Bot. Bull. Academia Sinica 26: 47-57 (1985)

CONSTRUCTION OF CHIMERIC PLASMIDS FOR MOLECULAR CLONING IN YEAST[1]

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(Received November 14, 1984; Accepted December 13, 1984)

Abstract

Chimeric plasmids containing 2μ *ori* or centromere sequences were constructed. Plasmids derived from yeast 2 μm plasmid are quite stable in laboratory strains: they are absent in about 40% from the population after ten generations' growth. Plasmid pYBH1, however, derived from centromere of yeast chromosome IV is not so stable: remaining only 15% of the total population under the same conditions. A foreign gene coding for hepatitis B surface antigen protein (HBsAg) was cloned into these plasmids and the activity of this gene in yeast was measured by radioimmunoassay. HBsAg was found in most of the yeast transformants. The levels of HBsAg are plasmid-dependent, but do not depend upon the stability of the plasmid. The highest amount of HBsAg in genetically engineered yeast was found to be around fifty micrograms per liter culture under our experimental conditions.

Key words: Yeast; molecular gene cloning; hepatitis B surface antigen; shuttle vector.

Introduction

Molecular cloning procedures used for yeast have been established by Hinnen *et al.* (1978) and been extensively studied ever since. Yeast *Saccharomyces cerevisiae* can be used to clone foreign genes which are not selectable in, or compatible with, bacteria systems. Among eucaryotes, *S. cerevisiae* has unique advantages because its fermentation techniques have been highly developed and it is the only organism that is known to carry a high copy number, autonomously replicating, extrachromosomal DNA element, *i.e.* the 2 μm DNA. Yeast cell has been chosen as a host to genetically engineer several foreign genes for the production of economically

[1] Paper No. 285 of the Scientific Journal Series, Institute of Botany, Academia Sinica, Taipei, Taiwan, Republic of China.

important proteins, such as interferons (Hitzemen *et al.*, 1983), and hepatitis B surface antigen (Miyanohara *et al.*, 1983; Valenzuela *et al.*, 1982).

In order to maintain plasmids in yeast cells during cell proliferation, cloning vectors are constructed to be either integrating or extrachromosomal replicating. Integrating vectors contain certain segment of cloned yeast genomic DNA sequences which directs the homologous recombination between vector and host chromosome, and extrachromosomally replicating vectors contain autonomous replicating sequences derived either from yeast chromosome (called *ars*) or from a naturally-occurring yeast plasmid (called 2μ *ori*). Because the integration event was the rate-limiting step in the yeast transformation process, the molecular cloning work in yeast has been concentrated in using extrachromosomally replicating vectors.

In this paper, we describe the construction of three kinds of extrachromosomally replicating vectors containing gene coding for hepatitis B surface antigen protein (HBsAg) and the activity of HBsAg gene in transformed yeast cells.

Materials and Methods

All of the restriction enzymes, *Echerichia coli* DNA polymerase large fragment (klenow polymerase I), T4 DNA ligase, *Eco*RI linker DNA were purchased either from Bethesda Research Laboratories or New England Biolabs and were used according to the prescribed reaction conditions.

Fine chemicals were obtained from Sigma Chemicals Co. Radioactive material and nitrocellulose filter Gene-Screen were purchased from New England Nuclear.

The procedures for isolating plasmid DNA, performing electrophoresis and *E. coli* cell transformation were performed as described (Maniatis *et al.*, 1982). The methods for yeast culture and transformation were performed according to published procedures (Hinnen *et al.*, 1978).

The procedures of *in vitro* labelling of probe DNA by nick-translation technique was provided by New England Nuclear. 100 μl of reaction solution containing 10 μl of 10x buffer (100 mM Tris-HCl, pH 7.8, 50 mM $MgCl_2$, 50 μg/ml BSA), 1 μg DNA, 0.2 mM dNTPs, 25 μCi α-^{32}P-TTP, 10 μg/ml DNase I and 2 units DNA polymerase I (Klenow fragment). After incubation at 15°C for 60 min, the reaction was terminated by adding 10 μl of 100 mM Na_2-EDTA solution. Proteins and deoxynucleotides were removed by gel filtration (Bio-Gel A-0.5 m, 0.5×50 cm). Colony-, dot-, and southern-blotting- hybridization were performed by the standard procedures as described (Maniatis *et al.*, 1983).

Plasmids used in this study are listed in Table 1. Plasmid pBH1 contains the structure gene for HBsAg from the *Bam*HI site at the upstream of the ATG codon through the HBsAg termination condon continuing to the *Hin*cII site in HBV DNA. This plasmid was provided by Dr. R.C. Huang of the Johns Hopkins University

Table 1. *Plasmids used in this study*

Plasmid	Molecular size, kbp	Replicating sequence	Selective markers	Source
pBH1	3.6	—	—	R-C Huang
pMA56	8.3	2μ *ori*	Trp1	B. Hall
pTY14-kan5	13.9	2μ *ori*	km-r	W-H Shu
YCp19	10.6	*ars*1, CEN4	Trp1, Ura3	M. Malavasic
pMK1	10.0	2μ *ori*	Trp1, km-r	this work
pAS32	9.3	2μ *ori*	Trp1	this work
pMKS1	10.9	2μ *ori*	Trp1, km-r	this work
pYBH1	11.6	CEN4	Trp1	this work

(Baltimore, USA). Plasmid pMA56 is a shuttle vector which can propagate both in *E. coli* and in yeast and its structure has been described (Valenzuela *et al.*, 1982). Plasmid pTY14-kan5 containing sequences coding for kanamycin-resistance determinant was obtained from Dr. W-H Shu (FIRDI, Hsin-Chu, ROC). Plasmid YCp19 containing yeast centromere sequence was provided by Dr. M. J. Malavasic (University of Chicago, USA). Both plasmids are shuttle vectors which can propagate both in *E. coli* and in yeast cells.

Hepatitis B surface antigen (HBsAg) production in yeast was detected by radioimmunoassay (RIA). The RIA kits for HBsAg measurement were from Abbott and all procedures were run by Dr. D. S. Chen of the National Taiwan University. The yeast extracts for RIA was prepared by glass-bead extraction method (Hitzeman *et al.*, 1983). RIA activities were given based on the same amount of cells.

Results

The Construction of Shuttle Vectors

The primary plasmid pAS32 containing HBsAg coding sequence and the promoter sequence of alcohol dehydrogenase I gene (ADH I-P) was derived from plasmids pBH1 and pMA56 as described (Fig. 1; Feng *et al.*, 1985). This plasmid can transform yeast cell using 2μ-*ori* as autonomous replicating sequence and active *trp*1 gene as a selective marker. This shuttle vector also contains ColEI-*ori* and penicillin-resistance determinant sequences which can be recognized as a replication origin and active gene respectively in *E. coli*.

Figure 2 shows the construction strategy of the plasmid pMKS1. Instead of using treptophan-requiring as selective marker in yeast transformation, pMKS1 was constructed by the combination of plasmids pAS32 and pMK1 such that an additional marker, kanamycin resistance can be used in doing yeast transformation.

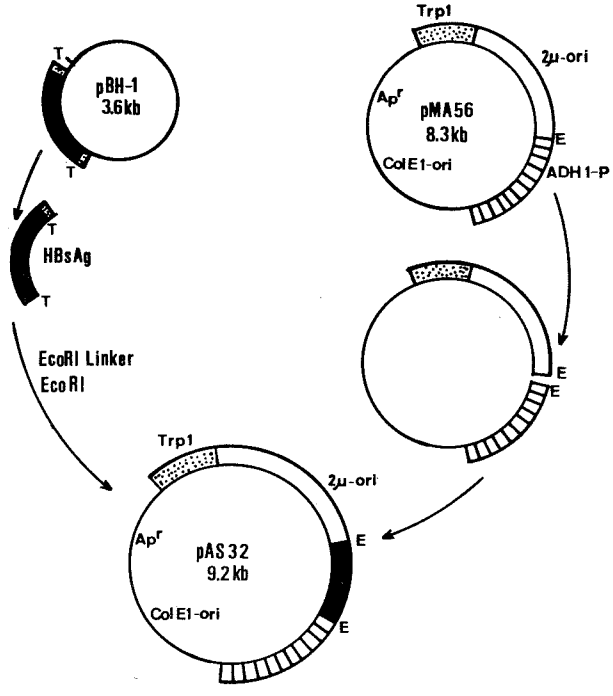

Fig. 1. Construction of plasmids for the expression of hepatitis B surface antigen gene in yeast. 10 μg of pBH-1 were digested with *Tha* I and the 900-bp fragment containing the HBsAg gene was isolated. 2 μg of the resulting 900-bp fragment was ligated to *Eco*RI linkers and cloned in the *Eco*RI site of pMA56. After transformation of *E. coli* HB101 cells, the transformant colonies were transferred to nitrocellulose and subjected to colony hybridization by using Tha-900 frament as a probe. Recombinant plasmids in which the HBsAg gene was in line with the ADH-I promoter were amplified in *E. coli* and used to transform yeast M1-2B, *α-trp*1-289, *ura*3-52, *gal*2. E=*Eco*RI, T=*Tha*I.

DNA fragment containing kanamycine-resistant determinant sequence (km-r) isolated from pTY14-kan5 was cloned in plasmid pMA56 (designated as pMK1) and then the fragment was re-isolated from *Hin*dIII-restricted pMK1 DNA. The *Hin*d III fragment of pAS32 which contains 5' part of *trp*1 gene and 2μ *ori* was replaced by the *Hin*dIII fragment of pMK1 which contains Km-r, 2μ *ori* and 5' part of *trp*1 gene. The resultant plasmid pMKS1 is suitable for yeast transformation without using auxotrophic marker.

Yeast centromere (CEN) and yeast nuclear autonomous replicating sequence (*ars*) were considered to replace 2μ *ori* as replication controlling element in yeast shuttle vector. Based on this idea, plasmid pYBH1 was constructed (Fig. 3). *Bam*HI-*Hin*dIII fragment of pAS32 containing ADHI-P and HBsAg coding sequence

was created by double digestion and inserted into double-digested plasmid YCp19 at *Bam*HI-*Hin*dIII. The resulting plasmid pYBH1 would maintain *trp*1, *ars*1 and CEN4 sequences plus active HBsAg gene. However, through unknown reason the *ars*1 and its adjacent sequences of the recombined plasmid were replaced by non-identified DNA, possiblly the host chromosomal DNA. Data from restriction pattern analysis (Fig. 4) revealed that pYBH1 is a plasmid having sequences of *ars*1 and small part of CEN4 deleted and replaced by unknown pieces of DNA with smaller in size. Due to the unexpected recombination the restriction sites of *Bgl*II, *Eco*RI and *Xho*I at the adjacent region of *ars*1 were eliminated and replaced by a new site of *Hin*dIII. *Trp*1 gene in pYBH1 was found to be less active than that in pAS32.

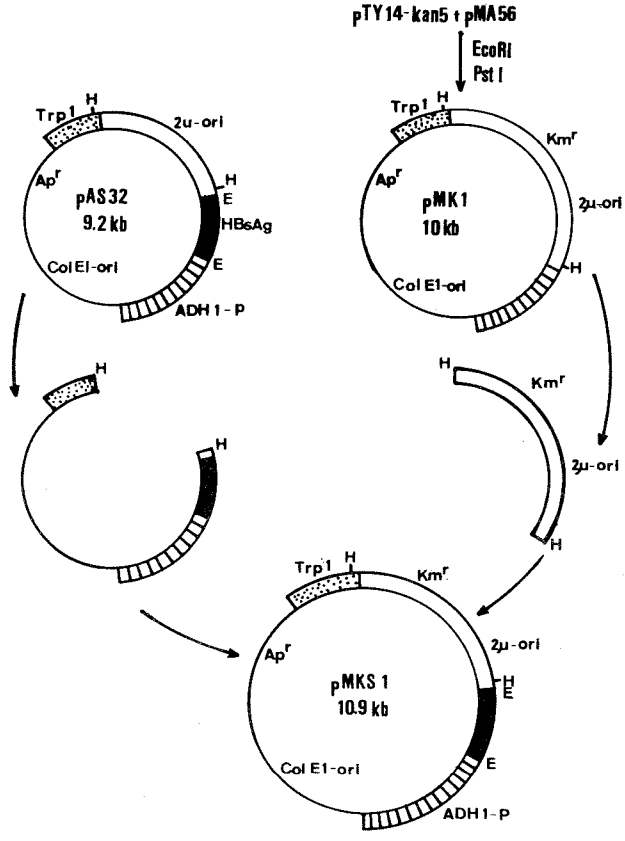

Fig. 2. Construction strategy of plasmid pMKS1. pMKS1 is a plasmid derived from pAS32 and has km-r gene from pMK1. pMK1 is a recombinant plasmid derived from pMA56 and pTY14-Kan5. The plasmid pMKS1 has advantage to transform antibiotic G418-sensitive yeast to be resistant to G418. H=*Hin*dIII, B=*Bam*HI.

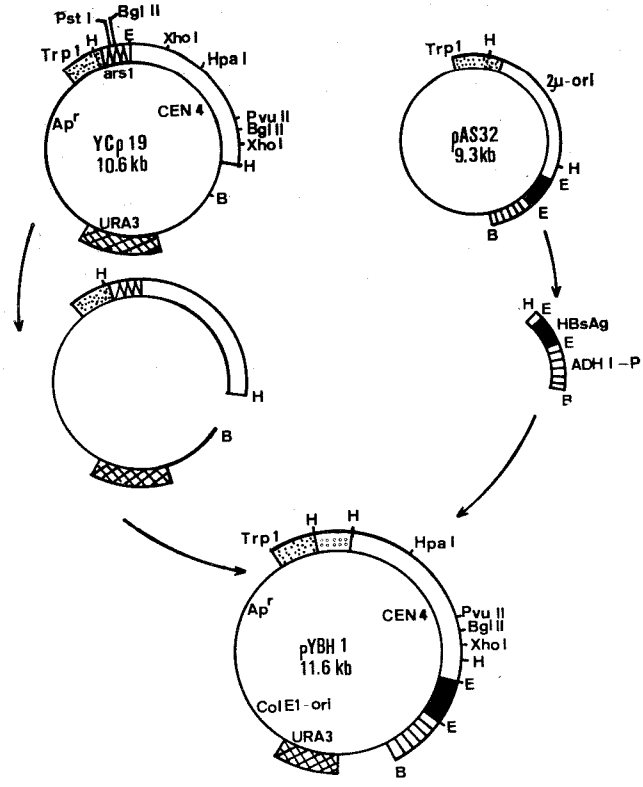

Fig. 3. Construction of plasmid pYHB1. pYBH1 is a plasmid derived from YCp19 and has expression unit for HBsAg from pAS32.

Activity of HBsAg Gene in Yeast

Three plasmids pAS32, pMKS1 and pYBH1 were used to transform yeast *Saccharomyces cerevisiea* and the production of HBsAg protein was measured by radioimmunoassy.

The frequency of yeast transformants containing active HBsAg gene varied considerably (Fig. 5). The transformants harboring pAS32 or pMKS1 behaved similar in the spectra of HBsAg production. The activity of HBsAg gene in most of the transformants was too low to be detected. Few of the HBsAg-producing transformants contained variable level of HBsAg content, while most of the pYBH1 transformed cells produced high level of HBsAg protein. Data of restriction pattern analysis indicated that the integrity of the ADHI promoter and HBsAg sequences were not altered during cell transformation (data not shown).

We have demonstrated that one isolate of the pAS32 transformants produced 17.5 μg of HBsAg protein per liter overnight culture (Feng *et al.*, 1985). This isolate has been stored and subcultured in our laboratory for more than a year and

the variation of HBsAg level was found to be very limited. The maximum level of HBsAg produced in pYBH1 transformed cells increased about three folds of that found in pAS32 transformed cells, while the content of HBsAg in pMKS1 transformed cells was in between, *i.e.* about 35 μg/l (Fig. 4).

Stability of Plasmids in Yeast

Transformansts harboring plasmids were maintained in selective medium and cultured in nonselective medium (YPD) for various time. The frequency of plasmid lost from host during cell division was measured as the percentage of cell number in selective plates over that in nonselective replica plates. In case of the pAS32 or pMKS1 transformed cells cultured in selective medium, the loss of kanamycin resistance phenotype was undetectable under our experimental conditions. However, only 60% of transformants under nonselective conditions for 18 hours were still kanamycin-resistance (Table 2). It was down to about 30% of the transformants which were able to keep kanamycin resistance phenotype after 36 hours cultured under nonselective conditions. Compared to the stability of pMKS1 in yeast, the plasmid pYBH1 behaved much less stable under the same conditions. This might reflect the loss of *ars*1 sequence during plasmid construction.

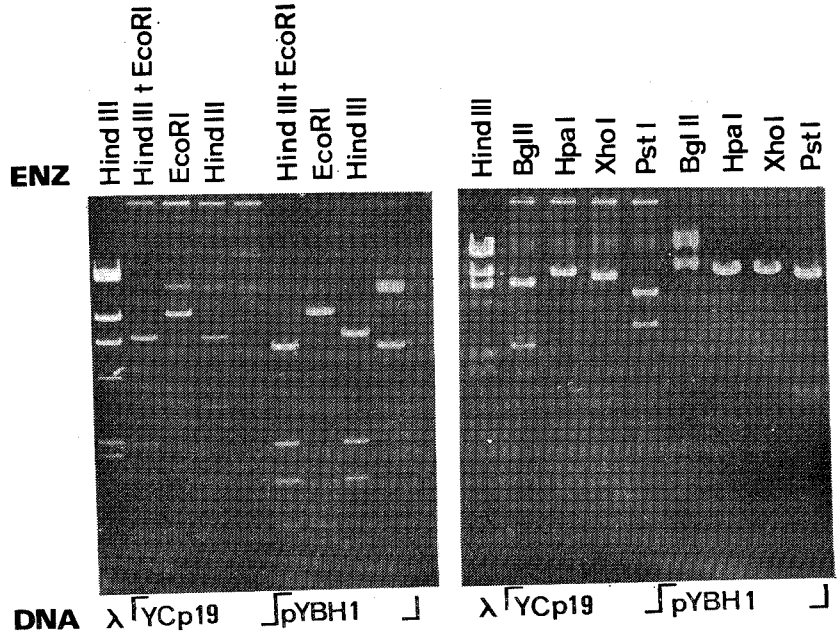

Fig. 4. Restriction patterns of plasmids YCp19 and pYBH1. YCp19 DNA contains unique sites for *Bgl*II, *Xho*I, and *Hpa*I in yeast sequences. The recombinant plasmid pYBH1 DNA was found to have one fragmenet less than the counterpart of YCp19 restricted DNA.

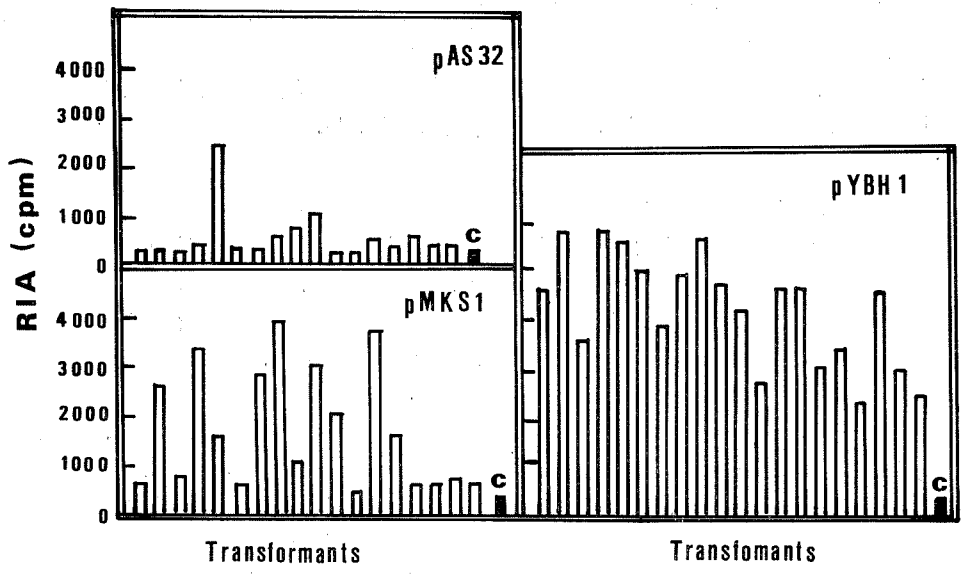

Fig. 5. HBsAg gene activity of yeast cells transformed by 2μ-containing plasmid pAS32, pMKS1 or by centromere-containing plasmid pYBH1. The TRP+ transformants containing HBsAg gene were cultured in rich medium (YPD) for 16 hours at 28°C. Cell extract from each transformant was subjected to HBsAg measurement. c represents HBsAg activity in the transformant which contains pMA56 or YCp19 as the control experiment.

Table 2. *Stability of plasmids in* ***Saccharomyces cerevisiae***

Yeast cells were transformed by plasmid and then single colonies derived from transformants were isolated from selective medium and cultured in YPD medium (non-selective) at 28 °C for 18 hours or 36 hours. The stability of plasmid in host was measured as percentage of cells which survived in selective medium plates over that in nonselective replica plates. The data shown below are the average of triplicate.

Plasmid	Host	Stability (%) after	
		18 hr	36 hr
pAS32	M1-2B	66	30
pMKS1	TL154	60	28
pYBH1	TL154	15	7.5

Discussion

Both plasmids pAS32 and pMKS1 contain the origin of replication (2μ *ori*) and 3′-termini of FLP gene (FLP-t) derived from 2 μm plasmid. 2μ *ori* functioned as a sequence for autonomous replication and FPL-t functioned as a terminator of HBsAg gene transcription. It has been reported that the endogenous 2 μm plasmid

is extremely stable in laboratory strains throughout many subculturings. Many of its derives were not so stable (Warren 1983). Although autonomous replication is conferred by the 2 μm circle origin of replication, stable propagation of the plasmid requires, in addition, two proteins encoded by the plasmid itself and STB sequence (Kikuchi, 1983). For those plasmids with less stability were due to the absence of the stablizing components. Plasmids pAS32 and pMKS1 were constructed to be 2μ *ori*-containing plasmids, and remained in laboratory strains (*S. cerevisiae* M1-2B or TL154) with equal loss rates throughout two subculturings (Table 2). The loss rates for 2μ derived plasmids were found to be in the range between 1% and 7% loss per generation (Table 2; Warren, 1983). The centromere-containing plasmid, however, was much less stable under the same conditions. The low stability of pYBH1 was an additional evidence of *ars*1 deletion.

HBsAg gene has been cloned in many organisms and its activities in different hosts have been studied (Feng *et al.*, 1985). Here we demonstrated that through genetic engineering procedures transformed yeast cells were able to produce HBsAg protein. The HBsAg protein produced in yeast was proved to be assembled in particle form similar to that identidfied in human serum (Feng *et al.*, 1985). Using an identical transcriptional unit (containing ADHI promoter and HBsAg coding sequence) the maximal activities of HBsAg gene in yeast differed about three folds between 2μ-containing plasmid and CEN-containing plasmid. Under the experimental conditions, the low stability of plasmid pYBH1 seems no essential effect on the production of HBsAg protein.

Colony hybridization and restriction pattern analysis have been employed to confirm that the introduced plasmids in all transformants which produced HBsAg were physically intact. Data from the restriction fragment pattern analysis were unable to resolve the possible alteration of incoming plasmid in detail. Furthermore, one of the pAS32 transformed cells, AK7, was included in the HBsAg content measurements to make sure the reliability of the RIA procedures. Therefore, the integrity of plasmid, the extraction procedures and the skillfulness of HBsAg detection were not considered as the causes of variations of HBsAg content among transformants. The elucidation of the variation of HBsAg production in yeast needs further investigation.

In this study, the highest level of HBsAg content in genetically engineered yeast was about 50 micrograms per liter culture. It has been reported that without changing the plasmid-host system the yield of HBsAg from yeast culture could be overall increased 100- to 1000-folds simply by modifying the fermentation and extraction procedures (Burnette *et al.*, 1984). It is reasonable to think that the highest number we obtained may not be the actual value in yeast. Therefore, to know the potential of using yeast to produce vaccine against hepatitis B virus infection the extraction procedures need to be improved and the fermentation

techniques require us to pay more attention.

Acknowledgements

We thank Drs. B.D. Hall, R-C. Huang, W-H. Shu and M.J. Malavasic for providing plasmids pMA56, pBH1, pTY14-kan5 and YCp19 respectively; Dr. D.S. Chen (National Taiwan University, Taipei) for the radioimmunoassy. This work was supported by a grant (NSC72-0416-B001-02) from the National Science Council, Republic of China.

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酵母菌中分子純系化質體之構築

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本實驗構築包含 2μ *ori* 或中心節序列之質體。由酵母菌 2μm 質體所演變而成的質體在實驗室菌株內相當穩定：經十代繼代培養後，約40%自族羣中消失，然而，源自酵母菌第四條染色體中心節之質體 pYBH1 就並不如此穩定：在相同的培養狀況下則只剩下羣族總數的15%。將一段含有B型肝炎表面蛋白（HBsAg）之外來基因純殖入這些質體，以放射性免疫偵測法測量此基因在酵母菌中的活性。結果在大多數的酵母菌轉型菌株中均可發現 HBsAg，其量的高低與質體種類有關，而與質體穩定度無關，經遺傳工程後之酵母菌中 HBsAg 的最高量在我們的實驗條件下約爲每公升菌液 50 μg。