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# Molecular cloning and sequence analysis of a *ras* gene from *Schizosaccharomyces pombe*

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**We have cloned a *ras* gene homologue from fission yeast *Schizosaccharomyces pombe* and determined its nucleotide sequence. A putative coding sequence for 219 amino acids was found. The sequence contained one set of splicing signals: GTAAGT for a donor sequence, ACTAA for a unique sequence found in introns of yeast genes and TAG for an acceptor sequence, indicating the existence of an intron. The amino-terminal one third of the predicted *S. pombe ras* protein was nearly perfectly homologous and the next one third moderately homologous to those of mammalian *ras* proteins. The carboxy-terminal one third showed no homology but terminated with a short conserved sequence Cys-X-X-Z (X being a hydrophobic amino acid) as in other *ras* proteins. The result of Southern analysis of *S. pombe* DNA under non-stringent hybridization conditions using our clone as a probe indicated that no other closely related gene may be present in the *S. pombe* genome. The transcript of this gene could be detected by Northern analysis.**

*Key words: ras* gene/*S. pombe*/DNA sequence/molecular cloning/intron

## Introduction

*Ras* genes have been identified as the oncogenes of Harvey and Kirsten sarcoma viruses (Ellis *et al.*, 1981). Transfection of NIH3T3 cells with DNAs from various human tumors showed that in many cases cellular homologues of *ras* may play a role in the formation of tumors (Der *et al.*, 1982; Parada *et al.*, 1982; Santos *et al.*, 1982; Shimizu *et al.*, 1983). In mammalian cells, at least three cellular *ras* genes, Ha-, Ki-, and N-*ras* genes, are present, each coding for a 21-kd protein (p21) (Capon *et al.*, 1983; McGrath *et al.*, 1983; Shimizu *et al.*, 1983; Taparowsky *et al.*, 1983). Mutations at the 12th, 13th, 59th, 61st or 63rd amino acid residue, can activate the transforming potential of the *c-ras* protein (Fasano *et al.*, 1984). The p21 lipoprotein is localized at the inner surface of the plasma membrane (Willingham *et al.*, 1980; Furth *et al.*, 1982; Sefton *et al.*, 1982). It binds guanine nucleotides (Scolnick *et al.*, 1979) and has an intrinsic GTPase activity (Sweet *et al.*, 1984; McGrath *et al.*, 1984).

Recently, two genes (*RAS1* and *RAS2*), which are highly homologous to mammalian *ras* genes were found in yeast, *Saccharomyces cerevisiae* (De Feo-Jones *et al.*, 1983, Powers *et al.*, 1984). The *S. cerevisiae ras* proteins (309 and 322 amino acids) are about twice as large as mammalian p21s (188 and 189 amino acids) but have many features common to p21 proteins. They showed nearly 90% homology to the mammalian p21 in the amino-terminal 80 amino acid sequence, nearly 50% homology in the next 80 amino acids, and possessed the unique sequence Cys-X-X-Z at the carboxy terminus. The ability to bind guanine nucleotides was also detected in the *RAS2* protein overproduced in *S. cerevisiae* (Tamanoi *et al.*, 1984). Destruction of one of the two *RAS* genes did not affect the growth of yeast, but when both of them were destroyed the cells were incapable of vegetative growth (Tatchell *et al.*, 1984; Kataoka *et al.*, 1984).

Here we report the molecular cloning and nucleotide sequence determination of a *ras* gene from fission yeast *Schizosaccharomyces pombe*. The predicted *S. pombe ras* protein is more similar to the mammalian *ras* proteins in size (219 amino acids) than *S. cerevisiae RAS1* and *RAS2*, and displays typical feature of mammalian *ras* proteins. It contains an intron structure in the neighborhood of its $NH_2$ terminus. On Southern hybridization analysis of *S. pombe* DNA, we could detect only a single band using the *S. pombe ras* gene as a probe.

## Results

### *Cloning of S. pombe ras gene*

*S. pombe* DNA was digested with several restriction endonucleases and analyzed by Southern hybridization using nick-translated *S. cerevisiae RAS1* and *RAS2* fragments as probes under non-stringent conditions. One faint band could be detected in each lane (Figure 1), of which the 4-kb *Sal*I fragment was

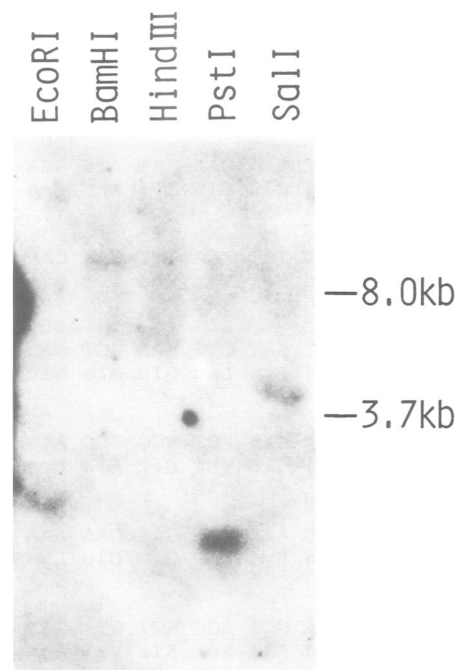


**Fig. 1.** Southern blot analysis of the *S. pombe* DNA. DNA from *S. pombe* strain JY282 was digested with several restriction endonucleases as indicated, and electrophoresed in a 1% agarose gel. DNA was denatured and transferred to a nitrocellulose membrane. Hybridization was carried out using nick-translated *S. cerevisiae RAS1* fragments under non-stringent conditions. The mobility of the size markers is shown on the right.

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cloned into a vector pBR327. For this, 50 μg of *S. pombe* DNA was digested with *Sal*I and electrophoresed on an agarose gel. Fragments with the sizes between 3.5 and 4.5 kb were prepared by electroelution and ligated to the *Sal*I-digested and alkaline phosphatase-treated pBR327. Transformation of *E. coli* with the hybrid plasmids yielded ~3000 colonies. These colonies were divided into several groups, and cultured. Plasmid DNAs were prepared from each culture, digested with restriction endonucleases and analyzed by Southern hybridization, using *RAS1* DNA as a probe. Existence of a fragment homologous to *RAS1* was confirmed in one group. Then the colonies in the group were divided into smaller groups and analyzed in the same way. The analysis was repeated until a single colony harboring a *ras* candidate clone was isolated. The clone hybridized not only with *S. cerevisiae RAS* but also with retroviral *ras* genes such as v-Ha-*ras* or v-Ki-*ras*.

100bp.
E S A A A S H AAASHAA A A SH

**Fig. 2.** The restriction map of *S. pombe ras* gene and the strategy of nucleotide sequencing. The 1.3-kb *S. pombe* genomic DNA fragment containing *SPRAS* is shown. The bold line indicates the coding sequences for the *SPRAS* protein. The single letters indicate the recognition sites of the following restriction endonucleases; A, *Alu*I; H, *Hae*III; E, *Eco*RI; and S, *Sau3A*I. The strategy for DNA sequencing is also shown. Arrows indicate the region and direction of DNA sequencing by the dideoxy method using the M13 system.

### Nucleotide sequence analysis

To minimize the sequence which is hybridizable with *ras* genes, the plasmid was digested with several restriction endonucleases and analyzed by Southern hybridization using $^{32}$P-labelled *RAS1* and *RAS2* probes. A 1.4-kb *Pst*I-*Eco*RI fragment was found to hybridize with *ras* probes. A restriction map of the fragment was constructed and the nucleotide sequence was determined by the dideoxy method using the M13 system as shown in Figure 2.

Figure 3 shows the nucleotide sequence and the predicted amino acid sequence of 219 amino acids. The coding sequence is interrupted by a 67-bp intervening sequence between codons 2 and 3. The sequences GTAAGT and TAG were found as splice donor and acceptor sequences, respectively. Recently, the sequence TACTAACA was proposed as a splice consensus sequence for

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   -50        *        *        *        *      -1+1         *        *        *
GACCAACCTAAAGAAATATAACGATTAAACTTAGTGTGTGCTTTATAAAATAGTGAAT ATG AGG GTAAGTCTAAGCAATGACTTGGGGTTATCTTT
                                                           Met Arg
                                                            1
 *       50        *        *         *         *        100
TATTACAACAAACTAATCAACGATAATATTTATAG TCT ACC TAC TTA AGA GAG TAC AAA TTC GTA GTT GTA GGT GAT GGT GGT
                                    Ser Thr Tyr Leu Arg Glu Tyr Lys Leu Val Val Val Gly Asp Gly Gly
                                                                 10
                                  150
GTT GGT AAA AGT GCT TTG ACA ATT CAA TTA ATT CAA TCG CAT TTT GTT GAC GAA TAC GAT CCT ACA ATC GAA GAT
Val Gly Lys Ser Ala Leu Thr Ile Gln Leu Ile Gln Ser His Phe Val Asp Glu Tyr Asp Pro Thr Ile Glu Asp
     20                                      30                                      40
 200                                                                250
TCA TAT CGG AAA AAG TGT GAA ATT GAT GGT GAA GGT GCG GTT TTA GAT CTA TTG GAC ACG GCC GGT CAA GAG GAA
Ser Tyr Arg Lys Lys Cys Glu Ile Asp Gly Glu Gly Ala Leu Leu Asp Val Leu Asp Thr Ala Gly Gln Glu Glu
                         50                                      60
                                  300
TAT TCC GCT ATG CGT GAA CAA TAT ATG AGA ACC GGA CAG GGT TTT TTA TTA GTT TAT AAT ATT ACC TCT CGG TCC
Tyr Ser Ala Met Arg Glu Gln Tyr Met Arg Thr Gly Glu Gly Phe Leu Leu Val Tyr Asn Ile Thr Ser Arg Ser
     70                                      80                                      90
 350                                                                400
TCT TTT GAC GAA ATT TCT ACA TTT TAT CAA CAA ATC CTT AGA GTT AAG GAT AAG GAT ACA TTT CCA GTA GTT TTA
Ser Phe Asp Glu Ile Ser Thr Phe Tyr Gln Gln Ile Leu Arg Val Lys Asp Lys Asp Thr Phe Pro Val Val Leu
                        100                                     110
                                  450
GTG GCT AAT AAA TGC GAT CTT GAA GCT GAA CGT GTA GTT TCA AGA CGT GAA CGC GAA CAA CTT GCA AAA AGC ATG
Val Ala Asn Lys Cys Asp Leu Glu Ala Glu Arg Val Val Ser Arg Arg Glu Gly Glu Gln Leu Ala Lys Ser Met
    120                                     130                                     140
 500                                                                550
CAT TGT TTA TAT GTT GAA ACT TCG GCG AAG CTA AGG TTA AAT GTA GAA GAA GCT TTT TAT AGT TTG GTT CGC ACC
His Cys Leu Tyr Val Glu Thr Ser Ala Lys Leu Arg Leu Asn Val Glu Glu Ala Phe Tyr Ser Leu Val Arg Thr
                        150                                     160
                                  600
ATC CGT CGC TAT AAC AAA TCA GAA GAA AAA GGT TTT CAT AAT AAG CAG GCG GTC CAG ATT GCC CAA GTT CCA GCT
Ile Arg Arg Tyr Asn Lys Ser Glu Glu Lys Gly Phe Gln Asn Lys Gln Ala Val Gln Ile Ala Gln Val Pro Ala
    170                                     180                                     190
 650                                                                700
TCA ACT GCT AAA AGA GCT AGC GCT GTA AAT AAC AGT AAA ACC GAA GAT GAG GTT TCA ACT AAA TGT TGT GTT ATA
Ser Thr Ala Lys Arg Ala Ser Ala Val Asn Asn Ser Lys Thr Glu Asp Glu Val Ser Thr Lys Cys Cys Val Ile
                        200                                     210
               *        *       750       *        *        *        *       800        *
TGT TAG CAAGTATTATTGCAGCAATATTTGAGCTGTTTGGTGAATGCACCGTTCCTGTCTTTTGTTTCATCTCTTTCTTTTCATTTCAATAA
Cys
219
```

**Fig. 3.** Nucleotide and predicted amino acid sequences of the *SPRAS* gene. The numbers indicate the positions of nucleotides or amino acid residues starting at the initiation codon. The underlined sequences are the splice consensus sequences.

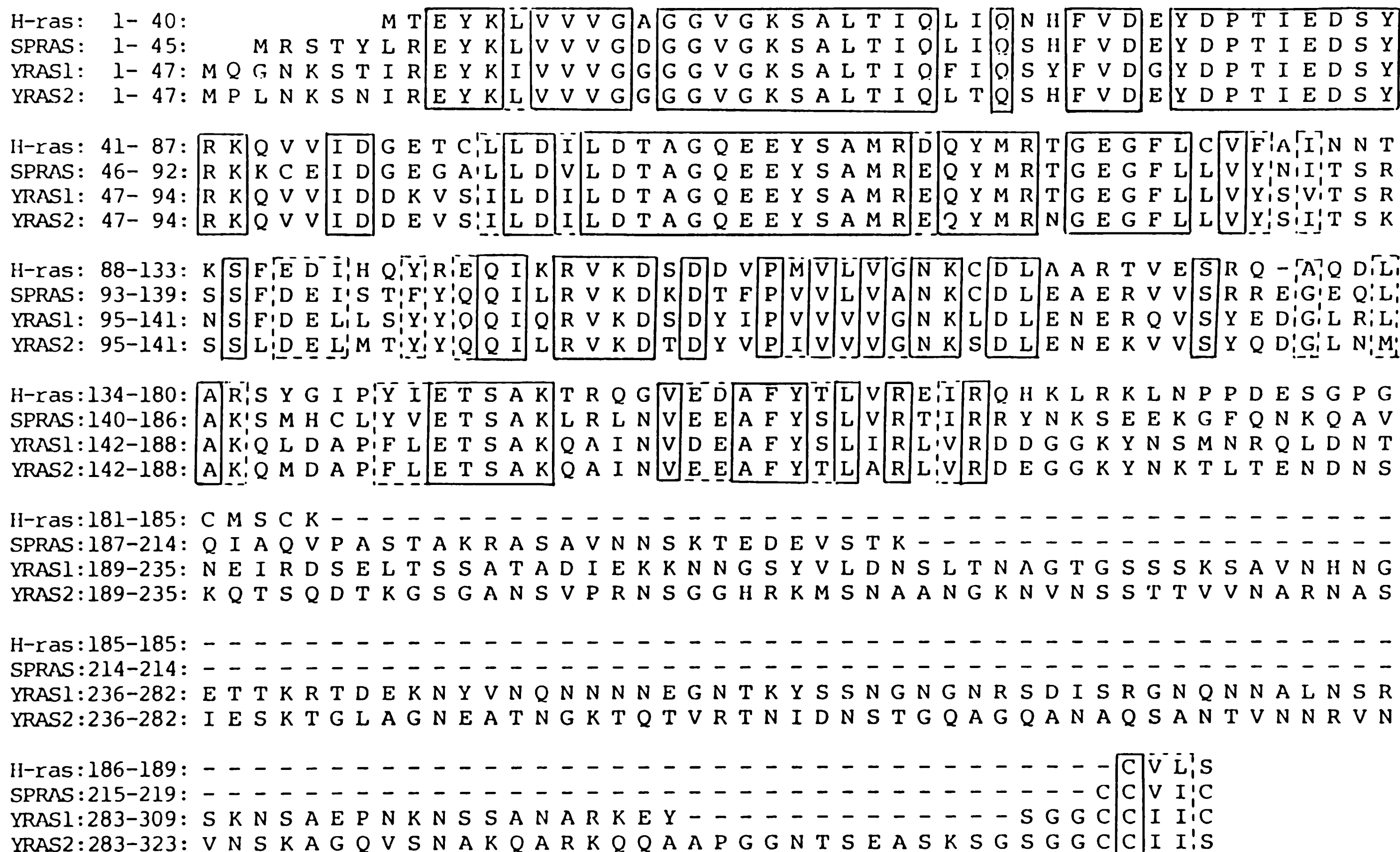


**Fig. 4.** Comparison of the amino acid sequences of *ras* proteins. The amino acid sequences of human Ha-*ras*, *S. pombe SPRAS*, *S. cerevisiae RAS1 (YRAS1)*, and *RAS2* (*YRAS2*) are shown. Sets of four identical amino acid residues are enclosed within solid lines and sets of four residues regarded as favored substitutions are enclosed within dotted lines. Favored amino acid substitutions are defined in pairs of residues belonging to one of the following groups: S, T, P, A and G; N, D, E and Q; H, R and K; M, I, L and V; F, Y and W (Dayhoff *et al.*, 1978). Hyphens show the gaps introduced to give maximal homology.

splicing for several *S. cerevisiae* genes (Langford *et al.*, 1984). We could find a sequence AACTAATCA which was similar to the typical one. The mol. wt. of the predicted polypeptide was calculated to be 28 727. We designated this gene as *SPRAS*.

### *Comparison of amino acid sequences between S. pombe and other ras proteins*

To determine the relationship between *SPRAS* and other *ras* genes, their amino acid sequences were compared. As shown in Figure 4, the amino acid sequence of residues 8 – 88 of *SPRAS* protein was nearly perfectly homologous to the human Ha-*ras* protein residues 3 – 83. For the next 80 amino acids they were moderately homologous. Beyond this region, the two sequences diverged, but the carboxy-terminal sequence Cys-X-X-Z which has been proposed as the signal for linkage with lipid moieties (Willumsen *et al.*, 1984) was conserved. These relationships were also found when the *SPRAS* gene was compared with *S. cerevisiae RAS1* and *RAS2* genes (Figure 4).

As pointed out by Leberman and Egner (1984), *E. coli* EF-Tu and p21 contain a homologous region in their amino acid sequences. It is of interest to note that the structure of the guanine nucleotide binding site of *E. coli* EF-Tu (Asn-Lys-Cys-Asp at positions 135 – 138) (Arai *et al.*, 1980) is conserved in the sequence of *SPRAS* protein at positions 121 – 124 (Figure 4).

### *Southern analysis of S. pombe DNA*

In *S. cerevisiae*, two *ras* homologues were detected. To investigate whether *S. pombe* harbors other ras genes like *S. cerevisiae*, a Southern blot analysis was carried out using the

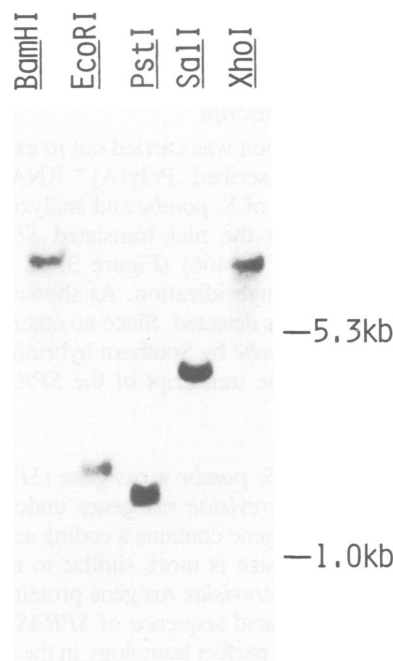


**Fig. 5.** Southern blot analysis of *S. pombe* DNA. DNA from *S. pombe* strain JY282 was digested with several restriction endonucleases as indicated. Southern blot hybridization was performed as in the case of Figure 1, except that the nick-translated *SPRAS* fragment was used as probe. The locations of the size markers are shown on the right.

**Fig. 6.** Northern blot analysis of *S. pombe* poly(A)$^+$ RNA. 20 $\mu$g of poly(A)$^+$ RNA was electrophoresed in a 1% agarose gel in a buffer containing 1.1 M formaldehyde and 10 mM sodium phosphate buffer (pH 7.4). RNA was transferred to a nitrocellulose membrane and hybridized with the nick-translated *SPRAS* fragment (positions 171–466). The mobility of the DNA size markers is shown on the right.

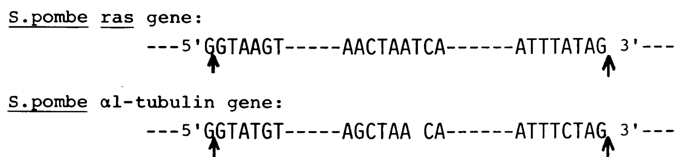


**Fig. 7.** Comparison of splice signal sequences. The predicted splicing signal sequences of the *SPRAS* gene are compared with those of the *S. pombe* $\alpha$1-tubulin gene (Toda *et al.*, 1984). The arrows indicate putative splicing sites.

nick-translated *SPRAS* probe under non-stringent conditions of hybridization (Figure 5). Several restriction endonucleases were tested but only a single band was detected in each case suggesting that only a single copy of *ras*-related gene may exist in *S. pombe*.

### *Detection of the ras transcript*

Northern blot hybridization was carried out to examine whether this gene is actively transcribed. Poly(A)$^+$ RNA was prepared from a log phase culture of *S. pombe* and analyzed by Northern blot hybridization using the nick-translated *SPRAS* fragment (nucleotide positions 171–466) (Figure 3) as a probe under stringent conditions for hybridization. As shown in Figure 6, a single band of 1.2 kb was detected. Since no other closely related gene was found in *S. pombe* by Southern hybridization, we consider this RNA to be the transcript of the *SPRAS* gene.

## Discussion

We have isolated from *S. pombe* a *ras* gene (*SPRAS*) by cross hybridization with *S. cerevisiae ras* genes under non-stringent conditions. The *SPRAS* gene contains a coding sequence for 219 amino acids. Thus, its size is more similar to mammalian *ras* p21 proteins than to *S. cerevisiae ras* gene proteins. Comparison of the predicted amino acid sequence of *SPRAS* with other *ras* proteins revealed almost perfect homology in the amino-terminal 80 amino acids and moderate homology in the following 80 amino acids. In mammalian *ras* proteins, mutation of the cellular p21s at specific sites results in the activation of its transforming potential assayed in NIH3T3 cells (Fasano *et al.*, 1984). In the *S. pombe ras* gene all of the above amino acid residues are conserved. At the carboxy terminus, a short consensus sequence Cys-X-X-Z, which may be required for linkage with a lipid moiety (Willumsen *et al.*, 1984), is found in all *ras* proteins including *SPRAS*.

Mammalian *ras* proteins (Scolnick *et al.*, 1979) as well as *S. cerevisiae RAS2* protein (Tamanoi *et al.*, 1984) are able to interact with guanine nucleotides. We assume that they may play an important role in modulating the signal transduction of growth factors by conformational transitions associated with ligand change from GDP to GTP or *vice versa*, in a manner analogous to that reported for other guanine nucleotide-binding proteins (Kaziro, 1978, 1980, 1983). In this respect, it is noteworthy that the sequence Asn-Lys-Cys-Asp (amino acid residues 121–124 of the *SPRAS* protein) is identical with the sequence of *E. coli* EF-Tu (Asn-Lys-Cys-Asp, amino acid residues 135–138), the region which is required for interaction with guanine nucleotides (Nakamura *et al.*, 1982; Arai *et al.*, 1980). Although the modification of Cys-137 of *E. coli* EF-Tu resulted in inactivation of its GDP binding activity, the cysteine residue itself may not be directly involved in interaction with guanine nucleotides, since it can be replaced with other amino acid residues as in the case of yeast cytosolic EF-1$\alpha$ and yeast mitochondrial EF-Tu (Nagata *et al.*, 1984). This is again in line with the fact that Cys-123 in *SPRAS* is conserved in Ha-*ras*, but is replaced by leucine and serine residues in *RAS1* and *RAS2*, respectively.

In the case of G-protein in the hormone-sensitive adenylate cyclase system (see Gilman, 1984), the inhibition of GTPase activity by NAD and cholera toxin leads to the persistent activation of the cyclase. Therefore, the hydrolysis of GTP is apparently required for the shut-off of the activation of adenylate cyclase. In this connection, it is interesting to note that the intrinsic GTPase activity of *ras* proteins is much lower in the transforming *ras* proteins as compared with the normal cellular p21s (Sweet *et al.*, 1984; McGrath *et al.*, 1984). Transformation may be caused by the defect of the shut-off of the growth factor stimulus due to the decrease of GTP hydrolysis.

The coding sequence of *SPRAS* protein contains the 67-bp intervening sequence in the vicinity of its amino terminus and the signals for splicing are similar to those of *S. pombe* $\alpha$1-tubulin gene (Toda *et al.*, 1984) as shown in Figure 7. The common sequence TACTAACA found in the intron of *S. cerevisiae* (Langford *et al.* 1984) is replaced by a similar sequence AACTAATCA, which resembles the sequence AGCTAACA found in the intron of the $\alpha$1-tubulin gene.

In Northern analysis of *S. pombe* mRNAs under stringent conditions, a single discrete band was detected using $^{32}$P-labelled *SPRAS* DNA. This suggests that the *SPRAS* gene is actively expressed during the vegetative growth. The function of *SPRAS* protein in the growth of *S. pombe* cells is under investigation.

## Materials and methods

### *Strains and media*

*S. pombe* strain JY282 ($h^+$, *ura4*) was cultured in a medium containing 10% glucose, 2% polypeptide, 1% yeast extract, and 50 $\mu$g/ml uracil. *S. cerevisiae* strain A364A (*ade1, ade2, ura1, tyr1, his1, lys2, gal1*) used for isolation of *RAS1* and *RAS2* was grown in the same medium supplemented with 50 $\mu$g/ml adenine. *E. coli* strain MC1061 was used for transformation studies with the cloned plasmids.

### *Preparation of DNA and RNA*

Yeast DNA was extracted from a log phase culture of *S. pombe* as described by Cryer *et al.* (1975). Poly(A)$^+$ RNA was prepared as follows. Spheroplasts, prepared in the same manner as for preparation of DNA, were lysed by the addition of an equal volume of a solution containing 2% SDS, 7 M urea, and 0.4 M NaCl. The mixture was extracted twice with phenol-chloroform (saturated with

50 mM Tris-HCl, pH 7.5) and twice with chloroform. Two volumes of ethanol were added and the precipitates were dissolved in buffer containing 150 mM Tris-HCl, (pH 7.5), 0.3 M NaCl, 15 mM EDTA, and 0.2% SDS. Poly(A)$^+$ RNA was obtained by the oligo(dT)-cellulose column chromatography.

*Isolation of RAS1 and RAS2 genes from S. cerevisiae*

DNA from *S. cerevisiae* strain A364A was digested with appropriate restriction endonucleases and the fragments were ligated with pBR327 digested with the same enzymes. *E. coli* cells were transfected with the hybrid plasmid and the clones harboring *S. cerevisiae ras* genes (*RAS1* and *RAS2*) were selected by cross-hybridization with a DNA fragment containing the V-Ha-*ras* gene (kindly provided by Dr. M.Shibuya). The clones were identified by comparing their restriction maps with those of the *ras* genes previously described (Defeo-Jones *et al.*, 1983; Powers *et al.*, 1984).

*Southern and Northern blot analysis*

Southern blotting was carried out as described by Southern (1979). The hybridization was carried out in a buffer containing 6 x SSC, 20% formamide, 0.1% SDS and 100 $\mu$g/ml heat-denatured calf thymus DNA at 37°C. For Northern blotting, 20 $\mu$g of poly(A)$^+$ RNA was electrophoresed on an agarose gel with a buffer containing 1.1 M formaldehyde and 10 mM sodium phosphate buffer (pH 7.4), and directly transferred to a nitrocellulose membrane filter using 20 x SSC. The hybridization was performed in a buffer containing 5 x SSC, 50% formamide, 0.1% SDS and 100 $\mu$g/ml heat-denatured calf thymus DNA at 37°C. In both cases, the filters were washed with 2 x SSC containing 0.1% SDS at 37°C.

*Restriction mapping and DNA sequencing*

Restriction sites were determined by mapping of the labeled fragments (Smith and Birnstiel, 1976). The fragments to be sequenced were cloned into M13 mp9 (Messing and Vieira, 1982) and the nucleotide sequences were determined by the dideoxy method of Sanger *et al.* (1977).

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