RT-PCR method for selective detection of silent gene transcripts in silencing mutants in homothallic strains of *Schizosaccharomyces pombe*

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Here we describe a method that allows selective detection of silent copy transcripts in homothallic strains of *Schizosaccharomyces pombe* in the presence of the active cassettes. The method involving RT-PCR (reverse transcriptase polymerase chain reaction) exploits our observation that the silent copy transcripts extend beyond the regions of homology to the flanking sequences specific for the donor cassettes, thus allowing design of oligos that are specific for the different donors. The results are validated using a known silencing mutant *swi6*.

With the emergence of *Schizosaccharomyces pombe* as a model system for gene silencing (1), there is a need for simpler and faster methods for detection of silent copy transcripts. The common biochemical approach for detecting the silent gene transcripts in the silencing mutants of *S. pombe* involves Northern hybridization, wherein a strain with stable *M* mating type is used and the transcripts from the *mat2P* locus are detected by probing the Northern blots with plus (+) allele-specific probe (2). Alternatively, the RT-PCR (reverse transcription polymerase chain reaction) approach was used to amplify either *mat2P* or *mat3M* specific transcripts in a strain carrying a deletion of the *mat1* locus (3). However, detection of silent gene transcripts in silencing mutants with a homothallic genetic background by the abovementioned approaches is not feasible because of contribution of similar plus (+) and minus (-) allele-specific products by the *mat1* locus as well.

We have arrived at a solution to this problem on the basis of our finding that the *mat2P*- (and possibly *mat3M*-) specific transcripts extend beyond the homology regions *H2* and *H3* (Figure 1). It has been shown earlier that the *mat1*-specific

transcripts *mat1Pc* and *mat1Mi* both extend beyond the *H2* region of *mat1* in the centromere-proximal direction, yielding 750 and 800 nucleotide-long transcripts, respectively (4). We asked the question whether the transcripts originating from the *mat2P* (*mat2Pc* transcript) and *mat3M* (*mat3Mi*) also extend beyond the homology region *H3*, which is common to both these cassettes. To test this, an oligo (dT) primer was used to make the cDNA

copy of the total RNA and the [γ^{32} P]-labeled plus or minus allele-specific oligos were used to PCR amplify the *Pc* and *Mi* transcripts both from the expressed *mat1* locus and the silent locus *mat2P*. We found that in addition to the 750 bp product specific for *mat1Pc* (4), another band of about 800 bp was also observed in the silencing mutants, which presumably originated from the donor locus *mat2P* and extended about 300 bp outside the *H3* region (data summarized in Figure 1). Although a similar mapping was not done for *mat3M*, the results shown below indicate that *Mi* transcript from *mat3M* also extends well past the *H3* box. From these data, we infer that the transcript *Pc* transcribed from *mat2P*, and possibly the transcript *Mi* transcribed from *mat3M* (this prediction was corroborated by the experiments below), extend far outside not only the *H2* box but the *H3* box as well. Therefore, without having to map the 3' ends of the donor-specific transcripts, it should be possible to amplify the *mat2Pc* and *mat3Mi* transcripts specifically by using the oligos located immediately outside the *H3* box in the *mat2P* and *mat3M* loci, respectively. It has been shown that *Pc* transcript is constitutively expressed but stimulated further

Figure 1. Schematic representation of the mating type loci *mat1P/M*, *mat2P*, and *mat3M*. The lengths of the *Pc* and *Mi* transcripts from *mat1* and PCR products from *mat2P* and *mat3M* are indicated. The relative location of the oligos within and outside the donor cassettes used for PCR and the predicted sizes of the PCR fragments (500 bp for *mat2Pc* and 366 bp for *mat3Mi*) are indicated.

by nitrogen starvation (4). On the other hand, the *Mi* transcript is not expressed at all in the nitrogen-rich medium and is induced upon nitrogen starvation (4). Further, it has been shown that the silent loci are also derepressed in the *swi6* mutant (5). Therefore, we chose *swi6* mutant as a representative silencing mutant.

Suitable antisense oligos were designed that are located immediately proximal to the *H3* region in the donor loci (Table 1). The whole procedure was carried out in the following steps:

1. Total RNA was isolated from homothallic (*h⁹⁰*) wild-type and *swi6* mutant strains grown in nitrogen-rich and nitrogen-free medium (6) by the hot phenol method (7).
2. cDNA synthesis was carried out with 10–15 µg RNA using the oligo (dT) primer (Table 1) with MuLV reverse transcriptase (New England Biolabs, Ipswich, MA, USA), using the manufacturer's protocol.
3. The cDNA product was diluted 10-fold and PCR reactions were carried out in 50 µl, using the primer pair 1 and 2 for *mat2Pc* and primer pair 3 and 4 for *mat3Mi* (Table 1). The PCR conditions were: 94°C, 1 min; 55°C, 1 min; and 72°C, 2 min; 15–20 cycles. Under these conditions, we have found that we can compare the quantities of the RNA levels as this condition occurs during the logarithmic phase of the amplification. The PCR products were resolved by agarose gel electrophoresis, followed by Southern blotting (8). Hybridization was carried out with the random primer labeled probes (9) prepared from the plasmids containing the *mat2P* and *mat3M* fragments. The protocol is outlined in Table 2.

Results are shown in Figure 2. An interesting observation is the presence of the PCR product of 500 bp specific for *mat2Pc* transcript, at a low level in nitrogen-rich medium, which is significantly elevated under nitrogen starvation conditions in wild-type samples (Figure 2, lane 2).

Figure 2. RT-PCR analysis of the *mat2Pc* and *mat3Mi* transcripts in wild-type, *h⁹⁰* (homothallic) and *h⁹⁰, swi6* strains. RNA was isolated from wild-type (lanes 1, 2, 5, 6) and *swi6* (3, 4, 7, 8) mutant cells grown in nitrogen-rich medium (lanes 1, 3, 5, 7; indicated by + sign) and nitrogen-free medium (lanes 2, 4, 6, 8; indicated by - sign), and cDNA synthesis and *mat2Pc*- and *mat3Mi*-specific PCR reactions were carried out as described earlier (4,7). The PCR products were resolved by agarose gel electrophoresis, subjected to Southern blotting and hybridization as described (8). The probes used were prepared from the plasmid DNAs of *mat2P* and *mat3M* by PCR using the same oligo pairs; oligos 1 and 2 for *mat2Pc* and oligos 3 and 4 for *mat3Mi*.

Table 1. List of the Oligos Used for cDNA Synthesis and PCR Reaction

Name of the Oligo	5'- Sequence of the oligo -3'
Oligo (dT) primer	AATTCTGCAGTGATATCTTTTTTTTTTTTTTTTTT
Oligo 1 (<i>mat2Pc</i> sense)	GATTAAGAGCACCTATTTTCTTGCC
Oligo 2 (<i>mat2Pc</i> antisense)	CAACGGATTACTAAAAACAGTTTAAATG
Oligo 3 (<i>mat3Mi</i> sense)	CATACTAATAATGTCAGCAGAAGAC
Oligo 4 (<i>mat3Mi</i> antisense)	CCAATCAACTTAACATGAAGCAACTCCTGATAC

Interestingly, a high level of *mat2Pc* transcript was observed in *swi6* mutant even in nitrogen-rich medium, which is similar to that of wild-type sample under nitrogen starvation conditions (Figure 2, compare lane 3 to lane 2), and is further elevated in the nitrogen minus medium (Figure 2, lane 4). More dramatic results are obtained with *mat3Mi* transcript. Very low levels of *mat3Mi* PCR product (estimated size 366 bp) are observed both in nitrogen-rich (Figure 2, lane 5) and nitrogen-free medium (Figure 2, lane 6). However, in *swi6* strain, while the nitrogen-rich sample shows a barely detectable level of the *mat3Mi* product (Figure 2, lane 7), nitrogen starvation leads to substantially higher levels of the product (Figure 2, lane 8). Controls for the expression of a normally expressed gene such as DNA pol α showed no change (not shown). Moreover, the specificity of the PCR product as *mat2Pc* or *mat3Mi* was confirmed by the absence of any signal when only RNA was used instead of the cDNA, ruling out the templating of the PCR by the genomic DNA that might still be

present in the RNA (data not shown). Quantitation by densitometric scanning shows that under nitrogen-rich conditions the *mat2Pc* transcript is elevated by 7.7-fold in *swi6* mutant as compared with the wild-type strain; this level was further elevated by 1.7-fold in the nitrogen-free medium. Likewise, *swi6* mutant shows a 13.8-fold induction in the level of *mat3Mi* transcript in nitrogen-free medium as compared with the nitrogen-rich medium. Using the conditions described above, we have been able to successfully detect and quantitate the silent gene transcripts in other silencing mutants as well, even in the homothallic background. The leakiness of the *mat2Pc* transcript from the silent locus under nitrogen-rich conditions is surprising but has been reported earlier (10); it has been speculated that this may result from the partial unfolding of the donor cassette *mat2P* during the process of switching in homothallic (*h⁹⁰*) strain (10).

In summary, we have devised a method that allows specific amplification of donor-specific transcripts in the silencing mutants in homothallic

Table 2. Steps Involved in the RT-PCR Protocol

Grow the strains (*h⁹⁰* and *h⁹⁰ swi6*) in nitrogen-rich medium at 30°C.

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Shift duplicate cultures to nitrogen-rich and nitrogen-free medium at 30°C.
Grow for 4 hours.

↓

Prepare total RNA by hot-phenol method.

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cDNA Synthesis:
Take 10–15 µg total RNA.
Add 5 µl 10× M-MuLV reverse transcriptase buffer (NEB).
5 mM dNTPs
80 ng Oligo (dT) primer
25 U M-MuLV reverse transcriptase (NEB)
Final volume = 50 µl

↓

Incubate at 42°C for 1–2 h.

↓

PCR reaction: [94°C, 1 min, 55°C, 1 min, 72°C, 2 min; 15–20 cycles]
1 µl cDNA
5 µl 10X PCR buffer (Promega)
2.5 mM MgCl₂
50–100 µM dNTPs each
20 ng oligo (dT) primer
20 ng *mat2Pc* or *mat3Mi*-specific primer
2 U Taq polymerase (Promega)
Final volume = 50 µl

Resolve the PCR product by agarose gel electrophoresis, followed by Southern blotting and hybridization.

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background in *S. pombe* and that allows quantitation of the relative message levels in different mutants. Further multiplexing can facilitate simultaneous detection and quantitation of transcripts from both the donor loci. This approach should be useful for research workers working in the field of gene regulation of the mating type loci in fission yeast.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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