Characterization of an Immuno-dominant Variable Surface Antigen from Pathogenic and Nonpathogenic *Entamoeba histolytica*

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Summary

A 125-kD surface antigen of *Entamoeba histolytica* is recognized by 73% of immune sera from patients with amoebic liver abscesses. Using pooled human immune sera a cDNA clone (λcM17) encoding this antigen (M17) has been isolated from a λgt11 expression library of the virulent stain *E. histolytica* HM1:IMSS. Monospecific antibodies, purified by binding to phage lysate of λcM17, and mAb FA7 reacted exclusively with the 125-kD antigen by Western blot analysis. Surface binding and cap formation are observed with patient sera, purified monospecific antiserum, and mAb FA7. Corresponding genomic clones (pBSgM17-1/2/3) were isolated by hybridization with the cDNA clone. These contained an open-reading frame of 3345 bp, which is in good agreement with the mRNA size of ~3.0 kb as revealed by Northern hybridization with λcM17. The inferred amino acid sequence predicts a 125,513 dalton protein that contains 17 potential N-linked glycosylation sites and is unusually rich in tyrosine and asparagine residues. A distinctly hydrophobic NH_2-terminal region may serve as membrane anchor or signal sequence. In contrast to conservation of an immunodominant epitope recognized in pathogenic and nonpathogenic strains by monoclonal FA7 and human immune sera, amplification and sequence analysis of a 1,400-bp fragment of this gene from a fresh nonpathogenic isolate by use of the PCR demonstrate regions of significant sequence divergence in this antigen. A 1% sequence variability among different isolates of the pathogenic strain HM1:IMSS and a 12–13% variability between pathogenic and nonpathogenic strains are revealed by comparison to published partial amino acid sequences (Tannich, E., R.D. Horstmann, J. Knobloch, and H.H. Arnold. 1989. *Proc. Natl. Acad. Sci. USA*. 86:5118.). Some restriction enzymes were found that allowed PCR diagnosis of nonpathogenic and pathogenic isolates with the exclusion of *E. histolytica*–like Laredo, suggesting that a detailed study of nonpathogenic and pathogenic isolates in relation to the M17 antigen sequence will provide a basis of differentiating isolates.

Entamoeba histolytica is a common human pathogen that causes a spectrum of disease ranging from a commensal state in asymptomatic carriers to fulminant diarrhea or extra-intestinal abscess formation. Virulent amoebae cause ulceration of the intestinal epithelium and may penetrate the bowel wall to form extra-intestinal abscesses, primarily in the liver. Several molecular activities thought to correlate with the virulent phenotype has been partially characterized. These include a sulfhydryl protease (1–3), a pore-forming protein (4–6), an *N*-acetyl-galactosamine–specific adherence lectin (7–13), a 220-kD *N*-acetyl-glucosamine lectin (14, 15), and a 96-kD surface antigen (16–18); however, the role of each of these in pathogenesis remains ill defined. Most importantly, it is still unclear whether in a given strain invasiveness is a stable (19) or a variable (20–22) genotypic characteristic. Standard methods of differentiating between potentially virulent strains of *E. histolytica* include host symptomatology and serology and the pattern of a number of parasite isoenzymes which together constitute its zymodeme. It is this latter criterion that has been generally used in the current classification of *E. histolytica* isolates; pathogenic and nonpathogenic zymodemes are differentiated on the basis of polymorphisms in the electrophoretic mobility of the glycolytic enzymes phosphoglucomutase (PGM),[1] hexokinase (HK), and phosphoglucoisomerase (PGI) (19, 23–26). At least 18 zymodemes

[1] *Abbreviations used in this paper:* HK, hexokinase; PGI, phosphoglucoisomerase; PGM, phosphoglucomutase.

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have been described in pathogenic and nonpathogenic amoebae, but the majority of clinical isolates fall into zymodemes I, II, and III.

More recently several other probes, based either on DNA sequences (27–29), or the detection of specific antigens (30), have been suggested as diagnostic reagents. However, in most cases, axenic cultivation (20) and cloning (31) of amoebae directly from fresh stool samples before assay with any of these probes have not been achieved and none of these probes have been validated by large scale screening of clinically defined isolates that have also been compared with extant criteria, such as zymodeme patterns. Since axenization and cloning of amoebae from patient isolates appear to favor outgrowth of the less fragile pathogenic strains and are known to result in reversible attenuation of virulence, it is especially important to develop probes that can directly discriminate virulent amoebae in fresh isolates. Clearly, the existence of such probes will be invaluable in clinically distinguishing infections caused by a mixture of strains or those that may result from phenotypic interconversion of pathogenic to nonpathogenic strains as has been suggested by Mirelman (22). Presented here is the isolation and characterization of the gene encoding a 125-kD surface antigen from *E. histolytica* which is immunodominant in patients with invasive disease. The 125-kD antigen exhibits significant sequence variation among amoebal isolates and is potentially useful in differentiating nonpathogenic and pathogenic strains of *E. histolytica*.

Materials and Methods

Entamoeba Isolates and Cell Culture. Trophozoites of the axenized *E. histolytica* strains (HM1:IMSS, NIH:HK9) and *E. histolytica*-like Laredo were grown in TYI-S-33 media as described by Diamond et al. (32). Polyxenic isolates were grown in liquid Robinson's medium supplemented with 10% bovine serum and containing 5 μg/ml of medium of each of the following antibiotics: kanamycin, erythromycin, and ampicillin. Amoebae were pelleted by centrifugation at 900 rpm and washed twice with PBS, pH 7.5. Polyxenic amoebae were further purified by centrifugation through a Percoll/PBS cushion at 3,000 rpm in a refrigerated Accuspin centrifuge. Isolates SD4 (pathogenic, zymodeme II) and REF 291 and SD116 (nonpathogenic, zymodemes III and I), were a generous gift of Dr. Sharon Reed from the University of California, San Diego. Nonpathogenic isolates Nos. 43 and 44 and pathogenic isolate No. 46, classified by zymodeme analysis using gradient PAGE (33), were isolated in Mexico City. They correspond to Sargeaunt zymodemes I, I, and II, respectively.

Human Immune Sera and Western Blot Analysis. Sera from 108 patients with amoebic liver abscesses were obtained from Drs. A. Isibasi and R. Landa at the Instituto Nacional de la Nutricion and La Raza-IMSS Hospitals, Mexico City. Diagnosis of hepatic abscess in patients was established by clinical symptoms, countercurrent immunoelectrophoresis, ELISA, and rectosigmoidoscopy. Human sera from donors without history of amoebiasis and negative for anti-amoebic antibodies as tested by immunoblot served as controls. Western blots of whole trophozoites were prepared by suspending washed cells in PBS containing 10 mM *p*-hydroxymercuribenzoate and Laemmli sample buffer, boiling for 5 min, fractionation by 10% or 5–15% gradient SDS-PAGE, and electrophoretic transfer to nitrocellulose filters. All sera were evaluated by Western blot analysis on extracts of whole amoebae. 29 sera with the highest titer were selected from the 108 samples and were pooled.

Antimembrane fraction serum. This serum was obtained by immunizing mice with 300 μg of membrane fraction, prepared as described previously (34) and diluted 1:1 with PBS and CFA. Mice were injected intraperitoneally every 2 wk until titers reached 1:5,000 as assayed by Western blot.

mAb FA7. Whole amoebic extract from 2×10^6 amoebae was fractionated by preparative 5–15% gradient SDS-PAGE. After electrophoretic transfer to nitrocellulose the 125-kD region was excised from the blot, ground to a powder, and suspended in PBS. 100 μl of the suspension were diluted 1:9 with PBS and injected three times intraperitoneally into mice at 2-wk intervals with a final boost before the fusion. Hybridomas were selected by positive reaction with the 125-kD band in Western transfers of *E. histolytica* extracts. Harvest fluid from clone FA7 was used at a 1:1,000 dilution in Western blot analysis.

Antibody Capping by Live Trophozoites. Human immune serum, hybridoma harvest fluid from clone FA7, and purified monospecific antiserum, were added to live trophozoites at 1:500, 1:2,000 and undiluted, respectively. After formation of caps (10 min at 37°C), cells were fixed with 3.7% formaldehyde, washed with PBS, and stained with FITC-labeled anti-human or anti-mouse IgG. Undiluted harvest fluid from an anti-actin-producing clone was used as control for a nonsurface antigen (15, 35).

Preparation and Screening of Libraries. Genomic DNA and poly(A)$^+$ RNA isolation and construction of the λgt11 cDNA library from strain *E. histolytica* HM1:IMSS have been described previously (36). For construction of the genomic library from *E. histolytica* HM1:IMSS, 600 μl of NaI (GeneClean kit; Bio101) were added to 200 μl (~20 μg DNA) of agarose-embedded nuclei (36) in an Eppendorf tube and melted by incubation at 60°C for 5 min. 20 μl of glassmilk were added, suspended well, and the mixture was incubated at room temperature for 5 min. The sample was vortexed for 1 min to shear the DNA and spun in a microfuge for 5 s. After removal of the supernatant the pellet was suspended in 1 ml wash buffer by vortexing for 30 s. The glassmilk was pelleted by a 5-s spin in the microfuge, and the supernatant was removed. The wash was repeated twice and the sheared and purified DNA was eluted into 100 μl 10 mM Tris-HCl (pH 8), 1 mM EDTA (TE) by incubation at 37°C for 5 min. Recovery and degree of shearing were assessed by agarose gel electrophoresis. All subsequent steps including addition of EcoRI linkers, methylation, ligation into the vector λZAPII, and packaging reaction were performed as described previously (37, 38). The λgt11 cDNA library (3×10^5 phage) was screened with the pool of 29 patient sera at a 1:200 dilution (39, 40). The genomic library was screened with the α-[^{32}P]dCTP-labeled EcoRI fragment of λcM17. Plasmids were rescued from genomic λZAPII clones as described previously (41). Phage DNA and plasmid DNA were purified by standard methods (42).

Sequence Analysis. With the exception of the first 207 bp, the entire sequence of gene M17 presented in Fig. 3 was determined on both strands in genomic clone pBSgM17-1 and on one strand in genomic clone pBSgM17-2. The internal EcoRI fragment representing the cDNA insert was also sequenced on both strands using nested deletion templates created with the Promega Biotech system (Madison, WI). Double-stranded sequence was also determined for two PCR fragments obtained by amplification of genomic DNA from isolate REF 291, Zymodeme III, derived from an asymptomatic Costa Rican refugee and kindly provided by Dr. S.L. Reed. Several oligonucleotides were used as primers in single-stranded DNA (M13mp18/19) and double-stranded DNA [pBSKS(+)] sequencing reactions with the Sequenase system (U.S. Biochemical

Corp., Cleveland, OH) or ABI Sequencer (Applied Biosystems Inc., Foster City, CA).

Primer Extension Sequence Analysis. Primer extension sequence analysis was performed by reverse transcriptase–mediated extension of oligonucleotide primer SRO9 (5'AACTACTCCTGTGACTATTGCAGAAG3') annealed to 10 μg poly(A)$^+$ enriched RNA in the presence of deoxyadenosine 5'-[α-[^{35}S]thio]triphosphate as described previously (36).

Polymerase Chain Reaction. The PCR was performed using a Cetus Corp./Perkin-Elmer DNA thermocycler. Reaction mixtures (50 μl) contained 25 pmol of each of the two oligonucleotide primer pairs SRO18 [5'GCAACTAGTGTTAGTTATAC3'] + SRO21 [5'GGTGGAATTTGGAATTCTGG3'] and SRO19 [5'GTATAACTAACACTAGT3'] + SRO22 [5'GCTGTTACACTTGAAAATAT3'], ~500 ng of genomic DNA, all four dNTPs each at 1.5 mM, 60 mM KCl, 25 mM Tris·HCl (pH 8), 0–20 mM $MgCl_2$, 0.1% BSA, and 10% DMSO. The reaction mixture was overlaid with a drop of paraffin oil and denatured at 94°C for 10 min, and amplification was initiated by addition of 2.5 U of *Thermus aquaticus* DNA polymerase (Cetus Corp., Emeryville, CA). PCR parameters were 35 thermal cycles consisting of a 1-min denaturation of 94°C followed by a 3-min annealing period at 42°C, a 3-min ramp, and a 4-min extension period at 72°C. The amplification products were restricted with EcoRI and SpeI endonucleases and purified for subcloning into M13 by 2% low-melting-point agarose gel electrophoresis.

Results

Western Blot Analysis. Sera from 108 different patients, diagnosed with amoebic liver abscess, were each reacted with *Entamoeba* whole cell extracts in Western blots; seven antigens (220, 190, 160, 125–129, 96, 75, 46 kD) were detected by >62% of the sera (43). Among these seven, a 125-kD antigen was immunodominant, reacting strongly and being recognized by >70% of the serum samples. We assume, based on their molecular weight and serological reactivity, that the 220-kD, the 160- and the 96-kD antigens represent the *N*-acetyl-glucosamine adherence lectin (14, 15), the *N*-acetyl-D-galactosamine adherence lectin (7–13), and the 96-kD integral membrane protein (16–18), respectively. Because it appeared that the 125-kD antigen had not been characterized, we chose to study this immunodominant antigen in more detail. A Western blot of whole cell extracts of axenically or polyxenically propagated pathogenic and polyxenically propagated nonpathogenic *E. histolytica* isolates was assayed with the pooled subset of 29 human immune sera (Fig. 1); the sera reacted strongly with a 125-kD antigen in all isolates regardless of source. Polyspecific antiserum prepared against amoebic plasma membrane (34) also reacted strongly with the 125-kD antigen (Fig. 1). The mAb FA7, prepared against partially purified 125-kD antigen, reacted specifically with an epitope of the 125-kD antigen; by Western analysis with FA7 this epitope was detected in different strains and species of *Entamoeba* (Fig. 1). In the Western blot with mAb FA7 additional bands of lower molecular weight and varying intensity are apparent in most of the isolates. Because potent proteases are present in whole amoebic extracts (1–3) we assume that these are degradation products of the 125-kD antigen, although processing intermediates of unknown origin can not be ruled out.

Localization of the 125-kD Antigen to the Surface of Amoebae. Live trophozoites will cap antibody-antigen complexes bound to their surface. Antibody-antigen caps were induced in HM1:IMSS trophozoites by incubation with above pooled patient serum, mAb FA7, or monospecific antibody recovered after specific binding and elution of pooled patient sera to phage lysates of cDNA clone λcM17 (see below) (Fig. 2); a negative control antibody (anti-actin mAb) neither bound to trophozoite surfaces nor induced cap formation.

Isolation and Characterization of a cDNA Clone Encoding Part of the 125-kD Antigen. The pooled sera from amoebic abscess patients were used to screen a λgt11 expression library

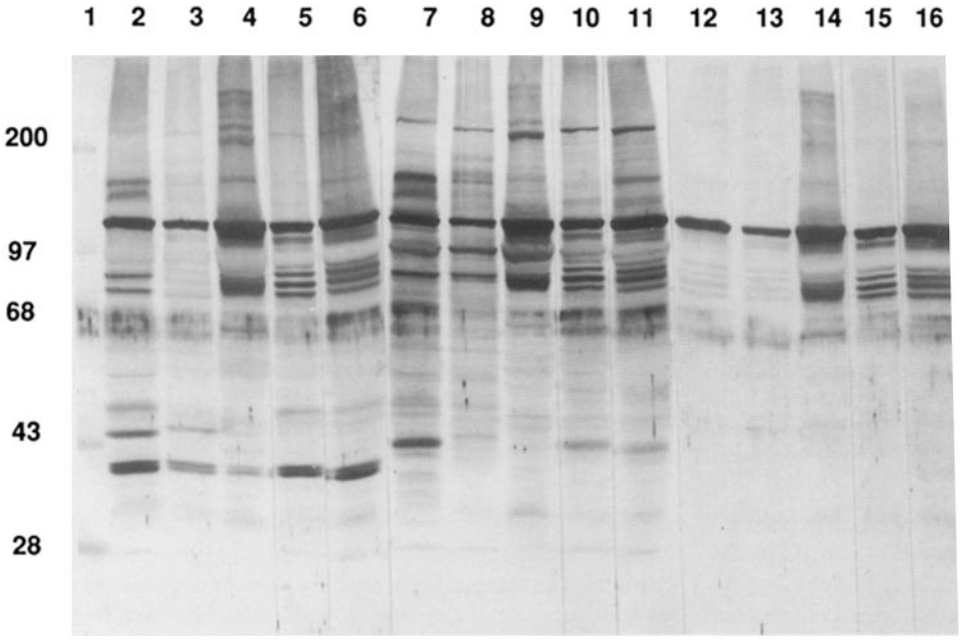

Figure 1. Western blot of whole *Entamoeba* extract fractionated by 5–15% SDS-PAGE, (lanes *2*, *7*, and *12*) polyxenic pathogenic *E. histolytica* isolate SD-4, (lanes *3*, *8*, and *13*) polyxenic nonpathogenic *E. histolytica* isolate SD116, (lanes *4*, *9*, and *14*) *E. histolytica*–like Laredo, (lanes *5*, *10*, and *15*) *E. histolytica* HK-9, (lanes *6*, *11*, and *16*) *E. histolytica* HM1:IMSS probed with anti-membrane fraction serum (lanes *2–6*), pooled human immune sera (lanes *7–11*), and mAb FA7 (lanes *12–16*); molecular masses are given in kilodaltons (molecular mass standards lane *1*: 200, 97, 68, 43, 28 kD).

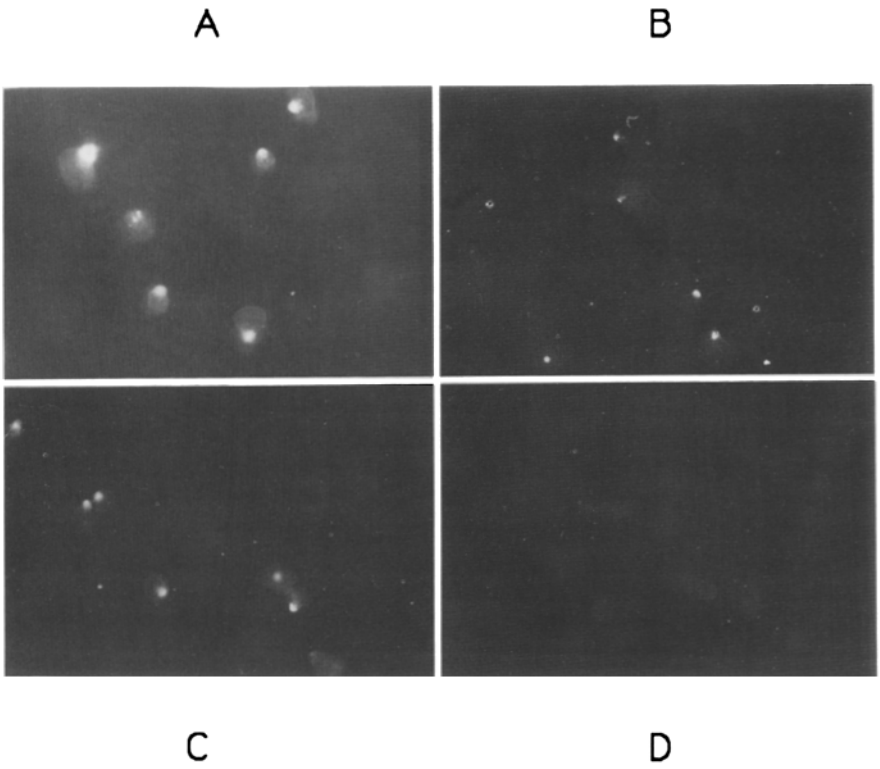

Figure 2. Photographs (×800) of *E. histolytica* HM1:IMSS trophozoites labeled in vivo with primary antibodies. (*A*) Pool of human anti-*E. histolytica* immune sera at 1:500 dilution; (*B*) pool of human anti-*E. histolytica* immune sera purified by binding to λcM17 phage lysates; (*C*) monoclonal FA7 harvest fluid at 1:1,000 dilution; (*D*) monoclonal anti-*E. histolytica* actin antibody at 1:1,000 dilution; secondary antibodies, FITC goat-anti-human and FITC goat-anti-mouse.

from *E. histolytica* HM1:IMSS; 46 reactive clones were each plaque purified and tested for recognition by each of the 29 patient sera included in the serum pool and by the anti-membrane antibody. Clone λcM17 strongly reacted with 26 of 29 patient sera as well as with the anti-membrane serum. Monospecific antibody was selected from the pooled human sera by elution from filter-bound phage lysate of λcM17. This eluate reacted with a single polypeptide of 125 kD by Western blot analysis of whole amoebic extracts; phage lysate of λgt11, serving as negative control, did not bind antibodies reacting with amoebic antigens (data not shown). After nucleotide sequence analysis, the λcM17 1.9-kb insert revealed an ORF spanning the entire insert (Fig. 3). The lack of a 5′ initiating methionine, the absence of a poly(A)-tail, and hybridization to a ~3 kb mRNA by Northern blot analysis (Fig. 4) indicated that NH_2- and COOH-terminal sequences were lacking in λcM17.

Isolation and Characterization of Genomic Clones Encoding the 125-kD Antigen. To isolate a genomic clone, a λZAPII library from *E. histolytica* HM1:IMSS was screened using the 1.9-kb insert of λcM17 as a probe. Three genomic clones were identified, and two of these were sequenced using oligonucleotide primers derived from the cDNA sequence. The nucleotide sequence of the cDNA was identical in both genomic clones. An additional 556 bp of 5′ and 870 bp of 3′ sequence yielded an ORF of 3,345 bp which was also identical in both genomic clones (Fig. 3). The size of this ORF (gene M17) is in reasonable agreement with the mRNA size of ~3,000 bp determined by Northern blot analysis (Fig. 4). The inferred amino acid sequence predicts a 125-kD protein.

5′ Flanking Sequence Comparison. The 5′ flanking sequence of gene M17 shares striking similarities with the 5′ flanking region of both actin and ferredoxin genes (Fig. 5), the only other genes of *E. histolytica* where sequence has been determined. The transcriptional start site of M17 was mapped to an adenine residue 17 bp 5′ of the start codon by primer extension sequence analysis using oligonucleotide SRO9 (data not shown). 5′ untranslated regions of actin (11 bp) (36, 44) and ferredoxin (9 bp) (45) genes were likewise very short as compared with other eukaryotic gene transcripts. A common sequence motif, 5′ATTCA3′, is present at the transcriptional start site of both M17 and actin genes, the initiating nucleotide being an adenine residue as is most frequently found in other eukaryotes. While the same motif is also present in the flanking sequence of the ferredoxin gene, its cap site was mapped to the 3′ thymidine rather than the 5′ adenine residue (45) (Fig. 5). An additional sequence motif shared among these genes is YATTTAAA present at -29, -31, and -32 for the M17, actin, and ferredoxin gene flanking sequences, respectively. This sequence motif does not conform with the Goldberg-Hogness promoter consensus sequence TATAAATA, which in eukaryotic genes is located 25–30 bp upstream of the transcriptional start site. Nevertheless, this sequence is similar in the three *E. histolytica* genes, both in sequence and relative position, suggesting a consensus which in *E. histolytica* serves as the entry point for RNA polymerase.

Gene Copy Number. Southern blot and sequence analysis of the M17 gene and limited flanking regions indicate that this surface antigen is encoded by a single copy gene. When a Southern blot of genomic DNA from *E. histolytica*, restricted

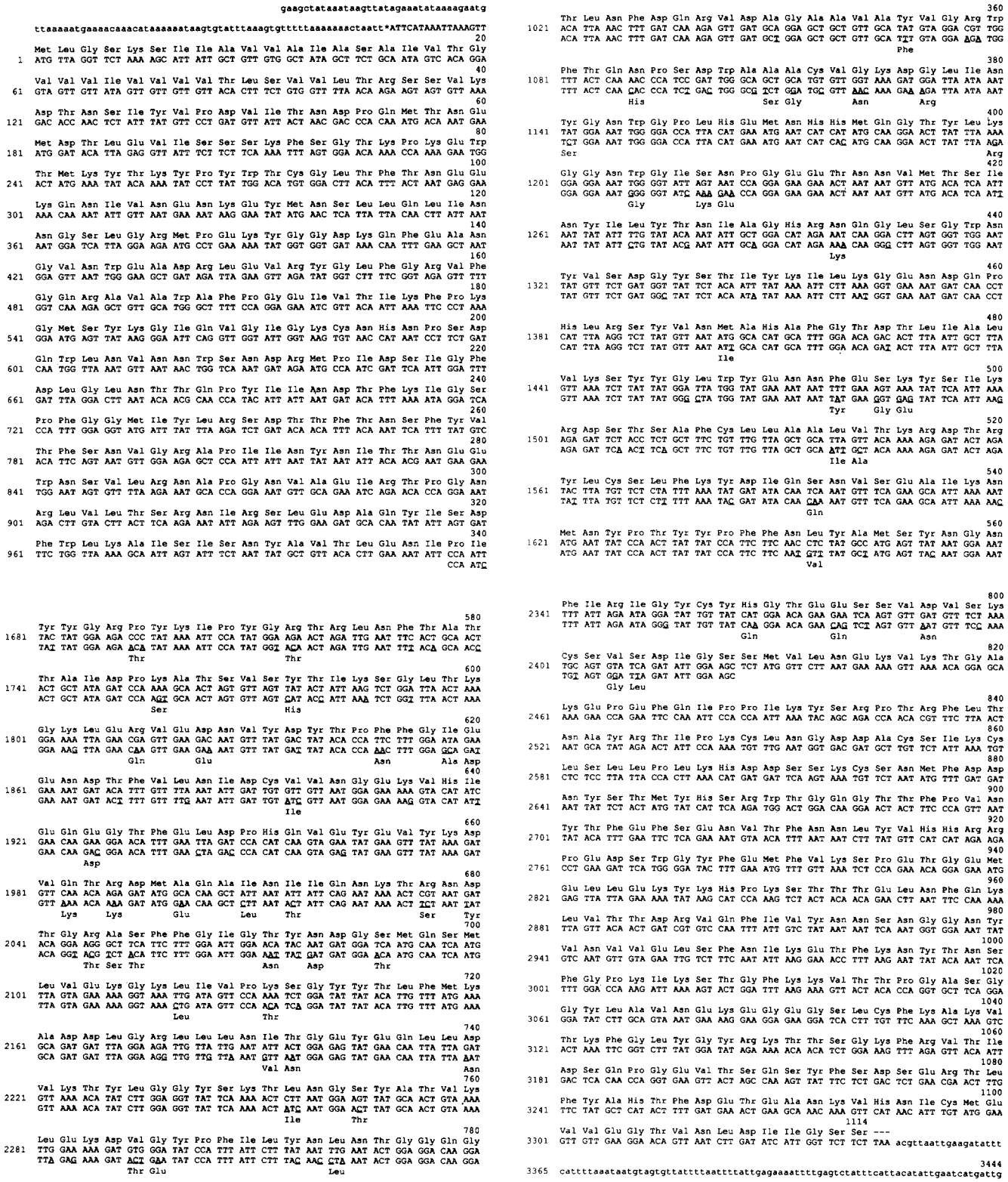

Figure 3. Inferred amino acid sequence and nucleotide sequence of coding region and flanking region obtained from genomic clone pBSgM17-1. The sequence of the internal EcoRI fragment was identical in both genomic clones (pBSgM17-1/2) and the cDNA clone λcM17. Shown below is the partial nucleotide sequence of PCR amplification products derived from nonpathogenic isolate REF291. Nucleotide substitutions are underlined and amino acid substitutions are indicated below the partial sequence derived from REF291.

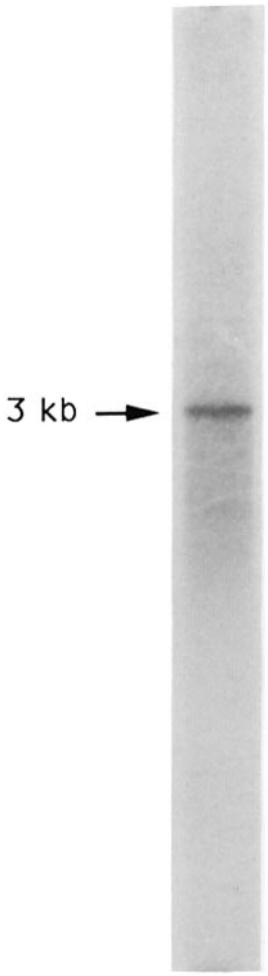

Figure 4. Transfer blot of *E. histolytica* HM1:IMSS RNA probed with cDNA clone λcM17 indicates a single hybridizing band migrating at ~3 kb. Hybridization conditions were 50% formamide, 0.2× SSC, 42°C. Autoradiography shown required a 72-h exposure.

with BglII and EcoRV in single and double digests, was probed with BamHI-BglII, BglII-EcoRV, and EcoRV fragments of λcM17, only unique restriction fragments hybridized with each probe (data not shown). Furthermore, the nucleotide sequence of both genomic clones and the cDNA clone is identical.

Detection of Sequences Related to Gene M17 in Nonpathogenic E. Histolytica *and Mapping of RFLPs.* Western blot analysis suggested that the 125-kD antigen or a closely related antigen that shared the epitope recognized by poly- and monoclonal antisera was found in both pathogenic and nonpathogenic *E. histolytica* isolates as well as *E. histolytica*–like Laredo. By Southern blot analysis, even under low stringency hybridization and wash conditions (25% formamide, 2 × SSC, 37°C) sequences related to M17 were difficult to detect in nonpathogenic *E. histolytica* isolates and *E. histolytica*–like Laredo (data not shown). To confirm the presence of a closely related gene in nonpathogenic amoebae, two fragments spanning most of the sequence contained within the cDNA clone λcM17 were amplified in a PCR using oligonucleotide pairs SRO19/SRO22, and SRO18/SRO21 as primers on genomic template DNA derived from nonpathogenic isolate REF 291. By nucleotide sequence analysis of the two subcloned PCR amplification products, REF 291 had 145 nucleotide substitutions over 1410 residues (10.3%) as compared with the sequence of λcM17 (HM1:IMSS) (Fig. 3). These substitutions result in 57 amino acid differences per 470 residues (12.1%). A computer search of published protein sequences with the entire 3,345 bp M17 gene sequence revealed that the internal gene fragment represented by the λcM17 insert encoded a protein sequence similar to that deduced for a DNA fragment isolated from nonpathogenic and pathogenic strains of *E. histolytica* by Tannich et al. (29) and proposed by these authors as a potential diagnostic probe for strain differentiation. Specifically, when we compared the amino acid sequences of Tannich et al. with that of λcM17 we detected five substitutions between pathogenic HM1:IMSS isolates (1%) (Fig. 6). As the nucleotide sequence of the DNA fragment was not published by Tannich et al., we infer from the amino acid sequence that at least three of these five differences between the *E. histolytica* HM1:IMSS laboratory strains must have arisen from more than one nucleotide substitution and are therefore unlikely to represent cDNA synthesis or sequencing artifacts. When the 470 amino acid sequence derived from the PCR product of nonpathogenic isolate REF291 was compared with isolate SAW 1734 (29), six amino acid substitutions (1.3%) were detected (Fig. 6). Over the same 470 amino acids, 61 amino acid residues (12.9%) differ among the pathogenic HM1:IMSS (29) and nonpathogenic SAW 1734 (29) strains (Fig. 6). Overall there are 65 variable residues over a stretch of 470 amino acids (13.8%) when these four isolates were compared (Fig. 6).

As the partial M17 amino acid sequences of nonpathogenic strains SAW 1734 (29) and REF 291 were significantly more similar to one another than to their pathogenic counterparts, PCR amplification of the same gene fragments from six additional strains was undertaken to examine the possibility of defining RFLPs that could reliably differentiate pathogenic from nonpathogenic amoebal isolates. Using oligonucleotide primers SRO19, SRO22, SRO18, and SRO21, PCR products of the same size were amplified from genomic DNA of strains SD116, SD4, Nos. 43, 44, 46, and HK9. Based

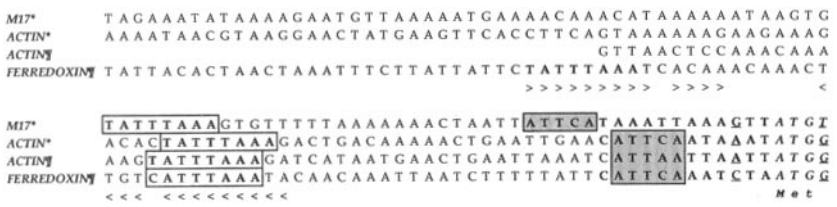

Figure 5. Alignment of the 5′ flanking sequences from genes M17, actin*(36), ¶(44) and ferredoxin (45); a likely Goldberg-Hogness consensus sequence at −29, −31, −32, respectively, is boxed and in bold face. The 5′ end of the mRNA is in bold face and italics and sequence similarities around the cap site are boxed and shaded.

on our nucleotide sequence of these fragments from HM1:IMSS and REF291, we predicted that restriction endonucleases EcoRV, SspI, PvuII, AccI, and HincII among others would cleave the PCR products into restriction fragments which might be expected to correlate with the pathogenic or nonpathogenic phenotype of the isolate. An example of such an analysis with EcoRV and SspI is presented in Fig. 7. A restriction site for EcoRV is absent in nonpathogenic No. 43, No. 44, and REF291 but present in nonpathogenic SD116 and Laredo as well as pathogenic HM1:IMSS, HK9, SD4, and No. 46 (Fig. 7). Digestion with restriction endonuclease SspI shows a distinct pattern for pathogenic (HM1:IMSS, HK9, SD4, 46) versus non-pathogenic (No. 43, No. 44, SD116, REF291) strains with the exception of *E. histolytica*–like Laredo, which would appear pathogenic by this criterion (Fig. 7). Similarly, restriction with AccI distinguishes pathogenic from nonpathogenic isolates with the exception of Laredo, which appears to have an additional restriction site for the enzyme. HincII digestion shows the same restriction fragments in nonpathogenic isolates No. 43, No. 44, and REF291 and pathogenic isolate SD4 but no restriction sites in commensal Laredo and pathogenic isolates No. 46, HM1:IMSS, and HK9.

Discussion

It has long been known that amoebiasis is a spectral disease, asymptomatic infections with "nonpathogenic" amoebae and life-threatening infections with "pathogenic" amoebae defining opposite ends of the spectrum. With the availability of effective treatment regimens, early diagnosis is crucial for the prevention of disease and transmission. However, much controversy has centered on benefits and drawbacks of initiating therapy in asymptomatic infections. Thus, recent investigations have focussed on a molecular genetic analysis of virulence and the definition of marker molecules that have high predictive value and can be applied in a clinically feasible fashion.

We have identified a variable, immunodominant 125-kD surface antigen in *E. histolytica* HM1:IMSS. The amino acid sequence inferred from the nucleotide sequence of the coding region of the 125-kD antigen is unusual with respect to its high Asn (90 = 8.2%), Tyr (70 = 6.3%), and hydroxyl amino acid residue (Ser, 85 = 7.6%; Thr, 90 = 8.1%) content. While a total of 17 *N*-linked glycosylation sites suggests that the 125-kD antigen may be glycosylated, Western blot analysis shows that this antigen migrates as a compact band on SDS-PAGE. A distinctly hydrophobic NH_2-ter-

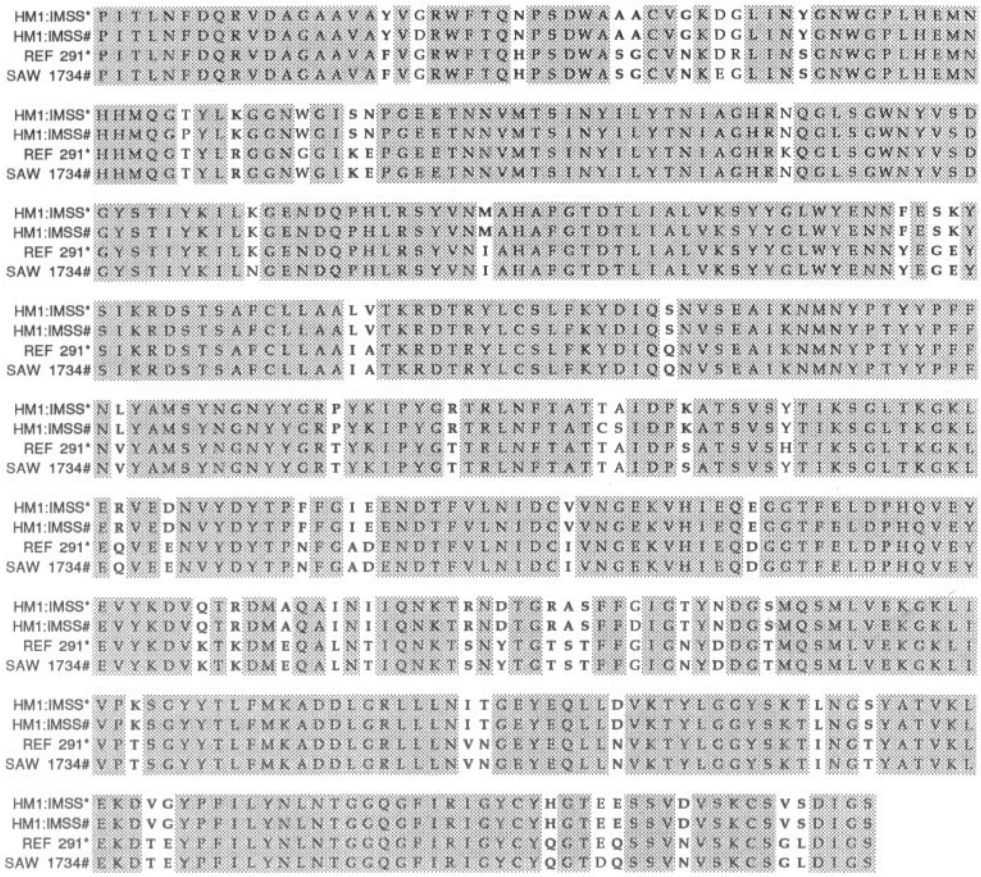

Figure 6. Alignment of the amino acid sequences inferred from nucleotide sequences of cDNA and genomic clones (HM1:IMSS*) and of PCR amplification products (REF291*) with those published by Tannich et al. (HM1:IMSS#, SAW 1734#) (29). Conserved amino acids are shaded, variable residues differentiating pathogenic from nonpathogenic isolates are in bold face, and additional variable residues are in plain text.

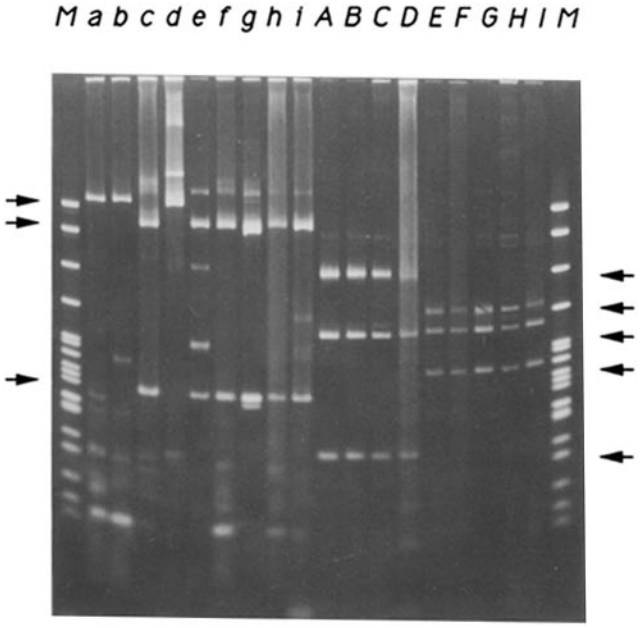

Figure 7. Restriction endonuclease (EcoRV lower case letters, SspI capital letters) digests of PCR products generated by amplification of genomic DNA from *E. histolytica* isolates/strains using oligonucleotide primers SRO 18 + 21 and SRO19 + 22. (*a/A*) No. 43, (*b/B*) No. 44, (*c/C*) SD116, (*d/D*) REF291, (*e/E*) *E. histolytica*–like Laredo, (*f/F*) No. 46, (*g/G*) HK9, (*h/H*) = HM1:IMSS.

minal region of 35 amino acids may serve as anchor or signal sequence. Compared with known prokaryotic and eukaryotic signal sequences this region contains an unusually long (20 amino acids) NH_2-terminal (n) region with a single positively charged residue, an 8 amino acid long hydrophobic core (h) region and a 7 amino acid long polar COOH-terminal (c) region with an amino acid composition similar to those seen in other signal sequences (46). By extrapolation this would imply that the antigen is either a peripheral membrane protein or it may be anchored in the membrane by other means such as a glycophospholipid anchor. Alternatively, the hydrophobic NH_2-terminal itself may serve to anchor the antigen in the membrane with the COOH-terminal externally exposed as no additional *trans* membrane domains could be discerned. From searches of protein and nucleic acid sequence data banks, a small domain was identified sharing sequence similarity with the β chain of the human fibronectin receptor, the β-1 chain of the mouse integrin, and band 3 precursor of the chicken integrin (Fig. 8). However intriguing, the functional significance of this similarity will need to be assessed by generation of antibodies to this domain for use in attachment or invasion assays.

Tannich et al. (29) had reported a sequence of 1.9 kb that differed substantially between pathogenic and nonpathogenic *E. histolytica* and also suggested that RFLPs in this gene fragment would allow the differentiation of pathogenic from nonpathogenic *E. histolytica*. Examination of the Tannich sequence revealed that it was derived from an internal fragment of M17, the gene sequenced in its entirety in this paper. While human sera and mAb FA7 demonstrated the presence of the 125-kD antigen in a number of nonpathogenic and pathogenic amoebal isolates (Fig. 1), DNA hybridization data with the λcM17 probe suggested that there were regions of substantial sequence variability in other portions of this molecule. For this reason, fragments of this antigen within a variable sequence region were amplified by the PCR to search for RFLPs that correlated with phenotype. If in fact RFLPs could be used to differentiate pathogenic from nonpathogenic strains, then in combination with FA7, PCR could provide a potent diagnostic protocol.

Based on the nucleotide sequence differences of strains HM1:IMSS and REF291, presented in Fig. 3, five restriction endonucleases were chosen that should have yielded RFLPs which correlate with the pathogenic and nonpathogenic phenotype of the isolate from which the PCR fragments were derived. PCR fragments from four fresh polyxenic nonpathogenic isolates of zymodeme I (SD116, No. 43, No. 44) or III (REF291), from axenized Laredo, from two fresh polyxenic pathogenic isolates (SD4, No. 46) of zymodeme II and two axenized established pathogenic laboratory strains (HM1:IMSS, HK9) of zymodeme II were subjected to this analysis. Our results provide strong evidence that the M17 antigen is a highly variable protein and that distinct sets of amino acid substitutions exist in pathogenic versus nonpathogenic strains of *Entamoeba*. These differences provide the basis for RFLPs which, in the limited sampling of this study, are correlated with pathogenic and nonpathogenic isolates. The exception to this correlation is the *E. histolytica*–like Laredo strain that is often used as a prototype nonpathogen in laboratory studies. Although Laredo was first isolated as a human commensal it appears morphologically more like free-living amoebae and belongs to an unusual zymodeme that is rarely found in patient isolates. Thus, Laredo's RFLP pattern may not preclude the use of this criterion in the clinical context.

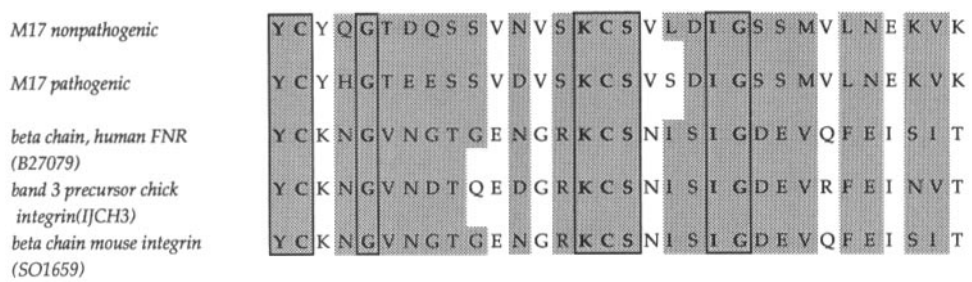

Figure 8. Similarity of a small region of the amino acid sequence inferred from the M17 nucleotide sequence to the β chain of the human fibronectin receptor, the band 3 precursor of the chick integrin, and the β-chain of the mouse integrin.

Given the rather limited sample size tested here, it is, however, noteworthy that none of the pathogenic isolates revealed a nonpathogenic RFLP pattern in this analysis.

While gene fragments from HM1:IMSS analyzed in these experiments were derived from the same original isolate, they have been propagated in different laboratories for some time. The extent of sequence differences indicates that a low degree of variation probably occurs within this gene family in the absence of selective pressure by the host immune system. Although the position and nature of most of the amino acid substitutions are conserved, it is clear that overall extensive interstrain variability and the modest intrastrain variability among strains with the same phenotype necessitate that RFLPs be validated on large numbers of amoebal isolates. Zymodeme characterization and the use of additional diagnostic markers will be required to develop a reliable set of criteria for the differentiation of pathogenic from nonpathogenic *E. histolytica*.

To further understand the significance of the 125-kD surface antigen sequence variation and its potential role in pathogenesis, sequence analysis of the entire gene from several nonpathogenic and pathogenic isolates has been undertaken. Evolutionary analysis of the small ribosomal subunit gene sequences from several nonpathogenic *E. histolytica* isolates and comparison to the known small ribosomal subunit gene sequences of *E. histolytica* HM1:IMSS, *E. histolytica*–like Laredo and *E. invadens* (manuscript in preparation) will improve our ability to interpret the significance of the variability in the 125-kD antigen in view of the degree of evolutionary divergence between these different *Entamoeba* strains/species.

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