RNA dependent RNA polymerase activity associated with the double-stranded RNA virus of *Giardia lamblia*

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ABSTRACT

***Giardia lamblia*, a parasitic protozoan, can contain a double-stranded RNA (dsRNA) virus, GLV (1). We have identified an RNA polymerase activity present specifically in cultures of GLV infected cells. This RNA polymerase activity is present in crude whole cell lysates as well as in lysates from GLV particles purified from the culture medium. The RNA polymerase has many characteristics common to other RNA polymerases (e.g. it requires divalent cations and all four ribonucleoside triphosphates), yet it is not inhibited by RNA polymerase inhibitors such as α-amanitin or rifampicin. The RNA polymerase activity synthesizes RNAs corresponding to one strand of the GLV genome, although under the present experimental conditions, the RNA products of the reaction are not full length viral RNAs. The *in vitro* products of the RNA polymerase reaction co-sediment through sucrose gradients with viral particles; and purified GLV viral particles have RNA polymerase activity. The RNA polymerase activities within and outside of infected cells closely parallel the amount of virus present during the course of viral infection. The similarities between the RNA polymerase of GLV and the polymerase associated with the dsRNA virus system of yeast are discussed.**

INTRODUCTION

Giardia lamblia, a parasitic protozoan, is one of the most prevalent parasitic infections in the world today. Its effect on the host can range from asymptomatic infections to chronic nutritional malabsorption and diarrhea. Currently available drugs, such as metronidazole, have adverse side effects and do not prevent reinfection, a common problem in developing countries (reviewed in 2).

Several years ago, our laboratory identified a *Giardia lamblia* virus (GLV; 1) which, after modification, may prove useful as a 'magic bullet' against *G. lamblia*. The GLV virus contains a 7 kb dsRNA genome encapsulated within an icosahedral protein shell composed predominantly of a 100 kD protein (1, 3). The virus is extruded into the medium from infected cells and virus isolated from either cell lysates or from filtered culture medium is infectious to uninfected *G. lamblia* cells (4). However, the viral infection does not affect the growth rate of cells in culture nor does it actively lyse the cells. GLV infected cells have been isolated from a variety of hosts with or without clinical symptoms (4). Thus the presence of the virus does not appear to be associated with pathogenesis.

Recently, we have identified a single-stranded RNA (ssRNA) present in GLV infected cells which appears to represent a full length version of one of the two strands of the GLV viral genome (5). We suggest that this RNA is a replication intermediate and/or an mRNA of GLV.

In our continuing investigation of the GLV life cycle, we have now identified an RNA dependent RNA polymerase activity in crude whole cell lysates from GLV infected cells, as well as in medium containing GLV and in purified GLV preparations. Properties of this RNA polymerase appear to be similar to those of other well characterized RNA polymerases. The GLV RNA polymerase is virus-associated and, under the present experimental conditions, synthesizes only one strand of the GLV genome, the same strand as the ssRNA described above. The identification of this activity may help to elucidate the life cycle of GLV and allow us to use GLV to interfere with *Giardia* infections in mammalian hosts.

MATERIALS AND METHODS

Strains, Growth Conditions, and Infection Conditions

In this analysis, we used two strains of *Giardia lamblia*, strain WB which is not infected with the virus and strain Portland 1 (P1) which is chronically infected with the virus. The origin of these two cloned strains and their growth conditions have been described previously (3). In addition, we infected strain WB with a GLV virus preparation (purified through two cesium chloride buoyant density gradient centrifugations; 3) at a multiplicity of infection (M.O.I.) of 1000 at cell densities between 1 to 5×10^5 cells per ml. These infected WB cells were then cultured and passaged by routine procedures. Cell densities were determined using a Coulter Counter Model ZF with a 100 μ aperture.

Preparation of Lysates

The preparation of lysates and the RNA polymerase assay were both modifications of procedures developed for the identification of RNA polymerase activity associated with the dsRNA killer virus of yeast (6, 7, 8). Briefly, a culture of *G. lamblia* strain WB was infected with a purified virus preparation at an M.O.I. of 1000. After 24 to 48 hours of incubation, the culture tubes

were placed on ice for 20 minutes and the cells were collected by centrifugation (2500 rpm, 10 min.). The cells were washed once in phosphate buffered saline (PBS) and then resuspended in Lysis Buffer [50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 150 mM NaCl, 100 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol and 20% glycerol.] The cells were disrupted in this buffer using a 1 minute continuous pulse at a setting of 4 on a Sonicator cell disruptor with microtip (Heat Systems−Ultrasonics, Inc.). After sonication, the lysates were immediately frozen at −70°C and stored until use.

Lysates of virus particles released into the culture medium were prepared in a similar manner. Culture medium in which the cells had been growing was filtered through a 0.22 μm filter. The filtrate was then centrifuged at 80,000 rpm for 1 hour in a TLA 100.3 rotor in a TL100 Beckman ultracentrifuge. The viral pellet was resuspended in lysis buffer and sonicated as above.

RNA Polymerase Assay

In a standard RNA polymerase assay, 15 μl of lysate was mixed with 15 μl of polymerase mix and incubated at 37°C for one hour. Polymerase mix consisted of 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 20 mM β-mercaptoethanol, 1mM ATP, 1mM GTP, 1mM UTP, 1μM non-radioactive CTP, 0.5 units/μl of RNAsin, and 0.4 μCi/μl of α ^{32}P CTP (Amersham, SP6/T7 grade, 800 Ci/mmol.). After incubation, the entire reaction mixture was spotted onto Whatman GF/C filters and washed extensively with cold 5% trichloroacetic acid/1% sodium pyrophosphate (TCA-PPi), followed by two washes with 95% ethanol. After drying, the filters were placed in vials with 5 ml of Ecolume (ICN) scintillation fluid and counted in a Beckman LS 3801 scintillation counter.

Characterization of the Reaction Products

The product of the RNA polymerase reaction was purified by repeated extractions with phenol, phenol/chloroform and chloroform, followed by ethanol precipitation, as described (9). After resuspension, the products were mixed with a Ficoll loading dye and electorphoresed through a 0.8% agarose gel in 1×TBE [89 mM Tris borate (pH 8.0), 89 mM boric acid, 2 mM EDTA] containing 0.5 μg/ml ethidium bromide as described (9). After photographing the gel on a 300 nm U.V. light box, the gel was soaked briefly in 10% TCA-PPi to fix the nucleic acids and the gel was dried by placing it between layers of Whatman 3MM filter paper until it was paper thin. The gel was then exposed to X-ray film for autoradiography.

Gels for Northern blots were prepared under non-denaturing conditions that allow separations of ssRNA and intact dsRNA. Subsequent denaturation of the gel separates the two strands of the dsRNA and allows both the ssRNA and the dsRNA to be blotted to nitrocellulose (5). The Northern strips were hybridized and washed as described (5) and exposed to X-ray film for autoradiography.

Sucrose density gradients (5−20%) were prepared using sucrose solutions containing the components of the lysis buffer. The gradients were run for 6 hours at 104,000×g (24,000 rpm) in an SW 28 swinging bucket rotor. After the run, the bottom of the tube was punctured and fractions were collected with a Gilson microfractionator. The refractive index was measured using a Zeiss refractometer.

Materials

Total RNA was prepared as previously described (3). Non-radioactive nucleotides were obtained from Pharmacia, RNAsin was obtained from Promega and RNA polymerase inhibitors were obtained from Sigma.

RESULTS

Identification of RNA Polymerase Activity

We have recently shown that a ssRNA is a potential mRNA or replication intermediate of the GLV genome and is synthesized intracellularly during GLV infection of *Giardia lamblia* (5). The highest level of ssRNA is produced approximately 24−48 hours after infection. Since this time period may be the optimal time for the detection of viral RNA polymerase activity, we made crude cell lysates of infected and uninfected cells 48 hours after infection (or mock infection) of the GLV free WB strain of *G. lamblia* (see Materials and Methods). Lysates of the infected cells were tested for RNA polymerase activity, defined as TCA-PPi precipitable radioactivity derived from α-^{32}P CTP. Time courses of the RNA polymerase activities in infected and uninfected lysates are shown in Figure 1. Substantial TCA-PPi precipitable counts are detected only in GLV infected cell lysates, and these counts increase with time, leveling off after one hour of incubation. The results in Figure 1 suggest that we are detecting a polymerase activity in lysates of GLV infected cells.

Characterization of the RNA Polymerase Activity

To characterize this putative RNA polymerase, we tested a variety of conditions which could alter this activity, defined as TCA-PPi precipitable α-^{32}P CTP counts after one hour incubation. As seen in Table 1, the activity is concentration and temperature dependent with an optimal temperature (37°C) which parallels the optimal growth temperature of *G. lamblia*. Most importantly, the TCA-PPi precipitable counts are dependent on the presence of the three other ribonucleotides (ATP, GTP and UTP; Table 1). The absence of any one of the three ribonucleotides drastically reduces the activity; the absence of all three ribonucleotides essentially eliminates the activity. Consistent with these observations, incorporation of radioactively labeled CTP can be diluted by the addition of unlabeled CTP to the reaction mixtures (Table 1). All of these observations suggest that we have identified a polymerase activity specific for GLV infected cells.

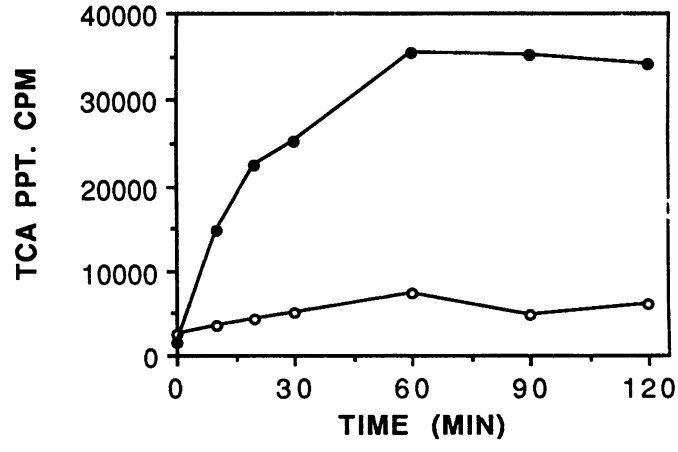

Figure 1. Time Course of RNA Polymerase Assay. Lysates from infected (closed circles) and uninfected (open circles) cells were tested in the RNA polymerase assay as described (Materials and Methods). At the indicated times, samples were spotted onto Whatman GF/C filters, TCA-PPi precipitated, and counted as described. Graph shows average of two independent assays for each point.

As would be expected of RNA polymerases, the activity is dependent on divalent cations since the reaction was inhibited by EDTA (Table 1; the Mg^{++} conc. in the reaction was 5 mM). Moreover, the culture medium in which the cells have been grown and removed was found to contain RNA polymerase activity associated with extruded virus. This activity was only detectable when Mg^{++} was added to the reaction (see below). Mg^{++} is usually supplied to the reaction mixture in the cell lysis buffer (see Materials and Methods). RNA polymerases are also expected to produce pyrophosphate since monophosphate nucleotides are polymerized from triphosphate nucleotides. Therefore, high levels of pyrophosphate could cause product inhibition of the enzyme. Indeed, the polymerase activity was highly sensitive to 5 mM pyrophosphate while a ten fold higher level of phosphate (50 mM) had much less of an inhibitory effect (Table 1).

Several compounds are known to inhibit RNA polymerases and some of these compounds were assayed for their effect on the GLV RNA polymerase activity. Ethidium bromide (and to some extent Actinomycin D) decreased the RNA polymerase activity as one would expect since these compounds interact with double-stranded nucleic acids (10). On the other hand, α-amanitin and rifampicin, which are known to inhibit by interaction with RNA polymerase molecules (11, 12, 13), had much less of an inhibitory effect (Table 1; compare rifampicin in DMSO to DMSO alone). These data suggest that this RNA polymerase activity represents a unique RNA polymerase.

This RNA polymerase activity has been seen in a variety of GLV infected cell lysates and in viral lysates from the culture medium. All lysates can be repeatedly thawed and refrozen without significant loss of activity. The reaction is not dependent on the addition of β-mercaptoethanol (Table 1) or of RNAsin, an RNAse inhibitor (up to 20 units/μl; data not shown). However, it is not clear if this latter result implies that there is no appreciable amount of RNAse in the lysates or that there is too much RNAse activity in the lysates to be affected by the large amounts of added RNAsin. Finally, while the RNA polymerase activity is dependent on the number of cells used to make the lysate, it is not dependent on the protein concentration of the lysate. This is because in all lysates examined, the predominant protein was the serum albumin from the culture medium which contaminates crude lysates as well as the culture medium filtrates and CsCl gradient purified viral preparations (data not shown, ref. 3).

Table 1. Characteristics of the RNA Polymerase

Conditions:		Percentage: [a]
Extract Conc. Dependence:	50%	100
	25%	57
	12.5%	29
	6.25%	13
Temperature Dependence:	23°C	46
	30°C	81
	37°C	100
	45°C	99
Nucleotides:	ATP, GTP, UTP	100
	no ATP	23
	no GTP	25
	no UTP	35
	no ATP, GTP, UTP	7
Cold CTP	none	100
	1.0 μM	93
	10 μM	69
	100 μM	<0
EDTA	1 mM	79
	10 mM	<0
Pyrophosphate (Na)	5 mM	4
Phosphate (Na)	5 mM	99
	50 mM	34
RNA Polymerase Inhibitors:		
Ethidium bromide	100 μg/ml	4
Actinomycin D	100 μg/ml	68
α-Amanitin	1 mg/ml	88
Rifampicin in DMSO	100 μg/ml	72
DMSO alone		76
No β-mercaptoethanol		107

[a] [(value − uninfected)/ (infected − uninfected)] × 100

Analysis of the Lysates and the Reaction Products

To further characterize the RNA polymerase activity, lysates were pretreated with a variety of conditions including DNAse, RNAse, proteinase K, sodium dodecyl sulfate (SDS) and 65°C (Table 2A). DNAse had no effect on the lysate suggesting that DNA is not involved in the reaction. Conversely, RNAse abolished all TCA-PPi precipitable counts suggesting that either the template and/or the product of the reaction is RNA. The activity is heat labile since pretreatment of the lysate at 65°C for 15 minutes inactivated the lysate by 50%. Pretreatment with SDS also eliminated most of the RNA polymerase activity. Heat lability and SDS sensitivity are consistent with the protein nature of RNA polymerases. However, the lysate was not sensitive to proteinase K pretreatment. One possible explanation for this resistance to protease digestion is that the RNA polymerase is not accessible to the proteinase, perhaps because it is internal to the viral particles.

The product of the reaction was purified by phenol/chloroform extraction and ethanol precipitation, and then subjected to the same battery of treatments as listed in Table 2A (Table 2B). Again, RNAse destroyed the product and DNAse had no effect, providing further proof that the product of the reaction is RNA. As one would expect, neither proteinase K, SDS nor 65°C had any appreciable effect on the RNA product.

For further characterizations, the products of the RNA polymerase assay were run on a 0.8% non-denaturing agarose gel (Fig. 2A). This type of electrophoresis was used because it separates dsRNA from full length ssRNA (5). Radiolabeled RNAs were only detected in assays of lysates from infected cells (Fig. 2A). When the labeled RNAs were run in a native form, most

Table 2. Treatment of extract and product

Condition:	Concentration:	Percentage:[a]
A. PRETREATMENT OF EXTRACT[b]		
DNAse 1	330 μg/ml	89
RNAse A	330 μg/ml	<0
Proteinase K	660 μg/ml	113
SDS	0.33%	10
65°C		51
B. TREATMENT OF PRODUCT[c]		
DNAse 1	500 μg/ml	80
RNAse A	500 μg/ml	2
Proteinase K	1 mg/ml	83
SDS	0.5%	76
65°C		99

[a] [(value − uninfected)/(infected − uninfected)] × 100

[b] Extract was treated for 15 minutes at 37°C (except 65°C treatment) under the listed condition prior to addition of the reaction mix.

[c] After the reactions were completed, the product was purified by phenol/chloroform extraction and ethanol precipitation. The resuspended product was then reacted for 15 minutes at 37°C (except 65°C treatment) under the listed condition.

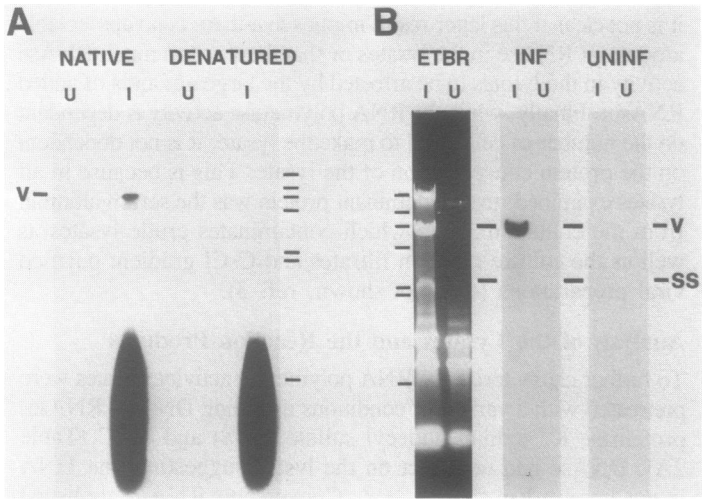

Figure 2A. Gel Electrophoresis of Products from the RNA Polymerase Assay. RNA polymerase assays (Materials and Methods) were run with lysates of virus infected (I) and uninfected (U) cells. After incubation, the reactions were stopped by adding SDS to a final concentration of 0.2%, followed by phenol/chloroform extraction, removal of excess nucleotides by running the reactions through a Sephadex G50 spun column and ethanol precipitation. After resuspension, the samples were either loaded directly on a 0.8% non-denaturing agarose gel (Native) or they were denatured for two minutes at 90°C in 10% formamide and then loaded on the non-denaturing gel (Denatured). After electrophoresis, the gel was dried and exposed to X-ray film for autoradiography. V in the figure represents the position of dsRNA virus as seen on an ethidium bromide stain of the gel (not shown). The bars at the right of the autoradiogram represent size markers of λ cut with Hind III (from top: 23.1, 9.4, 6.7, 4.4, 2.3, 2.0, 0.56 kb). **Figure 2B.** Hybridization of Northern Blots with RNA Polymerase Products. RNA polymerase assays were run on lysates of infected and uninfected cells. After incubation, the reactions were stopped by adding SDS to a final concentration of 0.2%, followed by phenol/chloroform extraction and removal of excess nucleotides by running the reaction through a Sephadex G50 spun column. The reactions were then used as probes against Northern strips of a non-denaturing gel which separates dsRNA from full length ssRNA. Each strip contains lanes of infected (I) and uninfected (U) total RNA blotted to nitrocellulose. The ETBR panel shows the ethidium bromide stain of a strip of the gel that was blotted. Panels INF and UNINF show the autoradiograms of strips probed with RNA polymerase products from infected and uninfected lysates respectively. V and SS represent the position of dsRNA virus and ssRNA respectively. Bars at the left of the autoradiogram represent size markers of λ DNA cut with Hind III (from top: 23.1, 9.4, 6.7, 4.4, 2.3, 2.0, 0.56 kb)

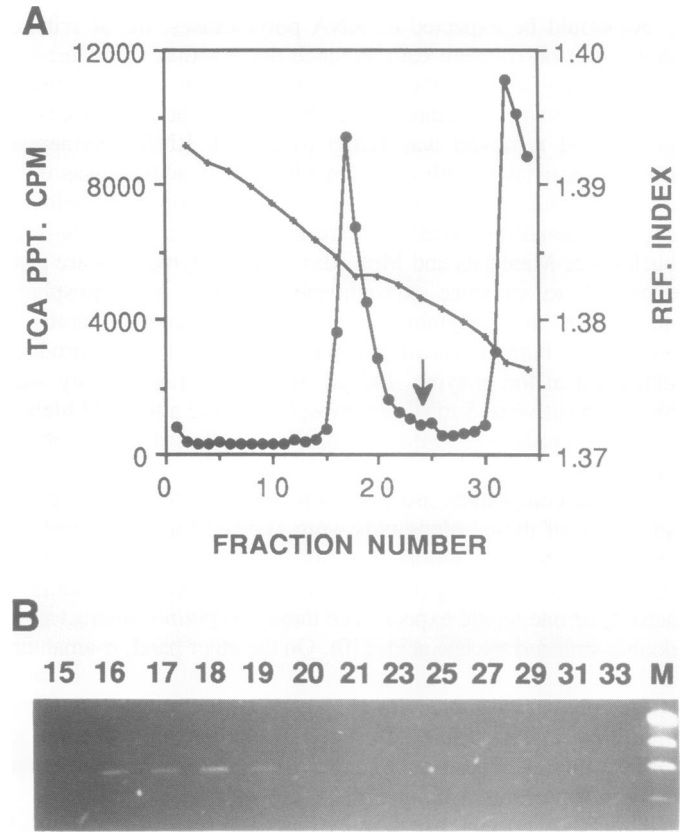

Figure 3. Sucrose Gradient Analysis of RNA Polymerase Products. **A.** RNA polymerase reactions were run on GLV infected cell lysates. The reaction was then layered on a 5–20 % sucrose gradient as described in the Materials and Methods. Each fraction was assayed for TCA-PPi precipitable counts (closed circles) and the refractive index was determined for alternate fractions (plus signs) to determine the quality of the gradient. The position of a fraction containing a large amount of rRNA is marked with an arrow. This fraction is presumed to contain ribosome monomers. **B.** RNAs from selected fractions in part A were extracted and run on an agarose gel. The numbers above each lane represent the fraction from which the RNA was extracted. The visible ethidium bromide stain of dsRNA viral genome clearly shows a peak of viral dsRNA centered on fraction 18, which closely corresponds to the peak of polymerase products seen in part A. Lane M shows lambda DNA cut with Hind III which was used as a size marker (the four bands represent, from top: 23.1, 9.4, 6.7 and 4.4 kb).

of the RNAs migrated at sizes less that 1 kb in length. However, a small proportion of the molecules migrated with the dsRNA viral genome (Fig. 2A, Native panel, lane I). When duplicate samples of labeled RNAs were denatured before electrophoresis, no labeled RNAs migrated with either dsRNA or ssRNA (Fig. 2a, Denatured panel, lane I). This suggests that the labeled RNA which comigrated with viral dsRNA under native conditions was non-covalently attached to dsRNA molecules. Our interpretation of these results is that a small proportion of the short labeled RNAs form an R loop in the viral genome. Denaturation then released these short labeled RNA molecules. These results also demonstrate that under the present conditions, either we are not yet able to synthesize full length viral RNAs with the viral RNA polymerase or the full length product is quickly degraded.

The presence of labeled RNA associated with the viral dsRNA suggests that the latter is the template for this reaction. To further verify this point, the labeled RNA product was used as a probe against Northern blots containing total RNA from infected or uninfected cells (Fig. 2B). The ethidium bromide stain of the non-denaturing agarose gel clearly shows the viral dsRNA (labeled V) and the viral ssRNA (labeled SS), both of which are only present in infected total RNA (1,3,4,5). When used as a probe, the labeled polymerase products only hybridized to the dsRNA viral genome (denatured after electrophoresis so that it could be blotted to nitrocellulose; ref. 5). Labeled RNA did not hybridize to the ssRNA which has been shown to be an mRNA/replication intermediate (5). Hybridization was not detected to ssRNA despite extra long exposures of the autoradiograph (data not shown) and despite the fact that under these Northern conditions, the ssRNA is transferred to nitrocellulose more efficiently than the dsRNA. This suggests that all of the labeled RNA synthesized in this reaction is the same strand as the ssRNA which is produced in viral infected cells (5) and it suggests that we are not detecting synthesis of the other strand of the viral genome.

RNA Polymerase Activity is Associated with Viral Particles

To determine if the RNA polymerase activity is present free in the cytoplasm or within viral particles, an RNA polymerase catalyzed reaction was performed and the entire reaction mixture was run on a 5–20 % sucrose gradient (Fig. 3). Roughly half of the TCA-PPi precipitable products were present at the top of the gradient whereas the other half was found in the gradient

in fractions containing viral particles (fractions 16–19). These fractions were identified as viral particles by two criteria. First, these fractions contained the GLV dsRNA genome, as determined by gel electrophoresis of each fraction (data not shown). Second, these fractions ran further down in the gradient than fractions which contained the large and small subunit rRNAs (arrow in Figure). The fractions containing the rRNAs in sucrose gradients are routinely interpreted as fractions containing 80S ribosomes (e.g. ref. 7). The fact that the fractions containing the viral dsRNA sediment faster than ribosomes suggests that we have identified the viral particle fractions. Thus, products of the RNA polymerase reaction remain associated (or become associated) with viral particles. Gel electrophoresis of the labeled products showed no difference between the RNA polymerase products isolated from the gradient fractions containing dsRNA and thus containing viral particles, and from the top of the gradient where the products have been released into the medium (both samples resembled Fig. 2A, Native panel, lane I; data not shown). Unfortunately, the RNA polymerase assay can not be performed on the collected fractions after running lysates on a sucrose gradient because the RNA polymerase activity was not recoverable from sucrose gradients. It is possible that the RNA polymerase activity from cell lysates is unstable in the storage buffer for the long periods necessary to run the gradient (6 hours at 4° C; data not shown).

The best evidence for the association of RNA polymerase activity with viral particles came from the purification of viral particles. The RNA polymerase activity found in the culture medium pelleted with the virus by ultracentrifugation (see Materials and Methods). Virus particles can be further pruified by banding in CsCl gradients (3). Virus particles purified through two CsCl gradients have RNA polymerase activity. In the first CsCl gradient, a band of virus particles can be found. In addition, a large amount of the contaminating proteins and cell debris migrate to the top of the gradient, along with a large fraction of virus particles. These trapped virus particles can be released from this protein band by sonication and recentrifugation through another CsCl gradient, where they band as would be expected of virus particles. The 'freed' virus particles contain dsRNA and RNA polymerase activity and are infective to uninfected *Giardia* cells, as were the first set of virus particles. All viral preparations pruified by CsCl gradients were stored in 50% glycerol at −20°C and retained RNA polymerase activity for at least six months (data not shown). RNA polymerase activity in these viral preparations also supports the fact that the dsRNA which purifies with these particles is being used as the template for the reaction.

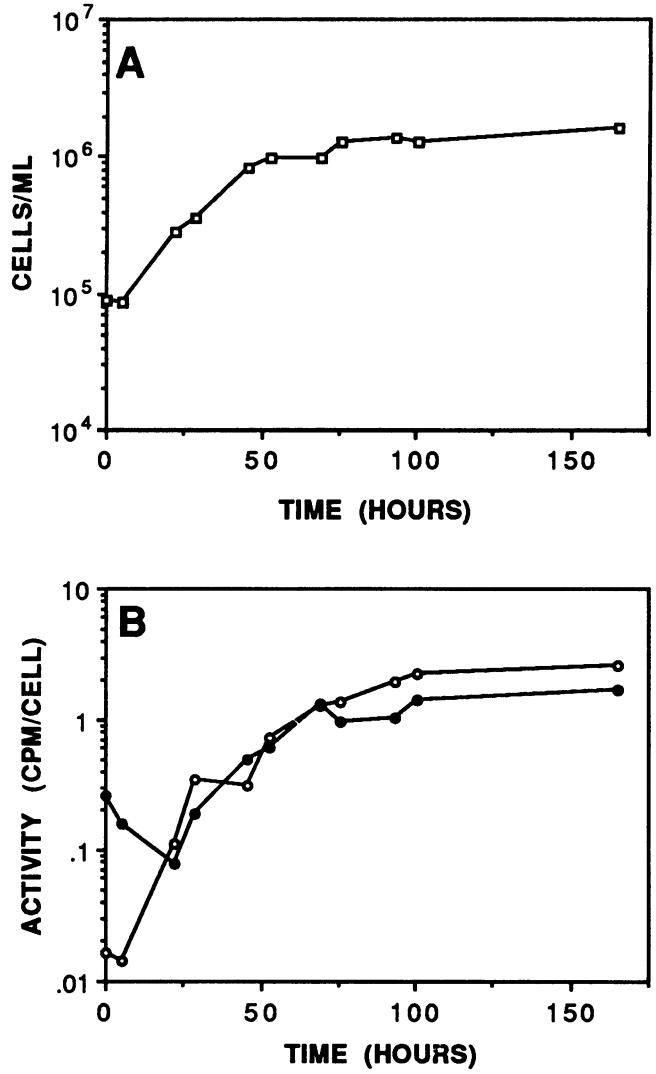

Figure 4. Time Course of RNA Polymerase Activity in Newly Infected Cells. Cells at a concentration of 10^5 cells/ml were inoculated with GLV at an M.O.I. of 1000. The cells were allowed to grow for the times indicated. At each time point, a sample was taken and the cell concentration was determined using a Coulter Counter (open squares). The cells were then pelleted, washed in PBS, resuspended and sonicated in lysis buffer, and stored at −70°C. The culture medium from each sample was filtered through a 0.22 mm filter and stored at 4°C. The cell lysates were assayed for RNA polymerase activity as described (Materials and Methods). The culture medium was assayed directly by mixing the medium 1:1 with polymerase mix containing 10 mM $MgCl_2$. The assays of the cell lysates (open circles) and the medium (closed circles) were expressed as TCA-PPi precipitable cpm per cell. The initial activity in the medium probably represents the virus added at the beginning of the infection.

RNA Polymerase Activity during GLV Infection and after Prolonged Culturing

Since GLV can infect virus-free cells, we were able to follow the level of RNA polymerase activity (and thus the amount of virus) during the course of infection (Fig. 4). The cells were infected at a density of 10^5 cells per ml. and an M.O.I. of 1000 and time points were taken for up to 170 hours. At each time point, we determined the cell density (Fig. 4A) and the RNA polymerase activity in the cells and in the culture medium (Fig. 4B). As can be seen in Figure 4B, the TCA-PPi counts per minute per cell increased 100 fold during the course of infection. Some RNA polymerase activity was initially detected in the culture medium and we assume that this activity was due to the RNA polymerase activity of the added virus. The calculated RNA polymerase activity per cell in cell lysates and in culture medium increased in a very similar pattern and plateaued at the same level after 75 hours. The level of RNA polymerase activity closely paralleled the level of viral dsRNA seen in the cells and in the medium (5; data not shown).

Finally, the RNA polymerase activities of four different samples of *Giardia lamblia* were determined after prolonged culturing (Fig. 5). As in the time course, the RNA polymerase activity in cell lysates and in viral lysates from the culture medium were very similar. Lysates from the WB cells had essentially no activity above background. Lysates from two samples of Portland 1, maintained in separate cultures for six months, had similar viral RNA polymerase activities at levels at least 100 fold higher than that in WB. In addition, a lysate from a sample of WB infected with GLV and cultured for six months had the highest

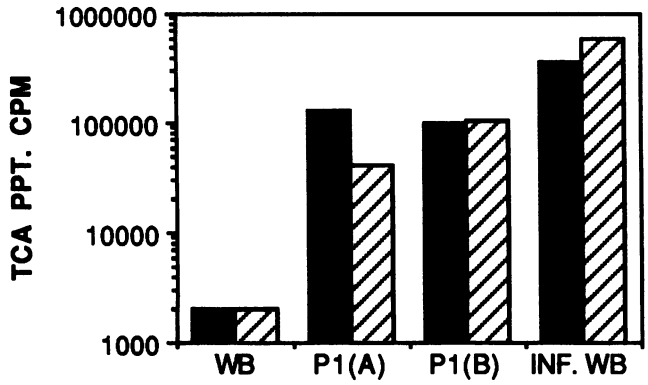

Figure 5. RNA Polymerase Activity in Long Term Cultures. Four samples of *Giardia*, which have been continuously cultured in our laboratory, were assayed for RNA polymerase activity. The samples include WB, two samples of Portland 1 [P1 (A) and P1 (B)] and a sample of WB infected with GLV approximately six months ago. For each sample, cell lysates (shaded boxes) and medium lysates (striped boxes) were prepared and assayed. The bar graphs show TCA-PPi precipitable cpm for each lysate.

RNA polymerase activity that we have been able to detect (Fig. 5). Thus the high levels of RNA polymerase activity seen after 100 hours post-infection in the time course (Fig. 4B) appear to persist through prolonged culturing of the infected cells, which is consistent with the presence and behavior of the virus in these cells (data not shown).

DISCUSSION

We have identified an RNA polymerase activity in crude cell lysates and in culture medium in which the cells have grown. This polymerase activity is dependent on the amount of lysate in the assay, and the time and temperature of the reaction. It requires divalent cations and all four ribonucleoside triphosphates; and it is inhibited by pyrophosphate and by molecules which interculate with double-stranded nucleic acids and interfere with polymerase activity (Table 1 and Fig. 1). The RNA product of this reaction corresponds to one strand of the GLV genome, the same strand that is present as ssRNA in the cytoplasm of GLV infected cells (Fig, 2, Table 2, and ref. 5). However, the products of the reaction are less than 1 kb. in length, either because these crude lysates contain a large amount of RNAse activity or because, under the present experimental conditions, one of the substrates of the reaction is limiting.

The RNA polymerase activity appears to be specifically associated with viral particles since the activity is present in extensively purified viral preparations and since almost half of the reaction products cosediment with viral particles (Fig. 3). Unfortunately, several attempts to recover RNA polymerase activity *after* sucrose gradient centrifugation of the crude lysates have been unsuccessful. It is possible that the polymerase activity from cell lysates is unstable in sucrose after long periods of time, that sucrose inhibits the activity or that the centrifugation releases factor(s) necessary for polymerase activity.

In many respects, the GLV virus of *Giardia* is highly analogous to the mycoviruses (15), of which the best studied is the L-A dsRNA virus of *Saccharomyces cerevisiae* associated with the killer phenotype (reviewed in 16, 17, 18, 3, 4, 5). Both GLV and L-A are dsRNA viruses which contain one RNA molecule per virus particle. In both cases, the virus particles are composed of predominantly one major capsid protein. Neither virus appears to adversely effect the growth of the host. Both viruses produce a ssRNA representing one strand of the viral genome. And we have now described an RNA polymerase activity with similar characteristics to the RNA polymerase of the L-A viruses of yeast (6, 7, 8, 19, 20, 21).

Replication of the L-A virus of yeast (reviewed in 17, 18) is similar to the replication of reovirus in mammalian cells (22). The dsRNA viral genome remains within the viral particles in the host cytoplasm. An RNA dependent RNA polymerase transcribes one of the strands of the dsRNA by conservative replication (23), producing a (+) strand which is released into the cytoplasm. This (+) strand then serves as the mRNA, producing the major capsid protein and the RNA dependent RNA polymerase. The polymerase is produced by frame shifting the coding sequence of the major capsid protein so that it continues into a second open reading frame (24, 25, 26). The polymerase molecule and the major capsid proteins then assemble a viral particle around a (+) strand RNA and the (−) strand is then synthesized within the new viral particle. The polymerase activity which synthesizes the (+) strand is referred to as a transcriptase and the activity which synthesizes the (−) strand is referred to as a replicase. However, it is clear that the RNA polymerase molecule is responsible for both activities and that the different syntheses depend on the components in the viral particle at that time.

GLV appears to have a similar replication cycle. A ssRNA is overexpressed in the cytoplasm of GLV infected cells. This RNA appears to be a full length copy of one strand of GLV and this ssRNA is synthesized with the kinetics one would expect for a replication intermediate or an mRNA from the virus (5). By analogy to the yeast system, this ssRNA may be the (+) strand synthesized by the transcriptase activity of the RNA polymerase.

This paper extends these observations by identifying the transcriptase activity of GLV which has similar properties to the yeast transcriptase. The GLV transcriptase only synthesizes one strand of the genome, the same strand as the ssRNA released in to media. *In vitro*, this transcriptase product appears to be released from the viral particles into the reaction buffer, at least to some extent (top of the gradient in Fig. 3).

We have not yet been able to identify the replicase activity of the polymerase since we have only detected labeled RNAs which represent the same strand of the genome as the ssRNA intermediate (Fig. 2B). Furthermore, in the crude fractions that we have used, new viral particles with only (+) strands would not be present in large enough amounts to be detectable. Therefore, further purification of empty or light viral particles may be necessary to detect the replicase activity of this RNA polymerase.

The one major difference between GLV and the L-A viruses of yeast is that GLV is extruded into the culture medium and can be used to infect uninfected *Giardia* cells. During the course of infection, the virus and the viral RNA polymerase activity in the cells and in the culture medium are very similar (Fig. 4 and ref. 5). The levels of polymerase activity closely parallel the amount of dsRNA during infection (4, 5). The polymerase activity does not match the levels of ssRNA in the cell, which peaks between 24 and 48 hours after infection (5). Furthermore, the presence of viral RNA polymerase activity in the culture medium suggests that mature viruses, capable of infection, contain all of the components necessary for transcriptase activity.

Our main interest in GLV is its potential for development as a transformation system in *Giardia* since efficient long-term transfection of parasitic protozoa is not yet possible (e.g. ref. 27). Before developing such a transformation system, we must

have a thorough knowledge of the replication cycle of GLV. We have now identified a ssRNA intermediate (5) and an RNA dependent RNA polymerase (this paper) which should allow us to produce full length cDNAs of the viral genome.

After modification, these RNAs may be incorporated into viral particles using the RNA polymerase activity. Recently, Fujimura and Wickner have shown that low salt incubations of viral particles releases endogenous viral genomes and allows the addition and subsequent replication of exogenous templates (14, 28). Similar experiments using GLV will be the next step towards developing a GLV transformation system in *Giardia.*

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