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Integration of heterologous genes into the chromosome of *Saccharomyces cerevisiae* using a delta sequence of yeast retrotransposon Ty

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Summary. Distribution of a delta (δ) sequence of the Ty element on a chromosome of the yeast *Saccharomyces cerevisiae* was analysed by pulsed-field gel electrophoresis. More than 100 copies of the δ sequence were non-randomly distributed on the chromosome. Using the δ sequence as a recombination site, mouse α-amylase and human β-endorphin genes were introduced into the chromosomal DNA. The integration occurred on a particular chromosome in each case and the copy number was estimated as three to five. It was also found that single- or multi-copy integration occurred at a single or multiple sites on the particular chromosome. The integrants secreted α-amylase and β-endorphin by three-to fivefold compared with single-copy integrants. This type of integration was mitotically stable over a period of 50 generations under non-selective conditions.

Introduction

The yeast *Saccharomyces cerevisiae* is a good host for heterologous gene expression and protein secretion. Recent works reveal that a higher copy number of plasmids bearing heterologous genes causes higher levels of transcription and therefore efficient production of heterologous proteins (Bitter et al. 1987). In many cases, heterologous genes were introduced into yeast cells using 2 μm-based (YEp) plasmids. The YEp plasmid, however, is not mitotically stable. The production of heterologous proteins requires rich medium in some cases (Kanaya et al. 1989) and mitotic stability of the plasmid is definitely required. The YCp plasmid containing a yeast centromere sequence is mitotically stable, but its copy number is only one per cell (Rose 1987). Another vector, YIp plasmid, is able to integrate heterologous genes into a chromosome. The chromosomal integration that takes place at a specific site by homologous recombination brings one to several copies of a heterologous gene (Szostak and Wu 1979; Orr-Weaver and Szostak 1983; Denis and Drouin 1987). The yeast retrotransposon Ty, the copy number of which is approximately 35 is dispersed throughout the genome of the yeast (Cameron et al. 1979). The Ty element has two terminal direct repeats (δ) and one of the δ sequences remains at the original sites after transposition of the Ty retrotransposon (Williamson 1983). It is estimated that more than 80 copies of the δ sequence are dispersed throughout the genome (Cameron et al. 1979).

In this paper, we have analysed the distribution of δ sequences in each chromosome, and have tried to integrate heterologous genes into the δ sequence of Ty as homologous recombination sites, aiming towards the higher production of heterologous proteins.

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Materials and methods

Strains. The yeast strains used in this work are listed in Table 1. Crosses, sporulation and tetrad dissection were performed as described previously (Sherman et al. 1986).

Escherichia coli strains HB101 and JM109 were employed as the hosts for propagating plasmids.

Media. The culture medium used for *S. cerevisiae* was a YPD medium containing 1% bacto-yeast extract, 2% bacto-peptone and 2% dextrose or a synthetic media containing 0.67% yeast nitrogen base without amino acids (Difco, Detroit, Mich, USA), 2% dextrose and amino acids required. The media were solidified with 2% bacto-agar for plates. To detect secreted amylase on plates, YPS plates were used which contained 1% bacto-yeast extract, 2% bacto-peptone, 2% starch and 2% bacto-agar. Luria broth (Moniatis et al. 1982) was used for the culture of *E. coli* and this was supplemented with ampicillin for the selection of the transformants.

Transformations. The transformation of *E. coli* was performed by the method of Hanahan (1983). The transformation of yeast was done as described by Ito et al. (1983).

DNA manipulation and Southern hybridization. These were performed as described by Maniatis et al. (1982).

Pulsed-field gel electrophoresis. Sample preparation for pulsed-field gel electrophoresis was performed by the method of Carle and Olson (1985). The apparatus for pulsed-field gel electrophoresis

Table 1. List of yeast strains

Strain	Genotype	Source
S288C	*MATα SUC2 mal mel gal2 CUP1*	YGSC[a]
AB972[b]	*MATα SUC2 mal mel gal2 CUP1*	M. Olson
A364A	*MATa ade1 ade2 ura1 his7 lys2 tyr1 gal1*	L. Hartwell
SH964[b]	*MATa ura3-52 lys2-801 ade2-102 trp1*	P. Hieter
YNN27	*MATα trp1 ura3 SUC2*	N. Gunge
A406[b]	*MATa can1 met14 trp1 ura3 SUC2*	This work
A448[b]	*MATα can1 aro7 trp1 ura3 SUC2*	This work

[a] Yeast Genetic Stock center
[b] Isogenic strains with S288C

was constructed by ourselves as described by Chu et al. (1986). Pulsed-field gel electrophoresis was performed according to the method of Chu et al. (1986) with a switching time of 80 s at 180 V for 24 h.

Enzyme assay. The activity of secreted amylase in the culture medium was determined as described previously (Sakai et al. 1988). The amount of human-β-endorphin in the culture medium was assayed using a radioimmunoassay kit from New England Nuclear, Boston, MA, USA. Protein concentrations were determined as described by Bradford (1976).

Construction of plasmids. Construction of plasmids are shown in Fig. 1. A 172-bp section of a *Bam*HI fragment containing the human β-endorphin gene of a multicopy secretion vector pREI078 (Kanaya et al. 1989) was eliminated by digesting it with *Bam*HI and re-ligation was done to construct pSAK027. The pSAK027 is a yeast multicopy secretion vector using a *PGK* promoter, a signal sequence of *MFα1* and terminator of *MFα1*. A single *Bam*HI site located after the sequence coding the signal sequence can be used for construction of the secretion vector.

The 1.8-kb DNA fragment containing a δ sequence of Ty element was isolated from the Ty-C1 DNA by digesting with *Bgl*II and *Eco*RI (Iida 1988). This was ligated with *Bam*HI/*Eco*RI digest of YIp5 to construct pSAK068. The 2.2-kb *Eco*RI fragment of pSAK027 bearing the promoter of the *PGK* gene, the signal sequence and the terminator of *MFα1* (*PGK*-secretion cassette) was ligated with *Eco*RI digest of pSAK068 to produce pSAK069. A mouse α-amylase gene whose own signal sequence was deleted was inserted into the *Bam*HI site of pSAK069 to obtain pSAK073.

The 172-bp DNA fragment bearing the human β-endorphin gene (Takahashi et al. 1981) was isolated by digesting pREI078 with *Bam*HI. The human β-endorphin gene was ligated in frame with the *Bam*HI site of pSAK069 to construct pSAK074. The mouse α-amylase gene and the human β-endorphin gene were inserted in the *Bam*HI site of pSAK028 DNA, in which the 2.2-kb DNA fragment of the *PGK* secretion cassette was inserted into the *Eco*RI site of YIp5, and the resultant plasmids were designated pSAK031 and pSAK017, respectively. For site-oriented integration of plasmids at the δ sequence on the chromosome, plasmids pSAK073 and pSAK074 were linearized by digestion with *Xho*I, the cleavage site of which was located within the δ sequence. To promote integration of plasmids into the *URA3* locus on the chromosome, plasmids pSAK031 and pSAK017 were linearized with *Stu*I, the cleavage site of which was located within *URA3* DNA.

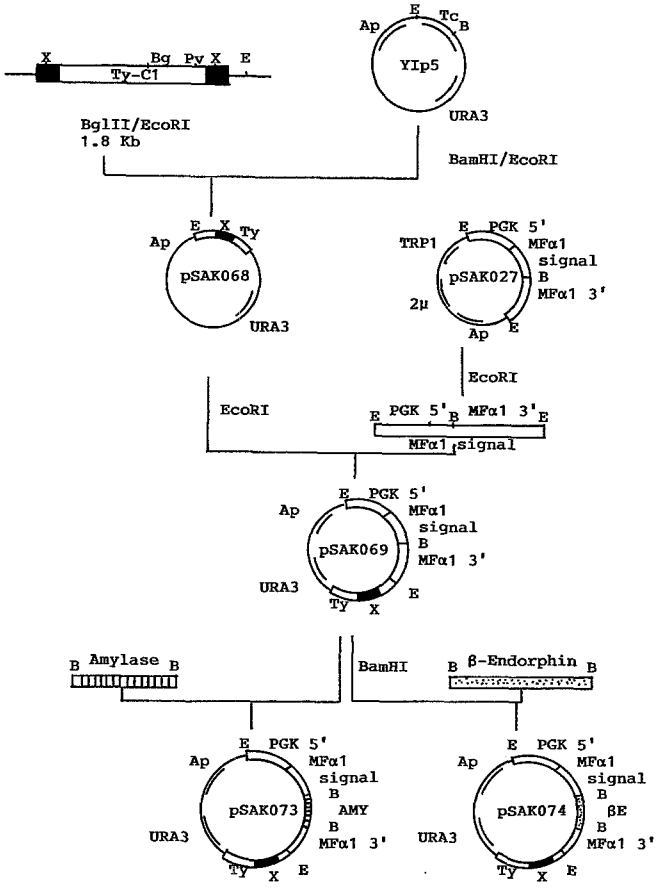

Fig. 1. Schematic illustration of plasmid construction: ■, delta (δ) sequence; ▥, mouse α-amylase gene; ▤, human β-endorphin gene. Restriction enzymes used were *B*, *Bam*HI; *Bg*, *Bgl*II; *E*, *Eco*RI; *Pv*, *Pvu*II; *X*, *Xho*I

Results

Distribution of the delta (δ) sequence on the yeast chromosome

Distribution of the δ sequence on the chromosome of *S. cerevisiae* was analysed by pulsed-field gel electrophoresis followed by Southern hybridization with a ^{32}P-labelled δ sequence (a 700-bp *Pvu*II-*Eco*RI fragment, see Fig. 1) as a probe. As shown in Fig. 2, all the laboratory yeast strains used in this experiment showed almost the same distribution pattern of the δ sequence, and the distribution of the δ sequence on the chromosome was found to be non-random. That is, several chromosomes did not contain the δ sequence, and some gave a strong hybridization signal.

The copy number of the δ sequence was estimated by densitometric scanning of the autoradiograph (Fig. 3)

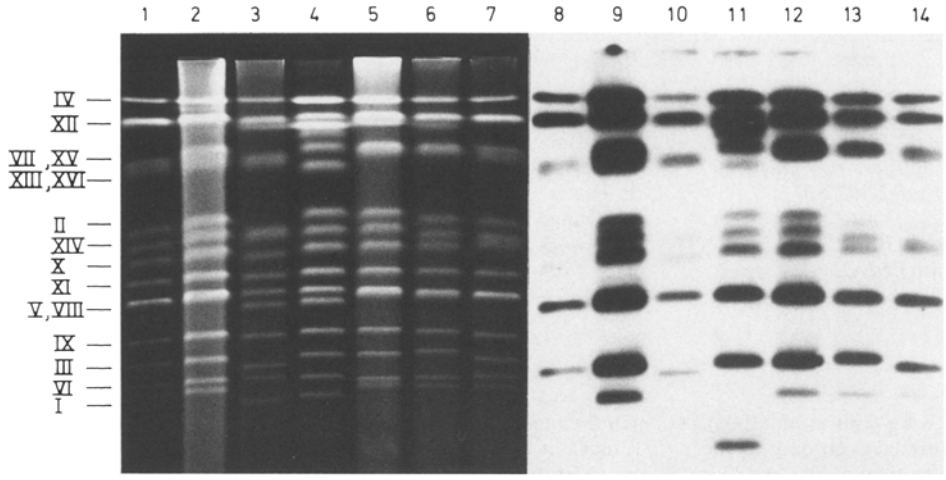

Fig. 2. Distribution pattern of δ sequences on the chromosome from various yeast strains. Chromosomes of various yeast strains were separated by pulsed-field gel electrophoresis followed by Southern hybridization as described in Materials and methods. A 700-bp *Pvu*II-*Eco*RI fragment containing the δ sequence (see Fig. 1) was nick-translated with [α^{32}-P]deoxycytidine triphosphate and used as a probe. *Lanes 1–7,* ethidium bromide staining; *lanes 8–14,* autoradiograph. *Lanes 1, 8,* AB972; *lanes 2, 9,* S288C; *lanes 3, 10,* A364A; *lanes 4, 11,* SH964; *lanes 5, 12,* YNN27; *lanes 6, 13,* A448; *lanes 7, 14,* A406. *Roman numbers* indicate chromosome number

using a Flying-Spot Scanner CS-9000 (Shimadzu, Kyoto, Japan). More than 100 copies of the δ sequence were present on haploid yeast chromosomes, but three classes of the copy number were present: chromosomes III, IV, V, VII, VIII, XII, XIII, XVI had more than five copies (5–20 copies) of the δ sequence (class I), chromosomes I, II, X, XIV, XV had one to two copies (class II) and chromosomes VI, IX, XI had no δ sequence (class III), indicating that distribution of the δ sequence is chromosome dependent.

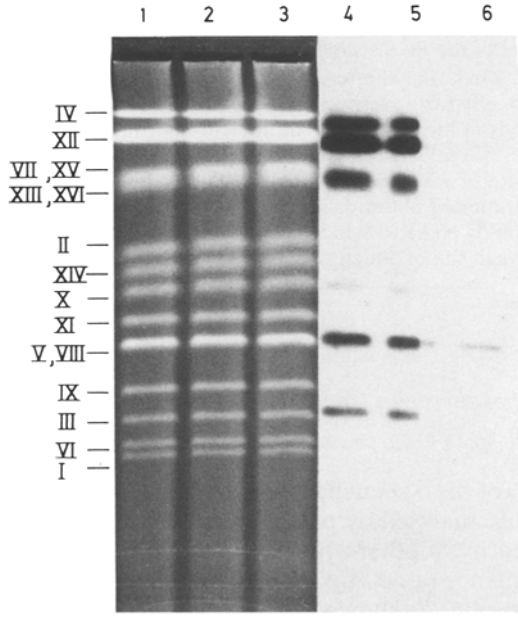

Fig. 3. Copy number estimation of δ sequence on yeast chromosomes. Chromosomes of strain A448 were separated by pulsed-field gel electrophoresis and Southern hybridization was performed using the δ sequence and the *URA3* DNA fragment as probes. *Lanes 1–3,* ethidium bromide staining; *lanes 4–6,* autoradiograph. *Lane 4* and the *left half of lane 5,* ^{32}P-labelled δ sequence was used as a probe; *right half of lane 5* and *lane 6,* ^{32}P-labelled *URA3* DNA fragment was used as a probe. *Roman numbers* indicate chromosome number

Integration of heterologous genes in the yeast chromosome by the use of homologous recombination

In order to use the δ sequence as a homologous recombination site, plasmids pSAK073 and pSAK074 were digested with *Xho*I, the cleavage site of which is present within the δ sequence. Integration of the linearized DNA could occur at the δ sequence. All the URA$^+$ transformants of pSAK073 showed amylase halos on YPS plates (data not shown). The transformants (integrants) could produce and secrete mouse α-amylase and human β-endorphin into the culture media. This type of integration was tentatively named δ-integration. The amounts of α-amylase and β-endorphin secreted from various integrants are summarized in Table 2. The δ-integrants secreted amylase and β-endorphin at 0.6–

Table 2. Secretion of heterologous proteins

Integration		Amylase (units)/ml	β-Endorphin (ng/ml)
δ-Integration			11.2[b]
	1	0.80[a]	
	2	1.26[a]	
	3	0.77[a]	
	4	0.60[a]	
URA3-Integration		0.27[c]	2.5[d]

A448 was used as host to the integrations
[a] pSAK073
[b] pSAK074
[c] pSAK031
[d] pSAK071

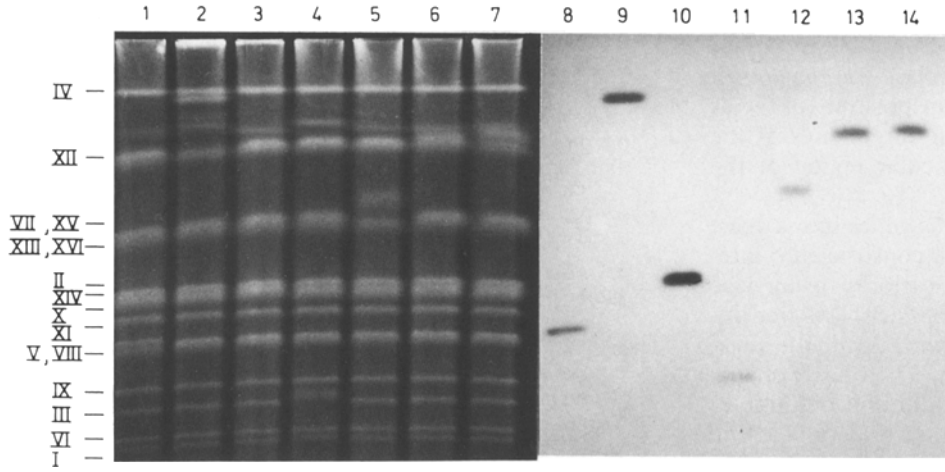

Fig. 4. Localization of δ-integrative plasmid on the yeast chromosomes. Chromosomes of the seven δ integrants of A448 with pSAK073 were separated by pulsed-field gel electrophoresis and Southern hybridization was performed using ^{32}P-labelled pBR322 as a probe. *Lanes 1–6,* ethidium bromide staining; *lanes 7–12,* autoradiograph. *Roman numbers* indicate chromosome number

1.3 units (U)/ml and 11.2 ng/ml, respectively. On the other hand, single-copy integrants at the *URA3* locus secreted amylase and β-endorphin at 0.27 U/ml and 2.5 ng/ml, respectively. The δ integrants could secrete heterologous proteins by three- to fivefold as compared with a single-copy integrant at the *URA3* locus.

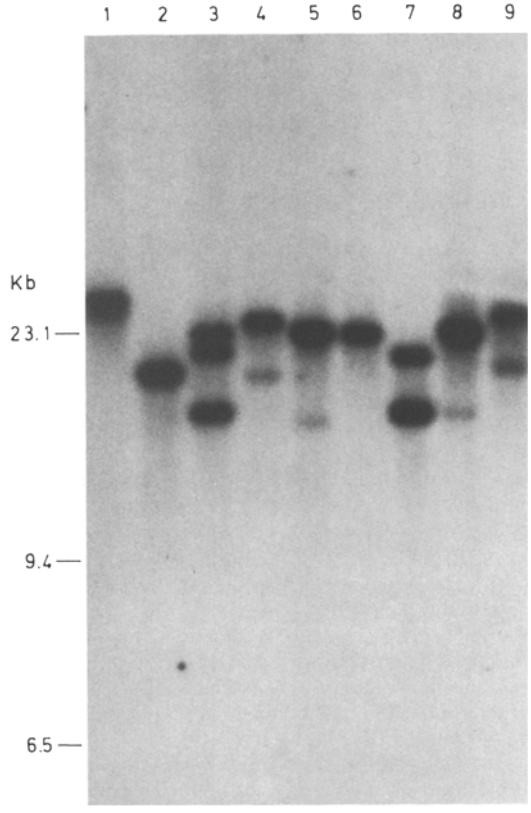

Fig. 5. δ-Mediated integration occurred at multiple sites. Chromosomal DNAs of nine δ integrants were prepared, digested with *Bgl*II and subjected to Southern hybridization using ^{32}P-labelled pBR322 as a probe. *Lanes 1–9,* nine independent δ integrants; *numbers at left,* DNA size markers

The stability of the integrated sequence was analysed. After growth of the δ-integrants for about 50 generations in non-selective medium, the size of the amylase halo did not change (data not shown), showing that the integrated sequences are mitotically stable. The chromosomal distribution of the integrated pSAK073 was analysed by pulsed-field gel electrophoresis. Seven independent δ-integrants had three to five copies of the plasmid, but the integration occurred on a different chromosome in each case (Fig. 4). Next, chromosomal DNAs of the δ-integrants were digested with *Bgl*II, the restriction site of which was not present in pSAK073, and subjected to Southern hybridization using a ^{32}P-labelled pBR322 as a probe. As shown in Fig. 5, more than two hybridization bands were detected in six cases out of nine integrants, indicating that the δ-mediated integration occurred at multiple sites on the particular chromosome. We did not detect multicopy integration on more than two chromosomes at the same time.

Discussion

In this paper we have described non-random distribution of the δ sequence of yeast retrotransposon Ty on a chromosome of *S. cerevisiae*. It has been reported that 30–50 copies of Ty are present in most laboratory strains of the yeast (Cameron et al. 1979; Williamson 1983). The average copy number of Ty on each chromosome would be expected to be two to three if the distribution is random. Pulsed-field gel electrophoretic analysis, however, showed that the distribution of the δ sequence in non-random (Figs. 2 and 3). The hydridization probe used in this experiment can hybridize with both δ and Ty itself, because Ty has two copies of δ at both ends. This result clearly indicates that distribution of Ty is also non-random. We also confirmed that the distribution of Ty is non-random using an ε-sequence as a probe (data not shown). It is interesting to note that the copy number of the δ sequence varies from each

chromosome and it can be classified into three categories: more than five copies (class I), one to two copies (class II) and no δ sequence present (class III). The result suggests the possibility that the transposition of Ty is chromosome dependent and that the target sites of the transposition are located on a particular region of the specific chromosome as "hot-spots".

In order to introduce heterologous genes into a yeast chromosome as multicopies, we have constructed δ-integrative plasmids which can secrete mouse α-amylase (pSAK073) or human β-endorphin (pSAK074). After linearizing the plasmids by digesting with *Xho*I, the plasmids were introduced into yeast cells. The transformants (integrants) secreted amylase or β-endorphin by three-to five-fold compared with a single-copy integrant at the *URA3* locus (Table 2), and the integrants were mitotically stable. We analysed the distribution of δ-integrative plasmid on the chromosomes.

Figure 4 shows that the δ-integration occurred on a particular chromosome in each case. Most of the δ-integration occurred on class I chromosomes and on class II in one case. No δ-integration was found to occur on class III chromosomes. It has been reported that multicopy integration occurs at a single site on a chromosome (Orr-Weaver and Szostak 1983). However, the δ-integration reported here occurred at multiple sites on a chromosome (Fig. 5). Mitotic loss of inserted sequences by δ-δ recombination or gene conversion events have been reported (Downs et al. 1985; Vincent and Petes 1986; Tschumper and Carbon 1986; Rothstein et al. 1987; Kupiec and Petes 1988). However, our results reveal that the δ-integrative plasmids are mitotically stable.

We could not rule out the possibility that the δ-integrative plasmid is relatively unstable and that only the transformant whose integration occurred on one chromosome is mitotically stable. This may be a reason why we could not detect multicopy integration on more than two chromosomes. The total copy number of the δ-integrative plasmids (three to five) was higher than that reported by Boeke et al. (1988) (two to three) using the transposition of the whole Ty element bearing a heterologous gene. Recently, high-copy-number integration of a heterologous gene into ribosomal DNA has been reported (Lopes et al. 1989). More than 100 copies of the integration were obtained when a defective *leu2-d* allele was used for selection of the plasmid. A defective allele of the selection marker will improve our δ-integration system.

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