this basis may prove useful in the investigation of native and of chemically modified active sites of enzymes.

The fact that the elution pattern of purified HMM S-1 in the presence of Ca^{2+} exhibits a single, symmetrical, and quite sharp peak suggests that both myosin heads can split bound ATP, apparently with the same activity. This can also account for the stronger affinity of binding of myosin in comparison to one-headed myosin under conditions in which ATP is split (as can be seen from Figure 6).

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Chemical Polymerization of Oligonucleotides Directed by a Complementary Polynucleotide. Preparation and Polymerization of Oligo(2′-*O*-methylinosine 3′-phosphate)†

S. Uesugi‡ and P. O. P. Ts'o*

ABSTRACT: Substantial quantities of oligo(2′-*O*-methylinosinates) with 3′-terminal phosphates and defined chain lengths (n = 2–12) have been prepared by controlled hydrolysis of poly(2′-*O*-methylinosinate) with micrococcal nuclease, followed by DEAE-cellulose column chromatography. Two oligonucleotide fractions, hexa(2′-*O*-methylinosine 3′-phosphate) and penta(2′-*O*-methylinosine 3′-phosphate), have been used as starting materials in a polymerization reaction directed by a poly(C) template. These reactions were carried out in aqueous solution at low temperature (0 or −15°) with a water-soluble carbodiimide as the activating agent. The absolute overall yield was 38–61%, and the relative overall yield based on the recovered material was 43–71%; the yield of the 30-mer fraction (product with 5–6 linkages) can be as large as 15%. The stability of the 1:1 oligo(2′-*O*-methylinosine)·poly(C) complex is an important factor in determining the extent of the polymerization and the chain length of the product.

Synthesis of long-chain polynucleotides of defined sequence in sufficient quantity has been a very formidable challenge. Development of synthetic chemistry in the past decade has provided the methodology for the synthesis of oligonucleotides (especially deoxyribosyl oligomers) with a chain length of less than ten (10-mer or smaller) in reasonable yield in return for the effort and the cost. One logical approach to the synthesis of longer polynucleotides (n = 50–100) is to condense the preformed oligomers (n = 5–10). An early attempt using this strategy of block condensation by the conventional method under anhydrous conditions was not promising (*e.g.*, see Jacob *et al.*, 1967). An alternative approach is to use a complementary polynucleotide as a template to concentrate the appropriate oligomer substrates into a small area with proper alignment. Such a reaction has to be carried out in aqueous solution usually with a water-soluble carbodiimide as the activating agent. In 1966, Naylor and Gilham reported that $d(pT)_6$ was condensed in the presence of a poly(A) template to afford mainly $d(pT)_{12}$ in 5% yield. Orgel and coworkers, with their interest principally in the mechanism of prebiotic synthesis of nucleic acid, have investigated the condensation of adenine nucleotides in the presence of complementary polyuridylate (Sulston *et al.*, 1968, 1969; Schneider-Bernloehr *et al.*, 1968; Weimann *et al.*, 1968; Renz *et al.*, 1971). In this series of studies, water-soluble carbodiimide was first used as an activating agent for the 5′-nucleotides; subsequently the preactivated nucleotides, such as imidazolides of adenosine 5′-monophosphates, and adenosine 2′,3′-cyclic phosphates were used. In 1970, Shabarova and Prokofiev reported the polymerization of the amino acid amidate of $d(pA)_2$ in the presence of a poly(U) template which gave a 10% yield of the polymerized products. Another alternative for the template-directed oligonucleotide polymerization recently developed is the use of the joining enzyme, the polynucleotide ligase. The successful use of this enzymic method not only allowed the joining of deoxyribosyloligonucleotides (Gupta *et al.*, 1968; Harvey and Wright, 1972) and ribosyl oligonucleotides (Kleppe *et al.*, 1970), but also the synthesis of

† From the Division of Biophysics, Department of Biochemical and Biophysical Sciences, The Johns Hopkins University, Baltimore, Maryland 21205. *Received December 21, 1973.* This work was supported in part by a grant from the National Institutes of Health (GM-16066-06) and a grant from the National Science Foundation (GB-30725X). This paper is the third in a series entitled Studies on 2′-*O*-Alkyl Polynucleotides.

‡ Present address: Faculty of Pharmaceutical Sciences, Osaka University, Toyonaka, Osaka, Japan.

a tRNA gene (Khorana *et al.*, 1972) and the addition of a deoxyribosyl dodecanucleotide to a phage DNA (Harvey *et al.*, 1973). This biosynthetic approach so far has been limited to a very small scale of synthesis and presumably only to the joining of naturally occurring nucleic acid linkages.

For the past 3 years, our laboratory has investigated extensively the preparation and the properties of oligoinosinates (Tazawa *et al.*, 1972c), the thermodynamics and the stoichiometry of the oligo(I)·poly(C) interaction (Tazawa *et al.*, 1972c), as well as the synthesis of 2′-*O*-methyl nucleoside 5′-diphosphates and 2′-*O*-alkyl polynucleotides in large quantities (Tazawa *et al.*, 1972a). From these studies we have developed a poly(C)-directed chemical condensation system for oligo(2′-*O*-methylinosinates) with 3′-terminal phosphates. The 5-mer [(2′MeIp)$_5$][1] and the 6-mer [(2′MeIp)$_6$] were used as starting materials. The reaction was conducted at low temperatures (0 or −15°) in aqueous solution with a water-soluble carbodiimide as the activating agent. The absolute overall yield was 38–61%, the relative overall yield based on the recovered material was 43–71%, and the yield of 30-mer or larger (products of 5–6 linkages or more) can be as high as 15%.

Results and Discussion

General Approach. The main goal of this research is to establish an effective procedure for the specific condensation of oligonucleotides to yield polynucleotides of defined sequences. Three requirements appear to be essential for the achievement of this goal. (1) The specific condensation of the oligonucleotides has to be directed by a suitable complementary template which, on the one hand, provides the instruction for the desired sequence and, on the other hand, serves as a device to concentrate the reacting oligonucleotides into a small area. (2) The condensation reaction will be more efficient if the more reactive 5′-hydroxyl group (a primary alcohol) of the furanose is used as the acceptor for the activated phosphate group rather than the less reactive 3′-hydroxyl group which is a secondary alcohol and has a greater steric restriction. (3) If the oligonucleotides with the 3′-terminal phosphate group are used in the condensation, the adjacent group attached to the 2′-carbon of the furanose cannot be an acceptor to the activated 3′-phosphate. In other words, ribosyl nucleoside 3′-phosphate *cannot* be used in the condensation process since the product from such a substrate will most likely be the nucleoside 2′,3′-cyclic phosphate, and not an internucleotidyl linkage. The oligonucleotides consisting of 2′-deoxyribosyl or 2′-*O*-methylribosyl backbone together with a 3′-terminal phosphate and a free 5′-terminal hydroxyl group are suitable substrates for the condensation.

We have investigated rather thoroughly the thermodynamics and stoichiometry of the oligoinosinate–poly(C) interaction (Tazawa *et al.*, 1972b). The results indicate that the stoichiometry of the oligo(I)·poly(C) complex in neutral solution is always 1:1. The necessary data concerning the stability of the oligo(I)·poly(C) complex as affected by the chain length and condensation of oligo(I) have been collected. We have also studied the problem of chemical synthesis of 2′-*O*-methylnucleoside 5′-diphosphates as well as the enzymic synthesis of 2′-*O*-alkyl polynucleotides in large quantities (Tazawa *et al.*, 1972a). A general strategy for preparing 2′-*O*-methyl oligonucleotides on a large scale has been developed in which the polynucleotide is first synthesized and then degraded selectively.

Preparation of Poly(2′-O-methylinosinic acid). 2′-*O*-Methyladenosine was phosphorylated with phosphoryl chloride according to the procedure of Yoshikawa *et al.* (1969). The initial nucleotide product (probably the phosphorodichloridate) was hydrolyzed in water instead of 0.1 M NaOH which gives 3′,5′-cyclic phosphate as a major product (Tazawa *et al.*, 1972a). Major products of the hydrolysis in water were 2′MeAMP (58%) and 2′-*O*-methyladenosine 3′,5′-diphosphate (28%). 2′MeAMP was converted into 2′MeADP in 70% yield according to the procedure of Moffatt and Khorana (1961). This compound was then deaminated with nitrous acid to give 2′MeIDP in 85% yield which was identical with an authentic sample previously prepared in our laboratory (Tazawa *et al.*, 1972a).

2′-*O*-Methylinosine 5′-diphosphate was polymerized by polynucleotide phosphorylase under conditions similar to those originally used by Zmudzka *et al.* (1969) for polymerization of 2′MeCDP and adopted by our laboratory (Tazawa *et al.*, 1972a) for polymerization of 2′MeIDP. The reaction conditions for the present experiments were investigated in terms on Mn^{2+} concentration, pH of buffer solution, salt form of EDTA, and temperature. The Mn^{2+} concentration has a large effect on the rate and yield of the polymerization reaction. After all these investigations, the combination of 2 mM Mn^{2+}, Tris-HCl buffer (pH 8.5), Na_2EDTA, and 35° was finally adopted. For deproteinization after the polymerization reaction, a chloroform–isoamyl alcohol mixture (Sevag *et al.*, 1938) was used instead of the usual phenol extraction. Acidic phenol causes the precipitation of the polynucleotides and also extracts the 2′-*O*-alkyl polynucleotides into phenol (Tazawa *et al.*, 1972a). After deproteinization, the polymer was isolated by precipitation from ammonium acetate with isopropyl alcohol and was extensively dialyzed to remove Mn^{2+}. Poly(2′-*O*-methylinosine) thus obtained showed the same properties as those described by Tazawa *et al.* (1972a). The solution of this polymer in water was stored in a refrigerator and was never frozen in order to avoid extensive aggregation.

Preparation of Oligonucleotides with 3′-Terminal Phosphate by Micrococcal Nuclease Hydrolysis of Polynucleotides. Micrococcal nuclease (EC 3.1.4.7) hydrolyzes both DNA and RNA to produce oligonucleotides with 3′-terminal phosphate and nucleoside 2′-monophosphates. Since DNA and 2′-*O*-methyl polynucleotides cannot be hydrolyzed by alkali to yield products with 3′-terminal phosphate, the use of this enzyme is the most logical procedure to obtain this type of oligonucleotide. This enzyme has both endonucleolytic activity and exonucleolytic activity (see review by Anfinsen *et al.*, 1971). Consequently, mononucleotide accumulates in the digest, and the ratio of dinucleotide to mononucleotide is always less than 1 (Alexander *et al.*, 1961; Sulkowski and Laskowski, 1969). Ca^{2+} is required for this enzyme and the optimum concentration is reported to be around 5 mM for RNA in Tris buffer (pH 8.8) (Cuatrecasas *et al.*, 1967). It is known that poly(2′MeA) can be hydrolyzed completely to nucleosides by the combination of micrococcal nuclease, venom phosphodiesterase, and alkaline phosphatase (Rottman and Heinlein, 1968). The hydrolysis reaction by micrococcal nuclease with poly(2′MeI) and poly(I) as substrates was examined. The chain-length distribution of the oligomers in the digest was analyzed by paper chromatography.

Two problems were encountered: (1) precipitation of the polynucleotides in the reaction mixture in the presence of Ca^{2+} at 5–10 mM concentration, and (2) the high yield of mononucleo-

[1] Abbreviations used are: (2′MeIp)$_5$, penta(2′-*O*-methylinosine 3′-phosphate); (2′MeIp)$_6$, hexa(2′-*O*-methylinosine 3′-phosphate); 2′MeAMP, 2′-*O*-methyladenosine 5′-monophosphate; 2′MeADP, 2′-*O*-methyladenosine 5′-diphosphate; 2′MeCDP, 2′-*O*-methylcytosine 5′-diphosphate; 2′MeIDP, 2′-*O*-methylinosine 5′-diphosphate; 2′MeA, 2′-*O*-methyladenosine; 2′MeI, 2′-*O*-methylinosine.

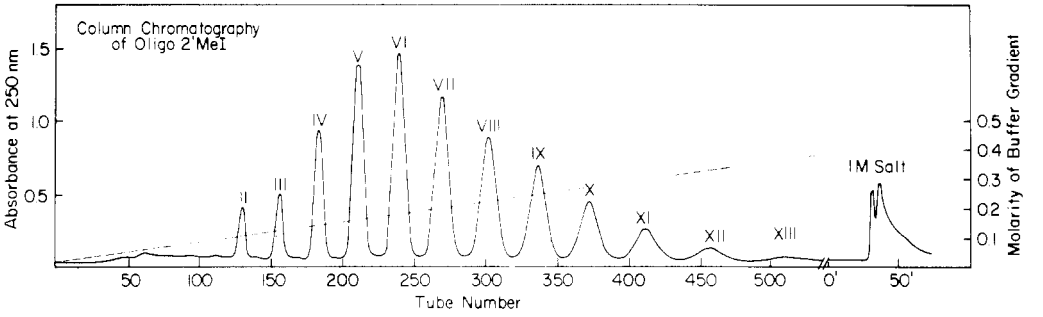

FIGURE 1: The hydrolytic products of poly(2'MeI) (1.34 mM) by micrococcal nuclease (50 units/ml). The oligomers in the hydrolysate were applied to a DEAE-cellulose column (3 × 62 cm) and eluted with a linear gradient from 0.02 to 0.50 M ammonium acetate and finally with 1 M ammonium acetate in 7 M urea at 4°. See Table I for peak identification and quantitative yield.

tides and dinucleotides relative to the oligonucleotides. These problems were overcome by reducing the Ca^{2+} concentration to *ca.* 0.3 mM and dialyzing the polymer extensively after precipitation with isopropyl alcohol. In the adopted procedure, poly(2'MeI) (1.34 mM) was hydrolyzed by micrococcal nuclease (50 units/ml) in the presence of $CaCl_2$ (0.33 mM) at 37° for 8.5 hr. The products were separated by DEAE-cellulose column chromatography in 7 M urea (step I) by the procedure of Tomlinson and Tener (1962). Twelve peaks (peaks II–XIII) and a 1 M salt fraction were obtained (Figure 1). The ratios of OD_T (total optical density) (250 nm) of these peaks are shown in Table I. Urea and salt in these fractions were removed by the usual procedure using small DEAE-cellulose columns (step II). Each desalted fraction was further purified by Sephadex column chromatography (step III). Some contaminant and residual triethylammonium bicarbonate were removed in this step. The recovery of OD_T (250 nm) from step I to step II was nearly quantitative. The apparent recovery of material from step II to step III is also shown in Table I. Interestingly, the recovery of each peak after peak VII was uniformly about two-thirds (Table I). As discussed later, this phenomenon was proved to be due to aggregation of the oligomers. Each oligomer fraction was finally lyophilized. Chain lengths of oligomers from peaks II–V were determined by digestion with venom phosphodiesterase after dephosphorylation with alkaline phosphatase. Since oligo(2'MeI) is fairly resistant to venom phosphodiesterase, a long incubation time is needed for complete digestion. Thus, it was necessary to remove phosphatase completely after dephosphorylation by paper chromatography of the products and also to use phosphodiesterase without 5'-nucleotidase activity. The results are shown in Table II. These oligomers showed a series of R_F values decreasing with increasing chain length on paper chromatography in solvent A (Table III). Upon dephosphorylation, these oligomers gave another series of R_F values higher than those of the original compounds (Table III). Together with the knowledge concerning the specificity of micrococcal nuclease, these results show that each of the original peaks contained oligo(2'MeI) with a 3'-terminal phosphate group and with a chain length equal to the number of the peak shown in Table I. Thus, a well-resolved series of $(2'MeIp)_n$ with a peak around $n = 6$ was obtained by controlled hydrolysis of poly(2'MeI) with micrococcal nuclease.

Poly(I) is hydrolyzed much faster than poly(2'MeI) by micrococcal nuclease. In this case, a low Ca^{2+} concentration relative to the polymer concentration causes inhibition of exonucleolytic activity and reduces the overall rate of hydrolysis. The

TABLE I: Separation and Purification of Oligo(2'MeI) from the Micrococcal Nuclease Hydrolysate.[a]

Peak No.[b]	Step I,[c] % Ratio of OD_T (250 nm)	Step II[d] to Step III,[e] % Recovery of OD_T (248 nm)
II	2.2	88
III	3.3	93
IV	7.3	90
V	13	90
VI	15.5	95
VII	14.5	66
VIII	12	68
IX	9.8	67
X	6.8	64
XI	3.8	64
XII	1.9	77
XIII	0.4	
1 M salt	9.5	

[a] The amount of poly(2'MeI) used in the hydrolysis was about 1040 OD (250 nm). [b] These numbers are shown in the chromatogram in Figure 1 and are identical with the chain length of the oligomers. [c] Separation by DEAE-cellulose column chromatography in 7 M urea. The sum of OD_T (250 nm) of these peaks is 996.4. [d] Desalting with small columns of DEAE-cellulose. Oligomers were eluted with 1 M triethylammonium bicarbonate. The recovery of the oligomers from step I was nearly 100%. [e] Purification by Sephadex column chromatography. Oligomers were eluted with water.

TABLE II: Chain-Length Determination of Oligo(2'-*O*-methylinosinic acid), $(2'MeIp)_n$, by Snake Venom Phosphodiesterase Hydrolysis after Removal of Terminal Phosphate Groups.[a]

Peak No.[b]	OD_T (240 nm) 2'MeI	OD_T (240 nm) p2'MeI	p2'MeI/2'MeI	Chain Length of $(2'MeIp)_n$
II	1.26	1.20	0.95	2
III	0.903	1.89	2.09	3
IV	0.497	1.64	3.30	4
V	0.618	2.36	3.82	5

[a] Compound from each peak was first treated with *Escherichia coli* alkaline phosphatase. The dephosphorylated product was separated by paper chromatography and incubated with venom phosphodiesterase for 20 hr at 35°. The products, 2'MeI and p2'MeI, were separated by paper chromatography. The amount of materials in these spots was measured by uv absorbance after correction from appropriate blanks. [b] Shown in Figure 1.

TABLE III: Paper Chromatographic Properties of Oligo-(2′MeI).[a]

Compound	R_F Solvent A	Solvent B	Solvent C
I	0.66	0.66	0.64
pI	0.37	0.23	0.35
2′MeI	0.77	0.76	0.76
p2′MeI	0.47	0.37	0.52
$2'MeI_2$	0.66	0.57	0.56
$(2'MeIp)_2$	0.42	0.22	0.32
$2'MeI_3$	0.53	0.36	0.37
$(2'MeIp)_3$	0.32	0.12	0.16
$2'MeI_4$	0.40	0.19	0.14
$(2'MeIp)_4$	0.23	0.04	0.06
$2'MeI_5$	0.26		
$(2'MeIp)_5$	0.14		
$2'MeI_6$	0.13		
$(2'MeIp)_6$	0.06		
$2'MeI_7$	0.08		
$(2'MeIp)_7$	0.04		

[a] Descending technique was used with Whatman No. 1 paper. For composition of solvent systems, see Materials and Methods.

exonucleolytic activity of micrococcal nuclease can be suppressed by a combination of slowing down the rate of hydrolysis and removing 3′-terminal phosphate groups of the product oligomers. Hence, poly(I) (13.6 mM) was hydrolyzed by micrococcal nuclease (15 units/ml) in the presence of $CaCl_2$ (5 mM) and alkaline phosphatase (0.1 mg/ml) at 37° for 1 hr. The products were separated by DEAE-cellulose column chromatography in 7 M urea. The elution profile is shown in Figure 2. The ratios of OD_T (250 nm) of the peaks are shown in Table IV. The chain lengths of the oligomers in peaks 3, 4, and 7–9 were determined by comparison with authentic samples on paper chromatography in solvent A. Thus, a well-resolved series of oligo(I), $(Ip)_{n-1}I$, with a peak of distribution around n = 7, 8 was obtained. Again, under these conditions, the rate of hydrolysis of poly(2′MeI) is much slower than that of poly(I). Hydrolysis of poly(2′MeI) (1.5 mM) by a combination of micrococcal nuclease (100 units/ml), $CaCl_2$ (3.3 mM), and alkaline phosphatase (0.06 mg/ml) in borate buffer at 37° for 23 hr gave a series of oligo(2′MeI), $(2'MeIp)_{n-1}2'MeI$, which has a sharp peak of distribution around n = 6.

Chemical Polymerization of $(2'MeIp)_6$ *Directed by Poly(C) Template.* In a probing experiment, the T_m of the 1:1 complex between $(2'MeIp)_6$ and poly(C) (0.6 mM each, base concentration) was measured in 0.7 M NaCl–0.05 M Tris-HCl buffer (pH 7.3) and found to be 11.3°. In consideration of the effects of oligomer concentration (Tazawa *et al.*, 1972c) and NaCl concentration (Szer and Shugar, 1966), the T_m of the complex (5 mM each, base concentration) in 1.5 M NaCl neutral solution can be estimated to be about 27°. As for the condensing agent, Naylor and Gilham (1966) originally used 1-cyclohexyl-3-(3-dimethylaminopropyl)carbodiimide *p*-toluenesulfonate for polymerization reaction in aqueous solution, and later 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride was employed by Sulston *et al.* (1968). The latter, a smaller carbodiimide, was adopted in this experiment.

An appropriate mixture of $(2'MeIp)_6$ (5 mM), poly(C) (5 mM), and the carbodiimide (125 mM) was incubated at 0°, pH 5.7, for 15 days. On the fourth day and again on the eleventh day, another similar amount of carbodiimide (125 mM) was added each time and the pH was readjusted to *ca.* 5.7. On the fifteenth day, the reaction mixture was treated with 0.3 N NaOH to hydrolyze poly(C), and the hydrolysate was fractionated on a Sephadex G-25 column. The oligomer fraction was treated again with 0.3 N NaOH to ensure the complete hydrolysis of poly(C), and the hydrolysate was fractionated again by the same column to give clearly resolved peaks of oligo(2′MeI) and Cp. The oligo(2′MeI) fractions were analyzed by Sephadex G-100 column chromatography at 51°. This procedure is based on the experiments of Imura *et al.* (1969), who used Sephadex G-100 at high temperature (56°) for the fractionation of tRNA fragments. We employed a solution of moderate salt concentration (0.02 M NaCl–0.01 M $NaPO_4$ buffer (pH 7.5)) for elution. This is a compromise condition for our experiment, since low salt concentration is required to avoid aggregation of oligomers while high salt concentration is needed for adequate chromatographic resolution. The column of Sephadex G-100

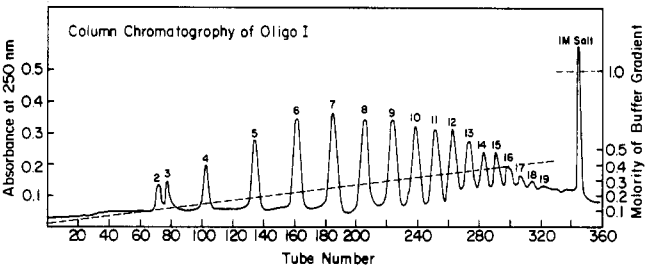

FIGURE 2: The hydrolytic products of poly(I) (13.6 mM) by micrococcal nuclease and *Escherichia coli* alkaline phosphatase. The oligomers in the hydrolysate were applied to a DEAE-cellulose column (1.5 × 40 cm) and eluted with a linear gradient from 0.02 to 0.52 M ammonium acetate and finally with 1 M ammonium acetate in 7 M urea at 4°. See Table IV for peak identification and quantitative yield.

TABLE IV: Distribution of Oligo(I)'s in the Micrococcal Nuclease and Phosphatase Hydrolysate of Poly(I).[a]

Peak No.[b]	% Ratio of OD_T (240 nm)	Peak No.[b]	% Ratio of OD_T (250 nm)
2	1.6	12	6.6
3[c]	1.9	13	5.6
4[c]	3.1	14	5.0
5	4.9	15	4.3
6	6.8	16	3.9
7[c]	8.2	17	3.5
8[c]	8.2	18	2.8
9[c]	7.8	19	2.6
10	7.2	1 M salt	8.8
11	7.1		

[a] Oligomers were separated by DEAE-cellulose column chromatography in 7 M urea. The hydrolysate from 125 OD (248 nm) of poly(I) was applied to the column. [b] These numbers are shown in the chromatogram in Figure 2 and are identical with the chain length of the oligomers. The sum of OD_T (250 nm) of these peaks is 152.4. [c] Oligomers in these peaks were identified by comparing paper chromatographic behavior in solvent A with those of authentic samples prepared by Tazawa *et al.* (1972b). R_F or R_I (R_F relative to inosine) are shown below in the following order: authentic sample and product: I_3, R_F 0.28, 0.29; I_4, R_F 0.18, 0.20; I_7, R_I 0.08, 0.12; I_8, R_I 0.05, 0.05; I_9, R_I 0.03, 0.02.

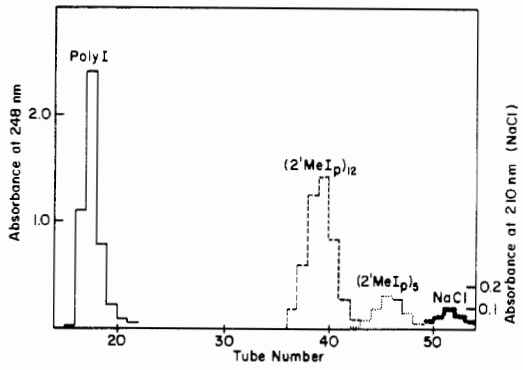

FIGURE 3: Elution position of poly(I), $(2'MeIp)_{12}$, $(2'MeIp)_5$, and NaCl in the Sephadex G-100 column (2 cm × 58 cm) at 51° with the buffer (0.02 M NaCl-0.01 M $NaPO_4$ (pH 7.5)).

was calibrated with poly(I), $(2'MeIp)_{12}$, $(2'MeIp)_5$, and NaCl. The elution positions of these compounds are shown in Figure 3. Note that each oligo(2'MeI) only shows a single peak; thus, aggregation is not observed under these conditions. The elution profile of polymerization products afer alkaline treatment is shown in Figure 4B. As a control, $(2'MeIp)_6$ was allowed to react with the carbodiimide in the *absence* of poly(C) under the same conditions. The reaction mixture was worked up and analyzed by Sephadex G-100 column chromatography in the same way. The elution profile of this control reaction is shown in Figure 4A. The recovery of OD_T (248 nm) from the polymerization reaction and the control reaction is 78 and 80%, respectively. The results in Figure 4 indicate that poly(C)-directed polymerization reaction of $(2'MeIp)_6$ took place in high yield (a yield of 23.4 OD units (248 nm) from a starting amount of 49 OD units (248 nm) of $(2'MeIp)_6$, or a relative yield of 61% as shown in Table V). In the absence of poly(C) template, the yield of the 12-mer fraction was about 5% or less (Figure 4A). Therefore, this reaction is critically dependent on the presence of poly(C) template.

In the elution profile shown in Figure 4B, at least five different fractions can be detected. These five different peaks were more obvious in the original recording of the chromatogram

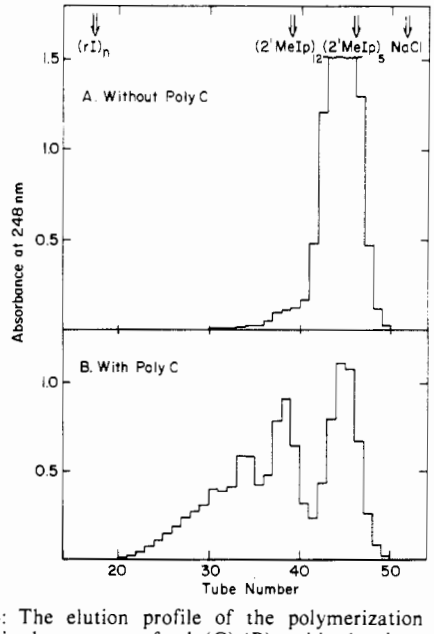

FIGURE 4: The elution profile of the polymerization products of $(2'MeIp)_6$ in the presence of poly(C) (B) and in the absence of poly(C) (A). The column and elution procedure are identical with that in Figure 3. The polymerization products have been treated with alkali to remove the template poly(C). See Table V for quantitative analyses of Figure 4B.

TABLE V: Yield of the Chemical Polymerization of $(2'MeIp)_6$ Directed by Poly(C).[a]

Tube No.	Chain Length of Oligomers Suggested by the Elution Positions	% Ratio of[b] OD_T (248 nm)
20-29	30	9.2
30-32	24	9.1
33-35	18	13.2
36-41	12	29.7
42-50	6	39.0

[a] The elution profile of the reaction products after alkaline hydrolysis of poly(C) is shown in Figure 4B. [b] OD_T (248 nm) of initial $(2'MeIp)_6$ was 49 and the recovery of OD_T (248 nm) was 78.2%.

monitored continuously. The tube number in each fraction, the relative yield of these fractions, and the chain length of the oligomers in these fractions are shown in Table V. For instance, the yields of $(Ip)_{12}$ and $(Ip)_{18}$ are 30 and 13%, respectively. The assignment of chain length of the oligomers in these fractions will be discussed in a later section.

In a preliminary experiment, polymerization of $(2'MeIp)_6$ [OD_T (250 nm) = 50] with poly(C) was conducted under the same conditions described earlier for 15 days. In this experiment, the reaction products were fractionated without alkaline hydrolysis of poly(C). The mixture was put on a DEAE-cellulose column. Elution by a linear gradient of ammonium acetate (pH 6.5, 0.02-0.08 M) in 7 M urea gave four peaks (peaks I-IV). Then the rest of the oligomer-polymer mixture was eluted by 1.5 M NaCl-7 M urea and alkaline 1.5 M NaCl-7 M urea solutions. These fractions were desalted, combined, and hydrolyzed in 0.3 N NaOH solution to give pure oligo(2'MeI). On examination by paper chromatography and Sephadex G-100 column chromatography conducted in a cold room, peaks I-IV were shown to contain $(2'MeIp)_52'MeI$, $(2'MeIp)_6$, $(2'MeIp)_{11}2'MeI$, and $(2'MeIp)_{12}$, respectively. The extent of dephosphorylation was 27% for the hexamer [15.5 OD units (250 nm)] and 23% for the dodecamer [6.96 OD units (250 nm)]. The fractions of oligomers with larger chain lengths [18.65 OD units (248 nm)] were analyzed by Sephadex G-100 column chromatography at 51° under the same conditions described earlier. Three major fractions corresponding to n = 30 (30%), n = 24 (34%), and n = 18 (28%) were obtained. Only 5.0 and 1.3% of OD_T (248 nm) were observed at the positions of 12-mer and 6-mer, respectively. The total recovery of OD_T (248 nm) in this experiment was about 82%, and the yield of the polymerization reaction was about 25.6 OD units (248 nm) (or a relative yield of 62%). Thus, the result of this polymerization experiment analyzed by a slightly different procedure is the same as that reported in Figure 4 and Table V.

Chemical Polymerization of $(2'MeIp)_5$ Directed by Poly(C) Template. Renz *et al.* (1971) reported that dimerization of adenosine 2',3'-cyclic phosphate in a frozen solution at −15° afforded a higher yield than that in a solution at 0°. Shabarova and Prokofiev (1970) heated the reaction mixture containing poly(U) and amino acid amidate of pdApdA during their attempt to polymerize the dinucleotide at −7°. It is conceivable that at a moderate temperature short oligomers are melted away from the complementary polynucleotide leaving behind only the relatively large oligomers on the surface of the template, since the large oligomer-polymer complex has a higher

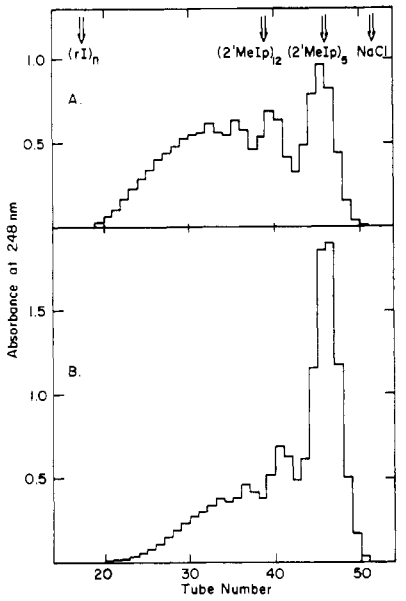

FIGURE 5: The elution profile of the polymerization products of $(2'MeIp)_5$ in two reaction conditions: (A) at 0° for 15 days and frozen at −15° for another 28 days; (B) at 0° for 11 days and another 4 days at 24°. The column and the elution procedure are identical with those in Figures 3 and 4. The polymerization products have been treated with alkali to remove the template poly(C). See Table VI for quantitative analyses.

T_m than the small oligomer-polymer complex. As a consequence, the number of the internucleotidyl linkages may be reduced at this higher temperature, but the products may be of longer chain length. Therefore, polymerization of $(2'MeIp)_5$ was conducted under two different conditions to elucidate the effect of temperature on the polymerization reaction. In the first experiment (A), $(2'MeIp)_5$ [50 OD units (248 nm)] and 1 equiv of poly(C) were treated in the same way as described in the $(2'MeIp)_6$–poly(C) experiment in the preceding section for 15 days. Then the reaction mixture was kept frozen at −15° for another 28 days. In the second experiment (B), $(2'MeIp)_5$ [50 OD units (248 nm)] and 1 equiv of poly(C) were also treated in the same way as in experiment A at 0° for 11 days. After the third addition of carbodiimide and readjustment of pH, the reaction mixture was kept at room temperature (24°) for an additional 4 days. After alkaline hydrolysis of poly(C), the product oligomers were analyzed by Sephadex G-100 column chromatography at 51° under the same conditions as described in the previous section. The elution profiles of the products from experiments A and B are shown in Figure 5. At least six fractions can be detected from each of these profiles and their corresponding original continuous recordings. The positions of these fractions can be correlated to those of fractions from the $(2'MeIp)_6$ polymerization shown in Figure 4. The tube numbers in these fractions, assignment of chain length of oligomers in these fractions, and relative yield of these fractions are given in Table VI. The total recoveries from reactions A and B are 86 and 88%, respectively. In experiment A, 30.6 OD units (248 nm) of polymerized products were obtained (relative yield 71%); in experiment B, 19.1 OD units (248 nm) of products were obtained (relative yield 43%). Obviously, both in terms of the quantities of the products and the sizes of the products, experiment A is superior to experiment B. In fact, the polymerization in experiment A was even more extensive than that occurring in the $(2'MeIp)_6$–poly(C) experiment described in the preceding section.

Identification and Characterization of the Polymerization Products. The polymerization products were first separated by

TABLE VI: Yield of the Chemical Polymerization of $(2'MeIp)_5$ Directed by Poly(C).

Tube No.	Chain Length of Oligomers Suggested by the Elution Positions	% Ratio of OD_T (248 nm)
A. 0°, 15 Days Followed by −15°, 28 Days		
20-28[a]	30	14.7[b]
29-31[a]	25	11.2[b]
32-34[a]	20	12.5[b]
35-37[a]	15	12.7[b]
38-42[a]	10	19.7[b]
43-50[a]	5	29.0[b]
B. 0°, 11 Days Followed by 24°, 4 Days		
21-29[c]	30	4.8[d]
30-32[c]	25	5.8[d]
33-35[c]	20	7.8[d]
36-38[c]	15	9.1[d]
39-42[c]	10	15.9[d]
43-51[c]	5	56.7[d]

[a] The elution profile of the reaction products after alkaline hydrolysis of poly(C) is shown in Figure 5A. [b] OD_T (248 nm) of initial $(2'MeIp)_5$ was 50 and the recovery of OD_T (248 nm) was 86.2%. [c] The elution profile of the reaction products after alkaline hydrolysis of poly(C) is shown in Figure 5B. [d] OD_T (248 nm) of initial $(2'MeIp)_5$ was 50 and the recovery of OD_T (248 nm) was 88.4%.

Sephadex G-100 column chromatography according to molecular size as described in the two previous sections. Next, some of the products were analyzed by DEAE-Sephadex A-25 column chromatography in 7 M urea. For this investigation, the same column was used for the analyses of all the oligomers, and these oligomers were eluted under identical conditions using a linear gradient of NaCl (0.1–0.5 M) in 7 M urea at pH 7.5. The 12-mer fraction (Figure 6c) from the $(2'MeIp)_6$–poly(C) polymerization (Table V) gave two peaks which are nearly identical with the two peaks of authentic $(2'MeIp)_{11}2'MeI$ (Figure 6B) and $(2'MeIp)_{12}$ (Figure 6A) in the elution profile. After dephosphorylation of this 12-mer fraction by alkaline phosphatase, the product gave only one single peak at the same position as the leading peak of the original 12-mer fraction (Figure 6D). A mixture of this product and the authentic $(2'MeIp)_{11}2'MeI$ afforded a single peak in the elution profile (Figure 6E). These results conclusively show that the 12-mer fraction (Table VVV) contains only $(2'MeIp)_{12}$ (about 57%) and its dephosphorylated compound (about 43%). The 18-mer fraction (Figure 6F) was eluted later than the 12-mer, indicating that this fraction has a longer chain length, while the 6-mer fraction was eluted earlier than the 12-mer and gave two peaks (Figure 6F). On examination by paper chromatography, these two peaks were proved to be $(2'MeIp)_6$ (about 25%) and its dephosphorylated derivative (about 75%). At present, not much is known about the cause and the time of this dephosphorylation reaction. It is possible that this process takes place only during the treatment of the products after polymerization, such as the alkaline hydrolysis, chromatography at 50–60°, etc. If the dephosphorylation process occurs during the polymerization reaction at pH 5.7, then it can be a serious problem.

The 12-mer fraction was hydrolyzed with a combination of snake venom and alkaline phosphatase at 36° for 20 hr. The

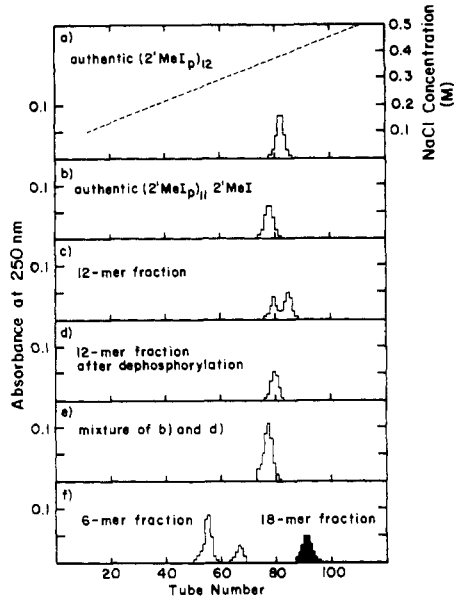

FIGURE 6: Identification and characterization of the polymerization product by DEAE-Sephadex A-25 column (1 × 12 cm) eluted with a linear gradient of 0.1 M–0.5 M NaCl in 0.01 M Tris-HCl (pH 7.5) and 7 M urea at 24°: (a) authentic $(2'MeIp)_{12}$ isolated from peak XII in Figure 1 after purification; (b) authentic $(2'MeIp)_{11}2'MeI$ which is a dephosphorylated product of a by alkaline phosphatase; (c) 12-mer fraction from Figure 4B; (d) dephosphorylated product of c by alkaline phosphatase; (e) mixture of b and d; (f) 6-mer fraction and 18-mer fraction from Figure 4B.

hydrolysate was analyzed by Sephadex G-25 column chromatography. At least 95% of the ultraviolet (uv) adsorbing material (about the limit of experimental error) eluted at the position of the monomer. Snake venom phosphodiesterase is an exonucleolytic enzyme cleaving polynucleotides from 3′-terminal ends. A compound in which two $(2'MeIp)_5$2′MeI groups are connected with a 3′-3′ phosphodiester linkage cannot be cleaved by this enzyme because it has no 3′-terminal end. Considering that oligo(2′MeI) is fairly resistant to this enzyme and oligo(2′MeI) tends to form an aggregate, the result of this experiment indicates the absence of such a 3′-3′ linked compound in the products.

The T_m values of the oligo(I)·poly(C) complex have been measured previously (Tazawa *et al.*, 1972c) in 10 mM

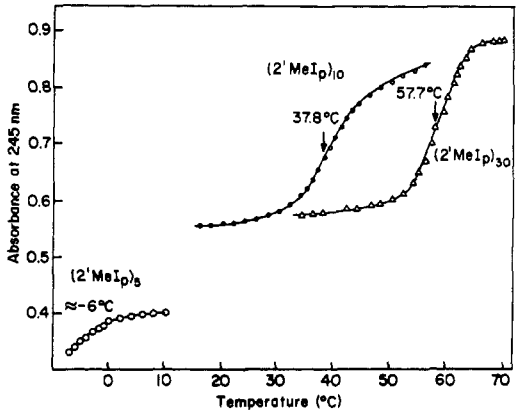

FIGURE 7: T_m measurement of poly(C) complexes (1:1) with the 5-mer, 10-mer, and 30-mer fractions of oligo(2′MeI). For the complexes with 5-mer and 10-mer fractions, 10 mM $MgCl_2$–10 mM $NaPO_4$ (pH 7.0) was used as solvent with an oligomer strand concentration of 5 × 10^{-6} M. For the complex with the 30-mer fraction, 1 mM $MgCl_2$–0.15 M NaCl–0.01 M $NaPO_4$ (pH 7.4) was used as solvent with the base concentration of 5 × 10^{-5} M. The 30-mer fraction was prepared from the polymerization of $(2'MeIp)_5$ as starting material.

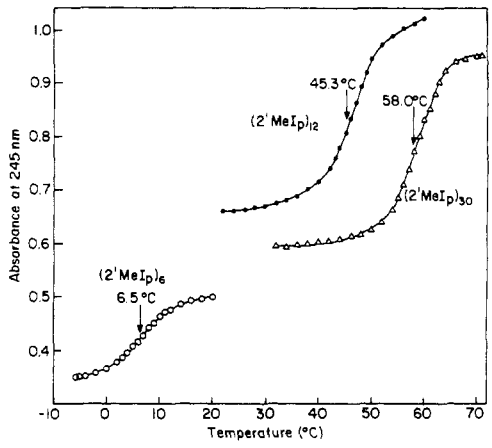

FIGURE 8: T_m measurement of poly(C) complexes (1:1) with 6-mer, 12-mer, and 30-mer fractions of oligo(2′MeI). For the complexes with 6-mer and 12-mer fractions, 10 mM $MgCl_2$–10 mM $NaPO_4$ (pH 7.0) was used as solvent with an oligomer strand concentration of 5 × 10^{-6} M. For the complex with the 30-mer fraction, 1 mM $MgCl_2$–0.15 M NaCl–0.01 M $NaPO_4$ (pH 7.4) was used as solvent with a base concentration of 5 × 10^{-5} M. The 30-mer fraction was prepared from the polymerization of $(2'MeIp)_6$ as starting material.

$MgCl_2$–10 mM $NaPO_4$ buffer (pH 7.0). For confirmation of the chain length of the polymerization products as well as for the examination of the effect of 2′-*O*-methyl groups, T_m values of oligo(2′MeI)·poly(C) complexes (1:1, 5 × 10^{-6} M, oligomer strand concentration) were measured with 5-, 6-, 10-, and 12-mer fractions under the same conditions. Each complex shows the same characteristic uv absorption spectrum as those of the oligo(I)·poly(C) complexes (Tazawa *et al.*, 1972c). The spectrum consists of two peaks, around 245 and 265 nm, of almost the same intensity. The melting profiles are shown in Figures 7 and 8. The T_m of the $(2'MeIp)_5$·poly(C) complex is too low to be measured directly under these conditions. From the spectrum of this solution at −7°, the fact that the absorbance of the intact complex at 245 and 265 nm is about equal, and from the expectation that the absorbance at 265 nm measured at −7° is close to that of the intact complex, the T_m values of this $(2'MeIp)_5$·poly(C) complex can be estimated to be around −6°. The T_m's of the 10-mev·poly(C) complex (37.8°) and of the 12-mer·poly(C) complex (45.3°) are considerably higher than those of the corresponding oligo(I)·poly(C) complexes (31.4 and 38.3°) measured previously (Tazawa *et al.*, 1972c). These T_m data of the poly(C) complexes with 5-, 6-, 10-, and 12-mers can be fitted into a straight line in a plot of $1/T_m$ *vs.* $1/n$ as shown in Figure 9, similar to that observed for the oligo(I)·poly(C) complexes (Tazawa *et al.*, 1972c). This linear

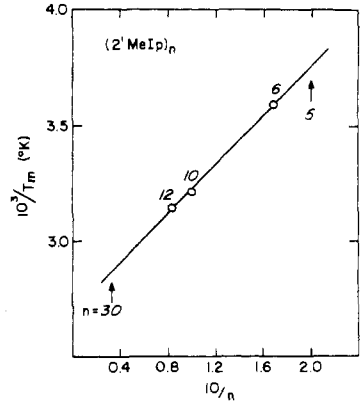

FIGURE 9: The results in Figures 7 and 8 analyzed on a $1/T_m$(°K) *vs.* $1/n$ plot.

plot of the T_m data confirms the chain-length assignments for the 10-mer and the 12-mer. Unfortunately, the T_m measurement of poly(C) complexes with oligomers having $n \geq 30$ (referred to as 30-mer, Tables V and VI) under the same condition was unsuccessful. In this solution, precipitation occurs near 52°, a temperature just before melting of the complex. It is most likely due to the precipitation of $(2'MeIp)_{30}$ in this high concentration of Mg^{2+} (10 mM). Such a phenomenon was also observed in solution of the poly(I)·poly(C) complex under the same conditions (Tazawa *et al.*, 1972c). Therefore, the T_m of the $(2'MeIp)_{30}$·poly(C) complex was measured instead in 0.15 M NaCl–0.01 M $NaPO_4$ (pH 7.4) and 1 mM $MgCl_2$ solution. As shown in Figures 7 and 8, essentially the same T_m (~58°) was observed for the poly(C) complex with the 30-mer (each in 5×10^{-5} M of base concentration) prepared from polymerization of $(2'MeIp)_5$ or with the 30-mer prepared from polymerization of $(2'MeIp)_6$.

The poly(2'MeI)·poly(C) complex has a T_m value of 75° under the same condition (J. Alderfer, I. Tazawa, S. Tazawa, and P. O. P. Ts'o, unpublished results). The T_m value of the 30-mer complex is much higher than that of the 12-mer complex even at lower Mg^{2+} and oligomer-strand concentrations. Thus, measurements of the T_m of oligo(2'MeI)))·poly(C) complexes provide evidence to confirm the chain length of the 10-mer, 12-mer, and 30-mer.

Aggregation of Oligo(2'-O-methylinosinate). It was observed in our laboratory (I. Tazawa, S. Tazawa, J. Alderfer, and P. O. P. Ts'o, unpublished data) that poly(2'MeI) forms a self-complex at relatively low ionic strength solution, especially upon freezing the solution. This tendency of aggregation was also observed for oligo(2'MeI). As described in the Experimental Section (see paragraph at end of paper regarding supplementary material), both 6-mer and 12-mer gave two fractions when chromatographed on a Sephadex G-100 column in a cold room. An authentic sample of $(2'MeIp)_{12}$, which afforded only a single peak on Sephadex G-100 column chromatography at 51°, gave two peaks when eluted near 5°. The first peak of the 6-mer was eluted earlier than the second peak of the 12-mer but later than the first peak of the 12-mer. This information suggests that the first peaks of the 6-mer and 12-mer observed at 5° are the self-complexes of these oligo(2'MeI)'s. This suggestion is again indicated in Table I where the recovery from step II (conducted at room temperature) to step III (conducted at 5°) in fractions of oligomers longer than 6-mer was uniformly about 65–70%, in contrast to the recovery of nearly 90% in fractions of oligomers shorter than 6-mer. The long oligomers exhibit hyperchromicity upon heating as indicated by the change of the $A_{max}/A_{270\,nm}$ ratio from about 2.2 to about 3, which is the $A_{max}/A_{270\,nm}$ ratio of the monomer. For example, the maximal absorbance of a solution of $(2'MeIp)_6$ at room temperature was found to be 0.810 ($A_{max}/A_{270\,nm} = 2.57$) immediately after thawing, and 0.915 ($A_{max}/A_{270\,nm} = 2.86$) immediately after heating briefly at 60–70°.

These results indicate that the oligo(2'MeI)'s longer than 6-mer form stable self-aggregates near 5° or upon freezing, even at relatively low ionic strength (about 0.02–0.04 M salt). Such a property is not known for oligo(I) or poly(I) and is related to the effect of the 2'-*O*-methyl group. The relatively high degree of stability of the oligo(2'MeI)·poly(C) complex presumably also contributes to the high yield of the condensation process of the oligo(2'MeI) on a poly(C) template.

A Comparison of Various Methods of Preparation of Oligonucleotides by Hydrolysis of Polynucleotides. For preparation of ribooligonucleotides, controlled alkaline hydrolysis is a well-known method (*e.g.*, Michelson and Monny, 1967; Tazawa *et al.*, 1972c). The products of a controlled alkaline hydrolysis of ribopolynucleotides contain considerable amounts of oligomers with terminal 2',3'-cyclic phosphate groups and an additional step of acid hydrolysis of these compounds is required (*e.g.*, see Tazawa *et al.*, 1972c). Acid treatment of oligonucleotides may be a serious problem for oligomers such as oligo(adenylic acids) which are precipitable in acid. Furthermore, the distribution of chain length of oligomer products is very broad; consequently the yields of oligomers with the desired chain length are small (I. Tazawa, S. Tazawa, and P. O. P. Ts'o, unpublished data).

For preparationnn of deoxyribooligonucleotides, the application of DNase I was reported by Bollum (1966). DNase I is an endonuclease, and its successive products are progressively more resistant substrates (Vanecko and Laskowski, 1961). This nature of DNase I, called "autoretardation," is highly advantageous for preparation of oligonucleotides because accumulation of relatively short oligonucleotides and very little formation of monomer are expected even in extensive digestion. As a matter of fact, Bollum obtained oligodeoxyadenylates with chain lengths 2–7 as major products from poly(deoxyadenylic acid) in high yield. In this case, relatively large amounts of enzyme were required, and unfortunately poly(deoxycytidylic acid) and poly(deoxyinosinic acid) were not hydrolyzed under the same conditions.

Micrococcal nuclease can be used for both ribopolynucleotides (Alexander *et al.*, 1961) and deoxyribopolynucleotides (Sulkowski and Laskowski, 1969) and even for the 2'-*O*-methyl derivative of ribopolynucleotides (Rottman and Heinlein, 1968). This enzyme is very stable and commercially available in high specific activity. However, it does have undesirable exonucleolytic activity in addition to endonucleolytic activity. Thus, if this exonucleolytic activity is suppressed, micrococcal nuclease could behave like DNase I. In our study, micrococcal nuclease hydrolysis of polynucleotides was proved to be an excellent procedure for preparation of oligonucleotides in intermediate chain length under certain conditions. The success in the application of this enzyme depends on the suppression of its exonucleolytic activity which can be achieved under two conditions: (1) lowering the Ca^{2+} concentration, and (2) removal of the terminal phosphate groups of nascent oligonucleotides in the reaction. For the first condition, Sulkowski and Laskowski (1968) examined the time course of micrococcal nuclease hydrolysis of DNA and RNA by alkali titration and showed that the apparent end point of hydrolysis occurred earlier with decreasing Ca^{2+} concentration. In other words, low Ca^{2+} concentration not only slows down the hydrolysis rate, but also reduces the extent of hydrolysis which was followed by alkali titration. For the second condition, it is also known that oligonucleotides without a 3'-terminal phosphate group are fairly resistant to micrococcal nuclease (Alexander *et al.*, 1961); thus, the removal of a 3'-terminal phosphate group renders the nascent oligonucleotides no longer susceptible to the exonucleolytic attack of the enzyme. Thus, a controlled micrococcal nuclease hydrolysis of polynucleotide can be a general method for preparation of oligonucleotides with a variety of sugar and base constituents (Cuatrecasas *et al.*, 1967).

Discussion of the Chemical Polymerization of Oligonucleotides Directed by Polynucleotide Templates in Aqueous Solution. So far, there have been only two reports on the chemical condensations of oligonucleotides directed by polynucleotide templates, one by Naylor and Gilham (1966) and the other by Shabarova and Prokofiev (1970). In the study by Naylor and Gilham, d-$(pT)_6$ (*ca.* 10 mM) was condensed in the presence of poly(A) (*ca.* 10 mM) by a water-soluble carbodiimide to give

d-$(pT)_{12}$ in 5% yield. In the study by Shabarova and Prokofiev, d-$(pA)_2$ (25 mM) preactivated in the form of an amino acid amidate was condensed in the presence of poly(U) (25 mM) to give products in 10% yield. In both cases, the reacting species are the 5'-phosphate group and the 3'-hydroxyl group of deoxyribooligonucleotides. In our present experiment, $(2'MeIp)_6$ or $(2'MeIp)_5$ (*ca.* 5 mM) was condensed in the presence of a poly(C) template by a water-soluble carbodiimide which is smaller in size than that used by Naylor and Gilham. The relative overall yield of the oligomer products was 43–71% in the present reaction. The reacting species in these experiments are the 3'-phosphate group and the 5'-hydroxyl group of 2'-*O*-methyl ribooligonucleotide. In general, the 5'-hydroxyl group is considered to be much more reactive than the 3'-hydroxyl group of a nucleoside.

In a chemical polymerization of oligonucleotides under anhydrous conditions (block polymerization), the yield of polymerized products is very poor and decreases abruptly for oligomers of longer chain length (*e.g.*, see Jacob *et al.*, 1967). In the present experiment of template-directed condensation in aqueous solution, $(2'MeIp)_6$ appears to be a better substrate than $(2'MeIp)_5$, even though the concentration of terminal groups (3'-phosphate and 5'-hydroxyl) is lower in the reaction with the hexamer for a given concentration of bases. In addition, the yield of 30-mer (a product with 5–6 linkages) is not much smaller than the yield of the 10-mer or 12-mer (a product with 2 linkages). Also, freezing the reaction mixture (−15°) enhances polymerization while raising the temperature from 0° to about 25° has an adverse effect. These facts suggest that in the present polymerization system, the stability of the complex between the oligonucleotides and the complementary template is perhaps the most important factor in determining the extent of the condensation.

It is challenging to consider the extension of the present template-directed, *chemical* condensation system for 2'-*O*-methyl ribosyl oligonucleotides to 2'-deoxyribosyl oligonucleotides. Template-directed polymerizations of deoxyribosyl oligonucleotides have been achieved in high yield (near 80%) by the use of polynucleotide ligase (Gupta *et al.*, 1968; Harvey and Wright, 1972). This technique has been used successfully in the synthesis of a tRNA gene (Khorana *et al.*, 1972) and addition of a dodecanucleotide to λ phage DNA (Harvey *et al.*, 1973). In comparison with the enzyme method, the present system of chemical condensation may have two advantages. First, it may be more amenable to a very large scale synthesis. Second, the chemical method may be applicable to the synthesis of polynucleotides of unnatural backbones (different types of sugars or different types of linkages, etc.) which probably cannot be achieved with the enzyme method.

Experimental Section

The supplement to this paper contains a detailed description of experimental procedures; see paragraph at end of paper regarding supplementary material.

Materials and Methods

2'-*O*-Methyladenosine (Broom and Robins, 1965), 2'MeAMP (Yoshikawa *et al.*, 1969), 2'MeADP (Tazawa *et al.*, 1972a), and 2'MeIDP (Tazawa *et al.*, 1972a) were prepared according to published procedures. The maximum molar extinction coefficients of 10.1×10^3 (water, pH 6.0), 9.9×10^3 (water, pH 6), and 6.5×10^3 (0.01 M Tris-HCl (pH 7.5)) were used for poly(I), poly(2'MeI), and poly(C), respectively. Descending paper chromatography on Whatman No. 1 paper employed the following solvent systems: (A) 1-propanol–concentrated ammonia–water (55:10:35, v/v); (B) ethanol–1 M ammonium acetate (7:3, v/v, pH 7.5); (C) ethanol–1 M ammonium acetate (7:3, v/v, pH 5) containing 3.3 mM EDTA; (D) saturated aqueous ammonium sulfate–1 M sodium acetate–2-propanol (80:18:2, v/v); (E) 2-propanol–concentrated ammonia–water (7:1:2, v/v). Paper electrophoresis was performed on Whatman No. 1 paper using 0.05 M triethylammonium bicarbonate buffer (pH 7.5) for 1 hr at 20 V/cm. The method of Baginski *et al.* (1967) was used to follow the release of inorganic phosphate during polymerization reactions catalyzed by polynucleotide phosphorylase. Ultraviolet spectra were recorded on a Cary 14 spectrophotometer. T_m measurements were carried out as previously described (Tazawa *et al.*, 1972c).

Poly(2'-O-methylinosinic acid). 2'-*O*-Methylinosine 5'-diphosphate (triethylammonium salt, 7 mM) in a solution containing 0.15 M Tris-HCl (pH 8.5), 2 mM $MnSO_4$, 0.4 mM Na_2EDTA, and 1 mM NaN_3 was polymerized for 69 hr at 35° by polynucleotide phosphorylase according to the method of Tazawa *et al.* (1972a–c). After work-up, 380 OD units (248 nm) (55%) of polymer and 377 OD units (249 nm) (43%) of starting diphosphate were obtained.

Enzymic Hydrolysis of Poly(2'MeI) and Preparation of Oligo(2'MeI). A solution containing 1.34 mM poly(2'MeI) in 83 mM Tris-HCl (pH 9)–0.33 mM $CaCl_2$ was incubated with micrococcal nuclease (50 units/ml) at 37° for 8.5 hr. The resulting oligonucleotides were separated on DEAE-cellulose column using 7 M urea–ammonium acetate as eluent. The elution profile is shown in Figure 1, and the relative yields of each oligomer are shown in Table I. After removal of urea by DEAE-cellulose chromatography and further purification by Sephadex gel filtration, the oligomers (R_F values, Table III) were characterized by dephosphorylation with alkaline phosphatase and digestion with snake venom phosphodiesterase (Table II).

Chemical Polymerization of $(2'MeIp)_6$ in the Presence of Poly(C). A solution containing 49 OD units (248 nm) of $(2'MeIp)_6$ and 4.5 μmol of poly(C) in 1.00 ml of water was heated for 2 min at 60°. Solid NaCl (88 mg), *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide (24 mg), and one drop of chloroform were added, and the pH was adjusted to 5.67. The solution was kept at 0° for 4 days. Additional carbodiimide (24 mg) was added, and after 7 days another 24 mg of carbodiimide was added, the pH being adjusted to 5.66 each time. After a total of 15 days at 0°, the reaction mixture was diluted with 1.5 ml of water and treated with 2.5 ml of 1 M sodium hydroxide for 24 hr at 36°. Following neutralization with 5 M HCl, the reaction mixture was fractionated on a Sephadex G-25 column (1.3 × 81 cm), using water as eluent. Fractions containing oligo(2'MeI) were further treated with 0.3 M NaOH to hydrolyze contaminating poly(C), followed by fractionation on Sephadex G-25 to give 40.4 OD units (248 nm) of pure oligo(2'MeI).

Chemical Polymerization of $(2'MeIp)_6$ in the Absence of Poly(C). The polymerization was carried out under the same conditions as described in the previous experiment except poly(C) was omitted. Treatment with sodium hydroxide was omitted and the reaction mixture was fractionated on a Sephadex G-25 column (1.5 × 76 cm).

Chemical Polymerization of $(2'MeIp)_5$ in the Presence of Poly(C). Two reactions under different conditions were carried out. (A) Fifty OD units (248 nm) of $(2'MeIp)_5$ were polymerized in the same way as $(2'MeIp)_6$ in the presence of poly(C) for 15 days at 0°. Then, the reaction mixture was kept frozen at −15° for 28 days. After desalting on a Sephadex G-25 column, the reaction mixture was treated with 0.3 N NaOH (3 ml) at 36° for 19 hr. After neutralization, the reaction mixture

was desalted again with a Sephadex G-25 column. This procedure of hydrolysis in 0.3 N NaOH and subsequent desalting with Sephadex G-25 was repeated twice. Finally, a clearly resolved peak of oligo(2'MeI) was obtained. The appropriate fractions were combined and concentrated to a small volume under reduced pressure. (B) Fifty OD units (248 nm) of $(2'MeIp)_5$ were polymerized in the same way as $(2'MeIp)_6$ in the presence of poly(C) for 11 days at 0°. After addition of the third portion of carbodiimide, the reaction mixture was kept at room temperature for 4 days. Then, the procedures of alkaline hydrolysis of poly(C) and desalting with Sephadex G-25 were repeated twice as in the reaction with $(2'MeIp)_6$. The desalted fractions of oligo(2'MeI) were combined and concentrated to a small volume.

Analysis of the Polymerization Products by Sephadex G-100 Column Chromatography at 51°. Sephadex G-100 and the buffer (0.02 M NaCl–0.01 M $NaPO_4$ buffer (pH 7.5)) for elution were degassed under vacuum and/or by boiling. Warmed Sephadex G-100 in the buffer solution was poured into a jacketed column of 2-cm diameter to a height of 58 cm. The column temperature was maintained at 51°. It was washed thoroughly with the buffer solution before use. The absorption and elution properties of this column were calibrated with poly(I), $(2'MeIp)_{12}$, $(2'MeIp)_5$, and NaCl. All solutions were preheated (60–90°) and applied to the column immediately. The elution profile of these compounds is shown in Figure 3. The elution profiles of the polymerization reaction mixtures are shown in Figures 4 and 5.

Analysis of the Chain Length of the Polymerization Products by DEAE-Sephadex Column Chromatography. Samples were chromatographed on a DEAE-Sephadex A-25 (Cl^- form) column (1 × 12 cm) equilibrated with 0.1 M NaCl–0.01 M Tris-HCl (pH 7.5)–7 M urea. Elution was carried out with a linear gradient of NaCl (0.1–0.5 M in 0.01 M Tris-HCl (pH 7.5)–7 M urea, 500 ml total). Fractions of 5 ml were collected at a rate of 12–35 ml/hr. The results are shown in Figure 6.

Preparation of Oligoinosinic Acid; Hydrolysis of Poly(I) by Micrococcal Nuclease and Alkaline Phosphatase. A solution (1 ml) containing poly(I) (13.6 mM), Tris-HCl (pH 9.0, 0.075 M), $CaCl_2$ (5 mM), alkaline phosphatase (0.1 mg/ml), and micrococcal nuclease (15 units/ml) was incubated 1 hr at 37°. After extraction with chloroform–isoamyl alcohol (5:2, v/v), the hydrolysate was fractionated on a DEAE-cellulose column using a linear gradient of ammonium acetate in 7 M urea. The elution pattern is shown in Figure 2. Yield and assigned chain length are shown in Table IV. Peaks 3, 4, 7, 8, and 9 were desalted using small DEAE-cellulose columns. Each compound was further purified by paper chromatography in solvent A. These compounds were identified by comparison of R_F values with those of authentic samples on paper chromatography in solvent A. The results are shown in the footnotes of Table IV.

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Supplementary Material Available

A detailed description of experimental procedures will appear following these pages in the microfilm edition of this volume of the journal. Photocopies of the supplementary material from this paper only or microfiche (105 × 148 mm, 24× reduction, negatives) containing all of the supplementary material for the papers in this issue may be obtained from the Journals Department, American Chemical Society, 1155 16th St., N.W., Washington, D. C. 20036. Remit check or money order for $4.00 for photocopy or $2.00 for microfiche, referring to code number BIO-74-0000.

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Deoxyribonucleic Acid Synthesis in Isolated Nuclei from Chicken Embryo Fibroblast Cell Cultures†

Lesley M. Hallick*,‡ and Masae Namba

ABSTRACT: A procedure for the isolation of high yields of nuclei from cultures of chicken embryo fibroblast cells is described and a detailed characterization of the DNA synthesis activity of these nuclei is presented. DNA synthesis by the isolated nuclei requires Mg^{2+}, ATP, and all four dNTPs. The activity is stimulated markedly by the addition of exogenous DNA template. If nuclei are isolated from various stages of the cell cycle, both the endogenous and exogenous activities *in vitro* are proportional to the rate of DNA synthesis *in vivo*. Further evidence that the DNA synthesis activity is replication rather than repair is given by the results from experiments with density markers. DNA synthesis is semiconservative and extends for at least 500 nucleotides. The DNA replicated *in vitro* is an extension of growing points previously initiated *in vivo*. Such a cell-free system capable of valid replication should offer a convenient way to study the mechanisms of the elongation and initiation of DNA snythesis.

The molecular mechanism of DNA replication and its initiation are poorly understood at the present time. In the last few years, the development of crude *in vitro* systems has provided a convenient way to dissect some of the components of the replication system in bacteria (Moses and Richardson, 1970; Vosberg and Hoffmann-Berling, 1971; Schaller *et al.*, 1972; Wickner *et al.*, 1972). These systems are capable of elongating bacterial chains on preexisting replication forks (Burger, 1971; Geider and Hoffmann-Berling, 1971; Matsushita *et al.*, 1971).

Nuclei isolated from eucaryotic cells have been shown to be capable of incorporating deoxyribonucleotides into DNA with activities proportional to the *in vivo* rates of DNA synthesis (Friedman and Mueller, 1968; Lynch *et al.*, 1970). Two principle approaches are being used in eucaryotic systems to characterize the DNA made *in vitro* and to attempt to distinguish between repair and replicative synthesis. The first is to study the replication of viral DNA in isolated nuclei so that the product can be characterized in detail by sucrose gradient sedimentation and hybridization (Winnacker *et al.*, 1972; Magnusson *et al.*, 1972; Hunter and Francke, 1974a; DePamphilis and Berg, personal communication). The second approach is to show that the DNA made in isolated nuclei is a continuation at the growing points of chains initiated *in vivo* (Kidwell and Mueller, 1969; Lynch *et al.*, 1970; Hershey *et al.*, 1973).

The purpose of the following experiments is to establish a cell-free system in which to study the regulation of the initiation and elongation of DNA replication in eucaryotic cells. With such a system, combined with knowledge of the events prior to initiation, it would be possible to study the mechanisms by which a tissue limits its final size in the differentiated state and yet retains the capacity to proliferate following injury. We feel that it is necessary to understand the control mechanisms of normal cells before it will be possible to understand the nature of cellular defects which result in failure to divide or in uncontrolled proliferation.

In this report we describe an isolation procedure for obtaining high yields of nuclei from cultures of partially synchronized chicken embryo fibroblasts, and the detailed characterization of the DNA synthesis activity of these nuclei is presented. In addition, we present several lines of evidence that the activity is likely to be replication rather than repair synthesis.

Experimental Section

Cell Culture Techniques. Primary cell cultures were prepared from 10-day-old chicken embryos as described previously (Rein and Rubin, 1968; Rubin, 1973). All experiments reported here were done with secondary chick fibroblast cultures. They were prepared by trypsinizing 4–5-day-old primary cultures (Rubin, 1973) and seeding the cells on 100-mm Falcon plastic petri dishes in growth medium 199 (Grand Island Biological) supplemented with 2% tryptose phosphate broth (TPB,[1] Difco) and 1% each of calf and chicken serum (Microbiological Associates) (designated 199 (2.1.1)). Unless other-

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‡ Present address: Department of Chemistry, University of California, Berkeley, Calif. 94720.

[1] Abbreviations used are: MalNEt *N*-ethylmaleimide; araCTP, cytosine β-D-arabinofuranonucleoside 5′-triphosphate; BrdUrd, bromodeoxyuridine; BrdUTP, bromodeoxyuridine 5′-triphosphate; TPB, tryptose phosphate broth.