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Amplification, Expression, and Packaging of a Foreign Gene by Giardiavirus in *Giardia lamblia*

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***Giardia lamblia* is an intestinal protozoan parasite and one of the earliest eukaryotic divergents. The trophozoite multiplies via asexual binary fission and lacks all natural means of lateral gene transfer. A system is developed here for long-term expression of a foreign gene in this organism by exploiting recombinant virions derived from the giardiavirus (GLV), a double-stranded RNA virus that infects many *Giardia* isolates. An in vitro transcript of the cloned GLV cDNA, comprising the firefly luciferase-encoding region flanked by 5′ and 3′ fragments of GLV positive-strand RNA, was electroporated into GLV-infected trophozoites. Luciferase activity in electroporated cells peaked on day 2 at levels 6 orders of magnitude above background. Expression of this foreign gene remained at 80% of its peak level after 30 days in the absence of selective pressure. The chimeric RNA was replicated as double-stranded RNA and packaged into virus-like particles. The recombinant virions were partially purified from the wild-type helper virus by CsCl equilibrium density-gradient centrifugation and used to superinfect *Giardia* trophozoites. At multiplicities of infection of 100 or higher, these chimeric virions were able to initiate new rounds of expression of luciferase activity in the superinfected cells. Thus, the engineered virion can be successfully used to introduce and efficiently express a heterologous gene in this eukaryotic microorganism.**

Giardia lamblia is a flagellate that commonly infects the upper intestines of many mammals, including humans (1). It lacks common organelles, such as mitochondria, peroxisomes, or hydrogenosomes, and is also missing a well-defined endoplasmic reticulum or Golgi (1). Sogin et al. (16) analyzed the sequences of the small subunit rRNA, and Hashimoto et al. (7, 8) studied the translation factors 1α and 2α from *G. lamblia* and other eukaryotes. Both studies placed *G. lamblia* at the earliest branching point from the prokaryotes. More recently, due to its possession of 70S instead of 80S ribosomes, *G. lamblia* was classified to a new kingdom Archezoa in the eight-kingdom system, separated from the protozoa (2). Preservation of these primitive features in present-day *Giardia* sp. suggests that many other characteristics bridging the prokaryotes and the eukaryotes might be retained in *Giardia* sp. as well. Understanding the regulation of gene expression in this organism is thus of particular interest.

The *G. lamblia* trophozoite lacks sexual differentiation as well as other means of genetic exchange, such as conjugation or transformation via transmissible DNA elements (1). The inability to perform genetic analysis on this flagellate has been a major impediment to the study of *G. lamblia*. The technique of electroporation was first used successfully to transfer a transcript of giardiavirus (GLV) into this protozoan (4). More recently, a DNA fragment from the upstream region of the *Giardia* glutamate dehydrogenase gene was introduced into *Giardia* sp. by the same technique to direct the transient expression of firefly luciferase (22). Viruses have been successfully used for expressing foreign genes in the past (3, 6, 9, 12–14). A similar approach by using GLV may lead to stable and permanent transfectants in *Giardia* sp. as well. Besides, genetically engineered virus carrying a lethal gene may also kill the infected cell, which could provide a new way of controlling giardiasis.

GLV is a double-stranded RNA (dsRNA) virus of the *Totiviridae* family that infects only *G. lamblia* (18). Its dsRNA genome of 6,277 bp encodes a 100-kDa capsid protein and a 190-kDa *gag-pol*-like fusion protein via a −1 ribosomal frameshifting (20). The fusion protein is presumed to be the viral RNA-dependent RNA polymerase (19, 20). Many features in GLV have made it a favorable candidate vector for transfecting *G. lamblia*. (i) It is small, stable, and easily purified (11, 18); (ii) it infects *Giardia* trophozoites very efficiently in vitro and has little cytotoxic effect on the cell (10, 17); and (iii) the GLV genome is replicated to a high copy number, and GLV capsid protein is the predominant protein synthesized inside the infected cells (17, 19). We recently generated an RNA transcript from a chimeric GLV cDNA/firefly luciferase gene construct and introduced it into GLV-infected *Giardia* trophozoites by electroporation. Luciferase activity in the electroporated cells peaked at 18 h postelectroporation to a level 10^2-fold above the background and declined to about 50% of the peak level by day 4 and to near background on day 5 (24). More cDNA constructs were made since then by varying the flanking GLV genomic fragments and tested in similar studies (25). The necessary *cis* elements in the GLV genome for optimal expression of luciferase gene in *G. lamblia* have been delineated in detail (25). In this article, we report further investigations of one of the best chimeric cDNA constructs described. Through introduction of the transcript of this construct into GLV-infected *Giardia* trophozoites, the luciferase activity was expressed at a level 3,000-fold beyond our previously reported result (24) and persisted for more than 30 days in the absence of any selective pressure. The chimeric RNA was continuously replicated as dsRNA. The latter was packaged into recombinant virus-like particles (VLP) that were capable of superinfecting GLV-harboring *Giardia* trophozoites and expressing luciferase activity at high levels.

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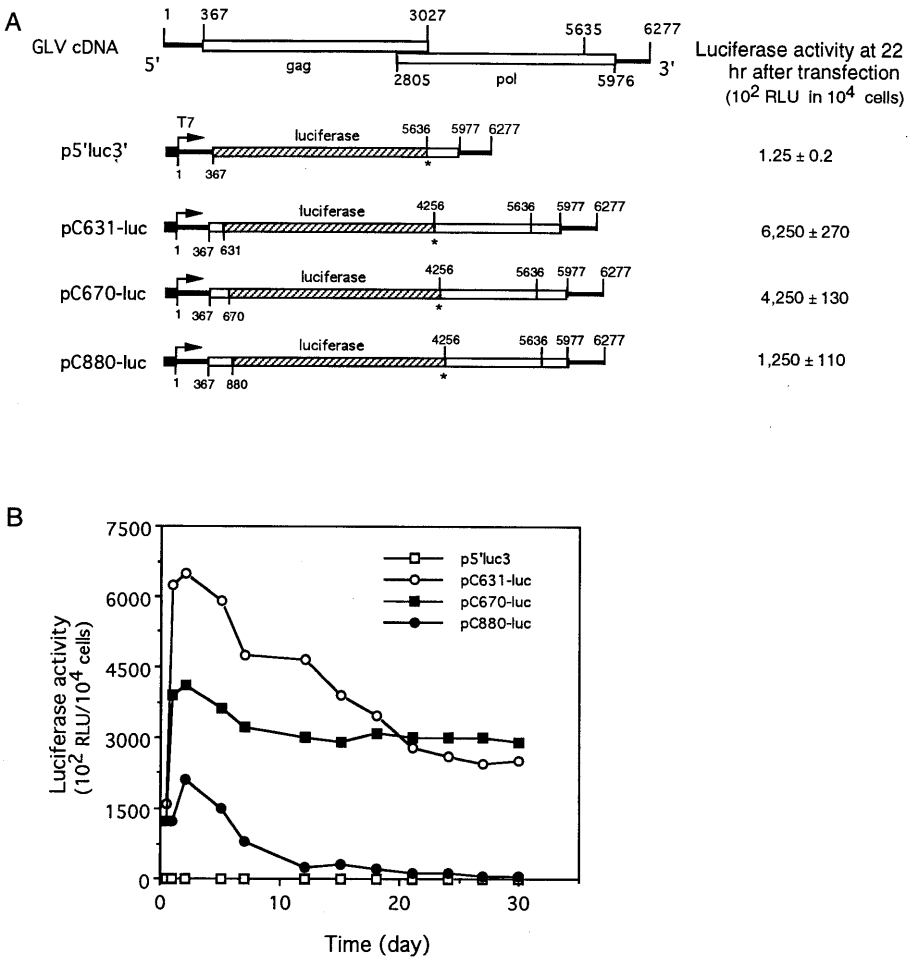

FIG. 1. Luciferase activity in the *Giardia* WBI trophozoites transfected with in vitro transcripts from the plasmid constructs. (A) Schematic descriptions of the expression plasmids (not to scale). The cassettes, containing the luciferase gene (hatched box) flanked by the 5′ and 3′ portions of GLV cDNA (coding region, open box; UTR, thick line), were located downstream from the T7 RNA promoter (black box). The in-frame stop codon of the luciferase gene is marked with an asterisk. The numbering of nucleotides was directly adopted from the numbering of GLV sense strand RNA (20, 21, 25). (B) Time course of luciferase activities expressed in *Giardia* WBI trophozoites transfected with transcripts from various plasmid constructs.

MATERIALS AND METHODS

Cell culture, electroporation, and luciferase assay. *G. lamblia* WB trophozoites (WB) or the trophozoites infected with GLV (WBI) were cultured as described before (11, 18). In vitro transcript (100 μg) of the plasmid was synthesized as previously (26) and used to transfect 4×10^6 WBI cells. Conditions for electroporation and luciferase assay have also been described previously (24). Each determination of luciferase activity presented in Results was from three independent experiments.

Construction of recombinant plasmids. Four plasmids, pSP-*luc*+, p5′luc3′, pM-GLV, and pM-GLVNcoI provided all the DNA fragments for the constructs reported here. pSP-*luc*+ (Promega) contributed the coding fragment of the luciferase gene, whereas pM-GLV was a derivative of pGEM5Zf(+) (Promega) carrying the 6,277-bp GLV cDNA (GenBank accession number L13218) (20, 21, 25). pM-GLVNcoI was created from pM-GLV by adding a *Nco*I site between nucleotides (nt) 365 and 370 by site-directed mutagenesis (25). For simplicity, the numbering of nucleotides throughout this report refers to the position in the GLV sense strand RNA. Among the plasmids shown in Fig. 1A, p5′luc3′ was the transient transfection vector described previously in which the firefly luciferase gene was flanked by the 5′ 366-nt untranslated region (5′-UTR) and the 3′ 642-nt portion from the GLV genome (24). In pC631-luc, the flanking GLV cDNA fragments were extended inward to 631 nt at the 5′ end and 2,022 nt at the 3′ end, respectively. It was constructed by first inserting the full-length firefly luciferase gene (the nt 45 to 1723 *Nco*I-*Xho*I fragment from pSP-*luc*+) into the *Nco*I-*Xho*I sites in pM-GLVNcoI (25), resulting in a new construct, pluc-Pol. To lengthen the 5′ GLV flanking sequence, a fragment from nt 103 to 631 of the GLV cDNA between the *Not*I and *Nco*I sites was generated by PCR, using pM-GLV (26) as template and synthetic oligonucleotides GLV-NI (5′-GTCCAGAGAAAGCGG CCGCTGTTAGG-3′) and GLV631 (5′-GATCCCATGGCGCTACCGCCCAG CG-3′) as primers. The insert from nt 103 to 366 in pluc-Pol was removed by *Not*I and *Nco*I and replaced with the PCR fragment double restricted with *Not*I and *Nco*I to produce pC631-luc. pC670-luc and pC880-luc were derived by the same procedure except that the 5′ domain of GLV cDNA was extended to nt 670 or 880 with the oligonucleotide GLV670 (5′-GATCCCATGGAGGCTGAGAAT CGTA-3′) or GLV880 (5′-GATCCCATGGTGGTTAATCTCCTG-3′) as PCR primer, respectively.

Assay for viral packaging and isolation of recombinant VLP. *Giardia* WBI cells, electroporated with the transcript of pC670-luc, were harvested after 2 days of incubation at 37°C. The trophozoites were lysed by sonication in the presence of 1% Nonidet P-40, 0.1 M NaCl, and 10 mM Tris HCl, pH 7.4. The cleared lysate was then subjected to CsCl equilibrium density-gradient ultracentrifugation in a Beckman SW41 rotor at 152,000 × *g* at 20°C for 18 h (11). Total RNA from each collected fraction was extracted with phenol-chloroform and analyzed by 1.3% agarose gel electrophoresis as described before (4). The gel was then blotted and hybridized with ^{32}P-labeled probes synthesized from either GLV cDNA or the luciferase gene. Conditions for RNA denaturation and hybridization were as described previously (5). Fractions containing the 6.3-kbp GLV dsRNA or the 4.3-kbp chimeric dsRNA were negatively stained with tungstic acetate and examined under the electron microscope.

Assay for infectivity of the recombinant VLP. The concentration of VLP containing the 4.3-kbp chimeric RNA was deduced by using known amounts of GLV dsRNA as standard to determine the amount of the 4.3-kbp chimeric dsRNA in agarose gel and assuming that only one molecule of dsRNA is encapsidated per VLP. Fractions collected from the CsCl density gradient were filtered through a 0.2-μm-pore-size filter, desalted in a Sephadex G-25 gel filtration column, and then used to infect 2×10^5 WB or WBI cells at a multiplicity

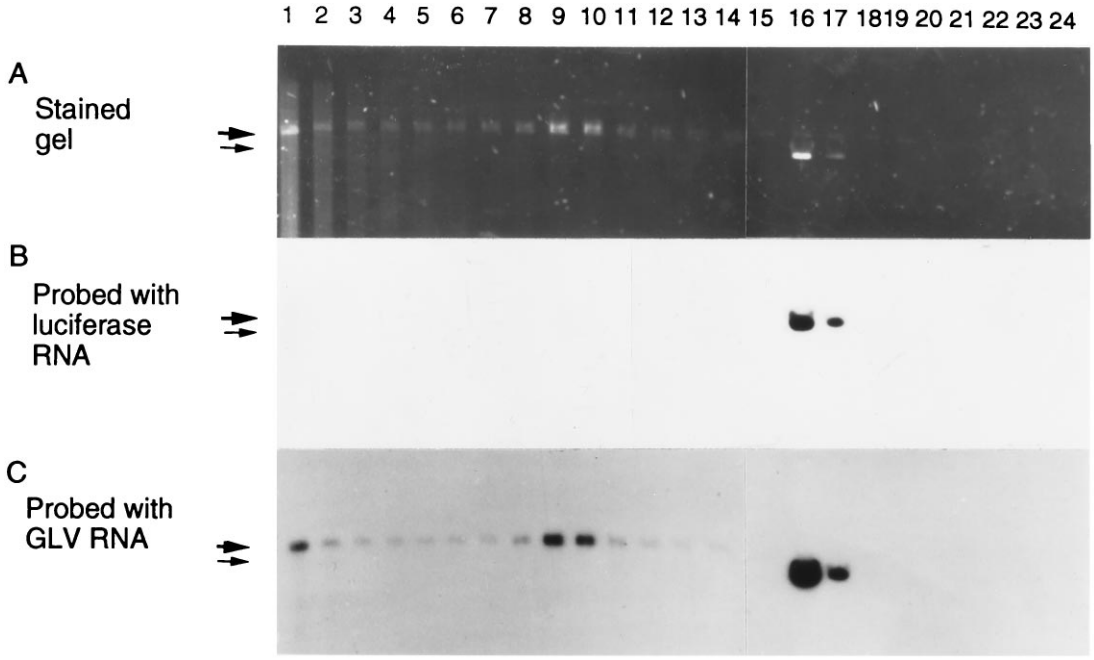

FIG. 2. Encapsidation of luciferase chimeric RNA. *Giardia* WBI trophozoites transfected with the transcript from pC670-luc were harvested after 48 h of incubation at 37°C. The trophozoites were lysed and subjected to ultracentrifugation. A total of 24 fractions (400 μl each) was collected through the bottom of the tube. RNA was resolved in agarose gel electrophoresis for Northern blots. (A) Ethidium bromide-stained gel of total RNA extracted from collected fractions. (B and C) Northern blots of the gel in panel A hybridized, respectively, with ^{32}P-labeled luciferase antisense RNA (B) or GLV negative-strand RNA (C). The large and small arrows mark, respectively, the positions of the 6.3-kbp GLV dsRNA and the 4.3-kbp chimeric dsRNA. Exposure time for the autoradiographs in panels B and C was 2 h.

of infection (MOI) of 10^2 or 10^3. Luciferase activity in the infected cells was monitored thereafter.

RESULTS

GLV-mediated amplification and expression of the luciferase gene. The luciferase activity expressed was several hundredfold above background and lasted for 3 to 4 days in our previous construct p5′luc3, in which luciferase gene was flanked by the 5′-UTR and the 3′ 642-nt fragment (nt 5636 to 6277) from the GLV genome (Fig. 1A) (24). Efforts to attain more efficient and stable expression of luciferase activity in transfected *Giardia* trophozoites led to the synthesis of a new series of cDNA constructs (25) in which the flanking 5′ GLV genomic fragment was extended stepwise downstream to include the GLV capsid-encoding region that fused with the N-terminal portion of the luciferase, while the 3′ flanking GLV fragment was also extended upstream to nt 4256. The transcripts thus obtained yielded progressively higher luciferase activities in the transfected cells until the 5′ GLV genomic fragment reached 631 nt (pC631-luc, Fig. 1A). Further lengthening of this flanking fragment resulted in decreased luciferase activity in transfected cells (25). Transfectants of pC631-luc showed the highest luciferase activity at 6.5×10^5 relative luminescence units (RLU) per 10^4 cells on day 2 after electroporation, which gradually declined to about 50% of the highest level after 15 days of serial passages. In comparison, transfectants of pC670-luc yielded luciferase activity of 4.3×10^5 RLU per 10^4 cells on day 2, but the activity remained stable upon continuous in vitro cultivation, with 1:13 dilutions at 3-day intervals. After 30 days of serial passages, 80% of the initial activity remained (Fig. 1B) in the absence of selective pressure. Both luciferase sense and antisense RNAs were detected by reverse transcriptase PCR throughout this period (data not shown), suggesting that the transcript from pC670-luc was continuously synthesized and replicated in the transfected cells. Because pC670-luc generated the most stable transfectants, it was chosen for further investigation.

Encapsidation of the luciferase gene into VLP. Since the chimeric GLV-luciferase RNA was replicated and maintained by the helper GLV in transfected cells, we investigated whether the chimeric RNA was also encapsidated into VLP by the GLV capsid protein. Lysate of pC670-luc-transfected WBI cells was centrifuged in CsCl density gradient, and the RNA in each fraction was examined in agarose gel. Two dsRNA species, 6.3 and 4.3 kbp, respectively, were detected in the ethidium bromide-stained gel and were separated by approximately five fractions (Fig. 2A). The 6.3-kbp RNA fragment peaked at fractions 9 and 10, whereas the 4.3-kbp fragment centered at fractions 16 and 17. ^{32}P-labeled luciferase sense and antisense RNA hybridized only with the 4.3-kbp fragment (Fig. 2B and data not shown), whereas the GLV RNA probe hybridized with both the 6.3- and the 4.3-kbp fragments (Fig. 2C). Since the 4.3-kbp species was recognized by both probes, it therefore could be the chimeric RNA transcribed from pC670-luc and replicated into the double-stranded form. Its size also agreed with the estimated sum of nearly 2.7 kbp of GLV sequences plus the 1.7-kbp luciferase gene. Portions from fractions 9 and 16 were negatively stained with tungstic acetate and examined under an electron microscope. Both samples showed the presence of icosahedral particles of 36 nm, resembling those of wild-type GLV (18) and of approximately equal abundance (Fig. 3). These results indicate that the recombinant RNA not only replicates as dsRNA in the transfected trophozoites (24) but is also packaged into progeny VLP (1.358 g ml^{-1}) that are lighter in density than GLV (1.368 g ml^{-1}), possibly due to the shorter dsRNA in the recombinant virion. Fraction 16, containing the recombinant VLP, was also desalted and assayed for luciferase activity. The results showed

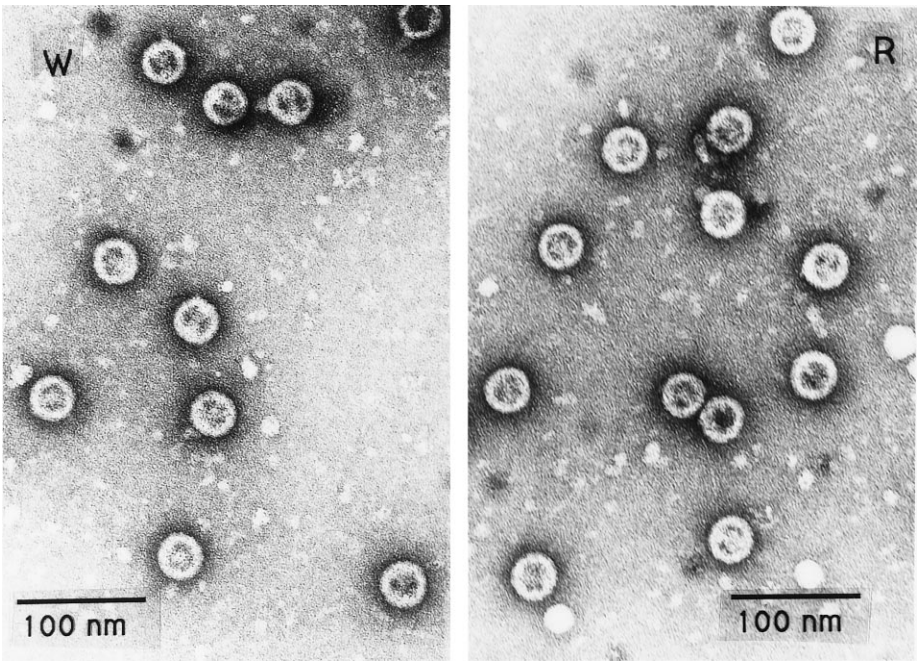

FIG. 3. Negative-stained GLV-like particles under electron microscope. W, wild-type GLV from fraction 9 in Fig. 2A; R, recombinant VLP from fraction 16 in Fig. 2A.

that a sample of recombinant VLP (200 μg of protein) exhibited no detectable intrinsic luciferase activity (data not shown), suggesting that the luciferase fusion protein synthesized in the transfected *Giardia* sp. is not packaged into viral particles.

Infectivity of the recombinant VLP. Fraction 16 was estimated to contain 3 ng of 4.3-kbp chimeric dsRNA per μl. Assuming that only one molecule of dsRNA is encapsided per VLP, this estimate translates into 6×10^8 VLP per μl in fraction 16. After infection, there was a lag period of 1 day for WBI and 2 days for WB cells before the luciferase activity became detectable. The activity then increased steadily with time (Fig. 4A). By day 6 postinfection, the luciferase activity was 10^3 RLU per 10^4 WBI cells and 5×10^2 RLU per 10^4 WB cells. These data indicate that the recombinant VLP are infectious and that gene transfer can therefore be accomplished by a simple viral infection.

Since the GLV-free WB trophozoites electroporated with the chimeric RNA did not express luciferase activity (24), the activity in WB cells infected with fraction 16 could be attributed to the presence of a minute amount of wild-type GLV in this fraction. This was in fact supported by the agarose gel electrophoresis and Northern (RNA) blot studies. A 6.3-kbp GLV dsRNA band faintly visible in Fig. 2A was recognized by the GLV probe when the autoradiograph was exposed overnight instead of 2 h (data not shown). Additionally, we also detected the presence of this 6.3-kbp GLV dsRNA in agarose gel electrophoresis of extracts from WB cells 2 days postinfection (data not shown). These observations confirm that expression of the luciferase gene in *G. lamblia* via recombinant VLP infection requires the concomitant establishment of wild-type GLV infection.

In separate experiments, when filtered culture supernatant from cells transfected with pC670-luc was pretreated with RNase (Boehringer Mannheim) at 37°C for 1 h and added to WB or WBI cells, luciferase activity could be detected after 2 days of incubation (data not shown). Since GLV dsRNA is noninfectious and GLV sense RNA is noninfectious without electroporation (4), the infectivity of filtered culture supernatant suggests that infectious recombinant VLP was also shed into the culture supernatant, probably by a mechanism similar to that used by the wild-type GLV (10, 18). The presence of these infectious VLP in the culture supernatant may contribute to the stable maintenance of recombinant VLP in *G. lamblia*.

DISCUSSION

We have shown that the RNA transcript from pC670-luc is able to direct the synthesis of luciferase in transfected *G. lamblia* trophozoites that showed luciferase activity more than a millionfold above the background and lasting at least 30 days through serial passages in the absence of selective pressure. The recombinant RNA was apparently packaged into VLP that are present both inside the cytoplasm and in the culture medium supernatant of the transfected cells. The ability of these recombinant VLP to infect and initiate subsequent rounds of luciferase expression in *G. lamblia* makes it possible, for the first time, to introduce foreign genes into these trophozoites without electroporation. This feasibility will be most helpful in certain circumstances when electroporation is impractical, such as transfection of *Giardia* trophozoites inside a mammalian host.

The results shown in Fig. 4A indicate that similar luciferase activities were observed in cells infected by recombinant VLP at MOIs of 10^2 and 10^3. This is probably due to the observation that *Giardia* WB trophozoites infected with wild-type GLV at MOIs of 10^2 and 10^3 result in a similar density of viruses at 5 and 10 per cell, respectively (10). A large percentage of the cell population remained apparently uninfected under these experimental conditions (17). Since the cells used in these experiments were not synchronized, it is also not known whether the susceptibility to viral infection is restricted to a certain phase(s) of the *Giardia* cell cycle.

The luciferase activity expressed via viral infections at 10^3 RLU per 10^4 cells appeared to be lower than that achieved via

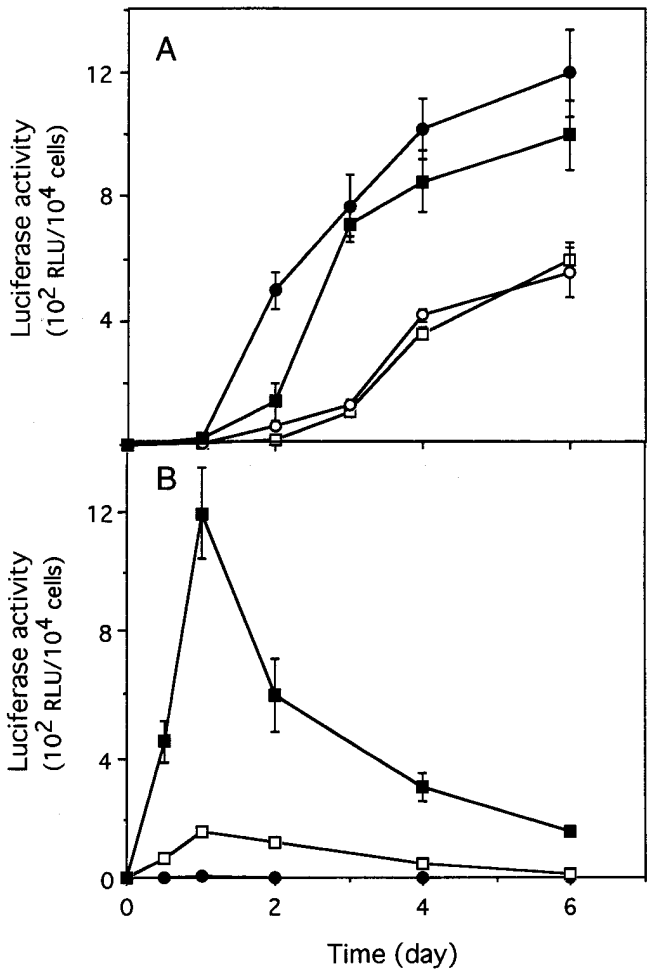

FIG. 4. Time course of luciferase expression in *Giardia* trophozoites infected with recombinant VLP (A) or transfected with low levels of transcript from pC670-luc (B). (A) WB cells infected with VLP at an MOI of 10^2 (□) or 10^3 (○) and WBI cells infected at an MOI of 10^2 (■) or 10^3 (●). (B) WBI cells transfected with the pC670-luc transcript at 10^3 (●), 10^4 (□), or 10^5 (■) RNA molecules per cell.

RNA transfection at 4.3×10^5 RLU per 10^4 cells (Fig. 1B and 4A). This discrepancy may be attributed to the fact that 100 μg of purified RNA synthesized in vitro was used to transfect 4×10^6 trophozoites, which was equivalent to a multiplicity of transfection of approximately 10^7 RNA molecules per cell, which is much higher than the MOIs of from 10^2 to 10^3 in Fig. 4A. When the transfection was conducted at 10^2 to 10^3 RNA molecules per cell, there was no detectable luciferase activity in the transfected *Giardia* trophozoites (Fig. 4B). When the multiplicity of transfection was increased to 10^4 and 10^5 RNA molecules per cell, luciferase activity expressed in the transfected cells reached its peak after 1 day and decreased rapidly in the ensuing 6 days (Fig. 4B). This rapid decrease was, however, not observed when the multiplicity of transfection was kept at 10^7 RNA molecules per cell, thus suggesting a threshold multiplicity of transfection for stable transfection by the transcript of pC670-luc (Fig. 1B). The highest activity achieved by transfection at 10^5 RNA molecules per cell is equivalent to that in cells infected by the recombinant VLP at an MOI of 10^3 after 6 days (compare panels A and B in Fig. 4). Transfection is thus much less efficient than infection. Furthermore, the rapid rise and fall of luciferase activity in the transfected cells contrast sharply with the slow but steady increase of activity in the infected cells. Although the mechanisms distinguishing these two phenomena are not known, this distinction favors infection as a better method of introducing and expressing a foreign gene in *G. lamblia*.

Most of the *G. lamblia* isolates from the field or patients are either infected with GLV or free of the virus but susceptible to GLV infection (10). There is, however, a small subset of *G. lamblia* isolates that is resistant to GLV infection due to the absence of a virus receptor but is supportive of the proliferation of GLV when GLV RNA is electroporated into these cells (15). Therefore, the usefulness of the transfection by transcripts from a pC670-luc vector can be extended to both GLV-sensitive and GLV-resistant isolates of *G. lamblia*. It is conceivable that stable gene transfer can be achieved through cytoplasmic inheritance of the recombinant VLP and GLV. We have recently replaced the luciferase gene with the neomycin phosphotransferase gene and obtained *G. lamblia* transfectants that were resistant to G418 at a level 150-fold above the 90% lethal dose value (23). This construct, pC670, is thus not confined to the transfer of luciferase gene but can be used most likely to transfer a broad spectrum of foreign genes into *G. lamblia* for efficient and stable expression. It is not clear why the transcript from pC670-luc can lead to stable expression of luciferase in transfectants without a favorable selective pressure, except for the obvious fact that the recombinant VLP and the wild-type GLV may replicate and infect at similar efficiencies. pC670-luc encodes a fusion protein between the N-terminal 101 amino acid residues of the GLV capsid protein and luciferase. Its extraordinarily high activity and longevity in the cytoplasm of the transfectants suggest that it is either vigorously synthesized or poorly degraded or both (25).

Thus, in summary, we have succeeded in introducing and expressing a foreign gene transcript in *G. lamblia* via viral infection. The recombinant virion may prove to be a simple and efficient tool for the introduction of heterologous or mutated genes into this Archebacteria-like eukaryotic organism.

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