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# Studies on the Organization and Expression of tRNA Genes in *Aspergillus nidulans* (V) The Molecular Structure of $tRNA^{Arg}$ in *Aspergillus nidulans*

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# *Aspergillus nidulans*의 tRNA 유전자의 구조와 발현에 관한 연구 (V) *Aspergillus nidulans*의 $tRNA^{Arg}$분자구조

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**Abstract :** We have determined the sequence of $tRNA^{Arg}$ of *A. nidulans* partially by enzymatic rapid RNA sequencing technique. The sequence was 5′GGCCGGCUGGCCCAAXUGGCAAGGXUCUGAXUACGAAXCAGGAGAUUGCACXXXXXGAGCXXUXXGUCGGUCACCA3′. The clover-leaf structure was made from above data. As a result, the anticodon sequence was identified as ACG. This result was confirmed with charging test. The complete sequence was proposed by supplementing the DNA sequence to and by assigning the position of minor bases to this RNA sequence.

**Key Words:** $tRNA^{Arg}$ gene, DNA sequencing, consensus sequences.

In the past, finger printing method was usually used for RNA sequence determination (Sanger and Brownlee, 1972; Brownlee, 1972), which is very tedious and requires high technique. But the advent of rapid RNA sequencing technique made it easier much more. Several methods were developed recently for RNA sequencing. The first is enzymatic partial digestion method; after labeling 5′-end 3′-end of RNA, the RNAs are digested partially with several base specific RNases (T1, U2, Phyl, B.cereus and CL3) and finally loaded onto polyacrylamide sequencing gel (Simoncsits et al., 1977; Donis-Keller et al., 1977; 1980). The second is formamide fragment analysis method; RNAs are labeled at 5′-end after single hit partial hydrolysis by heating in formamide, and each RNA band is separated on polyacrylamide-urea denaturating gel. After complete hydrodysis of each RNA fragment, PEI-cellulose TLC is finally employed (Standley and Vassilenko, 1978; Gupta and Randerath 1979; Randerath et al., 1980; Diamond and Ducock, 1983; Randerath, 1962; 1974). The third is mobility shift method; RNAs are partially digested with enzyme or heat treatment after labeling them with isotope and paper electrophoresis or homochromatography is employed to separate RNA fragments (Silberkland et al., 1983; Diamond and Dudock, 1983). Above three methods are used usually at the same time to determine over all sequence (Miller et al., 1983; Pirtle et al., 1981). In addition to this, a similar method to Maxam and Gilbert's DNA sequencing method was reported (Peattie, 1979) but this method is not used frequently.

In this experiment, we have determined the sequence of tRNA partially by enzymatic partial

digestion method. We have found that these sequences are enough to identify the specific tRNA by constructing clover-leaf structure and by reading the anticodon sequence and the accuracy of this sequence is confirmed by comparing RNA sequences with its cognate DNA sequence.

## MATERIALS AND METHODS

### Strains

*Aspergillus nidulans* used in this experiment was the wild type strain of Fungal Genetics Stock Center (FGSC).

### Isotopes and Enzymes

$^{32}$P-orthophosphate was purchased from New England Nuclear and $^{14}$C-arginine from Amersham. Various RNases were purchased from BRL and Sigma. Calf intestinal alkaline phosphatase and T4-polynucleotide kinase were bought from NEN and Takara.

### TLC plates

DEAE-cellulose and Cellulose TLC plates were purchased from Machery and Nagel Company and Merck Company respectively.

### Large Scale Isolation of Aspergillus nidulans tRNA

tRNAs were extracted by hot phenol methods with some modifications (Rubin, 1975; Hopper et al., 1978; Knapp et al., 1978; Kim et al., 1983). After inoculating Aspergillus spores to a concentration of 5×10 spore/m*l* in culture bottle, they were cultured with shaking for 12 hours and mycelia were harvested with filtering method. Harvested mycelia were washed by adding 40 m*l* of washing buffer per 500 m*l* culture (Knapp et al., 1978). Mycelia were disrupted by grinding in a pestle after adding 2 g of $Al_2O_3$/10 g of mycelia. Hot phenol extraction was done after being added 20 m*l* of extraction buffer. Specific tRNAs were separated by two-dimensional polyacrylamide gel electrophoresis (10%-4M urea in first, 20%-4M urea in second).

### Preparation of [r-$^{32}$P]-ATP

To label the 5'-end of tRNA, [r-$^{32}$P]-ATP was synthesized with phosphate-ATP exchange method (Maxam and Gilbert, 1980). After reaction, [r-$^{32}$P]-ATP synthesized was identified from DEAE-cellulose TLC (sodium phosphate, pH 3.5). The specific activity was measured by scintillation counting both the orthophosphate portion and [r-$^{32}$P]-ATP portion of the same chromatographic lane respectively.

### RNA Sequencing by Enzymatic Partial Digestion

Specific tRNA was eluted from the gel and dephosphorylated by treating calf intestine alkaline phosphatase (CIAP) at 55°C for 30 min. After several extraction with phenol and then with ether, tRNAs were labeled with [r-$^{32}$P]-ATP. Polyacrylamide dipping gel (20%-7M urea) electrophoresis (20V/cm) was followed to separate ethanol-precipitated intact tRNA from sheared ones. When xylene cyanol marker moved to 40 cm, the electrophoresis was stop and the bands showing strong radioactivity were cut out.

tRNAs eluted from each band of the gel were divided into six batches and they were partially cleaved by several RNases. Reaction conditions are indicated in Table 1. The mobility of tRNA base on the gel is not constant because of the presence of modified base. Therefore, each band must be identified by comparing it with ladder (Simoncsits et al., 1977).

From this RNA sequence clover-leaf structure was made and anticodon was identified as the result.

**Table 1.** *Partial Cleavage Condition.*

| | RNA[3] | Enzyme | Reaction[4] | Inactivation |
|---|---|---|---|---|
| T1 | 1 $\mu l$ | 0.5 U | 0°C, 15min | 10 $\mu l$ F.A. dye, 90°C, 2min |
| U2 | 1 $\mu l$ | 0.3 U | 0°C, 15min | 〃 |
| Phy 1 | 3 $\mu l$ | 0.4 U | R.T.[5] 15min | 〃 |
| B. cereus | 2 $\mu l$ | 1 U | 55°C, 15min | 〃 |
| CL3 | 1 $\mu l$ | 0.5 U | 37°C, 15min | 〃 |
| L[1] | 4 $\mu l$ | – | 100°C, 30min | 〃 |
| N[2] | 0.5 $\mu l$ | – | – | 〃 |

1 L : Ladder
2 N : No enzyme
3 RNA : 1 $\mu g/\mu l$, greater than 30,000 cpm/$\mu l$
4 Reaction : T1, U2 and Phyl reaction conditions were followed from Simoncsits et. al. (1977).
5 R. T. : room temperature

**Modified Base Analysis**

Specific tRNA was dissolved in 10$\mu l$ of deionized formamide solution and it was heated for 15 min. at 100°C to make single hit fragments. After labeling the RNAs at 5′-end with $^{32}P$, they were completely digested with RNA digestion mix (Diamond and Dudock, 1983) and treated with P1 RNase to make 32pN. Two-dimensional cellulose thin layer chromatography was applied to separate each bases (Silberklang et al., 1981).

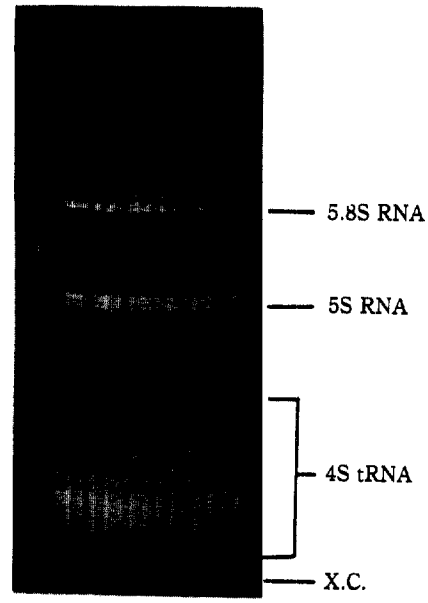
## RESULTS AND DISCUSSIONS

**tRNA Isolation**

About 25mg of RNA, measured by spectrophotometric method, was extracted from 500m$l$ culture broth. When mycelia were ground on pestle, more than 30 times of RNA were obtained without difference in RNA purity compared to those obtained by direct hot phenol extraction from intact mycelium. But we found that the temperature and grinding time affect the purity i.e. high temperature and long-time grinding lead to degradation of RNAs. Fig. 1 shows the profiles of tRNAs on the first and second dimensional gel. As shown in the figure, about 60 different tRNA spots were separated and of these, the number 2 spot (arrowed) was cut out. Eluted tRNAs from the gel were usually further purified on the denaturating polyacrylamide gel (20%-7M urea) to exclude any contaminating RNAs.

**[r-$^{32}$P]-ATP Synthesis**

To use [r-$^{32}$P]-ATP as substrate for the T4-kinase, it was synthesized by the phosphate exchange reaction. For [r-$^{32}$P]-ATP synthesis, there are two methods; one is phosphate exchange reaction of ATP, the other is foreward reaction of ADP. The latter method produces high specific activity of [r-$^{32}$P]-ATP but remaining ADP must be removed because residual ADP may cause reverse reaction in kination. Rf value of $^{32}$P-orthophosphate was 1 and that of [r-$^{32}$P]-ATP was 0.6 (Fig. 2). The spots of Pi and ATP were cut out and the radioactivity of each spot was counted. The specific activity was about 400 Ci/mmole which was calculated from the counting



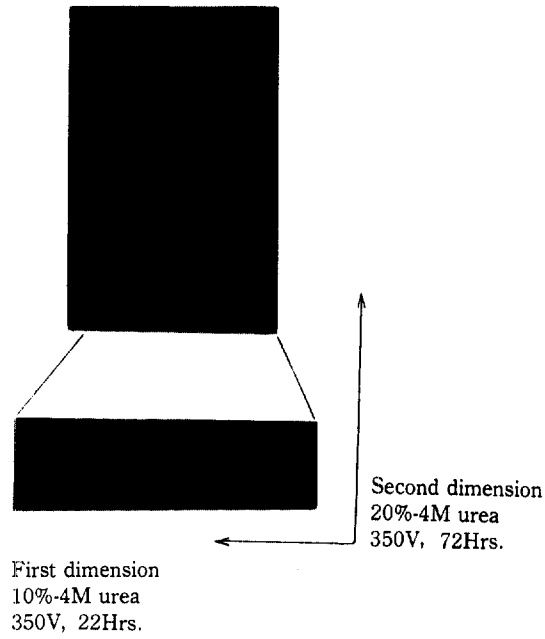




**Fig. 1.** *Separation of tRNAs by two dimensional PAGE. (A), the first dimensional gel (10%-4M urea) and (B), the separation pattern of tRNAs on the second dimensional gel (20%-4M urea). The arrow on the second dimensional gel indicates the number 2 spot ($tRNA^{Arg}$).*

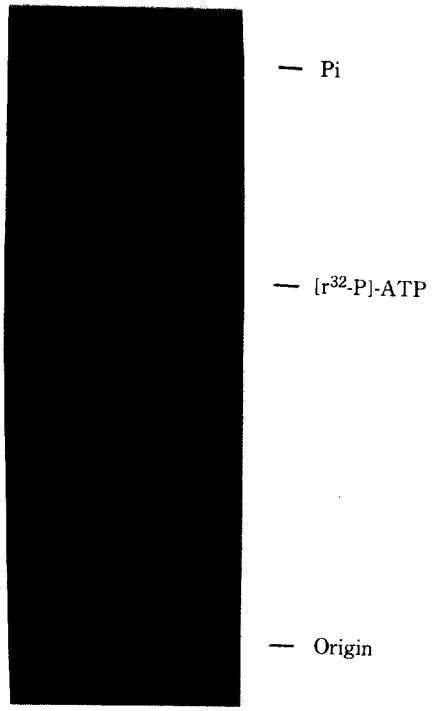



**Fig. 2.** *Synthesis of [r-32P]-ATP by phosphate exchange reaction.*

results.

**Labeling 5′-Ends with Polynucleotide Kinase**

Because the 5′-end is recessed in aminoacyl-stem of tRNA, it should be heated to give free end for dephosphorylation. Denaturation was carried out by heating tRNA at 65′C for 5 min. and quick-cooling. To inactivate CIAP, heating with SDS is usually done (Maniatis et al., 1982) but in this experiments direct phenol-chloroform extracton was used to prevent contamination of SDS and degredation of tRNA as much as possible. For the 5′-end of degraded RNA is -OH group and usually protruded, fragments are better substrate for labeling than intact tRNA. Therefore, the short fragments produced during dephosphorylation should be separated with denaturating gel electrophoresis (Fig. 3). The sheared fragments as well as the infact tRNA are useful for the 3′-end sequence determination, because the short fragment gives better resolution on the gel and it can encompass the secondary structure problem while digesting RNA with enzymes.

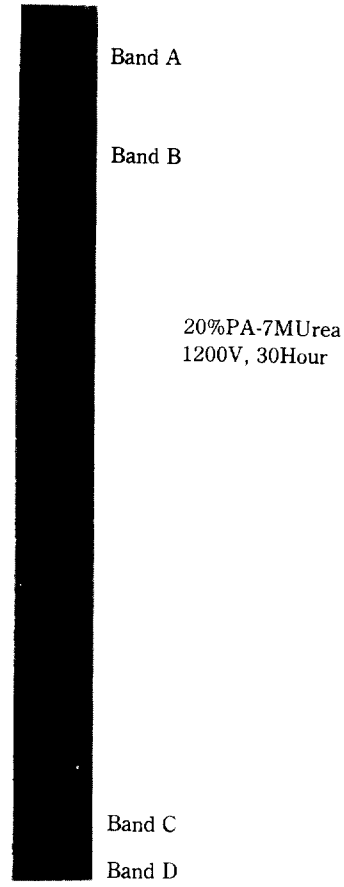



**Fig. 3.** *Separation of labeled tRNA fragments on polyacrylamide denaturating gel (20%-7M urea).*

**Enzymatic Partal Digestion**

Two methods have been reported for digesting RNA with T1 and U2. The one is that the reaction is carried out at 55′C in the presence of urea (Donis-Keller et al., 1977). The other is 0′C without urea (Sinoncsits et al., 1977). We adopted the latter methods because enzymes cut well although tRNAs prefer to maintain the secondary structure rather than heating. T1 cleaved tRNA at G, RNase of B. cereus at pyrimidine with high specificity but weak bands at G and C were produced by U2 and Phy1 respectively. And CL3 cut on C relatively well. Fig. 4 is the result of sequencing the number 2 spot. By constructing cloverleaf structure with these sequences, spot 2 was proved to be arginyl tRNA with anticodon ACG (Fig. 5).

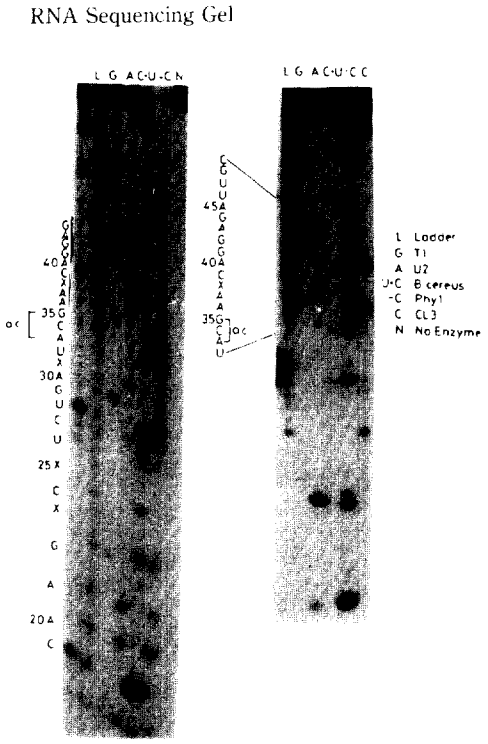



**Fig. 4.** *RNA sequencing gel. (A), 12% sequencing gel of oligonucleotide from Band A of Fig. 3. (B), 20% gel of Band B.*

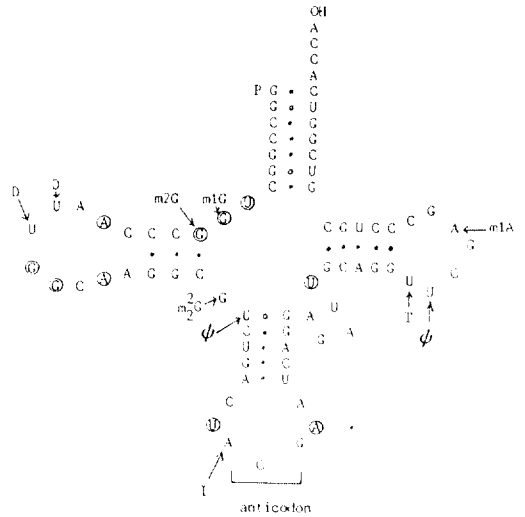



**Fig. 5.** *Clover-leaf structure of tRNA$^{Arg}$. Nomenclature of modified bases were followed from Sprinzl and Gauss (1984). The positions of modified bases were assigned by comparing the sequences of eukaryotic arginine-tRNAs with anticodon of ACG. Unidentified bases were supplemented from the DNA sequencing data (Lee and Kang, 1986). Watson-Crick's base pairing is designated as closed circle (•) and Crick's wobble base pairing as open circle (o). Conserved bases are surrounded with circle.*

When it was charged with $^{14}$C-arg, about 20 folds of radioactivities were precipitated than other negative control tRNAs. Almost all invariant sequences are occurred at the right position and bases in each stem were well paired. In this methods full sequences can not be determined because the modified bases are not identified on the sequencing gel. Thus we performed analysis of minor base.

**Minor Base Analysis**

We have analyzed the modified bases by Silberklang's methods (1981). Spots of major bases (pA, pG, pC and pU), pseudouridine (p$\psi$), dihydrouridine (pD), 1-methylguanosine (pm1G), 2-methylguanosine (pm2G) and Pi were easily identified. But it was difficult to identify exactly the spot W, X, Y and Z because of the lack of reference for Silberklang's method (Fig. 6). Lately the presence of $m_2^2$Gp, pGp and Tp were confirmed by Nishimura method (1972) but GmG was not found even in this method (data not shown).

Although inosine was not identified directly, one of unidentified spot (X in Fig. 6) seem to be pI with high probability because the first anticodon base of eukaryotic $tRNA^{Arg}_{ACG}$ are generally inosine (Sprinzl and Gauss, 1984). The position of each modified base was supposed from that of yeast's

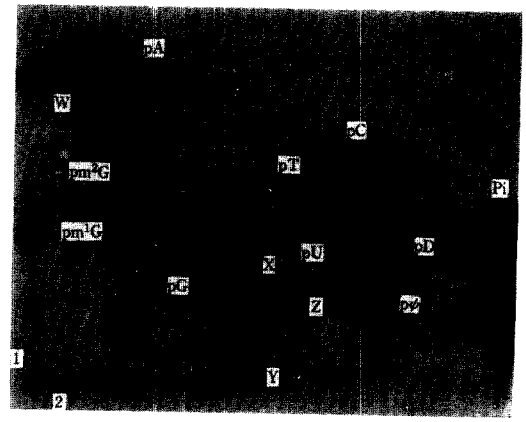



**Fig. 6.** *Modified base analysis on two-dimensional cellulose TLC*

**Table 2.** *Modified base of tRNA$^{Arg}$**

| Species | modified bases | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A1 | A2 | A7 | C2 | C3 | C5 | D | F | F1 | G1 | G2 | G4 | G7 | I | T | U4 | U7 | X |
| H. volcano | | | | | + | + | | + | + | + | + | + | | | | | | |
| E. coli | | + | | + | | | | + | | | | | + | + | + | + | | + |
| B. subtilis | | | | | | | + | + | | | | | + | | + | | | |
| yeast | + | | | | | + | + | + | | + | + | + | | + | + | | | |
| bovine (liver) | + | | + | | | | + | + | | + | + | + | | | + | | + | |
| mouse (leukemia) | + | | | | + | + | + | + | | + | + | + | | + | | | | |
| A. nidulans | | | | | | | + | + | | + | + | + | | + | + | | | + |

*All data except that of ***A. nidulans*** were followed from Sprinzl and Gauss (1984).

$tRNA^{Arg}_{ACG}$ (Fig. 5). We also compared the existence of modified bases among $tRNA^{Arg}$ species (Table 2). The existence of modified nucleoside seem to have intimate relationship with phylogeny. Namely, m1G, $m^2G$, $m^2_2G$ and m1A are appeared only in eukaryotes but m7G is found only in prokaryotes. *H. volcano* (an archaebacterium) shows very different aspects from prokaryotes ; it has preferably the eukaryotic characteristics. The sequence analysis of tRNA gene, indeed, showed the same features (Lee, 1986).

We have determined the full sequence of cognate $tRNA^{Arg}$ gene and found that this RNA sequences coincide with the DNA sequence. With the same methods as this, we have identified $tRNA^{Asp}$ and $tRNA^{Ser}$ (Kim et al., 1986; Jung, 1986) and determined their sequence partially. Although this sequence is not complete, this sequence is sufficient for identification of specific tRNA. The full sequence may be determined by formamide fragment analysis and by mobility shift method. If then, more insights for the molecular structure of $tRNA^{Arg}$ would be obtained.

## 적 요

*A. nidulans*의 $tRNA^{Arg}$의 염기순서를 효소절단 방법으로 결정하였다. 이 방법으로 염기순서를 결정한 결과 다음과 같았다. 5′GGCCGGCUGGCCCAAXUGGCAAGGCXUCUGAXUACGAAXCAGGAGAUUGCAXXXXXGAGCXXUXXGUCGGUCACCA3′. 위의 결과로 클로버잎 구조를 만들어본 결과 안티코돈이 ACG인 $tRNA^{Arg}$으로 판명되었고, 이 결과는 아미노산 부하검사(charging test)의 결과와 일치하였다. 이 tRNA의 유전자의 염기순서 결과와 비교하여 염기순서의 정확성을 검증하였고, minor base 분석을 통하여 전 염기순서를 추정하였다.

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