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In vivo determination of the *gap2* gene promoter activity in *Giardia lamblia*

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Abstract: A shuttle vector for *Escherichia coli* and *Giardia lamblia* was modified to produce a reporter plasmid, which monitors the expression of prescribed gene in *G. lamblia* by measuring its luciferase activity. Promoter regions of the *gap2* gene, one of the genes induced during encystation, were cloned into this plasmid, and the resultant constructs were then transfected into trophozoites of *G. lamblia*. Transgenic trophozoites containing one of the 3 *gap2-luc* reporters were induced to encystation, and characterized with respect to *gap2* gene expression by measuring their luciferase activities. Giardia containing a *gap2-luc* fusion of 112-bp upstream region showed full induction of luciferase activity during encystation.

Key words: *Giardia lamblia*, *gap2* gene expression, encystation, luciferase, transfection

INTRODUCTION

Giardia lamblia, a protozoan pathogen which causes a diarrheal disease in man, has a simple life cycle that is composed of 2 forms, trophozoites and cysts. Upon ingestion of the cysts, its infection is initiated and this is followed by excystation to the trophozoite form usually in the proximal duodenum of the host. Trophozoites are able to multiply by binary fission and colonize the proximal small intestine using adhesive discs. Some trophozoites undergo encystation, i.e., differentiation of trophozoites to the cyst form, in the distal small intestine. The distinct structural differences between these 2 forms imply that a series of genes are differentially expressed during these differentiations.

Previous studies on the encystation of *G. lamblia* identified several encystation-induced genes such as *cwp1*, *cwp2* (Mowatt et al., 1995), *bip/grp78* (Lujan et al., 1996), and *enc1* and *enc6* (Que et al., 1996). Knodler et al. (1999) reported that the expression of *gln6pi-b* for glucose-6-phosphate isomerase was also induced during encystation. In addition, the expression of the *myb2* gene encoding a putative transcriptional factor was found to be induced during encystation (Sun et al., 2002; Yang et al., 2003). Investigation of the 2 *gap* genes, *gap1* and *gap2* of *G. lamblia* (Rozario et al., 1996) indicated that Gap1 protein functions as a major glycolytic enzyme whereas *gap2* does not seem to encode a protein with GAPDH activity (Yang et al., 2002). Despite the isolation of the *gap2* gene as an induced clone in a differential display of encysting cells of *G. lamblia*, no detailed examination has been performed on its expression and function. In the present study, the expression of the *gap2* gene was monitored using

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Table 1. Oligonucleotides used in this study

Primer name and nucleotide sequences
Replacement of the *gfp* gene by the *luc* gene
LUC-F: 5′-GTAACCATGGCATTCCGGTACTGTTG-3′- *Nco*I site underlined
LUC-R: 5′-GAATGCGGCCGCATTTTACAATTTGGACTTTCC-3′- *Not*I site underlined
Construction of *gap2-luc* fusions
*gap2*P-F1: 5′-CCCAAGCTTGCGTAGATCTCCTCCACGGA-3′- *Hin*dIII site underlined
*gap2*P-F2: 5′-GCGCAAGCTTCGCTCCAGCGTTTCTCTTG-3′ - *Hin*dIII site underlined
*gap2*P-R: 5′-GCGCCCATGGCTAATTAGAGTGTTTATTTC-3′ - *Nco*I site underlined
*gap2*P-F3: 5′-AGCTTATTACACTAAAACAGGTTGGGGAAATAAACACTCTAATTAGC-3′ - *Nco*I site underlined and *Hin*dIII site double-underlined
*gap2*P-R3: 5′-CATGGCTAATTAGAGTGTTTATTTCCCCAACCTGTTTTAGTGTAATA-3′ - *Nco*I site underlined and *Hin*dIII site double-underlined

transgenic *G. lamblia* carrying a *gap2-luc* reporter system.

MATERIALS AND METHODS

Organisms and culture conditions

Trophozoites of *G. lamblia* WB strain (ATCC 30957, Washington DC., U.S.A.) were grown for 72 hr in normal TYI-S-33 medium (2% casein digest, 1% yeast extract, 1% glucose, 0.2% NaCl, 0.2% L-cysteine, 0.02% ascorbic acid, 0.2% K_2HPO_4, 0.06% KH_2PO_4 and 10% calf serum) (Keister, 1983) containing 0.5 mg/ml bovine bile at pH 7.1, and then transferred into an encystation medium containing 10 mg/ml of bovine bile at pH 7.8 (Kane et al., 1991). Portions of the encysting cells were sampled at various times by chilling on ice and centrifugation.

Construction of plasmids containing a *luc* reporter gene

To obtain a reporter system to allow us quantitative measurement of activity of the *gap2* gene promoter in *G. lamblia*, we made a new vector based on pGFP.pac (Singer et al., 1998). A 1,682-bp DNA fragment containing the full ORF of the *luc* gene was amplified from pGL2-Basic vector (Promega, Madison, U.S.A.). by PCR using 2 primers, LUC-F and LUC-R (Table 1). An *Nco*I and a *Not*I site located at either ends of the resultant *luc* DNA were used to clone this DNA into the corresponding sites of pGFP.pac, which resulted in plasmid pLUC.pac, in which the *gfp* gene was replaced by the *luc* gene (Fig. 1).

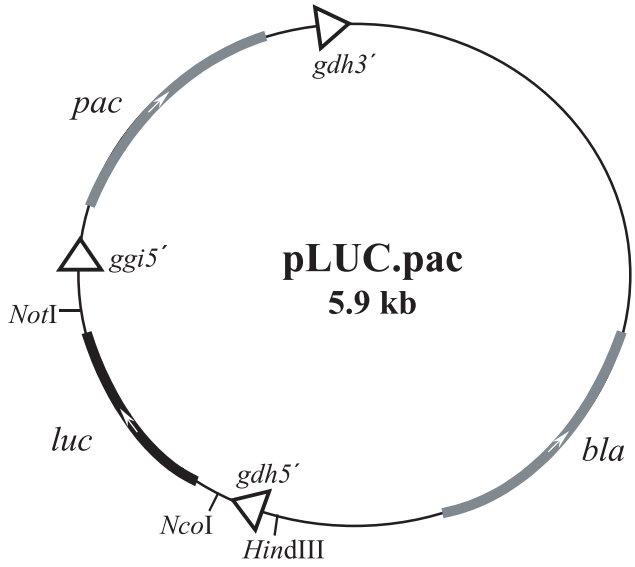

Fig. 1. Construction of pLUC.pac. The *gfp* gene of a shuttle vector of *E. coli* and *G. lamblia*, pGFP.pac (Singer et al., 1998), was replaced by a 1,682-bp *luc* DNA fragment of pGL2-Basic.

Based on the sequence published by Rozario et al. (1996) (GenBank database accession number, U31911), 3 different DNA regions of the *gap2* promoter were amplified and cloned into pLUC.pac (Fig. 2A). First a

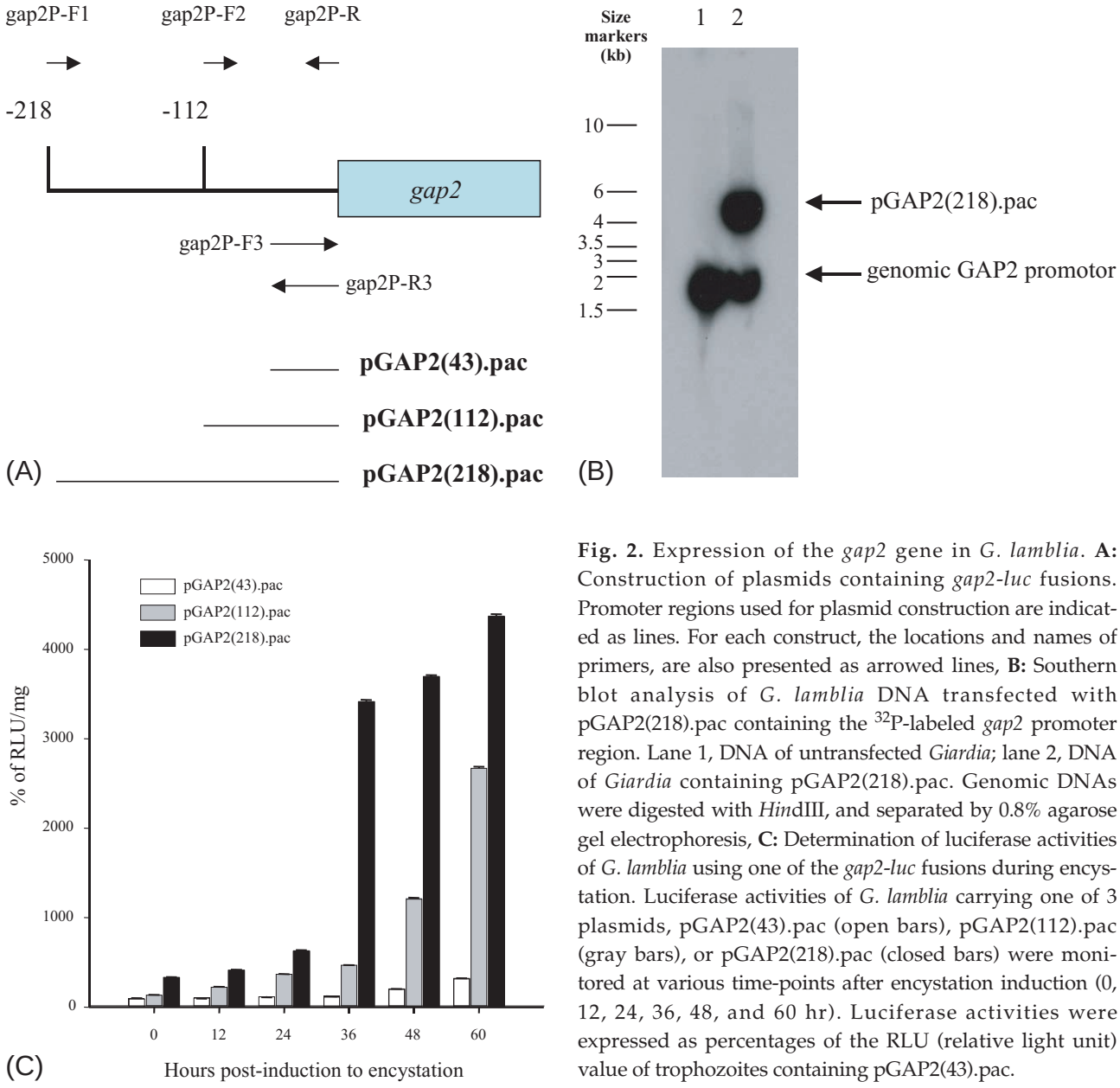

Fig. 2. Expression of the *gap2* gene in *G. lamblia*. **A:** Construction of plasmids containing *gap2-luc* fusions. Promoter regions used for plasmid construction are indicated as lines. For each construct, the locations and names of primers, are also presented as arrowed lines, **B:** Southern blot analysis of *G. lamblia* DNA transfected with pGAP2(218).pac containing the ^{32}P-labeled *gap2* promoter region. Lane 1, DNA of untransfected *Giardia*; lane 2, DNA of *Giardia* containing pGAP2(218).pac. Genomic DNAs were digested with *Hin*dIII, and separated by 0.8% agarose gel electrophoresis, **C:** Determination of luciferase activities of *G. lamblia* using one of the *gap2-luc* fusions during encystation. Luciferase activities of *G. lamblia* carrying one of 3 plasmids, pGAP2(43).pac (open bars), pGAP2(112).pac (gray bars), or pGAP2(218).pac (closed bars) were monitored at various time-points after encystation induction (0, 12, 24, 36, 48, and 60 hr). Luciferase activities were expressed as percentages of the RLU (relative light unit) value of trophozoites containing pGAP2(43).pac.

218-bp region of the DNA upstream of the *gap2* gene was amplified from the genomic DNA of *G. lamblia* using 2 primers, *gap2*P-R and *gap2*P-F1 (Table 1), and inserted into pLUC.pac pretreated with *Hin*dIII and *Nco*I, which resulted in a deletion of the *gdh* promoter. The second promoter studied was a 112-bp DNA upstream region of the *gap2* gene, which was made from the genomic DNA of *G. lamblia* with the primers, *gap2*P-R and *gap2*P-F2. The third *gap2-luc* reporter containing a 43-bp upstream region of ATG of the *gap2* ORF was constructed by cloning the annealed linker of 2 primers, *gap2*P-F3 and *gap2*P-R3, into the *Hin*dIII and *Nco*I site of pLUC.pac.

Transfection

Trophozoites were grown for 72 hr in normal TYI-S-33 medium. Fifteen μg of a *gap2-luc* reporter plasmid was transformed into 1 X 10^7 trophozoites by electroporation under the following conditions; 350 volts, 1,000 μF, and 700 Ω (Biorad Genepulser II, Hercules,

U.S.A.). Trophozoites harboring a *gap2-luc* plasmid were selected by adding puromycin to the TYI-S-33 medium to a final concentration of 100 μM.

Determination of luciferase activities

Luciferase activities of the harvested cells carrying one of the *gap2-luc* reporter plasmids were measured using a Luciferase Assay System (Promega). Briefly, collected cells were resuspended in a lysis buffer (25 mM Tris-HCl, pH 7.8, 2 mM EDTA, 2 mM DTT, 10% glycerol, and 1% Triton X-100), and frozen at -70°C at least for 1 hr Twenty μl of cell extracts were reacted with 100 μl of luciferase substrate, and light emission was measured for 5 min in a luminometer (TD20/20 DLReady, Turner Designs, Sunnyvale, U.S.A.).

Southern blot analysis

Genomic DNAs were purified from untransfected trophozoites as well as from trophozoites transfected with pGAP2(218).pac. Ten μg of genomic DNA was digested with *Hin*dIII and loaded into a 0.8% agarose gel. Upon separation by electrophoresis, digested genomic DNA was transferred to a nytran filter (Millipore, Billerica, U.S.A.), and fixed to the filter by UV-crosslinking (Hoefer, San Francisco, U.S.A.). The filter was then hybridized using the 218-bp *gap2* promoter region, which was labeled with ^{32}P by using a Random labeling kit (Takara, Otsu, Japan).

RESULTS

Construction of 3 *gap2-luc* reporter systems

We used the luciferase reporter system to examine the expression of the *gap2* gene, which has been identified to be induced during encystation (Yang et al., 2002). To define the essential *cis*-acting elements required for the full expression of the *gap2* gene, 3 different *gap2-luc* fusions were constructed, which contained *gap2* gene promoter regions of different sizes, as described in Fig. 2A.

Confirmation of transgenic *G. lamblia* containing a *gap2-luc* reporter plasmid

All of the 3 *gap2-luc* constructs were transfected into trophozoites as described above. For each construct, 2 independent transfectants were selected, and used for further studies. Maintenance of the *gap2-luc* reporter in puromycin-resistant *G. lamblia* was verified by Southern blot analysis (Fig. 2B). Genomic DNA of untransfected trophozoites displayed a band of ~1.7 kb in Southern blots with a *gap2* promoter region. In the case of genomic DNA of trophozoites transfected with pGAP2(218).pac, an additional band of 5.8 kb DNA was found in addition to the ~1.7 kb DNA band, which demonstrated that pGAP2(218).pac was maintained in puromycin-resistant *G. lamblia* clones.

Determination of the expression of the *gap2* gene in *G. lamblia*

For each *gap2-luc* construct, 2 independent transfectants were selected and their luciferase activities were measured. After establishing them as stable clones, the transfectants were induced to encystation by cultivating them in TYI-S-33 medium with high bovine bile at alkaline pH. At 5 different time-points of post-encystation, i.e, 12, 24, 36, 48, and 60 hr, portions of encysting cells were harvested.

In the trophozoite form, the 3 *gap2-luc* fusions displayed differential luciferase activities (Fig. 2C). Transfectants carrying pGAP2(43).pac showed lower luciferase activities than those of trophozoites carrying 1 of the other 2 constructs, pGAP2(112).pac and pGAP2(218).pac. Trophozoites of *G. lamblia* carrying pGAP2(112).pac showed 40% more luciferase activity than those carrying pGAP2(43).pac. Trophozoites carrying pGAP2(218).pac showed the highest luciferase activity, namely, over 3 fold higher than that of *Giardia* trophozoites containing pGAP2(43).pac.

Upon encystation, *Giardia* containing these 3 constructs showed significant increases in luciferase activity, even though the fold increases were different for each construct. Transfectants carrying pGAP2 (43).pac, also showed higher luciferase activities during encystation than that of trophozoites, i.e., gradual increase of up to 3-fold. *Giardia* containing pGAP2 (112).pac also showed a gradual increase in luciferase activity as cells proceeded to encystation (20-fold). *G. lamblia* carrying pGAP2(218).pac also displayed a dra-

matic increase in luciferase activity upon encystations; a 13-fold increase at 60 hr post-induction.

DISCUSSION

Despite the putative role of glyceraldehydes 3-phosphate dehydrogenase implied from the amino acid sequences of the *gap2* ORF, no enzymatic activity was demonstrated in the previous study (Yang et al., 2002). To identify the role of this putative *gap* gene, we confirmed encystation-induced expression of the *gap2* using a luciferase reporter system. There is no information on the *cis*-acting elements required for transcription in *G. lamblia* at the present level of knowledge. Therefore, we randomly constructed 3 *luc* fusions containing different sizes of the *gap2* promoter, i.e., 43, 112 and 218-bps.

When the constructed plasmids were transfected into *G. lamblia*, we found that the efficiency of transfection was very low, i.e., below 10^{-7}. Therefore, an experiment to detect the presence of the *gap2-luc* reporter in tranfected *G. lamblia* was performed to exclude a possibility that we could select puromycin-resistant clones of *G. lamblia* during transfection (Fig. 2B).

Luciferase activities of 3 different *gap2-luc* fusions clearly showed that 43-bp upstream region of the initiation codon of the *gap2* ORF did not contain all the essential elements required for expression of the *gap2* gene (Fig. 2C). In addition, the 112-bp upstream region of the *gap2* gene seemed to have *cis*-acting information for the full induction of this gene during encystation.

In this study, we constructed a reporter system in *G. lamblia*, which allows the quantitative measurement of the expression of a prescribed gene. This system was applied to monitor the expression of *gap2*, which was identified as one of the genes induced during encystation. Using reporter constructs containing different regions of the *gap2* promoter, we defined the *cis*-acting elements required for full induction of the *gap2* gene during encystation.

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