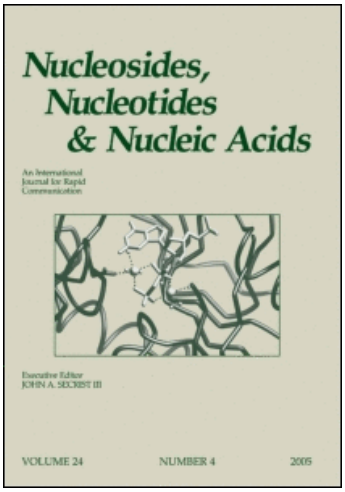

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Masahide Ishikawa[a]; Kazunori Ikebukuro[a]; Ichiro Hirao[a]; Kin-Ichiro Miura[a]
[a] Department of Industrial Chemistry, Faculty of Engineer-ing, The University of Tokyo, Tokyo, Japan

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SYNTHESIS OF FUSED OLIGORIBONUCLEOTIDES WITH TRIDEOXYRIBONUCLEOTIDE CONTAINING PHOSPHOROTHIOATE TO STABILIZE AGAINST NUCLEASE ACTIVITY

Masahide Ishikawa, Kazunori Ikebukuro, Ichiro Hirao, and Kin-ichiro Miura*.

Department of Industrial Chemistry, Faculty of Engineering, The University of Tokyo, Hongo, Tokyo 113, Japan

ABSTRACT: Fused oligonucleotides(21mer) consisting of RNA(18mer) and DNA(3mer) were synthesized by combined use of the phosphotriester and phosphoramidite methods. The RNA(18mer) corresponds to the leader sequence of phage f1 coat protein mRNA containing initiation codon. The RNA was stabilized against 3'-exonucleases by joining with trideoxyribonucleotides containing phosphorothioate linkages and it would be applied to the studies on the initiation complex formation in prokaryotic translation.

Messenger RNA(mRNA) is a key molecule in the biosynthesis of protein and is transcribed according to the genetic information of DNA. Its information is finally translated into a peptide on ribosomes. However, little is known about the regulation mechanism of translation in prokaryotes compared to that of transcription, one reason being the instability of mRNAs.

In general, a prokaryotic mRNA is polycistronic, namely, a single mRNA molecule carries messages of several peptides, and each peptide synthesis is controlled by the upstream sequence to the initiation codon, including the Shine-Dalgarno sequence(SD).[1] Translational efficiency depends on the stability of the initiation complex between mRNA and ribosomes in vitro.

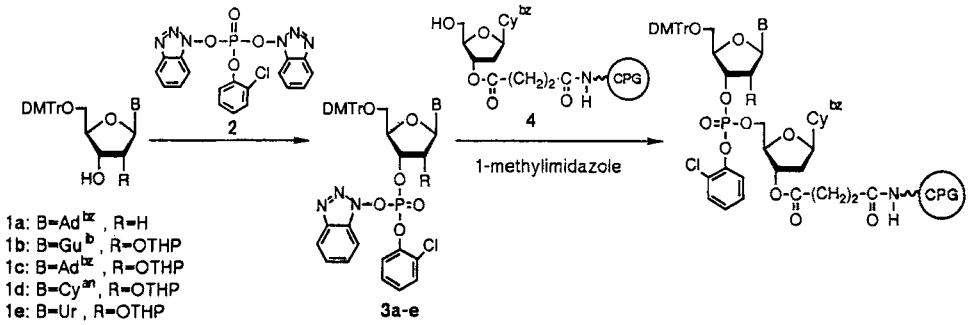
In a previous paper,[2] RNA fragments, octadecaribonucleotides corresponding to the leader sequence of phage f1 coat protein mRNA and its modified 18mer, were synthesized by the phosphotriester method. During the experiments for the in vitro initiation complex formation using E. coli lysate, 18mers were degraded slowly by the contamination of nucleases in the system.[3] By use of either 5'- or 3'-^{32}P-labeled 18mers, it was found that the degradation is caused by 3'-exonuclease activity. Based upon this fact, the RNA fragments(18mer) were modified by the addition of an oligodeoxyribonucleotide or its phosphorothioate analogue at the 3'-terminus of the 18mers to protect an RNA fragment against 3'-exonucleases.

In this paper, the syntheses of oligoribonucleotides (18mer) bearing trideoxyribonucleotide d(AAC) and its phosphorothioate analogue d(AsAsC) at the 3'-terminus are described as well as their stability under the conditions of the formation of the translational initiation complex in vitro.

RESULTS AND DISCUSSION

The fused oligonucleotides(21mer) were synthesized by the phosphotriester method by our modification[2] of the procedure of van Boom.[4] Initially, a fused 21mer, RNA_1-d(AAC) corresponding to the leader sequence of mRNA of f1 coat protein with the initiation codon AUG (RNA_1,18mer) bearing d(AAC) at the 3'-terminus was synthesized. The AAC sequence was chosen to avoid intramolecular hybridization with the SD sequence of the 18mer. In a similar manner, RNA_2-d(AAC), RNA_3-d(AAC), and RNA_4-d(AAC) were synthesized where the SD and surrounding sequence is altered.

The fully protected nucleosides(**1**) were phosphorylated with bifunctional phosphorylating agent(**2**)[4] in dioxane. The coupling reaction of **3** with N^4-benzoyldeoxycytidine 3'-succinate long chain alkylamino-CPG(controlled pore glass) (**4**) was carried out in acetonitrile at 30 °C for 15 min



Scheme 1

Table 1 Synthesis of the fully protected 21mers

RNA(18mer)-DNA(3mer)		Average Coupling yields/%	Isolated yields ($OD_{260}U$)
RNA_1-d(AAC)	r(ACUGGAAACUUCCUCAUG)d(AAC)	96	1.63
RNA_2-d(AAC)	r(ACUAAAAACUUCCUCAUG)d(AAC)	93	0.83
RNA_3-d(AAC)	r(ACUCCAAACUUCCUCAUG)d(AAC)	93	--[a]
RNA_4-d(AAC)	r(UAAGGAAACUUCCUCAUG)d(AAC)	93	--[a]
RNA_1-d(AsAsC)	r(ACUGGAAACUUCCUCAUG)d(AsAsC)	93[b]	0.66
RNA_2-d(AsAsC)	r(ACUAAAAACUUCCUCAUG)d(AsAsC)	92[b]	0.86

a) These oligomers were not isolated.
b) Yield indicates the coupling reactions of RNA synthesis.

(Scheme 1). The yield of each coupling reaction for internucleotidic bond formation was more than 90 % (Table 1).

After removal of whole protecting groups in the usual manner,[2] purification of RNA_1-d(AAC) was performed by reversed-phase HPLC, and the main peak was collected (Fig.1(a)). Further purification was carried out by electrophoresis on a 20% polyacrylamide gel containing 7 M urea. Similarly, RNA_2-d(AAC), RNA_3-d(AAC), and RNA_4-d(AAC) were synthesized. The results are summarized in Table 1 and Fig.2.

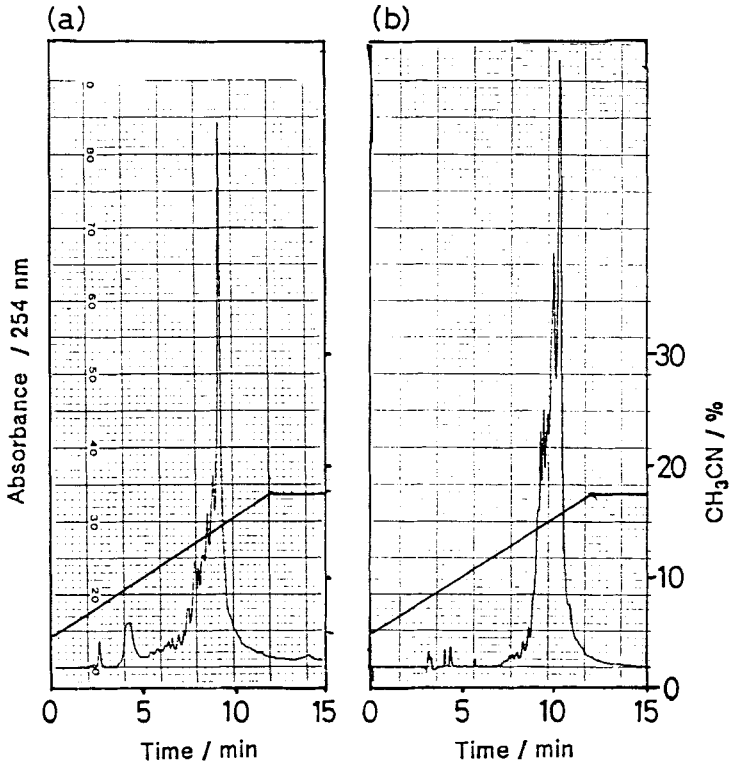


Fig.1 Reversed-phase HPLC profiles of deprotected RNA_1-d(AAC)(a) and RNA_1-d(AsAsC)(b).

Internucleotidic phosphorothioate linkages are known to be stable against nucleases[5] and the oligomers were used for antisense-DNA[6,7] and RNA.[8] Compared with d(AAC) the corresponding phosphorothioate analogue, d(AsAsC) seems to be also useful for the binding assay of these oligomers with ribosomes.

Next, fused 21mers, RNA_1-d(AsAsC) and RNA_2-d(AsAsC) were synthesized. Synthesis of d(AsAsC) on CPG was carried out by the phosphoramidite method (Scheme 2).[5]

After removal of the dimethoxytrityl(DMTr) group of the 5'-terminus of d(AsAsC) on CPG resin, RNA_1 or RNA_2 were constructed by the phosphotriester method as described above

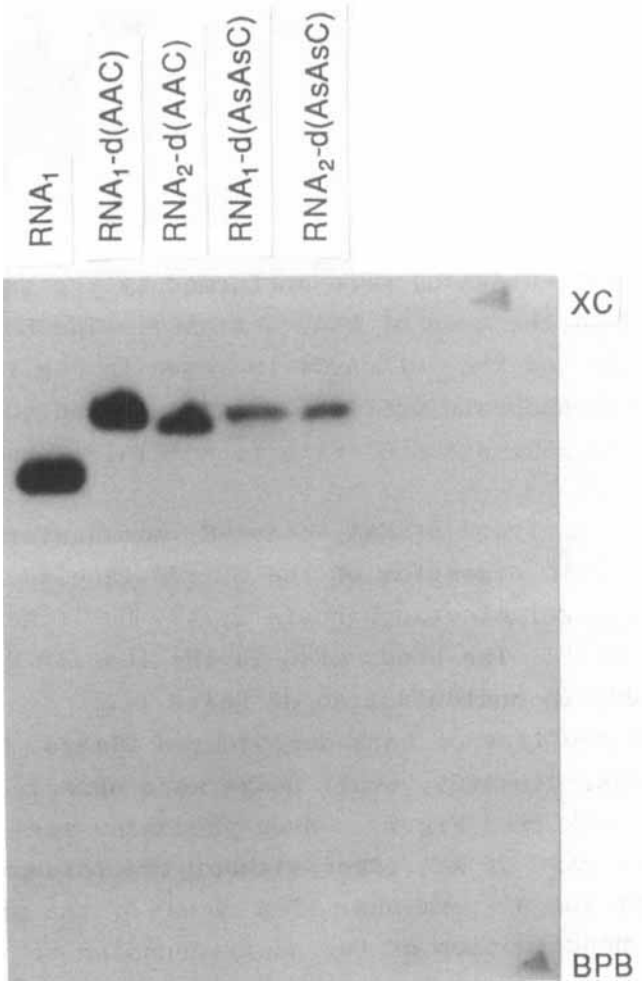
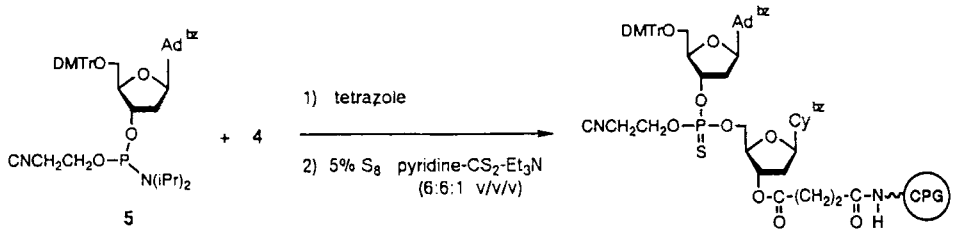


Fig.2 20% Polyacrylamide gel electrophoresis of the purified oligomers 5'-phosphorylated using [γ -^{32}P]ATP. XC: xylene cyanol, BPB: bromophenol blue.



Scheme 2

(Table 1). Deprotection and purification of either RNA_1-d(AsAsC) or RNA_2-d(AsAsC) were performed by the same method as described in the case of RNA_{1-4}-d(AAC). The HPLC profile of the deprotected RNA_1-d(AsAsC) is shown in Fig.1(b) and the gel electrophoresis profile of the purified and 5'-phosphorylated RNA_1-d(AsAsC) with [γ -^{32}P]ATP is shown in Fig.2.

Sequence analysis of RNA_1-d(AsAsC) was performed by partial enzymatic digestion of the 5'-^{32}P-labeled 21mer on polyacrylamide gel electrophoresis by the Donis-Keller method (Fig.3).[9] The bands of G in the lane of B. cereus RNase were due to contamination of RNase T_1.

On HPLC profiles of both deprotected 21mers, RNA_1-d(AAC) and RNA_1-d(AsAsC), small peaks were observed in front of the main peak (see Fig.1). Such phenomena were not observed in the case of RNA 18mer without the trideoxyribonucleotide at the 3'-terminus. The cause of the problem seems to be depurination of the deoxyadenosine residue under acidic conditions during removal of the 5'-DMTr and 2'-tetrahydropyranyl(THP) groups. The release of adenine under these conditions was ascertained independently by use of the trideoxyribonucleotides d(AAC) and d(AsAsC). The time-course of the depurination is shown in Table 2.

The internucleotidic phosphorothioate linkage also may be unstable under acidic conditions and be converted into the phosphate linkage. We examined the possibility of conversion from phosphorothioate to phosphate under acidic con-

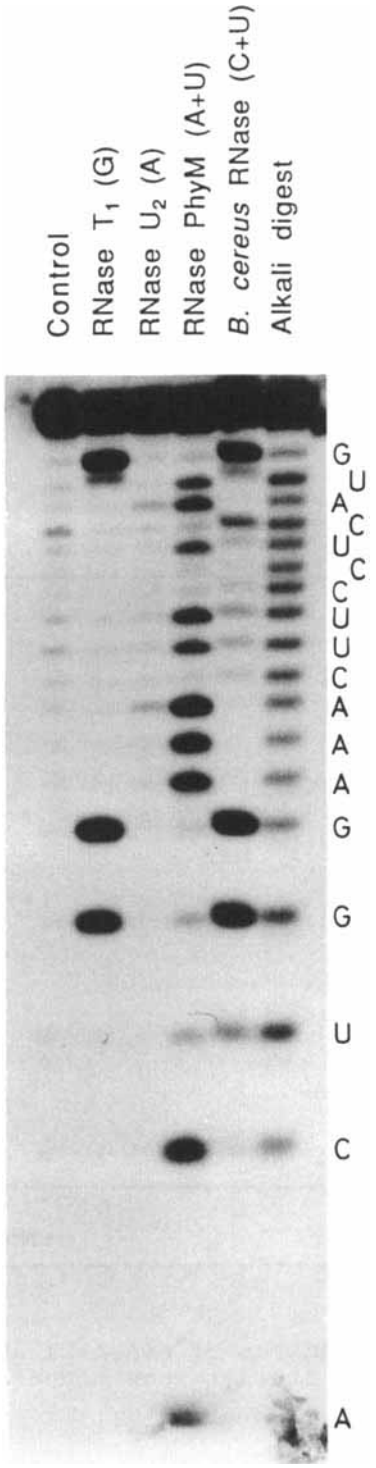


Fig.3 Sequence analysis of RNA_1-d(AsAsC) by the Donis-Keller method.

Table 2 Depurination of d(AAC) and d(AsAsC)

Time (h)	Formation of adenine(%)	
	d(AAC)	d(AsAsC)
24	28	36
48	52	64
72	73	94

The reaction was carried out in 0.01 M HCl at room temperature.

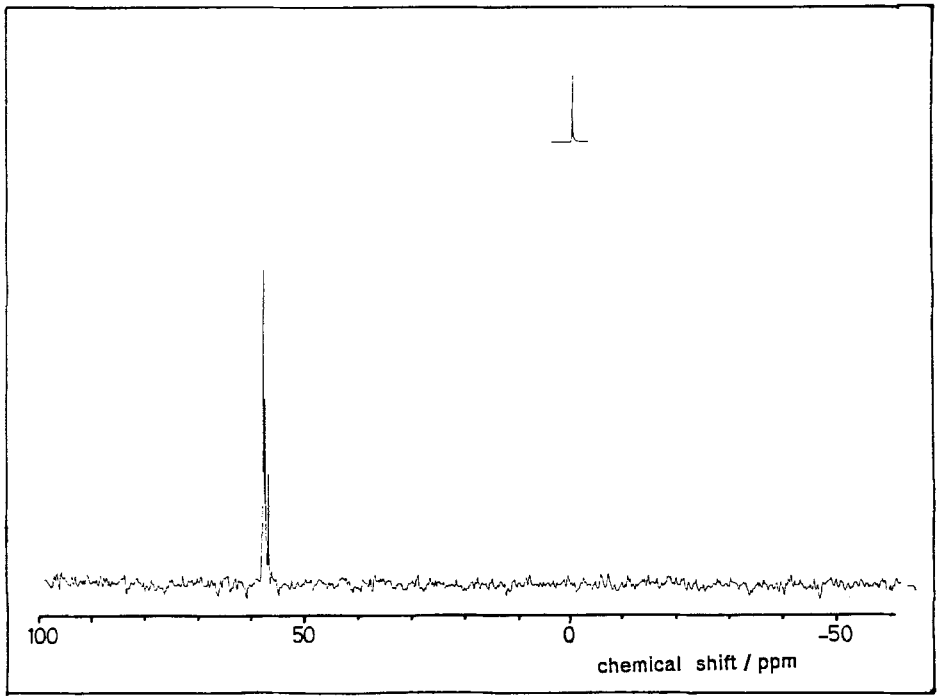


Fig.4 ^{31}P-NMR spectrum of d(AsAsC) after treatment with 0.01 M HCl(pH 2.0) at room temperature for 24 h.

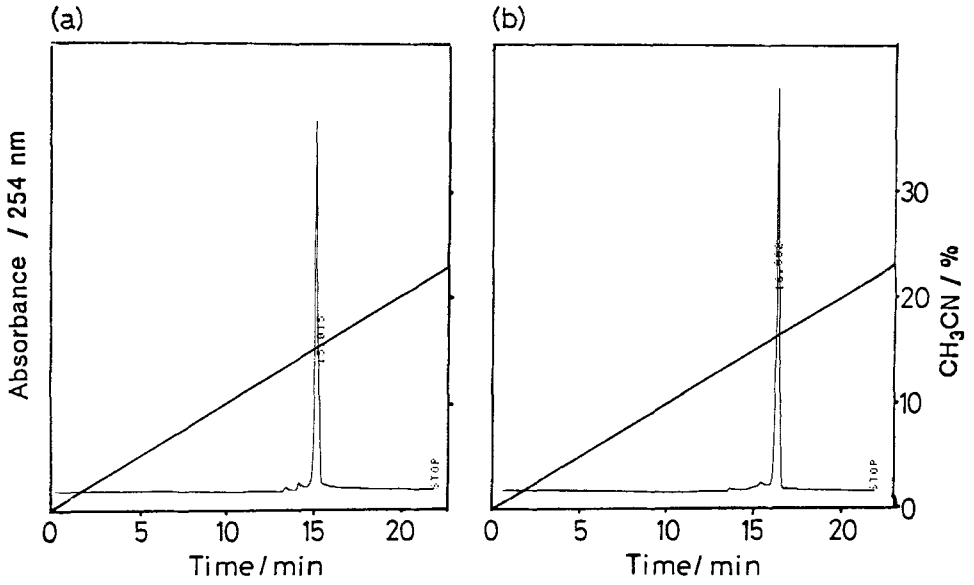


Fig.5 HPLC profiles of diastereoisomeric d(TsC)(a),(b) after treatment with 0.01 M HCl(pH 2.0) at room temperature for 3 days.

ditions. Synthetic d(AsAsC) was treated with 0.01 M HCl adjusted to pH 2.0 at room temperature for 24 h and analyzed by ^{31}P-NMR (Fig.4). The peaks between 55 and 58 ppm were assigned as the phosphorothioate and peaks of phosphate between -2 and 2 ppm were not observed at all. The HPLC profile was complicated by the depurination of deoxyadenosine residues.

To avoid the problem of depurination, thymidine-3'-yl deoxycytidine-5'-yl phosphorothioate, d(TsC) was synthesized as a model compound and the diastereoisomers derived from the chirality of phosphorothioate were separated. Each diastereoisomer was treated with 0.01 M HCl(pH 2.0) at room temperature for 3 days (Fig.5). However, no transformation of the phosphorothioate into the phosphate was observed. The phosphorothioate linkage 21mers also is not transformed to the phosphate under the conditions (1% dichloroacetic

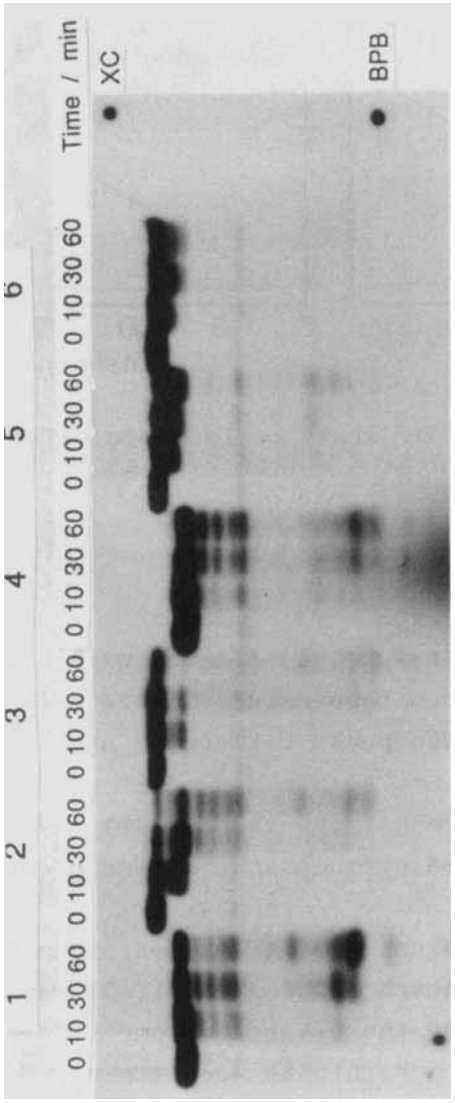


Fig.6 Stability of synthetic 21mers in the binding assay system, lane 1: RNA_1, lane 2: RNA_1-d(AAC), lane 3: RNA_1-d(AsAsC), lane 4: RNA_2, lane 5: RNA_2-d(AAC), lane 6: RNA_2-d(AsAsC). Each oligomer was labeled with [γ-^{32}P]ATP at the 5'-terminus.

acid) for removal of the 5'-DMTr group during the synthesis of RNA_1-d(AsAsC) and RNA_2-d(AsAsC).

Finaly, the purified 21mers were examined for their stability against nucleases under the optimized conditions in the binding assay. The 21mers were enzymatically phosphorylated with [γ -^{32}P]ATP to give the 5'-^{32}P-labeled 21mers. Then, 70S ribosome and crude initiation factors from E. coli Q13 strain were added. Under the conditions (see Experimental part), it was found that RNA_1-d(AsAsC) and RNA_2-d(AsAsC) having the phosphorothioate linkages were much more stable than the corresponding RNA_1-d(AAC) and RNA_2-d(AAC) (Fig.6).

In conclusion, it is noted in the binding assay system that RNAs can be stabilized against nucleases by addition of a trideoxyribonucleotide consisting of phosphorothioate linkages at the 3'-terminus. Therefore, this type of chemically synthesized oligonucleotide could be utilized for studies on translational mechanism.

EXPERIMENTAL

^{31}P-NMR spectra were recorded on a JEOL PS-100FT (40.50 MHz), and the chemical shifts are reported relative to an external capillary standard of 85% H_3PO_4. HPLC was performed on a M&S pack C_{18} column using 0.1 M triethylammonium acetate(pH 7.0) for purification of oligonucleotides, and a μ Bondasphere C_{18} column using 0.1 M ammonium acetate (pH 7.0) for analysis of depurination. 1,4-Dioxane and acetonitrile were purified by distillation from $LiAlH_4$ and calcium hydride, respectively. Pyridine was purified by distillation first from p-toluenesulfonyl chloride and then from calcium hydride. These solvents were stored over Molecular sieves 3A.

Preparation of fully protected nucleosides:

5'-O-Dimethoxytrityl-N^6-benzoyldeoxyadenosine(**1a**),[10] and 5'-O-dimethoxytrityl-2'-O-tetrahydropyranyl derivatives of N^2-isobutyrylguanosine(**1b**),[2] N^6-benzoyladenosine(**1c**),[11] N^4-anisoylcytidine(**1d**),[11] and uridine(**1e**),[11] were prepared according to the literature procedures.

5'-O-Dimethoxytrityl-N-acylnucleoside 3'-1-benzotriazoyl-2-chlorophenylphosphate (3):

To dried 5'-O-dimethoxytrityl-N^a-benzoyladenosine(**1a**) (66 μ mol/each coupling reaction) was added 0.2 M solution (0.3 mL) of **2** in dioxane followed by stirring at room temperature for 30 min. The solution was then concentrated and the residue was dissolved in dry pyridine(0.1 mL) and evaporated. The residue thus obtained was dissolved in dry acetonitrile(0.25 mL), and used without isolation.

5'-O-Dimethoxytrityl-2'-O-tetrahydropyranyl-N-acylribonucleosides (**1b,1c,1d,1e**) were also phosphorylated by the same method as **1a**.

Preparation of 5'-O-dimethoxytrityl-N^a-benzoyldeoxyadenosine 3'-2-cyanoethyl(N,N-diisopropyl)phosphoramidite(5):

Compound(**5**) was prepared from **1a** and bis(N,N-diisopropylamino)-2-cyanoethoxyphosphine by literature procedure.[12]

Preparation of CPG resin carrying deoxycytidine (4):

5'-O-Dimethoxytrityl-cytidine joined at the 3'-hydroxyl group on long chain alkylamino(LCA) CPG resin through a succinate linker was prepared according to the literature procedure.[13]

Synthesis of fused 21 mers, RNA_1-d(AAC), RNA_2-d(AAC), RNA_3-d(AAC), and RNA_4-d(AAC):

The fully protected deoxycytidine loading on LCA-CPG(**4**) (50 mg, 0.77 μ mol) was packed in a reaction vessel of a Zeon Genet DNA manual synthesizer(NIPPON ZEON Co.). After removal of the 5'-dimethoxytrityl group with 1% dichloroacetic acid at 30 °C for 1-2 min, the coupling reaction with **3a** or **3b-3e** was carried out at 30 °C for 15 min, and then the capping reaction was carried out. Repetition of the above procedure for elongation cycles gave the desired fully protected 21mers on the solid support. Coupling yields were listed in Table 1.

Synthesis of trideoxyribonucleotides d(AAC), d(AsAsC):

The fully protected deoxycytidine loading CPG (100 mg, 1.88 μ mol) was packed in a column for the manual synthesizer. Trideoxyribonucleotides were synthesized by the common phosphoramidite method.[14] In the case of d(AsAsC), sulfurization[5] was carried out with 5% S_8 in pyridine-CS_2-triethylamine (6:6:1 v/v/v) at room temperature for 15 min instead of oxidation in the synthetic cycle. The coupling reaction proceeded almost quantitatively by estimation on the colorimetry of dimethoxytrityl cation.

Synthesis of the fused 21 mers containing phosphorothioate linkages, RNA_1-d(AsAsC) and RNA_2-d(AsAsC):

RNA part of the fused 21 mers, RNA_1-d(AsAsC) and RNA_2-d(AsAsC), were constructed on the appropriately protected 5'-hydroxyl d(AsAsC) loading CPG (80 mg, 1.50 μ mol) by the phosphotriester method same as described in the case of RNA_{1-4}-d(AAC).

Deprotection and purification of Synthetic 21 mers:
Deprotection and purification procedures of the synthetic 21 mers were described previously.[2)]

Formation of adenine from d(AAC) and d(AsAsC):
Trideoxyribonucleotides d(AAC) or d(AsAsC) (0.25 A_{260} unit) were treated with 0.01 M HCl adjusted to pH 2.0 at room temperature. The solution was neutralized with aqueous ammonia, and analyzed by reversed-phase HPLC. The percentage of depurination was estimated on the basis of the peak areas of adenine and trideoxyribonucleotides using the values of the molar absorptivity of adenine 1.3×10^4(260 nm), and the hypochromicity of d(AAC) and d(AsAsC) estimated as 16%.[15)]

Preparation of 70S ribosome, crude initiation factors, and formylmethionyl transfer RNA(fMet-tRNA):
70S ribosome and crude initiation factors were obtained from E.coli Q13 according to the established procedure.[16)] fMet-tRNA was obtained by the literature procedure.[17)]

Stability of Synthetic 21mers under the Conditions of the Binding Assay:
The stability of synthetic 21mers was examined as follows. The mixture for the binding assay contained in a final volume of 15 μ L: 50 mM Tris-HCl(pH 7.4), 100 mM NH_4Cl, 5 mM $Mg(OAc)_2$, 1mM dithiothreitol, 10 pmol 70S ribosome, 2 μ L crude initiation factors (0.70 A_{260}/mL), 10 pmol fMet-tRNA, 15 nmol GTP, and 1 pmol of 5'-^{32}P-labeled oligomers, was incubated at 30 °C. The mixture(3 μ L) was picked up after 0, 10, 30, 60 min and incubated at 90 °C for 2 min with formamide dye, and then analyzed on 20% polyacrylamide gel electrophoresis (Fig.6).

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