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Cloning of RNA1 Gene from *Saccharomyces cerevisiae*

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Saccharomyces cerevisiae 에서 RNA1 유전자의 클로닝

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ABSTRACT: The temperature sensitive (ts) mutation on RNA1 gene of *Saccharomyces cerevisiae* prevents growth at restrictive temperature (36°C) by accumulation of precursor tRNA, rRNA and mRNA (Hutchison *et al.*, 1969; Shiokawa and Pogo, 1974; Hopper *et al.*, 1978). RNA1 gene was cloned by complementation of the temperature sensitive growth defect of an *rna1-1* mutant strain and identified by retransformation and concomitant loss of recombinant plasmid on non-selective condition. By deletion mapping, it was found that RNA1 gene resides within 3.5 kb of BglII fragment.

KEY WORDS □ *Saccharomyces cerevisiae*, splicing, RNA1, cloning

In *Saccharomyces cerevisiae*, some mutants which interfere the splicing of precursor RNA were isolated. Tmeperature sensitive *rna2-rna11* mutants accumulate intron-containing precursor mRNAs *in vitro*, and this proved that genes RNA2-RNA11 are essential in yeast mRNA splicing (Fried *et al.*, 1981; Rosbach *et al.*, 1981; Larkin and Woolford, 1983; Last *et al.*, 1984). Temperature sensitive and deletion mutation of the LOS1 locus that affect pre-tRNA splicing have been identified (Hopper *et al.*, 1980; Hurt *et al.*, 1987). Recently four more yeast genes which interfere splicing of precursor tRNA, SEN1, SEN2, PTA1 and TPD5 have been identified (Winey and Culberston, 1988). The temperature sensitive, conditionally lethal mutation *rna1-1* prevents growth at temperature of 34°C and above (restrictive temperature) by affecting the production of mature rRNA, tRNA and mRNA (Hutchison *et al.*, 1969; Shiokawa and Pogo, 1974; Hopper *et al.*, 1978). At restrictive temperature (uaually 36°C), *rna1-1* mutant exhibits a rapid decrease in the rate of accumulation of newly synthesized RNA (Hutchison *et al.*, 1969; Shiokawa and Pogo, 1974). After 20 min at 36°C, an *rna1-1* mutant accumulates about 60% of the total newly synthesized RNA that is accumulatd in the permissive temperature (23°C) control, and approximately 60% of this is localized in the nucleus (Hutchison *et al.*, 1969).

The effect of *rna1-1* mutation on mRNA and rRNA production is not clearly defined. It merely shows nuclear 35S rRNA precursor accumulation (Hopper *et al.*, 1978) and polyadenylated mRNA accumulation in nucleus (Shiokawa and Pogo, 1974). But there is no evidence that *rna1-1* mutation affects the splicing of RNA precursors direct-

ly. The effect of *rna1-1* mutation on tRNA production has been more cleaely defined. In nucelus, *rna1-1* mutant accumulates intron containing precursor tRNA species that have mature temini and the most of the nucleoside modifications found in the mature tRNA at the restrictive temperature (Kanpp *et al.*, 1978; O'Farrell *et al.*, 1978; Etcheverry *et al.*, 1979; Knapp *et al.*, 1979). But, with these data, we can not define the role of RNA1 gene precisely, and must investigate its effects not only on splicing itself but transport of RNA from nucleus to cytoplasm.

To elucidate the precise role of RNA1 gene, we have cloned the RNA1 gene by complementation of the temperature sensitive growth defect of the *rna1-1* mutant. And in this paper, we present the preparation strategy of suitable host strains which have high transformation efficiency.

MATERIALS AND METHODS

Strains

E. coli strains HB101 and JM109 were used for isolation of plasmid and as hosts for transformation. Yeast strains used for the present study are listed in Table 1.

Table 1. *Yeast strains used*

Strain		Genotype
1607	α	*ura*1, [KIL-K2], [NEX-0], K2+, R2+, excrutable
1058	a	*cyh2*, *met*13, *leu*1, *trp*5, *aro2*, *lys*5,
	α	*ade*2, Kl+, Rl+
ATCC38517	a	*ade*l, *trp*1, *pet*18
ATCC38625	a	*leu*2-3, *leu*2-112, *his*4-517, *can*1
ATCC42677	α	*ade*1, *trp*1, *ura*3
SL560-3A	a	*his*3-1, *leu*2-1, *met*8-1, *trp*1, *ura*3-52
ATCC44724	a	*lys*1, *mak*27-1
ATCC44382	α	*his*4-15, *ade*2-1, *can*1, *kar*1-1, ρ°
ATCC44717	α	*his*1, *ura*1, *ade2*, *lys*1, *ran*1-1, *kil-k*
ATCC44718	a	*his*1, *ura*1, *ade2*, *lys*1, *rna*1-1, *kil-k*
SHY3	*a*	*ste*-VC9, *ura*3-52, *leu*2-3, *leu*2-112, *his*3-1, *ade*1-101, *can*1-100
X464-24C	α	*trp*1, *ade2*, *his2*, *leu*1, *gal*1, *ura*3
MMY2	*a*	*ura*3, *sta*1, *sta*2, *sta*3, *sta*10, *ura*3
IS8	α	*rna*1-1, *ade2*

Mating and Sporulation

Grow each haploid yeast strain containing different mating type in YEPD (yeast extract 1%, peptone 2%, glucose 2%) broth to exponential phase and inoculate together 1% of each culture into freshly prepared YEPD media and further incubate to the stationary phase. Spread the culture broth onto the selective media to identify the complementation between two auxotrophic markers of each haploid yeast strains. The haploid yeast has been sporulated by adapting oxidative growth condition and depleting metabolic nitrogen source using yeast harvested during exponential growth (Roth and Halvorson, 1969). Grow the diploid yeast cell in YEPD broth at 23°C and incubate 1% of cell culture into pre-sporulation media (yeast nitrogen base with out amino acids 0.67%, Yeast extract 0.1%, potassium acetate 1%, pH 5.2). When it reachs to the mid-exponential phase ($2X10^7$ cells/m*l*), transfer 5% of culture to fresh pre-sporulation media with two or three times repeatedly for the adaptation of oxidative condition and continue the incubation until the absorbance at 600 nm reaches to 1-1.5. Harvest and wash with distilled water and adjust the cell density to $2X10^7$ cells/m*l* in 1% potassium acetate solution and further incubate with vigorous shaking for 60 hours. By the microscopic view, the formation of tetrad is determined. Ascus wall has been removed by the treatment with 1.5% of glusulase solution for 4 hours at 30°C. Harvest and wash with distilled water and resuspend in 5 m*l* of distilled water. The individual ascus has been dissected by the ultrasonic cell disruptor at maxium power and equal voluems of liquid paraffin (Siddigi, 1971) has been added to the single spore suspension. Mix vigorously and centrifuge briefly to divide the two phases. Spread the liquid phase containing haploid asci onto the YEPD media.

Isolation of yeast genomic DNA

The high molecular weight DNA from yeast for preparing the genomic DNA library was isolated by disrupting the cells with zymolase-60,000 (Rodriguez and Tait, 1983).

Transformation

The preparation of competent *E. coli* cell was carried out by following the standard procedure with rubidium chloride and MOPS (Kushner, 1978). The competent yeast cell was prepared by treatment with alkali cations (Ito *et al.*, 1983), or by spheroplast formation (Hinnen *et al.*, 1978).

Plasmid isolation

The isolation of the various kinds of plasmid DNAs from transformed *E. coli* was carried out by the method of alkaline lysis (Birnboim and Doly, 1979). For isolation of large amount of plasmid DNA, the two-step cesium chloride ethidium bromide gradient centrifugation method was used (Garger *et al.*, 1983).

The recombinant plasmid were isolated from transformed yeast as follows. A medium size yeast colony (approximately 3 mm in diameter) containing the plasmid was picked into 200 μl of 100 mM Tris-HCl pH 8.0, 10 mM EDTA, 0.1% SDS and then glass beads (0.45 nm in diameter) were added into the solution until just below the level of the cell suspension. Mix thoroughly on a vortex mixer at maxium speed for 1 min, extract the aqueous phase with an equal volume of Tris-saturated phenol pH 8.0, abjust salt concentration prior to ethanol precipitation and resuspend the dried pellet in 100 μl of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA).

Concomitant loss of the recombinant plasmid in transformed yeast

The concomitant loss of plasmid marker and mitotic stability carried on the transformed strains were assayed by growing the strains to stationary phase in YEPD broth at 23°C and plating the culture for single colonies upon YEPD plates at a density of 100-300 colonies per plate. These plates were incubated at 23°C and the colonies were replica plated to the selective media and grown under selective conditions to determine the frequencies of the loss of a particular phenotype.

RESULTS AND DISCUSSION

Construction of suitable host strain for the cloning of RNA1 gene

The yeast strain to be used as a host should have the *rna1-1* and *ura3* or *trp1* mutational maker in order to screen the yeast transformants and clone the RNA1 gene by genetic complementation using total genomic library which have been carried on yeast-*E. coli* shuttle vector YEp24 or YRp7. Since YEp24 contains URA3 gene and YRp7 contains TRP1 gene of *Saccharomyces cerevisiae* (Botstein *et al.*, 1979), only yeast transformants containing YEp24 or YRp7 plasmid grow on the synthetic media lacking uracil or tryptophan by the genetic complementation with *ura3* or *trp1* auxotrophic marker, while non-transformant cannot grow on the selective synthetic media depleting uracil or tryptophan.

Since SL560-3A strain is one of good transformable strains and contains *trp1* and *ura3-52* auxotrophic markers and that has a mating type opposite to *α* mating type of ATCC 44717 strain containing *rna1-1* temperature sensitive marker, these two auxotrophic markers of *trp1* and *ura3-52* can be introduced into ATCC 44717 by the general procedure of mating and sporulation. The overall strategy for preparing the suitable host strain is illustrated in Fig. 1. Since diploid strain constructed from SL560-3A (*ura3-52*) and ATCC 44717 (*rna1-1*) has grown well on the synthetic minimal media at 37°C, it has been concluded that *rna1-1* temperature sensitive marker was complemented by RNA1 wild type marker in SL560-3A and *ura3-52* auxotrophic marker in SL560-3A was com-

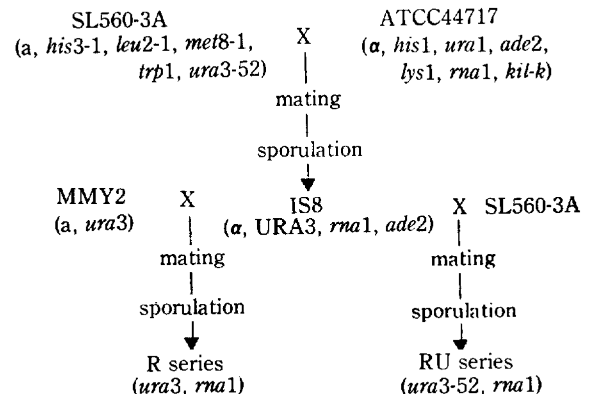

Fig. 1. *The strategy for the construction of suitable host strain for cloning of RNA1 gene.*

Table 2. *Marker test of RU series haploid strains*

	ADE	HIS	TRP	URA	MET	LEU	LYS	RNA1
RU3	–	–	–	+	–	+	+	ts
RU7	+	–	–	–	–	–	+	ts
RU11	+	–	–	–	–	–	+	ts
RU12	–	–	+	–	–	+	+	ts
RU14	–	–	–	–	–	+	+	ts
RU19	–	–	–	–	–	+	+	ts
RU21	–	–	–	–	–	–	+	ts
RU22	–	–	–	–	–	+	+	ts
RU27	–	–	–	–	–	–	+	ts
RU28	–	–	–	–	–	–	+	ts

plemented by URA3 wild type marker in ATCC 44717. From this diploid cell, various haploid strains to be used as a host for cloning of RNA1 gene could have been obtained by the liquid paraffin enrichment technique (see Materials and Methods).

According to the strategy for constructing the haploid strain as shown in Fig. 1, the resulting haploid strain, IS8 (α URA3, *rna1-1, ade2)* has been recrossed with SL560-3A, and the resulting diploid strain sporulated to obtain the haploid strain, named as strains of RU series, containing *ura3-52, rna1-1* marker. The genotypes of these RU series are exhibited in Table 2 and all the haploid strains contain *rna1-1* (temperature sensitive marker) and *ura3-52* marker except only RU3. The transformation efficiency of these RU strains is shown in Table 3 compared with SHY3 and X464-24C, using spheroplast method as well as alkali cation method (see Materials and Methods). Although the transformation efficiencies of RU7 and RU11 show 1-1.5 X 10^3 transformants/40 μg of YEp24 by sphoreplast method, it is nearly impossible to clone the yeast gene by complementation with such a low transformation efficiency, and what is worse, it is not easy to screen and recover the yeast transformants containing cloned RNA1 gene spheroplast method because the transformants are embedded in 2% overlayed agar (Hinnen *et el.*, 1978), When the yeast strain is treated with zymolase or glusulase to prepare the

Table 3. *Transformation frequencies of RU and R series haploid strains. Transformation was performed as described in Materials and Methods.*

Spheroplast Method

vector	host	spheroplast frequency	regeneration frequency	transformants (40μg DNA)
YRp7	X464-24C	85%	11%	2.5×10^5
YEp24	SHY3	95%	nd*	3.6×10^5
	RU7	80%	10%	1.0×10^3
	RU11	85%	11%	1.5×10^3

*nd = not determined

Alkali Cation Method

vector	host	transformants (1μg)
YEp24	RU11	10
	RU7	10
	R16	2×10^3
	R23	1×10^3

spheroplast, the mutation rate is increased more or less, thus the revertants may grew and appear at 37°C. Therefore, it is required to use the alkali cation method (Ito *et al.,* 1983) to clone RNA1 gene. The transformation efficiencies of RU7 and RU11, however, are extreamly low when the alkali cation method has been used (Table 3).

Because of these unsatisfied results, another set of haploid strains has been prepared by mating IS8 and MMY2 as following the general procedures for the sporulation of the diploid strains as illustrated in Fig. 1. These newly prepared haploid strains named R series exhibits very high transformation efficiency by the method of alkali cation as shown in Table 3, and the genotypes of these R series have *ura3-52* and/or *trp1* auxotrophic marker as well as *rna1* temperature sensitive marker (Table 4). So we have prepared suitable host strains, R series, for cloning RNA1 gene, which have high transformation efficiency.

Cloning of RNA1 gene

Total genomic library of yeast has been perpared by following the formula of Clarke and Carbon (1976) which relates the probability (P) of including any DNA sequence in a random library of

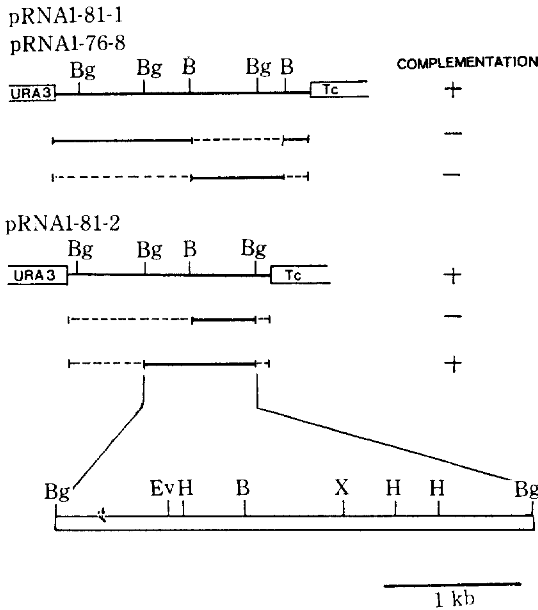

Fig. 2. *The restriction map and localization of RNA1 gene.*
The deletion mapping has revealed that the RNA1 gene resides within the BglII fragment (3.5kb) containing BamHI site. B: BamHI, Bg: BglII, Ev: EcoRV, H: HindIII, X: XbaI.

N (independent recombinants). Because the total genomic size of *Saccharomyces cerevisiae* has been estimated about 20,000 kb, it is necessary to be guaranteed the complete genomic library having a possibility of 99.99% over that about 20,000-25,000 transformants of *E. coli* in which each recombinant plasmid carry the yeast DNA fragments staggered Sau3A1 ends about 5 kb long to be prepared. Yeast DNA has been partially digested with Sau3A1 restriction endonuclease and ligated to the BamHI digated and CIP (calf intestinal alkaline phosphatase) treated plasmid YRp7 or YEp24. These ligated DNA molecules have been used as transforming DNA for preparing the yeast total genomic library in *E. coli*.

Using this library, we obtained 150,000 colonies of yeast transformants on the synthetic minimal media lacking uracil at 23°C. After all transformants have been replicated with velvet replica template on the three identical synthetic minmal media lacking uracil sucessively, one replicated plate has been

Table 4. *Marker test of R series haploid strains*

	ADE	HIS	LEU	MET	TRP	URA	LYS	RNA1
R4	+	−	+	−	−	−	+	ts
R8	+	−	−	−	−	−	+	ts
R10	−	−	−	+	+	−	+	ts
R12	+	+	−	+	−	−	+	ts
R16	+	+	+	−	−	−	+	ts
R18	+	−	+	+	+	−	+	ts
R21	+	−	−	−	+	−	−	ts
R23	+	+	+	−	+	−	+	ts
R29	+	+	+	+	−	−	+	ts
R35	−	+	+	+	−	−	+	ts
R46	+	+	+	+	−	−	+	ts
R49	+	+	+	+	−	−	+	ts
R54	+	−	−	−	−	+	+	ts
R55	+	+	−	−	+	−	+	ts
R59	+	−	−	−	−	−	+	ts

incubated at 23°C as a master plate while the other two plates have been incubated for 2 or 3 days at 37°C to screen the RNA1 gene clone. Only 17 olonies of 150,000 transformants have been complemented at 37°C and thought to be candidates for RNA1 gene clone. 13 clones out of 17 candidates for RNA1 gene clone showed a normal growth on the minmal media lacking uracil at 37°C, but 4 clones growed poorly on the same conditions. This may suggest that *rna1-1* mutation is a nonsense mutation and the 4 clones were transformed by a plasmid containing some suppressor gene. If a recombinant plasmid carrying a certain suppressor gene is introduced into the yeast host containing *rna1-1* mutational marker, the growth of the transformant harvoring such a recombinant plasmid will be retarded conspicuously. This phenomenon is resulted from the abnormal suppression of the intact genes save the *rna1-1* mutational gene, so that the other intact genes may not teminate at the original stop codon. Strains containing highly efficient UAA and UGA suppressors, class-I UGA suppressors or ribosomal suppressors grow poorly on nutrient medium, whereas normal or nearly normal growth rates are observed when these strains lose the suppressors or when

the suppressors are mutated to forms having lower levels of expression (Sherman, 1982). It was already reported that *rna1-1* mutation was suppressed by SRN1 gene (Pearson *et al.*, 1982). But we didn't investigate further the four slowly growing clones. The 13 clones showing a normal growth at 37°C were grown on YEPD media at 23°C and incubated at 23°C after plated on YEPD media. About 200 colonies from each candidate have been tested for the concomitant loss of plamid marker with the ts complementing activity. All of these clones lost the URA3 marker and the ts complementing activity simutaneously when grown on the non-selective condition. Therefore, these transformants have been idendified as clones for the RNA1 gene.

Localization of RNA1 gene

The recombinant plasmids have been isolated from three different yeast clones of RNA1 gene by method of quick isolation using glass (0.45 nm in diameter, see Materials and Methods). Each recombinant plasmids were introduced and amplified in *E. coli* HB101, and purified from the *E. coli* transformants, then reintroduced into *rna1-1* mutational yeast strain to convince the complementation with the temperature sensitive mutational marker when grown at 37°C, it has been proved that these recombinant plasmids, named pRNA1-76-8 pRNA1-81-1 and pRNA1-81-2, have carried the RNA1 gene. When these three plasmids containing RNA gene were digested by restriction enzymes (BamHI, BglII, EcoRV, HindIII and XbaI), it revealed that the two recombinant plasmids, pRNA1-81-1 and pRNA1-76-8, are exactly the same plasmid though they have been isolated from different yeast clones (Fig. 2). As shown in Fig. 2, pRNA1-81-2 has smaller yeast genomic DNA contaning RNA1 gene than the other two recombinant plasmids. For localization of the RNA1 coding region within the cloned sequence, deletions were constructed. Small BamHI fragment (3.0 kb) of pRNA1-81-1 was deleted and large fragment contaning YEp24 region was religated. This derivative didn't complement with *rna1-1* mutation. Plasmid contaning only 3.0 kb BamHI fragment also didn't complement with *rna1-1* mutation. Therefore, it is thought that RNA1 gene resides through the left BamHI site. To obtain positive derivative, 3.5 kb of BglII fragment of pRAN1-81-2 has been isolated and subcloned to the BamHI site of YEp24. This recombinant plasmid complemented with *rna1-1* mutational marker. The deletion mapping has revealed that the RNA1 gene resides within the BglII fragment (3.5 kb) containing BamHI site.

적 요

Saccharomyces cerevisiae 의 RNA1 유전자의 온도 감수성 돌연변이 균주는 성장허용 온도인 23℃에서는 정상적인 성장을 하나, 성장억제 온도인 36℃에서는 tRNA, rRNA 그리고 mRNA 의 선구물질들을 핵내에 축적함으로써 성장을 못한다. 본 실험에서는 complementation 에 의하여 RNA1 유전자를 클로닝하였으며 concomitant loss 실험에 의하여 이 유전자의 클로닝을 확인하였다. 유전자의 위치를 확인한 결과 3.5kb 의 Bgl II 조각내에 RNA1 유전자가 존재함을 알 수 있었으며, 2.1kb 에 해당하는 BamH I -Bgl II 조각에서는 RNA1 유전자에 의한 complementation 능력이 상실되는 것으로 보아 RNA1 유전자는 3.5kb 의 Bgl II 조각내에 포함되는 BamH I 자리 주위에 걸쳐 있음을 알 수 있었다.

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