Calcium Modulates Promoter Occupancy by the Entamoeba histolytica Ca\(^{2+}\)-binding Transcription Factor URE3-BP*

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The Entamoeba histolytica upstream regulatory element 5-binding protein (URE3-BP) binds to the URE3 sequence of the Gal/GalNAc-inhibitable lectin hgl5 and ferredoxin 1 (fdx) gene promoters. This binding can be inhibited in vitro by addition of calcium. Two EF-hand motifs, which are associated with the ability to bind calcium, are present in the amino acid sequence of URE3-BP. Mutation of the second EF-hand motif in URE3-BP resulted in the loss of calcium inhibition of DNA binding as monitored by electrophoretic mobility shift assay. Chromatin immunoprecipitation assays revealed that URE3-BP was physically bound to the hgl5 and fdx promoters in vivo. Parasite intracellular calcium concentrations were altered by changes in extracellular calcium. Promoter occupancy was lost when intracellular calcium levels were increased by coordinate increases in extracellular calcium. Increased intracellular calcium also resulted in decreased levels of URE3-BP mRNA. Together these results demonstrate that changes in extracellular calcium result in changes in URE3-BP mRNA and in the ability of URE3-BP to bind to URE3-containing promoters. Modulation of URE3-BP by calcium may represent an important mechanism of control of gene expression in E. histolytica.

The early branching eukaryote Entamoeba histolytica is a human parasite that is the etiologic agent of amebic dysentery and liver abscesses. Only one of every 10 infections leads to disease (1), and the parasite and host factors that control the outcome of infection are not well understood. Alteration in transcriptional control of certain crucial genes may contribute to the expression of a virulence phenotype. Padilla-Vaca et al. (1) demonstrated that co-cultivation of E. histolytica with Escherichia coli O55 resulted in increased virulence and a decrease in the expression of the Gal/GalNAc-inhibitable lectin light subunit. Ramakrishnan et al. (2) have shown alterations in the hgl genes transcribed in trophozoites derived from liver abscesses compared with those transcribed in established cell cultures. Bruchhaus et al. (3) have shown, in similar work, changes in the expression of over 55 other E. histolytica transcripts.

There has been considerable divergence in the mechanisms of transcription of the early branching E. histolytica from later branching eukaryotes such as Homo sapiens and Saccharomyces cerevisiae. For instance the E. histolytica core promoter for protein encoding genes consists of a novel GAAC element, in addition to a TATA and INR (4–6) and contains short regulatory 5’ and 3’ sequences (7). The RNA polymerase II is also unusual for a eukaryote in that it is resistant to α-amanitin (8). To increase our understanding of transcriptional regulation in this organism we investigated the mechanisms of transcriptional control of a well characterized virulence protein, the galactose- and N-acetyl-D-galactosamine-inhibitable lectin (Gal/GalNAc-inhibitable lectin), which is essential for parasite adherence and contact-mediated cytolysis. The promoter for 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 (4) and a GAAC sequence motif that predominantly influences the rate and site of transcription initiation (4–6). The hgl5 UREs differ in whether they regulate transcription via the TATA or the GAAC elements, with the URE3 exerting its effect on mRNA transcription via GAAC (9). Interestingly, mutation of the URE3 motif in the hgl5 promoter leads to an increase in transcription and in the fdx promoter decreased promoter strength (10). This indicates that, as is the case for the mouse context-dependant Pax-5 transcription factor, the URE3 sequence can mediate both positive and negative control in different milieu (11). Two different genes encode the ferredoxin proteins of E. histolytica. The fdx URE3-containing promoter regulates the gene that encodes the ferredoxin 1 protein. This transcript is down-regulated in metronidazole-resistant amebae, in contrast with the level of the ferredoxin 2 transcript (fd2), which is unaltered (12). Bruchhaus et al. (3) discovered that fd2 mRNA is one of the transcripts up-regulated in ameba cultured from liver abscesses. These two papers suggest that ferredoxins may be tightly regulated in E. histolytica. Investigation of the sequence surrounding the fd2 gene showed that a URE3 motif was located 1.2 kb 5’ of the initiation codon (International

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‡The abbreviations used are: URE, upstream regulatory element; BP, binding protein; EMSA, electrophoretic mobility shift assay; MUT, mutant; mAb, monoclonal antibody; ChIP, Chromatin immunoprecipitation; wt, wild-type; DREAM, downstream regulatory element antagonist modulator.
Entamoeba Genome Sequencing Project\(^2\), but it is unclear whether this motif would play any role in its regulation. A yeast-one-hybrid screen of an *E. histolytica* cDNA library using the URE3 element as “baits” identified the URE3-binding protein (URE3-BP) (14). Analysis of the URE3-BP amino acid sequence did not identify any canonical DNA binding motifs but did reveal the presence of two EF-hand motifs, which are consistent with the ability of a protein to bind to calcium (15). This suggested that the URE3-BP protein might function as both a calcium sensor and a transcription factor. The only other reported sequence-specific DNA-binding protein known to contain EF-hand motifs is DREAM. DREAM is a human neuronal protein (also known as Calsenilin and KChIP3) that functions both as a transcription factor (16, 17) and as a calcium sensor (16–20). DREAM contains four EF-hand sequence motifs. Typically, the binding of Ca\(^{2+}\) to EF-hand domains induces structural changes that alter the function of the protein. In the case of DREAM, binding of Ca\(^{2+}\) to its EF-hands leads to a reduced affinity for sequence-specific binding to its target downstream regulatory element sequence (16).

Previous analysis of the recombinant URE3-BP protein sequence-specific DNA binding by electrophoretic mobility shift assay (EMSA) showed that recognition of a URE3-containing oligonucleotide was blocked in *vitro* by calcium (14). In this report, we tested the *in vivo* role of the EF-hand motifs of URE3-BP in protein-promoter interactions.

**EXPERIMENTAL PROCEDURES**

**Cultivation of *E. histolytica*—** *E. histolytica* strain HM1-IMSS trophozoites were grown at 37 °C in TYI-S-33 medium containing penicillin (100 units/ml) (Invitrogen) and streptomycin (100 μg/ml) (Invitrogen) (21). In experiments examining the effect of calcium on transcription, *E. histolytica* was seeded at a concentration of 2–4 10\(^{5}\) ml\(^{-1}\) 18 h prior to the addition of medium containing 5 mM MgCl\(_2\) and 5 μM EDTA to sequester the serum calcium (1.4–2 mM Ca\(^{2+}\)) calculated using the dissociation constants of the chelating ligands and the CaLBuf Program (ftp.e.culeuven.ac.be/pub/droogmans/cabuf.zip) (22). The resulting calculated free calcium was in the order of 3–5 μM. The addition of 5.4 mM CaCl\(_2\) raised Ca\(^{2+}\) to 1 mM. Cells were harvested at the time points indicated under “Results.”

**RNA Isolation—** Approximately 2.4–5 10\(^{6}\) *E. histolytica* were lysed by the addition of 0.8 ml of TRIzol reagent (Invitrogen), and total RNA was purified according to the manufacturer’s directions. RNA of greater than 200 nucleotides in length was isolated from total RNA by the RNeasy protocol (Qiagen) after first treating the total RNA preparation with DNase I (Roche Molecular Biochemicals) to remove contaminating genomic DNA.

**Cloning and Expression of His\(_{6}\)-tagged URE3-BP and His\(_{6}\)-EF\(^{2}\)mut URE3-BP—**The wild-type URE3-BP His\(_{6}\)-tagged fusion protein expression vector was constructed as described previously (14). Mutagenesis of the EF-hand motif (2) of URE3-BP was done by a two-stage PCR procedure. The 3′ sequences of URE3-BP were amplified and mutated using the oligonucleotides GTTATGACCTGATCCGATAAGGT and AAATGGCATATTCCGAAGGAAAATGACG that replaced the conserved first and third amino acids of the EF-hand motif with two alanine residues. The resulting PCR product was denatured and used along with an oligonucleotide AAGGTCCTCTCAAGAATAACCACTGACGTCAATTTG to amplify the mutated copy of URE3-BP. This DNA was then subcloned into the pCR7/NT-TOPO expression vector (Invitrogen). The mutated gene was sequenced to confirm the presence of the desired mutation. The expression of the His\(_{6}\)-tagged fusion protein was induced in *E. coli* BL21(DE3) (F‘ ompT gal dcm [dcm] lon) hsdSB, (rB–mb–) cells grown in Luria-Bertani medium with 100 μM isopropyl-

**Calcium Calibration—** Amebae were prepared and labeled with the acetoxy-methyl ester of the Ca\(^{2+}\)-sensitive fluorescent dye indo-1 (indo/AM; Molecular Probes) as described by Carabajal et al. (25) with the following modifications. Labeling was performed in a buffer of 10 mM Heps-HCl, pH 7.2, 140 mM NaCl, 1 mM CaCl\(_2\), 1 mM MgCl\(_2\), 5 mM KCl, 10 mM glucose, 0.1% bovine serum albumin, and 0.2% pluronic F-127 (Molecular Probes) buffer containing 28.7 μM indo-1. The indo-1 was fixed, dried, and quantitated by PhosphorImager analysis.

**Immunolabeling—** Amebae were labeled with the mouse monoclonal anti-URE3-BP antibodies (described above) or the control antibodies of the same isotype (anti-lectin mAb 7F4) was bound to protein G Dynabeads (Dynal). They were then used to immunoprecipitate chromatin from amebic nuclear extracts prepared by a modification of methods described previously (10). Briefly, nuclei were harvested from amebae that had been cross-linked in 1% formaldehyde in either phosphate-buffered saline or Buffer B + 1 mM Ca\(^{2+}\) (5.7 mM CaCl\(_2\)). Nuclear extracts were prepared from 1 × 10\(^6\) amebae preincubated in Buffer A and the Basic Local Alignment Search Tool (BLAST) at NCBI (23, 24). Sequence data pertaining to the URE3-BP genomic context were produced by the TIGR Entamoeba histolytica Genome Project (www.tigr.org/db/e2k1/eha1/) and the Pathogen Sequencing Unit at the Sanger Institute (www.sanger.ac.uk/Projects/E_histolytica/blat_server.shtml), which are both part of the International Entamoeba Genome Sequencing Project. These sequences were obtained from ftp.sanger.ac.uk/pub/pathogens/entamoeba/GenBank. The University of Virginia Biomolecular Research Facility performed all other sequencing.
Fig. 1. Electrophoretic mobility shift assay of the recombinant (wt)URE3-BP and EF(2)mut URE3-BP. A, (wt)URE3-BP, B, EF(2)mut URE3-BP in which two key residues in EF-hand 2 have been altered. In both gels the first lane is an EMSA of the recombinant protein. In the second lane EMSA was performed in the presence of 6-fold excess unlabeled hgl5-URE3(wt), and in the third lane with 6-fold unlabeled hgl5-MUT (mut) oligonucleotide competitors. EMSA in buffer containing 6.7 μM (wt)URE3-BP or 86 μM free Ca2+ (EF(2)mut URE3-BP) is shown in the fourth lane.

Real Time Quantitative PCR—Real time quantitative PCR analysis of the hgl5 and fdx promoters and srehp coding region was performed in a Bio-Rad iCycler. The fluorescent dye SYBR Green 1 (Molecular Probes) was used to detect double strand DNA. Continuous SYBR Green I monitoring during amplification was done according to the manufacturer’s recommendations. The 5 μl of the immunoprecipitated DNA was subjected to 40 amplification cycles with Qiagen’s HotStar Taq. To amplify ~270 to ~3 bp of the hgl5 promoter (4) the primers CTACTGAAGCTTTAAAAATATAA were used. To amplify the region ~513 to +14 of the fdx promoter (29) oligonucleotides CTACTGAAGCTTTAAAAATATAA were used. To amplify the coding sequences +120 to +663 of the srehp gene (30) the oligonucleotides CTCTCTGAAAAGCTTGAAGC and GGTACTGATGAGCAATCAAGGT were used. All real time amplification reactions were performed in duplicate on each ChIP experiment, and the resulting fluorescent values were averaged.

Reverse transcription followed by real time PCR was implemented to quantitate the calcium-dependant expression of the fdx and URE3-BP genes and normalized to the level of the control transcript, L10. The reverse transcription was primed using random sequence hexamers and performed with the Superscript II enzyme (Invitrogen). Real time amplification was preformed as described above. To amplify the region between +3 to +151 of the URE3-BP cDNA the oligonucleotides AAAAGATCTATGGGATATGTGTTAAG7AATGGGAAACC and AAagggaagc...
RESULTS

Mutation of the EF-hand Motif in URE3-BP Abrogates Calcium Inhibition of in Vitro DNA Binding—Recognition of URE3 by URE3-BP is blocked in the presence of calcium, as measured by in vitro EMSA. URE3-BP contains two EF-hand motifs. We mutated the second EF motif to test its role in both DNA binding and calcium sensitivity. The Prosite consensus pattern of the EF-hand domain (PDPC00018) consists of 36 amino acids, with a twelve-residue loop, [x-DNS]-[ILVFYY]-[DEN-STG]-[DNQGHKR]-[GP]-[LVIMC]-[DENQSTAGC]-[LIVM][x]-[DE]-[LIVMFYY], flanked on both sides by twelve-residue α-helical domains (15). In an EF-hand loop the calcium ion is bound by the six residues in positions 1, 3, 5, 7, 9, and 12 or X, Y, Z, -Y, -X, and -Z (15). The amino acid sequence of the second EF-hand loop in URE3-BP was DRNRSGTLEPHF (conserved residues in bold). We replaced the residues at positions 1 and 3 in the second EF-hand loop to alter the sequence to ΔRΔRSGTLEPHF (changed residues underlined).

The resulting mutant protein was tested in an EMSA assay (Fig. 1). The mutated URE3-BP protein was able to form a protein-DNA complex with a radiolabeled double stranded hgl5-URE3 oligonucleotide and was competed by the addition of unlabeled double stranded oligonucleotide hgl5-URE3 (Fig. 1B). The DNA-protein complex was also inhibited to a lesser extent by the addition of hgl5-MUT, a double stranded DNA oligonucleotide, in which the URE3 DNA sequence was mutated to TTAGAATTCC from the wild-type sequence of TTATACTTCC. The partial inhibition of the protein-DNA complex with hgl5-MUT suggested some loss of specificity for DNA binding of the mutated URE3-BP.

Detection of in Vivo Promoter Occupation of URE3-BP—ChIP assays allow study of the interaction between nuclear proteins and DNA sequences in the context of the chromatin template (33). This technique was used to test whether nuclear URE3-BP was physically associated with the hgl5 gene promoter in the chromosomes. Trophozoites were treated with formaldehyde to cross-link nuclear proteins with chromosomal DNA. DNA-protein complexes were then purified by immunoprecipitation. The cross-links were reversed, and associated DNA was purified. Detection and measurement of the precipitated DNA was achieved by use of real time PCR (9). Figs. 2 and 3 show representative ChIP experiments in which DNA purified by immunoprecipitation of cross-linked nuclear DNA-protein complexes with anti-URE3-BP mAb was amplified by real time PCR for the hgl5 and fdx promoters. The graphs in Fig. 2A and Fig. 3A show the calibration of the Ct value of the primer pairs used to amplify the hgl5 and fdx E. histolytica promoters against DNA concentration. Each experiment was controlled with a ChIP using an irrelevant mAb. An additional control was PCR amplification of an irrelevant gene segment (the srehp coding sequence). There was no significant difference observed between the Ct for the srehp DNA amplified from the control or anti-URE3-BP mAb ChIPs (data not shown). Anti-URE3-BP mAb specifically immunoprecipitated DNA containing the hgl5 and fdx E. histolytica promoters (see Figs. 2 and 3). This was consistent with URE3-BP being physically associated with the DNA-chromatin complex at those promoters.
promoters (see Figs. 2 and 3).

**Manipulation of Intracellular Calcium in E. histolytica Trophozoites**—Because of the importance of calcium in the sequence-specific recognition of URE3 by URE3-BP, we wished to test the effect of intracellular calcium on promoter binding by URE3-BP. We estimated the level of intracellular calcium using the fluorescent dye indo-1. Trophozoites were suspended in Buffer B (25), which contained 5 mM MgCl₂ and EDTA to sequester calcium. The baseline internal Ca²⁺ was measured by the fluorescence ratio (398/480 nm) after excitation at 360 nm. The effects of altering the external [Ca²⁺]₀ concentration on internal Ca²⁺ was followed after the addition of 1.2 mM CaCl₂ (8 μM [Ca²⁺]₀), 5 mM CaCl₂ (608 μM [Ca²⁺]₀), and 5.7 mM CaCl₂ (1.1 mM [Ca²⁺]₀) (27). A rapid and consistent increase in internal Ca²⁺ was observed (as measured by the ratio of indo-1 fluorescence emission at 398/480 nm) when the external [Ca²⁺]₀ concentration was increased to 608 μM or 1.1 mM (Fig. 4). The change in indo-1 fluorescence induced by increases in extracellular calcium was not because of leakage of the dye from the trophozoites, as indo-1 fluorescence was not detected in the cell-free supernatant (data not shown). This indicates that in these conditions, changes in extracellular calcium resulted in alterations of intracellular calcium in the amebic trophozoites.

**Changes in URE3-BP Occupancy of the hgl5 and fdx Promoters in Vivo upon Alteration of Calcium Levels**—The in vitro EMSA experiments (Fig. 1A) demonstrated that the sequence-specific interaction of URE3BP with URE3 was blocked by calcium. To test the importance of calcium for URE3-BP binding to the promoter in **vivo** we performed ChIP after modulating intracellular calcium. Trophozoites were suspended in Buffer B + 5.7 mM CaCl₂ (1.1 mM [Ca²⁺]₀) before formaldehyde treatment, ChIP, and real time PCR analyses. As demonstrated in Figs. 5 and 6 the increase in intracellular calcium (caused by the addition of 5.7 mM extracellular calcium to the trophozoites) resulted in inhibition of URE3-BP binding to both the hgl5 and fdx promoters as measured by ChIP and real time PCR. The failure to immunoprecipitate the hgl5 and fdx promoters with the anti-URE3-BP mAb was not because of calcium-mediated interference in immunoprecipitation of the URE3-BP, as similar amounts of the protein were immunoprecipitated under both conditions (Fig. 6A). We therefore concluded that in the presence of elevated intracellular calcium URE3-BP was unable to occupy promoters that contained the URE3 DNA motif.

**URE3-BP Genomic Sequence Context**—The URE3-BP cDNA sequence was used to identify the equivalent genomic sequence in the International Entamoeba Genome Sequencing Project data bases (Fig. 7). A URE3 DNA consensus sequence was present in the 3’ sequence 1.3 kb distal to the URE3-BP gene stop codon. We have previously found functional regulatory sequences for other Entamoeba genes more than 1 kb from the...
open reading frame.\(^3\) There were no differences in genomic and cDNA sequences, which was not surprising, because most (about 80–90\%) \(E.\) histolytica genes lack introns. A sequence (ATTCG) that strongly resembled the INR consensus ATTCA was located immediately before the cDNA start site (7). The sequence GAACT, which is identical to the GAAC consensus (GAACT), was located 40 bp from the ATG start sequence (28 bp from the previously published cDNA sequence). The sequence (TGATATAAAG) with a very low similarity to the TATA consensus (GTATTTAAA(GyC) was located 46 bp from the ATG (6). A pentanucleotide sequence (TAATT) that was identical to the 3′-terminal consensus sequence TA(A/T)TT was located 42 bp after the translation stop codon (7). The 3′ non-coding sequence contained an open reading frame in the opposite orientation that terminated at 73 bp after the URE3-BP stop codon and initiated at 720 bp on the opposite strand and thus intervened between the termination of the URE3-BP open reading frame and the 3′ URE3 sequence motif. The intervening open reading frame could encode a highly basic 25-kDa protein but did not have an easily recognizable core promoter.

Calcium Dependence of URE3-BP

DISCUSSION

This work demonstrates that URE3-BP is a calcium-binding protein that can function as both a transcription factor and a calcium-binding protein. The presence of a URE3 motif in the URE3-BP non-coding region was consistent with the gene being under autoregulation.
Calcium Dependence of URE3-BP

![Graph showing calcium dependence of URE3-BP mRNA levels](image)

**Fig. 8.** Sequence expression of URE3-BP mRNA ± calcium. *E. histolytica* were seeded at a concentration of 2–4 × 10⁶/ml 18 h prior to the addition of 5 mM MgCl₂ and EDTA to sequester free calcium (calculated to reduce TYI-S-33 medium Ca²⁺ to 3–5 μM), and the addition of 5.4 mM CaCl₂ was calculated to raise the free calcium concentration to 1 mM and was added to calcium treated trophozoites. RNA was purified from the trophozoites at the times indicated, and the level of URE3-BP and L10 transcripts was quantified by real time reverse transcriptase PCR. URE3-BP mRNA values were then normalized to the level of L10 mRNA. The graph shows the ratio of URE3-BP mRNA isolated from trophozoites in 1 mM Ca²⁺ versus calcium-depleted medium at the times indicated. A value of one indicates that no change was observed. These results were obtained from three independent experiments. The asterisks (*) indicate that a statistical significant difference was observed from the L10 value at this time point (mean ± S.E., n = 3, p < 0.02).

Calcium sensor in *E. histolytica*. Only one other calcium binding eukaryotic transcription factor, DREAM, has been described. DREAM (also known as Calsenilin or KhChIP3) also contains EF-hand motifs and is similar to URE3-BP in that it exhibits sequence-specific binding to DNA. An increase in calcium levels in vitro blocks DNA binding by DREAM and URE3-BP. The mutation of EF(2) of URE3-BP had little effect on DNA binding but impeded the ability of Ca²⁺ to block in vitro DNA binding to URE3-BP. This showed that the second EF-hand motif of URE3-BP was essential for the calcium modulation of URE3-BP DNA binding.

Promoter occupancy of URE3-BP in vivo on the native chromatin of URE3-BP of the fdx and hgl5 promoters was tested in this work. To determine whether URE3-BP interacted with URE3-containing promoters in the nucleus we utilized the ChIP assay in conjunction with real time PCR. This allowed measurement of URE3-BP bound to URE3-containing promoters in intact trophozoites. Our results indicated that URE3-BP was located on both the fdx and hgl5 promoters in the nuclear environment.

Internal changes in [Ca²⁺], have been observed in response to various stimuli (25, 34, 35), and several important calcium-binding proteins have been discovered in *E. histolytica* including a protein similar to calmodulin, EhCaBP (36, 37). In addition, the multidrug resistance phenotype mediated by the up-regulation of the EhPgp1 and EhPgp5 genes of *E. histolytica* has been shown to be reversed by calcium-channel blocker verapamil (38–41). To investigate the role of URE3-BP as a calcium sensor protein we investigated the impact of altering [Ca²⁺], on URE3-BP promoter occupancy. A significant decrease in URE3-BP location at both the hgl5 and fdx promoters was observed upon an increase in [Ca²⁺]. Therefore, calcium not only prevented in vitro binding of URE3-BP to URE3 but also blocked the occupancy of URE3-containing promoters by URE3-BP in vivo.

Because calcium levels influenced the binding of URE3-BP to the URE3-containing promoters the effect of calcium on the steady state levels of fdx and URE3-BP mRNA was examined. The hgl5 mRNA was not analyzed, because it was not possible to design PCR primers that would amplify hgl5 mRNA without cross-hybridizing to other hgl genes that lack URE3-containing promoters (2). In the case of the fdx gene, its transcript was not significantly changed by increases in intracellular calcium. This perhaps was not surprising as changes in intracellular calcium would be predicted to have pluripotent effects on the trophozoite that could have disparate effects on fdx mRNA abundance.

In contrast to the situation for fdx mRNA, the addition of calcium sharply decreased the level of the URE3-BP mRNA. This decrease could be mediated by an autoregulatory mechanism where URE3-BP is displaced by calcium from the URE3 motif located at the 3′ sequence of URE3-BP. Mutational analysis of the URE3 motif in the 3′ noncoding region of URE-3BP will be required to test directly whether the gene is directly autoregulated, although one might expect, as is the case for the fdx gene, that URE-3BP will fall under complex regulatory responses to changes in intracellular calcium. In either case these results indicate that increases in intracellular calcium not only decrease the ability of URE-3BP to bind DNA but also decrease its mRNA abundance. This suggests that URE3-BP is both an effector and a responder in a cascade of calcium-regulated gene expression in the parasite.

One of the mysteries of amebiasis is why only a minority of infected individuals develop invasive disease. *E. histolytica* encounters differing levels of extracellular calcium in the human intestine where it lives. We have shown here that intracellular calcium levels in the parasite are exquisitely sensitive to changes in extracellular calcium. The extracellular calcium concentration in the intestinal lumen is influenced by the amount of calcium ingested, absorbed, and excreted. In the small intestine where the parasite excysts, luminal calcium concentrations are well above 1 mM (42). *E. histolytica* trophozoites colonizing the large intestine are exposed to extracellular calcium levels that vary depending upon the diet (13, 42). In hosts with a high calcium diet trophozoites would be exposed to >1.25 mM calcium. We would predict from our data that these calcium levels would be sufficient to block URE3-BP binding and decrease URE3-BP synthesis. In a low calcium diet, luminal calcium concentration may drop to <1.25 mM. In this circumstance calcium absorption in the large intestine is considerably enhanced, and free Ca²⁺ will be further decreased (42). URE3-BP-mediated gene regulation would then play a significant role in the regulation of transcription. It is possible that calcium and calcium sensors including URE3-BP influence the expression of the virulence phenotype of *E. histolytica* in the large intestine and impact the development of disease.

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