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Infection by Bacterial Pathogens Expressing Type III Secretion Decreases Luciferase Activity: Ramifications for Reporter Gene Studies

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ABSTRACT

Pathogenic microbes influence gene regulation in eukaryotic hosts. Reporter gene studies can define the roles of promoter regulatory sequences. The effect of pathogenic bacteria on reporter genes has not been examined. The aim of this study was to identify which reporter genes are reliable in studies concerning host gene regulation by bacterial pathogens expressing type III secretory systems. Human intestinal epithelial cells, T₈₄ Caco-2 and HT-29, were transfected with plasmids containing luciferase (*luc*), chloramphenicol acetyltransferase (*CAT*) or β -galactosidase (β -gal) as reporter genes driven by the inducible interleukin-8 (*IL-8*) or constitutively active simian virus 40 (*SV40*) promoter. Cells were infected with enteropathogenic *E. coli* or *Salmonella typhimurium* and the reporter activity was assessed. *Luc* activity significantly decreased following infection, regardless of the promoter. The activity of recombinant *luc* was nearly ablated by incubation with either EPEC or *Salmonella* in a cell-free system. Activity was partially preserved by protease inhibitors, and immunoblot analysis showed a decreased amount and molecular weight of recombinant *luc*, suggesting protein degradation. Neither β -gal nor *CAT* activity was altered by infection. Disruption of type III secretion prevented the loss of *luc* activity. We conclude that *CAT* or β -gal, but not *luc*, can be used as reliable reporter genes to assess the impact of pathogenic microbes, especially those expressing type III secretion on host cell gene regulation.

INTRODUCTION

Investigations concerning the interactions between pathogenic microbes

and host cells are receiving heightened attention in the scientific community. Infection with pathogenic bacteria alters many functions including gene expression. Studying the regulation of gene expression is crucial for understanding the cellular and molecular mechanisms underlying the pathophysiology associated with such infections. Reporter gene studies are necessary to assess the functional role of transcription factors in the regulation of gene expression in host epithelial cells during infection with bacterial pathogens. The aim of this study was to identify which reporter genes are best to use in model systems examining host-pathogen interactions. We focused on the interactions between enteric bacterial pathogens, in particular those expressing a type III secretory apparatus and host intestinal epithelial cells.

The most commonly used reporter genes for the study of promoter activity in eukaryotic cells are luciferase (*luc*), chloramphenicol acetyltransferase (*CAT*) and β -galactosidase (β -gal). Interestingly, despite the recent explosion in the number of studies concerning host-pathogen interactions, there are very few publications (1,2,7,14,16,20) in which standard reporter gene systems are used. Models investigating *Salmonella* used *CAT*, while *luc* was used in studies of *Helicobacter pylori*, which does not use type III secretion. The major disadvantage of *CAT* as a reporter gene is its low sensitivity. On the other hand, *luc* is quite sensitive, but its reliability as a reporter has not been systematically tested in host-pathogen models. For these studies, intestinal epithelial cells, including T₈₄, Caco-2 or HT-29, were transfected with constructs encoding either *luc*, *CAT* or β -gal driven by the inducible interleukin-8 (*IL-8*) (-135 to -46 bp) promoter or the constitutively active simian virus 40 (*SV40*) promoter. Transfected cells were then infected with either enteropathogenic *E. coli* (EPEC) or *S. typhimurium*, which are important enteric pathogens, or stimulated with *IL-1 β* . Each of these has been shown to upregulate *IL-8* expression in intestinal epithelial cells (16,19). We present here the systematic analysis of the impact of bacterial pathogens on the reporter activity of *luc*, *CAT* and β -gal.

MATERIAL AND METHODS

Cell Culture

T₈₄ cells were a generous gift from Dr. Kim Barrett, University of California, San Diego. Passages 35–55 were used for these studies and were grown in a 1:1 (vol/vol) mixture of Dulbecco-Vogt modified Eagle's medium and Ham's F-12 (both from Life Technologies, Rockville, MD, USA) as described previously (15). Caco-2 cells were grown in high glucose DMEM medium supplemented with 10% FCS (25). HT29 cells (ATCC, Manassas, VA, USA) were grown in DMEM medium and 10% FCS.

Bacterial Strains and Infection

EPEC (wild-type strain, E2348/69) and *S. typhimurium* (14028; ATTC) were used. Overnight bacterial cultures, grown in LB broth, were diluted (1:33) in serum- and antibiotic-free tissue culture medium containing 0.5% mannose and grown at 37°C to the mid-log growth phase. Cells grown in 6-well plates were infected with 5×10^7 bacteria per well for a period of 3 or 5 h. This model system of enteric infection has been well characterized using functional assays (6,21,26). After infection, the medium was removed and cells were washed three times with PBS. Proteins were extracted with buffers specified by the manufacturer of each assay.

EPEC mutant strains used in these studies harbored deletion mutations of genes involved in either type III secretion or intimate attachment. Strains with deletions of *espA* (11), *espB* (5), *espD* (13) or *escN* (6) exhibit nonfunctional type III secretion. The translocated intimin receptor (Tir), delivered using type III secretion into the host cells, serves as a receptor (4,10,12) for the outer membrane EPEC protein, intimin (8). The binding of intimin to Tir allows EPEC to attach intimately to intestinal host cells. The *invG* deletion mutant strain of *Salmonella* is ineffective in type III secretion (9).

Wild-type EPEC (strain E2348/69) and mutants for Tir, intimin and *escN* were generous gifts from James Kaper (Center for Vaccine Development, University of Maryland, Baltimore, MD,

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USA). The *espA*, *espB*, *espD* mutants were kindly provided by Michael Donnenberg (Section of Infectious Diseases, University of Maryland). *S. typhimurium invG* was kindly provided by Cathy Lee (Harvard University Medical School, Boston, MA).

Plasmids

Reporter plasmids used for these studies included an IL-8 luc reporter construct, -135 to -46 (IL-8) luc (24) and an IL-8 CAT reporter construct, a generous gift from Naofumi Mukaida,

Cancer Research Institute, Kanazawa University, Japan (17). SV40-luc, SV40-CAT and SV40- β -gal plasmids are commercially available (Promega, Madison, WI, USA). Each plasmid was amplified in *E. coli* strain JM109 and purified using the Plasmid Maxi Kit according to the manufacturer's directions (Qiagen, Valencia, CA, USA).

Transfection

Cells were plated at a density of 4×10^5 cells/cm² in 6-well plates containing complete medium. After 24 h for

Caco-2 and HT29 and 72 h for T84, cells were co-transfected with 1 μ g reporter plasmid and 0.5 μ g SV40- β -gal using LIPOFECTAMINE[®] Plus (Life Technologies) and following the manufacturer's protocol. Cells were infected 48 h later with enteric pathogens as described earlier.

Reporter Gene Assay

Luc activity in the cell extract was determined using the luc enzyme assay system (Promega). The light intensity was measured using a scintillation counter programmed for chemiluminescence. CAT activity was determined from 50 μ g cell extract by the commercially available CAT enzyme assay system (Promega). Following xylene extraction, radioactivity was measured in a scintillation counter. β -gal activity was determined using the commercially available β -gal enzyme assay system (Promega).

Figure 1. Time course of altered luc activity in response to infection (A) or IL-1 β stimulation (B). Caco-2 cells transfected with the construct -135(IL-8) luc were infected with EPEC or *S. typhimurium* or stimulated with IL-1 β for 1, 3 and 5 h. The data represent the mean $\pm$ SD (n = 4-6; $P < 0.005$ for t = 0 versus 1, 3, or 5 h infection) of luc activity in 20 μ L cell extract.

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Reporter Activity in a Cell-free System

Enteric pathogens were grown in serum- and antibiotic-free tissue culture medium in the presence of different concentrations of recombinant luc (0.01, 0.1, 1.0 and 10 $\mu\text{g/mL}$; Promega) and in the presence and absence of protease inhibitors: 0.1 mg/mL PMSF, 10 $\mu\text{g/mL}$ leupeptin and 10 mg/mL aprotinin, 100 mM DTT or β -gal (3 U; Promega). After 3 h, luc and β -gal activity were determined as described earlier.

Western Blot

Bacteria were grown for 3 h in tissue culture medium containing 1.0 $\mu\text{g/mL}$ recombinant luc (rluc). Bacteria were separated from the supernatant by centrifugation at $2000\times g$ for 10 min. The supernatant was diluted with sample buffer (30 mM Tris, pH 6.7, 3% SDS, 15% glycerol) and boiled for 5 min. Equal volumes of the samples were separated by SDS-PAGE (8% acrylamide). Proteins were transferred to a nitrocellulose membrane, blocked in TBS (20 mM Tris, pH 7.5, 150 mM NaCl) and immunodetected with luc antibody (Cortex Biochem, San Leandro, CA, USA) using an alkaline phosphatase-conjugated secondary antibody. Color development was achieved with a nitroblue tetrazolium/5-bromo-4-chloro-indolyl phosphate premixed solution (Zymed Laboratories, South San Francisco, CA, USA).

Quantitation of IL-8

T₈₄ cells were grown on Transwells (Corning, Corning, NY, USA). Basolateral medium from cells infected with EPEC or *Salmonella* was collected after 4 h. IL-8 was quantified using a dual-antibody ELISA kit from R&D Systems (Minneapolis, MN, USA), following the manufacturer's protocol.

Statistical Analysis

All data are represented as the mean $\pm$ SD. Data comparisons were made with Student's *t* test. Differences were considered significant when *P* was less than or equal to 0.05.

RESULTS

Infection by Enteric Pathogens Decreases Luc Activity

The initial experiment determined whether luc, commonly employed as a sensitive reporter gene system, is suitable for studying gene regulation in intestinal epithelial cells infected with enteric pathogens. Caco-2 cells were transfected with a construct encoding the *luc* gene under the control of the IL-8 promoter and then infected with either EPEC or *S. typhimurium* for 3 h. In contrast to the induction of IL-8 protein expression after 4 h (control 22 ± 13 pg/mL, EPEC 110 ± 16 pg/mL, *Salmonella* 623 ± 55 pg/mL) a decrease in luc activity was observed. In Figure 1, time course studies show gradually diminishing luc activity during infection with enteric pathogens, while over the

same time period, treatment with the IL-1 β positive control progressively increased luc activity. The same phenomenon was seen in the other intestinal epithelial cells, T₈₄ and HT-29, suggesting a cell type-independent event (data not shown).

Decreased Luc Activity in Response to Enteric Infection is Promoter Independent

The observed decrease in luc activity in the face of increasing IL-8 protein expression could be explained by either aberrant repression, specific only to -135 bp of the IL-8 promoter, or to protein degradation that inactivated enzymatic activity. To exclude the possibility that bacterial infection leads to aberrant repression specific to the IL-8 promoter, we studied a constitutively active independent promoter, SV40,

Figure 2. Neither β -gal nor CAT activity decreases after infection with enteric pathogens. β -gal and CAT activity were determined in the Caco-2 cells transfected with SV40- β -gal, SV40-CAT and T₈₄ cells transfected with -135(IL-8)-CAT. Cells were infected for 3 h with EPEC; 20 μL cell extract for β -gal or 100 μL cell extract for CAT were used for analysis. Data represent the mean $\pm$ SD ($n = 4$; $P > 0.8$ for uninfected controls versus infected for β -gal and CAT for SV40 promoter; $P < 0.007$ for CAT activity in uninfected controls versus infected for IL-8 promoter).

cloned in front of the *luc* gene in the same system. Again, luc activity from T₈₄ cells transfected with SV40-luc and infected with either EPEC or *Salmonella* decreased after 3 h (EPEC, $46 \pm 37 \times 10^4$; *Salmonella*, $124 \pm 52 \times 10^4$ versus control, $182 \pm 60 \times 10^4$ light units) and was completely abrogated after 5 h (data not shown). These findings suggest that decreased luc activity in response to bacterial infection is promoter independent.

The effect of enteropathogenic infection on CAT and β -gal reporter genes driven by the SV40 promoter was tested next. Neither CAT activity nor β -gal activity was altered during infection (Figure 2). In fact, CAT expression under the control of the IL-8 promoter was inducible (Figure 2). These findings demonstrate that this response is specific for luc because the other reporter assays were not affected.

Enteric Bacterial Infection Degrades Luc In Vitro

We next examined whether infection by enteric pathogens interferes with luc activity or degrades the luc protein. For these studies, we used a cell-free system. Bacteria were grown in cell culture medium to which recombinant luc had been added. After 3 h of incubation with either EPEC or *Salmonella*, luc activity from the culture medium was nearly ablated (Figure 3). Interestingly, the activity of this enzyme was partially protected by the addition of protease inhibitors (rluc alone $81.0 \pm 5.5 \times 10^5$; rluc $\pm$ EPEC $0.2 \pm 0.1 \times 10^5$; rluc + EPEC + inhibitors $23.0 \pm 4.5 \times 10^5$ light units). Bacteria did not interfere with the luc assay itself as the measurement of activity immediately following the addition of EPEC was no different from that in the absence of EPEC ($277 \pm 70 \times 10^5$ and $242 \pm 40 \times 10^5$ light units for rluc alone versus rluc with EPEC, respectively; $n = 6$; $P = 0.9$). In contrast, β -gal activity was not altered by EPEC (data not shown). These data suggest that bacteria degrade the luc protein.

To test this possibility, immunoblots using antibodies specific for luc were performed on samples containing 100 μ g/mL rluc in the absence and presence of bacteria for 3 h. Figure 3 shows a decrease in both the amount and the mol-

ecular weight of luc protein following incubation with EPEC and *Salmonella*. Intracellular protection of luc activity by extracellular proteases could not be demonstrated (data not shown), which suggests an intracellular host or bacterial enzyme is responsible. In addition, the amount of luc expressed by transfected cells was insufficient for detection by immunoblot analysis.

Functional Type III Secretion Required for Alteration in Luc Activity

These results pose the question of whether the degradation of luc is caused by general bacterial biology or specific bacterial virulence factors such as type III secretion. To address this question, specific mutant strains were used. As

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shown in the Table 1, EPEC strains that harbor a mutation affecting type III secretion or intimate attachment failed to alter luc activity. Similar results were obtained using a *Salmonella* mutant, *invG*, with defective type III secretion. These findings suggest that structural proteins comprising the type III secretory apparatus or, more likely, effector molecules delivered by this structure, may be directly involved in luc degradation or they may induce a host cell factor that results in degradation.

DISCUSSION

The use of reporter gene constructs is essential to characterize alterations in gene transcription. However, the impact of enteric bacterial pathogens on these models has not been examined. We therefore tested the effect of two pathogens with different virulence mechanisms, but both expressing a type III secretory system on commonly employed reporter gene systems. Our findings show that CAT and β -gal are stable in the presence of pathogenic bacteria, but luc activity progressively diminishes following infection. Induction with IL-1 β increased luc activity dramatically, demonstrating that this reporter system was functional in these cells. Our data showing decreasing luc activity and molecular weight suggest that this is likely the result of degradation of the luc protein.

Luciferase is the reporter most commonly used to study transcriptional regulation in eukaryotic cells, primarily because of its sensitivity; that is 10- to 1000-fold higher than CAT and its lack of interference with cellular functions. Despite its sensitivity as a reporter, luc has a short half-life of approximately 3 h and is unstable in vivo compared with other reporter proteins (22). Although there are no regions in the luc protein that suggest it may belong to the family of short-lived proteins (22), there is a strong suggestion that luc instability arises from its sensitivity to proteolysis both in vivo and in vitro (22). Reactive oxygen intermediates have been shown to increase the concentration of intracellular Ca²⁺ (19) and to activate proteolytic processes in cells (23). Previous studies from our laboratory suggest that

Table 1. Effect of Mutations in Type III Secretion on Luc Activity at 3 h after Infection

Pathogen	Luc Activity (% of Control)
Control	100
EPEC (wild-type)	1.5 $\pm$ 5.7
Δ escN	109 $\pm$ 30
Δ espA	159 $\pm$ 20
Δ espB	118 $\pm$ 13
Δ espD	99 $\pm$ 23
Δ eaeA (intimin)	119 $\pm$ 21
Δ tir	119 $\pm$ 36
<i>S. typhimurium</i> (wild-type)	48 $\pm$ 4.5
<i>invG</i>	145 $\pm$ 26

EPEC activates these intermediates in intestinal epithelial cells (18). Together, these data lead us to speculate that during EPEC infection there is activation of proteolytic processes that degrade luc but not β -gal or CAT.

Although gram-negative bacteria produce proteases that may be involved in pathogenicity, a functional type III

secretion was found to be required for luc degradation. It is possible that a bacterial protease enters the cell by the type III secretory apparatus. Alternatively, other bacterial products may induce a host cell factor that degrades luc. The other commonly used reporter genes, CAT and β -gal, were not affected in this system. Unfortunately, the

Figure 3. EPEC and *Salmonella* degrade luc in a cell-free system. The activity of rLuc was measured after dilution in cell culture medium in the absence and presence of pathogens for 3 h. EPEC (rLuc EPEC) and *Salmonella* (rLuc *Salmonella*) significantly decreased luc activity. Immunoblot analysis (inset) revealed decreased luc concentration and molecular weight in the presence of both pathogens.

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CAT reporter system is relatively insensitive in that it generates only a few-fold increase in enzyme activity. The same disadvantage exists for the standard β -gal assay. Fortunately, the new β -gal light-sensitive assay is more sensitive and generates increases in activity approaching that seen in other systems with luc (3). Based on the data presented here, in model systems exploring the impact of pathogenic bacterial infection (especially those using type III secretion on host epithelial gene expression), we recommend that β -gal (using the light sensitive assay) or CAT be employed as reporter genes.

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Suzana D. Savkovic, Athanasia Koutsouris, Gary Wu¹ and Gail Hecht
*University of Illinois
 West Side VA Medical Center
 Chicago, IL
¹University of Pennsylvania
 School of Medicine
 Philadelphia, PA, USA*