteworthy that *flp1* and *flp2* contained unusual regulatory elements. The significance of our results is discussed.

Materials and Methods

Culture and growth of microorganisms. Recombinant *Escherichia coli* strains InvαF′ and TOP10 containing the vectors pcDNAII, pCR2.1, or pTrcHis2A (Invitrogen), were grown in Luria broth (LB) or on LB agar plates containing 65 μg mL^{-1} ampicillin (23). Recombinant protein production in pcDNAII and pTrcHis2A was induced with a 3-h incubation of cultures in the presence of 1 mM isopropyl-β-D-galactopyranoside (IPTG, Sigma Chemical Co., St. Louis, MO, USA). The fresh, clinical *T. vaginalis* isolate T016 was grown in trypticase-yeast extract-maltose medium supplemented with 5% heat-inactivated horse serum at 37°C. High and low iron organisms were generated as described previously (11).

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0188-4409/01 $–see front matter.
PII S0188-4409(01)00262-4

Screening of T. vaginalis cDNA expression library. Recombinant *E. coli* strain InvαF′, containing the cDNA library previously generated (4), was screened for colonies binding to FN. Briefly, overnight (O/N) cultures were lifted onto nitrocellulose, induced, lysed, and blocked with TNT buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl and 0.05% Tween-20) containing 5% milk and 1.5% bovine serum albumin), as described previously (4). Colonies were incubated O/N with 50 μg mL^{-1} FN in TNT buffer at 4°C. Blots were washed and incubated O/N at 4°C in goat anti-FN IgG antibody (25 μg mL^{-1}). Finally, the nitrocellulose filters were incubated in rabbit anti-goat antibody coupled to alkaline phosphatase (1:6000), washed in TNT buffer, and developed (23). Reactive *E. coli* colonies were subjected to insert analysis (4). *E. coli* lysate was run on a 10% SDS-PAGE gel (16) and transferred to nitrocellulose. After blocking in TNT-5% milk, blots were handled as described previously (23).

Generation of full-length sequences for flp1 and flp2. The cDNA insert, C1, was sequenced using the T7 Sequenase® Version 7.0 7-deaza-dGTP DNA sequencing kit (Amersham Life Sciences, Arlington Heights, IL, USA). The remaining 5′-end sequence missing from C1 was obtained with the Universal GenomeWalker™ kit (Clontech Laboratories, Inc., Palo Alto, CA, USA) using the cDNA-derived primer flp1-P1 (5′-CATGCTTTGGATCGCCATATTCACCTGG-3′). The complete sequence of *flp2* was obtained using the Universal GenomeWalker™ kit (Clontech Laboratories, Inc.) and the primer flp2-P1 (5′TTATGGCACAGTACCAGGACGAAG-3′). PCR was performed using the conditions recommended by the manufacturer. Computer analyses of complete sequences utilized Omiga 1.1.3 (Oxford Molecular, Ltd., Oxford, UK). Blast homology searches were carried out using SwissProt, GenBank, and EMBL. The sequence data presented in this paper were submitted to GenBank under accession numbers AF153014 and AF153015.

Northern and Southern analyses. Total RNA was obtained using TRI REAGENT™ (Sigma) from lysates of 10^8 high- and low-iron-grown trichomonads according to the manufacturer's protocol and quantified spectrophotometrically. Genomic DNA (gDNA) was harvested from 10^8 parasites by phenol/chloroform extraction, as carried out previously (9), and quantitated. Northern and Southern analyses were performed as before (9). ^{32}P-radiolabeled probe was generated by nick translation of purified C1 insert. For Figure 5, part A, gDNA from isolate T016 was digested with *Blg*II, *Cla*I, *Not*I, *Mlu*I, *Pvu*II, and *Eco*RV (Boehringer Mannheim, Mannheim, Germany) (data not shown), and for part B, gDNA from five different isolates was digested using *Hinc*II restriction enzyme (12). Digested gDNA (10 μg per lane) was electrophoresed on a 0.7% agarose gel. Total RNA (3 μg per lane) was run on a 1% denaturing agarose gel containing 1X MOPS (morpholinepropanesulfonic acid) and 8% formaldehyde. Nucleic acids (DNA and RNA) were transferred onto a nylon membrane (ZetaProbe®, BioRad Laboratories, Hercules, CA, USA). Following a 1-h incubation of membranes in hybridization solution (50% formamide, 120 mM Na_2HPO_4, 250 mM NaCl, 7% SDS, 1 mM EDTA) at 42°C, the ^{32}P-labeled probe was added and blots were incubated overnight at 42°C. Membranes were washed for 30 min at room temperature with 2X SSC (1X SSC in 150 mM NaCl and 15 mM sodium citrate, pH 7.0)—0.1% SDS, followed by a wash in 0.5X SSC—0.1% SDS. A final wash was performed in 0.1X SSC—0.1% SDS for 30 min at 65°C. Autoradiography was performed on each probed membrane.

Cloning of flp1 into pTrcHis2A. pFLP1A and pFLP1B were generated by directional cloning of *flp1* into pTrcHis2A using *Bam*HI and *Hind*III and the sense primers flp1-P2 (5′-TTGGATCCGATGGGCGACAGAAG AGACAACAT ACGACC-3′) and flp1-P3 (5′-TTGGATCCGATGGCGACAGAAGAGACAACATACGACCTTCT TTCAAGACACG-3′), respectively. The antisense primer flp1-P4 (5′-TTAAGCTTGAGTGGTGTAACTTCAGTGCATGGGC-3′) was used for both. Products were amplified during 20 cycles of PCR at 94°C for 30 sec, 61°C for 30 sec, and 68°C for 3 min. Clones were sequenced as described previously. Recombinant lysates were electrophoresed and transferred to nitrocellulose (23), and FN-binding activity was determined as described previously.

Cloning of flp1 and flp2 into pcDNAII. Primers flp1-P2 and flp1-P5 (5′-TTGCGGCCGCTTAGAGTGGTGTAA CTTCAGTGCATGGGC-3′) and flp2-P1 and flp2-P2 (5′-TTGCGGCCGCTTATAGTGGTGTGACTTCTGTTTTGGACGTTCTGG-3′) were used to generate full-length clones of *flp1* and *flp2*, respectively. Genes were amplified during 25 cycles of PCR at 94℃ for 30 sec, 61℃ for 30 sec (55℃ for *flp2*), and 68℃ for 2 min. PCR products were digested with *Bam*HI and *Not*I and cloned into pcDNAII.

RT-PCR. RT-PCR was performed as follows: 0.2 μg low iron total RNA and 1 μL oligo(dT) primer (0.5 mM final) were incubated at 70°C for 2 min followed by 2 min on ice. To the reaction, 10 U AMV reverse transcriptase (Promega), 1 μL dNTP mix (10 mM each) (Boehringer Mannheim), and 2 μL 5X reaction buffer was added as described by the manufacturer. The reaction was incubated at 42°C for 1 h and at 70°C for 15 min, and placed on ice. Control reactions were also prepared but without addition of AMV reverse transcriptase. The primers used for the PCR reactions were those utilized for cloning as previously described.

Results

Analysis of *flp1* and identification of *flp2*. Functional screening of a cDNA expression library with FN identified one *E. coli* clone, C1, containing a ∼700-bp insert. After

SDS-PAGE and blotting of proteins onto nitrocellulose, the blot of total lysate of recombinant *E. coli* C1 was incubated with FN. A FN-binding protein was readily detected with anti-FN serum (data not shown). No similar reactivity was seen in control *E. coli* containing plasmid without insert regardless of blot pretreatment with FN. These results suggested that C1 encoded a FN-binding protein. We then sequenced C1 cDNA, which revealed a partial open reading frame (ORF) of 366 bp, of which 290 bp was a 3′-UTR followed by a poly(A) tail (data not shown). The recombinant fusion protein was predicted to be ~20-kDa.

As the cDNA lacked a start site, we performed chromosome walking using primer flp1-P1 (Figure 1) and the Universal GenomeWalker™ kit. The gene termed *flp1*, for reasons described later, consisted of 378 bp encoding a 125 amino acid protein of 14.8 kDa. Interestingly, an additional partial gene, *flp2*, was identified (Figure 1). A *flp2*-specific primer (flp2-P1) was then used similarly as for *flp1* to generate the complete gene. Figures 1 and 2 show the complete sequence comparisons between *flp1* and *flp2*, which share 67.5 and 70% identity at the nucleotide and amino acid levels, respectively. The *flp2* gene is 411 bp and encodes a 136 amino acid protein of 16 kDa. Both proteins have a pI of 6.2 and no predicted transmembrane domain. A prominent difference between the two genes and proteins was the presence at the N-terminus of FLP2 of two repeats, R1 and R2 (Figures 1 and 2) with little homology to FLP1. The *flp1* gene has one consensus Inr promoter-like sequence (18), while *flp2* possesses two Inrs, one of which is located after R1 (Figure 1). The 3′ UTR of *flp1*, but not *flp2*, contains two AU-rich elements (AREs) (13) represented by the RNA consensus sequences AUUUA and UUAUUUA(U/A)(U/A), which are known to regulate eukaryotic mRNA stability (25).

Finally, as presented in Figure 3, a BLAST search of the two protein sequences revealed that both FLP1 and FLP2 share some homology with two discontinuous peptides

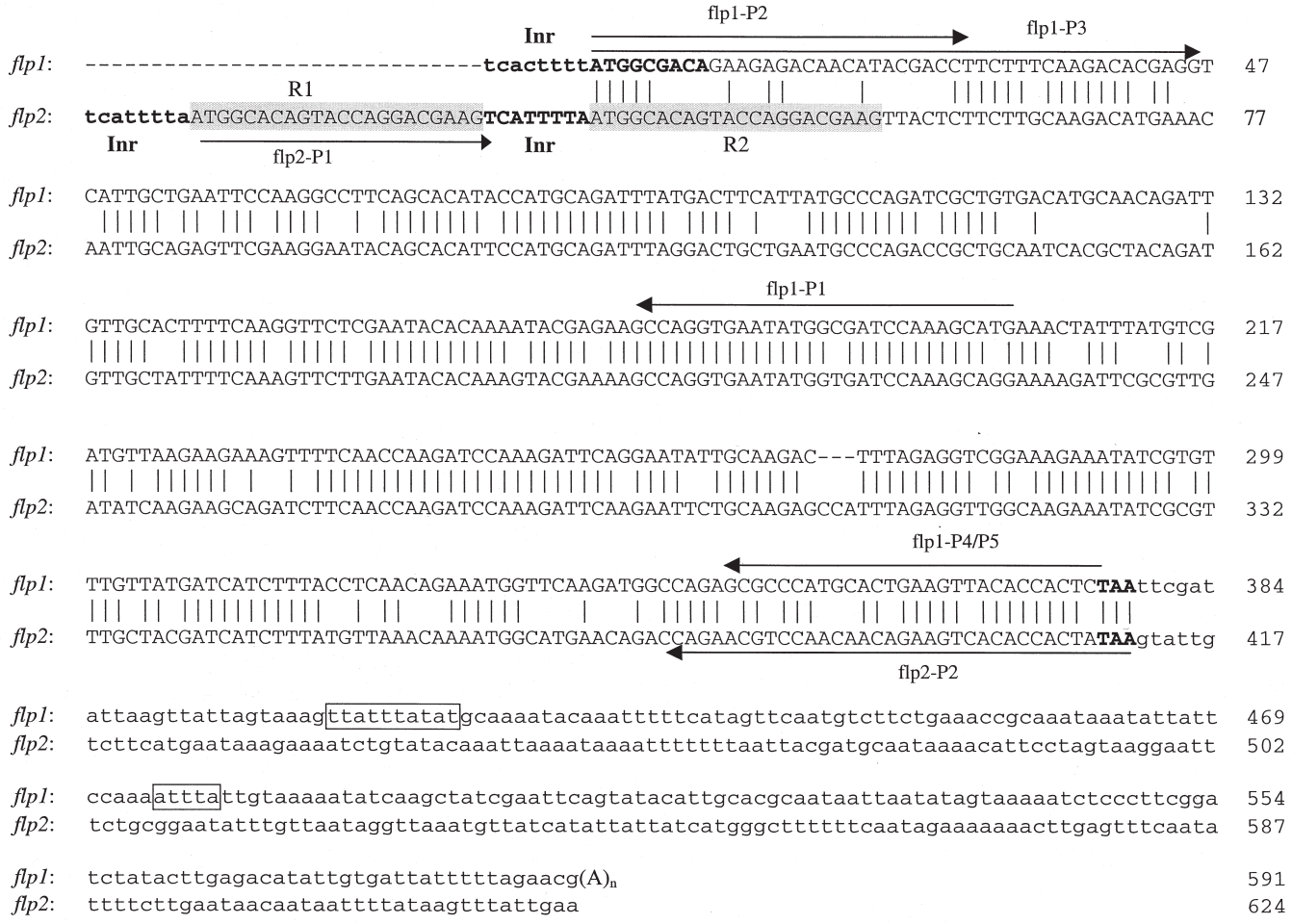

Figure 1. Complete nucleotide sequences of *flp1* and *flp2*. This shows the nucleotide sequence representing the complete transcript of *flp1* aligned with the *flp2* sequence obtained from genomic DNA. Small cap bold lettering at the 5′ end of each gene represents the *T. vaginalis* promoter element (Inr). The capitalized and bolded *flp2* sequence designates the repeated Inr element. The *flp1* sequence presented in bold represents the information acquired from the Universal GenomeWalker™ kit. The small cap sequence at the 3′ end of *flp1* designates the 3′-UTR and the downstream *flp2* sequence from genomic DNA. Shaded boxes represent the repeats R1 and R2 of *flp2*. Arrows represent the primers used throughout this study. The boxed sequences in the 3′-UTR of *flp1* represent the AU-rich elements. The stop codons for *flp1* and *flp2* are in bold at positions 376 and 409, respectively.

```
FLP1:   ----------MATEETTYDLLSRHEVIAEFQGLQHIPCRFMTSLC   35
                  ||       || ||| |||| |.||||||| |. |
FLP2:   MAQYQDEVILMAQYQDEVTLLARHETIAEFEGIQHIPCRFRTAEC   45
          R1        R2

FLP1:   PDRCDHATDVALFKVLEYTKYEKPGEYGDPKHETIYVDVKKKVFN   80
        |||| ||||||.|||||||||||||||||| | | ||.|| .||
FLP2:   PDRCNHATDVAIFKVLEYTKYEKPGEYGDPKQEKIRVDIKKQIFN   90

FLP1:   QDPKIQEYCKT-LEVGKKYRVCYDHLYLNRNGSRWPERPCTEVTPL  125
        ||||||| ||  |||||||||||||||.  ||   |||| ||||||
FLP2:   QDPKIQEFCKSHLEVGKKYRVCYDHLYVKQNGMNRPERPTTEVTPL  136
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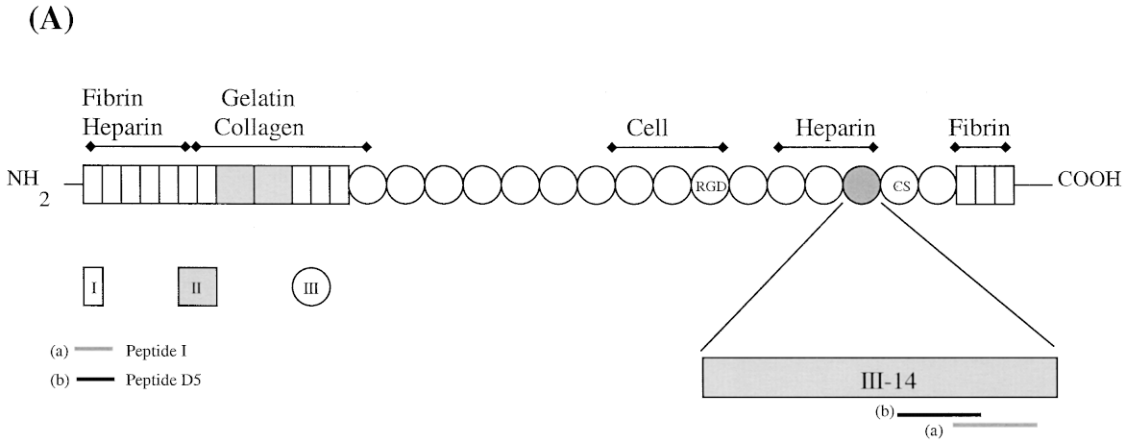
Figure 2. Amino acid sequence alignment of FLP1 and FLP2 showing the high degree of identity between the two proteins. Vertical dash (|) represents identity between two amino acids, and dot (.) designates homologous residues. The repeats R1 and R2 are lightly shaded, whereas the region of homology with FN is darkly shaded.

found within the type III-14 repeat of the heparin-binding domain at the C-terminus of FN (Figure 3A). Over the 37 residues presented, FLP1 and FLP2 share 51 and 46%, respectively, with that region of FN (Figure 3B).

flp1 is single copy and regulated by iron. We next performed Southern analysis on trichomonad genomic DNA restricted with enzymes that do not cut within the gene. One predominant band was present in each lane when probed with ^{32}P-labeled C1 cDNA (Figure 4A). Furthermore, a membrane containing DNA cut with *Hinc*II from different *T. vaginalis* isolates showed a single band in each lane with only one isolate (T068-II) showing a RFLP (Figure 4B). These results suggest that *flp1* is single copy and present in all isolates.

Trichomonads were then grown under iron-replete and iron-depleted medium conditions to determine whether *flp1* expression was influenced by iron as shown previously for other trichomonad genes (9). As seen in Figure 5A, Northern analysis on total RNA was performed using ^{32}P-labeled C1 as probe. Results showed two major bands present under low-iron conditions, the higher band possibly representing *flp2*. The bands on autoradiograms had mobilities as expected around 400 bp. Further, the overexposure clearly shows, albeit at an extremely low level, the transcription oc-



(B)

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FLP1   49KVLEYT-KYEKPGEY62   63G-DPKHE-TIYVDVKK76
         ..  |. ||||||       |. |  | ||||   |
FN   1899RITGYIIKYEKPGS1911  2053GLEPGTEYTIYVIALK2068
         ..  |. ||||||       |. |  |  | |  |
FLP2   59KVLEYT-KYEKPGEY72   73G-DPKQE-KIRVDIKK86
```

Figure 3. Comparison of FLP1 and FLP2 amino acid sequences with two discontinuous regions of FN. (A) Schematic representation of one subunit of FN with the type III-14 repeat and the two peptides with homology to the *T. vaginalis* FLP1 and *FLP2*. (B) Amino acid sequence comparisons between FLP1, FLP2, and FN.

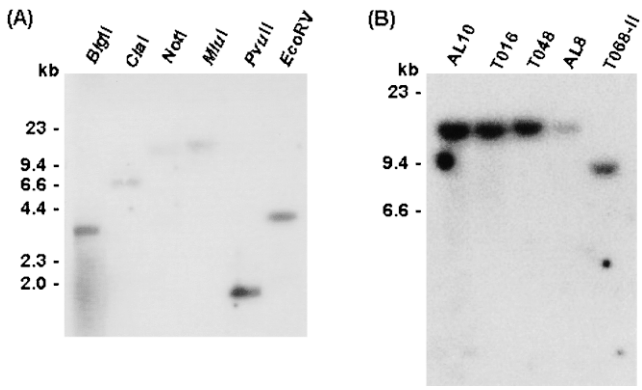

Figure 4. Southern analyses using C1 cDNA as probe. *T. vaginalis* isolate T016-restricted gDNA (A) and *Hinc*II-digested gDNA from five isolates, AL10, T016, T048, AL8, and T068-II (B) were run on a 0.7% agarose followed by transfer to Zetaprobe® membrane prior to probing with ^{32}P-labeled cDNA C1.

curring within high-iron-grown organisms (lane H). It is noteworthy that ethidium bromide stained total RNA gels used in Northern analysis had the two prominent ribosomal bands in equivalent amounts. These results suggest that *flp1* expression is upregulated under iron-restricted growth.

We performed RT-PCR to see whether *flp2* was also expressed under the same low-iron growth conditions. As seen in Figure 5B, a product was obtained for both *flp1* (lane 2) and *flp2* (lane 4). Furthermore, the higher migration of the *flp2* PCR product, compared to *flp1*, suggested that *flp2* transcription might start at the Inr preceding R1. Finally, in data not shown, the adhesin gene *ap65-1*, previously shown

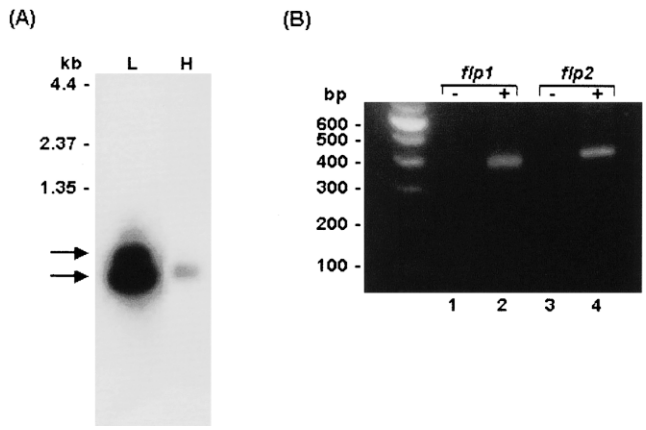

Figure 5. *flp1* and *flp2* expression is regulated by iron. (A) Equal amounts (15 μg) of total RNA of low (L)- and high (H)-iron-grown trichomonad were run on a 0.7% denaturing agarose gel, transferred to a Zetaprobe® membrane and probed with ^{32}P-labeled cDNA C1. RNA in both the L and H lanes contained equivalent amounts of total RNA, evidenced by the fact that ethidium bromide-stained patterns of duplicate gels showed identical amounts of the two prominent ribosomal bands. (B) RT-PCR showing expression of *flp1* and *flp2* under low-iron conditions. Total RNA was reverse-transcribed using AMV reverse transcriptase and the poly(dT) primer. Control lanes (-) represent reactions where the AMV was omitted. PCR was performed on the resulting cDNA using *flp1* (lanes 1 and 2) and *flp2* (lanes 3 and 4) specific primers.

to be upregulated by iron (9), was included as an additional control to confirm upregulation of *flp1* and *flp2* expression in low-iron growth conditions.

Discussion

In this report, we describe nucleic acid and protein sequences for two newly discovered *Trichomonas vaginalis* genes, *flp1* and *flp2*. Both *flp1* and *flp2* shared homology with two discontinuous regions of the type III-14 repeat of FN (Figure 3). This region of FN has been extensively studied and found to have multiple functions. For example, one peptide (Peptide I at 1899–1911) effectively binds heparin and promotes melanoma cell adhesion and spreading in an RGD-independent fashion (20). Within Peptide I, there is an identical hexapeptide common to both *flp1* and *flp2*. Another peptide called D5, encompassing a region similar to Peptide I, inhibited FN binding to heparin (21). Not surprisingly, hydropathy plot analyses of the FN peptide and the *flp1* and *flp2* fragments (Figure 3B) showed similar patterns, suggesting a commonality in exposed residues. However, this in itself may be meaningless without additional structure-function characterization of the trichomonad proteins. It must be further acknowledged that we do not yet have a role for either *flp1* or *flp2*. Nonetheless, their homology to FN must be kept in mind during investigations involving cytoadherence and interactions with substrates, such as ECM FN, a property that has recently been demonstrated for this protozoan (11).

These FN-like genes possess many features already reported for other *T. vaginalis* genes. The 5′ UTR is short and consists of a promoter-like initiator element (Inr) (18,19,22). This element, with the consensus TCA(T/C)T(T/A)(T/C)TCATTA, serves as a recognition sequence for both transcription and translation. It is similar to a motif overlapping the transcriptional start site of mammalian genes lacking a typical TATA box (14). Interestingly, *flp2* is the first *T. vaginalis* gene described to date possessing two potential start sites. Under the conditions used to perform RT-PCR, transcription of *flp2* appears to start at the first Inr (Figure 5B). However, we cannot rule out transcription from the Inr located after R1, as this promoter-like element might function under environmental conditions other than those examined in this study. With only one Inr, *flp1* is unusual for the length of its 3′-UTR. Although the coding region is only 378 bp, the 3′-UTR is 290 bp, making it the longest known 3′-UTR for any *T. vaginalis* genes identified to date. The *flp1* 3′-UTR also contains additional regulatory motifs not found in the downstream sequence of *flp2*. The AU-rich elements (ARE) are involved in the regulation of mRNA stability (13). Depending on the nature of the transcript, this element can either increase or decrease the half-life of mRNA (15). The presence of two AREs in *flp1* (AUUUA and UUAUUUAUA) and two Inr motifs for *flp2* suggests

strongly that differential regulation of expression occurs for these two genes. Although both are induced under low-iron growth conditions, it is plausible to hypothesize that the regulatory elements modulate *flp1* at the level of transcript stability and *flp2* at the level of transcription.

It is noteworthy that *flp1* and *flp2* are the first low-iron-regulated trichomonad genes to be reported. Amounts of transcript were greater in organisms grown in batch culture in low-iron medium. This is not surprising, because it was established that both low- and high-iron medium conditions induce expression of trichomonad immunogens (17). Therefore, *flp1* and *flp2* represent important genes in the delineation of low-iron regulatory sequences in the future. Furthermore, we demonstrated by PCR using primers specific for *flp1* and *flp2* that similar sequences are present in four other members of the *Trichomonadae* family (unpublished observations). This reinforces the idea that, despite the distinct niches and host tropisms, evolutionary conservation of these low-iron-induced genes might reflect a common feature in the biology of this genus. For example, the resemblance of FLP1 and FLP2 to the heparin-binding domain of FN may relate to the colonization of mucosal surfaces by the *Trichomonadae.* Clearly, more research is needed to delineate the *flp1* and *flp2* functions in *T. vaginalis* as well as in the other members of this family.

Finally, it is noteworthy that the FN-binding function of the original recombinant C1 protein labeled *flp1* resulted from the amino acids of the expression vector pcDNAII inserted by fusion at the *Ecor*RI site of the *LacZ* gene. Sequence analysis revealed that although in frame and producing a recombinant C-terminal *myc*-His_6 tagged fusion protein with FN-binding function, the clone pFLP1A was lacking nine bases (ATGGCGACA, data not shown) coding for the three N-terminal amino acids (M-A-T) of FLP1. There was instead a 31-bp insertion of the *LacZ* gene from pcDNAII located between the vector start site (ATG) and the *flp1* sequence. The insertion resulted in the addition of 10 β-galactosidase residues preceding FLP1. Removal of only 10 β-galactosidase residues at the N-terminal end of C1 abolished FN recognition. These results further demonstrate the need for caution in isolating putative functional proteins during screening of expression libraries.

Acknowledgments

This study was supported by Public Health Service grants AI-43940 and AI-39803 from the National Institute of Allergy and Infectious Diseases of the National Institutes of Health, Bethesda, MD, USA. MLC was supported in part by NIH Training Grant T32-AI-07271.

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