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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 $tRNA_f^{Met}$
E. Ohtsuka, T. Doi, H. Uemura, Y. Taniyama & M. Ikehara (1980) Nucleic Acids Res. 8, 3909
(E. coli $tRNA_f^{Met}$ 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 $tRNA_f^{Met}$ 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 $tRNA_f^{Met}$ のアンチコドントリプレットを終止コドン UAG, UAA に置換することによる修飾)

3) Replacement and insertion of nucleotides at the anticodon loop of E. coli $tRNA_f^{Met}$ by ligation of chemically synthesized ribooligonucleotides.
T. Doi, A. Yamane, J. Matsugi, E. Ohtsuka & M. Ikehara (1985) Nucleic Acids Res. 13, 3685
(化学合成リボオリゴヌクレオチドの ligation による E. coli $tRNA_f^{Met}$ 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 $tRNA_f^{Met}$ by ligation of chemically synthesized ribooligonucleotides.
T. Doi, H. Morioka, J. Matsugi, E. Ohtsuka & M. Ikehara (1985) FEBS Letters 190, 125
(化学合成リボオリゴヌクレオチドの ligation による E. coli $tRNA_f^{Met}$ のアミノ酸受容ステムの修飾)

5) The in vivo stability, maturation and aminoacylation 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$_f^{Met}$ の in vivo における安定性と成熟化及びアミノアシル化)

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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 を用いたリン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 formylmethionine 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$_f^{Met}$ 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$_2^{Gly}$.
E. Ohtsuka, A. Yamane, T. Doi & M. Ikehara (1984) Tetrahedron 40, 47
(E. coli tRNA$_2^{Gly}$ の 5'半分子の化学合成)

論文内容の要旨

博士論文題名

ホルミルメチオニンtRNA変換体
の合成と活性

学位申請者　　土井 健史

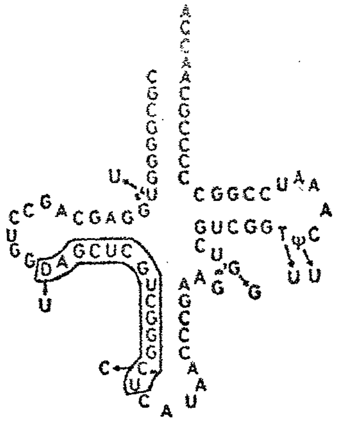
tRNA は鎖長 70～80 の機能を有する RNA としては最小の分子である。

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 ligase 1) を用いて合成した。この際 これらの RNA ligase 反応における塩基特異性を見い出した。

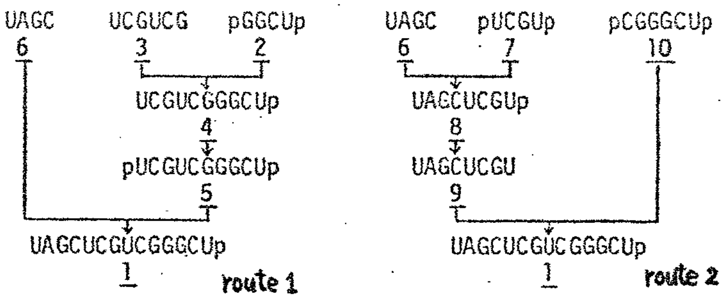
次に RNA ligase 反応を用い E. coli tRNA$_f^{Met}$ のアミノアシルステム, TΨループ, Dループ, アンチコドンループを変換した tRNA を合成し 主にメチオニル tRNA 合成酵素との相互作用について調べた。

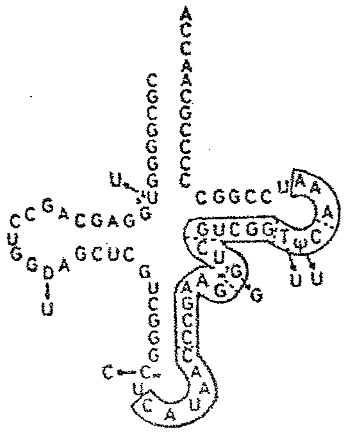
I RNA ligase を用いた長鎖オリゴリボヌクレオチドの合成

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 tRNA 半分子を合成した。4)

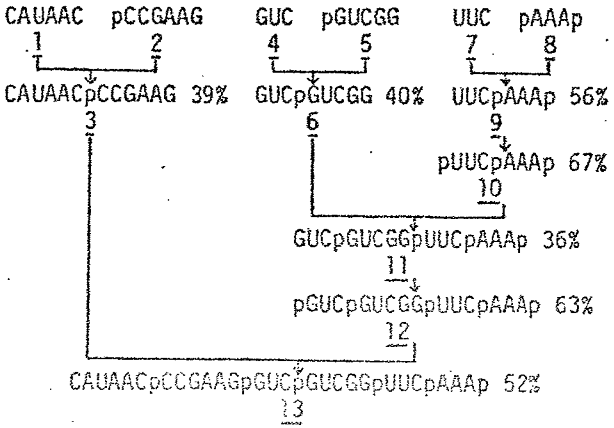


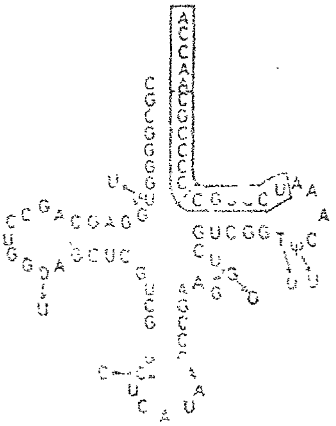
synthesis of 14mer (bases 21-)



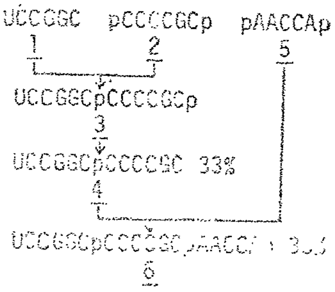


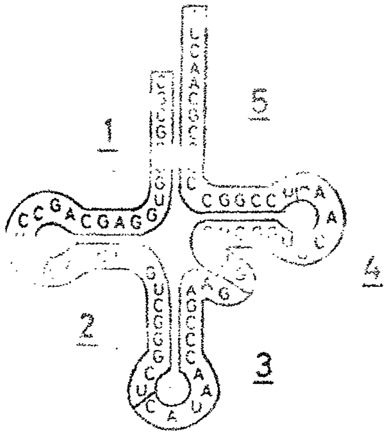
Synthesis of 26mer (bases 35-60)





Synthesis of 17mer (bases 21-34)





Synthesis of half molecules

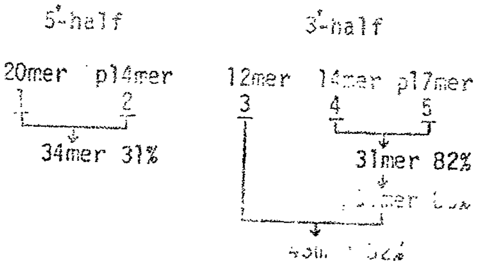


図1

表1　Reaction conditions for joining

acceptor nmol [μM]	donor nnol [μM]	ATP μM	RNA ligase [unit/ml]	time hr	Reaction extent[a)] %	Yield %
UCGUCG (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－プリン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$^{Met}_{f}$ 変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$^{Met}_{f}$ 変換体

を合成した。（図2）

1. アミノアシルステムの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

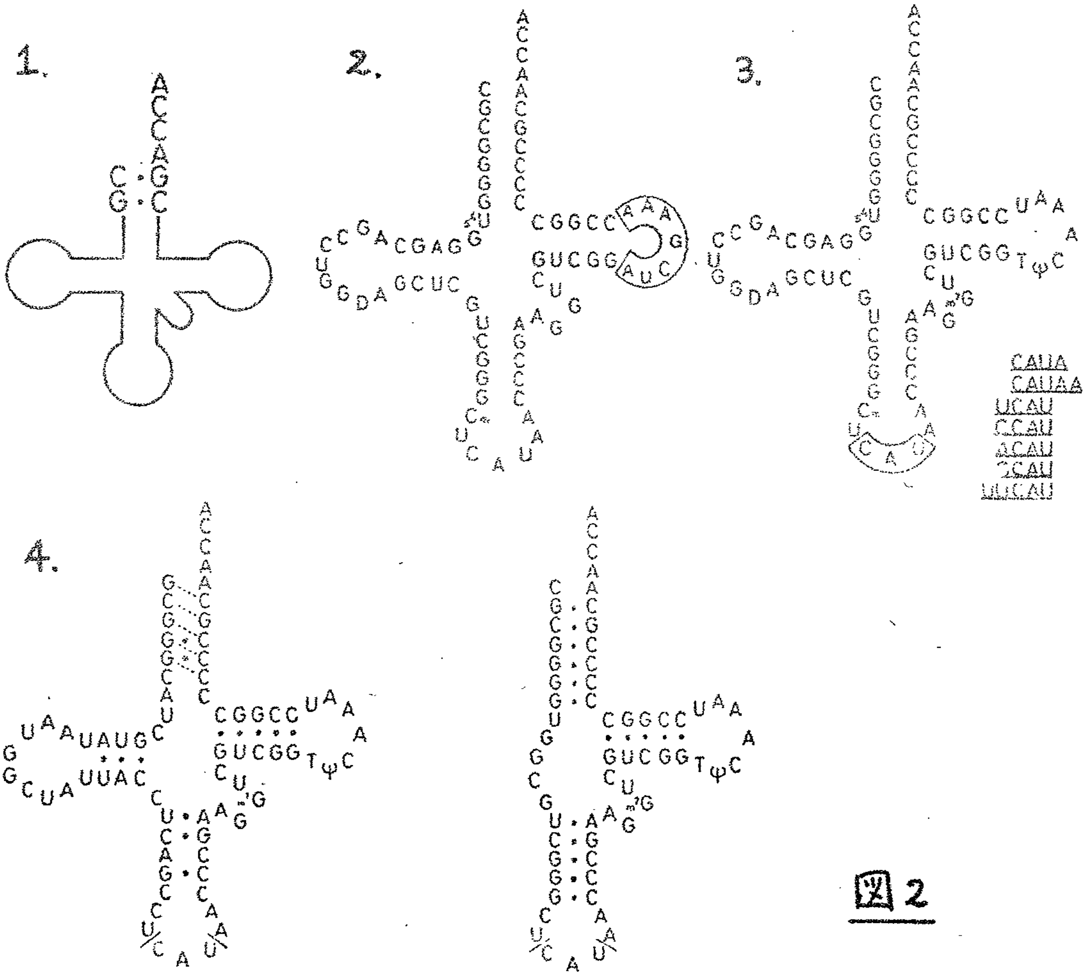


図2

ンループを拡大した$tRNA_f^{Met}$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た8)のでこの配列を残しループを拡大した。

4. Dループの欠除した$tRNA_f^{Met}$や5'側半分子にE. coli $tRNA_2^{Gly}$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あわせ、これらをRNA-ligaseで結合し行なった。

アミノアシル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、その他のループの変換には$tRNA_f^{Met}$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した。RNA ligase 反応後は nearest neighbor analysis で結合部位を確認した。

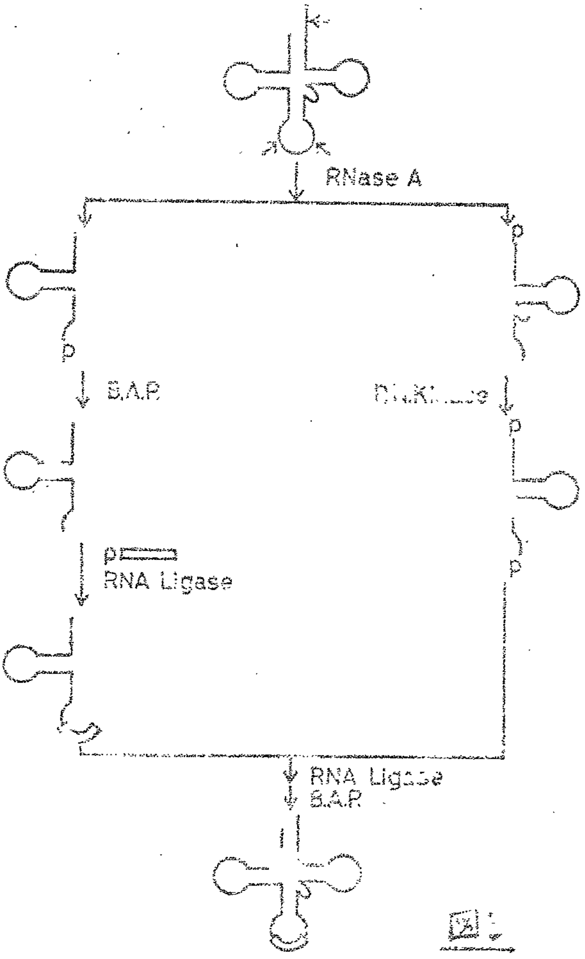


図1

合成したtRNAについてアミノ酸受容活性を測定した。

アミノアシルステムを変換したtRNAはもとのtRNAと同じ活性を示したが、TΨループ, Dループを変換した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、ループを拡大したtRNAについてはその付加塩基と位置により親和性に差がみら

れた。図4にその時のLineweaver－Burkプロットを示す。

アンチコドンの3'側と5'側で塩基のこみ具合が異なるために5'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'側への付加や2塩基付加では弱くなると考えられる。又，アンチコドンループを拡大したtRNAについてリボソーム上でどのmRNA（トリマー）と結合するかを調べたが，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。

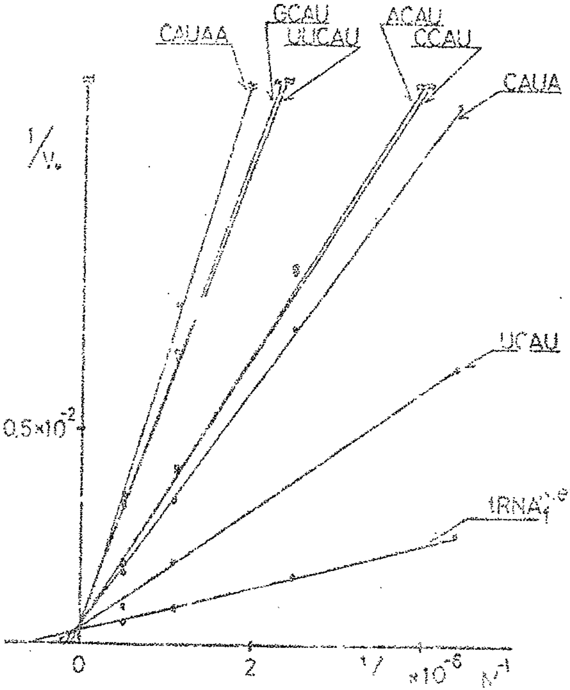


図4 テトラマー，ペンタマーはアンチコドンCAUをそれぞれの塩基配列に置換したtRNA$_f^{Met}$を示す。

2) EF-Tu との相互作用

アミノアシル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 に結合しないのに対し，合成した tRNA は一部複合体を形成することがわかった。

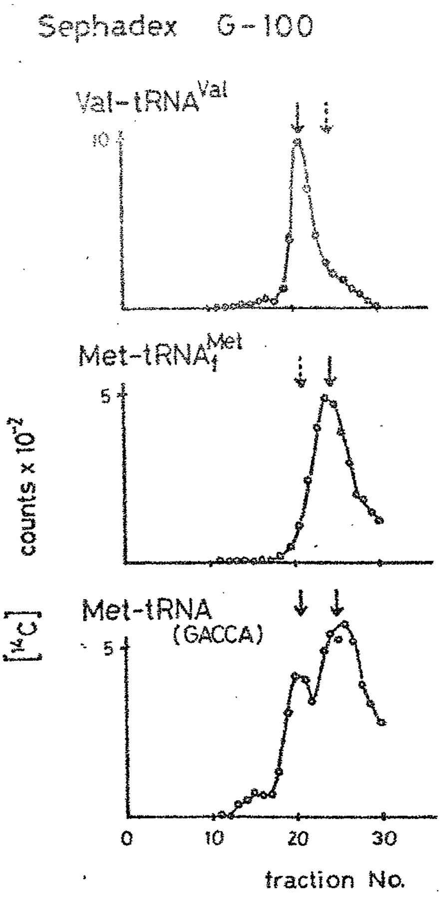


図5

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

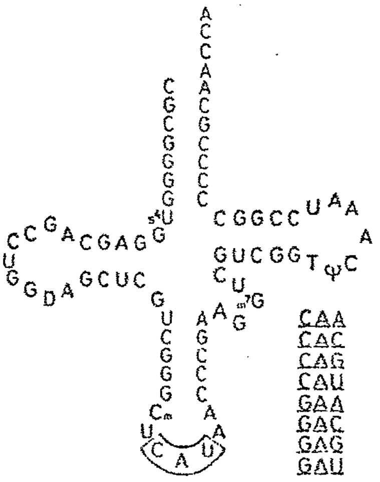


図6

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のみAがt^6A（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* $tRNA_f^{Met}$のアミノアシルステム，TΨ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TΨ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チオニンを受容した。

3. *E. coli* tRNA$_f^{Met}$のアミノアシル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	pseuduridine
s^4U	4-thiouridine
Cm	2'-O-methylcytidine
m^7G	7-methylguanosine
t^6A	N-[9-(β-D-ribofuranosyl) purin -6-ylcarbamoyl]-threonine
i^6A	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
*p	^{32}p
PEP	paper electrophoresis
PAGE	polyacrylamide slab gel electrophoresis
PAGE(disk)	polyacrylamide disk gel electrophoresis

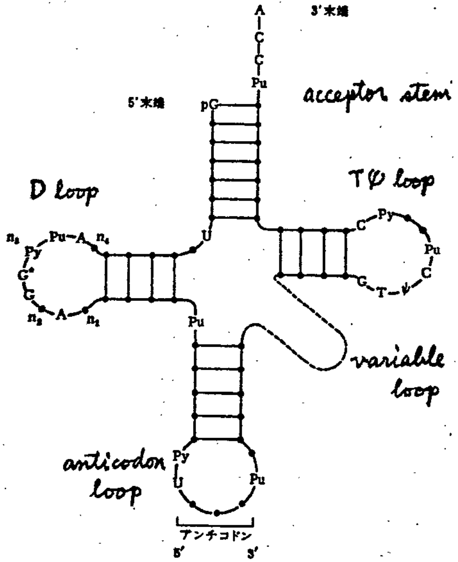
目次

本論

緒　論

近年のDNA合成技術の進歩はめざましく，E.coli $tRNA^{Tyr}_{su^+}$ の遺伝子が合成[1]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。Hurwitz等によりT4感染大腸菌から発見されたRNA ligase[2]は一本鎖オリゴヌクレオチドに作用し，5'末端リン酸基と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は得られない長鎖のRNAの合成が可能となる。

tRNAは鎖長70～80の機能を有するRNAとしては最小のもので人工合成の標的として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]，$tRNA^{Met}_{f}$[7]，E.coliでは $tRNA^{Met}_{f}$[8]の3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た。



[図1] tRNAのクローバーリーフ型二次構造

生体においてtRNAはmRNA上の情報に従いアミノ酸を運搬しタ

ンパク質合成を行なわせるが，特定の tRNA が特定のアミノ酸を受容し mRNA の情報どおりタンパク質が合成される機構は興味深い。tRNA にアミノ酸を受容させるアミノアシル 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]がなされているが今だに認識部位等に関して不明な点が多い。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いる[10]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し難い。

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 反応にはかなりの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 TΨループ，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

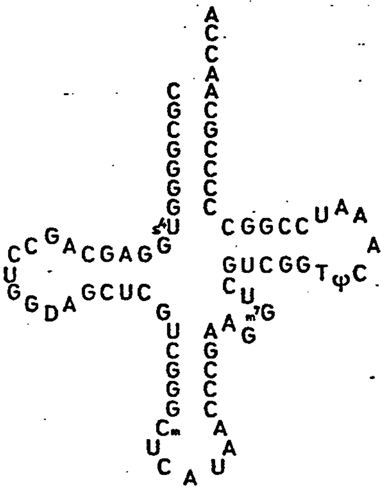
し，特にそのアンチコドンのCAUという配列が認識に重要であること，又 アンチコドン部のみならずtRNA全体の3次構造が認識に必要であることがわかった。

さらにアンチコドン部を変換したtRNAについては，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な系におけるtRNAの安定性と修飾について今まで知られていなかった新しい事実を得ることができた。

本論

第一章 化学合成 nascent E. coli tRNA$_f^{Met}$ フラグメントの RNA ligase による結合反応

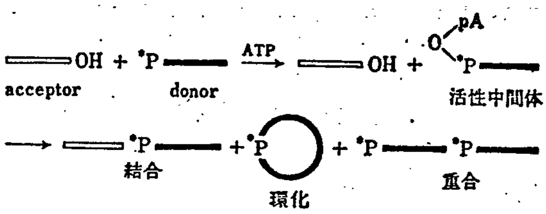
tRNA$_f^{Met}$（図2）は種々のアミノ酸に対応するtRNAの中でもタンパク質合成の開始に関与する特別な分子で種々のタンパク質から認識を受ける。このtRNAを人工的に合成することは核酸とタンパク質との相互作用を研究する上で意義があり，又任意の長鎖RNAが合成可能であることの証明となる。



[図2] E. coli tRNA$_f^{Met}$

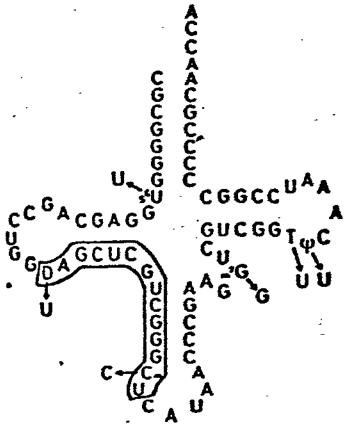
化学的にRNA鎖を伸長する場合，鎖長が10を越えると合成がかなり困難となる。tRNAの様な長鎖RNAの合成の場合比較的長鎖の化学合成フラグメントを用いそれらをRNA ligaseで結合する方法が適当であると考えられる。

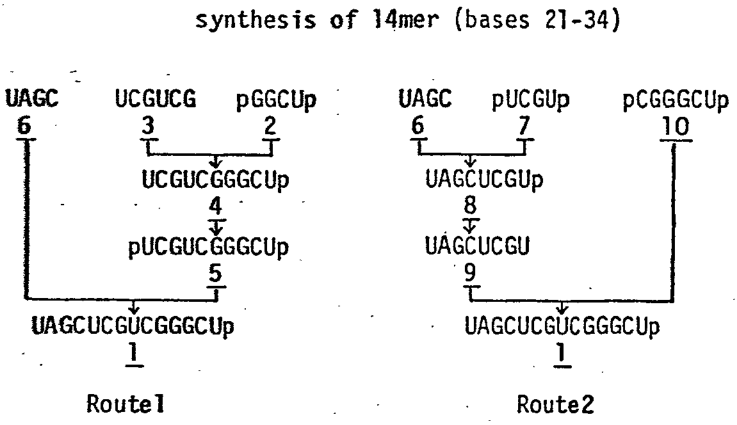
著者は図3の様な反応を触媒するRNA ligaseを用い比較的短鎖の化学合成フラグメントを出発原料にし結合を繰り返して，長鎖RNA分子を合成することを試みた。



［図3］ RNA リガーゼの反応

第一節 Dループ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ループからアンチコドン部位に至る14merを図5のような2種類のルートを用いて合成した。

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

1) ルート1による方法

2はGGCUpをkination（P.N. Kinaseによる5'末端リン酸化反応：特に記述しない限り[γ-^{32}P]-ATPを用いリン酸化した。）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った。20% polyacrylamide disk gel electrophoresis (PAGE(disk))

で単離し，3からの収率が4%であった。4はkination後Sephadex G-50で精製し76%の収率で5を得た後6とligationした。このligation反応液はそのまま次のkinationに使用し，20% PAGE (disk)でpUAGCUCGUCGGGCUpを単離した。収率はligation, kinationを通して11%であった。得られた14merのligation stepにおける結合位置が正しいことは，nearest neighbor analysis（後述）を行ない確認した。

2) ルート2による方法

7, 10はそれぞれUCGUp, CGGGCUpをkinationし，7はイオン交換カラムクロマトグラフィー，10はゲル濾過で精製したフラグメントで，ルート1の場合とは異なる結合位置を有するように設計した。

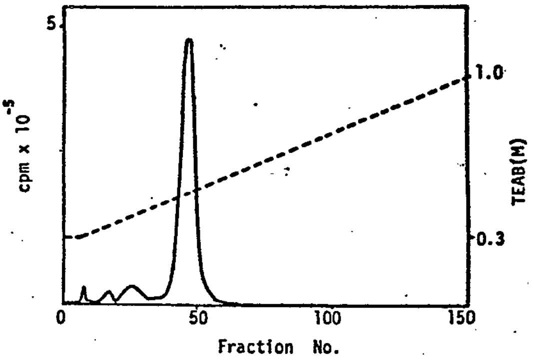


図6 UAGCUCGUp(8)の精製

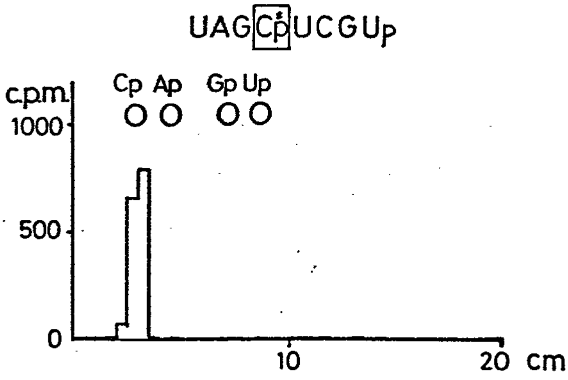
まず6と7をligationし，DEAE celluloseイオン交換カラムクロマトグラフィーで単離し（図6），8を好収率で得た。8はnearest neighbor analysis（ligation productをRNase T$_2$で完全分解した後pH 3.5の条件下PEPを行ない^{32}Pがdonor ^{32}pUCGUpからacceptor UAGCのCに移りC^{32}pとして検出され結合部位が正しいことが確認できる方法）により結合を確かめた。（図7）

8はBAP処理により3'位のリン酸基を除去し，7から通算71%の収率で9を得た。9は10とligationし20% PAGE (disk)により14mer (1)を9から31%の収率で単離した。（図8）

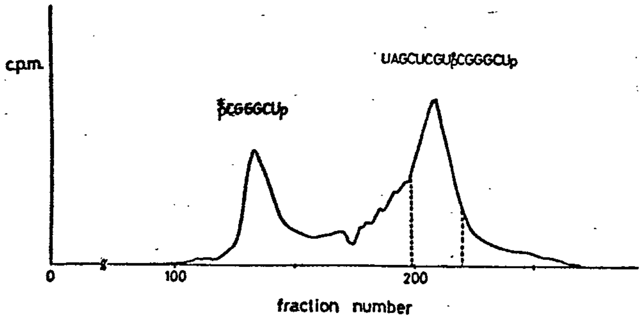
Nearest neighbor analysis で結合部位を, polyacrylamide slab gel electrophoresis (PAGE) で鎖長をそれぞれ確認した。

以上のように2つのルートで14mer を合成したが, ligation について条件と収率を表1にまとめた。

同じ14mer を合成する場合においてもフラグメントの組みあわせで収率が大きく異なる。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 nnol [µM]	ATP µM	RNA ligase [unit/ml]	time hr	Reaction extent$^{a)}$ %	Yield %
UCGUCG (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.

[表1] 14mer合成における ligation の条件と単離収率

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。Acceptor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)ープリンヌクレオシド(Pu)(ex. C−G)の順に並ぶとligationの収率が悪い。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。

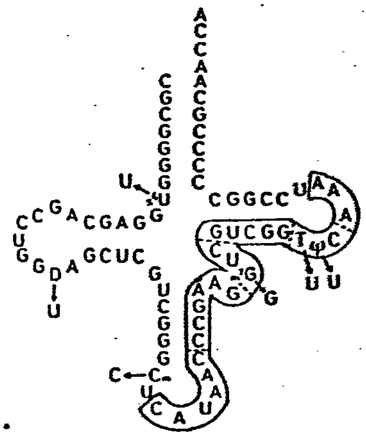
以上のことからacceptor分子の3′末端ヌクレオシドのconformationはligationに大きく影響を及ぼすと推論できる。

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分子がた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。

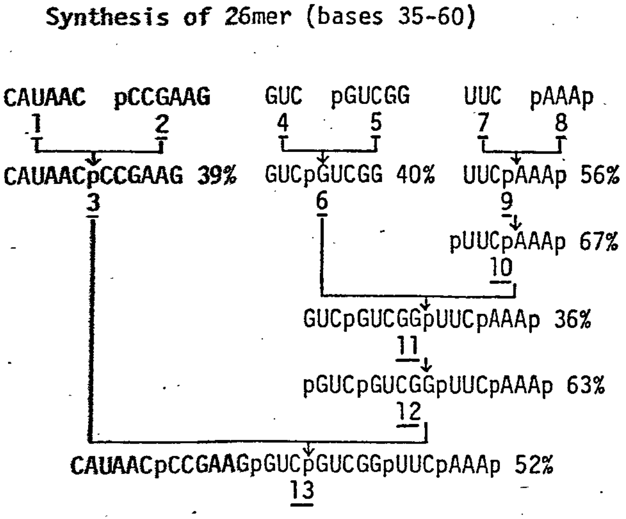
UCGUCG + pGGCUpというligationの場合，acceptor分子の構造のみならずdonor分子についても自己相補的なsequenceであるため反応が進行し難く4%という低収率であった。

第二節 アンチコドン部位からTΨ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TΨ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 $\underline{1}+\underline{2}$，$\underline{4}+\underline{5}$ 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く，副反応のdonor分子の重



［図9］ 26merの合成

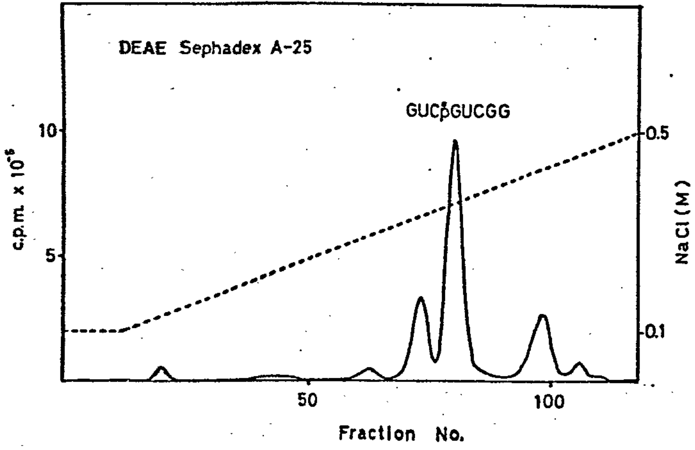


［図10］ 26merの合成経路

合化を防ぐためにそれぞれacceptorをdonorの10倍，20倍と過剰に用いた。1＋2では反応後20%PAGE(disk)により3を，4＋5では7M urea存在下45℃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donor分子が結合した副生成物のピークが見られる。

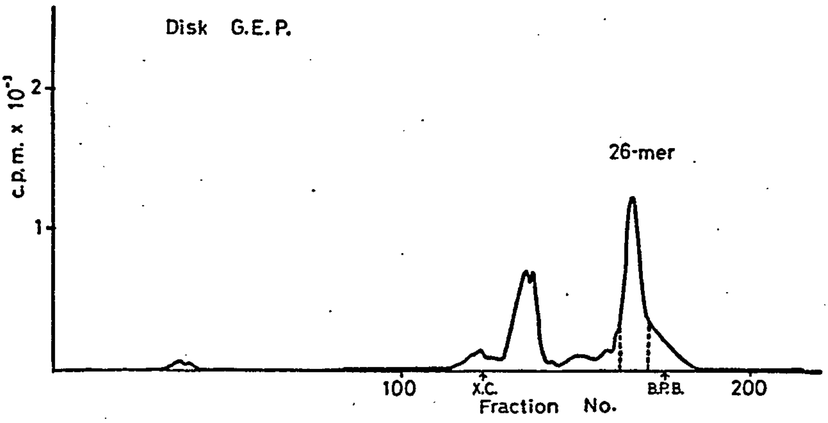
7＋8は収率良く反応が進み，イオン交換カラムクロマトグラフィーにより9を単離した。

9はkination後再びイオン交換カラムクロマトグラフィーを行ない定量的に10



［図11］ GUCGUCGG(6)の精製

を得た。 6 + 10 の ligation, 11 の kination, 3 + 12 の ligation も常法どおり行ない，反応後それぞれ urea 存在下イオン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。



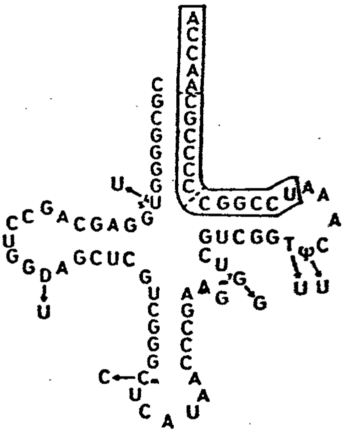
［図12］ 26mer の精製

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。

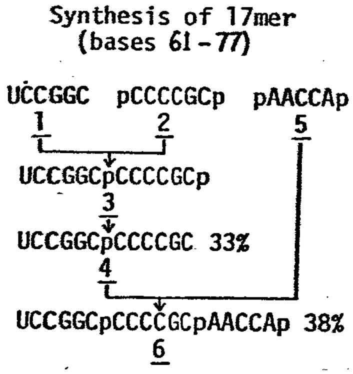
今回 3′末端を保護していない donor 分子を用いて ligation を試みたが acceptor 分子を大過剰用い反応すれば副反応をほとんどおさえ ligation を行なえることがわかった。

第三節 TΨ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13)，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 + CCCCGC + 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



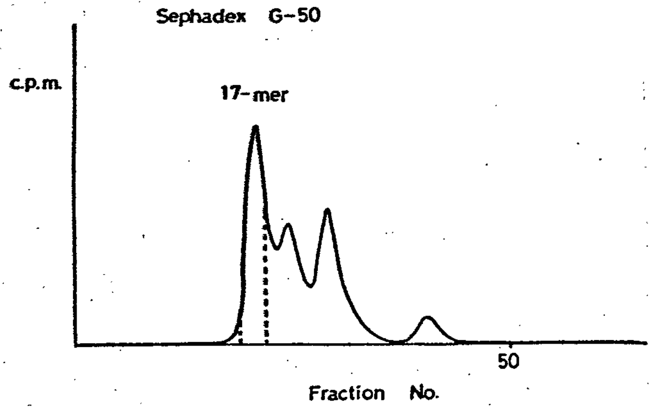
［図14］17merの合成経路

は nearest neighbor analysis で確認した。

2, 5 はそれぞれ CCCCGCp, AACCAp を kination し定量的に得られた。1 + 2 の ligation では 1 を 2 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 1 を 2 の2.4倍しか過剰に用いられずホモクロマトグラフィー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 2 のピークがはっきり分離せず、1回目の分離で純粋な 3 の部分のみを集め残りのピーク部分を再度 Sephadex G-50で分離する操作を行なった。3 はBAP処理により3'末端リン酸基を除去し、2 より通算33％の収率で 4 を得た。4 は 5 と ligation により結合し Sephadex G-50で単離し 38％の収率で 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

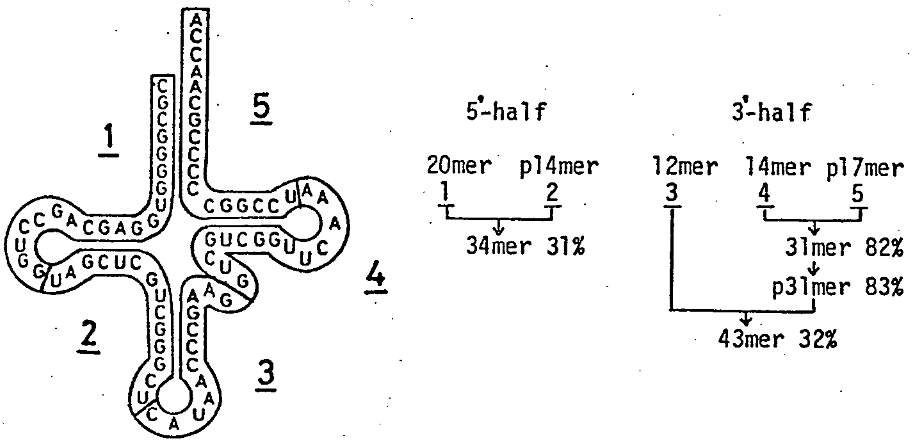


［図15］17merの精製

で生成物17merを原料の12mer, 5merより単離できた。

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（ただし末端のリン酸残基は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している分子同士では一度では分離できないかあるいは分離が不可能であることがわかった。しかし回収率に関してはこのゲル濾過法が最良と考えられる。なお、1＋2のligationにお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%の収率で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に示す様に5'半分子は20mer＋14mer、3'半分子は12mer

+ (14mer + 17mer) の ligation により それぞれ合成した。

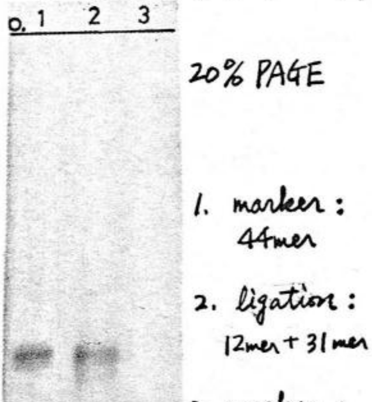
1) 5'半分子の合成

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) は第一節で合成した14merをkination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しては好ましいが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を加え末端のリン酸基を除去した後（全分子合成のacceptorとするため）nitrilotriacetic acid (NTA) を加え（最終濃度を5mMに調整）酵素を失活させ20% PAGEで分離した。生成物はゲルより抽出し31%の収率で5'半分子を得た。

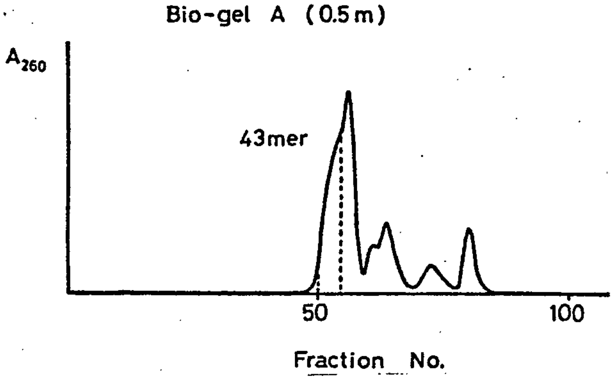
2) 3'半分子の合成

3の12merと4の14merは第二節で合成したフラグメントで14merは図10の11をBAP処理し調整した。5のp17merは第三節で合成した17merをkinationし調整した。

まず4+5のligationを行ない，Sephadex G-50で単離し82%の好収率で31merを得た。次にこの31merをkinationしSephadex G-50でATPを分離した後3とligationした。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43merと



[図17] 12mer + 31mer



[図18] 43merの精製

原料 p31mer の分離を試みた。(図18) しかし これらの 2つの ピークが 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'半分子としては p31mer から 32%の収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 kination を行ない Sephadex G-200 で精製し この操作でほとんど混在している 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った。これは RNA ligase の逆反応15)が原因と考えられた。RNA ligase の逆反応は基質に対し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生じていず 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'末端が修復される16)ため このまま次の反応に使用できた。

第二章　TΨループを変換したE. coli $tRNA_f^{Met}$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への結合に関与しているという報告17)もあり興味ある領域といえる。

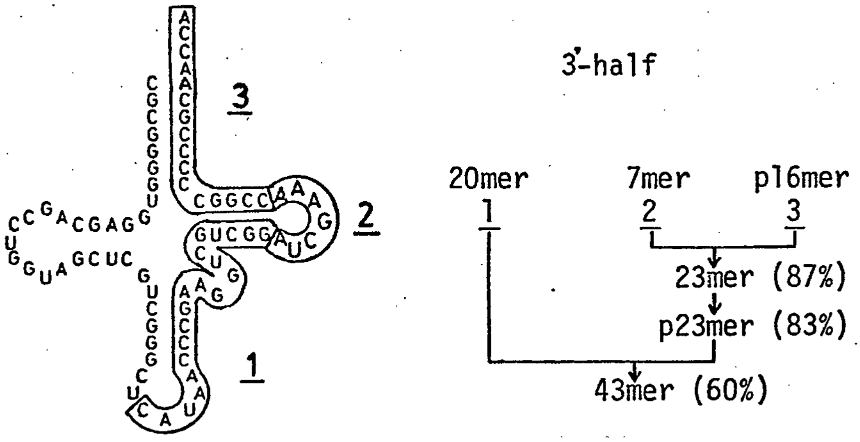
著者はE. coli $tRNA_f^{Met}$のTΨループを共通塩基配列TΨCから真核生物のinitiator tRNAに特異的に存在するAUCGへ変換することを試みた。

第一節　TΨループにeukaryotic sequenceを有するE. coli $tRNA_f^{Met}$の合成18)

E. coli $tRNA_f^{Met}$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AUCGm^1AAAであるが化学合成の都合上AUCGAAAとした）に変換した。

1)　3′半分子（43mer）の合成

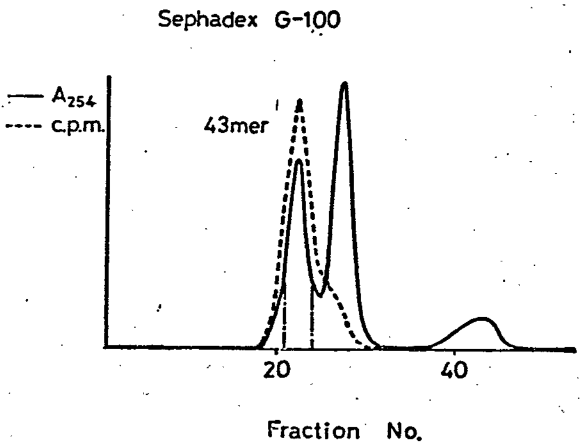
図19に示す様に化学合成フラグメント<u>1</u>, <u>2</u>, <u>3</u>より3′半分子を合成した。<u>3</u>は16merをkinationによりリン酸化しp16-merとした分子ではじめにTΨループsequence <u>2</u>とligationした。反応後Sephadex G-50で分離し87％の好収率で23mer



［図19］ 3'半分子の合成

を得た。次に23merをkinationし p23merとした後 20mer（1）とligationした。図20に示す様に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の進行を調べた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もkination, ligationをくり返したが，今回の様に長鎖化学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，

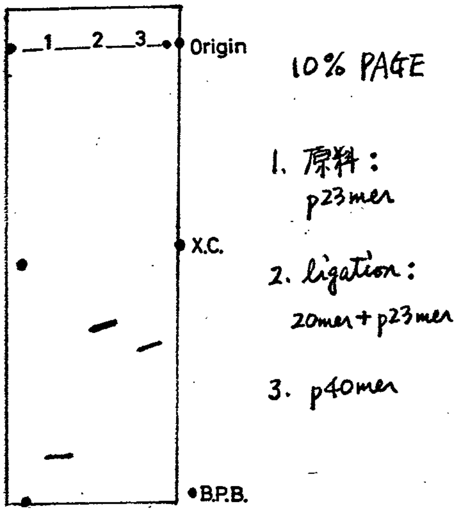


［図20］ 3'半分子の精製

化学合成の立場からは 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。)

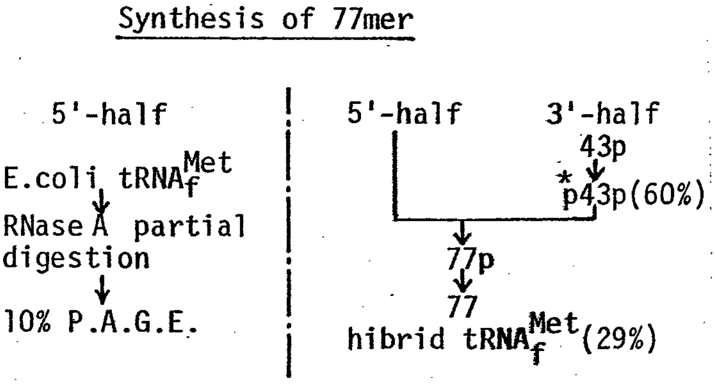
2) 全分子の合成

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

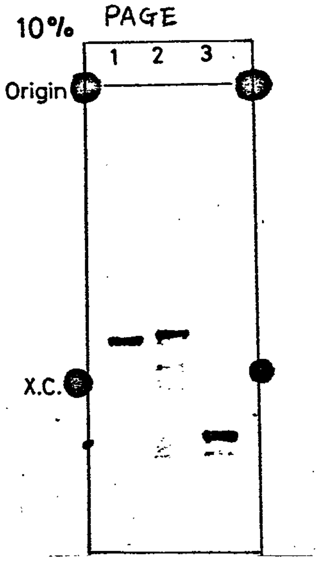


[図21] 20mer + p23mer

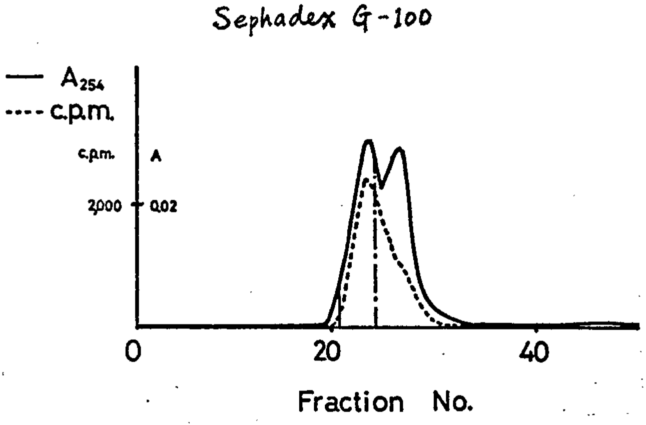
Donor分子については 1)で述べた3′半分子を kination し Sephadex G-50 を用い ATPから分離することにより調整した。半分子同士の ligation は acceptor を donor の約2倍過剰に用いて反応し, 10% PAGE で反応の進行を確認した。(図23) 反応混合物をフェノール処理で除タンパクし, Sephadex G-100 で脱塩した後 BAP処理を行なった。



[図22] 全分子の合成



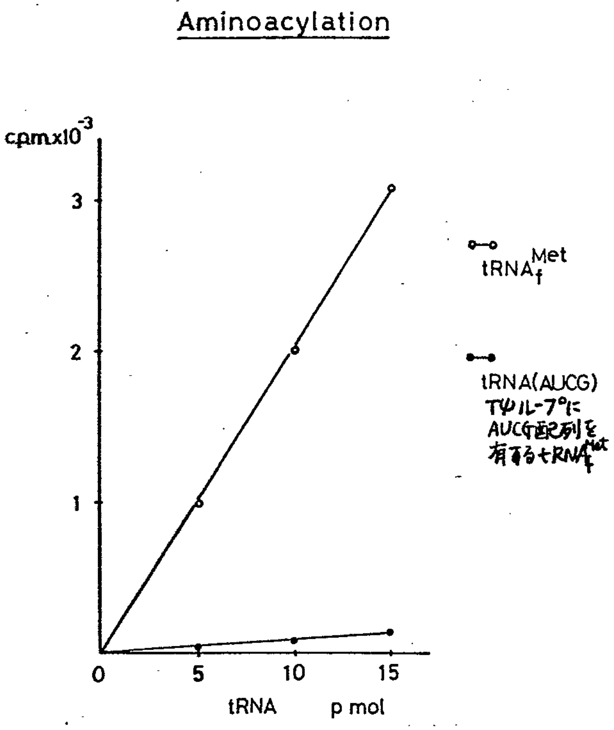
[図23] 5'半分子 + 3'半分子
1. marker : 75mer
2. ligation : 34mer + p43mer
3. 原料 : p43mer



[図24] 全分子の精製

BAP処理後 再びフェノ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ピークに分かれたが集めたフラクションのチェックを10% PAGEで行なったところ若干の短鎖生成物が混入していた。その比率の測定から9.3%の収率で目的の全分子を得たことがわかった。Nearest neighbor analysisによる結合部位の確認においてもCmUpが2次元TLC[21]により検出され半分子同士が結合していることがわかった。

次にこの分子のメチオニン受容活性を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[^{14}C]-メチオニンのうちtRNAと結合したメチオニンのみがフィ



［図25］メチオニン受容活性

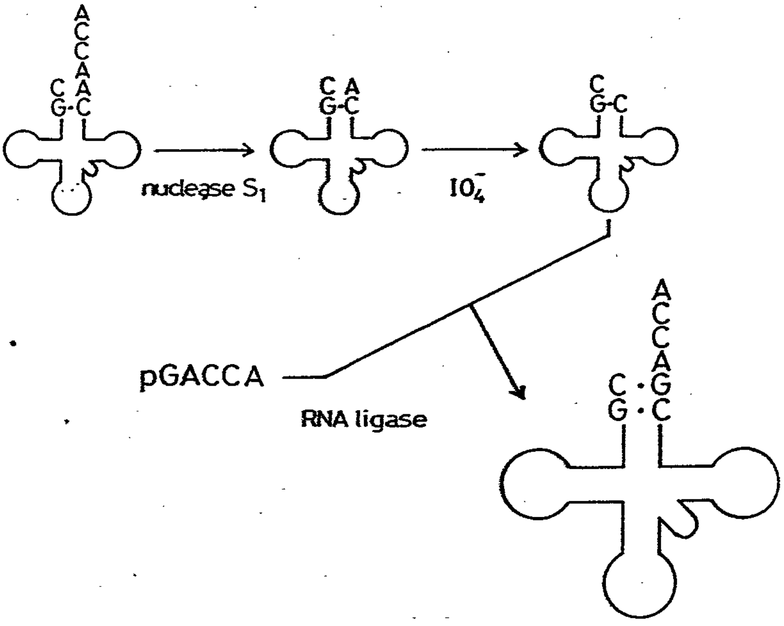
ルター上に残る。この放射能をアミノ酸受容活性として測定した。合成したtRNAは図25に示す様にほとんどメチオニンを受容できず天然のE. coli tRNA$_f^{Met}$に比べ4%の活性しか示さなかった。これはこのtRNAが安定な三次構造をとれないためにこのような結果が生じたと思われる。Dループと塩基対を形成すると考えられるTψループ（TψCAAAU）のψC配列はこの合成tRNAではUCに変換されているがこの配列でもDループと塩基対を組むことができる。しかしTψループ内でのT-A塩基対（TψCAAAU）は合成tRNAの場合（AUCGAAA）では組めない。この様な3次構造上の相異がtRNAを不安定化しMet RSaseとの相互作用を弱めたと考えられる。又この3′半分子の可変ループのm^{7}G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しれない。

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らはこのことを確かめるために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ない。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25)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G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$_f^{Met}$ の合成

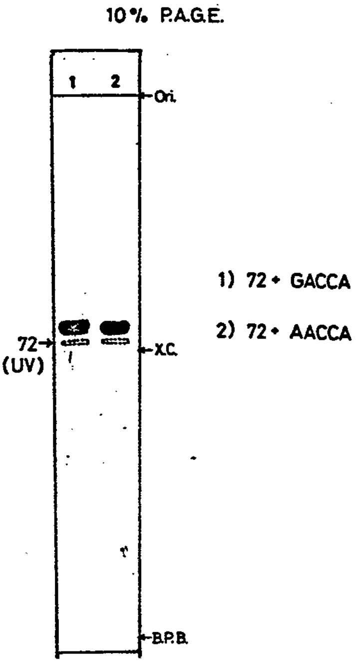


[図26] 5'末端に塩基対を形成する tRNA$_f^{Met}$ の合成

E. coli tRNA$_f^{Met}$ を立体構造を保持させたまま nuclease S1 で限定分解を行なうとアンチコドンループでの切断をおさ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させると 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 $NaIO_4$ 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 $NaIO_4$ 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 に調整したリジン溶液を用い遮光下 室温(約 20°C), 3.5 時間反応し

β脱離をおこした。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'末端分析を行なったが 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 で単離し次の 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)



[図27] 72mer + 5mer

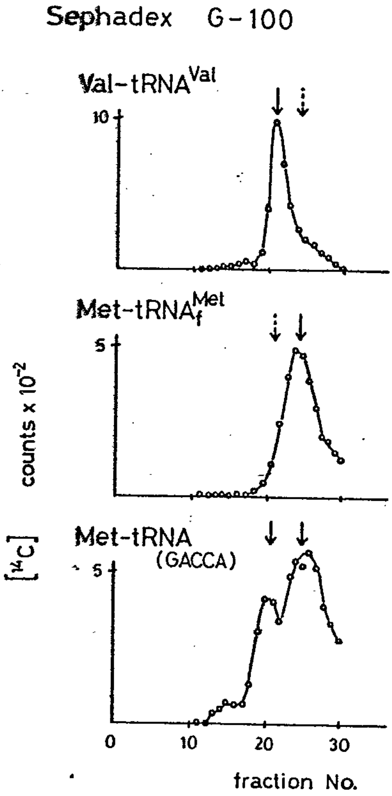
Nearest neighbor analysis で結合部位を確認した後 アミノアシル化反応を行なったが、塩基対を組むように GACCA に変換した tRNA も もとの配列を有する AACCA と同じメチオニン受容活性を示した。このことから $tRNA_f^{Met}$ と Met RSase との相互作用には この位置の塩基対は影響しない事がわかった。

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'末端をリン酸化し Sephadex G-50 で単離した後 [^{14}C]-メチオニンを用いてアミノアシル化を行なった。
[^{14}C]-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}を[^{14}C]-Val-tRNA^{Val}として，塩基対を組んでいない E.coli tRNA$_f^{Met}$を[^{14}C]-Met-tRNA$_f^{Met}$としてそれぞれ用いた。E.coli から精製された EF-Tu を用い[^{3}H]GTP binding により EF-Tu の活性を測定した。



[図28] アミノアシルtRNAと EF-Tu・GTPの相互作用

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°C，5分間のインキュベートで行なった。反応終了後 低温(4°C)下 Sephadex G-100 によるゲル濾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そ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$_f^{Met}$ 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$_f^{Met}$ (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

なかったが 塩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った。

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

以上のことからA-U塩基対であれ G-C塩基対であれ 5'末端での塩基対は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った。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しかちがわない28),29)ということから この塩基対形成はEF-Tuとの相互作用に対し本質的ではないかもしれない。

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'末端の塩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

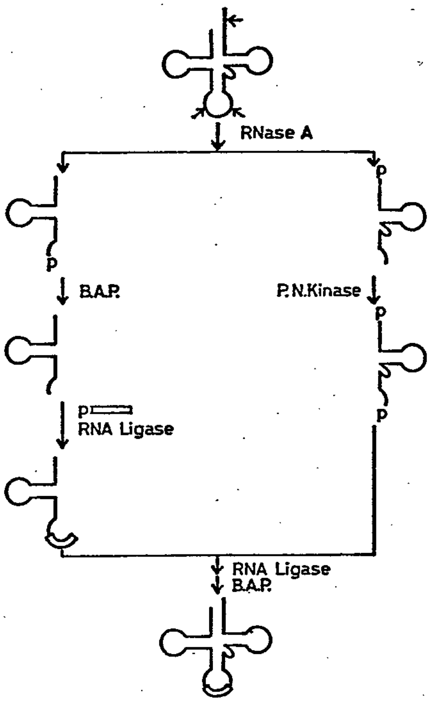
mRNA の情報を読みとる tRNA のアンチコドン部は tRNA が機能するための不可欠な領域であり tRNA 研究上の興味ある標的といえる。mRNA と塩基対を組むアンチコドントリプレットは tRNA の種類により異なるが，アンチコドンの 5′側隣接位はほとんどすべて U であり 3′側隣接位は Pu 又は Pu 誘導体がその位置を占める。E.coli tRNA$_f^{Met}$ の場合 アンチコドンの 5′側隣接位は一般的な U であるが 3′側隣接位に関しては修飾されていない A であり，これはアンチコドンの 3字目が U の tRNA ではこの位置が修飾されているのに比べ例外的であ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

アンチコドン トリプレットに関して天然に存在するサプレッサー 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$_f^{Met}$ をサプレッサー 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 人工サプレッサー 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 その認識のされ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た 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い検討を行なった。

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

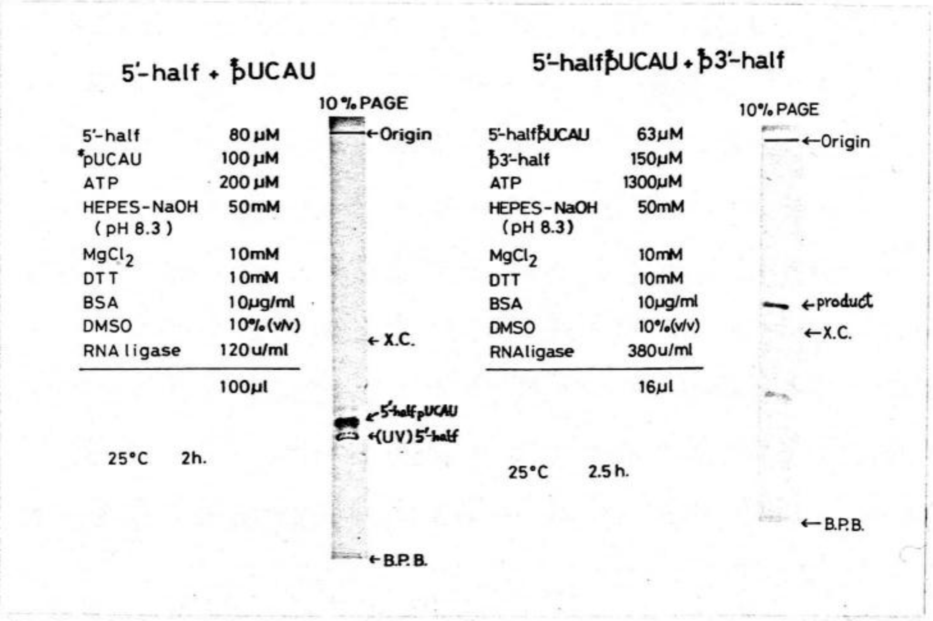
E. coli tRNA$_f^{Met}$ の場合 そのアンチコドントリプレットが他の配列に変化するとメチオニンを受容しなくなる。20) そこで著者は CAU というトリプレットは含んでいるが拡大されたアンチコドンループを有する tRNA$_f^{Met}$ を合成し，この tRNA が E. coli Met RSase にいかに認識されるかを調べた。

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 により限定分解し 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 処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, CAUAA）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 kination 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した。10% PAGEで単離し，酸処理(0.1N 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の欠除した目的の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によりCCAに修復される。

図30に一例としてCAUをUCAUに変換した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した。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)



5'-half + $\overset{*}{p}$UCAU

5'-half	80 µM
*pUCAU	100 µM
ATP	200 µM
HEPES-NaOH (pH 8.3)	50mM
$MgCl_2$	10mM
DTT	10mM
BSA	10µg/ml
DMSO	10% (v/v)
RNA ligase	120 u/ml
	100µl

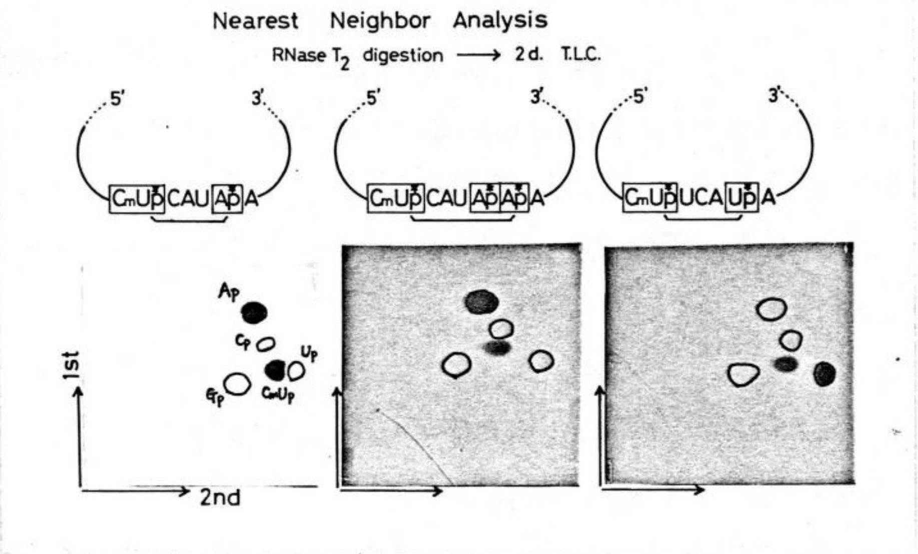
25°C 2h.

5'-half$\overset{*}{p}$UCAU + $\overset{*}{p}$3'-half

5'-half$\overset{*}{p}$UCAU	63µM
$\overset{*}{p}$3'-half	150µM
ATP	1300µM
HEPES-NaOH (pH 8.3)	50mM
$MgCl_2$	10mM
DTT	10mM
BSA	10µg/ml
DMSO	10% (v/v)
RNAligase	380u/ml
	16µl

25°C 2.5 h.

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



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

5'側半分子と合成オリゴマーとの ligation では収率はあまり良くないが（単離収率 11〜25%）, 半分子同士の ligation では収率良く進んだ。（単離収率 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'末端がリン酸基で保護されていず 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 が良く進行した事とが考えられる。

当初 この tRNA の合成順序として合成オリゴマーをまず 3'側半分子と結合させることを計画したが, 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)リボソーム上での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$_f^{Met}$ に比べ UCAU配列を有するtRNA（tRNA(UCAU)）では 53%, tRNA(CAUA)では 25%, tRNA(CAUAA)では 13%の活性を示した。

次に図32に示す様にKm, Vmax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、一定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た。図32の右下に Lineweaver-Burk プロットを示したが、このグラフから Vmax 値はいずれもほぼ同じであるのに対し Km 値が tRNA$_f^{Met}$, tRNA(UCAU), tRNA(CAUA), tRNA-(CAUAA) の順に増大

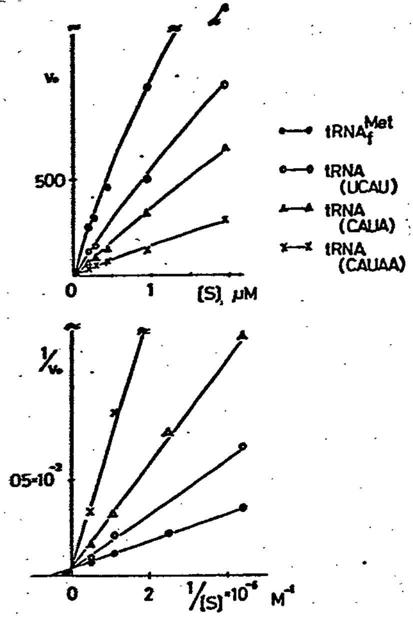
Aminoacylation

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
β-ME	10 mM
S-100	1 mg/ml

↓ 37°C, 30 min.
↓ 100°C, 2 min.

[^{14}C]-Met	18 μM
S-100	0.5 mg/ml
	10 μl

↓ 37°C, 20 min.



［図32］アンチコドンに付加塩基を有するtRNAのaminoacylation (Km, Vmax study)

し, Met RSase との親和性がこの順に減少している事がわかった。Km 値はそれぞれ 1.7 μM, 3.3 μM, 6.7 μM, 12.5 μM であった。

次にこれらの 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についてもアミノアシル tRNA は検出できなかった。

2) ホルミル化反応

Transformylase[32] 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っていない。

合成した3種の tRNA について 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 folinic 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った Met-tRNA を deacyl 化した。硫酸銅で処理したフラクションとしなかっ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) → 93%, tRNA (CAUA) → 88%, tRNA (CAUAA) → 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った。

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 について, 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] に基づき行なった。(図33) mRNA として合成オリゴマー AUG, UGA, UUA, UAU を用いリボソーム上での特異

tRNA-mRNA interaction

tRNA	0.5μM
mRNA	25 μM
Tris-HCl(pH7.5)	0.1 M
$Mg(OAc)_2$	20 mM
KCl	50 mM
E.coli ribosome 30S or 70S	1.8μM
	20 μl

25°C, 30min.
millipore filtration

mRNA	$tRNA_f^{Met}$	tRNA CAUA	tRNA CAUAA	tRNA UCAU
AUG	100	0	0	33
UGA	0	0	0	0
UUA	0	0	3	0
UAU	0	0	0	0

% relative to $tRNA_f^{Met}$-AUG

［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と結合しなかった。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であった。

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レッ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は付加塩基の存在によりトリプレット付近のコンホメーションが変化しmRNAと塩基対を形成することができなくなったと考えられる。

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はtRNAが認識を受けるにはアンチコドンの特定の立体構造とCAUという塩基配列が必要であることがわかった。CAUの5′側にUを付加したtRNAの方が3′側にAを付加したtRNAよりMet RSaseに対し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って機能を果たすために非常に重要であることがわかったが，一方ホルミル化

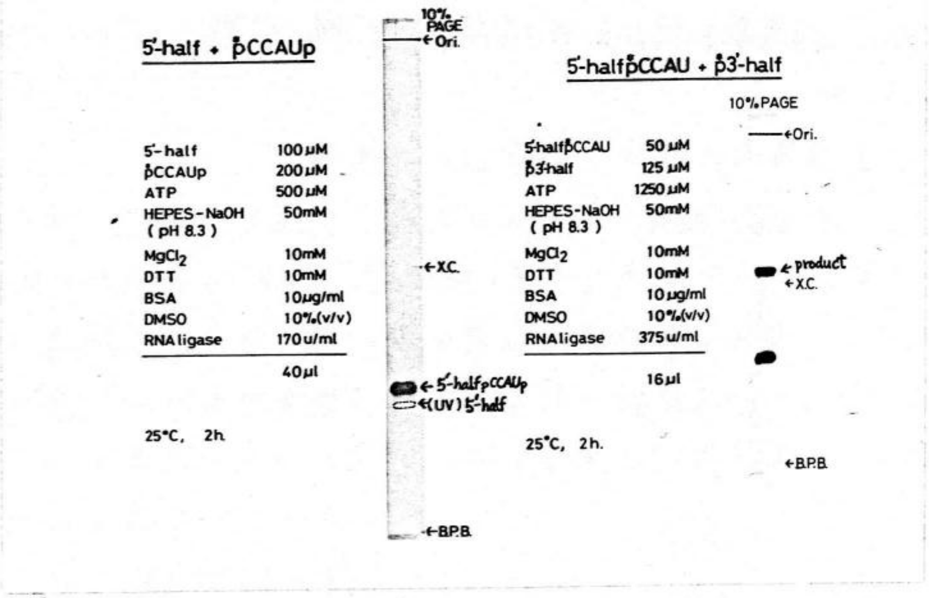
に関しては この部分は影響しないことも判明した。

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'側隣接位に付加塩基Uが存在してもアンチコドン付近のコンホ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った。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，Met RSaseによる認識はUの場合とどのように異なるかを調べた。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$_f^{Met}$ 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＋合成オリゴマーの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処理によりリン酸基を除去し次のligationのacceptorとした。



5'-half + $\overset{*}{p}$CCAUp

5'-half	100 μM
$\overset{*}{p}$CCAUp	200 μM
ATP	500 μM
HEPES-NaOH (pH 8.3)	50mM
$MgCl_2$	10mM
DTT	10mM
BSA	10μg/ml
DMSO	10%(v/v)
RNA ligase	170 u/ml
	40μl

25°C, 2h.

5'-half$\overset{*}{p}$CCAU + $\overset{*}{p}$3'-half

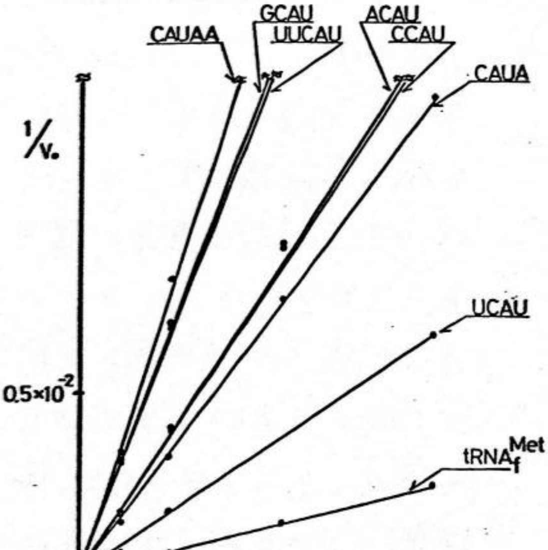
5'-half$\overset{*}{p}$CCAU	50 μM
$\overset{*}{p}$3'-half	125 μM
ATP	1250 μM
HEPES-NaOH (pH 8.3)	50mM
$MgCl_2$	10mM
DTT	10mM
BSA	10 μg/ml
DMSO	10%(v/v)
RNA ligase	375 u/ml
	16μl

25°C, 2h.

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

合成したtRNAについて第二節と同様にメチオニン受容活性を測定し，今までの拡大ループを有するtRNAとKm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 RSaseとの親和性が強く，他のC, A, Gの場合は

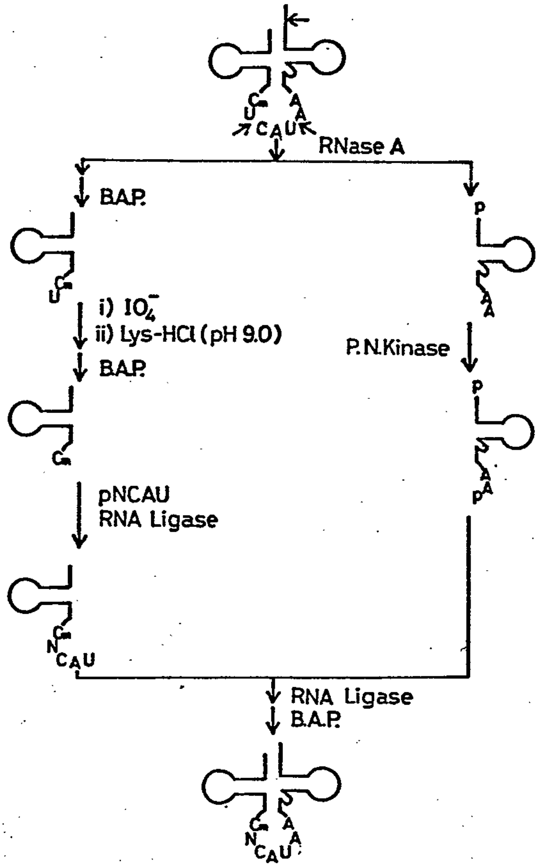


[図35] aminoacylation (Km, Vmax study), テトラマー，ペンタマーは アンチコドン CAUを それぞれのオリゴマーに変換したtRNAを示す。

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った。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った。

2) 5'側隣接位に存在する共通Uの変換

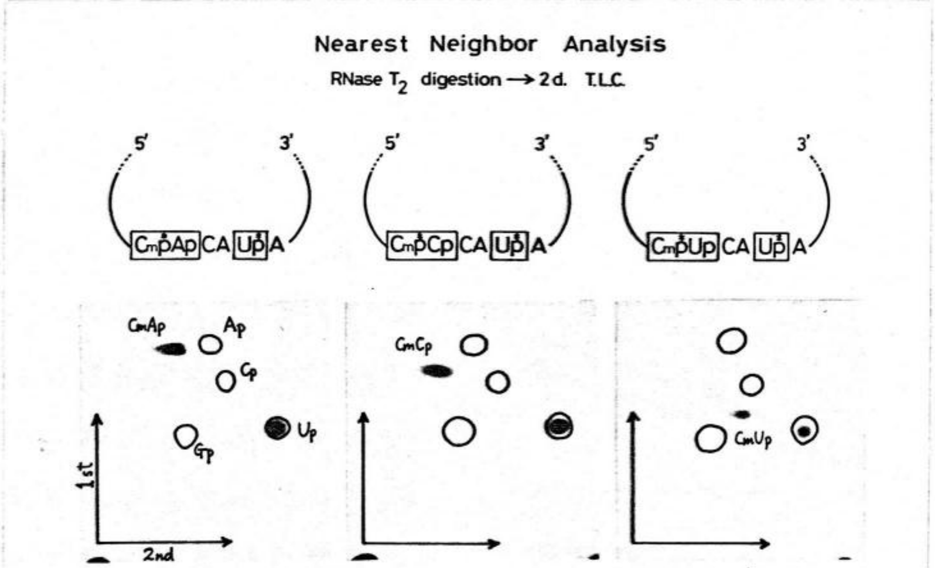
天然のE. coli tRNA$_f^{Met}$をRNase Aにより限定分解し，得られる5'側半分子をさらに過ヨウ素酸酸化し3'末端の共通Uの除去を行なった。過ヨウ素酸酸化は第三章で用いた方法と同様に行なった。図36に合成経路を示す。当初5'側半分子のみを過ヨウ素酸酸化し，経路に従い合成したが　得られたtRNAはコントロールとして合成した天然と同等の塩基配列を有するtRNAでさえも　そのメチオニン受容活性をほとんど示さなかった。これは5'側半分子単独の場合，tRNAの様に三次構造をとることができず修飾塩基等が障害を受けたためと考えられる。そこで過ヨウ素酸酸化の時に当量の3'側半分子を加え再構成させて（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みた。酸化後，BAP処理を行ない10% PAGE



［図36］共通U変換tRNAの合成経路

で3'末端の一塩基を除去した5'側半分子を単離した。3'末端分析を行ないU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kinationしイオン交換クロマトグラフィーで単離して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ン酸を開裂した3'側半分子はkination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処理を行ない目的のtRNAを得た。

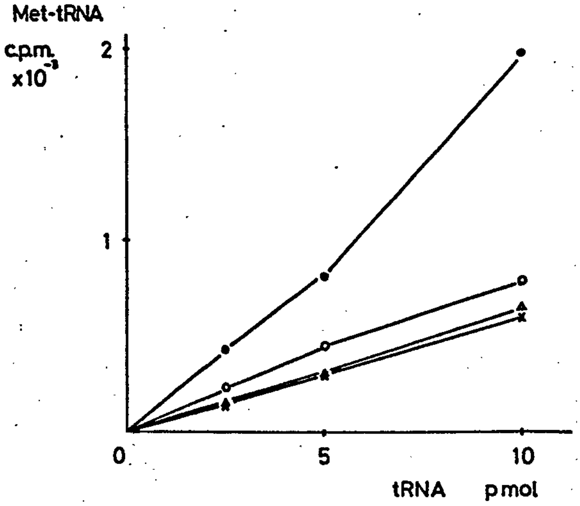
図37に全分子合成後のnearest neighbor analysisを示す。Donor分子の5'末端に位置していた^{32}Pがacceptor分子に移り、RNase T_2 digestionで□で囲んだモノマー、ダイマーが2次元TLCにより確認できた。最後のligationではいずれもCAUのU



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

由来の$U^{32}p$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)でそこにオリゴマーが結合するとRNase T_2ではCmの3'側では切断されずダイマー$Cm^{32}pNp$(N:A,C,U)で検出される。この$Cm^{32}pNp$は各々Nのちがいにより二次元TLCでその位置が異なる。この差異によりNを確認した。合成した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 $tRNA^{Tyr}$のアンチコドンとその5'側隣接位Uを変換したサプレッサー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した。この実験においてアミノアシル化の%が天然の$tRNA_f^{Met}$に比べ約半分しか回復していないが，これは過ヨウ素酸酸化の際にtRNAとしての三次構造が組めるように3'側半分子を加えたにもかかわらず完全に構造を回復できずに反応が進み



［図38］共通Uを変換したtRNAのaminoacylation
●–● $tRNA_f^{Met}$, ○–○ tRNA(CmU→CmU) control,
△–△ tRNA(CmU→CmC), ×–× tRNA(CmU→CmA)

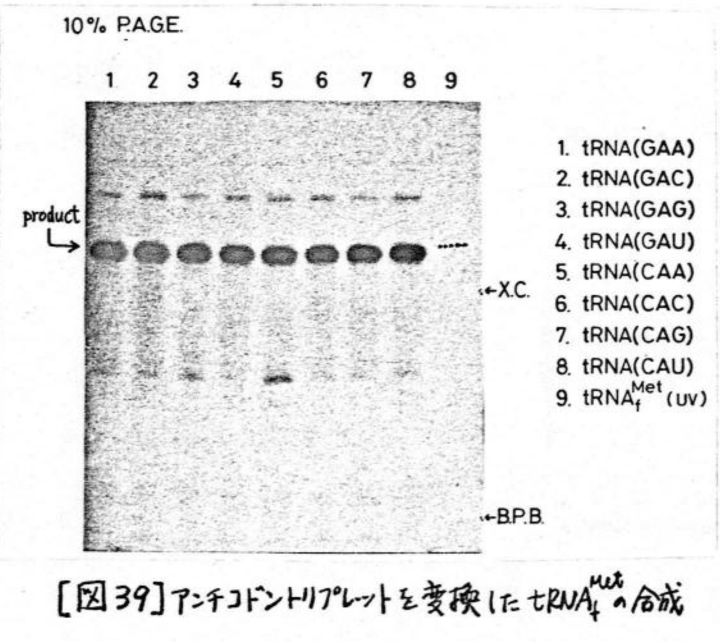
塩基に障害が生じたためと考えられる。

第四節 アンチコドントリプレットを種々の配列に変換した E. coli tRNA$_f^{Met}$ の合成

E. coli tRNA$_f^{Met}$ 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 中では修飾されない tRNA$_f^{Met}$ のこの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され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した 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$_f^{Met}$ の合成の場合と同様に行なった。各トリマーの kination は非放射性の ATP を用いて行ない, 3′側半分子の kination には比活性の高い [γ-^{32}P]-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 した。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サンプルあ

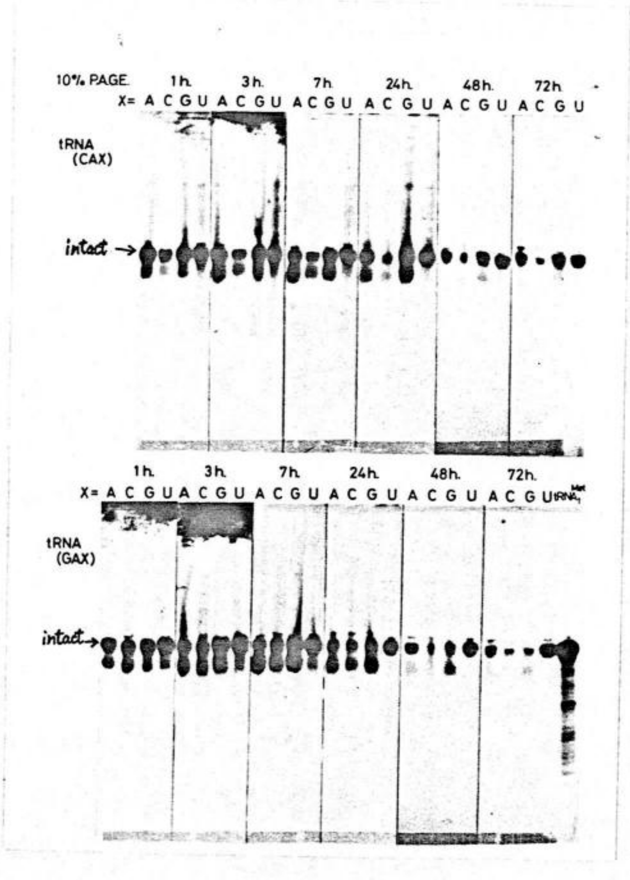


[図39] アンチコドントリプレットを変換した tRNA$_f^{Met}$ の合成

たり ^{32}P で 10^6 cpm 以上のカウントを有する tRNA を得た。

第五節 アンチコドン変換体の Xenopus laevis oocyte 中における生化学的性質

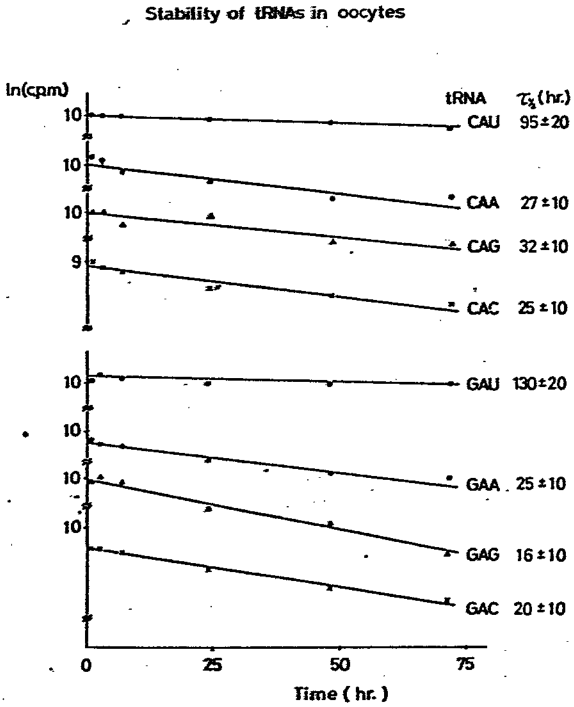
Xenopus laevis oocyte（アフリカツメガエル卵母細胞）は，mRNA の翻訳が非常に効率良く行なえる系36)として多くの研究者に利用されている。この系を用いると容易に in vivo での実験が行なえ，後処理も比較的簡単であるため著者はこの系を用い修飾の実験を行なった。



第四節で合成した8種の tRNA を Xenopus laevis oocyte の細胞質に microinjection した。一定時間おきに反応を止め oocyte を破砕し除タンパク後 10% PAGE に apply し tRNA の安定性を調べた。

図40にその泳動パターンを示す。過剰の核酸成分のため泳動が乱

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

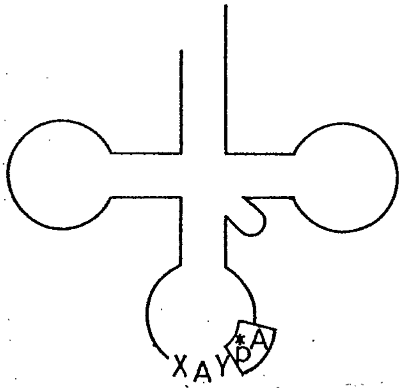


［図41］アンチコドン変換体のoocyte中における安定性

れているが，intact tRNAと3〜4塩基切断の生じたtRNAのバンドが検出された。

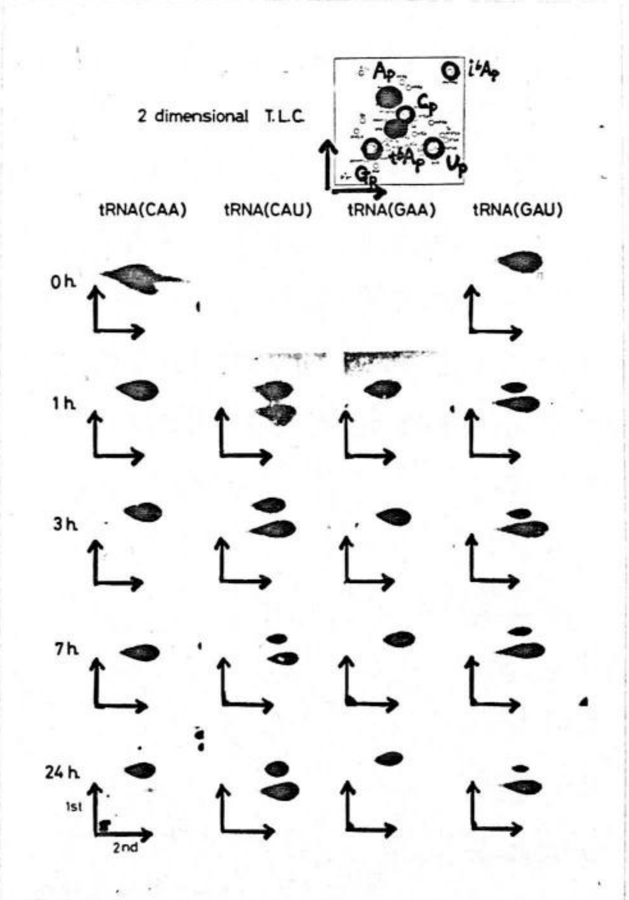
Intact tRNAのバンドを切り出しそのカウントを測定してこれらのtRNAsのoocyte中における安定性について調べた。（図41）

その結果アンチコドントリプレットが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時間であった。それに対し他のアンチコドントリプレットを有するtRNAはいずれも切断物が検出され$\tau_{1/2}$が20〜25±10時間と不安定であった。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（キャリアーとしてYeast tRNAを約0.2 A_{260} unit加えた。）を2回行ない脱塩した後，nuclease P_1による完全分解を行なった。反応後サンプルを2次元TLC上で展開し，^{32}pN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Aの修飾
X: C, G , Y: A, C, G, U

^{32}Pのラベルは^{32}pAもしくは^{32}pA*（A*は修飾されたA）としてTLC上に検出された。図43に2次元TLCの一部を示すが，Aの修飾が生じたのはtRNA(CAU)とtRNA(GAU)のみで他のtRNAに関しては何ら修飾を受けなかった。又tRNA(CAU)とtRNA(GAU)の場合 いずれもAはt^{6}Aに修飾を受けていた。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}Aに修飾を受けることから予想どおりの結果であった。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}Aなどに修飾を受けるが，このtRNA(CAA)とtRNA(GAA)の場合 全く修飾を受けなかった。これはi^{6}Aに修飾を受けるtRNAはアンチコドンステムの最もループ寄りの塩基対がA-Ψであるのに対しtRNA$_f^{Met}$の場合G-Cであるため修飾酵素に認識されなかったとも考えられる。



［図43］アンチコドンの3'側隣接位Aの同定

Xenopus laevis oocyte中でのtRNAの修飾に関してE. coli中ではアンチコドンの3'

側隣接位Aが修飾されない E.coli tRNA$_f^{Met}$でも 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t^6Aに修飾されることがわかった。Xenopus laevis oocyte中に存在するt^6A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t^6A修飾酵素の場合, tRNA$_f^{Met}$のどの部分を認識して修飾をしないのかは不明である。

次にXenopus laevis oocyte中のtRNAの安定性に関しては, 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してCAUとGAUを有するtRNA$_f^{Met}$のみが安定で 他の配列のtRNA$_f^{Met}$では不安定であった。これは修飾を受けたために安定になったとは考えられない。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たからである。

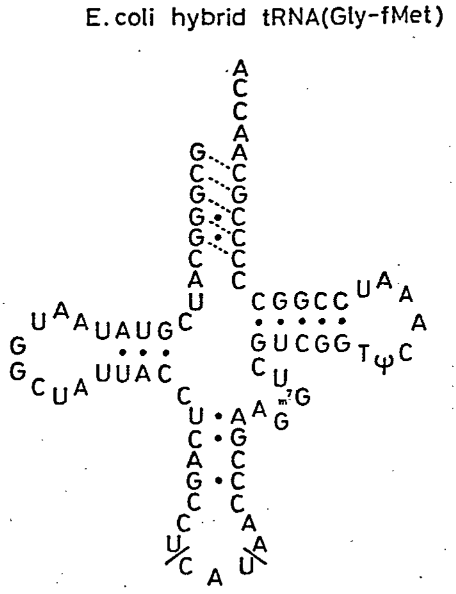
この場合, アンチコドンの3字目により安定性が規定されたが, なぜXenopus laevis oocyte中でE.coli tRNA$_f^{Met}$のこの位置がUの分子が安定で 他の分子が不安定であったかについては不明である。

第五章　5'側半分子を変換したE.coli tRNA$_f^{Met}$の合成37)

5'側半分子にE.coli tRNA$_2^{Gly}$ 38)a) の塩基配列を有するtRNA$_f^{Met}$ (tRNA(Gly-Met)) とミトコンドリア中のtRNA^{Ser} 38)b) などにみられるDループとステムの欠除した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 tRNA$_2^{Gly}$の塩基配列を有するE.coli tRNA$_f^{Met}$の合成

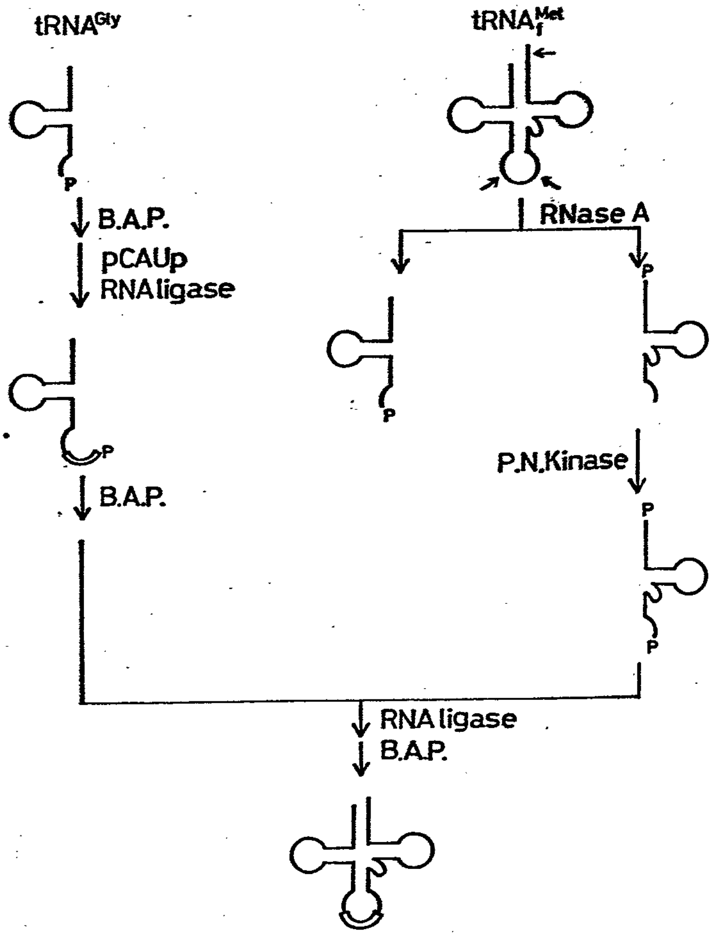
E.coli tRNA$_2^{Gly}$は修飾塩基が少なく5'側半分子には全く含まれていない。このことはこの分子を化学合成する上で非常に有利な点で，最近アンチコドンを含まないE.coli tRNA$_2^{Gly}$の5'側半分子（33mer）の化学合成が試みられ成功した。39)



[図44] tRNA(Gly-Met)

著者はこの合成されたtRNA$_2^{Gly}$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にpCAUpをligationにより結合し10% PAGEで分離した。BAP処理により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 $tRNA_f^{Met}$ より得られる分子を kination し調整した。

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 に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った。（第二節図49）又，グリシン受容活性についても調べたが活性を示さなかった。合成した tRNA は 3'末端 CCA の CA が欠除しているがアミノ酸受容活性を示さなかったのは CCA 修復が生じてい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った。修復反応を行なった後 [5'-^{32}P] pCp の single addition を行ない 10% PAGE で tRNA^{32}pCp を単離した。RNase T2 で完全分解し N^{32}p（N = A or C or G or U）を調べた結果，反応前で

は $\mathrm{C}^{32}\mathrm{p}$ が検出されたが 修復後 $\mathrm{A}^{32}\mathrm{p}$ に変化し 3'末端が修復されていることを確かめた。

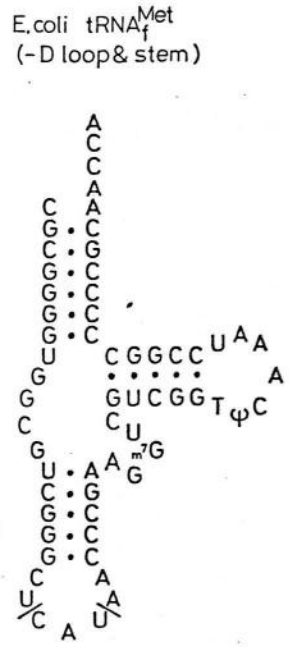
この hybrid tRNA は共通の二次構造に従って塩基対を配列するとアクセプターステムで二塩基対しか組むことができないが，図44の破線で示す様に一塩基ずらすと五塩基対組める。hybrid tRNA は安定な後者の構造を形成すると予想されるが全体の形が天然の tRNA_f^{Met} と異なるため このことが原因で ARSase に認識されなかったと考えられる。

第二節 Dループとステムの欠除した E.coli tRNA_f^{Met} の合成

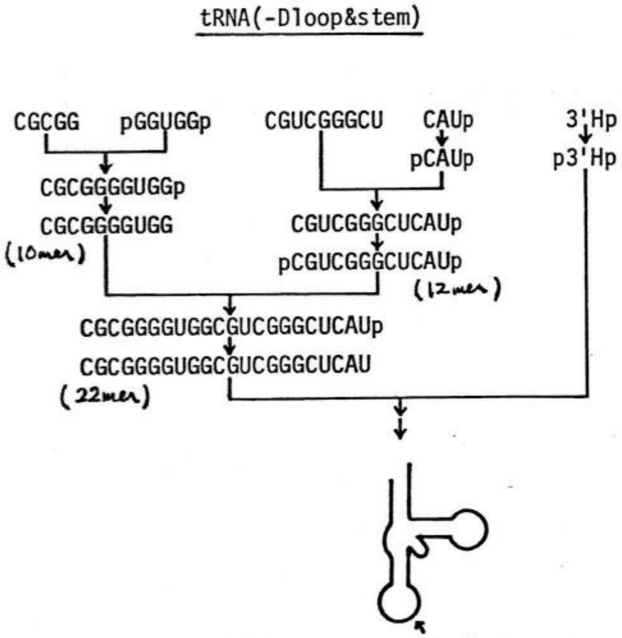
ミトコンドリアには一般的なクローバ葉型二次構造を有する tRNA と異なり Dループとステムが欠除した特異的な tRNA が存在する。tRNA^{Ser} がその例でこれらの部分が欠除しているにもかかわらずセリンを受容し tRNA としての機能を果たす。他のクローバ葉型二次構造を有する tRNA も Dループやステムがなくても機能するであろうか。著者は E.coli tRNA_f^{Met} について Dループとステムが欠除した tRNA を合成し，この分子がメチオニンを受容できるか否かについて調べた。

アクセプタ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続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おいた。(図46)

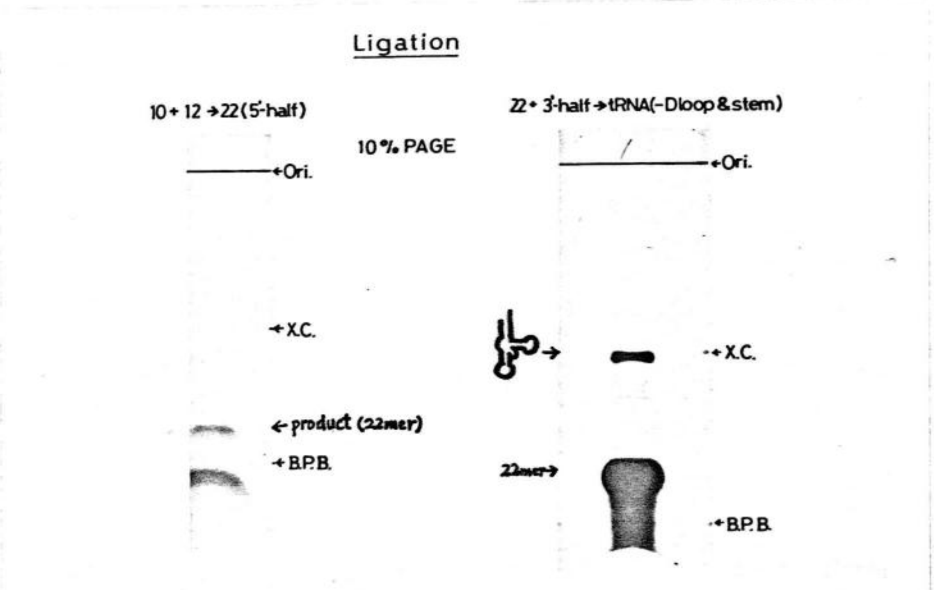
合成経路を図47に示す。5'側半分子は鎖長が3から9の化学合成フラグメントを RNA ligase で結合することにより合成した。3'側半分子は 天然の E.coli tRNA_f^{Met} の RNase A 限定分解



［図46］Dループとステムの欠除した E. coli tRNA$_f^{Met}$



［図47］Dループとステムの欠除した tRNA$_f^{Met}$ の合成経路



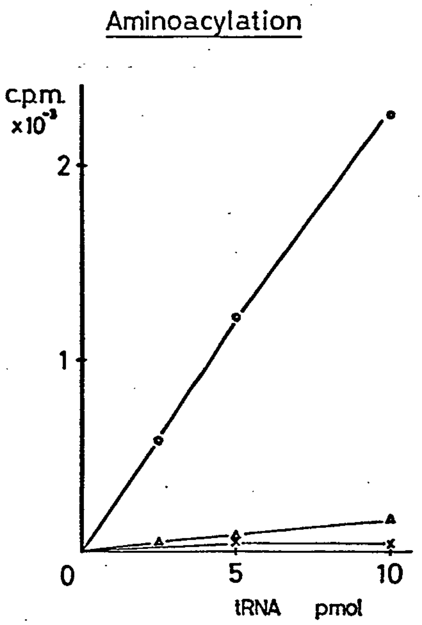
［図48］Dループとステムの欠除した tRNA$_f^{Met}$ 合成における ligation

より得られるフラグメントを用いた。

CGCGG＋pGGUGGp，CGUCGGGCU＋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Dループ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。Dループが欠除するとDループとTΨループの水素結合ができなくなり，tRNA として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かったと考えられる。Dループが欠除しているにもかかわらず天然の $tRNA_f^{Met}$ 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。



［図49］5'側半分子変換した $tRNA_f^{Met}$ のメチオニン受容活性
○—○：$tRNA_f^{Met}$，
△—△：tRNA（−D loop & stem），
×—×：tRNA（Gly-Met）

最近 Klug らによりミトコンドリアの $tRNA^{Ser}$ 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クセプターステムとアンチコドンステムの間の塩基を変換し 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。

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* $tRNA_f^{Met}$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* $tRNA_f^{Met}$のTψループの中で共通塩基配列TψCをAUCに変換した$tRNA_f^{Met}$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ったことより，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った。
3. *E. coli* $tRNA_f^{Met}$の5′末端位でG-C塩基対が組める修飾$tRNA_f^{Met}$を合成し調べたところ，天然の$tRNA_f^{Met}$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* $tRNA_f^{Met}$のアンチコドン部を変換した修飾$tRNA_f^{Met}$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った。
   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った。
5. *E. coli* $tRNA_f^{Met}$のアンチコドン部を変換した修飾$tRNA_f^{Met}$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$^{Met}_{f}$ の5′側半分子を E. coli tRNA$^{Gly}_{2}$ に変換した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$^{Met}_{f}$ 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
[^{32}P]-正リン酸	日本アイソトープ協会
[^{14}C]-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 T$_2$	三共株式会社
RNase A	Boehringer-Mannheim社
ヌクレアーゼ P$_1$	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

アビセル 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 $MgCl_2$, 10 μg/ml BSA, 10〜15% DMSO (v/v) を含む buffer中 25°Cでインキュベートした。

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 $MgCl_2$, 2.0mM スペルミン, 10mM DTT, 0.1M KCl を含む buffer中 37°Cでインキュベートした。

基質量, 酵素量については各章ごとに後述する。

◦ BAP処理

50mM Tris HCl (pH8.0) 中，基質 1nmol に対し酵素 0.005 unit 加え，基質 100～200 μM の条件で 50°C 1.5時間インキュベートして反応を行なった。反応後は反応液と同量の 50mM Tris HCl (pH7.5) 飽和のフェノール，クロロホルム混液 (1:1, v/v) によって2度除タンパクし，フェノール相をさらに 50mM Tris HCl (pH7.5) で3回逆抽出してから水相を約2倍量のエーテルで5回洗った。溶液中のエーテルを蒸発させた後 Sephadex G-50 で脱塩した。

◦ RNase T_2 による完全分解

40mM 酢酸アンモニウム buffer (pH4.5) 中，基質約 2 A_{260} unit (キャリア RNA を含む) に対して酵素約 0.05 unit 加え，10μl で 37°C 3時間インキュベートした。反応後 混合物は沪紙にスポットして pH3.5 の条件下 PEP を行なった。

◦ RNase A による限定分解

第二章の実験参照

◦ Nuclease P_1 による完全分解

40mM 酢酸アンモニウム buffer (pH5.0) 中，基質約 2 A_{260} unit (キャリア RNA を含む) に対して酵素約 0.1μg 加え，10μl で 37°C 2時間インキュベートした。

◦除タンパク

通常50mM Tris HCl (pH7.5)で飽和したフェノール，クロロホルム混液（1:1, v/v）を試料に対し同量加え2度抽出操作を行なった。フェノール相は50mM Tris HCl (pH7.5)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度洗いフェノール，クロロホルムを除去した。エーテルを留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え −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。

◦沪紙電気泳動（pH 3.5）

東洋沪紙No51Aを用い，0.2M酢酸モルホリンbuffer (pH 3.5)中，35 V/cmの電圧で泳動した。泳動後沪紙を風乾し，5mm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（アビセルSF, 10cm×10cm）にスポットし，一次元目をイソ酪酸−0.5Mアンモニア（5:3, v/v）で展開し，二次元目をイソプロパノール−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 サンプルの両脇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 沪紙を吸取紙としてクリップで止めて展開を行なった。(60～70 ℃) 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してラップで包み, X ray フィルムを接触し感光した。

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ピレーターで吹いこみ Cerenkov法[43]で測定し 算出した。

・二次元ホモクロマトグラフィー

Wu等,[42] 及びSilverklang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 Km, Vmax を求める時以外 後に示す条件で行なった。

反応後 全量をWhatmanグラスフィルターにスポットし, 冷5% TCA溶液にひたし 氷冷下10分間放置した。この洗浄を後2回計3回行なった後, EtOH : Et_2O = 1 : 1 (v/v) 溶液を加え氷冷下 5分間放置した。これを再度行なった後フィルターを赤外

線ランプ下で乾燥させ，トルエン系シンチレーターで ^{14}C のカウントを測定した。

Km, Vmax を求める時の条件は第四章の実験で記述する。

アミノアシル化反応の条件

HEPES-NaOH (pH8.0)	0.1 M
$Mg(CH_3COO)_2$	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
E. coli 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：ビスアクリルアミド＝30：1 (w/w), 7M urea, 45mM Tris borate buffer (pH8.4), 2mM EDTA）に過硫酸アンモンとTEMEDを加えてゲル化した。

Slab gel の場合は厚さが 0.5～2mm，巾 20～30 cm，長さ 40 cm の平板ゲルになるようにガラス板でわくを作りそこにアクリルアミドを流しこんで固めた。

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

サンプルの apply の前に2時間程 prerunning をしてゲルの状態を確かめてから，凍結乾燥したサンプルに loading solution (9M urea, 10mM EDTA, 40mM Tris borate buffer (pH8.4), ～0.1% X.C.－B.P.B.) を 2～5 μl 加え 100°C，2分間加熱し apply を行なった。

泳動後 slab gel ではガラス板をはずしてラップでおおい，X-ray フィルムを接触させフリーザー中で感光した。Disk gel の場合はポンプ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て 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) を少し変えて行なった。溶出用 buffer には SDS を加えずに調整した。溶出液を濃縮し，Sephadex G-50 に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鎖長）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. でフラクションとして約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

鎖長が 8～14 のオリゴマーを分離精製するのに用いた。7M urea, 20mM 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 5ml/h. でフラクションとして約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

ムを用い，kination後 ATPとオリゴマーとを分離する時には40cm程度，ligation後のオリゴマー同士の分離には 長さ90cm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た。溶出は50〜100mM TEAB bufferを用いた。流速は約15ml/hr.でフラクションとして約1mlずつ集めた。

◦ Sephadex G-100, Bio-Gel A(0.5m) カラムクロマトグラフィー

長さ90cmのカラムを用い，0.1M TEAB bufferで溶出した。流速は約4ml/hr.でフラクションとして約1mlずつ集めた。

◦ $[\gamma\text{-}^{32}P]$-ATPの調整

Glynn-Chappell法[46]により，杉浦ら[47]の方法を少し変え調整した。反応前に$[^{32}P]$-正リン酸を沸騰水浴上1時間加熱し，副生しているポリリン酸を分解した。

反応後はDowexカラムにapplyし 溶出した$[\gamma\text{-}^{32}P]$-ATPは次にDEAE-Sephadex A-25のカラムに吸着させた。50mM TEAB bufferで脱塩後0.5M TEAB bufferで溶出した。これを減圧乾固しTEABを完全に除いた後，10mM 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し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 を使用した。

◦ 放射能測定

通常 Aloka Liquid Scintillation spectrometer LSC-671 にて 0.4% diphenyloxazole 0.01% triphenyloxazole のトルエン溶液中で測定した。ただしカラムクロマトグラフィーのフラクションは試験管ごと Cerenkov 法により測定した。

第一章の実験

- kination

反応条件，単離収率を表2にまとめた。Bufferはmethodに示した。

Substrate (μM)		$[\gamma\text{-}^{32}p]$ATP (μM)	P.N. Kinase (units/ml)	Volume (μl)	Time (min.)	Yield (%)
1. UCGUp	100	152	50	500	60	46
2. GGCUp	100	150	100	1000	30 a)	19
3. CGGGCUp	50	100	69	720	45	85
4. UCGUCGGGCUp	23	75	47	20	60	76
5. CCGAAG	400	800	94	73	60	60
6. GUCGG	1000	1077	250	200	60	88
7. AAAp	101	187	64	692	30	100
8. UUCAAAp	100	143	179	140	45	53
9. GUCGUCGGUUCAAAp	80	160	64	25	30 a)	88
10. CCCCGCp	200	267	100	150	90	99
11. AACCAp	200	240	100	50	60	93
12. UAGCUCGUCGGGCUp	55	300	300	40	50 a)	95
13. UCCGGCCCCGC- AACCAp	68	150	100	40	90 a)	93
14. GUCGUCGGUUCAAA- UCCGGCCCCGC- AACCAp	74	148	111	27	90 a)	83

[表2] 第一章のkination

a) 酵素を加える前に60°C，5分間のpreheat処理を行なった。

単離の方法及び条件を次に示す。

1、 DEAE-Sephadex A-25 (φ0.7 × 15 cm)

TEAB buffer 0.3 M から 1 M まで total 200 ml の gradient で溶出した。

2、 DEAE-Sephadex A-25 (φ0.9 × 18 cm)

TEAB buffer 0.3 M から 0.9 M まで total 200 ml の gradient

で溶出した。

3. Sephadex G-25 (φ1.1 × 40 cm)

4, 8, 9, 10, 11, 12, 13, 14.
Sephadex G-50 (φ0.9 × 30 cm)

5. DEAE-Sephadex A-25 (φ0.7 × 20 cm)
TEAB buffer 0.3Mから0.9Mまで total 150 ml の gradient で溶出した。

6. DEAE-Sephadex A-25 (φ0.8 × 16 cm)
TEAB buffer 0.3Mから0.8Mまで total 200 ml の gradient で溶出した。

7. DEAE-Cellulose (DE23) (φ0.4 × 16 cm)
TEAB buffer 0.1Mから0.5Mまで total 150 ml の gradient で溶出した。

○ ligation

反応条件，単離収率を表3にまとめた。Bufferは method に示した。RNA ligase を加える前に 60°C，5分間 preheat した。

単離の方法及び条件を示す。

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

3. DEAE-Cellulose (DE23) (φ0.6 × 18 cm)
TEAB buffer 0.3Mから1Mまで total 200 ml の gradient で溶出した。

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

Acceptor (μM)		Donor (μM)		ATP (μM)	RNA Ligase (units/ml)	Volume (μl)	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. CAUAACCCGAAG	150	pGUCGUCGGUUCAAAp	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. UCCGGCCCCCGC	101	pAACCAp	116	229	114	70	3.0	38
13. CGCGGGGUGGAGCAGCCUGG	74	pUAGCUCGUCGGGCUp	111	227	535	12	2.0	31[b]
14. GUCGUCGGUUCAAA	200	pUCCGGCCCCGCAACCAp	125	200	150	20	2.0	82
15. CAUAACCCGAAG	159	pGUCGUCGGUUCAAAUCCGGCCCCCGCAACCAp	100	200	153	17	2.0	32

[表3] 第一章の ligation

a) kination後の単離収率
b) BAP処理後の単離収率

5. 20% PAGE (disk), 定電圧 300 V, ポンプ流速 40 ml/h. でフラクションを集めた。

6. urea 存在下 DEAE-Sephadex A-25 (φ0.3 × 38 cm) NaCl 0.1M から 0.5M まで total 80 ml の gradient (50 mM Tris HCl (pH 7.5) 存在下) で溶出した。

7. DEAE-Sephadex A-25 (φ0.6 × 18 cm) TEAB buffer 0.3M から 1 M まで total 150 ml の gradient で溶出した。

8\. 1) urea 存在下 DEAE-Sephadex A-25 (Φ0.3 x 40cm)
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% であった。

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 (Φ0.9 x 82 cm)

11\. urea 存在下 DEAE-Sephadex A-25 (Φ0.3 x 43 cm)
NaCl 0.2M から 0.6 M 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 (Φ0.9 x 82 cm, Φ1.0 x 90 cm)

15\. Bio-gel A (0.5m) (200 ~ 400 mesh) (Φ0.7 x 91 cm)

第二章の実験

◦ Kination

反応条件，単離収率を表4に示した。P.N. kinase を加える前に60°C 5分間 preheat した。

Substrate (μM)		$[\gamma\text{-}^{32}P]$ATP (μM)	P.N. Kinase (units/ml)	Volume (μl)	Time (min.)	Yield (%)
1. CCGGCCCCCGC-AACCApp	94	150	150	40	80	99
2. AUCGAAACCGGC-CCCCGCAACCAp	100	200	250	30	75	83
3. (43mer)p	100	300	100	12	45	55

[表4] 第二章の kination

単離はすべて Sephadex G-50 (φ0.9×30cm) により行なった。

◦ ligation

反応条件，単離収率を表5に示した。RNA ligase を加える前に60°C 5分間 preheat した。

Acceptor (μM)		Donor (μM)		ATP (μM)	RNA Ligase (units/ml)	Volume (μl)	Time (hr.)	Yield (%)
1. AUCGAAA	200	pCCGGCCCCCGC-AACCAp	100	200	100	32	2.0	87
2. CAUAACCC-GAAGGUC-GUCGG	148	pAUCGAAACCG-GCCCCCGCAA-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処理後の単離収率

単離の方法及び条件を示す。

1. Sephadex G-50 (Φ0.9 × 74 cm)

2, 3. Sephadex G-100 (Φ0.7 × 90 cm)

∘ E. coli $tRNA_f^{Met}$ の RNase A による限定分解20)

右に示す条件で行なった。

反応後 冷BSAを150 μg加え、冷buffer (50 mM Tris HCl (pH 7.5), 10 mM $MgCl_2$) で全量を300 μlに調整し、ただちに冷フェノール (50 mM Tris HCl (pH 7.5) 飽和) で除タンパクした (2回). 冷 50 mM Tris HCl (pH 7.5) で逆抽出を2回行ない、水相をエーテルで2回洗浄した後 エタノール沈殿を行なった。沈殿をさらに EtOH : Et_2O = 1 : 1 混液で洗った後、乾燥し 10% PAGE で 5′側半分子と 3′側半分子を単離した。

E. coli $tRNA_f^{Met}$	1,000 A_{260}
Tris HCl (pH 7.5)	50 mM
$MgCl_2$	10 mM
RNase A	25 μg/ml
	100 μl

incubate at 0°C for 30 min.

第三章の実験

○ kination

反応条件, 単離収率を表6に示した。

Substrate (μM)		$[\gamma\text{-}^{32}P]$ATP (μM)	P.N. Kinase (units/ml)	Volume (μl)	Time (min.)	Yield (%)
1. AACCAp	167	133	173	30	60	50
2. GACCA	100	157	167	30	60	70

[表6] 第三章の kination

単離の方法を示す。

1. Sephadex G-50 (φ0.8×23cm)
2. Sephadex G-25 (φ1.0×26cm)

○ ligation

反応条件, 単離収率を表7に示した。RNA ligase を加える前に60°C 5分間の preheat を行なった。

Acceptor (μM)		Donor (μM)		ATP (μM)	RNA Ligase (units/ml)	Volume (μl)	Time (hr.)	Yield (%)
1 72mer	50	pAACCAp	156	1250	212	16	2.0	16
2 72mer	50	pGACCA	131	1250	212	16	2.0	30

[表7] 第三章の ligation

単離はすべて10% PAGEにより行なった。

○ 過ヨウ素酸酸化による3'末端一塩基除去

Sprinzl らの方法27)に基づき行なった。

nuclease S1 処理により得られた E.coli tRNA$_f^{Met}$ の 3'末端より4塩基除去された分子(3 A$_{260}$ unit)を 50mM 酢酸ナトリウム(pH 5.2), 0.8mM NaIO$_4$ 中 (total volume 60μl) 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 容の 0.5M ラムノースを加え 遮光下 4°C 0.5時間反応し過剰の NaIO$_4$ を不活化した。 EtOH 沈殿後, さらに沈殿を EtOHで洗った後 乾燥し, ここに 0.25 M Lys-HCl (pH9.0) を加え遮光下, 室温(~20°C) 3.5時間反応した。 反応後 当容の 1M 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処理をし末端のリン酸を除去した。除タンパク後 10% PAGE で単離した。

生成物は [5'-^{32}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 T$_2$で完全分解した。沪紙電気泳動 (pH3.5) によりラベルが A^{32}p として検出され末端のCが除去できたことを確認した。

- アミノアシル tRNA - EF-Tu・GTP 三重複合体の Sephadex G-100 を用いた 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った。48)

Sephadex G-100 は buffer (50mM Tris HCl (pH7.8), 150mM NH$_4$Cl, 10mM Mg(OAc)$_2$, 10mM mercaptoethanol, 10mM GTP) で平衡化しておき, サンプルを apply 後 同じ buffer で溶出した。 各溶出フラクションを 500μl / 9分 で集めた後,

三重複合体形成の条件

Tris HCl (pH 7.8)	50 mM
$Mg(OAc)_2$	10 mM
NH_4Cl	150 mM
mercaptoethanol	10 mM
GTP	30 μM
phospho(enol) piruvate	13 mM
piruvate kinase	3 μg
EF-Tu・GDP	6.3 μg (30 pmol)
	30 μl

↓ incubate at 30°C for 30 min.

↓ add [^{14}C]-Met-$tRNA_f^{Met}$ or [^{14}C]-Val-$tRNA^{Val}$ or [^{14}C]-Met-tRNA (GACCA), 5~10 pmol

↓ incubate at 0°C for 5 min.

↓ gel filtration on Sephadex G-100 at 4°C (φ0.7 × 46 cm)

フラクションをグラスフィルターにスポットし乾燥後，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-$tRNA^{Val}$ は複合体を形成でき，Met-$tRNA_f^{Met}$ は形成できない。

第四章の実験

- kination

反応条件，単離収率を表8に示した。

Substrate (μM)		$[\gamma\text{-}^{32}P]$ATP (μM)	P.N. Kinase (units/ml)	Volume (μl)	Time (min.)	Yield (%)
1. 3'Hp	67	100	67	120	120	56
2. 3'Hp	78	117	122	50	70	85
3. CAUA	150	100	50	100	90	59
4. CAUAAp	99	150	99	80	90	63
5. UCAU	100	100	50	300	180	56
6. ACAUp	106	83	37	141	60	47
7. GCAUp	100	78	87	150	120	83
8. CCAUp	120	90	90	167	120	93
9. UUCAUp	104	80	80	125	120	89
10. CAA	340	260	180	50	90	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 (Φ0.8×23cm)

3. DEAE-Sephadex A-25 (Φ0.5×6cm)

TEAB buffer 0.2Mから0.7Mまで total 80ml の gradient で溶出した。

5, 10. DEAE-Sephadex A-25 (Φ0.5×6cm)

TEAB buffer 0.2Mから0.7Mまで Total 60ml の gradient で溶出した。

6, 11, 12, 14, 15, 16, 17. DEAE-Sephadex A-25 (φ0.5×6cm)

TEAB buffer 0.2Mから0.8Mまで total 60mlの gradient で溶出した。

7, 8. DEAE-Sephadex A-25 (φ0.5×6cm)

TEAB buffer 0.3Mから0.9Mまで total 60mlの gradient で溶出した。

13. DEAE-Sephadex A-25 (φ0.5×6cm)

TEAB buffer 0.1Mから0.6Mまで total 60mlの gradient で溶出した。

○ ligation

反応条件，単離収率を表9に示した。RNA ligase を加える前に60°C，5分間 preheat した。

単離はすべて10% PAGEにより行なった。

○ アミノアシル化反応 (K_m, V_{max} study)

右に示す条件で行なった。

tRNAの3′末端CCA配列を修復した後，$[^{14}C]$-Met を加えて反応した。

反応後の処理は method に記述した方法と同一に行なった。

tRNA	0.15~2.0μM
CTP	0.8mM
ATP	4mM
HEPES-NaOH (pH8.0)	100mM
$Mg(OAc)_2$	10mM
KCl	10mM
mercaptoethanol	10mM
E. coli S-100	1mg/ml

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

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

↓incubate at 37°C for 20min.

Acceptor (μM)		Donor (μM)		ATP (μM)	RNA Ligase (units/ml)	Volume (μl)	Time (hr.)	Yield (%)
1. CAUA	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	pCAUAAp	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	pACAUp	125	500	130	40	2.0	25 a)
9. 5'H	100	pGCAUp	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	pACAUp	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	pUCAUp	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	67	pCAA	300 ~ 433	667	200	30	2.0	17
23. 5'H		pCAC						17
24. 5'H		pCAG						10
25. 5'H		pCAU						22
26. 5'H		pGAA						30
27. 5'H		pGAC						12
28. 5'H		pGAG						13
29. 5'H		pGAU						28
30. 5'HpCAA	13	p3'Hp	3	600	300	3	2.0	
31. 5'HpCAC		p3'Hp						
32. 5'HpCAG		p3'Hp						
33. 5'HpCAU		p3'Hp						
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処理後の単離収率

- $tRNA_f^{Met}$ の RNase A による限定分解

第二章の実験で記述したとおり行なった。

- ホルミル化反応[49)]

右に示す条件で行なった。

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ポットし 他方は等量の 50mM $CuSO_4$-0.3M TrisHCl (pH7.5) 溶液を加え，37°C 10分間反応しグラスフィルターにスポットした。グラスフィルターは冷5% TCA 溶液で10分間3回，冷 $EtOH$-Et_2O (1:1, v/v) 液で5分間2回洗浄し，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。$CuSO_4$処理により fMet-tRNA は deacyl 化されないが Met-tRNA は deacyl 化されることから $CuSO_4$処理を行なった方のカウントの行なわなかった方のカウントに対する比をホルミル化の効率とし%で表わした。

tRNA	0.7 μM
HEPES-NaOH (pH8.0)	0.1 M
$Mg(OAc)_2$	10 mM
KCl	10 mM
mercapto-ethanol	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.

- リボソーム上での mRNA と tRNA の相互作用

Nirenberg らの方法[33)]により行なった。リボソームは E.coli A19 株より調整した。[50)] 5'末端を $[\gamma$-$^{32}P]$ATP と P.N.Kinase によりラベルした tRNA を用い右の条件で反応した。

tRNA	0.5 μM
mRNA	25 μM
TrisHCl (pH7.5)	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 としてトリマーを用いた。

反応後 冷 buffer (0.1M Tris HCl (pH7.5), 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。

非特異的な結合によるカウントを差し引き E.coli $tRNA_f^{Met}$ 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 $tRNA_f^{Met}$ 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おさえるために 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 microinjection 36) (図50)

アフリカツメガエルより 卵巣をとり出し，Barth solution 51) (2mM Tris HCl (pH7.6), 89mM NaCl, 1mM KCl, 0.33mM $Ca(NO_3)_2$, 0.41mM $CaCl_2$, 0.82mM $MgSO_4$, 2.4mM $NaHCO_3$, 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。

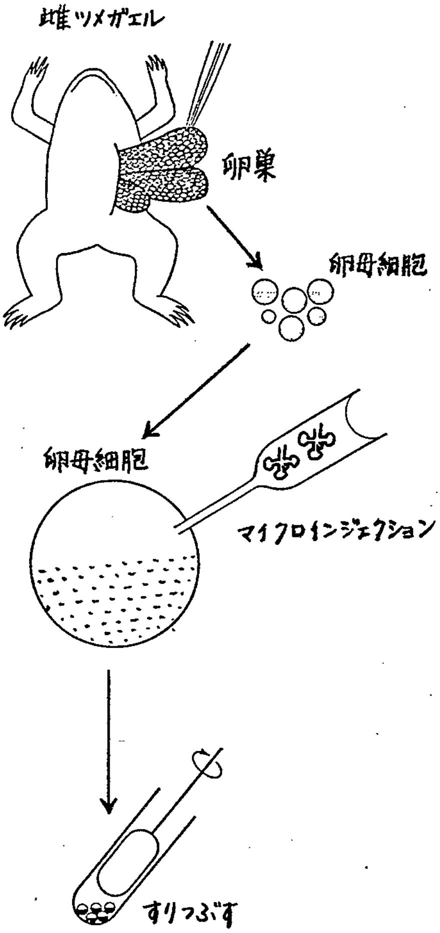
サンプルは injection を行なう直前に 13,000 rpm，5分間遠心し dust を除去した。(マイクロピペットがつまるのを防ぐた

め)

卵母細胞をBarth solutionの入ったシャーレに移し, 1つのサンプルにつき5個の卵母細胞の細胞質に50nlずつinjectした。(1卵母細胞あたり0.0025 pmolのtRNAをinjectした。)

Inject後エッペンドルフチューブ(1.5ml)に5個の卵母細胞とBarth solutionを移し, 20℃でインキュベートした。

1, 3, 7, 24, 48, 72時間それぞれインキュベート後Barth solutionを除き, ガラス棒で卵母細胞を破砕した。Buffer (0.2M NaOAc (pH4.5), 10mM $MgCl_2$, 1mM EDTA, 2% SDS(w/v)) 200μlを直ちに加えsuspendしフェノール:クロロホルム=1:1溶液(0.2M NaOAc飽和) 200μlで除タンパクした。



[図50] 卵母細胞へのmicroinjection

フェノール相を先ほどのSDSを含むbuffer 100μlで逆抽出し, あわせた水相にEtOH 700μl加えEtOH沈殿を行なった。沈殿をEtOH:0.3M NaOAc(pH5.2)=4:1溶液で洗浄し乾燥した後10% PAGEにapplyした。

第五章の実験

∘ kination

反応条件，単離収率を表10に示した。

Substrate (μM)		$[\gamma\text{-}^{32}P]$ATP (μM)	P.N. Kinase (units/ml)	Volume (μl)	Time (min.)	Yield (%)
1. CAUp	250	150	75	200	90	47
2. 3'Hp	81	119	81	62	90	44
3. CGUCGGGCUCAUp	100	334	100	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 (φ0.5×6 cm)

TEAB buffer 0.3Mから0.8Mまで total 60ml の gradient で溶出した。

2, 3. Sephadex G-50 (φ0.8×23cm)

∘ ligation

反応条件，単離収率を表11に示した。 RNA ligaseを加える前に60°C 5分間 preheatした。

単離の方法を示す。

1, 2, 5, 6. 10% PAGE

3, 4. Sephadex G-50 (φ0.7×90cm)

Acceptor (μM)		Donor (μM)		ATP (μM)	RNA Ligase (units/ml)	Volume (μl)	Time (hr.)	Yield (%)
1. 5'H(Gly)	100	pCAUp	200	400	100	70	2.0	7
2. 5'H(Gly)-pCAU	31	p3'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. CGCGGGGUGG	200	pCGUCGGGCUCAUp	100	600	260	50	2.0	20
6. CGCGGGGUGGCGUCGGGCUCAU	75	p3'Hp	150	1000	340	10	2.0	3

[表11] 第五章の ligation

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te base sequences for RNA ligase reactions in the synthesis of a rresponding to bases 21-34 of E. coli $tRNA_f^{Met}$ [1]

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ABSTRACT

A tetradecanucleotide U-A-G-C$(U-C-G)_2$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-

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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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MATERIALS AND METHODS

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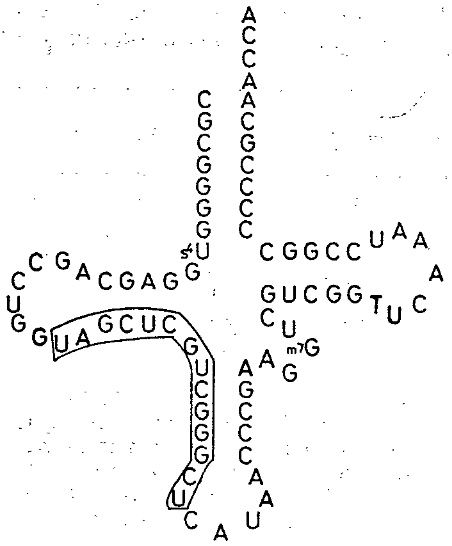

Fig. 1

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-^{32}P]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 $MgCl_2$ 10 µg/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] Homochromatography[8] was carried out using Homomix I-VI[9] 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 aparatus.[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-G-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 $[\gamma-^{32}P]$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 $[\gamma-^{32}P]$ATP. The overall yield was 11%. The elution profile from the gel is shown in Fig. 2.

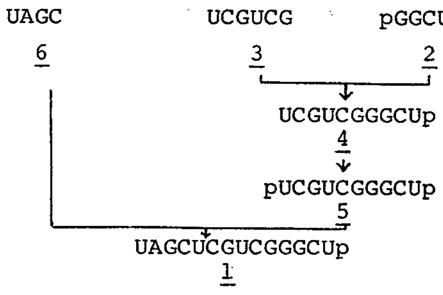

Chart 1

Table I Reaction conditions for joining

acceptor nmol [µM]	donor nnol [µM]	ATP µM	RNA ligase [unit/ml] Volume	time hr	Reaction extent[a)] %	Yield %
UCGUCG (3) 12[100]	pGGCUp (2) 20[167]	333	250 120 µl	1	15	4
UAGC(6) 1.2[240]	pUCGUCGGGCUp(5) 0.4[80]	200	180 5	2	27	11[b)]
UAGC(6) 30[150]	pUCGUp(7) 20[100]	200	100 200	3.5	88	71
UAGCUCGU(9) 7[100]	pCGGGCUp(10) 8[114]	200	214 70	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

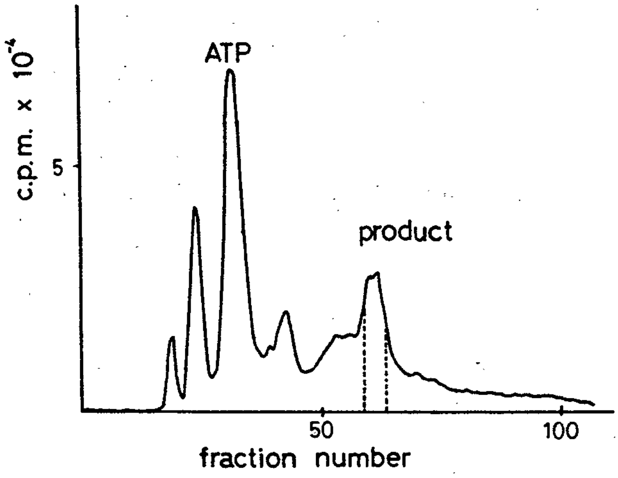

Fig. 2 Elution profile of the products obtained by joining of U-A-G-C and *pU-C-G-U-C-G*pG-G-C-Up, followed by phosphorylation with polynucleotide kinase and ATP, from a preparative disk gel (70 mm^2 x 80 mm) at 600 V with a suction rate of 32 ml/hr. The last peak contained *pU-A-G-C*pU-C-G-U-C-G*pG-G-C-Up.

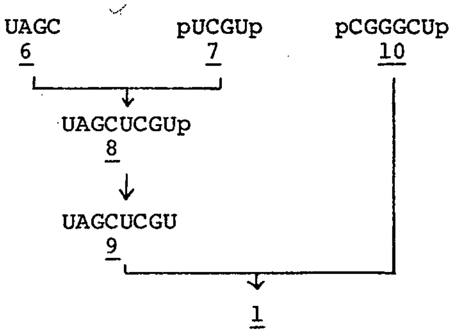

Chart 2

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

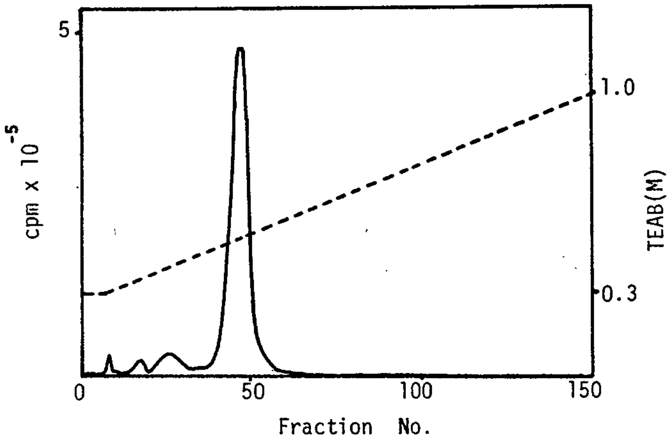

Fig. 3 Chromatography of the joined product, U-A-G-C*pU-C-G-Up on a column (0.6 x 18 cm) of DEAE-cellulose (bicarbonate) equilibrated with 0.15 M triethylammonium bicarbonate. Elution was performed with a linear gradient of triethylammonium bicarbonate (0.3-1 M, total 200 ml). The main peak contained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

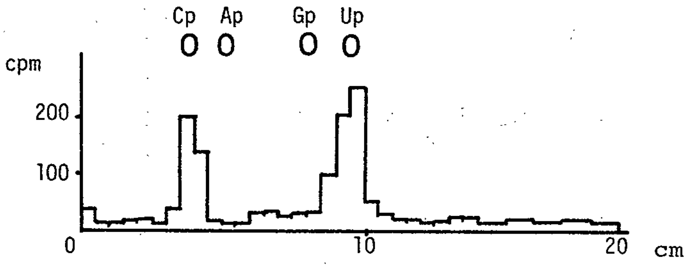

Fig. 4 Nearest neighbor analysis of the joined product U-A-G-C-*pU-C-U-C-G-G-G-C-Up with RNase T2. The digested mixture was electrophoresed at pH 3.5 with nucleotide UV markers.

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-

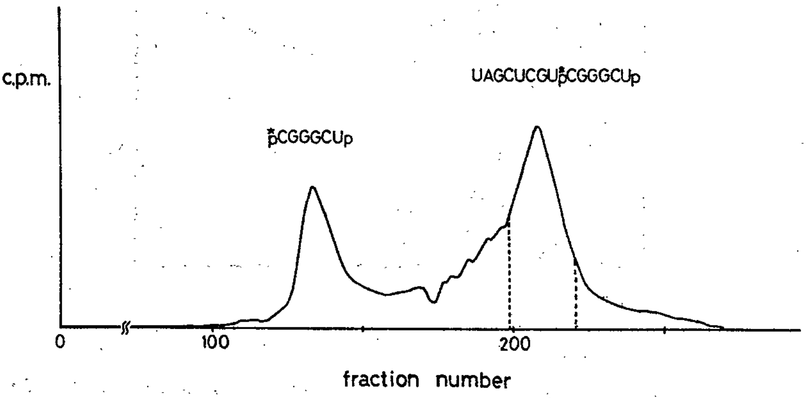

Fig. 5 Elution profile of the tetradecamer (1) from a disk gel. The conditions for electrophoresis was as described in Fig. 2.

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 substrates[4] 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 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 extent[10] and this conformation may be unfavorable for RNA ligase reactions if it exist 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 comformers.[11]

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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 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 $tRNA_f^{Met}$ 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 $tRNA_f^{Met}$ 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]

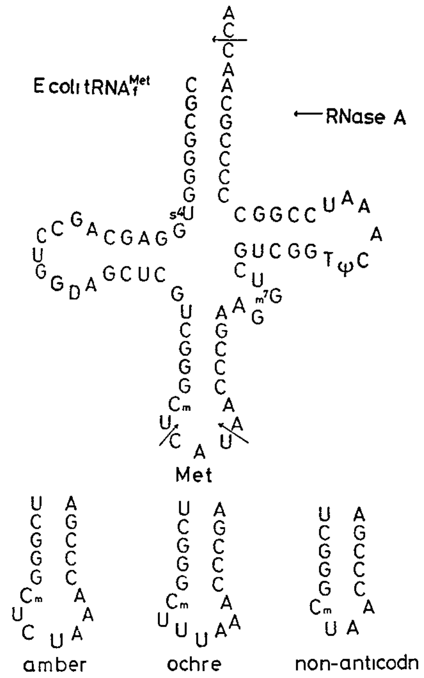

Fig. 1 Cleavage sites in partial digestion with RNase A and modified structures at the anticodon.

MATERIALS AND METHODS

Materials

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/A_{260} 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. ^{14}C 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 µl solution. RNAase A (1 µg), carrier RNA (0.3 A_{260} 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 A_{260}) 50 mM Tris-HCl (pH 7.5) and EDTA 1 mM at 37° for 20 min, nuclease P1 (0.2 µg), carrier RNA $1A_{260}$ in 50 mM ammonium acetate (pH 5.0) at 37° for 1 hr; RNase T2 (2U) carrier RNA (0.8 A_{260}) in 50 mM sodium acetate (pH 4.5) at 37° for 30 min. Oligonucleotides recovered after homochromatography contained ca. 200 µg of RNA/cm^2.

Partial digestion of tRNA (100 A_{260}) was performed in 50 mM Tris-HCl(pH 7.5)-20 mM $MgCl_2$ at 0° for 30 min using RNase A (25 µg). 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 desalted by gel filtration on Sephadex G-50. The yields was ca. 20%.

Aminoacylation of tRNA was performed in 50 mM HEPES-NaOH (pH 8.0), 10mM magnesium acetate, 10 mM potasium chloride 10 mM β-mercaptoethanol, 4mM ATP, 0.5 mM CTP, 9 μM L-U-^{14}C Met, 0.5 μM tRNA and E.coli S-100[13] at 37°C for 40 min in 20 μl 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 treatement with phos-

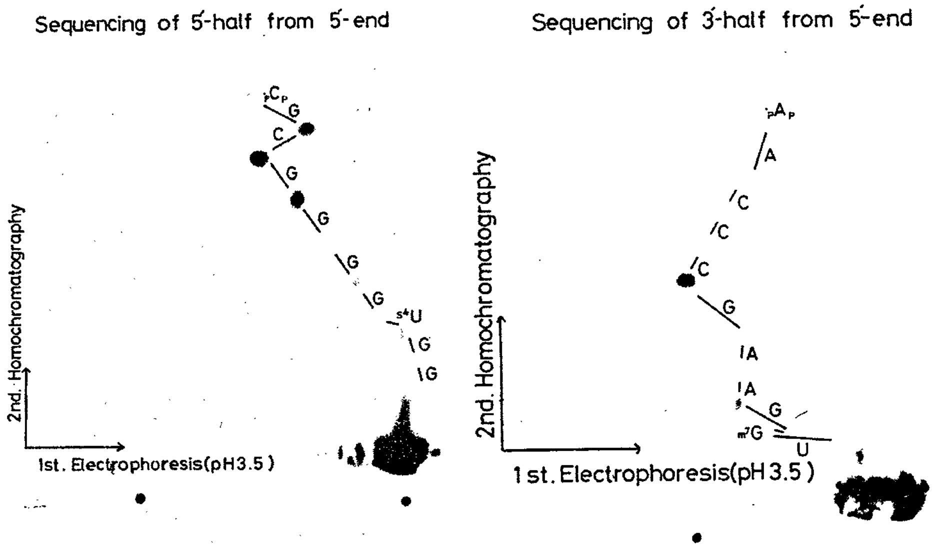

Fig. 2 Mobility shift analyses of the 5'-half (a) and the 3'-half (b) by labeling at the 5'-end.

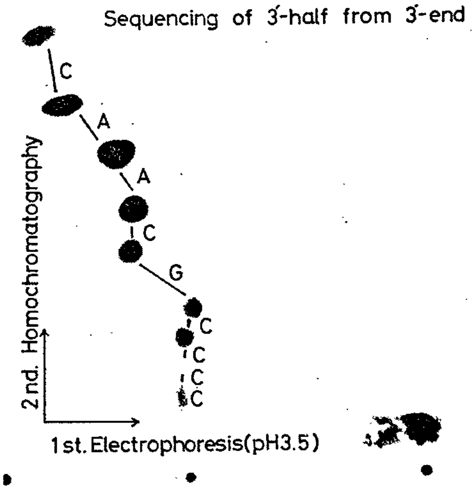

Fig. 3 The sequence of the 3'-end of 3'-half (38-75) developed after labeling at the 3'-end with 5'-^{32}p pCp and RNA ligase.

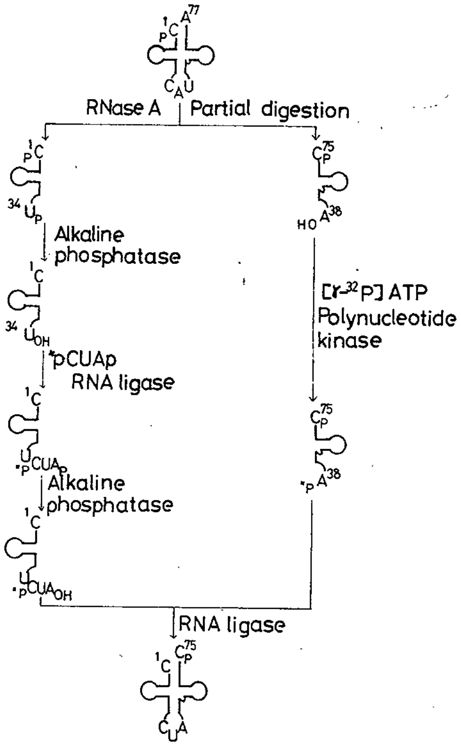

Fig. 4 Scheme for reconstruction of $tRNA_f^{Met}$.

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 ocher suppressor tRNA (UUA 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 analys by digestion with RNase T2 is shown in Fig. 6. The $tRNA_f^{Met}$ lacking the anticodon CAU (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

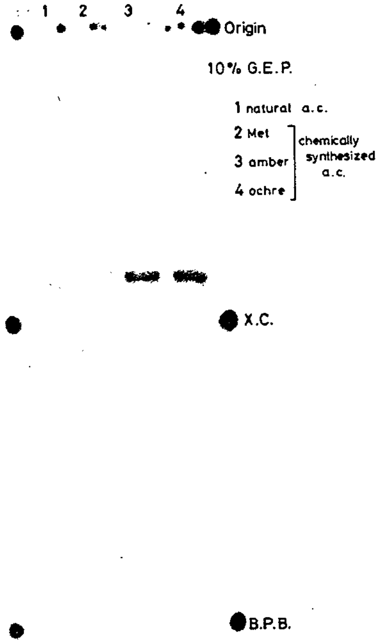

Fig. 5 Electrophoresis of tRNA$_f^{Met}$ and modified molecules: 1, intact molecule; 2, reconstructed tRNA$_f^{Met}$ (CAU); 3, amber tRNA$_f^{Met}$ (CUA); 4, ochre tRNA$_f^{Met}$ (UUA).

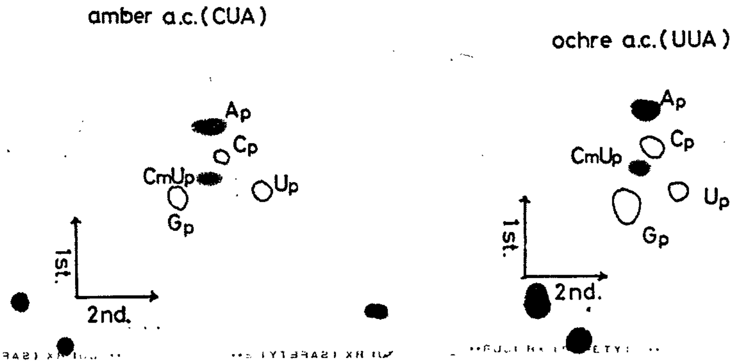

Fig. 6 Nearest neighbor analyses of modified tRNAs.

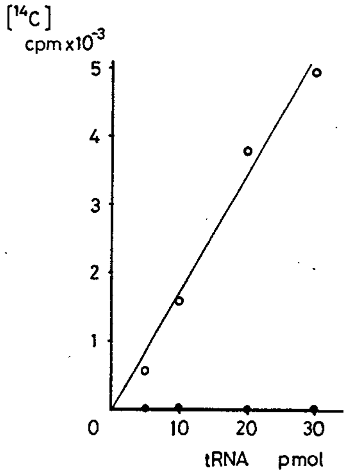

Fig. 7 Charging of methionine to the intact $tRNA_f^{Met}$ (o--o) and the amber $tRNA_f^{Met}$ (CAU) (o--o).

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'-^{32}P pCp and RNA ligase.[15] It was found that more than 95% count was transfered to pA. As summarized in Table I, the reconstructed

Table I ^{14}C 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)}, ocher 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$_f^{Met}$ 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$_f^{Met}$ 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$_f^{Met}$ 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 $\mathrm{tRNA}^{\mathrm{Phe}}$ 20,21) and E. coli $\mathrm{tRNA}_{\mathrm{f}}^{\mathrm{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 $\mathrm{tRNA}^{\mathrm{Tyr}}$ was not essential for amber suppression.13)

In this paper, we describe the synthesis of several mutant E. coli $\mathrm{tRNA}_{\mathrm{f}}^{\mathrm{Met}}$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-^{32}P]ATP (specific activity; 2,900 Ci/mmol) and L-[U-^{14}C]Methionine (specific activity; 282 mCi/mmol) were obtained from NEN and Amersham, respectively. E. coli $\mathrm{tRNA}_{\mathrm{f}}^{\mathrm{Met}}$ was a gift of Dr. D. Söll. (specific activity; 1,400 pmol/A_{260} 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 T_2 and Nuclease P_1 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 previously[1)] 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 potasium chloride,10 mM β-mercaptoethanol, 4 mM ATP, 0.8 mM CTP, 0.15-2.0 μM tRNA and 1 mg/ ml E. coli S-100 at 37°C for 30 min in 10 μl. 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-^{14}C]

Met and 2 µg 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 µl. 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 $CuSO_4$-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'-^{32}P 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

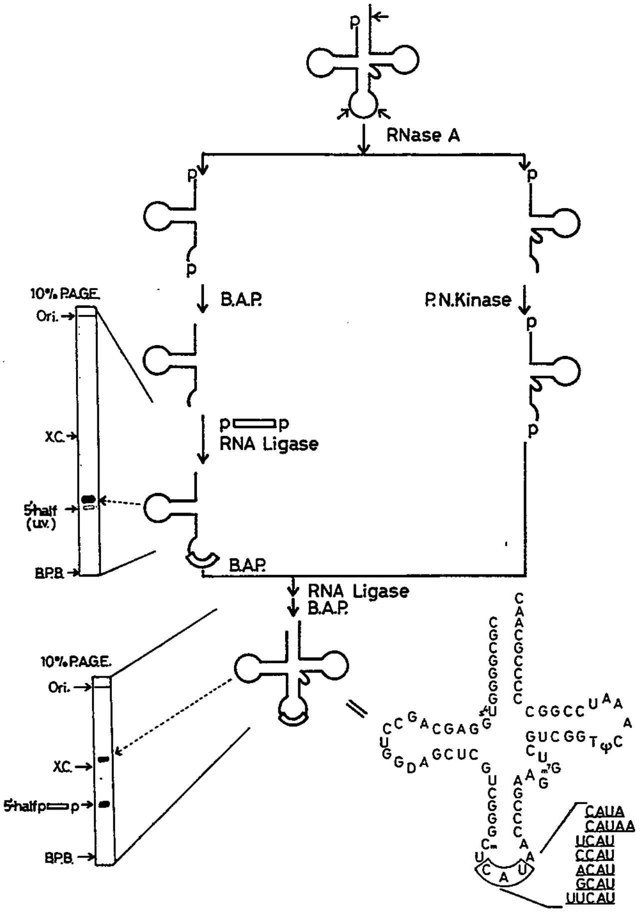

Fig. 1 Scheme for syntheses of modified *E. coli* initiator tRNAs and the pattern of 10% PAGE in each ligation step. Open square shows the synthesized tetra- or penta-ribooligomers corresponding to the anticodon sequence. These autoradiographs of 10% PAGE show the case in which C-C-A-U is the anticodon sequence. In the cases of other ribooligomers, all the PAGE patterns 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 $tRNA_f^{Met}$ with limited amounts of RNase A was treated with phosphatase to remove the 3'-phosphate and with $NaIO_4$ 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. $Cm^{32}pNp$ from the first step of the ligation and $U^{32}p$ 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 $Cm^{32}pNp$ 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

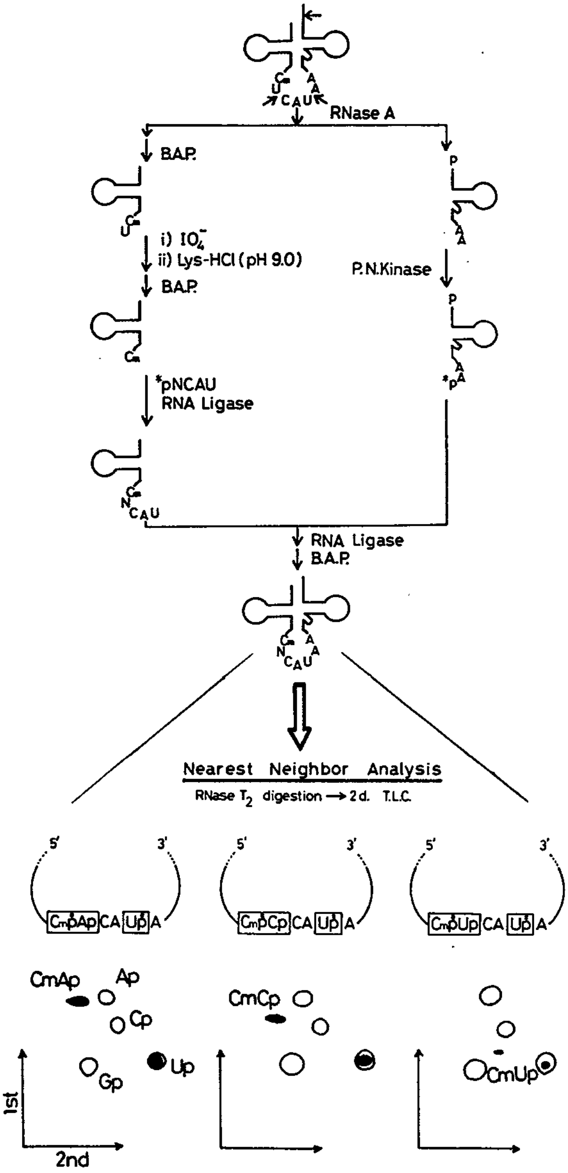

Fig. 2 Scheme for the replacement of constant U and nearest neighbor analyses of these products. The asterisk denotes ^{32}P-phosphate.
N; A, C, U.

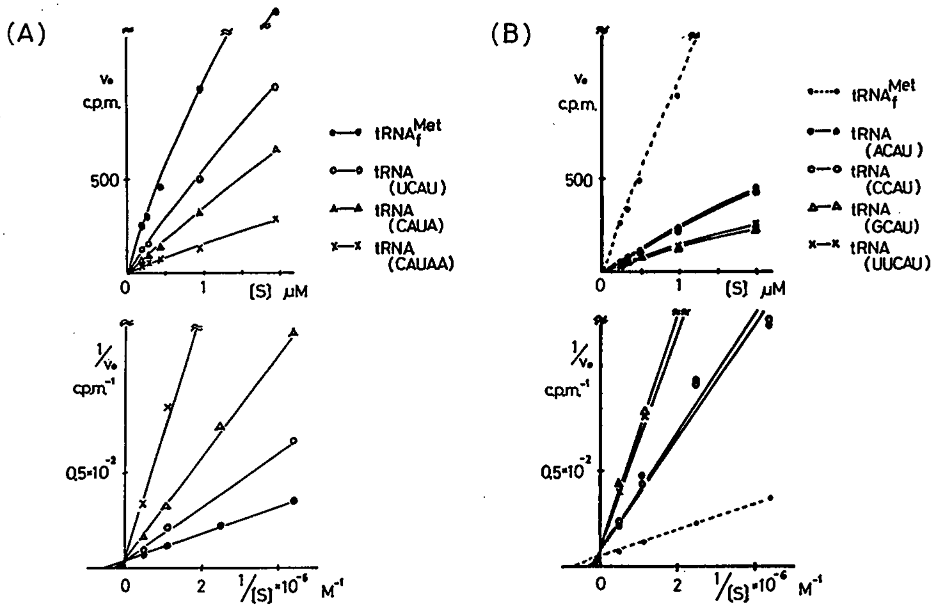

Fig. 3 Aminoacylation of synthesized tRNAs. tRNA(UCAU), tRNA(CAUA), tRNA(CAUAA), tRNA(ACAU), tRNA(CCAU), tRNA(GCAU) and tRNA(UUCAU) indicate the tRNAs whose anticodon sequences are U-C-A-U, C-A-U-A, C-A-U-A-A, A-C-A-U, C-C-A-U, G-C-A-U and U-U-C-A-U, respectively. Lower graphs show the Lineweaver-Burk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

methionyl tRNA synthetase considerably; the Km value was 12.5 μM (the Km value of natural tRNA$_f^{Met}$ 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 Km value was 3.3 μM. On the other hand, A at the 3'-side increased the Km value; the Km 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 Km 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 tRNA$_f^{Met}$, whose constant U was first removed by periodate treatment then replaced. However this control tRNA$_f^{Met}$ did not attain the same level of activity as natural _E. coli_

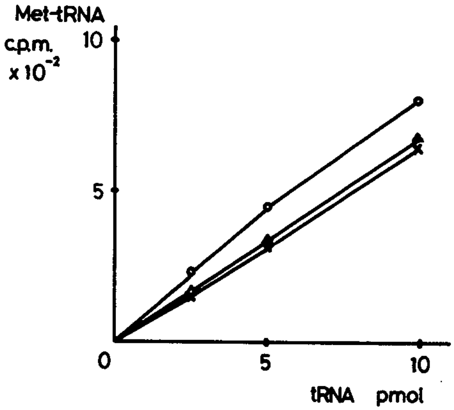

Fig. 4 Methionine acceptor activity of tRNAs with changed constant U. o-o; control tRNA$_f^{Met}$, Δ-Δ; tRNA(C-33) meaning that the constant U is changed to C, x-x; tRNA(A-33).

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(CAUA) 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 ^{32}P-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 1 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 $CuSO_4$-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 $CuSO_4$ treated sample radioactivity to non-treated sample radioactivity was defined as the formylation ratio. The percentages for tRNA(CAUA), 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 bulkyness 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-stranged 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.

Aminoacylation *Formylation* *Elongated aminoacylation end* *Truncated aminoacylation end ligation* *Synthetic oligonucleotide*

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].

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-^{32}P]ATP and L-[U-^{14}C]-methionine were obtained from NEN and Amer-

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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 μl formylation reaction mixture. Half of the mixture was treated with the same volume of 50 mM $CuSo_4$, 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-^{32}P]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)

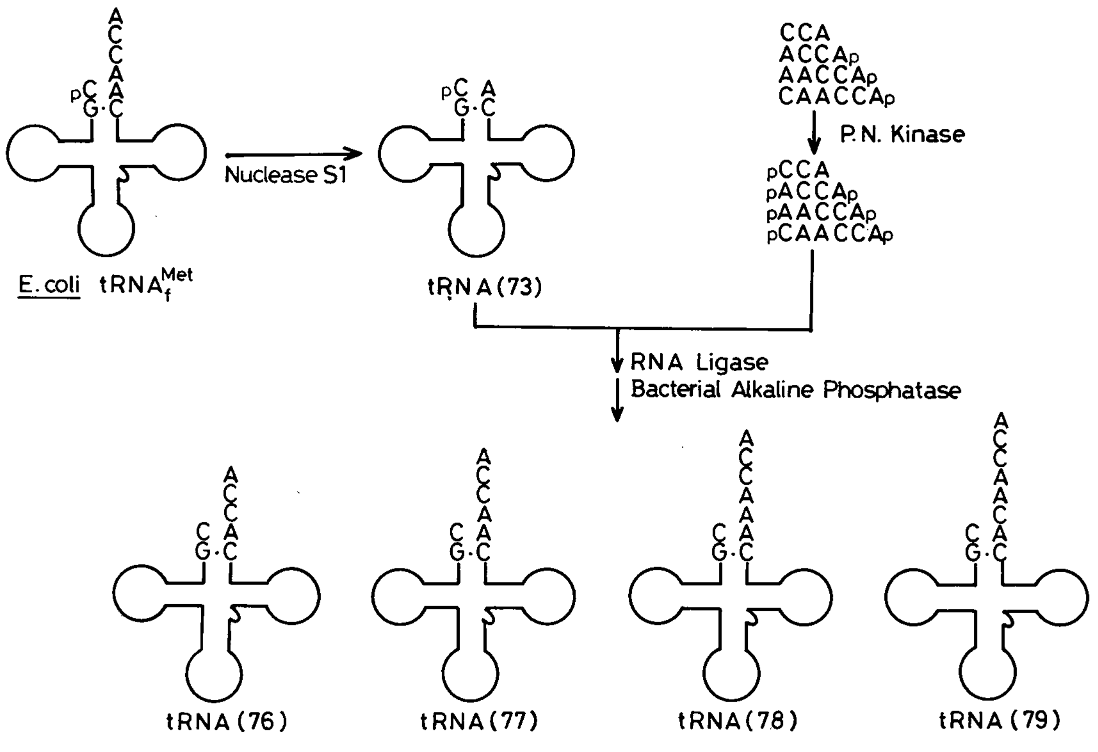

Fig.1. Scheme for the modification of the amino acid acceptor stem of *E. coli* tRNA$_f^{Met}$.

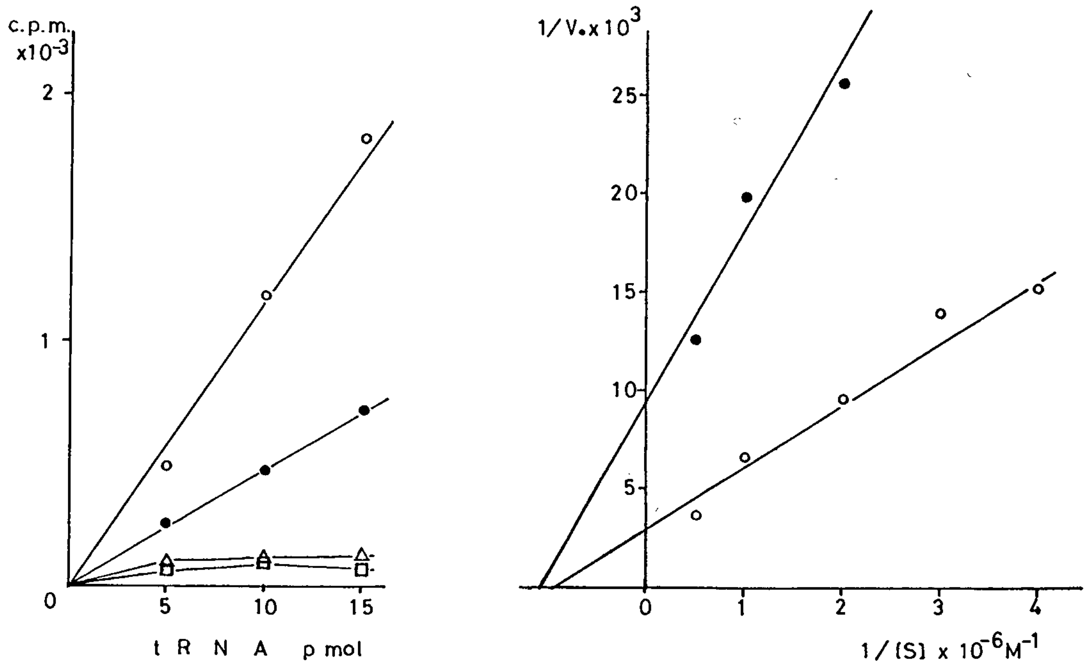

Fig.2.Aminoacylation of modified tRNAs. Methionine acceptor activities of tRNAs (left) and kinetic studies for aminoacylation (right). (○—○) Control tRNA(77), (△—△) tRNA(76), (●—●) tRNA(78), (□—□) tRNA(79).

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^6A) 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^6A, *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.

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]. [γ-^{32}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-

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Abbreviations. t^6A, *N*-[*N*-(9-β-D-ribofuranosylpurine-6-yl)carbamoyl]threonine; i^6A, 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).

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 orizae* and nuclease P1 from *Penicillium citrinium* 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-^{32}P]ATP; this product was then ligated to the 5′ half molecule having the attached trinucleotide [9]. The reconstructed ^{32}P-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 200000 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 $MgCl_2$, 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 ^{32}P (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′-^{32}P-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 cm^2, 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 $MgCl_2$ 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 $MgCl_2$ 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

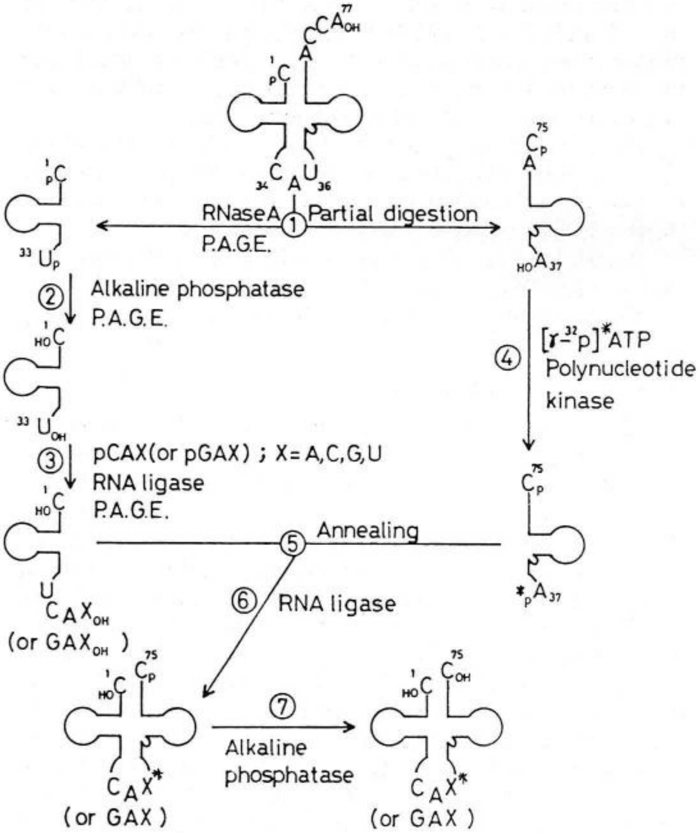

Fig. 1. *Synthetic scheme for the preparation of* E. coli *initiator Met-tRNA variants.* Numbers and names identify the successive steps and molecules used (see text). Each fragment was isolated by electrophoresis on polyacrylamide gel. The asterisk signifies the position of an internal ^{32}P label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

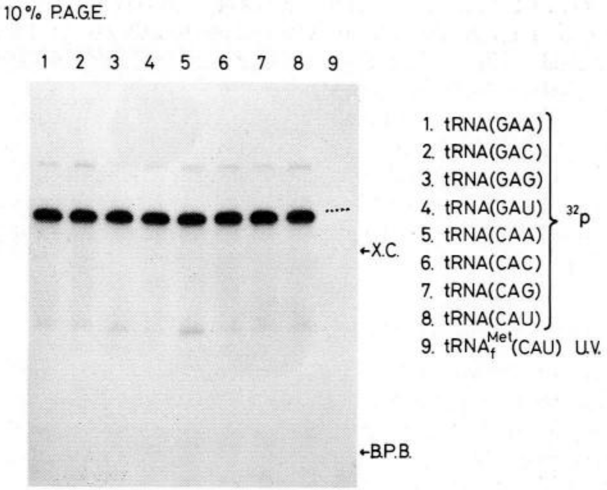

Fig. 2. *Autoradiogram of the purification by electrophoresis on polyacrylamide gel (10% in 7 M urea) of eight tRNA chimery.* This is the gel after step 6 of Fig. 1. The tRNAs lack the 5′ terminus and the C^{75}-A^{76} of the 3′ terminus; therefore, they run correspondingly faster than the authentic 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-lifes of approximately 95 h and 130 h respectively. All other tRNAs had half-lifes 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

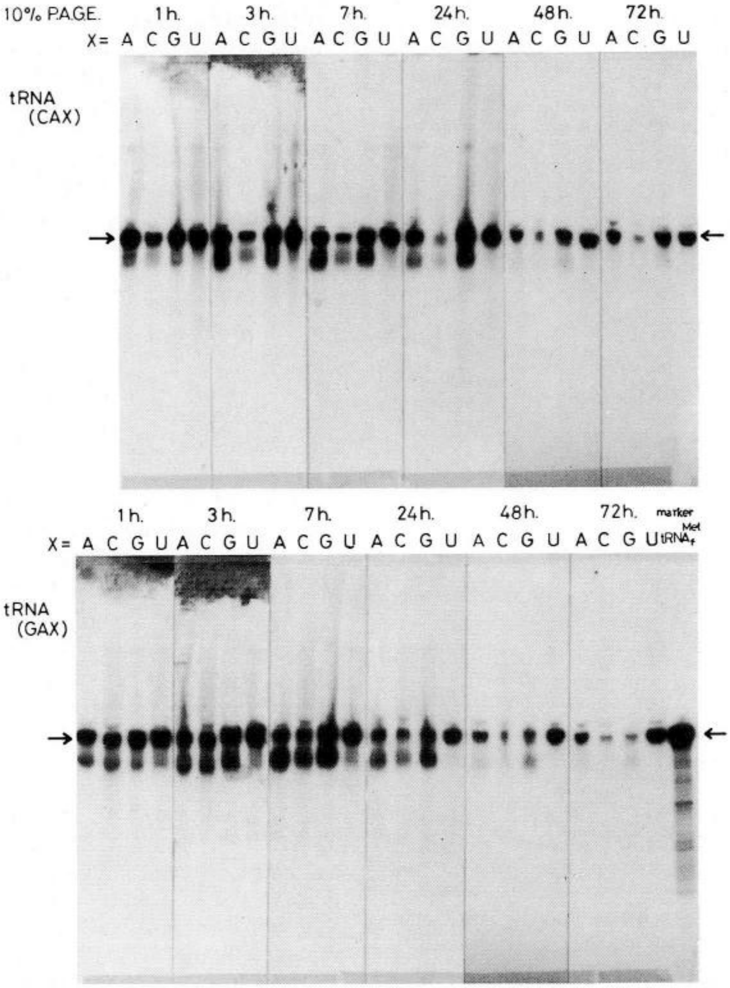

Fig. 3. *Stability of microinjected tRNAs in the* X. laevis *oocytes.* A given amount (fmol level) of purified ^{32}P-labelled tRNA (natural or chimeric) was microinjected into the cytoplasm of a series of five oocytes. At the indicated time after microinjection, total RNA was extracted from the oocytes 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}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}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

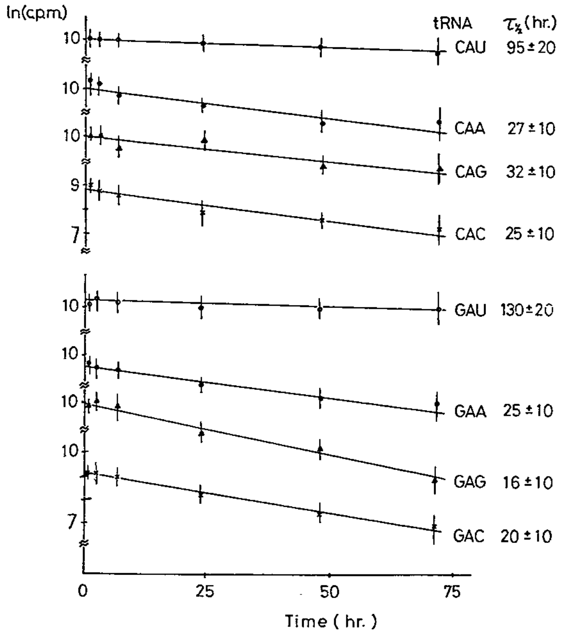

Fig. 4. *Stability of microinjected tRNA (natural or chimeric) in the* X. laevis *oocytes.* The results of Fig. 3 were further analyzed by counting the radioactivity recovered from the oocytes in bands corresponding to the full-length tRNA and the tRNA lacking the terminal nucleotides of the amino acid 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-lifes $t\frac{1}{2}$ 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 underivatized 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-lifes, are the only tRNAs where the A-37 is converted to t^{6}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}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}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}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}A and those ending with A, a i^{6}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 unambigiously 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].

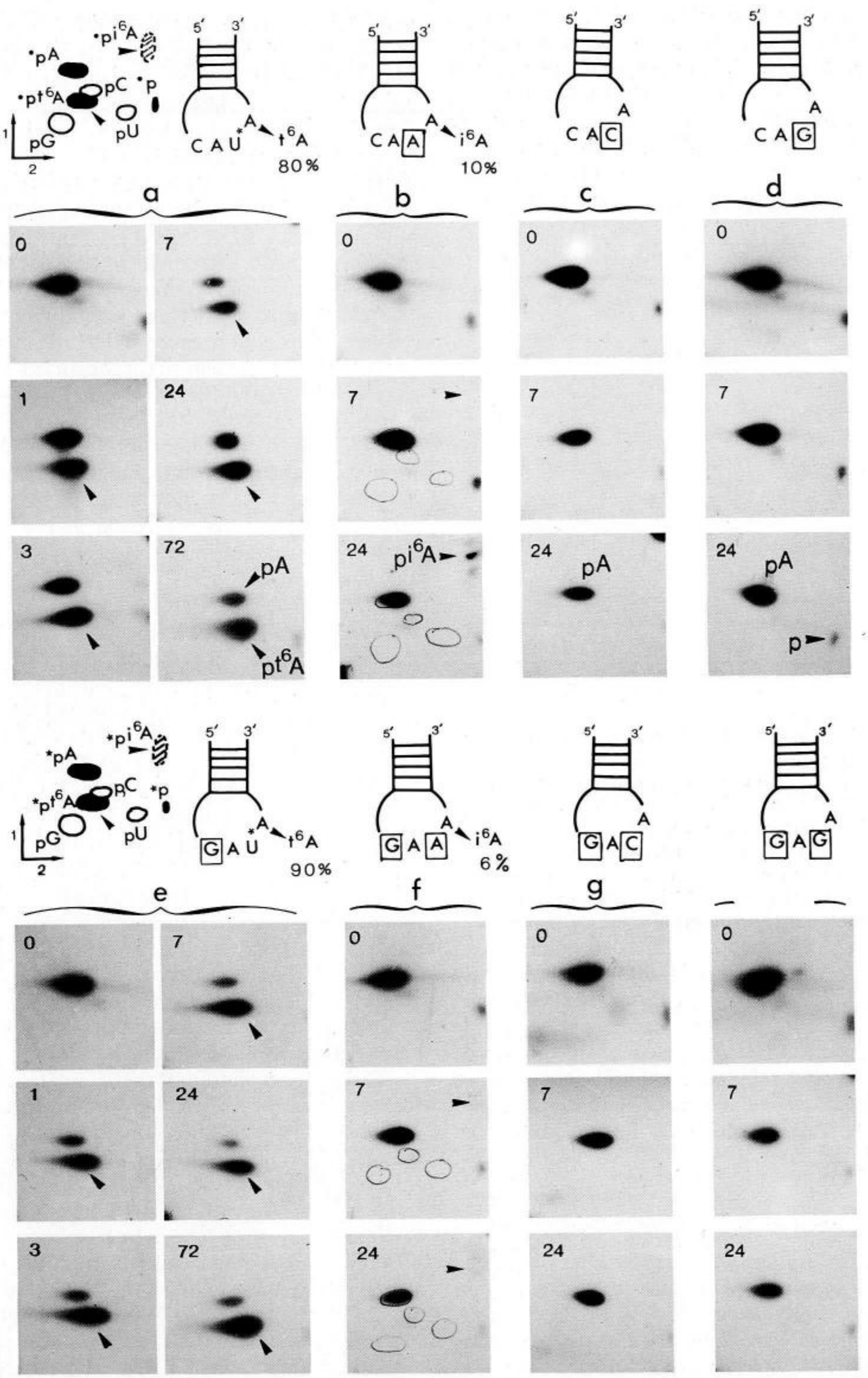

Fig. 5. *Autoradiograms of the separation of 5'-nucleosides by chromatography on thin-layer cellulose plates.* After isolation from the oocytes and purification by electrophoresis on polyacrylamide gel as shown in Fig. 3, the ^{32}P-labelled fMet-tRNA recovered from the gel was completely digested into 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^{6}A and pi^{6}A, are identified by arrows. Percentages correspond to molar yield of transformation (mol/100 mol) of A-37 to t^{6}A-37 or i^{6}A-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 (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 [11 b], 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].

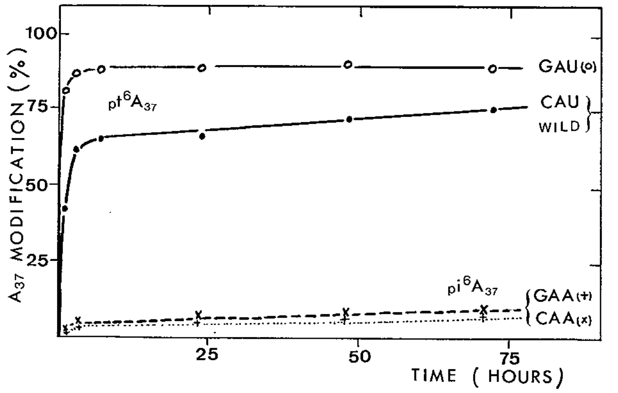

Fig. 6. *The kinetics of A-37 modification*. Results shown in Fig. 5a–h were analyzed by radioactive counting of the various spots on the thin-layer cellulose plates. From these data were calculated the quantities of pt^6A and pi^6A as a function of time, expressed as percentages of the total radioactivity 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

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

P^1-Adenosine 5'-P^2-o-nitrobenzyl pyrophosphate (nbzlppA) has been synthesized as a substrate for T4 RNA ligase catalyzed 3'-phosphorylation. Incubation of oligoribonucleotides and nbzlppA 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 $[P^2\text{-}{}^{32}P]$nbzlppA, 3'-termini of oligoribonucleotides could be labelled with ^{32}P. 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-Gpnbzl and phosphorylated with polynucleotide kinase. pU-A-Gpnbzl was then joined to an acceptor trinucleotide A-U-G to yield A-U-G-U-A-Gp.

INTRODUCTION

T4 RNA ligase[2] 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 chemical[8] 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'-phosphate[13] 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-

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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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MATERIALS AND METHODS

nbzlppA. o-Nitrobenzyl phosphate was synthesized by condensation of o-nitrobenzyl alcohol with phosphoric acid using trichloroacetonitrile[15] and adenylated by treatment with adenosine 5'-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)x2, 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 λmax (H_2O) 261, λmax (H^+) 256, λmax (OH^-) 261 nm. The yield of nbzlppA was 1382 A_{260} 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.

nbzl*ppA. The [^{32}P] 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 $MgCl_2$, 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. Homochromatography[19] was performed using Homomix I-V.[20] Partial digestion with Nuclease P1 and venom phosphodiesterase were as described previously.[9]

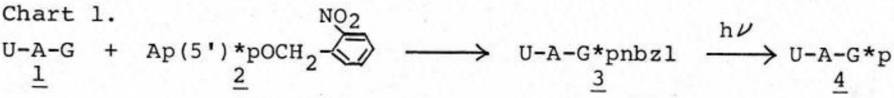
For the removal of the o-nitrobenzyl group, compounds were placed in a pyrex tube (1.5 mm thick) and irradiated through a pyrex filter (2 mm thick) inserted in a water jacket. The photolysis apparatus had a 300 W high pressure mercury lamp (Eikosha Model PIH 300) with a quartz water circulating jacket.

RESULTS

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

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

Chart 1.



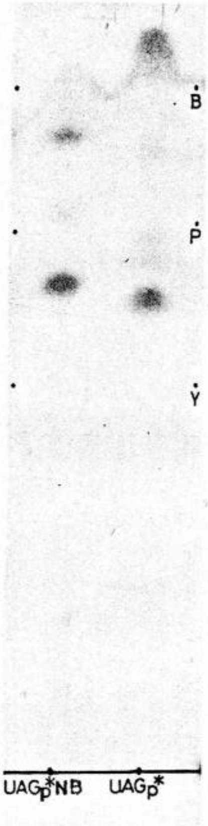


Figure 1. Homochromatography of the products after phosphorylation of U-A-G with nbzl*ppA. Before photoirradiation, U-A-G-*pnbzl (the slower spot); after photoirradiation, U-A-G*p (the slowest spot). nbzl*ppA travels behind the blue marker.

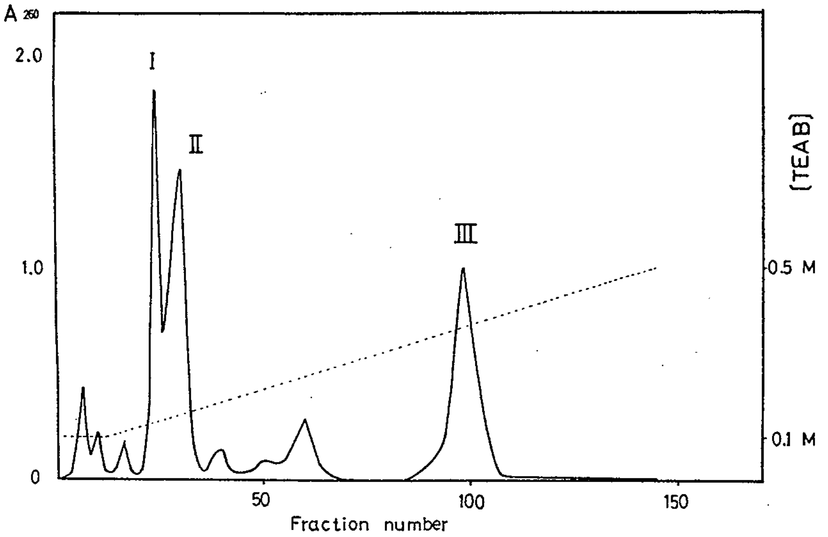


Figure 2. Chromatography of the products in the RNA ligase reaction (605 µl) of nbzlppA (1025 nmol) with U-A-G (532 nmol) using RNA ligase (28 units) on a column (0.6 x 15 cm) of DEAE-Sephadex A25. Elution was performed with a linear gradient of triethylammonium bicarbonate (pH 7.5, 0.1 M to 0.5 M, total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

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-Gpnbzl (5) was prepared in quantity using a two fold excess of unlabelled nbzlppA and RNA ligase and isolated by chromatography on Sephadex as shown in Fig. 2. 5

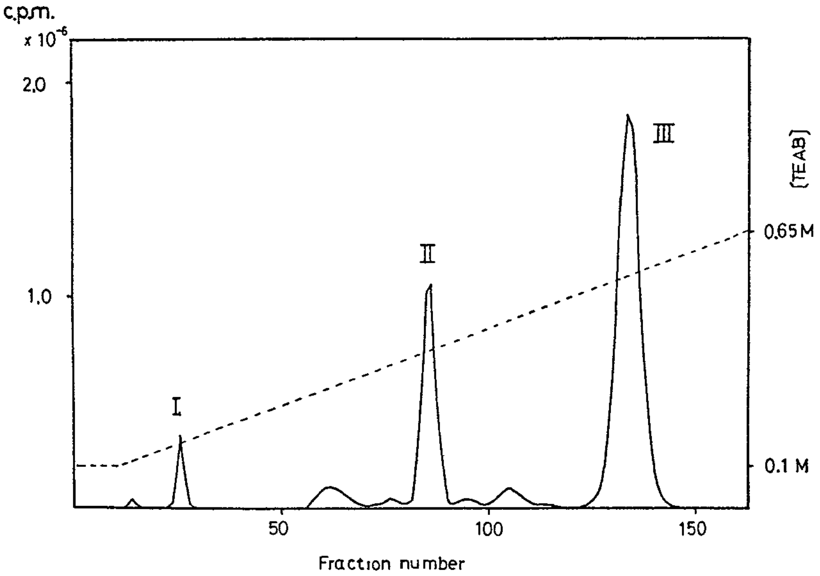


Figure 3. Chromatography of the product in the phosphorylation of U-A-Gpnbzl(292 nmol) with [γ-^{32}P]ATP(480 nmol) and polynucleotide kinase (15 units) on DEAE-Sephadex A25 using a linear gradient of triethylammonium bicarbonate pH 7.5 from 0.1 M to 0.65 M (total, 220 ml). Fractions of 1.6 ml were collected every 10 min. Radioactivity was counted by Cerenkof'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

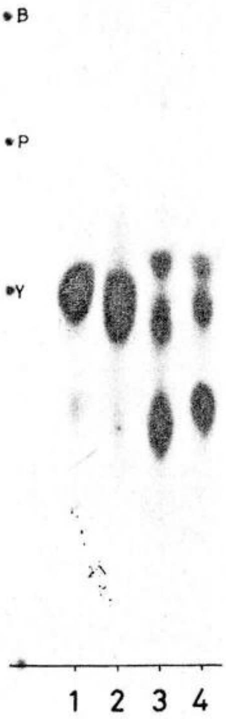


Figure 4. Homochromatography of the products in the joining of *pU-A-Gpnbzl to A-U-G. 1, *pU-A-Gpnbzl; 2, *pU-A-Gp; 3, A-U-G*pU-A-Gp (the slowest spot, after photoirradiation); 4, A-U-G*pU-A-Gpnbzl (the slowest spot, before photoirradiation).

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.

$$\underset{5}{\text{U-A-Gpnbzl}} + [\gamma\text{-}^{32}\text{P}]\text{ATP} \longrightarrow \underset{6}{\text{*pU-A-Gpnbzl}} \xrightarrow[\text{RNA ligase}]{\text{A-U-G}}$$

$$\underset{7}{\text{A-U-G*pU-A-Gpnbzl}} \xrightarrow{h\nu} \underset{8}{\text{A-U-G*pU-A-Gp}} \longrightarrow \underset{9}{\text{A-U-G*pU-A-G}}$$

The 3'-modification by o-nitrobenzyl phosphorylation of

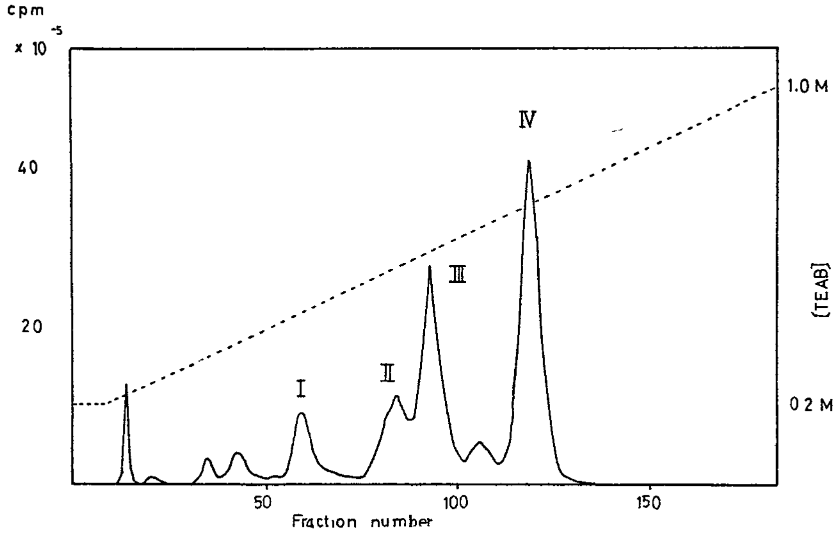


Figure 5. Chromatography of the deblocked product, A-U-G-U-A-Gp after joining of *pU-A-Gpnbzl (277 nmol) to A-U-G (278 nmol) using RNA ligase (14 units in total volume of 450 µl) on a column of DEAE-Sephadex A25. The conditions for elution were the same as described in Fig. 2. Peaks: I, unidentified, II, mainly dephosphorylated product, A-U-G*pU-A-G; III, the reaction intermediate, Ap(5')pU-A-Gp; IV, A-U-G*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 o-nitrobenzyl phosphate (nbzlppA) was recognized by RNA ligase as a donor molecule and that o-nitrobenzyl phosphate was transfered 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 o-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 irradiation with UV light of wavelength longer than 280nm.[22]

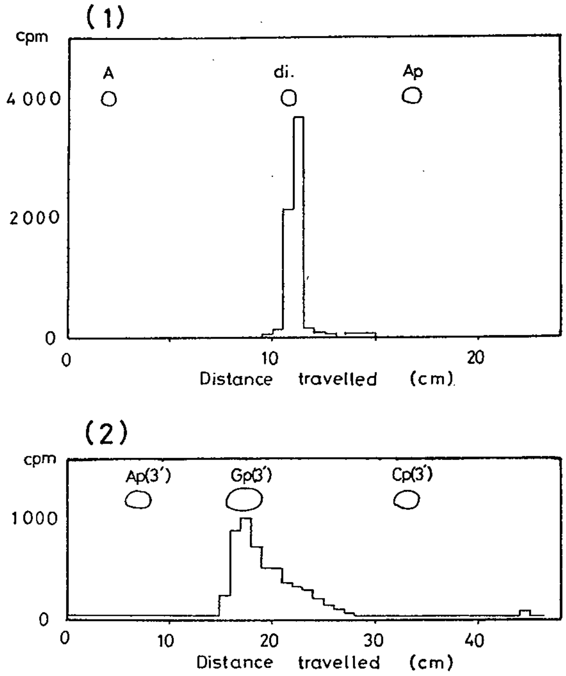


Figure 6. Characterization of A-U-G*pU-A-G. (1) Paper electrophoresis of the digested product (G*pU) after RNase A plus phosphatase treatment. A, Ap and dimer indicate markers detected by UV absorption (2) Paper chromatography of the digested product (G*p) in an RNase M hydrolysis. Ap, Gp and Cp show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

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

Acceptor	(nmol)	nbzlppA (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-Zpnbzl from homochromatogram after the reaction mixture was phosphorylated with [γ-^{32}P]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 (nbzl*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 kinase[24] may overcome this problem, it is desirable to protect the 3'-phosphate during 5'-kination. This type of reaction was examined in our kination 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'-kination 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.

ACKNOWLEDGEMENT

The authors thank Drs. M. Sugiura and A. Matsushiro for advices and provision of facilities for RNA ligase purification. Also thanks are due to Dr. A. F. Markham for reading the manuscript. This work was supported by Grant-in-Aid from the Ministry of Education, Science and Culture, Japan.

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Biochemistry

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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Communicated by H. Gobind Khorana, June 8, 1981

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-^{32}P]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 $MgCl_2$/10% (vol/vol) dimethyl sulfoxide containing bovine serum albumin at 10 μg/ml. The 3′-phosphate was removed by treatment with *E. coli* alkaline phosphatase in 50 mM Tris·HCl (5 μl, pH 8.1) at 55°C for 30 min. The enzyme was inactivated by treatment with 1 μl

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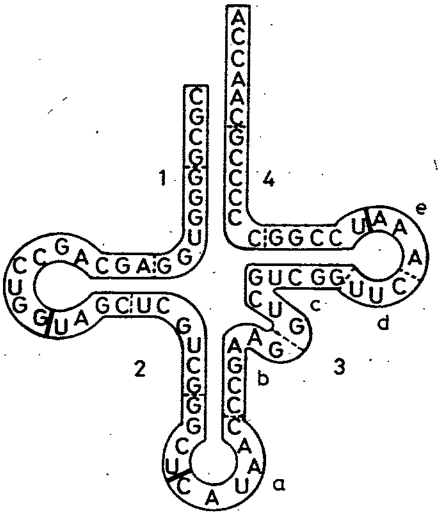

FIG. 1. Structure of *E. coli* tRNA$_f^{Met}$. The quarter molecules 1, 2, and 4 were obtained by joining chemically synthesized oligonucleotides with RNA ligase. Quarter molecule 3 was joined as shown in Fig. 2.

of 40 mM EDTA at room temperature for 30 min and then at 100°C for 2 min (28) and extracted twice with 2 μl of phenol saturated with 50 mM Tris·HCl (pH 8.1). The phenol layer was washed twice with water (20 μl) and the aqueous phase was subjected to gel filtration on a Sephadex G-50 column (1.1 × 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 4 μg/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 μM L-[^{14}C]methionine (582 Ci/mol; 1 Ci = 3.7×10^{10} becquerels) in total volume of 20 μl 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 μl) and the mixture was applied to a column (0.8 × 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 [^{14}C]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 ^{32}P radioactivity became negligible.

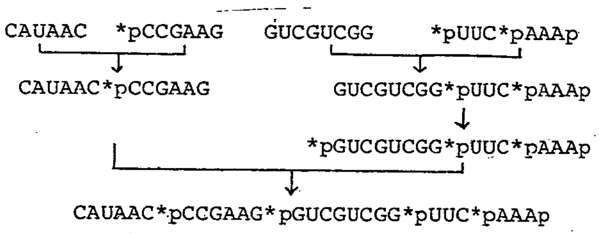

FIG. 2. Synthesis of quarter molecule 3 (bases 35–60).

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 (μM)	Donor, nmol (μM)	ATP, μM	Enzyme, μg/ml	Temp., °C	Time, hr	Isolated yield, %
CAUAAC 80 (1000)	pCCGAAG 8 (100)	200	100	25	1	39
GUCGUCGG 15 (150)	pUUCAAAp 10 (100)	200	140	25	1	36
CAUAACCCGAAG 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

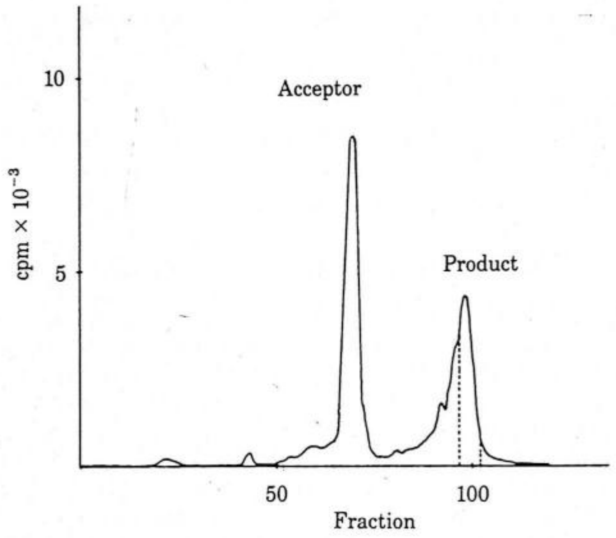

FIG. 3. Disk gel electrophoresis of the 26-nucleotide-long segment **3** (C-A-U-A-A-C*pC-C-G-A*pG-U-C-G-U-C-G-G-U-U-C-A-A-Ap) (Fig. 2) on 20% acrylamide. The first peak contained the acceptor (C-A-U-A-A-C*pC-C-G-A) and the last peak contained the product.

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-^{32}P]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. 5*A*). 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′-phosphorylated with polynucleotide kinase and [γ-^{32}P]ATP and joined to the 5′-half molecule at reduced temperature (Table 1).

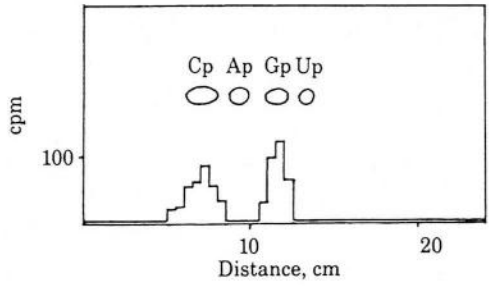

FIG. 4. Nearest-neighbor analysis of **3** after digestion with RNase T2 and paper electrophoresis at pH 3.5.

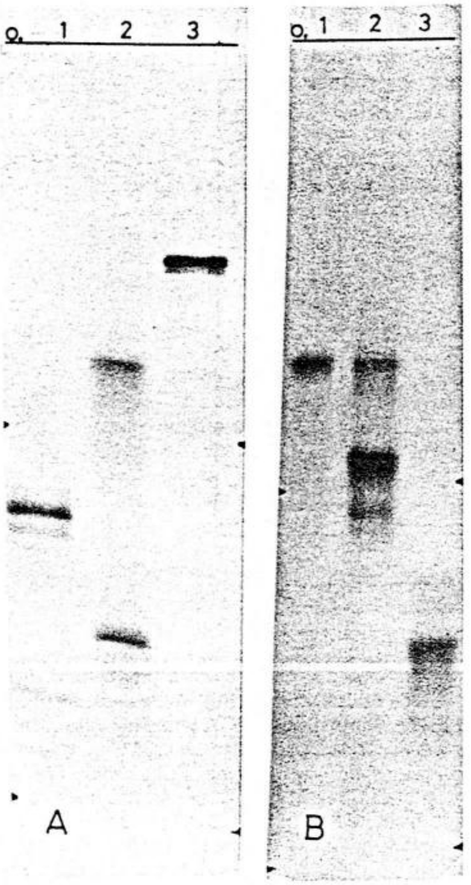

FIG. 5. Polyacrylamide gel electrophoresis of products. (*A*) Synthesis of the 5′-half molecule from **1** and **2** (lane 2). Lane 1: the 5′ quarter molecule, **1**. Lane 3: the three-quarter molecule. (*B*) Synthesis of the 3′-half molecule from bases 35–46 and bases 41–77 (lane 2). Lane 1: bases 35–77.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

The reaction mixture was subjected to gel filtration (Fig. 6*A*). 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 ^{32}P-labeling (with polynucleotide kinase and [γ-^{32}P]ATP) and RNase T2 digestion yielded [^{32}P]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. 5*B*) 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

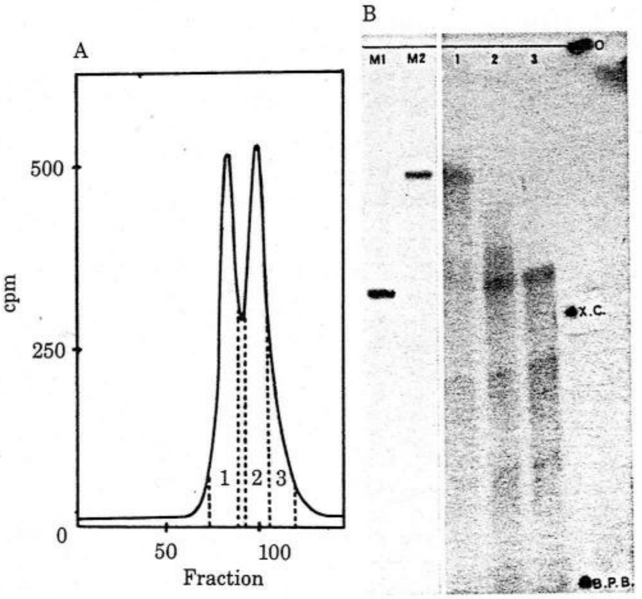

FIG. 6. (*A*) Gel filtration of products in the synthesis of the total molecule from the 3'- and 5'-half molecules on a column (0.7 × 90 cm) of Sephadex G-200 equilibrated with 50 mM potassium phosphate; pH 7.5/0.1 mM EDTA. Elution was at 50°C with flow rate 1.2 ml/hr; 0.22-ml fractions were collected. 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 kination. 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-[^{14}C]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 [^{14}C]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 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.

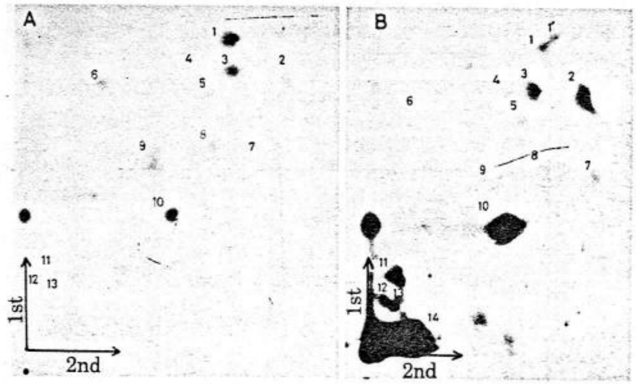

FIG. 7. Two-dimensional thin-layer chromatography of RNase T1 fragments of the natural tRNA$_f^{Met}$ (*A*) and of the synthetic nascent molecule (*B*) on PEI-cellulose plates (20 × 20). The plates were irrigated with 1.4 M lithium formate, pH 3.5/7 M urea for 10 cm, and then with 2.3 M lithium formate, 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, ps^{4}U-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$_i$; 11, pCmU-C-A-U-A-A-C-C-C-G or pC-U-C-A-U-A-A-C-C-C-G; 12, pm^{7}G-U-C-G or pG-U-C-G; 13, pT-Ψ-C-A-A-A-U-C-C-G or pU-U-C-A-A-A-U-C-C-G; 14, ATP.

The chemical synthesis of oligoribonucleotides that have sequences of *E. coli* tRNA$_f^{Met}$ and their analogs (e.g. U-G-C-C-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 ^{32}P 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 NH_2-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 HA_0 into the HA_1 and HA_2 subunits during infection with the HA–SV40 viral recombinant. This was not surprising as cleavage of HA_0 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* tRNA$_2^{Gly}$

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Abstract—A tritriacontanucleotide which has the sequence of the 5′-half molecule of *E.coli* glycine tRNA$_2$, 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-benzoylcytidine, N-benzolyadenosine 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 arenesulfonyl 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* tRNA$_2^{Gly}$.[6] The synthesis involved phosphotriester block condensations using a combination of tetrahydrofuranyl[7] and dimethoxytrityl[8] 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 problems and various groups have been used in combination with selectivity removable 5′-O-protecting groups. Tetrahydropyranyl,[8] 4-methoxytetrahydropyranyl,[11] tert-butyldimethylsilyl,[12] and *o*-nitrobenzyl[13] groups have been used for synthesis of larger ribooligonucleotides: octadecamer,[14] nonadecamer[15] 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 derivatives[22] and 5′-chloroquinolinyl[23] 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-tetrahydrofuranylnucleosides (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

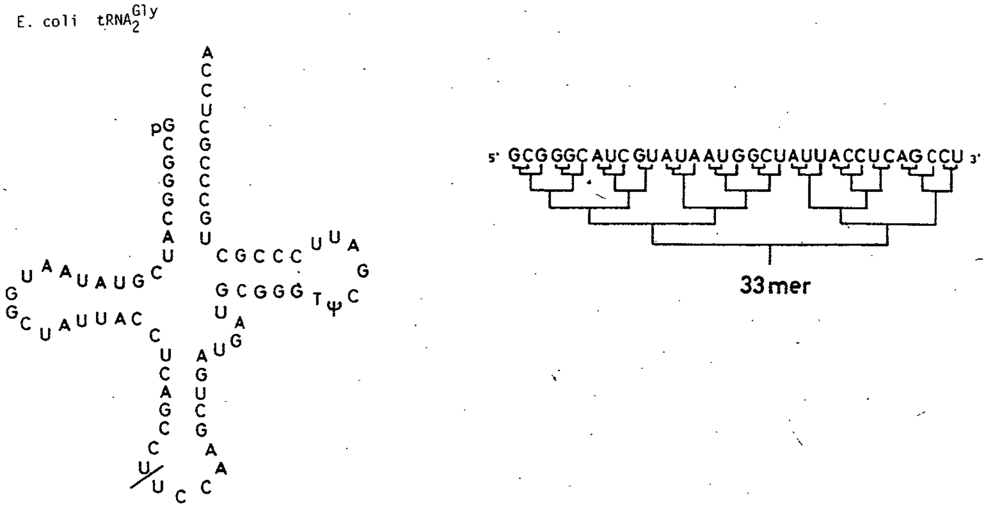


Fig. 1. Structure of the *E.coli* tRNA$_2^{Gly}$ and the 5′-half sequence (1–33).

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) phosphate[27] using mesitylenesulfonyl tetrazolide (MSTe, 1-(2,4,6-trimethylbenzenesulfonyl)-1H-tetrazole).[28] For further 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.

Trimers were prepared from the above dimers by

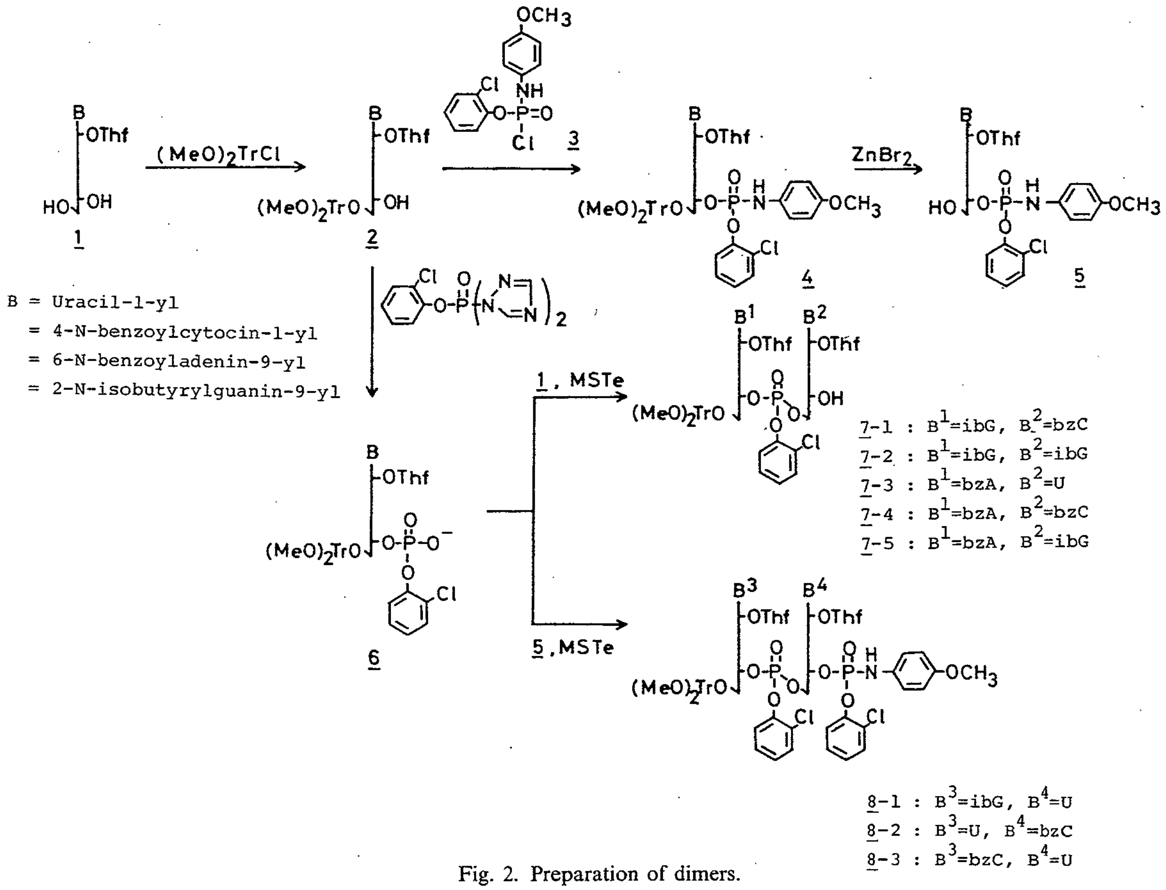


Fig. 2. Preparation of dimers.

Table 1. Reaction conditions for the synthesis of dimers

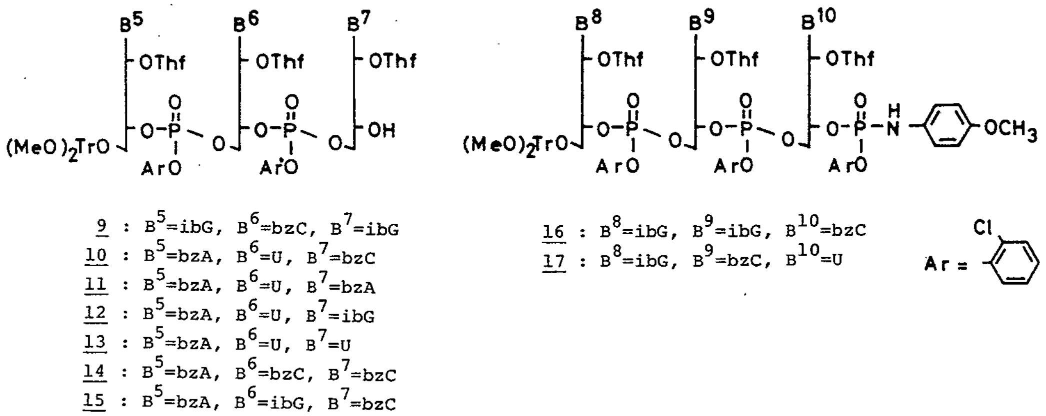
3'-Phosphodiester Component* (mmol)		5'-OH Component (mmol)		MSTe (mmol)	Time (min)	Product		Yield (%)
DT[G]OH	(8.00)	HO[C]OH	(11.23)	15.90	25	DT[GC]OH	(7-1)	62
DT[G]OH	(2.81)	HO[G]OH	(3.93)	5.60	25	DT[GG]OH	(7-2)	72
DT[A]OH	(10.13)	HO[U]OH	(13.99)	19.91	25	DT[AU]OH	(7-3)	75
DT[A]OH	(2.45)	HO[C]OH	(3.29)	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 = $NH\phi\text{-}p\text{-}OCH_3$, 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 H_2O.

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 tritriacontemer 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*-anisidate 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 tritriacontamer (**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-carboaldoximate (TMG-PAO),[29] (3) ammonium hydroxide, (4) anion-exchange resin Dowex 50 W × 2 (pyridinium form), and (5) dilute hydrochloric acid (pH 2). The deblocked product was



9 : B^5=ibG, B^6=bzC, B^7=ibG
10 : B^5=bzA, B^6=U, B^7=bzC
11 : B^5=bzA, B^6=U, B^7=bzA
12 : B^5=bzA, B^6=U, B^7=ibG
13 : B^5=bzA, B^6=U, B^7=U
14 : B^5=bzA, B^6=bzC, B^7=bzC
15 : B^5=bzA, B^6=ibG, B^7=bzC

16 : B^8=ibG, B^9=ibG, B^{10}=bzC
17 : B^8=ibG, B^9=bzC, B^{10}=U

. 3

Fig. 3. Structure of trimers.

Table 2. Reaction conditions for the synthesis of trimers

3'-Phosphodiester 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 = NHϕ-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-tetrahydrofuranyl 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 electrophoresis[30] of the 5′-labeled products. For mobility shift analysis[31] and polymers were labeled at either end with polynucleotide kinase[32] and $[\gamma\text{-}^{32}P]$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 T2[34] followed by labeling with polynucleotide kinase and $[\gamma\text{-}^{32}p]$ ATP. The results of two dimensional thin layer chromatography on cellulose[35] 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[AUGGCU]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[AUAAUGGCU]pAn (44)	56
47	(0.064)	10	2	HO[AUUACCUCAGCCU]pAn (49)	66

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

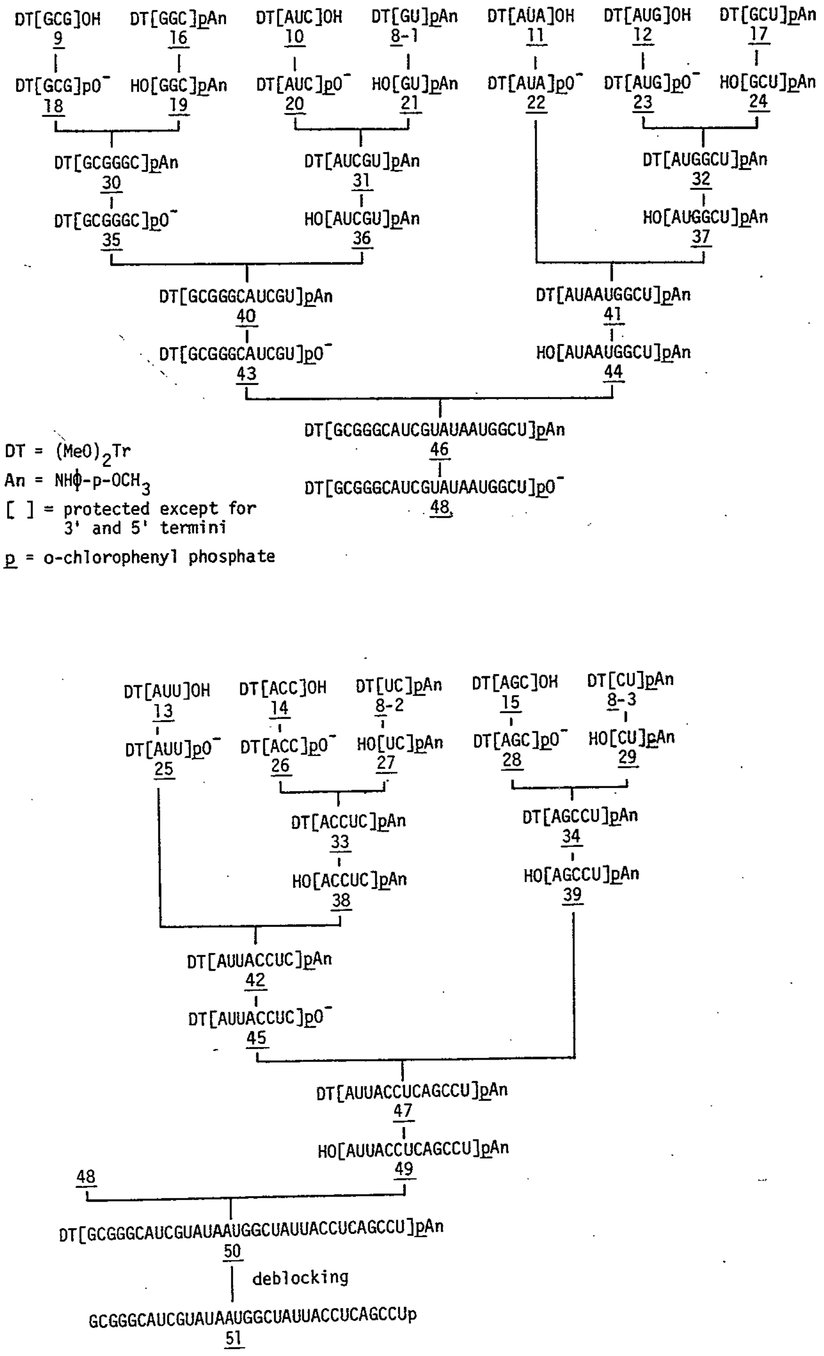


Fig. 4. Synthesis of the 33 mer.

EXPERIMENTAL

TLC was performed on plates of silica gel (Kieselgel 60 HF$_{254}$, Merck) using a mixtures of $CHCl_3$ and MeOH. For reversed phase TLC (RPTLC), silanized silica gel (Kieselgel 60 HF$_{250}$ Silanisiert, Merck) was used with a mixture of acetone–water. For columns, silica gel (type 60 or 60 H, Merck) was used with a mixtures of $CHCl_3$–MeOH. For preparative reversed phase chromatography, 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, Toyosoda).

Two dimensional homochromatography[31] was performed as described previously.[36]

Triethylammonium bicarbonate (TEAB) buffer (pH 7.5)

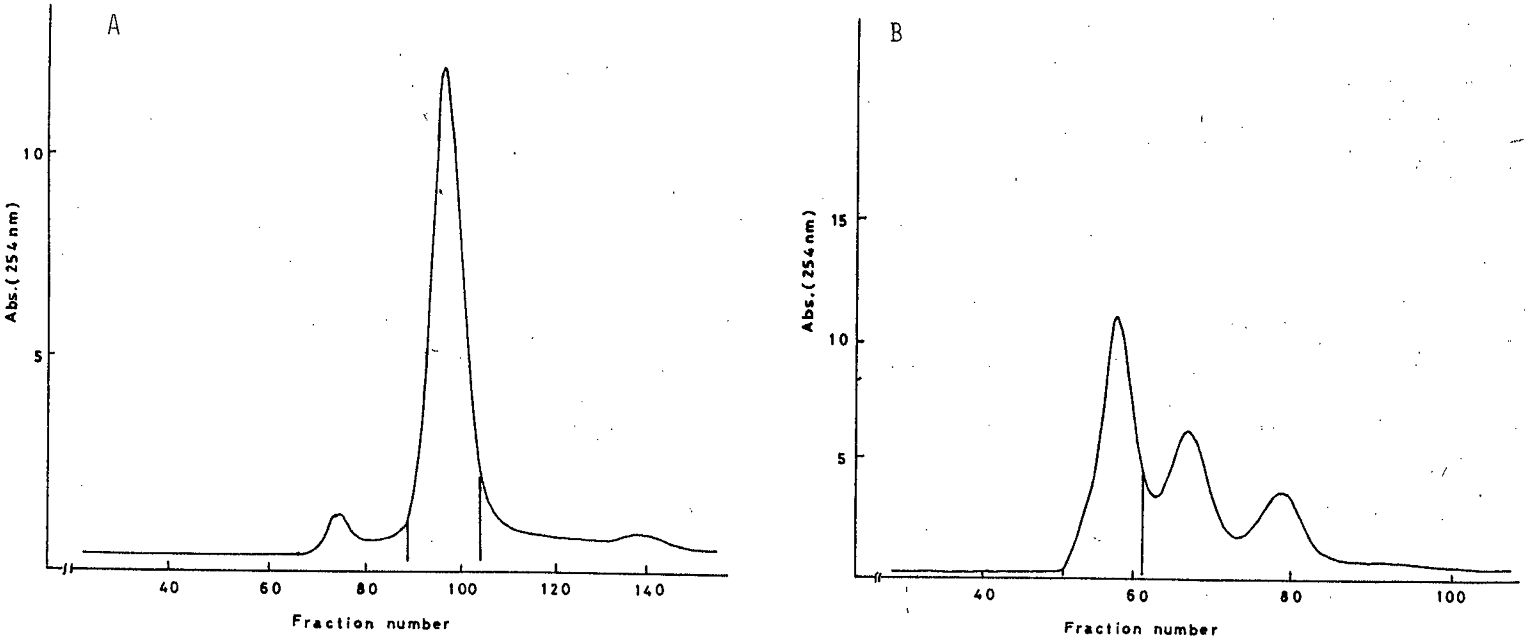


Fig. 5. Gel filtration of the 22 mer (A) and 33 mer (B) on a column (2.8 × 117 cm) of Sephadex G-50 equilibrated with 0.1 M TEAB. A, Fractions of 2.7 ml were collected every 5 min. B, Fractions of 3.9 ml were collected every 6 min.

Table 4. Conditions for block condensation

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

a) : DT[NNN]OH was converted to 3'-phosphodiester component by phosphorylation with o-chlorophenyl phosphoroditriazolide followed by treatment with H_2O.

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 $CHCl_3$ (150 ml). The aqueous phase was reextracted with $CHCl_3$-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 $CHCl_3$ and washed with sat $NaHCO_3$ 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 $CHCl_3$, and precipated with hexane from its soln in $CHCl_3$, 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

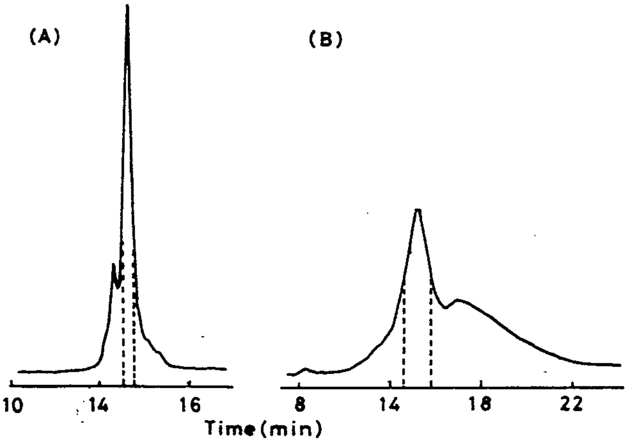


Fig. 6. Purification of the 22 mer (A) and 33 mer (B) by HPLC on silica gel (TSK-LS410) with a flow rate of 2 ml/min. A, a linear gradient of acetonitrile (5–25% during 30 min) in 0.1 M triethylammonium acetate. B, a linear gradient of acetonitrile (11–15% during 03 min) in 0.1 M triethylammonium acetate.

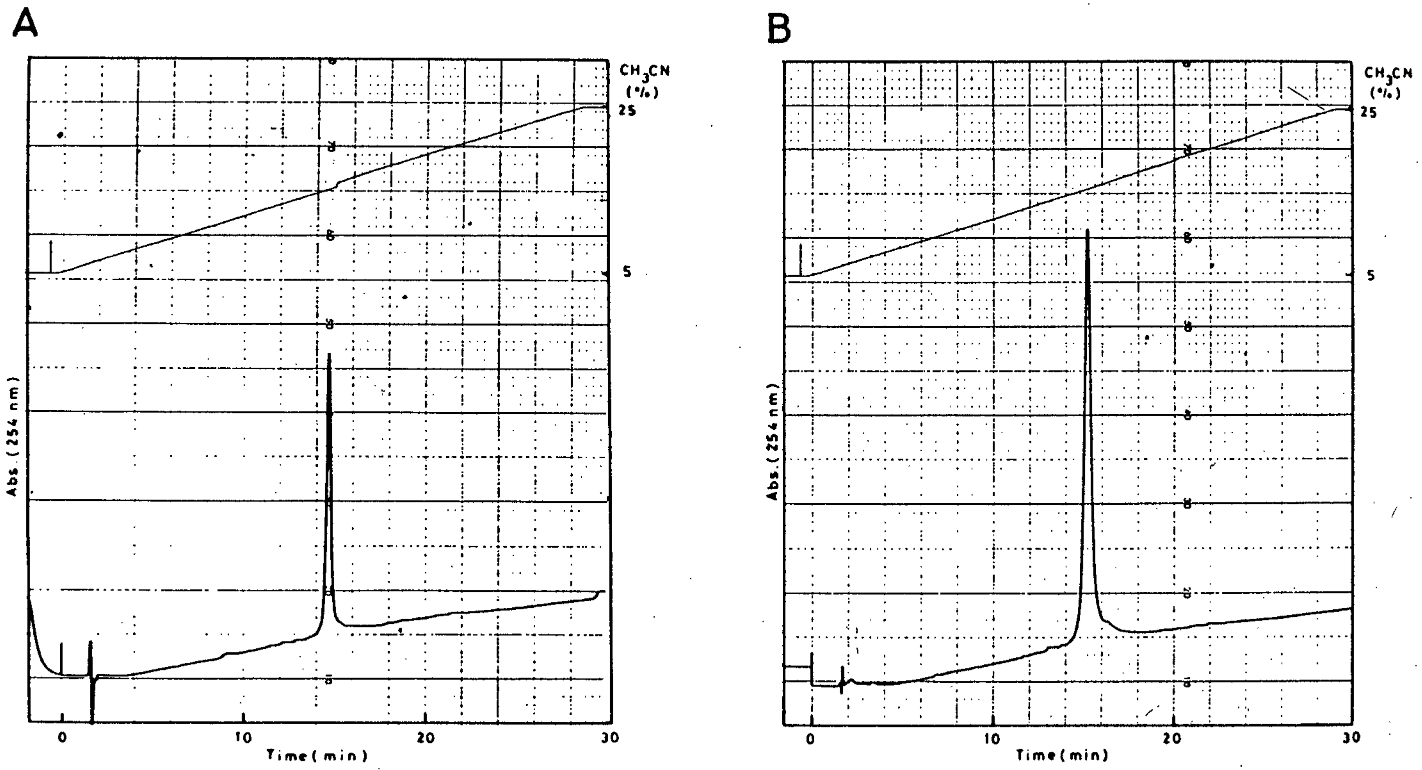


Fig. 7. Analysis of the purified 20 and 33 mer by HPLC on C-18 silica gel (TSK-LS410) in 0.1 M triethylammonium acetate.

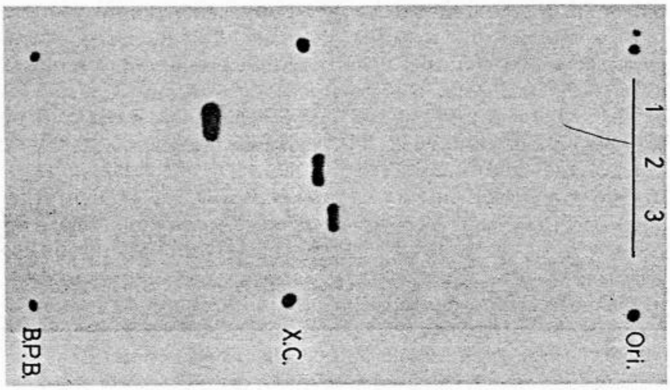


Fig. 8. Gel electrophoresis of the 20 mer (1), 33 mer (2) and a marker (3, 34 mer from the *Ecoli* tRNA$_f^{Met}$) on 10% polyacrylamide.

(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 CHCl$_3$ and washed twice with sat NaHCO$_3$ aq (70 ml). The product (8-2) was isolated by reversed phase chromatography on C-18 silica gel (ϕ 4 × 9.5 cm) using a gradient of acetone in 0.2% pyridine and precipated with hexane from its soln in CHCl$_3$. 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 × 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 ZnBr$_2$ in CH$_2$Cl$_2$-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 CH$_2$Cl$_2$ (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 × 5.5 cm) of Kieselgel 60 H (40 g). The oligonucleotide was eluted with a gradient of MeOH in CHCl$_3$ and precipated with hexane from its soln in CHCl$_3$. 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 CHCl$_3$ (140 ml), washed three times with 0.2 M TEAB (100 ml), applied to a column (ϕ 4 × 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 precipated 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 × 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 × 7 cm) of C-18 silica gel. The product **46** was purified by chromatography on a column (ϕ 3 × 2.8 cm) of silica gel (Kieselgel 60 H, 7 g) using a gradient of MeOH in CHCl$_3$. 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 CHCl$_3$-pyridine (2:1, 45 ml), washed 4 times with 0.2 M TEAB (50 ml) and precipated with ether–pentane (1:4, 50 ml) from its soln in CHCl$_3$ (2.5 ml). The eicosamer **48** was reprecipated, 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 CHCl$_3$-pyridine (5:2, 70 ml), washed twice with 0.1 M TEAB (50 ml) and precipated with pentane from its soln in CHCl$_3$. 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 CHCl$_3$–pyridine (2:1, 30 ml). The eicosamer was precipated 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°

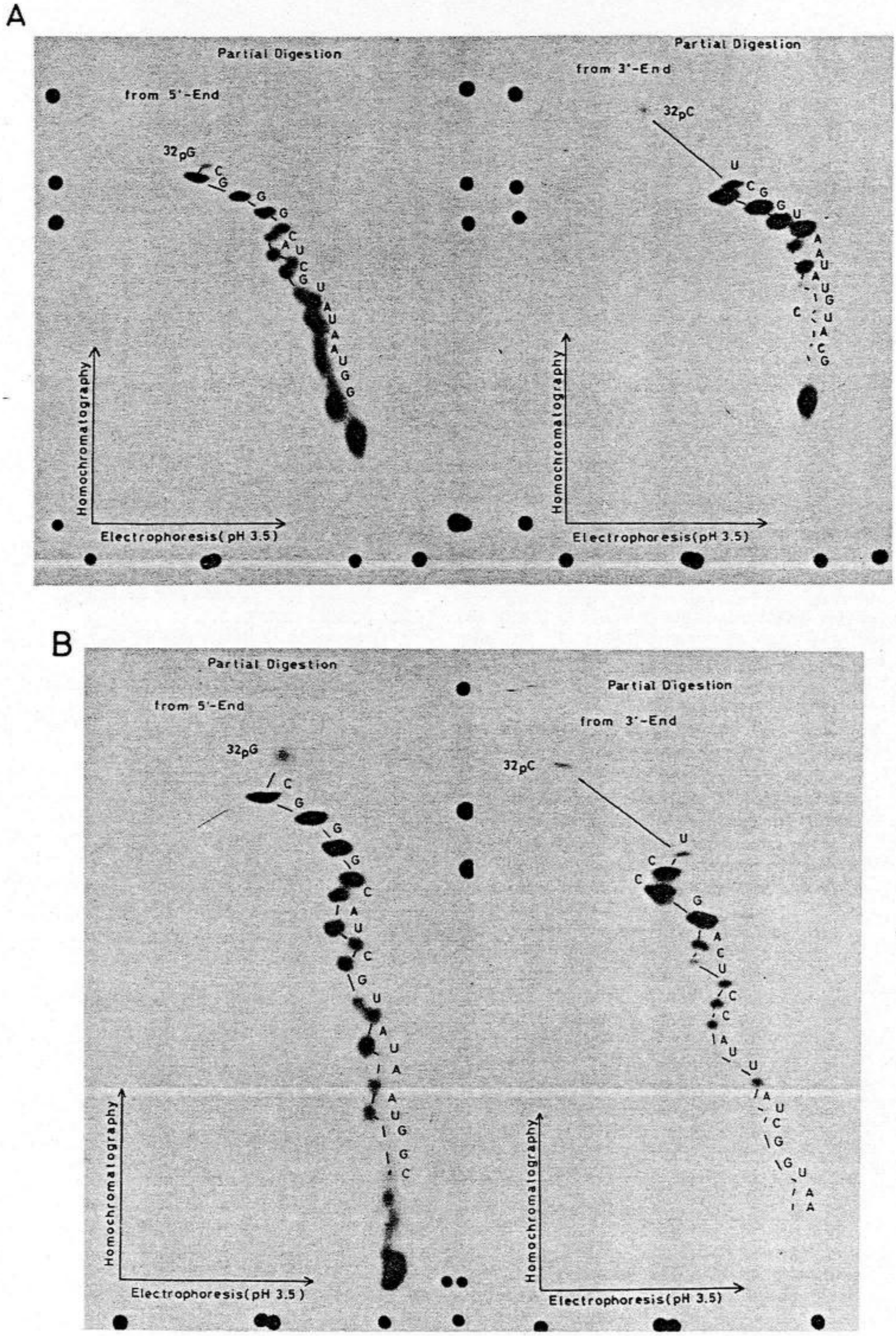


Fig. 9. Mobility shift analysis of the 20 mer (A) and 33 mer (B) using Homo-mix I.[31b]

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 × 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 m). 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 A_{260}) was

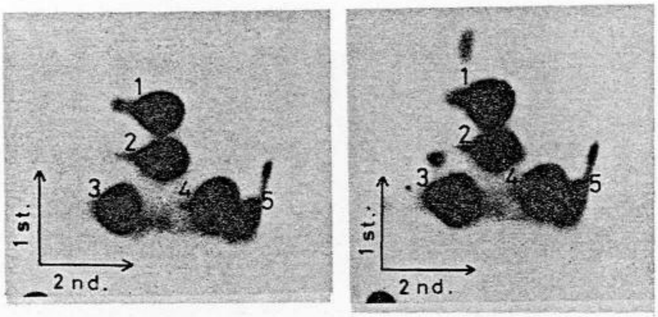


Fig. 10. Two dimensional TLC of mononucleotides obtained by digestion with RNase T2 of the 20 mer (A) and 33 mer (B) Spots: 1, Ap, 2, Cp; 3, Gp; 4, Up; 5, Pi. Solvents: 1st dimension, isobutyric acid-0.5 M ammonium hydroxide (5:3, v/v); 2nd dimension, isopropanol-conc. hydrochloric acid-water (70:15:15,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

collected. An aliquot (6.8 A_{260}) was subjectected to HPLC (TSK LS 410) (Fig. 6) and 1.7 A_{260} 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 × 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 A_{260}). HPLC analysis showed incomplete removal of protecting groups. The product (220 A_{260}) 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 A_{260}) was further purified by HPLC (TSK-LS 410) as shown in Fig. 6.

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