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Purification of hyperexpressed *Bacillus subtilis* $tRNA^{Trp}$ cloned in *Escherichia coli*

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ABSTRACT

To study the effects of evolutionary sequence changes on the molecular interactions of tRNA inside the cell, the *Bacillus subtilis* $tRNA^{Trp}$ gene has been cloned into *Escherichia coli* JM109 under the control of the *lac* promotor. Hyperexpression of the gene in minimal medium upon induction yielded 28% of total tRNA in the form of *B. subtilis* $tRNA^{Trp}$. The $tRNA^{Trp}$ gene product was purified by the use of a single Vydac C_4 high-performance liquid chromatography (HPLC) matrix. This experimental system provided a valuable system for the hyperexpression and purification of a heterologous tRNA for studies *in vitro*. Moreover, because HPLC fractionation of the heterologous $tRNA^{Trp}$ gene product yielded multiple peaks, the system made possible an analysis of the molecular mechanisms for the transcriptional modifications of the $tRNA^{Trp}$ gene product *in vivo*.

INTRODUCTION

The accurate incorporation of amino acids into proteins is made possible by the high fidelity of aminoacyl-tRNA synthetases in their recognition of cognate tRNA and amino acid substrates. The mechanisms by which the enzymes discriminate between structurally similar tRNA molecules to ensure the correct translation of genetic information are only beginning to be defined. An important experimental requirement in this regard has been the investigation of variant tRNA molecules mutagenized at defined sites. Although *in vitro* transcription using *T7* RNA polymerase can be used to generate such molecules [1], the method yields only tRNAs lacking in all nucleotide modifications and cannot yield insight into the behaviour of variant tRNAs *in vivo*. The alternative is to clone the variant tRNA genes into a homologous or a heterologous host [2–4]. Moreover, when a heterologous host with an appropriate phylogenetic distance is employed, such that the cloned tRNAs display substantial sequence dissimilarity from the host tRNAs without abolishing all interactions with host enzymes, the system will make possible an investigation of the *in vivo* effects of evolutionary change on tRNA interactions with aminoacyl-tRNA synthetases, as well as processing and modifying enzymes.

Because the Trp content of cellular proteins is low, the endogenous $tRNA^{Trp}$ level is low in cells [5]. It is therefore particularly difficult to purify. Previously, Vold [6] and Vold and Green [7] have succeeded in expressing a number of *B. subtilis* tRNAs in *Escherichia coli*. However, the expres-

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sion level of the cloned tRNAs was largely comparable to that of their host tRNA counterparts, rendering it difficult to overcome the problem posed by the poor expression of such tRNA as Trp acceptors. Accordingly, in the present study conditions are sought that enable the hyperexpression of *B. subtilis* $tRNA^{Trp}$, as well as its HPLC purification in order to facilitate the preparation of large quantities of $tRNA^{Trp}$, which is otherwise difficult to obtain on account of its low endogenous levels in most organisms. This approach utilising heterologous hyperexpression coupled with HPLC purification might also be applicable to the investigation of other tRNAs with a low level of endogenous expression.

EXPERIMENTAL

Plasmids and strains

The pGEM-9Zf(−) vector supplied by Promega (Madison, WI, USA) contained an inducible *lac* promoter as well as *T7* and *SP6* promoters. Insertion of a synthetic *B. subtilis* $tRNA^{Trp}$ gene bearing the $tRNA^{Trp}$ gene sequence as determined within the *trrnD* gene cluster [8] and a *T7* promoter sequence between the *Sfi*I and *Hind*III sites of the vector yielded the recombinant pWWT plasmid [9], where the synthetic gene was placed under the control of two promoters, the inducible *lac* promoter on the vector for *in vivo* expression and the *T7* promoter for *in vitro* run-off transcription.

E. coli strain JM109 [10] served as host cell for plasmid transformation and expression. The growth media employed included the enriched 2YT medium (16 g Bacto-tryptone, 10 g Bacto-yeast extract and 5 g NaCl per litre) and the M9-glycerol minimal medium (6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, 2 mmol $MgSO_4$, 0.1 mmol $CaCl_2$, 10 μmol $FeCl_3$ per litre, with 1% glycerol and 0.01% thiamine). These media were supplemented with 100 μg/ml ampicillin. Frozen *B. subtilis* cell paste was obtained from Sigma.

Induction and extraction of $tRNA^{Trp}$

JM109 cells transformed by pWWT were grown in either 2YT or M9-glycerol medium. For the 2YT medium, they were grown to an absorbance of 0.3 at 600 nm, at which point the inducer isopropylthiogalactoside (IPTG) was added to 1 m*M*. After 2 h, cells were harvested by centrifugation. For the M9-glycerol medium, cells were grown to an absorbance of 0.15 before addition of IPTG, and harvested 3 h later.

Isolation of total tRNA

To extract tRNA from freshly harvested cells, the cells were suspended in TM buffer (0.01 *M* magnesium acetate, 0.01 *M* Tris–acetate, pH 6.0), 1 g/ml. An equal volume of ice-cold phenol saturated with TM buffer was added, and the mixture was shaken for 45 min at 4°C prior to centrifugation at 27 000 *g* for 30 min. NaCl was added to the upper phase to 2 *M* in order to precipitate the ribosomal RNA, and the mixture was swirled and kept in cold for 30 min and again centrifuged at 27 000 *g* for 30 min. The supernatant was mixed with 2.5 volumes of cold ethanol and left at −20°C for several hours. The precipitate containing mainly tRNA was collected by centrifugation at 27 000 *g* for 30 min. The tRNA was further purified by adsorption on a DE52 ion-exchange column at 0.15 *M* NaCl, pH 6.0, and elution at 0.65 *M* NaCl. For small preparations, a prepacked Sep-Pak Plus C_{18} cartridge was employed instead of DE52, and the tRNA, loaded in water, was eluted by 20% methanol.

Transfer RNA fractionation by HPLC

Separation of tRNAs was achieved by modifications of the HPLC method previously described [11]. Vydac C_4-derivatized silica columns were obtained from the Separations Group (Hesperia, CA, USA) with a particle size of 10 or 5 μm. A column of 250 mm × 4 mm I.D. was employed for a 0.1–1 mg tRNA sample, and one of 250 mm × 10 mm I.D. for a 1–5 mg tRNA sample. Fractions accepting Trp were identified by charging with [^{3}H]tryptophan (from Amersham) and purified TrpRS from *B. subtilis*. Both charging [12] and TrpRS purification [13–15] were performed as described.

Mode I. To separate the multiple species of

tRNA in the cell extract by HPLC, the sample was loaded in loading buffer (10 m*M* sodium phosphate, pH 5.5, 3 *M* sodium formate, 8 m*M* $MgCl_2$) and eluted at 22°C with a 60-min linear gradient (curve 6 of Waters 720 System) from buffer A (10 m*M* sodium phosphate, pH 5.5, 1 *M* sodium formate, 8 m*M* $MgCl_2$) to buffer B (10 m*M* sodium phosphate, pH 5.5, 10% methanol), followed by isocratic elution with 100% buffer B for 20 min.

Mode II. To improve separation of the expressed $tRNA^{Trp}$ peaks that emerged in the latter half of the gradient from surrounding peaks, especially in the case of the 5-μm column on which the retention times of tRNAs were more prolonged, the linear gradient employed in mode I was replaced by a convex gradient (curve 5 of Waters 720 system).

Mode III. To further spread out the peaks on the chromatogram, the curve 5 convex gradient

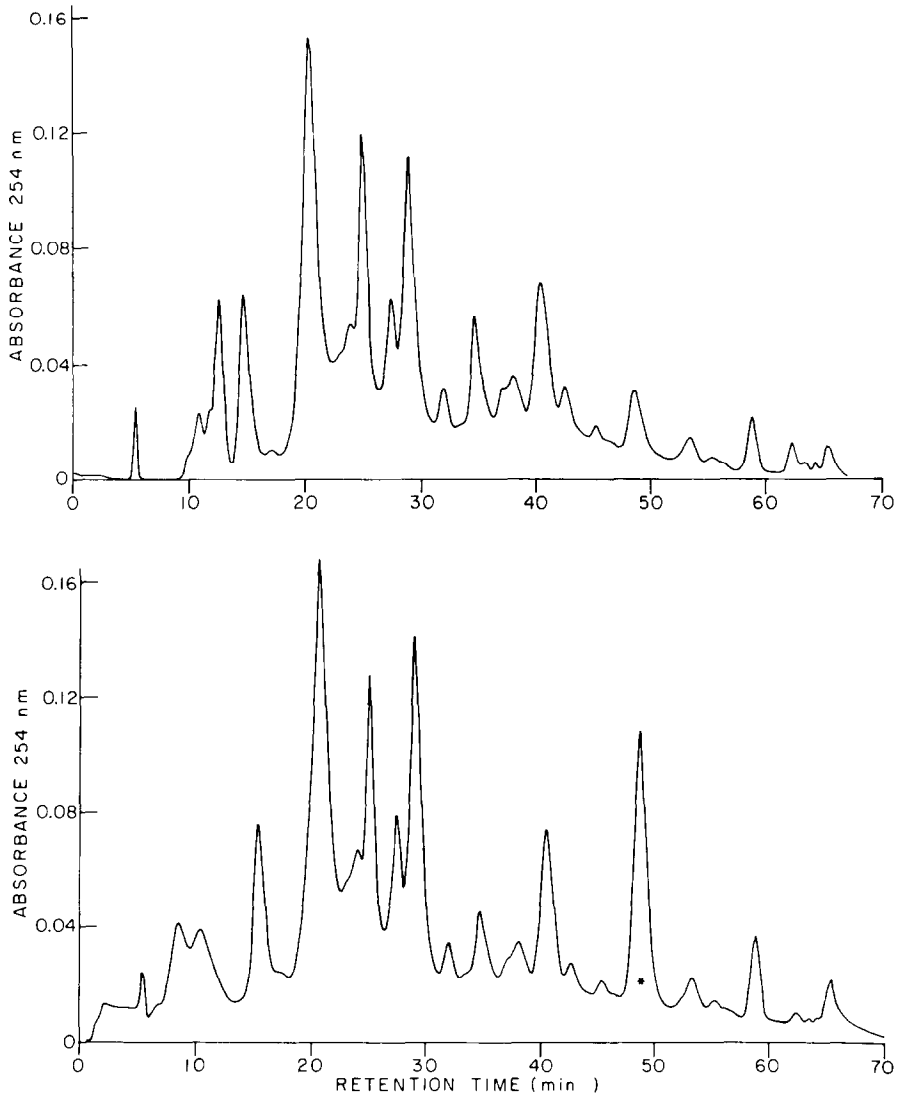

Fig. 1. Expression of $tRNA^{Trp}$ in 2YT medium. HPLC with mode I elution was performed for tRNA (400 μg each) from *E. coli* cells grown in 2YT medium bearing parental plasmid pGEM-9Zf(−) as control (top) or pWWT plasmid with *B. subtilis* $tRNA^{Trp}$ gene (bottom). The Trp acceptor peak in the bottom diagram (indicated by asterisk) is located at 49 min.

of mode II was interrupted with an isocratic elution at 40% buffer B for 20 min, before resuming with the curve 5 gradient.

To provide a specific step in the HPLC purification of $tRNA^{Trp}$, the mobility–retardation method of Maxwell *et al.* [16], based on enhanced hydrophobicity conferred on the tRNA upon tryptophanylation and thereby retardation on the HPLC column, was found to be applicable to both *E. coli* and *B. subtilis* $tRNA^{Trp}$ [17]. For this purpose, the peak $tRNA^{Trp}$ fractions from the mode I chromatogram were combined and precipitated overnight by 2 volumes of ethanol. The precipitate was collected by centrifugation, dried, and charged with [^{3}H]tryptophan. After phenol treatment, the aqueous phase was treated with 2 volumes of ethanol at −80°C for 30 min. The precipitate was collected by centrifugation and lyophilized for 60 min to ensure a thorough removal of ethanol prior to HPLC. Even small amounts of ethanol could interfere with the HPLC.

To store the HPLC peak fractions of $tRNA^{Trp}$ in the preceding procedures, these were dialysed

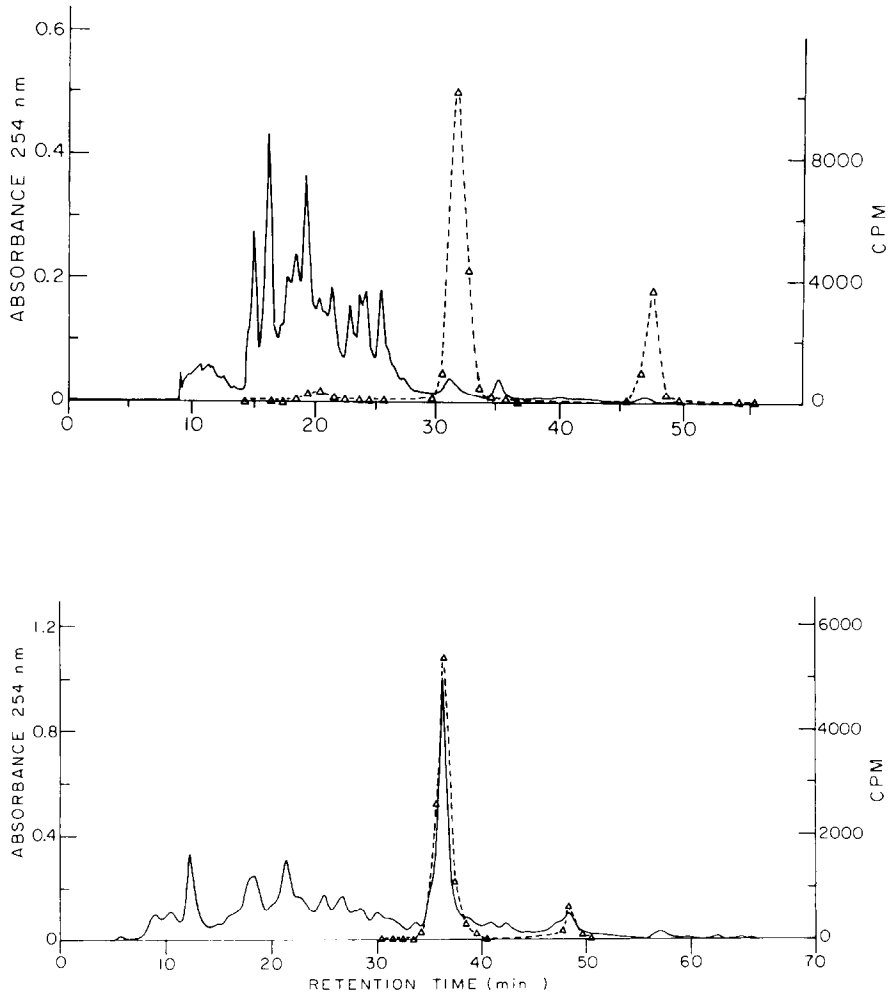

Fig. 2. Expression of $tRNA^{Trp}$ in M9-glycerol medium. HPLC with mode II elution was performed for 280 μg total tRNA from *E. coli* cells bearing parental plasmid pGEM-9Zf(−) (top) or 600 μg total tRNA from cells bearing pWWT plasmid with cloned *B. subtilis* $tRNA^{Trp}$ gene (bottom). Trp acceptor activity expressed in cpm is indicated by dashed line. From the HPLC fractions, 50 μl were assayed for acceptor activity in the top graph, and 5 μl in the bottom graph.

and concentrated using a Centriprep-3 concentrator (Amicon). Afterwards they were lyophylized and stored in powder form at −20°C.

RNA sequencing

This was performed according to Donis-Keller *et al.* [18]. Purified $tRNA^{Trp}$ was 5′-end labelled with ^{32}P and electrophoresed for 15 h at 1500 V in a 0.08-cm-thick 15% polyacrylamide–7 *M* urea gel. The single gel band containing $tRNA^{Trp}$ was cut out and extracted. RNA sequencing was carried out by means of enzymatic hydrolysis of the 5′-end labelled $tRNA^{Trp}$ with RNases T1, U2, A and Phy-M [19].

RESULTS

Hyperexpression of the heterologous $tRNA^{Trp}$ gene

When tRNAs extracted from cells grown in the rich 2YT medium carrying either the parental plasmid pGEM-9Zf(−) or the recombinant pWWT plasmid was separated on the Vydac C_4 column, the chromatographic profiles were largely similar in the two instances, except that the Trp-accepting peak was evidently elevated for pWWT relative to the parental plasmid (Fig. 1). The new Trp-accepting peak accounted for about 9.3% of total tRNA.

Since the *B. subtilis* $tRNA^{Trp}$ gene was regulated by the *lac* promoter on the pWWT plasmid, conditions minimizing catabolite repression might be expected to increase expression. Indeed, when the cells were grown in M9-glycerol, the expression of the new $tRNA^{Trp}$ peak was substantially enhanced, such that it represented 28% of total tRNA in Fig. 2, where tRNA separation was also improved in the vicinity of the enhanced peak by use of Mode II elution.

In Fig. 3 the enhanced tRNA peaks extracted from cells carrying the pWWT vector grown in M9-glycerol were further spread apart on the HPLC by mode III elution. One major Trp-accepting peak I and two minor peaks II and III were detected. Dot-blots hybridization indicated that all three Trp acceptor peaks in the pWWT profile, but not material from pGEM-9Zf(−) carrying cells, contained transcripts from the *B. subtilis* $tRNA^{Trp}$ gene.

Characterization of heterologous $tRNA^{Trp}$

To purify further the main heterologous $tRNA^{Trp}$ peak, the mobility–retardation method of Maxwell *et al.* [16] was employed (Fig. 4). The rightshifting of this peak on HPLC brought about its separation from other tRNAs that originally co-migrated with $tRNA^{Trp}$, thus leading to a further purification of the $tRNA^{Trp}$. The se-

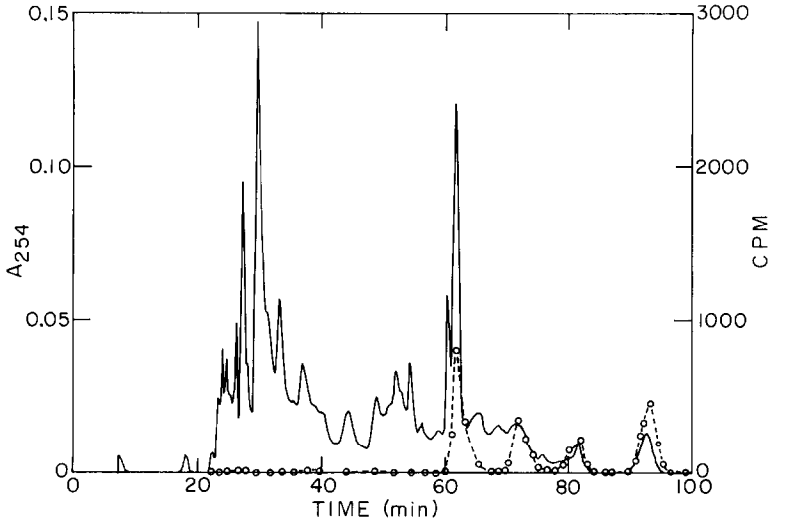

Fig. 3. HPLC with mode III elution. Sample consisted of 250 μg of total tRNA extracted from cells grown in M9-glycerol medium and induced by 0.2 m*M* IPTG for 4 h. Trp acceptor activity expressed in cpm is indicated by dashed line.

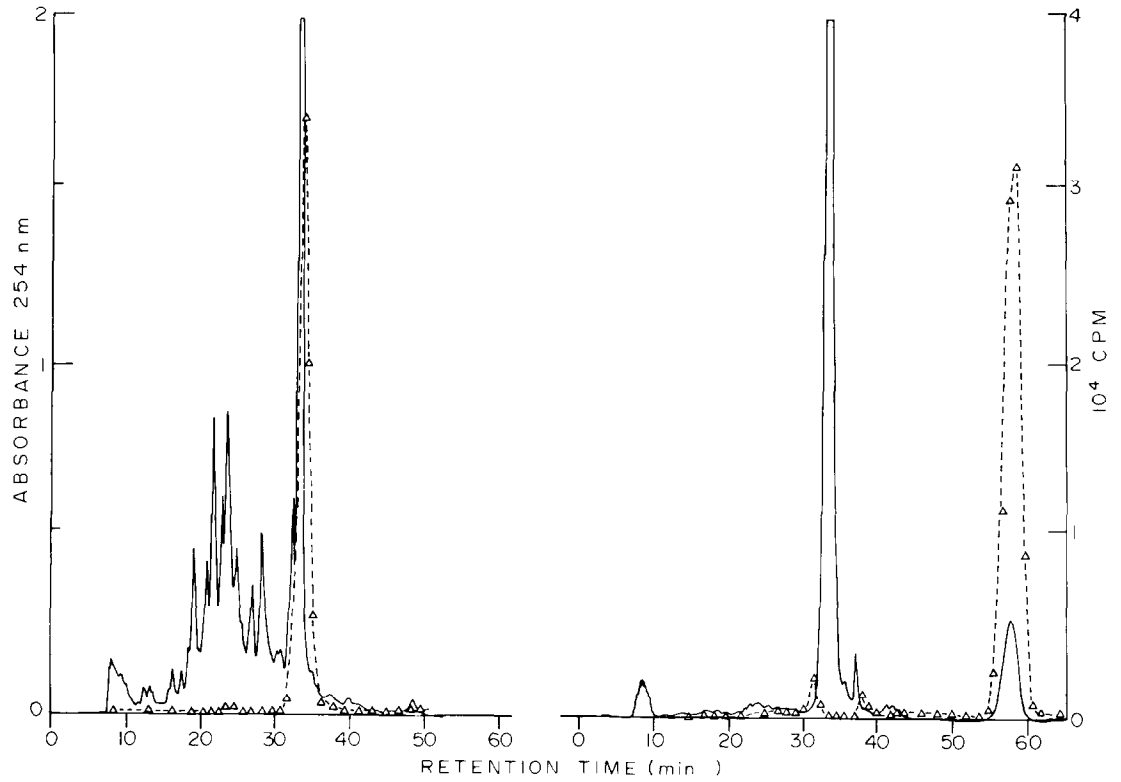

Fig. 4. Retardation of $tRNA^{Trp}$ elution by aminoacylation with tryptophan. HPLC (mode II elution) was performed for 1.5 mg total tRNA (left), and 0.25 mg of the Trp-accepting peak from the chromatogram on the left after tryptophanylation with 1.4 mg TrpRS (right).

quence of nucleotides 1–49 of the $tRNA^{Trp}$ after purification by rightshifting was determined (Fig. 5). The sequence together with corresponding portions of the unmodified $tRNA^{Trp}$ sequences derived from the gene sequences of *B. subtilis* and *E. coli* [20] are as follows:

cloned: AGGGGCAUANUUUAAC - GGUAGAACAGAGGUCUCCAAAACCUCCGG - UGUG
B.subtilis: AGGGGCAUAGUUUAAC - GGUAGAACAGAGGUCUCCAAAACCUCCGG - UGUG
E. coli: AGGGGCGUAGUUCAAUUGGUAGAGCACCGGUCUCCAAAACCGGGUGUUGGG

Thirteen out of the first 49 nucleotides in the $tRNA^{Trp}$ of *B. subtilis* are different from those of *E. coli*. On this basis, the major $tRNA^{Trp}$ peak expressed in IPTG-induced JM109[pWWT] cells was clearly transcribed from the *B. subtilis* rather than *E. coli* $tRNA^{Trp}$ gene.

The kinetics of aminoacylation for the three Trp acceptor HPLC peaks gave rise to linear Eadie plots (Fig. 6). The K_m and k_{cat} values determined from these plots are presented in Table I.

DISCUSSION

Because of the low $tRNA^{Trp}$ content of cells [5], the purification of $tRNA^{Trp}$ for physical and enzymic studies presents a formidable problem.

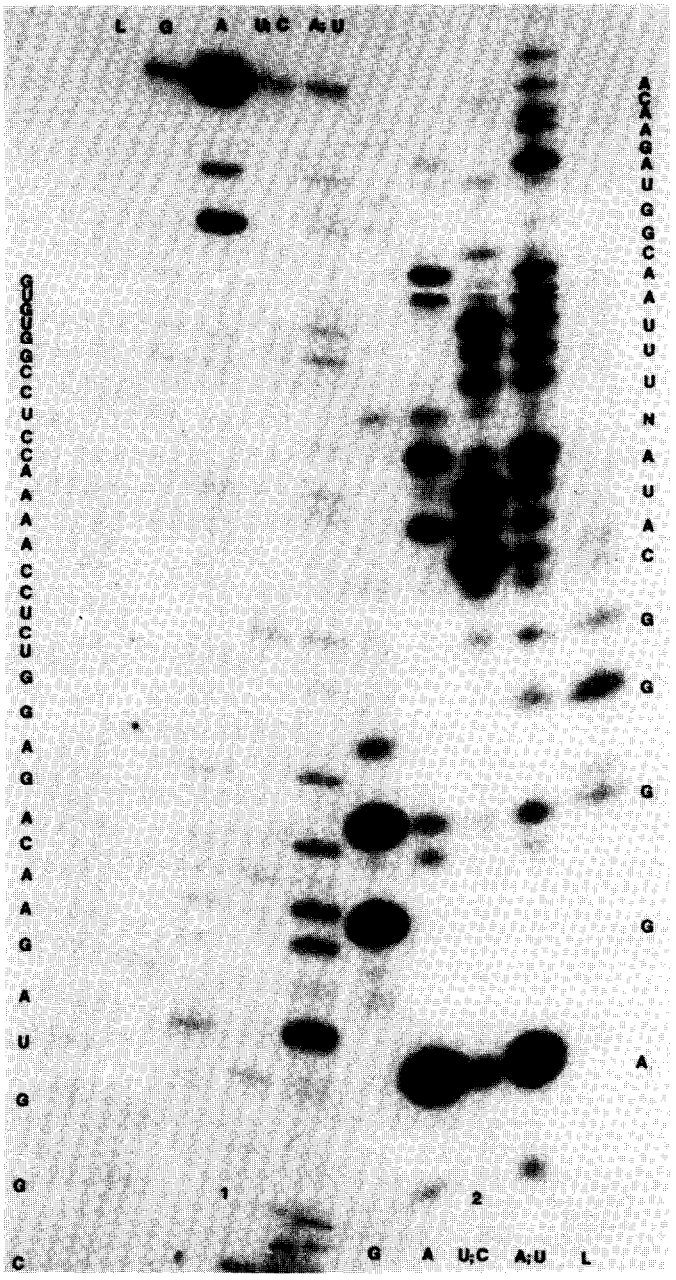

Fig. 5. The 5′ partial RNA sequence of cloned $tRNA^{Trp}$. RNA sequencing was carried out by hydrolysis of the 5′-end ^{32}P-labelled $tRNA^{Trp}$ with RNase T1 (cuts at G), RNase U2 (cuts at A), RNase A (cuts at U and C), RNase Phy-M (cuts at A and U), or chemically with formamide (ladder, L). The five lanes on the left are the results of run 1, and the five lanes on the right are the results of run 2 initiated 2 h after run 1.

Conventional chromatographic procedures yield only about 1 mg $tRNA^{Trp}$ from 100 g cells [21]. The hyperexpression attained in this study provides an effective solution to this problem of paucity of $tRNA^{Trp}$.

Previously, in the field of tRNA fractionation

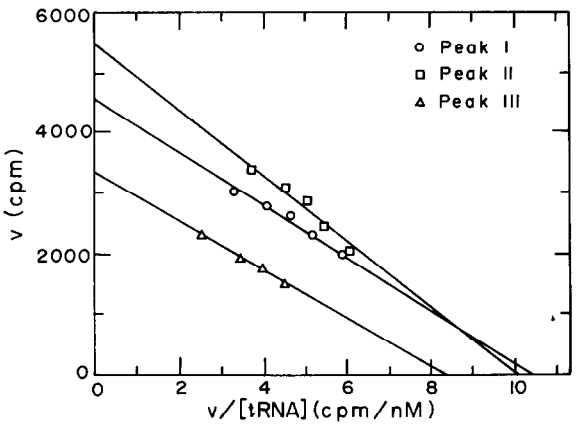

Fig. 6. Eadie plots of the variation of aminoacylation rates with [$tRNA^{Trp}$] for the three Trp acceptor peaks.

by HPLC, an early application of RPC-5 by Dion and Cedergren [21] was followed by more highly resolving systems developed by Pearson *et al.* [22] and Bischoff and McLaughlin [23]. Subsequently, a reversed-phase HPLC system on a Vydac C_4 matrix was developed that resolves *E. coli* tRNA into more than 20 peaks [11]. In the present study, resolution was further increased by the combination of a convex gradient with isocratic elutions. Total *E. coli* tRNA was fractionated into about 30 detectable peaks (Fig. 3). This method together with the mobility–retardation procedure [16] brought about the purification of the hyperexpressed tRNA using only the single Vydac C_4 matrix. Carter *et al.* [24] pointed out that the mobility–retardation method is highly demanding of TrpRS, and proposed instead a two-step procedure for purifying the $tRNA^{Trp}$ of *Bacillus stearothermophilus* by means of chromatography on BD-cellulose and Sepharose 4B. With the cloning and high-level expression of the TrpRS of *B. subtilis* rendering TrpRS plentiful [14,15], this shortcoming of the mobility–retardation procedure is overcome for $tRNA^{Trp}$ species that are aminoacylatable by this enzyme. It will be of considerable interest to determine if the hyperexpression of heterologous tRNAs in *E. coli* cells could be extended to the acceptor tRNAs for amino acids other than Trp, and to tRNA genes from species besides *B. subtilis*, so that the present heterologous expression–HPLC system might accomplish the large-scale preparation of additional species of tRNA, even those that are expressed normally at low or trace levels.

TABLE I

AMINOACYLATION KINETICS OF THE HYPEREXPRESSED $tRNA^{Trp}$

$tRNA^{Trp}$ peak	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (relative)
I	0.43	11.5	1
II	0.64	15.0	0.88
III	0.41	8.5	0.78

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