



Title	ホルミルメチオニンtRNA変換体の合成と活性
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論文目録

氏名 土井 健史

博士論文題名

ホルミルメチオニンtRNA変換体の合成と活性

- 1) Comparison of substrate base sequences for RNA-ligase reactions in the synthesis of a tetradecanucleotide corresponding to bases 21-34 of E. coli $\text{tRNA}_f^{\text{Met}}$
E. Ohtsuka, T. Doi, H. Uemura, Y. Taniyama & M. Ikehara (1980) Nucleic Acids Res. 8, 3909
(E. coli $\text{tRNA}_f^{\text{Met}}$ の 21番目から 34番目の塩基に対応する 14mer の合成における)
(RNA ligase 反応の基質塩基配列の比較)
- 2) Modification of the anticodon triplet of E. coli $\text{tRNA}_f^{\text{Met}}$ by replacement with trimers complementary to non-sense codons UAG and UAA.
E. Ohtsuka, T. Doi, R. Fukumoto, J. Matsugi & M. Ikehara (1983) Nucleic Acids Res. 11, 3863
(E. coli $\text{tRNA}_f^{\text{Met}}$ の アンチコドントリプルレットを 終止コドン UAG, UAA に 置換する)
(これによる修飾)
- 3) Replacement and insertion of nucleotides at the anticodon loop of E. coli $\text{tRNA}_f^{\text{Met}}$ by ligation of chemically synthesized ribooligonucleotides.
T. Doi, A. Yamane, J. Matsugi, E. Ohtsuka & M. Ikehara (1985) Nucleic Acids Res. 13, 3685
(化學合成リボオリゴヌクレオチドの ligation による E. coli $\text{tRNA}_f^{\text{Met}}$ の アンチコドン)
(ループの置換と 塩基の挿入)
- 4) Modification of the amino acid acceptor stem of E. coli $\text{tRNA}_f^{\text{Met}}$ by ligation of chemically synthesized ribooligonucleotides.
T. Doi, H. Morioka, J. Matsugi, E. Ohtsuka & M. Ikehara (1985) FEBS Letters 190, 125
(化學合成リボオリゴヌクレオチドの ligation による E. coli $\text{tRNA}_f^{\text{Met}}$ の アミノ酸受容)
(システムの修飾)

5) The in vivo stability, maturation and amino-acylation of anticodon-substituted *Escherichia coli* initiator methionine tRNAs

H. Grosjean, S. DeHenau, T. Doi, A. Yamane, E. Ohtsuka, M. Ikehara, N. Beauchemin, K. Nicoghosian & R. Cedergren (1987) *Eur. J. Biochem.* 166, 325

(アンチコドンを置換した *E. coli* tRNA^{Met} の in vivo における安定性と成熟化)
(及びアミノアシル化)

参考論文

1) A new method for 3'-labelling of polyribonucleotides by phosphorylation with RNA ligase and its application to the 3'-modification for joining reactions.

E. Ohtsuka, H. Uemura, T. Doi, T. Miyake, S. Nishikawa & M. Ikehara (1979) *Nucleic Acids Res.* 6, 443

(RNA ligase を用いたリボ核酸化によるポリリボヌクレオチドの 3'標識の新法と
その結合反応に対する 3'修飾の応用)

2) Total synthesis of a RNA molecule with sequence identical to that of *Escherichia coli* formyl-methionine tRNA.

E. Ohtsuka, S. Tanaka, T. Tanaka, T. Miyake, A. F. Markham, E. Nakagawa, T. Wakabayashi, Y. Taniyama, S. Nishikawa, R. Fukumoto, H. Uemura, T. Doi, T. Tokunaga & M. Ikehara (1981) *Proc. Natl. Acad. Sci. U.S.A.* 78, 5493

(*E. coli* tRNA^{Met} の塩基配列を持つ RNA 分子の全合成)

3) Chemical synthesis of the 5'-half molecule of *E. coli* tRNA^{Gly}.

E. Ohtsuka, A. Yamane, T. Doi & M. Ikehara (1984) *Tetrahedron* 40, 47

(*E. coli* tRNA^{Gly} の 5'半分子の化学合成)

論文内容の要旨

博士論文題名

ホルミルメテオニンtRNA変換体
の合成と活性

学位申請者

土井 健史

tRNA は鎖長 70~80 の機能を有する RNA と
しては最小の分子である。

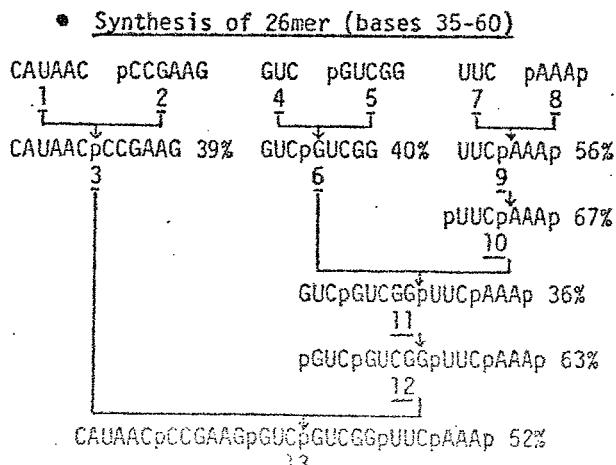
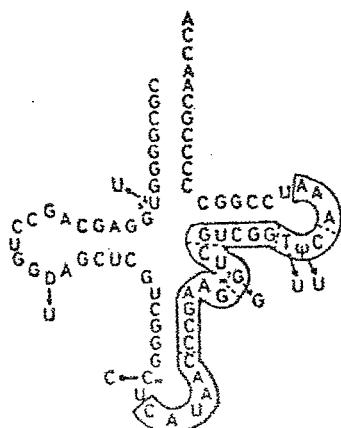
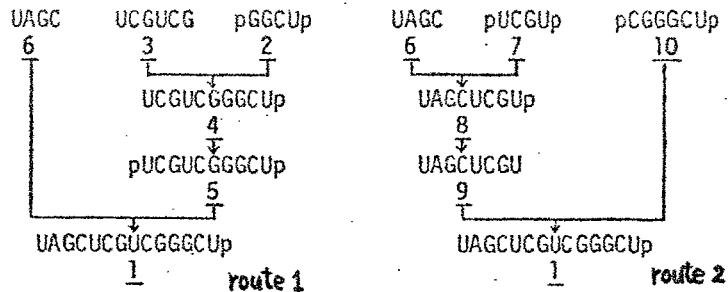
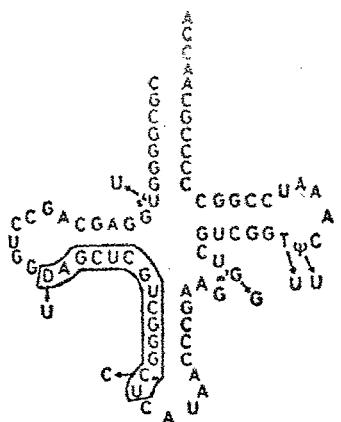
著者は $E. coli$ tRNA_f^{Met} nascent strand 分子の
3'側半分子、5'側半分子を化学合成フラグメ
ントより RNA ligase¹⁾ を用いて合成した。
この際 これら tRNA ligase 反応における塩基
特異性を見い出した。

次に RNA ligase 反応を用いて $E. coli$ tRNA_f^{Met}
のアミノアシルシステム、T₄ ループ、D ルー
プ、アンチコドンループを変換して tRNA を
合成し 主にメチオニル tRNA 合成酵素との
相互作用について調べた。

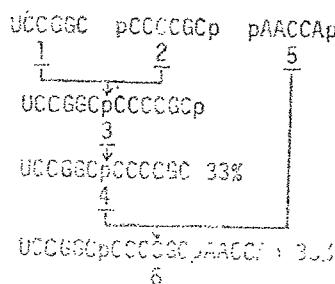
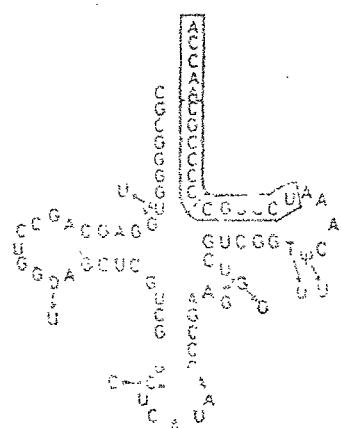
I RNA ligase を用いた長鎖オリゴリボ ヌクレオチドの合成

図 1 に示す様に比較的短鎖の化学合成オリ
ゴリボヌクレオチドより tRNA 1/4 鎖長分子
を合成した。²⁾³⁾ さらにこれらを用いて tRNA 半
分子を合成した。⁴⁾

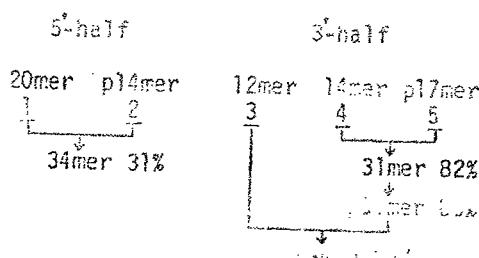
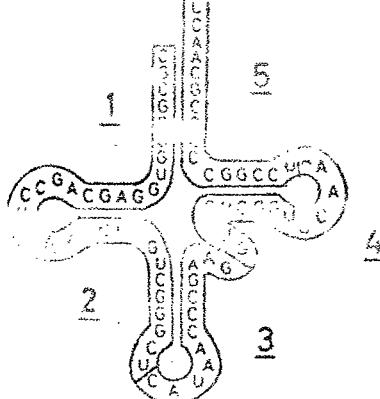
• Synthesis of 14mer (bases 2)-1



• Synthesis of 17mer (bases 21-34)



• Synthesis of half molecules



1

表1

Reaction conditions for joining

acceptor nmol [μ M]	donor nmol [μ M]	ATP μ M	RNA ligase [unit/ml]	time hr	Reaction extent ^{a)} %	Yield %
UCGUUCG (3) 12 [100]	pGGCUp (2) 20 [167]	333	250	1	15	4
UAGC (6) 1.2 [240]	pUCGUCGGGCUp (5) 0.4 [80]	200	180	2	27	11 ^{b)}
UAGC (6) 30 [150]	pUCGUp (7) 20 [100]	200	100	3.5	88	71
UAGCUCGU (9) 7 [100]	pCGGGCUp (10) 8 [114]	200	214	2	52	31 ^{b)}

Incubation mixtures contained 10% DMSO and the temperature was 25°.

a) Estimated by homochromatography.

b) The compound was isolated after kination.

RNA ligase 反応において表1に示す様に、acceptor 分子では 3'末端がピリミジン-アリノ配列を有するものが、donor 分子では自己相補的な構造を有するものが それぞれ反応し難いことがわかった。³⁾

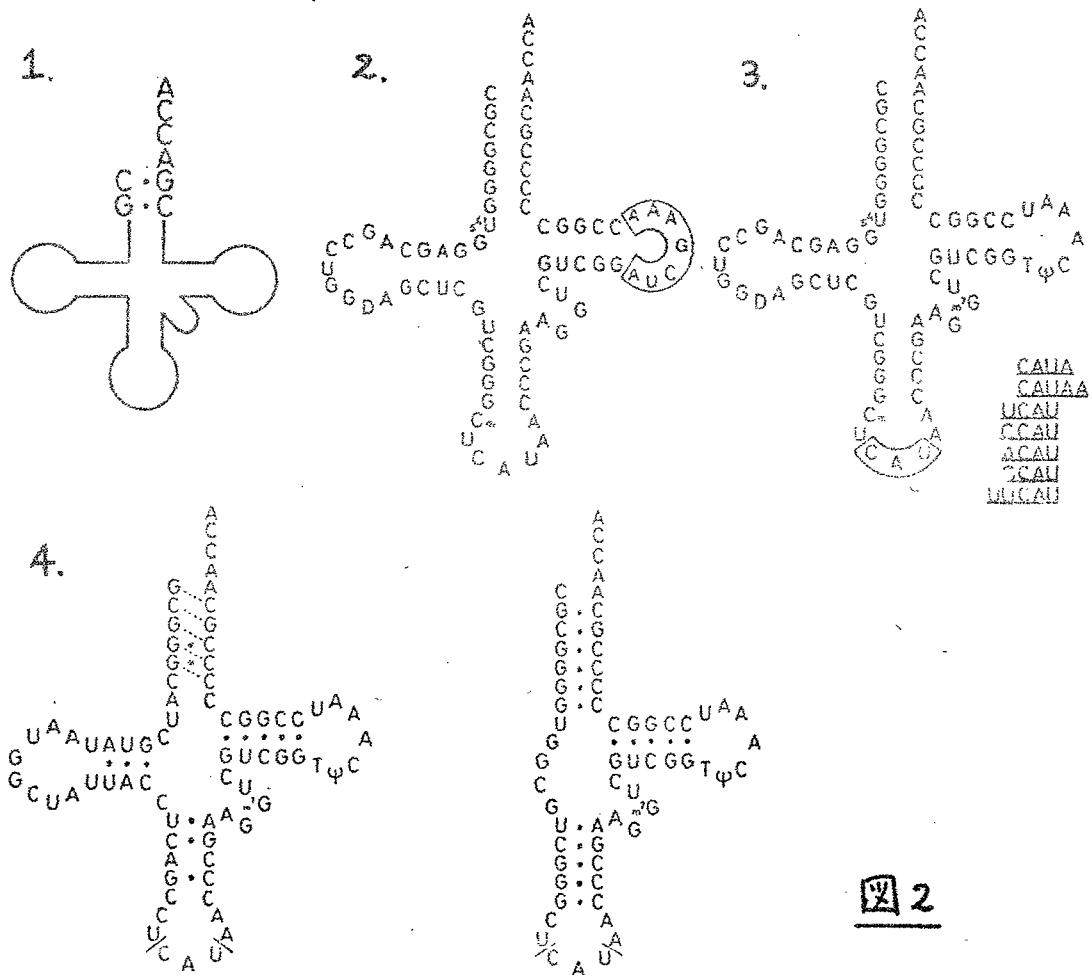
II E. coli tRNA^{Met} 变換体の合成とその性質

1) E. coli メチオニル tRNA 合成酵素との相互作用

E. coli メチオニル tRNA 合成酵素との相互作用を調べるために以下の tRNA_f^{Met} 变換体

至合成 1 T. (図 2)

1. アミノアシルシステムの 5'末端で G-C 塩基対を形成する $tRNA_f^{Met}$
2. Tψループの TψC を真核生物のイニシエーター $tRNA$ に特有の AUC に変換した $tRNA_f^{Met}$ 5)
3. アンチコドンの 5'側隣接位の共通塩基 U を他の塩基に変換した $tRNA_f^{Met}$ やアンチコド



2

ンルーフ^oを拡大した tRNA_f^{Met}を合成した^{6), 7)}
メチオニル tRNA 合成酵素のアンチコドン
認識についてにはアンチコドン配列 CAU が
重要であることが調べられていて⁸⁾この
配列を残しルーフ^oを拡大した。

4. Dルーフ^oの欠陥 tRNA_f^{Met}や 5'側单分子
に E. coli tRNA₂^{Met}の塩基配列を有する tRNA
を合成した。⁹⁾

合成は天然の tRNA_f^{Met}フラグメントと化学合
成オリゴマーとを組み合わせ、これらを RNA-
ligase で結合し行なった。

アミノアシルシステムでの変換には天然の
tRNA_f^{Met}の nuclease S1 限定分解フラグメント¹⁰⁾を、
その他のルーフ^oの変換には tRNA_f^{Met}の RNase A 限
定分解フラグメント^{8), 11)}を用い合成した。又、
RNA の 3'末端より一塩基を除去する方法とし
て過ヨウ素酸酸化¹²⁾を用いた。一例として ア
ンチコドンを変換した時の合成 scheme を図 3
に示す。

生成物はポリア
クリルアミドゲル
電気泳動で単離！

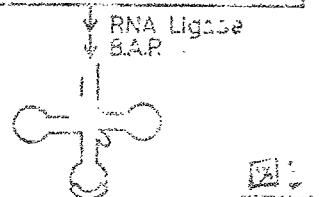
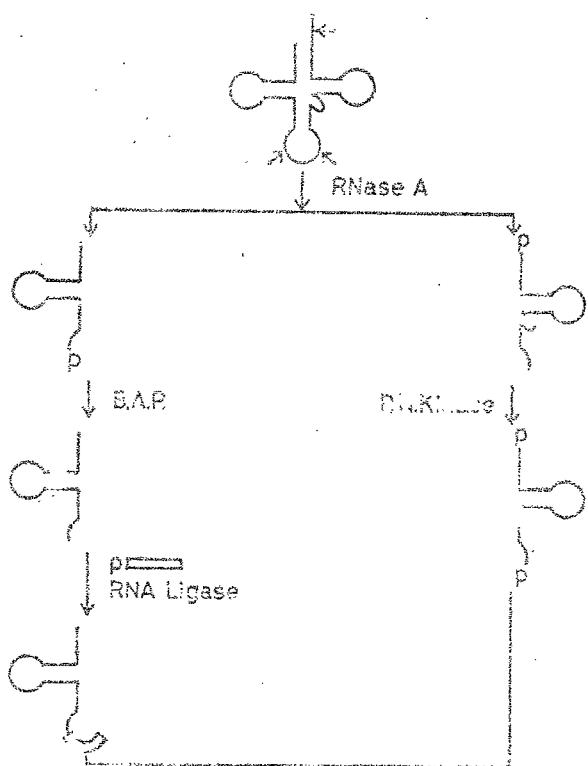
下。 RNA ligase 反応
後 18 nearest neighbor
analysis で結合部
位を確認した。

合成した tRNA
についてアミノ酸
受容活性を測定！

下。

アミノペルルス
チムを変換した

tRNA はもとの tRNA と同じ活性を示したが、
T⁴ル-ア¹、Dル-ア¹を変換した tRNA は
ほとんど活性を失った。アンチコドンを変換
した tRNA については、アンチコドンの 5'側
Uを変換した tRNA は Uの分子と変わりなか
ったが、ル-ア¹を拡大した tRNA については
その伸縮位置と位置により親活性に差がある



図

れた。図4にその時の Lineweaver-Burk $\text{F}^{\circ}\text{E}^{-}\text{A}^{-}$ プロットを示す。

アンチコドンの3'側と5'側2'塩基の組み合が異なるため(2'側にUを付加したtRNAがメチオニルtRNA合成酵素と強い親和性を示し, 3'側へのU添加や2'塩基付加を行くすると考えられる)。又、アンチコドンループを拡大したtRNAについてリポソーム上でのmRNA(トリマー)と結合するか調べたが、5'側にUを付加した時のみもとのAUGと結合できることがわかった。このことよりアンチコドンの5'側にCAUの並びをあまり変化させずにUが入りこむことが可能と考えられる。

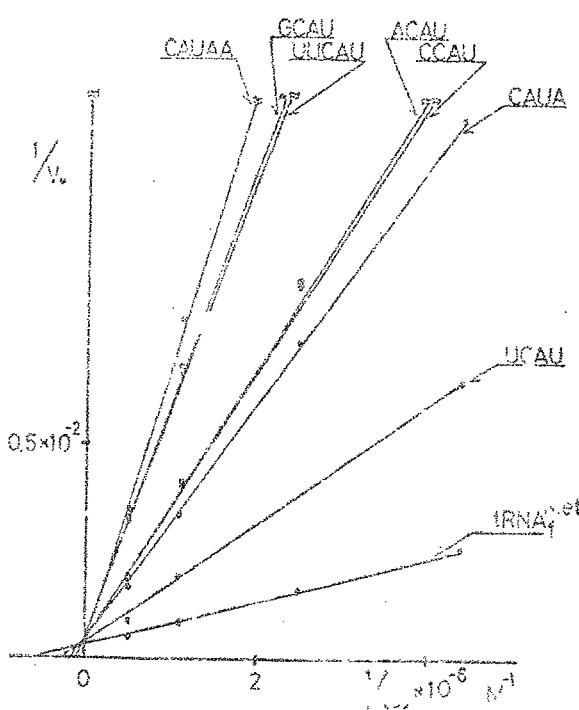


図4 テラヌ-、ハンヌ-はアンチコドン CAU とそれからの塩基配列に結合した tRNA^{Met} を示す。

2) EF-Tu との相互作用

アミノアシルシステムで塩基対を形成した $tRNA_f^{Met}$ について EF-Tu との結合実験を行なった。図 5 に示す様に塩基対を形成しない天然の E. coli $tRNA_f^{Met}$ は EF-Tu に結合しないのに對し、合成した $tRNA$ は一部複合体を形成することがわかつた。

Sephadex G-100

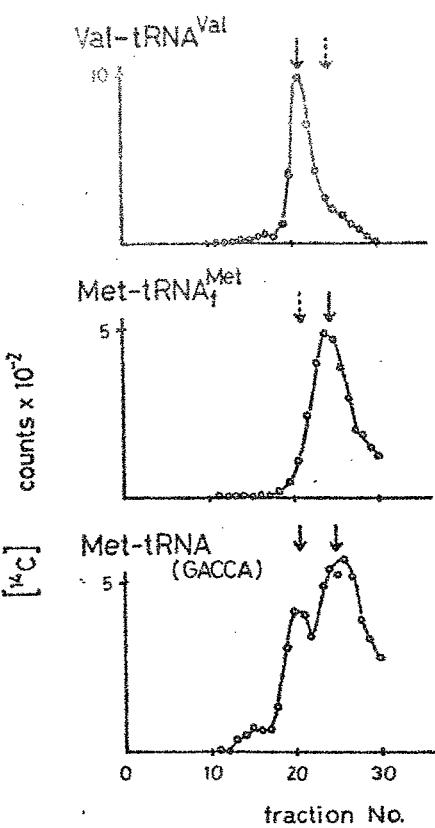


図 5

3) アンチコドンの 3'側隣接位の塩基修飾

E. coli $tRNA_f^{Met}$ のアンチコドンを図 6 に示す 8 種類の塩基に変換した $tRNA_f^{Met}$ を

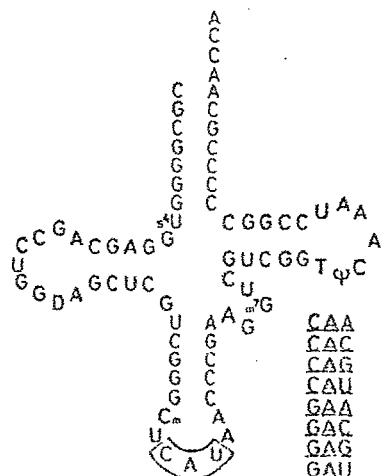


図 6

合成した。これらの tRNA を *Xenopus laevis* oocyte の細胞質にマイクロインジェクションしアンチコドンの 3'側が修飾されるか否かを調べた。CAU (もとの tRNA_f^{Met} と同じ) と GAU 配列を有する tRNA の 3'端が t⁶A (*N*-[9-(β -D-ribofuranosyl)purin-6-yl carbamoyl]threonine) に修飾を受けることがわかった。又、これら 2 種の tRNA のみ *Xenopus oocyte* 中で安定に存在できることがわかった。

結論

1. 化学合成フラグメントと RNA ligase を用い種々の長鎖 RNA フラグメントを合成した。又、 RNA ligase 反応の基質特異性を見出した。
2. *E. coli* tRNA_f^{Met} のアミノアシルヌクレオチド、T ループ、アンチコドンループ、D ループを変換した tRNA_f^{Met} を合成しメチオニン受容活性を調べた。D ループと T ループを

変換した tRNA はほとんど活性がなくなり、アミノアシルシステムの 5'末端で塩基対を形成した tRNA は本来の $tRNA_f^{Met}$ と同様の活性を示した。アンチコドンループを拡大した tRNA は CAU 配列が存在すれば付加塩基の種類や位置により活性に差はあるがメテオニンを受容した。

3. $E. coli tRNA_f^{Met}$ のアミノアシルシステムで塩基対を形成した tRNA は塩基対を形成しない tRNA に比べ $E. coli$ EF-Tu と複合体を形成しやすいことがわかった。
4. アンチコドン配列のちがいにより tRNA の安定性が異なることを $E. coli tRNA_f^{Met}$ 変換体を用い *Xenopus laevis oocyte* の系で見出した。又、アンチコドンの 3'側隣接位の A がこの系で修飾されることを見出した。

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ホルミルメチオニンtRNA
変換体の合成と活性

土井 健史

略語表

Py	pyrimidine
Pu	purine
D	5,6-dihydrouridine
T	5-methyluridine
U	pseudouridine
⁴ U	4-thiouridine
Cm	2'-O-methylcytidine
⁷ G	7-methylguanosine
⁷ A	N-[9-(β-D-ribofuranosyl)purin-6-ylcarbamoyl]-threonine
¹ A	6-(Δ ² -isopentenyl)adenosine
P,N.kinase	polynucleotide kinase
BAP	bacterial alkaline phosphatase
ARSase	aminoacyl-tRNA synthetase
Met RSase	methionyl-tRNA synthetase
Gly RSase	glycyl-tRNA synthetase
EF-Tu	elongation factor Tu
BSA	bovine serum albumine
Tris	tris(hydroxymethyl)aminomethane
HEPES	N-2-hydroxymethylpiperazine-N'-2-ethanesulfonic acid
TEAB	triethylammonium bicarbonate
DTT	dithiothreitol
β-ME	β-mercaptoethanol
EDTA	ethylenediaminetetraacetic acid
NTA	nitrilotriacetic acid
TCA	trichloroacetic acid
DMSO	dimethyl sulfoxide
SDS	sodium dodecylsulfate
TEMED	N,N,N',N'-tetramethylethylenediamine
X.C.	xylene cyanol FF
B.P.B.	bromophenol blue
³² p	
PEP	paper electrophoresis
PAGE	polyacrylamide slab gel electrophoresis
PAGE(disk)	polyacrylamide disk gel electrophoresis

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緒論

近年のDNA合成技術の進歩にめざましく、*E. coli tRNA^{Tyr}*の遺伝子が合成されて以来数多くの遺伝子が合成されている。一方RNAはその2'水酸基の存在のためDNAに比べ合成が困難で長鎖オリゴマーの合成には長い時間と労力を要する。Hunwitz等によりT4感染大腸菌から発見されたRNA ligase²⁾は一本鎖オリゴヌクレオチドに作用し、5'末端リニ酸基と3'末端水酸基とを結合する酵素で比較的短鎖のRNAフラグメントをこの酵素を用いてくり返し結合すれば化学合成だけで得られない長鎖のRNAの合成が可能となる。

tRNAは鎖長30~80の機能を有するRNAと1つは最小のもので人工合成の標的と1つ非常に興味ある対象である。現在まで多くの研究者により数百に及ぶ

tRNAの一次構造が明らかにされ³⁾

一部のtRNAを除き全て共通の

クローバーリーフ型二次構造を組むと考えられている。(図1)又、

X線結晶構造解析によりYeastではtRNA^{Phe}^{4,5)}, tRNA^{Asp}⁶⁾, tRNA^{Met}⁷⁾

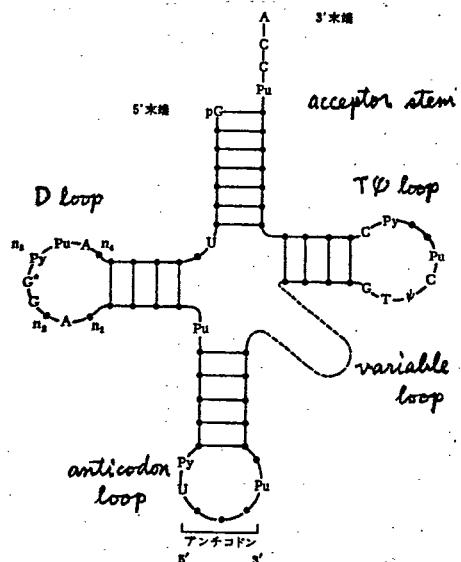
*E. coli*ではtRNA^{Met}⁸⁾の三次構造が

明らかにされ、それらはいずれも

ほぼ共通のL字型構造を有するこ

とがわかっている。

生体内においてtRNAはmRNA上の情報を従いアミノ酸を運搬する



[図1] tRNAのクローバーリーフ型
二次構造

ンペア複合体を経るが、特異的tRNAが特異のアミノ酸を受容し mRNAの情報どおりタンペア複合体が合成される機構は興味深い。tRNAにアミノ酸を受容するアミノアシルtRNA合成酵素(ARSase)はこの特異的な認識を行なう酵素で、アミノ酸の種類によりそれぞれ独自の酵素が存在する。現在までこの酵素とtRNAとの相互作用について種々の研究⁹⁾がなされていろが、今だに認識部位等に関する不明な点が多い。tRNAの一部を変換した分子を合成し、ARSaseとの相互作用を調べればtRNAにおけるその部位の役割がより明確になると考えられる。

著者は化学合成オリゴマーと天然のtRNAフラグメントを組みあわせ RNA ligaseで結合し種々の E. coli tRNA_f^{Met}変換体を合成した。そしてこれらの分子と主として E. coli メチオニルtRNA合成酵素(Met RSase)との相互作用を調べた。

以前に Schulman らにより tRNA分子を化学修飾し Met RSaseとの相互作用が調べられて¹⁰⁾いるが、この方法では目的の箇所以外でも修飾が生じ特異的な部位の相互作用は説明困難。

著者ははじめに RNA ligaseと化学合成オリゴマーを用い、修飾塩基の欠除した E. coli tRNA_f^{Met}の長鎖フラグメントの合成を試みた。その過程において RNA ligase 反応における塩基配列特異性があることがわかった。

次に化学合成オリゴマーと天然の E. coli tRNA_f^{Met}より得られるフラグメントを用い T₄ルード、アンチコドンループ、Dループ、アクセプターステムなどを変換したtRNAを合成し、Met RSaseとの相互作用を中心に tRNA変換体の性質を調べた。その結果 Met RSase は E. coli tRNA_f^{Met}のアンチコドン部を認識

し、特にそのアンチコドンの CAU という配列が認識に重要であること、又 アンチコドン部のみならず tRNA 全体の 3 次構造が認識に必要であることがわかった。

さらにアンチコドン部を変換した tRNA について、アフリカツメガエルの卵母細胞にマイクロインジェクションを行ないこの系での tRNA の修飾について調べた結果、hetero 分系における tRNA の安定性と修飾について今まで知られていないか、新しい事実を得ることことができた。

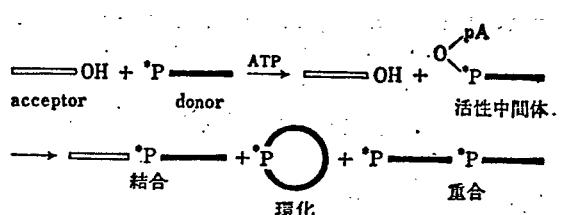
本論

第一章 化學合成 nascent E. coli tRNA_f^{Met} 737'X> + o RNA ligase I = F₃ 組合及亦

$tRNA_f^{Met}$ (図2) は種々のアミノ酸に対応する $tRNA$ の中でもタンパク質合成の開始に関与する特別な分子で種々のタンパク質から認識を受ける。この $tRNA$ を人工的に合成することは核酸とタンパク質との相互作用を研究する上で意義があり、又任意の長鎖RNAが合成可能であるとの証明となる。

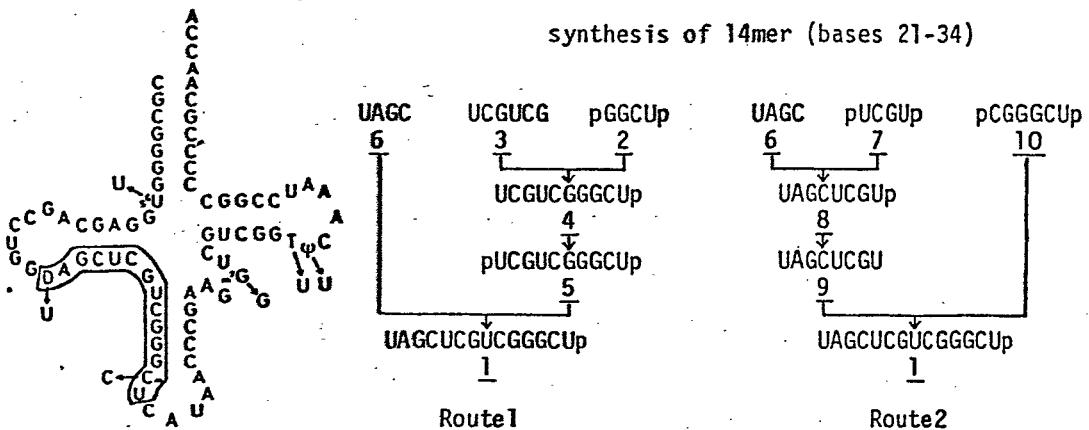
化学的に RNA鎖を伸長する場合、鎖長が 10 を越えると合成がかなり困難となる。tRNA の様な長鎖 RNA の合成の場合比較的長鎖の化学合成フラグメントを用いそれらを RNA ligase で結合する方法が適当であると若々られてる。

著者は図3の様な反応を触媒する RNA ligase を用い比較的短鎖の化学合成フラグメントを出発原料に(結合互繋り)遙12, 長鎖RNA分子を合成することを試行した。



[図3] RNAリガーゼの反応

第一節 ダル-フ⁹からアンチコドン部位に至る 14mer (bases 21-34) の合成¹¹⁾



[図4] 14merの合成

[図5] 14merの合成経路

図4に示す D-アラニンからアンチコドン部位に至る 14mer を図5のようなく 2 種類のルートを用いて合成した。

はじめにルート 1 に 3' 結合反応を試みたが 2 と 3 の結合收率が非常に悪く数% を越えなかつた。そこでルート 2 の様に結合部位を変えて反応を行なつた結果、各段階の ligation (RNA ligase による結合反応) の收率は大きく向上した。

1) ルート 1 による方法

2 は GUCUp を kinase (P.N. Kinase I = よる 5' 末端リン酸化反応: 特に記述しない限り $[\gamma-32P]$ -ATP を用いリン酸化) し得られた分子で、次の ligation における副反応を防ぐため 3' 末端をリン酸基で保護している。この 2 を 3 と ligation したが反応の進行が悪く最良の条件下でも 15% 程度しか進行しなかつた。20% polyacrylamide disk gel electrophoresis (PAGE(disk))

で単離し、3から $5'$ の收率が4%である、T。4 IF kinaseation 後 Sephadex G-50 で精製し 76% の收率で $5'$ を得た後 6と ligation 1 である。この ligation 反応液はそのまま次の kinaseation に使用し、20% PAGE (disk) で p UAGCUCGU_nGGCUp を単離する。收率は ligation, kinaseation を通じて 11% である、T。得られた $T=14$ mer の ligation step 1 における結合位置が正しいことは、nearest neighbor analysis (後述) を行ない確認する。

2) ルート 2 による方法

7, 10はそれぞれ UCGUp, CGGGCUp を kinaseation 1, 7はイオン交換カラムクロマトグラフィー、10はゲル泳動で精製するフランメントで、ルート 1 の場合とは異なる結合位置を有するよう設計する。

まず 6と 7を ligation 1, DEAE cellulose イオン交換カラムクロマトグラフィーで単離し (図 6), 8を好收率で得る。8は nearest neighbor analysis (ligation product を RNase T₂ で完全分解する後 pH 3.5 の条件下 PEP を行ない 32 P が donor 32 p UCGUp から acceptor UAGC の C に移り C 32 p として検出され 結合部位が正しいことが確認できる方法) により結合を確かめた。 (図 7)

8 IF BAP 处理により 3' 位のリノ酸基を除去し、7から通算 71% の收率で 9を得る。9 IF 10と ligation (20% PAGE (disk)) により 14mer (1) を 9から 31% の收率で単離する。 (図 8)

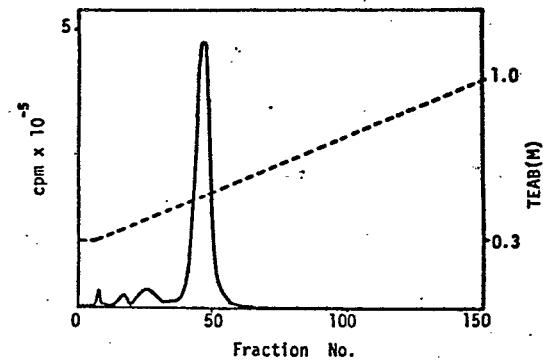
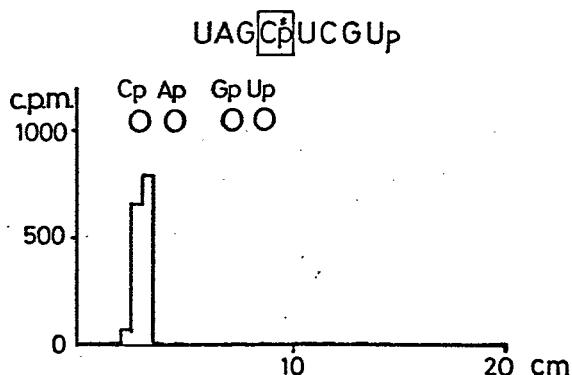


図 6 UAGCUCGU_nUp (8) の精製

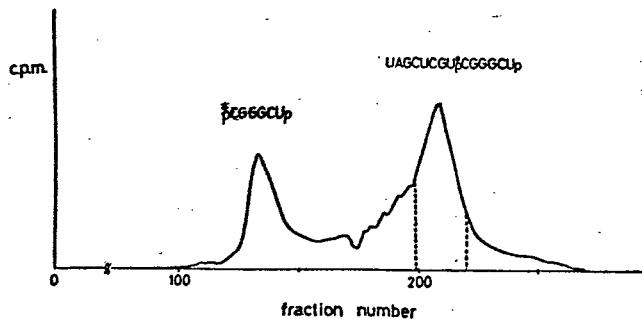
Nearest neighbor analysis
で結合部位を、polyacrylamide
slab gel electrophoresis
(PAGE) で鎖長をそれぞれ
確認した。

以上のように 2, のルー
トで 14mer を合成したが、
ligation について条件
と收率を表 1 にまとめ
た。

同じ 14mer を合成す
る場合における 77
グメントの組合あわせ
で收率が大きく異なる。
この原因は RNA ligase



[図7] UAGCUCGUp の nearest neighbor analysis



[図8] 14mer の精製 PAGE (disk)

Reaction conditions for joining

acceptor nmol [μM]	donor nmol [μM]	ATP μM	RNA ligase [unit/ml]	time hr	Reaction extent ^{a)} %	Yield %
UCGUUCG (3) 12 [100]	pGGCUp (2) 20 [167]	333	250	1	15	4
UAGC (6) 1.2 [240]	pUCGUCGGCUp (5) 0.4 [80]	200	180	2	27	11 ^{b)}
UAGC (6) 30 [150]	pUCGUp (7) 20 [100]	200	100	3.5	88	71
UAGCUCGU (9) 7 [100]	pCGGGCUp (10) 8 [114]	200	214	2	52	31 ^{b)}

Incubation mixtures contained 10% DMSO and the temperature was 25°.

a) Estimated by homochromatography.

b) The compound was isolated after kinivation.

[表1] 14mer 合成における ligation の条件と単離收率

の acceptor 分子や donor 分子に対する認識の差によるものと考えられる。Accepter 分子については、3'末端が 5'側からピリミジンヌクレオシド (Py) - プリンヌクレオシド (Pu) (ex. C-G) の順に並ぶと ligation の收率が悪い。これは RNA 分子が右巻き構造をとると 3'末端の Py-Pu の base stacking が弱く末端の Pu の conformation が固定され難くなり RNA ligase との親和性が低下したためと考えられる。これに対し 3'末端が Pu-Py (ex. G-C) の場合 base stacking が Py-Pu よりも強い構造を有し 3'末端分子が固定され RNA ligase に対する親和性が増したと思われる。

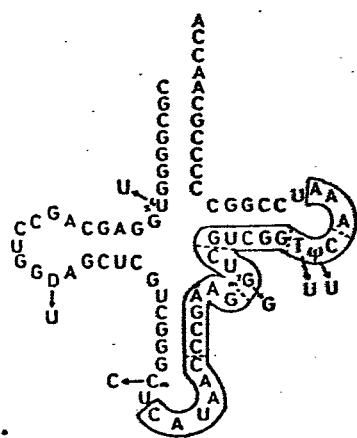
以上のことから acceptor 分子の 3'末端ヌクレオシドの conformation は ligation に大きく影響を及ぼすと推論できる。

Donor 分子については sequence による特異性はあまりみられないが、donor 分子が T などとえば“自己相補的な sequence”である場合 分子同士が凝集し ligation が進行しなくなる。

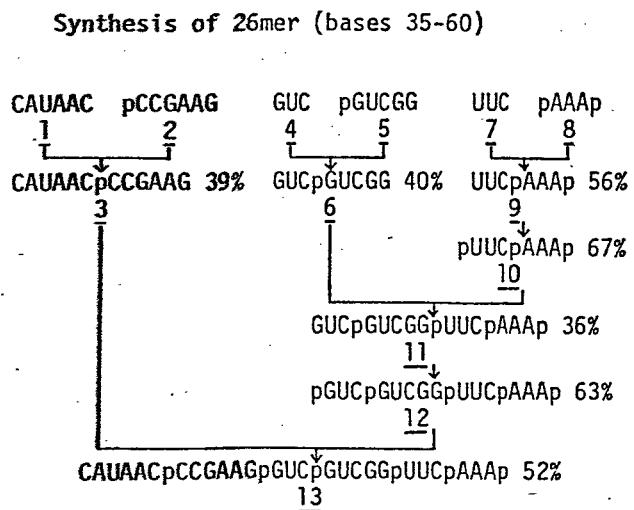
UCGUCG + pGAGCUp という ligation の場合、acceptor 分子の構造のみならず donor 分子についても自己相補的な sequence であるため 反応が進行し難く 4% という低收率である。

第二節 アンチコドン部位から T4 ループに至る 26mer (bases 35-60) の合成¹²⁾

図 9 に示す様にアンチコドンループから T4 ループに至る修飾塩基を含まない 26mer を図 10 に示す順序で合成した。これらの結合反応で 1+2, 4+5 については donor 分子の 3'末端がリン酸基で保護されていないが、副反応の donor 分子の重



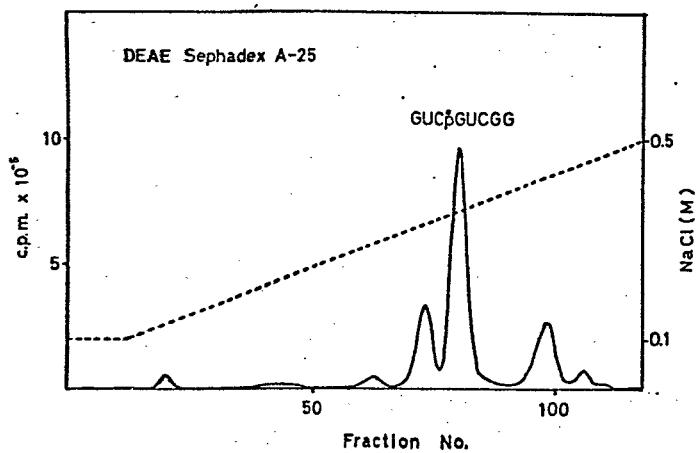
[図9] 26merの合成



[図10] 26merの合成経路

合化を防ぐためにそれぞれ acceptor と donor の 10倍, 20倍と過剰に用いた。1 + 2 では反応後 20% PAGE (disk) に F1 と F2 が見られ、7M urea 存在下 45°C でのイオン交換カラムクロマトグラフィーにより 6 をそれぞれ単離した。図11は 6 を得た時のカラムクロマトグラフィーの溶出パターンで目的物のピークの後にさらには donor 分子が結合した副生成物のピークが見られる。7 + 8 は收率良く反応が進み、イオン交換カラムクロマトグラフィー - 1 に F1 と F2 が単離した。

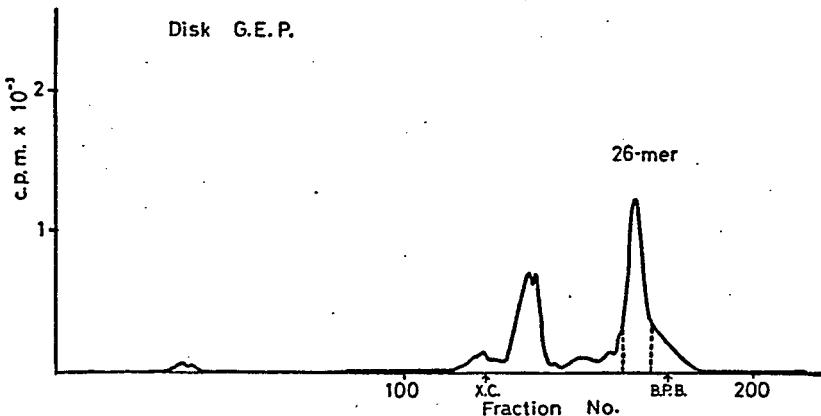
9 は *kination* 後
再びイオン交換カラム
クロマトグラフー¹
を行なう定量的に¹⁰



[図11] GUCGUCGG(6)の精製

を得た。

6 + 10 の
ligation, 11
or formation,
3 + 12 の
ligation が
常法どおり
行ない、反



[図12] 26merの精製

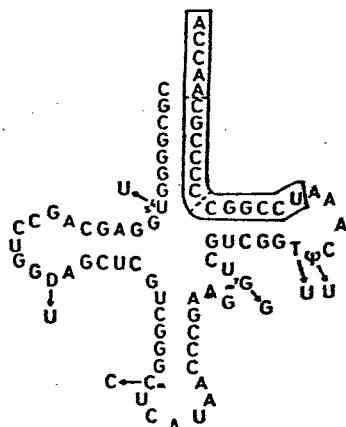
応後これら urea 存在下 1オニ交換カラムクロマトグラフィー, Sephadex G-50, 20% PAGE (disk) (図12) で単離した。

Ligation 後はすべて nearest neighbor analysis を行ない, 結合部位が正しいことを確認した。

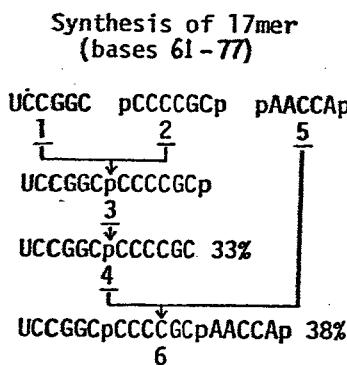
今回 3' 末端を保護しない donor 分子を用いて ligation を試みたが acceptor 分子を大過剰用い反応すれば副反応をほとんどおさえ ligation を行なうことがわかった。

第三節 T4 ループから 3' 末端に至る 17mer (bases 61-77) の合成

図13 に示す 3' 末端部 17mer を図14 の順序で合成した。以前にこの 17mer は当教室で UCCGG + CCCCCG + CAA + CCA というフラグメントの組みあわせにより合成されているが、3' 末端で Py-Pu の配列を持つ CCCCCG が acceptor として用いられた時 ligation がほとんど進行しなかった。そこで今回著者は UCCGGC + CCCCCG + AACCA という組みあわせで行なった。Ligation 後の結合部位が正しい事



[図13] 17merの合成



[図14] 17merの合成経路

1' nearest neighbor
analysis で確認

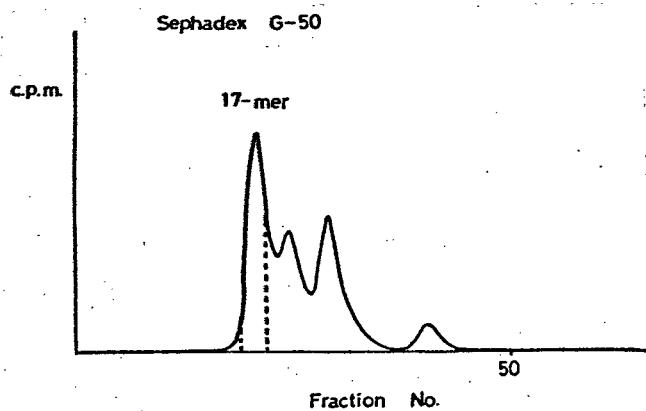
た。

3, 5 は 3' で
4 は CCCCCGCP,
AACCAp を termination
定量的に得られ
た。 1 + 2 の
ligation で 1 を

3 の 20~30 倍過剰に用いるとほぼ定量的に反応が進行した。
しかし、この条件で大量スケールで反応するには 1 の量が不足して
いたため上を 3 の 2.4 倍しか過剰に用いられず、HPLC による定量によると約 40% しか反応が進行しなかったことがわかった。

Sephadex G-50 による分離においても生成物 3 と原料 3 のピク
クが下、3' が分離せず、1 回目の分離で純粋な 3 の部分のみを
集め残りのピクク部分を再度 Sephadex G-50 で分離する操作を行
なった。3 は BAP 处理により 3' 末端リシン酸基を除去し、3 より
通算 33% の收率で生じ
得た。4 は 3 と ligation
により結合し Sephadex
G-50 で単離し 38%
の收率で 6 を得た。

図 15 は 6 を単離する
ために行なった Sephadex
G-50 の溶出パターン

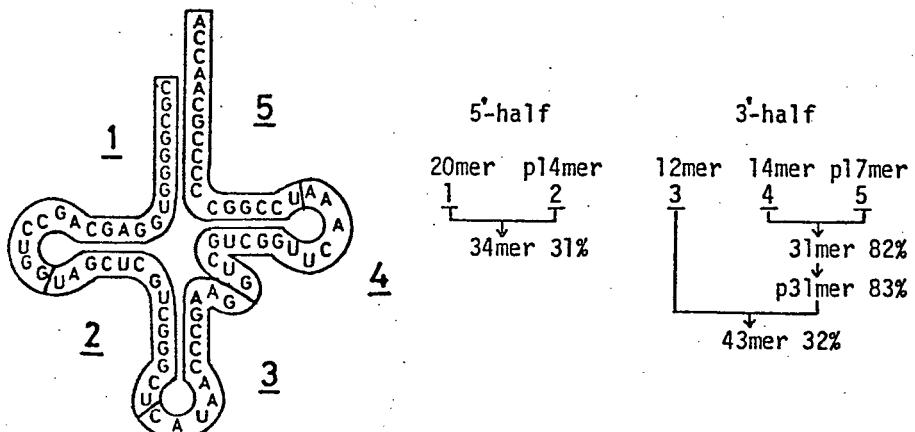


[図15] 17mer の精製

で、生成物 17mer を原料の 12mer, 5mer 及び 単離できました。

以上のように 17mer の合成ではすべて Sephadex G-50 による分離を行なったが、原料と生成物の鎖長(ただし末端のリノ酸残基は 1 鎖長の効果に相当する。)が相当異なれば分離は良いが、鎖長の接近 1-2 の分子同士では一度では分離できなかろうことは分離が不可能であることがわかつた。しかし回収率に關してはこのゲル沈過法が最も良と考えられる。なお、上と 2 の ligation について以後の実験より基質濃度、酵素濃度を共に上げれば ligation は良く進行し、7M urea 存在下イオン交換カラムクロマトグラフィーにより 65% の收率で 3 を得ることができた。(実験の部参照)

第四節 5' 半分子 (34mer: bases 1-34) 及び 3' 半分子 (43mer: bases 35-77) の合成



[図16] 5' 半分子 及び 3' 半分子 の合成

図16 1-示す様に 5' 半分子 は 20mer + 14mer, 3' 半分子 は 12mer

+ (14mer + 17mer) or ligation 1 = より それぞれ 合成 1 T=。

1) 5'半分子の合成

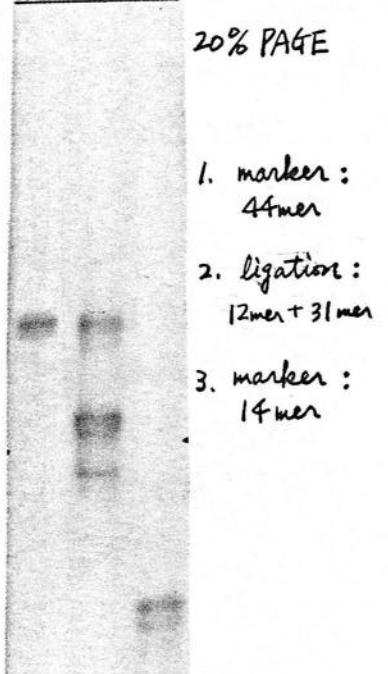
Acceptor 20mer (1) は以前に当教室で合成されたフラグメント¹⁴⁾を用い、donor p14mer (2) は第一節で合成 1 T= 14mer を termination しこれを用いた。Donor 分子に対し acceptor 分子が過剰の方が反応条件と 1 2 は好み いが acceptor 20mer の量が限られていたため逆に donor 分子を 1.5 倍過剰に用い反応した。反応後この反応液に直接 BAP を加え末端のリニン酸基を除去 1 T= 後 (全分子合成の acceptor とするため) nitrilotriacetic acid (NTA) を加え (最終濃度を 5 mM に調整) 酵素を失活させ 20% PAGE で分離 1 T=。生成物はゲルより抽出 31% の收率で 5'半分子を得た。

0.1 2 3

2) 3'半分子の合成

3 の 12mer と 4 の 14mer は第二節で合成 1 T= フラグメント 2 14mer は 図 10 の 11 を BAP 处理 (調整 1 T=)。5 の p17mer は第三節で合成 1 T= 17mer を termination 1 調整 1 T=。

まず 4 + 5 の ligation を行ない、Sephadex G-50 で単離 82% の好收率で 31mer を得た。次に 2 の 31mer を termination 1 Sephadex G-50 で ATP を分離 1 T= 後 3 と ligation 1 T=。反応の進行を 図 17 に示す様に 20% PAGE で調べた後 Bio-gel A (0.5m) によると 43mer と



[図 17] 12mer + 31mer

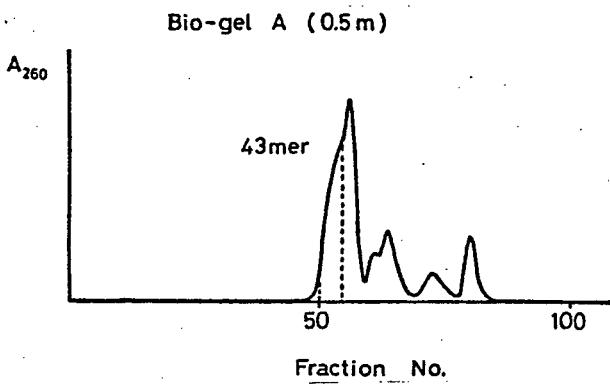
原料 p31mer の分離を試

みた。(図18) しかし

これらの 2 の C^o-T が
明確に分離せず p31mer が
少し混在する 43mer を図
に示す範囲で集めた。

20% PAGE で p31mer の
混在の程度を調べ (~30%)

純粋な 3' 半分子と 12 は p31mer から 32% の收率で反応し集め
られていることがわかった。次に混合物のまま fraction を行な
う Sephadex G-200 で精製。この操作でほとんどの混在する
p31mer を除くことができた。しかし 43mer を分析したところ
若干の 3' 末端 A の欠除した 42mer が混入していることがわかつ
た。これは RNA ligase の逆反応が原因と考えられた。RNA ligase
の逆反応は基質に対する酵素を過剰に用いると生じやすいが、今
まで用いてきた条件下ではほとんどの生じてはいなかった。今回初めて検
出された。この 43mer については次の全分子合成後に ATP-
(CTP): tRNA nucleotidyl transferase により 3' 末端が修復され
たためこのまま次の反応に使用できた。



[図18] 43mer の精製

第二章 Tψループを変換し T= E. coli tRNA^{Met}_f の合成

tRNA の Tψループは一部の tRNA を除いて共通の塩基配列 TψC が存在する。(図1)

原核生物の initiator tRNA はこの領域が共通配列と同じ TψC であるのに対し、真核生物では例外的に AUCG である。またこの Tψループは 5S ribosomal RNA と相補的な塩基配列を有することから tRNA の ribosome への結合に関するといふ報告¹⁷⁾もあり興味ある領域といえる。

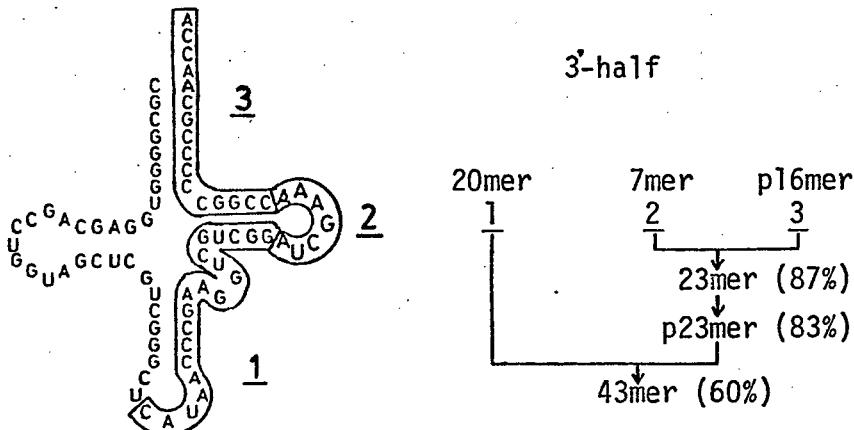
著者は E. coli tRNA^{Met}_f の Tψループを共通塩基配列 TψC から真核生物の initiator tRNA に特異的に存在する AUCG へ変換することを試めた。

第一節 Tψループに eukaryotic sequence を有する E. coli tRNA^{Met}_f の合成¹⁸⁾

E. coli tRNA^{Met}_f の Tψループの塩基配列 TψCAAAU を真核生物の initiator tRNA に存在する AUCGAAA (正確には AUCG_mAAA であるが化学合成の都合上 AUCGAAA と T=) に変換し T=。

1) 3' 単分子 (43mer) の合成

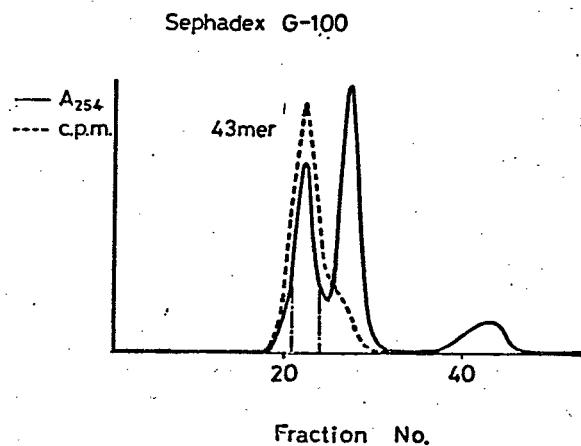
図19に示す様に化学合成フラグメント 1, 2, 3 より 3' 単分子を合成した。3 は 16mer を formation 1 でリニン酸化 (p16mer) と 1' ト分子で 1 はじめに Tψループ sequence 2 と ligation した。反応後 Sephadex G-50 で分離し 87% の好収率で 23mer



[図19] 3'半分子の合成

を得た。次に 23mer を formation 1 p23mer と 1 で後 20mer (1) と ligation 1 で。図20は示す様に Sephadex G-100 で分離し、60% の収率で目的の 43mer を得た。図21にこの時の ligation の進行を調べて 20% PAGE のパターンを示すが 3'半分子が生成していることが確認できた。各 ligation 後は nearest neighbor analysis で結合を確かめ、20% PAGE で鎖長を確認した。

第一章の nascent strand tRNA_f^{Met} 合成の場合、短鎖フラグメントから出発し何度か formation, ligation をくり返してが、今回の様に長鎖化合成フラグメントを用いると短期間にしかも比較的容易に目的物を得ることができた。(ただし、



[図20] 3'半分子の精製

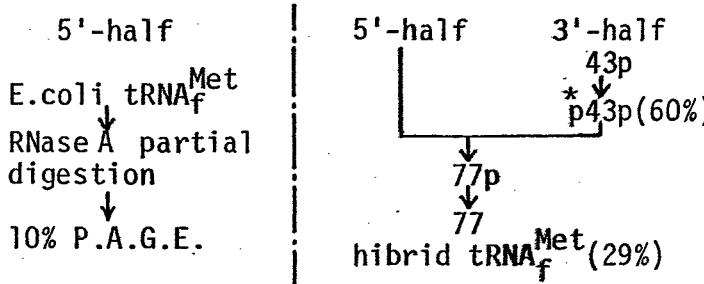
化學合成の立場からいへばこの様な長鎖オリゴマーの合成には多大の時間と労力を要する。)

2) 全分子の合成

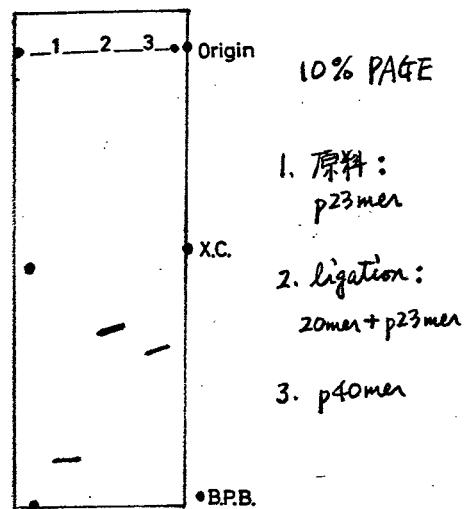
図22に示す様に全分子の合成を行なう。す。5'半分子は *E. coli* tRNA^{Met}_f を RNase A で限定分解^{(19), (20)}することにより調整す。す。得られた5'半分子は塩基配列を2次元不モクロマトグラフィーで確認後 3'末端のリソ酸基を BAP 处理で除去し ligation の acceptor とす。す。 (BAP 处理の前 1 = 3' 末端環状リソ酸を完全に開裂す) す。ため 0.1 N HCl で 4°C 14 時間反応させた。)

Donor 分子については 1) で述べた 3'半分子を formation し Sephadex G-50 を用い ATP から分離することにより調整す。す。半分子同士の ligation は acceptor を donor の約2倍過剰に用いて反応し、10%

Synthesis of 77mer

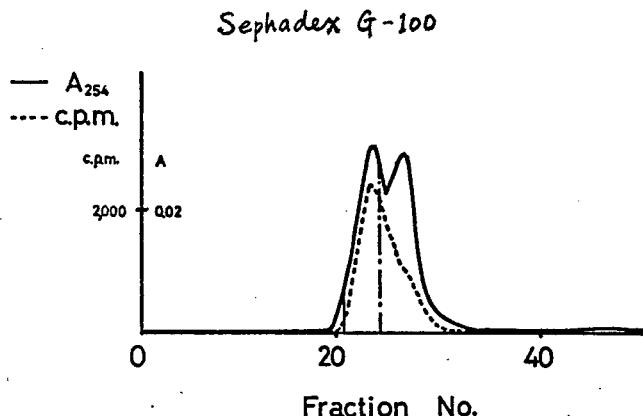
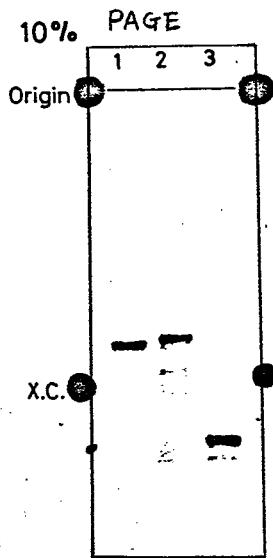


[図22] 全分子の合成



[図21] 20mer + p23mer

PAGE で反応の進行を確認した。(図23)
反応混合物を フィルター処理で除タンパクし、Sephadex G-100 で脱塩し E で BAP 处理を行なう。す。



[図24] 全分子の精製

[図23] 5'半分子 + 3'半分子

1. marker: 75mer
2. ligation: 34mer + p43mer
3. 原料: p43mer

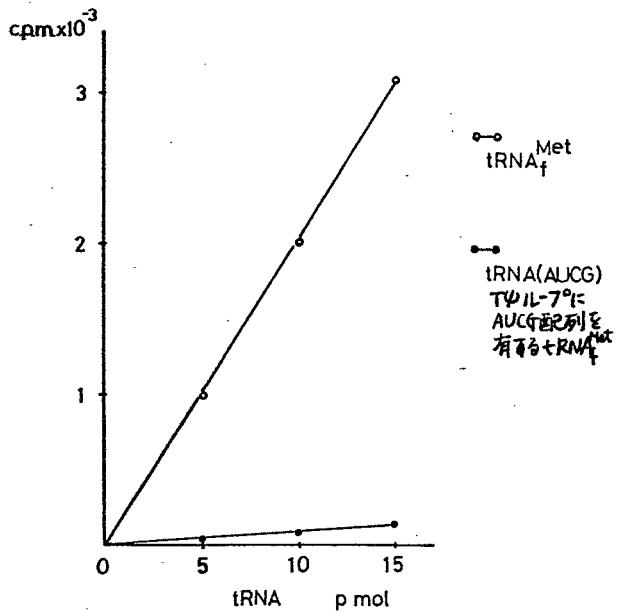
BAP処理後 再びフェリール処理で除タンパクし、全長約1mの Sephadex G-100 で生成物と原料の分離を試みた。図24に示す様に 2ピーカーに分かれながら集めたフラクションのチェックを 10% PAGE で行なうと 23% 若干の短鎖生成物が混入している。その比率の測定から 9.3% の收率で目的の全分子を得たことがわかつた。Nearest neighbor analysis による結合部位の確認においても CmUp が 2次元 TLC²¹⁾ により検出され半分子同士が結合していることがわかつた。

次にこの分子のメチオニン残基活性を *E. coli* の粗酵素系²²⁾ (核酸成分を除去した S-100 フラクション) を用いて調べた。アミノアシル化後反応液をグラスフィルターにスポットし 冷 5% trichloroacetic acid (TCA) で洗浄すると系に加えられた [³C]-メチオニンのうち tRNA と結合したメチオニンのみがア

Aminoacylation

ルター上に残る。この放射能をアミノ酸受容活性として測定した。合成したtRNAは図25に示す様にほとんどメチオニンを受容できず天然のE.coli tRNA^{Met}に比べ4%の活性しか示さなかった。これはこのtRNAが安定な三次構造をとれないとめにこのような結果が生じたと思われる。Dループと塩基対を形成すると考えられるTψループ。

(TψCAAUAU)のψC配列はこの合成tRNAではUCに変換されているがこの配列でもDループと塩基対を組むことができる。しかしTψループ内でのT-A塩基対(TψCAAUAU)は合成tRNAの場合(AUCGAAA)では組めない。この様な三次構造上の相異がtRNAを不安定化しMetRSaseとの相互作用を弱めたと考えられる。又この3'单分子の可変ループのm⁷Gが合成の都合上非修飾のGでありこのことも影響しているかもれない。



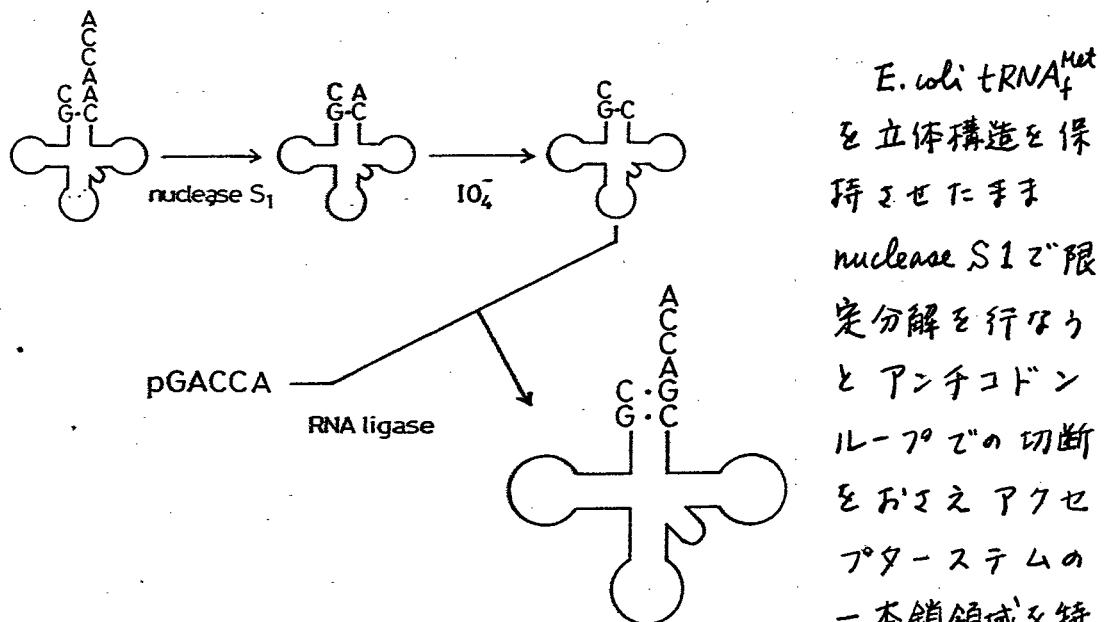
[図25]メチオニン受容活性

第三章 アクセプターステムを変換した *E. coli tRNA_f^{Met}* の合成

tRNAのアクセプターステムはその3'末端にアミノ酸が直接結合する部位でタンパク質合成に関与する種々の因子との相互作用が考えられる。

一般にtRNAはその5'末端で塩基対を形成しているが、原核生物のinitiator tRNAだけは形成していない。ポリペプチド鎖延長因子EF-Tuはinitiator tRNAよりもelongator tRNAにより強く結合する²³⁾といわれ、これはtRNAの5'末端の塩基対に依存すると思われる。Schulmanらはこれを確かめるために5'末端塩基をCからUに化学修飾し塩基対が組めるように変換した。そしてEF-Tuとの結合実験を行ない結合能力が上昇したと報告している。²⁴⁾しかしこの化学修飾の方法では5'末端のみならず他の部位にも修飾が生じている可能性があり5'末端の特異的な影響はわからず。そこで著者はこの5'末端で塩基対が形成できるような特異的tRNAを合成し、EF-Tuとの結合を調べた。我々の研究室ではすでに5'末端CをUに変換した5'末端から鎖長20のRNAを合成し、天然のその部分が欠除した*E. coli tRNA_f^{Met}*との再構成を行ないEF-Tuとの結合が増加したという報告を行なった。著者は再構成によるのではなくRNA ligaseによる結合反応を用いて *tRNA_f^{Met}*の5'末端塩基Cに対応する3'側フラグメントAをUに変換したtRNAを合成し、同様のEF-Tuとの結合実験を行なった。

第一節 5'末端に塩基対を形成する $E. coli$ tRNA^{Met} の合成



[図26] 5'末端に塩基対を形成する tRNA^{Met} の合成

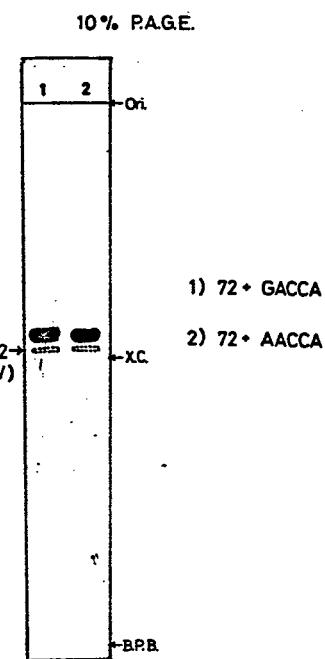
$E. coli$ tRNA^{Met} を立体構造を保持せたまま nuclease S₁ で限定分解を行なうとアンチコドンルート²⁶の切断を防ぎえアクセプターステムの一本鎖領域を特異的に切断し、

3'末端 ACCA の欠除した tRNA が得られる。²⁶⁾ この tRNA をさらに修飾塩基が障害を受けない条件で 3'末端シスジオールを過ヨウ素酸で酸化し β 脱離をおこすと 3'末端の一塩基が除去できる。²⁷⁾ この分子と化学合成オリゴマーとを結合せると 5'末端に塩基対を有する tRNA が得られる。(図26) 著者はこのルートに従って tRNA 変換体を合成した。Nuclease S₁ 限定分解により得られる四塩基欠除した tRNA は我々の研究室で調整された試料を用いた。²⁶⁾ この tRNA を pH 5.2 の条件下 NaIO₄ で遮光下 4°C, 2 時間反応させた。次に過剰のラムノースを加えさらに遮光下 4°C, 0.5 時間反応し未反応の NaIO₄ を不活化した。エタノール沈殿により脱塩した後 HCl で pH を 9.0 に調整しリジン溶液を用い遮光下 室温(約 20°C), 3.5 時間反応し

β 脱離をおこし T_c 。エタノール沈殿後 BAP 処理を行ない除タンパクした後 10% PAGE で精製した。この分子 (72mer) の 3' 末端分析を行なうと T_c が C であり A が除去されていることが確認できた。

化学合成オリゴマー GACCA と AACCA は kination 後 それぞれ Sephadex G-25, Sephadex G-50 で単離し次に ligation の donor とした。この 5mer と 72mer を ligation し 10% PAGE で単離して目的の全分子を得た。(図27)

Nearest neighbor analysis で結合部位を確認した後 アミノアシル化反応を行なうと T_c が、塩基対を組むように GACCA に変換した tRNA がもとの配列を有する AACCA と同じメチオニン受容活性を示した。このことから tRNA_f^{Met} と Met RSase との相互作用にはこの位置の塩基対は影響しない事がわかった。



第二節 塩基対を形成した変換体と EF-Tu・GTP の相互作用

塩基対を形成した tRNA 変換体 (tRNA_f^{Met} (C-G)) は kination により 5' 末端をリン酸化 (Sephadex G-50 で単離した後 [¹⁴C]-メチオニンを用いてアミノアシル化を行なうと T_c 。 [¹⁴C]-Met-tRNA_f^{Met} (C-G) を低温下 Sephadex G-50 でメチオニン

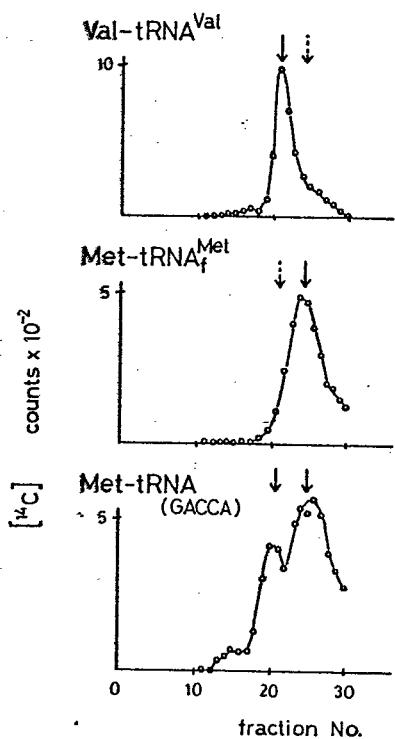
より分離し、結合実験に用いた。

コントロールとして 5' 末端に塩基対を組んでいる $E. coli$ tRNA^{Val} と [^{14}C]-Val-tRNA^{Val} として、塩基対を組んでいない $E. coli$ tRNA^{Met} と [^{14}C]-Met-tRNA^{Met} としてそれをお用いた。 $E. coli$ から精製された EF-Tu を用い [3H]GTP binding により EF-Tu の活性を測定した。

結合反応は EF-Tu-GDP を EF-Tu-GTP に変換した後、アミノアシル tRNA を加え $0^\circ C$ 、5 分間のインキュベーションを行なった。反応終了後 低温 ($4^\circ C$) 下 Sephadex G-100 によるゲル沈過を行ない三重複合体を形成しているか否かを調べた。もし三重複合体を形成していれば EF-Tu の分子量が 43,000 dalton であることから、複合体の Sephadex G-100 における溶出位置がアミノアシル tRNA のそれよりも先行する。Sephadex G-100 の溶出フラクションをグラスフィルターにスポットし ^{14}C のカウントを測定することによりこれらの溶出位置を検出した。

図 28 に示す様にコントロールの Val-tRNA^{Val} は三重複合体を形成し早く溶出されるが、Met-tRNA^{Met} は形成できず遅れて溶出する。合成により塩基対を形成した Met-tRNA^{Met} (C-G) は Val-tRNA^{Val} のようにすべて三重複合体を形成することはでき

Sephadex G-100



[図 28] アミノアシル tRNA と EF-Tu·GTP の相互作用

なかつたが 嘌基対を生じたことにより確かに EF-Tu と相互作用することがわかつた。

以前に我々の研究室で行なわれた A-U 嘌基対を有する $tRNA_f^{Met}$ ²⁵⁾ 再構成分子の実験においても同様の結果であった。

以上のことから A-U 嘌基対であれ G-C 嘌基対であれ 5' 末端での 嘌基対は $tRNA$ の EF-Tu・GTP との三重複合体形成能に影響していることがわかつた。しかし その寄与の程度に関して $tRNA_f^{Met}$ の EF-Tu に対する解離定数は elongator $tRNA$ の場合に比べ数倍程度（かちがわない^{28), 29)} といふことから この 嘌基対形成は EF-Tu との相互作用に対する本質的ではないかも知れない。

EF-Tu は 5' 末端以外に $tRNA$ 全体の構造も認識していると考えられるので 5' 末端の 嘌基対の有無による数倍程度の親和性の差をどのように評価するかは問題である。

第四章 アンチコドンループを変換した *E. coli* tRNA_f^{Met} の合成

mRNAの情報を読み取るtRNAのアンチコドン部はtRNAが機能するための不可欠な領域でありtRNA研究上の興味ある標的といえる。mRNAと塩基対を組むアンチコドントリプトットはtRNAの種類により異なるが、アンチコドンの5'側隣接位はほとんどすべてUであり3'側隣接位はPu又はPu誘導体がその位置を占める。*E. coli* tRNA_f^{Met}の場合 アンチコドンの5'側隣接位は一般的なUであるが3'側隣接位に関しては修飾されていないA^{2'}であり、これはアンチコドンの3字目がUのtRNAではこの位置が修飾されていらないのに比べ例外的である。

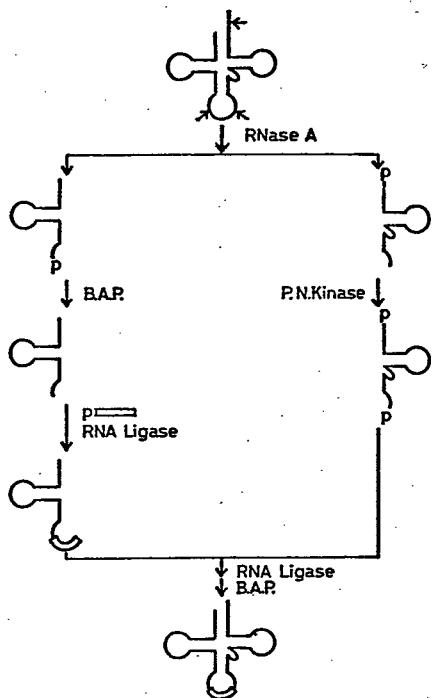
アンチコドントリプトットに関して天然に存在するサブレッサーtRNAは多くはこの部分に変化が生じたとえば終止コドンを読めるようになり活性を示す。これらのtRNAはアンチコドンが変化してもアミノ酸を受容できる。

*E. coli*のinitiator tRNAについてアンチコドンCAUをamber, opalに対応するアンチコドンに変換したtRNAを合成し、*E. coli* ARSaseによりアミノ酸受容活性を調べたがいずれも受容しなかった。²⁰⁾ tRNA_f^{Met}をサブレッサーtRNAに変換する試みはこの系においては成功しなかった。他のelongator tRNAを用いた人工サブレッサーtRNAを合成する試みがなされたがこのtRNAでは活性を示した。³⁰⁾ これはtRNAのアンチコドン部を変化させてもARSaseに認識されたためでtRNAやARSaseが異なればその認識の仕方が異なることを示す。

著者は $E. coli$ $tRNA_f^{Met}$ のアンチコドン部を種々変換して $tRNA$ を合成し Met RSase との相互作用を調べ $tRNA_f^{Met}$ のアンチコドン部の働きについて詳しく述べを行なった。

第一節 アンチコドンの隣接位に付加塩基を有する

$E. coli$ $tRNA_f^{Met}$ の合成³¹⁾



[図29] アンチコドン変換体の合成経路

$E. coli$ $tRNA_f^{Met}$ の場合 そのアンチコドントリプトレットが他の配列に変化するとメチオニンを受容し得くなる。²⁰⁾ そこで著者は CAU というトリプトレットは含んでいなければ拡大されたアンチコドンループを有する $tRNA_f^{Met}$ を合成し、この $tRNA$ が $E. coli$ Met RSase にいかに認識されるかを調べた。

図29に全体の合成経路を示した。

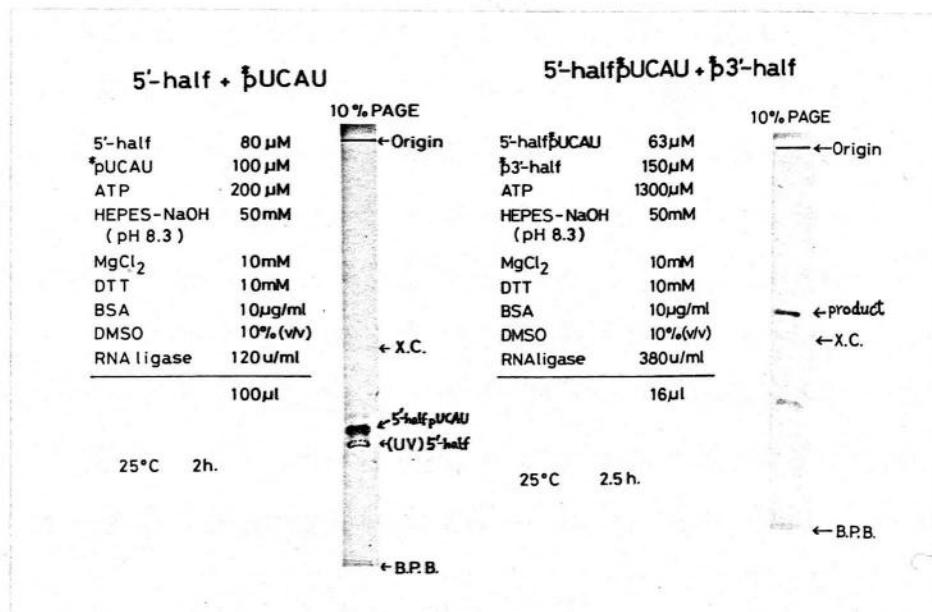
まず 天然の $E. coli$ $tRNA_f^{Met}$ を RNase A に作用させた結果²⁰⁾ 5'側半分子 (1-34) と 3'側半分子 (38-75) を 10% PAGE で単離した。

5'側半分子については BAP 处理を行ない 除タンパク、脱塩後、化学合成により得られたアンチコドン部オリゴマー (UCAU, CAUA, CAAUA) と ligation し 10% PAGE で付加塩基を有する 5'側半分子を単離した。3'側半分子については IF kinase 作用後 Sephadex

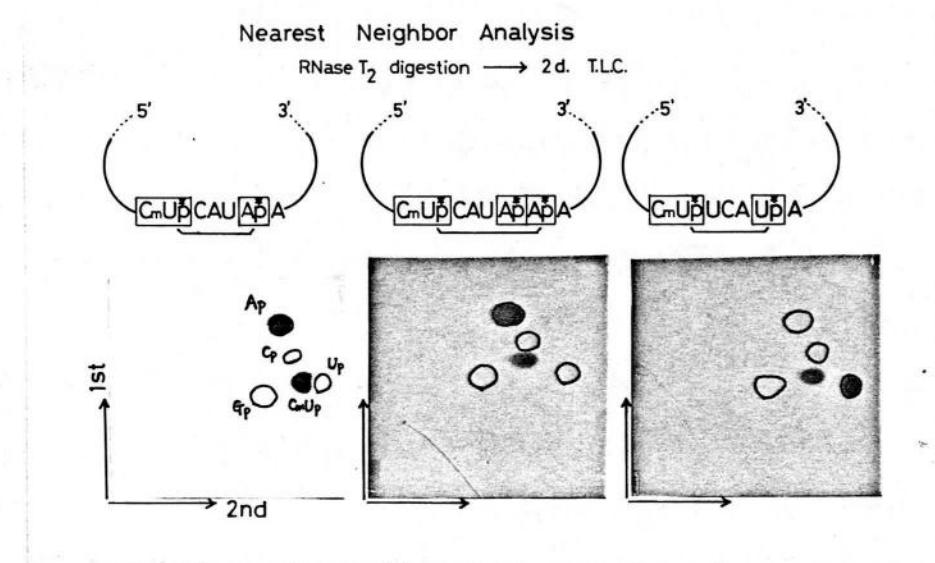
G-50で"ATPから分離し、先ほど得られた5'側半分子と ligation し T_c。10% PAGE で"分離し、酸処理(α IN HCl で4°C, 12時間反応), BAP処理を行ない 3'末端CCA配列のうち CAの欠除しを目的の tRNAを得た。この CA部分は後に *E. coli S-100* フラクション中に含まれる ATP(CTP):tRNA nucleotidyl transferase で 5'CCAに修復される。

図30は一例と 12 CAUとUCAUを変換したtRNAの合成における ligationの条件と PAGEのオートラジオグラフを示す。他の2種についても全く同様のパターンを示す。

各 ligation後は nearest neighbor analysisを行ない結合部位を確認した。(図31)



[図30] アンチコドン CAUとUCAUを変換したtRNAの合成 (ligation条件と PAGE)



[図31] アンチコドント付加塩基を有するtRNAのnearest neighbor analysis

5'側半分子と合成オリゴマーとの ligation では收率はあまり良くないが(単離收率 11~25%), 半分子同士の ligation では收率良く進んだ。(単離收率 39~49%) これは前者の反応において donor の合成オリゴマーの 3'末端がバリン酸基で保護されていて、そのため わずかではあるが重合体が生じた事と、後者の反応において 半分子同士が 3次構造を組み、結合部位が接近したため ligation が良く進行した事が考えられる。

当初 この tRNA の合成順序として 合成オリゴマーを用いて 3'側半分子と結合させる事を計画したが、この場合ほとんど ligation が進行せず、図29に示す様な合成経路に変更した。

第二節 アンチコドンに付加塩基を有する合成tRNAの 生化学的性質³¹⁾

第一節で合成したtRNAについて 1)アミノアシル化反応, 2)ホルミル化反応, 3)リボソーム上でコドンの認識を調べた。

1)アミノアシル化反応

E. coli 粗酵素系 (S-100 フラクション) を用いてメチオニン受容活性を調べた結果 天然の E. coli tRNA^{Met} に比べ UCAU配列を有する tRNA (tRNA(UCAU)) では 53%, tRNA(CAUU) では 25%, tRNA(CAUAA) では 13% の活性を示した。

次に 図32 に示す様に K_m , V_{max} 値を求めた。まず合成tRNAの 3'末端を CCA に修復した後 ^{14}C -メチオニンを加え反応を開始し、一気時間後 グラスフィルターにスポットし 酸不溶性放射能を測定し

Aminoacylation

tRNA	0.15~2.0 μ M
CTP	0.8 mM
ATP	4 mM
HEPES-NaOH (pH 8.0)	100 mM
Mg(OAc) ₂	10 mM
KCl	10 mM
B-ME	10 mM
S-100	1 mg/ml

↓ 37°C, 30 min.
↓ 100°C, 2 min.

^{14}C -Met	18 μ M
S-100	0.5 mg/ml

10 μ l

↓ 37°C, 20 min.

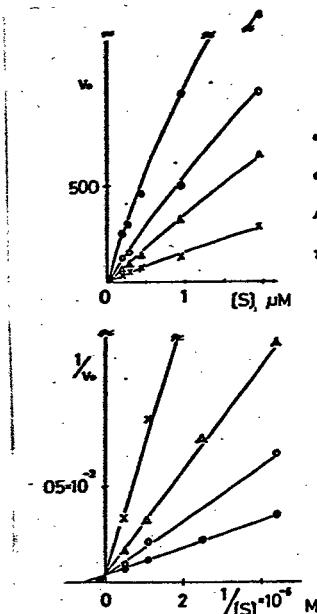


図32の右下に
Lineweaver-Burk
プロットを示した
が、このグラフか
ら V_{max} 値はいつ
れもほぼ同じであ
るのにに対し K_m 値が
tRNA^{Met}, tRNA(UCAU),
tRNA(CAUU), tRNA-
(CAUAA) の順に増大

[図32] アンチコドンに付加塩基を有するtRNAの aminoacylation (K_m , V_{max} study)

1) Met RSase との親和性がこの順に減少してゐる事がわかつた。
 K_m 値はそれぞれ $1.7 \mu M$, $3.3 \mu M$, $6.7 \mu M$, $12.5 \mu M$ である, したがつて。

次にこれらのおtRNA が他のアミノ酸を受容するか否かを 16 種のアミノ酸 (Ala, Val, Leu, Ile, Phe, Pro, Gly, Ser, Thr, Tyr, Asn, Asp, Glu, Lys, His, Arg) を用い検討したが、いずれのアミノ酸も $1.7 \mu M$ TM_3 / アシルtRNA は検出できなかつた。

2) ホルミル化反応

Transformylase³²⁾ は原核生物の initiator tRNA と特異的に反応する酵素で、その認識部位についてはほとんどわかつてない。

合成した 3 種の tRNA に $1.7 \mu M$ Methionyl-tRNA がホルミル化を受けるか否かを調べた。反応条件はアミノアシル化の条件とほぼ等しく、ホルミルドナーとして folic acid-SF を加え E. coli S-100 フラクションで反応した。反応後 硫酸銅で処理し、ホルミル化を受けなかつた Met-tRNA を deacylize した。硫酸銅で処理したフラクションとしなかつたフラクションのカウントの比をホルミル化の比として百分率で表わし、tRNA(UCAU) $\rightarrow 93\%$, tRNA(CAUU) $\rightarrow 88\%$, tRNA(CAUAA) $\rightarrow 98\%$ という結果を得た。アミノアシル化の場合と異なり transformylase はアンチコドン配列を認識していないことがわかつた。

3) リボソーム上でのコドンの認識

アンチコドンループを拡大したこれらの tRNA に $1.7 \mu M$, mRNA 上のコードを認識するか否かを調べた。

1964 年 Nirenberg らが RNA codeword を決定した時に用いた方法³³⁾に基づき行なつた。(図 33) mRNA と 12 合成オリゴマ - AUG, UGA, UUA, UAU を用い リボソーム上での特異

tRNA-mRNA interaction						
tRNA	0.5 μ M	mRNA	tRNA _f ^{Met}	tRNA _{CAUA}	tRNA _{CAUAA}	tRNA _{UCAU}
mRNA	25 μ M		AUG	100	0	0
Tris-HCl (pH7.5)	0.1 M		UGA	0	0	0
Mg(OAc) ₂	20 mM		UUA	0	0	3
KCl	50 mM		UAU	0	0	0
E.coli ribosome	1.8 μ M					
30S or 70S						
	20 μ l					
% relative to tRNA _f ^{Met} -AUG						
25°C, 30min.						
millipore filtration						

[図33] リボソーム上でのコドンの認識

的結合を測定した結果、
tRNA (UCAU) のみが
AUG と結合し他の合成
tRNA についてはいず
れも mRNA と結合しな
い。一方 tRNA (UCAU)
の AUG への結合も天

然の tRNA_f^{Met} の AUG への結合の約 1/3 である。

tRNA のアンチコドンと mRNA が塩基対を形成するためには、
アンチコドントリプトロートがループの外側に出て 3 塩基が並ば
なければならないが、これらのループを拡大した tRNA は何加
塩基の存在によりトリプトロート付近のコンホメーションが変化
(mRNA と塩基対を形成することができなくなる) たと考えら
れる。

以上の実験結果を基にしてまとめると、Met RSase との相互
作用について tRNA が認識を受けるにはアンチコドンの持た
立体構造と CAU という塩基配列が必要であることがわかつ
た。CAU の 5'側に U を何加した tRNA の方が 3'側に A を何
加した tRNA より Met RSase に対するより強い親和性を示したが、
これは CAU の 5'側に CAU のコンホメーションをある程度保
持したまま U が入りこめる余地が存在したためと考えられる。

このことは mRNA との塩基対形成実験においても tRNA (UCAU)
のみが AUG と塩基対を形成できた結果からも推測できる。

tRNA_f^{Met} のアンチコドン部はこの分子にとて機能を果た
すために非常に重要であることがわかつたが、一方ホルミル化

に関するところはこの部分は影響しないことを判明した。

第三節 アンチコドンの 5'側隣接位塩基変換³⁴⁾

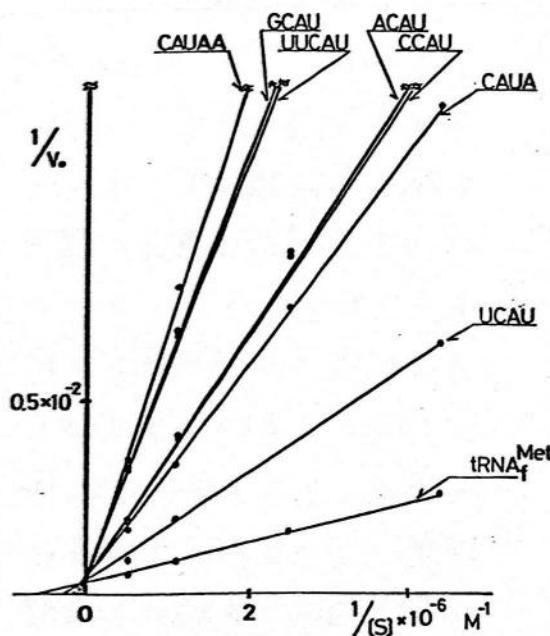
アンチコドンの 5'側隣接位に何か塩基 U が存在してもアンチコドン付近のコンヌメーションがあまり変化しないことが第二節で明らかになつた。そこでこの付加塩基 U を他の塩基 A, G, C, UU に置きかえた tRNA を合成し, MetRSase による認識は U の場合とどのように異なるか調べた。さらにアンチコドンの 5'側隣接位は tRNA に共通の U であるが、この塩基を特異的に他の塩基に変換した tRNA の合成も試み、そのメチオニン受容活性を調べた。

1) 5'側隣接位に U 以外の付加塩基を有する tRNA の合成

合成方法は第二節で用いた方法と同じく RNase A 限定分解より得られた天然の *E. coli* tRNA^{Met}_f 5'側半分子に化学合成オリゴマー - ACAU, GCAU, CCAU, UUCAU をそれぞれ RNA ligase で結合させ、最後にこの分子と 3'側半分子とを結合させて全分子を得た。結合部位が正しいことは nearest neighbor analysis により確認した。例として図 34 に CCAU の場合における ligation の条件と 10% PAGE のオートラジオグラフを示す。今回は合成オリゴマーの 3'末端がリン酸基で保護されていたため、5'半分子 + 合成オリゴマー + ligation においてポリメリゼーションは防ぐことができた。アンチコドンを有する 5'側半分子は 10% PAGE で単離し、BAP 处理によりリン酸基を除去し次の ligation の acceptor とした。

5'-half + β CCAUp		5'-half β CCAUp + β 3'-half	
5'-half	100 μ M	5'-half β CCAUp	50 μ M
β CCAUp	200 μ M	β 3'-half	125 μ M
ATP	500 μ M	ATP	1250 μ M
HEPES-NaOH (pH 8.3)	50mM	HEPES-NaOH (pH 8.3)	50mM
MgCl ₂	10mM	MgCl ₂	10mM
DTT	10mM	DTT	10mM
BSA	10 μ g/ml	BSA	10 μ g/ml
DMSO	10% (v/v)	DMSO	10% (v/v)
RNA ligase	170 u/ml	RNA ligase	375 u/ml
	40 μ l		16 μ l
25°C, 2h.		25°C, 2h.	
		← B.P.B.	
		← XC.	← XC.
		← 5'-half β CCAUp	← product
		← (UV) 5'-half	← XC.
		← B.P.B.	

[図34] アンチコドン CAU と CCAU に変換した tRNA の合成 (ligation の条件と PAGE)



合成した tRNA は 112 第二節と同様にメチオニン受容活性を測定し、今までのループを有する tRNA と K_m 値の比較を行なった。(図35)

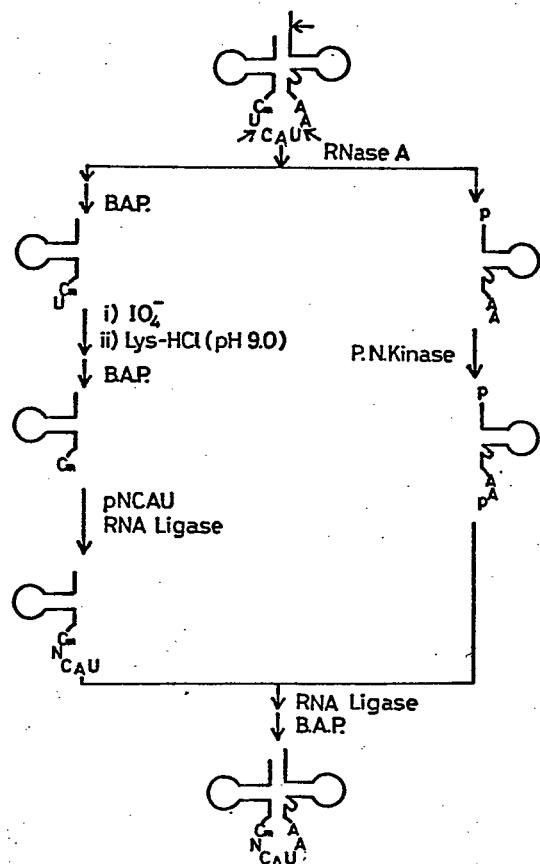
その結果 アンチコドンの 5' 側への付加塩基は U の時が最も Met R.S.ase との親和性が強く、他の C, A, G の場合には

[図35] aminoacylation (K_m , V_{max} study), テトラマー, ペンタマーはアンチコドン CAU をそれぞれのオリゴマーに変換した tRNA を示す。

3'側に A を付加した時と変わらないか それよりも弱い程度である, T. 2 塩基を付加した場合は 3'側, 5'側いずれも親和性は弱か, T.

2) 5'側隣接位に存在する共通Uの変換

天然の *E. coli* tRNA^{Met} と RNase A により限定分解し, 得られる 5'側半分子を Mg^{2+} に過ヨウ素酸酸化 (3'末端の共通Uの除去) を行なう, T. 過ヨウ素酸酸化は第三章で用いた方法と同様に行なう, T. 図36に合成経路を示す。当初 5'側半分子のみを過ヨウ素酸酸化し, 経路に従い合成したが 得られた tRNA はコントロ

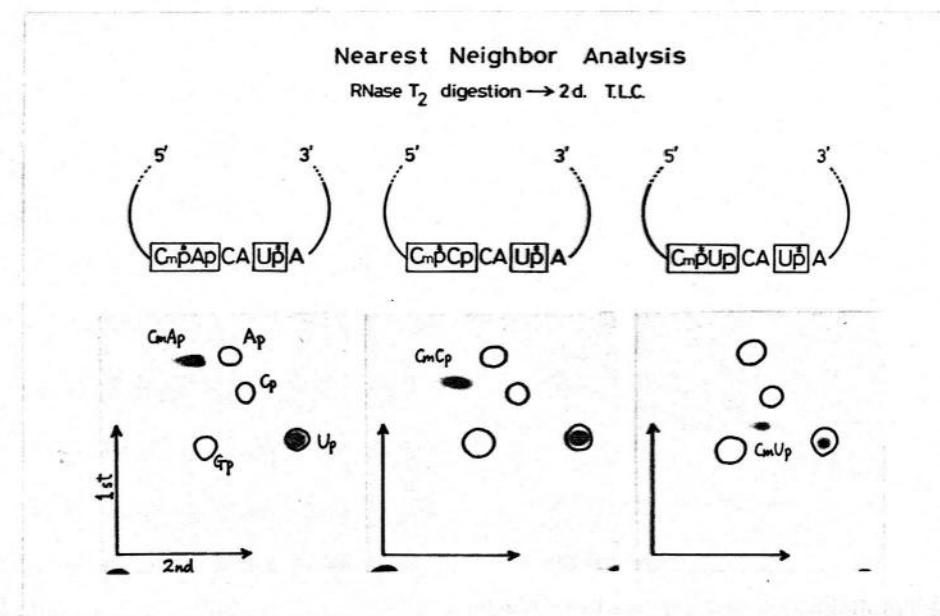


[図36] 共通U変換tRNAの合成経路

ールとして合成した天然と同等の塩基配列を有する tRNA であるも そのメチオニン受容活性をほとんど示さなかつた。これは 5'側半分子単独の場合, tRNA の様に三次構造をとることができず修飾塩基等が障害を受けてためと考えられる。そこで過ヨウ素酸酸化の時に当量の 3'側半分子を加え再構成させて (アンチコドンは欠いているが) tRNA の構造を保持させ過ヨウ素酸酸化を試めた。酸化後, BAP 处理を行なう 10% PAGE

で 3' 末端の一塩基を除去した 5' 側半分子を単離した。3' 末端分析を行なうのが除去できた事を確認した後 化学合成オリゴマーと ligation した。化学合成オリゴマー UCAU, CCAU, ACAU は formation 1 イオン交換クロマトグラフィーで単離した ligation の donor とした。Ligation 後 10% PAGE で単離した。酸処理 (0.1N HCl で 4°C, 6 時間反応) により 末端サイクリックリソリン酸を開裂した 3' 側半分子は formation 後 Sephadex G-50 による精製を経て アンチコドンを含む 5' 側半分子と ligation した。10% PAGE で 単離し BAP 処理を行なう目的の tRNA を得た。

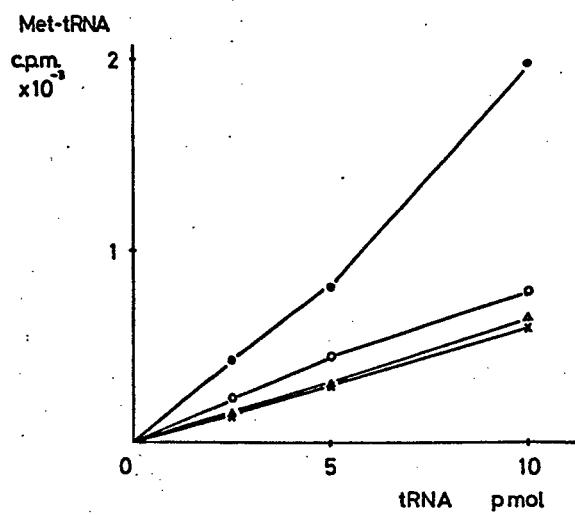
図37に全分子合成後の nearest neighbor analysis を示す。Donor 分子の 5' 末端に位置1 2 11 T= ^{32}P が acceptor 分子に移り, RNase T₂ digestion で □で囲んだモノマー, ダイマーが 2 次元 TLC により確認できた。最後の ligation で IF いずれか CAU の U



[図37] 共通Uを変換したtRNAの nearest neighbor analysis

由来の $U_{3'p}$ が検出されるが、5'側半分子と化学合成オリゴマーの ligation では 5'側半分子の 3'末端は 2'-O-メチル C (Cm) で、ここにオリゴマーが結合すると RNase T₂ では Cm の 3'側では切断されず ダイマー-Cm^{3'p}Np (N: A, C, U) で検出される。この Cm^{3'p}Np は各々 N の方がいにより二次元 TLC での位置が異なる。この差異により N を確認した。合成 I で tRNA についてメチオニン受容活性を測定した。その結果を図 38 に示す。アンチコドンの隣接位を U 以外の塩基に変換した tRNA においても U の tRNA と同等のメチオニン受容活性を示し、アンチコドンの 5'側隣接位の共通 U はアミノアシル化に影響を及ぼしていないことが判明した。

最近 Uhlenbeck らにより Yeast tRNA^{Trn} のアンチコドンとその 5'側隣接位 U を変換したサブレッサー tRNA が合成され translation ではこの U は本質的な役割を果たしていないことが示された。³⁵⁾ 今回の著者の実験も同様の結果を示しておりアミノアシル化の段階で差がないことを示す。



[図 38] 共通 U を変換した tRNA の aminoacylation

○—○ trnA^{Met}, ○—○ tRNA (CmU → CmU) control,

△—△ tRNA (CmU → CmC), X—X tRNA (CmU → CmA)

わらず完全に構造を回復できずには反応が進み

塩基に障害が生じたためと考えられる。

第四節 アンチコドントリプレットと種々の配列に 変換 I T= $E. coli$ tRNA^{Met} の合成

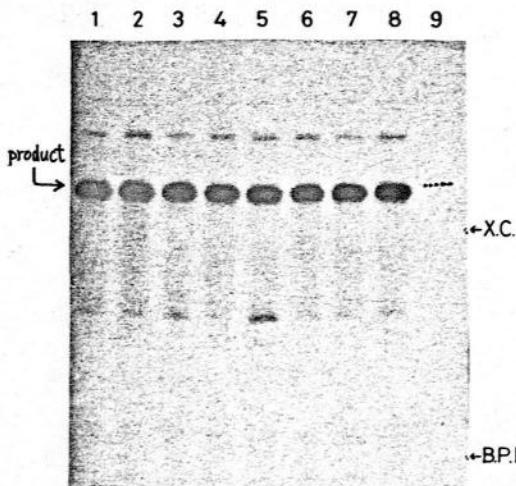
$E. coli$ tRNA^{Met} のアンチコドンの 3'側隣接位は例外的に未修飾の A である。 $E. coli$ 中で 3' は修飾されない tRNA^{Met} の 3' の A は真核生物の系では修飾されるか否か、又アンチコドンの配列により修飾の工方が変化するかどうか等を調べるためにアンチコドントリプレット CAU を CAA, CAC, CAG, CAU, GAA, GAC, GAG, GAU の各合成オリゴマーに変換 I T= tRNA を合成した。

合成方法は第一節、第三節で記述した拡大ループを有する tRNA^{Met} の合成の場合と同様に行なう。各トリマーの formation は非放射性の ATP を用いて行ない、3'側半分子の formation には

比活性の高い $[\gamma-^3P]$ -ATP を用いた。トリマーと 5'側半分子との ligation 後 10% PAGE で単離し 3'側半分子と ligation I T=。図 39 に半分子同士の ligation における 10% PAGE の泳動パターンを示す。

生成物をゲルより切り出し 1 サンプルを

10% PAGE



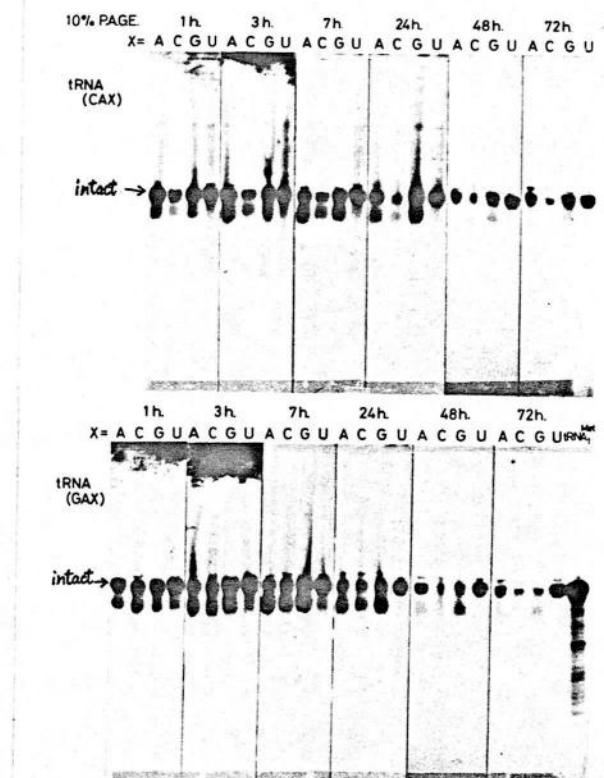
1. tRNA(GAA)
2. tRNA(GAC)
3. tRNA(GAG)
4. tRNA(GAU)
5. tRNA(CAA)
6. tRNA(CAC)
7. tRNA(CAG)
8. tRNA(CAU)
9. tRNA^{Met} (UV)

[図 39] アンチコドントリプレットを変換 I T= tRNA^{Met} の合成

$T=4$ ^{32}P 2" 10^6 cpm 以上のカウントを有する tRNA を得た。

第五節 アニチコドン変換体の *Xenopus laevis* oocyte 中にみける生化学的性質

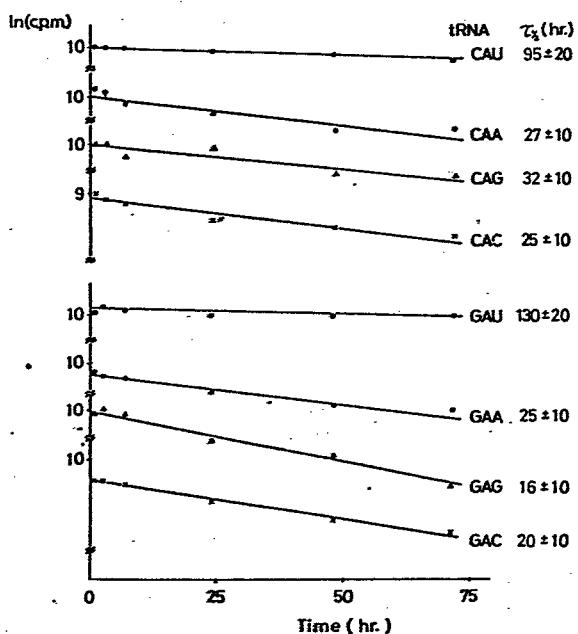
Xenopus laevis oocyte (アフリカツメガエル卵母細胞) は, mRNA の翻訳が非常に効率良く行なえる系³⁶⁾として多くの研究者に利用されている。この系を用いると容易に *in vivo* の実験が行なえ, 後処理も比較的簡単であるため著者はこの系を用い修飾の実験を行



[図40] oocyte 中に injection したアンチコドン導入体 (インキュベーション時間ごとの PAGE) を示す。

第四節で合成した 8 種の tRNA を *Xenopus laevis* oocyte の細胞質に microinjection (T. 一定時間過ぎに反応を止め oocyte を破碎し 除タンパク後 10% PAGE に apply し tRNA の安定性を調べた。 図 40 にその泳動ペターンを示す。 過剰の核酸成分のため泳動が乱

Stability of tRNAs in oocytes



[図41] アンチコドン変換体の oocyte 中に
おける安定性

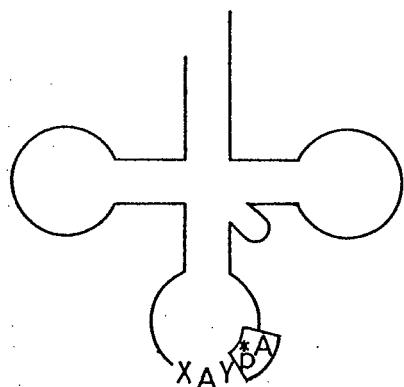
たとえば、intact tRNA と
3~4 塩基切断の生じた tRNA
のバンドが検出された。

Intact tRNA のバンドを
切り出し、そのカウントを測
定してこれらの tRNAs の
oocyte 中における安定性に
ついて調べた。(図41)

その結果アンチコドント
リフロレットが CAU, GAU
の tRNA は 3~4 塩基切断
されたバンドがほとんど検
出されず非常に安定で、そ
れぞれ半減期 $\tau_{1/2}$ が 95 ±

20 時間、130 ± 20 時間である。それに対し他のアンチコドントリフロレットを有する tRNA はいずれも切断物が検出され $\tau_{1/2}$
が 20~25 ± 10 時間と不安定である。
次に切り出したメインバンドか
ら tRNA を抽出し、エタノール沈殿

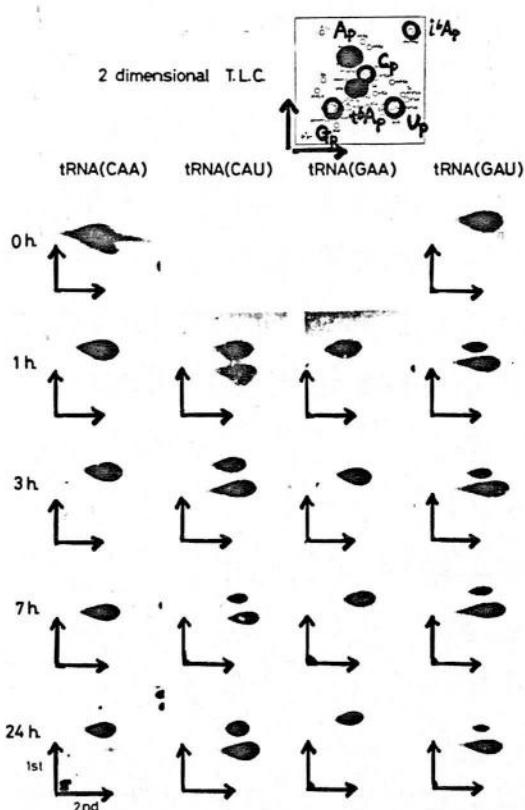
(キャリアーと 12 Yeast tRNA を約
0.2 A₂₆₀ unit 加えた) を 2 回行ない
脱塩した後、nuclease P₁ による完全
分解を行なう。反応後サンプルを
2 次元 TLC 上で展開し、³²P N の位置
を確認した。図42 からわかる様に



[図42] アンチコドンの 3'側隣接位
の修飾

X: C, G, Y: A, C, G, U

^{32}P のラベルは ^{32}pA と $^{32}\text{pA}^*$ (A^* は修飾された A) と 12 TLC 上に検出された。図43は2次元TLCの一部を示すが、Aが修飾が生じるのは tRNA(CAU) と tRNA(GAU) のみで他の tRNA に関する何ら修飾を受けなかつた。又 tRNA(CAU) と tRNA-(GAU) の場合、いずれも A は $t^6\text{A}$ に修飾を受けている。一般にアンチコドンの3字目が U である tRNA では 3'側隣接位の A はほとんど $t^6\text{A}$ に修飾を受けることから予想どおりの結果である。又、アンチコドンの3字目が A である tRNA では 3'側隣接位 A は $i^6\text{A}$ などに修飾を受けるが、この tRNA(CAA) と tRNA(GAA) の場合、全く修飾を受けない。これは $i^6\text{A}$ に修飾を受けた tRNA はアンチコドンシステムの最もループ寄りの塩基対が A-U であるのに對し tRNA_f^{ret} の場合 G-C であるため修飾酵素に認識されなかつたとも考えられる。



[図43] アンチコドンの3'側隣接位Aの同定

Xenopus laevis oocyte 中の tRNA の修飾に関する E. coli 中ではアンチコドンの3'

側隣接位Aが修飾されるE. coli tRNA^{Met} 2' 3' oocyte 中では他のアンチコドンの3字目がUであるtRNAと同様このAがt⁶Aに修飾されることがわかった。Xenopus laevis oocyte 中に存在するt⁶A修飾酵素はE. coli の場合と異なり全体のtRNA構造を認識するのではなくアンチコドンの3字目の塩基に影響されることがわかった。E. coli のt⁶A修飾酵素の場合, tRNA^{Met} のどの部分を認識して修飾を行なうかは不明である。

次にXenopus laevis oocyte 中のtRNAの安定性に関するでは、今回初めてアンチコドンの配列がtRNAの安定性に影響を及ぼすことが確認された。アンチコドン配列と1' CAUとGAUを有するtRNA^{Met}のみが安定で他の配列のtRNA^{Met} 2' 3' は不安定である。これは修飾を受けたために安定になるためとは考えられる。なぜなら tRNA(CAU), tRNA(GAU)は修飾を受けたがこれら分子すべてが修飾を受けたわけではなく未修飾の分子も存在し、それでもなお安定性を保っているからである。

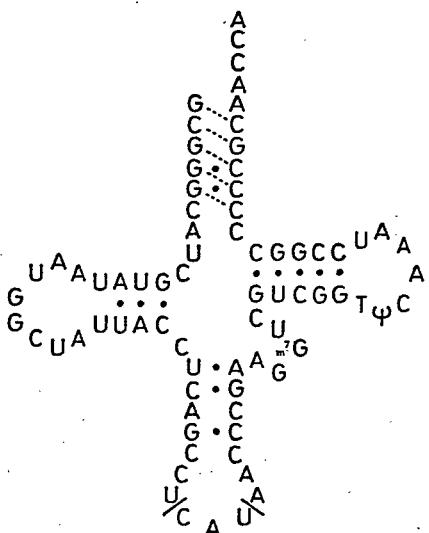
この場合、アンチコドンの3字目により安定性が規定されるが、なぜXenopus laevis oocyte 中でE. coli tRNA^{Met} の位置が1'の分子が安定で他の分子が不安定であるかは不明である。

第五章 5'側半分子を変換 I Tc E. coli tRNA_f^{Met} の合成³⁷⁾

5'側半分子に E. coli tRNA₂^{Gly}^{38) a)} の塩基配列を有する tRNA_f^{Met} (tRNA(Gly-Met)) と 3'側半分子の tRNA^{Ser}^{38) b)} などに 2つされる DIL-70 リストムの欠陥 I Tc tRNA_f^{Met} を合成し、そのアミノ酸受容活性を調べた。

第一節 5'側半分子に E. coli tRNA₂^{Gly} の塩基配列を有する E. coli tRNA_f^{Met} の合成

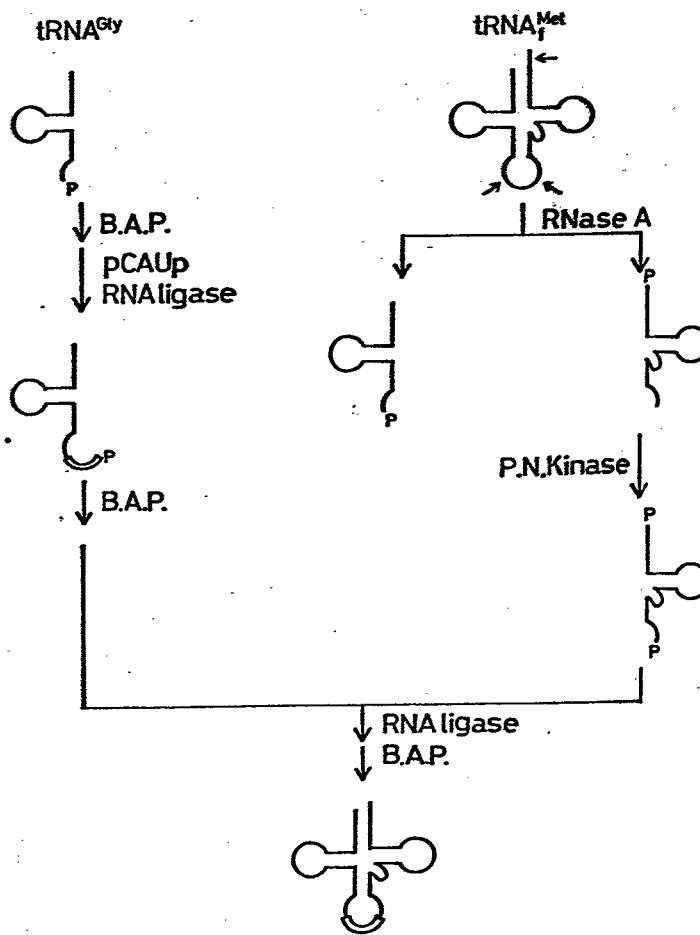
E. coli tRNA₂^{Gly} は修飾塩基が少なく 5'側半分子には全く含まれていない。このことはこの分子を化学合成する上で非常に有利な点で、最近アンチコドンを含まない E. coli tRNA₂^{Gly} の 5'側半分子 (33mer) の化学合成が試みられ成功した。³⁹⁾



[図44] tRNA(Gly-Met)

著者はこの合成された tRNA₂^{Gly} の 5'側半分子の 33mer を用い、アンチコドンと 3'側半分子に E. coli tRNA_f^{Met} の塩基配列を有する hybrid tRNA (図44) の合成を行なった。

合成経路を図45に示す。33mer = pCAUp と ligation により結合し 10% PAGE で分離した。BAP 处理により 3'末端リン酸基を除去し 5'側半分子 (アンチコドンを含む)



[図45] tRNA(Gly-Met)の合成経路

を得た。3'側半分子は天然の *E. coli* tRNA^{Met}より得られる分子を termination (調整) した。これらの半分子同士を RNA ligase で結合させ 10% PAGE で精製し hybrid tRNA を得た。結合部位は nearest neighbor analysis で正しい事を確認した。

合成した tRNA はついてメチオニン受容活性を測定したが受容活性を示さなかつた。(第二節図49) 又、アリシン受容活

性についても調べたが活性を示さなかつた。合成した tRNA は 3'末端 CCA の CA が欠損しているがアミノ酸受容活性を示さなかつた。これは CCA 修復が生じてない事が原因ではないかと考え *E. coli* S-100 フラクションで tRNA の 3'末端修復の確認を行なつた。修復反応を行つた後 $[5'-3'P]pCp$ の single addition を行ない 10% PAGE で tRNA^{3'P}Cp を単離した。RNase T₂ で完全分解し N^3P ($N = A, G, C, T$) を調べた結果、反応前で

は $C_{3'p}$ が検出されたが 修復後 $A_{3'p}$ に変化し 3'末端が修復されることを確かめた。

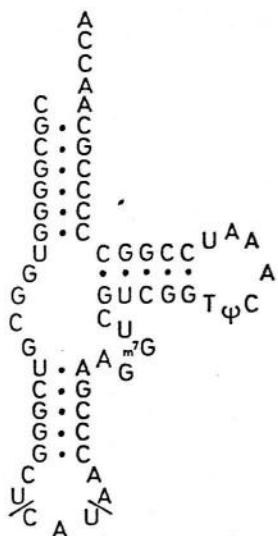
この hybrid tRNA は共通の二次構造に従って塩基対を配列すると アセトアラバーステムで二塩基対しか組みこむことができないが、図44の破線で示す様に一塩基対と五塩基対組める。 hybrid tRNA は安定な後者の構造を形成すると予想されるが全體の形が天然の $tRNA_f^{Met}$ と異なりためこのことが原因で ARSase I に認識されなかつたと考えられる。

第二節 DIL- γ とステムの欠除した $E. coli tRNA_f^{Met}$ の合成

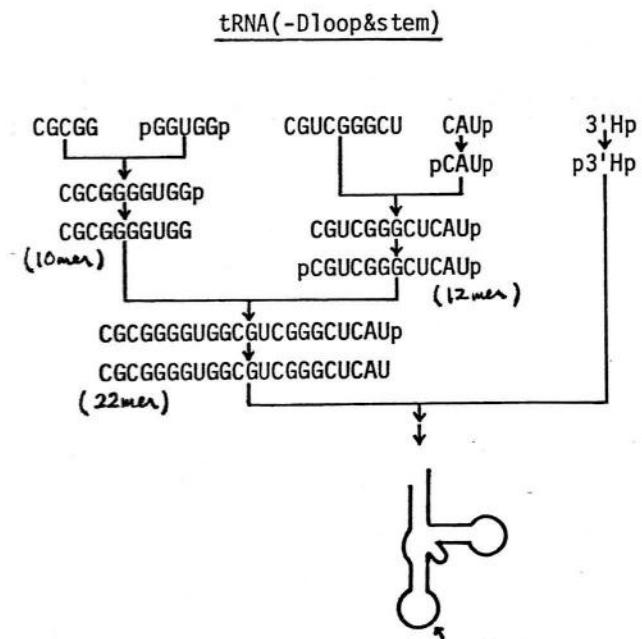
ミトコンドリアには一般的なクローバー葉型二次構造を有する tRNA と異なり DIL- γ とステムが欠除した持異的な tRNA が存在する。 $tRNA^{Ser}$ がその例でこれらの部分が欠除して 3'にもかかわらずセリンを受容する tRNA としての機能を果たす。他のクローバー葉型二次構造を有する tRNA も DIL- γ やステムがなくとも機能するであろうか。著者は $E. coli tRNA_f^{Met}$ について DIL- γ とステムが欠除した tRNA を合成し、この分子がメチオニンを受容できるか否かについて調べた。

アセトアラバーステムとアンチコドンステムの間は それぞれのステムに統く塩基配列を各三塩基、二塩基延長させ、ミトコンドリア $tRNA^{Ser}$ の場合と同様五塩基の間隔をあいた。(図46) 合成経路を図47に示す。 5'側半分子は鎖長が 3 から 9 の化学合成フラグメントを RNA ligase で結合することにより合成した。 3'側半分子は 天然の $E. coli tRNA_f^{Met}$ の RNase A 限是分解

E.coli tRNA_f^{Met}
(-D loop& stem)



[図46] DIL-7°とステムの欠除した
E.coli tRNA_f^{Met}



[図47] DIL-7°とステムの欠除したtRNA_f^{Met}の合成経路

Ligation

10 + 12 → 22 (5'-half)

→ Ori.

22 + 3'-half → tRNA(-Dloop & stem)

→ Ori.

10% PAGE

→ X.C.



→ X.C.

← product (22mer)

→ B.P.B.



22mer

→ B.P.B.



[図48] DIL-7°とステムの欠除したtRNA_f^{Met}合成におけるligation

より得られるフラグメントを用いた。

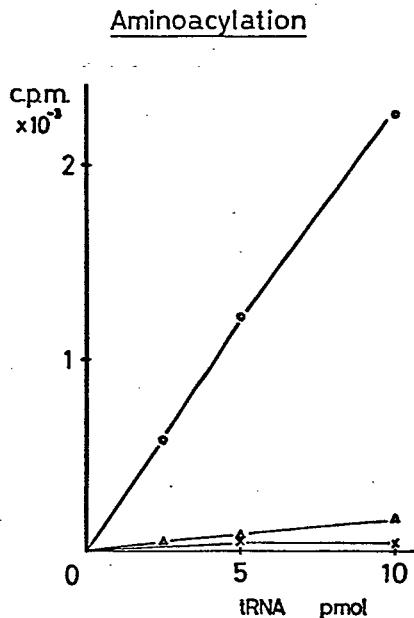
$CGCGG + pGUGGp$, $CGUCGGCUT + pCAUp$ の各 ligation より得られる 10mer, 12mer はいずれも Sephadex G-50 により精製した。10mer + 12mer, 22mer + 3'側半分子の各 ligation ではいずれも 10% PAGE (図48) で単離し、目的の tRNA を得た。

合成した tRNA についてメチオニン受容活性を測定した。

図49に結果を示すが DIL- f^0 とステムの欠除した tRNA は全くチャージしないわけではなく低いながらもわずかにメチオニンを受容することがわかった。DIL- f^0 が欠除すると DIL- p と T ψ IL- f^0 の水素結合ができなくなり、tRNA と 1 つの安定な三次構造が組めないと予想される。それ故 Met RSase に認識されにくくなりほとんどチャージしないだと考えられる。

DIL- f^0 が欠除しているにもかかわらず天然の tRNA $^{\text{Met}}$ と同様の L 字形三次構造をもつ分子がわずかに存在することが推定できる。

最近 Klug らによりミトコンドリアの tRNA $^{\text{Ser}}$ も安定な構造をとれる三次構造モデルが提出された。⁴⁰⁾ このモデルに従いアセプターステムとアンチコドンステムの間の塩基を交換し tRNA を合成すれば、安定な三次構造を有する tRNA が得られメチオニン受容活性が上昇することが予測される。



[図49] 5'側半分子変換した tRNA $^{\text{Met}}$ のメチオニン受容活性

○—○: tRNA $^{\text{Met}}$,
 △—△: tRNA(-D loop & stem),
 ×—×: tRNA(Gly-Met)

結論

1. 種々の化学合成フラグメントを用いて RNA ligase 反応を行ない *E. coli* tRNA^{Met} の 5'側半分子, 3'側半分子を合成した。又、多くの ligase 反応の結果から RNA ligase に認識され難い基質の塩基配列を見出した。
2. *E. coli* tRNA^{Met} の TΨループの中で共通塩基配列 TΨC を AUC に変換した tRNA^{Met} を合成し、そのメチオニン受容活性を調べたところほとんど活性を示さなかつたことより、TΨループの塩基配列が *E. coli* Met RSase の認識に関与することがわかつた。
3. *E. coli* tRNA^{Met} の 5'末端位で G-C 塩基対が組める修飾 tRNA^{Met} を合成し調べたところ、天然の tRNA^{Met} と同様のメチオニン受容活性を示した。又、*E. coli* EF-Tu との親和性がこの塩基対により強まることを示した。
4. *E. coli* tRNA^{Met} のアンチコドン部を変換した修飾 tRNA^{Met} を合成し、Met RSase による認識には CAU 配列が必要で、この配列が存在すれば他の塩基を 3'側あるいは 5'側に付加しても親和性の差はあるにせよ認識されることが明らかとなつた。又、アンチコドンの 5'側隣接位に共通して存在する塩基 U を他の塩基に変換しても Met RSase による認識には影響しないことがわかつた。
5. *E. coli* tRNA^{Met} のアンチコドン部を変換した修飾 tRNA^{Met} は、*Xenopus laevis oocyte* 中でその配列により安定性が異なることを見出した。すなわちアンチコドン配列が tRNA の安定性に影響することを初めて示した。又、*E. coli* 中では修

飾を受けない塩基が *Xenopus laevis* oocyte 中では修飾を受けることを示した。

6. *E. coli* tRNA_f^{Met} の 5'側半分子を *E. coli* tRNA₂^{Gly} に変換して tRNA を合成し、この分子が Met RSase, Gly RSase に認識されないことがわかった。
7. *E. coli* tRNA_f^{Met} の Dループとステムの欠除した tRNA を合成し、Met RSase によりわずかにメチオニンを受容することがわかった。

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実験の部

Materials

・基質

種々のリボオリゴマー 薬化学生教室で合成されたもの
 $E. coli$ tRNA_f^{Met} アメリカ合衆国 Yale大学 D. Söll博士より
供与されたもの

[³²P]-正リン酸 日本アイソトープ協会

[¹⁴C]-アミノ酸 Amersham

Folinic Acid-SF (leucovorin) Lederle製, 武田薬品工業株式
会社

・酵素

RNA ligase 及び P.N. kinase (3'-phosphatase free)
薬化学生教室で精製されたもの

BAP Worthington Biochemical Corp. (BAPF), 及び
宝酒造株式会社

RNase T₂ 三共株式会社

RNase A Boehringer-Mannheim 社

ヌクレアーゼ P₁ ヤマサ醤油株式会社

ヌクレアーゼ S₁ 三共株式会社

$E. coli$ 粗酵素系 S-100 分画 薬化学生教室で調整

$E. coli$ EF-Tu·GDP 東京大学医科学研究所 上代淑人
博士より供与されたもの

Xenopus laevis oocyte ベルギー Université Libre de Bruxelles
H. Grosjean博士より供与されたもの

◦ 樹脂

DEAE Sephadex A-25 Pharmacia 社

DEAE Cellulose (DE 23) Whatman 社

Sephadex G-25, G-50, G-100 Pharmacia 社

Bio-Gel P-2 Bio Rad 社

Bio-Gel A (0.5m) (200~400 mesh) Bio Rad 社

DEAE Cellulose plate (polygram cell 300 DEAE/HR-2/15)
Macherey Nagel 社

アセトセル SF (セルロース薄層プレート) フナコシ薬品株式会社

Method

◦ RNA ligase 反応

通常 50mM HEPES-NaOH (pH8.3), 10mM DTT, 10mM MgCl₂,
10μg/ml BSA, 10~15% DMSO (v/v) を含む buffer 中 25°C 2~
1ンキュベート 1Tc。

基質量, 酵素量については各章ごとに後述する。

◦ P.N. Kinase 反応

通常 50mM Tris HCl (pH9.6), 10mM MgCl₂, 2.0mM スペルミン,
10mM DTT, 0.1M KCl を含む buffer 中 37°C 2~1ンキュベート 1Tc。

基質量, 酵素量については各章ごとに後述する。

• BAP 处理

50 mM Tris HCl (pH 8.0) 中, 基質 1 nmol に對し 酵素 0.005 unit 加え, 基質 100 ~ 200 μ M の条件で 50°C 1.5 時間インキュベート 12 反応を行なう。反応後は 反応液と同量の 50 mM Tris HCl (pH 9.5) 飽和の フェノール, フロロブルム混液 (1:1, v/v) に加え 2 度除タンパクし, フェノール相を 5' に 50 mM Tris HCl (pH 9.5) で 3 回逆抽出してから水相を約 2 倍量の エーテルで 5 回洗う。溶液中のエーテルを 蒸発させた後 Sephadex G-50 で脱塩する。

• RNase T₂ による完全分解

40 mM 酢酸アンモニウム buffer (pH 4.5) 中, 基質約 2 A₂₆₀ unit (キャリヤ RNA を含む) に對して 酵素約 0.05 unit 加え, 10 μ l で 37°C 3 時間インキュベートする。反応後 混合物は 液紙にスポット 12 pH 3.5 の条件下 PEP を行なう。

• RNase A による限定分解

第二章の実験参照

• Nuclease P₁ による完全分解

40 mM 酢酸アンモニウム buffer (pH 5.0) 中, 基質約 2 A₂₆₀ unit (キャリヤ RNA を含む) に對して 酵素約 0.1 μ g 加え, 10 μ l で 37°C 2 時間インキュベートする。

• 除タンパク

通常 50 mM Tris HCl (pH 7.5) で飽和したエタノール、クロロホルム混液 (1:1, v/v) を試料に対して同量加之 2 度抽出操作を行なう。エタノール相は 50 mM Tris HCl (pH 7.5) で逆抽出を 3 度行ないはじめの水相とあわせた。この水相はエーテルで 5 度洗いエタノール、クロロホルムを除去した。エーテルを除去後エタノール沈殿、又は Sephadex G-50 により脱塩した。

• エタノール沈殿

3 倍容のエタノールを加之 -80°C で 5 分以上冷やし 13,000 rpm, 10 分間遠心し上清を除去した。

• 液紙電気泳動 (pH 3.5)

東洋液紙 No 51A を用い、0.2 M 酢酸モルホリン buffer (pH 3.5) 中、35 V/cm の電圧で泳動した。泳動後液紙を風乾し、5 mm の巾に切って液体シンチレーションカウンターでカウントを測定した。

• 二次元 TLC

西村の方法²¹⁾に従う。試料をセルロース薄層プレート (アセセル SF, 10 cm \times 10 cm) にスポットし、一次元目をイソ酪酸 - 0.5 M アンモニア (5:3, v/v) で展開し、二次元目を 1% パラバーチル - 濃塩酸 - 水 (70:15:15, v/v) で展開した。

・ホモクロマトグラフィー

Sanger等⁴¹⁾ 及び Wu等⁴²⁾ の方法に従って homomixture を調製した。サンプルを DEAE-Cellulose plate にスポットした後、plate を MeOH で洗い サンプルの両端に色素のマーカー混液 (1% キシレンシアノール FF (青), 2% オレンジ G (黄), 1% 酸性フクシン (桃)) をスポットし、上端には Whatman 3MM 液紙を吸収紙としてクリップで止めて展開を行なう。 (60~70 °C) 青色マーカーが plate の先端に達した時点で展開を終え、風乾後 原点及び色素マーカーの位置に ^{32}P のマーカーをスポット (2 ラップ) で包み、 X-ray フィルムを接触感光する。

ホモクロマトグラムから各スポットの量比を算出する場合は、スポット部分の薄層をかきとりこれをプラスチック製ミクロチューブの底に穴をあけ脱脂綿をつめたものにアスロレーターで吹いこみ Cerenkov 法⁴³⁾ で測定し 算出する。

・二次元ホモクロマトグラフィー

Wu等⁴²⁾ 及び Silverkelang 等⁴⁴⁾ の方法に従う。

・アミノアシル化反応

合成した tRNA のアミノ酸受容活性は K_m , V_{max} を求める時以外 後に示す条件で行なう。す。

反応後 全量を Whatman グラスフィルターにスポットし、冷 5% TCA 滴液にひたし 氷冷下 10 分間放置する。この洗浄を後 2 回計 3 回行なう。その後、EtOH : Et₂O = 1 : 1 (v/v) 滴液を加え 氷冷下 5 分間放置する。これを再度行なう。その後フィルターを赤外

線ラン 7°C 下で乾燥させ,
トルエン系シンチレータ
-2" ^{14}C のカウントを測
定した。

K_m , V_{max} を求める時
の条件は第四章の実験で
記述する。

アミドアセチル化反応の条件

HEPES-NaOH (pH 8.0)	0.1 M
$\text{Mg}(\text{CH}_3\text{COO})_2$	10 mM
KCl	10 mM
mercaptoethanol	10 mM
ATP	4 mM
CTP	1 mM
L-[U- ^{14}C]-amino acid	9 μM
tRNA	0.3 ~ 2 μM
<i>E. coli</i> S-100	1 mg/ml
	15 μl

incubate at 37°C for 40 min.

・ポリアクリルアミドゲル電気泳動

10 ~ 20% のアクリルアミド溶液 (アクリルアミド : ピースアツ
1) ルアミド = 30 : 1 (w/w), 7M urea, 45 mM Tris borate
buffer (pH 8.4), 2 mM EDTA) に過硫酸アンモニウムと TEMED を
加えてゲル化した。

Slabgel の場合は厚さが 0.5 ~ 2 mm, 幅 20 ~ 30 cm, 長さ 40 cm
の平板ゲルに左右にガラス板でわくを作り 3 : 1 アクリル
アミドを流し入れて固めた。

Disk gel の場合は Miles Laboratories Inc. 製の PREP-DISC
を用い 70 mm^2 × 8 cm のゲルを調製した。

サンプルの apply の前に 2 時間程 prerunning を 12 ゲルの
状態を確かめてから, 凍結乾燥したサンプルを loading solution
(9M urea, 10 mM EDTA, 40 mM Tris borate buffer (pH 8.4), ~0.1
% X.C.-B.P.B.) を 2 ~ 5 μl 加え 100 $^{\circ}\text{C}$, 2 分間 加熱 (apply
を行なう, た。

泳動後 slab gel ではガラス板をはずしてラッピングしない、X-ray フィルムを接触させ フリーザー 中で感光した。Disk gel の場合はボンブでくみ出されたフラクションをそのままバイアルに入れ Cerenkov 法により測定した。

• ゲル片からの核酸の溶出

Maxam-Gilbert の方法⁴⁵⁾を少し変更を行なった。溶出用 buffer 1 = 1% SDS を加えて pH 調整した。溶出液を濃縮し、Sephadex G-50 1 = よる脱塩を行ない核酸を単離した。

• DEAE-Cellulose (DE23) カラムクロマトグラフィー、urea 非存在下 DEAE-Sephadex A-25 カラムクロマトグラフィー
短鎖長 (3~8 鎮長) のオリゴマーを分離精製するのに用いた。TEAB buffer の直線濃度勾配により溶出した。流速は約 12 ml/h. で フラクションと 12 約 1.2 ml ずつ集めた。

• urea 存在下 DEAE-Sephadex A-25 カラムクロマトグラフィー

鎮長が 8~14 のオリゴマーを分離精製するのに用いた。7M urea, 20 mM Tris HCl (pH 7.5) 存在下 (約 50°C), NaCl の直線濃度勾配により溶出した。流速は約 5 ml/h. で フラクションと 12 約 0.8 ml ずつ集めた。

• Sephadex G-25, G-50 カラムクロマトグラフィー
脱塩の目的で使用する時は、長さが 20~30 cm 程度のカラム

ムを用い, formation 後 ATP とオリゴマーとを分離する時に
は 40 cm 程度, ligation 後のオリゴマー同士の分離には 長さ
90 cm 程度のカラムを用いた。

カラムにサンプルを apply する時は できる限り容量を少なく
し 1 ml 。溶出は 50~100 mM TEAB buffer を用いた。流速は約 15
ml/h. で フラクションと 1 つ約 1 ml ずつ集めた。

• Sephadex G-100, Bio-Gel A (0.5 m) カラムクロマトグラフィー
長さ 90 cm のカラムを用い, 0.1 M TEAB buffer で溶出した。
流速は約 4 ml/h. で フラクションと 1 つ約 1 ml ずつ集めた。

• $[\gamma-^{32}\text{P}]\text{-ATP}$ の調整

Glynn-Chappell 法⁴⁶⁾により, 杉浦らの方法⁴⁷⁾を少しあげて調整した。反応前 $[\gamma-^{32}\text{P}]\text{-ATP}$ を沸騰水浴上 1 時間加熱し,
副生する不溶性リン酸を分解した。

反応後は Dowex カラムに apply し 溶出した $[\gamma-^{32}\text{P}]\text{-ATP}$
は次に DEAE-Sephadex A-25 のカラムに吸着させた。50 mM
TEAB buffer で脱塩後 0.5 M TEAB buffer で溶出した。 $\gamma\text{射}$
を減圧乾固し TEAB を完全に除いた後, 10 mM Tris HCl (pH
7.8) に溶かして凍結保存した。

• UV 吸収の測定

サンプルは水溶液とて 日立ダブルビーム分光光度計 124 型,
又は 200-10 型で測定した。

カラムから溶出されたサンプルの測定には ALTEX Biochemical

UV-VIS Monitor を使用して。

・放射能測定

通常 Aloka Liquid Scintillation spectrometer LSC-671
に 2 0.4% diphenyloxazole 0.01% triphenyloxazole の
トルエン溶液中で測定して。T=70℃カラムクロマトグラフ
のフラクションは試験管ごと Cerenkov 法により測定して。

第一章の実験

• Kination

反応条件、単離收率を表2に示す。Buffer is method 1を示す。

Substrate (μ M)	[γ^{32} P]ATP (μ M)	PN. Kinase (units/ml)	Volume (μ l)	Time (min.)	Yield (%)
1. UCGUp	100	152	50	500	60
2. GGCUp	100	150	100	1000	30 ^{a)}
3. CGGGCUp	50	100	69	720	45
4. UCGUCGGGCUp	23	75	47	20	60
5. CCGAAG	400	800	94	73	60
6. GUCGG	1000	1077	250	200	60
7. AAAp	101	187	64	692	30
8. UUCAAAp	100	143	179	140	45
9. GUCGUCGGUUCAAAp	80	160	64	25	30 ^{a)}
10. CCCCCGp	200	267	100	150	90
11. AACCAp	200	240	100	50	60
12. UAGCUCGUCGGGCUp	55	300	300	40	50 ^{a)}
UCCGGCCCCCGC-AACCAp	68	150	100	40	90 ^{a)}
GUCCGUCGGUUCAAA-AACCAp	74	148	111	27	90 ^{a)}
14. UCCGGCCCCCGC-AACCAp					83

[表2] 第一章の Kination

a) 酶素を加え3前に 60°C , 5分間 a preheat 处理を行ふ。

単離の方法及び条件を次に示す。

1. DEAE-Sephadex A-25 ($\Phi 0.7 \times 15 \text{ cm}$)

TEAB buffer 0.3M から 1M まで total 200 ml a gradient で溶出する。

2. DEAE-Sephadex A-25 ($\Phi 0.9 \times 18 \text{ cm}$)

TEAB buffer 0.3M から 0.9M まで total 200 ml a gradient で溶出する。

2"溶出 1 Tc。

3. Sephadex G-25 ($\Phi 1.1 \times 40\text{cm}$)
4, 8, 9, 10, 11, 12, 13, 14.
Sephadex G-50 ($\Phi 0.9 \times 30\text{cm}$)

5. DEAE-Sephadex A-25 ($\Phi 0.7 \times 20\text{cm}$)
TEAB buffer 0.3M から 0.9M まで total 150ml お gradient 2"
溶出 1 Tc。

6. DEAE-Sephadex A-25 ($\Phi 0.8 \times 16\text{cm}$)
TEAB buffer 0.3M から 0.8M まで total 200ml お gradient 2"
溶出 1 Tc。

7. DEAE-cellulose (DE 23) ($\Phi 0.4 \times 16\text{cm}$)
TEAB buffer 0.1M から 0.5M まで total 150ml お gradient 2"
溶出 1 Tc。

◦ ligation

反応条件、单離収率を表3にまとめた。Buffer は method 1 に示す 1 Tc。RNA ligase を加える前に 60°C, 5分間 preheat 1 Tc。
单離の方法及び条件を示す。

1, 2. 20% PAGE (disk), 定電圧 400V, ポンプ流速 30ml/h で フラクションを集めた。

3. DEAE-cellulose (DE 23) ($\Phi 0.6 \times 18\text{cm}$)
TEAB buffer 0.3M から 1M まで total 200ml お gradient 2"
溶出 1 Tc。

4. 20% PAGE (disk), 定電圧 400V, ポンプ流速 60ml/h で フラクションを集めた。

Acceptor (μ M)	Donor (μ M)		ATP (μ M)	RNA Ligase (units/ml)	Volume (μ l)	Time (hr.)	Yield (%)	
1. UCGUCG	100	pGGCUp	167	333	250	120	1.0	4
2. UAGC	240	pUCGUCGGGCUp	80	200	180	5	2.0	11 ^{a)}
3. UAGC	150	pUCGUp	100	200	100	200	3.5	71
4. UAGCUCGU	100	pCGGGCUp	114	200	214	70	2.0	31 ^{a)}
5. CAUAAC	1000	pCCGAAG	100	200	100	80	1.0	39
6. GUC	2000	pGUCGG	100	200	50	1730	0.7	40
7. UUC	400	pAAAp	200	400	100	165	1.0	73
8. GUCGUCGG	150	pUUCAAAp	100	200	140	320	1.0	36
9. CAUAAC- CCGAAG	150	pGUCGUCGG- UUCAAAp	100	200	115	12	1.0	52
10. UCCGGC	24	pCCCCGCP	10	40	50	2500	2.0	33 ^{b)}
11. UCCGGC	132	pCCCCGCP	100	200	100	280	2.0	65
12. UCCGGC- CCCCGC	101	pAACCAp	116	229	114	70	3.0	38
13. CGCGGGG- UGGAGC- AGCCUGG	74	pUAGCUCGUCG- GGCUp	111	227	535	12	2.0	31 ^{b)}
14. GUCGUCG- GUUCAA CAUAACC-	200	pUCCGGCCCCG- CAACCAp pGUCGUCGGUUC-	125	200	150	20	2.0	82
15. CGAAG	159	AAAUCGGGCC- CCGCAACCAp	100	200	153	17	2.0	32

[表3] 第一章の ligation

a) kinase後の単離收率

b) BAP処理後単離收率

5. 20% PAGE (disk), 実電圧300V, ノンフローフ流速40ml/h.
で73クションを集めた。
6. urea 存在下 DEAE-Sephadex A-25 ($\phi 0.3 \times 38$ cm)
NaCl 0.1M から 0.5M まで total 80ml on gradient (50mM
Tris HCl (pH 7.5) 存在下) で溶出 T=。
7. DEAE-Sephadex A-25 ($\phi 0.6 \times 18$ cm)
TEAB buffer 0.3M から 1M まで total 150ml on gradient で
溶出 T=。

8. 1) urea 存在下 DEAE-Sephadex A-25 ($\phi 0.3 \times 40\text{cm}$)
NaCl 0.25M から 0.65M まで total 120 ml の gradient (50
mM Tris HCl (pH 7.5) 存在下) で溶出した。
2) 20% PAGE (disk), 定電圧 400V, ポンプ流速 56 ml/h.
でフラクションを集めた。この時の単離収率は 24% である。
IC.

9. 20% PAGE (disk), 定電圧 300V, ポンプ流速 44 ml/h.
でフラクションを集めた。

10, 12. Sephadex G-50 ($\phi 0.9 \times 82\text{cm}$)

11. urea 存在下 DEAE-Sephadex A-25 ($\phi 0.3 \times 43\text{cm}$)
NaCl 0.2M から 0.6M まで total 100 ml の gradient (50
mM Tris HCl (pH 7.5) 存在下) で溶出した。

13. 20% PAGE

14. Sephadex G-50 ($\phi 0.9 \times 82\text{cm}$, $\phi 1.0 \times 90\text{cm}$)

15. Bio-gel A (0.5m) (200~400 mesh) ($\phi 0.7 \times 91\text{cm}$)

第二章の実験

• Kinase

反応条件、単離收率を表4に示す。P.N. kinaseを加之る前に 60°C 5分間 preheat し $T=$ 。

Substrate (μM)	[$\gamma^{32}\text{P}$]ATP (μM)	P.N. Kinase (units/ml)	Volume (μl)	Time (min.)	Yield (%)
1. CCGGCCCCGC-AACCApp	94	150	150	40	80
2. AUCGAAACCGGC-CCCCGCAACCAp	100	200	250	30	75
3. (43mer)p	100	300	100	12	45

[表4] 第二章の kinase

単離は可べて Sephadex G-50 ($\phi 0.9 \times 30\text{cm}$) により行なう。 $T=$ 。

• ligation

反応条件、単離收率を表5に示す。 $T=$ 。RNA ligaseを加之る前に 60°C 5分間 preheat し $T=$ 。

Acceptor (μM)	Donor (μM)	ATP (μM)	RNA Ligase (units/ml)	Volume (μl)	Time (hr.)	Yield (%)
1. AUCGAAA 200	pCCGGCCCCGC-AACCApp	100	200	100	32	2.0 87
2. CAUAACCC-GAAGGUC-148 GUCGG	pAUCGAAACCG-GCCCCGCAA-CCAp	100	200	100	21	2.0 60
3. 5'H 79	p(43mer)p	39	94	122	16	2.0 9 ^{a)}

[表5] 第二章の ligation

a) BAP処理後の単離收率

単離の方法及び条件を示す。

1. Sephadex G-50 ($\Phi 0.9 \times 74 \text{ cm}$)
- 2, 3. Sephadex G-100 ($\Phi 0.7 \times 90 \text{ cm}$)

• $E. coli$ tRNA_f^{Met} on RNase A 1:5 3'末端分解²⁰⁾

右に示す条件で行う, $T=$ 。

反応後 冷 BSA を $150 \mu\text{g}$ 加え,

冷 buffer (50mM Tris HCl (pH 7.5),

10mM MgCl₂) で全量を $300 \mu\text{l}$ に

調整し, たとちに 冷 フェノール

(50mM Tris HCl (pH 7.5) 鮫和) で除

タンパクを (2回). 冷 50mM Tris HCl (pH 7.5) で逆抽出を 2回行

うる, 水相をエーテルで 2回洗浄した後 エタノール沈殿を

行う, $T=$. 沈殿をさらに EtOH: Et₂O = 1:1 混液で洗, その後,

乾燥し 10% PAGE で 5'側单分子と 3'側单分子を単離する。

$E. coli$ tRNA _f ^{Met}	1,000 A ₂₆₀
Tris HCl (pH 7.5)	50mM
MgCl ₂	10mM
RNase A	25 $\mu\text{g}/\text{ml}$

100 μl

incubate at 0°C for 30 min.

第三章の実験

◦ Kination

反応条件、単離收率を表6に示す。

Substrate (μ M)	[γ^{32} P]ATP (μ M)	P.N. Kinase (units/ml)	Volume (μ l)	Time (min.)	Yield (%)
1. AACCAp	167	133	173	30	60
2. GACCA	100	157	167	30	70

[表6] 第三章の Kination

単離の方法を示す。

1. Sephadex G-50 ($\Phi 0.8 \times 23$ cm)
2. Sephadex G-25 ($\Phi 1.0 \times 26$ cm)

◦ ligation

反応条件、単離收率を表7に示す。RNA ligaseを加える前は 60°C 5分間の preheat を行なう。

Acceptor (μ M)	Donor (μ M)	ATP (μ M)	RNA Ligase (units/ml)	Volume (μ l)	Time (hr.)	Yield (%)
1 72mer	50	pAACCAp	156	1250	212	16
2 72mer	50	pGACCA	131	1250	212	16

[表7] 第三章の ligation

単離はすべて 10% PAGE により行なう。

◦ 過ヨウ素酸酸化による 3'末端一塩基除去

Springl の方法²⁷⁾に基づき行なう。

nuclease S1 处理により得られた *E. coli* tRNA_f^{Met} の 3'末端より 4 塩基除去された分子 (3 A₂₆₀ unit) を 50 mM 酢酸ナトリウム (pH 5.2), 0.8 mM NaIO₄ 中 (total volume 60 μ l) 遮光下, 4°C 2 時間反応し 3'末端塩基の 2', 3' cis-diol を開裂した。次に 1/60 倍の 0.5 M ラムノースを加え 遮光下 4°C 0.5 時間反応し 過剰の NaIO₄ を不活化した。EtOH 沈殿後, さらに沈殿を EtOH で洗い, 焼き乾燥し, これに 0.25 M Lys-HCl (pH 9.0) を加え遮光下, 室温 (~20°C) 3.5 時間反応した。反応後当量の 1 M AcONa (pH 4.6) を加え EtOH 沈殿を行ない脱塩した。沈殿を EtOH で洗い乾燥した後 BAP 处理を 1' 末端のリノ酸を除去した。除タンパク後 10% PAGE で単離した。

生成物は [$5'-^{32}\text{P}$]-pCp と RNA ligase による single addition により 3'末端にラベルを導入し, この分子を 10% PAGE で単離後, RNase T₂ で完全分解した。沪紙電気泳動 (pH 3.5) によりラベルが A₂₆₀ と 1/2 検出された末端の C が除去できたことを確認した。

アミノアシル tRNA - EF-Tu · GTP 三重複合体を Sephadex G-100 を用いて assay

EF-Tu の活性を測定した後, 後に示す条件で三重複合体形成反応を行なう (Fig. 4b)

Sephadex G-100 IT buffer (50 mM Tris HCl (pH 7.8), 150 mM NH₄Cl, 10 mM Mg(OAc)₂, 10 mM mercaptoethanol, 10 mM GTP) で平衡化しておき, サンプルを apply 後 同じ buffer で溶出して。各溶出フラクションを 500 μ l / 9 分で集めた後,

三重複合体形成の条件

Tris HCl (pH 7.8)	50mM
Mg(OAc) ₂	10mM
NH ₄ Cl	150mM
mercaptoethanol	10mM
GTP	30μM
phosphoenol pyruvate	13mM
pyruvate kinase	3μg
EF-Tu · GDP	6.3μg (30pmol)

30μl

↓ incubate at 30°C for 30 min.
 ↓ add [¹⁴C]-Met-tRNA_f^{Met} or [¹⁴C]-Val-tRNA_f^{Val}
 or [¹⁴C]-Met-tRNA(GACCA), 5~10 pmol
 ↓ incubate at 0°C for 5 min.
 ↓ gel filtration on Sephadex G-100
 at 4°C (Φ0.7 x 46cm)

フラクションを グラスフィルター
 に スポンジー 乾燥後, ト
 ルエン系シンチレーターで
 カウントを測定した。

この条件では Val-tRNA^{Val}
 は 複合体を形成せず, Met-
 tRNA_f^{Met} が 形成せず ない。

第四章の実験

• kination

反応条件、単離收率を表8に示す。

Substrate (μ M)	[γ^{32} P]ATP (μ M)	P.N. Kinase (units/ml)	Volume (μ l)	Time (min.)	Yield (%)
1. 3'Hp	67	100	67	120	56
2. 3'Hp	78	117	122	50	85
3. CAUA	150	100	50	100	59
4. CAUAp	99	150	99	80	63
5. UCAU	100	100	50	300	180
6. ACAUp	106	83	37	141	60
7. GCAUp	100	78	87	150	120
8. CCAUp	120	90	90	167	120
9. UUCAUp	104	80	80	125	120
10. CAA	340	260	180	50	70 99
11. CAC					
12. CAG					
13. CAU					
14. GAA					
15. GAC					
16. GAG					
17. GAU					

[表8] 第四章の kination

単離の方法及び条件を示す。

1, 2, 4, 9. Sephadex G-50 ($\phi 0.8 \times 23$ cm)

3. DEAE-Sephadex A-25 ($\phi 0.5 \times 6$ cm)

TEAB buffer 0.2M or 0.7M ± 2 " total 80ml a gradient 2" 滴出1Tc。

5, 10. DEAE-Sephadex A-25 ($\phi 0.5 \times 6$ cm)

TEAB buffer 0.2M or 0.7M ± 2 " Total 60ml a gradient 2" 滴出1Tc。

6, 11, 12, 14, 15, 16, 17. DEAE-Sephadex A-25 ($\phi 0.5 \times 6\text{cm}$)

TEAB buffer 0.2M \rightarrow 0.8M $\pm 2^\circ$ total 60ml on gradient 2"
溶出 1 T \circ

7, 8. DEAE-Sephadex A-25 ($\phi 0.5 \times 6\text{cm}$)

TEAB buffer 0.3M \rightarrow 0.9M $\pm 2^\circ$ Total 60ml on gradient 2"
溶出 1 T \circ

13. DEAE-Sephadex A-25 ($\phi 0.5 \times 6\text{cm}$)

TEAB buffer 0.1M \rightarrow 0.6M $\pm 2^\circ$ total 60ml on gradient 2"
溶出 1 T \circ

• ligation

反応条件、単離収率を表9に示す。RNA ligaseを加える前
は 60°C , 5分間 preheat 1 T \circ 。

単離はすべて 10% PAGE はより行なう, T \circ 。

• アミノアシル化反応 (K_m, V_{max} study)

左に示す条件で行なう, T \circ 。

tRNA の 3'末端 CCA 配列を修復する

後, [¹⁴C]-Met を加えて 反応 1 T \circ 。

反応後の処理は method 1 に記述

方法と同一で行なう, T \circ 。

tRNA	0.15~2.0 μM
CTP	0.8 mM
ATP	4 mM
HEPES-NaOH (pH 8.0)	100 mM
Mg(OAc) ₂	10 mM
KCl	10 mM
mercaptoethanol	10 mM
E. coli S-100	1 mg/ml

↓ incubate at 37°C for 30 min.
↓ heat at 100°C for 2 min.

[¹⁴ C]-Met	18 μM
E. coli S-100	0.5 mg/ml
	10 μl

↓ incubate at 37°C for 20 min.

Acceptor (μ M)	Donor (μ M)	ATP (μ M)	RNA Ligase (units/ml)	Volume (μ l)	Time (hr.)	Yield (%)		
1. CUA	1425	p3'Hp	95	250	143	40	2.0	4
2. 5'H	66	pCAUA	66	133	108	80	2.0	11
3. 5'H	100	pCAUAp	125	300	100	40	2.0	20
4. 5'H	80	pUCAU	167	300	120	100	2.0	16
5. 5'HpCAUA	30	p3'Hp	120	480	241	25	2.0	42
6. 5'HpCAUAA	50	p3'Hp	125	875	376	16	2.0	40
7. 5'HpUCAU	75	p3'Hp	156	938	375	16	2.0	49
8. 5'H	100	pACAU _p	125	500	130	40	2.0	25 ^a
9. 5'H	100	pGCAU _p	240	500	130	40	2.0	23 ^a
10. 5'H	100	pCCAUp	200	500	173	40	2.0	25
11. 5'H	100	pUUCAUp	200	500	173	40	2.0	25
12. 5'HpACAU	63	p3'Hp	125	1250	376	16	2.0	15
13. 5'HpGCAU	56	p3'Hp	125	1250	376	16	2.0	7
14. 5'HpCCAU	50	p3'Hp	125	1250	375	16	2.0	21
15. 5'HpUUCAU	50	p3'Hp	125	1250	375	16	2.0	30
16. 5'H-1	67	pACAU _p	400	670	201	30	2.0	30
17. 5'H-1	67	pCCAUp	400	670	201	30	2.0	25
18. 5'H-1	67	pUCAU _p	200	670	201	30	2.0	5
19. 5'H-1-pACAU	60	p3'Hp	96	200	344	10	2.0	17
20. 5'H-1-pCCAU	50	p3'Hp	96	200	344	10	2.0	17
21. 5'H-1-pUCAU	10	p3'Hp	48	200	344	10	2.0	21
22. 5'H		pCAA						17
23. 5'H		pCAC						17
24. 5'H		pCAG						10
25. 5'H	67	pCAU	300					22
26. 5'H		pGAA	433	667	200	30	2.0	30
27. 5'H		pGAC						12
28. 5'H		pGAG						13
29. 5'H		pGAU						28
30. 5'HpCAA		p3'Hp						
31. 5'HpCAC		p3'Hp						
32. 5'HpCAG		p3'Hp						
33. 5'HpCAU	13	p3'Hp		600	300	3	2.0	
34. 5'HpGAA		p3'Hp						
35. 5'HpGAC		p3'Hp						
36. 5'HpGAG		p3'Hp						
37. 5'HpGAU		p3'Hp						

[表9] 第四章の ligation

a) BAP処理後の単離收率

- tRNA_f^{Met} の RNase A による 限局分解

第二章の実験で記述したとおり行なう。Tc。

- ホルミル化反応⁴⁹⁾

右に示す条件で行なう。Tc。

反応後 反応液を 2 等分し、一方はそのまま グラスフィルターにスコットト + 1 他方は等量の 50mM CuSO₄

0.3M Tris HCl (pH 7.5) 溶液を加え、37°C 10 分間 反応 | グラスフィルター

にスコットト + Tc。グラスフィルターは

冷 5% TCA 溶液で 10 分間 3 回、

冷 EtOH-Et₂O (1:1, v/v) 液で 5 分間 2 回 洗浄 | Tc。CuSO₄ 处理により fMet-tRNA_f deacyl 化されないが Met-tRNA_f deacyl 化されるところから CuSO₄ 处理を行なう。Tc のカウントの行なわぬか、Tc のカウントに対する比をホルミル化の効率と 12 % で表わす。Tc。

tRNA	0.7 μM
HEPES-NaOH (pH 8.0)	0.1 M
Mg(OAc) ₂	10 mM
KCl	10 mM
mercaptoethanol	10 mM
ATP	4 mM
CTP	1 mM
[¹⁴ C]-Met	9 μM
calcium leucovorin	0.7 mM
E. coli S-100	1 mg/ml
	30 μl

incubate at 37°C for 30 min.

- リボソーム上の mRNA と tRNA の相互作用

Nirenberg らの方法³³⁾ により行

なう。リボソームは E. coli A19

株より調整 | Tc。5' 末端を [³²P]

ATP と P.N. kinase によりラベル | Tc

tRNA を用い右の条件で反応 | Tc。

tRNA	0.5 μM
mRNA	25 μM
Tris HCl (pH 7.5)	0.1 M
Mg(OAc) ₂	20 mM
KCl	50 mM
E. coli ribosome 30S or 70S	1.8 μM

20 μl

incubate at 25°C for 30 min.

mRNA と TCA + TTR - を用いた。

反応後 冷 buffer (0.1M Tris HCl (pH 7.5), 20mM Mg(OAc)₂, 50mM KCl) を 850μl 加え ミリポアフィルターで沪過した。同様の buffer 2ml で 3 回フィルターを洗浄した後 乾燥し、トルエン系シンチレーターでカウントを測定した。

非特異的結合によるカウントを差し引く *E. coli* tRNA^{Met} と AUG の特異的結合を 100% とし、それぞれの特異的結合を示した。

• 過ヨウ素酸酸化による tRNA^{Met} の 5' 側半分子の 3' 末端一塩基除去

第三章の実験で記述した方法に従った。

5' 側半分子の他の箇所の損傷を防ぐために 反応前に当量の 3' 側半分子を加え tRNA の三次構造を組ませた後 反応した。
(3' 側半分子は 10% PAGE で 5' 側半分子と反応後分離できる。)

• *Xenopus laevis* oocyte への micro injection³⁶⁾ (図 50)

アフリカツメガエルより 卵巣を取り出し, Barth solution⁵¹⁾ (2mM Tris HCl (pH 7.6), 89mM NaCl, 1mM KCl, 0.33mM Ca(NO₃)₂, 0.41mM CaCl₂, 0.82mM MgSO₄, 2.4mM NaHCO₃, 0.01g/l penicilline, 0.01g/l streptomycine) 中でピンセットを用いて卵母細胞に分離し、この solution 中 20°C 下 injection を行なうまで保存した。

サンプルは injection を行なう直前に 13,000 rpm, 5 分間遠心し dust を除去した。 (マイクロビペットがつまらぬを防ぐた

め)

卵母細胞を Barth solution に
入し, $T=$ シャーレに移し, 1 \times a
サンプルに 7 つ 5 個の卵母細胞
の細胞質に 50 nl ずつ inject
 $1 T=$ (1 卵母細胞あたり 0.0025
pmol α tRNA を inject 1 $T=$)

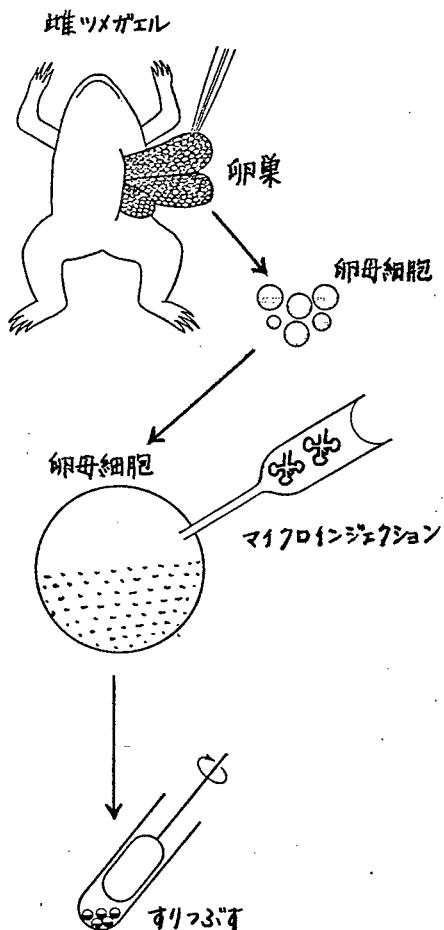
Inject 後 エッヘンドルフ
エ-7" (1.5 ml) に 5 個の卵母細胞
を Barth solution に移し, 20°C
で 1 キュベート 1 $T=$ 。

1, 3, 7, 24, 48, 72 時間 30°C
で 1 キュベート 後 Barth
solution を除き, ガラス棒で卵
母細胞を破碎 1 $T=$ Buffer

(0.2 M NaOAc (pH 4.5), 10 mM
MgCl₂, 1 mM EDTA, 2% SDS (%))

200 μ l を直ちに加え suspend 1 Tエール: フロウセルム =
1:1 溶液 (0.2 M NaOAc 鮑和) 200 μ l を除き $-10^{\circ}C$ 1 $T=$

Tエール相を先ほどの SDS を含む buffer 100 μ l で逆抽出
し, あわせて水相に EtOH 200 μ l を加え EtOH 沈殿を行なう $T=$
沈殿を EtOH: 0.3 M NaOAc (pH 5.2) = 4:1 溶液で洗浄 乾燥
1 $T=$ 後 10% PAGE に apply 1 $T=$



[図50] 卵母細胞への microinjection

第五章の実験

• kination

反応条件、単離收率を表10に示す。

Substrate (μ M)	[γ - 32 P]ATP (μ M)	R.N. Kinase (units/ml)	Volume (μ l)	Time (min.)	Yield (%)
1. CAUp	250	150	75	200	90
2. 3'Hp	81	119	81	62	90
3. CGUCGGGCUCAUUp	100	334	100	90	80

[表10] 第五章の kination

単離の方法及び条件を示す。

1. DEAE-Sephadex A-25 ($\phi 0.5 \times 6$ cm)

TEAB buffer 0.3M から 0.8M まで total 60ml on gradient で溶出する。

2, 3. Sephadex G-50 ($\phi 0.8 \times 23$ cm)

• ligation

反応条件、単離收率を表11に示す。RNA ligase E 加え
反前に 60°C 5分間 preheat する。

単離の方法を示す。

1, 2, 5, 6. 10% PAGE

3, 4. Sephadex G-50 ($\phi 0.7 \times 90$ cm)

Acceptor (μ M)	Donor (μ M)	ATP (μ M)	RNA Ligase (units/ml)	Volume (μ l)	Time (hr)	Yield (%)		
1. $5'$ H(Gly)	100	pCAUp	200	400	100	70	2.0	7
2. $5'$ H(Gly)- pCAU	31	p $3'$ H(Met)p	138	1000	375	16	2.0	2
3. CGUCGGGCU	67	pCAUp	77	267	113	300	2.0	25
4. CGCGG	300	pGGUGGp	150	400	150	200	2.0	33
5. CGCGGGGU- GG	200	pCGUCGGGCU- CAUp	100	600	260	50	2.0	20
6. CGCGGGGU- GGCGUCG- GGCUAU	75	p $3'$ Hp	150	1000	340	10	2.0	3

[表11] 第五章 o ligation

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ABSTRACT

A tetradecanucleotide U-A-G-C(U-C-G)G-G-C-Up corresponding to bases 21-34 of a nascent sequence of formylmethionyl tRNA of *E. coli* has been synthesized by the joining of two combinations of chemically synthesized oligonucleotides: 1) U-A-G-C + U-C-G-U-C-G + G-G-C-Up and 2) U-A-G-C + U-C-G-U + C-G-G-G-C-Up. In reaction 1) the extent of joining *pG-G-C-Up to U-C-G-U-C-G was only 15.4% and the last ligation of the decamer to U-A-G-U proceeded to 27%. In reaction 2) joining between U-A-G-C and pU-C-G-Up gave a high yield (88%). The ligation of this octamer and *pC-G-G-G-C-Up also gave a satisfactory yield (52%). These reactions suggest that sequence preferences in RNA ligase reactions may arise from the structure of the 3'-end of acceptor molecules.

INTRODUCTION

We have previously reported the joining of chemically synthesized tRNA fragments with RNA ligase to yield 3'²- and 5'³-quarter molecules of *E. coli* tRNA_f^{Met}. In the present paper we describe syntheses of a tetradecanucleotide U-A-G-C-U-C-G-U-C-G-G-G-C-Up which correspond to bases 21-34 of a nascent sequence of tRNA_f^{Met} of *E. coli* (Fig. 1). Two different sets of synthetic oligonucleotides were used as substrates for RNA ligase to yield the tetradecanucleotide. As has been observed in previous joining reactions of synthetic ribooligonucleotides,^{4,5} the extent of joining differed in each case. Some structural preferences of acceptor molecules in ligase reactions were observed in these reactions.

MATERIALS AND METHODS

Nucleotides

All oligonucleotides used in this study have been synthe-

Comparison of substrate base sequences for RNA ligase reactions in the synthesis of a tetradecanucleotide corresponding to bases 21-34 of *E. coli* tRNA_f^{Met 1}

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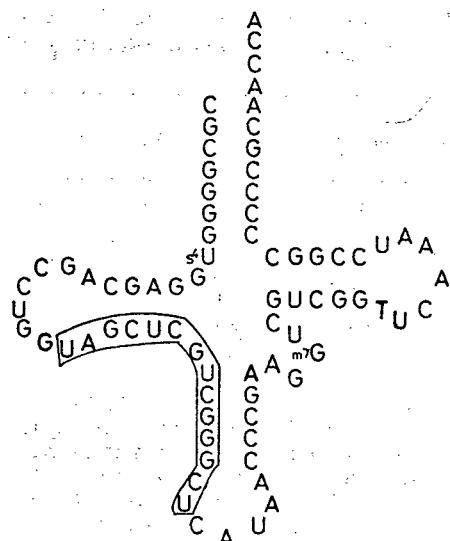


Fig. 1

sized chemically.⁶

Enzymes

RNA ligase was purified as described previously.⁷ Polynucleotide kinase was isolated from E. coli infected with T4 PseT1-amN8SP62 which was a generous gift of Dr. A. Sugino. Other enzymes were obtained as described previously.^{2,4}

Kination and Ligation

Reaction conditions for 5'-phosphorylation using polynucleotide kinase and [γ -³²P]ATP were as described previously. A two fold excess of ATP with respect to donor molecules was used in RNA ligase reactions unless otherwise specified, in the presence of 50 mM Hepes-NaOH (pH 8.3), 10 mM DTT, 10 mM MgCl₂, 10 μ g/ml BSA.

Chromatography, Electrophoresis and Other Methods

Paper chromatography of nucleotides in nearest neighbor analysis, gel electrophoresis and paper electrophoresis for purification and identification of nucleotides were described previously.^{2,3} Homochromatography⁸ was carried out using Homomix I-VI⁹ and partial digestion with nuclease P1 was as described previously.² The joined products were isolated either by ion-exchange chromatography on DEAE-cellulose (DE-23, Whatman) or

electrophoresis on 20% acrylamide gel using a disk apparatus.³

Desalting was performed by gel filtration on Sephadex G-50 equilibrated with 0.05 M triethylammonium bicarbonate (pH 7.5).

Enzymatic digestion of oligonucleotides for characterization was as described previously.^{2,3}

RESULTS

Synthesis of the tetradecanucleotide U-A-G-C-U-C-G-U-C-G-G-C-C-Up (1) using the tetranucleotide pG-G-C-Up (2)

The oligonucleotides 2, 3 and 6 were used for the synthesis of the tetradecanucleotide 1 as shown in Chart 1. The 3',5'-bisphosphorylated tetranucleotide (2) was prepared by phosphorylation of G-G-C-Up with [γ -³²P]ATP and polynucleotide kinase. The 3'-phosphomonoester served as a blocking group to prevent self-polymerization in the next RNA ligase reaction. The tetramer (2) was joined to the hexamer with RNA ligase using the conditions shown in Table I. The extent of the reaction was not higher than 15% in spite of an excess of the enzyme. Alteration of temperature and other conditions did not improve the yield. The decamer 4 was isolated by a disk gel electrophoresis and phosphorylated enzymatically to give 5. The decamer (5) was then joined to the tetramer (6). The result and reaction conditions are shown in Table I. The tetradecamer (1) was isolated by a preparative disk gel electrophoresis after treatment with polynucleotide kinase and [γ -³²P]ATP. The overall yield was 11%. The elution profile from the gel is shown in Fig. 2.

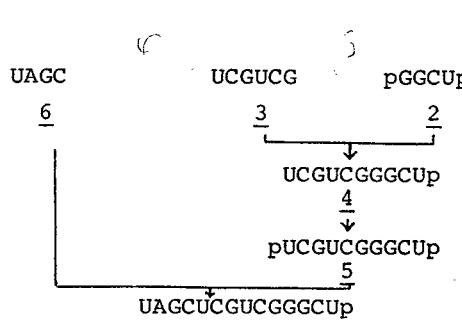


Chart 1

Table I Reaction conditions for joining

acceptor nmol [μ M]	donor nmol [μ M]	ATP μ M	RNA ligase [unit/ml]	time hr	Reaction extent ^{a)} Volume	Yield % %
UCGUCC (3) 12 [100]	pGGCUp (2) 20 [167]	333	250	1	15	4
UAGC (6) 1.2 [240]	pUCGUCGGGCUp (5) 0.4 [80]	200	180	2	27	11 ^{b)}
UAGC (6) 30 [150]	pUCGUp (7) 20 [100]	200	100	3.5	88	71
UAGCUCGU (9) 7 [100]	pCGGGCUp (10) 8 [114]	200	214	2	52	31 ^{b)}

Incubation mixtures contained 10% DMSO and the temperature was 25°.

a) Estimated by homochromatography.

b) The compound was isolated after kination.

Alternative synthesis of the tetradecanucleotide (1) using the hexanucleotide pC-G-G-G-C-Up (10)

The tetradecanucleotide (1) was prepared by using a different set of synthetic oligonucleotides as shown in Chart 2. The yield

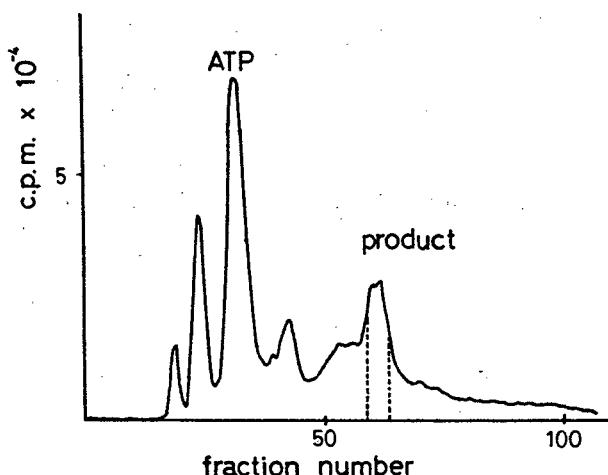


Fig. 2 Elution profile of the products obtained by joining of U-A-G-C and *pU-C-G-U-C-G*pG-G-C-Up, followed by phosphorylation with polynucleotide kinase and ATP, from a preparative disk gel (70 mm² x 80 mm) at 600 V with a suction rate of 32 ml/hr. The last peak contained *pU-A-G-C*pU-C-G-U-C-G*pG-G-C-Up.

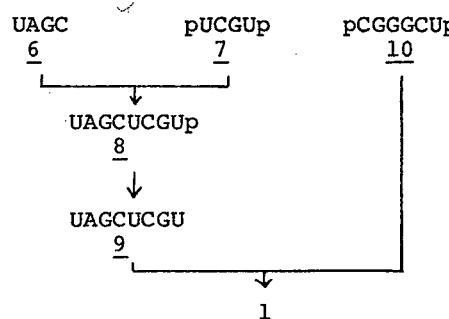


Chart 2

1

in joining 6 and 7 was nearly quantitative and that in joining the octamer (9) and the hexamer (10) was also satisfactory (Table I). The first joining product (8) was isolated by ion-exchange chromatography on DEAE-cellulose as shown in Fig. 3 and the 3'-phosphate was removed by phosphatase treatment to give 9. The nearest neighbor analysis of 1 was performed by digestion with RNase T2 followed by identification of C*p and U*p in acidic electrophoresis (Fig. 4). The result showed complete transfer of the 5'-phosphate to the tetramer (7) to the 3'-end of 6 and the 5'-phosphate of 10 to the 3'-terminal U of

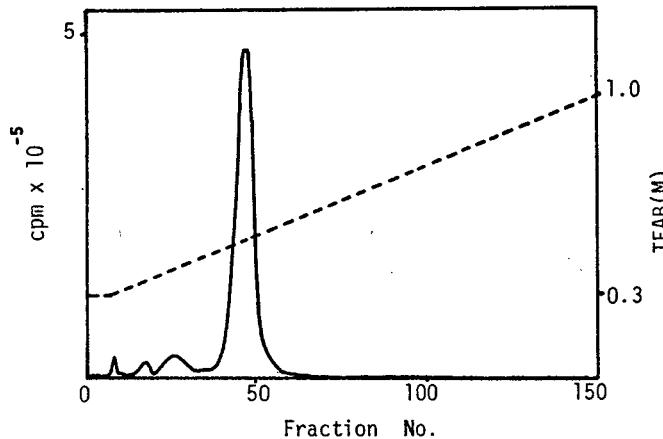


Fig. 3 Chromatography of the joined product, U-A-G-C*pU-C-G-Up on a column (0.6 x 18 cm) of DEAE-cellulose (bicarbonate) equilibrated with 0.15 M triethylammonium bicarbonate. Elution was performed with a linear gradient of triethylammonium bicarbonate (0.3-1 M, total 200 ml). The main peak contained the product.

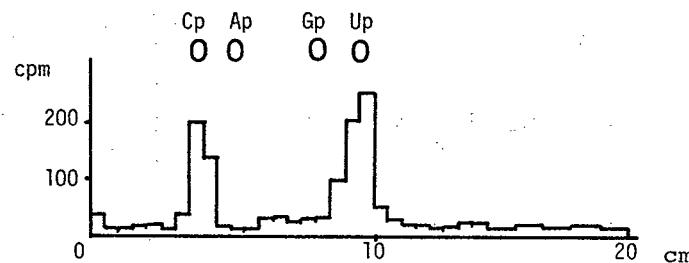


Fig. 4 Nearest neighbor analysis of the joined product U-A-G-C-*pU-C-U-C-G-G-C-Up with RNase T2. The digested mixture was electrophoresed at pH 3.5 with nucleotide UV markers.

9. The isolation of the joined product (1) in the reaction in the reaction between 9 and 10 was performed using preparative disk gel electrophoresis. The elution profile is shown in Fig. 5. The tetradecamer (1) was characterized by slab gel electrophoresis for comparison of chain length.

DISCUSSION

As shown in Table I there were large differences in yields between the reactions shown in Chart 1 and 2. This may indicate that ribooligonucleotide fragments used as substrates for RNA ligase in the synthesis of larger RNA fragments should be pre-

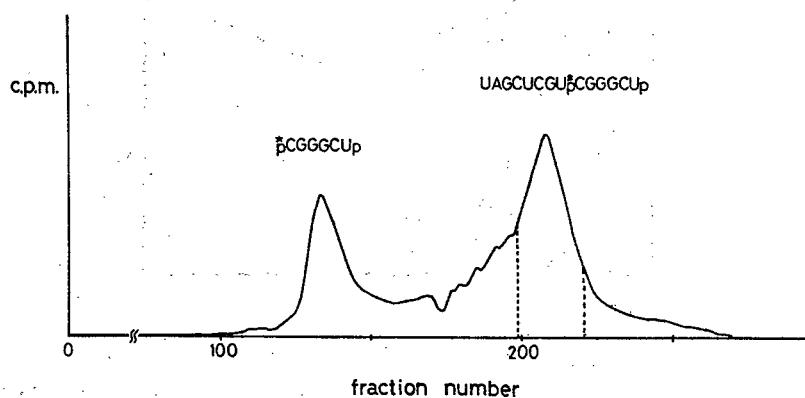


Fig. 5 Elution profile of the tetradecamer (1) from a disk gel. The conditions for electrophoresis was as described in Fig. 2.

pared according to the preferences in recognition between the enzyme and oligonucleotides. It was found that the 3'-hydroxy groups of oligoadenylates were much better acceptor substrates⁴ than those of oligouridylates and various trimers showed different extents of joining when they were treated with the same 5'-phosphorylated trinucleotide donors in RNA ligase reactions.⁵ In those trimer reactions C-C-G was the poorest acceptor when *pC-C-A was used as a donor. The trinucleotide *pC-C-A itself was also a poor acceptor molecule, since no polymerization occurred in the conditions used and C-C-A was joined to *pC-C-A in a yield of 38% which was about one half the efficiency compared to C-A-A or C-C-C. Thus the pyrimidine-purine sequences at the 3'-termini effect the efficiency of reactions with 5'-phosphorylated oligonucleotides. This may be due to unstacked structures at the 3'-ends of acceptors when a 3'-linked pyrimidine nucleoside is present next to a 5'-linked purine nucleoside (e.g. C-G). In a right-handed helical strand the pyrimidine ring of the 3'-linked pyrimidine nucleoside is overlapped with the imidazole ring of the 5'-linked purine nucleoside. This overlapping is less favorable than the comparable overlapping between pyrimidine rings, which may result from purine-pyrimidine sequences (e.g. G-C). Guanosine is known to adopt the syn conformation to a significant extent¹⁰ and this conformation may be unfavorable for RNA ligase reactions if it exists at the 3'-terminus of acceptors. However oligonucleotides having the sequence G-G at the 3'-terminus have been good acceptor molecules in the RNA ligase reactions so far tested. Thus purine-purine sequence seems to be function well on acceptor molecules. Oligonucleotides bearing C-G at the 3'-end, e.g. C-G-C-G,³ C-C-C-C-G,² have been found to be poor acceptors in the synthesis of tRNA_f^{Met} quarter molecules. It has also been reported previously that in single-stranded oligonucleotides guanine has a tendency for unstacking especially when adjacent to uridine residues probably due to the formation of syn and anti conformers.¹¹

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Nucleic Acids Research

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Modification of the anticodon triplet of *E.coli* tRNA_f^{Met} by replacement with trimers complementary to non-sense codons UAG and UAA

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ABSTRACT

E. coli tRNA_f^{Met} was hydrolyzed with RNase A using a limited amount of the enzyme to give two half molecules lacking the anticodon trimer and 3'-terminal dimer. Chemically synthesized trimers CUAp and UUAp were joined to the 5'-half molecules by phosphorylation with polynucleotide kinase plus ATP followed by treatment with RNA ligase. These modified tRNA_f^{Met} species had anticodons complementary to the termination codons UAG and UAA. Two half fragments were joined by a similar procedure to yield a molecule lacking the anticodon trimer and the 3'-dimer. Methionine acceptor activity of these tRNA was tested under conditions in which the CAU inserted control tRNA_f^{Met} accepted methionine. It was found that all three modified molecules were not recognized by the methionyl-tRNA synthetase from *E.coli*. The other sixteen amino acids were not incorporated with partially purified aminoacyl-tRNA synthetases.

INTRODUCTION

We have previously reported enzymatic joining of chemically synthesized ribooligonucleotides with T4 RNA ligase to yield the *E.coli* formylmethionine tRNA.² The basic methodology used in the synthesis of RNA of this size can be applied to the synthesis of modified tRNAs which are useful for studies on structure-function relationship of tRNAs. Anticodon triplets in tRNAs play an essential role in decoding messenger RNAs and suppression of nonsense mutations is known to occur by the action of aminoacyl-tRNAs having anticodon triplets complementary to non-sense codons.³ Synthesis of tRNAs with anticodons complementary to nonsense codons is of interest in testing properties in decoding systems. Replacement of the anticodon of yeast phenylalanine tRNA was reported by Uhlenbeck and his coworkers by removal of Y base and partial RNase A digestion followed by ligation of a new oligonucleotide.^{4a} They found a

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sequence-specific contact between the anticodon loop of yeast tRNA^{Phe} and the phenyl alanyl-tRNA synthetase.^{4b} Recognition of tRNA by aminoacyl-tRNA synthetases seems to differ in each amino acid and cognate tRNA. Modification of the anticodon loop does not affect aminoacylation of tRNA in certain cases.⁶ The wobble position of E.coli tRNA^{Met}_f has been converted to uridine and this modification seemed to affect the aminoacylation.^{5a} Replacement of the anticodon of E.coli formylmethionine tRNA in the present study by joining of RNase A fragments of E.coli tRNA^{Met}_f with CUA or UUA abolishes the recognition with the methionyl-tRNA synthetase of E.coli. (Fig. 1). Removal of the anticodon triplet also caused no-charging. Recently replacement of the wobble position and resulting inhibition of aminoacylation of the same tRNA was reported.^{5b}

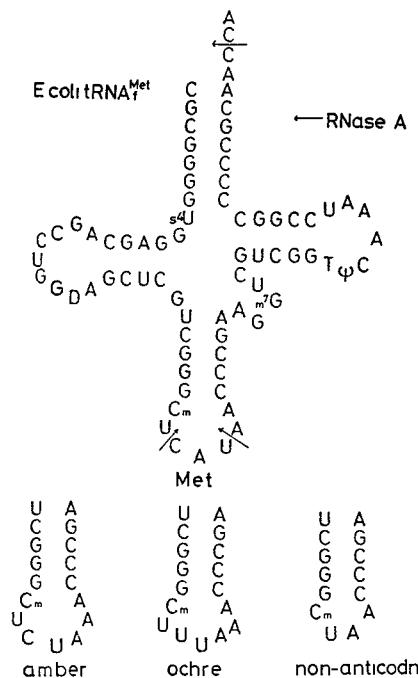


Fig. 1 Cleavage sites in partial digestion with RNase A and modified structures at the anticodon.

MATERIALS AND METHODSMaterials

Nucleotides. Trinucleotides CAU, CUA and UUA were synthesized either by the phosphodi- or triester methods.² E.coli tRNA_f^{Met} was a gift of Dr. D. Söll and had a specific acceptor activity of 1.4 nmol/A₂₆₀ unit.

Enzymes. T4 RNA ligase was purified as described.⁷ Polynucleotide kinase and E.coli alkaline phosphatase were obtained from Takara Shuzo Co.. 3'-Phosphatase-free kinase was isolated from T4 Pse T1-amN82SP62 as described.⁸ RNase T1, RNase T2 and Nuclease S1 were obtained from Sankyo Co. Nuclease P1 was obtained from Yamasa Shoyu Co.. Other enzymes for characterization of products were obtained as described.⁸⁻¹²

Amino acids. ¹⁴C L-Amino acids were purchased from Amersham International plc: Specific activities were Ala, 285 Ci/mol; Val, 285; Leu, 150; Ile, 150; Phe, 225; Pro, 125; Gly, 50; Ser, 165; Thr, 100; Tyr, 225; Asn, 100; Lys, 340; His, 150; Arg, 150; Asp, 225; Glu, 255.

Methods

Phosphorylation, ligation and dephosphorylation were performed as described previously.⁸⁻¹² Complete digestion by nucleases was performed in the presence of carrier RNA (yeast RNA, sigma Co. Type IV) in 10 μ l solution. RNAase A (1 μ g), carrier RNA (0.3 A₂₆₀ unit) in 50 mM Tris-HCl (pH 7.5) and EDTA 1 mM at 37° for 1 hr; RNase T2 (2U), carrier RNA (0.2 A₂₆₀) 50 mM Tris-HCl (pH 7.5) and EDTA 1 mM at 37° for 20 min, nuclease P1 (0.2 μ g), carrier RNA 1A₂₆₀ in 50 mM ammonium acetate (pH 5.0) at 37° for 1 hr; RNase T2 (2U) carrier RNA (0.8 A₂₆₀) in 50 mM sodium acetate (pH 4.5) at 37° for 30 min. Oligonucleotides recovered after homochromatography contained ca. 200 μ g of RNA/cm².

Partial digestion of tRNA (100 A₂₆₀) was performed in 50 mM Tris-HCl(pH 7.5)-20 mM MgCl₂ at 0° for 30 min using RNAase A (25 μ g). The enzyme was removed by extraction with phenol and nucleotides were precipitated with ethanol. The precipitate was subjected to acrylamide gel electrophoresis. Two bands were eluted from gel and desaltsed by gel filtration on Sephadex G-50. The yields was ca. 20%.

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Aminoacylation of tRNA was performed in 50 mM HEPES-NaOH (pH 8.0), 10mM magnesium acetate, 10 mM potassium chloride 10 mM β -mercaptoethanol, 4mM ATP, 0.5 mM CTP, 9 μ M L-U- 14 C Met, 0.5 μ M tRNA and E.coli S-100¹³ at 37°C for 40 min in 20 μ l and counted as described previously.²

RESULT AND DISCUSSION

Removal of the anticodon triplet of tRNA_f^{Met} with RNase A

A single strand specific nuclease, nuclease S1, has been used to hydrolyze anticodon loops of tRNAs.¹⁴ Later it was reported by Wrede et al. that E.coli tRNA_f^{Met} was hydrolyzed at the unique position presumably because of a different conformation at the anticodon region.¹⁵ We found that digestion of E.coli tRNA_f^{Met} with nuclease S1 gave heterogeneous products. On the other hand partial digestion of the same tRNA with a limited amount of RNase A occurred at specific points. Two large fragments with chain length 34 and 38 were isolated by 20% acrylamide gel electrophoresis in a yield of 20%. The 5'-terminal sequence of these oligonucleotides was determined by the mobility shift analysis as shown in Fig. 2. The 5'-ends of the 5'- and 3'-halves were found to have cytidine and adenosine, respectively. The 3'-end of the 5'-half molecule was analyzed by two dimensional thin layer chromatography of the complete digest after labeling with 5'- 32 P pCp and RNA ligase. It was found to be CmU*p. The 3'-terminus of the 3'-half was analyzed after the 3'-labeling as shown in Fig. 3, which indicate a loss of two nucleotides from the terminus.

Construction of tRNA_f^{Met} with modification at the anticodon

Two ribotrinucleotides pCUAp and pUUAp which were complementary to nonsense codons UAG and UAA were inserted between the 5' and 3'-half molecules obtained above. The methionine anticodon pCAUp was also inserted to obtain a control molecule. The 3', and 5'- halves were also joined to yield a tRNA_f^{Met} analog lacking the anticodon triplet.

A typical example of ligation is shown in Fig. 4. The 3',5'-diphosphorylated trimer pCAUp was joined to the dephosphorylated 5'-half molecule and the product (37 nucleotides) was isolated by 20% gel electrophoresis after treatment with phos-

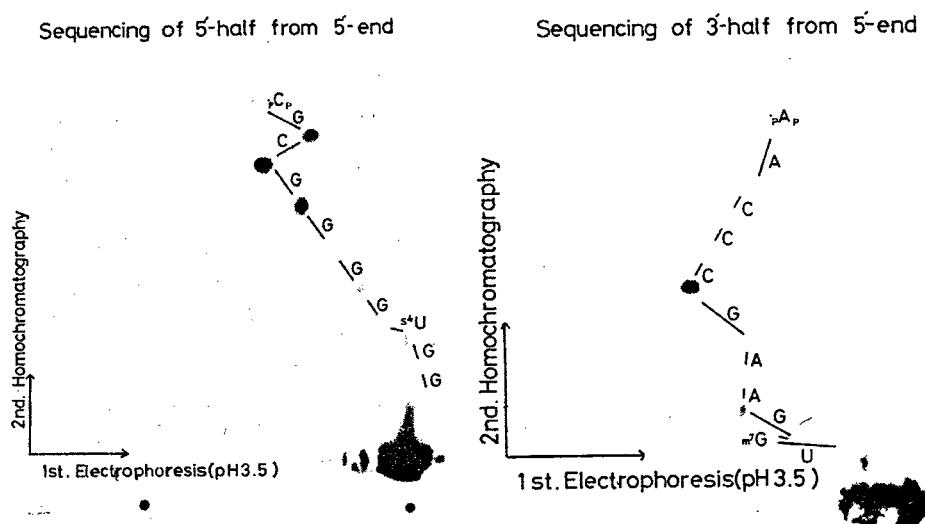


Fig. 2 Mobility shift analyses of the 5'-half (a) and the 3'-half (b) by labeling at the 5'-end.

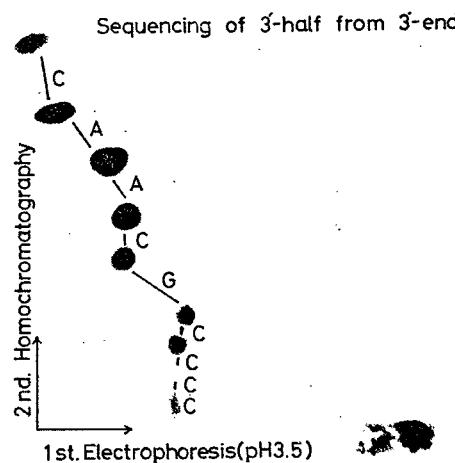


Fig. 3 The sequence of the 3'-end of 3'-half (38-75) developed after labeling at the 3'-end with 5'-³²P pCp and RNA ligase.

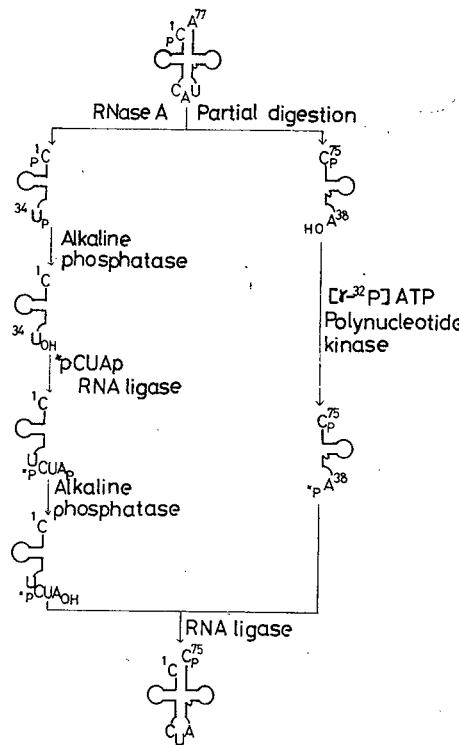


Fig. 4 Scheme for reconstruction of $tRNA_f^{Met}$.

phatase. The 3'-half was 5'-phosphorylated with polynucleotide kinase and isolated by 20% gel electrophoresis. These two fragments were joined and treated with phosphatase. The molecule lacking CA at the 3'-position (75 nucleotides long) was purified by 20% gel electrophoresis and identified by nearest neighbor analysis.

The amber suppressor tRNA containing CUA anticodon and ochre suppressor tRNA (UUU anticodon) were constructed by the same procedure. Electrophoretic mobilities on 10% acrylamide gel of these molecules are shown in Fig. 5 and the result of nearest neighbor analysis by digestion with RNase T2 is shown in Fig. 6. The $tRNA_f^{Met}$ lacking the anticodon CUA (72 nucleotides) was also isolated by electrophoresis on 20% acrylamide gel.

Aminoacylation of Modified $tRNA_f^{Met}$

Incorporation of methionine to these tRNA was tested using

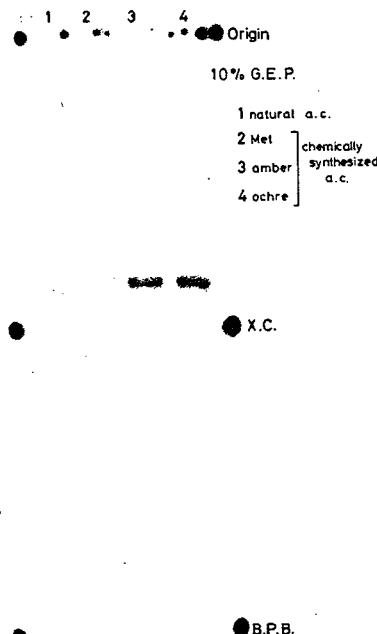
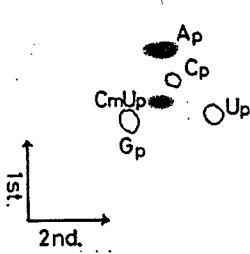


Fig. 5 Electrophoresis of tRNA^{Met} and modified molecules:
 1, intact molecule; 2, reconstructed tRNA^{Met}_f (CAU);
 3, amber tRNA^{Met}_f (CUA); 4, ochre tRNA^{Met}_f (UUA).

amber a.c.(CUA)



ochre a.c.(UUA)

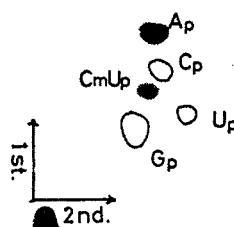


Fig. 6 Nearest neighbor analyses of modified tRNAs.

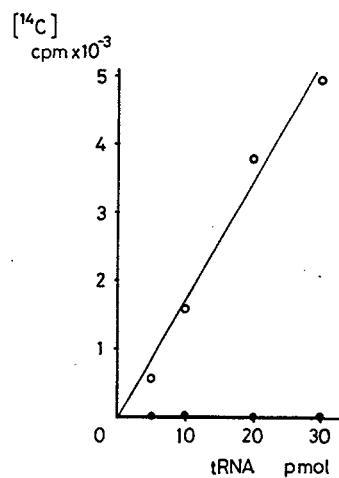


Fig. 7 Charging of methionine to the intact tRNA_f^{Met} (o--o) and the amber tRNA_f^{Met} (CAU) (o--o).

partially purified E.coli aminoacyl tRNA synthetase containing ATP(CTD) tRNA nucleotidyl transferase to repair the 3'-terminal CA. The 3'-terminus was analyzed by labeling with 5'-³²P pCp and RNA ligase.¹⁵ It was found that more than 95% count was transferred to pA. As summarized in Table I, the reconstructed

Table I ¹⁴C Met-acceptor activity of modified tRNAs

tRNA	pmol ¹⁴ C Met charged per 10 pmol	% relative to reconstituted tRNA _f ^{Met}
tRNA _f ^{Met}	4.1	-
reconstituted (CAU)	2.3	100
amber (CUA)	0.04	2
ochre (UUA)	0.14	6
non-anticodon	0.06	3
3' (38-75) and 5' (1-34) -halves	0.08	3
3'-half (38-75)	0.09	4

$tRNA_f^{Met}$ { $tRNA(CAU)$ } was charged 55% with respect to the natural molecule. The tRNA containing CUA anticodon {amber $tRNA(CUA)$ }, other tRNA (UUA), non-anticodon tRNA a mixture of two halves and the 3'-half fragment were not aminoacylated by the synthetase preparation. An example of charging experiment using increasing amounts of amber $tRNA(CUA)$ is shown in Fig. 7 together with the natural $tRNA_f^{Met}$ control. This result indicated that the E.coli methionyl-tRNA synthetase did not recognize $tRNA_f^{Met}$ modified at the anticodon loop. We have observed previously that replacement of the fourth nucleotide from the 3'-end did not affect the charging by the E.coli methionyl-tRNA synthetase significantly.¹⁷ The enzyme may recognize the shape of the anticodon region more restrictively than that of the amino acid acceptor stem part.

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Replacement and insertion of nucleotides at the anticodon loop of *E. coli* tRNA^{Met} by ligation of chemically synthesized ribooligonucleotides

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ABSTRACT

Insertion of the four major nucleotides at the 5'-side of the anticodon triplet of *E. coli* tRNA^{Met} was performed by joining of the half molecules obtained by limited digestion with RNase A and the chemically synthesized tetranucleotide pN-C-A-U using RNA ligase. Insertion of U-U at the 5'-side or A and A-A at the 3'-side of the anticodon were also performed using U-U-C-A-U, C-A-U-A and C-A-U-A-A. The constant U next to the 5'-side of the anticodon was replaced with A and C by ligation of A-C-A-U and C-C-A-U to the 5'-half molecule which had been treated with periodate plus lysine, followed by joining to the 3'-half. These modified tRNAs were tested for their ability to accept methionine with the methionyl-tRNA synthetase of *E. coli*. The affinity of these analogs for the synthetase decreased more extensively when the insertion was at the 3'-side of the anticodon triplet. Insertion of mononucleotides at the 5'-side or replacement of the constant U next to the 5'-side of the anticodon did not affect aminoacylation drastically. This may mean that the 3'-side of the anticodon loop of tRNA is one of the major recognition sites for the methionyl-tRNA synthetase.

INTRODUCTION

Recognition of tRNA by aminoacyl-tRNA synthetases is a particularly good example of the specific interaction of proteins with nucleic acids. By using tRNA analogs with specific modifications at certain regions, information on the recognition of tRNA by the synthetase should be obtainable. We have previously reported that the anticodon triplet of *E. coli* tRNA^{Met} could be replaced by ligation of chemically synthesized trimers to partial RNase A digestion products of the tRNA and that the C-A-U sequence of the anticodon was indispensable for recognition by *E. coli* methionyl-tRNA synthetase.¹⁾ This methodology using RNA oligomers and T4

RNA ligase to obtain tRNA analogues is very useful for studies on structure-function relationships. Since the discovery of T4 RNA ligase, this methodology has been used in various studies.²⁻¹⁹⁾

The anticodon is one of the most important regions in the interaction of tRNA with mRNA during protein synthesis. There are more than 20 species of tRNAs each with their own anticodon which can form hydrogen bonds with complementary codons in mRNA. Furthermore, the 5'-side of the anticodon is almost always occupied by uridine and the 3'-side is a purine or a purine derivative. It is not clear why the residue on the 5'-side of the anticodon should be uridine. In the X-ray crystal structures of tRNAs for yeast tRNA^{Phe}^{20,21)} and E. coli tRNA^{Met}²²⁾, the 5'-side of the anticodon consists of the so called "U-turn" structure. Recently Uhlenbeck et al. showed that this uridine in yeast tRNA^{Tyr} was not essential for amber suppression.¹³⁾

In this paper, we describe the synthesis of several mutant E. coli tRNA^{Met}_f's having additional bases at the 3'- or 5'- side of the anticodon, and investigate whether these additional bases have an influence on the methionine acceptor activity. In addition we have changed the constant U to the 5'-side of the anticodon in order to ascertain the importance of this structural requirement for aminoacylation of the tRNA.

MATERIALS AND METHODS

Materials

Trinucleotides; A-U-G, U-A-U, U-G-A, U-U-A, tetranucleotides; C-A-U-A, A-C-A-U, C-C-A-U, G-C-A-U, U-C-A-U, pentanucleotides; C-A-U-A-A, U-U-C-A-U, were prepared by the triester method.³⁾ [γ -³²P]ATP (specific activity; 2,900 Ci/mmol) and L-[U-¹⁴C]Methionine (specific activity; 282 mCi/mmol) were obtained from NEN and Amersham, respectively. E. coli tRNA^{Met}_f was a gift of Dr. D. Söll. (specific activity; 1,400 pmol/A₂₆₀ unit) Folinic Acid-SF as a formyl-donor was obtained from Lederle. T4 RNA ligase was purified from E. coli A19 strain infected with T4 phage as

described.²³⁾ Polynucleotide kinase and alkaline phosphatase from E. coli A19 were obtained from Takara Shuzo Co., RNase A, RNase T₂ and Nuclease P₁ were obtained from Boehringer Co., Sankyo Co. and Yamasa Shoyu Co., respectively. Crude E. coli aminoacyl-tRNA synthetase was prepared as described by Nishimura et al.³⁵⁾ E. coli ribosomes were prepared as described by Nishizuka et al.²⁴⁾

Methods

Phosphorylation, dephosphorylation and ligation were performed as described previously.²⁵⁻²⁹⁾ Phosphorylated tetra and pentanucleotides corresponding to the anticodon were purified by DEAE Sephadex A-25 ion exchange column chromatography and Shephadex G-50 gel filtration, respectively. 5'- and 3'-half molecules of E. coli tRNA_f^{Met} were prepared by partial digestion with RNase A followed by purification by 10 % polyacrylamide gel electrophoresis (PAGE), and sequenced by mobility shift analysis as described previously.¹⁾ After RNA ligase reactions, the products were purified by 10% PAGE and the binding sites between two RNA molecules were identified by nearest neighbor analysis.

Removal of one base from the 3'-end of the 5'-half using periodate plus lysine was performed as described by Sprinzl et al.³⁰⁾ We added an equal amount of 3'-half to the 5'-half in the reaction mixture to form a complex with the secondary structure of tRNA_f^{Met}. After the reaction, the 5'-half lacking one base at the 3'-end was purified by 10% PAGE.

Aminoacylation of tRNA was performed as described previously¹⁾ except for measurement of the Km. Km studies were carried out by two step reactions; restoring the C-C-A end and aminoacylation. The C-C-A restoring reaction on the synthesized tRNA was performed in 100mM HEPES-NaOH (pH 8.0), 10 mM magnesium acetate, 10 mM potassium chloride, 10 mM β -mercaptoethanol, 4 mM ATP, 0.8 mM CTP, 0.15-2.0 μ M tRNA and 1 mg/ ml E. coli S-100 at 37°C for 30 min in 10 μ l. After the C-C-A restoring reaction the mixture was heated at 100°C for 2 min and slowly cooled to room temperature.

Aminoacylation was started by adding 200 pmol of L-[U-¹⁴C]

Met and 2 μ g of E. coli S-100. This reaction mixture was incubated at 37°C for 20 min and quantified as described previously.³⁾

Formylation was performed in aminoacylation buffer containing 0.7 mM Folinic Acid-SF at 37°C for 30 min in 30 μ l. After the reaction one half of the reaction mixture was spotted onto a glassfilter disk and to the other half was added the same volume of 50 mM CuSO₄-0.3 M Tris-HCl (pH 7.5). Incubation was continued at 37°C for 10 min³¹⁾ and this mixture was also spotted on another glassfilter disk. These disks were washed three times with cold 5% trichloroacetic acid for 10 min, twice with cold ethanol-ether (1:1, v/v) for 5 min and dried and counted.

A binding study involving the codon-anticodon interaction on E. coli ribosomes was performed as described by Nirenberg et al.³²⁾ except that 5'-³²P labelled tRNAs were used instead of aminoacyl-tRNAs.³³⁾

RESULTS

Synthesis of modified tRNAs with extra bases at either side of the anticodon triplet

We synthesized several E. coli tRNA_f^{Met} derivatives having additional bases either side of the anticodon as shown in Fig. 1. We removed the anticodon trimer of E. coli tRNA_f^{Met} by partial digestion with RNase A. In these conditions we could obtain a 5'-half molecule (bases 1-33) and a 3'-half molecule (bases 37-75) as major products. These halves were purified on 10% PAGE. Both halves were sequenced as described previously.¹⁾ The 3'-end phosphate of the 5'-half was removed by treatment with bacterial alkaline phosphatase after opening the 2',3'-cyclic phosphate.

Tetra or pentaribooligonucleotides containing the anticodon sequence were synthesized by the triester method. Three kinds of RNAs; the 5'-half, an anticodon sequence and the 3'-half, were joined with T4 RNA ligase to reconstruct the whole structure of a tRNA. Firstly, phosphorylated anticodon-oligomers were ligated to the 5'-half molecules and the products were isolated on 10% PAGE. After

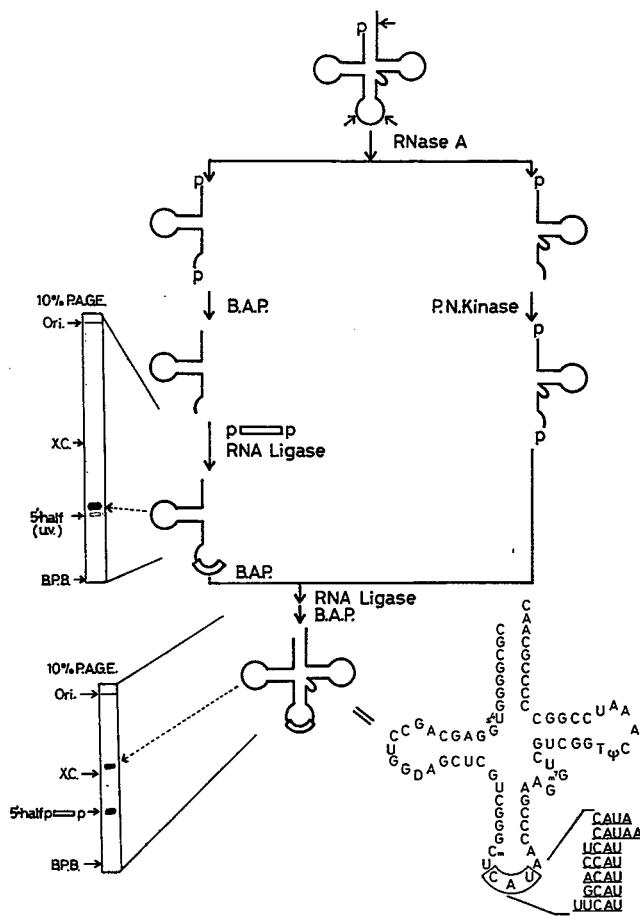


Fig. 1 Scheme for syntheses of modified *E. coli* initiator tRNAs and the pattern of 10% PAGE in each ligation step. Open square shows the synthesized tetra- or penta-riboligomers corresponding to the anticodon sequence. These autoradiographs of 10% PAGE show the case in which C-C-A-U is the anticodon sequence. In the cases of other riboligomers, all the PAGE patterns³² were the same as that of C-C-A-U. The asterisk denotes ^{32}P -phosphate.

dephosphorylation, 5'-half plus anticodon-oligomer molecules were ligated to the phosphorylated 3'-half molecules and the final product was isolated on 10% PAGE. After each step of ligation, we carried out nearest neighbor analysis to confirm that the binding site between the donor and the acceptor was

not incorrect. By this strategy we obtained seven kinds of tRNA which have anticodon sequences of A-C-A-U, C-C-A-U, G-C-A-U, U-C-A-U, C-A-U-A, U-U-C-A-U and C-A-U-A-A instead of C-A-U. The yield of ligation between 5'- and 3'-halves was about 50% after isolation on 10% PAGE. This was relatively satisfactory compared to the yield for ligation of the 5'-half molecule and anticodon sequences (20-25%).

Replacement of constant U

The 5'-half molecule which was generated by digestion of *E. coli* tRNA^{Met}_f with limited amounts of RNase A was treated with phosphatase to remove the 3'-phosphate and with NaIO₄ at pH 5.2. The 3'-uridine was removed by β -elimination with lysine. The 3'-terminal analysis showed that the U residue was removed and that the Cm residue to the 5'-side of U was the new 3'-terminal base. Three tetramers; U-C-A-U, C-C-A-U and A-C-A-U, were phosphorylated with polynucleotide kinase and ATP, and joined to the 5'-half lacking the 3'-terminal U. These 5'-half molecules plus tetramer and the phosphorylated 3'-half molecule were joined and the products were purified on 10% PAGE. Fig. 2 shows the scheme for the replacement of constant U and nearest neighbor analysis of these products. Cm³²pNp from the first step of the ligation and U³²p from the second step were detected on two dimensional TLC.³⁴⁾ These experiments also showed that the 3'-side of Cm was changed to the planned base as judged by the position of Cm³²pNp on TLC.

Aminoacylation of synthesized tRNAs

Synthesized tRNAs which have extra bases in the anticodon loop and another base at the 5'-side of the anticodon instead of constant U were investigated for their methionine acceptor activity using partially purified *E. coli* aminoacyl-tRNA synthetase. This crude enzyme contained ATP(CTP)tRNA nucleotidyl transferase to repair the 3'-terminal C-A sequence of the synthesized tRNAs and in fact this repair was almost complete as described previously.¹⁾ All tRNAs having extra bases in the anticodon loop accepted methionine. Kinetic studies showed that the addition of two bases beside the anticodon increased the Km value of these tRNAs for the

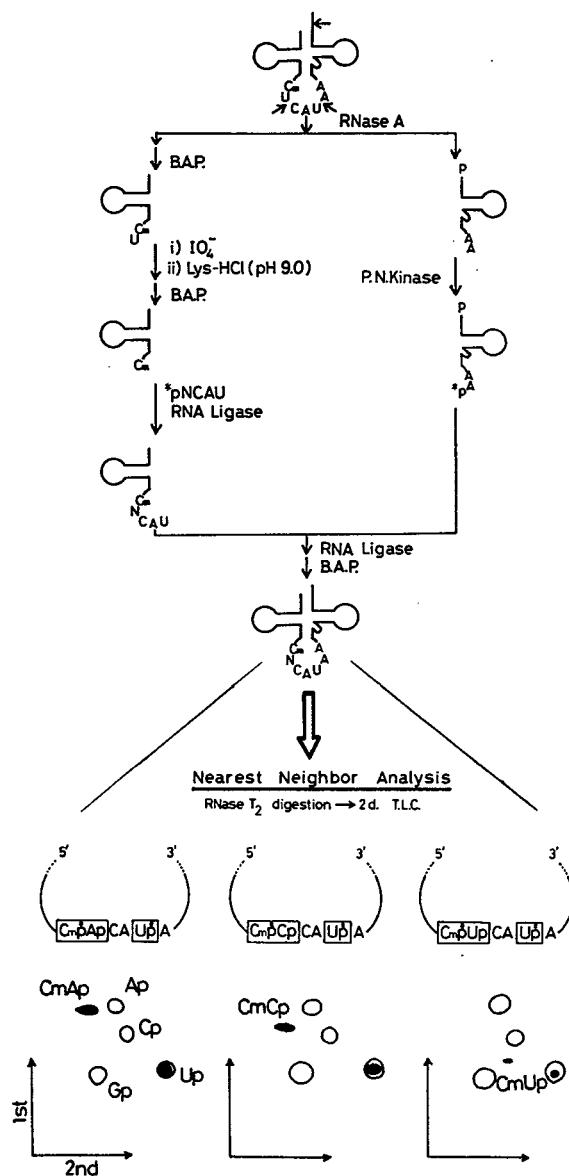


Fig. 2 Scheme for the replacement of constant U and nearest neighbor analyses of these products. The asterisk denotes ^{32}P -phosphate.

N; A, C, U.

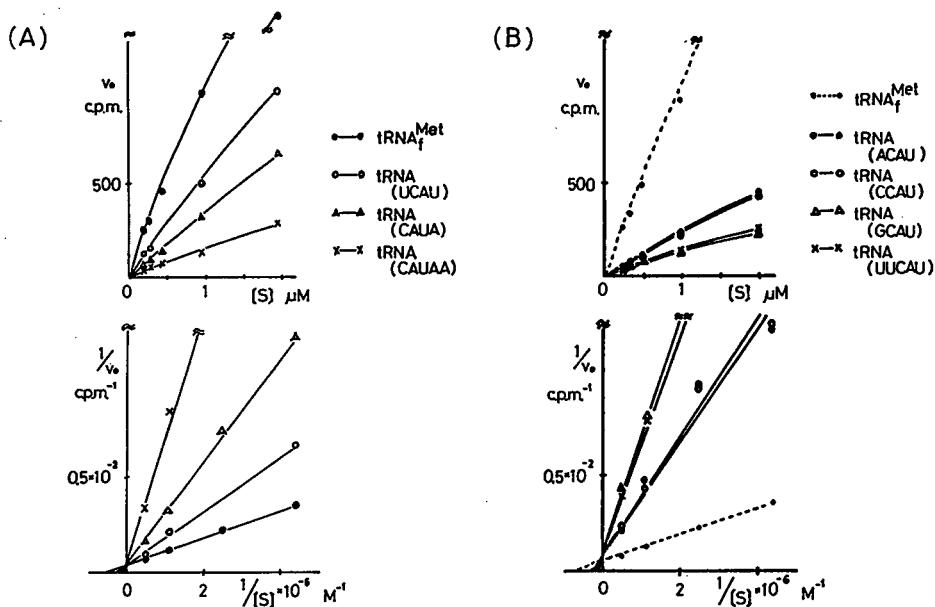


Fig. 3 Aminoacylation of synthesized tRNAs. $t\text{RNA}(\text{UCAU})$, $t\text{RNA}(\text{CAUA})$, $t\text{RNA}(\text{CAUAA})$, $t\text{RNA}(\text{ACAU})$, $t\text{RNA}(\text{CCAU})$, $t\text{RNA}(\text{GCAU})$ and $t\text{RNA}(\text{UUCAU})$ indicate the tRNAs whose anticodon sequences are U-C-A-U, C-A-U-A, C-A-U-A-A, A-C-A-U, C-C-A-U, G-C-A-U and U-U-C-A-U, respectively. Lower graphs show the Lineweaver-Burk plots.

methionyl tRNA synthetase considerably; the K_m value was 12.5 μM (the K_m value of natural $t\text{RNA}_{\text{f}}^{\text{Met}}$ was 1.7 μM). In cases where one base was added, the addition of U at the 5'-side of the anticodon preserved the high affinity for the synthetase; the K_m value was 3.3 μM . On the other hand, A at the 3'-side increased the K_m value; the K_m value was 6.7 μM . (Shown in Fig. 3.A) The addition of A, C or G at the 5'-side of the anticodon also increased the K_m value. (Shown in Fig. 3.B) The synthesized tRNAs replacing the constant U with A or C at the 5'-side of the anticodon were also tested for their methionine acceptor activity. (Fig. 4) Both tRNAs had the same level of methionine acceptor activity as the control $t\text{RNA}_{\text{f}}^{\text{Met}}$, whose constant U was first removed by periodate treatment then replaced. However this control $t\text{RNA}_{\text{f}}^{\text{Met}}$ did not attain the same level of activity as natural *E. coli*

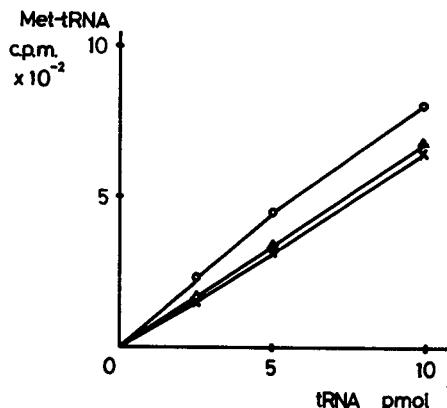


Fig. 4 Methionine acceptor activity of tRNAs with changed constant U. \circ - \circ ; control tRNA_f^{Met}, Δ - Δ ; tRNA(C-33) meaning that the constant U is changed to C, \times - \times ; tRNA(A-33).

tRNA_f^{Met}. This may be due to periodate treatment of the 5'-half molecule. Initially we carried out the periodate treatment of the 5'-half molecule without adding the 3'-half reconstructed tRNAs using these periodate treated 5'-halves had little activity as methionine acceptors. Therefore for subsequent periodate treatments we added 3'-half molecules to the reaction mixture in order to protect the 5'-half by forming a secondary structure as in tRNA. Following this procedure, the methionine acceptor activity of reconstructed tRNAs increased but did not reach the natural level. It seems that not all 5'-half molecules were protected by secondary structure formation as in tRNA_f^{Met} during the periodate treatment and that some bases were damaged to decrease the methionine acceptor activity.

The interaction on ribosomes between triribonucleotides and the anticodons of tRNA_f^{Met} sequences having larger anticodon loop

We examined the interaction between ribotrinucleotides and some synthesized tRNAs with larger anticodon loops on E. coli ribosomes to investigate the structural role of the anticodon. Using the chemically synthesized ribooligomers, A-U-G, U-G-A, U-U-A, or U-A-U as mRNA, we measured the

Table I

	tRNA _f ^{Met} pmol(%)	tRNA(CAUUA) pmol(%)	tRNA(CAUAA) pmol(%)	tRNA(UCAU) pmol(%)
-mRNA	0.45	0.50	0.42	0.36
AUG	0.95(100)	0.49(0)	0.41(0)	0.51(33)
UGA	0.46(∞0)	0.50(0)	0.38(0)	0.37(0)
UAU	-	0.45(0)	0.39(0)	0.35(0)
UUA	-	0.49(0)	0.43(∞0)	0.33(0)

Codon-anticodon interactions on E. coli ribosomes. Each ³²P-labeled tRNA (10 pmol) was added to the reaction mixture containing mRNA and E. coli 70S ribosomes. -mRNA indicates the condition of no mRNA in this reaction mixture. % when the specific binding of natural tRNA_f^{Met} to A-U-G is 100%.

specific binding of tRNAs whose anticodon sequences were C-A-U-A, C-A-U-A-A, U-C-A-U instead of C-A-U to mRNA on E. coli ribosomes. Table I shows that only tRNA_f^{Met} having U-C-A-U at the anticodon; tRNA(UCAU), binds specifically to A-U-G and that this binding was 33% with respect to the binding of natural tRNA_f^{Met} to A-U-G. This result showed that the structure of the anticodon trimer in tRNA(UCAU) is similar to that in natural tRNA_f^{Met} notwithstanding the insertion of U at the 5'-side of the anticodon. It is presumed that three bases of the anticodon in tRNA(UCAU) stand in line outside the phosphate backbone of the loop.

Formylation of synthesized tRNA_f^{Met} species

Formylation is an event specific to the initiator tRNA in prokaryotes. The part of the initiator tRNA recognized by the transformylase is unknown. Three tRNAs having enlarged anticodon loop were tested for formylation by adding the formyl donor to the aminoacylation system. After the reaction, the mixture was divided to two parts. One part was treated with 50 mM CuSO₄-0.3 M Tris HCl (pH 7.5) in order to deacylate non-formylated methionyl-tRNA, and then spotted on a glass filter. The other was spotted on a glass filter without treatment. The ratio of CuSO₄ treated sample radioactivity to non-treated sample radioactivity was defined as the formylation ratio. The percentages for tRNA(CAUUA), tRNA(CAUAA) and tRNA(UCAU) were 88%, 98% and 93%,

respectively. All these values were considered to represent quantitative formylation within the limits of experimental error. These values suggest that the transformylase does not interact with the anticodon area of $tRNA_f^{Met}$.

DISCUSSION

We have synthesized seven kinds of tRNAs which have larger anticodon loops. In the synthetic procedure, we first tried to join the anticodon sequences to 3'-half molecules but the yields in ligation were too low for the expected product to be obtained. This may be due to the bulkiness of the 3'-half as a donor in the RNA ligase reaction. In cases of ligation of the 5'-halves containing the anticodon sequence to the 3'-half, we could obtain relatively high yields. In this reaction, the 5'-half and 3'-half molecules form tRNA secondary structure and therefore the 3'-end of the 5'-half is located close to the 5'-end of the 3'-half. Essentially this ligation between the two halves behaves as an intramolecular joining reaction across the anticodon.

Modified tRNAs having large anticodon loops were previously shown to accept methionine.¹⁶⁾ We have further modified the structure around the anticodon of E. coli $tRNA_f^{Met}$. The additional U at the 5'-side of the anticodon does not change the conformation of the anticodon loop more than the additional A at the 3'-side of the anticodon. Even if U was added to the 5'-side of the anticodon, this tRNA could form a complex with A-U-G on ribosomes. It may be assumed that the additional U does not disturb the so called U-turn structure with the anticodon trimer in line.

The 5'-side of the anticodon in tRNAs is almost always occupied by a U residue. Uhlenbeck et al. showed previously that this constant U and the anticodon sequence in yeast $tRNA^{Tyr}$ could be substituted by another base.¹³⁾ We have showed similar results in this report. Thus the $tRNA_f^{Met}$ which had other bases instead of constant U could be charged with methionine to the same level as $tRNA_f^{Met}$ having constant U. We conclude that the constant U at the 5'-side of the anticodon is not indispensable for the amino acid acceptor

activity of tRNA. Presumably even if the 5'-side of the anticodon is another base than U, the anticodon structure is maintained as in natural tRNA_f^{Met}.

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Modification of the amino acid acceptor stem of *E. coli* tRNA_f^{Met} by ligation of chemically synthesized ribooligonucleotides

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The single-stranded region of the amino acid acceptor stem corresponding to the 3'-end of *E. coli* tRNA_f^{Met} was replaced by ligation of chemically synthesized ribooligonucleotides, in order to change the length of the single-stranded CCA terminus. The chemically synthesized ribooligomers, CCA, ACCA, AACCA and CAACCA, were ligated to nuclease-treated *E. coli* tRNA_f^{Met}, which lacked the ACCA sequence at the 3'-end. The methionine acceptor activities of these modified tRNAs were examined using *E. coli* methionyl-tRNA synthetase. Ligation of the chemically synthesized pentamer (AACCA) to the acceptor terminus restored the methionine acceptor activity, whereas ligation of the hexamer (CAACCA) or trimer (CCA) to the acceptor terminus did not. Modification of the acceptor terminus had no effect on the formylation of accepted methionine.

<i>Aminoacylation</i>	<i>Formylation</i>	<i>Elongated aminoacylation end</i>	<i>Truncated aminoacylation end ligation</i>
		<i>Synthetic oligonucleotide</i>	

1. INTRODUCTION

Transfer RNA is one of the smallest nucleic acids that has a biological function in living cells. In many laboratories structure requirements for the recognition of tRNAs by aminoacyl-tRNA synthetases have been concerned with the interaction between nucleic acids and proteins [1-14].

We have synthesized various analogues of *E. coli* tRNA_f^{Met} by ligating chemically synthesized ribooligomers to natural tRNA_f^{Met} fragments using T4 RNA ligase [12-14].

Replacement of the anticodon trimers in *E. coli* tRNA_f^{Met} caused a large decrease in the amino acid acceptor activity with *E. coli* methionyl-tRNA synthetase [14]. On the other hand, changes in the discriminator position of the amino acid acceptor stem had little influence on the affinity for the synthetase [13].

* To whom correspondence should be addressed

E. coli tRNA_f^{Met} has a unique structure at the anticodon loop and the amino acid acceptor stem [15,16] where the last 5 bases from the 3'-terminus curl back in contrast to other tRNAs [16]. We assumed that the acceptor end directly binds methionine, and therefore investigated the correlation between the length of the protruding 3'-terminus and the methionine acceptor activities of analogs having a different protruding end. Here, we describe the syntheses of several modified *E. coli* tRNA_f^{Met} formed by ligating CCA, ACCA, AACCA or CAACCA to a tRNA_f^{Met} lacking the 3'-ACCA. The methionine acceptor activities of these analogues were examined using the *E. coli* methionyl-tRNA synthetase.

2. MATERIALS AND METHODS

Ribooligonucleotides were synthesized by the triester method [17]. [γ -³²P]ATP and L-[U-¹⁴C]-methionine were obtained from NEN and Amer-

sham, respectively. *E. coli* tRNA_f^{Met} was a gift from Dr D. Söll. T4 RNA ligase was purified as described [18]. Polynucleotide kinase and *E. coli* alkaline phosphatase were obtained from Takara Shuzo and nuclease S1 from Sankyo. Other enzymes used for the analyses of products were obtained as in [14,19,20]. Crude *E. coli* aminoacyl-tRNA synthetase was purified as described [21]. Folinic acid-SF as a formyl donor was obtained from Lederle.

Partial digestion of *E. coli* tRNA_f^{Met} with nuclease S1 and aminoacylation reaction were performed as in [13]. Phosphorylation with polynucleotide kinase, dephosphorylation with alkaline phosphatase and ligation with T4 RNA ligase were performed according to [12,14]. Formylation was performed under the conditions for aminoacylation containing 0.7 mM folinic acid-SF at 37°C for 30 min in 30 μ l formylation reaction mixture. Half of the mixture was treated with the same volume of 50 mM CuSO₄, 0.3 M Tris-HCl (pH 7.5) at 37°C for 10 min.

3. RESULTS AND DISCUSSION

A scheme for the synthesis of modified *E. coli* tRNA_f^{Met} is shown in fig.1. *E. coli* tRNA_f^{Met} was treated with a limited amount of nuclease S1 to remove the 3'-terminal ACCA. The remaining fragment with 73 nucleotides [tRNA(73)] was isolated on 10% polyacrylamide gel electrophoresis (PAGE). Four ribooligonucleotides, CCA, ACCA, AACCA, CAACCA, were synthesized by the triester method as in [17]. These chemically synthesized ribooligonucleotides were phosphorylated with [γ -³²P]ATP and polynucleotide kinase and then joined to the tRNA(73) with T4 RNA ligase. The ligated products were isolated on 10% PAGE and subjected to a nearest neighbor analysis to confirm the junction point. The yields of these ligase reactions were below 10%.

The ligated molecules, tRNA(76), tRNA(77), tRNA(78) and tRNA(79), were dephosphorylated with alkaline phosphatase and tested for their methionine acceptor activities (fig.2). The tRNA(77)

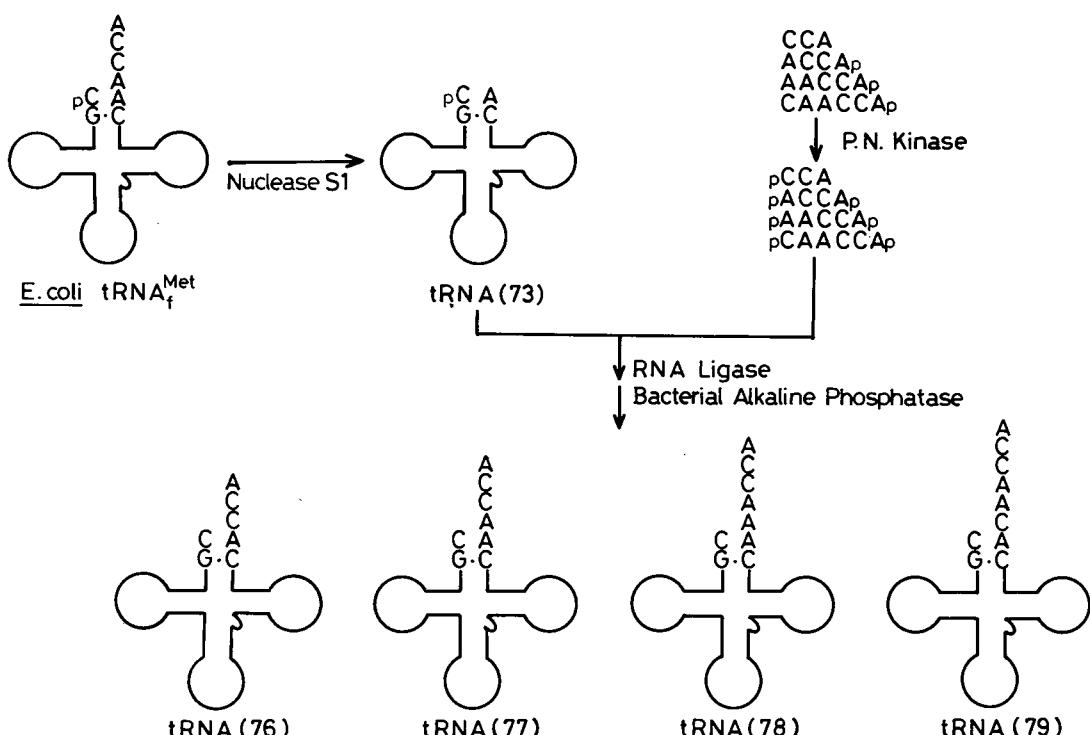


Fig.1. Scheme for the modification of the amino acid acceptor stem of *E. coli* tRNA_f^{Met}.

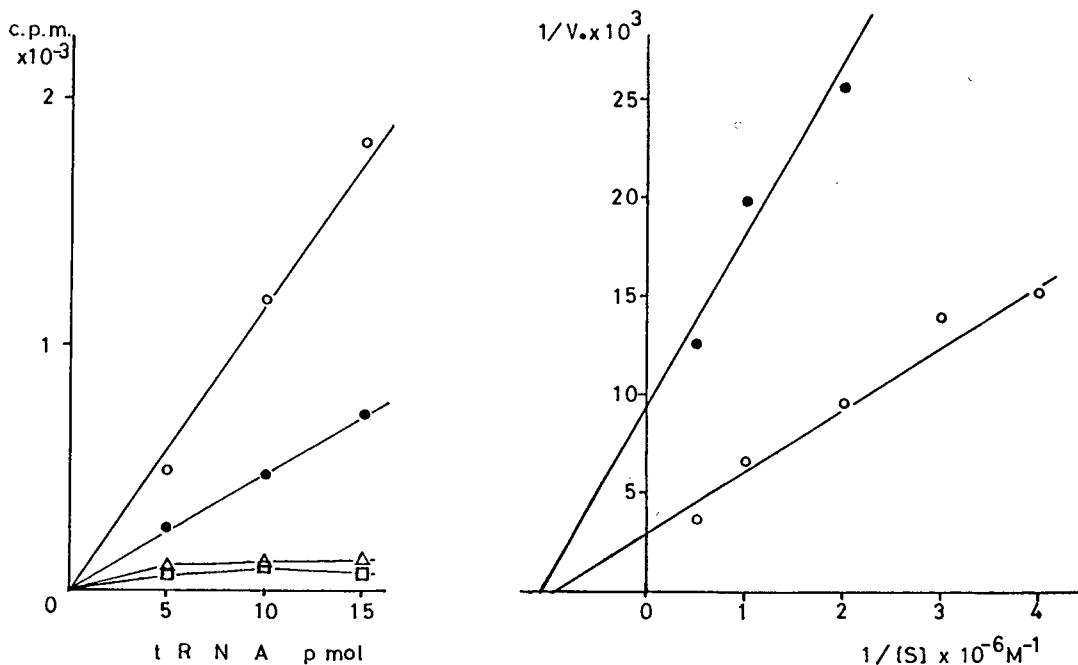


Fig.2. Aminoacylation of modified tRNAs. Methionine acceptor activities of tRNAs (left) and kinetic studies for aminoacylation (right). (○—○) Control tRNA(77), (Δ—Δ) tRNA(76), (●—●) tRNA(78), (□—□) tRNA(79).

molecule was synthesized as a control since it had the same sequence as the natural tRNA_f^{Met}. Fig.2 shows that tRNA(76), 1 base shorter, and tRNA(79), 2 bases longer, had no methionine acceptor activity, but tRNA(78), 1 base longer, could accept methionine. The kinetic studies on aminoacylation showed that the differences in aminoacylation activity were reflected only in the V_{max} values since the K_m values of these tRNAs were almost the same. Therefore, both tRNA(77) and tRNA(78) had similar affinities for *E. coli* methionyl-tRNA synthetase. It appears that the 3'-terminus of tRNA_f^{Met} does not influence the affinity of methionyl-tRNA synthetase. Consequently we can assume that both tRNA(76) and tRNA(79) presumably form a complex with the synthetase, but are unable to position the 3'-end adenosine moiety at the catalytic site of the synthetase. One extra base in the 3'-terminus region may not prevent the required contact of the 3'-end adenosine with the catalytic site.

The recognition site of methionyl-tRNA_f^{Met} by the transformylase in prokaryotes is still unclear. We have investigated the formylation of the tRNA(78) under the same assay system conditions

as for the aminoacylation reaction except for the presence of formyl donor. The formylation ratio for tRNA(78) and tRNA(77) was 0.87 and 0.74, respectively. This may indicate that the addition of 1 extra base in the 3'-terminus region of tRNA_f^{Met} does not affect the shape of the molecule or that the transformylase may recognize not only the 3'-terminal region but also another area as can be seen by comparison of the sequence of tRNA_f^{Met} [22] and tRNA_m^{Met} [23].

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The *in vivo* stability, maturation and aminoacylation of anticodon-substituted *Escherichia coli* initiator methionine tRNAs

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We have constructed eight anticodon-modified *Escherichia coli* initiator methionine (fMet) tRNAs by insertion of synthetic ribotrinucleotides between two fragments ('half molecules') derived from the initiator tRNA. The trinucleotides, namely CAU (the normal anticodon), CAA, CAC, CAG, GAA, GAC, GAG and GAU, were joined to the 5' and 3' tRNA fragments with T4 RNA ligase. The strategy of reconstruction permitted the insertion of radioactive ^{32}P label between nucleotides 36 and 37. tRNAs were microinjected into the cytoplasm of *Xenopus laevis* oocytes, and the following properties were evaluated: (a) the stability of these eubacterial tRNA variants in the eukaryotic oocytes; (b) the enzymatic modification of the adenosine at position 37 (3' adjacent to the anticodon) and (c) aminoacylation of the chimeric tRNAs by endogenous oocyte aminoacyl-tRNA synthetases.

In contrast to other variants, the two RNAs having CAU and GAU anticodons were stable and underwent quantitative modification at A-37. These results show that the enzyme responsible for the modification of A-37 to *N*-(*N*-(9- β -D-ribofuranosylpurine-6-yl)carbamoyl)threonine ($t^6\text{A}$) is present in the cytoplasm of oocytes and is very sensitive to the anticodon environment of the tRNA. Also, these same GAU and CAU anticodon-containing tRNAs are fully aminoacylated with the heterologous oocyte aminoacyl-tRNA synthetases *in vivo*. During the course of this work we developed a generally applicable assay for the aminoacylation of femtomole amounts of labelled tRNAs.

Some time ago a correlation between the anticodon sequence of a tRNA and the identity of neighbouring modified nucleosides was noted [1] (reviewed in [2]). Thus, the hypermodified nucleoside $t^6\text{A}$, *N*-(*N*-(9- β -D-ribofuranosylpurine-6-yl)carbamoyl)threonine, or a derivative thereof is located in position 37 of tRNAs having anticodons terminating in a uridine. One enigmatic exception is the initiator methionine tRNA of *Escherichia coli*, for most other tRNAs including the eubacterial elongator Met-tRNA, which has the same anticodon sequence, and the eukaryotic initiator tRNA contain the A-37 modification [3].

To study the effect of structural modifications in the anticodon loop on the modification of A-37, we have turned to recombinant RNA methods based on T4 RNA ligase. These techniques are particularly well-suited to the preparation of related tRNA chimera, which have substitutions in or near the anticodon, since fragments serving as starting material for the tRNA variant can be readily obtained from controlled

nucleolytic cleavage of tRNA. Several variant tRNA molecules have been constructed in this way from *E. coli* and yeast tRNA species; these have been useful in evaluating the effect of nucleotide substitution on the interaction of tRNA with aminoacyl-tRNA synthetases, nucleoside modification enzymes and codons during protein synthesis [4–13] (reviewed in [14]). In the present work we prepared eight anticodon-substituted fMet-tRNA chimera. These anticodon variants of the type CAX and GAX (where X is each of the four nucleosides A, C, G and U; CAU is the normal Met-tRNA anticodon) were synthesized by inserting trinucleotides between 3' and 5' half molecules and analyzed after injection into *Xenopus laevis* oocytes. Several of these same variants were prepared previously for studies on *E. coli* methionyl-tRNA synthetase recognition [8–12]. Using the *Xenopus* oocyte system [15] we were able to evaluate *in vivo* the heterologous charging of these variants, the stability of the chimeric tRNA and the structural requirements for nucleoside modification.

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Abbreviations: $t^6\text{A}$, *N*-(*N*-(9- β -D-ribofuranosylpurine-6-yl)carbamoyl)threonine; $i^6\text{A}$, *N*⁶-isopentenyladenosine; BD-cellulose, benzoylated DEAE-cellulose.

Enzymes (IUB Recommendations 1984). Methionyl-tRNA synthetase of L-methionine:tRNA ligase (AMP) (EC 6.1.1.10); T4 RNA ligase (EC 6.5.1.3); T4 polynucleotide kinase (EC 2.7.1.78); alkaline phosphatase (EC 3.1.3.1); pancreatic RNase A (EC 3.1.27.6); RNase T2 (EC 3.1.27.1); nuclease P1 (EC 3.1.30.1); tRNA nucleotidyltransferase (EC 2.7.7.25).

MATERIALS AND METHODS

The ribotrinucleotides CAA, CAC, CAG, CAU, GAA, GAC, GAG and GAU were synthesized by the triester method [16]. Their sequences were determined by complete digestion with RNase T2 and nuclease P1 followed by paper electrophoresis at pH 3.5 [16]. $[\gamma-^{32}\text{P}]$ ATP (spec. act. > 3000 Ci/mmol) was obtained from the Amersham International (England). The fMet-tRNA (spec. act. 1400 pmol/ A_{260} unit) was a gener-

ous gift of Dr D. Söll of Yale University. T4 RNA ligase was purified from *E. coli* strain A19 infected with phage T4 as described [17]. T4 polynucleotide kinase and alkaline phosphatase from *E. coli* strain A19 were obtained from Takara Shuzo Co. (Japan). Pancreatic RNase A, RNase T2 from *Aspergillus oryzae* and nuclease P1 from *Penicillium citrinum* were obtained from Boehringer, Mannheim (FRG), Sankyo Co. (Japan) and Yamasa Shoyu Co. (Japan) respectively. Other products were obtained as described earlier [9, 16, 18].

Preparation of E. coli fMet-tRNA half molecules

E. coli fMet-tRNA was digested by pancreatic RNase A under limiting conditions previously described [9]. The resulting 5' and 3' half-molecule products were purified by 10% polyacrylamide gel electrophoresis under denaturing conditions (7 M urea), and their sequences were determined by mobility-shift analysis [9].

Ligation, phosphorylation and dephosphorylation

Enzymatic assay conditions for ligation, phosphorylation and dephosphorylation of oligoribonucleotides or tRNA fragments were described previously [16–20]. Trinucleotides, after phosphorylation using T4 polynucleotide kinase, were purified by DEAE-Sephadex A-25 ion-exchange chromatography [19]. First the trinucleotide was ligated to the 5' half molecule. The 3' half molecule of fMet-tRNA was 5' phosphorylated using T4 polynucleotide kinase and [γ -³²P]ATP; this product was then ligated to the 5' half molecule having the attached trinucleotide [9]. The reconstructed ³²P-labelled fMet-tRNA variants were purified by 10% polyacrylamide gel electrophoresis in 7 M urea. The gel mobility of each tRNA was compared to that of a genuine sample of *E. coli* fMet-tRNA detected by ultraviolet shadowing [52]. The variant tRNAs were eluted from the gel. The solutions were centrifuged to remove small pieces of acrylamide and 2.5 volumes of ethanol were added. After redissolution in buffer, the product was reprecipitated in ethanol.

Complete digestions of RNA molecules with RNase T2 or nuclease P1 were carried out for nearest-neighbour analysis as previously described [18–20].

Microinjection and fate of tRNA chimera in oocytes

Microinjection into the cytoplasm of *X. laevis* oocytes was performed as described [15, 18], using 50 nl aqueous labelled tRNA solution at a concentration which gave approximately 200 000 cpm (Cerenkov)/nl. This is equivalent to about 1 fmol foreign tRNA/oocyte and corresponds to about 1% of the normal amount of oocyte Met-tRNA. Groups of five oocytes were injected with each sample (reproducibility in tRNA microinjections is about 20%) and then incubated in saline solution [21] at 19°C for the desired period of time (from 1 h to 72 h after injection). At the end of the incubation the oocytes were homogenized in 0.2 ml cold buffer containing 0.2 M sodium acetate (pH 4.5, 4°C), 0.01 M MgCl₂, 1 mM disodium EDTA and 1% (w/v) of sodium dodecyl sulfate. The nucleic acids were immediately extracted by phenol/chloroform treatment and ethanol-precipitated as described [18]. Each tRNA precipitate was then redissolved in the same volume of electrophoresis buffer. Given aliquots (the same for each tRNA sample) were then loaded side by side, on a 10% polyacrylamide gel containing 7 M urea. Electrophoresis

was performed at constant voltage for 5–7 h. Location of the ³²P (labelled Met-tRNA and the putative degradation products) was performed by autoradiography of the gel, and the band corresponding to intact full-length tRNA was cut out, eluted and its radioactivity counted again.

Identification of the 5'-³²P-labelled nucleotide at position 37 of the eluted tRNA was carried out by exhaustive nuclease P1 digestion [22] and analysis of the products by two-dimensional chromatography on thin-layer cellulose plates (6.6 × 6.6 cm², see [1]). After detection of the labelled nucleotides on the plates, they were eluted with water and their radioactivity was determined by liquid scintillation.

Assay of in vivo aminoacylation

tRNA was extracted from oocytes using phenol saturated with 50 mM sodium acetate buffer, pH 4.5, and precipitated with ethanol [23]. It was dissolved in 50 µl 0.1 M triethanolamine/HCl buffer at pH 8.0, and 1 mg phenoxyacetyl ester of *N*-hydroxysuccinimide in 10 µl of anhydrous tetrahydrofuran was added at 0°C [24]. After 10 min the reaction was stopped by dilution with 60 µl cold 20 mM sodium acetate buffer at pH 4.5 containing 20 mM MgCl₂ and 100 mM NaCl. This solution was then applied to a 1-ml column of benzoylated DEAE-cellulose (BD-cellulose), which was pre-equilibrated in 10 mM sodium acetate buffer pH 4.5 containing 10 mM MgCl₂ and 50 mM NaCl. The sample was applied, and the column was washed with 3 ml starting buffer and then with 2 ml of the same buffer containing 1.0 M NaCl. A final wash was composed of the starting buffer containing 1.0 M NaCl and 20% ethanol. All fractions (0.5 ml) were counted in a scintillation counter, and the extent of aminoacylation in the original tRNA preparation was estimated by comparison of the radioactivity of the ethanol fraction to the radioactivity applied to the column.

RESULTS

Construction of anticodon-substituted E. coli fMet-tRNA

The enzymatic replacement of the *E. coli* fMet-tRNA anticodon by several oligoribonucleotides was reported previously [9, 12] and similar procedures have been developed independently by Schulman et al. [10, 11a, b]. In this paper we concentrate on the anticodon variants of the type CAX and GAX (where X is any of the four nucleotides). The reconstruction scheme in Fig. 1 is essentially the same as that previously described [9] except for a simplification of step 3 and the addition of step 7 (see below). Thus, the digestion of fMet-tRNA with pancreatic RNase A generated the two half molecules less the three anticodon nucleotides and the dinucleotide CA of the 3' terminus. The 5' half molecule, composed of pC-1 to Up-33, and the 3' half, A-37 to Cp-75, were separated by 10% polyacrylamide gel electrophoresis under denaturing conditions. The 5' half molecule was then treated by alkaline phosphatase to remove the two terminal phosphates and the product was isolated again by denaturing polyacrylamide gel electrophoresis (step 2). In step 3, synthetic ribonucleotides were joined to the 5' half molecule using RNA ligase. In the earlier procedure [9] this step was performed with synthetic trinucleotides, which were phosphorylated at both ends to prevent self-polymerization. In the present procedure trinucleotides phosphorylated at only the 5' terminus were used; this simplification saves a 3'-dephosphorylation step which would be necessary subsequently to link this

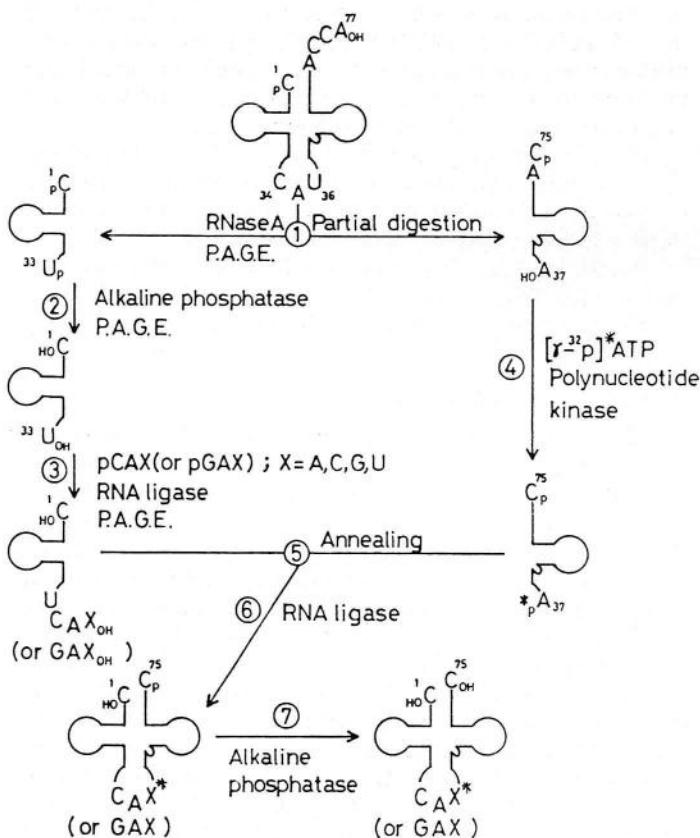


Fig. 1. Synthetic scheme for the preparation of *E. coli* initiator Met-tRNA variants. Numbers and names identify the successive steps and molecules used (see text). Each fragment was isolated by electrophoresis on polyacrylamide gel. The asterisk signifies the position of an internal ^{32}P label introduced in step 4 on the 5'-phosphate of A-37

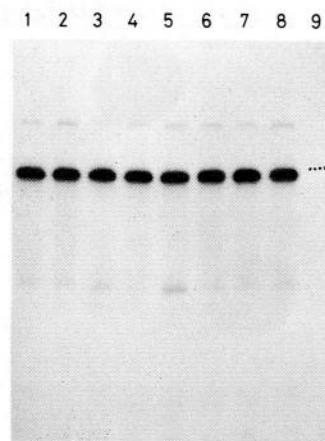
oligomer to the 3' half molecule. The modification gives yields equivalent to the original procedure, but it is preferable when large numbers of tRNA variants must be prepared.

In step 4 the 3' half molecule was phosphorylated with kinase and $[\gamma-^{32}\text{P}]$ ATP. This radioactive oligomer was then annealed with a ten-times excess of the unlabelled extended 5' half molecule (step 5). The large excess was used to consume all the radioactive fragment and is probably not needed to obtain respectable yields in the ligation step. Ligation of the two molecules in step 6 gave nearly a quantitative yield of the tRNA product, which lacks only the 5' phosphate and the 3' CA terminus. Purification by polyacrylamide gel electrophoresis is shown in Fig. 2. Dephosphorylation by alkaline phosphatase (step 7), a necessity for proper CCA terminal transferase repair in the oocyte [40], was followed by a final gel purification.

Stability of the fMet-tRNA chimera

Each of the eight tRNA chimera were microinjected into the cytoplasm of *X. laevis* oocytes. The degradation of these eubacterial variants in the eukaryotic cytoplasm was measured after incubation of the oocytes at 19°C for different periods of time by quantitative analysis of the remaining tRNAs. This analysis consisted of the gel electrophoresis of tRNA samples extracted from oocytes. As shown in Fig. 3, only products having a few less nucleotides than the tRNA are seen: they migrate more quickly than the full-length molecules (compare with the position of the arrow on Fig. 3).

10% PAGE.



1. tRNA(GAA)	^{32}P
2. tRNA(GAC)	
3. tRNA(GAG)	
4. tRNA(GAU)	
5. tRNA(CAA)	
6. tRNA(CAC)	
7. tRNA(CAG)	
8. tRNA(CAU)	
9. tRNA ^{Met} (CAU) UV.	

Fig. 2. Autoradiogram of the purification by electrophoresis on polyacrylamide gel (10% in 7 M urea) of eight tRNA chimeras. This is the gel after step 6 of Fig. 1. The tRNAs lack the 5' terminus and the C⁷⁵-A⁷⁶ of the 3' terminus; therefore, they run correspondingly faster than the authentic fMet-tRNA sample in lane 9 (indicated by the dashed line). The absence of low-molecular-mass fragments reflects a greater than 95% yield of the cyclization reaction (step 6). Migration is from the top to the bottom of the gel as indicated by the bromophenol blue marker (B.P.B.); X.C. corresponds to the position of xylene cyanol

These shorter molecules could represent the tRNA without the 3'CCA terminus while the longer molecules probably correspond to the fMet-tRNA having their 3'CCA end repaired by the tRNA nucleotidyltransferase which is present in the cytoplasm of *X. laevis* oocytes [40, 41]. Interestingly enough, degradation products corresponding to small pieces of tRNA are not apparent on the gel after electrophoresis. This suggests that the very first cleavage in tRNA by oocyte nucleases is the rate-limiting step, after which full degradation of the tRNA into nucleotides occurs rapidly. A similar observation was already made with microinjected yeast tRNA-Asp, where the anticodon loop was cleaved prior to microinjection [18].

The overall rate of degradation of full-length tRNA was evaluated by determination of the amount of radioactivity remaining in the corresponding band on the polyacrylamide gel. These results are summarized in the degradation curves of Fig. 4. The rate of degradation is seen to be, to a first approximation, a simple exponential function, and each tRNA variant has a characteristic half-life in the oocyte. Clearly the fMet-tRNA with a CAU anticodon (the native anticodon) and the GAU anticodon (the anticodon of isoleucine belonging to the same genetic-code quartet) are the most stable having half-lives of approximately 95 h and 130 h respectively. All other tRNAs had half-lives on the order of 16–32 h.

Maturation of tRNA chimera in oocytes

In order to determine the state of modification of the A-37 in the above experiments, the full-length tRNA was extracted from the gels, and each sample was digested by nuclease P1 producing nucleoside 5'-phosphates. Owing to the location of the radioactive phosphate between nucleotides 36 and 37, only the nucleotide in position 36 was labelled, the

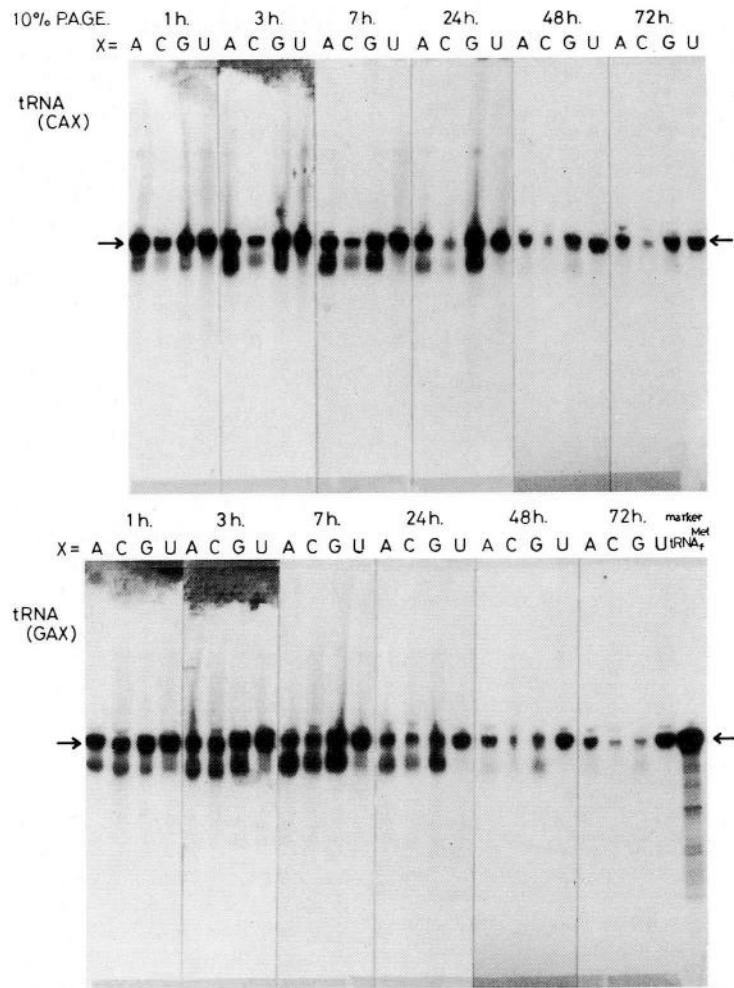


Fig. 3. *Stability of microinjected tRNAs in the *X. laevis* oocytes.* A given amount (fmol level) of purified ^{32}P -labelled tRNA (natural or chimeric) was microinjected into the cytoplasm of a series of five oocytes. At the indicated time after microinjection, total RNA was extracted from the oocytes by phenol/chloroform treatment and ethanol precipitation. RNA samples were then redissolved in electrophoresis buffer and analysed for the presence of degradation products by electrophoresis on a 10% polyacrylamide gel in 7 M urea. The figure shows the resulting autoradiography for *E. coli* fMet-tRNA with anticodon CAX (upper part) or with anticodon GAX (lower part). The last lane (lower part) shows the degradation products of an authentic sample of *E. coli* fMet-tRNA incubated in formamide for 30 min at 100°C (control experiment). The arrows indicate the position of a genuine sample of *E. coli* fMet-tRNA (having its terminal CCA end) detected by ultraviolet shadowing [52]. RNA bands migrating faster than this authentic *E. coli* fMet-tRNA, probably correspond to fMet-tRNA lacking the terminal nucleotides of the amino acid stem (see text)

identity of which could be determined by two-dimensional thin-layer chromatography.

Fig. 5a–h shows representative autoradiograms of these experiments and illustrates clearly that A-37 was rapidly modified to $t^6\text{A}$ in the tRNA variants having the native CAU (Fig. 5a) and GAU (Fig. 5e) anticodon. Trace amounts of the isopentenyl modification of A-37 was detected in the variants having CAA (Fig. 5b) or GAA anticodons (Fig. 5f), and no modification was detected in variants harboring the CAC, CAG, GAC and GAG anticodons (Fig. 5c, g and h) even after incubations as long as 48 h.

Quantification of the radioactivity in the different spots from the thin-layer chromatogram allows the calculation of the kinetics of *in vivo* modification (Fig. 6). The modification of A-37 to $t^6\text{A}$ occurs very rapidly for the CAU and GAU chimera reaching a plateau at 80–90% transformation. The apparent higher rate of the GAU chimera could be due to sequestering of significant quantities of the natural initiator CAU chimera. Isopentenylation of A-37 in the CAA or GAA anticodon variants is considerably slower: only 6–10%

conversion after 72 h. Moreover, the rates of conversion are essentially the same whether the tRNA is injected in the oocyte cytoplasm or nucleus (data not shown).

A microassay of tRNA aminoacylation

With the set of tRNA variants in hand, it was also of interest to determine which, if any, of them could be aminoacylated in the oocyte. Allende and his coworkers [25a, b] were the first to devise a microassay to evaluate the biological activity of small amounts of tRNA microinjected into amphibian oocytes. This procedure was based on the ability of aminoacylated tRNA, but not uncharged tRNA, to bind to *E. coli* elongation factor EF-Tu. Later on, Corbo et al. [26] adapted this procedure in order to quantify small amounts of ^{32}P -labelled tRNAs produced after transcription of a tRNA gene in the *Xenopus* oocyte nucleus. These procedures, however, are unwidely at best, since they depend on a Sephadex G-100 separation of charged and uncharged tRNA. Another technique was based on the use of radioactive

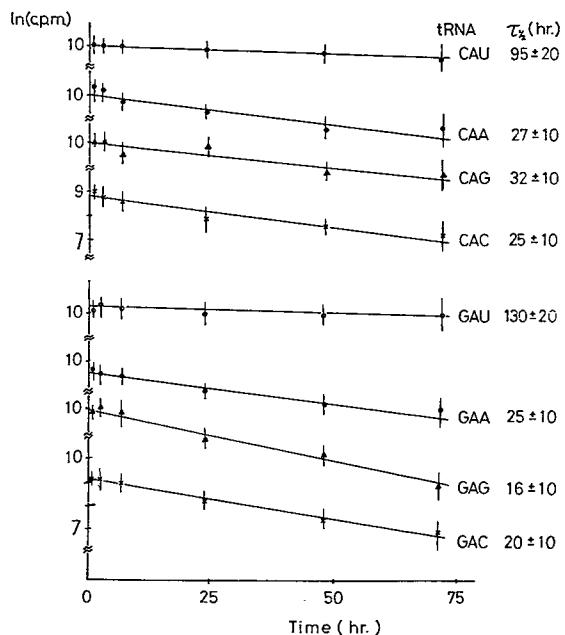


Fig. 4. Stability of microinjected tRNA (natural or chimeric) in the *X. laevis* oocytes. The results of Fig. 3 were further analyzed by counting the radioactivity recovered from the oocytes in bands corresponding to the full-length tRNA and the tRNA lacking the terminal nucleotides of the amino acid stem (see Fig. 3). These data were then plotted in a semilogarithmic mode as a function of time. In the right-hand column are listed the calculated half-lives $t_{1/2}$ of each of the eight tRNA variants assuming an exponential decay law $-dN/dt = kN$

amino acids [25b] (see also [15]). In all, none of these techniques was particularly well-suited to the determination of variant tRNA aminoacylation for this study; therefore, we set out to establish an alternative method. The technique selected is based on the well-known chromatographic behaviour of phenoxyacetylated aminoacyl-tRNA to be absorbed strongly to BD-cellulose [24]. The derivatized charged tRNA is eluted only in an alcoholic buffer, whereas uncharged and underderivatized charged tRNAs are eluted anteriorly in a salt buffer.

The in vitro aminoacylation of tRNA chimera

Using the above technique we were able to determine the aminoacylation level of each microinjected tRNA variant (Table 1) after various times of incubation in the oocytes. As above, only the natural CAU and the GAU (isoleucine) anticodon-containing tRNAs were aminoacylated to a reasonable extent. Presumably both are aminoacylated with methionine, although the technique based on the detection of ^{32}P in the RNA does not distinguish between aminoacylating groups. Also, only the fraction of Met-tRNA in which the 3' ends have been fully repaired by the oocyte tRNA nucleotidyltransferase [40] can be charged with the amino acid.

DISCUSSION

In this paper we have presented evidence that, among the eight *E. coli* fMet-tRNA anticodon chimera, the GAU and the native CAU variants have the longest half-lives, are the

only tRNAs where the A-37 is converted to $t^6\text{A}$ and are aminoacylated to a significant degree in amphibian oocytes. This surprising coincidence must be related to the functionality of these tRNAs; tRNAs which can be aminoacylated could be sequestered by proteins and thus be protected from cytoplasmic nucleases (see [15]).

It is indeed remarkable that the A-37 of *E. coli* fMet-tRNA is modified to $t^6\text{A}$ in the oocyte, since this modification does not take place in *E. coli* [39]. Thus, there is no intrinsic structural reason, relating to an unusual anticodon conformation for example [27], that this tRNA cannot be modified. The eubacterial modifying enzyme would seem to be more selective in its action. Since the tRNAs variants were injected into the oocyte cytoplasm, we conclude that the $t^6\text{A}$ modifying enzyme is located in the cytoplasm. This result is fully consistent with the fact that this modification has not been found in nuclear tRNA precursors [28]. Considering the cytoplasmic presence of the queuosine (a modified base found at position 34) and the $i^6\text{A}$ -37 modification enzymes shown by the microinjection of yeast tyrosine tRNA [29], it may be that most anticodon-loop-modifying enzymes are cytoplasmic (see also [42]).

Most eukaryotic tRNAs having anticodons ending with U have a $t^6\text{A}$ and those ending with A, a $i^6\text{A}$, 1-methylguanosine or a Y base at position 37 [1, 30, 31]. A remarkable illustration of these correlations is that mutations resulting in the replacement of C-36 by A-36 or U-36 as well as U-36 by A-36 in glycine or lysine *E. coli* tRNAs is accompanied by a change in the nature of the modification at A-37 [32–34]. Results of Murgola and coworkers [34, 35], however, point to the fact that the molar yield of such modifications may be quite low, and caution must be used in interpreting the existing correlative data [36]. The low or non-existent conversion of A-37 to the 6-isopentenyl derivative in the CAA and GAA chimera demonstrates that several correlations observed in natural tRNAs do not necessarily hold for synthetic variants. In any case the determinants of nucleotide modification by a given enzyme acting in the anticodon loop are much more complex than the anticodon correlations would lead one to believe. For more discussion, see [36, 42].

The aminoacylation of the *E. coli* fMet-tRNA variants by the oocyte aminoacyl-tRNA synthetases is also of interest. In previous work it has been unambiguously demonstrated that the anticodon nucleotides of *E. coli* fMet-tRNA were crucial for recognition by the corresponding homologous enzyme. Indeed, aminoacylation is reduced to levels below experimental detection *in vitro* by base substitution in the wobble position, and lesser, although dramatic, effects result from structural changes at the other two positions (positions 35 and 36) of the anticodon [9–11a]. The mechanism by which just one base substitution in the wobble position reduces the aminoacylation rate of *E. coli* fMet-tRNA by at least five orders of magnitude remains, however, unclear [11a, 37]. On the other hand, base substitution at position 33 (the constant uridine) or at position 37 (the unmodified adenosine) as well as enlargements of the anticodon by one or two nucleotides at its 5' or the 3' side do not affect drastically aminoacylation of *E. coli* fMet-tRNA by *E. coli* methionyl-tRNA synthetase [11a, 12]. From these results it has been concluded that recognition of *E. coli* fMet-tRNA by its cognate homologous enzyme requires specific interactions of the methionyl-tRNA synthetase with functional groups of the nucleotide base of the anticodon and that the spatial arrangement of those bases in the anticodon loop is less critical than their chemical nature [11a, 37].

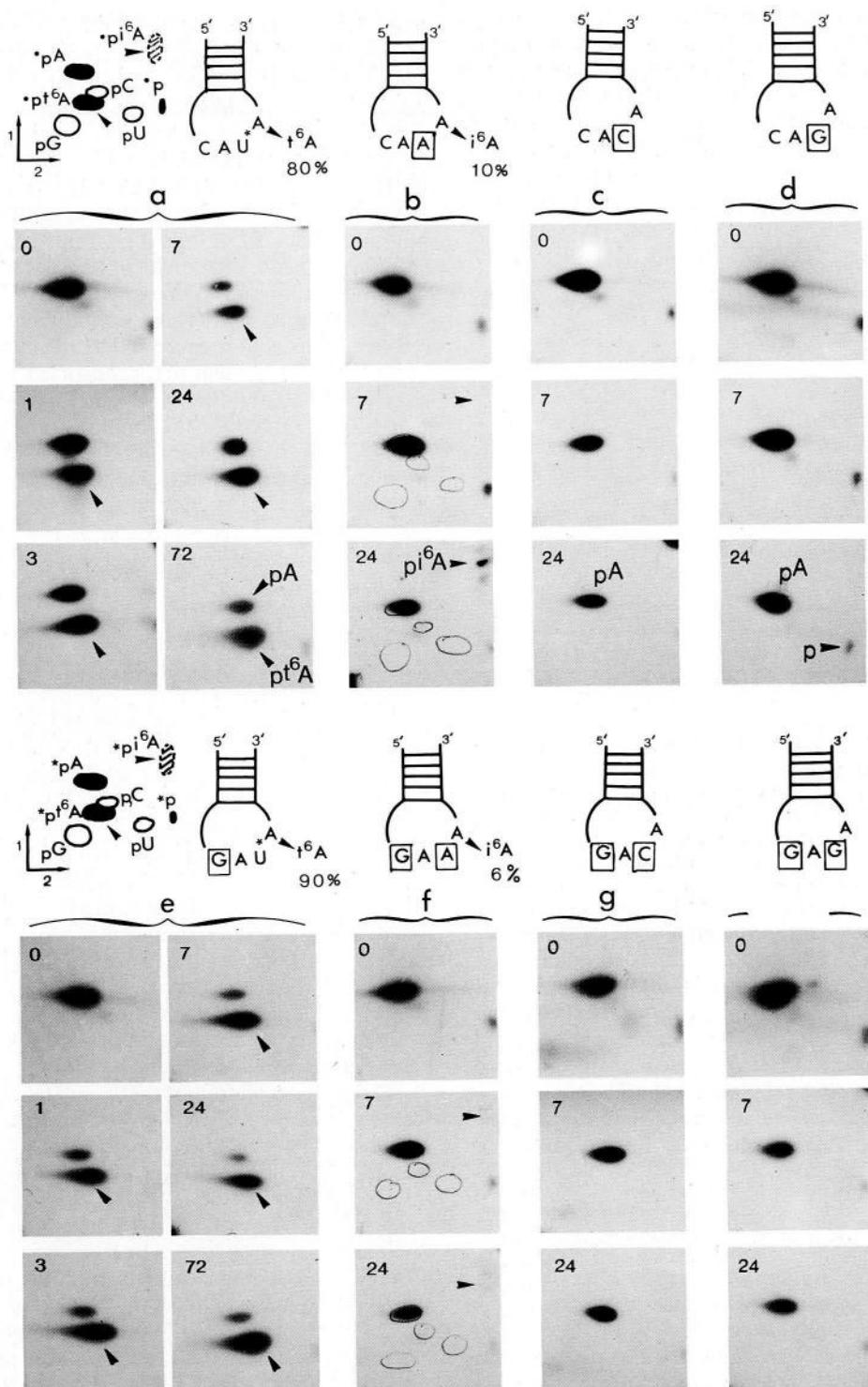


Fig. 5. Autoradiograms of the separation of 5'-nucleosides by chromatography on thin-layer cellulose plates. After isolation from the oocytes and purification by electrophoresis on polyacrylamide gel as shown in Fig. 3, the ^{32}P -labelled fMet-tRNA recovered from the gel was completely digested into nucleosides with nuclease P1. The resulting nucleoside 5'-monophosphates were separated on thin-layer cellulose plates as described in Materials and Methods. The pattern of nucleosides is given in the upper left-hand corner and each sample is identified by the schematic drawing of its anticodon loop: from (a) to (h) for each of the eight tRNA variants. Nucleotides in boxes are those which differ from the parent *E. coli* fMet-tRNA. Numbers in the corner of each autoradiogram correspond to the time of incubation (h) of the tRNA in the oocytes at 19°C; zero corresponds to tRNA before the microinjection (control experiments). Characteristic spots, which correspond to modified nucleotides pt^6A and pi^6A , are identified by arrows. Percentages correspond to molar yield of transformation (mol/100 mol) of A-37 to t^6A -37 or i^6A -37 in *E. coli* fMet-tRNA after 72 h incubation in the oocytes. Asterisk indicates which compounds are labelled with ^{32}P in the nuclease hydrolysate of the tRNA.

Clearly these rules, which applied to the *E. coli* methionyl-tRNA synthetase, cannot be extended to the same enzyme from the *X. laevis* oocyte. Indeed, in the experiments reported here the GAU variant of *E. coli* fMet-tRNA (with a base substitution in the wobble position) is at least as active in aminoacylation as the native fMet-tRNA (with normal anticodon CAU): even the reaction rates were so fast *in vivo* that we cannot differentiate between the two tRNAs. It should be pointed out that the non-stoichiometric aminoacylation could result from either the lack of a 5'-phosphate on the tRNA, even though this feature is not thought to effect aminoacylation.

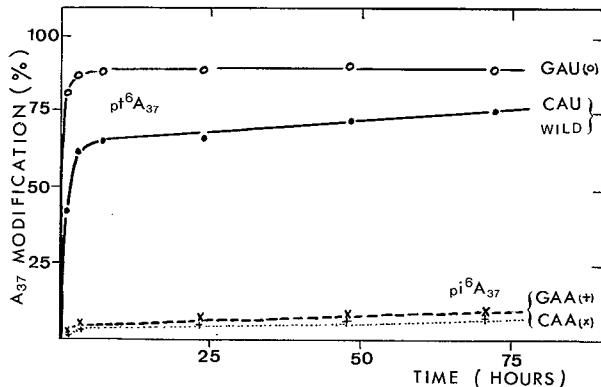


Fig. 6. The kinetics of A-37 modification. Results shown in Fig. 5a-h were analyzed by radioactive counting of the various spots on the thin-layer cellulose plates. From these data were calculated the quantities of pi^6A and pi^6A as a function of time, expressed as percentages of the total radioactivity on the thin-layer cellulose plates. The nucleotide sequences of the novel synthetic anticodons in tRNAs corresponding to each curve are given. The kinetics correspond to the post-transcriptional modifications of A-37 in microinjected *E. coli* fMet-tRNA under physiological conditions of a living cell

Table 1. In vivo aminoacylation level of tRNAs

Activity is expressed as the percentage of radioactivity (^{32}P) retained on the BD-cellulose column during salt washes (1 M NaCl/sodium acetate buffer at pH 4.5) and eluted in the same salt buffer containing 20% ethanol; the total radioactivity recovered from the column being taken as 100%. These radioactivities correspond to the fraction (%) of Met-tRNA labelled with ^{32}P containing a phenoxyacetyl group, because of the presence of an aminoacyl group at the 3' termini. The error in the evaluations is considered to be 15–20%. The control experiment corresponds to about 15–20 min incubation *in vivo*; it represents the minimal time required to complete the microinjections in one series of five oocytes and to proceed in order to recover the ^{32}P -labelled tRNA from the oocytes by phenol/chloroform extraction as described in Materials and Methods

Anticodon	Aminoacylation after incubation for		
	control	3 h	12 h
%			
CAU	23	52	54
CAA	3	5	7
CAC	6	10	10
CAG	4	8	10
GAU	24	55	64
GAA	4	8	9
GAC	8	6	14
GAG	5	10	11

tion (at least with *E. coli* methionyl-tRNA synthetase) [38], or the incomplete repair of the CCA terminus in the oocyte [40, 41]. Although misacylation of the *E. coli* fMet-tRNA by an amino acid other than methionine can not be ruled out [11b], generally misacylation rates never reach the proportions of aminoacylation by cognate amino acid [11b, 43, 44]. Also there is no evidence for aminoacylation of a yeast initiator Met-tRNA having an arginine CCU (instead of CAU) anticodon using the homologous partially purified yeast methionyl-tRNA synthetase [45]. Since this variant of yeast Met-tRNA was not aminoacylated by the *E. coli* enzyme, in contrast to the natural yeast tRNA [45, 46], we conclude that the tRNA recognition by methionyl-tRNA synthetase of eukaryotic cells must be less dependent on the anticodon than is the activity of its *E. coli* counterpart. Experiments indicating the involvement of the anticodon in tRNA recognition by several aminoacyl-tRNA synthetases are often debated. In several instances it is clear that functional groups of anticodon nucleotides are important in the interaction but this might not be a general rule: see for example [37, 43, 47–49, reviewed in 53].

Finally, a method for the determination of femtomole quantities of aminoacylated tRNA is presented. This rapid assay is insensitive to the presence of other (endogenous) unlabelled tRNAs and is therefore particularly well-suited to microinjection techniques. Previous methods had drawbacks relative to the detection limit of radioactive amino acids or tritiated tRNA and to their ease of analysis. The method presented here could be even more easily applied to aromatic amino acids, since their aminoacyl-tRNAs are strongly adsorbed to BD-cellulose without the use of the phenoxyacetyl group [50]. Two cautionary notes must be added, however. First, while the technique used in the assay of microinjected tRNA does determine which fraction of the tRNA is charged, it cannot identify the amino acid involved. The heterologous tRNAs used here could be subject to misaminoacylation, although demonstration of this phenomenon *in vivo* remains to be made. Also, occasionally a modified nucleotide, present in the labelled tRNA sequence, could react with the phenoxyacetyl group leading to the adsorption of non-charged tRNA to BD-cellulose. This situation could prevail for tRNAs containing 5-carboxymethylaminomethyluridine and 3-(amino-3-carboxypropyl)uridine [51]. In spite of these drawbacks we feel that this method will find many applications in the evaluation of the biological activity of small amounts of tRNAs, especially those produced by recombinant technology.

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elling of polyribonucleotides by phosphorylation with RNA ligase and
modification for joining reactions 1

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ABSTRACT

¹-Adenosine 5'-P²-O-*o*-nitrobenzyl pyrophosphate (nbz1ppA) has been synthesized as a substrate for T4 RNA ligase catalyzed 3'-phosphorylation. Incubation of oligoribonucleotides and nbz1ppA with RNA ligase yielded oligoribonucleotides having a 3'-O-(*o*-nitrobenzyl)phosphate. Photochemical removal of the *o*-nitrobenzyl group provided the free 3'-phosphate. Using [P²-³²P]nbz1ppA, 3'-termini of oligoribonucleotides could be labelled with ³²P. This reaction was applied to modify the 3'-end of donor molecules in joining reaction with RNA ligase. A trinucleotide U-A-G was converted to U-A-Gnbz1 and phosphorylated with polynucleotide kinase. pU-A-Gnbz1 was then joined to an acceptor trinucleotide A-U-G to yield A-U-G-U-A-Gp.

INTRODUCTION

T4 RNA ligase² has been demonstrated to be a useful reagent for joining oligonucleotides.³⁻⁹ For intermolecular reactions the 3'-termini of the 5'-phosphorylated components (donor molecules) have to be modified to prevent self-polymerization and/or cyclization.¹⁰ Several approaches have been reported for preventing these undesired reactions by substitution of various groups at the 2'- or 3'-hydroxyl group. A 2'-substituted mononucleotide can be linked to the 3'-end of oligoribonucleotides by either chemical⁸ or polynucleotide phosphorylase catalyzed reactions.⁶ Single addition reactions of nucleoside 3',5'-diphosphate catalyzed by RNA ligase have provided 3'-phosphorylated oligonucleotides.^{11,12} It was also found that alkyl phosphates and sugar phosphates were recognized by the enzyme if they were linked with adenosine 5'-phosphate¹³ in the way found in the active intermediate for the ligase reaction.^{5,6,14} In the present paper we wish to report 3'-phosphorylation of oligoribonucleotides using an adenylated photolabile *o*-nitro-

A new method for 3'-labelling of polyribonucleotides by phosphorylation with RNA ligase and its application to the 3'-modification for joining reactions ¹

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benzyl phosphate (P^1 -adenosine 5'- P^2 -o-nitrobenzyl pyrophosphate, nbz1ppA) and RNA ligase. Substitution of the alkyl phosphate with ^{32}P enabled us to label the 3'-terminus of oligonucleotides in the same way. The 3'-phosphorylated oligonucleotides could serve as donor molecules after 5'-phosphorylation with polynucleotide kinase. Using these techniques a hexanucleotide was synthesized as a model messenger RNA.

MATERIALS AND METHODS

nbz1ppA. o-Nitrobenzyl phosphate was synthesized by condensation of o-nitrobenzyl alcohol with phosphoric acid using trichloroacetonitrile¹⁵ and adenylated by treatment with ^{32}P -phosphoromorpholidate.¹⁶ Phosphoric acid (85%, 0.116 ml, 1.7 mmol), o-nitrobenzyl alcohol (4.25 g, 27.7 mg) and triethylamine (0.56 ml, 4 mmol) were dissolved in acetonitrile (10 ml) and treated with trichloroacetonitrile (1 ml, 10 mmol) in acetonitrile (1 ml) at 75° for 4 hr in the dark. The extent of reaction was examined by paper electrophoresis. The volatile materials were removed by evaporation and water (20 ml) was added to the residue. The excess of o-nitrobenzyl alcohol was removed with ether (15 ml) x 2, and the aqueous layer was concentrated in vacuo, then dissolved in water (14 ml) and applied to a column of Dowex 50X2 (H^+) (1.7 x 10 cm). o-Nitrobenzyl phosphate was recrystallized from chloroform (20 ml)-acetone (5 ml). The yield was 53%, 0.209 g, 0.89 mmol. Anal. calcd. for $C_7H_8NO_6P$: C, 36.07; H, 3.56; N, 6.01. Found: C, 35.80; H, 3.54, N, 5.81. o-Nitrobenzyl phosphate (0.2 mmol) was mixed with tri-n-octylamine (0.2 ml), coevaporated with pyridine three times and allowed to react with adenosine 5'-phosphoromorpholidate (0.2 mmol) in dry DMF (1 ml) at room temperature for 3 days. Paper electrophoresis showed a pyrophosphate which could be converted to ADP by irradiation with UV light. The reaction was stopped after another 3 days by addition of water. DMF was removed and the residue was dissolved in aqueous pyridine. The aqueous pyridine solution was passed through a column (2.7 x 5 cm) of Dowex 50X2 (pyridinium form) to remove tri-n-octylamine. The eluent and washings (200 ml, 10% pyridine) were applied to a column (1.7 x 19 cm) of DEAE-Sephadex A-25 (bicarbonate form).

After the column was washed with water (1 L), the product was eluted with a linear gradient of triethylammonium bicarbonate (0 to 0.2 M, total 2 L). The appropriate fractions (0.13 M) were examined by paper electrophoresis and desalted by evaporation. The relative mobility of the product to pA was 0.94. The spectral properties of the product were $\lambda_{\text{max}} (\text{H}_2\text{O})$ 261, $\lambda_{\text{max}} (\text{H}^+)$ 256, $\lambda_{\text{max}} (\text{OH}^-)$ 261 nm. The yield of nbz1ppA was 1382 A₂₆₀ units, 33%. The pyrophosphate was characterized by enzymic digestion with venom phosphodiesterase to yield o-nitrobenzyl phosphate and pA. The product could be converted to ADP by irradiation with UV light.

nbz1*ppA. The [³²P] labelled pyrophosphate was synthesized as above except that *Pi (1 mCi) was diluted with 0.05 mmol of phosphoric acid. The yield was 5.3 μmol , 11%.

Enzymes.

RNA ligase was purified according to a procedure of Cranston et al.¹⁷ with a modification using affinity chromatography on ADP-Sepharose.¹⁸ Polynucleotide kinase reactions and other enzymes for characterization of products were described previously.^{6,8}

RNA ligase reaction.

Unless specified otherwise 50 mM HEPES-NaOH (pH 8.3), 10 mM MgCl₂, 10 mM DTT, 0.1 mM ATP and 0.5-2 μg BSA were used in 10 μl at 25°. Substrate concentration was 0.28-1.5 mM when the enzyme concentration was 140 units/ml.

Other methods.

Paper chromatography was performed by the descending technique using solvent systems: A, isopropyl alcohol-concentrated ammonia-water (7 : 1 : 2, v/v); B, n-propyl alcohol-concentrated ammonia-water (55 : 10 : 35, v/v); C, 0.1 M phosphate (Na, pH 6.8)-ammonium sulfate-n-propyl alcohol (100 : 60 : 2, v/w/v). Paper electrophoresis was performed using 0.05 M triethylammonium bicarbonate (pH 7.5). Ion-exchange chromatography was performed with DEAE-Sephadex A25 (Pharmacia Co.) and triethylammonium bicarbonate. Homochromatography¹⁹ was performed using Homomix I-V.²⁰ Partial digestion with Nuclease P1 and venom phosphodiesterase were as described previously.⁹

For the removal of the o-nitrobenzyl group, compounds were

placed in a pyrex tube (1.5 mm thick) and irradiated through a pyrex filter (2 mm thick) inserted in a water jacket. The photolysis apparatus had a 300 W high pressure mercury lamp (Eikosha Model PIH 300) with a quartz water circulating jacket.

RESULTS

3'-Labelling by phosphorylation with nbz1*ppA (2) and RNA ligase.

A trinucleotide U-A-G (**1**) was phosphorylated using a two fold excess of nbz1*ppA and RNA ligase as shown in Chart 1.

Chart 1.

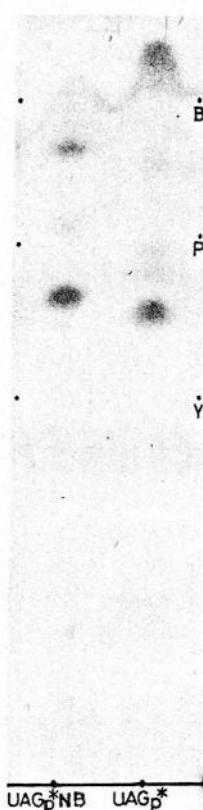
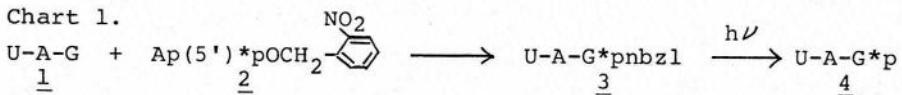


Figure 1. Homochromatography of the products after phosphorylation of U-A-G with nbz1*ppA. Before photoirradiation, U-A-G-*nbz1 (the slower spot); after photoirradiation, U-A-G-*p (the slowest spot). nbz1*ppA travels behind the blue marker.

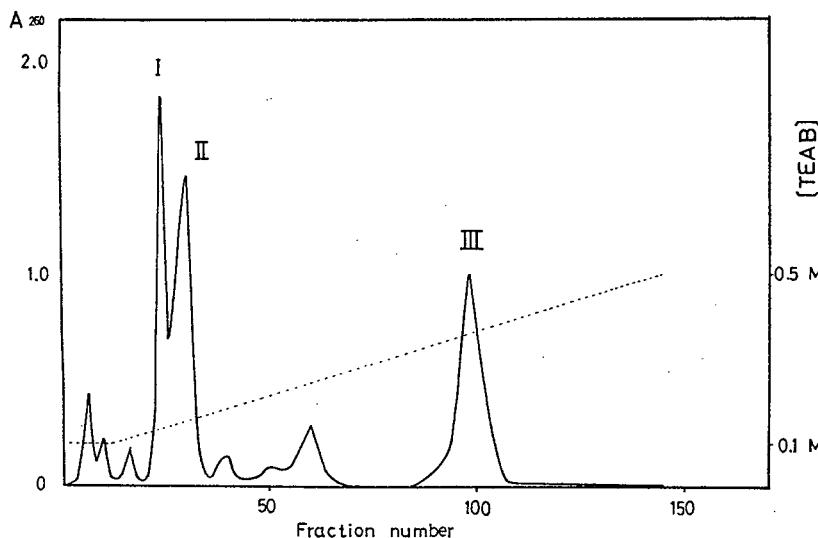


Figure 2. Chromatography of the products in the RNA ligase reaction (605 μ l) of nbz1ppA (1025 nmol) with U-A-G (532 nmol) using RNA ligase (28 units) on a column (0.6 x 15 cm) of DEAE-Sephadex A25. Elution was performed with a linear gradient of triethylammonium bicarbonate (pH 7.5, 0.1 M to 0.5 M, total 220 ml). Fractions of 1.6 ml were collected every 10 min. Peaks: I, pA; II, nbz1ppA; III, U-A-Gpnbz1.

The 3'-(o-nitrobenzyl)phosphorylated product (3) was detected by homochromatography. After 4hr, the reaction was almost complete. Fig. 1 shows 3 as a slower moving compound compared with the pyrophosphate (2) which travels behind the blue marker. Removal of the o-nitrobenzyl group from 3 was effected by irradiation with UV light of wavelength longer than 280 nm. The 3'-phosphorylated trinucleotide (4) travelled slightly slower than the benzylated starting material (3) as shown in Fig. 1.

Preparation of the 3'-modified trinucleotide and its use in joining reactions.

For joining of pU-A-G to the 3'-end of other oligonucleotides U-A-G was modified at the 3'-hydroxyl group before 5'-phosphorylation. U-A-Gpnbz1 (5) was prepared in quantity using a two fold excess of unlabelled nbz1ppA and RNA ligase and isolated by chromatography on Sephadex as shown in Fig. 2. 5

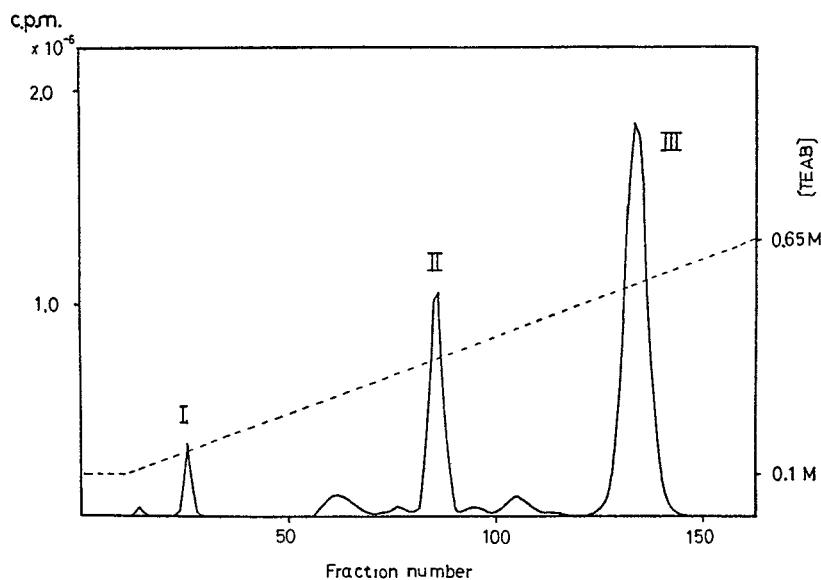
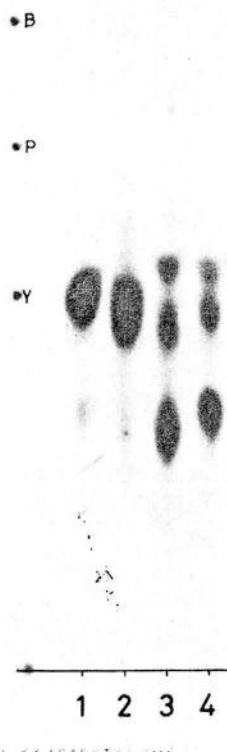


Figure 3. Chromatography of the product in the phosphorylation of U-A-GpnBzL (292 nmol) with [γ - 32 P]ATP (480 nmol) and polynucleotide kinase (15 units) on DEAE-Sephadex A25 using a linear gradient of triethylammonium bicarbonate pH 7.5 from 0.1 M to 0.65 M (total, 220 ml). Fractions of 1.6 ml were collected every 10 min. Radioactivity was counted by Cerenkov's method. Peaks: I, *Pi; II, [32 P]ATP; III, *pU-A-GpnBzL.

was phosphorylated using [γ - 32 P]ATP with low specific activity by polynucleotide kinase. The 5'-phosphorylated product (6) was isolated by the similar chromatography (Fig. 3) and joined to A-U-G by treatment with RNA ligase as illustrated in Chart 2. The joined product (7) was analyzed by homochromatography and the reaction mixture was irradiated with UV light to remove the o-nitrobenzyl group. Fig. 4 shows mobilities of the 3'-phosphorylated products before and after photoirradiation. The deblocked hexanucleotide (8) was also isolated by ion-exchange chromatography on DEAE-Sephadex (Fig. 5) and characterized by nearest neighbor analysis (Fig. 6-1) as well as RNase A plus phosphatase treatment (Fig. 6-2). An aliquot of 8 was treated with phosphatase and 9 was isolated by paper chromatography in solvent C. The dephosphorylated hexanucleotide (9) could be an acceptor molecule in a subsequent ligation if the chain is

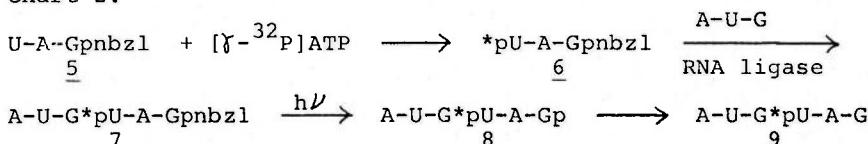
Figure 4. Homochromatography of the products in the joining of $^3\text{pU-A-GpnBz1}$ to A-U-G. 1, $^3\text{pU-A-GpnBz1}$; 2, $^3\text{pU-A-Gp}$; 3, A-U-G \cdot $^3\text{pU-A-Gp}$ (the slowest spot, after photoirradiation); 4, A-U-G \cdot $^3\text{pU-A-GpnBz1}$ (the slowest spot, before photoirradiation).



to be elongated in the 3'-direction.

Thus U-A-G was joined to A-U-G by successive 3'-modification, 5'-phosphorylation, ligation and 3'-deblocking to yield A-U-G-U-A-G which could serve as a model messenger RNA in protein synthesizing systems.

Chart 2.



The 3'-modification by o-nitrobenzyl phosphorylation of

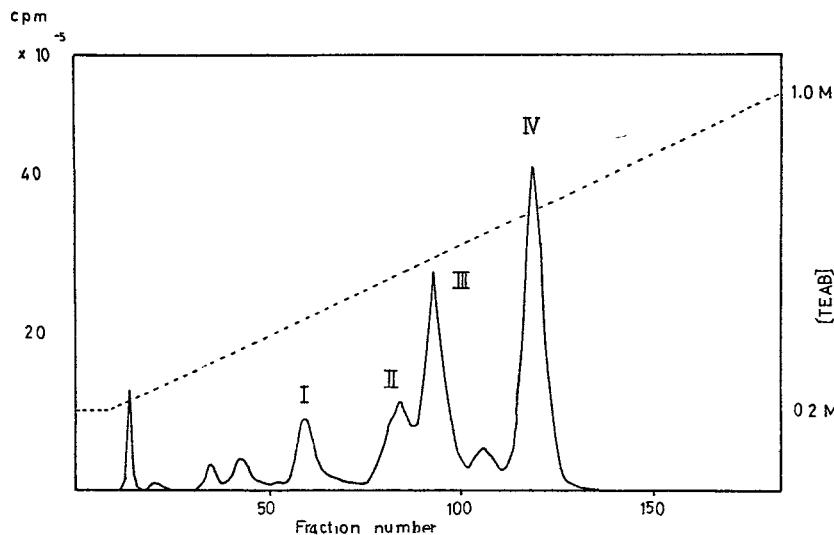


Figure 5. Chromatography of the deblocked product, A-U-G-U-A-Gp after joining of 3 pU-A-GpnBz1 (277 nmol) to A-U-G (278 nmol) using RNA ligase (14 units in total volume of 450 μ l) on a column of DEAE-Sephadex A25. The conditions for elution were the same as described in Fig. 2. Peaks: I, unidentified, II, mainly dephosphorylated product, A-U-G \cdot pU-A-G; III, the reaction intermediate, Ap(5')pU-A-Gp; IV, A-U-G \cdot pU-A-Gp.

C-C-A, A-U-C gave similar results. The conditions for these reactions are summarized in Table I.

DISCUSSION

The present study indicates that the adenylated α -nitrobenzyl phosphate (nbz1ppA) was recognized by RNA ligase as a donor molecule and that α -nitrobenzyl phosphate was transferred to the 3'-hydroxyl group of ribooligonucleotides. This result is consistent with the previous finding that adenylated cyanoethyl phosphate served as a substrate in RNA ligase reactions.¹³ The α -nitrobenzyl group was introduced as a photolabile protecting group for amino acids, carbohydrates and phosphates.²¹ Extensive use of this group as protection for the 2'-hydroxyl group of nucleotides in oligonucleotide synthesis has shown no detectable photochemical side reactions during deblocking by UV irradiation with wavelength longer than 280nm.²²

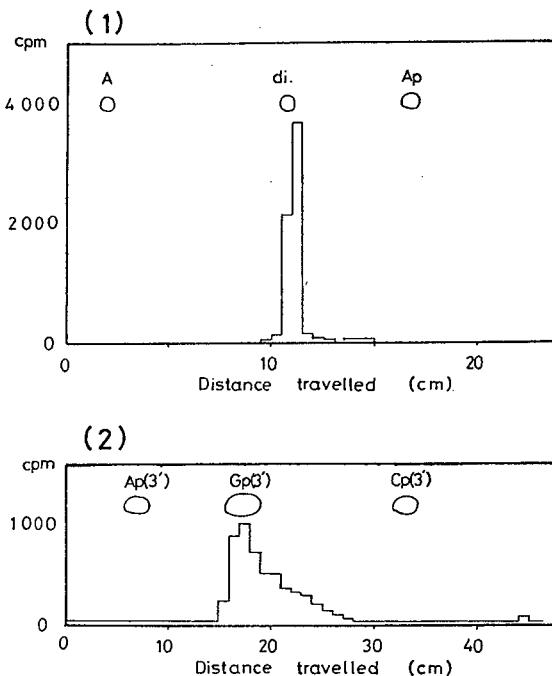


Figure 6. Characterization of A-U-G*pU-A-G. (1) Paper electrophoresis of the digested product (G*pU) after RNase A plus phosphatase treatment. A, Ap and dimer indicate markers detected by UV absorption (2) Paper chromatography of the digested product (G*p) in an RNase M hydrolysis. Ap, Gp and Cp show markers detected by UV absorption.

Table I
The 3'-(o-Nitrobenzyl)phosphorylation of Ribotrinucleoside Diphosphate.

Acceptor (nmol)	nbz1ppA (nmol)	RNA ligase (unit)	Total vol. (μl)	Time (hr)	Temp. (C°)	Yield ^a %	
C-C-A	14.1	70.4	7.0	50	26	25	82
C-C-A	14.1	70.4	5.3	50	17	37	66
U-A-G	14.1	70.4	7.0	50	26	25	49
U-A-G	139	705	14.0	100	4	25	100
U-A-G	530	1025	28.0	605	19	25	70
A-U-C	13.9	70.5	1.4	10	21	25	97

a, The yield was estimated by counting radioactivity of *pX-Y-Z and *pX-Y-Zpnbz1 from homochromatogram after the reaction mixture was phosphorylated with [γ -³²P]ATP and polynucleotide kinase.

The *o*-nitrobenzyl group on the 3'-phosphate of oligonucleotides can therefore be removed to give the 3'-phosphomonoester end group. The present procedure provides a new method of 3'-phosphorylation of ribooligonucleotides. When the radioactive pyrophosphate (nbz1*ppA) was used in this reaction, the 3'-end of ribooligonucleotides was labelled with ^{32}P .

The 3'-*o*-nitrobenzylphosphorylation of oligonucleotides is also useful as method for modification of the 3'-end. If a 3'-modified oligonucleotide is phosphorylated at the 5'-hydroxyl group, a good donor molecule for RNA ligase reactions is obtained. During 5'-phosphorylation with polynucleotide kinase and ATP, a 3'-phosphate may be removed by an activity which co-chromatographs with polynucleotide kinase.²³ Although 3'-phosphatase free kinase²⁴ may overcome this problem, it is desirable to protect the 3'-phosphate during 5'-kinination. This type of reaction was examined in our kinination of the trinucleotide (5). The 5'-phosphorylated product (6) was obtained in a high yield (Fig. 3). [γ - ^{32}P]ATP was used to simplify product analysis in both the phosphorylation and the subsequent RNA ligase reaction. (6) was joined with A-U-G to yield (7) and the protecting group was removed at this stage to yield (8) (Fig. 5). If the chain is to be elongated in the 5'-direction it can be retained for the next 5'-kinination reaction. The hexanucleotide with a 3'-phosphomonoester (8) was dephosphorylated by treatment with alkaline phosphatase to obtain (9). By this treatment (9) can be elongated in the 3'-direction.

This 3'-modification by *o*-nitrobenzylphosphorylation provides a method for facile introduction of a 3'-phosphomonoester which can be removed at later stages by phosphatase treatment if necessary. Once ribotriplets are obtained by whatever means, they can be modified at the 3'-end without the need for addition of mononucleotides and used for RNA ligase reactions in a blockwise fashion. This technique would appear especially useful in synthesis of messenger RNA consisting of trinucleotide codons.

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Total synthesis of a RNA molecule with sequence identical to that of *Escherichia coli* formylmethionine tRNA[†]

(chemical synthesis of tRNA fragments/RNA ligase/methionyl-tRNA synthetase)

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ABSTRACT A RNA molecule has been synthesized that is identical in sequence to *Escherichia coli* tRNA_f^{Met} except that it lacks the base modifications present in the *E. coli* tRNA. This was achieved by enzymatic joining of chemically synthesized oligonucleotides with chain lengths of 3-10 which were synthesized by the phosphodiester or phosphotriester method. First, quarter molecules of tRNA were constructed by joining of chemically synthesized fragments with RNA ligase. The 5'-quarter molecule (bases 1-20) served as an acceptor in joining reactions with the 3',5'-bisphosphorylated donor molecule (bases 21-34). The 5'-half molecule thus obtained was treated with phosphatase and joined to the 3'-half molecule which was prepared by ligation of the other quarter molecules (bases 35-60, acceptor; bases 61-77, donor) followed by 5'-phosphorylation with polynucleotide kinase. The synthetic tRNA was characterized by oligonucleotide pattern and was partially active in aminoacylation with *E. coli* methionyl-tRNA synthetase.

Chemical synthesis of nucleic acids has been a challenging problem in organic chemistry since the structure of the nucleic acids was elucidated. Chemical methods to synthesize short ribo- and deoxyribopolynucleotides with defined sequences were established in early 1960s, and those oligonucleotides were important in the elucidation of the genetic code (1). Discovery of DNA ligase allowed the synthesis of bihelical DNAs from chemically synthesized deoxyribopolynucleotides. With this chemical-enzymatic method the genes for yeast alanine tRNA (2) and *Escherichia coli* tyrosine tRNA precursor (3) have been synthesized; the latter was the first synthetic functional DNA molecule. Genes for peptides have also been synthesized by the same approach, and the methods for joining double-stranded DNA pieces with protruding ends have been used in various recently developed reactions for genetic manipulations.

Although tRNAs are the smallest nucleic acids with unique functions, their synthesis has been difficult until recently, mainly because of the lack of good synthetic methods for larger oligoribonucleotides as well as a lack of joining enzymes. After the primary structure of yeast alanine tRNA had been determined (4), the nona- and hexanucleotide corresponding to the terminal sequence of this tRNA were synthesized by phosphodiester block condensation. These fragments in turn were used to form reconstituted molecules with natural tRNA fragments derived by RNase digestions. However, aminoacylation was not possible because the synthetic fragments were too small to form sufficiently stable complexes for recognition by the alanyl-tRNA synthetase (5). The discovery of RNA ligase (6) and its ability to join single-stranded oligoribonucleotides (7) made it possible

to elongate synthetic RNA fragments to yield larger molecules such as tRNAs.

The initiator methionine tRNA of prokaryotes has a special role in protein biosynthesis, which manifests itself in several unique properties of that tRNA (8). It was also the subject of detailed modification studies to explain its structure-function relationship (9). Because a RNase T1-digested one-quarter molecule of *E. coli* tRNA_f^{Met} reconstituted methionine acceptor activity when mixed with the corresponding three-quarter molecules (10), this tRNA seemed an appropriate target for chemical synthesis. The final aim would be to modify systematically the functionally important parts of the molecule.

We began by synthesizing terminal fragments of the tRNA (11-16) and examined the ability of RNA ligase (17-19) to join these fragments. The 5'-terminal icosanucleotide (20), the tetradecanucleotide (bases 21-34) (21), and the 3'-heptadecanucleotide (22) have been obtained by this method. The 5'-quarter molecule here was found to reconstitute methionine acceptor activity when it was combined with the natural RNase T1-generated three-quarter molecule (20). Oligonucleotides corresponding to the rest of the molecule and certain of their analogs have been synthesized either by the phosphodiester method (23, 24) or by the triester method (25, 26).

In this paper we report total synthesis of a RNA molecule with a sequence identical to that of *E. coli* tRNA_f^{Met} obtained by the enzymatic joining of chemically synthesized fragments with RNA ligase.

MATERIALS AND METHODS

Enzymes. T4 RNA ligase was purified as described (27). Polynucleotide kinase and *E. coli* alkaline phosphatase were gifts of M. Sugiura. 3'-Phosphatase-free kinase was isolated from *E. coli* infected with T4 PseT1-amN82SP62 as described (20). Other enzymes for characterization of the products were obtained as described (17-20).

Kinase Treatment, Ligation, and Dephosphorylation. 5'-Phosphorylation by using polynucleotide kinase and [γ -³²P]ATP was performed as described (20). All 5'-phosphorylations of 3'-phosphorylated oligonucleotides were performed by using the 3'-phosphatase-free kinase unless otherwise specified. Ligation was carried out in the presence of a 2-fold excess of ATP with respect to donor molecules in 50 mM Hepes (made pH 8.3 with NaOH)/10 mM dithiothreitol/10 mM MgCl₂/10% (vol/vol) dimethyl sulfoxide containing bovine serum albumin at 10 μ g/ml. The 3'-phosphate was removed by treatment with *E. coli* alkaline phosphatase in 50 mM Tris-HCl (5 μ l, pH 8.1) at 55°C for 30 min. The enzyme was inactivated by treatment with 1 μ l

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[†] This is paper no. 36 in a series. Paper 35 is ref. 22.

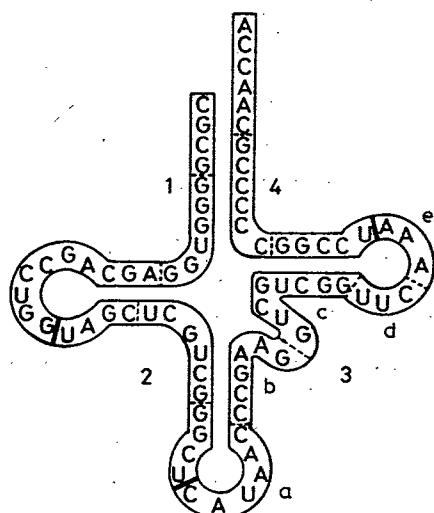


FIG. 1. Structure of *E. coli* tRNA^{Met}. The quarter molecules 1, 2, and 4 were obtained by joining chemically synthesized oligonucleotides with RNA ligase. Quarter molecule 3 was joined as shown in Fig. 2.

of 40 mM EDTA at room temperature for 30 min and then at 100°C for 2 min (28) and extracted twice with 2 μ l of phenol saturated with 50 mM Tris-HCl (pH 8.1). The phenol layer was washed twice with water (20 μ l) and the aqueous phase was subjected to gel filtration on a Sephadex G-50 column (1.1 \times 21 cm).

Isolation and Characterization of Joined Products. Paper chromatography was performed with 0.1 M sodium phosphate, pH 6.8/ammonium sulfate/1-propanol, 100:60:2 (vol/wt/vol), as the solvent system. Paper electrophoresis was performed at 900 V/40 cm with 0.05 M triethylammonium bicarbonate (pH 7.5) or 0.2 M morpholinium acetate (pH 3.5). Homochromatography (29) was performed with Homo-mix I-IV (30). Two-dimensional chromatography on cellulose plates was as described (31). Polyethylenimine-cellulose plates (Macherey-Nagel, Polygram Cell 300 PEI) were treated as described (32). Polyacrylamide gel electrophoresis was performed on slab gels or on a disc apparatus as described (20).

Nearest-neighbor analysis (17), 3'- and 5'-terminal analysis (20), and partial nuclease P1 digestion (22) for mobility shift analysis were as described. For complete RNase T1 digestion of the product (1 pmol), RNase T1 (1 unit) was used in the presence of phosphatase (180 microunits) in 10 mM Tris-HCl (pH 7.5) at 27°C for 4 hr.

Aminoacylation of the Joined Product. The purified *E. coli* methionyl tRNA synthetase (a gift of J. P. Waller) was used at

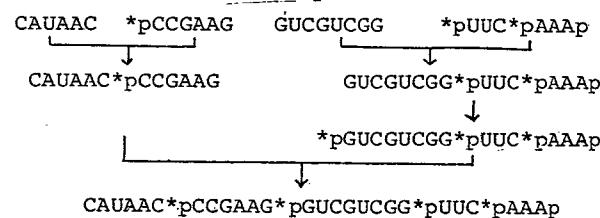


FIG. 2. Synthesis of quarter molecule 3 (bases 35-60).

4 μ g/ml for 10 pmol of the tRNA in 100 mM Hepes, pH 8.0/10 mM Mg(AcO)₂/10 mM KCl/4 mM ATP/10 mM 2-mercaptoethanol/6 μ M L-[¹⁴C]methionine (582 Ci/mol; 1 Ci = 3.7 \times 10¹⁰ becquerels) in total volume of 20 μ l at 37°C for 30 min. Aminoacylation with a crude mixture of *E. coli* synthetases (33) (0.12 mg/ml) was performed in the presence of 0.1 mM CTP at 37°C for 60 min. The reaction was stopped by addition of 1 M pyridinium acetate (pH 5.0; 5 μ l) and the mixture was applied to a column (0.8 \times 23 cm) of Sephadex G-50 equilibrated with 50 mM pyridinium acetate (pH 5.0). The aminoacylated tRNA was eluted with the equilibration buffer, assayed by Cerenkov's method, desalted by gel filtration on Sephadex G-50 in 0.05 M triethylammonium bicarbonate, and treated with Tris-HCl (pH 9.0) at 37°C for 1 hr to hydrolyze the amino acid. The mixture was applied to a column of Sephadex G-50 to resolve the tRNA and [¹⁴C]methionine. Fractions were assayed using a scintillation counter. An aliquot (10 pmol) of the tRNA was assayed for methionine acceptor activity by acid precipitation as described (20) after ³²P radioactivity became negligible.

RESULTS

Chemical Synthesis of tRNA Fragments. Most of the synthetic methods used for fragments shown in Fig. 1 have been described previously. The phosphodiester method was used for the synthesis of fragments consisting of bases 1-4 (12), bases 5-10 (13), bases 41-57 (23), bases 58-60 (24), and bases 61-71 (14, 23). The phosphotriester method was applied for synthesis of fragments consisting of bases 11-20 (15), bases 21-34 (unpublished work), bases 35-40 (26), and bases 72-77 (16).

Preparation of Quarter Molecules. Segment 1 (bases 1-20) was prepared by joining three fragments as described (20) and the 3'-terminal phosphate was removed. The next quarter, molecule 2 (bases 21-34), was synthesized by two different approaches (21).

Segment 3 (bases 35-60) was prepared by the joining of five synthetic fragments (Fig. 2). The dodecamer C-A-U-A-A-C-C-C-G-A-A-G (bases 35-46) was synthesized by using 10-fold excess of the acceptor molecule (3a, see Fig. 1) as summarized in

Table 1. Reaction conditions for joining of oligoribonucleotides with RNA ligase

Acceptor, nmol (μ M)	Donor, nmol (μ M)	ATP, μ M	Enzyme, μ g/ml	Temp., °C	Time, hr	Isolated yield, %
CAUAAC 80 (1000)	pCCGAAG 8 (100)	200	100	25	1	39
GUCGUCGG 15 (150)	pUUCAAp 10 (100)	200	140	25	1	36
CAUAACCGAAG. 2.1 (150)	pGUCGUCGGUU. CAAp, 1.5 (100)	200	115	25	1	52
1 0.88 (74)	2 1.3 (111)	227	535	25	2	31
3 4 (100)	4 2.5 (63)	200	150	25	2	15
5'-half 0.14 (4.7)	p3'-half 0.20 (6.7)	100	200	4	17	42

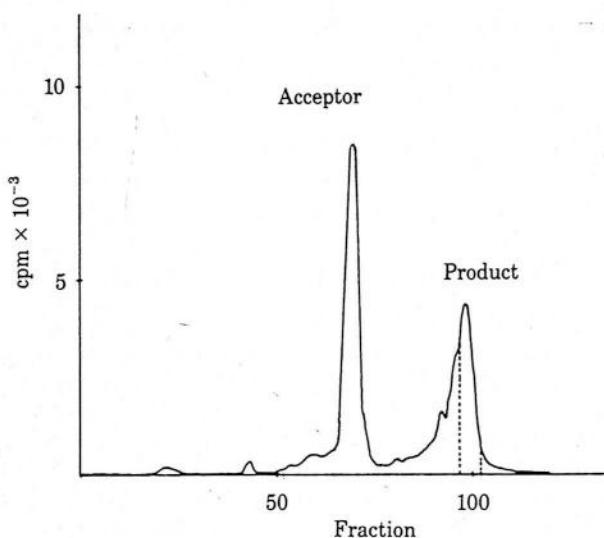


FIG. 3. Disk gel electrophoresis of the 26-nucleotide-long segment 3 (C-A-U-A-A-C*pC-C-G-A*pG-U-C-G-U-C-G-G-U-U-C-A-A-Ap) (Fig. 2) on 20% acrylamide. The first peak contained the acceptor (C-A-U-A-A-C*pC-C-G-A) and the last peak contained the product.

Table 1. The tetradecamer (bases 47–60) was synthesized by joining 3c to the hexanucleotide *pU-U-C-A-A-Ap which had been obtained by phosphorylation with polynucleotide kinase and [γ -³²P]ATP of the joined product from U-U-C (3d) and pA-A-Ap (3e). After 5'-phosphorylation, the tetradecamer was joined to the dodecamer under the condition shown in Table 1. The 26-nucleotide-long segment 3 was isolated by electrophoresis on a 20% acrylamide gel disc as illustrated in Fig. 3. The nearest-neighbor analysis of the product is shown in Fig. 4. The chain length was confirmed by slab gel electrophoresis.

The 3'-phosphorylated heptadecamer 4 was prepared as described (22). The 3'-phosphorylation of C-A-A-C-C-A (16) was done with P1-adenosine-P2-(*o*-nitrobenzyl) pyrophosphate and RNA ligase (19).

Joining of Quarter Segments to Yield the tRNA Molecule. The 5'-half molecule was synthesized by joining of quarter molecules 1 and 2. The reaction conditions are summarized in Table 1. The mixture was separated by polyacrylamide gel electrophoresis, and the product was detected by autoradiography (Fig. 5A). The extent of the reaction was 73% as measured by assaying gel slices at the appropriate positions. However, the isolated yield after elution from the gel was 31% based on 1.

For the synthesis of the 3'-half molecule, heptadecamer 4 was phosphorylated and joined to the 3 by using the conditions shown in Table 1. The product was isolated as described for the 5'-half molecule in a yield of 20%. It was characterized by nearest-neighbor and terminal analyses. The 3'-half was then 5'-

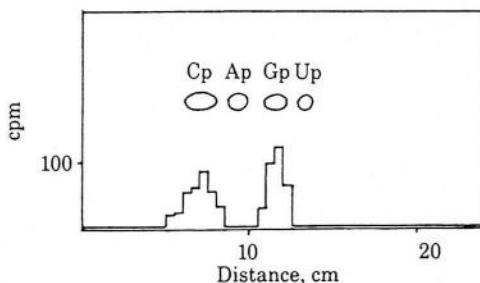


FIG. 4. Nearest-neighbor analysis of 3 after digestion with RNase T2 and paper electrophoresis at pH 3.5.

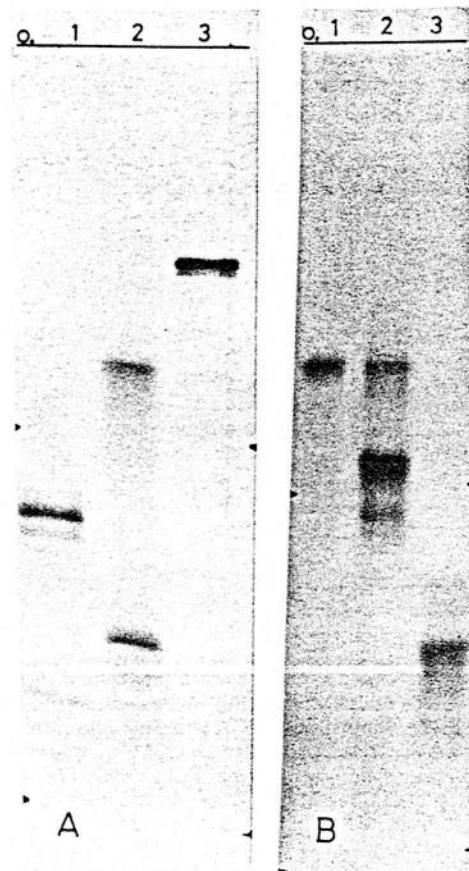


FIG. 5. Polyacrylamide gel electrophoresis of products. (A) Synthesis of the 5'-half molecule from 1 and 2 (lane 2). Lane 1: the 5' quarter molecule. Lane 3: the three-quarter molecule. (B) Synthesis of the 3'-half molecule from bases 35–46 and bases 41–77 (lane 2). Lane 1: bases 35–77. Lane 3: bases 47–60.

phosphorylated with polynucleotide kinase and [γ -³²P]ATP and joined to the 5'-half molecule at reduced temperature (Table 1).

The reaction mixture was subjected to gel filtration (Fig. 6A). The joined product was found in peak 1 whereas the acceptor and the donor eluted together in peak 2. This was verified by polyacrylamide gel electrophoresis of the fractions. The molecular weight of the product was estimated to be 2.6×10^4 from a plot of logarithm of molecular weight against mobility in polyacrylamide gel electrophoresis. As expected, 5'-end group analysis of the product after ³²P-labeling (with polynucleotide kinase and [γ -³²P]ATP) and RNase T2 digestion yielded [³²P]pCp. The 3'-end analysis was performed by transferring the labeled 5'-phosphate by circularization with RNA ligase followed by hydrolysis with RNase T2. C*p (instead of A*p) was identified as the 3'-end by two-dimensional chromatography. This may be due to removal of pAp by a reverse reaction of RNA ligase which has been observed with large excesses of the enzyme (R. I. Gumport and O. C. Uhlenbeck, personal communication). To avoid this side reaction, the 3'-half molecule was prepared (Fig. 5B) and the 3'-phosphate of the 43-unit segment was removed during the 5'-phosphorylation by using polynucleotide kinase with 3'-phosphatase activity. Although the presence of a 3'-OH group on the donor molecule could lead to the donor molecule joining onto itself, it was hoped that the secondary structure would prevent circularization of the 3'-half molecule (donor) during the joining reaction of the halves at the anticodon loop.

Joining of this 43-unit segment to the 5' half was performed under the same conditions as described above, and the product

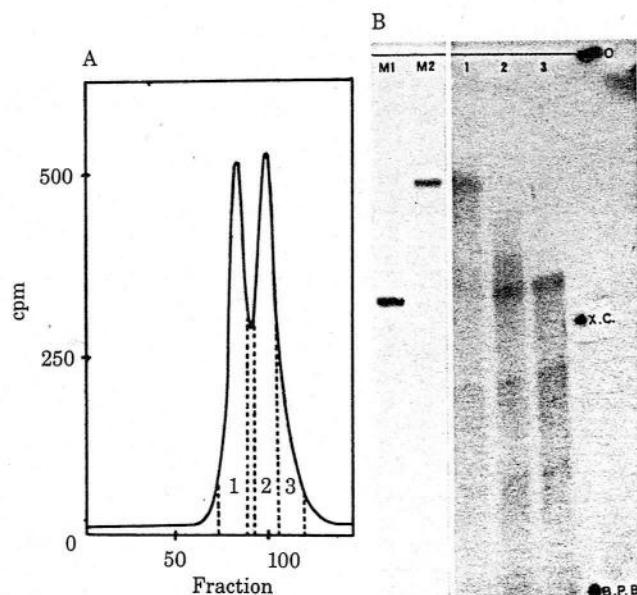


FIG. 6. (A) Gel filtration of products in the synthesis of the total molecule from the 3'- and 5'-half molecules on a column (0.7 × 90 cm) of Sephadex G-200 equilibrated with 50 mM potassium phosphate; pH 7.5/0.1 mM EDTA. Elution was at 50°C with flow rate 1.2 ml/hr; 0.22-ml fractions were collected. Column volume, 34.6 ml; void volume, 11.5 ml. Peak 1 contained the total molecule; the half molecules were eluted in peaks 2 and 3. (B) Polyacrylamide gel electrophoresis of the compound in each peak. M1 and M2 indicate markers of segment 1 and tRNA_f^{Met}, respectively. Lanes 1, 2, and 3 correspond to peak 1, 2, and 3.

was isolated in a yield of 17% by gel filtration. The joined product was analyzed as previously and again partial removal of pA from the 3' end was observed. Possibly, dephosphorylation did not go to completion during kinination. The whole molecule was then treated with phosphatase to remove the 3'-phosphate residue and then phosphorylated at the 5' end with unlabeled ATP and polynucleotide kinase.

This tRNA molecule was tested for methionine acceptor activity by using L-[¹⁴C]methionine and purified or crude methionyl-tRNA synthetase. Aminoacylation was measured by isolating the aminoacyl-tRNA formed by gel filtration in acidic medium followed by hydrolysis and quantitation of the [¹⁴C]methionine produced. With the purified enzyme aminoacylation was 6%; with the crude enzyme it was 4%.[‡] The deacylated recovered tRNA was then subjected to RNase T1 digestion for further structural analysis. The T1 fragments were labeled by phosphorylation (28) and mapped by two-dimensional thin-layer chromatography on PEI-cellulose. As shown in Fig. 7, the synthetic tRNA gave essentially the same pattern as the natural tRNA_f^{Met}. The 3'-fragment *pC-A-A-C-C-A (spot 1) was accompanied by *pC-A-A-C-C (spot 1') in chromatography of the product.

DISCUSSION

Examination of tRNA structure-function relationship may lead to an understanding of an interesting example of the specific

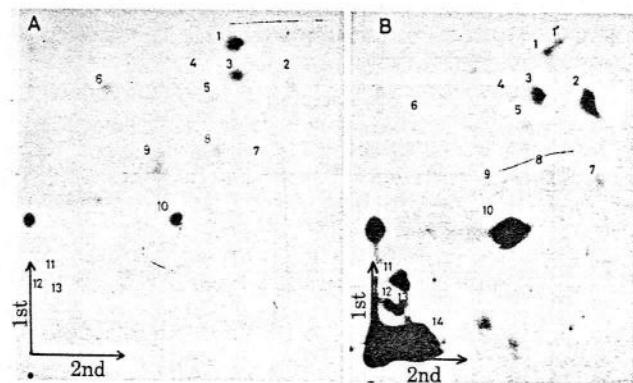


FIG. 7. Two-dimensional thin-layer chromatography of RNase T1 fragments of the natural tRNA_f^{Met} (A) and of the synthetic nascent molecule (B) on PEI-cellulose plates (20 × 20). The plates were irrigated with 1.4 M lithium formate, pH 3.5/7 M urea for 10 cm, and then with 2.3 M lithium formate, pH 3.5/7 M urea (1st dimension) and with 0.6 M lithium chloride/20 mM Tris-HCl, pH 8.0/7 M urea (2nd dimension) (33). Spots: 1, pC-A-A-C-C-A; 1', pC-A-A-C-C; 2, ps⁴U-P or pU-G; 3, pC-G; 4, pC-A-G; 5, pA-G; 6, pC-C-C-C-G; 7, pD-A-G or pU-A-G; 8, pU-C-G; 9, pC-U-C-G, pC-C-U-G, and pA-U-C-G; 10, P; 11, pCmU-C-A-U-A-A-C-C-C-G or pC-U-C-A-U-A-A-C-C-C-G; 12, pm⁷G-U-C-G or pG-U-C-G; 13, pT-Ψ-C-A-A-U-C-C-G or pU-U-C-A-A-U-C-C-G; 14, ATP.

recognition of a nucleic acid by a protein. Chemical modifications of tRNAs or genetic approaches to find mutants with base substitutions have been used previously for recognition studies. However, these approaches have certain limitations. Chemical synthesis should provide defined alterations which would be useful in structure-function relationship studies of tRNAs.

The chemical synthesis of oligoribonucleotides that have sequences of *E. coli* tRNA_f^{Met} and their analogs (e.g. U-G-C-G-G) (25) has provided suitable substrates for the construction of tRNA molecules by joining with RNA ligase. This paper reports the total synthesis of tRNA_f^{Met} from synthetic oligonucleotides with chain lengths 3–10. Oligonucleotides containing modified bases can be joined to other synthetic fragments by methods similar to those described herein. Even though RNA ligase can join short oligonucleotides and is a convenient tool for substituting fragments, it would be desirable to reduce the number of joining steps so as to obtain tRNA molecules in sufficient quantity to provide enough material for biological studies. Chemical synthesis of oligonucleotides as long as quarter molecules of tRNA would yield whole molecules after three ligations. Recently, we synthesized an icosaribonucleotide corresponding to bases 35–54 of the tRNA_f^{Met} by the phosphotriester method (unpublished data) and this fragment will be joined to fragments including modified bases to obtain tRNAs with partial modifications.

The tRNA synthesized in the present work is recognized to a limited extent by *E. coli* methionyl-tRNA synthetase. We do not know the tertiary structure of this tRNA. If modified nucleotides are necessary for forming the correct conformation required for synthetase recognition, then the low acceptor activity would be explained. Otherwise, a particular nucleotide modification may be required for direct interaction with the enzyme. Further synthetic investigation to identify the levels of modification that increase amino acid acceptor activities should aid in elucidating the mechanisms of these interactions.

We are indebted to Dr. M. Sugiura for suggestions and a gift of polynucleotide kinase and phosphatase. We thank Dr. J.-P. Waller for a gift of the purified methionyl-tRNA synthetase of *E. coli* and Oak Ridge National Laboratory for formylmethionine tRNA from *E. coli* K-12.

[‡] Acid precipitation of the amino acid carried by the aliquot of the synthetic tRNA (10 pmol) was measured after ³²P radioactivity became negligible with a control (-tRNA_f^{Met}, 66 cpm); it was found to be 25/cpm (0.32 pmol after subtraction of background) when the natural tRNA_f^{Met} (10 pmol) accepted methionine (2866 cpm, 4.8 pmol). Thus, the aminoacylation of the synthetic tRNA was 6.7% with respect to the intact tRNA.

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conjugated anti-sheep serum (directed against the globulins in the sheep H3 HA antiserum) and rhodamine-conjugated anti-hamster serum (directed against SV40 large-tumor antiserum). These cells showed coordinate expression of the HA and large-tumor antigen, indicating the specificity of the observed immunofluorescent reactions. Therefore, we conclude that the HA synthesized in HA-SV40-infected AGMK cells is expressed on the cell surface in the absence of influenza virus infection.

DISCUSSION

We have described the construction of a recombinant viral genome consisting of an SV40 vector and a cloned full-length DNA coding for the HA protein of influenza virus. Infection of AGMK cells with this recombinant virus produced a putative HA polypeptide that was immunoprecipitable with HA antiserum. The polypeptide showed a molecular size corresponding to that of uncleaved influenza HA and it was glycosylated, as shown by incorporation of radioactive labeled sugars. Furthermore, the putative HA product appeared to be functionally active; extracts from cells infected with the HA-SV40 recombinant exhibited specific hemagglutination not seen in control cell extracts. The HA product of HA-SV40 exhibited, in the absence of other influenza viral functions, properties characteristic of a surface glycoprotein. These observations suggest that the HA product of HA-SV40 is similar to the HA synthesized in cells infected with influenza virus. This is expected as our cloned HA DNA contains full-length sequences that code for the entire polypeptide sequence. Analysis of amino acid sequences suggests that the HA molecule includes three functional domains: an NH₂-terminal prepeptide signal for transport of the polypeptide from the cytoplasm to cell membranes, a COOH-terminal hydrophobic peptide for anchorage of the polypeptide in the cell membrane, and an internal "hinge" peptide region necessary for activation of viral infectivity through specific cleavage (30, 31). Our finding of HA synthesis demonstrates that amino acid sequences encoded by cloned HA DNA are sufficient for expression of the HA on the surface of eukaryotic cells. We did not observe, however, cleavage of the HA₀ into the HA₁ and HA₂ subunits during infection with the HA-SV40 viral recombinant. This was not surprising as cleavage of HA₀ occurred at a low level in AGMK cells without added trypsin during a productive infection of these cells with influenza A virus (Fig. 2A).

Only one of the nine HA-SV40 isolates examined synthesized HA. There are several possible explanations for the failure of other isolates to produce the polypeptide. Some of the HA-SV40 recombinants may have sustained deletion of DNA sequences that are required for transcription or translation. Also, some HA-SV40 hybrids may contain HA DNA inserted in an opposite orientation so that the sense (+) HA RNA strand is not transcribed. In the latter case, the (-) HA RNA strand that is present in genomic RNA would be synthesized. Analysis of nucleotide sequences at the junctures of SV40 and HA DNA should help to differentiate between these possibilities.

Our HA DNA recombinant should be useful in elucidating several interesting properties of the influenza HA. Individual domains that specify polypeptide functions can be rigorously tested through introduction of deletions or site-specific mutations. The regions that are associated with cell-receptor binding

and, similarly, the separate antibody-binding sites that are defined by classes of monoclonal antibodies can be dissected at the molecular level. Experiments involving phenotypic mixing should answer the question of whether hemagglutinin coded for by cloned DNA is expressed normally on the surface of viral particles. If so, it would then be possible to seek evidence for complementation between HA-SV40 recombinant DNA and influenza viral mutants defective in HA function.

We are very grateful to Dr. Robert Chanock for his advice, suggestions, encouragement, and support. We would also like to thank Messrs. Ron Mayner and Michael Phelan for preparing hemagglutinin antiserum, Mses. JoAnn Berndt and Salome Kruger for excellent assistance, and Eleanor Rice for typing the manuscript.

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CHEMICAL SYNTHESIS OF THE 5'-HALF MOLECULE OF *E. coli* tRNA₂^{Gly}

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Abstract—A tritriacontanucleotide which has the sequence of the 5'-half molecule of *E. coli* glycine tRNA₂, was synthesized by the phosphotriester method involving *p*-anisidate protection for the 3'-phosphate ends. Di- and trinucleotide units were prepared from 5'-dimethoxytrityl-2'-O-tetrahydrofuranyl-3'-O-(*o*-chlorophenyl)phosphoryl derivatives of uridine, N-benzoylcystidine, N-benzoyladenosine and N-isobutyrylguanosine by condensation with 3',5'-unprotected nucleosides followed by phosphorylation to give 3'-phosphodiester blocks. The 3'-terminal dimers and trimers were synthesized by using 3'-(*o*-chlorophenyl)phosphoro-*p*-anisidates instead of 3',5'-unprotected nucleosides. The 3'-phosphodiesters of oligonucleotides with a chain length of larger than 5 were obtained by removal of the 3'-phosphoro-*p*-anisidate with isoamyl nitrite. The 5'-dimethoxytrityl group was removed by treatment with zinc bromide under anhydrous conditions. Fragments were designed to use common dimer blocks and to reduce the step for 5'-deblocking of larger fragments. Finally a 3'-phosphodiester block with a chain length of 20 was condensed with a 5'-OH component (tridecanucleotide). The fully protected 33 mer was deblocked and purified by chromatography. The structural integrity of the product was confirmed by mobility shift analysis and complete digestion with RNase T2.

Chemical synthesis of ribooligonucleotides is an important subject in organic chemistry. It also provides a useful approach for studies on biological and physico-chemical properties of ribonucleic acids. In the early 1960s short oligomers such as all possible ribotriplets were synthesized by the phosphodiester method and used to elucidate the genetic code.¹ Later phosphodiester ribooligonucleotide blocks were prepared for the synthesis of tRNA fragments.² For the synthesis of larger oligonucleotides, the phosphotriester method becomes a method of choice with introduction of phenyl derivatives as protecting groups for internucleotidic phosphates and arnesulfonyl azolides as activating reagents for phosphodiester groups.³ We have been synthesizing ribooligonucleotides either by the phosphodi- or triester method, and have performed the synthesis of *E. coli* formyl methionine tRNA by enzymatic joining of the chemically synthesized fragments with RNA ligase.⁴ The methodology used in this study can be applied to replacement of functional parts of the tRNA molecule with synthetic oligonucleotides. Synthesis of larger fragments is advantageous in reducing enzymatic joining steps to construct modified tRNAs. Larger quantities of oligonucleotides, which can only be obtained chemically, are very useful for studies on interaction of nucleic acids with proteins, such as aminoacyl-tRNA synthetases.⁵ Completely chemical synthesis of RNA of the size of tRNA is a challenging subject in chemistry and has its own value. In the present paper we report a synthesis of a tritriacontanucleotide having the sequence of the 5'-half molecule (1-33) of *E. coli* tRNA₂^{Gly}.⁶ The synthesis involved phosphotriester block condensations using a combination of tetrahydrofuranyl⁷ and dimethoxytrityl⁸ groups for the 2'- and 5'-OH functions, respectively.⁹ The abbreviated scheme of the synthesis is shown in Fig. 1. The 5'-dimethoxytrityl group was shown to be removed selectively in the

presence of the 2'-O-tetrahydrofuranyl group on treatment with zinc bromide,⁹ although this reaction required anhydrous conditions, in contrast to the conditions used with deoxyoligonucleotides.¹⁰ In the phosphotriester synthesis, protection of the 2'-OH group is an essential problem and various groups have been used in combination with selectivity removable 5'-O-protecting groups. Tetrahydrofuranyl,⁸ 4-methoxytetrahydropyranyl,¹¹ tert-butyl-dimethylsilyl,¹² and *o*-nitrobenzyl¹³ groups have been used for synthesis of larger ribooligonucleotides: octadecamer,¹⁴ nonadecamer¹⁵ and eicosamer.¹⁶ The present tetrahydrofuranyl group has previously been used and shown to have the properties required for a 2'-O-protecting groups, e.g. facile introduction, stability during synthesis and complete removal at the final stage.^{9,17-19}

Preparation of di- and trinucleotide blocks. For the synthesis of larger oligonucleotides, condensation of protected oligonucleotides has obvious advantages. Preparation of oligonucleotides with phosphotriester internucleotidic phosphate requires two kinds of protecting groups for phosphates. One of those has to be removed selectively. A variety of combinations has been reported for the phosphotriester synthesis. Phenyl derivatives,²⁰ 2-cyanoethyl,²¹ phenylethyl derivatives²² and 5'-chloroquinolinyl²³ have been used for protection for internucleotide phosphates. As removable protecting groups, 2-cyanoethyl,²⁴ 2,2,2-trichloroethyl,²⁵ anilido,^{26a} and anisido^{16,26b} groups are used at the terminal phosphate. In the present synthesis 3'-(*o*-chlorophenyl)-*p*-anisido phosphoryl derivatives (5) were synthesized by phosphorylation of 5'-dimethoxytrityl-2'-O-tetrahydrofuranyl nucleosides (2) with *o*-chlorophenyl *p*-anisidophosphorochloridate^{26b} (3) as the phosphorylating reagent followed by removal of the 5'-dimethoxytrityl group with zinc bromide as illustrated in Fig. 2 using the condensations described

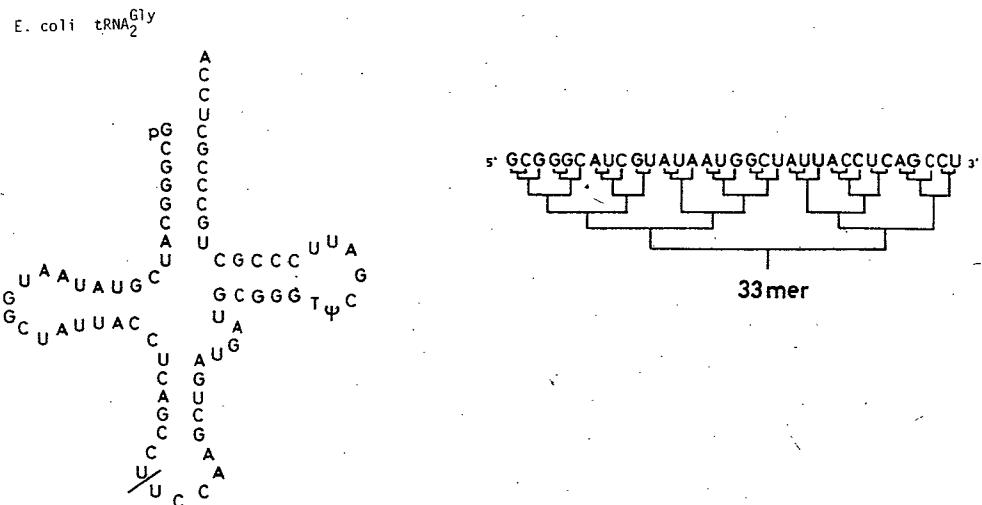


Fig. 1. Structure of the *E. coli* tRNA₂^{Gly} and the 5'-half sequence (1-33).

previously.⁹ Dimer units (7) were prepared by condensation of 3',5'-unprotected nucleoside (1) with 5'-dimethoxytrityl-2'-O-tetrahydrofuranyl-nucleoside 3'-(*o*-chlorophenyl)phosphates (6), which in turn were prepared by phosphorylation of 2 with *o*-chlorophenyl bis-(1H-1,2,4-triazol-1-yl) phosphate²⁷ using mesitylenesulfonyl tetrazolide (MSTe, 1-(2,4,6-trimethylbenzenesulfonyl)-1H-tetrazole).²⁸ For fur-

ther elongation in the 3'-direction, 7 was phosphorylated. The terminal dimer blocks (8) were obtained by condensation of the 3'-phosphodiesters (6) with the 5'-free nucleotides (5). Reaction conditions for the preparation of dimers are summarized in Table 1. The dimers were isolated by chromatography on silica gel or alkylated silica gel.

Trimmers were prepared from the above dimers by

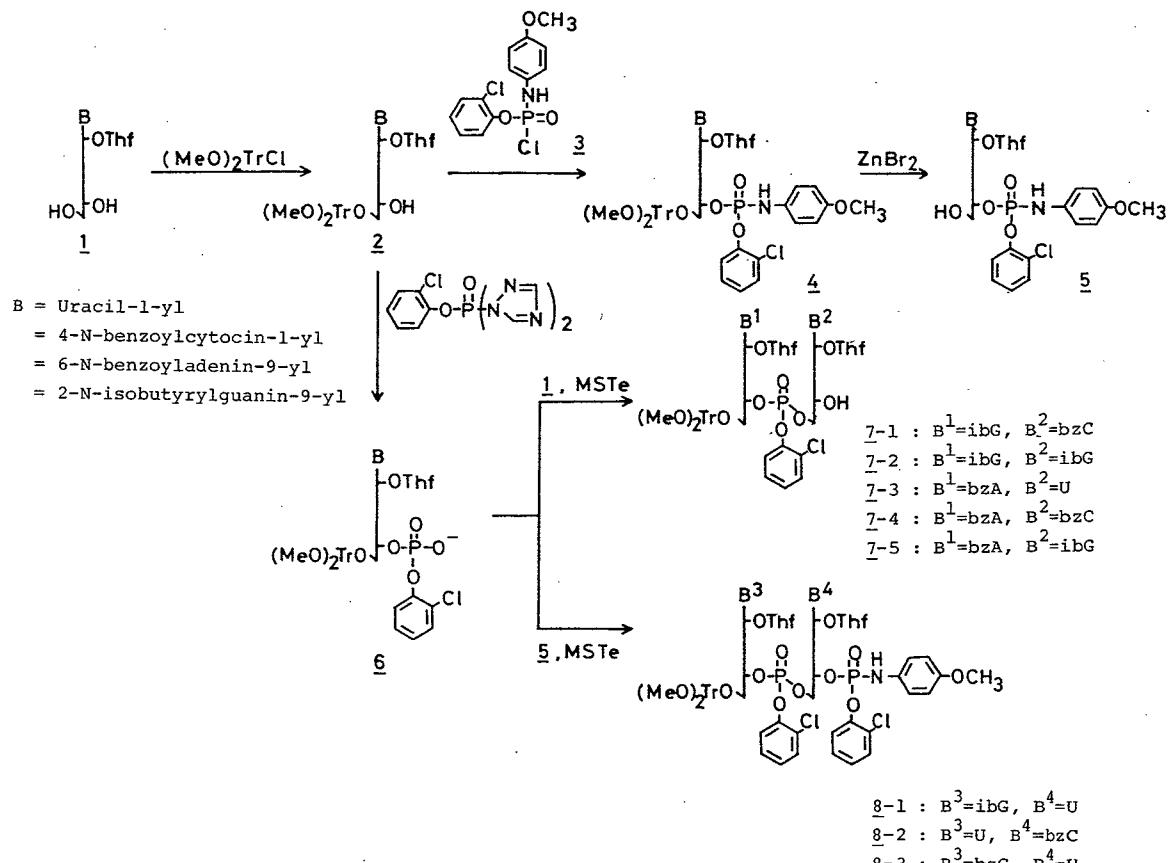


Fig. 2. Preparation of dimers.

Table 1. Reaction conditions for the synthesis of dimers

3'-Phosphodiester Component * (mmol)	5'-OH Component (mmol)	MSTe (mmol)	Time (min)	Product	Yield (%)
DT[G]OH (8.00)	HO[C]OH (11.23)	15.90	25	DT[GC]OH (7-1)	62
DT[G]OH (2.81)	HO[G]OH (3.93)	5.60	25	DT[GG]OH (7-2)	72
DT[A]OH (10.13)	HO[U]OH (13.99)	19.91	25	DT[AU]OH (7-3)	75
DT[A]OH (2.45)	HO[C]OH (3.29)	4.71	35	DT[AC]OH (7-4)	64
DT[A]OH (2.02)	HO[G]OH (2.59)	3.99	35	DT[AG]OH (7-5)	88
DT[G]OH (2.00)	HO[U]pAn (1.70)	3.40	25	DT[GU]pAn (8-1)	78
DT[U]OH (1.50)	HO[C]pAn (1.40)	2.96	25	DT[UC]pAn (8-2)	85
DT[C]OH (0.60)	HO[U]pAn (0.50)	1.01	30	DT[CU]pAn (8-3)	74

DT = (MeO)₂Tr, An = NH₂-p-OCH₃, p = o-chlorophenyl phosphate, [] = protected except for 3' and 5' termini, * : DT[N]OH was converted to 3'-phosphodiester component by phosphorylation with o-chlorophenyl phosphoroditriazolide followed by treatment with H₂O.

essentially the same procedure. Structures of the trimers are shown in Fig. 3 and reaction conditions are summarized in Table 2. The trimers were separated by reversed phase chromatography on alkylated silica gel. These dimers and trimers were designed to have purines at the 5'-terminal position, since removal of the dimethoxytrityl group of purine nucleosides was found to be easier.

Synthesis of tritriaccontamer by condensation of oligonucleotide blocks. All 3'-diesterified trinucleotide intermediates used in this synthesis were prepared by phosphorylation of trinucleoside diphosphates listed in Table 2. As shown in Fig. 4 the 5'-OH components were derived by removal of the 5'-dimethoxytrityl groups. Yields and conditions of these conversions are summarized in Table 3. Reaction conditions of these nucleotide blocks are given in Table 4. The 3'-phosphoro-p-aniside of penta- and hexanucleotides were converted to the phosphate by treatment with isoamyl nitrite when elongation was in the 3'-direction. The eicosamer (48) was obtained as the 3'-phosphodiester form by elongating the chain in the

3'-direction. It was activated by the condensing reagent to react with the tridecamer (49). Removal of the 5'-dimethoxytrityl of larger oligonucleotides was avoided as far as possible. Complete removal of the 5'-protecting group of the hexamers and tridecamer was not intended. Unchanged dimethoxytritylated oligonucleotides were recovered when the reaction had been slow. Yields listed in Table 4 varied mainly due to decomposition during chromatography on reversed-phase support. The eicosamer (46) was partially lost by conversion to polar compounds. The fully protected tritriaccontamer (50) was not purified by chromatography. It was collected by preparation and isolated after deblocking.

Deblocking of the product was performed by a procedure similar to that reported previously,⁹ by treatment with: (1) isoamyl nitrite in pyridine-acetic acid (5:4), (2) 0.5 M 1,1,3,3-tetramethylguanidinium *syn*-pyridine-2-carboxaldoximate (TMG-PAO),²⁹ (3) ammonium hydroxide, (4) anion-exchange resin Dowex 50 W \times 2 (pyridinium form), and (5) dilute hydrochloric acid (pH 2). The deblocked product was

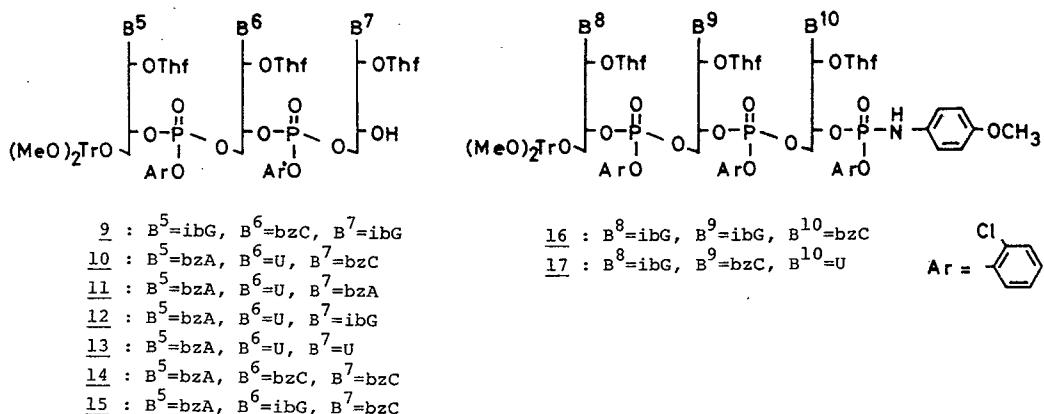


Fig. 3. Structure of trimers.

Table 2. Reaction conditions for the synthesis of trimers

3'-Phospho-diester Component* (mmol)	5'-OH Component (mmol)	MSTe (mmol)	Time (min)	Product	Yield (%)
DT[GC]OH (1.72)	HO[G]OH (2.43)	3.45	30	DT[GCG]OH (9)	76
DT[AU]OH (1.72)	HO[C]OH (2.42)	3.23	30	DT[AUC]OH (10)	72
DT[AU]OH (1.08)	HO[A]OH (1.43)	2.04	30	DT[AUA]OH (11)	69
DT[AU]OH (2.92)	HO[G]OH (4.21)	6.03	25	DT[AUG]OH (12)	73
DT[AU]OH (0.66)	HO[U]OH (0.93)	1.30	30	DT[AUU]OH (13)	59
DT[AC]OH (1.35)	HO[C]OH (1.89)	2.71	30	DT[ACC]OH (14)	58
DT[AG]OH (0.56)	HO[C]OH (0.79)	1.23	25	DT[AGC]OH (15)	70
DT[GG]OH (1.66)	HO[C]pAn (1.67)	3.34	25	DT[GGC]pAn (16)	73
DT[GC]OH (3.04)	HO[U]pAn (3.05)	6.02	30	DT[GCU]pAn (17)	76

DT = $(MeO)_2Tr$, An = $NH_2-p-OCH_3$, p = o-chlorophenyl phosphate, [] = protected except for 3' and 5' termini, * : DT[NN]OH was converted to 3'-phosphodiester component by phosphorylation with o-chlorophenyl phosphoroditriazolide followed by treatment with H_2O .

separated by gel filtration on Sephadex G-50. Figure 5 shows profiles of gel filtration of the eicosamer (20 mer) and tritriacontamer (33 mer). The product were analyzed by reversed-phase high pressure liquid chromatography (HPLC) and the 33 mer was found to be contaminated with partially protected compounds. Acid treatment for removal of the 2'-O-tetrahydrofuran group was repeated and the product was fractionated by reversed-phase HPLC after gel filtration as shown in Fig. 6. The fractionated products were found to be homogeneous (Fig. 7).

The 20 and 33 mer were identified by analysis of the chain length (Fig. 8) and mobility shift method (Fig. 9). Figure 8 shows a radio-autograph of a 20%

polyacrylamide gel electrophoresis³⁰ of the 5'-labeled products. For mobility shift analysis³¹ and polymers were labeled at either end with polynucleotide kinase³² and $[\gamma-^{32}P]ATP$ or with RNA ligase plus 5'-labeled pCp.³³

Complete removal of the protecting groups and maintaining of the 3'-5' internucleotide linkages were confirmed by complete digestion with RNase T2³⁴ followed by labeling with polynucleotide kinase and $[\gamma-^{32}P]ATP$. The results of two dimensional thin layer chromatography on cellulose³⁵ of the mixture of 5'-labeled pNp are shown in Fig. 10. This test indicated that the 20 mer and 33 mer were digested with RNase T2 to give nucleoside 3'-phosphates.

Table 3. Removal of the 5'-dimethoxytrityl group

Substrate (mmol)	1M $ZnBr_2$ (ml)	* Time (min)	Product	Yield (%)
16 (1.195)	40	2.5	HO[GGC]pAn (19)	67
8-1 (1.289)	40	3	HO[GU]pAn (21)	84
17 (2.250)	70	2.5	HO[GCU]pAn (24)	68
8-2 (1.146)	30	20	HO[UC]pAn (27)	73
8-3 (0.354)	10	12	HO[CU]pAn (29)	76
31 (0.791)	35	2	HO[AUCGU]pAn (36)	70
32 (1.129)	50	1	HO[AUGGU]pAn (37)	57
33 (0.603)	30	5	HO[ACCUC]pAn (38)	81
34 (0.221)	11	5	HO[AGCCU]pAn (39)	67
41 (0.380)	25	2	HO[AUAUAGGGCU]pAn (44)	56
47 (0.064)	10	2	HO[AUUAUCCUCAGCCU]pAn (49)	66

An = $NH_2-p-OCH_3$, p = o-chlorophenyl phosphate, [] = protected except for 3' and 5' termini, * : CH_2Cl_2 :iso-PrOH = 85:15.

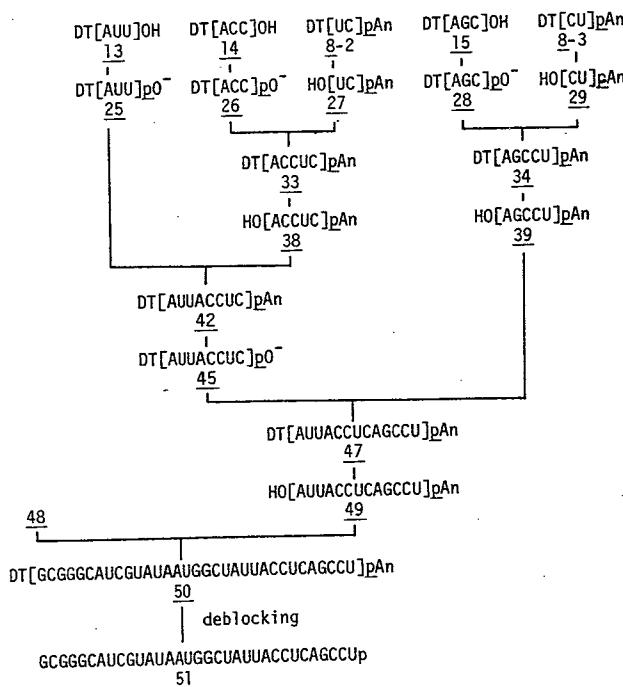
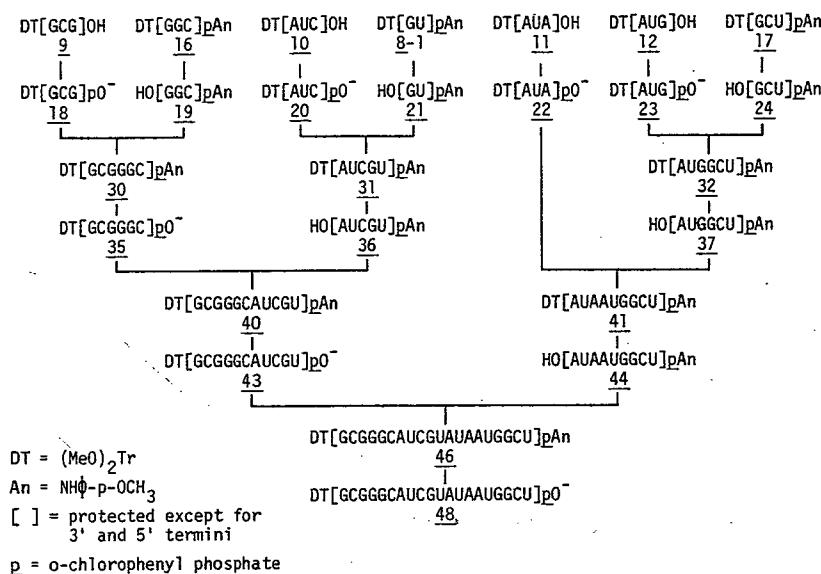


Fig. 4. Synthesis of the 33 mer.

EXPERIMENTAL

TLC was performed on plates of silica gel (Kieselgel 60 HF₂₅₄, Merck) using a mixture of CHCl_3 and MeOH . For reversed phase TLC (RPTLC), silanized silica gel (Kieselgel 60 HF₂₅₀ Silanisiert, Merck) was used with a mixture of acetone-water. For columns, silica gel (type 60 or 60 H, Merck) was used with a mixture of CHCl_3 - MeOH . For preparative reversed phase chro-

matography, alkylated silica gel (C-18, 35-105 μ , Waters) was packed with 60-70% acetone and elution was performed with a gradient of acetone (60-80%) in 0.2% aqueous pyridine. HPLC was carried out on an Altex 332 MP apparatus using a reversed phase column (TSK-LS410, Toyo Soda).

Two dimensional homochromatography³¹ was performed as described previously.³⁶

Triethylammonium bicarbonate (TEAB) buffer (pH 7.5)

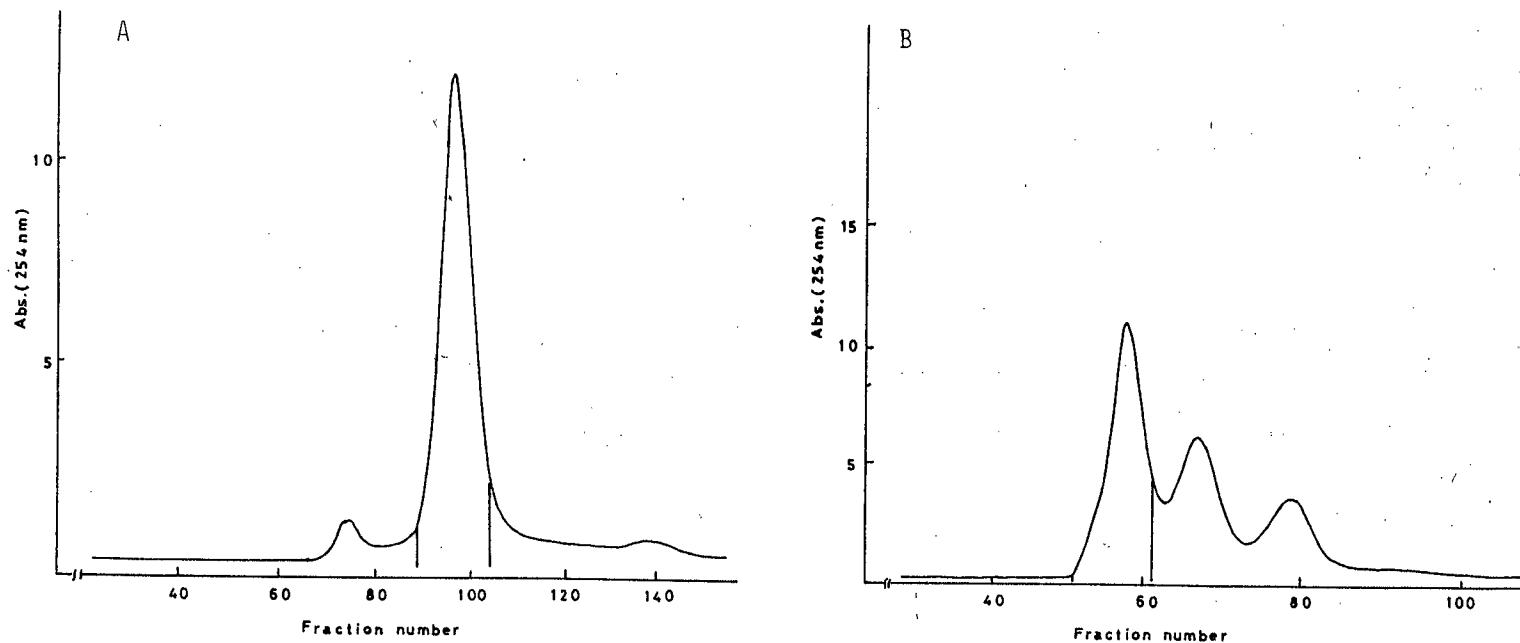


Fig. 5. Gel filtration of the 22 mer (A) and 33 mer (B) on a column (2.8×117 cm) of Sephadex G-50 equilibrated with 0.1 M TEAB. A, Fractions of 2.7 ml were collected every 5 min. B, Fractions of 3.9 ml were collected every 6 min.

Table 4. Conditions for block condensation

3'-Phosphodiester Component (mmol)	5'-OH Component (mmol)	MSTe (mmol)	Time (min)	Product (Chain length)	Yield (%)		
<u>9</u> a)	(0.903)	<u>19</u>	(0.791)	1.988	40	<u>30</u> (6 mer)	59
<u>10</u> a)	(1.061)	<u>21</u>	(1.060)	2.620	40	<u>31</u> (5 mer)	79
<u>12</u> a)	(1.702)	<u>24</u>	(1.492)	3.702	35	<u>32</u> (6 mer)	77
<u>14</u> a)	(0.778)	<u>27</u>	(0.794)	1.660	25	<u>33</u> (5 mer)	84
<u>15</u> a)	(0.365)	<u>29</u>	(0.277)	0.650	35	<u>34</u> (5 mer)	82
<u>30</u> b)	(0.456)	<u>36</u>	(0.466)	1.008	40	<u>40</u> (11 mer)	49
<u>11</u> a)	(0.675)	<u>37</u>	(0.646)	1.693	40	<u>41</u> (9 mer)	74
<u>13</u> a)	(0.352)	<u>38</u>	(0.356)	1.062	55	<u>42</u> (8 mer)	64
<u>40</u> b)	(0.222)	<u>44</u>	(0.183)	0.720	50	<u>46</u> (20 mer)	22
<u>45</u>	(0.155)	<u>39</u>	(0.146)	0.464	55	<u>47</u> (13 mer)	63
<u>46</u> b)	(0.020)	<u>49</u>	(0.020)	0.187	90	<u>50</u> (33 mer)	

a) : DT[NNN]OH was converted to 3'-phosphodiester component by phosphorylation with *o*-chlorophenyl phosphoroditriazolide followed by treatment with H₂O.

b) : Fully protected ribooligonucleotide was converted to 3'-phosphodiester component by treatment with isoamyl nitrite.

was used to wash organic layers containing protected nucleotides.

Dinucleoside monophosphates 7; Table 1, Example (7-1)
General methods for phosphorylation and condensation. 5'-Dimethoxytrityl-2'-O-tetrahydrofuranyl-N-isobutyrylguanosine (2, B = ibG; 5.805 g, 7.998 mmol) was dried by evaporation of pyridine and dissolved in pyridine (3 ml).

o-Chlorophenyl phosphoroditriazolide (12.0 mmol in 40 ml of dioxane) was added and the mixture was shaken for 20 min at 30°. TLC and RPTLC showed disappearance of the starting material. After 25 min, 0.1 M TEAB (150 ml) and pyridine (70 ml) were added. The product (6) was extracted with CHCl₃ (150 ml). The aqueous phase was reextracted with CHCl₃-pyridine (3:1, 40 ml) and the combined organic layer was washed twice with TEAB (150 ml).

After evaporation of organic solvents the residue was dried by evaporation of pyridine 3 times and mixed with 2 (B = bzC) (higher isomer, 4.686 g, 10.15 mmol). The mixture was dried as above and treated with MSTe (4.012 g, 15.90 mmol) in pyridine (40 ml) at 30° for 15 min. Completion of the reaction was confirmed by TLC and RPTLC. After 25 min, water (3 ml) was added and evaporated. The residue was dissolved in CHCl₃ and washed with sat NaHCO₃ aq. The product (7-1) was separated by chromatography on silica gel (Kieselgel 60 H, 150 g, ϕ 10 × 5.4 cm) using a gradient MeOH in CHCl₃, and precipitated with hexane from its soln in CHCl₃, yield was 62%, 6.512 g, 4.95 mmol.

Dinucleotide 8. Table 1, Example (8-2). Compound 2 (B = U) (0.927 g, 1.50 mmol) was phosphorylated as described above and 6 (B = U) was condensed with 5 (B = BzC) (0.996 g, 1.40 mmol) in the presence of MSTe

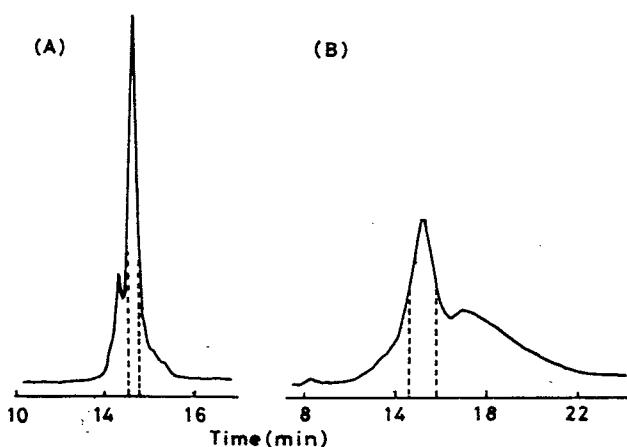


Fig. 6. Purification of the 22 mer (A) and 33 mer (B) by HPLC on silica gel (TSK-LS410) with a flow rate of 2 ml/min. A, a linear gradient of acetonitrile (5-25% during 30 min) in 0.1 M triethylammonium acetate. B, a linear gradient of acetonitrile (11-15% during 03 min) in 0.1 M triethylammonium acetate.

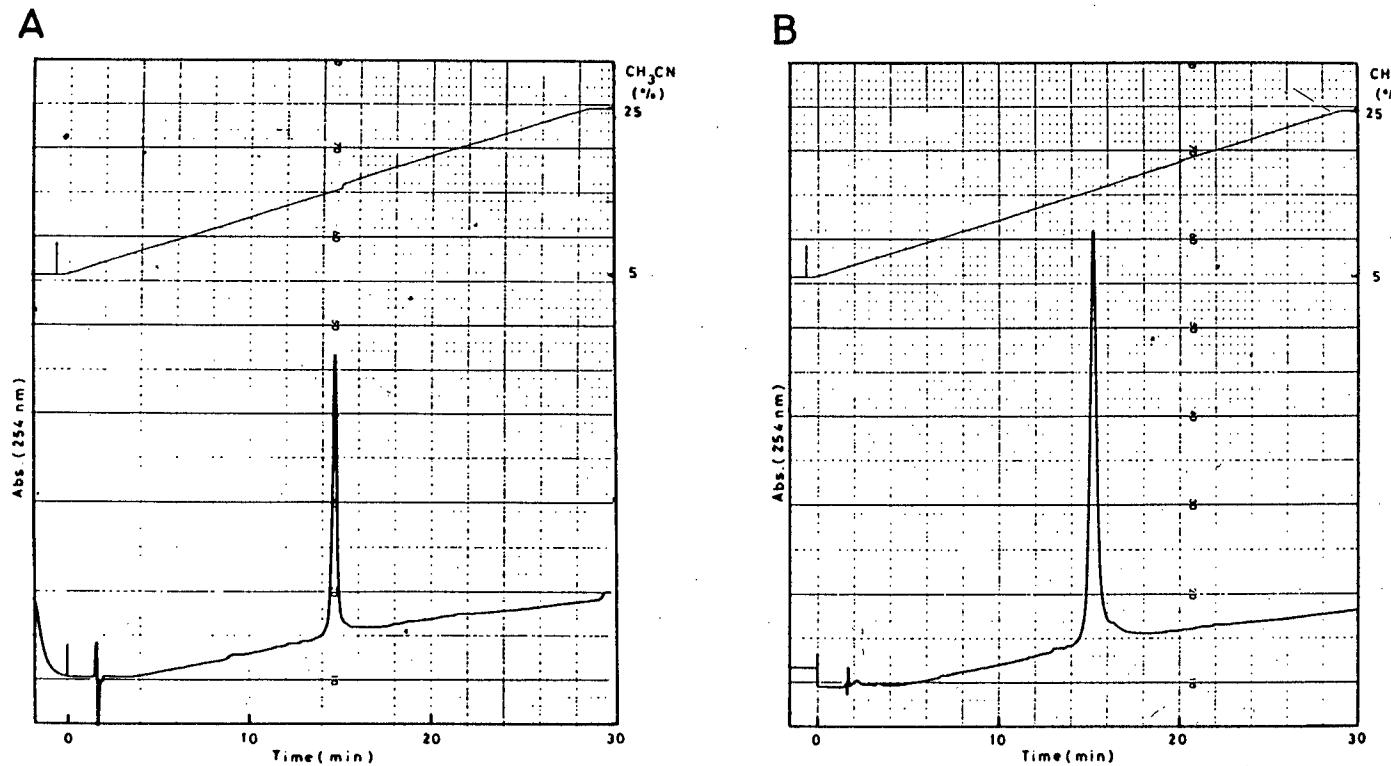


Fig. 7. Analysis of the purified 20 and 33 mer by HPLC on C-18 silica gel (TSK-LS410) in 0.1 M triethylammonium acetate.

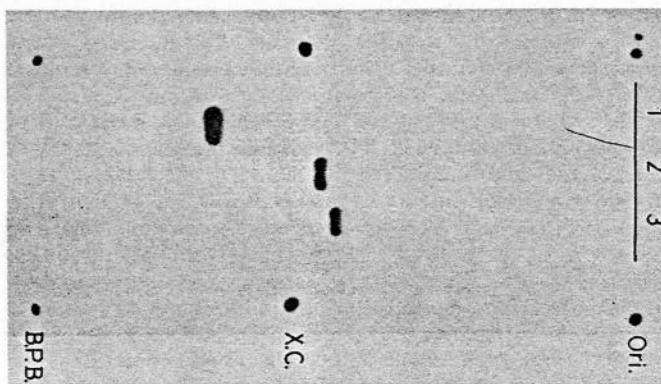


Fig. 8. Gel electrophoresis of the 20 mer (1), 33 mer (2) and a marker (3, 34 mer) from the *E. coli* tRNA_f^{Met} on 10% polyacrylamide.

(0.746 g, 2.96 mmol) at 30° for 15 min. The reaction was checked by TLC and RPTLC. After 25 min water (2 ml) was added and the mixture was concentrated. The residue was dissolved in CHCl₃ and washed twice with sat NaHCO₃ aq (70 ml). The product (8-2) was isolated by reversed phase chromatography on C-18 silica gel (ϕ 4 \times 9.5 cm) using a gradient of acetone in 0.2% pyridine and precipitated with hexane from its soln in CHCl₃. The yield was 85%, 1.789 g, 1.193 mmol.

Synthesis of the trimers (9-17) Table 2. Trimers (9-15) were synthesized from 7 by phosphorylation as described for the preparation of 6 followed by condensation N, 2'-protected nucleosides (1). For the synthesis of trinucleotides (16, 17), nucleotides (5) were used. Synthetic procedures were the same described for dimers (7, 8) and trimers were isolated by reversed phase chromatography on C-18 silica gel (ϕ 4 \times 9 cm) using a gradient of acetone in 0.2% pyridine.

Removal of the 5'-dimethoxytrityl group (Table 3). The trinucleotide (protected GCUp, 17) (4.723 g, 2.25 mmol) was dried by evaporation three times with pyridine, three times with toluene and shaken with 70 ml of 1 M ZnBr₂ in CH₂Cl₂-PrOH (85:15, v/v) for 2.5 min at room temp. A soln of 1 M ammonium acetate (200 ml) was added with shaking and the product was extracted with CH₂Cl₂ (100 ml). The organic soln was washed twice with 1 M ammonium acetate (100 ml) and concentrated. The residue was applied to a column (ϕ 5 \times 5.5 cm) of Kieselgel 60 H (40 g). The oligonucleotide was eluted with a gradient of MeOH in CHCl₃ and precipitated with hexane from its soln in CHCl₃. The yield was 68%, 2.728 g, 1.521 mmol.

Condensation of oligonucleotide blocks

(1) *The nonanucleotide (41)*. The trimer 11 (1.245 g, 0.675 mmol) was phosphorylated by the procedure described for the synthesis of 6, and condensed with 37, (3.254 gm 0.646 mmol) using MSTe (4.27 mg, 1.69 mmol) at 30° for 25 min. Completion of the reaction was checked by TLC and RPTLC and the product was isolated by reversed phase chromatography as described for the trimers.

(2) *The undecamer 40 (Removal of the anisido group)*. The protected 30 (1.817 g, 0.456 mmol) was treated with isoamyl nitrite (3.1 ml, 23 mmol) in pyridine-AcOH (5:4, 14 ml) at 30° for 5 hr. The mixture was added with 0.2 M TEAB (100 ml), pyridine (75 ml), ether-pentane (1:1, 100 ml) on the aqueous phase was washed with ether-pentane (1:1, 100 ml). The product (35) was extracted with CHCl₃ (140 ml), washed three times with 0.2 M TEAB (100 ml), applied to a column (ϕ 4 \times 7 cm) of C-18 silica gel and eluted with a gradient of acetone in 0.2% pyridine. The

hexamer 35 was collected, dried by evaporation with pyridine and condensed with 36 (1.348 g, 0.466 mmol) using MSTe (254 mg, 1.01 mmol) at 30° for 40 min. The product was isolated by reversed phase chromatography as above and precipitated with pentane. The yield was 49%, 1.505 g, 0.223 mmol. The *R*_f values in TLC (10:1) and RPTLC (7:3) were 0.29 and 0.23, respectively.

(3) *The tridecamer (47)*. The octamer 45 (0.756 g, 0.155 mmol) and 39 (0.437 g, 0.146 mmol) were condensed using MSTe (0.117 g, 0.464 mmol) at 30° for 55 min and the product was isolated by reversed phase chromatography on a column (ϕ 3 \times 8 cm) of C-18 silica gel. The *R*_f values in TLC (10:1) and RPTLC (8:2) were 0.43 and 0.70, respectively.

(4) *The eicosamer (46)*. The undecamer 43 and 39 were condensed using conditions shown in Table 4 and 46 was isolated first by reversed phase chromatography on a column (ϕ 4 \times 7 cm) of C-18 silica gel. The product 46 was purified by chromatography on a column (ϕ 3 \times 2.8 cm) of silica gel (Kieselgel 60 H, 7 g) using a gradient of MeOH in CHCl₃. The yield was 22%, 0.481 g, 0.041 mmol. The *R*_f values in TLC (10:1) and RPTLC (8:2) were 0.35 and 0.62, respectively.

(5) *The tritriacotamer (50)*. The eicosamer 46 (239 mg, 0.020 mmol) was treated with isoamyl nitrite (0.15 ml, 1.11 mmol) in pyridine-AcOH (5:4, 1.5 ml) at 30° for 5.5 hr. Completion of the reaction was checked by TLC and RPTLC. The eicosamer 48 was extracted with CHCl₃-pyridine (2:1, 45 ml), washed 4 times with 0.2 M TEAB (50 ml) and precipitated with ether-pentane (1:4, 50 ml) from its soln in CHCl₃ (2.5 ml). The eicosamer 48 was reprecipitated, dried by evaporation with pyridine and condensed with 49 (150 mg, 0.020 mmol) in pyridine (0.5 ml) using MSTe (27 mg, 0.11 mmol) at 30° for 50 min. Starting materials were detected after 50 min in TLC and RPTLC. The mixture was treated with MSTe (20 mg, 0.079 mmol) for 40 min and added with water (0.5 ml). The product was extracted with CHCl₃-pyridine (5:2, 70 ml), washed twice with 0.1 M TEAB (50 ml) and precipitated with pentane from its soln in CHCl₃. The yield of the crude product was 463 mg.

The deblocked 20 mer. The protected 46 (25 mg, 0.002 mmol) was treated with isoamyl nitrite (0.027 ml, 0.2 ml) in pyridine-AcOH (5:4, 0.3 ml) at 30° for 5 hr and the product was extracted with CHCl₃-pyridine (2:1, 30 ml). The eicosamer was precipitated with ether-pentane (1:4, 50 ml), treated with 1 M TMG-PAO in dioxane (2 ml) and water (2 ml) at 30° for 60 hr. The volatile materials were removed and the residue was dissolved in pyridine (2 ml). The mixture was treated with conc ammonia (10 ml) at 55°

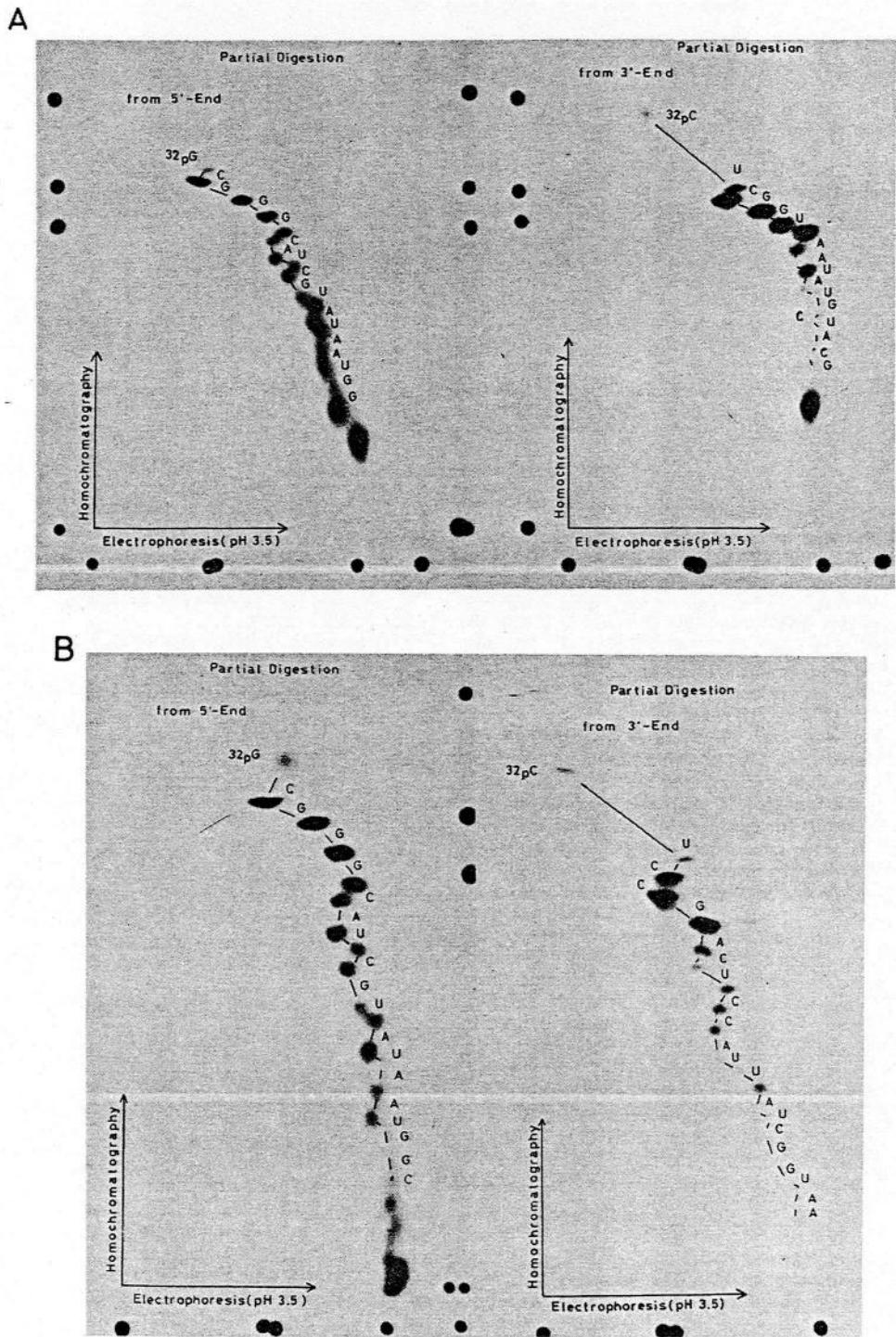


Fig. 9. Mobility shift analysis of the 20 mer (A) and 33 mer (B) using Homo-mix I.^{31b}

for 6 hr and concentrated. The product was dissolved in aqueous pyridine (30%, 20 ml) and passed through a column (5 ml) of Dowex 50 W \times 2 (pyridinium form). The column was washed with 30% pyridine (100 ml) and combined solns were concentrated. The residue was dissolved in 0.1 M TEAB (50 ml), washed twice with ether (40 ml), evaporated

three times with added toluene and mixed with 0.1 N HCl (15 ml). The solution was adjusted to pH 2 with 0.1 N HCl, kept at 25° for 9 hr, neutralized with 0.1 M ammonium hydroxide, washed twice with ether (40 ml) and concentrated. The residue was applied to a column of Sephadex G-50 (Fig. 5a) and the product in peak I (272 A₂₆₀) was

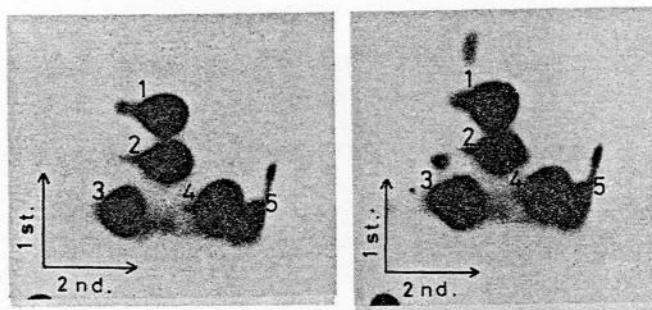


Fig. 10. Two dimensional TLC of mononucleotides obtained by digestion with RNase T2 of the 20 mer (A) and 33 mer (B). Spots: 1, Ap; 2, Cp; 3, Gp; 4, Up; 5, Pi. Solvents: 1st dimension, isobutyric acid-0.5 M ammonium hydroxide (5:3, v/v); 2nd dimension, isopropanol-conc. hydrochloric acid-water (70:15:15, v/v).

collected. An aliquot (6.8 A₂₆₀) was subjected to HPLC (TSK LS 410) (Fig. 6) and 1.7 A₂₆₀ units of the pure eicosamer was obtained. The estimated yield from 46 was 16% assuming ϵ of the eicosamer being 20×10^4 .

The deblocked 33 mer (51). The protected 33 mer 50 (crude, 77 mg) was treated with isoamyl nitrite (0.018 ml, 0.6 mmol) in pyridine-AcOH (1:1, 1 ml) at 30° for 6 hr and treated with 0.5 M TMG-PAO (16 ml) using procedures described for the deblocking of the 20 mer. The 33 mer was then treated with conc ammonia (20 ml) at 55° for 6 hr, concentrated and passed through a column of Dowex 50 W \times 2 (10 ml, pyridinium form). Acid treatment was performed as described for the 20 mer using 0.1 N HCl (30 ml) and 0.01 N HCl. The product was applied to gel filtration (Fig. 5b) and fractions containing the 33 mer were combined (234 A₂₆₀). HPLC analysis showed incomplete removal of protecting groups. The product (220 A₂₆₀) was retreated at pH in HCl (15 ml) at 25° for 8 hr and neutralized. The 33 mer was subjected to gel filtration and a part of the product (fraction No. 65, 18 A₂₆₀) was further purified by HPLC (TSK-LS 410) as shown in Fig. 6.

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