Original

Isolation and Characterization of Genes Showing Altered Expression Specific to Autogamy in *Paramecium tetraurelia*

Hiroyuki TANABE*, Masaki FUJIIKE and Sachiko YOSHIOKA
Laboratory of Biochemistry, Faculty of Agriculture, Kinki University, Nara 631-8505, Japan.

SUMMARY

Autogamy is a sexual process of meiosis and self-fertilization which occurs in unpaired *Paramecium* cells and is considered to be coupled to the *Paramecium* clonal life span. In order to identify gene expression which may underlie the initiation of autogamy, we screened for genes differentially expressed before and after the onset of autogamy by using a subtraction strategy and obtained two species of difference genes. Both genes were found to be the ones increasingly expressed with clonal age that we have cloned previously. One was repressed upon commitment to autogamy and identified to be the α-51D surface protein gene. The other, progressively activated while paramecia traversed the autogamy pathway, was identical to the gene named MS2. Although we failed to structurally characterize the MS2 gene, it was suggested that continuous delivery to paramecia of double stranded RNA corresponding to a part of this gene impaired normal development of autogamy, implying direct involvement of MS2 in the process of autogamy.

*Corresponding author
Tel: +81-742-43-1511
Fax: +81-742-43-1445
E-mail: tanabe@nara.kindai.ac.jp

Received 29 Oct 2002; Accepted 24 Dec 2002

INTRODUCTION

The ciliated protozoan *Paramecium tetraurelia* undergoes two forms of sexual reproduction, both of which include meiosis, fertilization, and macronuclear development (Sonneborn, 1974). One, conjugation, develops from surface interaction between sexually competent cells of complementary mating types. The other, autogamy, is an intracellularly initiated process of self-fertilization in single cells which is triggered by food deprivation. Termination of the *Paramecium* clonal life span can be defined by either clonal death or entry into these sexual pathways. Paramecia count a definite number of cell fissions to traverse the period from the clonal origin to clonal death. Likewise, undergoing sexual reproduction requires a certain number of fissions after the preceding fertilization, the duration of which is called the immaturity period. Conjugation and autogamy have their own period for immaturity, sexual immaturity and autogamy immaturity, respectively, and thereafter paramecia become mature, that is, capable of entering each sexual pathway.

A positive correlation has been established between the ciliate clonal life span and the length of the sexual immaturity period (Smith-Sonneborn, 1981). Clonal age of parent cells has an adverse effect on the duration of sexual immaturity (Siegel, 1961) and of autogamy immaturity (Iizima et al.,

1997) in their progeny. Autogamy immaturity is similarly affected by parental cultural age as well (Ishikawa et al., 1998). In the *jumyo* mutant of *P. tetraurelia*, which has an extremely short life span, the immature period for autogamy is also shortened (Takagi et al., 1987; 1989). These findings allow the speculation that the mechanism for setting the clonal life span and that for initiating sexual reproduction are tightly linked (Takagi, 1999).

The molecular aspects of the control of the *Paramecium* sexual reactivity have been elucidated to some extent. Immaturin, a small cytoplasmic protein, was identified as the factor responsible for sexual immaturity (Miwa et al., 1975; Haga and Hiwatashi, 1981; Miwa, 1984). There seems to exist the autogamy version of immaturin. Transplantation of macronuclear material or the entire macronuclei between autogamy-immature and mature cells significantly influences the occurrence of autogamy in *P. tetraurelia* (Kosciuszko and Koizumi, 1983; Mikami and Koizumi, 1983). Together with the cell-cycle-stage-dependence of commitment to autogamy (Berger, 1986), these experiments indicate that a nuclear factor(s) is directly involved in the regulation of the autogamy pathway and hence particular alterations in gene expression must be associated with this sexual event. We have compared gene expression profiles between the short-lived *jumyo* mutant and its wild-type parent stock and isolated differentially expressed genes for the final purpose of identification of the causal determinant of the *Paramecium* clonal life span (Tanabe and Yoshioka, 2000; Tanabe et al., 2002). In view of the above argument, investigation of autogamy-specific gene expression would be another important approach to our goal. This paper describes an initial step in the second approach.

MATERIALS AND METHODS

Culture of paramecia

P. tetraurelia wild-type stock 51 and the *jumyo* mutant d4-SL4 (Takagi et al., 1987) were the strains used in this study. Daily isolation line culture (Sonneborn, 1954) was developed from exautogamonts of the wild-type stock as a source of cells mature for autogamy. To obtain preautogamous, autogamy-committed, and postautogamous cells at the age of about 35 fissions, cultures derived from the isolation culture were allowed to reach 400-500, 1,200-1,500, and 4,000-5,000 cells/ml, respectively. The clonal age and population density for each autogamy stage were determined on the basis of kinetic experiments by Berger and Rahemtullah (1990). We detected occurrence of autogamy by cell staining (Dippell, 1955) and observed that the percentages of autogamonts in each culture phase were 0, about 5%, and about 80%, respectively. The cell density of 1,200-1,500 cells/ml has been estimated to be that at the median time of initial commitment to autogamy for 35-fission old paramecia (Berger, 1986; Berger and Rahemtullah, 1990). The committed cells undergo autogamy after the following cell fission, the “critical fission” (Mikami and Koizumi, 1983). Actually, the percentage of autogamy was raised to about 60% after one fission, *i.e.*, at about 3,000 cells/ml, indicating that more than half of paramecia at 1,200-1,500 cells/ml were in the state of autogamy commitment under the conditions used here. Therefore, we considered paramecia at each of the above three culture phases to be satisfactory in the present study as a cell material for the corresponding autogamy stage. Liquid medium and other cultivation conditions were mentioned before (Tanabe and Yoshioka, 2000).

cDNA subtraction

Construction and screening of subtractive cDNA libraries were carried out according to our protocol (Tanabe et al., 2002). In the present experiment, mRNAs prepared from matured wild-

type cells in a preautogamous state and those committed to autogamy were used as the starting materials. Two subtraction experiments were designed to enrich genes expressed preferentially either in the preautogamous or in the autogamy-committed cells: the forward subtraction to recover preautogamy-specific cDNAs (tester, preautogamous cell population; driver, autogamy-committed cell population) and the reverse subtraction to leave cDNAs specific to autogamy commitment (tester, autogamy-committed cell population; driver, preautogamous cell population).

Plasmid DNA and host bacteria for dsRNA expression

DNA fragments were generated by PCR amplification (Tanabe and Yoshioka, 2000) using the entire cDNA sequence of MS2 (DDBJ accession no. AB059619) as the template. The primers used here were tagged with the T7 promoter sequences to allow synthesis of double stranded RNA (dsRNA) from the resulting PCR products by T7 RNA polymerase (Tsuboi et al., 2002). The amplification products (see Fig. 2) were inserted into the pGEM-T cloning vector (Promega) and introduced into *Escherichia coli* HT115 (DE3) (Takiff et al., 1989; Dasgupta et al., 1998) used as a food bacterium for delivering the dsRNA to *Paramecium* cells.

Feeding of bacterially expressed dsRNA to paramecia

The above *E. coli* strain harboring plasmid was cultivated in the *Paramecium* liquid medium at 37°C to an optical density at 600 nm of approximately 0.4. To induce T7 RNA polymerase, isopropyl-β-D-thiogalactopyranoside was added to 0.4 mM and further cultivation was done for 2 h. Synthesis of the dsRNA was confirmed by Northern blotting using a sense and an antisense cDNA of MS2 as probes. Two *Paramecium* cells were transferred into 2 ml of the RNA-feeding culture thus prepared and their phenotypic response was observed after a 5-day successive cultivation at 25°C.

Other experiments

Preparation of mRNA, Northern blotting, DNA sequence analysis and rapid amplification of cDNA ends were described before (Tanabe and Yoshioka, 2000; Tanabe et al., 2002).

RESULTS AND DISCUSSION

Subtractive cloning of genes expressed differentially during autogamy

In the conviction that underlying transcriptional changes precede the prosecution of autogamy, we performed isolation of genes up- or down-regulated on conversion from preautogamous to autogamy-committed stages by means of cDNA subtraction. Following a series of screening steps, one each of cDNA clone was finally isolated from the forward- and the reverse subtractions. The resulting preautogamy- and autogamy- specific clones were tentatively designated as NS and AS, respectively. Fig. 1 depicts their transcription patterns during the development of autogamy. The expression of the preautogamy-specific NS clone declined abruptly to a basal level when cells were committed to autogamy. On the contrary, the AS clone from autogamy-committed stage is positively regulated over the course of the autogamy process. Its transcriptional level remained elevated until the completion of autogamy.

Identification of autogamy-specific genes

A search of the DDBJ database revealed that the two clones were identical to the α-51D gene and MS2. These genes are two of the three genes

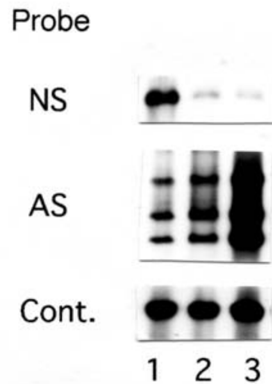

Fig. 1. Northern blot analysis of autogamy-dependent gene expression in *Paramecium tetraurelia*. mRNA samples (1 μg each) derived from preautogamous (lane 1), autogamy-committed (lane 2), and postautogamous (lane 3) mature cells of stock 51 were separated by electrophoresis and transferred onto nylon membranes to prepare a triplicate blot. Each blot was hybridized with a cDNA probe for NS, AS, or control for mRNA load (from top to bottom). The control gene has proven to be transcribed independent of the autogamy stage in a preliminary experiment.

isolated in our preceding study, where they showed much higher expression in the short-lived *jumyo* mutant than in its wild-type parental strain as well as increasing expression during clonal aging (Tanabe et al., 2002): We found the nucleotide sequence of the NS clone in the gene that encodes the α-51D surface protein specifying the D serotype of *P. tetraurelia* (Brener et al., 1996). The unique multiply banded profile of the AS transcripts (Fig. 1) is reminiscent of the MS2 gene with the accession number AB059619. Indeed, both DNA sequences were aligned with a perfect homology.

Since aged paramecia are prone to undergo autogamy even when they are well fed and the same is the case for the *jumyo* mutant (Takagi et al., 1987), it was conceivable that the α-51D gene up-regulated in these cells is similarly regulated in the autogamy process. However, Fig. 1 shows a result to the contrary. Although the observed autogamy-dependent repression of this serotype gene is difficult to reconcile with its aging-dependent activation, one possible explanation for the two opposite expression pattern is that the aging pathway and the autogamy pathway involve their respective independent steps, to each of which α-51D contributes in a different manner. In any case, it is likely that this cellular surface protein receives adverse environmental stimuli as food restriction and regulates the subsequent intracellular transmission of the external signals. This notion is supported by a structural analysis (Nielsen et al., 1991) indicating that *Paramecium* surface proteins are potentially capable of intermolecular protein interactions.

While, the MS2 gene with little known function is worth noting. Its transcription mode in the course of autogamy (AS in Fig. 1) is parallel with that during clonal aging (Tanabe et al., 2002). This close transcriptional association between the two events could reflect their intimate interrelation (Takagi, 1999) as introduced above, in which the MS2 gene product may play an important role. Thus, our interest was focused on the relationship between the MS2 gene and the *Paramecium* autogamy.

Structural analysis of MS2

As shown in Fig. 1, the MS2 gene generates three distinct transcripts, for which several interpretations have been offered: diversity in the splicing point, in the transcription starting point or in the polyadenylation point (Tanabe et al., 2002). To discriminate between those possibilities, we analyzed 30 independent cDNA clones of this gene isolated by rapid amplification of cDNA ends. They were sorted into three groups according to size: about 1.8, 2.4 and 4.0 kb, which were close to the size of the MS2 transcripts estimated from their Northern blot. Sequencing of representative

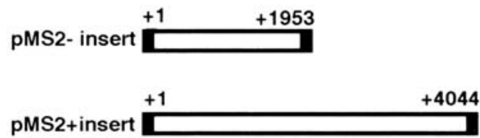

Fig. 2. Maps of MS2 transgene sequences used in dsRNA-feeding experiments. Two DNA segments of MS2, the 5' half of cDNA (the upper open box) and the full-length cDNA (the lower open box), carrying tags of the T7 promoter (solid boxes) on both ends were cloned into pGEM-T and the resulting constructs were named pMS2- and pMS2+, respectively. Nucleotide positions are given relative to the first transcriptional start (+1) of MS2.

clones belonging to each group revealed that the 1.8-kb and 2.4-kb clones are shorter isoforms representing the 3' portion of the 4.0-kb clone, the full-length cDNA (4,044 bp), and have the start sites located 2,255 bp (+2,255) and 1,606 bp (+1,606) downstream the 5' terminus of the full-length clone. Therefore, we concluded that these differentially transcribed variants of MS2 resulted from use of alternative transcriptional start.

We next searched the 4,044-bp cDNA sequence for protein coding frames. In *Paramecium*, TAA and TAG specify glutamine and TGA alone is used as a translational stop (Caron and Meyer, 1985; Preer et al., 1985). The three possible reading frames, however, all contained scattered TGA (stop) codons, making it difficult to define the translated domain. So, we applied to this study the method for protein frame determination from purine/pyrimidine information (Shepherd, 1981): (1) Codon usage in all organism is restricted such that the ratio of RNY to YNR codons, where R is purine, N any base and Y a pyrimidine, is > 2 for true reading frames, and < 1 for noncoding frames. (2) In organisms having A+T-rich genomes, coding regions have a much lower A+T content than noncoding regions and are characterized by a 3-bp periodicity in A+T richness, *i.e.*, the third base of each codon shows the strong bias for A and T. Among numerous ATG-TGA frames present in the MS2 cDNA sequence, only the 192 bp starting at position +707 counting from the third transcriptional initiation point (+2,255 relative to the 5' terminus) followed the first rule: its RNY/YNR ratio is 2.1, but this DNA segment did not satisfy the second criterion: it has 65% A+T at the third position versus 70% A+T in the entire sequence, displaying no such periodicity in A+T content. In addition, the 192-bp frame shares only about one tenth the shortest transcribed portion (1.8 kb) of MS2, which seems to be unlikely for a translation moiety, and the amino acid sequence of 63 residues deduced from its nucleotide sequence showed little homology with other known proteins. Together with these data, it is doubtful whether the DNA sequence of MS2 actually encodes a certain translation product(s).

Functional characterization of MS2

From the above results, we did not obtain any information about the role of MS2 in the autogamy event, so we investigated phenotypic influence of repression of this cryptic gene on autogamy by means of RNA interference (RNAi). RNAi is a posttranscriptional gene-silencing phenomenon in which exogenous dsRNA triggers degradation of homologous cellular mRNA of target gene, thereby the target gene is repressed (Brantl, 2002; Hannon, 2002), and has been proven to work for *Paramecium* as well (Kung et al., 2000). In this study, we employed bacterially mediated RNAi (Timmons and Fire, 1998; Timmons et al., 2002; Galvani and Sperling, 2002): Transformation was performed by feeding autogamy-competent paramecia with food bacteria producing high levels of dsRNA derived from MS2 transgenes.

We first assessed phenotypic changes with respect to autogamy in matured wild-type paramecia (about 50 fissions old) that were fed bacteria expressing dsRNA from pMS2- lacking the 3' half of MS2 or pMS2+ containing the entire MS2 (Fig. 2). Previous studies demonstrated that gene silenc-

Table 1. Phenotypic characterization of paramecia transformed with MS2 transgenes*

Plasmid	Host strain	
	Wild-type	d4-SL4
pGEM-T	58.5% (20)	90.2% (18)
pMS2-	51.1% (20)	91.7% (14)
pMS2+	62.8% (20)	92.0% (19)

*The dsRNA-feeding experiment was done with 20 independent cultures for each combination of host strain and plasmid. Results were expressed as the fraction of autogamonts in the cell population inspected for macronuclear fragmentation using Dippell's stain after a 5-day consecutive cultivation. The percentages were the average of the results of the 20 cultures which did not differ by more than 4%. The values in parenthesis indicate the number of the cultures that survived the 5-day cultivation out of the 20 cultures.

ing takes place by transforming paramecia with transgenes lacking the 3' noncoding region of target genes (Ruiz et al., 1998), but the effect can not be obtained with transgenes bearing the flanking regulatory region (Galvani and Sperling, 2001). If MS2 intimately participates in the autogamy pathway, paramecia transformed with pMS2- should present a silenced phenotype connected with autogamy and the interference phenomenon should not appear with pMS2+. Data in Table 1 seem to agree with this assumption: the wild-type cells that fed on bacteria harboring pMS2- displayed a weak but distinct resistance to undergoing autogamy in comparison to feeding of pMS2+ as well as the control plasmid pGEM-T.

We then carried out feeding of bacteria engineered to express the MS2 constructs to the *jumyo* mutant d4-SL4. In this case, clonal descendants that were exposed to pMS2- showed a reduction in viability with a significant difference from that in reference experiments using pGEM-T and pMS2+ (14 cultures for pMS2- versus 18 cultures for pGEM-T and 19 cultures for pMS2+), while liability to autogamy was unaffected as for surviving cells which were supposed to successfully escape the transformation by pMS2- (91.7% versus 90.2% and 92.0%). The *jumyo* mutant terminates its clonal life span either in early death or in preferred autogamy (Takagi et al., 1987; Takagi et al., 1989), *i.e.*, escapes clonal death by causing autogamy. Consequently, genetic interference of the autogamy process should cause a lethal effect on the mutant cells in the vegetative growth. The lowered viability of the mutant for pMS2- can be explained straightforwardly by assuming that repression of MS2 adversely affected the occurrence of autogamy in the mutant cells, which resulted in their clonal death.

In this study, we obtained presumptive evidence for functional participation of MS2 in the *Paramecium* autogamy, but the susceptibility of paramecia to silencing transgene observed in our feeding experiments was significantly lower than the case of other *Paramecium* genes so far reported (Galvani and Sperling, 2002), so that assignment of the inhibitory effect of pMS2- on autogamy to the RNAi phenomenon is a matter of debate. For certain target genes and experimental regime, higher RNAi efficiency has resulted from transgenes directly injected into host cells than from those delivered by feeding (Timmons and Fire, 1998; Timmons et al., 2002). Delivery of the MS2 transgene by microinjection may trigger more distinct inhibitory effect and make the assignment conclusive. Besides, fine mapping of the MS2 sequence required for the phenotypic change should help the molecular understanding of the role of MS2 in the *Paramecium* autogamy pathway.

ACKNOWLEDGMENTS

We thank Dr. Yoshiomi Takagi for valuable suggestions.

REFERENCES

Berger, J.D. (1986) Autogamy in *Paramecium*: cell cycle step-specific commitment to meiosis. Exp. Cell Res., 166, 475-485.

Berger, J.D. and Rahemtullah, S. (1990) Commitment to autogamy in *Paramecium* blocks mating reactivity: implications for regulation of the sexual pathway and the breeding system. Exp. Cell Res., 187, 126-133.

Brantl, S. (2002) Antisense-RNA regulation and RNA interference. Biochim. Biophys. Acta, 1575, 15-25.

Breuer, M., Schulte, G., Schwegmann, K.J. and Schmidt, H.J. (1996) Molecular characterization of the D surface protein gene subfamily in *Paramecium tetraurelia*. J. Euk. Microbiol., 43, 314-322.

Caron, F. and Meyer, E. (1985) Does *Paramecium primaurelia* use a different genetic code in its macronucleus? Nature, 314, 185-188.

Dasgupta, S., Fernandez, L., Kameyama, L., Inada, T., Nakamura, Y., Pappas, A. and Court, D. L. (1998) Genetic uncoupling of the dsRNA-binding and RNA cleavage activities of the *Escherichia coli* endoribonuclease RNaseIII-the effect of dsRNA binding on gene expression. Mol. Microbiol., 28, 629-640.

Dippell, R.D. (1955) A temporary stain for *Paramecium* and other ciliate protozoa. Stain Technol., 30. 69-71.

Galvani, A. and Sperling, L. (2001) Transgene-mediated post-transcriptional gene silencing is inhibited by 3’ non-coding sequences in *Paramecium*. Nucl. Acids Res., 29, 4387-4394.

Galvani, A. and Sperling, L. (2002) RNA interference by feeding in *Paramecium*. Trends in Genetics, 18, 11-12.

Haga, N. and Hiwatashi, K. (1981) A protein called immaturin controlling sexual immaturity in *Paramecium*. Nature, 289, 177-179.

Hannon, G.J. (2002) RNA interference. Nature, 418, 244-251.

Iizima, S., Numata, M. and Miwa, I. (1997) Carry over effects on the immaturity of autogamy in *Paramecium tetraurelia*. Zool. Sci., 14 (Suppl.), 27.

Ishikawa, Y., Suzuki, A. and Takagi, Y. (1998) Factors controlling the length of autogamy-immaturity in *Paramecium tetraurelia*. Zool. Sci., 15, 707-712.

Kosciuszko, H. and Koizumi, S. (1983) Induction of autogamy by transfer of macronuclear karyoplasm in *Paramecium tetraurelia*. Exp. Cell Res., 146, 426-438.

Kung, C., Saimi, Y., Haynes, W.J., Ling, K-Y. and Kissmehl, R. (2000) Recent advances in the molecular genetics of *Paramecium*. J. Eukaryot. Microbiol., 47, 11-14.

Mikami, K. and Koizumi, S. (1983) Microsurgical analysis of the clonal age and the cell-cycle stage required for the onset of autogamy in *Paramecium tetraurelia*. Dev. Biol., 100, 127-132.

Miwa, I., Haga, N. and Hiwatashi, K. (1975) Immaturity substances: material basis for immaturity in *Paramecium*. J. Cell Sci., 19, 369-378.

Miwa, I. (1984) Destruction of immaturin activity in early mature mutants of *Paramecium caudatum*. J. Cell Sci., 72, 111-120.

Nielsen, E., You, Y. and Forney, J. (1991) Cysteine residue periodicity is a conserved structural feature of variable surface proteins from *Paramecium tetraurelia*. J. Mol. Biol., 222, 835-841.

Preer, J.R., Jr., Preer, L.B., Rudman, B.M. and Barnett, A.J. (1985) Deviation from the universal code shown by the gene for surface protein 51A in *Paramecium*. Nature, 314, 188-190.

Ruiz, F., Vayssie, L., Klotz, C., Sperling, L. and

Madeddu, L. (1998) Homology-dependent gene silencing in *Paramecium*. Mol. Biol. Cell, 9, 931-943.

Shepherd, J.C.W. (1981) Method to determine the reading frame of a protein from the purine/pyrimidine genome sequence and its possible evolutionary justification. Proc. Natl. Acad. Sci., USA, 78, 1596-1600.

Siegel, R.W. (1961) Nuclear differentiation and transitional cellular phenotypes in the life cycle of *Paramecium*. Exp. Cell Res., 24, 6-20.

Smith-Sonneborn, J. (1981) Genetics and aging in protozoa. Int. Rev. Cytol., 73, 319-354.

Sonneborn, T.M. (1954) The relation of autogamy to senescence and rejuvenescence in *Paramecium aurelia*. J. Protozool., 1, 38-53.

Sonneborn, T.M. (1974) *Paramecium tetraurelia*. *In:* Handbook of Genetics Vol. 2. Meyer, E (ed.). Plenum, New York/London, pp. 469-594.

Takagi, Y., Suzuki, T. and Shimada, C. (1987) 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.

Takiff, H.E., Chen, S-M. and Court, D.L. (1989) Genetic analysis of the *rnc* operon of *E. coli.* J. Bacteriol., 171, 2581-2590.

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.

Timmons, L. and Fire, A. (1998) Specific interference by ingested dsRNA. Nature, 395, 854.

Timmons, L. Donald, L.C. and Fire, A. (2002) Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. Gene, 263, 103-112.

Tsuboi, D., Qadota, H., Kasuya, K., Amano, M. and Kaibuchi, K. (2002) Isolation of the interacting molecules with GEX-3 by a novel fractional screening. Biochem. Biophys. Res. Commun., 292, 697-701.