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Transient transfection of the enteric parasite *Entamoeba histolytica* and expression of firefly luciferase

(electroporation/amebiasis/galactose-binding adhesin/lectin/*hgl*)

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ABSTRACT **Development of DNA-mediated transfection in *Entamoeba histolytica* will facilitate basic research toward the control of this protozoan parasite. A transient transfection system was established by using the firefly luciferase gene ligated to the 5′ and 3′ flanking regions of the amebic *hgl1* gene. The optimal construct tested encoded an hgl1–luciferase fusion protein and contained 1 kb of 5′ flanking sequence with 16 bases of coding sequence from the *hgl1* gene ligated in-frame to the luciferase start codon and 2.3 kb of 3′ flanking sequence from *hgl1* ligated 3′ to the luciferase stop codon. Optimal electroporation conditions in strain HM-1:IMSS trophozoites when using this construct were 500 μF and 500 V/cm, which resulted in luciferase activity up to 5000-fold above background 9–12 hr after electroporation. Constructs that contained the luciferase gene without amebic flanking sequences or that contained a simian virus 40 promoter, enhancer, and polyadenylylation signal produced only background levels of luciferase activity. The ability to introduce and express genes in amebae will now permit a genetic analysis of the virulence of this organism, which remains a serious threat to world health.**

The enteric protozoan *Entamoeba histolytica* is the cause of amebiasis, a disease that is surpassed only by malaria and schistosomiasis as a parasitic cause of death (1). As the improvements in sanitation necessary to prevent the fecal–oral spread of this parasite in the developing world are only slowly being made, control of amebiasis is dependent upon advancements in diagnosis, treatment, and immunoprophylaxis.

The development of a vaccine has been hampered by an incomplete understanding of *E. histolytica* pathogenesis. Although several proteins have been identified that appear to be involved in colonization and virulence, in most cases their specific functions and roles in pathogenesis are poorly defined. The ability to manipulate the parasite genome via DNA transfection would allow a more detailed analysis of the amebic factors responsible for virulence.

E. histolytica presents a challenge to genetic analysis because there is no known sexual cycle or method to introduce foreign DNA. We sought to develop a transfection system that would maximize DNA internalization without destroying the fragile trophozoite. Conserved sequences in the 5′ and 3′ flanking regions of amebic genes suggested that promoter and polyadenylylation signals diverge significantly from standard eukaryotic sequences (2, 3). In the absence of definitive information about amebic regulatory sequences, we presumed that the 5′ and 3′ flanking regions from the amebic *hgl1* lectin gene (4) must contain the necessary elements for proper gene expression and used these regions to express firefly luciferase in *E. histolytica*. We report the successful introduction and expression of foreign DNA in an enteric parasite.

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MATERIALS AND METHODS

Cell Culture Conditions. *E. histolytica* strain HM-1:IMSS trophozoites were grown in TYI-S-33 medium (5) containing penicillin (100 units/ml) and streptomycin sulfate (100 μg/ml) in 75-cm^2 flasks at 37°C. Amebae in logarithmic-phase growth were used for transfection experiments after they had grown to 5.3–6.6 × 10^4 trophozoites per ml. Simian kidney cell line CV1 (ATCC CCL 70) was grown in 25-cm^2 flasks in 5 ml of minimal essential medium (MEM; GIBCO/BRL) containing 10% (vol/vol) fetal bovine serum at 37°C and in an atmosphere containing 5% CO_2.

Plasmid Construction. Plasmid pGEM-luc, which contains the luciferase gene, and plasmid pGL2-Control, which contains the luciferase gene flanked by a simian virus 40 (SV40) promoter, polyadenylylation signal, and enhancer, are commercially available (Promega). To make plasmid ΔR8 (all constructs are illustrated in Fig. 1), the 3′ portion of the luciferase gene from pGEM-luc was amplified by using the polymerase chain reaction (PCR) with the primers 94 and 95 (all nucleotides used for plasmid construction are described in Table 1), which added a synthetic *Xho* I site in the amplified product two bases 3′ of the stop codon of luciferase. The amplified product and pGEM-luc were digested with *Cla* I and *Xho* I and were ligated together with T4 DNA ligase (GIBCO/BRL). By effectively deleting 53 bases between the stop codon of the luciferase gene and the *Xho* I site in the multicloning site of pGEM-luc, 3′ amebic sequences could be ligated in close proximity to the 3′ terminus of the reporter gene. A short 3′ untranslated region in amebic mRNA is typical and may prove critical to message stability.

To make the AΔ2R8 construct, approximately 1 kb of the 5′ flanking region of *hgl1* was PCR-amplified from a genomic clone containing the 5′ coding region and flanking region of *hgl1* (6) by using the primers 96 and 98. The amplified product and ΔR8 were digested with *Bam*HI and *Hin*dIII and subsequently ligated, producing plasmid AΔ2R8. This placed the 5′ noncoding region of *hgl1* 5′ of the reporter gene at the expense of replacing bases −1 through −8 of the *hgl1* gene with the restriction site *Bam*HI.

Plasmid BΔ1R8 was constructed by PCR amplification with primers 96 and 118 of approximately 1 kb of the 5′ flanking region of *hgl1* and the first 16 bases of the *hgl1* coding region. This product and ΔR8 were digested with *Hin*dIII and *Bam*HI and ligated. This construct contained (in 5′ to 3′ orientation) an unaltered 5′ noncoding region of *hgl1*, the first 5 codons of *hgl1*, three in-frame codons created by the ligation of the synthetic *Bam*HI restriction site to *hgl1* and

Abbreviations: E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; E-64c, (2*S*,3*S*)-*trans*-epoxysuccinyl-L-leucylamido-3-methylbutane; SV40, simian virus 40.

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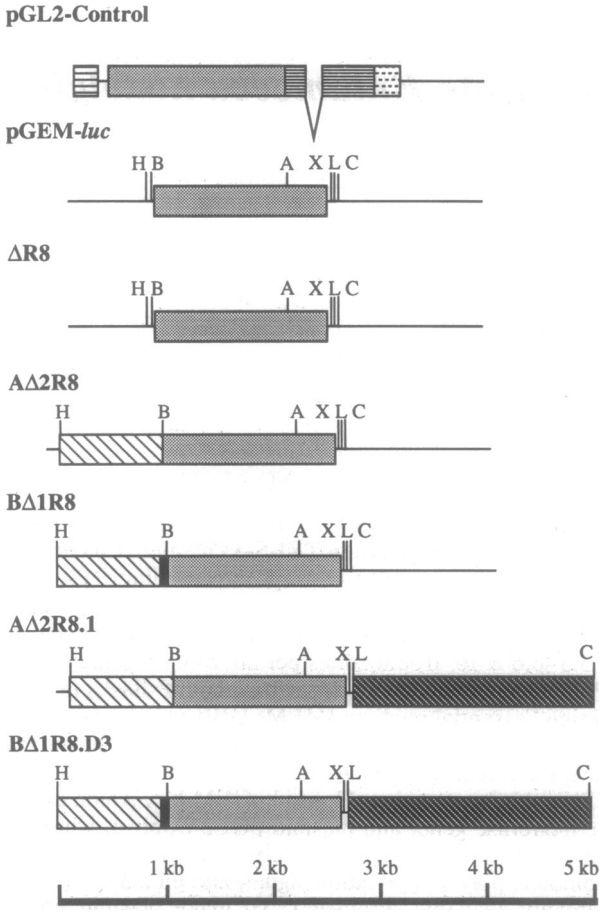

FIG. 1. Plasmid constructs used to transiently transfect *E. histolytica*. ▤, SV40 promoter; ■, SV40 polyadenylylation signal; V, intron; ▨, SV40 enhancer; ▧, luciferase coding region; ▩, 5′ flanking region of *hgl1*; ■, coding region of *hgl1*; ▩, 3′ flanking region of *hgl1*; —, plasmid sequence; H, *Hin*dIII; B, *Bam*HI; A, *Cla* I; X, *Xho* I; L, *Sal* I; C, *Sac* I. See *Materials and Methods* for details of plasmid construction.

luciferase DNA, and the in-frame methionine codon of luciferase. Not only is the 5′ flanking region unaltered in this construct, but a hgl1–luciferase fusion protein should result, allowing the amebic ribosome to initiate by using the amebic codon bias before beginning translation of the foreign protein.

To construct BΔ1R8.D3, the 3′ flanking region of *hgl1* was PCR-amplified from a genomic clone containing the 3′ coding and flanking region of *hgl1* (6) by using the primers 131 and 99. The product and BΔ1R8 were digested with *Sal* I and *Sac* I and ligated together. This placed the 3′ noncoding region of *hgl1* 14 bases 3′ of the reporter gene. Plasmid AΔ2R8.1 was constructed by restriction digestion of BΔ1R8.D3 and AΔ2R8 with *Sal* I and *Sac* I. The 2.3-kb insert (3′ flanking region of *hgl1*) from BΔ1R8.D3 and the AΔ2R8 plasmid were purified on an agarose gel and ligated together.

The structures of all constructs were confirmed by restriction digestion, and all points of ligation were confirmed by DNA sequence analysis. Plasmids used for electroporation were isolated via alkaline lysis followed by purification on an anion-exchange column (either Maxi tip-500 or Mega tip-2500) according to the manufacturer's instructions (Qiagen, Chatsworth, CA). All preparations were assayed for purity and plasmid concentration by spectrophotometer. No luciferase activity was detectable in the purified plasmid preparations prior to transfection.

Electroporation. Logarithmic-phase trophozoites were incubated on ice for 15 min in TYI-S-33 medium, centrifuged at 200 × *g* for 5 min, and washed once in incomplete cytomix buffer [120 mM KCl/0.15 mM $CaCl_2$/10 mM K_2HPO_4/KH_2PO_4, pH 7.5/25 mM Hepes/2 mM EGTA/5 mM $MgCl_2$, final pH 7.8–7.9]. Complete cytomix buffer, containing also 2 mM ATP and 5 mM glutathione (7), was used for comparison. Trophozoites were resuspended in incomplete cytomix buffer at a concentration of 2.6–2.8 × 10^6 per ml, and 0.8 ml was placed into 0.4-cm electroporation cuvettes (Bio-Rad) on ice. Forty micrograms of plasmid or distilled water and 2.5 μl of DEAE-dextran (8) at 1 mg/ml were added, and the medium was mixed immediately prior to electroporation. Standard electroporation conditions were 500 μF and 500 V/cm with a Gene Pulser augmented with a capacitance extender (Bio-Rad), resulting in a time constant of 9.7–10.6 msec. Cuvettes were placed back on ice for 15 min, after which the electroporated trophozoites were added to 11 ml of TYI-S-33 medium containing penicillin, streptomycin sulfate, and 8 μM (2*S*,3*S*)-*trans*-epoxysuccinyl-L-leucylamido-3-methylbutane (E-64c; Sigma) in capped glass tubes; 100–150 μl of preelectroporated cytomix buffer and postelectroporated trophozoites were spread on a Luria broth (LB) bacterial plate, and aliquots of cytomix buffer were added to LB broth and TYI-S-33 medium to confirm the lack of bacterial contamination.

For control experiments, 18–36 units of RNase (Boehringer Mannheim) was added prior to electroporation, cycloheximide (Sigma) was added (100 μg/ml) to culture media (9), amebae were treated the same without electroporation, or cytomix buffer was electroporated alone. In each case, cefotaxime (Claforan; Hoechst–Roussel) was added (100 μg/ml) to culture media.

Luciferase Assay. Transfected trophozoites in TYI-S-33 medium were centrifuged at 200 × *g* for 5 min and washed once in PBS (pH 7.5). The trophozoite pellet was resuspended in an equal amount of 1× lysis buffer [25 mM Tris phosphate, pH 7.8/2 mM 1,4-dithiothreitol/2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid; 10%

Table 1. Oligonucleotide primers used in the construction of plasmids shown in Fig. 1

Name	Sequence	Description
94	TGGCCCCCGCTGAATTG	Nucleotides 393 to 409 of luciferase coding region of pGEM-luc
95	gcgcgc*ctcgag*TTTTACAATTTGGACTT	Nucleotides 116 to 100 of luciferase coding region of pGEM-luc, *Xho* I site, reverse primer
96	gcgcgc*aagctt*TTTGATAAGTCATGAGT	Approximately −1000 bases 5′ of *hgl1* start codon, *Hin*dIII site
98	gcgcgc*ggatcc*CTTTCTAGTTCATTGTC	Nucleotides −9 to −25 relative to the start codon of *hgl1*, *Bam*HI site, reverse primer
99	gcgcgc*gagctc*ACGATGTAACTCAATAA	Approximately 2300 bases 3′ of the *hgl1* stop codon, *Sac* I site, reverse primer
118	gcgcgc*ggatcc*ATAATAATAATTTCATAT	Nucleotides +16 to −2 relative to the start codon of *hgl1*, *Bam*HI site, reverse primer
131	gcgcgc*gtcgac*GAACAATAATTAAGAGAATT	Nucleotides 1 to 18 3′ of the *hgl1* stop codon, *Sal* I site

All nucleotides are in 5′ to 3′ orientation. Primers that are reverse antisense to the coding strand are referred to as "reverse primers." Lowercase letters indicate nonhomologous sequences, with restriction sites italicized and identified under Description.

(vol/vol) glycerol, 1% Triton X-100] containing 75 μM *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64; Sigma) and 0.75 μg of leupeptin (Sigma) per ml. Samples were immediately frozen at −20°C for a minimum of 1 hr, thawed on ice for 10 min, centrifuged briefly to pellet debris, and returned to ice for an additional 10 min. After warming to room temperature for 10 min, 20 μl of the amebic lysate was assayed in 100 μl of luciferase assay reagent [20 mM Tricine/1.07 mM $(MgCO_3)_4 \cdot Mg(OH)_2 \cdot 5H_2O$/2.67 mM $MgSO_4$/0.1 mM EDTA/33.3 mM dithiothreitol/270 μM coenzyme A/470 μM luciferin/530 μM ATP, final pH 7.8] (Promega) with a Turner Luminometer model TD-20e (Promega). Background luminescence on the luminometer was calibrated to zero immediately prior to all assays with *E. histolytica* electroporated without plasmid. The luciferase activity was calculated from a standard curve obtained before each experiment by using the same substrate and exogenous firefly luciferase (ca. 1×10^7 luciferase light units/mg of luciferase, Boehringer Mannheim). To assay for luciferase secretion, growth medium was assayed for luciferase activity with negative results.

Transfection of CV1 Cells. Twenty micrograms of plasmid DNA was precipitated with calcium phosphate in a total of 1 ml according to the manufacturer's instructions (5 Prime → 3 Prime, Inc.). Precipitated plasmid solution (0.8 ml) was added to CV1 cells in 25-cm^2 flasks (80% confluent) that had been washed with 5 ml of serum-free MEM (GIBCO/BRL). After 30 min at room temperature, 5 ml of MEM containing 10% fetal bovine serum were added, and the CV1 cells were incubated at 37°C in an atmosphere containing 10% CO_2 for 3.5 hr. The medium was replenished, and the flasks were incubated 48 hr at 37°C in 10% CO_2/90% air. CV1 cells were assayed for luciferase activity according to the manufacturer's protocol (Promega).

RESULTS

Optimal Electrical Conditions of Electroporation. Amebic trophozoites harvested from logarithmic-phase growth cultures were electroporated with the BΔ1R8.D3 plasmid (40 μg per 2.1–2.3 × 10^6 trophozoites) under a wide variety of electrical settings to determine the conditions yielding maximal luciferase expression. The optimal voltage and capacitance were determined to be 500 μF and 500 V/cm (200 V per 0.4-cm cuvette) (Fig. 2). At these conditions, the average time constant (related to capacitance and resistance) was 10.0 msec, with the trophozoite rate of survival based upon visual inspection being 25–35%. Electroporation with 125-μF, 25-μF, and 3-μF capacitances resulted in suboptimal levels of luciferase activity at all voltages assayed (data not shown).

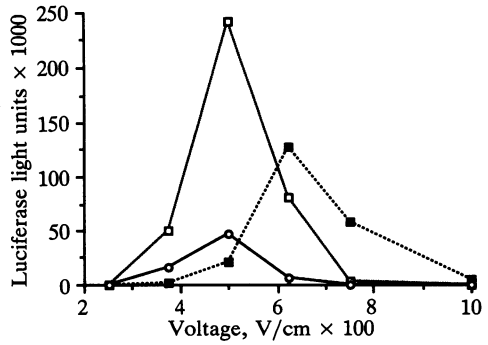

FIG. 2. Optimal voltage and capacitance for electroporation. Amebae in logarithmic-phase growth were electroporated at various combinations of voltage and capacitance in the presence of 40 μg of BΔ1R8.D3. They were harvested after 6 hr and assayed for luciferase activity. Results are expressed as luciferase light units per 2.1–2.3 × 10^6 amebae transfected. Each determination is representative of between three and five similarly performed experiments. ○, 960 μF; □, 500 μF; ■, 250 μF.

Optimal Electroporation Buffer. The amebae were resuspended in incomplete cytomix buffer for all electroporation experiments reported. Electroporation with complete cytomix buffer (7) resulted in luciferase activity 10–15% of that seen when incomplete cytomix was used (data not shown). DEAE-dextran, which is thought to increase the local concentration of DNA at the cell surface (8), was added to each cuvette prior to electroporation (3.1 μg/ml), as it resulted in luciferase activity 58% greater than the activity observed when no DEAE-dextran was present. Final DEAE-dextran concentrations of 10 μg/ml and 5 μg/ml decreased luciferase activity by 96% and 5%, respectively, from the activity observed when no DEAE-dextran was present (data not shown). Luciferase activity increased linearly with plasmid concentrations of 10 μg per cuvette to 60–80 μg per cuvette when using the described conditions (data not shown).

Time Course of Luciferase Expression. Amebae were electroporated at the optimal electrical and buffer conditions, and the luciferase activity was assayed at different time points. The pGEM-luc plasmid, which lacked amebic sequences, resulted in background levels of luciferase activity at each assay (Fig. 3). Luciferase activity after electroporation with the BΔ1R8.D3 construct was not detectable prior to 3 hr; peaked 9–12 hr after electroporation, with luciferase activity 200- to 5000-fold greater than that seen with the pGEM-luc plasmid; and decreased to 20-fold over background by 24 hr.

Protease Inhibitors. *E. histolytica* produces significant amounts of cysteine proteases (10). The addition of up to 40 μg of exogenous luciferase to amebic lysate resulted in only background levels of activity because of rapid digestion of luciferase (data not shown). Protease inhibitors were tested to determine the concentration that would maximally inhibit amebic proteases while minimally inhibiting luciferase. This was accomplished by resuspending trophozoites in lysis buffer, adding protease inhibitors alone or in combination and at different concentrations, adding exogenous luciferase, and assaying for activity. Of the protease inhibitors assayed [phenylmethylsulfonyl fluoride, EDTA, *p*-hydroxymercuribenzoic acid, EGTA, trypsin inhibitor, *p*-chloromercuriphenylsulfonic acid, *N*-ethylmaleimide, E-64, 4-(2-aminoethyl)benzenesulfonyl fluoride, and leupeptin] it was found that concentrations of 37.5 μM of E-64 and 0.375 μg of leupeptin per ml were optimal, retaining approximately 50% of the exogenous luciferase activity (data not shown).

As these protease inhibitors were not present prior to lysis, it was reasonable to assume that luciferase degradation was also occurring in the trophozoites prior to this step. Thus

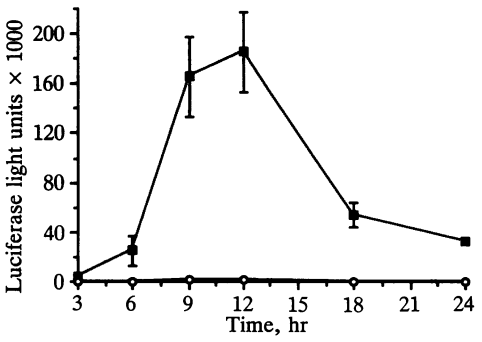

FIG. 3. Time course of luciferase expression after electroporation. Amebae in logarithmic-phase growth were electroporated at 500 μF and 500 V/cm with 40 μg of BΔ1R8.D3 or pGEM-luc, harvested at various time points, and assayed for luciferase activity. Results are expressed as luciferase light units per 2.1–2.3 × 10^6 amebae transfected. Each point represents three assays. ■, BΔ1R8.D3; ○, pGEM-luc.

E-64c, an inhibitor similar to E-64 but able to cross cellular membranes, was added to the TYI-S-33 medium in which the amebae were placed immediately after electroporation. Concentrations of E-64c between 1.4 μM and 10.6 μM increased luciferase activity after electroporation, with the optimal concentration of 8 μM increasing luciferase activity by 550%.

Control Transfections. The addition of the broad-spectrum antibiotic cefotaxime (100 μg/ml) to amebic culture media after transfection of BΔ1R8.D3 resulted in no decrease in luciferase activity, suggesting that electroporation of contaminating bacteria was not an explanation for observed luciferase activity. Additionally, bacteria were not detected when the electroporation buffer was spread on rich bacterial plates or inoculated into rich bacterial media. The addition of RNase (18–36 units per cuvette) prior to transfection of amebae with BΔ1R8.D3 did not decrease luciferase activity, indicating that this activity was not due to contamination of plasmid DNA by *E. coli*-produced luciferase mRNA transcripts. In fact, the addition of RNase increased luciferase activity from 86.3 milliunits ± 12.3 (± SE; $n = 3$) to 479.6 milliunits ± 152.5 because of glycerol in the RNase stock, which increased amebic survival. When glycerol alone was added to amebae prior to electroporation, luciferase activity increased by a similar amount. The addition of cycloheximide (100 μg/ml) after transfection of BΔ1R8.D3 resulted in luciferase activity of 0.0 milliunits ± 0.2, indicating that luciferase is dependent upon eukaryotic protein synthesis machinery. Unelectroporated amebae or cytomix buffer electroporated without amebae resulted in only background levels of activity (1.6 milliunits ± 0.1 and 0.6 milliunits ± 0.3, respectively), indicating that bacterial contamination of buffers or contamination of plasmid or amebae with luciferase protein was unlikely.

Dependance on Amebic Flanking Sequences for Luciferase Expression. To determine which regions of *hgl1* were required for expression of the luciferase gene, 2.1–2.3 × 10^6 amebae per cuvette were electroporated with 40 μg of each plasmid construct at the optimal electroporation conditions and harvested after 9 hr. The resultant luciferase activity is shown in Table 2. The pGL2-Control construct, which contains an SV40 promoter, enhancer, and polyadenylylation signal, is readily expressed in most eukaryotic systems. However, this construct resulted in no detectable luciferase activity in *E. histolytica*. The construct that lacked any promoter or polyadenylylation sequences, pGEM-luc, also resulted in background levels of luciferase activity. The addition of the 5′ flanking region of *hgl1* to the pGEM-luc construct (AΔ2R8) resulted in luciferase activity slightly over background (Table 2). As this construct required the replacement of *hgl1* bases −1 through −8 with a restriction site, which destroyed part of a conserved region (2), plasmid BΔ1R8 was constructed that contained all of the 5′ flanking region of *hgl1* present in AΔ2R8, the conserved sequence that had been altered in AΔ2R8, and 16 bases of *hgl1* coding region 5′ of the start codon of luciferase. This effectively moved the restriction site out of the 5′ flanking region of *hgl1* and placed it 5 amino acids into a hgl1–luc fusion protein. Transfection with BΔ1R8 resulted in an average luciferase activity 10-fold greater than that observed for AΔ2R8 (Table 2).

Table 2. Expression of transfected plasmid constructs

Plasmid	Luciferase activity, microunits per transfection	SE	*n*
pGL2-Control	0.0	0.1	3
pGEM-luc	0.0	0.1	3
AΔ2R8	0.8	2.4	3
BΔ1R8	8.5	7.5	3
AΔ2R8.1	1171.3	180.9	6
BΔ1R8.D3	2619.4	291.0	6

Amebae were electroporated in cytomix buffer containing 0.375% glycerol and assayed for luciferase activity. Activity is expressed as luciferase light units per 2.1.–2.3 × 10^6 amebae transfected. SE, standard error; *n*, number of determinations.

In an attempt to further increase luciferase expression, 2.3 kb of the 3′ flanking region of *hgl1* was ligated 3′ of the stop codon of luciferase in the construct AΔ2R8, creating construct AΔ2R8.1. When this construct was electroporated into amebae, luciferase activity increased to 1171.3 milliunits per reaction or >2000-fold greater than background levels of luminescence. A similar finding was observed when the same 2.3 kb of *hgl1* 3′ flanking sequence was ligated 3′ of the luciferase stop codon in construct BΔ1R8, creating construct BΔ1R8.D3. Luciferase activity resulting from transfection of amebae with BΔ1R8.D3 was >300-fold greater than that observed from BΔ1R8.

A comparison of the level of luciferase activity resulting from transfection with BΔ1R8.D3 and AΔ2R8.1 was also informative. These constructs differ only at the fusion point between the 5′ *hgl1* flanking sequence and the start codon of the luciferase gene. The AΔ2R8.1 construct possesses the 5′ flanking region of *hgl1* with an altered conserved sequence, while the BΔ1R8.D3 construct contains an unaltered 5′ flanking region and 16 bases of *hgl1* coding region. BΔ1R8.D3 resulted in the highest levels of luciferase expression noted—2.2-fold greater than that observed from AΔ2R8.1 and >5000-fold over background.

Transfection and Expression of Luciferase Constructs in CV1 Cells. The finding that *E. histolytica* transfected with the pGL2-Control plasmid containing the SV40 promoter, enhancer, and polyadenylylation signals resulted in background levels of luciferase activity suggested that amebic gene regulation and/or expression differs significantly from other eukaryotic gene regulation/expression. This had been expected because conserved sequences identified 5′ of amebic genes diverge significantly from promoter sequences in higher eukaryotes (2, 3). To determine if the BΔ1R8.D3 construct containing 5′ and 3′ amebic flanking DNA could be expressed in higher eukaryotic cell lines, BΔ1R8.D3, pGL2-Control, and pGEM-luc were transfected via calcium phosphate precipitation into the primate cell line CV1. The luciferase activity resulting from the transfection of the pGEM-luc plasmid was not significantly greater than the luminescence of buffer alone (data not shown). Transfection of CV1 cells with the pGL2-Control plasmid containing the SV40 promoter, enhancer, and polyadenylylation signal resulted in 567 milliunits of luciferase activity per flask, which is 2153 fold over background. The optimal plasmid in amebae, BΔ1R8.D3, in contrast, resulted in only 2 milliunits of luciferase activity per flask, which is 6.3-fold over background.

DISCUSSION

We present here a DNA-mediated transient transfection system for the enteric parasite *E. histolytica*. By using a construct that encodes an amebic lectin–firefly luciferase fusion protein ligated both 5′ and 3′ to flanking sequences from the amebic *hgl1* gene, luciferase activity as great as 5000-fold over the background level was observed. Both 5′ and 3′ flanking regions, including a conserved sequence located 5′ of the *hgl1* start codon, appeared to be required for optimal expression of luciferase activity. These constructs resulted in low levels of expression in a primate cell line, suggesting that they require *E. histolytica*-specific factors for optimal expression.

Peak luciferase activity in *E. histolytica* was 0.97 microunit per trophozoite electroporated, which compares favorably with *Trypanosoma brucei* which, when transfected with a luciferase construct containing the highly active *PARP* (pro-

cyclic acidic repetitive protein) promoter, resulted in peak luciferase levels of 0.04 microunit per organism electroporated (11). The successful electroporation of *E. histolytica* required several unique conditions. Because of the greater volume of the amebic trophozoite, concentrations of 2.1–2.3 $\times 10^6$ amebae per cuvette were used compared with 0.5–8.6 $\times 10^8$ for *Trypanosoma* (12, 13), 1×10^8 in *Leishmania* (14, 15), and 1–3 $\times 10^7$ for *Dictyostelium* (16). Of the plasmid constructs available, BΔ1R8.D3, which contained both 5′ and 3′ amebic flanking regions of *hgl1* and an hgl1–luc fusion protein, resulted in the highest luciferase activity. A shorter time course was required as the time of peak luciferase activity in *E. histolytica* was 9–12 hr compared with *Toxoplasma gondii*, which produced peak activity within the first 24 hr but retained 50% of maximal activity after 3 days (9). Incomplete cytomix buffer was more effective than complete cytomix. This may be due to the unique biochemistry of *E. histolytica*, which lacks glutathione and utilizes pyrophosphate in several steps of glycolysis (17). Finally, because of the high level of amebic protease activity (10, 18), the cysteine protease inhibitors E-64 and E-64c were required to prevent degradation of the expressed luciferase.

The possibility that the observed luciferase activity is due to electroporation of contaminating bacteria is highly unlikely for a number of reasons. The electrical parameters used were nonideal for bacterial electroporation, which requires higher voltages and lower capacitances. The cytomix buffer was assayed for contamination on rich LB bacterial plates and in LB medium with none being found. Aliquots of postelectroporation cytomix buffer/trophozoite medium were spread on LB bacterial plates with no evidence of contamination. The amebic culture medium contained both penicillin and streptomycin sulfate to prevent bacterial contamination. The addition of a third broad spectrum antibiotic, cefotaxime, resulted in no decrease in activity. Finally, cycloheximide, an inhibitor of eukaryotic protein synthesis, reduced luciferase activity to background levels.

The possibility that luciferase activity was due to contamination of the plasmid preparation by *E. coli*-produced luciferase or luciferase-encoding mRNA is equally unlikely. All plasmids were purified on an anion-exchange column that is very efficient at isolating plasmid DNA without protein or RNA contamination. When aliquots of the purified plasmid preparations were assayed separately for luciferase activity, none was present. Mock electroporation of amebae with luciferase-producing plasmids at 0 V produced no luciferase activity. The addition of RNase (18–36 units per cuvette) into the electroporation buffer prior to electroporation did not decrease luciferase activity. Finally, no luciferase activity was detectable prior to 3 hours after electroporation.

Transfection of *E. histolytica* with the different plasmid constructs suggests a number of interesting conclusions. First, while we have not yet determined the point of transcription initiation and polyadenylylation, both the 5′ and 3′ *hgl1* flanking regions appear to be required for optimal expression of luciferase, suggesting that they are required for proper expression of the lectin gene. Second, the creation of an hgl1–luc fusion protein by inclusion of the conserved sequence just 5′ of the *hgl1* start codon and 16 bases of *hgl1* coding sequence produced the highest levels of luciferase activity. This finding suggests (*i*) that the conserved 5′ sequence altered in plasmids AΔ2R8 and AΔ2R8.1 (Fig. 1) was required for optimal expression or (*ii*) that foreign proteins are translated more efficiently if the amebic ribosome can begin translation with its own codon bias or (*iii*) that the eight amino acids added to the amino terminus of luciferase increase protein stability. Third, electroporation with pGL2-Control, which contained an SV40-regulated luciferase gene, resulted in no detectable luciferase activity in *E. histolytica*, whereas constructs containing 5′ and 3′ amebic sequences were expressed. As the reverse was found when the same constructs were transfected into CV1 cells, these flanking regions may contain amebic regulatory sequences that are sufficiently divergent from higher eukaryotic regulatory regions to require amebic-specific factors for proper expression. Similar findings have been observed in the protozoan parasite *Leishmania*, which also appears to be unable to recognize 3′ SV40 regulatory sequences (15, 19).

The establishment of a transient transfection system for *E. histolytica* now allows a definitive characterization of regulatory regions required for proper gene expression and enables a genetic approach to the study of virulence in an enteric parasite.

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