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Effect of the higher-order structure of tRNAs on the stability of hybrids with oligodeoxyribonucleotides: separation of tRNA by an efficient solution hybridization

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ABSTRACT

In the course of developing a method to purify a single tRNA species efficiently, we have examined hybridization effciencies between some tRNAs and short oligodeoxyribonucleotide probes both by the filter and solution hybridization methods without denaturants. The hybridization efficiencies varied considerably among probes which are complementary to different regions of the tRNAs, although there was little efficiency variation in the probes toward DNA substrates including the same nucleotide sequence. This efficiency variation was shown to be due to tRNA-specific higher-order structures as well as a hypermodified nucleotide in the anticodon loop. Characterization of the tRNA-probe hybrids by both nondenaturing gel electrophoresis and chemical modification showed the existence of two stable hybridizing states as a function of ionic strength. Our results indicate that RNA molecules with a number of intramolecular base pairings are able to form stable hybrids with complementary sequences under nondenaturing conditions. On the basis of these data, an appropriate probe was designed to successfully purify yeast $tRNA^{Phe}$ by making a $tRNA^{Phe}$–probe hybrid, which has a longer retention time in hydroxyapatite high performance liquid chromatography than the $tRNA^{Phe}$ itself.

INTRODUCTION

Purification of a single tRNA species is important for investigating its structure and function. tRNA purification has been carried out mostly by successive column chromatography using an assay of radioactive amino acid-accepting activity with its cognate aminoacyl-tRNA synthetase [1]. However, effective combination of columns differs from tRNA to tRNA, and thus has to be chosen by trial and error. An alternative one-step isolation method described by Gillam and Tener [2] is for cognate tRNAs to be selectively aminoacylated by synthetases, naphthoxyacethylated and retained on a benzoylated DEAE-cellulose column. This method is generally applicable, but problematic in the quantitative recovery of tRNA, as aminoacylation and derivatization usually do not proceed to completion. In particular, both the methods using the aminoacylation procedure are not suited to purifying scanty quantities of minor isoacceptor tRNAs, such as animal mitochondrial tRNAs, because, in addition to the problem of the small amount of tRNA, aminoacylation assay is unable to discriminate isoacceptor tRNAs and occasionally-contaminating cytosolic tRNAs from one another [3, 4].

Another method is based on the hybridization principle, which should be able to discriminate any tRNA species from others if the tRNA contains a unique sequence. Indeed, tRNA purification has been achieved via hybridization to its gene DNA sequence which was cloned into a plasmid, amplified in *E. coli*, and immobilized to an appropriate support [5, 6]. Nevertheless, there are still several shortcomings hampering the more widespread use of this method: 1) slow kinetics of hybridization to a solid phase, and thus the necessity for a large amount of immobilized DNA to achieve efficient hybridization; 2) conversely, the requirement to limit the DNA amount for active immobilization to a support; 3) non-specific interaction of tRNAs with support materials; 4) deterioration of the tRNA recovery rate when the columns are used repeatedly; and 5) the need to make different immobilized columns for each tRNA species following careful procedures.

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Notwithstanding these difficulties, we consider that tRNA purification via hybridization may be potentially useful and worth improving. In this regard, we have already reported specific and quantitative detection of a single tRNA species by a filter hybridization method, and the purification of some mitochondrial tRNAs by fusing this sensitive assay system and conventional open column chromatography [3, 4]. This system was also used by others in order to quantitatively detect cellular suppressor serine tRNAs in dystrophic mouse muscle and in *E. coli* [7, 8]. However, in the course of applying this procedure to other mitochondrial and nonmitochondrial tRNAs, some oligodeoxyribonucleotide probes were found not to hybridize to the corresponding tRNA species. We have thus studied the hybridization efficiencies of various probes complementary to different portions of tRNAs, and the results are reported in this communication.

We found that higher-order structures of a tRNA critically modulate the hybridization efficiency, warning of the importance of carefully designing complementary probes for RNAs with a number of intramolecular base pairings such as tRNAs, rRNAs and self-cleaving intron RNAs. We discuss the relationship between the higher-order structures and the hybridization efficiency in the case of yeast $tRNA^{Phe}$ whose higher-order structures have been well characterized [9]. Finally, the efficient formation of tRNA-probe hybrids in solution was applied to the development of a new tRNA separation procedure with the aid of hydroxyapatite high performance liquid chromatography (HAP HPLC).

MATERIALS AND METHODS

Materials

Oligodeoxyribonucleotide probes were chemically synthesized by an Applied Biosystems DNA synthesizer model 391, and highly purified by a reversed phase HPLC system. Probes used in the present study are listed in Table 1. Yeast cytosolic $tRNA^{Phe}$ and yeast $tRNA^{Mix}$ were purchased from Boehringer Mannheim. Bovine mitochondrial $tRNA^{Ser}$(AGY) and $tRNA^{Ser}$(UCN) were prepared according to the literature [3]. *In vitro* transcribed RNA having the same nucleotide sequence as bovine mitochondrial $tRNA^{Ser}$(AGY), except for a modified base, was provided through the courtesy of Dr. T.Ueda (Tokyo Institute of Technology). An *E. coli* expression vector, pMK-2, was a gift from Dr. T.Hibino and Mr. S.Misawa (Univ. of Tokyo).

Filter Hybridization

The procedure for filter hybridization was essentially the same as described previously [3, 4]. In the present study, UV irradiation of tRNA spotted on a nylon membrane filter was performed for 2 min. The hybridization temperatures in Table 1 were determined according to work by Wallace *et al.* , which mostly considered the GC content and the chain length of probes [10]. Hybridization efficiency in this study is defined in terms of how much each probe hybridized to a certain amount of tRNA at the above-mentioned hybridization temperature. In experiments comparing the relative hybridization efficiency among probes

	Synthetic Oligodeoxyribonucleotides (5'→3')	a) Hybridization temperature (°C)	b) Calculated Tm (°C)
Yeast cytosolic $tRNA^{Phe}$			
A1	CTGAGCTAAATCCGC	39	78
A2	TCTGGCGCTCTCCC	41	86
A3	CCAGATTTTCAGTCTGG	41	79
A4	ATCGAACACAGGACCT	39	80
A5	TGTGGATCGAACACAG	39	80
A6	TGCGAATTCTGTGGA	39	78
A7	TGCGAATTCTGTGGATCGAACACAGGACCT	—	—
Bovine mitochondrial $tRNA^{Ser}$(AGY)			
B1	TTCTTGCATACTTTTTC	39	71
B2	TAGAATTAGCAGTTCTT	39	67
B3	ATATGGGAGCATAGAA	39	71
B4	CCATACTATTAGATATG	39	61
B5	CGAAAAAGCCATACTA	39	72
Bovine mitochondrial $tRNA^{Ser}$(UCN)			
C1	ATCATAACCTCTATGTC	41	68
C2	TCAAGCCAACATCAT	37	76
C3	TTGGTTTCAAGCCAAC	41	81
C4	AAGGAATCGAACCCC	41	81
C5	TAAGAAAGGAAGGAATCG	45	80

Table 1 Oligodeoxyribonucleotide probes used for the hybridization experiments. In deciding nucleotides complementary to modified residues, tRNA gene sequences were referred to [32]. a) Hybridization temperatures used for the filter hybridization experiments in Fig. 1, as determined by the method of Wallace *et al.* [10]. b) Melting temperatures calculated between the probe DNAs and complementary DNA sequences. Since the nearest neighbor thermodynamic parameters of the DNA-RNA duplex have not yet been reported, we used the parameters of the DNA-DNA duplex under 1M NaCl and pH7 conditions [14, 15], assuming that the initial concentration of each complementary DNA sequence was 1 μM.

complementary to the same tRNA species, special care was taken to ensure equal conditions for each probe. Quantitative comparison of the filter hybridization efficiency was carried out by the Cerenkov method to measure [^{32}P]radioactivity retained on each filter piece excised after hybridization. Only unsaturated spots, where the residual radioactivity was proportional to the concentration of a spotted tRNA, were used for this purpose.

Solution Hybridization

DNA probes were 5′-[^{32}P]labeled with T4 polynucleotide kinase (Takara) and [γ-^{32}P]ATP (Amersham) [11]. The medium for hybrid formation contained 90 mM Tris-HCl (pH 8.0 at 25°C), 6 mM EDTA-NaOH (pH 7.5), 900 mM NaCl, 3.3 μM tRNA^{Phe}, and 20 nM [^{32}P]labeled DNA probe. 5 μl of the medium was combined with the same volume of a dye mixture containing 40% glycerol, 0.01% (w/v) bromephenolblue (BPB) and 0.01% xylene cyanol (XC), and incubated in a small Eppendorf tube (500 μl capacity) at 39°C for 20 min. 2 μl of the reaction mixture was quickly loaded on a 10% polyacrylamide nondenaturing gel. After electrophoresis (100 V constant at 25°C) in 1× TBE buffer containing 50 mM Tris, 50 mM boric acid and 1 mM EDTA (pH 8.3 at 25°C), the gel was dried and then autoradiographed for 6 hours.

Chemical Modification

Yeast tRNA^{Phe} was labeled at the 3′ terminus with [5′-^{32}P]pCp (Amersham) and T4 RNA ligase (Pharmacia) [12], purified by 15% polyacrylamide/7M urea gel electrophoresis, and ethanol-precipitated with glycogen as a carrier. Solution hybridization was carried out at 37°C for 30 min in 100 μl of a reaction mixture containing 45 mM Tris-HCl (pH 8.0), 3 mM EDTA-NaOH (pH 7.5), 0.17 μM labeled tRNA (50,000 cpm by the Cerenkov counting), and 3.4 μM unlabeled DNA probe in the presence or absence of 900 mM NaCl. Chemical modifiers were then added to the reaction mixture at the same temperature. The modification procedure was essentially the same as described by Peattie and Gilbert [13]. Diethylpyrocarbonate (DEPC) and dimethylsulfate (DMS) reactions were carried out at 37°C for 45 min and 10 min, respectively. Samples after strand-scission treatment by aniline were analyzed by 20% polyacrylamide/7M urea gel electrophoresis.

Purification of tRNA by Hybridization

Yeast tRNA^{Mix} was separated by HAP HPLC and each eluted fraction was assayed for the existence of tRNA^{Phe} using the filter hybridization method described above. A fraction containing tRNA^{Phe} (Fraction A of Fig. 8d) was dialyzed against sterile water and lyophilyzed. 100 μl of a hybridization medium containing 45 mM Tris-HCl (pH 8.0), 3 mM EDTA-NaOH (pH 7.5), 22 μM A7 probe and the lyophilized Fraction A, was incubated at 39°C for 40 min and subsequently injected to HAP HPLC under the conditions described in the legend of Fig. 8. A fraction corresponding to tRNA^{Phe}-A7 hybrid (Fraction C of Fig. 8e) was desalted in the same way as above and applied to

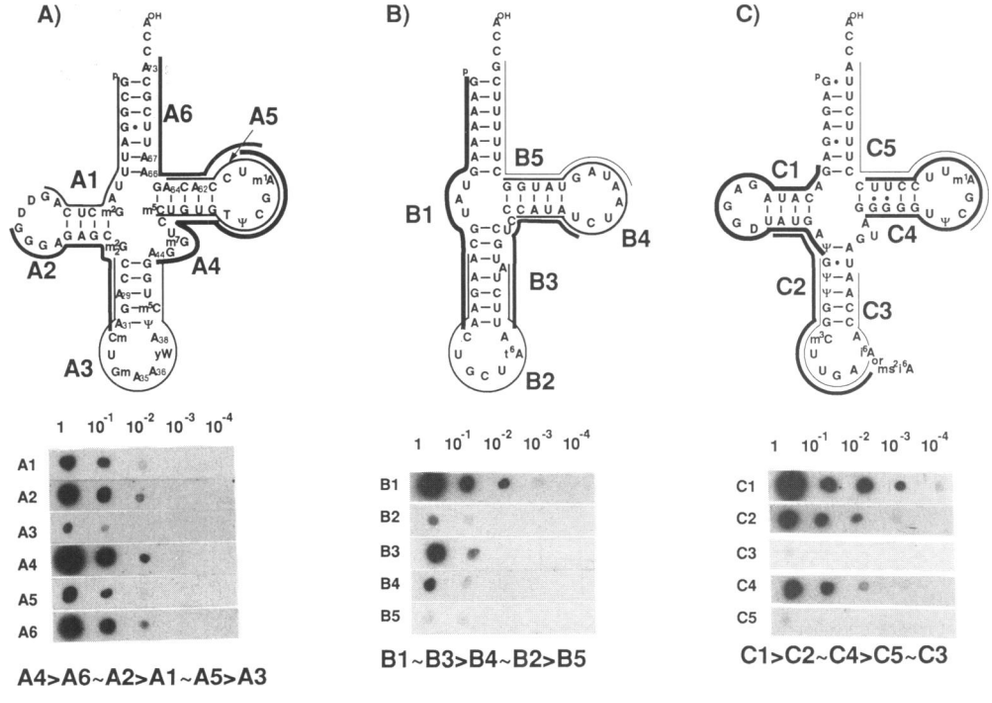

Fig. 1. Efficiency comparison in filter hybridization. Clover-leaf representations of (A) yeast cytosolic tRNA^{Phe}, (B) bovine mitochondrial tRNA^{Ser}(AGY), and (C) bovine mitochondrial tRNA^{Ser}(UCN), showing regions complementary to their hybridization probes listed in Table 1. The tRNA sequences are cited from Sprinzl *et al.* [32] except that for tRNA^{Ser}(UCN)[20]. The secondary structure of tRNASer(AGY) was drawn as proposed by de Bruijn and Klug [21]. The autoradiograms shown below the representations are the results of the efficiency comparison. 1.7 μM tRNA^{Phe}, 2.0 μM tRNA^{Ser}(AGY), and 7.6 μM tRNA^{Ser}(UCN) were diluted with H_2O by 10^1-10^4 times. 1 μl of each diluted solution was spotted on a nylon membrane filter, dried and UV-irradiated for 2 min. Incubation with each [^{32}P]labeled probe was carried out in a separate plastic bag for 12 hrs with a medium containing 90 mM Tris-HCl (pH 8.0), 900 mM NaCl, 6 mM EDTA-NaOH (pH 7.5) and 0.3% sodium dodecyl sulfate [3, 4]. Hybridization and autoradiography of all the spots within (A), (B) and (C) were simultaneously performed using the same materials. The specific activities as well as the final concentrations of individual probes in the hybridization medium were carefully adjusted to be the same. The resultant relative efficiency is indicated in the clover-leaf structures by the thickness of the lines.

a 15% polyacrylamide/7 M urea denaturing gel (10 cm×0.5 mm), where the hybrid was readily dissociated. A single band for yeast $tRNA^{Phe}$ was excised and extracted from the gel.

RESULTS

Efficiency Variation in Filter Hybridization

Fig. 1A shows an efficiency comparison in filter hybridization among six probes (A1−A6) which were designed to be complementary to different portions of yeast $tRNA^{Phe}$. Among the probes, A4 showed the highest efficiency followed by A6, A2, A1, A5 and A3. The efficiencies of A3 and A4 differed by two orders of magnitude. This efficiency variation was reproducible and was not peculiar to yeast $tRNA^{Phe}$. Among five probes complementary to bovine mitochondrial $tRNA^{Ser}(AGY)$, B1 was the most efficient while B5 was over 10^3 times less efficient than B1 (Fig. 1B). Probes for bovine mitochondrial $tRNA^{Ser}(UCN)$ showed a similar efficiency variation to those for the $tRNA^{Ser}(AGY)$ (Fig. 1C); *i.e.* C1 covering the D arm was the most efficient, whereas C5 corresponding to the 3′ quarter of the tRNA was inefficient. In these filter hybridization experiments, crude tRNAs with various degrees of purity gave the same efficiency variation as did the purified tRNA species (data not shown), indicating that each probe hybridized specifically to the corresponding tRNA species even in the presence of other tRNA species. Melting temperatures calculated from parameters for the nearest neighbor thermodynamics of the DNA-DNA duplex [14, 15] were similar among the probes sharing the same tRNA target (Table 1), albeit with some exceptions (A2, B4 and C1). Accordingly, the significant efficiency variation in Fig. 1 appeared not to be explained by the inferred stability of the duplexes. These results addressed questions concerning what major factors are responsible for the efficiency variation, and practically how probe sequences should be designed to attain high hybridization efficiency.

First, it was shown that the efficiency variation is a phenomenon due to tRNA-specific structural characteristics. When probes complementary to mitochondrial $tRNA^{Ser}(AGY)$ were hybridized to a single-stranded tDNA-like fragment and a double-stranded plasmid to which a gene for $tRNA^{Ser}(AGY)$ was inserted (Fig. 2 a-c), the B1-B5 probes showed little efficiency variation toward either of the DNA targets. While the efficiency difference of these probes toward the DNA targets was within one order of magnitude (Figs. 2b and 2c), that toward the $tRNA^{Ser}(AGY)$ was by 3−4 orders of magnitude (Fig. 2e and Fig. 1B). Alkali treatment of these DNA targets prior to spotting did not change the outcome at all (data not shown). These results indicate that the significant difference in efficiency toward $tRNA^{Ser}(AGY)$ was not caused by problems on the probes but on the tRNA. Although the probes differ from each other in base composition and self-complementarity, these factors do not dictate the hybridization efficiency under the conditions we used.

Bovine mitochondrial $tRNA^{Ser}(AGY)$ is known to have only one modified nucleotide, N^6(N-threonylcarbamoyl)adenosine (t^6A), at position 23 [16]. In order to examine the effect of this modification on the hybridization efficiency, *in vitro* transcribed $tRNA^{Ser}(AGY)$ lacking the modified base was used (Fig. 2d). Whereas B1 and B3 hybridized to both the $tRNA^{Ser}(AGY)$ and the transcript with similar efficiencies, B2 showed an approximately 20-fold higher efficiency toward the transcript than toward the $tRNA^{Ser}(AGY)$. The efficiency of B4 was slightly enhanced in the case of the transcript. These results indicated that the existence of the bulky modification at the N^6 position of A_{23}, which should have participated in Watson−Crick hydrogen bonding with the B2 probe, greatly contributed to the inefficiency of the B2 probe.

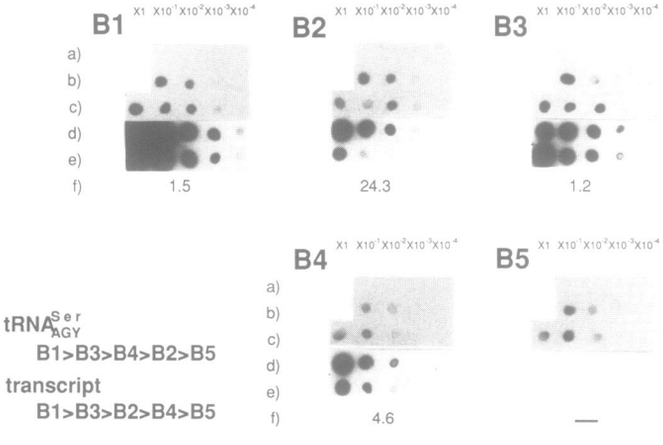

Fig. 2. Filter hybridization of bovine mitochondrial $tRNA^{Ser}(AGY)$ analogues. $tRNA^{Ser}(AGY)$ and its analogues were diluted by 10^1-10^4 times. 1 μl of the diluted samples was spotted on a nylon filter, and hybridized with probes B1-B5 as described in Fig. 1. a) *E. coli* plasmid pMK-2 (1.2 μM) used as a control, b) pMK-2 plasmid to which the $tRNA^{Ser}(AGY)$ gene was inserted (1.1 μM), c) a single-stranded synthetic DNA (68 mer) having the corresponding sequence to $tRNA^{Ser}(AGY)$, except that it extends $^{5'}AATTC^{3'}$ at the 5′ end in order to create an EcoR1 site for its subcloning into pMK-2 (4.5 μM), d) unmodified RNA transcript corresponding to the $tRNA^{Ser}(AGY)$ (2.7 μM), and e) native $tRNA^{Ser}(AGY)$ (3.0 μM). f) The ratio of hybridization efficiency (transcript vs. $tRNA^{Ser}(AGY)$) was quantitated by the Cerenkov method, using only the unsaturated spots where the residual radioactivity correlated with a change in tRNA concentration. The ratio for the B5 probe was not determined because of the low residual radioactivity.

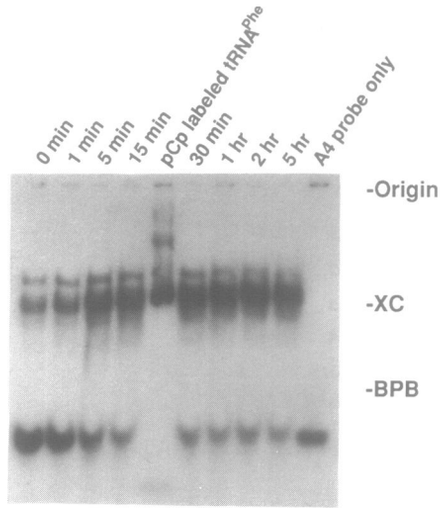

Fig. 3. Time course of hybrid formation in solution. Solution hybridization was carried out using yeast $tRNA^{Phe}$ and [^{32}P]labeled A4 probe in 40 μl of reaction mixture as described in MATERIALS AND METHODS. The incubation tube was occasionally spun down to prevent evaporation. After each interval, 2 μl was withdrawn and immediately frozen with dry ice. After 5 hrs, the frozen aliquots were thawed and analyzed by 10% polyacrylamide gel electrophoresis together with 3′-[^{32}pCp]labeled $tRNA^{Phe}$ and 5′-[^{32}P]labeled A4 probe as size markers.

Another possible cause of efficiency variation is that UV irradiation may preferentially immobilize some regions of tRNAs to a nylon membrane filter, or damage specific nucleotides of tRNAs through making pyrimidine dimers or crosslinking structures. In fact, the hybridization efficiency was found to be quite sensitive to the extent of UV irradiation. An experiment examining the relationship between the irradiation time and the hybridization efficiency showed that the smaller amount of yeast $tRNA^{Phe}$ spotted, the shorter was the irradiation time giving the highest efficiency (data not shown). This indicated that excessive UV irradiation actually results in overattachment of tRNAs to a filter and/or degradation of the tRNAs.

Solution Hybridization

In order to examine whether the UV irradiation was a major factor causing the efficiency variation of Fig. 1, we adopted a solution hybridization procedure which does not need UV irradiation. First, the time course of the hybridization reaction was analyzed using yeast $tRNA^{Phe}$ and its most efficient probe A4 (Fig. 3). Since the [^{32}P]label was introduced only to the probe, radioactive bands with a lower migration than the probe must represent a stable complex between the tRNA and the probe. Fig. 3 shows that the hybridization reaction proceeded quickly in a solution environment, and that the formation of a band with a lower mobility reached a plateau in only 15−30 min. This plateau formation means either that the reaction reached a steady-state equilibrium at the plateau level, or that a small part of the complex was dissociated during the electrophoresis. The data presented in Fig. 6 support the former interpretation, as shown below.

In Fig. 3, two major bands representing $tRNA^{Phe}$-A4 complexes were observed. One of them with a higher migration had almost the same mobility as $tRNA^{Phe}$ itself, indicating that there are two major states of the hybrid between the tRNA and the probe, and that the one with a higher mobility may have a conformation with similar compactness to the $tRNA^{Phe}$ itself. As the concentration of NaCl varied from zero to 600 mM in Fig. 4, the higher ionic strength shifted the hybridized band from the one with a lower mobility to the other with a higher mobility. When no NaCl was added, the complex was almost exclusively the former, whereas the latter band was predominant at the high NaCl concentration.

As shown in Fig. 4, the hybridization reaction in the 1×TBE buffer was inefficient enough to discount the possibility that the hybridization proceeded during the period of loading samples and/or electrophoresis. It is known that the hybridization reaction rate is generally enhanced by monovalent cations [17]. The lack of monovalent cations thus seems to be a major reason why the hybrid was not formed efficiently in the 1×TBE buffer. On the other hand, the medium for the solution hybridization contained 6 mM EDTA whose pH had been preadjusted to be 7.5 with NaOH, giving rise to a final 20 mM Na^+ in the hybridization medium, even if no exogenous Na^+ was supplied.

Efficiency Comparison in Solution Hybridization

Fig. 5 shows the result of solution hybridization using yeast $tRNA^{Phe}$ and its probes A1-A6. A2, A4 and A6 were found to form stable hybrids with the $tRNA^{Phe}$, whereas no hybridized form was detected with A1, A3 and A5. Incubation for a shorter period (30 min) gave essentially the same results (data not shown). The hybrids with A2, A4 and A6 consisted of two bands, as seen in Fig. 3. The order of efficiency in solution (A4>A2>A6>>A1,A3,A5) was very consistent with that in the filter method (Fig. 1A). The same experiments using bovine mitochondrial $tRNA^{Ser}$(AGY) and its probes also confirmed that the order of hybridization efficiency is consistent between the solution and filter methods (data not shown). These observations indicate that neither the manner of tRNA attachment to a filter nor the damage of tRNA by UV irradiation is a major cause of the efficiency variation in the filter hybridization, which is now likely to reflect how efficiently each probe makes a hybrid with a tRNA taking a nearly native conformation in an aqueous solution on the basis of a fact that yeast $tRNA^{Phe}$ preserves a native-like higher-order structure under our hybridization condition with no Mg^{++} but high Na^+ [18,19]. In order to quantitate the stability of the hybrid between yeast $tRNA^{Phe}$ and each probe, the following formula was assumed;

$$\text{tRNA} + \text{probe} \rightleftharpoons \text{tRNA} - \text{probe}$$

If this reaction reaches a steady state equilibrium,

$$[\text{tRNA-probe}] / [\text{tRNA}][\text{probe}] = K \text{ (constant)}.$$

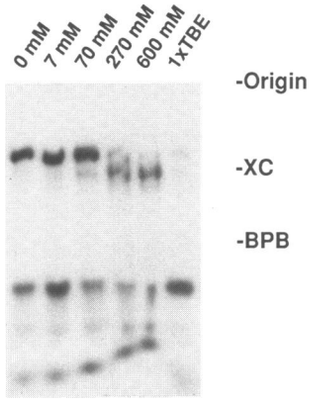

Fig. 4. Effect of Na^+ concentration on solution hybridization. Solution hybridization using yeast $tRNA^{Phe}$ and [^{32}P]labeled A4 probe was carried out at 39°C for 20 min by adding the indicated concentrations of NaCl. In the far right lane, a sample in which the hybridization medium was substituted by 1×TBE, was also incubated and loaded. 0 mM NaCl contained 20 mM Na^+, as described in the text.

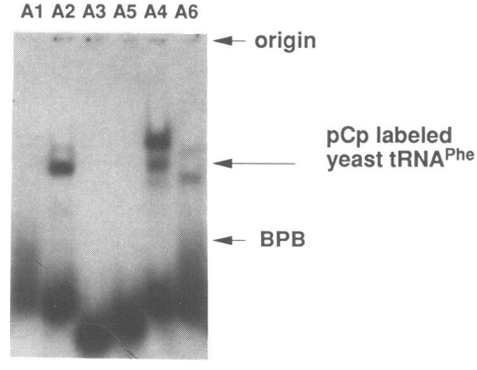

Fig. 5. Efficiency comparison in solution hybridization. Solution hybridization using yeast $tRNA^{Phe}$ and each of the probes A1−A6 was carried out at 39°C for 6 hrs. Each probe was labeled with the same specific activity and used at the same concentration.

Fig. 6 examines doses of $tRNA^{Phe}$ and A4 probe in hybrid formation under low Na^+ conditions. In Fig. 6A, where A4 was [^{32}P]labeled, the increasing concentration of $tRNA^{Phe}$ encouraged the incorporation of free A4 probe into the $tRNA^{Phe}$-A4 complex. When [A4] appeared to be almost the same as [$tRNA^{Phe}$-A4], [$tRNA^{Phe}$] was estimated to be 450−700 nM, where [$tRNA^{Phe}$] was able to be approximated to the initial concentration of $tRNA^{Phe}$:

$$K = 1 / [tRNA^{Phe}] = 1.8 \pm 0.4\ [\mu M^{-1}].$$

In Fig. 6B, where $tRNA^{Phe}$ was [^{32}P]labeled, the addition of the A4 probe gradually shifted the radioactive band representing $tRNA^{Phe}$ itself to that representing the hybrid. When [$tRNA^{Phe}$] became approximately the same as [$tRNA^{Phe}$-A4], [A4] was 300−400 nM:

$$K = 1 / [A4] = 2.9 \pm 0.4\ [\mu M^{-1}].$$

The K values from Figs. 6A and 6B were not very far from each other. The K values, which were determined from different initial concentrations of A4 probe by quantitating the radioactivity of each band in the same experiments as Fig. 6B, coincided with each other within 25% difference (data not shown). These results support a nearly steady state equilibrium under our conditions for hybridization (39°C, 20 min). The observation that almost all the labeled probes were incorporated into the hybrid in the presence of a large amount of $tRNA^{Phe}$ (Fig. 6A) means that dissociation of the hybrid during electrophoresis was negligible.

When [^{32}P]labeled $tRNA^{Phe}$ was mixed with increasing concentrations of A3 or A5 probe, as in Fig. 6B, hybrids between the $tRNA^{Phe}$ and these probes were finally formed, but the K values were found to be two orders of magnitude lower than that for the A4 probe (data not shown). This showed that the $tRNA^{Phe}$-A4 hybrid was much more stable than the $tRNA^{Phe}$-A3 and the $tRNA^{Phe}$-A5 ones.

Characterization of the Hybridized State

In order to see how higher-order structures of $tRNA^{Phe}$ were altered by providing a complementary region to each probe, hybrids were characterized by chemical modification [13]. Fig. 7A shows modification patterns of yeast $tRNA^{Phe}$-probe hybrids by DEPC, which monitors the susceptibility of the N^7 position as well as the stacking state of adenine bases [13]. First, the modification pattern of the tRNA itself was distinct between the low and high Na^+ concentrations (lanes 4 and 9, respectively). Although A_{35}, A_{36} and A_{38} in the anticodon loop, which are not involved in base pairings, showed a similar intensity of modification between the two lanes, A_{44}, A_{62}, A_{64}, A_{66}, A_{67} and A_{73} were modified more intensely at the low than at the high Na^+ concentration. These observations indicate that the

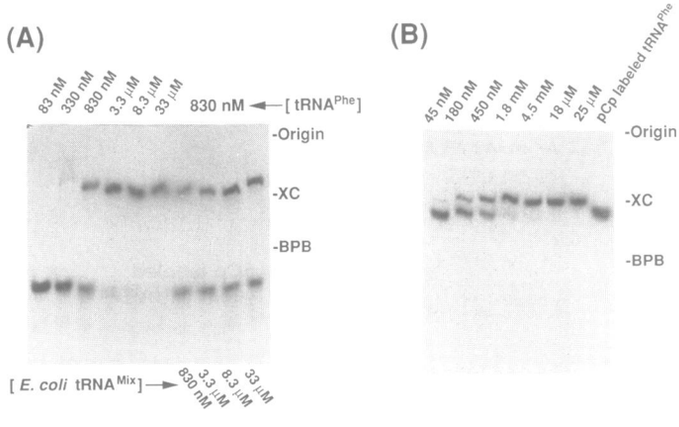

Fig. 6. Variation of (A) yeast $tRNA^{Phe}$ and (B) A4 probe doses in solution hybridization. (A) Variation of $tRNA^{Phe}$ concentration against 5 nM 5′-[^{32}P]labeled A4 probe. On the right 4 lanes, inhibition of the hybrid formation by *E. coli* $tRNA^{Mix}$ was examined with 830 nM $tRNA^{Phe}$. (B) Variation of the A4 probe concentration against 13 nM 3′-[^{32}pCp]labeled $tRNA^{Phe}$. Since the hybrid had the same migration as $tRNA^{Phe}$ itself at high Na^+ concentrations (refer to Fig. 3 and Fig. 4), the experiments of (A) and (B) were carried out without adding NaCl. Incubation was carried out at 39°C for 30 min.

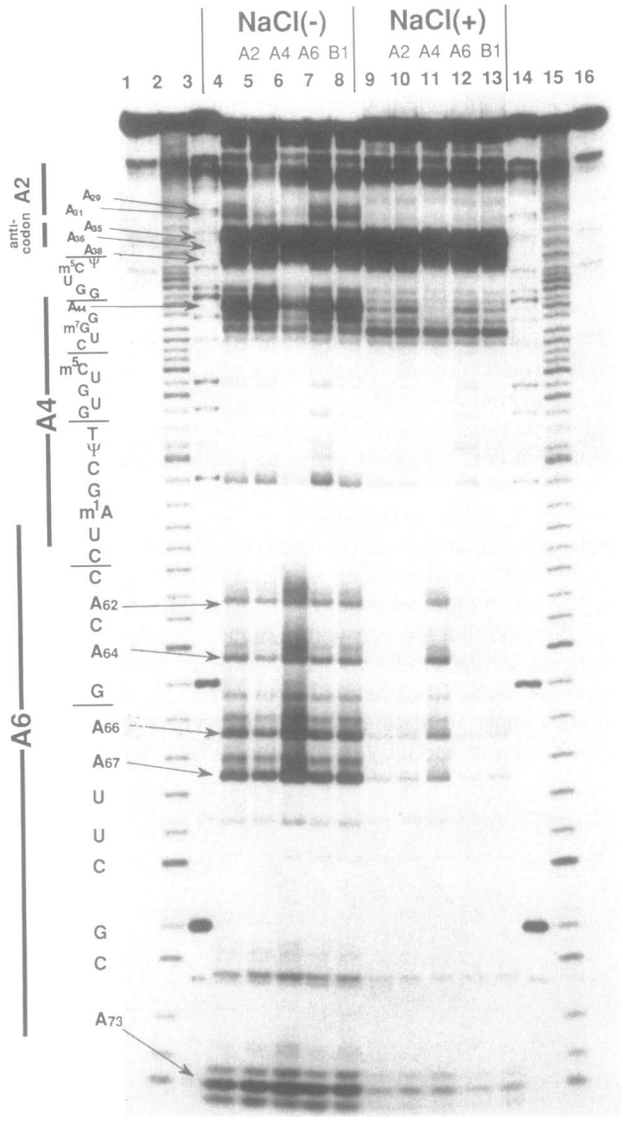

Fig. 7. Chemical modification of yeast $tRNA^{Phe}$ hybridized with its probes. Chemical modification by DEPC, followed by subsequent strand-scission was carried out as described by Peattie and Gilbert [13]. Samples in lanes 4−8 and in lanes 9−13 were hybridized and modified in the presence of 0 mM (however, 20 mM Na^+, see text) and 900 mM NaCl, respectively. Lanes 4 and 9, with no probe; lanes 5 and 10, with A2 probe; lanes 6 and 11, with A4 probe; lanes 7 and 12, with A6 probe; and lanes 8 and 13, with B1 probe as controls. Lanes 1 and 16, controls with no modifier; lanes 2 and 15, alkali ladder; and lanes 3 and 14, RNase T1 ladder. Since $tRNA^{Phe}$ was labeled at the 3′ terminus with [5′-^{32}P]pCp, nonspecific cleavage at this cytidine caused weak extra bands in this figure.

higher-order structures of $tRNA^{Phe}$, including the stacking state of bases in the stem regions, were considerably loosened at the low Na^+ concentration.

When the modification was carried out after $tRNA^{Phe}$ was hybridized with A2, A4 and A6 probes, the modification patterns became different from that of the $tRNA^{Phe}$ itself, whereas the control B1 probe did not affect the modification pattern at all (lane 13). In lane 11 at the high Na^+ concentration, the A4 probe complementary from A_{44} to U_{59} weakened the modification at A_{44} but enhanced those at A_{62}, A_{64}, A_{66}, and A_{67}. This suggested that the probe hybridized accurately to the corresponding region of $tRNA^{Phe}$, disrupting preexisting base pairs and stacking structures of the T stem, and making A_{62} and A_{64} more susceptible to DEPC. Since the acceptor and T stems have been shown to form a successive helical structure by crystallographic studies [9], the disruption of the T stem is likely to have affected the stacking state of A_{66} and A_{67}. On the contrary, A_{29} and A_{31} in the anticodon stem were still not modified in the hybrid (lane 11). The stem structure in the anticodon arm thus seems to be taken, even though large-scale structural alteration is brought to the T arm of the $tRNA^{Phe}$ by hybridizing to the A4 probe.

In the hybrid with A2, modification at A_{44} was slightly enhanced (lane 10). A_{44} is known not only to have a base pair with $m^2 2G_{26}$ (the N^7 position of A_{44} is not involved in the base pair with $m^2 2G_{26}$.), but also to be stacked on the first base pair in the anticodon stem [9]. The enhanced modification at A_{44} therefore means the disruption of this stacking structure. On the other hand, A_{62}, A_{64}, A_{66} and A_{67} were not significantly affected. The acceptor and T stems thus seem to be intact in the hybrid with A2. Similarly, in the hybrid with A6, the anticodon stem appeared to be held, the stacking state at A_{44} being disturbed (lane 12).

The modification patterns of the hybrids at the low Na^+ concentration (lanes 4–8) were mostly similar to those at the high Na^+ concentration. A_{62}, A_{64}, A_{66} and A_{67} became more intensely modified in the hybrid with A4 (lane 4 and 6). The acceptor and T stems appeared to be intact in the hybrid with A2 (lane 5), as was the case in lane 10. Since the stacking structure at A_{44} has already been disrupted in lane 4, it seems reasonable that modifications in this position were not significantly enhanced in the hybrids with A2 and A6 (lanes 5 and 7). The fact that modifications at A_{62}, A_{64}, A_{66} and A_{67} did not change between lanes 4 and 7 as well as between 9 and 12, meant that the stacking states of these adenosines were similar in the acceptor and T stems of $tRNA^{Phe}$ and in the hybrid with A6. A striking difference in the modification pattern of the hybrids by the Na^+ concentration was only the intensity of modification. Modifications of the hybrids were generally weaker at the high than at the low ionic strength, although adenosines in the anticodon loop were modified with a similar intensity. Chemical modifications using DMS-hydrazine monitoring N^3 of cytidines and DMS-sodium borohydride monitoring N^7 of guanosines were also carried out as described by Peattie and

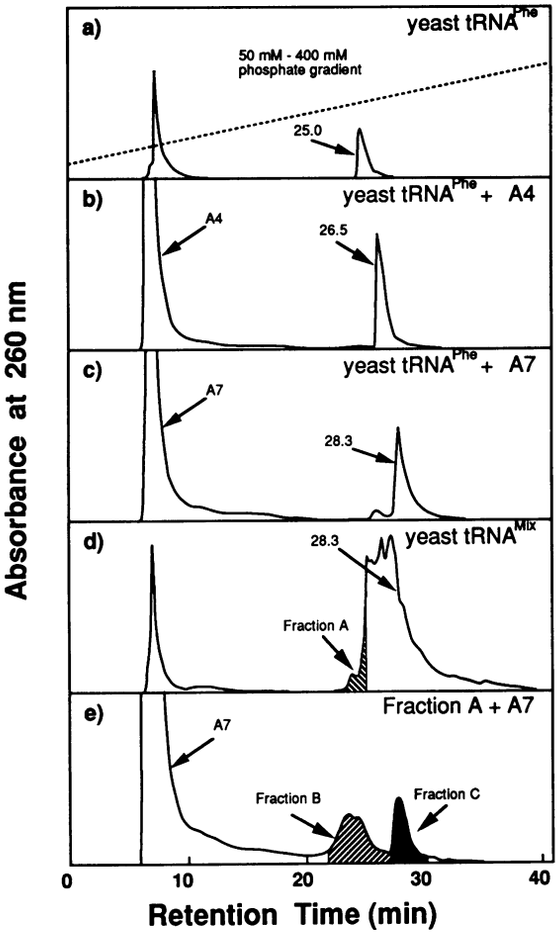

Fig. 8. Purification of yeast $tRNA^{Phe}$ by the HAP HPLC. a)–c): Small-scale analyses of $tRNA^{Phe}$-probe hybrids; a) yeast $tRNA^{Phe}$ alone, b) $tRNA^{Phe}$ and A4 probe, and c) $tRNA^{Phe}$ and A7 probe. Solution hybridization was carried out at 39°C for 30 min in 10 μl of a reaction mixture containing 45 mM Tris-HCl (pH 8.0), 3 mM EDTA-NaOH (pH 7.5), 1.7 μM yeast $tRNA^{Phe}$ and 50 μM of a DNA probe. 5 μl of the reaction mixture was quickly injected to a Shimadzu HPLC system model LC-6A using a HAP column TAPS-020810, (7.5 mm×100 mm) (Tonen). Elution was performed at a constant flow rate of 0.5 ml/min at 25°C by sodium phosphate linear gradient at pH 7.0 (50 mM–400 mM in 40 min). d)–e): Large-scale purification of yeast $tRNA^{Phe}$ by hybrid formation with the A7 probe; d) yeast $tRNA^{Mix}$ alone, and e) Fraction A hybridized with the A7 probe. The hybridization reaction using Fraction A and A7 is described in MATERIALS AND METHODS.

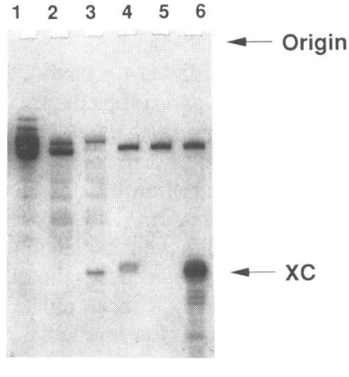

Fig. 9. Analysis of the HAP-purified yeast $tRNA^{Phe}$. Fractions shown in **Fig. 8** were applied to 15% polyacrylamide-7M urea gel electrophoresis using 1×TBE buffer, and stained with toluidineblue: 1) yeast $tRNA^{Mix}$ (2.5 μg), 2) Fraction A (0.8 μg), 3) Fraction B (0.5 μg), 4) Fraction C (0.3 μg), 5) purchased yeast $tRNA^{Phe}$ (0.3 μg), and 6) hybrid formed between $tRNA^{Phe}$ and A7 (5 μl of the reaction mixture for solution hybridization without adding NaCl at 39°C for 20 min).

Gilbert [13], and supported the conclusions from the DEPC modification (data not shown).

Purification of Yeast tRNA^{Phe} by hybridization

Fig. 6A showed that addition of *E. coli* tRNA^{Mix} did not inhibit the tRNA^{Phe}-A4 hybrid formation, and that the solution hybridization is highly specific and efficient enough to be applied to the separation of a single tRNA species from an excess of other tRNA species. For this purpose a HAP HPLC system was used, because nucleic acids with a greater content of double-stranded region are known to be eluted the more slowly on a HAP column [17].

The tRNA^{Phe}-A4 hybrid was actually found to be eluted more slowly than the tRNA^{Phe} itself (Fig. 8a and 8b). Dissociation of the complex during HPLC was very little. In order to shift the peak for the hybrid more to the right, we designed a longer probe (A7) which overlapped two of the most efficient probes, A4 and A6 (Table 1). It was found that A7 formed a hybrid with the tRNA^{Phe} even more stably than A4; *i.e.* the K value for A7 was 5 times that for A4 at 39°C (data not shown). The tRNA^{Phe}-A7 hybrid, in fact, showed a longer retention time on HAP HPLC than tRNA^{Phe}-A4 one (Fig. 8c).

For the actual purification of tRNA^{Phe} from yeast tRNA^{Mix}, 500 μg of yeast tRNA^{Mix} was applied to the HAP column, from which 11 μg of Fraction A containing tRNA^{Phe} was collected (Fig. 8d). Although this fraction still contained other tRNA species, as shown in Fig. 9, tRNA^{Phe} in this fraction was specifically incorporated into the tRNA^{Phe}-A7 hybrid to make a discrete peak as Fraction C of Fig. 8e. Analysis of this peak in Fig. 9 confirmed the apparent homogeneity of the purified tRNA^{Phe} (lane 4), and no tRNA^{Phe} was observed in Fraction B (lane 3). Some parts of the unused probe tailed in HPLC and contaminated Fraction B (Fig. 8e), but the probe was mostly recovered from the initial large peak of Fig. 8e for recycled use. The urea-containing gel of Fig. 9 did not allow comigration of tRNA^{Phe} with the probes (lane 6), and thus became a convenient system for separating the tRNA^{Phe} from the tRNA^{Phe}-A7 hybrid. 2 μg of yeast tRNA^{Phe} was finally recovered.

DISCUSSION

The present study showed distinctive hybridization efficiency among DNA probes toward the same tRNA targets, both by the filter and solution hybridization methods. The melting temperatures of these probes and complementary DNA sequences were inferred to be similar in most cases (Table 1), which was consistent with the observation that the B1−B5 probes did not show as much efficiency variation toward DNA substances as toward tRNA^{Ser}(AGY) (Fig. 2). The experiment shown in Fig. 2 identified a hypermodified nucleotide in the anticodon loop (t^6A) as one of the major factors causing the efficiency variation among the B1−B5 probes. Since most tRNAs possess hypermodified nucleotides at the immediate 3′ side of the anticodon triplet, it seems reasonable that probes covering the anticodon loop were commonly inefficient toward all the three tRNAs tested (A3, B2 and C3 in Fig. 1). It is also possible that other modified nucleotides in the mitochondrial tRNA^{Ser}(UCN) and yeast tRNA^{Phe} influenced the variation. Nevertheless, higher-order structures specific to tRNAs were crucial for the efficiency variation, because the variation was still observed toward the transcript (Fig. 2).

A straightforward interpretation of this is that the stability of the hybrids (the K value) depends mostly on the balance of free energy between that to be decreased by hybrid formation and that to be increased by disrupting the higher-order structures of tRNAs. In this respect, it is noteworthy that the chemical modification detected stable structures remaining in such parts of tRNA^{Phe} that were not directly interacting with its probes (Fig. 7). Therefore, it is logical to design complementary probes which destroy as few hydrogen bonds of a tRNA as possible. When only the base pairs of stem regions are concerned in a simplification, A1 appears to gain 2 G-C pairs (2 A-T pairs) by hybridizing with tRNA^{Phe}; A2, 4 G-C (1 A-T) pairs; A3, 5 G-C (7 A-T) pairs; A4, 5 G-C (6 A-T) pairs; A5, 5 G-C (6 A-T) pairs; and A6, 1 G-C (2 A-T) pairs. These figures simply imply that the stability of the hybrids is as follows; A3 ~ A4 ~ A5 > A2 > A1 ~ A6. However, experimental results showed that the hybrids with A3 and A5 probes were much more unstable than expected (Fig. 1A and Fig. 5), indicating the existence of another factor crucial to hybridization efficiency.

One idea for this factor is that characteristic higher-order structures in tRNAs differentiate the rate of effective nucleations between probes and complementary regions of the tRNAs. Under the hybridization condition with no Mg^{2+} but high Na^+, yeast tRNA^{Phe} preserves a native-like higher-order structure [18, 19]. According to the crystal structure of yeast tRNA^{Phe} [9], coaxial helical structures are taken between the acceptor and T stems, as well as between the D and anticodon stems. A6 and A2 probes, complementary to these extended helices, respectively, seem to access the targeted regions without radically reshaping the L-shaped higher-order structures. In contrast, regions complementary to A3 and A5 probes are sharply turned in the middle. This non-linear conformation of the targeted sequences would decrease the chance of effective nucleations with these probes, so that a decreased velocity of hybrid formation reaction as compared to that of hybrid dissociation reaction should lead to lowered K values.

Animal mitochondrial tRNA^{Ser}s belong to the most structurally deviated tRNAs that have been sequenced so far. These tRNA^{Ser}s have longer anticodon stems than the usual 5 base pairs. tRNA^{Ser}(AGY) lacks the entire D arm, while tRNA^{Ser}(UCN) has been shown to have only one nucleotide spacer at the junction of the acceptor-D stems [20]. Although de Bruijn and Klug [21] have proposed a structural model for the tRNA^{Ser}(AGY), very little has been elucidated about their higher-order structures as compared to yeast tRNA^{Phe}. The apparently dissimilar tRNA^{Ser} isoacceptors have to share unknown structural features which are recognized by a common aminoacyl-tRNA synthetase. Thermal melting experiments demonstrated that both the mitochondrial tRNA^{Ser}s had significantly weak higher-order structures [3]. Even at our hybridization temperature (37°-45°C), in the absence of Mg^{2+} the tRNA^{Ser}s were 20−40% melted [3]. Since some *E. coli* tRNAs were shown to melt first from the D stem [22], the high efficiency of the C1 probe may reflect a common structural weakness in the D arm region of the mitochondrial tRNA^{Ser} isoacceptors.

The largest difference in hybridization efficiency was observed between the probes complementary to the 5′ side and the 3′ side of tRNA^{Ser}s (Figs. 1B and 1C). When the corresponding sequences were examined, a characteristic stretch of purine-pyrimidine pairs was found in the acceptor and anticodon stems of tRNA^{Ser}(AGY) and in the acceptor and T stems of

$tRNA^{Ser}$(UCN). It is widely known that sequences of homopurine and homopyrimidine take a triple helix with a 1 : 2 molar ratio, where the third pyrimidine strand parallels the purine strand by Hoogsteen base pairs [23–25]. Therefore, it seems possible that the B1 probe first interacts with the acceptor and anticodon stems of $tRNA^{Ser}$(AGY) by taking advantage of the free N^7 position of purines in the stems, partially destabilizes the higher-order structures of the tRNA, and facilitates the effective nucleations of another probe molecule with the target. This interpretation was supported by preliminary experiments showing that *E. coli* $tRNA^{Ser}$(AGY) also inefficiently hybridized with a probe complementary to a similar pyrimidine stretch at the 3′ terminus, but that bovine cytosolic $tRNA^{Ser}$(UCN) and suppressor $tRNA^{Ser}$s with more purine content at the same 3′ side showed much improved efficiencies with the corresponding probes (data not shown).

This study has shown that the complementary region of probes with the highest efficiency differs from tRNA to tRNA. How, then, should hybridization probes for other tRNAs be designed? Our findings provide the following suggestions: (a) Probes covering the anticodon arm should be avoided. Hayase *et al.* have recently reported a novel method to site-specifically cleave *E. coli* formylmethionyl tRNA which was hybridized with complementary 2′-*O*-methyl (partially 2′-deoxy) oligodeoxyribonucleotides by RNase H [26]. Their observation that the cleavage by probes covering the anticodon arm was very inefficient, although this tRNA exceptionally has no modification in base moiety of the anticodon loop, is consistent with our conclusion. (b) It is desirable to avoid regions containing any modification that impairs the Watson–Crick hydrogen bondings. Otherwise, it seems better not to include it in the central part of the probe sequence, because mismatch pairs in the central part of DNA-DNA duplexes were shown to reduce the duplex stability more than those in the terminal part [10, 27]. (c) Probes should not be designed toward a long pyrimidine stretch in stem regions as well as toward a whole stem-loop structure, which may decrease the chance of effective nucleations. (d) If even information about modification is not available, probes spanning two different arms of a tRNA, such as A2, A4 and A6 in Fig. 1, should be tested. All the other tRNAs that we have tested so far (bovine mitochondrial $tRNA^{Phe}$, $tRNA^{Leu}$(UUR) and $tRNA^{Met}$, bovine cytosolic $tRNA^{Phe}$ and *E.coli* $tRNA^{Leu}$(UUR)) were successfully detected by such probes (data not shown).

It was an unexpected finding that two discrete states of hybrids were observed in response to the Na^+ concentration (Fig. 4). According to a number of previous studies on the effects of ionic conditions on tRNA structures (reviewed in [19, 28]), lowering the ionic concentration leads to a transition of the native tRNA conformation to a semi-stable one, named as an 'extended' form, although its precise structure is unknown. This is presumably because the electrostatic shielding between phosphates is reduced under low ionic conditions, resulting in an overall structural expansion of tRNA in order to reduce free energy [19, 28]. This is consistent with our chemical modification data in that higher-order structures of both $tRNA^{Phe}$ itself and the hybrids were considerably loosened at the low Na^+ concentration (Fig. 7). Therefore, it is likely that the hybrid formed at low ionic strength and run slowly in native gel electrophoresis (Fig. 4) represents the expanded structure of the hybrid.

Finally, we applied the efficiency comparison data to the purification of a tRNA. We employed the A7 probe, which was the composit of A4, A5 and A6 probes. Although the $tRNA^{Phe}$-A7 hybrid became more stable than the $tRNA^{Phe}$-A4 one, employing further long probes would not necessarily be the better from a practical standpoint; *i.e.* it would be more difficult to separate these longer probes from tRNA fractions by the HAP HPLC, owing to the self-complementarity of the probes. To the best of our knowledge, this method of tRNA separation using efficient solution hybridization is unique in that a complementary probe is not immobilized to a solid phase. Solution hybridization has overcome the problems of the previous method using a double-stranded DNA-immobilized column [5, 6] (refer to INTRODUCTION). Hybrid formation in our procedure was quick, specific and quantitative in the solution environment. Probes were able to be recovered and reused. Considering the present capacity of the HAP HPLC column, this technique will be useful for purifying small amounts of tRNAs whose gene sequence has been revealed. In the meantime, since some researchers have recently reported technical advances in specifically and quantitatively immobilizing short single-stranded DNAs to a solid support at a terminal residue [29–31], we do not deny that some of the previous problems experienced in separating tRNAs via hybridization may be solved by the immobilized DNA method. Rather, our data showing hybridization efficiency variation will be useful as a direction indicator for designing probes in such an approach, too. Since recent technical advances in DNA sequencing are leading to the accumulation of tRNA gene sequences as a data base [32], tRNA purification via hybridization should become more important than ever.

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