The ancient eukaryotic human pathogen, *Entamoeba histolytica*, is a nucleo-base auxotroph (*i.e.* lacks the ability to synthesize purines or pyrimidines *de novo*) and therefore, is totally dependent upon its host for the supply of these essential nutrients. In the current study, we identified two unique, 28 kDa, DTT-sensitive, nucleases and showed that they are constitutively released/secreted by parasites during axenic culture. Using several different molecular approaches, we identified and characterized the structure of *EhNucI* and *EhNucII*, genes that encode Ribonuclease T2 family proteins. Homologous episomal expression of epitope-tagged *EhNucI* and *EhNucII* chimeric-constructs were used to define the functional and biochemical properties of these released/secreted enzymes. Results of coupled immunoprecipitation-enzyme activity analyses demonstrated that these “secretory” enzymes could hydrolyze a variety of synthetic polynucleotides, as well as the natural nucleic acid substrate, RNA. Further, our results demonstrated that sera from acutely infected amebiasis patients recognized and immunoprecipitated these parasite secretory enzymes. Based on these observations, we hypothesize that within its host, these “secretory” nucleases could function, at a distance away from the parasite, to harness (*i.e.* hydrolyze/access) host-derived nucleic acids to satisfy the essential purine and pyrimidine requirements of these organisms. Thus, these enzymes might play an important role in facilitating the survival, growth and development of this important human pathogen.

The ancient eukaryotic human parasite, *Entamoeba histolytica*, is the causative agent of invasive amebiasis in humans infecting 10% of the world’s population, and resulting in over 100,000 deaths annually, chiefly from amebic liver abscess (1). Infection is acquired through the ingestion of the quadrinucleate cyst form of the organism from fecally contaminated food or water supplies. Excystation occurs in the small intestine, releasing immature trophozoites, which move into and colonize the nutrient poor large intestine.

As ancient eukaryotes, *Entamoeba* parasites contain only rudimentary endoplasmic reticulum and Golgi apparatus (2,3). Despite this, vesicle trafficking pathways appear to be essential to the pathogenesis of these parasites as (i) uptake and digestions of nutrients by mature trophozoites in the large intestine, (ii) invasion of the intestinal epithelium, (iii) delivery of cyst wall components during encystation, and (iv) dissemination and establishment of extra-intestinal infections all rely on endocytosis and secretion. In addition, these parasites lack many traditional eukaryotic biochemical pathways, including the *de novo* biosynthesis of purine and pyrimidine nucleobases. As a result, these parasites are completely dependent upon their host for the supply of these essential preformed nutrients. In that regard, it has been previously reported that *Entamoeba* are capable of salvaging both purines and pyrimidines from the extracellular milieu during *in vitro* culture, presumably through the action of membrane transporters (4,5). However, to date, no report exists to indicate that these organisms might be capable of producing and releasing/secreting nucleotide salvage enzymes (*e.g.* nucleotide hydrolases, nucleases, or other nucleic acid hydrolases) into their host environments to aid in accessing these vital nutrients.

To that end, in the current report, we identified, for the first time, unique nuclease activities that were released/secreted by *Entamoeba histolytica* parasites during their
Based on those observations, we used several molecular approaches to identify and characterize the structure of two genes, *EhNucI* and *EhNucII*, which encode T2 family ribonucleases from these parasites. Epitope-tagged *EhNucI* and *EhNucII* chimeric constructs were used in a homologous expression system to delineate the functional and biochemical properties of these unique parasite released/secreted enzymes.

**EXPERIMENTAL PROCEDURES**

**Reagents**- All reagents and chemicals used in this study were of analytical grade and were obtained from Sigma-Aldrich Chemical Co. unless otherwise specified. Enzymes used for molecular studies were obtained from Roche Molecular Biochemicals; DNA and RNA molecular mass standards were from Invitrogen, Inc. Protein molecular mass standards used in zymogram gels were purchased from GE Healthcare Bio-Sciences Corp (Amersham) and those used for Western blots were from Invitrogen, Inc.

**Parasites**- *E. histolytica*, strain HM-1:IMSS were originally obtained from American Type Culture Collection (Manassas, VA, strain accession # 30459). Trophozoites of this strain were maintained under axenic conditions in 15 mL glass screw cap tubes in trypticase-yeast extract iron-medium (TYI-S-33) supplemented with 15% (v/v) heat-inactivated adult bovine serum (ABS; BioSource, Rockville, MD) at 37°C as previously described (6). Such cells were routinely microscopically assessed by trypan-blue dye exclusion assay to ensure >99% viability prior to use in experiments. Culture supernatants from serum-grown cells were examined to determine whether trophozoites released/secreted any nuclease activity into their growth medium during *in vitro* cultivation. To that end, when cultures reached a density of ~1.5 X 10⁵ cells ml⁻¹ (i.e. mid-log phase) tubes were placed on ice for 10 min to release adherent parasites, and then centrifuged at ~500 X g for 10 min at 4°C. To ensure the complete absence of cells, the supernatants from these were carefully removed and recentrifuged at 6000 X g for 10 min at 4°C. Following this, the cell-free supernatants were carefully removed, frozen and stored at -80°C until assayed.

For isolation of nucleic acids and proteins, parasite cell cultures were grown to ~mid-log phase and harvested as above. The resulting cell pellets were washed three times in ice-cold PBS by centrifugation as above and finally resuspended in buffers appropriate for the extraction of DNA, RNA or proteins (see below).

**Parasite Cell Lysates**- For zymogram gel analyses, washed cell pellets of *E. histolytica* were solubilized in SDS-PAGE sample buffer (7) lacking any reducing agents and heated in a boiling water bath for 5 min. Following this, the solubilized samples were cooled to RT, frozen and stored at -80°C until analyzed. The protein concentration in these cell lysates was determined using the bicinchoninic acid method according to manufacturer’s instructions (Micro BCA, Pierce). In preliminary experiments, to test the effects of reducing agents on nuclease activity, cell lysates were also prepared in SDS-PAGE sample buffer containing 50 mM DTT (final concentration).

**In situ Nuclease Activity/ Zymogram Gels**- Parasite cell lysates, cell free culture supernatants, and samples from immunoprecipitation and affinity binding assays were separated in SDS-PAGE mini-gels and processed for *in situ* staining of nuclease activity essentially according to Joshi and Dwyer (8). Briefly, samples were separated by SDS-PAGE containing polyadenyllic acid [poly(A), 300 μg ml⁻¹, final concentration] and a discontinuous buffer system (9). Following separation, gels were washed twice at RT with 100 mM HEPES, 0.1% (v/v) Triton X-100 (Protein Grade® Detergent, Calbiochem, EMD Biosciences, Inc.), pH 8.5 on an orbital shaker. Following this, they were washed two additional times (15 min ea.) in buffer containing 50 mM sodium acetate, 0.1% (v/v) Triton-X-100, pH 6.0. Subsequent to washing, these gels were incubated in sodium acetate buffer above for 2 h at 37°C with gentle agitation in a rocking-hybridization oven (Shake ‘N’ Bake, Boekel Scientific, Feasterville, PA). Following incubation, gels were fixed in 7.5% (v/v) aqueous acetic acid, stained with Toluidine Blue O [0.2% (w/v) in 10 mM HEPES, pH 8.5] and de-stained using multiple changes of deionized water (10). Nuclease activity in these gels was readily apparent as distinct, clear/colorless bands of substrate hydrolysis in an otherwise uniformly stained, dark blue background of un-hydrolyzed polynucleotide
For some experiments, zymogram gels were prepared as above except that various other individual synthetic polynucleotides [i.e. polyguanylic acid, poly(G), polyinosinic acid, poly(I) or polyuridylic acid, poly(U) all from Sigma] were substituted for the poly(A) substrate. Oligonucleotide primers, PCR and Cloning- In preliminary experiments, we found that during their growth in vitro, *E. histolytica* cells released/secreted two nuclease activities of ~ 24 and 25 kDa into their culture medium. Based on these observations, we searched the *E. histolytica* GeneDB (at http://www.genedb.org/genedb/ehistolytica/) database for potential “secretory” nucleases having the following properties: 1) a predicted signal peptide sequence defined using the SignalP 3.0 Server (at http://www.cbs.dtu.dk/services/SignalP/), 2) lack of any apparent membrane-anchoring motifs and 3) a predicted molecular mass of ~ 20-30 kDa. Amongst those meeting these criteria, two were chosen for further analyses. The deduced proteins encoded by these two open reading frames (ORFs) were annotated in the database as: putative ribonucleases, (Gene DB Identifier: 177.m00126 and 100.m00129) and designated here as: *EhNucI* and *EhNucII*, respectively. Comparisons of these two *E. histolytica* sequences were done using the Gap-Global Alignment program of Genetic Computer Group (GCG) via NIH Helix (at http://molbio.info.nih.gov/molbio/gcglite/compare.html).

Primers were designed against each of these sequences towards amplifying them using PCR. To that end, to amplify *EhNucI*, a forward primer (Fwd 5’-ATGATTTCATTGTGTTATTAC - 3’) with a methionine initiation codon (boldface type) corresponding to aa1-aa7 and a reverse primer (Rev 5’-TAAATACAAACATCCACC -3’) corresponding to aa247-aa251 were synthesized as above.

For PCR amplifications, these primers were used with 500 ng of *E. histolytica* HM-1 strain genomic (g) DNA as template and a high fidelity polymerase mix (High Fidelity PCR Master Mix, Roche). After an initial “hot start” at 94°C for 2 min, the conditions used for amplification were: 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min (35 cycles) and a final step at: 72°C for 5 min. The resulting ~750-bp amplified-products were cloned into the pCR®2.1-TOPO vector (Invitrogen). The resulting plasmid clones of *EhNucI* and *EhNucII* were subjected to nucleotide sequencing using vector encoded M13 -forward and -reverse primers. Results of those analyses demonstrated that their nt sequences were identical to those annotated in the *E. histolytica* GeneDB.

Nucleotide Sequencing and Analyses- DNA was sequenced using the fluorescent di-deoxy chain terminator cycle sequencing method (11) at the Johns Hopkins University DNA Analysis Facility (Baltimore, MD). Sequence data obtained from both strands were analyzed using the GCG software package (12) running on a NIH Unix System and Sequencher™3.0 software (Gene Codes Corp., Ann Arbor, MI). Further, such sequences were also subjected to BLAST-N and BLAST-P analyses using the NCBI BLAST-link (http://www.ncbi.nlm.nih.gov/BLAST/). Signal peptide sequence and protease cleavage sites were predicted using the SignalP link available at the worldwide ExPASy (Expert Protein Analysis System) proteomics server available at: http://www.expasy.ch/tools. Protein domain analysis was done using the Worldwide Web based Simple Modular Architecture Research Tool (SMART, at http://smart.embl-heidelberg.de) available via EMBL-EBI, European Bioinformatics Institute at http://www.ebi.ac.uk). Protein multiple sequence alignments were done using the ClustalW program (13) using a MacVector® 7.0 software package (Accelrys).
recommendations (Tel-Test, Inc., Friendswood, TX). To verify that the isolated parasite RNA was free of DNA contamination it was tested in a control reaction that lacked reverse transcriptase. Reverse transcription of *E. histolytica* total RNA was done using the GeneAmp Kit (Applied Biosystems) with oligo-d(T)16 to generate cDNA according to manufacturer’s instructions. PCR amplification was carried out using conditions as above but with the following oligonucleotide primer pairs: for *EhNucI*: Fwd 5’-GGCTCTTAAATCAGGATT-3’ and Rev 5’-GAAAGAGAAGCCCCAGTG-3’, and for *EhNucII*: Fwd 5’-GGCTACTAAACCAGGATT-3’ and Rev 5’-GTTAATGAAGCCCAATTG-3’. In addition, a set of forward and reverse oligonucleotide primers corresponding to a portion of an *E. histolytica* actin gene (16) were synthesized and used as a positive control in these reactions. The PCR amplified products resulting from these reactions were separated and analyzed using 1.2% E-Gels® (Invitrogen). These PCR fragments were cloned into the pCR®2.1-TOPO vector (Invitrogen) and subjected to nucleotide sequencing using vector encoded M13 -forward and -reverse primers.

**Generation of Epitope Tagged Expression Constructs** - A homologous episomal system was used to express epitope-tagged *EhNucI-HA* and *EhNucII-HA* chimeric proteins in *E. histolytica* trophozoites. To that end, individual constructs were designed that contained the complete open reading frame of the *EhNucI* and *EhNucII* genes (including 5’-ends encoding putative signal peptides) joined, at the 3’-ends, with a nine amino acid sequence encoding the influenza virus hemagglutinin (-HA) epitope (Roche). These constructs were generated by PCR using *E. histolytica* gDNA as template with the following forward primers: for *EhNucI*: Fwd 5’-GGTACCATGATTTGTTGATTTAC-3’ (containing a *KpnI* restriction site shown in bold) and for *EhNucII*: Fwd 5’-GGTACCATGATTTGTTGATTTAC-3’. A common reverse primer was used for both *EhNucI* and *EhNucII* in these reactions, *i.e.*: Rev 5’-GGATCCTTAAAGCGTAATCTGGAAACATCGTATGGGTAATACAACACATCCACC-3’ (containing a *BamHI* restriction site shown in bold; stop codon in bold italics; and the -HA epitope sequence [underlined]). The conditions used for these PCR were: a “hot start” at 94°C for 5 min, followed by 10 cycles of amplification: 94°C for 30 sec, 50°C for 30 sec, 72°C for 30 sec, followed by 25 cycles of amplification: 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec and a final step at 72°C for 5 min. The resulting ~800-bp amplified fragments (*EhNucI-HA* and *EhNucII-HA*) were gel purified and cloned into the pCR®2.1-TOPO vector (Invitrogen) to generate pCR2.1::*EhNucI-HA* and pCR2.1::*EhNucII-HA* plasmids. The inserts were excised from these plasmids using *KpnI* and *BamHI*. Subsequently, the excised inserts were ligated into the *KpnI* and *BamHI* -sites of the *Entamoeba histolytica* expression-vector *ExEhNeo* ([17], kindly provided by Dr. Egbert Tannich, Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany) to generate *ExEhNeo::EhNucI-HA* and *ExEhNeo::EhNucII-HA* plasmid constructs. The nt sequences of these constructs were verified by DNA sequence analysis.

**Homologous Episomal Expression of *EhNucI* and *EhNucII* -** The *ExEhNeo::EhNucI-HA* and *ExEhNeo::EhNucII-HA* plasmid constructs as well as the [ExEhNeo] control plasmid were transfected into *E. histolytica* trophozoites using electroporation conditions modified from those described by Nickel and Tannich (18). Briefly, mid-log phase trophozoites (~1.5 X 10⁵ cells ml⁻¹) were detached by chilling on ice for 10 min, pelleted by centrifugation at 500 X g at 4°C for 10 min, washed twice in ice-cold PBS and once in electroporation buffer (120 mM KCl, 0.15 mM CaCl₂, 25 mM HEPES, 2 mM EGTA, and 5 mM MgCl₂, 10 mM K₂HPO₄/KH₂PO₄, pH 7.6). Subsequently, 2.4 X 10⁶ cells were resuspended in 0.8 mL of electroporation buffer supplemented immediately before use with 3µg/mL (final concentration) DEAE-Dextran (ProFection®, Promega), and transferred to 4-mm gap, electroporation cuvettes (BTX, Harvard Apparatus) in the presence or absence of 100µg of plasmid DNA. Cells were pulsed once using a BTX-ECM600 electroporator under conditions of 500V/cm charging voltage, 800 µF capacitance, and 129 Ohms resistance. Subsequently cells were place on ice for 5 min and then subjected to a second electroporation pulse. Following this, they were placed in complete culture medium as above and allowed to recover for 48 hours at 37°C before
being selected for their growth in the same medium containing 5µg ml⁻¹ Geneticin® (G418, Invitrogen). Once drug-resistant parasites emerged, they were further selected for growth in increasing concentrations of G418 up to 10µg ml⁻¹. For routine purposes, these transfectants were maintained and grown at 37°C in complete growth medium containing 10 µg ml⁻¹ of G418. Both cell lysates and cell-free culture supernatants were prepared from such transfectants as described above.

Growth Kinetics of Transfected Parasites-
ExEhNeo control, ExEhNeo::EhNucI-HA and ExEhNeo::EhNucII-HA transfected parasites were monitored at regular intervals during the course of their growth in vitro. Parasite cultures used for growth kinetic studies were initiated at 3 X 10^3 cells ml⁻¹ from stock cultures in their exponential phase of growth. Aliquots of the resulting cultures were taken at regular intervals, diluted appropriately and counted using disposable cell counting chambers (Cellometer™, Nexcelom Bioscience, Lawrence, MA).

Temporal release assays- Temporal release assays were used to determine whether transfected trophozoites constitutively released/secreted any episomally-expressed proteins into their extracellular environment over time. For these experiments, mid-log phase transfected trophozoites were harvested by centrifugation at ~500 X g as above, resuspended and washed twice with phosphate buffered saline (PBS, 10mM sodium phosphate, 145 mM NaCl, pH 7.4) by centrifugation and finally resuspended at 3 X 10^5 cells ml⁻¹ in fresh complete growth medium. Following incubation at 37°C for various periods (i.e. 30, 60, 90, and 180 min), aliquots were removed, centrifuged as above to produce cell-free supernatants for Western blot analyses.

Western Blots- Lysates of E. histolytica episomal-transfected parasites, as well as aliquots of their cell-free culture supernatants (all in 1X LDS sample buffer, Invitrogen) were separated in SDS-PAGE gels (10 %, pre-cast, Bis-Tris polyacrylamide, NuPAGE® gels, Invitrogen). These gels were trans-blotted onto polyvinylidene difluoride (PVDF) membranes (Invitrogen), and the membranes were blocked and washed as described previously (19). Such blots were probed with a rabbit anti-HA polyclonal antibody (Sigma) as described (8). Subsequent to incubation and washing, the blots were reacted with a donkey anti-rabbit-horse radish peroxidase (HRP)-conjugated secondary antibody (GE Healthcare Bio-Sciences) as previously described (19). Immuno-detection was carried out using ECL Western Blot Kit reagents according to manufacturer’s recommendations (GE Healthcare Bio-Sciences) and images were captured using BIOMAX™-MR X-ray film (Kodak).

Subcellular fractionation- Trophozoites of E. histolytica transfected with EhNucI-HA and EhNucII-HA were subjected to subcellular fractionation using Dounce homogenization and differential centrifugation essentially as described by Sato et al. (20).

Light Microscopy- The subcellular localization of EhNucI-HA and EhNucII-HA was investigated using indirect immunofluorescence microscopy. For these assays, episomally-transfected trophozoites were chilled on ice for 10 min, pelleted by centrifugation at 500 X g for 10 min, and then washed 3 times in ice cold PBS. These cells were resuspended in fresh culture medium lacking bovine serum, and aliquots were placed onto glass coverslips and allowed to adhere for 30 min at 37°C. For some experiments, the medium was supplemented with either 100µg ml⁻¹ Texas Red Dextran (10,000 MW, Molecular Probes, Invitrogen) to label pinosomes, or with 6 X 10^8 Texas Red labeled E. coli BioParticles® (Invitrogen) to label phagosomes and cells incubated for up to 3 hr at 37°C. Subsequently, the adherent cells were washed 3 times with pre-warmed PBS, fixed in pre-warmed 2% paraformaldehyde (Polysciences, Inc., Warrington, PA) in PBS, incubated for 30 min at room temperature (RT) and then post-fixed for 5 min in -20°C methanol. For immunofluorescence, fixed cells were blocked with 0.5% (w/v) bovine serum albumin (MP BioMedicals), 0.045% (w/v) fish gelatin (Sigma) in PBS, pH 7.2 at RT for 30 min. These fixed and blocked cells were then reacted with a rabbit anti-HA polyclonal antibody (Sigma) appropriately diluted in the blocking buffer as above for 1 hr at RT. Following washing with PBS, these cells were reacted for 30 min at RT with a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (H +L) secondary antibody (Jackson ImmunoResearch Laboratories) diluted in blocking buffer. Subsequently, cells were washed three times with PBS and once with
distilled water. Stained, washed coverslips were mounted on glass slides with VECTASHIELD® mounting medium containing 15 μg/ml 4’-6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Inc., Burlingame, CA) and sealed with nail polish.

Confocal and z-series images were obtained using a Leica model TCS-AOBS/SP2 confocal microscope with a 63X, 1.4 NA objective. Images were adjusted for contrast in Adobe Photoshop CS2 (Adobe Systems). Some z-series were processed for deconvolution with Huygens®Essential, version 2.9.1p0 (Scientific Volume Imaging BV, The Netherlands). Three-dimensional reconstructions and isosurface models were assembled with Imaris®, version 5.5.2 (Bitplane AG, Zurich, Switzerland).

Immunoprecipitation and Detection of Parasite Nuclease Activity- To test whether our episomally expressed EhNucI-HA and EhNucII-HA actually possessed functional nuclease activity, we immunoprecipitated these proteins from whole cell lysates of E. histolytica trophozoites. Immunoprecipitations were performed using the Catch and Release® v2.0 system (Upstate-Cell Signaling Systems, Millipore) using 1mg of total cellular protein and a rabbit anti-HA antibody (Sigma) according to the manufacturer’s instructions. Following binding to this bead-matrix, the immunoprecipitated proteins were washed and eluted using buffers according to manufacturer’s recommendations. Similarly, cell-free culture supernatants from these transfectants were subjected to immunoprecipitations as above to determine whether the expressed EhNucI-HA and EhNucII-HA released/secreted by these parasites also possessed functional nuclease activity. For these assays, parasite cell-free culture supernatants were analyzed for their nuclease activity using SDS-PAGE-poly(A) zymogram gels in the presence or absence of DTT as described above.

For some experiments, aliquots of cell free culture supernatants were also subjected to immunoprecipitations with either sera obtained from patients suffering from acute amebic disease or sera from normal, uninfected volunteers in conjunction with a Protein A/G affinity bead matrix, washed and eluted as above. The resulting immune complexes were subjected to SDS-PAGE and subsequent Western blotting using a rabbit anti-HA polyclonal antibody.

Nuclease Assays using Nucleic Acid Substrates- Total RNA was isolated from Leishmania donovani as previously described (8), and was used as substrate to evaluate the ribonuclease activity of the E. histolytica EhNucI-HA and EhNucII-HA episomally expressed nucleases. Aliquots of this RNA were incubated with immunoprecipitates of EhNucI-HA and EhNucII-HA obtained from whole cell lysates as described above. Such reactions were carried out in 100mM MES buffer at pH 6.0 or 100mM HEPES buffer at pH 8.0 in a total assay volume of 20 µl. Following incubation at 37°C for 30 minutes, the reaction products were mixed with RNA-loading buffer containing ethidium bromide (Sigma), boiled for 5 min and then resolved in 1.2% agarose gels containing 0.7% formaldehyde (v/v) in MOPS buffer (Quality Biosciences, Inc.). Subsequently, the hydrolysis products in these gels were visualized using an ultraviolet trans-illuminator with an UVP-Biochemi Imaging System (UVP-Bioimaging Systems, UVP, Inc., Upland, CA) and images were processed using Adobe Photoshop CS2.

To further evaluate the nuclease activities of the EhNucI-HA and EhNucII-HA expressed proteins they were reacted with both an ~ 7, 249 nt, single stranded (ss-) DNA (M13mp18 phage, Sigma), and a circular 7,249 bp, double stranded (ds-) DNA (M13mp18 RF I, Sigma) as substrates. For experiments, aliquots of such ssDNA (0.5 µg) or dsDNA (0.5 µg) were incubated with anti-HA immunoprecipitates of EhNucI-HA and EhNucII-HA obtained from cell lysates (as above) in a total reaction volume of 25 µl containing 100 mM MES, pH 6.0. Following incubation (30 min at 37°C), the hydrolysis products were resolved in
0.8% E-Gels® (Invitrogen) according to the manufacturer’s instructions. Subsequently, images were captured using an ultraviolet trans-illuminator and an UVP-Biochem Imaging System (UVP-Bioimaging Systems, UVP, Inc., Upland, CA) and processed using Adobe Photoshop CS2, as above.

RESULTS

Identification of *E. histolytica* released/secreted nuclease activity. Previously, it has been shown experimentally that *Entamoeba histolytica* parasites are incapable of the *de novo* biosynthesis of purine nucleo-bases (i.e. purine auxotrophs) (21). Further, recent analysis of the *E. histolytica* genome has revealed that these parasites also lack the essential enzymes for pyrimidine biosynthesis (22,23). Thus, these organisms are totally dependent upon their human hosts to provide these essential nutrients for their growth and survival. In that regard, while it has been shown that axenically cultured *E. histolytica* parasites are capable of taking up such nutrients from their growth medium in *vitro* (5,24,25), the means by which these parasites might access these essential compounds *in vivo* within their human host is unknown. One potential mechanism by which *Entamoeba* might access exogenous nucleic acids present in their host environment is via the release/secretion of nucleic acid hydrolases (e.g. nucleases). To examine this concept further, experiments were designed to evaluate whether *E. histolytica* trophozoites released any nuclease activity into their culture medium during their growth in *vitro*. This was accomplished by analyzing cell-free culture supernatants from parasites for their nuclease activities using SDS–PAGE, poly(A)-containing substrate zymogram gels. For these assays, samples were prepared in the presence of dithiothreitol (DTT, 50mM final concentration (19,26)) or absence of this reducing agent (27).

Results obtained from these *in situ* activity gel assays indicated that under non-reducing conditions, cell-free culture supernatants of *E. histolytica* contained two distinct zones of nuclease activity of ~24 and ~25 kDa (Fig. 1A, lanes 1 and 2). However, no nuclease activity was observed when these samples were first treated with the reducing agent DTT (Fig. 1B, lanes 1 and 2). In addition, no nuclease activity was observed from similarly treated (+/−DTT) control samples of fresh parasite culture medium (data not shown). Taken together, results of these activity gel assays demonstrated that *E. histolytica* trophozoites possess at least two DTT-sensitive nuclease activities. Further, these nuclease appear to be constitutively released by these parasites into their culture medium during their growth *in vitro*.

Identification and characterization of EhNucl and EhNuclII genes. In preliminary experiments we used a variety of different affinity-based bead matrices (e.g. binding to concanavalin A and the nucleotide-dye mimic, Reactive Red) in attempts to isolate sufficient quantities of these nucleases for functional characterization including direct amino acid sequencing (data not shown). However, we were unable to obtain sufficient quantities of these proteins for such purposes. In that regard, it is important to point out that while the functional activity of the parasite secreted nucleases could be readily detected in the *in situ* activity gels above using cell-free culture supernatants, the actual amount of protein present in these samples was far below the level of detection. These observations suggested that the parasite secreted nucleases might exhibit high levels of specific activity. Therefore, we adopted a molecular approach to determine the possible identity of these secreted nucleases. To accomplish this, an annotated protein database of *E. histolytica* (Gene DB) was examined for putative secretory nucleases using the follow search criteria: (i) a predicted molecular mass of ~20 - 30 kDa; (ii) possession of a predicted signal peptide; and (iii) the lack of any apparent membrane-anchoring motif(s). Amongst the 51 putative nucleases present in the *E. histolytica* database, five met our search criteria, and two of these were selected for further investigation. These two deduced proteins were annotated in Gene DB as 177.m00126 and 100.m00129, and both were designated as putative (i.e. no demonstrated functional activities) ribonucleases. To amplify genes encoding these two putative *E. histolytica* nucleases, oligo-nucleotide primers were synthesized complementary to the sequences of their 5’- and 3’-ends and used in PCR with *E. histolytica* genomic DNA as template. The resulting ~750 bp products obtained from these amplification reactions were gel purified, cloned.
and subjected to nucleotide sequencing, and designated as \(EhNucI\) and \(EhNucII\). These sequences were subjected to both BLAST-N and BLAST-P analyses. Those analyses showed that the PCR clones contained open reading frames (ORF) that had complete nucleotide and deduced amino acid sequence identity with the putative \(E.\ histolytica\ 177.m00126\) and 100.m00129 ribonucleases above.

The deduced amino acid sequences encoded by the open reading frames of \(EhNucI\) and \(EhNucII\) were compared to all available, non-redundant databases using BLAST-P. Results of these comparisons showed that they had homologies to nucleases from diverse sources (28-31). Further, analyses of the \(EhNucI\) and \(EhNucII\) deduced-proteins using the Pfam database showed that they had high homology with members of the T2 family of ribonucleases. Members of the T2 RNase family have been isolated from a variety of bacteria, fungi, plants and animals and typically show an acid pH preference [reviewed in (32)]. While \(Aspergillus\ oryzae\), the proto-typical T2 RNase family member, shows a preference for adenylic acid (33), most members of this family show no specific base preference [reviewed in (32)].

Based on the von Heijne algorithm (34,35), the hydrophobic, N-terminal, 14 amino acids of both the \(EhNucI\) and \(EhNucII\) deduced-proteins constitute putative signal peptides (Fig. 2). Cleavage at these sites, presumably within the parasite endoplasmic reticulum-like structure(s), would result in mature proteins with Lys15 as the N-terminal amino acid residue. Such cleavages in \(EhNucI\) and \(EhNucII\) would result in mature proteins consisting of 237 amino acids with calculated molecular masses of 26,340 and 26,339 Da, respectively. Both proteins have a calculated pl of 6.56. The deduced proteins were also analyzed using various other structural algorithms. Those analyses indicated that both of these enzymes lacked any apparent hydrophobic transmembrane domains or glycosylinositol phosphate (GPI-) anchor signature sequences (36). Similarly, no KDEL- or analogous endoplasmic reticulum (ER) retention sequences (37) or any other intracellular organelle specific-targeting sequences were identified in either deduced protein. The overall hydrophilicity, the presence of N-terminal signal peptides and the absence of both membrane anchors and ER-retention motifs suggest that both \(EhNucI\) and \(EhNucII\) represent soluble, released/secreted proteins. Interestingly, many microbial members of the T2 ribonuclease family have been shown to function as extracellular enzymes (38-40).

Previously it has been reported that members of the T2 family of ribonucleases posses two conserved amino acid sequences (CAS I and II), each containing a single histidine residue essential for catalytic activity \(41,42\). Analyses of the \(EhNucI\) and \(EhNucII\) deduced proteins showed that they both posses the CAS I and II domains including the two critical histidine residues (Fig. 2, boxed aa), further solidifying the identity of these \(Entamoeba\) enzymes as T2 ribonucleases. Additionally, all members of the T2 ribonuclease family possess at least four structurally conserved cysteine residues involved in intra-chain disulfide bridges and such bridges are essential for the enzymatic functions of these nucleases (43). In that context, our analyses showed that the mature \(EhNucI\) and \(EhNucII\) deduced proteins also possess four similarly conserved cysteine residues that could be involved in forming disulfide bridges (Fig. 2. Cys\(^{81}\), Cys\(^{128}\), Cys\(^{195}\) and Cys\(^{232}\)). Such disulfide bonds appear to be essential for the function of the endogenous \(E.\ histolytica\) wild-type released/secreted enzymes, as treatment with the reducing agent, DTT, abolished all parasite nuclease activity (cf. Fig. 1 panels A and B).

Expression of \(EhNucI\) and \(EhNucII\). Reverse transcriptase (RT-) PCR was used to determine whether \(EhNucI\) and \(EhNucII\) were indeed transcribed by \(E.\ histolytica\) parasites. To accomplish this, total RNA was extracted from \(E.\ histolytica\) trophozoites and reverse transcribed using reverse transcriptase and Oligo d(T)\(_{16}\). Aliquots (100ng) of the resulting cDNA were used as template in PCR with primers \((RT-F\) and \(RT-R)\) specific to \(EhNucI\) and \(EhNuc II\), respectively (Fig. 3, panel A). A control reaction that lacked reverse transcriptase was used to verify that the isolated parasite RNA was free of DNA contamination. In addition, a set of oligonucleotide primers corresponding to a portion of an \(E.\ histolytica\) actin gene \((16)\) was used as a positive control in these reactions. Products from these reactions were separated and visualized in 1.2% agarose E-Gels\(^{\circ}\). Results of these analyses...
showed that no PCR product was generated from a control reaction that lacked reverse transcriptase verifying that the isolated parasite RNA was free of DNA contamination (Fig. 3B, lane 1). As anticipated, a single amplified product of \( \sim 220 \) bp was obtained from the \( E. \) histolytica actin positive control (Fig. 3B, lane 4). More importantly, a single amplified product of ~180 bp was generated for both \( \text{EhNucl} \) and \( \text{EhNuclII} \) (Fig. 3B, lanes 2 and 3, respectively). These results indicated that \( E. \) histolytica trophozoites were actively synthesizing message for each of these genes. To analyze this further, the products of these reactions were gel purified, cloned and sequenced. Results of such sequence analyses verified that mRNAs for each of these nucleases (i.e., \( \text{EhNucl} \) and \( \text{EhNuclII} \)) were in fact readily transcribed by these parasites during their growth in vitro.

**Transfection of** \( E. \) histolytica trophozoites with epitope-tagged gene constructs. We used a homologous episomal expression system to functionally characterize the proteins encoded by the \( \text{EhNucl} \) and \( \text{EhNuclII} \) genes. To accomplish this, individual chimeric constructs were generated that contained the complete ORF of either \( \text{EhNucl} \) or \( \text{EhNuclII} \), in frame, at the 3'-end with a sequence encoding a nine aa HA-epitope of the influenza virus (designated as: \( \text{EhNucl-HA} \) and \( \text{EhNuclII-HA} \), respectively). Following ligation into the \( \text{ExEhNeo Entamoeba} \) expression vector, these constructs were used to transfect \( E. \) histolytica trophozoites (Fig. 4A). Cells transfected with the \( \text{ExEhNeo} \) vector alone served as controls in all transfection experiments. Transfected parasites were selected for their growth in complete medium containing increasing concentrations of G418 (i.e. up to 10 \( \mu \)g ml\(^{-1} \)). Following drug selection, the growth kinetics of these transfected parasites were compared. For experiments, triplicate cultures of \( \text{EhNucl-HA}, \text{EhNuclII-HA} \), and \( \text{ExEhNeo} \) control transfectants were initiated at 4 \( \times \) 10\(^3 \) cells ml\(^{-1} \) from stock cultures in their exponential phase of growth. Aliquots from such cultures were taken at 24 hr intervals, diluted appropriately and counted using Cellometer™ disposable cell counting chambers. Results of these in vitro assays showed that cells transfected with either the \( \text{EhNucl-HA} \), \( \text{EhNuclII-HA} \) chimeric constructs or the \( \text{ExEhNeo} \) control plasmid had very similar growth kinetics (Fig. 4B). These observations indicated that these episomal-transfections did not appear to alter the characteristic growth kinetics of the parental \( E. \) histolytica cell line.

Subsequently, such transfected cells were analyzed for the expression of the \( \text{EhNucl-HA} \) and \( \text{EhNuclII-HA} \) chimeric proteins using Western blots, in situ activity gel assays and immunofluorescence confocal microscopy.

**Expression of** \( \text{EhNucl-HA} \) and \( \text{EhNuclII-HA} \) **chimeric proteins in transfected parasites.** Western blot analyses were performed to determine whether transfected parasites synthesized and released/secreted the chimeric proteins during their growth in vitro. For these experiments, cells transfected with either the \( \text{EhNucl-HA} \) or \( \text{EhNuclII-HA} \) constructs or \( \text{ExEhNeo} \) control plasmid were grown in complete medium containing 10 \( \mu \)g ml\(^{-1} \) of G418. Lysates and cell-free culture supernatants from such cells were subjected to SDS-PAGE and subsequent Western blotting using a rabbit anti-HA polyclonal antibody. Results of such assays showed that the anti-HA antibody reacted with only a single ~28 kDa protein present in lysates of both \( \text{EhNucl-HA} \) and \( \text{EhNuclII-HA} \) transfected cells (Fig. 5A, lanes 2 and 3). In parallel blots, the anti-HA antibody reacted most strongly with a ~28 kDa protein present in the cell-free culture supernatants of both \( \text{EhNucl-HA} \) and \( \text{EhNuclII-HA} \) transfected cells (Fig. 5B, lanes 2 and 3). This antibody also reacted to a lesser extent with several lower apparent molecular weight proteins present in the cell-free culture supernatants of these transfecteds. Presumably, the latter represent proteolytic degradation products of the released/secreted \( \text{EhNucl-HA} \) and \( \text{EhNuclII-HA} \) expressed proteins. In contrast, the anti-HA antibody showed no reactivity with either lysates (Fig. 5A, lane 1) or cell-free culture supernatants (Fig. 5B, lane 1) obtained from control \( \text{ExEhNeo} \)-transfected cells.

Having demonstrated that the chimeric proteins were in fact synthesized and released by transfected parasites into their culture supernatants, it was of interest to investigate the temporal release of both \( \text{EhNucl-HA} \) and \( \text{EhNuclII-HA} \). To that end, 3 \( \times \) 10\(^5 \) cells transfected with either the \( \text{EhNucl-HA} \) or \( \text{EhNuclII-HA} \) construct were incubated for up to 3 hours in complete medium. At defined intervals, cells were
harvested and their resulting cell-free culture supernatants were subjected to SDS-PAGE and subsequent Western blotting as above. Results from such blots showed that the transfected parasites released/secreted detectable quantities of both EhNucI-HA and EhNucII-HA into their culture medium as little as 30 min post incubation (Fig. 5C, 30min). In addition, both of these expressed proteins appear to accumulate in the culture medium over time (Fig. 5C, 60min-180min).

Taken together, results of these Western blot experiments demonstrated that: 1) the EhNucI-HA and EhNucII-HA chimeric-gene constructs were readily transcribed and translated into single ~28 kDa chimeric proteins by transfected E. histolytica cells; 2) both proteins were released/secreted by these transfectants during their growth in vitro; and 3) both proteins appeared to accumulate in the culture medium of these transfectants over time.

Intracellular distribution of EhNucI-HA and EhNucII-HA. In preliminary experiments, subcellular fractionations were carried out to determine the intracellular localization of the EhNucI-HA and EhNucII-HA expressed proteins. For such experiments, transfected parasites were Dounce homogenized, subjected to differential centrifugation and the resulting pellets and supernatants analyzed by SDS-PAGE and Western blotting with an anti-HA antibody as above. Results obtained from these experiments demonstrated that the EhNucI-HA and EhNucII-HA expressed proteins were pelletable and appeared to fractionate with vesicular compartments (data not shown). Similar results have been obtained with other compartmentalized proteins in E. histolytica (20).

Subsequently, we used indirect immunofluorescence microscopy to visualize the subcellular distribution of EhNucI-HA and EhNucII-HA in E. histolytica transfected parasites. For these experiments, EhNucI-HA, EhNucII-HA and ExEhNeo control transfected cells were fixed, permeabilized and reacted with a rabbit anti-HA polyclonal antibody. Subsequently, cells were reacted with a FITC-conjugated secondary antibody and examined by confocal-fluorescence microscopy. Results obtained from microscopic evaluation revealed that the EhNucI-HA and EhNucII-HA expressed proteins were present in vesicular compartments dispersed throughout the cytoplasm of EhNucI-HA and EhNucII-HA transfected cells (Fig.6A and B). Such staining is consistent with processing of the EhNucI-HA and EhNucII-HA proteins through the rudimentary endoplasmic reticulum present in these parasites for entry into the secretory system. Importantly, very similar immunofluorescence staining patterns have been reported for other secretory proteins in these parasites (44,45). In contrast to the above, no fluorescence was detected in cells transfected with the ExEhNeo control plasmid (data not shown). Results of these experiments showed that the EhNucI-HA and EhNucII-HA expressed proteins were readily synthesized and localized in vesicular compartments in cultured E. histolytica trophozoites.

To further characterize the nature of the vesicular compartments seen in the above microscopic experiments, both phagosomes and pinosomes were labeled in the EhNucI-HA and EhNucII-HA transfected cells. To label pinosomes, cells were allowed to ingest 10 kDa Texas Red labeled dextran for 60 min. To label phagosomes, cells were allowed to ingest Texas Red labeled E. coli for 60 min. Following such incubations, cells were fixed, permeabilized, and reacted with a rabbit anti-HA polyclonal antibody as above, and then reacted with a FITC-conjugated secondary antibody and examined by confocal-fluorescence microscopy. Results obtained from deconvolved z-series images showed that the EhNucI-HA and EhNucII-HA expressed proteins did not co-localize with either compartments containing labeled dextran (i.e. pinosomes, Fig. 7A and B) or those containing labeled bacteria (i.e. phagosomes, Fig.7C and D). In addition, some of these images were subjected to three-dimensional projections and isosurface modeling. Videos of these were assembled using Imaris®, version 5.5.2 (Bitplane AG, Zurich, Switzerland). Results obtained from analysis of the latter further confirmed that the EhNucI-HA and EhNucII-HA expressed proteins did not colocalize with compartments containing endosomal markers. A representative video illustrating the three dimensional intracellular distribution of EhNucI-HA and Texas-red labeled pinosomes is shown in Supplemental Fig. S1.

Similarly, EhNucI-HA and EhNucII-HA expressed proteins did not co-localize with
compartments containing various other endosomal markers (i.e. latex beads, human red blood cells, and Chinese Hamster ovary cells (data not shown)).

Results of these immunofluorescence experiments showed that: 1) the EhNucl-HA and EhNuclII-HA expressed proteins were synthesized and processed by the transfectedants and 2) the expressed proteins were localized in putative secretory vesicles (i.e. vesicular compartments distinct from either phagosomes or pinosomes).

Functional enzyme activity analyses of the EhNucl-HA and EhNuclII-HA expressed proteins. Results of our Western blots and subcellular localization studies above demonstrated that both EhNucl-HA and EhNuclII-HA chimeric proteins were synthesized and released/secreted by transfected cells. It was important, however, to also demonstrate that these expressed proteins actually possessed functional nuclease activity. To accomplish this, parasites transfected with the EhNucl-HA construct, the EhNuclII-HA construct, or the ExEhNeo control plasmid were grown in complete medium and their cell-free culture supernatants were harvested. Aliquots of these supernatants were subjected to immunoprecipitations using a rabbit anti-HA polyclonal antibody in conjunction with an affinity matrix and the resulting immune complexes were analyzed for their nuclease activity using poly(A)-containing SDS-PAGE zymogram gels. Results of these assays demonstrated that the anti-HA antibody specifically immunoprecipitated a major ~25 kDa band of nuclease activity from the cell-free culture supernatants of both EhNucl-HA and EhNuclII-HA transfectedants (Fig. 8A, lanes 2 and 3, respectively). These immunoprecipitates also contained several additional bands of nuclease activity of higher apparent molecular mass, which presumably reflect aggregates of these 25 kDa nucleases. As expected, however, no activity was detected in anti-HA immunoprecipitates obtained from culture supernatants of ExEhNeo control transfectedants (Fig. 8A, lane 1). Further, no detectable nuclease activity was observed with immunoprecipitates obtained from parallel samples incubated with the reducing agent DTT (50 mM final concentration) and then analyzed using a poly(A)-containing SDS-PAGE zymogram gel (Fig. 8B).

It is important to mention that the nuclease activities immunoprecipitated above appeared to have relative molecular masses different than those observed in our Western blots. To reconcile these apparent differences, parallel immunoprecipitated samples were separated in poly(A) containing SDS-PAGE gels, and then transferred to PVDF and subsequently analyzed by Western blot as above. Results of these analyses showed that inclusion of poly(A) in these SDS-PAGE zymogram gels altered the relative mobility of both the EhNucl-HA and EhNuclII-HA expressed proteins (data not shown). Taken together, results of these coupled immunoprecipitation/zymogram analyses demonstrated that the EhNucl-HA and EhNuclII-HA expressed proteins released/secreted by transfected parasites did in fact possess functional nuclease activities that, like the endogenous wild-type parasite enzymes, were completely inhibited by treatment with DTT.

Activity of the EhNucl-HA and EhNuclII-HA expressed proteins using synthetic polynucleotide substrates. As shown above, the wild-type parasite released/secreted nucleases and the EhNucl-HA and EhNuclII-HA expressed enzymes readily hydrolyzed the poly(A) substrate in zymogram gels. In preliminary experiments, we found that the nuclease activities released/secreted by wild-type parasites were also capable of hydrolyzing various other polynucleotide substrates. Therefore, it was of interest to determine whether the EhNucl-HA and EhNuclII-HA expressed nucleases were also capable of hydrolyzing such substrates. To that end, anti-HA immunoprecipitates obtained from lysates of EhNucl-I-HA, EhNuclII-HA, and ExEhNeo control transffected parasites were analyzed for their nuclease activity using SDS-PAGE zymogram gels containing various individual polynucleotide substrates [poly(A), poly(U), poly(I) or poly(G)]. Results of such analyses demonstrated that the anti-HA immunoprecipitates obtained from EhNucl-I-HA and EhNuclII-HA transfectedants each possessed a predominant ~25 kDa nuclease activity, capable of hydrolyzing three of the polynucleotide substrates tested: poly(U) > poly(A) > Poly(I) (Fig. 9, panels A-C lanes 2 and 3, respectively). In contrast, such immunoprecipitates failed to show any detectable nuclease activity with poly(G) containing...
zymogram gels (data not shown). As expected, the anti-HA immunoprecipitates obtained from ExEhNeo control transfectants showed no nuclease activity with any of the polynucleotide substrates tested in these assays (Fig. 9, lane 1 in panels A-C).

Results of these coupled anti-HA immunoprecipitation-zymogram assays demonstrated that the EhNucl-HA and EhNucII-HA expressed proteins, similar to the native wild-type released/secreted nucleases, were capable of hydrolyzing various different polynucleotide substrates.

**Activity of the EhNucl-HA and EhNucII-HA expressed enzymes with nucleic acid substrates.** The activities of the Entamoeba expressed nucleases were also evaluated using several different nucleic acid substrates. For these assays, RNase activity was assessed using total RNA isolated from an unrelated eukaryotic organism as substrate, while DNase activities were evaluated using both a single stranded M13mp18 phage DNA (ssDNA) and double stranded M13mp18 RF I DNA (dsDNA) as substrates. To determine whether EhNucl-HA and EhNucII-HA possessed RNase or DNase activities, these expressed proteins were immunoprecipitated from cell lysates using an anti-HA antibody as above. Aliquots of the eluted immune complexes were reacted with the RNA, ssDNA and dsDNA substrates above in either a pH 6.5 or pH 8.0 buffer for 30 min at 37°C. Following incubation, RNA containing samples were separated in 1.2% agarose gels containing formaldehyde and ethidium bromide and DNA containing samples were separated in 0.8 % E-Gels®. Subsequently, all samples were visualized and evaluated using UV trans-illumination. Results of these experiments demonstrated that immunoprecipitates obtained from lysates of both the EhNucl-HA and EhNucII-HA transfectants readily hydrolyzed the RNA substrate (Fig. 10A, lanes 2 and 3, respectively). In contrast such immunoprecipitates showed no activity with either the ssDNA or the dsDNA substrates (Fig. 10, panels B and C, lanes 2 and 3, respectively). Interestingly, the RNA substrate seemed to be more efficiently hydrolyzed at acidic pH (pH 6.5) than under alkaline conditions (pH 8.5, data not shown). As expected, anti-HA immunoprecipitates obtained from control ExEhNeo transfected parasites failed to hydrolyze any of these RNA, ssDNA or dsDNA substrates (lanes 1 in Fig. 10A, B, and C, respectively).

Results of these coupled anti-HA immunoprecipitation-nucleic acid hydrolysis assays demonstrated that the EhNucl-HA and EhNucII-HA expressed proteins possessed functional RNA hydrolase activity consistent with them being members of the T2 family of ribonucleases.

**DISCUSSION**

According to World Health Organization (WHO) estimates, Entamoeba histolytica, a major enteric protozoan pathogen of humans, is the etiologic agent responsible for causing up to 50 million cases of amebic dysentery and more than 100,000 deaths per year worldwide (1,46). These parasites typically colonize, and some cases invade, the large intestine of their human hosts. While the human small intestine represents an environment relatively rich in nutrients and enzymatic activities, the large intestine produces no digestive enzymes and is generally nutrient poor (47). E. histolytica parasites are purine auxotrophs (i.e. they are incapable of synthesizing the purine ring de novo) (21). In addition, it has been recently suggested, based on the cumulative data from the completed E. histolytica genome project, that these parasites are also incapable of de novo pyrimidine biosynthesis (22,23). Thus, these parasites are completely reliant on salvaging these essential compounds from their host environment for their own survival, growth and development. We hypothesized that one mechanism by which Entamoeba might access such nucleo-bases from their host environment is through the release/secretion of enzymes capable of hydrolyzing nucleic acids (i.e. nucleases). It is of interest to note that E. histolytica is unique amongst eukaryotes in that it contains only rudimentary endoplasmic reticulum and Golgi, (2,3). However, homologues to important ER-associated proteins such as BiP, calreticulin and protein disulfide isomerase (PDI) and Golgi-associated proteins such as ERD2 have been identified in this pathogen (48-51). Further, several Entamoeba cell surface and secreted proteins (i.e. Gal/GalNac lectin, amebapores, and cysteine proteases) contain typical signal
sequences similar to higher eukaryotes for targeting through the ER and Golgi (52-54). In fact, the secretion of cysteine proteases is essential for the pathogenicity of E. histolytica (55-57). Thus, it appears that Entamoeba is quite capable of secretion despite the apparent lack of traditional eukaryotic secretory machinery. In that regard, in preliminary experiments, using SDS-PAGE, poly(A)-zymogram gels, we demonstrated that E. histolytica did, in fact, constitutively synthesize and release/secrete soluble, DTT-sensitive, nuclease activities into their culture supernatants during their growth in vitro.

Having found these nuclease activities, it was of interest to further characterize their properties. Consequently, in preliminary experiments, multiple approaches were used in attempts to purify these secretory nucleases from parasite culture supernatants. Results of those experiments showed, however, that the actual amount of protein obtained from such samples was far below the level needed for these analyses. Therefore, we adopted an alternative, molecular approach to identify E. histolytica genes encoding putative secretory nucleases and subsequently expressed them to examine some of their biochemical and functional properties. To that end, using a PCR-based approach, we isolated and characterized two highly similar open reading frames (EhNucI and EhNucII) encoding 251 aa deduced proteins, both with calculated molecular masses of ~28 kDa. Analyses of the EhNucI and EhNucII deduced proteins, using various algorithms, suggested that they both possessed features typical of soluble, secreted proteins (i.e. the presence of putative N-terminal signal peptides, overall hydrophilicity, and the absence of membrane anchoring domains or organelle retention signals). In addition, BLAST-P analyses of the deduced aa sequences showed that both EhNucI and EhNucII had homologies with a variety of nucleases from diverse sources (28-31). Significantly, results of Pfam database comparisons revealed that both EhNucI and EhNucII deduced proteins belong to the T2 family of ribonucleases [Reviewed in (32)]. It has been reported that all T2 ribonuclease family members possess two conserved blocks of aa residues designated as CAS I and CAS II, each containing a histidine residue involved in the catalytic activity of the enzymes (41,42). Results of our sequence analyses showed that these domains were also present in both EhNucI and EhNucII deduced proteins. Additionally, all T2 ribonuclease family members possess at least two intra-chain disulfide bonds, which may be critical for the enzymatic activity of these proteins (43). In that regard, structural analyses of the mature EhNucI and EhNucII deduced proteins showed they both possess four cysteine residues, which could be involved in intra-chain disulfide bonding. Interestingly, disulfide bonds were found to be essential for the function of the native released parasite enzymes since treatment with reducing agents, such as DTT, completely abolished parasite nuclease activity. In addition, many members of the T2 family are soluble, secreted enzymes, which function extracellularly to cleave nucleic acids [reviewed in (32)]. These properties are in good agreement with those we determined experimentally for the native, wild-type, E. histolytica secreted nucleases.

Having identified and cloned the genes encoding EhNucI and EhNucII, it was important to determine whether or not both genes were actually transcribed by parasites. To that end, we developed primers specific to each of these genes, used them in RT-PCR with total E. histolytica RNA as template, and the products of such reactions were cloned and sequenced. Results of these experiments demonstrated that both EhNucI and EhNucII were indeed transcribed by E. histolytica trophozoites during their growth in vitro.

While the foregoing data suggested that the cloned EhNucI and EhNucII genes were both transcribed by E. histolytica, and both possessed sequences characteristic of nucleases, it was necessary to demonstrate that these genes actually encoded functionally active nucleases. To accomplish this, we used a homologous E. histolytica episomal-expression system to produce HA-tagged EhNucI and EhNucII chimeric proteins. Results of our Western blot analyses using an anti-HA antibody demonstrated that both EhNucI-HA and EhNucII-HA transfected parasites synthesized and released/secreted the 28 kDa EhNucI-HA and EhNucII-HA chimeric proteins into their culture medium during their growth in vitro. Further, results of our coupled immunoprecipitation/SDS-PAGE, poly(A)-zymogram activity gel assays showed that both the
EhNucI-HA and EhNucII-HA expressed proteins did, in fact, possess functionally active, DTT-sensitive, nuclease activities. Moreover, results of these coupled immunoprecipitation/zymogram gel analyses also demonstrated that the EhNucI-HA and EhNucII-HA chimeric enzymes could hydrolyze, in addition to poly(A), a variety of other synthetic polynucleotide substrates [i.e. poly(U) and poly(I), but not poly(G)]. The apparent inability of the parasite EhNucI-HA and EhNucII-HA enzymes to hydrolyze poly(G) may be due to the secondary structure of this polynucleotide substrate. Interestingly, similar results have been reported for several other T2 ribonuclease family members (58-61). While the use of synthetic polynucleotide substrates to demonstrate nuclease activity is well established, it was essential to demonstrate that the parasite EhNucI-HA and EhNucII-HA expressed enzymes could, in fact, hydrolyze naturally occurring nucleic acids. In that regard, the results of our coupled anti-HA immunoprecipitation/substrate hydrolysis assays clearly demonstrated that the EhNucI-HA and EhNucII-HA expressed nucleases were capable of hydrolyzing RNA. Further, the RNA substrate was more efficiently hydrolyzed by EhNucI-HA and EhNucII-HA at acidic pH, a property characteristic of many Ribonuclease T2 family member enzymes [reviewed in (32)]. In contrast, however, neither EhNucI-HA nor EhNucII-HA showed any detectable activity with ssDNA or dsDNA as substrates. The absence of nuclease activity with DNA substrates is also typical of T2 Ribonucleases [reviewed in (32)].

In summary, in the current report, we showed, for the first time, that the human intestinal pathogen, Entamoeba histolytica synthesizes and releases extracellular nucleases. Using a molecular approach we identified and characterized genes coding for two closely related nucleases, EhNucI and EhNucII, which are constitutively transcribed by parasites during their in vitro culture. Further, we showed when episomally expressed in parasites, the functionally active protein products of these genes are constitutively released/secreted by E. histolytica. As indicated above, E. histolytica parasites are both purine and pyrimidine auxotrophs that reside and multiply primarily within the large intestine of their infected human host and extra-intestinally in more advanced disease states. We hypothesize that within such environments, these secretory nucleases could function at a distance away from the parasite to hydrolyze host-derived nucleic acids. Presumably, these nucleases could act in concert with other purine/pyrimidine salvage enzymes and nucleoside/nucleo-base transporters to facilitate the acquisition of such essential nutrients by these parasites during the course of human disease. In that regard, it has been shown recently using micro-array technology that the nucleases annotated in Entamoeba Gene DB as 177.m00126 and 100.m00129, and characterized here as EhNucI and EhNucII, were expressed by parasites isolated directly from experimental animal infections (62). In that context, results of our preliminary experiments have shown that sera derived from acute human amoebiasis patients recognized and immunoprecipitated both the EhNucI-HA and EhNucII-HA nucleases expressed and released/secreted by transfected E. histolytica trophozoites. Cumulatively, these data suggest that EhNucI and EhNucII are expressed by parasites in situ during the course of both experimental and naturally occurring (human) host infections. Taken together, results of the current study should facilitate future investigations into the role(s) that the EhNucI and EhNucII secretory nucleases might play in the survival, growth and development of this important pathogen.
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KEY WORDS: nuclease; *Entamoeba*; secretory enzyme; ribonuclease; human enteric pathogen

ABBREVIATIONS: aa, amino acid; Ab, antibody; nt, nucleotide; DTT, dithiothreitol; ORF, open reading frame; PBS, phosphate buffered saline; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl-sulfate polyacrylamide gel electrophoresis.

FIGURE LEGENDS

**FIGURE 1.** Detection of *E. histolytica* released/secreted nuclease activities in SDS-PAGE zymogram gels. Panel A, cell-free culture supernatants (Cult Sup, 10 µl, lane 1; 20 µl, lane 2) obtained from cells grown in complete medium, prepared in SDS-PAGE sample buffer in the absence of reducing agent (-DTT) and separated in a poly(A)-containing SDS-PAGE substrate gel. Following renaturation and staining with Toluidine Blue O, nuclease activity was visualized in these gels as a clear/colorless zone of substrate hydrolysis in an otherwise uniformly-stained, dark blue background of un-hydrolyzed poly(A) substrate. Arrow indicates the two zones ~24 and ~25 kDa of nuclease activity observed in these samples. Panel B, samples as in panel A (lanes 1 and 2) prepared in SDS-PAGE sample buffer but containing reducing agent (+DTT, 50 mM, final conc.) and analyzed as above. Note the absence of any activity in the presence of the reducing agent. Protein molecular mass standards in kDa are shown on the left of each panel.

**FIGURE 2.** The deduced amino acid (aa) sequence and structural features of the genes encoding *EhNucl* and *EhNuclII*. The *E. histolytica* *EhNucl* and *EhNuclII* genes encode deduced polypeptides of 251 aa residues each. The shaded boxed sequences delineate the putative 14 aa signal peptide (SP, Met1 – Ala14) and the predicted cleavage sites are indicated by the arrow for each deduced protein. The unshaded boxes delineate the two conserved amino acid sequence domains (CAS I and CAS II) present in all members of the T2-RNase family. The histidine residues (asterisks) within these sequences have been shown to be necessary for the catalytic activity of various members of this enzyme family. In addition,
several conserved cysteine residues have been shown to be responsible for the formation of disulfide bridges in all T2-RNase family members. The predicted disulfide bridges in EhNucI and EhNucII involve Cys\textsuperscript{81}-Cys\textsuperscript{126}, black-filled flags and Cys\textsuperscript{195}-Cys\textsuperscript{232}, white flags.

FIGURE 3. RT-PCR of EhNucI and EhNucII. Panel A, diagrammatic representation of the complete 753 nt open reading frames of EhNucI and EhNucII. The black box (nt 1 - nt 42) represents the sequence encoding the predicted signal peptide (SP) of both EhNucI and EhNucII. The unshaded boxes indicate the relative positions of the sequences encoding the CAS I and CAS II domains of both EhNucI and EhNucII (see Fig. 2 above). Arrows represent the annealing positions of the specific EhNucI and EhNucII forward and reverse primers (RT-F and RT-R, respectively) used for RT-PCR experiments. These specific primers were designed to amplify the most variable region between EhNucI and EhNucII. Panel B, ethidium bromide stained agarose gel showing the amplification products of RT-PCR. Total RNA extracted from *E. histolytica* trophozoites was reverse transcribed using reverse transcriptase and Oligo (dT)\textsubscript{16} as primer. Aliquots (100ng) of the resulting cDNAs were used as templates in PCR with EhNucI and EhNucII specific RT-F and RT-R primers, respectively. Products of these reactions were separated and visualized in 1.2% agarose E-gels\textsuperscript{86} containing ethidium bromide. No reaction products were observed from controls in which reverse transcriptase was omitted (lane 1). A single amplified product of approximately 160bp was obtained for EhNucI with its specific set of primers (lane 2), and a similar 160bp product was obtained for EhNucII with its specific set of primers (lane 3). The specific identity of each of these PCR products was independently verified by nt sequence analyses. In addition, a single ~250bp amplified product was obtained using primers specific for an *E. histolytica* actin gene (lane 4), which served as a positive control in these RT-PCR experiments. DNA standards in bp are shown on the left.

FIGURE 4. Episomal expression constructs and growth kinetics of transfected parasites. Panel A, map of the EhNucI-HA and EhNucII-HA chimeric constructs. A diagrammatic representation of the complete open reading frame (*i.e.* nt 1 - nt 753, minus the terminal TAA stop codon) of the EhNucI and EhNucII genes fused at their 3'-ends with a 27-nt sequence encoding the influenza virus hemagglutinin (HA) epitope (light gray box). The black box at the 5'-end represents nt-1 to nt-42 encoding the putative 14 aa signal peptide (SP) of the EhNucI and EhNucII proteins. The thick black line represents the ExEhNeo expression plasmid vector backbone and the *Kpn*I and *BamH*I restriction endonuclease sites used for cloning the chimeric inserts. Panel B, Growth kinetics of transfected parasites. *E. histolytica* trophozoites transfected with the EhNucI-HA ( ), EhNucII-HA ( ) or the ExEhNeo control plasmid ( ) were monitored at regular intervals during the course of their growth in vitro. Triplicate cultures of parasites were initiated at 4 X 10\textsuperscript{8} cells ml\textsuperscript{-1} from stock cultures in their exponential phase of growth. Aliquots of the resulting cultures were taken at regular intervals as indicated, diluted appropriately and counted using a hemocytometer. Values shown represent the results obtained from a typical experiment.

FIGURE 5. Episomal expression of EhNucI-HA and EhNucII-HA chimeras in *E. histolytica*. Panel A, expression of the EhNucI-HA and EhNucII-HA chimeric proteins in *E. histolytica* transfected cells. *E. histolytica* cells transfected with the EhNucI-HA, EhNucII-HA constructs or the control ExEhNeo plasmid were grown in complete medium. Whole cell lysates (Cell Lys., 10 µg total protein, lanes 1, 2, and 3) of these transfectants were subjected to SDS-PAGE and reacted in Western blots with a rabbit anti-HA polyclonal (primary) antibody and an HRP-conjugated, goat anti-rabbit IgG (secondary) antibody. The anti-HA antibody showed no reactivity with any protein in ExEhNeo controls (lane1) but showed strong reactivity with a single ~28 kDa chimeric protein expressed in both the EhNucI-HA and EhNucII-HA transfected cells (arrow lanes 2 and 3). Protein molecular mass standards (in kDa) are shown on the left. Panel B, detection of EhNucI-HA and EhNucII-HA chimeric proteins released into the culture supernatants of *E. histolytica* transfected cells. Cell-free culture supernatants (Cult. Sup, 20 µl, lanes 1, 2, and 3) obtained from *E. histolytica* cells transfected with the EhNucI-HA, EhNucII-HA constructs or the control ExEhNeo plasmid were subjected to SDS-PAGE and Western blotting. Blots were probed with a
rabbit anti-HA polyclonal (primary) antibody and an HRP-conjugated, goat anti-rabbit IgG (secondary) antibody, as above. The anti-HA antibody showed no reactivity with any protein in supernatants of the ExEhNeo controls (lane 1) but showed strong reactivity with a single ~28 kDa chimeric protein present in the culture supernatants of both the EhNucI-HA and EhNucII-HA transfected cells (arrow lanes 2 and 3). The additional proteins of lower apparent molecular mass (<17 kDa) observed in lanes 2 and 3 presumably represent proteolytic degradation products of the released/secreted EhNucI-HA and EhNucII-HA expressed proteins. Protein molecular mass standards (in kDa) are shown on the left. Panel C, release of EhNucI-HA and EhNucII-HA chimeric proteins over the course of a temporal in vitro incubation assay. Log-phase transfected parasites were harvested, washed and resuspended to 3 X 10^5 cells ml^-1 in fresh culture medium and incubated for 30, 60, 90, and 180 min at 37°C. Cell free aliquots (20 µl) of such supernatants were subjected to SDS-PAGE and Western blotting as above. The anti-HA antibody reacted with a single ~28 kDa chimeric protein present in the supernatants of both the EhNucI-HA and EhNucII-HA transfected cells. Further, the ~28 kDa chimeric proteins were detectable in as little as 30 min post incubation and appeared to accumulate in the supernatants of each of these transfected cell lines over time.

FIGURE 6. Localization of chimeric EhNucI-HA and EhNucII-HA by fluorescence microscopy. Log phase E. histolytica transfected with either the EhNucI-HA or the EhNucII-HA construct were allowed to adhere to glass coverslips, fixed, permeabilized, and reacted with a primary anti-HA rabbit polyclonal antibody (α-HA) followed by a FITC-labeled goat anti-rabbit secondary antibody. To visualize the nuclei in these cells, coverslips were mounted on glass slides using mounting medium containing DAPI (Vector Labs). Differential interference-contrast (DIC) images show the overall loboid morphology of these transfected parasites as well as some of their intracellular compartments (e.g. nuclei and various sized vesicles). The fluorescence images shown are projected confocal z-series processed using deconvolution software. Both the EhNucI-HA (A) and the EhNucII-HA (B) expressed proteins showed very similar patterns of vesicular distribution throughout the cytoplasm of these transfected cells. In the merged images, DAPI stain is blue and α-HA staining is green. Bar = 10 μm.

FIGURE 7. Intracellular distribution of the chimeric EhNucI-HA and EhNucII-HA expressed proteins in relation to pinosomes and phagosomes by fluorescence microscopy. E. histolytica transfected with the EhNucI-HA or the EhNucII-HA construct were allowed to endocytose Texas Red labeled ~10kDa dextran to visualize pinosomes (A and B) or Texas Red labeled E. coli to visualize phagosomes (C and D). Cells were fixed, permeabilized and reacted with a primary rabbit anti-HA polyclonal antibody (α-HA) followed by a FITC-labeled goat anti-rabbit secondary antibody. All images are projected confocal z-series processed using deconvolution software. Results of such image analyses indicated that both the EhNucI-HA and EhNucII-HA expressed proteins occupied compartments distinctly separate from either pinosomes containing dextran or phagosomes containing E. coli. In the merged images, the endosomal markers are red and α-HA staining is green. Bar = 10 μm.

FIGURE 8. Zymogram analyses of EhNucI-HA and EhNucII-HA released/secreted by transfected parasites. Panel A, cell-free culture supernatants from parasites transfected with either the ExEhNeo control plasmid, the EhNucI-HA construct or the EhNucII-HA construct were subjected to immunoprecipitation with an α-HA antibody. The resulting immunoprecipitates (IP) were solubilized in SDS-PAGE sample buffer in the absence of reducing agent (-DTT) and analyzed for their nuclease activity in a poly(A) containing SDS-PAGE zymogram gels. Arrow indicates the major ~25 kDa nuclease activity immunoprecipitated from the supernatants of EhNucI-HA (lane 2) and EhNucII-HA (lane 3). The additional nuclease bands of higher apparent molecular mass seen in lanes 2 and 3 presumably reflect aggregates of the 25 kDa immunoprecipitated nucleases. Note the absence of any nuclease activity in the immunoprecipitates obtained from the ExEhNeo transfected controls (lane 1). Panel B, Samples as in panel A (lanes 1-3) prepared in SDS-PAGE sample buffer containing reducing
agent (+DTT, 50 mM, final conc.) and analyzed as above. Note the absence of any activity in the presence of the reducing agent. Protein molecular mass standards in kDa are shown on the left of each panel.

**FIGURE 9. Detection of EhNucI-HA and EhNucII-HA expressed nuclease activity with various synthetic polynucleotide substrates.** Whole cells lysates (1 mg total cellular protein) of parasites transfected with either the ExEhNeo control plasmid, the EhNucI-HA construct or the EhNucII-HA construct were subjected to immunoprecipitation with an α-HA antibody. The resulting immunoprecipitates (IP) were solubilized in SDS-PAGE sample buffer in the absence of reducing agent (-DTT) and analyzed for their nuclease activity in SDS-PAGE zymogram gels containing various synthetic polynucleotide substrates: Panel A. poly(A), Panel B. poly(U) and Panel C. poly(I). Arrows indicate that the major ~25 kDa nuclease immunoprecipitated from cell lysates of EhNucI-HA had activity against poly(A), poly(U) and poly(I) (lane 2 in Panels A, B and C). The immunoprecipitates from EhNucII-HA showed similar nuclease activity with each of these polynucleotide substrates (lane 3 in Panels A, B and C). The additional nuclease bands of higher apparent molecular mass seen in lanes 2 and 3 presumably reflect aggregates of the 25 kDa immunoprecipitated nucleases. Note the absence of any nuclease activity in the immunoprecipitates obtained from the ExEhNeo transfected controls (lane 1 in Panels A, B and C). Molecular mass standards (in kDa) are shown on the left.

**FIGURE 10. Activity of EhNucI-HA and EhNucII-HA expressed nucleases with various nucleic acid substrates.** Whole cells lysates (1 mg total cellular protein) of parasites transfected with either the ExEhNeo control plasmid, the EhNucI-HA construct or the EhNucII-HA construct were subjected to immunoprecipitation with an α-HA antibody. The resulting immunoprecipitates (IP) were analyzed for their nuclease activity with several different nucleic acid substrates. Panel A, immunoprecipitates were incubated at pH 6.0 with total RNA as substrate and the resulting reaction products were separated and visualized in EtBr stained formaldehyde agarose gel. Note that the immunoprecipitates obtained from parasites transfected with the ExEhNeo control plasmid (lane 1) showed no nuclease activity with the RNA substrate (Arrow). In contrast, immunoprecipitates obtained from EhNucI-HA (lane 2) and EhNucII-HA (lane 3) transfectants readily hydrolyzed the RNA substrate. Panel B, immunoprecipitates as in panel A were incubated at pH 6.0 with a single-stranded circular DNA (ssDNA) substrate and the reaction products separated in a 0.8% agarose, EtBr-stained, E-Gel®. Neither immunoprecipitates from ExEhNeo control transfectants (lane 1) nor those obtained from the EhNucI-HA (lane 2) and EhNucII-HA (lane 3) transfectants showed any nuclease activity with the ssDNA substrate (Arrow). Panel C, the immunoprecipitates as in panel A were incubated at pH 6.0 with a double-stranded DNA (dsDNA) substrate and the reaction products separated and visualized in a 0.8% agarose, EtBr-stained, E-Gel®. None of the immunoprecipitates obtained from the control, ExEhNeo (lane 1), EhNucI-HA (lane 2) or EhNucII-HA (lane 3) transfectants showed any nuclease activity with the dsDNA substrate. Arrow indicates the unhydrolyzed dsDNA substrate.

**SUPPLEMENTAL FIGURE LEGEND**

**FIGURE S1. Intracellular distribution of the chimeric EhNucI-HA expressed protein by three dimensional isosurface imaging.** E. histolytica transfected with the EhNucI-HA construct was allowed to endocytose Texas Red labeled ~10kDa dextran to visualize pinosomes. Cells were fixed, permeabilized and reacted with a primary rabbit anti-HA polyclonal antibody (α-HA) followed by a FITC-labeled goat anti-rabbit secondary antibody. Images were acquired using confocal fluorescence microscopy, and three dimensional isosurface models of z-series were constructed using Imaris®, version 5.5.2 (Bitplane AG, Zurich, Switzerland) to compile this video. In this video, the pinosomal marker is red and EhNucI-HA is green.
Fig. 1.

A. Cult Sup  
1 2 
(-) DTT  

B. Cult Sup  
1 2 
(+) DTT
Fig. 3.

A.

![Image of a diagram showing SP, CAS I, CAS II, and RT-F, RT-R positions.]

B.

![Image of a gel showing bands for Control, EhNucl, EhNucl, and Actin.](http://www.jbc.org/)
Fig. 5.

A. Cell Lys.

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B. Cult. Sup

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Fig. 6.
Fig. 8.

A. 

Sup: α-HA IP

ExEhNeo | EhNucl-HA | EhNuclL-HA

1 | 2 | 3

(-) DTT

B. 

Sup: α-HA IP

ExEhNeo | EhNucl-HA | EhNuclL-HA

1 | 2 | 3

(+) DTT
Identification and biochemical characterization of unique secretory nucleases of the human enteric pathogen, Entamoeba histolytica
Glen C. McGugan, Jr., Manju B. Joshi and Dennis M. Dwyer

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