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Virus-Mediated Expression of Firefly Luciferase in the Parasitic Protozoan *Giardia lamblia*

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***Giardia lamblia*, a prevalent human pathogen and one of the lineages that branched earliest from prokaryotes, can be infected with a double-stranded RNA virus, giardiavirus (GLV). The 6,277-bp viral genome has been previously cloned (A. L. Wang, H.-M. Yang, K. A. Shen, and C. C. Wang, Proc. Natl. Acad. Sci. USA 90:8595–8599, 1993; C.-H. Wu, C. C. Wang, H. M. Yang, and A. L. Wang, Gene, in press) and was converted to a transfection vector for *G. lamblia* in the present study. By flanking the firefly luciferase gene with the 5′ and 3′ untranslated regions (UTRs) of the GLV genome, transcript of the construct was synthesized in vitro with T7 polymerase and used to transfect *G. lamblia* WB trophozoites already infected with GLV (WBI). Optimal electroporation conditions used for the transfection were set at 1,000 V/cm and 500 μF, which resulted in expression of significant luciferase activity up to 120 h after electroporation. Furthermore, the mRNA and the antisense RNA of the luciferase gene were both detected by reverse transcription and PCR from 6 to 120 h postelectroporation, whereas no antisense RNA of luciferase was observed in the electroporated virus-free *Giardia* WB trophozoites. The mRNA of luciferase was detectable in the virus-free trophozoites by reverse transcription and PCR only up to 20 h after the electroporation, indicating that the introduced mRNA was replicated only by the viral RNA-dependent RNA polymerase inside the WBI cells. This expression of luciferase was dependent on the presence of UTRs on both ends of the viral genome transcript, including a putative packaging site that was apparently indispensable for luciferase expression. This is the first time that a viral vector in the form of mRNA UTRs has been successfully used in transfecting a protozoan.**

Giardia lamblia, an anaerobic, flagellated protozoan, is a common inhabitant of the upper small intestines of humans and other mammals. It lacks mitochondria, a normal endoplasmic reticulum, and Golgi bodies (7) and possesses the smallest rRNAs yet reported for any eukaryotic organism (2). The sequence of the small-subunit rRNA has retained many of the features that may have been present in the common ancestor of eukaryotes and prokaryotes, such as its 3′-terminal sequence that resembles the Shine-Dalgarno binding site for bacterial mRNA (25). A sequence homologous to the Shine-Dalgarno element was identified upstream from a *G. lamblia* surface protein gene (11). *G. lamblia* is apparently the best preserved among eukaryotes and the lineage that branched earliest from the prokaryotes. Thus far, our understanding of *Giardia* genetics has been obtained by analysis of the few *Giardia* genes that have been cloned. An in-depth understanding of the regulations of gene expression in *G. lamblia* would increase our knowledge of the gap between prokaryotes and eukaryotes during evolution.

One technical hindrance to a thorough investigation into this interesting subject lies in our inability to perform genetic studies with *Giardia* organisms. The lack of a defined growth medium with nutritional auxotrophs or drug-resistant *Giardia* mutants has made classic genetic analysis difficult. However, successful transient (1, 4, 12, 16, 21, 22, 26) as well as stable (14, 15, 17) expression of extrachromosomal DNA by homologous recombinations has been reported for other protozoa. To the best of our knowledge, there has not yet been any effective means of performing genetic transformation or transfection for *Giardia* organisms. Viruses would be particularly helpful in this aspect because they can function as vectors of transfection (20). Furthermore, a genetically engineered virus carrying a lethal gene to the infected host cell may be a killer of the cell, which in the case of *Giardia* cells could become a new way of controlling giardiasis.

In 1985, we came across the abundant presence of a 7-kb linear double-stranded RNA (dsRNA) species in the trophozoites of a *G. lamblia* strain, Portland I (28), which turned out to be the genome of a nonsegmented, dsRNA virus that was named the *G. lamblia* virus (GLV) thereafter. The virus belongs to the family *Totiviridae* and is assigned to the genus *Giardiavirus* (29). Two aspects of GLV distinguish it from the rest of the totiviruses: (i) its ability to infect *Giardia* trophozoites (19) and (ii) the fact that a single-stranded transcript of the GLV dsRNA genome can be isolated and introduced into *Giardia* trophozoites by electroporation to initiate GLV infection and replication (10). These features make GLV a promising candidate for conversion into a transfection vector for *Giardia* organisms.

As a first step toward developing a vector for *G. lamblia*, a 6,277-bp full-length cDNA clone of the GLV dsRNA genome was recently pieced together from partial cDNA clones (30, 33). The GLV genome contains two large open reading frames (ORFs) that overlap by 220 nucleotides (nt) and are separated by a −1 frameshift (30). The coding region is flanked by a 367-bp 5′ untranslated region (5′ UTR) and a 300-bp 3′ UTR. The 5′ UTR of the positive-strand viral RNA contains five separate Shine-Dalgarno-like elements (30), some of which may be required for translating the RNA inside the infected *Giardia* trophozoite. The same 5′ UTR may also contain the sequence complementary to the promoter at the 3′ end of the negative-strand RNA for the viral RNA-dependent RNA polymerase to perform its transcriptase function. According to our

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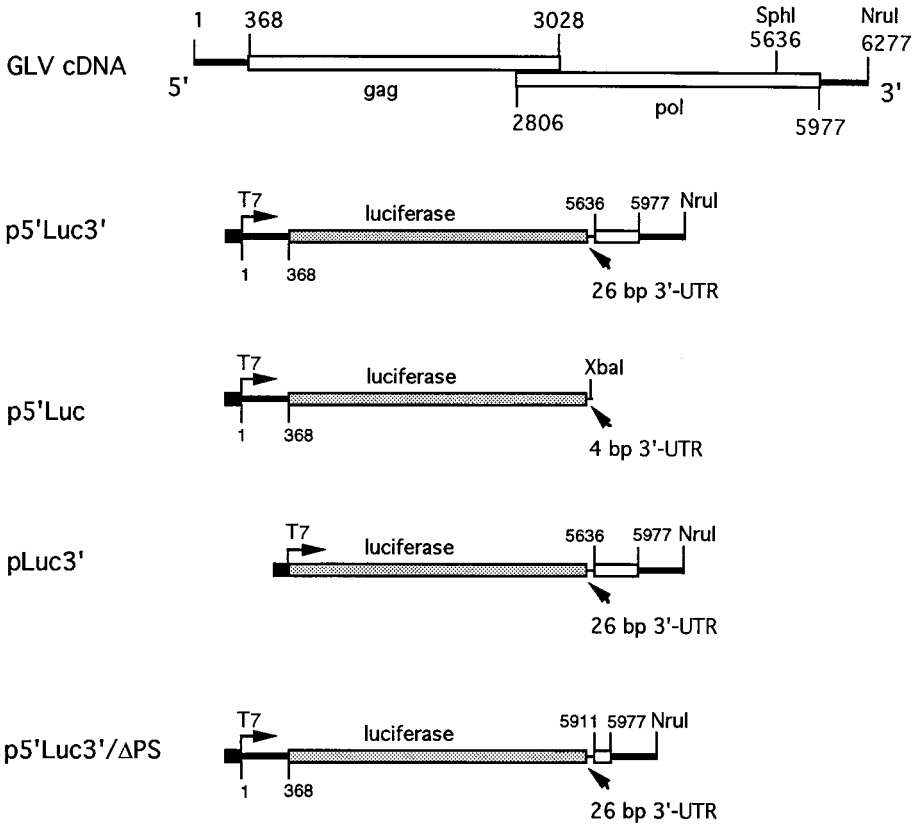

FIG. 1. Schematic diagrams of the cDNAs encoding the GLV-luciferase chimeric RNAs for transfection of *G. lamblia* (not to scale). The cassettes, containing the luciferase ORF (light stippling) flanked by the GLV 5′ UTR (thick line) on the upstream end and the 26-bp 3′ UTR from the luciferase gene (thin line) plus 341 bp of the 3′ terminus of GLV ORF2 (open box) and the GLV 3′ UTR (thick line) on the downstream end, were located downstream from the T7 RNA promoter (black box). The restriction endonucleases, *Nru*I and *Xba*I, for linearization of plasmid DNA are indicated. Numbering of nucleotides is adopted from the GLV genome (30, 33).

current knowledge of the mechanism of propagation of totiviruses (32), the positive RNA strand must first be packaged into the viral procapsid before it can be replicated to generate the negative-strand RNA to form the dsRNA. This key process demands that a specific site(s) in the viral positive-strand RNA be recognized and bound by the viral RNA-dependent RNA polymerase. The only totivirus whose RNA packaging site has been identified in the positive-strand RNA is the dsRNA virus of *Saccharomyces cerevisiae* (ScV L-A) (24, 32). The site consists of a stem-loop located about 400 nt upstream from the 3′ terminus of the positive strand. The stem structure, the specific sequence of the loop, and the budge protruding from the 5′ side of the stem-loop are all necessary for binding the RNA to the viral RNA-dependent RNA polymerase to initiate the process of packaging (5, 8, 24). There is an additional, yet smaller stem-loop structure at the very 3′ end of the ScV L-A positive-strand RNA that is required for the initiation of synthesis of the negative-strand RNA (6, 9).

In the present investigation, we constructed a cDNA clone consisting of the firefly luciferase gene flanked by the 5′ and 3′ UTRs of GLV cDNA. The in vitro transcript of the construct was introduced by electroporation into *G. lamblia* WB trophozoites already infected with GLV (WBI). The results indicated that the mRNA thus introduced into the cells was replicated and transcribed to produce the antisense RNA as well as more mRNA up to 120 h after electroporation. Substantial luciferase activity was also observed in the transfected cells up to 120 h following electroporation. A reporter gene has therefore been successfully introduced into and expressed in *G. lamblia*.

MATERIALS AND METHODS

Cell culture and virus infection. *G. lamblia* WB clone 1 was obtained from R. L. Berens, University of Colorado. It is GLV free and susceptible to GLV infection (19). Culture conditions have been described previously (28). Unless otherwise specified, *G. lamblia* trophozoites were infected with GLV at a multiplicity of infection of 1,000 and incubated at 37°C. The cells were harvested after 20 h and used for the electroporation experiments.

Construction of GLV-Luc recombinant plasmids. The sequence of the full-length GLV genome has GenBank accession number L13218. By site-directed mutagenesis with an in vitro mutagenesis kit (Amersham), the G residue at position 367 of GLV cDNA (pGEMGLV [30, 34]) was changed to C, thus converting the original sequence CCGATGG to CCCATGG to create an *Nco*I restriction site between nt 366 and 371 and allowing a clean separation between the 5′ UTR and ORF1. This mutant construct, pGLVNcoI, was digested with *Sph*I, filled in with T4 DNA polymerase, and digested with *Nco*I to remove the insert nt 368 to 5635. Plasmid pSP-Luc+ (Promega) carrying the firefly luciferase gene from nt 45 to 1694 was digested with *Xho*I and filled in with T4 DNA polymerase to generate a blunted 3′ end at nt 1723 and digested with *Nco*I to free a DNA fragment from nt 45 to 1723. This fragment was ligated to the *Nco*I-linearized pGLVNcoI to produce a new construct, p5′Luc3′ (Fig. 1), containing the entire structural gene of luciferase flanked upstream by the 367-bp 5′ UTR of the GLV genome (the sequence in the junction was converted from the mutant CCCATGG to the original CCGATGG by site-directed mutagenesis) and downstream by the 26-bp luciferase 3′ UTR from pSP-Luc+ and followed by nt 5636 to 6277 of the GLV cDNA consisting of the 341-bp 3′ domain of ORF2 and the entire 300-bp GLV 3′ UTR.

Construct p5′Luc was derived from p5′Luc3′ by digestion with *Sma*I and *Eco*RV, followed by self-ligation. It consists of the entire luciferase ORF flanked by the 367-bp GLV 5′ UTR at the 5′ side and by 4 bp of the original pSP-Luc+ multiple cloning site at the 3′ end. Similarly, pLuc3′ was derived from p5′Luc3′ by digestion with *Spe*I and filling in with T4 DNA polymerase to generate a blunted 5′ end, followed by *Pst*I digestion to free a DNA fragment from nt 367 to 2703 which consists of the entire ORF of the firefly luciferase gene and the same 3′ UTR from p5′Luc3′. This fragment was ligated to pGEM-5Zf(+) (Pro-

mega), which had been linearized with *Apa*I, filled in with T4 DNA polymerase, and digested with *Pst*I, to produce pLuc3′.

p5′Luc3′/ΔPS was constructed by deletion of nt 5636 to 5911 of the GLV cDNA from p5′Luc3′ by digestion with *Bsm*I followed by self-ligation (Fig. 1). This construct lacks the putative packaging site (nt 5870 to 5912) in the GLV positive-strand RNA (see Results).

In vitro synthesis of GLV-Luc chimeric mRNAs. The plasmids were linearized (*Nru*I for p5′Luc3′, p5′Luc3′/ΔPS, and pLuc3′ and *Xba*I for p5′Luc) at the 3′ end of each cloned cDNA. Runoff transcription reactions with T7 RNA polymerase were performed as described previously (34). The synthesized RNA was purified by phenol extraction and isopropanol precipitation in the presence of 1 M ammonium acetate and dissolved in diethyl pyrocarbonate-treated water. The purified RNA was analyzed by 1.0% agarose-formaldehyde gel electrophoresis and used in the electroporation experiments.

Electroporation. Log-phase *G. lamblia* trophozoites, newly infected with GLV, were incubated on ice for 30 min in the *Giardia* culture medium, centrifuged at 750 × *g* for 10 min, and washed twice with cold phosphate-buffered saline (PBS) and once in complete cytomix buffer [10 mM K_2HPO_4-KH_2PO_4 (pH 7.6), 120 mM KCl, 0.15 mM $CaCl_2$, 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 2 mM ethylene glycol-bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 5 mM $MgCl_2$ 2 mM ATP, 4 mM glutathione] (27). These washed trophozoites were resuspended in complete cytomix buffer at a concentration of 1×10^7 to 2×10^7 cells per ml. A 0.8-ml sample was placed into a 0.4-cm electroporation cuvette (Bio-Rad) and kept on ice. Fifty to 100 μg of the in vitro-synthesized RNA mixed with yeast tRNA (Gibco BRL) to a total of 200 μg of RNA was added to diethyl pyrocarbonate-treated water containing 200 U of RNase inhibitors (Promega) to a total volume of 40 μl. The RNA solution was mixed with 0.8 ml of the suspension of trophozoites immediately prior to electroporation. Standard electroporation conditions were set at 1,000 V/cm and 500 μF with a Gene Pulser augmented without a capacitance extender (Bio-Rad), resulting in an average time constant of 6.4 ms. The rate of survival among the electroporated trophozoites was estimated to be 30 to 50%. Cuvettes were placed on ice afterwards for an additional 15 min, after which the electroporated trophozoites were added to 13 ml of the *Giardia* culture medium containing penicillin and streptomycin sulfate in capped glass tubes. The in vitro cultivation of electroporated trophozoites was carried out at 37°C. There was usually an initial lag in cell growth extending up to 8 h before the trophozoites resumed a normal growth rate of 18 h per generation. Serial passages of the in vitro culture were carried out every 2 days to maintain a continuous logarithmic cell growth.

Detection of mRNA and antisense RNA by RT-PCR. The electroporated *G. lamblia* trophozoites (~14 ml at an estimated density of 3×10^4 viable cells per ml) were pelleted, washed twice in PBS (both Mg^{2+} and Ca^{2+} free), and suspended in 0.1 mM sodium acetate (pH 4.8). Total RNA was extracted as previously described (28) and used as templates in reverse transcription-PCR (RT-PCR) after a digestion with RNase-free DNase I (Boehringer Mannheim). Two primers were used in the RT-PCRs; primer 1 (5′-TCCTCTGGATCTACTGG-3′) represents the sequence of the sense strand from nt 591 to 608, whereas primer 2 (5′-GGAAGCGTTTTGCAACC-3′) is reversely complementary to nt 977 to 994 of the sense strand of the luciferase gene. Thus, when luciferase mRNA was monitored, primer 2 was used to prime the RT reaction and, following denaturation of the reverse transcriptase at 95°C, primer 1 was added to complete the PCR. When the antisense RNA of the luciferase gene was examined, primer 1 was used to initiate the RT reaction whereas primer 2 was used to finish the PCR. By the recommendations of the manufacturer (Invitrogen) with minor modifications, 10 μg of the purified RNA in 12 μl of diethyl pyrocarbonate-treated water was mixed with 1 μl of undiluted primer and heated at 65°C for 10 min in the presence of 4 mM sodium PP_i to eliminate the secondary structure of RNA. Five units of avian myeloblastosis virus reverse transcriptase was added, and the mixture was incubated at 42°C for 60 min. The sample was then incubated at 95°C for 3 min to denature the RNA-cDNA hybrids, centrifuged briefly, and placed immediately on ice. This cDNA was then amplified by using *Taq* DNA polymerase (Gibco BRL) for 35 cycles of 1 min at 95°C, 40 s at 52°C, and 1 min at 72°C. The amplified DNA was resolved by 1.0% agarose gel electrophoresis and visualized by ethidium bromide staining.

Luciferase activity assay. The *Giardia* trophozoites (3×10^6 cells) were examined microscopically, pelleted, and suspended in 50 μl of luciferase lysis buffer (100 mM K_2HPO_4-KH_2PO_4 [pH 7.8], 1 mM dithiothreitol, 2 mM EDTA, 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 −70°C for more than 1 h and thawed on ice for 10 min. After warming to room temperature, 10 μl of the cell lysate was assayed in 100 μl of luciferase assay reagent (30 mM Tris-HCl [pH 7.8], 3 mM ATP, 15 mM $MgSO_4$, 10 mM dithiothreitol, 0.2% Triton X-100) by injecting 100 μl of 0.5 mM D-luciferin (Sigma) to initiate the reaction according to the manufacturer's instructions. The data thus obtained were in relative light units as measured with a luminometer (Monolight 2010; Analytical Luminescence Laboratory, San Diego, Calif.).

RESULTS

Tentative assignment of the packaging and initiation sites in GLV dsRNA. There are 367- and 300-bp UTRs at the 5′ and 3′ termini, respectively, of the sense strand of the GLV dsRNA genome (30, 33). With the aid of the computer program Mulford (35), we have tentatively identified two stem-loops in the 3′-terminal region of the GLV positive-strand RNA, 400 and 33 nt upstream from the 3′ end (Fig. 2). The similarities between these two stem-loops and those found in the ScV L-A positive-strand RNA are striking (5, 6, 8, 9). In addition to their similar locations and sizes, important features such as the direct repeats and the protruding A in the stem of the ScV L-A packaging site are all present in GLV. They could very well be the packaging site and the replication site in GLV. Additionally, we also tentatively identified a unique stem-loop at the very 3′ end of the GLV negative-strand RNA that is not found in ScV L-A, which could be analogous to the cloverleaf structure at the 5′ UTR of poliovirus and could constitute the transcription initiation site in GLV (Fig. 2). The tentative identification of the packaging site was supported by experimental evidence in our subsequent studies (see below).

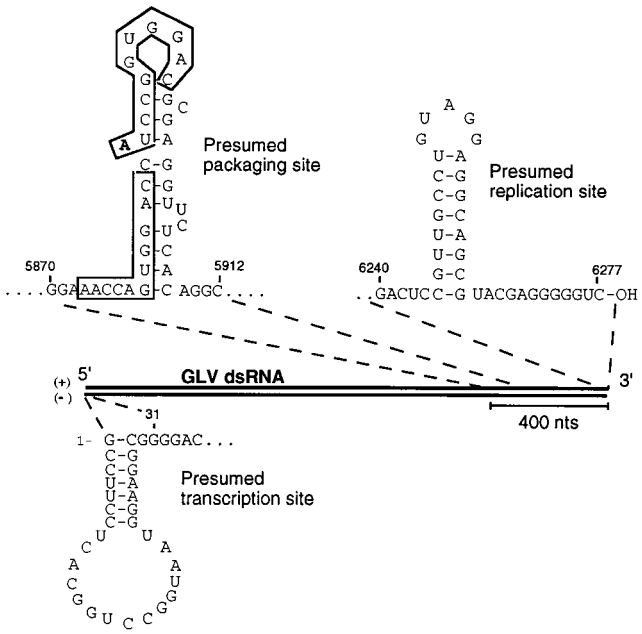

FIG. 2. Predicted secondary structure in the 5′- and 3′-terminal regions of GLV genomic RNA. Sites that may be involved in transcription initiation, packaging (for polymerase binding to the RNA template), and replication initiation are marked. Boxed sequences in the putative packaging site and the protruding A are analogous to the direct repeats and the unpaired A found in ScV L-A and M molecules (5, 6, 8, 9).

Luciferase expression in transfected *G. lamblia*. The GLV-Luc chimeric RNAs transcribed in vitro from the constructs presented in Fig. 1 were electroporated into GLV-infected *G. lamblia* (WBI) trophozoites. An RT-PCR assay was used to monitor the presence of the mRNA and antisense RNA of the luciferase gene (see Materials and Methods). Figure 3A shows that in the WBI trophozoites transfected with the transcript of p5′Luc3′, an ~400-bp DNA fragment, representing most likely nt 591 to 994 of the luciferase gene, was amplified with either the sense or the antisense primer in the RT-PCR, suggesting that both the positive and negative strands of the transcripts of the luciferase gene were present in the electroporated *Giardia* trophozoites. These transcripts persisted in the transfected trophozoites up to 5 days after the electroporation.

To verify whether the detected antisense RNA in electroporated WBI cells was newly replicated from the mRNA intro-

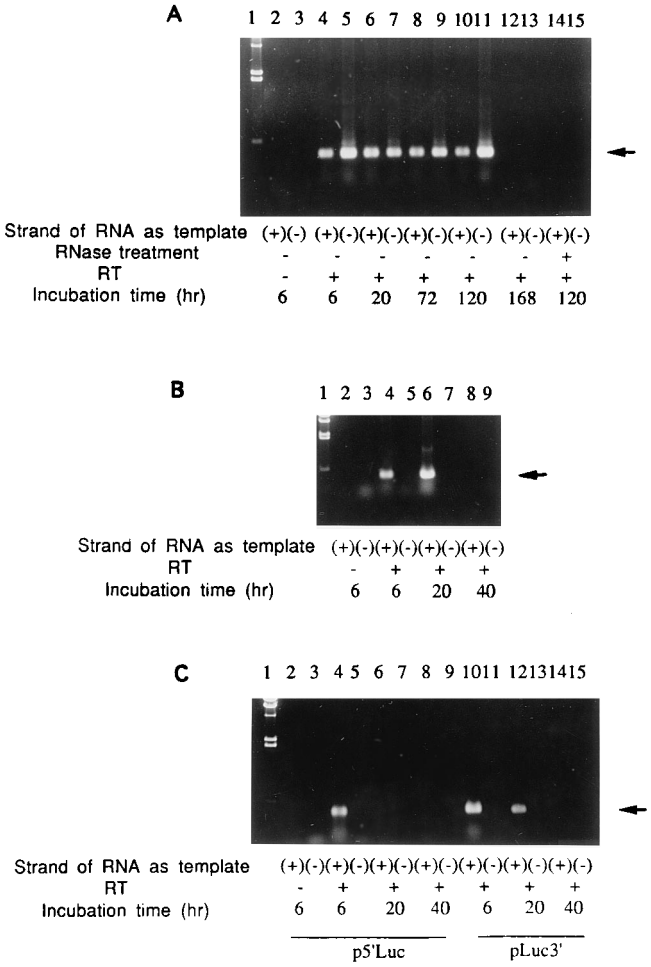

FIG. 3. Detection of the luciferase mRNA and antisense RNA by RT-PCR in transfected *Giardia* trophozoites. RNAs transcribed in vitro from various cDNA constructs were introduced into *G. lamblia* trophozoites by electroporation. Total RNA, extracted from the electroporated trophozoites at various time points after electroporation, was monitored for luciferase mRNA and antisense RNA by RT-PCR as described in Materials and Methods. The amplified DNA fragments (~400 bp) are indicated by arrows. (A) p5′Luc3′ transcript transfection of GLV-infected *Giardia* WB trophozoites; (B) p5′Luc3′ transcript transfection of uninfected *Giardia* WB trophozoites; (C) p5′Luc or pLuc3′ transcript transfection of GLV-infected *Giardia* WB trophozoites. Lanes 1, lambda phage DNA *Hin*dIII digests.

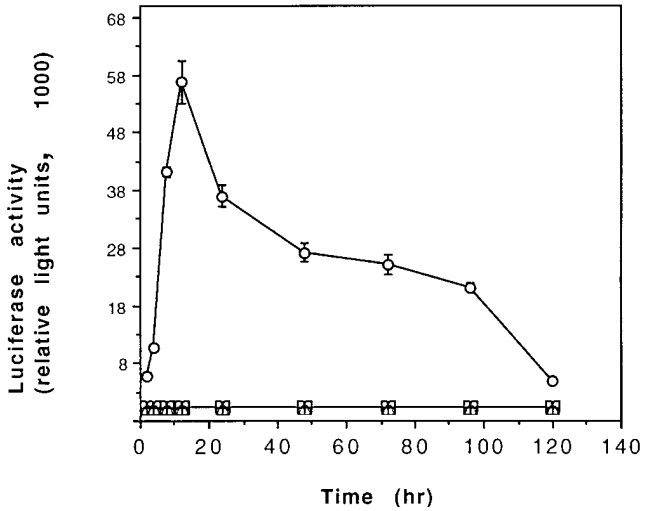

FIG. 4. Luciferase activity in transfected *Giardia* trophozoites (3×10^6 cells). ○, p5′Luc3′ transcript transfecting GLV-infected *Giardia* WBI trophozoites; □, p5′Luc3′ transcript transfecting uninfected *Giardia* WB trophozoites; ▲ and ✣, p5′Luc and pLuc3′ transcripts transfecting GLV-infected *Giardia* WBI trophozoites; ◆, p5′Luc3′/ΔPS transcript transfecting GLV-infected *Giardia* WBI trophozoites.

duced by electroporation or from contaminating plasmid DNA, total RNAs extracted from the electroporated WBI trophozoites were first treated with RNase A (Boehringer Mannheim) prior to RT-PCR. No DNA fragment of the expected size of ~400 bp was observed with the subsequent RT-PCR (Fig. 3A, lanes 14 and 15). Similarly, no detectable DNA products were obtained when RT-PCR was replaced by PCR (Fig. 3A, lanes 2, 3), suggesting that the RT-PCR product was indeed derived from the RNA template. As part of the established protocol, we routinely treat the in vitro transcript with RNase-free DNase I and examine it by PCR to ensure the absence of contaminating plasmid DNA prior to electroporation. Thus, the data shown in Fig. 3A strongly suggest that we have successfully introduced mRNA into the GLV-infected *Giardia* trophozoites and that it was replicated to produce the negative-strand RNA inside the trophozoites thereafter.

When the *G. lamblia* WB trophozoites were transfected with the transcript from p5′Luc3′ without prior GLV infection, data generated by RT-PCR (Fig. 3B) indicate that, in contrast to what was observed with WBI trophozoites, only the sense-strand RNA of luciferase was detectable for up to 20 h in the cells but vanished by the 40th hour (Fig. 3B, lane 8), suggesting that the RNA molecules introduced into the cells did not replicate and survived only up to 20 h. Thus, the replication of luciferase mRNA should require the presence of GLV.

As the luciferase mRNA flanked by the 5′- and 3′-UTR sequences from GLV persists in the transfected trophozoites for 5 days, it is expected that the mRNA may be translated by the host cell to produce luciferase. Time samples of p5′Luc3′ transcript-transfected *Giardia* WBI trophozoites (3×10^6 cells) were collected each in 50 μl of luciferase lysis buffer. The results, presented in Fig. 4, demonstrate a rapid rise of luciferase activity to a significantly high level of 60,000 relative light units in 3×10^6 trophozoites and a peak at about 18 h following electroporation, indicating that the transcript from p5′ Luc3′ can be translated efficiently by the protein-synthesizing machinery in the transfected *G. lamblia*. The activity declined gradually over the next 4 to 5 days of in vitro cultivation of the transfected trophozoites, agreeing well with the duration of the presence of luciferase mRNA in the same transfected cells (Fig. 3A). In contrast, only background level luciferase activity was detected in the transfected WB trophozoites (Fig. 4), suggesting that the expression of luciferase activity is also dependent on the presence of GLV.

Requirement of the GLV UTR sequences for luciferase expression in *G. lamblia*. To determine if any of the GLV genomic sequences included in the construct p5′Luc3′ are required for the RNA replication and the expression of luciferase in transfected, GLV-infected *Giardia* trophozoites, two additional constructs, p5′Luc and pLuc3′, lacking the 3′ or 5′ UTR, respectively, were tested in the electroporation experiments. The data obtained from subsequent RT-PCR (Fig. 3C) indicate that (i) for p5′Luc, only the sense-strand RNA was present in the transfected cells up to 6 h postelectroporation and (ii) the same was true with the transcript from pLuc3′ except that it survived in the cells a bit longer, up to 20 h postelectroporation. Both the 5′ UTR and the 3′-terminal region of the sense strand of the GLV dsRNA are thus necessary for the synthesis of the

antisense RNA. The shorter survival of the p5′Luc transcript, compared with that of the pLuc3′ transcript, may suggest that the sense RNA becomes less stable without its 3′ end. Meanwhile, only background level luciferase activity was detected in the p5′Luc or pLuc3′ transcript-transfected WBI trophozoites, suggesting no expression of luciferase (Fig. 4).

When the in vitro transcript from p5′Luc3′/ΔPS was used in the transfection of WBI trophozoites, no appreciable luciferase activity could be detected in the cells thereafter (Fig. 4). Since p5′Luc3′/ΔPS differs from p5′Luc3′ only by a deletion of nt 5636 to 5911 of the GLV gene, this 275-nt stretch in the chimeric mRNA must be essential for replication of the mRNA and/or the translation of the mRNA. This 275-nt fragment in the mRNA consists of the postulated packaging site for GLV presented in Fig. 2. The experimental data thus lend support to our postulation on the existence of this packaging site and its essential role in initiating the replication of the mRNA (see Discussion).

DISCUSSION

An in vitro transcript of the firefly luciferase gene flanked with the UTRs of GLV cDNA was introduced into GLV-infected *Giardia* trophozoites by electroporation, resulting in the synthesis of both the mRNA and the antisense RNA of luciferase, as well as the expression of luciferase activity, up to 5 days after the transfection. This is the first time, as far as we are aware, that transfection and expression of a foreign gene have been successfully carried out in *G. lamblia* trophozoites, that a viral UTR transcript has been used to express a reporter gene in a protozoan, and that the transcript of a totivirus dsRNA genome has been converted to a transfection vector to express a foreign gene in the host cell. In a similar study carried out previously (13), *S. cerevisiae* expressing brome mosaic virus RNA replication genes 1a and 2a was found to support RNA-dependent replication and transcription of the viral RNA3 derivatives containing a reporter gene subsequently maintained as persistent RNA episomes.

The successful transfection of *G. lamblia* has several prerequisites. It is necessary that the *G. lamblia* trophozoite be infected with GLV in order to serve as a suitable host. There are at least two possible mechanisms involved in expressing the luciferase activity in the transfected parasites. One possibility is that the luciferase mRNA introduced into the cells is first recognized and packaged into the GLV procapsid and then replicated by the GLV RNA-dependent RNA polymerase to form the dsRNA within the viral capsid (31). The *gag-pol*-like protein inside the particle may then transcribe the dsRNA template to synthesize and extrude the mRNA from the viral particle into the cytoplasm. The mRNA is then recognized by the ribosomes in *G. lamblia* and subsequently translated by the parasite translation machinery. Thus, by this working hypothesis, the transfecting luciferase mRNA must contain signals in the flanking regions for viral packaging, replication, transcription, and *G. lamblia* ribosome binding for successful translation. Since the in vitro transcripts from p5′Luc and pLuc3′, which lack either the 3′-terminal region or the 5′ UTR from GLV, both fail to produce the antisense RNA and luciferase activity in the transfected WBI trophozoites, both the 5′ UTR and the 3′-terminal region are apparently involved in expressing the luciferase. Furthermore, the negative outcome from transfecting the WBI trophozoites with the transcript from the mutant construct p5′Luc/ΔPS, in which the putative packaging site (Fig. 2) in the sense transcript of p5′Luc3′ had been deleted from the 3′-terminal region, is consistent with the working hypothesis and supportive of the location of our tentatively identified GLV packaging site. The much-extended presence of the luciferase mRNA in WBI trophozoites of up to 120 h compared with that in WB of only 20 h provides the evidence suggesting de novo synthesis of luciferase mRNA only in the transfected WBI trophozoites. However, an alternative explanation could be that GLV is capable of decapping the cellular mRNA in the infected *G. lamblia* trophozoites. Thus, the decapped cellular mRNA may compete with the uncapped luciferase mRNA for degradation by the cellular exo-RNase specific for uncapped mRNA, thus prolonging the half-life of the luciferase mRNA. This cellular exo-RNase, which has been identified in *S. cerevisiae* (SKI1/XRN1), is known to lose its effectiveness in degrading ScV L-A uncapped positive-strand RNA, because ScV L-A coat protein binds m^7GMP from 5′ capped cellular mRNA and creates decapped cellular mRNA in the infected *S. cerevisiae* (18). This hypothesis, however, cannot explain the appearance of the negative-strand RNA of luciferase in transfected *G. lamblia*, which may still have to rely on viral packaging and RNA replication.

The relatively short life span of pLuc3′ mRNA and the absence of antisense RNA of luciferase in the pLuc3′-transfected WBI trophozoites differ somewhat from our anticipation that only the 3′-terminal region in the sense RNA would be needed for synthesizing the antisense RNA. The observed survival time of 20 h for pLuc3′ mRNA, compared with that of 20 h for the nonreplicating p5′Luc3′ mRNA in uninfected WB cells, strongly suggests that transcription of new chimeric RNA did not take place without the GLV 5′ UTR. A stem-loop similar to that at the 3′ terminus of the GLV negative-strand RNA shown in Fig. 2 is also present in the complementary sequence at the 5′ terminus of the sense-strand RNA, resulting in a hammerhead structure, not unlike the cloverleaf in poliovirus (23), at one end of the GLV genome. The 5′ UTRs in some of the other RNA viruses, such as picornavirus and poliovirus, have been shown to be essential for viral RNA synthesis (3, 23), because of a requirement of compatible interactions between the cloverleaf 5′ UTR and the coding and/or 3′ noncoding regions of the genome for efficient replication (23). Recently, Borman et al. provided direct evidence showing that sequences essential for viral RNA synthesis are located in the 3′ region of the poliovirus internal ribosome entry segment at the 5′ UTR and predicted that sequestration of the internal ribosome entry segment could block the replication of the viral RNA at the level of negative-strand synthesis (3). We are currently looking into the possibility of a similar involvement of an internal ribosome entry segment in the synthesis of GLV negative-strand RNA.

Many factors could have contributed to the transient nature of the present transfection scheme with *G. lamblia*. First of all, there is no selective pressure in favor of the transfected cells in our present system. The transfected cells may be overtaken by the untransfected ones upon prolonged in vitro cultivation. One way to overcome this difficulty is to replace the luciferase gene with the neomycin phosphotransferase gene (*neo*). *Giardia* trophozoites are susceptible to G418, a neomycin analog, with an estimated 95% lethal dose of 300 μg/ml in in vitro culture (unpublished observation). Cells expressing the *neo* gene transcript could be selected under such a drug pressure for enrichment. Another possible explanation for the transient nature of the transfection is that the 3′ UTR in p5′Luc3′ needs further refinement. Its current chimeric composition of 26 bp of the 3′ UTR from the luciferase gene and 641 bp of the 3′-terminal region of the GLV genome may not exhibit an optimal binding affinity to GLV *gag-pol* protein during the viral packaging process and may thus gradually be blocked by the bona fide GLV mRNA. Further analysis of the 3′-UTR structure may lessen this difficulty. It is also unsatisfactory to rely on

RT-PCR to demonstrate the presence of mRNA or the negative RNA strand (see Fig. 3) instead of the more quantitative measure of Northern (RNA) blottings. The difficulty lies in the limited size of the postelectroporation sample, which is confined to a total of 0.5×10^7 cells at time zero, not enough for one Northern blot in its entirety. By continuously improving the chimeric construct, we expect that higher levels of the two RNA species in the transfected cells and more persistent transfection may be achieved in the near future for a detailed quantitative analysis of the viral RNAs.

The establishment of a transfection system for *G. lamblia* provided a sensitive method for characterizing GLV gene replication and expression. Further analysis of the 5′ and 3′ UTRs currently attached to the luciferase gene will reveal specifically and precisely the packaging, replication, and transcription sites needed in GLV propagation and the 5′-UTR sequence of the mRNA required by the *G. lamblia* translational system for protein synthesis. In view of the close similarities between the prokaryotic and *G. lamblia* rRNAs (25), this future investigation should reveal much interesting information. The experimental evidence collected thus far points to encapsulation of the luciferase gene in the form of dsRNA in GLV particles. Our next study will be to isolate these viral particles to verify the presence of the dsRNA luciferase gene. A positive outcome from this study will lead to infection of *Giardia* trophozoites with these viral particles together with GLV for expression of luciferase. These investigations may eventually lead to transfection of GLV-infected *G. lamblia* with a gene transcript whose protein product is lethal to the protozoan. GLV particles containing this lethal gene in the dsRNA form may have a useful therapeutic effect in treating giardiasis when administered together with GLV.

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ADDENDUM IN PROOF

J. Yee and T. E. Nash have also recently reported succesful transient DNA transfection of *G. lamblia* via electroporation (Proc. Natl. Acad. Sci. USA **92:**5615–5619, 1995).

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