Original

Paramecium tetraurelia Genes Expressed in an Age-Dependent Manner

Hiroyuki TANABE*, Masaki FUJIIKE and Sachiko YOSHIOKA

Laboratory of Biochemistry, Faculty of Agriculture, Kinki University, Nara 631-8505, Japan.

SUMMARY

In search of the *jumyo* gene, a putative gene that specifies the clonal life span of *Paramecium tetraurelia*, we previously compared gene expression profiles between young wild-type stock 51 and the short life span *jumyo* mutant d4-SL4 by the differential display technique, and detected four differentially expressed genes. In this study, assuming that expression of the *jumyo* gene is continuously altered throughout the clonal life span, we examined the transcriptional level of the four genes in the process of clonal aging, but none of them presented such an alteration. Thus, we again screened expressed genes of the two strains using another strategy, cDNA subtraction, and successfully isolated three different genes that show age-dependent expression patterns as expected. Of these three genes, one was specific to young wild-type cells and found to code for the 51A surface protein. The other two were preferentially transcribed in the *jumyo* mutant, of which one was the α-51D surface protein gene.

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

INTRODUCTION

Aging and a limited span of longevity are fundamental realities of organic life and it is clear that one or more genetic components mediate these complex phenomena (Martin, 2000 and a series of reviews in the same issue). One promising approach for identification of these life-controlling factors is to look for genetic differences that discriminate long-lived from short-lived species. The *jumyo* gene has been reported to be responsible for the shortened clonal life span of a mutant strain, d4-SL4, of the ciliated protozoan *Paramecium tetraurelia* (Takagi et al., 1989). In our attempt to elucidate the molecular basis for the *Paramecium* life span, we previously screened expressed genes of the short-lived *jumyo* mutant and its wild-type parental strain for the prevalence of specific gene transcripts, using the differential display technique (Tanabe and Yoshioka, 2000). Although we isolated four genes that showed differential expression patterns between the two strains, their involvement in life span determination was not elucidated.

Normal somatic cells of higher eukaryotes have a finite number of divisions and telomeres are considered to be a molecular clock marker for such limited cellular life span: progressive telomere shortening at each replicative cycle is associated with replicative senescence (Dhaene et al., 2000; Ishikawa, 2000). Similarly, irreversible changes

must be recorded in the expression profile of the *jumyo* gene as a function of the number of cell fissions, which may underlie the limited longevity of *Paramecium*, because the metric of clonal life span in *Paramecium* is the number of cell fissions rather than chronological age (Smith-Sonneborn and Reed, 1976; Takagi and Yoshida, 1980; Takagi et al., 1987a). A speculative model to explain the fission-counting machinery in the *Paramecium* life-maintenance program is as follows: The *jumyo* gene normally serves to extend the clonal life span. Hence, its expression is prominent when paramecia are young but thereafter declines as cell fission number increases until the termination of expression and consequent clonal extinction. The *jumyo* mutant would be defective in this gene. An alternative model is that the *jumyo* gene operates against life extension. In this model, its expression, at background level in young paramecia, is gradually enhanced with each cell fission to a threshold, beyond which clonal death results. In this case, a mutational constitutive activation of this gene takes place in the *jumyo* mutant.

In this study, we screened genes differentially expressed between young wild-type paramecia and the *jumyo* mutant and isolated three genes expressed in correlation with clonal aging. We show the characterization of these genes and discuss some of their biological consequences in the cell.

MATERIALS AND METHODS

Strains and cultivation

P. tetraurelia wild-type stock 51 and the *jumyo* mutant d4-SL4 (Takagi et al., 1987b) were used and cultivated as done previously (Tanabe and Yoshioka, 2000).

Clonal aging of paramecia

Six cell lines of stock 51 were propagated by daily single cell isolation (Sonneborn, 1957) until exautogamous cells senesced and died; the experiments were repeated four times with almost identical results. During cultivation, cells that had freshly undergone autogamy were clonally aged by daily transfer of single cells into fresh culture medium, and absence of autogamy was confirmed by inspection of the culture for senescence characteristics such as gradual decrease in growth rate, clonal extinction at ~ 200 fissions, and changes in cell morphology and behavior (Takagi, 1988) in parallel with autogamy check by cell staining (Dippell, 1955).

For RNA preparation, a portion of the daily isolation culture was removed at five stages during the clonal growth and transferred to mass cultivation to reach ~ 300 cells/ml. Average cell fissions postautogamy and fission rate (frequency per day ± standard deviation) at each point of the sampling were: the 1st, 22.1 fissions and 3.8 ± 0.32; the 2nd, 60.6 fissions and 3.8 ± 0.29; the 3rd, 95.3 fissions and 3.2 ± 0.37; the 4th, 139.0 fissions and 2.7 ± 0.77; the 5th, 167.6 fissions and 2.1 ± 1.00.

Construction and screening of subtractive libraries

Subtracted cDNA libraries were generated through suppression subtractive hybridization (Diatchenko et al., 1996; Gurskaya et al., 1996), an improved version of cDNA subtraction. Fig. 1 presents a brief overview of the procedure. "tester" (the population containing the cDNAs of interest) and "driver" (the reference population) cDNAs were synthesized from their respective mRNA populations and digested with *Rsa*I. An age-synchronized cell population of young stock 51 (about 15 fissions old) and cells of the *jumyo* mutant (1-12 fissions old) were used as sources of starting mRNA. Two different adaptor DNAs were separately ligated to the tester cDNAs. After two rounds of hybridization of the tester with excess of the driver, through which tester cDNA species shared by the driver were subtracted against the

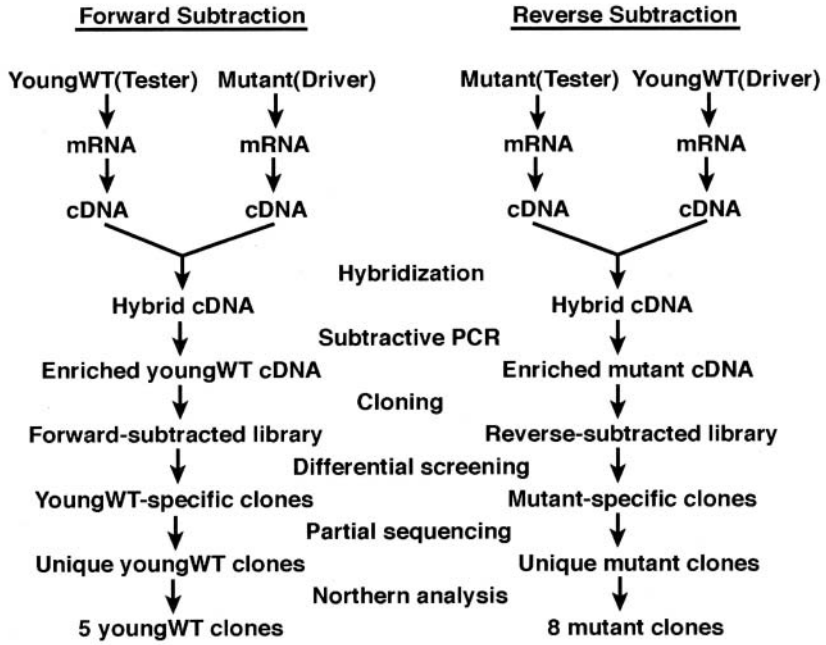

Fig. 1. Schematic diagram of the subtractive cloning method.

driver cDNAs, the resulting subtractive tester cDNAs were selectively amplified by PCR using primers complementary to the sequences of the adaptors and inserted into the vector pBluescript (Stratagene).

We performed subtractions in both directions: In the forward subtraction for leaving cDNAs specific to young stock 51, cDNAs derived from young stock 51 were used as tester and the *jumyo* mutant cDNAs served as driver. The reverse subtraction, a second subtraction done in reverse, was designed to obtain cDNAs present in the mutant (tester) but not in young stock 51 (driver). The resulting subtractive cDNA pools were greatly enriched for sequences found exclusively in the tester population, from which two subtractive cDNA libraries were constructed: the forward-subtracted library rich in young-paramecia-specific clones and the reverse-subtracted library dominated by mutant-specific ones.

These libraries were then subjected to differential screening to remove cDNA species common to tester and driver which were still present after subtraction: 200 cDNA clones randomly picked from each library were dotted (100 ng per spot) onto two pieces of nylon membrane for duplicate screening. Each membrane was probed with either of the two kinds of the subtracted cDNA pool: one piece with the forward-subtracted cDNA pool and the other with the reverse-subtracted one. The filters were then washed under stringent conditions and exposed to signal detection as previously described (Tanabe and Yoshioka, 2000).

Further screening was carried out by partial DNA sequencing for removal of redundant clones and Northern analysis to confirm expression specificity.

Other experimental techniques

Full-length cDNA sequences were obtained by

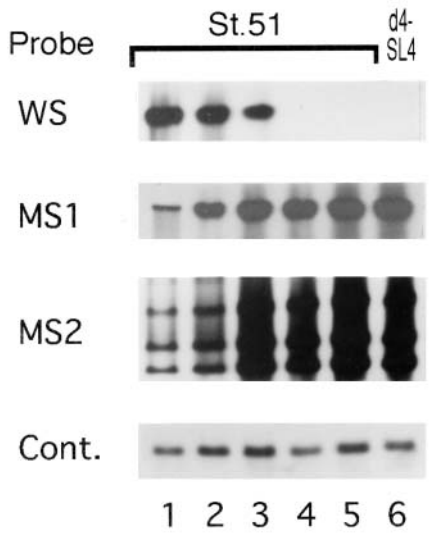

Fig. 2. Transcriptional time course of differentially-expressed genes during the clonal aging of *Paramecium tetraurelia*. A series of cultures of increasing clonal age was prepared from exautogamous stock 51. One μg of mRNAs derived from each culture were electrophoresed in a denaturing agarose gel and blotted to a nylon membrane. Lanes 1 through 5 contain the RNA samples from the first through the fifth aging stages, respectively (see MATERIALS AND METHODS for details). mRNA of the *jumyo* mutant d4-SL4 of 1-12 fissions old (lane 6) was run in parallel with these wild-type samples. Each of the four identical RNA blots was probed with the WS clone specific to young stock 51, either of two cDNA clones, MS1 and MS2, specific to the *jumyo* mutant, or a loading control cDNA (from top to bottom). The gene from which the control probe was generated has proven to be expressed in a constant degree throughout the *Paramecium* life span (data not shown).

rapid amplification of cDNA ends (Chenchik et al., 1996) using cDNA inserts from the subtractive libraries as templates. Preparation of mRNA, Northern blotting, and DNA sequence analysis were done as previously described (Tanabe and Yoshioka, 2000). The nucleotide sequence referred to here as MS2 has been deposited in the DDBJ database with the accession number AB059619.

RESULTS AND DISCUSSION

Expression of differentially displayed genes during clonal aging

As aforementioned, it is our interest whether the four genes we have cloned by differential display follow the expression mode of the *jumyo* gene in our model: As clonal age of stock 51 increases, the young-paramecia-specific W gene becomes depressed, whereas the M1 to M3 genes preferentially expressed in the short-lived mutant become activated. To address this question, we analyzed the transcript prevalence of these genes at various points in the course of clonal aging. mRNA was prepared at intervals during the daily isolation of exautogamous stock 51 and subjected to Northern blotting with probes for these genes. However, the result was inconsistent with the expected one: the W gene maintained the initial level of transcription throughout the life span and expression of the three genes specific to the mutant remained negligible until clonal termination (data not shown). We have detected only the four clones in a differential display employing 3 x 20 combinations of PCR primer (Tanabe and Yoshioka, 2000). Our target gene transcripts could be displayed with other pairs of primer, but a theoretical calculation (Bauer et al., 1993) estimated that screening all species of the *Paramecium* transcripts requires too many primer combinations for us to deal with.

Cloning of differentially-expressed genes by subtractive hybridization

Although lack of the transcriptional variation during clonal aging does not necessarily rule out the four genes for further study, we preferred isolation of other transcriptional differences between young paramecia and the *jumyo* mutant by suppression subtractive hybridization in light of our model for the life-span and obtained two subtractive cDNA libraries: the forward- and reverse-subtracted libraries (Fig. 1). Differential screening revealed that about one-fifth of the screened clones produced a

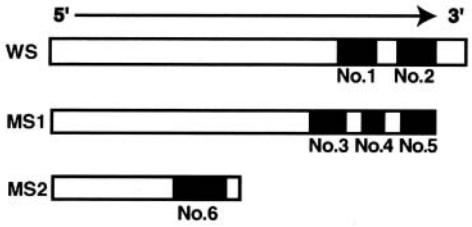

Fig. 3. Physical map of differentially expressed clones. The filled segments indicate the relative location and length of the cDNA fragment of No. 1- 6 clones in the full-length WS (8.2 kb), MS1 (7.6 kb), or MS2 (4.0 kb) cDNAs. The direction of transcription is arrowed.

markedly and reproducibly stronger hybridization signal with the corresponding cDNA probe than with the other probe in either library (data not shown). The clones that gave the differential signal indeed represent differentially expressed genes.

We partially sequenced the cDNA insert of the positive clones to see whether they were unique or redundant, and accordingly assigned them to several groups representing distinct genes or gene families. These positive clones were checked for expression specificity by probing a Northern blot containing mRNAs from young stock 51 and the *jumyo* mutant with cDNA inserts from the individual groups. Every probe hybridized preferentially to the corresponding mRNA sample, supporting the result of differential screening (compare lines 1 and 6 in Fig. 2 for representative clones). Thus, it was confirmed that representation of the quantitatively different transcripts was well retained in our experimental conditions. Overall, five cDNA clones were isolated as young-wild-type-specific from the forward-subtracted library and eight clones as mutant-specific from the reverse-subtracted library.

Screening for gene expression dependent on clonal age

We analyzed the differential clones thus obtained for expression throughout the clonal life span of stock 51 by Northern blotting as with the previously isolated four clones (Fig. 2). Of the five young-paramecia-specific clones, two (clone Nos. 1 and 2) had progressively lowered transcription levels with increasing clonal age to be almost undetectable later in the aging stage (WS in Fig. 2). In contrast, gene expression continued to increase during clonal aging in four clones (clone Nos. 3 - 6) out of the eight derived from the mutant-specific library (MS1 and MS2 in Fig. 2). As is evident from this figure, the transcriptional kinetics in these six clones is almost superposable on either of the two patterns of the *jumyo* expression predicted in our model. As for the remaining clones, little transcriptional change accompanied clonal aging, narrowing our target genes to the above six clones.

During determination of the complete cDNA sequences corresponding to the six clones (Fig. 3), the two young-wild-type-specific sequences, Nos. 1 and 2, appeared separately in the same gene tentatively named WS in Fig. 2. Similarly, three of the four mutant-specific clones, Nos. 3 - 5, were found to represent three different regions of one common gene, MS1 in Fig. 2. The remaining one, No. 6, was assigned to MS2.

Characterization of target genes

Sequencing the full-length cDNA for the three genes indicated that WS and MS1 scored perfect matches with the genes for *P. tetraurelia* surface proteins 51A (Nielsen et al., 1991) and α-51D (Brener et al., 1996), respectively, but no meaningful alignment was found for MS2 with available DNA and protein databases.

The *Paramecium* surface protein, or immobilization antigen, is a class of proteins with unknown function and a phenotype resulting from its presence is called serotype (Caron and Meyer, 1989; Bleyman, 1996). *P. tetraurelia* stock 51 is able to express at least 11 different serotypes including 51A and 51D, and the 51D gene comprises a family

of isogenes, named α-, γ_1-, γ_2-, δ-, and ε-51D, in which α-51D is expressed in conditions where the D serotype is detected. Serotype expression occurs in a mutually exclusive manner with only one surface protein present on a cell surface at a time and transforms to other serotypes in response to environmental changes. Among the *P. tetraurelia* serotypes, 51A is dominant in standard cultivation (Preer et al., 1987) as done in this study. Our results indicate that cells of stock 51 present a decreasing 51A expression with a concurrent increasing α-51D expression as clonal aging proceeds. Although other laboratory has reported serotype transformation as a senescence characteristic of paramecia (Sundararaman and Cummings, 1983), the present study is the first to identify serotypes showing an age-associated expression pattern.

Despite its expression associated with *Paramecium* clonal aging, the 51A serotype is unlikely to causally participate in the aging process, but is supposed to be expressed as a consequence of senescence, because *P. tetraurelia* d48, a mutant lacking 51A, is healthy (You et al., 1991) and passes through the normal aging process (Gilly and Blackburn, 1994). The same holds for α-51D. The dominant serotype in a double mutant unable to express the 51A and 51B serotypes appears to be 51D (Preer et al., 1987), making it a plausible idea that the senescent up-regulation of α-51D is a reciprocal transformation for the down-regulation of 51A. These surface protein genes constituted a great majority of the subtractive library after the differential screening. This high redundancy is accounted for by abundance of mRNAs for surface proteins. The amount of surface proteins has been estimated to be about 3.5% of the total protein of *Paramecium* and their mRNAs are also present in high concentration (Preer et al., 1981). In contrast, genes playing a key role in cellular functions are in general weakly expressed and therefore poorly represented in mRNA populations, which is probably the case with the *jumyo* gene. Although the subtraction strategy introduces equalization of proportions of particular transcripts during the subtractive process in favor of representation of rare transcripts and the subsequent cloning of their cDNAs (Gurskaya et al., 1996), the quantitative predominance of the two serotype genes even after the subtraction suggests that the exceptionally high abundance of the serotype transcripts may adversely affect the equalization step and thereby make direct cloning of the *jumyo* gene extremely difficult. Still, given that there is a causal relationship between the serotype switch and the control of *Paramecium* longevity, we may be able to isolate the *jumyo* gene as the ultimate agent functioning most upstream the cellular pathway for the serotype expression by means of genetic and/or biochemical approaches. Our next targets are regulatory factors immediately involved in the serotype expression.

At present, we have little information about the function of the MS2 gene. In consideration of its representation in subtractive libraries, this clone is presumably highly transcribed, similar to serotype genes. The high abundance of the MS2 transcript is also supported by its strong Northern blot signal (Fig. 2). Furthermore, MS2 gave rise to multiple sizes of transcript (Fig. 2). Such a multi-banded pattern is yielded via at least three mechanisms: alternative splicing (Smith and Valcarcel, 2000), use of different transcription initiation sites, and use of different polyadenylation sites. Alternatively, MS2 may be a member of a multigene family. In either case, involvement of this polymorphism in the senescent expression of MS2 is not clear. Transformational overexpression in wild-type paramecia and transcriptional repression in the *jumyo* mutant of this gene should provide insights into its role and significance in the aging process and the life-controlling mechanism.

Subtractive cDNA hybridization has been a powerful approach to identify and isolate cDNAs of differentially expressed genes. The incorporation of PCR into the procedure gave a strong increase in

sensitivity and made it possible to detect differential hybridization signals derived from only a few transcripts. One potential disadvantage of this technique, however, is that the strategy is biased for high copy number mRNA and might be inappropriate in experiments in which only a few genes are expected to vary. The original disproportion in expression level between the group of unusually high abundance, *i. e.*, serotype genes and the newly isolated MS2, and that of rare transcripts is preserved in the *Paramecium* system, resulting in the escape of the latter from subtractive cloning. To overcome this problem, we must extensively examine the equalization of the abundance of cDNAs within the target population after subtraction.

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