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Partial Synthesis of Leader Sequence of Phage f1 Coat Protein mRNA

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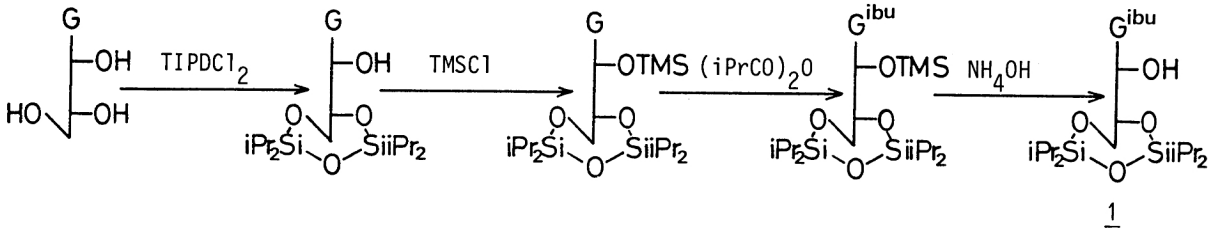
Tetra to undecaribonucleotides of the leader sequence in messenger RNA(mRNA) of f1 coat protein were synthesized on a polystyrene solid support through simple phosphotriester approach.

The process for protein biosynthesis starts by association of mRNA with ribosome. Shine and Dalgarno propose for prokaryote cell that the polypyrimidine sequence at the 3' terminus of 16S rRNA in a smaller subunit of ribosome interacts with the purine-rich sequence (SD sequence) preceding the initiation codon of mRNA in translational initiation.[1] In some papers the effects of altering the nucleotide sequence of the SD region in DNA gene have been examined.[2] However, the experiments carried out directly on the association between mRNA leader sequence and ribosome for the efficiency of protein synthesis has scarcely been studied.[3] In order to examine this idea, we first synthesized RNA fragments (4-11 mer) of the leader sequence of f1 coat protein mRNA (Fig. 1). Our aim is to join them enzymatically to construct several large fragments (22 mer) with modified sequence.

The nucleosides used in the synthesis were protected at the 2'-position with the tetrahydropyranyl (THP) group and at the 5'-position with the 4,4'-dimethoxytrityl (DMTr) group. Exocyclic amino groups of adenosine, cytidine, and guanosine were also protected with the benzoyl, the anisoyl, and the isobutyryl (ibu) groups, respectively.[4] These nucleosides were prepared from 3',5'-O-(tetraisopropyldisiloxane-1,3-diyl) derivatives.[4] N^2-Isobutyryl-3',5'-O-(tetraisopropyldisiloxane-1,3-diyl) guanosine (1) was synthesized from guanosine by the rapid one pot reaction. Guanosine was first treated with 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane ($TIPDSC1_2$) in a DMF - pyridine mixture (5:1 v/v). Then trimethylchlorosilane[5] was added and the mixture was immediately reacted with isobutyric anhydride, followed by addition of dilute ammonia solution. This method produced crystalline 1 in 62% yield from guanosine.



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We have previously reported[6] that the selective deprotection of the 5'-O-DMTr groups of oligoribonucleotides by treatment with 1% dichloroacetic acid in dichloromethane at room temperature, without removal of the 2'-O-THP group, and the synthesis of trinucleotides on polystyrene support using the bifunctional reagent 2-chlorophenyl-O,O-bis (1-benzotriazolyl) phosphate (2). This bifunctional reagent has been studied by J.H. van Boom and co-workers and is employed for liquid[7] and solid[8] phase synthesis of DNA as well as liquid phase synthesis of short length oligoribonucleotides.[9] We then applied this method to solid phase synthesis of RNA.

The polymer support (aminomethylated polystyrene, 2% cross-linked by divinyl benzene) was derivatized with uridine (384 μmol/g) or adenosine (228 μmol/g) unit according to established procedure.[10] The activated nucleotides were prepared by the addition of 0.2 M dioxane solution of 2 (1.0 mol equiv.) to the protected nucleoside (1.1 mol equiv.) and the solution was stirred for one hour under argon atmosphere. Synthesized oligoribonucleotides corresponding to the leader sequence of mRNA of coat protein of phage f1 and the reaction cycle in the solid phase synthesis are shown in Fig. 1 and Table 1, respectively. Condensation reactions using 0.2 M dioxane solution (500 μl) of activated nucleotides, 1-methylimidazole (50 μl), and polystyrene support (20 mg) attached with nucleoside unit were carried out at 30 °C for 30 min. Coupling yield of each fragment is shown in Table 2, the relatively low yields are due to the sensitivity to moisture of activated nucleotides and also to technical problems.

Deblocking was performed by the following procedure. Polystyrene support attached with 5'-O-DMTr-oligoribonucleotides, which is fully protected, was treated with 0.5 M 2-pyridinealdoxime - tetramethylguanidine in a mixture of pyridine and water (9:1 v/v) at 37 °C for 24 h. The resin was filtered and washed with a mixture of pyridine and water (1:1 v/v). This filtrate was then concentrated and treated with conc. NH_4OH at 55 °C for 12 h. The resulting 5'-O-DMTr-2'-O-THP-oligoribonucleotides were purified by high performance liquid chromatography (HPLC) with reverse phase C-18 column (M&S Co.)(Fig. 2a). The eluted oligoribonucleotides were treated with aq. HCl (pH 2.0) at room temperature for 24 h to remove the 2'-O-THP and the terminal 5'-O-DMTr groups. The deblocked crude oligomers were analyzed (Fig. 2b) and purified (Figs. 2c and 2d) by HPLC.

All of the fragments were completely degraded with nuclease P1 to nucleosides and nucleotides and then analyzed by HPLC technique (Table 2). The 5'-^{32}P labeled oligomers were also analyzed by gel electrophoresis (Fig. 3). The mobilities differ from UpCpCpU to UpApApU, due to their different molecular weights.

This solid phase RNA synthesis is simple, and it seems to be suitable for synthesis of longer chain length oligoribonucleotides. But THP group protected at the 2'-OH position of uridine was removed in mere trace amounts by treatment with 1% dichloroacetic acid, and this matter is remained to be solved.

To prepare full length of mRNA leader sequences, these synthetic fragments will be ligated with T4 RNA ligase.

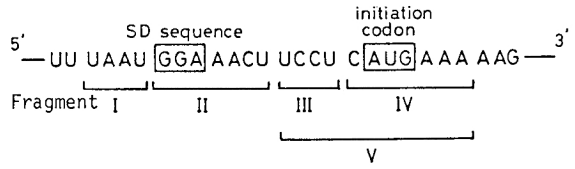

Fig. 1. Leader sequence in the mRNA of f1 coat protein.

Table 1. Condition of coupling cycle[a)]

Step	Reagent or solvent	Time/min
1	1% $Cl_2CHCOOH$-CH_2Cl_2, 30 °C	2.0 - 3.0
2	CH_2Cl_2-MeOH (8:2 v/v)	0.2 (3 times)
3	Pyridine	0.2 (3 times)
4	Argon (flush), 40 °C	10
5	0.2M Activated nucleotide-dioxane (500 μℓ), 1-methyl-imidazole (50 μℓ), 30 °C	30
6	Pyridine	0.2 (3 times)
7	10% Ac_2O-0.1M DMAP- Pyridine	3.0
8	Pyridine	0.2 (3 times)
9	CH_2Cl_2-MeOH (8:2 v/v)	0.2 (3 times)

a) Synthesizer; ZEON Genet DNA SYNTHESIZER.

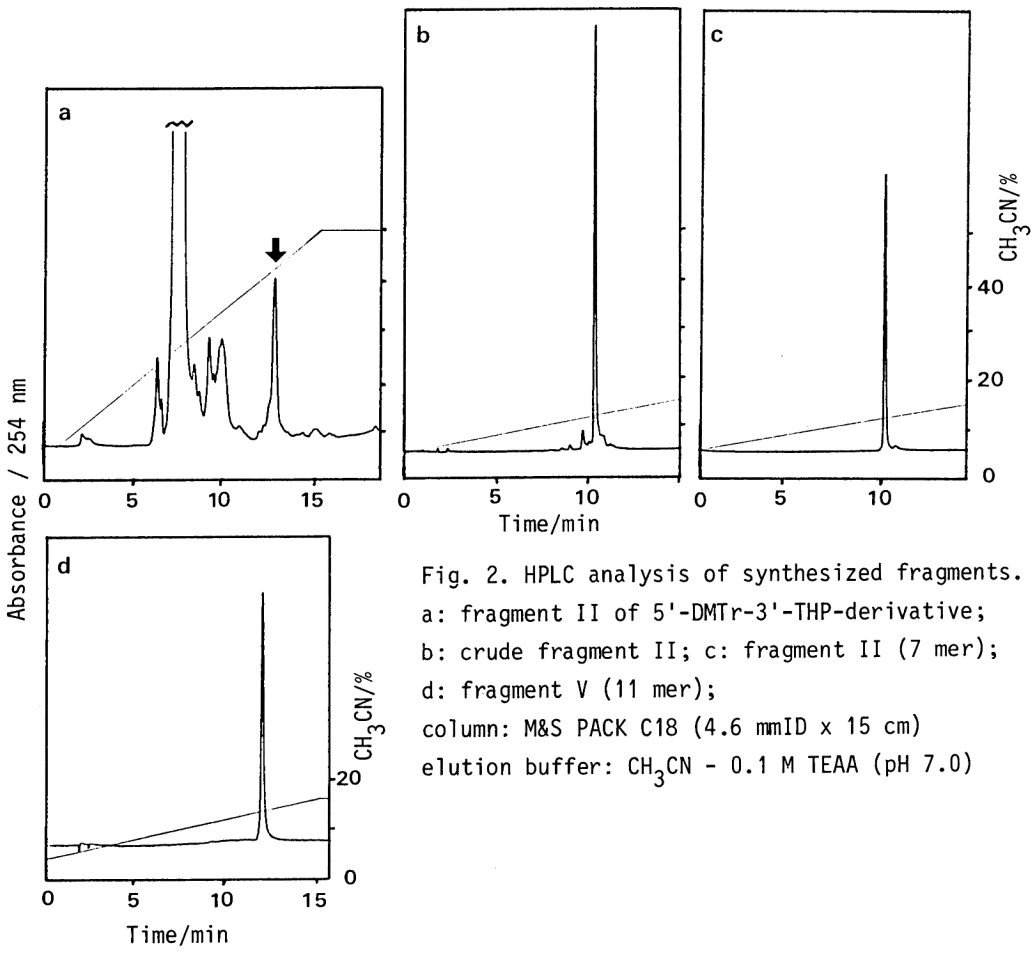

Fig. 2. HPLC analysis of synthesized fragments.
a: fragment II of 5'-DMTr-3'-THP-derivative;
b: crude fragment II; c: fragment II (7 mer);
d: fragment V (11 mer);
column: M&S PACK C18 (4.6 mmID x 15 cm)
elution buffer: CH_3CN - 0.1 M TEAA (pH 7.0)

Table 2. Summary of yields and analysis of enzyme digestion

Fragments	Coupling yields/% average	overall	Nuclease P1 digestion
I (4 mer)	85	71	pU:U:pA=1:1.07:2.03
II (7 mer)	76	25	pC:pU:pG:pA:G=1.06:0.98:1.10:3.01:1
III(4 mer)	91	75	pC:pU:U=2.05:1:1.06
IV (7 mer)	71	18	pU:pG:C:pA=1:1.10:1.06:4.02
V (11 mer)	79	11	pC:pU:pG:U:pA=3.00:2.10:1.11:1:3.97

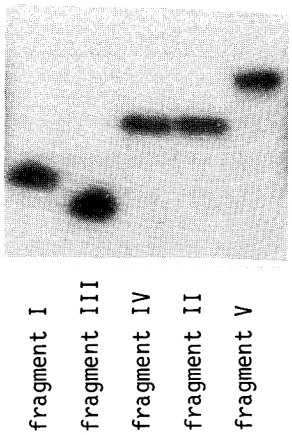

Fig. 3. Gel electrophoresis of 5'-^{32}P labeled oligomer.

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