Kor. J. Appl. Microbiol. Biotechnol.
Vol. 19, No. 3, 215-221 (1991)

Distinctive Characteristics of an Autonomous Replication Sequence of *Cephalosporium acremonium* in Yeast

Lee, Kyoung, Dae-Ook Kang, Byung-Dae Yoon, In-Kyu Hwang, Jeong-Seog Ahn and Tae-Ick Mheen*
Genetic Engineering Research Institute
Korea Institute of Science and Technology, Taejeon 305-333, Korea

*Cephalosporium acremonium*의 자율복제 기점의 특성

이 경 · 강대욱 · 윤병대 · 황인규 · 안종석 · 민태익*
한국과학기술연구원 유전공학연구소 미생물공학연구실

Abstract — An autonomous replication sequence (ARS) derived from *Cephalosporium acremonium* ATCC 20339 was cloned in *Saccharomyces cerevisiae* SHY 3 using YIp5 as a cloning vector. A new recombinant plasmid, designated pCY-2, which contained a 3.7 kb *Bam*HI fragment of *C. acremonium* DNA showed the highest stability among the 40 recombinant plasmids composed of the YIp5 and ARS of *C. acremonium*. Also, Southern hybridization and transformation of *E. coli* with DNA purified from yeast transformants verified that pCY-2 autonomously replicates in yeasts. Transformation efficiency and plasmid stability of pCY-2 in yeast were higher than those of YRp 7 containing ARS which originated from yeast. Detailed studies by subcloning revealed that two ARSs existed within 2.6 kb of the insert, which is a novel discovery. However, it was concluded that these two ARSs were ligated during the gene manipulation *in vitro*.

The idea that eukaryotic DNA replication may involve specific sequences was strengthened by the discovery that a specific group of replication fragments from yeast chromosomal DNA can be isolated and preform replication independent of the chromosome when cloned into integrative plasmids (1, 2). The minimum subfragment capable of conferring these properties has been reported to be an autonomous replication sequence or ARS. ARSs have been identified in DNA obtained from yeast (1, 3, 4), *Neurospora crassa, Drosophila melanogaster, Zea mays* (2, 5), human (6), *C. acremonium* mitochondria (7, 8) and *C. acremonium* chromosome (9).

Many reports suggest that the DNA fragments with ARS activity may contain biologically significant sequences which are important for the initiation and/or regulation of DNA replication. For example, estimates of the number of ARSs in *Saccharomyces cerevisiae* genome agree with the number of initiation sites estimated by fiber autoradiography and electron microscopy (10). Also, the average replicon size of yeast according to the determination by the precentage of random yeast DNA fragments that contain these functionally defined ARSs is about 30 to 40 kb (1, 11). Several ARSs have been sequenced and all have been found to contain the sequence A/T TTTAT Pu TTT A/T (5, 6, 12). This 11 bp core consensus sequence is necessary but not sufficient for function as the ARS. Deletion experiments have demonstrated that addition of sequences to the ARS are needed to promote high frequency transformation of the vector (3, 5, 6).

Key words: *Cephalosporium acremonium*, autonomous replication sequence (ARS), southern hybridization, transformation efficiency, plasmid stability
*Corresponding author

A fungus, *C. acremonium* is used in the manufacture of many clinically important semi-synthetic cephalosporin antibiotics (13). In this report, we have cloned a 3.7 kb *Bam*HI fragment containing ARS of *C. acremonium* chromosome in YIp5 vector, a yeast integrative vector as the first step to develop a secretion vector in *C. acremonium*. If we succeeded in developing it, we would clone and express the genes involved in cephalosporin synthesis in order to produce this antibiotics in higher yield in *C. acremonium*. The characteristics such as transformation frequency and plasmid stability of vector with *C. acremonium* ARS were compared with those of YRp7 containing yeast ARS. The 3.7 kb *Bam*HI insert was analyzed for the localization of ARS by restriction endonuclease mapping and subcloning.

Materials and Methods

Strains and plasmids

C. acremonium ATCC 20339 was the wild type strain used as a source of the ARS gene. *E. coli* JM83 (*ara*, del (*lac*-proAB), rpsL, φ80, del (*lac* Z) M 15) served as a host for gene amplification. *S. cerevisiae* SHY-3 (a, *ste*-Vc9, *ura* 3-52, *trp* 1-289, *leu* 2-3, *leu* 2-112, *his* 3-1, *ade* 1-101, *can* 1-100) was used for the transformation of hybrid plasmids containing ARS. Plasmid YIp5 (5.5 kb, *tet*R, *amp*R, URA 3, ARS1) (2) was used as an ARS cloning vector in this study.

Media

E. coli were grown in Luria-Bertani broth (1% Bacto-tryptone, 1% NaCl, 0.5%, Bacto-yeast extract). The YPD (2% Bacto-peptone, 2% glucose, 1% Bacto-yeast extract) and YNBD (0.67% Bacto-yeast nitrogen base without amino acid, 2% glucose) medium for yeast culture were prepared as described by Johnston (14). *C. acremonium* was grown in TS broth (3% tryptic soy broth, 0.005% potassium phosphate, pH 6.7).

Isolation and manipulation of DNA

Plasmid DNA from *E. coli* was prepared by cesium chloride-ethidium bromide density gradient centrifugation and by rapid minipreparation of cleared lysates (15). Total yeast DNA was rescued according to the method of Johnston (14). *C. acremonium* genomic DNA was isolated from protoplasts which were prepared by β-glucuronidase treatment (Sigma, USA) (16).

For gene library construction, total DNA from *C. acremonium* was partially digested with *Bam*HI and digested DNA was ligated to the *Bam*HI cloning site of the YIp5 vector. These hybrid vectors were amplified by transformation to *E. coli*. Plasmid DNA was isolated from all *E. coli* transformants which were resistant to ampicillin and sensitive to tetracycline. And then, *S. cerevisiae* SHY-3 was transformed to uracil prototroph with amplified hybrid plasmids.

Transformation and plasmid stability

Yeast was transformed according to the method of Ito (17). *E. coli* competent cells were prepared by Ca^{2+} treatment and transformation was carried out by the method of Maniatis (15). Plasmid stability of yeast transformant was determined as mentioned in a previous paper (18).

Southern hybridization analysis

The electroeluted 3.7 kb *Bam*HI insert of pCY-2 was labelled by nick translation using [α-^{32}P] dATP for the preparation of the probe (15). Restriction endonuclease digested DNAs were fractionated by agarose gel electrophoresis and were transferred to a nitrocellulose filter by the Southern procedure (19). Hybridization was carried out in 50% formamide, 0.8 M NaCl, 0.1% SDS, 0.5% polyvinylpyrrolidone, 1 mM EDTA, 0.05% BSA, 0.05% Ficoll, 100 μg/ml denatured salmon sperm DNA, 50 mM sodium phosphate (pH 6.5) at 42℃ for 12 hours.

Results and Discussion

Isolation of ARS from *C. acremonium* genome and construction of pCY-2

A yeast integrative plasmid, YIp5, which contained the *S. cerevisiae* URA3 gene and bacterial pBR 322 sequences, did not have replication origin of yeast (4). Therefore, YIp5 can not exist in an extrachromosomal state in yeast. This is the reason why

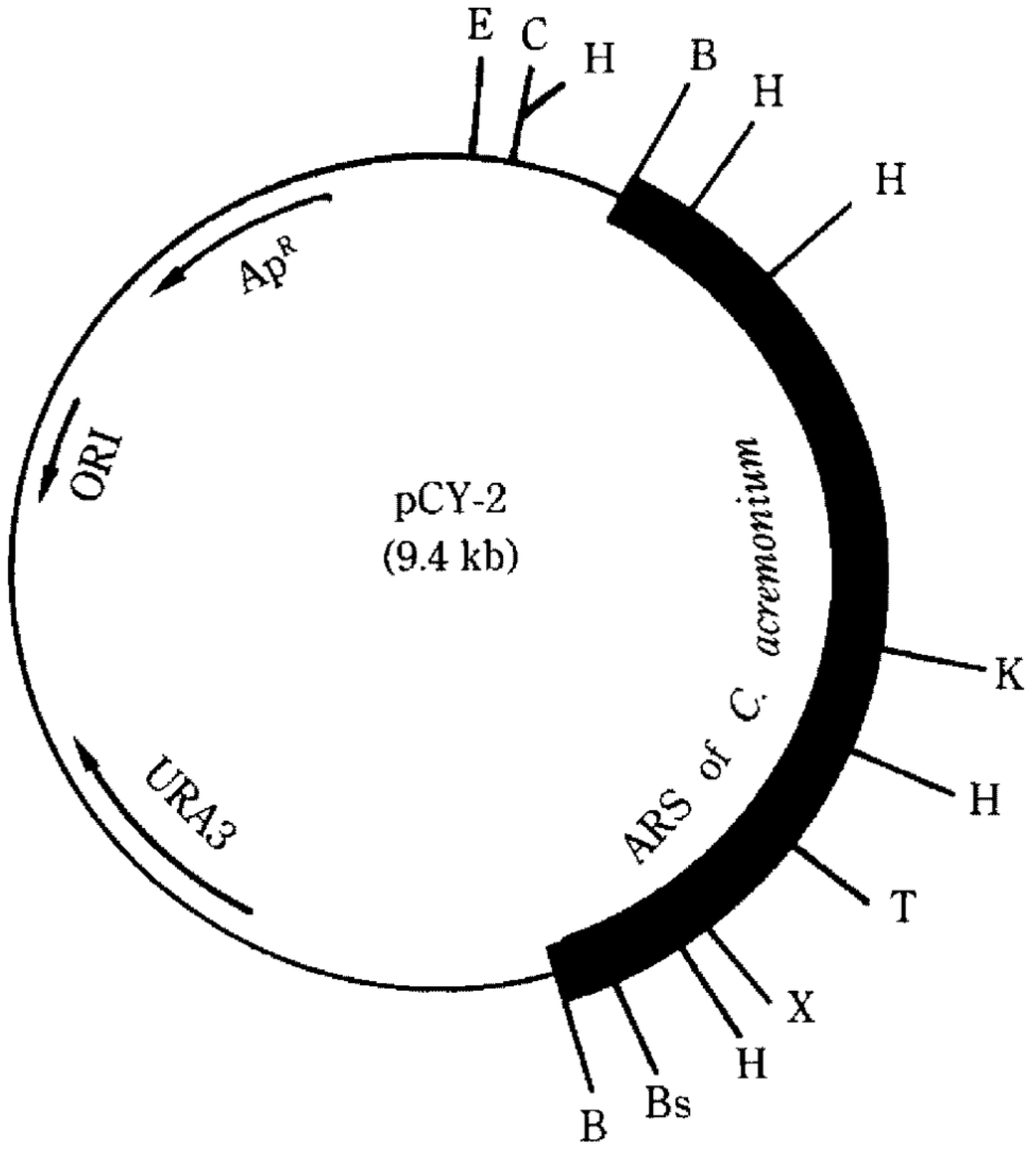

Fig. 1. Schematic diagram of pCY-2 plasmid.
In diagram, YIp5 sequence is represented by thin line, and 3.7 kb of the insert containing *C. acremonium* replication origin is represented by thick bar.
C: *Cla*I, H: *Hin*dIII, B: *Bam*HI, T: *Taq*I, K: *Kpn*I, X: *Xba*I, Bs: *Bst*EII, E: *Eco*RI.

YIp5 is particularly well-suited for ARS cloning.

In the present study, *C. acremonium* DNA/YIp5 plasmids were transformed to *S. cerevisiae* SHY-3 to select for the independence of uracil in YNBD medium (without uracil). The resulting transformants were assumed to possess the hybrid plasmid containing YIp5 and ARS of *C. acremonium*. An ura$^+$ transformant which showed the highest stability among 40 yeast transformants was selected for this research. Plasmid DNA was rescued from the retransformant of *E. coli* and restriction enzyme analysis showed the plasmid, designated pCY-2, contained the 3.7 kb *Bam*HI fragment in YIp-5 (Fig. 1). The insert DNA was not digested by *Eco*RI, *Pst*I, *Bam*HI, *Cla*I and *Sma*I (data not shown here). Because *C. acremonium* mitochondrial ARS was reported to have a *Eco*RI site in the 1.9 kb *Pst*I fragment (7), this 3.7 kb *Bam*HI ARS fragment was assumed to have been derived from *C. acremonium* chromosomal DNA, not mitochondrial DNA. Also, this ARS is different from the proviously reported ARS of *C. acremonium* (9) because the latter has

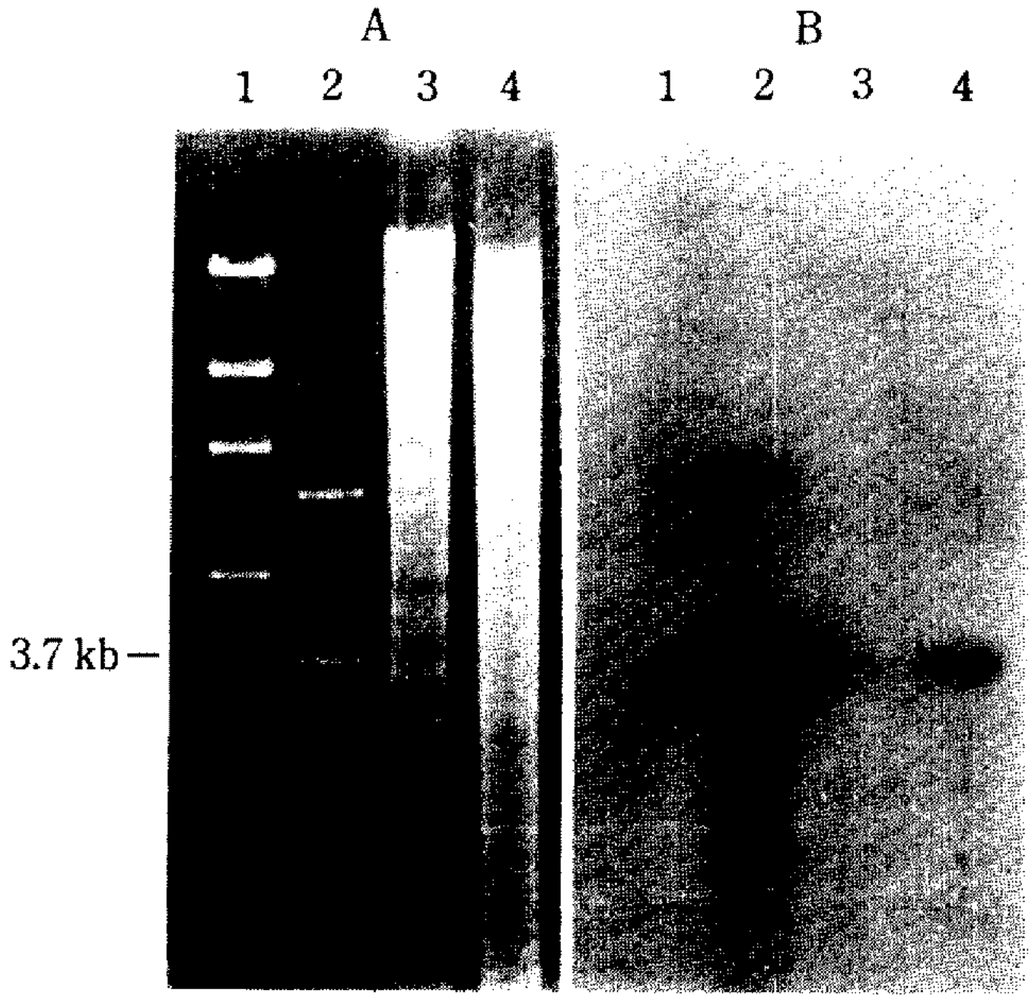

Fig. 2. Southern hybridization of the *Bam*HI-digested *C. acremonium* chromosomal DNA, *S. cerevisiae* SHY-3 chromosomal DNA and recombinant plasmid pCY-2. The 3.7 kb *Bam*HI-digested DNA fragment of pCY-2 was used as a probe.
A. Agarose gel electrophoresis pattern
Lane 1: λ DNA digested with *Hin*dIII as a molecular weight marker
Lane 2: pCY-2 digested with *Bam*HI
Lane 3: *S. cerevisiae* SHY-3 chromosomal DNA digested with *Bam*HI
Lane 4: *C. acremonium* ATCC 20339 chromosomal DNA digested with *Bam*HI
B. Hybridization pattern of A

the *Cla*I and *Sma*I site.

Southern hybridization was conducted to determine if the 3.7 kb *Bam*HI insert was drived from *C. acremonium* chromosomal DNA using nick-translated 3.7 kb *Bam*HI inset as a probe. Fig. 2A represents the agarose gel electrophoresis pattern of pCY-2, *S. cerevisiae* SHY 3 chromosomal DNA and *C. acremonium* ATCC 20339 chromosomal DNA digested with *Bam*HI respectively. The results of Southern hybridization are also shown in Fig. 2B. The signal was detected at *C. acremonium* DNA (lane 4 of Fig. 2B), not at *S. cerevisiae* DNA (lane 3 of Fig. 2B) and its size was the same as that of pCY-2 digested with *Bam*HI

From these results, it was concluded that the ARS fragment of pCY-2 originated from *C. acremonium* chromosomal DNA.

Analysis of yeast transformant by Southern hybridization

Southern hybridization was performed in order to verify whether transformed pCY-2 plasmid exists extrachromosomally or is integrated into chromosomal DNA in yeast. Fig. 3A represents agarose gel electrophoresis pattern of pCY-2 and total DNA of yeast transformants digested with *Eco*RI. Also, the result of Southern hybridization by using nick translated 3.7 kb *Bam*HI insert as a probe, is shown in Fig. 3B. The signal in yeast transformants was detected at the same size of pCY-2 linearized by *Eco*RI digestion. This result confirms that pCY-2 autonomously replicates in yeast because pCY-2 has a unique site for *Eco*RI as shown in Fig. 1. If pCY-2 was integrated into yeast chromosomal DNA, the size of two signals would have been different, not same.

In addition to the above evidence, to independently prove that pCY-2 is maintained extrachromosomally in yeast transformants, plasmid DNA from yeast transformants was retransformed to *E. coli* JM83. The plasmid from *E. coli* retransformants was the same size as pCY-2 (data not shown here). This is a common phenomenon that yeast vectors, except for integration vectors, are maintained in extrachromosomal state before and after transformation in yeast (8, 14).

From the results, it could be concluded that pCY-2 autonomously replicates in yeast.

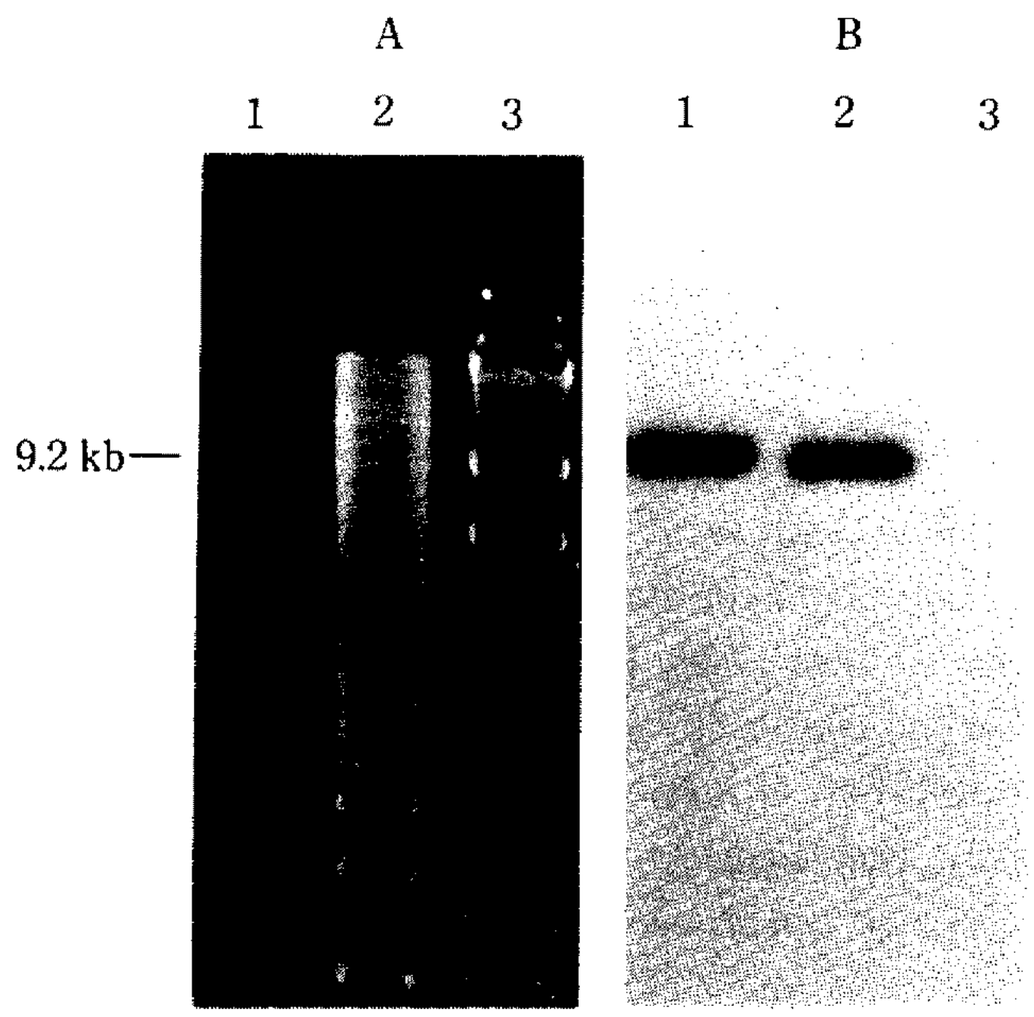

Fig. 3. Southern hybridization of *Eco*RI digested total DNA of transformed yeast and recombinant plasmid pCY-2. The 3.7 kb *Bam*HI-digested DNA fragmemt of pCY-2 was used as a probe.

A. Agarose gel electrophoresis pattern
  Lane 1: pCY-2 digested with *Eco*RI
  Lane 2: total DNA of transformed yeast, digested with *Eco*RI
  Lane 3: λ DNA digested with *Hin*dIII
B. Hybridization pattern of A

Transformation efficiency and plasmid stability of pCY-2 in yeast

To characterize the pCY-2 vector, the transformation efficiency in yeast was compared with those of YIp5 and YRp7 under transformation conditions by the method of Ito *et al.* (17). Plasmid YRp7, which contains a yeast ARS, replicates autonomously in yeast and transforms yeast at a high frequency (4). The selection marker for pCY-2 and YIp5 was uracil prototrophy and for YRp7 was tryptophan prototrophy. The transformation frequency per 10 μg DNA of pCY-2, YIp5, and YRp7 was 9.1×10^3, 10 and 3.3×10^3 respectively. Thus, the transformation frequency of pCY-2 was about 10^3 times and 3 times as high as those of YIp5 and YRp7 respectively.

Also, among yeast vectors, it was reported that the YRp type vector containing yeast ARS was mitotically unstable even under selection pressure (2). Tryptophan-independent yeast cells transformed with the yeast replicative plasmid, YRp7, have been observed to be similarly unstable (20).

In the present study, plasmid stability of pCY-2 was estimated by measuring yeast transformants independent of uracil after 48 hr growth in selective medium (without uracil) and after 25 hr growth in non-selective medium (uracil present). For comparison, the stability of a YRp7 was estimated by measuring the number of yeast transformants independent of tryptophan in both selective medium (no tryptophan present) and in non-selective medium (tryptophan present). The average retentions of pCY-2 in *S. cerevisiae* SHY3 in selective and non-selective medium were 10.0% and 0.5% respectively, compared with retentions of 4.3% and 0.1% for YRp7 as shown in Table 1. It could be conclu-

Table 1. Comparison of plasmid stability of pCY-2 and YRp7[a] in *S. cerevisiae* SHY-3

Plasmids	% Retention of plasmid phenotype[b]	
	Non-selective growth	Selective growth
pCY-2	0.5	10.0
YRp7	0.1	4.3

[a] Stability was estimated after 48 hr and 25 hr cultivation in selection and non-selection medium, respectively.
[b] Uracil prototrophy for pCY-2
Tryptophan prototrophy for YRp7

ded that the retention of pCY-2 is more stable in yeast than that of TRp7.

Localization of the functional region of the ARS

Subcloning of the 3.7 kb *Bam*HI insert containing ARS was performed to find the functional region of pCY-2. *Hin*dIII-digested fragments of the 3.7 kb *Bam*HI insert were ligated to *Hin*dIII-digested YIp 5. The *Hin*dIII site in YIp5 is not directly related to the tet^r promoter and structural gene. YIp5 vector containing an insert at *Hin*dIII site, however, would block the expression of tet^r gene because the *Hin*dIII site in YIp5 is so closely located at to disturb the function of the promoter of the tet^r gene. This is the rationale of using of the *Hin*dIII site of YIp5 as a cloning site. Therefore, it was easy to select transformants containing an insert at *Hin*dIII site of YIp5 because they cannot grow at 15 μg/m*l* of tetracycline in medium.

Plasmid DNA was rescued from these transformants and analysis by restriction enzyme digestion showed the plasmids, designated pCYH-1 and pCYH-2, contained 1.5, 1.1 kb *Hin*dIII fragment in the 3.7 kb *Bam*HI insert. These fragments were subsequently designated H-1 and H-2 respectively (Fig. 4A). The transformation frequencies of each clone in yeast were investigated. These two plasmids, pCYH-1 and pCYH-2, transformed *S. cerevisiae* SHY-3 to uracil prototroph at an extraordinary frequency of 3.8×10^3 per 10 μg DNA and 1.3×10^3 per 10 μg DNA respectively.

To identify the presence of pCYH-1 and pCYH-2 in the transformant uracil prototroph, total DNA isolated from the yeast transformants was transformed into *E. coli* JM83. The restriction pattern of the plasmids from the retransformant of *E. coli* was investigated. Fig. 4B represents restiction patterns of subclones. Lane 2 shows the control marker by linearized YIp5, H-1, H-2 fragments. The H-1 fragment in lane 3 and H-2 fragment in lane 5 are shown by digestion of pCYH-1 and pCYH-2 with *Hin*dIII. Also, a single band is shown in lane 4 and lane 6 by digestion with *Kpn*I and *Xba*I which is unique site for pCYH-1 and pCYH-2 respectively. This result indicates that both pCYH-1 and pCYH-2 transform *S. cerevisiae* SHY3 to uracil prototroph. In other words, each H-1 and H-2 fragment contains ARS activity.

However, it is concluded that these two fragme-

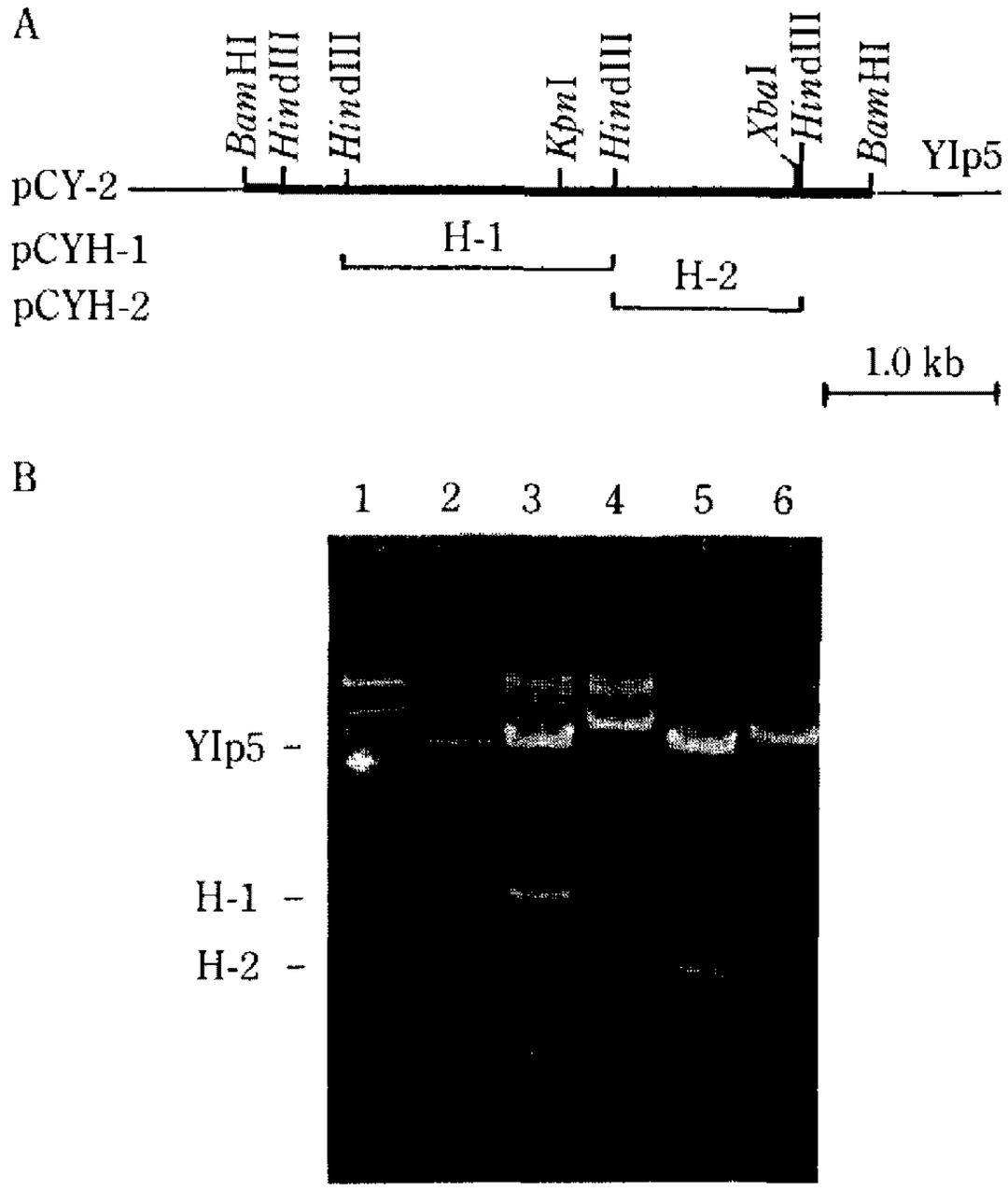

Fig. 4. Demostration of ARS activities of H-1 and H-2 fragment, separatively. Restiction maps (A) and digestion pattern (B) of subclones isolated from retransformant.
Lane 1: λ DNA digested with *Hin*dIII
Lane 2: Standard marker (linearized YIp5, H-1, H-2)
Line 3: pCYH-1 digested with *Hin*dIII
Line 4: pCYH-2 digested with *Kpn*I
Line 5: pCYH-2 digested with *Hin*dIII
Line 6: pCYH-2 digested with *Xba*I

Table 2. Transformation efficiencies and plasmid stabilities of pCY-2, pCYH-1 and pCYH-2 in yeast

Vector	Transformation[a] efficiency	% Retention of plasmid phenotype[b]
pCY-2	9.1×10^3	10.0
pCYH-1	3.8×10^3	5.8
pCYH-2	1.3×10^3	2.0

[a] Numbers of transformants which were observed per 10 μg DNA.
[b] Uracil-independence portion was estimated after 25 hr cultivation in selective medium.

nts (H-1 and H-2) were ligated during the ligation reaction of *Bam*HI-digested *C. acremonium* DNA and YIp5 because, as shown in Fig. 2, single band in *C. acremonium* DNA was observed in Southern hybridization by using 3.7 kb *Bam*HI insert as a probe. These results indicate that two adjacent ARSs are located in *C. acremonium* chromosomal DNA *in vivo*.

Plasmid stabilities were also estimated by measuring the number of transformants independent of uracil after 25 hr cultivation in non-selective medium. The average retentions of pCYH-1 and pCYH-2 in *S. cerevisiae* SHY-3 were 5.8% and 2.0% respectively.

As summarized in Table 2, it can be seen that transformation efficiency is directly related to plasmid stability. Also the efficiency of trasformation and plasmid stability increased in the other of pCYH-2<pCYH-1<pCY-2. It may be concluded from this result that H-1 and H-2 ARS fragments cumulatively work to reach the replication efficiency of pCY-2. Nontheless, the colony size of yeast transformant harboring pCYH-1 and pCYH-2 was the same as that harboring pCY-2.

Many ARSs have been reported to contain multiple copies of sequences that have at least 10 out of 11 bases homologous to a previously reported 11 bp core consensus sequence (5, 6, 9) but each ARS is separated at the spacing of 30~40 kb in yeast genome. This vector, pCY-2, will be the first reported case that two ARSs existed within a distance of about 2.6 kb *in vivo*. This clone may also serve as a useful probe for the study of replication events initiated from adjacent replication origins.

요 약

YIp5를 사용하여 *Cephalosporium acremonium* ATCC 2033으로부터 *Saccharomyces cerevisiae* SHY3에서 발현되는 자율복제 기점(ARS)를 분리하였다. 자율복제 기점을 갖는 40종의 vector 가운데 가장 안정성(stability)이 높은 plasmid를 pCY-2라 명명하였으며, pCY-2는 *C. acremonium*에서 유래하는 3.7 kb의 단편을 갖고 있었다. 또 Southern hybridization과 재형질전환을 통해 pCY-2가 효모내에서 자율적으로 복제되고 있다는 것을 확인하였다. 또한 pCY-2는 형질전환율과 안정성에 있어 효모의 ARS를 갖는 YRp7 vector보다 우수하였다.

이와 같은 성질은 2.6 kb의 DNA 단편내에는 두 자율복제 기점이 공존하기 때문으로 추측되어졌다. 그리고 pCY-2는 근접해 공존하는 두 복제 기점의 연구에 중요한 재료가 되리라 추측된다.

References

1. Beach, D., M. Piper and S. Shall: *Nature*, **284**, 185 (1980)
2. Stinchcomb, D.T., M. Thomas, J. Kelly, E. Selker and R.W. Davis: *Proc. Natl. Acad. Sci.*, **77**, 4559 (1980)
3. Chan, C.S.M. and B.K. Tye: *J. Mol. Biol.*, **168**, 505 (1983)
4. Struhl, K., T.D. Stinchcomb, S. Scherer and R.W. Davis: *proc. Natl. Acad. Sci.*, **76**, 1035 (1979)
5. Mills, J.S., A.J. Kingsman and S.M. Kingsman: *Nucl. Acids. Res.* **14**, 6633 (1986)
6. Montiel, J.F., C.J. Norbury, M.T. Tuite, M.J. Dohson, J.S. Mills, A.J. Kingsman and S.M. Kingsman: *Nucl. Acids Res.*, **12**, 1049 (1984)
7. Oeser, B., P. Tudzynski and K. Esser: *Appl. Microbiol. Biotechnol.*, **23**, 280 (1986)
8. Skatrud, PlL. and S.W. Queener: *Curr. Genet.*, **8**, 155 (1984)
9. Isogai, T., M. Yoshida, M. Tanaka and H. Aoki: *Agr. Biol. Chem.*, **51**, 2321 (1987)
10. Campbell, J.L.: in Genetic Engineering, Setlow, J. and A. Hollaender Eds., Vol. 5, 109 Plenum Publishing, NY.
11. Chan, C.S.M. and B.-K. Tye: *Cell*, **33**, 563 (1983)
12. Palzkill, T.G., S.G. Oliver and C.S. Newlon: *Nucl. Acids Res.*, **15**, 6247 (1986)
13. Lee, Kyoung, S.H. Choi, J.J. Lee and T.I. Mheen:

Kor. J. Appl. Microbiol. Bioeng., **15**, 361 (1987)
14. Johnston, J.R.: Yeast I, (Campbell ed.) IRL Press, London (1988)
15. Maniatis, T., E.F. Fritch and J. Sambrook: *Molecular Cloning: A Laboratory Mannual*, Cold Spring Harbor Laboratory (1982)
16. European Patent, 85, 306, 765, 0 (1986)
17. Ito, H., Y. Fukuda, K. Murada and A. Kimura: *J. Bacteriol.*, **153**, 163 (1983)
18. Ahn, J.-S., I.K. Hwang, M.-S. Jeong and T.I. Mheen: *Kor. J. Appl. Microbiol. Bioeng.*, **17**, 606 (1989)
19. Souithern, E.M.: *J. Mol. Biol.*, **98**, 503 (1975)
20. Xakian, V.A. and J.F. Scott: *Mol. Cell Biol.*, **2**, 221 (1982)

(Received March 6, 1991)