MOLECULAR AND CELLULAR BIOLOGY, July 1990, p. 3659–3663
0270-7306/90/073659-04$02.00/0

Vol. 10, No. 7

Transfection of the *Giardia lamblia* Double-Stranded RNA Virus into *Giardia lamblia* by Electroporation of a Single-Stranded RNA Copy of the Viral Genome

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Received 13 February 1990/Accepted 24 April 1990

The development of a genetic vector for protozoan parasites is a major hurdle yet to be crossed in the study of the molecular and cellular biology of these parasites. We have identified and isolated a double-stranded RNA virus (*G. lamblia* virus [GLV]) from certain strains of the intestinal parasitic protozoan *Giardia lamblia* (A. L. Wang and C. C. Wang, Mol. Biochem. Parasitol. 21:269–276, 1986), which is capable of infecting other virus-free strains of *G. lamblia* (R. L. Miller, A. L. Wang, and C. C. Wang, Exp. Parasitol. 66:118–123, 1988). Here we demonstrate that *G. lamblia* can be infected with GLV by electroporating uninfected cells with purified single-stranded RNA (E. S. Furfine, T. C. White, A. L. Wang, and C. C. Wang, Nucleic Acids Res. 17:7453–7467, 1989) representing a full-length copy of one strand of the GLV double-stranded RNA genome. To the best of our knowledge, this is the first demonstration in vivo that a single-stranded RNA is a competent replicative intermediate for this class of double-stranded RNA virus. In addition, this result represents the first long-term transfection of a protozoan by a single species of RNA and will hopefully expedite the development of GLV as a genetic transfecting vector.

Giardia lamblia, an intestinal parasite, is responsible for many cases of debilitating and chronic diarrhea worldwide (12). As much as 7% of the U.S. population are asymptomatic carriers (12). Like that of most protozoan parasites, the molecular and cellular biology of this organism is not well understood, largely because of the lack of a permanent transfecting vector. We have identified and isolated a double-stranded (ds) RNA virus (*G. lamblia* virus [GLV]) from certain strains of the intestinal parasitic protozoan (15), which resembles the *Saccharomyces cerevisiae* dsRNA killer virus and many fungal dsRNA viruses (for a review, see references 3 and 17) both structurally and in its apparent replication mechanism. The purified virus is capable of infecting other virus-free strains of *G. lamblia* (11). Subsequent studies of virus-infected *G. lamblia* cells have led to the identification of a single-stranded (ss) RNA representing a full-length copy of one strand of the GLV dsRNA genome (5), but no DNA species were observed to be homologous to the 7-kilobase viral genome in the infected cells (15). Recently, RNA viruses have also been observed in other protozoan parasites, such as *Trichomonas vaginalis* (14) and *Leishmania braziliensis* (13). The results of RNA electroporation described in this paper may expedite the development of a genetic vector in *Giardia* spp., as well as in these other organisms.

MATERIALS AND METHODS

Electroporation and RNA analysis. Trophozoites of virus-free *G. lamblia* WB were grown to confluency and harvested by centrifugation (15). The cells were washed three times and suspended (10^7/ml) in phosphate-buffered saline. The cell suspension (0.8 ml) was incubated for 10 min on ice with the total RNA (0.5 mg/ml), extracted from either the WB strain or the virus-infected Portland I (PI) strain, and then electroporated (Bio-Rad Laboratories gene pulser apparatus) at 2.5 kV, 25 mF, 400 Ω, and a 0.6 time constant in a 0.2-cm electrode gap cuvette. The cells were incubated for an additional 15 min on ice after electroporation and transferred to 13 ml of the culture medium. Control samples were treated similarly but without electroporation. Experiments were done in duplicate. The cells were grown to confluence (about 3 days) and passed at a 1:12 dilution seven times. Cultures were harvested, and total RNA was phenol extracted from the cells and electrophoresed in 0.8% agarose (5).

ssRNA preparation and electroporation. To isolate the ssRNA copy of the GLV genome, PI total RNA (2 mg) was electrophoresed in a 0.8% agarose gel (14 by 10 by 0.5 cm) in Tris-borate-EDTA buffer (TBE). Gel slices which contained ssRNA were excised, and the RNA was eluted from the slice by the Schleicher & Schuell, Inc., Elutrap with 100 mg of *S. cerevisiae* tRNA as the carrier. The WB cells were electroporated with ssRNA (25 ng) plus 25 mg of tRNA carrier under the conditions described above.

RESULTS

Total RNA was prepared from the cloned PI and WB strains of *G. lamblia* (5). Our PI strain was infected with GLV so that the total RNA contained both the 7-kilobase dsRNA viral genome and the ssRNA copy of the genome, whereas WB was not infected but was susceptible to infection by GLV (11). WB cells were electroporated in the presence of PI or WB total RNA, whereas WB cells incubated with the PI RNA without electroporation were used as a control (Fig. 1A and B). After the electroporation (or incubation) with RNA, the cells were transferred to the standard culture medium and grown until confluent. Each serial transfer resulted in a 13-fold increase in cell number after confluence was reached. The confluent cultures were

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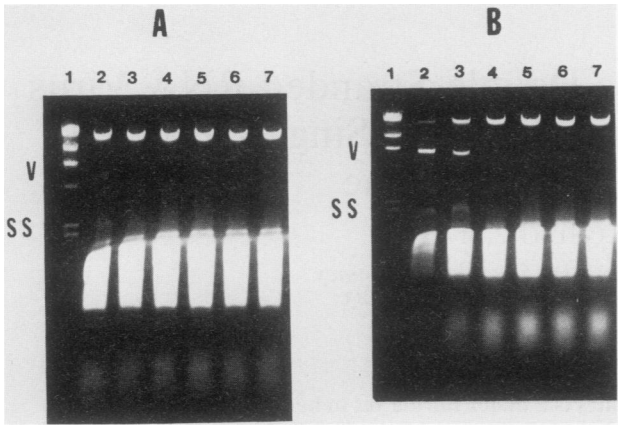

FIG. 1. Total RNA extracted from electroporated and nonelectroporated WB cells. Photographs of the ethidium bromide-stained agarose gel are shown. The cells were electroporated and serially transferred as described in Materials and Methods. The third (A) and eighth (B) cultures were harvested. The total RNA was extracted from the cells and electrophoresed in 0.8% agarose. Lanes 1, *Hin*dIII-cut λ phage DNA; lanes 2 and 3, PI RNA, electroporation; lanes 4 and 5, PI RNA, no electroporation; lanes 6 and 7, WB RNA, electroporation. V, dsRNA; SS, ssRNA.

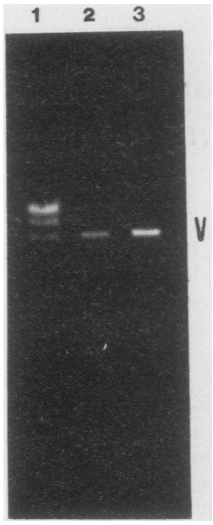

FIG. 2. GLV in filtered culture medium of *G. lamblia* WB cells transfected with PI total RNA by electroporation. The filtered culture medium from the duplicate WB cultures (serially transferred for the eighth time) which were electroporated with PI total RNA (Fig. 1) was analyzed for the presence of GLV by isolating and electrophoresing the dsRNA (5) in a 0.8% agarose gel. A photograph of the ethidium bromide-stained gel is shown. Lane 1, *Hin*dIII-cut λ phage DNA (300 ng); lanes 2 and 3, RNA from the filtered medium of the duplicate cultures. V, dsRNA

serially passaged seven consecutive times. The third (Fig. 1A) and eighth (Fig. 1B) culture samples were harvested, and the total RNA from these samples was isolated and electrophoresed in an agarose gel (5). Analysis for the presence of the GLV dsRNA genome and the ssRNA genome copy in the total RNA extracts (Fig. 1) indicated that only WB cells electroporated with PI total RNA became infected with GLV. WB cells electroporated with WB total RNA or merely incubated with PI total RNA did not lead to the appearance of the dsRNA or the ssRNA in the electroporated WB cells upon subsequent cultivations. The total RNA extracts prepared from the eighth culture of the electroporated WB cells produced similar results. These data suggest that the observed GLV infection was as stable as that observed in the original PI strain (11). Since the cells which were incubated with PI total RNA without electroporation did not become infected after seven transfers, it seems unlikely that the electroporated RNA extracts could have contained a small number of intact GLV particles responsible for the infection. To determine whether the cells were indeed infected by GLV after electroporation, the culture medium of the electroporated cells was analyzed for the presence of virus. Figure 2 shows the presence of the dsRNA in the filtered culture medium in which the PI RNA-electroporated WB cells were grown. This dsRNA could not be due to a carry-over from the dsRNA in the total RNA of the PI extract used for electroporation, because the RNAs are diluted away during the serial transfers. Therefore, the data suggest that the electroporation of WB cells with PI RNA resulted in the observation of GLV inside the cell as well as in the growth medium (as determined by the presence of the dsRNA), whereas the ssRNA was found only inside the cell, as in the original PI isolate (5, 10, 11). In addition, the results obtained with the electroporated cells are comparable to the results obtained when authentic GLV is used to infect uninfected WB cells (10). Although complete growth studies to monitor the production of virus inside and outside the cell after the transfection experiments were not performed, it is unlikely that the ssRNA could ever be observed in the culture medium at any time, according to our previous studies (5).

It is difficult to determine the frequency of transformation of the cells, because there is not a satisfactory method for examining clonal populations of *Giardia* spp. It is likely that the transformation efficiency is very low. However, the reproducibility of the transformation was shown by performing this experiment three times without significant deviation in the results.

We did not attempt to purify the viruses from the culture medium of a transfected cell line and use them to infect WB cells. However, it is highly likely that these virus particles are infective, since infectivity of freed virus particles appears to be a requirement for the maintenance of an infected cell line (5). This requirement for infectivity is suggested by the fact that infected cell lines do not have a selective advantage over uninfected cell lines, since their growth rates are very similar (10).

Next, we determined the specific nucleic acid requirement for GLV infection by electroporation. The ssRNA copy of the GLV genome was purified from PI total RNA by agarose gel electrophoresis and electroelution of the gel slice containing the ssRNA (Fig. 3). WB cells were electroporated in the presence of the purified ssRNA and tRNA carrier or incubated just with the RNA mixture without electroporation. After growth and passage of the electroporated cells, total RNA was extracted from these cultured cells and electrophoresed in an agarose gel (Fig. 3). Only the WB cells electroporated with the purified ssRNA copy of the GLV genome became infected with GLV. Those cells incubated simply with the ssRNA did not become infected. In addition, cells electroporated with purified dsRNA or dsRNA denatured with 10% formamide at 90°C or a combination of native and denatured dsRNA did not become infected with GLV (data not shown). Therefore, the only nucleic acid required to transfect WB cells with GLV by electroporation is the

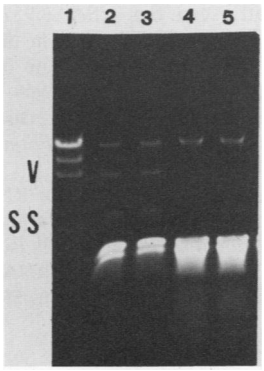

FIG. 3. Electroporation of WB cells with purified ssRNA (SS). A photograph of the ethidium bromide-stained agarose gel is shown. Lane 1, *Hin*dIII-cut λ phage DNA (300 ng); lanes 2 and 3, total RNA of WB cells which were electroporated with the ssRNA; lanes 4 and 5, total RNA from WB cells which were incubated but not electroporated with ssRNA. V, dsRNA.

ssRNA copy of the dsRNA viral genome under the present experimental conditions (see below).

DISCUSSION

We have described a method of infecting *G. lamblia* with GLV by electroporation with an isolated ssRNA copy of the viral genome. The fact that the ssRNA alone is capable of transfecting cells by electroporation confirms the evidence for our hypothesis (5) that the ssRNA is both a viral replication intermediate and the message strand of the viral genome. Since many yeast and fungal dsRNA viruses are similar to GLV (3, 17), it is interesting to note that previous in vitro studies also indicated that the ssRNAs of those viruses are replicative intermediates and message strands of the viral genomes (2, 4, 6). The fact that *G. lamblia* cells electroporated with purified ssRNA can become infected with GLV may be the first in vivo demonstration that the viral ssRNA is the competent replicating intermediate and message strand for this class of dsRNA viruses.

The data also suggest that the denatured dsRNA is not a viable transfecting vector. Since this class of virus requires a virally encoded RNA-dependent RNA polymerase for replication (2), it seems likely that after entry of the ssRNA by electroporation, the first requirement for a successful infection is the translation of the ssRNA to produce the polymerase and required proteins for viral replication. In the case of the denatured dsRNA, the message strand might not be translated efficiently, because of its partial or complete reannealing to its complementary strand which is still present in the mixture. However, it is possible that some further modifications of the denaturation conditions may allow the denatured mixture to become a viable transfection vector.

We have identified a GLV RNA-dependent RNA polymerase activity (16) similar to that described for the yeast virus (2). The electroporation results strongly suggest that the ssRNA must code for this polymerase activity. However, although the ssRNA was purified by agarose gel electrophoresis, we cannot completely exclude the possibility that some contaminating viral protein or a small amount of the dsRNA, which may be somehow copurified with the ssRNA, was partially responsible for the infection process. To ensure that only the ssRNA is required, we need a full-length cDNA copy of the viral genome. Such a clone will enable us to make an ssRNA copy of the viral genome in vitro with bacteriophage RNA polymerases and perform the transfection without the contamination of any GLV nucleic acids or proteins. So far, our efforts to obtain a full-length cDNA have yielded 1.6 kilobases of the viral genome as a cDNA copy (A. L. Wang, M. Wang, and C. C. Wang, unpublished results).

Recently, Bellofatto and Cross (1) temporarily expressed a foreign gene in *Leptomonas seymouri*, and Laban and Wirth (9) temporarily expressed a chloramphenicol acetyltransferase gene in *Leishmania enriettii*. Even more recently, Laban et al. (8) and Kapler et al. (7) succeeded in performing stable expression of foreign genes in *Leishmania* spp. via electroporation with artificial DNA constructs. Infections of *G. lamblia* with GLV are stable. The ssRNA copy of the GLV viral genome is a competent molecule for the stable transfection of the WB cells and the production of GLV. Given the ease with which an ssRNA copy of recombinant DNAs can be generated and the ease with which a cloned gene can be genetically manipulated, GLV and other dsRNA viruses of the protozoan parasites (13, 14) are promising candidates for future development as genetic transfecting vectors in these organisms. In vitro transcripts of a DNA copy of the viral promoter plus a foreign gene may be introduced via electroporation into the cells already infected with the virus, and the foreign gene is expected to be expressed. The RNA stability problems originally associated with RNA-transfecting vectors seem less impeding, given these new technologies.

ACKNOWLEDGMENTS

We thank Andrew Peterson and Doris Yang for their technical assistance with the electroporation apparatus, Ted White for helpful criticism of the procedures and manuscript, and Heather Wilkie for help with the preparation of the manuscript.

This work was supported by Public Health Service grant AI 19391 from the National Institutes of Health.

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