Identification and Characterization of an Entamoeba histolytica
Upstream Regulatory Element 3 Sequence-specific DNA-binding
Protein Containing EF-hand Motifs

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The hgl5 gene of Entamoeba histolytica is negatively regulated through the upstream regulatory element 3 (URE3) DNA motif TATTCCTATT. This motif is also present and significant in the function of the E. histolytica fdx gene promoter. A yeast one-hybrid screen was used to identify an E. histolytica cDNA encoding a protein (URE3-BP) that recognized this DNA motif. Analysis of the predicted amino acid sequence demonstrated the presence of two EF-hand motifs but identified no canonical DNA binding motifs. URE3-BP, expressed in bacteria, demonstrated Ca²⁺-dependent and sequence-specific recognition of the URE3 DNA sequence as assessed by electrophoretic mobility shift assays. Antibodies raised against URE3-BP blocked the formation of the URE3 DNA-protein complex by native nuclear extracts. The URE3-BP protein was present in the E. histolytica nucleus and cytoplasm with an apparent molecular mass of 22.6 kDa. Our results represent the first use of a yeast genetic screen to identify, on the basis of function, a DNA-binding protein of an early branching eukaryote. Since the URE3 DNA can modulate gene expression in both a positive and negative manner, this protein may have more than one mechanism of interaction with transcriptional machinery. Characterization of URE3-BP should provide insight into transcription regulation and virulence control in this parasite.

The eukaryotic parasite Entamoeba histolytica is a major cause of morbidity and mortality worldwide. There are an estimated 50 million cases of invasive amebiasis annually, with an estimated 40,000–110,000 deaths each year (1). The most common illness from E. histolytica infection is amebic dysentery, but amebic abscesses in liver, lung, and brain also occur. Disease occurs in only a minority of infections and requires parasite invasion of the intestinal epithelium. The factors responsible for the E. histolytica trophozoite invasion of the host gut cell wall, subsequent entry into the blood stream, and the formation of life-threatening tissue abscesses are not well understood. Changes in E. histolytica virulence could be mediated by changes in the transcription of genes encoding proteins involved in the pathogenicity of the organism.

A well characterized virulence factor of E. histolytica, the galactose- and N-acetyl-d-galactosamine-inhibitable lectin (Gal/GalNAc-inhibitable lectin), is essential for parasite adherence and contact-mediated cytosis. One of the genes encoding the lectin heavy subunit (hgl5), contains five major regulatory regions (upstream regulatory elements 1–5 (URE1–5)) upstream of the core promoter (2). Mutation of the URE3 sequence TATTCCTATT (−80 to −51 base pairs upstream of the site of transcription initiation) within the 5′ hgl5 DNA results in an increase in relative promoter activity (2). In contrast, mutation of this sequence (−60 to −51 base pairs relative to site of transcription initiation) in the E. histolytica ferredoxin (fdx) promoter results in a decrease in promoter activity (3), indicating that this sequence can mediate both positive and negative control depending on the promoter context. We have previously shown that E. histolytica nuclear protein(s) exhibit sequence-specific binding to the URE3 motif in electrophoretic mobility shift assays (EMSA). Amebic nuclear proteins had a higher affinity for oligonucleotides containing the URE3 motif, TATTCCTATT, than oligonucleotides of identical base composition but in which this sequence had been altered (3).

Identification of the protein(s) that bind to the URE3 DNA is important not only for the elucidation of the mechanism of regulation of virulence but also for the insight this may provide into transcriptional regulation in an early branching eukaryote. The E. histolytica core promoter for protein-encoding genes is unusual in that it consists of a novel “GAAC” element in addition to a TATA and INR (2, 4, 5). E. histolytica UREs differ in whether they regulate transcription via the TATA or the GAAC elements; URE3 appears to utilize GAAC.² The protein(s) binding to URE3 therefore may be particularly interesting due to potential interaction with the most divergent portion of the amebic core promoter.

Here we present the first application of a yeast one-hybrid screen to identify E. histolytica sequence-specific DNA-binding proteins. The URE3 DNA sequence was used as the “bait” to identify amebic cDNAs encoding proteins capable of binding to this DNA motif. Validation of the screen was accomplished by characterization of the binding specificity of the recombinantly expressed URE3-BP. The predicted amino acid sequence of this protein (+1 to 667) contained two EF-hand motifs, characteristic of DNA binding proteins of an early branching eukaryote. The hgl5 gene of E. histolytica is expressed during early trophozoite invasion of the host, consistent with a role in the initial steps of invasion. No amebic lectin heavy chain proteins have been described previously. Immunoblot analysis confirmed that URE3-BP is present in E. histolytica trophozoites, and antibodies raised against URE3-BP blocked the formation of the URE3 DNA-protein complex by native nuclear extracts. The predicted amino acid sequence of this protein (+1 to 667) contained two EF-hand motifs, characteristic of DNA binding proteins of an early branching eukaryote. Identification of the protein(s) that bind to the URE3 DNA is important not only for the elucidation of the mechanism of regulation of virulence but also for the insight this may provide into transcriptional regulation in an early branching eukaryote. The E. histolytica core promoter for protein-encoding genes is unusual in that it consists of a novel “GAAC” element in addition to a TATA and INR (2, 4, 5). E. histolytica UREs differ in whether they regulate transcription via the TATA or the GAAC elements; URE3 appears to utilize GAAC.²

¹ The abbreviations used are: URE, upstream regulatory element; URE3-BP, URE3-binding protein; EMSA, electrophoretic mobility shift assay(s); 3-AT, 3-aminotriazole; GST, glutathione S-transferase; TEV, tobacco etch virus; DREAM, downstream regulatory element antagonist modulator.
² C. A. Gilchrist, U. Singh, and W. A. Petri, Jr., unpublished results.
expressed cDNA. The novel structure of the URE3-binding protein is discussed.

**EXPERIMENTAL PROCEDURES**

*Cultivation of E. histolytica and Nuclear Extract Preparation—* E. histolytica strain HM1-IMSS trophozoites were grown at 37°C in TYI-S-33 medium containing penicillin (100 units/ml) (Life Technologies, Inc.) and streptomycin (100 μg/ml) (Life Technologies) (6). Amebas in logarithmic phase growth (~ 5 × 10⁷ trophozoites/ml) were used for nuclear extract preparation. Crude nuclear extracts were prepared by the method previously described (3) with the following modifications: the protease inhibitors 2 mM (2S,3S)-trans-epoxyacucyclic-1-leucyl-amiido-3-methylbutane and 2 mM 4-(2-aminoethyl) benzenesulfonylfluoride, HCl were added to both cell and nuclear lysis buffers, and dithiothreitol was omitted from the nuclear lysis buffer.

**Yeast Transformation, Bait Construction, and Yeast One-hybrid Screen of an E. histolytica cDNA Library—** Yeast-one-hybrid screening for DNA sequence-specific binding proteins was used to identify proteins recognizing the URE3 motif, as previously described (7). In brief, three URE3 DNA motifs (underlined) AGCTTATTATATTCTATTGATATA-TTCTATGGGTTCTTAAGTT CTTTTATTTTCTTC were cloned between the EcoRI and XhoI restriction sites of pHS-1 (CLONTECH), placing upstream of the yeast GAL4 minimal promoter to generate the plasmid p3X-URE3. This vector was linearized and integrated into the yeast strain YEM271 (MATa, ura3-52, his3-200, aded-101, lys2-801, leu2-3, 112, trp1-903, tyr1-501, gal4-D512, gal80-D358, ade5::hisG) (CLONTECH) by homologous recombination with the yeast genomic copy of the mutant HIS3 gene to generate the URE3-bait yeast strain 1.4. A Gal4-cDNA fusion expression library was generated from E. histolytica cDNA. The cDNA was synthesized by the ZAP-cDNA Synthesis Kit (Stratagene) from E. histolytica mRNA isolated using the PolyATtract® 1000 system (Promega). The cDNA was then ligated via EcoRI and XhoI linkers into the HybriZAP-2.1 vector, placing it in frame with and downstream of the Gal4 activation domain in this construct. The primary λ library was amplified and converted by in vitro mass excision into a PAD-GAL4–2.1 library according to the manufacturer’s directions (Stratagene). This plasmid expression library of Gal4 activated expressed proteins recognizing the URE3 motif, as previously described (7). In brief, to create the radiolabeled probe, complementary oligonucleotides were annealed and then labeled with the large DNA polymerase I subunit (Klenov) and [α-²⁵P]dATP. Reactions contained 0.003 pmol of radiolabeled probe, 2 μg of poly(dC·dH), and 2 μg of protein from E. histolytica crude nuclear extract (3). The protein-nucleic acid interactions occurred in band shift buffer (10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 1 mM EDTA, 0.05% nonfat milk powder (Carnation), 3% glycerol, 0.05 mg of bromphenol blue). The reaction was incubated at room temperature (20 °C) for 20 min prior to electrophoresis on a nondenaturing polyacrylamide gel for 2–3 h. The gel was then fixed, dried, and quantitated by PhosphorImager analysis (Molecular Dynamics, Inc., Sunnyvale, CA).

**Northern Blot Analysis—** Total RNA from HML-IMSS trophozoites was isolated using the total RNA isolation system (Promega). Ten micrograms of RNA was separated on a formaldehyde-containing agarose gel (12) and transferred to Zeta-probe GT Genomic blotting membrane (Bio-Rad). Prehybridization, hybridization, and washes were performed according to the manufacturer’s instructions. DNA probes were labeled using random primers ([α-²⁵P]dATP, and the Klenow fragment of DNA polymerase I (Life Technologies). Message levels were determined by PhosphorImager exposure.

**Immunization and Immunodetection—** A female BALB/c-strain mouse was immunized intraperitoneally with 100 μg of TEV protease-cleaved URE3-BP emulsified in complete Freund’s adjuvant. Two weeks and 4 weeks later, the mouse was boosted with 100 μg of TEV-cleaved URE3-BP in incomplete Freund’s adjuvant. One week after the last boost, ~0.2 ml of blood was obtained by retro-orbital puncture. Nonimmune serum was obtained from a BALB/c mouse that had not been immunized.

Immunoblots were performed by first electrophoresing 5 ng of recombinant protein or 60 μg of amebic nuclear or cytoplasmic extracts through a 12% SDS-polyacrylamide side gel. Proteins were transferred to a PVDF membrane (Millipore Corp.), incubated for 1 h at room temperature in 5% nonfat dry milk in blot wash buffer (50 mM Tris, pH 7.4, 200 mM NaCl, 0.1% Tween 20). The blot was then incubated for 1 h at room temperature with mouse antiserum diluted 1:750 in 2% nonfat dry milk in blot wash buffer. After 3–5 min washes, the membranes were incubated for 1 h with sheep anti-mouse IgG horseradish peroxidase-conjugated antibody (Amersham Pharmacia Biotech) at a dilution of 1:1000. The secondary antibody was detected using the ECL Western blotting detection system according to the manufacturer’s directions (Amersham Pharmacia Biotech) and was visualized by exposure of the blot to BioMax MR-1 film (Eastman Kodak Co.).

Immunofluorescence and confocal laser microscopy were performed on E. histolytica trophozoites by the methods of Voigt et al. (13) and Vines et al. (14). Approximately 2 × 10⁶ amebas/sample were washed in M199S medium (M199 medium (Life Technologies, Inc.) supplemented with 25 mM HEPES, pH 6.8, 5 mM l-cysteine, and 0.5% bovine serum albumin) and allowed to adhere onto acetic-washed coverslips for 15 min at 37°C. Amebas were washed with warmed phosphate-buffered saline once, fixed in 3.75% paraformaldehyde for 30 min at 37°C, and permeabilized with 0.2% Triton X-100 for 60 s at room temperature. Samples were washed twice with phosphate-buffered saline and once with 50 mM ammonium chloride. Amebas were incubated with blocking agent (5% bovine serum albumin with 20% goat serum (Sigma) in phosphate-buffered saline) for 60 min. Mouse anti-URE3-BP serum was used at a dilution of 1:100 and incubated for 60 min. Bound antibody was detected using a 1:64 dilution of fluorescein isothiocyanate-conju-
DNA and Deduced Amino Acid Sequence of URE3-BP—The complete sequence of the 0.7-kilobase insert contained in pGAL4-URE3-BP is shown in Fig. 2. We found considerable identity (98%) between 512 base pairs of URE3-BP and an E. histolytica expressed sequence tag entry AB002727 (Fig. 2, underlined region), previously identified by Tanaka et al. (17) during analysis of E. histolytica cDNA.

An open reading frame of 670 base pairs encoded a protein with a calculated mass of 25.8 kDa. The presence of EF-hand motifs (Fig. 2, boldface type) was identified by use of the ExPASy ScanProsite Program (15). The presence of this motif has been strongly correlated with calcium binding activity (18). A potential tyrosine kinase phosphorylation site (19–21) and serine phosphorylation sites (22, 23) were also recognized by this program (Fig. 2). Northern blot analysis of E. histolytica total RNA with URE3-BP probes identified a unique message of 0.67 kilobases in size (Fig. 3A). The close agreement between the observed mRNA size and the size predicted from the E. histolytica URE3-BP cDNA clone (0.7 kilobases) were consistent with the cDNA clone being full-length. The short 5′- and 3′-untranslated regions of the URE3-BP mRNA are a characteristic of E. histolytica mRNAs (2, 24).

Cellular Localization of URE3-BP—The URE3-binding protein (URE3-BP) was synthesized in E. coli with an amino-terminal GST fusion protein. TEV cleavage of the purified protein removed the GST domain; the remaining 1.49 kDa of linker peptide increased the calculated protein mass to 27.2 kDa. The observed molecular mass of this protein in both Coomassie Blue-stained gels (data not shown) and Western blots was 24.3 kDa (L-URE3-BP, Fig. 3B). Serum raised against this URE3-BP fusion protein identified a protein of ~22.6 kDa in both nuclear and cytoplasmic extracts of E. histolytica proteins (Fig. 3B). A comparison of band intensities of a known amount of synthesized URE3-BP protein with those that resulted after loading a known quantity of nuclear extract permitted the rough estimation that URE3-BP constituted 0.01% of the protein present in nuclear extracts.

Since the method used to prepare the nuclear extracts does not completely exclude cytoplasmic protein contamination, immunofluorescence techniques were used to confirm the location of URE3-BP within the cell. The Gal/GalNac-inhibitable lectin, which is located on both the cell surface and the cytoplasm but not in the trophozoite nucleus (14), was used as a positive control (Fig. 4B). The URE3-BP (Fig. 4A) was present in both the cytoplasm and the nucleus. URE3-BP also appeared to be membrane-associated (Fig. 4D).

Synthesized URE3-BP Binds to the hgl5 URE3 Motif—EMSA was used to determine whether the E. coli-expressed URE3-BP could bind in vitro to DNA containing the URE3 sequence (Fig. 5). The URE3-BP protein was incubated with a double-stranded radiolabeled oligonucleotide containing the DNA sequence spanning the URE3 binding motif present within the hgl5 promoter. Increasing concentrations (10× and 60×) of unlabeled hgl5-URE3, hgl5-MUT, or a nonspecific oligonucleotide (Olig-1) were added as competitors to the binding reactions as indicated. Competition with unlabeled self was demonstrated at a 10-fold excess of radiolabeled oligonucleotide (Fig. 5, lane 3). The URE3-mutated oligonucleotides, in which the sequence TTGAGATTTC replaced the URE3 motif TTATCTTAT, competed less well and had to be added at higher concentrations to interfere with the formation of the specific protein complex (lane 6). The amount of DNA-protein complex formed was dependent on the concentration of recombinant URE3-BP added to the assay (data not shown). The addition of an irrelevant oligonucleotide control (Olig-1) (Fig. 5, lanes 7 and 8) did not affect the formation of the DNA-protein complex.

RESULTS

Isolation of the cDNA Clone Encoding a URE3 DNA-binding Protein—To use the yeast one-hybrid system to identify a URE3-binding protein, we first inserted an oligonucleotide containing three copies of the URE3 DNA motif upstream of the yeast minimal promoter that controls the expression of the HIS3 gene in the plasmid pHis-1. The oligonucleotide containing the trimer had been shown to compete for URE3-specific binding in crude E. histolytica nuclear extracts with an affinity at least equal to the URE3 monomer (data not shown). We integrated the new construct into the genome of the histidine- (His) auxotrophic strain YM4271 to create the bait yeast strain 1.4. An increase in minimal promoter activity due to binding of a protein to the 5′ URE3 sequences that contain the Gal4 activation domain was monitored on plates containing the competitive inhibitor (3-AT) of the product of the HIS3 gene His3p. The yeast Gal4 activation domain-E. histolytica cDNA fusion proteins were introduced into strain 1.4, and approximately three genome equivalents of E. histolytica (1.5 × 106 fusion constructs) were screened for proteins that can bind to the URE3 DNA trimer. In the first screen, 281 positive clones were identified, but only four were capable of reconstituting the selectable phenotype (growth on his−, leu−, ura− with or without 10 mM 3-AT (as indicated).

Gate anti-mouse IgG antibody (Sigma). Nonimmune serum was used as a negative control was also used as a 1:100 dilution. As a positive control, rabbit polyclonal antibodies raised against the E. histolytica Gal/GalNac-inhibitable lectin were diluted 1:100 and detected by Cy3-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch Laboratories). Amebas were washed twice with phosphate-buffered saline and mounted on glass slides using GelMount (BioMeda). Amebas were visualized using a Zeiss LSM 410 laser-scanning confocal microscope equipped with an argon/krypton laser. To generate final images, four averages at 4 s each were compiled using a Zeiss LSM 410 laser-scanning confocal microscope.

Protein—To use the yeast one-hybrid system to identify a URE3 Sequence-specific DNA-binding Protein

Fig. 1. Yeast one-hybrid assay of the URE3-BP cDNA and URE3 DNA motif. Growth of yeast bait strain URE3 1.4 transformants expressing either the Gal4 activation domain-URE3-BP fusion protein (pGAD-URE3-BP) or the Gal4 activation domain (control plasmid pGAD424) on his−, leu−, ura− with or without 10 mM 3-AT (as indicated).
Control extracts identically prepared from bacteria transformed with either pRSET-Eh1 (an irrelevant pRSETA-E. histolytica cDNA construct) or vector alone had no gel shift ability (data not shown). The results from these experiments indicated that URE3-BP exhibited sequence-specific binding to the URE3 DNA motif.

Since analysis of the URE3-BP protein had revealed the presence of two EF-hand domains (Fig. 2), we next examined the effect of Ca\(^{2+}\) on the ability of URE3-BP to form DNA-protein complexes. Electrophoretic mobility shift analysis performed in buffer containing 50 mM NaCl, 3 mM MgCl\(_2\) and 1 mM EDTA showed that the addition of Ca\(^{2+}\) markedly decreased the formation of the recombinant DNA-protein complex (Fig. 6).

URE3-BP Is a Component of the URE3 Binding Activity Present in E. histolytica Nuclear Extracts—To determine whether URE3-BP was a component of the gel shift complex of URE3 DNA with native E. histolytica nuclear protein(s), antibodies raised against URE3-BP were incubated with the hgl\(_5\)-URE3 DNA-E. histolytica nuclear extract complex. An antibody binding to the transcription factor may form a larger antibody-protein-DNA complex and hence a more highly retarded “supershift” in the EMSA, or it may prevent or destabilize the DNA/protein interaction, resulting in an inhibition EMSA. The addition of increasing concentrations (0.25 and 1 \(\mu\)l) of antiserum from mice immunized with URE3-BP but not with non-immune serum interfered with the formation of URE3-DNA-E. histolytica nuclear protein complexes (Fig. 7). (For these gel shifts, dithiothreitol was excluded from the gel shift buffer, so as not to reduce and denature the antibodies. This resulted in two DNA-protein complexes on EMSA as opposed to the single complex seen in previous work (3).

DISCUSSION

The main conclusion of this paper is that URE3-BP, an E. histolytica protein, binds specifically to the TATTCTATT (URE3) DNA motif. This sequence is important in the regulation of at least two E. histolytica genes, the hgl\(_5\) encoding the heavy subunit of the Gal/GalNAc-inhibitable lectin (2) and the fdx gene encoding ferredoxin (3). We have tested and validated the use of a genetic approach to identify the E. histolytica proteins that bind to a target sequence. We have also confirmed that URE3-BP is present in the trophozoite nucleus, and therefore its sequence-specific DNA binding activity is of biological significance.

Proof that URE3-BP bound to the URE3 sequence was determined by 1) the ability of the Gal4-URE3-BP fusion protein to reconstitute the selectable phenotype (growth on 3-AT-containing media in the yeast bait strain); 2) the ability of recombinant URE3-BP to bind to URE3 DNA in vitro in electrophoretic mobility shift assays; and 3) the ability of antibodies raised
The amino acid sequence deduced for URE3-BP has little similarity to other known DNA-binding proteins. It has potential phosphorylation sites that may be important in its function. The EF-hand protein motifs that occur in URE3-BP are present in the calcium-binding human transcription factor, downstream regulatory element antagonist modulator (DREAM) (28). DREAM, like URE3-BP, is a DNA-binding protein that does not contain an easily recognized consensus DNA binding motif. There are, however, no other significant regions of similarity between the two proteins that might suggest the location of a new DNA binding motif in EF-hand-containing proteins.

Fluxes in the amount of intracellular ions are important signals and determinants of gene expression in later branching eukaryotes. DNA binding of the DREAM transcription factor is blocked by calcium. Calcium also decreased the affinity of recombinant URE3-BP for DNA as measured by EMSA. Relatively high concentrations of Ca\(^{2+}\) were required to block the binding of DNA by URE3-BP in comparison with DREAM (100–500 \(\mu\)M versus 5–10 \(\mu\)M). Electrophoretic mobility shift analysis of native nuclear extracts showed that 500 \(\mu\)M Ca\(^{2+}\) was also required to block binding of the nuclear protein to URE3 DNA (data not shown), consistent with the recombinant His-tagged URE3-BP protein behaving in a manner similar to the nuclear URE3 binding complex. Calcium fluxes have been observed in \textit{E. histolytica} (29) and therefore could regulate URE3-BP possibly via direct binding to the EF-hand motifs or, as in later branching eukaryotes, by calcium-dependent phosphorylation of the URE3-BP protein (30, 31).

The \textit{in situ} localization of URE3-BP indicated that this protein was located not only in the nucleus and cytoplasm but also associated with the trophozoite membrane (indicated by the colocalization of the lectin and URE3-BP staining (Fig. 4D)). The presence of URE3-BP in the cytoplasm and cell membrane may enable it to act as an intracellular messenger and transcription factor. Examples of transcription factors with this dual function include the estrogen receptor transcription factor ERF, membrane-associated transcription factor \(\sigma^K\) (32), and the sterol regulatory element-binding protein (33). Cytoplasm to nuclear translocation of transcription factors can be regulated by several different mechanisms, such as proteolytic processing (\(\sigma^K\) and sterol regulatory element-binding protein (32, 33)), phosphorylation or dephosphorylation (NF-AT4 (34), and association with cofactors (NF-\(\kappa\)B (35)). Because transcriptional activation mediated through the URE3 DNA motif in the \(fdx\) promoter is modulated by serum deprivation (3), it is possible that the activity of the URE3-BP may be controlled by...
environmental and intracellular factors. It is tempting to speculate that URE3-BP activity may be regulated in part by its location within the cell. The elucidation of URE3-BP regulation promises to provide some of the first insights into the transcriptional regulation of virulence in this early branching eukaryote.

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