# Studies on the Organization and Transcription of *Aspergillus nidulans* tRNA Genes

## II. Cloning of *A. nidulans* total tRNA genes in *Escherichia coli*

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# *Aspergillus nidulans*의 tRNA 유전자의 구성과 발현에 관한연구

## II. *Aspergillus nidulans* 총 tRNA 유전자의 cloning

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## ABSTRACT

Total tRNA genes from *Aspergillus nidulans* were cloned for the further investigation of the structure and expression of Aspergillus tRNA genes.

Aspergillus DNA was isolated from spores and cloned into pBR322 plasmid DNA using *Bam*HI and $T_4$ ligase. The recombinant hybrid DNA was transformed into *E. coli* HB101 and some 30,000 transformants were initially selected. Of these, about 5,300 *E. coli* clones containing Aspergillus DNA inserted into plasmid pBR322 at *Bam*HI site have been isolated.

The hybridization data obtained from the labeled Aspergillus $^{32}P$-tRNA indicated that 105 colonies carried the total tRNA genes.

From the data above and cohybridization experiment, tRNA genes of *Aspergillus nidulans* seem to be twice more clustered than those of yeast.

## INTRODUCTION

Recent development of recombinant DNA technology (Cohen *et al.*, 1972, Cohen *et al.*, 1977, Helinski 1977, Morrow, 1979), along with the establishment of DNA sequencing method (Maxam and Gilbert, 1977, Sanger *et al.*, 1977) have made it possible to understand the structure and the expression mechanism of genes in molecular terms (Abelson, 1979, Breathnach and Chambon, 1981). One of the important achievements enabled by these techniques was the discovery of intervening sequences (intron).

It is now widely accepted that almost all eucaryotic genomes have introns and the introns may play a role in the expression of genes (Vanin *et al.*, 1980, Nishioka *et al.*, 1980) and in the arrangement of genes (Heilig *et al.*, 1980, Breathnach and Chambon, 1981).

Eucaryotic genes do show altered mechanisms of expression when it is transcribed by RNA polymerase III instead of RNA polymerase II (Sakonju and Bogenhagen, 1980, DeFranco *et al.*, 1980, Goldberg, 1979, Rosenberg *et al.*, 1979).

In case of RNA polymerase II, transcription

initiates from the promotor at 5'-end of a gene. RNA polymerase III appears, however, in the mid of genes when it is involved in the transcription of 5S or tRNA genes, suggesting the strong probability of the existence of the promoter somewhere other than proximal regions. Several experiments supporting this fact have been issued (Hall *et al.*, 1982, Hofstetter *et al*, 1981).

tRNA genes have notable advantages over other genes in studying the organization and expression of gene by the merit of their small size and the abundence in organisms. By the shot-gun method, we have cloned the total tRNA genes from *Aspergillus nidulans*.

## MATERIALS AND METHODS

### 1. Strains

Haploid wild type *Aspergillus nidulans* was purchased from FGSC. *E. coli* HB101($r^-$, $m^-$, recA. suII, $leu^-$, $Bl^-$, $thi^-$, $pro^-$) and Rd103 (HB101 harboring pBR322 as vector) were used as a cloning host and for vector isolation respectively. E1150($T_4$lig $CI_{857}$nin5 Sam100) used for $T_4$ligase and *Bacillus amyloliquifaciens* H for *Bam*HI were donated from Dr. J.B. Yim and Dr. H.W. Lee, respectively.

### 2. Preparation of *A. nidulans* DNA

Nuclear DNA of *A. nidulans* was prepared by the modified method of Morris' (Morris, 1978). Conidial sproes were inoculated onto complete medium (CM) (Cho, *et al.*, 1982) plate were incubated at 37°C for 4~5 days until the conidial spores were fully developed. Conidial spores were harvested with spatula in 0.08% tween 80 sterile solution, and filtered through several folds of gauze. Spore suspension was spinned down and the pellet was washed with cold lysis buffer (10mM Tris-HCl, pH7.4, 2M Sucrose, 10mM KCl, 5mM Spermidine). The washed spores were transferred to 30ml siliconized corex tube and mixed with cold lysis buffer and glass beads (0.14~0.17mm diameter) in the ratio of 2:2.5:10 (w/v/w). Spores were vortexed at the maximum velocity observing intermittantly with phase-contrast microscope until 70% of spores were disrupted. Then 4ml per 2g spores of cold lysis buffer was added, mixed well, and stood for a few minutes.

Supernatant was decanted and added with sodium dodecyl sulfate (SDS) to the final concentration of 1%, extracted by phenol twice at 60°C, and precipitated with ethanol. DNA was pruified further by CsCl-EtBr isopycnic centrifugation and treatment with 200 $\mu$g/ml of proteinase-K and repeated extraction with phenol.

### 3. Purification of Vector DNA

Purification of pBR322 DNA was done according to the method previously described (Clewell, 1972, Clewell and Helinski, 1972).

### 4. Purification of *Bam*HI

*Bam* HI restriction endonuclease was purified by the method of Greene (Greene *et al.*, 1978). $\lambda$DNA was used as a substrate and the assay was done in 30$\mu l$ of reaction solution (60mM NaCl, 20mM Tris-HCl, pH7.5, 2mM $\beta$-mercaptoethanol, 7mM $MgCl_2$, 0.5$\mu$g of DNA).

### 5. Purification of $T_4$ DNA ligase

E1150($T_4$lig $CI_{857}$nin5 Sam100) was grown at 32°C, induced by shifting temperature to 43°C in log phase and amplified 4hrs at 37°C. Subsequent procedures were followed with the modification of other methods (Amagase *et al.*, 1981, Davis *et al.*, 1980). Cells were harvested and sonicated to obtain the cell extracts. It was fractionated with 0.33g/ml of ammonium sulfate. The precipitate was slowly dissolved in a small volume of ligase column buffer (20mM Tris-HCl, pH7.5, 0.1M NaCl, 10mM mercaptoethanol, 5% glycerol) and then loaded onto the phosphocellulose column. The column was developed with a linear gradient from 0.1M to 0.7M NaCl in ligase column buffer.

Ligase activity was measured. Fractions which

have enzyme activity were pooled and applied to DEAE-cellulose chromatography between 0.02M and 0.5 M NaCl linear gradient in ligase column buffer. The isolated enzyme was tested for the existence of undesirable enzyme such as DNase.

**6. Restriction and Ligation**

Aspergillus DNA and pBR322 DNA were digested with *Bam*HI at 37°C, extracted twice with phenol, three times with ether and precipitated by ethanol. Pellets were dissolved in ligation buffer. Passenger DNA and vector DNA were mixed in 3:1 ratio, heated at 65°C for 1min., transferred quickly to ice bath. Finally, ATP and $T_4$ ligase were added and incubated at 14°C for 12~16hrs.

**7. Transformation and Screening**

Transformation methods are described in many literatures (Mandel *et al.*, 1970, Cohen *et al.*, 1973, Bergmans *et al.*, 1981), however, we have adopted the method using $MgCl_2$ and $CaCl_2$. After transformation, colonies harboring foreign DNA inserted plasmids ($Ap^r$, $Tc^s$) were selected by selective antibiotic medium.

**8. Preparation of $^{32}P$-labeled tRNA Probe**

Aspergillus spores were inoculated to CM broth ($1\sim5\times10^6$ spores/ml) and cultured at 37°C for 6 hrs. Mycelia were harvested by centrifugation, washed with low phosphate complete medium broth (LPCM; CM lacking only $Na_2HPO_4$) and grown in LPCM broth for 2hrs until mycelia were depleted for phosphate. Then, carrier free $^{32}P$-orthophosphate was added, and if necessary, pH was adjusted to 6.0. Mycelia were grown for 2hrs until 60~70% of $^{32}P$ was incorporated. tRNA was isolated from the mycelia by hot phenol method (Knapp *et al.*, 1978) which was modified from Rubin's method (Rubin, 1975).

**9. Colony Hybridization**

In order to select the tRNA gene clones from Aspergillus gene bank, we used the colony hybridization technique (Beckmann *et al.*, 1977) which was originally developed by Grunstein and Hogness (1975).

## RESULTS AND DISCUSSIONS

**1. Cloning Strategy of tRNA Genes**

The cloning strategy is illustrated in Fig. 1. tRNA gene copies are abundant in a cell and extraction procedure of tRNA has been established in various organisms. Therefore shot-gun experiment is very easy and useful. It has been known that some restriction enzymes, *Bam* HI and *Hind* III, do not have restriction site at the 5S or tRNA genes. Therefore we used *Bam*HI which cuts the tetracycline resistance gene of plasmid, pBR322. Clones harboring foreign DNA could be selected by insertional inactivation ($Ap^r$. $Tc^s$ clones) and the total tRNA gene clones by *in situ* colony hybridization techniques.

We decided to purify the enzymes needed in

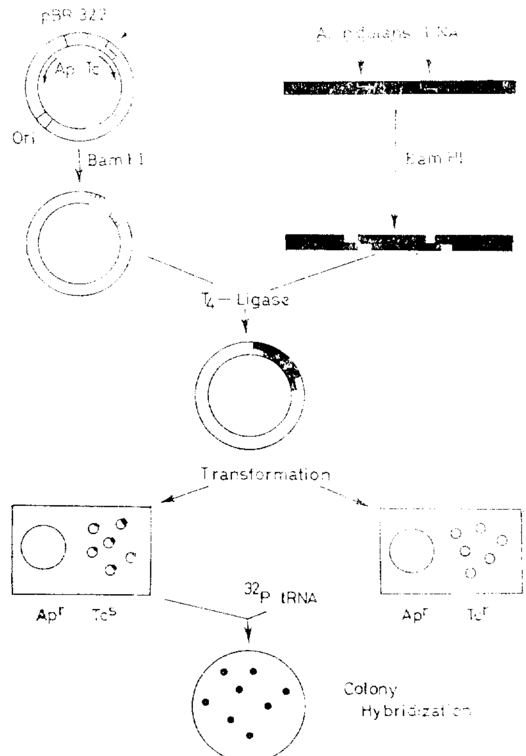



**Fig. 1.** The cloning strategy of *A. nidulans* tRNA gene.

**Fig. 2.** Photograph of disrupted and intact spores observed with phase-contrast microscope (x600). Black spores are the disrupted, and white spores indicate the intact ones.

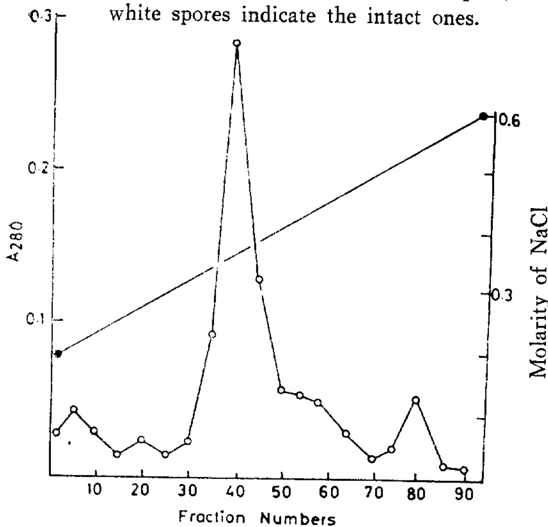


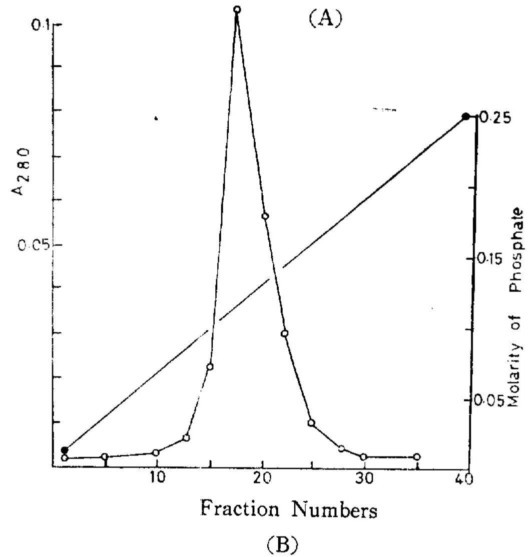

**Fig. 3.** P-II column chromatography for Bam HI purification. Endonuclease activities were eluted between 13th and 34th fractions (0. 25 ~0. 35).

this experiment in order to establish the fundamental techniques of genetic engineering in our laboratory.

**2. DNA Isolation**

pBR322 was isolated from *E. coli* C600 and transformed into *E. coli* HB101, in order to maintain the plasmid in monomer state.

About 80~100ug of plasmid DNA was obtained from 500ml culture. $A_{260}/A_{280}$ ratio of vector DNA was 1. 9, when measured with Gilford spectrophotometer. $A_{260}/A_{280}$ ratio is quite important in evaluating the purity of nucleic acids. In order for the DNA to be digested readily by restriction enzyme, the ratio should be greater than 1. 80.

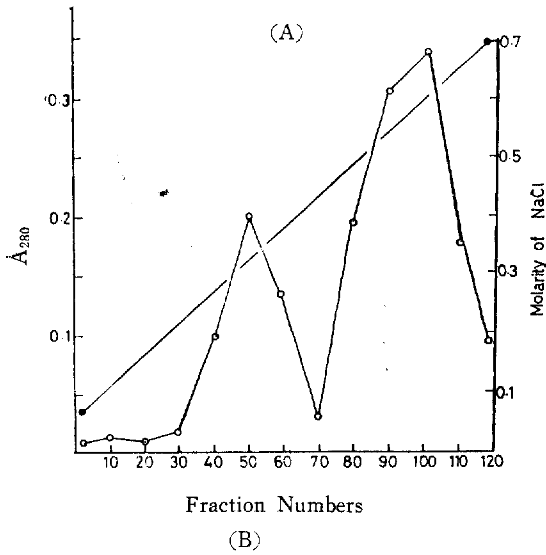



(B)

**Fig. 4.** (A) Hydroxylapatite column chromatography of *Bam*HI endonuclease activities were eluted between 16th and 30th fractions (0. 1M~0. 2M).

(B) Assay of *Bam*HI restriction endonuclease activity after hydroxylapatite chromatography.

*Aspergillus nidulans* DNA was isolated from spores, which were generated and harvested as described in the Materials and Methods. The relative amounts of spores, glass beads and buffer was very critical for spore disruption. When the ratio was adjusted to the optimum condition more than 90% of spores were disrupted with in one minute of vortexing. The ruptured spores could be easily distinguished




(B)

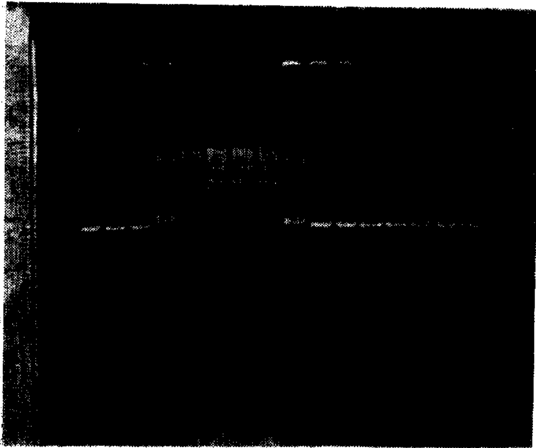


**Fig.** 5. (A) P-II column chromatography for the purification of $T_4$ DNA ligase. Ligase activities were eluted between 38th and 66th fractions (0.3M~0.4M).

**Fig.** 5. (B) $T_4$ ligase assay of fractions after P-II column chromatography. The substrate was linear pBR322 DNA. 5$\mu$l of of the fractions were added to reaction mixture and incubated at 37°C for 1hr.

from intact ones by phase-contrast microscope (Fig. 2). CsCl-EtBr centrifugation was carried out to eliminate the possibility of mitochondrial DNA and RNA contaminations. Isolated DNA was deproteinized by proteinase-K treatment (200ug/ml at 55°C for 1 hr) and extracted with phenol three times. Then the phenol was removed by ether extraction. In this case, the $A_{260}/A_{280}$ ratio was greater than 1.80. About

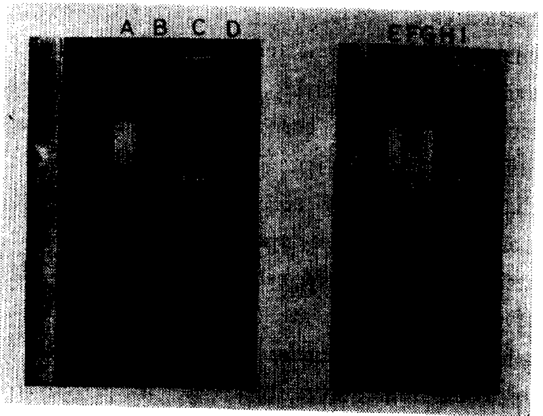



**Fig.** 6. *Bam*HI digestion and ligation of pBR 322 and *A. nidulans* DNA. Lane A.E: *A. nidulans* DNA B: *A. nidulans* DNA+*Bam*HI C.H: pBR322+*Bam*HI D.I: pBR 322 F: mixture of B and C G: ligated product of F

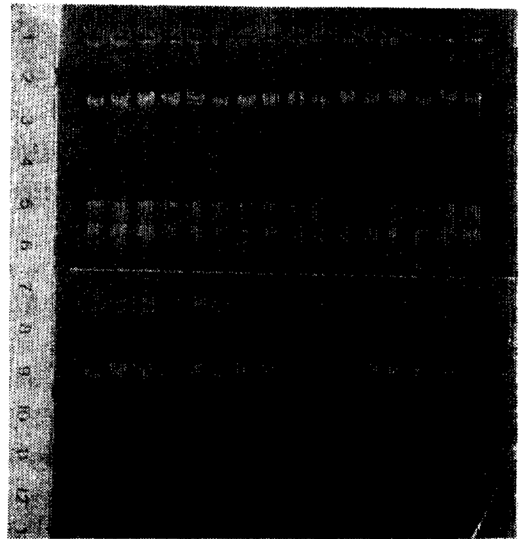


**Fig.** 7. Cracking of $Ap^s$, $Tc^r$ colonies. The first line from left of lower gel stands for isolated YRP7 (5.7kb).

200ug of DNA was obtained from 1g of spores.

### 3. The Purification of *Bam*HI Restriction Enzyme and $T_4$ DNA Ligase

24g of *Bacillus amyloliquifaciens* H cells were obtained from 6 liter culture in SLBH broth (Greene et al., 1978). The cell extract was subjected to phosphocellulose column chromatography (0.01M~0.6M NaCl gradient). Endonuclease activities were eluted between 0.25M and 0.35M of NaCl concentration (Fig. 3).

Wilson *et al* and Greene *et al* reported that the enzyme activities were eluted at 0.3M~0.36M and 0.35M, respectively. The differences in salt molarity may have been caused by the technical differences (Wilson and Young, 1975). Fractions that have enzyme activity were pooled and loaded onto hydroxylapatite column. Enzyme activities were eluted between 0.1M and 0.2M of potassium phosphate (Fig. 4A and 4B). The contaminating exonuclease activity was checked by incubating it with linearized pBR322 overnight and ligating the DNA with $T_4$-ligase. The enzyme preparation was essentially exonuclease free. About 4,000 units of enzyme were obtained from 6 liter culture.

$T_4$-DNA ligase was extracted from *E. coli* harboring lysogenic phage in which $T_4$-DNA ligase gene was cloned (Panasenko *et al.*, 1977, Wilson and Murrey 1979, Murrey *et al.*, 1979, Amagase, 1981). After cell culture, sonication and ammonium sulfate fractionation, the crude extract was applied to phosphocellulose chromatography. Ligase activities were eluted between 0.3M and 0.4M NaCl (Fig. 5A and 5B). The active fractions were subjected to DEAE-cellulose chromatography. Enzyme activities were eluted between 0.15M and 0.25M of NaCl. In order to confirm the absence of undesirable enzyme such as DNase, the enzyme was reacted overnight at 37°C without ATP and the mixture was electrophorased on agarose gel. About 3,000 units of ligase were purified from 2 liters culture. The qualities of *Bam*HI and $T_4$-ligase purified in this experiment were also demonstrated by other experimenters (Kim *et al.*, 1983).

**4. Construction of Recombinant DNA**

The ratio $A_{260}/A_{280}$ of Aspergillus DNA was 1.5. Therefore we have deproteinized by repeated treatment of proteinase-K and phenol extraction. Fig. 6 shows that the restriction and ligation of Aspergillus DNA and pBR322 DNA. DNA concentraction of ligation mixture was 20ug/ml and the relative amount of Aspergillus

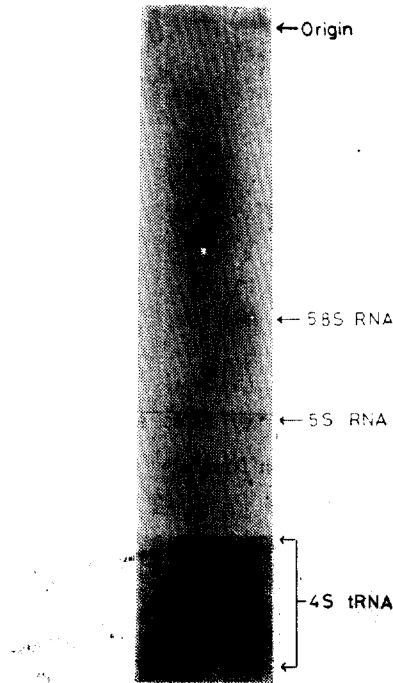



**Fig. 8.** Autoradiogram of $^{32}$p labeled tRNAs from *A. nidulans*. Sample was loaded on 4M urea-10% polyacrylamide gel and run at 250V for 8 hrs. The gel was exposed for 10 min, then developed.

DNA and pBR322 DNA was 1 : 3.

**5. Transformation**

Transformation efficiency was about 1330 transformants per ug of the ligated DNA. We performed 15 times of transformation independently and obtained 39,000 transformants ($Ap^r$) among which 5,320 $Ap^r$, $Tc^s$ colonies were selected, showing 13% frequency. This is lower than the frequency of yeast in other experiment (Beckmann *et al.*, 1977), and it might be caused by fragmentation of Aspergillus DNA in the process of purification. The sizes of the inserted foreign DNA were varied from 4kb to 20kb (Fig. 7).

**6. Preparation of Probes**

Crude extracts of $^{32}$P-labeled tRNAs were purified by DEAE-cellulose column chromatography and 10% polyacrylamide gel analysis (Fig. 8). The final product eluted from gel slices

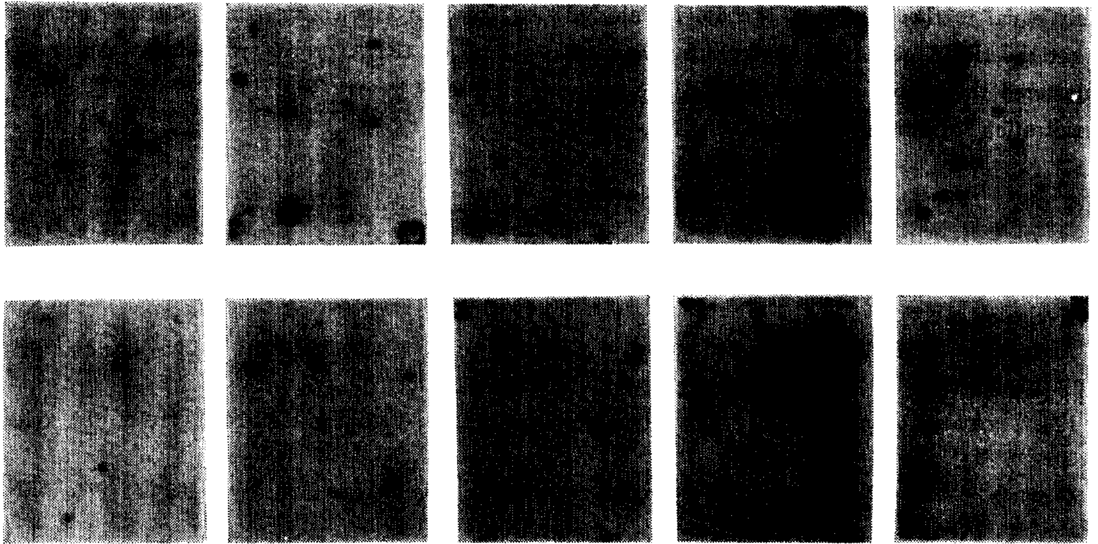


**Fig. 9.** Selection of clones carrying *Aspergillus nidulans* tRNA genes with colony hybridization.

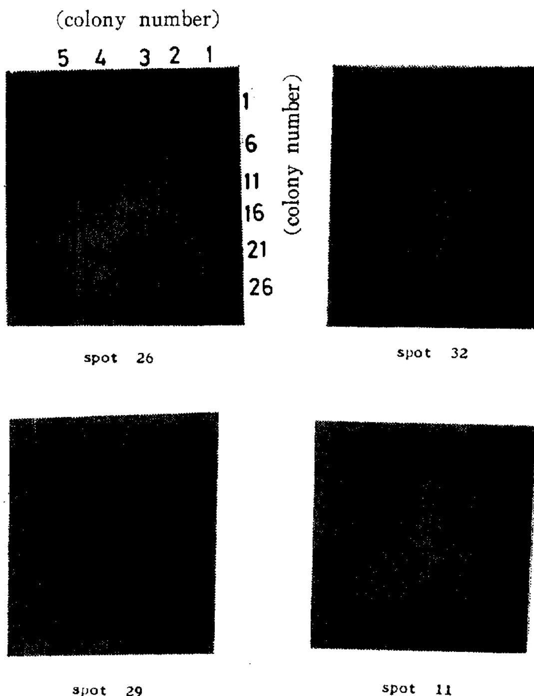



**Fig. 10.** Cohybridization test of clones carrying Aspergillus tRNA genes. Spot 11, 26, 29, 32 means the spots on the two dimensional polyacrylamide gel. Each spot was not contaminated by others. Clones of 2, 7, 14, 15, 16, 23 are cohybridized with two or three specific tRNAs.

showed 150,000cpm of radioactivity when 1 mCi of $^{32}$P-orthophosphate was used. The eluted tRNAs were reelectrophoraised before being used in hybridization in order to test that they were intact or not.

**7. Colony Hybridization**

150 colonies were grown on each Whatman No. 540 paper circle (9 cm in diameter). Colonies were lysed and hybridized with $^{32}$P-labeled tRNA probes as described in Materials and Methods. 105 total tRNA gene clones were detected by colony hybridization (Fig. 9). In the experiment of Beckman *et al* (1977) with yeast, 175 clones out of approximately 4,000 foreign DNA inserted colonies were selected (approximately 4.4%) which is about twice the frequency of our's (Table 1). Assuming the total DNA content and copy numbers of tRNA gene of Aspergillus to be approxima-

**Table 1.** Comparison of *A. nidulans* of gene bank and total tRNA gene clones with yeast's.

| | Aspergillus | yeast |
|---|---|---|
| total transformants | 30,000 | 12,000 |
| $Ap^r$, $Tc^s$ (foreign DNA inserted) colonies | 5,300 | 4,000 |
| insertion frequency | 13% | 33% |
| frequency of tRNA genes from total clones | 2% | 4.4% |

tely equal to those of yeast, it could be inferred that the tRNA genes in Aspergillus are more clustered than those in yeast. Indeed the cohybridization of cloned Aspergillus tRNA genes (probably indicating gene clusters) with some specific tRNA probes were more efficient than those of yeast (Fig. 10).

## 적 요

*Aspergillus nidulans*의 tRNA 유전자의 구성과 발현기작을 연구하기 위하여 우선 Aspergillus의 총 tRNA 유전자를 cloning 하였다.

Aspergillus의 핵 DNA를 포자로 부터 분리해 내고 본 실험실에서 분리한 *Bam*HI과 $T_4$ DNA ligase를 사용하여 pBR322플라스미드에 재조합시켜서 cloning하였다.

15번의 transformation을 하여 30,000개의 transformants얻었고, 이중 Aspergillus DNA를 가지고 있는 colony는 5,300여개 였다.

*In vivo*에서 $^{32}P$로 표지된 total tRNA를 probe로 하여 colony hybridization을 한 결과, 105개의 total tRNA유전자 clone을 얻었다.

위의 결과와 cohybridization 실험 결과를 분석해 보면, Aspergillus의 tRNA 유전자는 yeast의 그것보다는 좀 더 밀집되어 존재한다고 생각된다.

## ACKNOWLEGEMENT

We would like to thank S.T. Lee and U.J. Kim for their excellent technical aids and also B.G. Kang for tireless help during colony picking. Part of this research was supported by grant from Korea Science and Engineering Foundation from 1981 to 1983.

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