Quantitative Analysis of Transcripts from Two Zygote-specific *zys1* Genes in *Chlamydomonas reinhardtii*

Hidehito Uchida^{1,*} and Yoshiaki Hara^{1,2}

¹ Regional Joint Research Project of Yamagata Prefecture, Yamagata Public Corporation for the Development of Industry R and D Department, Yamagata 990–2473, Japan

² Department of Biology, Faculty of Science, Yamagata University, Yamagata 990–8560, Japan

Accepted January 18, 2001

Summary Primer extension analysis using 10 µg of polyA RNA extracted from zygotes of *Chlamydomonas reinhardtii* 10 min after mating was carried out to elucidate relative amounts of transcripts of early zygote-specific (*zys1*) genes. A novel minor extension product as well as a major product for each of the *zys1A* and *zys1B* genes was detected. Quantification of band intensity showed that relative accumulation amounts of *zys1A* major, *zys1A* minor, *zys1B* major and *zys1B* minor transcripts were in the ratio of 1.78 : 0.171 : 1.00 : 0.730. This result suggests that the *zys1A* promoter is slightly stronger than that of *zys1B* at an initial stage of gene expression.

The unicellular green alga *Chlamydomonas reinhardtii* has been widely used as a model organism. Because of the large amount of accumulated mutants and the well established mating system (Harris 1989), this alga has been widely used for genetic and biotechnology studies. Nuclear transformation of the alga was first attained by rescuing auxotrophic mutants with corresponding wild-type genes (Debuchy *et al.* 1989, Kindle *et al.* 1989). The introduction of drug resistance markers to wild-type strains of *C. reinhardtii* further developed the transformation system (Stevens *et al.* 1996, Cerutti *et al.* 1997a). Though foreign genes integrated into the nuclear genome, they were often not expressed (Cerutti *et al.* 1997b). To overcome this problem, codon bias change (Fuhrmann *et al.* 1999), or intronic enhancer was adopted (Lumbreras *et al.* 1998).

Another strategy that would aid in efficient expression of transgenes, for example reporter genes, would be to drive expression of these genes with an inducible promoter, which would be silent during genome integration, and would respond only to external stimuli. Such candidate promoters include the promoters of zygote-specific genes, carbonic anhydrase genes, or heat shock protein genes. Of these candidates, zygote-specific promoter is easily induced and the corresponding stimulus does not damage cells or gene products. The zygote-specific genes named as *zys1* to *zys4* have been isolated from zygotes 10 min after mating (Uchida *et al.* 1993). Though quantitative analysis of *zys1*, *zys2*, *zys3* and *zys4* transcripts is yet to be analyzed, the *zys1* transcripts (expression of *zys1A* and *zys1B* genes is similar in temporal pattern after mating) accumulate earliest of all the *zys* gene transcripts. A foreign gene product driven by *zys1* promoter, if successfully expressed, might be modified by transgenes expressed using the other later expressing *zys* promoters.

In order to estimate which of *zys1A* and *zys1B* promoter is suited better for driving foreign gene, quantitation of 2 endogenous *zys1* gene transcripts was performed using polyA RNA which was extracted from young zygotes formed in highly synchronized mating. In this study, we show that a novel minor band and a major band in extension products for each *zys1* gene were detected using 10 µg of polyA RNA that was isolated from cells of *C. reinhardtii* 10 min after mating. Relative amounts of each product were also determined from intensity of bands on an autoradiogram.

* Corresponding author, e-mail: uchida@ckk.ymgt-techno.or.jp

Materials and methods

The wild-type strain 137c of *Chlamydomonas reinhardtii*, which originated from the *Chlamydomonas* Genetic Center, Duke University, was used in this study. Mating condition was the same as in a previous paper (Uchida *et al.* 1992).

PolyA RNA was isolated from cells 10 min after mixing the 2 mating types of gametes. Primer extension was performed using Primer Extension SystemTM (Promega, Madison, WI, U.S.A.). SuperScriptTM RNase⁻ Reverse transcriptase was also provided with this kit. In each extension reaction, 10 μ g of polyA RNA was used. Nucleotide sequence of the primer, which anneals to the 2 genes, is complementary to *zys1A* sequence (Genbank accession number of AB001485) at +171 to +140, and to *zys1B* sequence (Genbank accession number of AB001486) at +179 to +148. The primers specific for each gene are mentioned in the previous report (Uchida *et al.* 1999). An imaging analyzer, BAS2000 (Fuji Film, Tokyo, Japan), was used to monitor images of sequence ladders and primer extension products.

Fig. 1. Differential-interference-contrast images of cells forming zygotes. Cells before (a, b) and 1 (c, d), 2 (e), 3 (f), 5 (g), and 10 (h) min after mixing 2 types of gametes. Images (a, c) and (b, d) show mt^+ and mt^- gametes, respectively. Arrowheads and an arrow designate gametic cell walls and a fertilization tubule, respectively. Bar represents 5 μ m.

Results and discussion

Mating of *Chlamydomonas*

In a previous preliminary analysis of transcription initiation sites of *zys1* genes, polyA RNA was extracted from 10-min old zygotes (Uchida *et al.* 1999). In this analysis little attention was paid on synchronizing mating of cells from which the RNA was extracted. In the present study, in order to check synchronous mating of cells, aliquots from cell suspension mixture were withdrawn successively and morphology of cells was monitored under light microscopy. After suspension of mt^+ (Fig. 1a) and mt^- (Fig. 1b) gametes were mixed, cell wall was lysed in either type of cells (Fig. 1c,d). In this stage, fully developed mating tubule was observed at the anterior of the mt^+ gamete (Fig. 1c). Pairing of opposite types of gametes is initiated by flagellar tipping (Fig. 1e). Cell fusion was performed to produce quadriflagellate zygotes (Fig. 1f,g,h). These morphological changes observed were the same as in previous reports (Harris 1989), and 0%, 82% and 90% of cells formed to be quadriflagellated zygotes in 0, 8 and 10 min after the mixing, respectively.

Primer extension analysis

A previous report showed that a transcription initiation site was detected 22 bp and 24 bp downstream of a TATA box-like sequence for *zys1A* and *zys1B* genes, respectively (Uchida *et al.* 1999). In this analysis, this major extension product was detected, when primer extensions were performed using 2 μ g of polyA RNA. Further analysis using 10 μ g of polyA RNA was also carried out. As a result, we recognized a novel minor extension product for each gene (Fig. 2). When a *zys1A*-specific primer was used, a minor band was detected 5 bp downstream of the major band. When a *zys1B*-specific primer was used, a novel minor band was detected 4 bp upstream of the major band. No other bands were detected in the entire region on the autoradiograms analyzed for both genes. The same results were obtained using the same amount of RNA in another experiment. These results showed that each of *zys1A* and *zys1B* gene transcription starts from 2 adjacent positions located about 20–30 bases downstream of the TATA box-like sequence (Fig. 3). Multiple transcription initiation sites has been suggested in *Chlamydomonas* zygote-specific class IV gene (Woessner and Goodenough 1989). Multiplicity of transcripts might be due to evolution of different function, as indicated in higher plant genes (Dehesh *et al.* 1994). Hereafter, transcripts corresponding to a major, or a minor band was referred to as a major, or a minor transcript.

In order to examine the expression of *zys1A* and *zys1B* genes at an early stage of expression, further analysis was performed using the primers which anneal with the 2 *zys1* genes. Using 10 μ g polyA RNA isolated from zygotes 10 min after mating, the major and minor bands were again the

Fig. 2. Primer extension analysis of *zys1A* and *zys1B* genes. A primer specific to either *zys1A* (a) or *zys1B* (b) gene was annealed to mRNA and extended with reverse transcriptase to yield major and minor bands (arrows) seen in lane E. The same primer was used to generate the adjacent sequence ladder (lanes G, A, T, C). The nucleotide sequence complementary to the coding strand is presented outside the ladder.

Fig. 3. Transcription initiation sites and sequences of primers used for extension analyses. Aligned nucleotide sequences of *zys1A* and *zys1B* genes; nucleotide identity is shown with black boxes. Nucleotide positions of major and minor transcription initiation sites for *zys1A* and *zys1B* genes (Amaj, Amin, Bmaj, Bmin), and primers specific to *zys1A* (Primer A), to *zys1B* (Primer B), and to both *zys1A* and *zys1B* (Primer A & B) are indicated by arrows. Numbers show nucleotide positions when the major transcription initiation site is defined as +1. TATA box-like sequence (TAAAA) and initiation codon (ATG) are underlined.

only products detected and located at positions mentioned above, and no other bands were detected in the entire region. Relative accumulation amounts of major and minor *zys1* transcripts 10 min after mating were estimated from intensities of the bands on an autoradiogram using an image analyzer (Table 1). When the major *zys1B* transcript amount was set to 1.00, the relative amounts of *zys1B* minor, *zys1A* major and *zys1A* minor transcripts were calculated as 0.730, 1.78 and 0.171, respectively. These results show that total amount of transcripts driven by the *zys1A* upstream region (*zys1A* major plus *zys1A* minor transcripts) is more than that of *zys1B* 10 min after mating. In this context, the *zys1A* promoter is stronger and would be better suited for driving foreign gene than that of *zys1B*. We are now investigating whether this promoter power difference might be related with positive effect of cycloheximide on *zys1A* transcript accumulation in young zygote, or distinct nucleotide sequence difference in 3' UTR of the *2zys1* genes (Uchida *et al.* 1993).

Table 1. Relative amounts of *zys1* transcripts detected in the primer extension reaction using polyA RNA extracted from cells 10 min after mating

Extension products		Mean ($n=4$) $\pm$ S.D.
<i>zys1A</i>	major	1.78 $\pm$ 0.11
	minor	0.171 $\pm$ 0.044
<i>zys1B</i>	major	1.00 ²
	minor	0.730 $\pm$ 0.062

¹ Mean values in 4 reactions, 2 extension reactions were performed using 2 independent RNA samples.

² The relative intensity of *zys1B* major band was defined as 1.00.

Acknowledgments

The authors appreciate the kindness of Prof. Dr. Tsuneyoshi Kuroiwa, University of Tokyo, for his encouragement, and Dr. Joe Zuccarello, University of New South Wales, for his critical reading of the manuscript. This work was partly supported by a grant for the Regional Joint Research Pro-

ject of Yamagata Prefecture from Japan Science and Technology Corporation.

References

- Cerutti, H., Johnson, A. M., Gillham, N. W. and Boynton, J. E. 1997a. A eubacterial gene conferring spectinomycin resistance on *Chlamydomonas reinhardtii*: integration into the nuclear genome and gene expression. *Genetics* **145**: 97–110.
- , —, — and —. 1997b. Epigenetic silencing of a foreign gene in nuclear transformants of *Chlamydomonas*. *Plant Cell* **9**: 925–945.
- Debuchy, R., Purton, S. and Rochaix, J.-D. 1989. The argininosuccinate lyase gene of *Chlamydomonas reinhardtii*: an important tool for nuclear transformation and for correlating the genetic and molecular maps of the ARG7 locus. *EMBO J.* **8**: 2803–2809.
- Dehesh, K., Franci, C., Sharrock, R. A., Somers, D. E., Welsch, J. A. and Quail, P. H. 1994. The *Arabidopsis* phytochrome A gene has multiple transcription start sites and a promoter sequence motif homologous to the repressor element of monocot phytochrome A genes. *Photochem. Photobiol.* **59**: 379–384.
- Fuhrmann, M., Oertel, W. and Hegemann, P. 1999. A synthetic gene coding for the green fluorescent protein (GFP) is a versatile reporter in *Chlamydomonas reinhardtii*. *Plant J.* **19**: 353–361.
- Harris, E. 1989. The *Chlamydomonas* Sourcebook. Academic Press, San Diego. pp. 1–780.
- Kindle, K. L., Schnell, R. A., Fernández, E. and Lefebvre, P. A. 1989. Stable nuclear transformation of *Chlamydomonas* using the *Chlamydomonas* gene for nitrate reductase. *J. Cell Biol.* **109**: 2589–2601.
- Lumbreras, V., Stevens, D. R. and Purton, S. 1998. Efficient foreign gene expression in *Chlamydomonas reinhardtii* mediated by an endogenous intron. *Plant J.* **14**: 441–447.
- Stevens, D. R., Rochaix, J.-D. and Purton, S. 1996. The bacterial phleomycin resistance gene *ble* as a dominant selectable marker in *Chlamydomonas*. *Mol. Gen. Genet.* **251**: 23–30.
- Uchida, H., Kawano, S., Nakamura, S. and Kuroiwa, T. 1992. A study on the effects of UV on preferential digestion of chloroplast nuclei in young zygote of *Chlamydomonas reinhardtii*. *Cytologia* **57**: 395–399.
- , —, Sato, N. and Kuroiwa, T. 1993. Isolation and characterization of novel genes which are expressed during the very early stage of zygote formation in *Chlamydomonas reinhardtii*. *Curr. Genet.* **24**: 296–300.
- , Suzuki, L., Anai, T., Doi, K., Takano, H., Yamashita, H., Oka, T., Kawano, S., Tomizawa, K., Kawazu, T., Kuroiwa, H. and Kuroiwa, T. 1999. A pair of invertedly repeated genes in *Chlamydomonas reinhardtii* encodes a zygote-specific protein whose expression is UV-sensitive. *Curr. Genet.* **36**: 232–240.
- Woessner, J. P. and Goodenough, U. W. 1989. Molecular characterization of a zygote wall protein: an extensin-like molecule in *Chlamydomonas reinhardtii*. *Plant Cell* **1**: 901–911.
-