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Transcriptional Regulation of the MS2 Gene of *Paramecium tetraurelia*

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SUMMARY

MS2 of *Paramecium tetraurelia* is the gene that we have cloned as one expressed at much higher levels in a short-lived mutant than in its parental wild-type stock and have characterized its expression to be enhanced with increased clonal age of the wild-type stock. This gene has been found to be preferentially expressed also in paramecia undergoing autogamy which is one of the termination modes of the *Paramecium* clonal life span. Here we investigated the transcriptional mechanism for the MS2 expression in light of its possible causal relationship to the *Paramecium* clonal life span. DNA-protein binding analyses of the upstream of the MS2 gene identified a stretch of DNA sequence that interacted specifically with macronuclear proteins prepared from the MS2-expressing mutant. The DNA sequence was mapped to the 33 bp between – 335 and – 303, counting from the initiation position of MS2 transcription. A protein, 15,000 in molecular mass, with a binding ability for this DNA element was purified to homogeneity from the macronuclear proteins by a chromatography using the specific DNA-protein interaction. *In vitro* transcriptional analyses revealed that both the purified protein and its target DNA domain are essential for increased synthesis of the MS2 transcript. These results showed that the DNA-protein interaction is required for induction of the MS2 expression.

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INTRODUCTION

For the final purpose of identifying the causal determinant of the *Paramecium* clonal life span, the *jumyo* gene (Takagi et al., 1989), we examined differences in gene expression between wild-type stock 51 and its mutant d4-SL4 with a short clonal life span, the *jumyo* mutant, and cloned three genes that showed expression patterns dependent on clonal age (Tanabe et al., 2002). Structural analyses (Tanabe et al., 2002) revealed that two of the three were genes for serotype (Caron and Meyer, 1989; Bleyman, 1996), 51A and α-51D. The remaining one was a novel gene named MS2. The MS2 gene, isolated as one expressed predominantly in the short-lived mutant, was gradually activated in the wild-type paramecia as their clonal age increased (Tanabe et al., 2002). *Paramecium tetraurelia* terminates its clonal life span either in clonal death

or in causing autogamy (Takagi, 1999). In the course of another strategy for identification of the *jumyo* gene, molecular understanding of the autogamy mechanism, we showed that the MS2 gene became up-regulated in the wild-type stock through the process of autogamy (Tanabe et al., 2003) The parallel transcriptional patterns of MS2 observed during clonal aging and during autogamy suggest that the two events may share a certain stage in the genetic program for determination of the *Paramecium* life span, in which the gene product of MS2 is functionally participated.

Thus, MS2 is expected to become the first molecular basis of the fundamental correlation between clonal life span and autogamy and the eventual setting of the *Paramecium*-specific longevity. In support of this notion, a transformation experiment gave a result suggesting that targeted repression of the MS2 gene caused a phenotypically silencing effect on the occurrence of autogamy (Tanabe et al., 2003), arguing for an intimate participation of MS2 in the autogamy pathway. However, the cDNA sequence of MS2 is unlikely contain some genetic information for translation products (Tanabe et al., 2003), so it is doubtful whether MS2 is our final target. Instead, we suppose that the gene product of *jumyo* serves as the nuclear factor controlling the gene expression that specifies the clonal life span by ultimately governing essential cellular functions accompanied with age-dependent phenomenon like the occurrence of autogamy. If this holds true, elucidation of the mechanism for MS2 expression would give some clues for isolation of the *jumyo* gene. Since expression of *Paramecium* genes is considered to be usually regulated at the level of transcription (Preer, 1997), our another interest was directed toward the underlying molecular process for transcriptional regulation of MS2. Here we dealt with the transcriptional mechanism for the MS2 gene.

MATERIALS AND METHODS

Culture of paramecia

P. tetraurelia wild-type stock 51 and the *jumyo* mutant d4-SL4 (Takagi et al., 1987b) were used and cultivated as done previously (Takagi et al., 1987a; Tanabe et al., 2002).

Preparation of macronuclear proteins

An age-synchronized cell population of young stock 51 (about 15 fissions old) and cells of the *jumyo* mutant were used as sources of protein. Cells were harvested from a culture of 1,200 - 1,500 cells/ml and subjected to cellular fractionation for isolation of macronuclei (Shiomi et al., 1980). Nuclear proteins were extracted from the macronuclear fraction with the NE-PER nuclear extraction reagent (PIERCE) and dialyzed overnight against the undermentioned binding buffer using a dialysis membrane with a molecular weight cut-off of 5,000. The dialyzed samples were used as the macronuclear extract in the following experiments.

Cloning of genomic DNA segment

The *Paramecium* genomic DNA was prepared by a phenol method (Forney et al., 1983). The upstream segment flanking the MS2 transcribed region was isolated from the genomic DNA by inverse PCR (Ochman et al., 1988; Triglia et al., 1988) and the nucleotide sequence of the resulting 897-bp fragment was submitted to the DDBJ database with the accession number AB094989.

Gel retardation assay

Probe DNAs longer than 100 bp were generated by PCR using the above 897-bp DNA as the template. Shorter probe DNAs were chemically synthesized. These probe DNAs were labeled with biotin using the biotin 3' end DNA labeling kit (PIERCE).

The DNA-binding experiment (Fried and Clothers, 1981) was carried out nonisotopically as

follows: Protein (10 µg in the macronuclear extract or 10 ng of purified preparation) was preincubated with 400 ng of denatured salmon sperm DNA (0.5 - 1 kb) and, when necessary, 100 ng of unlabeled probe DNA as a competitor in 19 µl of the binding buffer (10 mM HEPES-KOH, pH 7.5, 50 mM NaCl, 8 mM $MgCl_2$, 1 mM dithiothreitol and 10% glycerol) for 20 min on ice, and then 1 µl of biotin-labeled probe DNA (1 ng) was mixed with the reaction. After an additional 30 min, the mixture was applied to a 6% polyacrylamide gel (8 cm × 8 cm × 1 mm) containing 0.25 × TBE (22.5 mM Tris, 22.5 mM boric acid and 1 mM EDTA) and electrophoresed at 100 V until bromophenol blue reached the bottom of the gel. The labeled probe DNA was then electroblotted to a nylon membrane in 0.25 × TBE at 4 mA/cm^2 for 1 h and detected using the LightShift chemiluminescence kit (PIERCE) and an ECL Mini-camera (Amersham).

DNA affinity chromatography

The principle of this procedure is based on a specific interaction between biotinylated ligand DNA immobilized on streptavidine-conjugated agarose beads (PIERCE) and proteins recognizing the nucleotide sequence of the ligand DNA (Chodosh et al., 1986). After immobilization of ligand, the streptavidine gel, 0.5 ml, was packed into a column and equilibrated with 2.5 ml of the above binding buffer. The macronuclear extract, 50 mg in protein, was incubated with 2 mg of denatured salmon sperm DNA (0.5 - 1 kb) for 20 min on ice and loaded onto the column. Following a thorough washing of the column with 2.5 ml of the binding buffer containing 0.2 M NaCl, proteins bound to the ligand DNA were eluted with 1.5 ml of the binding buffer containing 0.5 M NaCl. The purified proteins were dialyzed against the binding buffer as described above for use in the subsequent experiments.

In vitro run-off transcription

Two µl of the macronuclear extract was added singly or in combination with 10 ng of purified protein to 18 µl of the binding buffer containing 100 ng of template DNA, 1 mM each of ATP, CTP and GTP, 650 µM of UTP, 350 µM of biotin16-UTP and 40 units of the ribonuclease inhibitor RNasin. Transcription was carried out at 25°C for 1 h. Electrophoresis of the transcript (2 µl) and the subsequent transfer to nylon membranes followed our protocol for Northern blotting (Tanabe and Yoshioka, 2000). Signals from biotinylated transcripts were detected as described under "Gel retardation assay".

Other experiments for protein

Protein concentration was determined by the method of Bradford (1976) with bovine γ-globulin as the standard protein. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed following Laemmli (1970). Gels after the run were stained with a fluorescent dye (Steinberg et al., 1996; 1997).

RESULTS AND DISCUSSION

Protein-DNA interaction on the upstream of the MS2 gene

In our preceding study, the MS2 gene was found to be transcribed at a low level when wild-type paramecia were young but its transcription was prominent in the *jumyo* mutant (Tanabe et al., 2002). This differential gene expression is supposed to result from transcriptional regulation taking place upstream of this gene: there should exist a *trans*-acting nuclear protein factor(s) that responds to a *cis*-active DNA element(s) located anywhere on the upstream regulatory region, by which the gene expression is up- or down-regulated.

Assuming that such an intermolecular regulatory interaction occurs preferentially in young wild

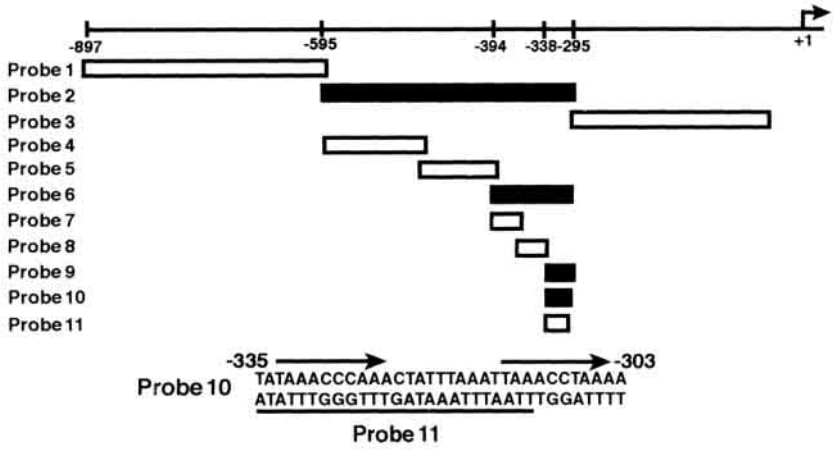

Fig. 1. Schematic representation of strategy for location of MS2-specific responsive DNA elements. The 897-bp macronuclear genomic region (accession No.: AB094989) 5' to the MS2 transcribed region is presented at the top. Nucleotides are numbered with the first transcriptional initiation site at position + 1. The direction of transcription is arrowed. Shown below are a set of DNA fragments used as probes in gel retardation assays with their relative sizes and positions depicted. The filled and open boxes represent probe DNAs testing positive and negative, respectively, in the assays. The nucleotide sequence of Probe 10 is given at the bottom along with the nucleotide positions of its 5'- and 3'-ends. The direct repeat within this segment is indicated by arrows facing in the same direction. The nucleotide sequence of Probe 11 is underlined.

type or the *jumyo* mutant, we searched the macronuclear proteins of the two stocks for DNA-binding activities specific to the MS2 gene by a gel retardation assay using eleven kinds of DNA fragments covering the upstream nontranscribed region as the probe (Fig. 1). First, three fragments, Probes 1 to 3, spanning – 897 to – 35 relative to the start of the transcription were examined for interaction with proteins in the macronuclear extract. In the assay using the proteins of the mutant in which MS2 is actively expressed, Probe 2 (– 595 to – 295) caused an upward band shift indicating the formation of a protein-DNA complex (lane 2 in Fig. 2), but the mobilities of Probes 1 and 3 were not affected with the same protein preparation (data not shown). The shifted DNA band disappeared when an excess of unlabeled Probe 2 was added as a competitor in the reaction mixture (lane 3), which demonstrated that the DNA-protein interaction is sequence-specific. On the other hand, proteins of young wild type did not show such an affinity for Probe 2 (lane 1) as well as for Probes 1 and 3 (not shown). Thus, we detected a DNA-binding activity toward the MS2 upstream region only in macronuclear proteins of the *jumyo* mutant and thereafter exclusively investigated the mutant macronuclear extract.

We next divided the Probe 2 region into three parts (Probes 4 to 6 in Fig. 1) and assayed them for response to the mutant macronuclear proteins. In this screening, the protein sample was found to preferentially retard the mobility of Probe 6, located between positions – 394 and – 295 (data not shown). We did further mapping by subdividing the DNA sequence of Probe 6 into three (Probes 7 to 9 in Fig. 1), and the subsequent deletion analysis of the resulting positive probe, Probe 9 (– 338 to – 295), finally narrowed down the target sequence to Probe 10, the 33 bp present ahead of position – 303 (not shown).

A close examination of the 33-bp domain

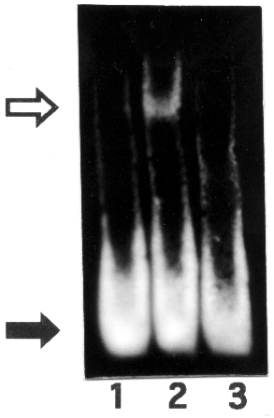

Fig. 2. Gel-mobility shift profile of an MS2-specific responsive DNA element. A gel retardation assay was done using either biotin-labeled Probe 2 (see Fig. 1) and macronuclear proteins either of young wild-type stock 51 (lane 1) or of the *jumyo* mutant (lanes 2 and 3) in the presence (lane 3) or absence (lane 2) of a competitor DNA, unlabeled Probe 2. The filled and open arrows indicate a free and a protein-bound form of the probe DNA, respectively.

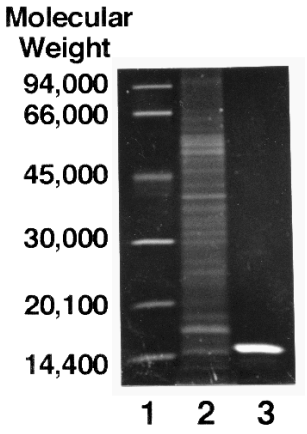

Fig. 3. SDS-PAGE profile of the affinity-purified protein. Proteins were electrophoretically size-fractionated and stained by UV fluorescence. Lanes: 1, molecular weight markers; 2, the macronuclear extract prepared from the *jumyo* mutant; 3, purified preparation after the second chromatography. The molecular weights of the marker proteins in lane 1 are given to the left of the lane.

detected a direct repeat of nucleotide sequence, which is comprised of the consensus sequence 5'-TAAACC(C/T)AAA-3' (Fig. 1). Since many transcriptional regulator proteins function through binding to repeated DNA sequences in their target regions, the repeat of the decameric sequence is likely to participate in the direct contact with the mutant macronuclear proteins. To investigate this possibility, deletion in Probe 10 was assayed for retaining the affinity with the proteins: a partial deletion in the 3'-half of the repeat made the resultant DNA fragment, Probe 11 (Fig. 1), inactive in the binding assay (not shown). Consequently, the direct repeat was identified as the target sequence on the MS2 gene for the mutant-specific macronuclear factor. A DNA database search did not find the decameric sequence in any transcriptional regulatory element which has so far been reported.

Purification of a DNA-binding protein specific to the MS2 gene

We attempted purification of proteins with the binding ability for the DNA sequence of Probe 10 by affinity chromatography using the probe DNA as the ligand. Macronuclear proteins of the mutant stock were separated by chromatography and proteins retained in the affinity column were eluted with the running buffer with increased salt concentration.

The profile of the eluted proteins was made visible by SDS-PAGE: the track in which the proteins were run had one major protein band and a few faint ones (not shown). To differentiate between all the eluted proteins participating in the specific contact with the ligand DNA or the eluted fraction being contaminated with non-specifically bound proteins, this fraction was put through the second round of chromatography and the new eluate was electrophoretically inspected for purity: the faint multiple band seen after the first purification was lost, while the major band of a protein with molecular weight of 15,000 appeared again (Fig. 3).

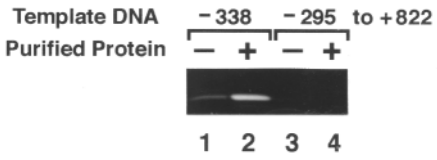

Fig. 4. *In vitro* transcriptional analyses of the MS2 gene. Run-off transcriptional reactions were carried out on a template DNA covering the macronuclear genomic MS2 region from – 338 (lanes 1 and 2) or – 295 (lanes 3 and 4) to + 822. The numbering of nucleotide position is the same as used in Fig. 1. The macronuclear extract of the *jumyo* mutant was put into the reactions either alone (lanes 1 and 3) or with the affinity-purified protein (lanes 2 and 4). The resulting biotinylated transcripts were subjected to Northern blotting and made visible by chemiluminescence. The size of the transcript detected here was estimated to be approximately 0.8 kb by comparison with the migration positions of co-electrophoresed size marker RNAs (not shown).

Therefore, we ascribed the DNA-binding activity detected in the binding assay to this protein obtained after the repeated purification. In fact, the second purified preparation proved to retard the electrophoretic mobility of Probe 10 in DNA-binding assays as observed for the mutant macronuclear extract (data not shown), which seems to verify this assignment, but final conclusion must await structural analyses of this protein.

A titration experiment showed that about 2.5 ng of the purified protein is equivalent in the ability to retard DNA migration to 10 μg of the mutant macronuclear proteins, indicating that about four thousandfold purification on the basis of DNA-binding activity was attained through the chromatography.

Transcriptional activation of the MS2 gene

The molecular interaction between the protein thus purified and its 33-bp target DNA was functionally characterized by *in vitro* transcription reactions on the MS2 gene (Fig. 4). As a positive control, the mutant macronuclear extract was incubated alone with a DNA fragment of the MS2 gene beginning 338 bp ahead of and ending 822 bp past the first transcriptional start site (– 338 to + 822) which contains the 33-bp sequence of interest (see Fig. 1), after which synthesis of RNA was begun. The transcript was run on a denaturing gel, transferred to a membrane, and detected as a chemiluminescent signal. Successful RNA synthesis would generate a transcript of about 0.8 kb long. Imaging of the signal gave a single band at a position corresponding to the expected size (lane 1), confirming that the nuclear extract can elongate nascent RNA chains while incorporating ribonucleoside-triphosphates including a biotin-labeled one. When the affinity-purified protein was added to the reaction, the transcription was found to be stimulated several times (lane 2). Since the input of the purified protein, 10 ng, is about four times the equivalent of the nuclear extract used here in terms of DNA-binding ability (see above), these two data together are represented as the synthesis of the MS2 transcript in proportion to the DNA-binding activity, which is best explained by assigning the transcriptional increase to the binding of the protein to its target element. Actually, in the transcription experiments using a template DNA devoid of the 33-bp domain (the region – 295 to + 822), the relative intensity of signals from the resulting transcripts was below detectable levels with (lane 4) or without (lane 3) the protein.

Taken together, we concluded that the protein purified in this study serves as a *trans*-acting factor for positive regulation of the MS2 transcription through the specific interaction with the – 335 to – 303 domain, and thereby induces the MS2 expression in the *jumyo* mutant. Extended investigations of the DNA-protein interaction in the MS2 expression as well as structural characterization of the *trans*-acting factor should elucidate the underlying regulatory mechanism and its role in setting the *Paramecium* clonal life span.

REFERENCES

Bleyman, L.K. (1996) Serotypes: the immobilization antigens. *In:* Ciliates -Cells as Organisms-. Hausmann, K. and Bradburg, P.C (ed.). Guster Fischer, Stuttgart, pp. 300-303.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72, 248-254.

Caron, F. and Mayer, E. (1989) Molecular basis of surface antigen variation in paramecia. Ann. Rev. Microbiol., 43, 23-42.

Chodosh, L.A., Carthew, R.W. and Sharp, P.A. (1986) A single polypeptide possesses the binding and transcription activities of the adenovirus major late transcription factor. Mol. Cell. Biol., 6, 4723-4733.

Forney, J.D., Epstein, L.M., Preer, L.B., Rudman, B.M., Widmayer, D.J., Klein, W.H. and Preer, J.R., Jr. (1983) Structure and expression of genes for surface proteins in *Paramecium*. Mol. Cell. Biol., 3, 466-474.

Fried, M. and Clothers, D.M. (1981) Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. Nucl. Acids Res., 9, 6505-6525.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 207, 682-685.

Ochman, H., Gerber, A.S. and Hartl, D.L. (1988) Genetic applications of an inverse polymerase chain reaction. Genetics, 120, 621-623.

Preer, J.R., Jr. (1997) Whatever happened to *Paramecium* genetics? Genetics, 145, 217-225.

Shiomi, Y., Higashinakagawa, T., Saiga, H. and Mita, T. (1980) Metrizamide isopycnic centrifugation for the isolation of macro- and micronuclei for *Paramecium*. J. Univ. Occup. Environ. Health., 2, 323-330.

Steinberg, T.H., Jones, L.J., Hangland, R.P. and Singer, V.L. (1996) SYPRO Orange and SYPRO Red protein gel stains. Anal. Biochem., 239 223-227.

Steinberg, T.H., White, H.M. and Singer, V.L. (1997) Optimal filter combination for photographing SYPRO Orange or SYPRO Red dye-stained gels. Anal. Biochem., 248 168-172.

Takagi, Y., Nobuoka, T. and Doi, M. (1987a) Clonal lifespan of *Paramecium tetraurelia*: effect of selection on its extension and use of fissions for its determination. J. Cell Sci., 88, 129-138.

Takagi, Y., Suzuki, T. and Shimada, C. (1987b) Isolation of a *Paramecium tetraurelia* mutant with short clonal life span and with novel life features. Zool. Sci., 4, 73-80.

Takagi, Y., Izumi, K., Kinoshita, H., Yamada, T., Kaji, K. and Tanabe, H. (1989) Identification of a gene that shortens clonal life span of *Paramecium tetraurelia*. Genetics, 123, 749-754.

Takagi, Y. (1999) Clonal life cycle of *Paramecium* in the context of evolutionally acquired mortality. *In:* Progress in Molecular and Subcellular Biology Vol. 24. Macieira-Coelho, A (ed.). Springer-Verlag, Berlin/ Heidelberg, pp. 81-101.

Tanabe, H. and Yoshioka, S. (2000) Molecular cloning of genes differentially expressed in *Paramecium tetraurelia* with different life spans. Jpn. J. Protozool., 33, 29-34.

Tanabe, H., Fujiike, M. and Yoshioka, S. (2002) *Paramecium tetraurelia* genes expressed in an age-dependent manner. Jpn. J. Protozool., 35, 143-150.

Tanabe, H., Fujiike, M. and Yoshioka, S. (2003) Isolation and characterization of genes showing altered expression specific to autogamy in *Paramecium tetraurelia*. Jpn. J. Protozool., 36, 1-8.

Triglia, T., Peterson, M.G. and Kemp, D.J. (1988) A procedure for in vitro amplification of DNA segments that lie outside the boundaries of known sequences. Nucl. Acids Res., 16, 8186.