NONENZYMATIC TEMPLATE-DIRECTED CONDENSATION OF SHORT-CHAINED OLIGOURIDYLATES ON A POLY(A) TEMPLATE

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Abstract. An oligouridylate with chain-length of more than three was condensed on a polyadenylate [poly(A)] template with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) in imidazole buffer. The condensation reaction proceeds via the phosphorimidazolide of the oligouridylate as an intermediate. Pentauridylate [$(pU)_5$] was converted to decauridylate [$(pU)_{10}$] in 10% yield at 0 °C for 7 days in the presence of the poly(A) template, while no coupling product was obtained in the absence of the poly(A) template. The resulting linkage of the $(pU)_{10}$ was mainly $2'$–$5'$ linkage.

Keywords: nonenzymatic template-directed condensation, oligouridylates, poly(A)

1. Introduction

It has been proposed that RNA could store and transfer information, and could play the role of a catalyst in the prebiotic era (Gilbert, 1986; Joyce and Orgel, 1993). Non-enzymatic replication of RNA is considered to occur based on Watson-Crick base-pairing at some stage of the origin of life. Orgel and co-workers have demonstrated the non-enzymatic template-directed synthesis of oligoribonucleotides on complementary oligo- and polynucleotides and their analogues (Orgel and Lohrmann, 1974; Orgel, 1986, 1992, 1995). They have shown that adenylic acid or an adenylic acid derivative oligomerizes on a poly(U) template to give oligoriboadenylates (Sulston *et al.*, 1968; Orgel and Lohrmann, 1974). Formation of a helical complex has been confirmed between poly(U) and adenylic acid or its derivative under the reaction conditions (Sulston *et al.*, 1968; Shim *et al.*, 1974). In a similar way, guanylic acid derivatives oligomerize on a poly- or oligocytidylate (Sulston *et al.*, 1969; Inoue and Orgel, 1981, 1982, 1983; Kanavarioti *et al.*, 1990). Information transfer from purine nucleotides to pyrimidine nucleotides, and vice versa, is required for the successive replication of RNA. However, template-directed synthesis of oligomers of uridylate or cytidylate cannot take place non-enzymatically on a poly(A) or on a poly(G) template (Orgel and Lohrmann, 1974; Orgel, 1986). An attempt to oligomerize an activated uridylate on a poly(A) template under eutectic conditions proved unsuccessful (Stribling and Miller, 1991a, b). This limitation could be overcome by template-directed ligation of di- or short oligoribonucleotides containing pyrimidine nucleotides. This approach has been demonstrated

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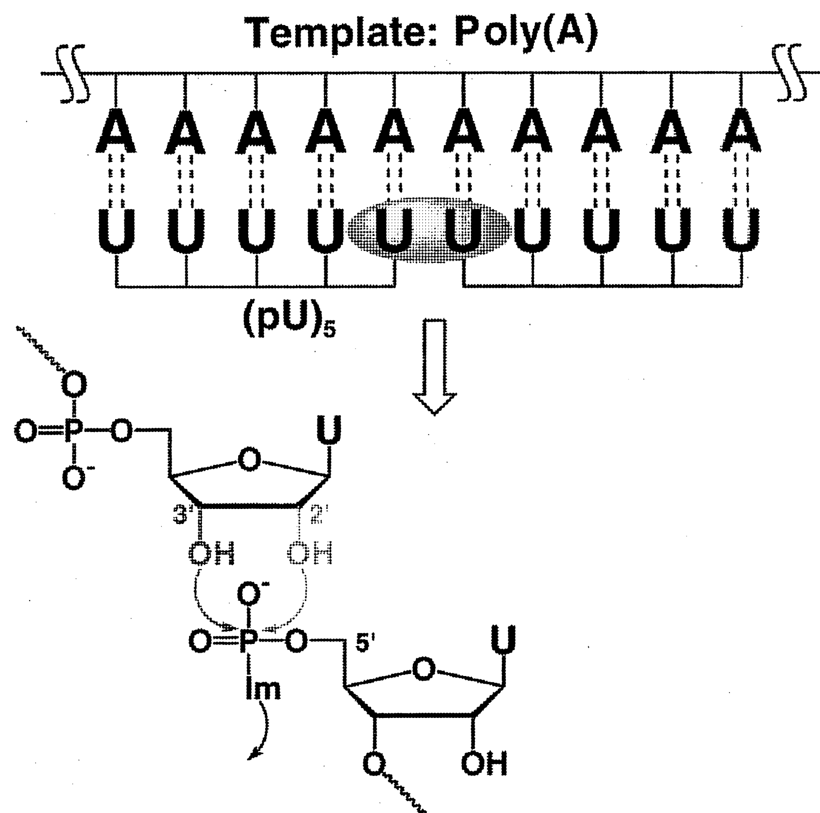

Scheme 1. Condensation of oligouridylate on a poly(A) template.

by several workers (Nino and Orgel, 1978; Rohtagi *et al.*, 1996a, b) since the first report by Naylor and Gilham who showed the template-directed condensation of $(pT)_6$ on a poly(A) template (Naylor and Gilham, 1966). Previously we showed that oligo(U) with a chain length of more than three can form a helix with poly(A) or long-chained oligo (A), but not di- nor monouridylate (Sawai *et al.*, 1997). The result of the helix formation suggests that triuridylate or longer oligouridylate could condense on a poly- or long oligo(A) template to form the corresponding longer oligouridylates. Thus, we undertook the ligation of $(pU)_3$, $(pU)_4$ or $(pU)_5$ on a poly(A) template in the presence of a water-soluble condensing agent to gain information on the minimum requirement of the template-directed condensation of the pyrimidine nucleotides on a polypurine template.

2. Experimental

2.1. Materials

Poly (A) and poly(U) were purchased from Yamasa Co. Imidazole and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) were from Wako Pure Chemicals. All other compounds were of reagent grade.

2.2. Prepration of poly(A) with chain length of 40–60 and oligo(U)s

A solution of poly (A) (52.8 mg) in 10.6 mL of distilled water was sonicated for 24 hr at room temperature to degrade the long-chained polyA. The solution was poured in a Spectropore dialysis tube (MW cut-off: 10 000) and dialysed for 6 days at 4 °C against 1000 mL of distilled water by replacing the water once a day. The chain length of the resulting poly(A) was estimated to be 40–60, which was confirmed by a denatured polyacrylamide gel (7 M urea 20% polyacrylamide) electrophoresis using t-RNA as a size marker and by HPLC on an RPC-5 column (Sawai, 1989). Oligouridylates, $(pU)_2$, $(pU)_3$, $(pU)_4$ and $(pU)_5$, were prepared by partial digestion of poly(U) with nuclease SW and purified as described previously (Sawai *et al.*, 1996a, b). The concentrations of poly(A) and oligo(U)s were determined by UV absorption at 260 nm using the residual molar coefficients of A and U, $\varepsilon_{260} = 15.3 \times 10^3$ and 10.0×10^3 M^{-1} cm^{-1}, respectively, after correcting the hypochromicity of each oligonucleotide. The hypochromicities of poly(A), $(pU)_2$, $(pU)_3$, $(pU)_4$ and $(pU)_5$, which were estimated from the ratio of absorbance at 260 nm before and after alkaline hydrolysis, were 33.5, 5.6, 6.7, 7.3 and 7.8%, respectively. Concentrations were expressed on a nucleotide residue basis.

2.3. Oligomerization reactions

Condensation of oligo(U) was carried out in a 0.5 mL Eppendorf tube in a total volume of 20 μL at 0 °C. A reaction mixture containing 0.02 M poly(A), 0.04 M oligo(U), 0.02 M $MgCl_2$, 0.2 M NaCl and 1.0 M EDAC in 0.4 M imidazole-HCl buffer (pH 6.0) was prepared at 0 °C and kept for 14 days at 0 °C. Aliquots of the mixture were taken after appropriate intervals and analysed by HPLC. A control reaction without poly(A) template was also carried out under the same conditions for the template-directed condensation.

2.4. HPLC analysis

HPLC analyses were performed on an RPC-5 column with a linear gradient elution of $NaClO_4$ (0–0.05 M in 30 min then 0.05–0.2 M in 30 min) buffered with 2 mM Tris-acetate (pH 7.5) and 0.1 mM EDTA or on an ODS-silicagel column with a

linear gradient of acetonitrile (2–30%) buffered with 0.1 M triethyl ammonium acetate (pH 7.0).

2.5. CHARACTERIZATION OF PRODUCTS

Identification of the condensation products of oligo(U), long-chained oligouridylates, was carried out by comparison of HPLC retention times with those of authentic samples prepared from partial digestion of poly(U) with nuclease SW (Sawai *et al.*, 1996a, b). An intermediate phosphorimidazolide of oligouridylate was identified by comparison of HPLC retention time with that of an authentic sample prepared from the corresponding oligouridylate and imidazole using di-pyridyl disulfide and triphenylphosphine as a condensing agent. For characterization of the dimerized products from $(pU)_5$ on a poly(A) template, the product $(pU)_{10}$ was isolated by preparative HPLC on an ODS-silicagel column. The isolated $(pU)_{10}$ was digested with nuclease P1 to determine the ratio of the resulting linkage isomers. The nuclease P1 digestion was carried out in a solution (20 mL) containing 4 μg of nuclease P1 and 0.3 ODU_{260} of the substrate in 0.02 M ammonium acetate (pH 5.3) at 37 °C for 6 hr. Nuclease P1 degrades only 3′–5′ linkage of the oligonucleotides, leaving only 2′–5′ linkage. The digested products, pU and pU2′p5′U were analysed by HPLC. Authentic pU2′p5′U was obtained as described previously (Sawai *et al.*, 1996a, b).

3. Results and Discussion

3.1. CONDENSATION OF OLIGO(U) IN THE PRESENCE OF A POLY(A) TEMPLATE

The complex formation between complementary oligonucleotides depends on the concentration and chain-length of the strands, temperature and the presence of metal ions. The template-directed condensation of oligo(U) was carried out under the triple helix forming condition between $(pU)_5$ and poly(A), whose concentrations were 40 and 20 mM, respectively, in the presence of Mg^{2+} ion (Sawai *et al.*, 1997).

Figures 1A and 1B illustrate the HPLC profiles of the reaction products from pentauridylate, $(pU)_5$, using EDAC on a poly(A) template in the presence of imidazole buffer (pH 6.0) and magnesium chloride at 0 °C after 7 days and 1 day, respectively. After 1 day, the formation of 5′-phosporimidazolide of pentauridylate and a small amount of $(pU)_{10}$ was observed in addition to the starting $(pU)_5$. The subsidiary peak before the main is considered to be 5′-dephosphorylated or cyclic pentauridylate. The formation of decauridylate reached 10% after 7 days as shown in Figure 1A. Figure 1D demonstrates that a very small amount of $(pU)_{10}$ was formed after 7 days in the control reaction where no template was used. The yield of $(pU)_{10}$ decreased to less than 1% when the condensation reaction was carried

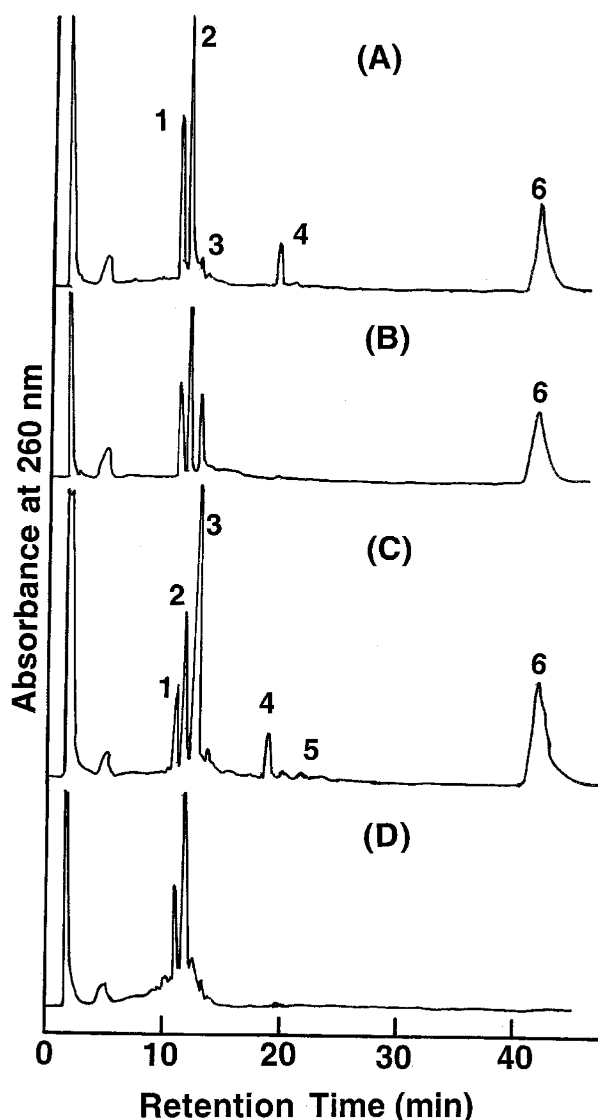

Figure 1. HPLC profiles of the reaction products from template-directed condensation of $(pU)_5$. HPLC was taken on an RPC-5 column under the condition described in the experimental section. (A) Reaction on a poly(A) template for 7 days. The reaction was performed in 20 mL solution containing 40 mM $(pU)_5$, 20 mM poly(A), 20 mM $MgCl_2$, 0.2 M NaCl and 1.0 M EDAC in 0.4 M imidazole-HCl buffer (pH 6.0) for 7 days at 0 °C. (B) Reaction on a poly(A) template for 1 day. The reaction was carried out under the same condition as described above except for the reaction time. (C) Reaction on a poly(A) template at –25 °C for 60 days. The reaction was carried out under the same condition as that for (A) except for the temperature and reaction time. (D) Control Reaction without template. The reaction was performed under the same condition as that for (A). Peak identification; Peak 1, 5′-dephosphorylated and/or cyclic pentauridylate (not fully characterised), 2, $(pU)_5$, 3, 5′-phosphorimidazolide of pentauridylate, 4, $(pU)_{10}$, 5, $(pU)_{15}$, 6, poly(A).

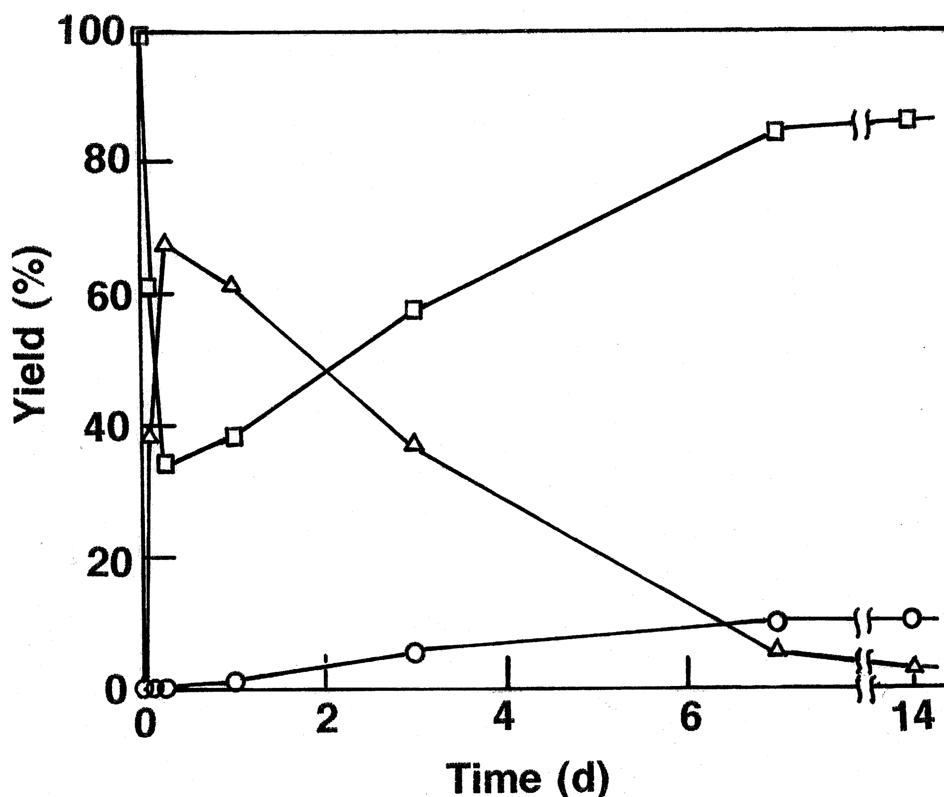

Figure 2. Time course of template-directed condensation of $(pU)_5$ on poly(A). The reaction was performed in 20 mL solution containing 40 mM $(pU)_5$, 20 mM poly(A), 20 mM $MgCl_2$, 0.2 M NaCl and 1.0 M EDAC in 0.4 M imidazole-HCl buffer (pH 6.0) at 0 °C. □: $(pU)_5$ (5′-dephosphorylated and cyclic pentauridylate are included), △: $Im(pU)_5$, ○: $(pU)_{10}$.

out at 25 °C even in the presence of poly(A). On the other hand, poly(A) template-directed condensation reaction at –25 °C under a eutectic condition resulted in increase in yield of $(pU)_{10}$, although both condensation and hydrolysis reactions of the intermediate phosphorimidazolide of $(pU)_5$ were retarded largely under the eutectic conditions. Thus the yields of $(pU)_{10}$ and $(pU)_{15}$ were, 6.6 and 0.4%, respectively, and the intermediate phosphorimidazolide survived in a large amount at –25 °C after 60 days (Figure 1C). The results indicate that helix formation between $(pU)_5$ and poly(A) takes place at low temperatures and is necessary for the coupling reaction of $(pU)_5$.

Figure 2 shows the time course of the poly(A) template-directed coupling reaction of $(pU)_5$ with EDAC at 0 °C in the presence of an imidazole buffer. The reaction was almost complete in 7 days. The condensing agent, EDAC, promoted the reaction of 5′-phosphate of $(pU)_5$ with imidazole initially to convert the phosphorimidazolide of pentauridylate, which condensed on a poly(A) template to form decauridylate. The intermediate phosphorimidazolide hydrolysed in aqueous solu-

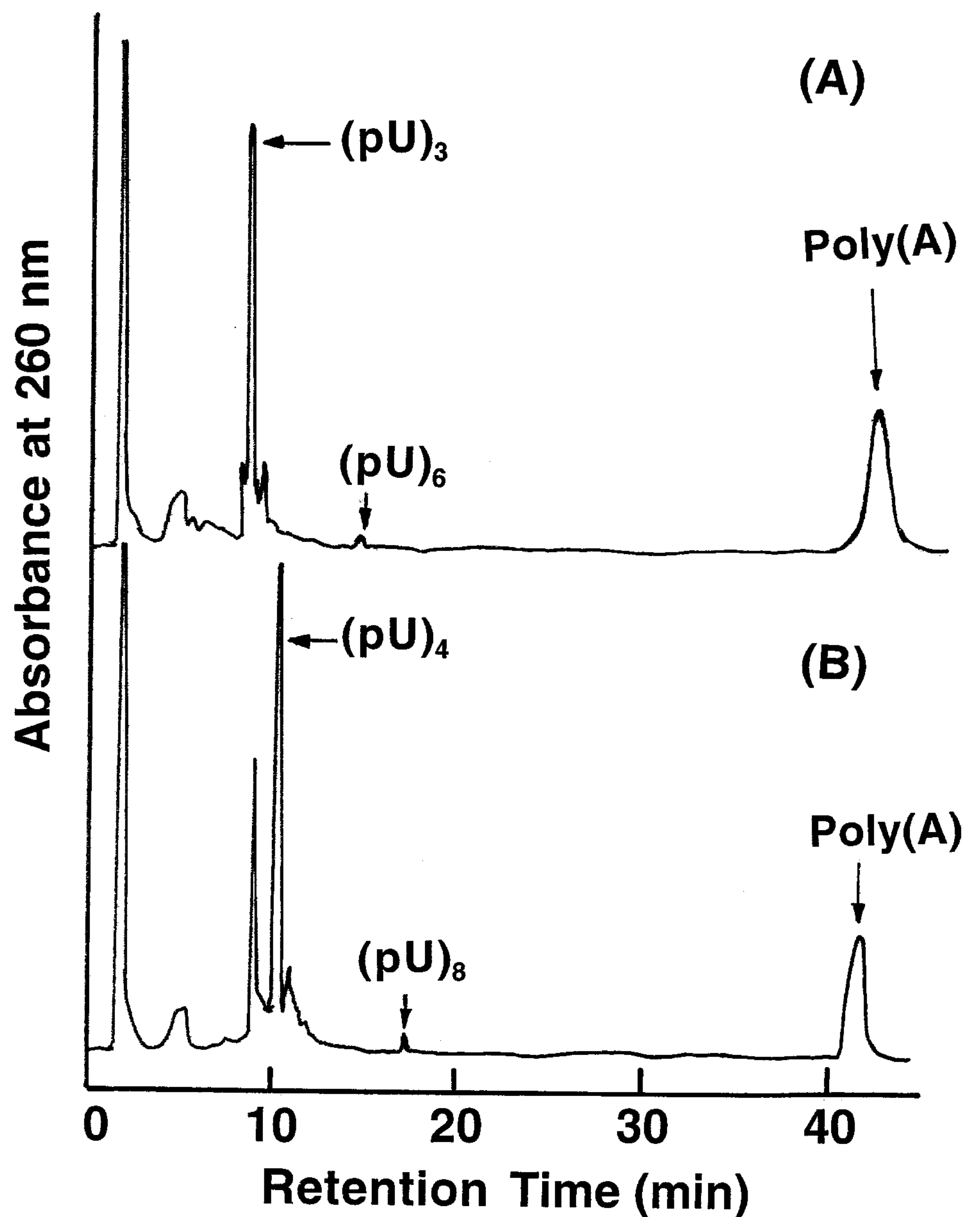

Figure 3. HPLC profiles of the reaction products from template-directed condensation of $(pU)_3$ and $(pU)_4$. (A) Condensation of $(pU)_3$ on a poly(A) template. The reaction was performed in 20 mL solution containing 40 mM $(pU)_3$, 20 mM poly(A), 20 mM $MgCl_2$, 0.2 M NaCl and 1.0 M EDAC in 0.4 M imidazole-HCl buffer (pH 6.0) for 7 days at 0 °C. (B) Condensation of $(pU)_4$ on a poly(A) template. The reaction was carried out under the same condition as described above.

tion, giving the starting $(pU)_5$. The intramolecular reaction of phosphorimidazolide of pentauridylate may also take place to form cyclic pentauridylate.

The resulting $(pU)_{10}$ was isolated by HPLC on an ODS-silicagel and digested with nuclease P1 to determine the ratio of the linkage isomers. Nuclease P1 degrades only the 3′–5′ linkage leaving only 2′–5′ linked oligouridylate. HPLC analysis of the reaction mixture of the nuclease P1 digestion revealed that pU and pU2′p5′U were formed in 8.16:1.84 ratio on a nucleotide residue basis. The result indicates that the ratio of the linkage isomers of decauridylate, pUpUpUpUpU-2′p5′pUpUpUpUpU to pUpUpUpUpU3′p5′UpUpUpUpU, was 92 to 8. Thus, the 2′–5′ internulceotide linkage was mainly formed in the template-directed reaction. The preferential formation of the 2′–5′ linkage was also observed in the template-directed oligoadenylate formation on a poly(U) template (Orgel and Lohrmann, 1974; Usher and MacHale, 1976; Orgel, 1986; Kanaya and Yanagawa, 1986; Sawai *et al*., 1998).

Figures 3A and 3B shows the HPLC profiles of the condensation of $(pU)_3$ and $(pU)_4$, respectively, in the presence of a poly(A) template at 0 °C. The reactions were carried out under the same condition using EDAC as that for $(pU)_5$. The coupling products, $(pU)_6$ and $(pU)_8$ were obtained in small amounts; however, their yields were higher than those obtained in the control reactions where no template was used in the reactions. On the other hand, the presence of poly(A) did not promote the coupling reaction of $(pU)_2$ (Data not shown). Essentially, no coupling product, $(pU)_4$, was obtained from $(pU)_2$ in both cases in the presence and in the absence of poly(A).

The results of the coupling reaction indicate that the oligouridylate with chain length of more than three is required for the nonenzymatic template-directed condensation on a poly(A) template. Previously, we have reported that 50 mM of uridylic acid or diuridylic acid cannot form a helix with 25 mM of poly(A) or oligo(A) at 0 °C although adenylic acid, di- or triadenylic acid can form helix with poly(U) or oligo(U) under the same conditions (Sawai *et al*., 1997). We have also demonstrated that triuridylic acid, or the longer oligo(U) forms a helix with oligo(A) or poly(A) (Sawai *et al*., 1997). The results of the poly(A) template-directed condensation of oligo(U) presented in this report confirm that the non-enzymatric template-directed condensation of oligoribonucleotides correlates well with the results of the helix formation of complementary oligoribonucleotides. The observation suggests that a trimer is the minimum unit as an incorporating nucleotide for conducting any set of non-enzymatic directed synthesis, $A \rightarrow U$ and $U \rightarrow A$.

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