Lang, D. (1970), *J. Mol. Biol. 54*, 557.
Lazdunski, C., and Lazdunsky, M. (1969), *Eur. J. Biochem. 7*, 294.
Plocke, D. J., Levinthal, C., and Vallee, B. L. (1962), *Biochemistry 1*, 373.
Polsinelli, M., and Barlati, S. (1967), *J. Gen. Microbiol. 49*, 267.
Riva, S., Polsinelli, M., and Falaschi, A. (1968), *J. Mol. Biol. 35*, 347.
Rothman, F., and Byrne, R. (1963), *J. Mol. Biol. 6*, 330.
Rushinzky, G. W., and Miller, W. W. (1967), *Anal. Biochem. 20*, 181.
Sabbioni, E., Clerici, L., Campagnari, F., Girardi, F., and Bartolini, P. (1971), *Biochemistry 10*, 4185.
Sarfert, E., and Venner, H. (1965), *Hoppe-Seyler's Z. Physiol. Chem. 340*, 157.
Simpson, R. T., and Vallee, B. L. (1968), *Biochemistry 7*, 4343.
Soave, C., Galante, E., Torti, G. (1970), *Bull. Soc. Chem. Biol. 52*, 857.
Strohmaier, K. (966), *Anal. Biochem. 15*, 109.
Studier, F. W. (1965), *J. Mol. Biol. 11*, 373.
Van der Schans, G. P., Aten, G. B. T., and Blok, J. (1969), *Anal. Biochem. 32*, 14.
Weiss, B., Live, T. R., Richardson, C. C. (1968), *J. Biol. Chem. 243*, 4530.

Properties of Oligodeoxynucleotides That Determine Priming Activity with *Escherichia coli* Deoxyribonucleic Acid Polymerase I†

Mehran Goulian,* Susan H. Goulian, Ellen E. Codd, and Aija Z. Blumenfield

ABSTRACT: The requirements for priming of DNA synthesis by oligonucleotides with *Escherichia coli* DNA polymerase I and single-stranded templates have been examined with respect to sequence and chain length. Sequences containing only pyrimidines have very little activity, whereas purine sequences are highly active. The highest activity in an oligonucleotide of defined sequence was found with $d(pApG)_4$. Maximum priming activity at 20° occurs with chain lengths of 8–12, but activity can be detected even with trimers and tetramers, suggesting that the enzyme plays a role in stabilizing the association between template and primer. Oligo(dG) had only slight activity, whereas there was no activity for the homo-oligonucleotides of dA, dT, or dC. Some requirements for activity are probably based on general features of oligonucleotide–polynucleotide interactions, but it is not clear that all of the requirements have this explanation.

Studies (*in vitro*) with *Escherichia coli* DNA polymerase I have established that the purified enzyme is unable to initiate new polynucleotide chains (Goulian, 1968a). The enzyme appears to commence synthesis only by extension of existing chains at the 3′-OH termini. Oligodeoxynucleotides can provide this priming function with single-stranded templates (Goulian 1968a), an observation that had been made earlier for animal cell DNA polymerases (Keir, 1962; Smellie, 1963; Furlong, 1966; Bollum, 1967). It is postulated that the oligonucleotides associate with template in a short region of sequence homology, and nonpaired residues at the 3′-OH terminus are trimmed back by the 3′→5′ exonuclease associated with DNA polymerase I, providing thereby a suitable 3′-OH priming site for the enzyme. The oligonucleotides are incorporated into the product in covalent form although as synthesis proceeds they are largely removed, presumably by the 5′→3′ nuclease associated with polymerase I (Goulian, 1968b).

Preliminary studies on the properties of oligonucleotide primers for *E. coli* DNA polymerase I suggested little or no specificity; fragments active in priming could be produced by digestion of DNA from several different sources with pancreatic DNase. It also appeared that fragments could be quite short and still retain primer activity although this was not firmly established.

In the present study the requirements for primer activity are defined in greater detail, with specific attention to the influence of chain length, base sequence, nucleotide sugar, enzyme, and incubation conditions.

Materials and Methods

Single Strands of DNA

Escherichia coli. *E. coli* 15T⁻ was grown at 37° with aeration in M-9 medium (Adams, 1959) containing, per milliliter, [³H]thymidine, 0.5 μg (1 μCi/μg). The cells were lysed by the lysozyme–Brij procedure of Cozzarelli *et al.* (1968). After centrifugation (60,000*g*, 15 min) and removal of the supernatant containing the minicircular plasmid DNA, the pellet DNA was resuspended in 50 mM EDTA–1% Sarkosyl and incubated with Pronase (1 mg/ml) for 18 hr, 37°. The mixture was centrifuged in 12-ml alkaline sucrose gradients in the

† From the Department of Medicine, University of California—San Diego, La Jolla, California 92037, and the Departments of Medicine and Biochemistry, University of Chicago, and Argonne Cancer Research Hospital, Chicago, Illinois (operated by the University of Chicago for the U. S. Atomic Energy Commission). *Received September 5, 1972.* This research was supported in part by the U. S. Public Health Service (Grant No. 5-RO1-CA11705), the American Cancer Society (Grant No. P-567), and the U. S. Atomic Energy Commission (Argonne Cancer Research Hospital, University of Chicago).

Spinco SW-40 rotor (40,000 rpm, 20°, 140 min) and a fraction of mol wt ~2×10^6 was selected using M13 DNA as marker.

Bacillus subtilis. B. subtilis 168 *thy⁻try⁻* (obtained from Dr. K. Bott) was grown at 37° with aeration in a medium containing, per milliliter, minimal medium Davis (Difco) (21.2 mg), glucose (5 mg), $FeSO_4 \cdot 7H_2O$ (10 μg), $MnCl_2$ (1.3 μg), DL-tryptophan (40 μg), [^{3}H]thymidine (4 μg) (0.5 μCi/μg). The lysate prepared with lysozyme and EDTA was incubated with Sarkosyl and Pronase, and single strands were isolated as described for tritiated *E. coli* DNA.

Micrococcus luteus. M. luteus (obtained from Dr. Sam Weiss) was grown overnight with aeration at 30° in a medium containing, per milliliter, glucose (20 mg), yeast extract (10 mg), salts (Steiner and Beers, 1961), and [^{3}H]thymidine (4 μg) (2.5 μCi/μg). The cells were lysed and the DNA purified by the procedure described for *B. subtilis*.

M13. ^{3}H-Labeled and unlabeled DNA from M13 phage were prepared by the procedure of Mitra *et al.* (1967).

Oligonucleotides

Unlabeled Oligodeoxynucleotides from DNA. Overnight cultures of *E. coli* B, *B. subtilis* 168 *thy⁻trp⁻*, and *M. luteus* were prepared in H broth, Penassay broth (Difco), and the medium described above for *M. luteus* (see section on *E. coli*), respectively. Cells suspended in 5 vol of buffer (0.01 M Tris-HCl (pH 8.1)–1 mM EDTA) were broken by sonication and heated for 5 min at 100°, followed by recentrifugation (20,000*g*, 15 min). The clear supernatant was extracted three times with phenol that had been saturated with water and neutralized with NH_3. The phenol was extracted with ether and the ether removed with N_2, following which the extract was made 5 mM in $MgCl_2$ and incubated with pancreatic DNase (50 μg/ml) for 1 hr at 37°. The reaction was stopped with EDTA (20 mM) and, after another cycle of phenol, ether, and N_2 to remove DNase, the extract was treated with NaOH (0.3 M) for 18 hr at 22°. It was then neutralized with KH_2PO_4 and chromatographed on Sephadex G-50 in 0.1 M NH_4HCO_3, selecting a fraction that is estimated to have a mean chain length of 15.2 (Hohn and Schaller, 1967).

^{32}P-Labeled Oligodeoxynucleotides from DNA. E. coli B was grown overnight at 37° in the low phosphate medium of Garen and Levinthal (1960), without peptone, and containing 0.1 μmol/ml of $K_2H^{32}PO_4$ (1 mCi/μmol).

B. subtilis 168 *thy⁻trp⁻* was grown overnight at 37° in a minimal medium (Young and Spizizen, 1961) supplemented with, per milliliter, DL-tryptophan (40 μg), thymidine (40 μg), and $K_2H^{32}PO_4$ (0.3 μmol) (0.3 mCi/μmol).

M. luteus was grown overnight at 30° in the medium described above (see section on *M. luteus*) except for the reduction of yeast extract to 2.5 mg/ml, the addition of carrier-free $H_3{}^{32}PO_4$ (0.1 mCi/ml), and omission of thymidine.

Oligodeoxynucleotides were prepared from the ^{32}P-labeled *E. coli*, *B. subtilis*, and *M. luteus* cells (see above section on unlabeled oligodeoxynucleotides). Specific activities (calculated back to the first day of preparation) were 1000 cpm/pmol[1] for *E. coli*, 580 cpm/pmol for *B. subtilis*, and 390 cpm/pmol for *M. luteus*.

Oligodeoxynucleotides of Defined Chain Lengths from DNA Calf thymus DNA (100 mg) (Worthington) was incubated in a solution (10 ml) containing Tris-HCl (20 mM, pH 8.1), $MgCl_2$ (5 mM), and DNase (0.1 mg/ml). After 30 min at 37°, the mixture was made 10 mM in EDTA and 0.1 M in KOH, heated to 70° for 10 min, and then neutralized with HCl. The mixture was chromatographed on DEAE-Sephadex using a neutral chloride gradient containing urea, as described by Tomlinson and Tener (1963). Pooled fractions were desalted as described in the next section. The number average chain length was determined by the ratio of total phosphorus to phosphorus released by bacterial alkaline phosphatase.

Purine and Pyrimidine Tracts from DNA. Purine sequences were prepared from calf thymus DNA by hydrazinolysis, according to the procedure described by Sedat and Sinsheimer (1964). The procedure was also followed for alkaline chain scission and removal of terminal phosphates with bacterial alkaline phosphatase. Alkaline phosphatase was removed by extraction with phenol and retreatment with alkali. The oligonucleotides were fractionated according to chain length by chromatography on DEAE-Sephadex A-25 with a chloride gradient at pH 7.6 in 7 M urea (Tomlinson and Tener, 1963; Sedat and Sinsheimer, 1964). The pooled fractions were desalted on DEAE-Sephadex A-25 using triethylammonium bicarbonate (Sedat and Sinsheimer, 1964).

Pyrimidine sequences were prepared from calf thymus DNA by the method of Burton and Peterson (1960). Terminal phosphates were removed with bacterial alkaline phosphatase and the enzyme inactivated as described above. The oligonucleotides were fractionated on DEAE-Sephadex A-25 using a gradient of triethylammonium bicarbonate (Hall and Sinsheimer, 1963).

The purine and pyrimidine oligonucleotides were each shown to be free of contamination with the other bases by digestion and paper chromatography (see section on chemically synthesized oligodeoxynucleotides below). The chain length was determined for both purine and pyrimidine oligonucleotides by the ratio of nucleotide base (A_{260}) to total phosphorus.

From Enzymatically Synthesized Polymers. Poly[d(A·T)] was synthesized by the procedure of Riley *et al.* (1966), poly[d(G·C)] by the procedure of Radding *et al.* (1960), and poly[d(pApG·pCpT)] by the procedure of Byrd *et al.* (1965). The primer template for the latter was kindly provided by Dr. H. G. Khorana. Half of each was converted to apurinic acid and half to apyrimidinic acid by the procedure described above (under purine and pyrimidine tracts) to yield the strands of each pair. Oligonucleotides were prepared by digestion with pancreatic DNase to an extent sufficient to make 15–30% acid soluble.

Chemically Synthesized Oligodeoxynucleotides. Chemically synthesized oligodeoxynucleotides of defined sequence and chain length were purchased from Collaborative Research, Inc., and were treated with 1 M NaOH before use to ensure freedom from nucleases. Characterization of the oligonucleotides included digestion to bases (12 M perchloric acid, 100°, 1 hr) followed by chromatography on S&S Orange Ribbon paper in isopropyl alcohol–water–concentrated HCl (65:18.3:16.7, v/v/v) (Bendich, 1957). Ratios were calculated from the ultraviolet absorption of the eluted bases. Oligonucleotides containing more than one type of base were digested with micrococcal nuclease and spleen phosphodiesterase (Josse *et al.*, 1961) and chromatographed on Whatman 1 paper in water-saturated *n*-butyl alcohol–concentrated ammonia (100:1, v/v) to identify the 3′-terminal nucleotide. Chain length was determined from the ratio of total phosphorus to phosphorus released by bacterial alkaline phosphatase.

Oligoribonucleotides. Purified RNAs from *E. coli* ribosomes

[1] Moles or molarity of DNA, polynucleotide, or oligonucleotide refer to nucleotide residues.

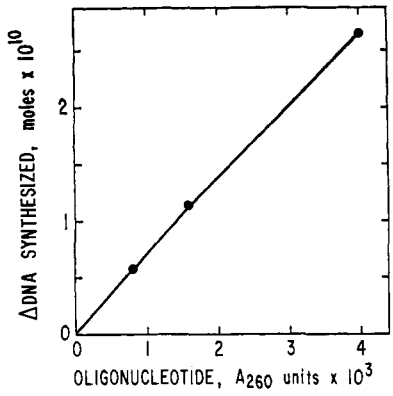

FIGURE 1: A sample assay for primer activity. The procedure is described under Materials and Methods. The ordinate refers to stimulated synthesis in 30 min (total synthesis minus synthesis in the absence of primer oligonucleotide). The oligonucleotide source was *E. coli* DNA.

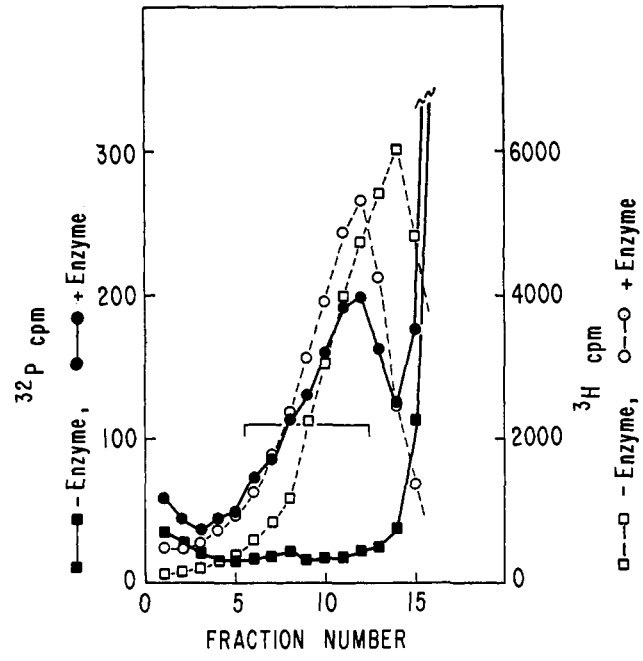

FIGURE 2: Sucrose gradient sedimentaion of ^{32}P-labeled oligonucleotide-primed synthesis. Conditions for synthesis and centrifugation are described under Materials and Methods. In this instance template was single-stranded tritiated *B. subtilis* DNA and primer was oligonucleotide prepared from ^{32}P-labeled *E. coli* DNA. The results from paired incubations, with and without DNA polymerase, are plotted together. The bracket indicates the fractions pooled for subsequent digestion and base analysis.

and MS2 phage were the kind gift of Dr. S. Weiss. Preparation of the RNA included treatment with DNase, extraction with phenol, and passage over Sephadex G-100. Oligonucleotides were prepared by treating with 0.1 M KOH to an extent sufficient to make 15–30% acid soluble. After neutralization with acetic acid the terminal 2′- and 3′-phosphates were removed by treatment with bacterial alkaline phosphatase, followed by extraction with phenol at 70°.

Enzymes

E. coli DNA polymerase I was prepared by the method of Jovin *et al.* (1969). The preparation of *E. coli* DNA polymerase II followed the procedures of Moses and Richardson (1970) and Kornberg and Gefter (1971). T4 polymerase was prepared by the method of Goulian *et al.* (1968). Pancreatic DNase, venom phosphodiesterase, and bacterial alkaline phosphatase were purchased from Worthington Biochemical Corp. Lysozyme and Pronase were from Calbiochem.

Assay for Initiator Activity

The assay mixture (22 μl) contained Tris-HCl (20 mM, pH 7.6), $MgCl_2$ (5 mM), KCl (0.1 M), bovine plasma albumin (20 μg/ml), M13 DNA (2.5 nmol), dCTP, dATP, dGTP, and [^{3}H]dTTP (30 μM each) (120,000 cpm/nmol), oligonucleotide primer, and *E. coli* DNA polymerase I (3 units). All steps were carried out at 20°. Primers and templates were heated at 95° for 2 min just prior to assay to minimize effects due to secondary structure (Studier, 1969). Enzyme was added 30 sec after primer and, after an additional 30 min at 20°, the reaction was terminated with EDTA (50 mM). Acid-insoluble radioactivity was retained and counted on glass fiber filters. Subtraction of the synthesis in a mixture without primer (usually less than 5 pmol) gives a measure of the stimulatory effect of the primer, or "priming activity." The results are essentially linear in the range 30–300 pmol of stimulated synthesis[2] (Figure 1). Each primer was also treated in an incubation that lacked template but in no instance was there detectable synthesis with the oligonucleotides described in this paper. The unit of initiator activity is defined as 1 nmol of stimulated synthesis in 30 min. The specific activity of an oligonucleotide primer is expressed as (initiator units/A_{260} units)[3] × chain length.

Base Frequencies of Primer Incorporated into Product

Synthesis was carried out in a mixture (42 μl) containing Tris-HCl (25 mM, pH 7.6), $MgCl_2$ (5 mM), KCl (0.1 M), bovine plasma albumin (50 μg/ml), dATP, dCTP, dTTP, and dGTP (30 μM each), ^{3}H-labeled single-stranded DNA template (20 nmol), ^{32}P-labeled oligonucleotide primer (40 nmol), and *E. coli* DNA polymerase I (2.5 units). After incubation for 10 min at 20°, the mixture was made 10 mM in EDTA, and centrifuged (Spinco SW-56 rotor) for 180 min at 56,000 rpm, 20°, in a linear 5–20% sucrose gradient, 10 mM Tris-HCl (pH 8.1)–0.1 mM EDTA. An incubation and centrifugation identical with the first except for omission of enzyme was carried out in parallel.

Aliquots from each fraction were plated on paper disks, dried, and counted. Comparison of the results in the paired incubations (with and without enzyme) identified the fractions that contained primer incorporated into product (Figure 2). These fractions were pooled and dialyzed against 0.2 mM Tris-HCl (pH 8.1), and the water was removed by rotary evaporation. The residue was dissolved in water and incubated in the following mixture (80 μl): Tris-HCl (50 mM, pH 8.5), $MgCl_2$ (5 mM), calf thymus DNA (25 μg), and pancreatic DNase (10 μg). After 60 min at 37° 10 μg of pancreatic DNase and 10 μg of venom phosphodiesterase were added. After an additional 60 min at 37° the mixture was made 10 mM in EDTA, chromatographed with deoxynucleoside 5′-monophosphate markers on S&S Orange Ribbon paper in isobutyric acid–water–concentrated NH_4OH (66:33:1), and counted.

[2] The calculations assume that all of the synthetic product is a complement of M13 DNA, which would have 24% thymidylate.

[3] An A_{260} unit is the amount that has an absorption of 1.0 in a 1-ml volume, at 260 nm in 0.1-cm light path.

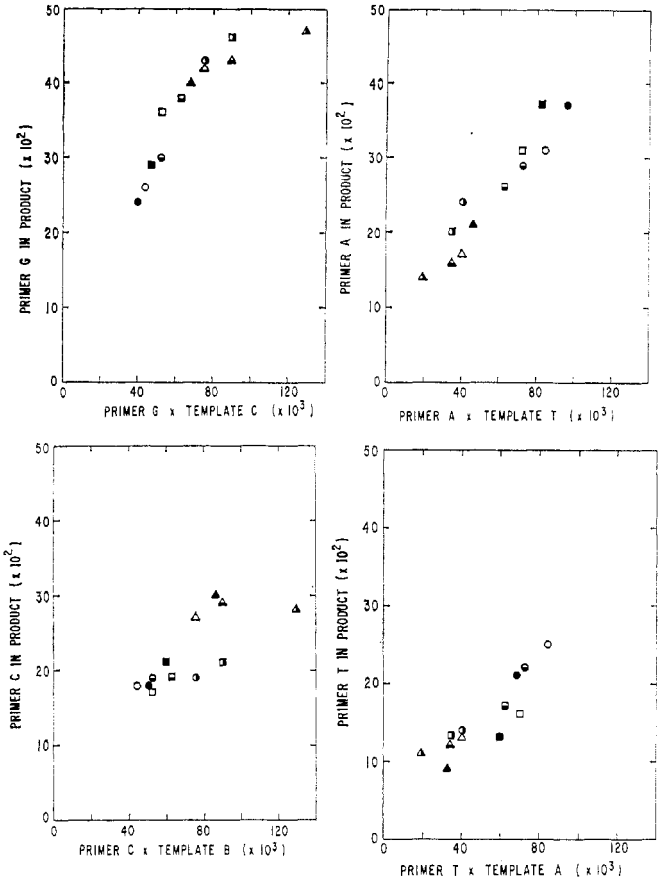

FIGURE 3: Correlation of frequency of primer base incorporated into product with the product of the base frequency in primer and frequency of complement in template. Base frequencies of oligodeoxynucleotides incorporated into the synthetic product were determined for the ^{32}P-labeled oligonucleotides from *B. subtilis*, *E. coli*, and *M. luteus* DNA used separately with ^{3}H-labeled single-stranded template DNA from *B. subtilis*, *E. coli*, *M. luteus*, and phage M13. The data for all 12 combinations are plotted together for each base. The ordinate gives the base frequency for the material incorporated into product; the abscissa, the product of the published figures for the frequency of that base in primer and of its complement in the template. The numbers refer to mole fractions. The base frequencies in the DNAs are as follows

	A	T	G	C
B. subtilis	28.0	28.0	22.0	22.0
E. coli	24.5	24.5	25.5	25.5
M. luteus	19.0	19.0	36.0	36.0
Phage M13	24.0	33.0	24.0	19.0

(from Schildkraut *et al.* (1962) for *B. subtilis*, *E. coli*, and *M. luteus*, and from Salivar *et al.* (1964) for phage M13). Symbols used are

	Template			
Primer	M13	*subtilis*	*luteus*	*coli*
B. subtilis	●	○	◑	◒
M. luteus	▲	△	◭	◮
E. coli	■	□	◨	◪

Other Materials

Nonradioactive nucleotides were purchased from P-L Biochemicals. Methyl ^{3}H-labeled thymidine and [^{3}H]dTTP were from Schwarz/Mann Bioresearch. [α-^{32}P]dCTP was purchased from International Chemical & Nuclear Corp. $H_3{}^{32}PO_4$ was from New England Nuclear. Sarkosyl (NL-30) was a gift from Geigy. Sephadex and DEAE-Sephadex were purchased from Pharmacia.

TABLE I: Comparison of Priming Activities with Different Templates.

	Primer				
Template	*E. coli*	*B. subtilis*	*M. luteus*	*Calf thymus*	d(pApG)$_4$
M13	3610[a]	7430	5190	4090	8360
E. coli	1140	1840	3640	2760	770
B. subtilis	1210	2500	4090	4780	3200
M. luteus	120	370	2210	490	50
Calf thymus	1210	1360	3310	710	2570

[a] Figures represent specific activities, expressed as (units of activity/A_{260} units) $\times$ chain length. The oligonucleotides had an average chain length of 15.2. The assay for primer activity is as described under Materials and Methods except that the labeled substrate was [^{32}P]dCTP rather than [^{3}H]-dTTP because most of the templates were labeled with ^{3}H.

Results

Comparison of Synthetic Activity with Different Templates and Oligonucleotides. The stimulation by oligodeoxynucleotides was compared in both homologous and nonhomologous primer–template pairs from different sources (Table I). *M. luteus* DNA fragments were the most consistently active as primers; however, this same DNA was least effective as template. With the possible exception of *M. luteus* DNA homology in pairing template with primer did not appear to enhance activity and, in fact, with calf thymus DNA the opposite seemed to be true. Coliphage M13 DNA was the most generally active template. The remainder of the figures show no clearly discernible pattern; the preparations used make it unlikely that this was because of uncontrolled variations in size of template or primer.

Base Frequency of Oligonucleotides Incorporated into Product. Oligodeoxynucleotides labeled with ^{32}P were prepared from *B. subtilis*, *E. coli*, and *M. luteus*, and used as a primer with *E. coli* DNA polymerase I and single-stranded template DNAs. Template DNAs, labeled with ^{3}H, had been prepared from the same three DNAs plus M13. The incubation conditions favored retention of incorporated oligonucleotides in the product by use of short incubations, limiting enzyme, and excess primer (Goulian, 1968b). The incorporated ^{32}P from oligomer was isolated on alkaline sucrose gradients and the base frequencies were determined (Figure 2).

The relative frequency of a given mononucleotide in the product appears to be correlated with the product of its frequency in the primer and the frequency of the complementary nucleotide in the template (Figure 3). The correlation appears fairly good for incorporated deoxyguanylate, deoxyadenylate, and deoxythymidylate (correlation coefficients 0.87, 0.95, and 0.88) but less so for deoxycytidylate (correlation coefficient 0.75). The frequency of incorporation of a primer-derived nucleotide showed little or no correlation with either the frequency of that nucleotide in the primer, or with its complement in the template, alone. When the figures for the four nucleotides are compared, a distinct difference in the overall frequency of incorporation is evident (Figure 3). The order of decreasing frequency is deoxyguanylate, deoxyadenylate, deoxycytidylate, and deoxythymidylate and this is superimposed on the variation in incorporation discussed previously.

Primer residues in the synthetic product represent what has

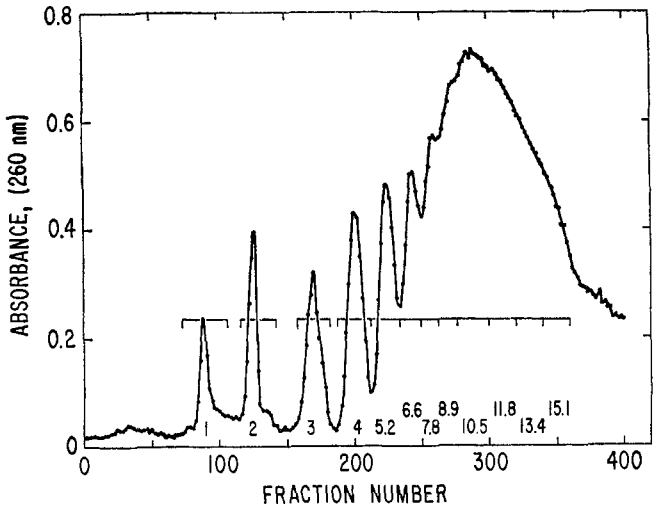

FIGURE 4: Fractionation of oligonucleotides from calf thymus DNA. Oligonucleotides from a DNase digest of calf thymus DNA were chromatographed on a column of DEAE-Sephadex (0.64 cm² × 90 cm) using a linear salt gradient (4 l., 0–0.5 M NaCl, pH 7.6) containing 7 M urea (Materials and Methods). Brackets indicate fraction pools, and the number for each pool refers to the average chain length.

escaped the removal process, which may be carried out by the 5′→3′ nuclease activity of DNA polymerase I (Goulian, 1968b). Therefore, the possibility arises that observed nucleotide frequencies could represent, in part at least, a bias in the removal process. This has not been excluded in these experiments; however, the assumption that the frequencies reflect utilization in primer fits well with the observed correlation (Figure 3). It remains possible that some of the deviations from this correlation, *e.g.*, for deoxycytidylate, may be traced to variable removal rates.

Activity of Oligonucleotides from Calf Thymus DNA as a Function of Chain Length. Oligodeoxynucleotides were prepared from calf thymus DNA by digestion with pancreatic DNase and fractionated according to chain length (Figure 4). Initiating activity is detectable at chain length 3 and increases progressively with increasing length to a maximum activity with chain lengths of 10–12 (Table II). The activity of the shorter chain lengths, *e.g.*, 3 or 4 residues, is of interest since under these conditions a stable association with the template strand would not ordinarily form. This result is consistent with the activity of tetramers in a primer–template system of complementary synthetic oligodeoxynucleotides (Wells *et al.*, 1965). It also confirms earlier experiments with random oligonucleotides from DNA, fractionated by gel filtration, which suggested that active primers may have as few as 4 or 5 residues (Goulian, 1968a). At the highest chain lengths activity again appears to decline somewhat (Table II). The conditions of these experiments may be relatively unfavorable for longer chain lengths because the latter require higher "annealing" temperatures to unfold regions of self-association, and proportionally more time to form the complex (Studier, 1969). Some evidence to support the former is described below.

TABLE II: Influence of Chain Length on Priming Activity of Oligonucleotides from Calf Thymus DNA.

Chain Length	Act.	Chain Length	Act.
1	NM[a]	7.8	2280
2	NM	8.9	5890
3	0.4	10.5	7990
4	5.5	11.8	6950
5.2	23.2	13.4	6490
6.6	603	15.1	3720

[a] The oligonucleotide fractions are from the experiment shown in Figure 4. The figures refer to specific priming activities (Table I); NM, not measurable.

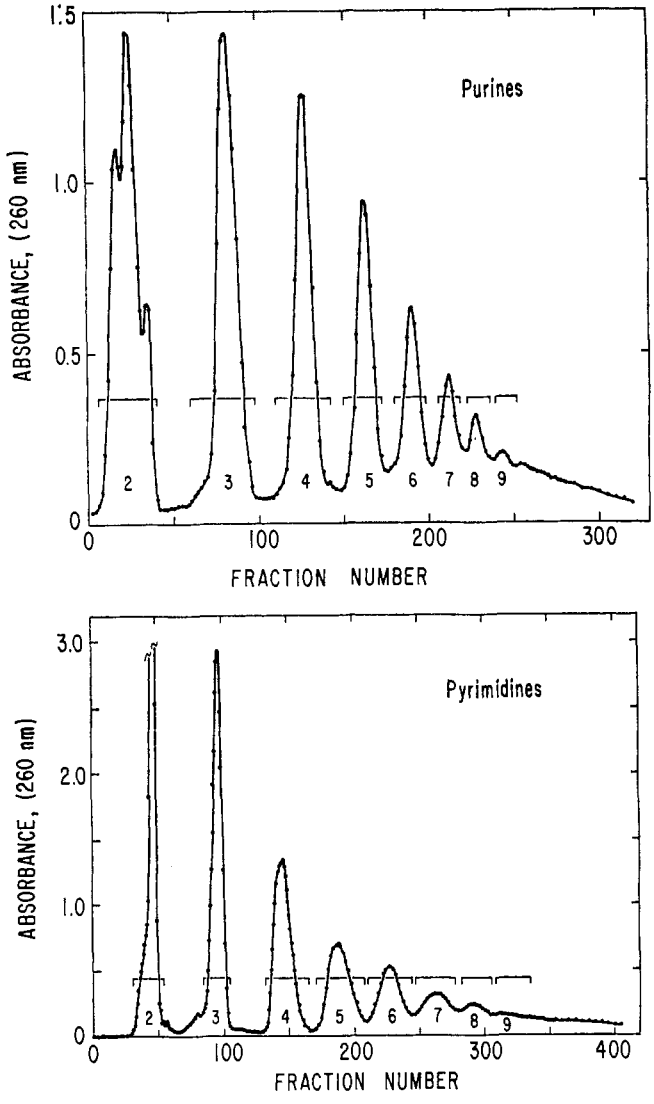

FIGURE 5: Fractionation of DNA purine and pyrimidine sequences by chain length. Purine and pyrimidine tracts from calf thymus DNA were chromatographed on DEAE-Sephadex (Materials and Methods). NaCl gradient in urea (see Figure 4) was used for the purines, and triethylammonium bicarbonate gradient (0.001–1.3 M) for the pyrimidines. Numbers on the fraction pools (brackets) refer to chain length.

Purine and Pyrimidine Sequences from DNA. Purine and pyrimidine tracts lacking terminal phosphates were prepared from calf thymus DNA and fractionated on DEAE-Sephadex (Figure 5). Comparison of the two shows that the pyrimidine sequences have very little activity compared to the purine sequences (Table III). In the purine series, as with the oligonucleotides prepared with DNase (Table II), activity is present even in trimers and increases progressively with chain length. The highest initiating activity with both purine and

TABLE III: Influence of Chain Length on Priming Activity of DNA Purine and Pyrimidine Sequences.

	Chain Length							
	2	3	4	5	6	7	8	9
Purine[a]	NM	6.3	(0.4)[b]	45	1630	6920	6880	5180
Pyrimidine[a]	NM	NM	NM	NM	32	188	162	55

[a] Purine and pyrimidine sequences are fractions shown in Figure 5. Figures refer to specific activity (Table I). [b] This fraction was inhibitory; the value shown is a minimum activity.

pyrimidine tracts is found with chain lengths of 7 and 8, in contrast to the DNase fragments, in which highest activity was found with chain lengths of 10–12. Again, there is some decline in activity in both for the highest chain lengths.

Oligonucleotides of Defined Sequence and Length. Synthetic oligodeoxynucleotides of defined sequence and chain length, obtained from a commercial source, provide additional information concerning primer requirements in this system (Table IV). By far the most active series was the alternating deoxyadenylate–deoxyguanylate copolymer, $d(pApG)_n$. For the series $d(pApG)_n$, the shortest with activity was chain length 4, optimal activity was with chain length 8, and longer fragments had less activity.

No activity was detected in any oligonucleotides that contained only pyrimidines nor was there any activity in homooligonucleotides of deoxyadenylate. The only homooligonucleotides with activity were those of deoxyguanylate and these generally had less activity than corresponding oligonucleotides in which deoxyguanylate was present with other nucleotides. The alternating copolymers $d(pApC)_n$ and $d(pTpG)_n$ also had some activity. The single trimer available contained only pyrimidines and was inactive. The self-complementary alternating sequences, $d(pApT)_n$, $d(pTpA)_n$, $d(pGpC)_n$, and $d(pCpG)_n$, were not tested in chain lengths greater than the dimer because of their potential activity as primer–templates (Kornberg *et al.*, 1964). Only one oligonucleotide was tested that had more than one type of base not in a strictly alternating sequence; d(pApApApApCpApC) had modest activity (sp act. = 136) but nevertheless distinctly more than would have been expected from the results with $d(pCpA)_3$, $d(pApC)_4$, $d(pApA)_4$, and $d(pCpC)_5$ (Table IV).

These results, obtained with chemically synthesized polymers, were confirmed in part with enzymatically synthesized polymers. Oligonucleotides were prepared by DNase digestion of enzymatically synthesized poly(dA), poly(dT), poly(dC), poly(dG), poly[d(pApG)], and poly[d(pCpT)]. The unfractionated digest of poly[d(pApG)] was active, that of poly(dG) had a small amount of activity, and the remainder were without detectable activity.

The high priming activity of $d(pApG)_4$ was not a feature unique to the M13 DNA template since it was evident with some other DNA templates, *e.g.*, *B. subtilis* or calf thymus; however, it was much lower with *E. coli* and *M. luteus* DNA templates (Table I). Also similar to the results with M13 DNA, no priming activity could be found for $d(pTpC)_4$ with template DNAs from *B. subtilis*, *E. coli*, *M. luteus*, and calf thymus.

Activity of Oligonucleotides from RNA. Oligoribonucleotides were prepared from RNA by limited alkaline digestion,

TABLE IV: Priming Activity of Defined Oligodeoxynucleotides.

	Chain Length						
	2	4	6	8	10	12	12–18
$d(pApA)_n$	NM			NM	NM		
$d(pGpG)_n$	NM	NM	NM	42[a]	73		
$d(pCpC)_n$	NM				NM		
$d(pTpT)_n$	NM		NM		NM		
$d(pApG)_n$	NM	19.9	641	8180	6550		3240
$d(pGpA)_n$	NM						
$d(pCpT)_n$	NM	NM	NM				
$d(pTpC)_n$	NM	NM					
$d(pApC)_n$	NM			103			
$d(pCpA)_n$	NM	NM	NM		324		
$d(pTpG)_n$	NM	NM					
$d(pGpT)_n$	NM						
$d(pApT)_n$	NM						
$d(pTpA)_n$	NM						
$d(pGpC)_n$	NM						
$d(pCpG)_n$	NM						

[a] Figures refer to specific activities (Table I).

followed by treatment with alkaline phosphatase to remove terminal 2′- and 3′-phosphates. Oligoribonucleotides had definite priming activity but, although no direct comparison can be made with the activities of oligodeoxynucleotides of known size, the figures suggest a distinctly lower activity than for the latter (Table V). The activity of oligoribonucleotides was eliminated by treatment with alkali thus excluding the possibility that the activity was due to contamination of the RNA by DNA fragments. The activity of oligoribonucleotides was enhanced somewhat by addition of Mn^{2+} or replacement of Mg^{2+} by Mn^{2+}. The latter effect may be related to the requirement for Mn^{2+} in the incorporation of ribonucleotides into DNA by this enzyme (Berg *et al.*, 1963). In contrast to its effect with oligoribonucleotides Mn^{2+} caused considerable depression of the activity of oligodeoxynucleotides (Table V).

Influence of Incubation Conditions. The effect of temperature of incubation was tested by comparing, at three different temperatures, the relative activities of three fractions of calf thymus DNA fragments of different chain lengths (Table VI). A distinct effect of lowering temperature was observed, with augmentation of the initiating activity of the short chain fragments, in contrast to a decrease in activity of long chain fragments. For the short oligonucleotides this is assumed to reflect the relationship between melting temperature and chain length. It is possible that, beyond a minimum chain length sufficient for stability at a given temperature, a further increase in length permits self-association introducing thereby a requirement for higher temperatures to unfold and allow "annealing" between the fragments and templates. By this reasoning an effect on activity would also be expected from alterations in salt concentrations or preincubation time; however, no systematic effect was observed from variations in KCl concentration between 0.01 and 0.2 M. This may have been due, in part, to opposing effects on the activity of the enzyme. Likewise, varying preincubation times from 5 sec to 30 min caused no change in results, apparently because the incubation time with enzyme was long compared to the maximum time required for association of primer with template.

TABLE V: Priming Activity of Oligoribonucleotides.

	Mg^{2+}	Mn^{2+} [a]	Mg^{2+}-Mn^{2+} [b]
$d(pApG)_n$	9570[c]	505	337
Purine oligodeoxynucleotides, chain length 9[d]	5910	3960	2450
MS2 RNA digest	6.2[e]	8.1	8.2
rRNA digest	3.4	7.1	7.1

[a] $MgCl_2$ replaced by 1 mM $MnCl_2$; assay otherwise as described in Materials and Methods. [b] 1 mM $MnCl_2$ added to usual reaction mixture. [c] Figures refer to specific activities (Table I). [d] See Table III. [e] The RNA digests were not fractionated and chain length is not known. The activities are given as initiator units/A_{260} units (Materials and Methods) and cannot be compared between the two digests or with the chain length corrected specific activities.

TABLE VI: Influence of Temperature on Priming Activity.

Temp of Incubation[a] (°C)	Duration of Incubation (min)	Ratio of Activities[c] for Primer Chain Length of 5.2[b]	10.5	15.1
3	180	91×10^{-4}	1	0.11
20	10	21×10^{-4}	1	0.33
37	5	6.8×10^{-4}	1	0.46

[a] Assays followed the procedures described under Materials and Methods except that all steps from addition of the primer to end of incubation with enzyme were at the temperature indicated, and incubation with enzyme was for the times indicated. [b] Oligonucleotides from the experiment shown in Figure 4 and Table II. [c] Figures represent relative specific activities (Table I) for a given temperature, the value for the 10.5 chain length fragment being arbitrarily set to equal 1.

Other DNA Polymerases. Neither *E. coli* DNA polymerase II nor coliphage T4[4] DNA polymerase showed detectable enhancement of activity on M13 DNA template with oligonucleotides, including a fraction prepared from *E. coli* DNA, $d(pApG)_4$, and $d(pCpT)_4$. It is possible that this result reflects a difference between the enzymatic (polymerase) properties of these two enzymes and polymerase I. Alternatively, in the case of the T4 enzyme the difference could be accounted for by the nuclease activity that is associated with the enzyme and which is particularly active against oligodeoxynucleotides (Oleson and Koerner, 1964). The possibility that the nuclease results in hydrolysis of the fragments before priming can take place was not tested for in these studies. Differential susceptibility to digestion may also account for some of the differences in activity between various oligomers with *E. coli* DNA polymerase I, although this latter enzyme has a much lower hydrolytic activity on oligodeoxynucleotides than the T4 enzyme.

Discussion

A high degree of specificity in the properties of primer fragments seemed unlikely from the earlier studies (Goulian, 1968b) and this has been confirmed in the experiments reported here. DNA primer fragments and templates from diverse sources are active when paired, and both purine and pyrimidine sequences can function as primer. The base frequencies of incorporated primer fragments indicate that selection of bases and, by interference, sequences is strongly influenced by the overall abundance of the complementary pair in the interacting primer–template mixture.

Although specificity that restricts activity to one or only a few sequences is excluded, the requirements for primer activity are also not totally nonspecific. The current studies point to certain features that determine activity. The large contribution by deoxyguanylate is indicated by its relative abundance in primer fragments incorporated into product. It is also presumed that the content of deoxyguanylate plays a part in the high activity of primers from the G-C-rich DNA of *M. luteus*.

Homooligonucleotide showed surprisingly little activity, none at all being detected with oligo(dA), oligo(dC), and oligo(dT). The activity for oligo(dG) was much less than for oligodeoxynucleotides in which dG was present along with other residues. The fact that oligo(dG) did have some initiating activity whereas the other homooligonucleotides were inactive is additional support for the importance of deoxyguanylate in this system.

Pyrimidine tracts from DNA had very little initiating activity compared to purine tracts, and none of the defined pyrimidine oligodeoxynucleotides had detectable activity. The incorporation frequency for deoxyadenylate was actually higher than for deoxycytidylate. In view of the inactivity of oligodeoxycytidylate, the higher energy of association of G·C, as compared to A·T base pairs, can only partially account for the activity of deoxyguanylate.

It is likely that at least some of the specific requirements for priming have a physical basis determined by general requirements for oligonucleotide–polymer interactions. The general preference for purine over pyrimidine is an example. In studies with model systems, oligo(dC) binds to poly(dG) less well than oligo(dG) binds to poly(dC) (Uhlenbeck, O., personal communication; Lewis, 1971). It has also been observed that the melting temperature of oligo(dT)–poly(dA) is lower than oligo(dA)–poly(dT) of the same size; in that case the three-stranded structure of the latter was considered to explain the difference (Cassani and Bollum, 1969).

It is also possible that specificities reflect something special about DNA, in the exposed or repetitive nature of certain sequences, perhaps having some biological function. Still another possibility is bias in primer selection introduced by requirements of the polymerase enzyme itself. The enzyme may have base or sequence preferences; the types of preference under discussion here are reminiscent of the pyrimidine richness of RNA polymerase-binding strands and the initiation of RNA chains exclusively by adenylate or guanylate. Although there is the expected effect of low temperature in enhancing the activity of short oligonucleotides, the fact that tetramers and apparently even trimers are active at 20° suggests that the enzyme stabilizes the initial complex. In a recent report even a dinucleotide, d(pGpG), was found to act as

[4] *E. coli* DNA polymerase II was tested both under the conditions used for polymerase I (with the addition of 0.1 mM dithiothreitol) and at the salt concentration optimal for polymerase II (Kornberg and Gefter, 1971). Conditions used with T4 polymerase were the same as for *E. coli* polymerase I except for inclusion of 10 mM β-mercaptoethanol.

initiator with calf thymus DNA polymerase on a poly(dC) template (15°, 0.045 M potassium phosphate) (Hayes *et al.*, 1971). However, that result may not be directly comparable with the results described here since there could be additional stability in tandem or clustered association of the dinucleotide with the homopolymer template.

The limited examination of sequences in this study leaves many active sequences unidentified. Thus, a mixture of random fragments from thymus DNA, in spite of many inactive runs, is essentially as active as the most active oligonucleotide studied. The potential activity of irregular sequences was indicated by the significant activity of d(pApApApCpApC) as compared to the lower or absent activities of $d(pApC)_3$, $d(pCpA)_3$, oligo(dC), and oligo(dA).

E. coli DNA polymerase II, like DNA polymerase I, cannot initiate new chains and requires a primer strand (Gefter *et al.*, 1972). Synthetic oligonucleotides act as primers for DNA polymerase II with complementary synthetic polynucleotides (Gefter *et al.*, 1972); however, no priming activity was detected in the current study using a DNA template. This may have resulted, at least in part, from the inability of *E. coli* DNA polymerase II to replicate long stretches of single-stranded DNA (Wickner *et al.*, 1972a).

Recent work indicates that, at least under some circumstances, the initiation of DNA chains *in vivo* actually occurs by extension of oligoribonucleotides (Brutlag *et al.*, 1971; Sugino *et al.*, 1972). However, the latter are probably synthesized by RNA polymerase at sites on the DNA template, rather than provided in a free form as in the experiments described here (Wickner *et al.*, 1972b; Schekman *et al.*, 1972).

It has been shown previously by Wells *et al.* (1972) that *E. coli* DNA polymerase can employ a free polyribonucleotide primer in synthetic polynucleotide primer–template systems. The current study confirms this for random oligonucleotides from RNA with a DNA template, and *in vitro* experiments have been reported by Keller (1972) using DNA polymerases from *M. luteus* and mammalian cells, with RNA primer synthesized *in situ* by an RNA polymerase.

In spite of earlier evidence to the contrary (DeLucia and Cairns, 1969), *E. coli* DNA polymerase I now appears to have a function in replication (Kuempel and Veomett, 1970; Kingsbury and Helinski, 1970; Okazaki *et al.*, 1971; Goebel and Schrempf, 1972; Lehman and Chien, 1973), but the details of that function must be more clearly identified before it is known whether or not there is an *in vivo* counterpart to the facile initiation by free oligonucleotides that occurs *in vitro*. Studies thus far have failed to detect priming activity in cell extracts that could be ascribed to oligodeoxynucleotides present *in vivo*.

Acknowledgment

The author wishes to thank Dr. O. Uhlenbeck for helpful discussions.

References

Adams, M. H. (1959), Bacteriophages, New York, N. Y., Interscience.

Bendich, A. (1957), *Methods Enzymol. 3*, 715.

Berg, P., Fancher, H., and Chamberlin, M. (1963), *in* Informational Macromolecules, Vogel, H. J., Bryson, V., and Lampen, J. O., Ed., New York, N. Y., Academic Press, p 467.

Bollum, F. J. (1967), *in* Genetic Elements, Shugar, D., Ed., New York, N. Y., Academic Press, p 2.

Brutlag, D., Schekman, R., and Kornberg, A. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 2826.

Burton, K., and Peterson, G. B. (1960), *Biochem. J. 75*, 17.

Byrd, C., Ohtsuka, E., Moon, M. W., and Khorana, H. G. (1965), *Proc. Nat. Acad. Sci. U. S. 53*, 79.

Cassani, G. R., and Bollum, F. J. (1969), *Biochemistry 8*, 3928.

Cozzarelli, N. R., Kelly, R. B., and Kornberg, A. (1968), *Proc. Nat. Acad. Sci. U. S. 60*, 992.

DeLucia, P., and Cairns, J. (1969), *Nature (London) 224*, 1164.

Furlong, N. B. (1966), *Biochim. Biophys. Acta 114*, 491.

Garen, A., and Levinthal, C. (1960), *Biochim. Biophys. Acta 38*, 470.

Gefter, M. L., Molineux, I. J., Kornberg, T., and Khorana, H. G. (1972), *J. Biol. Chem. 247*, 3321.

Goebel, W., and Schrempf, H. (1972), *Biochem. Biophys. Res. Commun. 49*, 591.

Goulian, M. (1968a), *Cold Spring Harbor Symp. Quant. Biol. 33*, 11.

Goulian, M. (1968b), *Proc. Nat. Acad. Sci. U. S. 61*, 284.

Goulian, M., Lucas, Z. J., and Kornberg, A. (1968), *J. Biol. Chem. 243*, 627.

Hall, J. B., and Sinsheimer, R. L. (1963), *J. Mol. Biol. 6*, 115.

Hayes, F. N., Hansbury, E., Mitchell, V. E., Ratliff, R. L., Smith, D. A., and Williams, D. L. (1971), *J. Biol. Chem. 246*, 3631.

Hohn, T., and Schaller, H. (1967), *Biochim. Biophys. Acta 138*, 466.

Josse, J., Kaiser, A. D., and Kornberg, A. (1961), *J. Biol. Chem. 236*, 864.

Jovin, T. M., Englund, P. T., and Bertsch, L. L. (1969), *J. Biol. Chem. 244*, 2996.

Keir, H. M. (1962), *Biochem. J. 85*, 265.

Keller, W. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 1560.

Kingsbury, D. T., and Helinski, D. R. (1970), *Biochem. Biophys. Res. Commun. 41*, 1538.

Kornberg, A., Bertsch, L. L., Jackson, J. F., and Khorana, H. G. (1964), *Proc. Nat. Acad. Sci. U. S. 51*, 315.

Kornberg, T., and Gefter, M. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 761.

Kuempel, P. L., and Veomett, G. E. (1970), *Biochem. Biophys. Res. Commun. 41*, 973.

Lehman, I. R., and Chien, J. R. (1973), *in* DNA Synthesis *in vitro*, Wells, R. D., and Inman, R. B., Ed., Baltimore, Md., University Park Press, in press.

Lewis, J. B. (1971), Ph.D. Thesis, Harvard University.

Mitra, S., Reichard, P., Inman, R. B., Bertsch, L. L., and Kornberg, A. (1967), *J. Mol. Biol. 24*, 429.

Moses, R. E., and Richardson, C. C. (1970), *Biochem. Biophys. Res. Commun. 41*, 1557.

Okazaki, R., Arisawa, M., and Sugino, A. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 2954.

Oleson, Q. E., and Koerner, J. M. (1964), *J. Biol. Chem. 239*, 2935.

Radding, C. M., Josse, J., and Kornberg, A. (1962), *J. Biol. Chem. 237*, 2869.

Riley, M., Maling, B., and Chamberlin, M. J. (1966), *J. Mol. Biol. 20*, 359.

Salivar, W. O., Tzagoloff, H., and Pratt, D. (1964), *Virology 24*, 359.

Schekman, R., Wickner, W., Westergaard, O., Brutlag, D., Geider, K., Bertsch, L., and Kornberg, A. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 2691.

Schildkraut, C. L., Marmur, J., and Doty, P. (1962), *J. Mol. Biol. 4*, 430.

Sedat, J., and Sinsheimer, R. L. (1964), *J. Mol. Biol. 9*, 489.
Smellie, R. M. S. (1963), *Exp. Cell Res., Suppl. No. 9*, 245.
Steiner, R. F., and Beers, R. F., Jr. (1961), Polynucleotides; Natural and Synthetic Nucleic Acids, Amsterdam, Elsevier.
Studier, F. W. (1969), *J. Mol. Biol. 41*, 199.
Sugino, A., Hirose, S., and Okazaki, R. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 1863.
Tomlinson, R. V., and Tener, G. M. (1963), *Biochemistry 2*, 697.
Wells, R. D., Flügel, R. M., Larson, J. E., Schendel, D. F., and Sweet, R. W. (1972), *Biochemistry 11*, 621.
Wells, R. D., Ohtsuka, E., and Khorana, H. G. (1965), *J. Mol. Biol. 14*, 221.
Wickner, R. B., Ginsberg, B., and Hurwitz, J. (1972a), *J. Mol. Biol. 247*, 498.
Wickner, W., Brutlag, D., Schekman, R., and Kornberg, A. (1972b), *Proc. Nat. Acad. Sci. U. S. 69*, 965.
Young, F. E., and Spizizen, J. (1961), *J. Bacteriol. 81*, 823.

Preferred Sites of Digestion of a Ribonuclease from *Enterobacter sp.* in the Sequence Analysis of *Bacillus stearothermophilus* 5S Ribonucleic Acid†

Charles A. Marotta,‡ Carl C. Levy,§ Sherman M. Weissman,* and Frederick Varricchio ¶

ABSTRACT: The preferred sites of digestion of a ribonuclease from *Enterobacter sp.* have been characterized by analysis of the oligonucleotides produced after digestion of 5S [^{32}P]-RNA from *Bacillus stearothermophilus* with this enzyme. The major degradation products obtained contain cytidylic acid at the 3′-phosphorylated end and adenylic acid at the 5′-hydroxylated end. Less commonly, cleavage occurred between cytidylic and guanylic or between uridylic and adenylic acid phosphodiester bonds. There was no cleavage between two purines or between two pyrimidines and the phosphodiester bonds between uridylic and guanylic acid residues remained intact. The ribonuclease has proved valuable in determining the primary structure of *B. stearothermophilus* 5S RNA and is useful in general for nucleotide sequence analysis of RNA.

Recently progress has been made in determining the nucleotide sequences of a wide variety of RNA molecules including such large molecules as the genomes of Qβ (Billeter *et al.*, 1969) and R17 (Nichols, 1970; Jeppesen *et al.*, 1970), RNA phages (Adams and Cory, 1970), and the 16S rRNA of *Escherichia coli* (Fellner, 1969) as well as discrete RNA species transcribed from DNA *in vitro* (Lebowitz *et al.*, 1971). Although continuing advances are being made in the preparation and isolation of discrete RNA fragments which contain genetic information of substantial biological interest, a number of serious technical problems remain in the sequence analysis of even moderately long RNA chains. Among the common problems in nucleotide sequence determination are the analysis of long stretches of pyrimidines and purines, the analysis of very large oligonucleotides produced by T_1 ribonuclease digestion, and the ordering within an RNA chain of fragments produced by specific nucleases.

Because of these problems we have continued to examine a wide variety of nucleases which may prove to be of use in this area. We report here that an enzyme ("C" ribonuclease)[1] isolated originally by Levy and Goldman (1970) from *Enterobacter sp.* grown on poly(cytidylic acid), has particular utility since it produces clean, discrete, and relatively large oligonucleotides from a wide variety of RNAs. The products obtained are useful in arranging in order oligonucleotides produced by T_1 ribonuclease digestion. Furthermore this nuclease has potential utility in the sequence analysis of large oligonucleotides lacking internal guanylic acids. The 5S RNAs are convenient to prepare in reasonably pure forms and are complex enough to provide a range of sequences with various amounts of secondary structures and yet simple enough so that all the products of a nuclease digest can be analyzed. We have used 5S [^{32}P]RNA from *Bacillus stearothermophilus* to determine the preferred sites of digestion of this nuclease.

Materials and Methods

B. stearothermophilus 1430R (kindly provided by Dr. P. Lengyel) was grown at 60° with vigorous shaking initially in a rich medium containing 1% yeast extract, 1% Casamino acids, 1% glucose, $FeCl_3 \cdot 5H_2O$, 0.016 g; NH_4Cl, 2.0 g; Na_2HPO_4, 6.0 g; $NaH_2PO_4 \cdot H_2O$, 3.0 g; Na_2SO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; in a total volume of 1 l.; the pH was adjusted to 7.2. Cells from a log-phase culture were harvested by centrifugation and resuspended in a low phosphate medium containing 0.5% glucose; Tris-HCl, 14.5 g; $(NH_4)_2SO_4$, 2.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $FeSO_4 \cdot 7H_2O$, 0.005 g; 0.1 g of aspartic and glutamic acids; 0.005 g of methionine, phenylalanine, and glutamine. The pH of the medium was adjusted to 7.5 and the final volume was 1 l.

The generation time in the low phosphate medium was 70

† From the Departments of Human Genetics, Internal Medicine and Molecular Biophysics & Biochemistry, Yale University, New Haven, Connecticut 06520. *Received February 5, 1973.*

‡ National Institutes of Health Postdoctoral Fellow. A portion of this work was submitted by C. A. M. to Yale University in partial fulfillment of requirements for the degree of Doctor of Philosophy.

§ National Cancer Institute, Baltimore Cancer Research Center, National Institutes of Health, Baltimore, Md. 21211.

¶ Present address: Sloan-Kettering Institute for Cancer Research, New York, N. Y. 10021; supported by grants from National Institutes of Health (USPHS AM 5035-02) and the American Cancer Society (ACS VC-ID).

[1] "C" ribonuclease refers to the *Enterobacter sp.* ribonuclease.