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Chemical Synthesis of an Octanucleotide Complementary to a Portion of the Cohesive End of P2 DNA and Studies on the Stability of Duplex Formation with P2 DNA†

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ABSTRACT: A pyrimidine octanucleotide complementary to one of the cohesive ends of P2 DNA was chemically synthesized. Its sequence, d(C-T-T-T-C-C-C-C-OH), was verified by labeling it at the 5′ end, followed by partial enzyme digestion and separation by a two-dimensional fingerprinting system. A single ribo-G residue was added to its 3′ end using calf thymus deoxynucleotidyl terminal transferase. The resulting nonanucleotide primer was used in a detailed study on the stability of the duplexes formed in the partial as well as complete repair synthesis catalyzed by DNA polymerase I, at 5 °C in the presence of 70 mM potassium phosphate and 70 mM NaCl. The nonanucleotide primer was able to form a stable duplex with P2 DNA template only in the presence of DNA polymerase I. When the chain lengths of pyrimidine oligonucleotides were varied from 4 to 8 to test their abilities to serve as primers for the enzymatic repair synthesis, it was revealed that the minimum length required for the primer function is 8. Using the nonanucleotide as the primer and the right-hand cohesive end of the DNA as the template, repair synthesis was initiated simultaneously at the 3′ end of the primer as well as at the right-hand 3′ end of the DNA. This resulted in a decrease in the efficiency of repair synthesis at the 3′ end of the primer, possibly due to the displacement of the primer by the enzyme. The enzyme was unable to displace the primer, when the primer was extended to a 13-mer prior to the initiation of repair synthesis at the 3′-OH end of the DNA. These data suggest that the strand displacement by DNA polymerase I at 5 °C in the presence of 70 mM potassium phosphate and 70 mM NaCl is not significant when the duplex is at least 13 nucleotides long. The efficiency of the repair synthesis at the 3′-OH end of the DNA–primer duplex could be increased by blocking the repair synthesis at the 3′-OH end of the DNA by converting it to 3′- phosphate. This method could be useful in DNA sequence analysis, where such specfic repair synthesis is desired.

The complete sequences of the cohesive ends of *Escherichia coli* bacteriophage DNA molecules such as λ, 186, φ80, and P2 have been reported previously (Wu and Taylor, 1971; Padmanabhan and Wu, 1972; Bambara et al., 1973; Padmanabhan et al., 1974a; Murray and Murray, 1973). These DNA molecules have been very useful as models to test new methods for DNA sequencing or to study the properties of enzymes such as polymerases or nucleases (Ghangas and Wu, 1975; Uyemura et al., 1975).

Binding studies involving short synthetic oligonucleotide duplexes have been published previously (Gupta and Khorana, 1968; Kleppe et al., 1970, 1971). It was found that the minimum length of short oligonucleotides required to form stable duplexes with complementary oligo- or polynucleotides varied from 6 to 12 depending on the composition and concentration of the oligonucleotides used. Duplexes with uneven 3′-hydroxyl ends can be repaired by DNA polymerase (Goulian et al., 1973).

A method was proposed for DNA sequence analysis using a short oligonucleotide primer which could be bound to a specific location on the single-stranded region of a DNA molecule (Wu et al., 1972; Wu, 1972). The nucleotide sequence beyond the 3′ end of the primer could be determined

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after extension of the primer with radioactive nucleotides using the DNA polymerase catalyzed repair reaction (Wu, 1972; Padmanabhan et al., 1972).

In order to study in more detail the stability of duplexes consisting of DNA and oligonucleotide primers of varying length under the same conditions as used for in vitro extension of the primer, the pyrimidine octanucleotide, d(C-T-T-T-C-C-C-C) was chemically synthesized using the procedures developed by Khorana et al. (Weber and Khorana, 1972; Agarwal et al., 1971). The sequences of the intermediate products, d(C-T-T-T) and d(C-T-T-T-C-C) and the final octanucleotide product were verified by the methods described previously (Wu et al., 1973; Jay et al., 1974). The right-hand cohesive end of P2 DNA was chosen as the template because of the following special properties of the system. (1) Due to the location of the complementary sequence on the cohesive end template (Padmanabhan et al., 1974b), the octanucleotide primer could be extended stepwise at its 3′ end to give 9-, 11-, 13-, and 16-mers by varying the nucleoside triphosphates present, thus enabling the study on the stability of these oligonucleotide duplexes (see Figure 5a). (2) The complete repair of the right-hand cohesive end of P2 DNA catalyzed by DNA polymerase I requires only dGTP, dTTP, and dCTP; under these conditions, the left-hand cohesive end is partially repaired. For the complete repair of the left-hand cohesive end, dGTP, dATP, and dCTP are required. Thus, at the cohesive end to which the primer is bound addition of dGTP, dTTP, dCTP, and polymerase I resulted in a simultaneous initiation of repair synthesis at both the natural 3′ ends of the DNA as well as the 3′ end of the primer, the former reaction competing with the latter. It was shown that the repair synthesis at the 3′ end of the DNA template competing with the primer extension can be blocked by conversion of DNA-3′-OH to DNA-3′-P. Finally, by using deoxynucleotidyl terminal transferase (Roychoudhury et al., 1971), a single ribonucleotide can be added to the 3′ end of the octanucleotide primer to form a nonanucleotide which can still serve as the primer for copying the remaining portion of the template, but with added advantage for DNA sequence analysis. The nucleotides added to the 3′ end of the primer-rG-OH can be cleaved off from the primer by using the specific T_1 ribonuclease or alkali, making the sequence analysis of the labeled region easier (Sekiya et al., 1975).

Materials and Methods

DNA. Phage P2 grown and purified according to published procedures (Bertani and Bertani, 1970; Lengyel et al., 1975) was a generous gift of Dr. Richard Calendar, Department of Molecular Biology, University of California, Berkeley, Calif. DNA was extracted by phenol and further purified by sucrose gradient sedimentation as described elsewhere (Wu et al., 1974).

Enzymes. Purified spleen phosphodiesterase which is free of phosphatase activity was a gift of Dr. G. Bernardi, Institut de Biologie Moleculaire, Université Paris VII. Deoxynucleotidyl terminal transferase was a gift of Drs. Roychoudhury and Kossel. DNA polymerase I was purified according to Jovin et al. (Jovin et al., 1969). Bacterial alkaline phosphate was from Worthington Biochemical Co. Venom phosphodiesterase (Worthington) was further purified to remove 5′-nucleotidase (Sulkowski and Laskowski, 1971). Labeled deoxynucleoside triphosphates—tritiated dATP, dTTP, and dCTP—were purchased from Schwartz BioResearch, Inc., and tritiated dGTP was from Amersham Searle Co. α-^{32}P labeled deoxynucleoside triphosphates were purchased from New England Nuclear.

Chemical Synthesis of d(C-T-T-T-C-C-C-C). The starting material for the synthesis of octanucleotide (protected dinucleoside monophosphate; see Table I) was a generous gift of Dr. Ray Wu. The octanucleotide was chemically synthesized following the general methods developed by Khorana et al. (Weber and Khorana, 1972; Agarwal et al., 1971). A summary of conditions for the synthesis and the yield of the oligonucleotides is given in Table I. A small amount of the protected compound was treated with ammonium hydroxide (28% NH_3) followed by acetic acid to remove the protecting groups. The composition and 3′-end-group analyses of synthetic tetramer and octamer were verified by digesting with spleen phosphodiesterase in an incubation mixture (0.26 mL) containing 2.5 mM potassium phosphate, pH 6.0, 0.23 mM EDTA,[1] 0.004% Tween 80 and 3–5 μg of the enzyme. Incubation was for 8 h at 37 °C.

5′-End-Group Labeling of d(C-T-T-T), d(C-T-T-T-C-C), and d(C-T-T-T-C-C-C-C). The oligonucleotides were labeled at the 5′ end with ^{32}P using polynucleotide kinase (Richardson, 1965) in an incubation volume of 15 μL containing 200 pmol of oligonucleotide, 66 mM Tris-HCl (pH 7.8), 6.6 mM $MgCl_2$, 15 mM dithiothreitol, 0.1 mM [γ-^{32}P]ATP, and 5 units of the enzyme. The incubation was carried out for 2 hours at 37 °C. The labeled products were purified by PEI chromatography (Randerath and Randerath, 1967; Southern and Mitchell, 1971).

3′-End-Group Labeling of d(C-T-T-T) and d(C-T-T-T-C-C-C-C). Two-hundred-twenty picomoles each of tetramer and octamer in 24 μL of final incubation volume containing 240 mM potassium cacodylate, 50 mM Tris (pH 7.6), 8.3 mM $MgCl_2$, 0.83 mM $CoCl_2$, 0.16 mM dithiothreitol, 33 μM [α-^{32}P]GTP, and 30 units of calf thymus deoxynucleotidyl terminal transferase were incubated at 37 °C for 6 h. Any additional ribonucleotides incorporated after the first were removed by the procedure described by Roychoudhury et al. (1971). The reaction mixture containing the transferase addition product of tetramer was purified directly by PEI-cellulose chromatography. The product of the octamer was first purified on a Whatman No. 1 paper using a solvent system of 0.5 M ammonium acetate–95% ethanol (3:7, v/v, pH 3.5). The material which stayed at the origin was eluted and purified by PEI-cellulose chromatography using 1 M LiCl in 7 M urea as solvent.

Binding of Pyrimidine Octanucleotide d(C-T-T-T-C-C-C-C-OH) and 3′-labeled Pyrimidine Octamer–prG-OH to P2 DNA followed by Repair Synthesis. The pyrimidine octanucleotide was isolated by depurination of P2 DNA labeled at the cohesive ends by DNA polymerase I catalyzed repair synthesis, dephosphorylated with bacterial alkaline phosphatase (BAP-F), and further purified by one-dimensional electrophoresis on DEAE-cellulose at pH 3.5 (Sanger et al., 1965). The binding of a complementary oligonucleotide with the native P2 DNA and the separation of free oligonucleotide from the DNA–primer hybrid were carried out essentially as described elsewhere (Padmanabhan et al., 1972).

Dissociation of DNA–Oligonucleotide Hybrid. After repeated evaporation to remove NH_4HCO_3, the DNA–oligonucleotide duplex in 50 μL of water was heated in a boiling

[1] Abbreviations used are: anC, *N*-anisoyldeoxycytidine; mmt, monomethoxytrityl-; TPS, triisopropylbenzenesulfonyl chloride; N indicates a tritium-labeled nucleotide or nucleoside, p*N indicates a ^{32}P-labeled nucleotide; PEI, poly(ethylenimine); EDTA, (ethylenedinitrilo)tetraacetic acid; DEAE, diethylaminoethyl; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

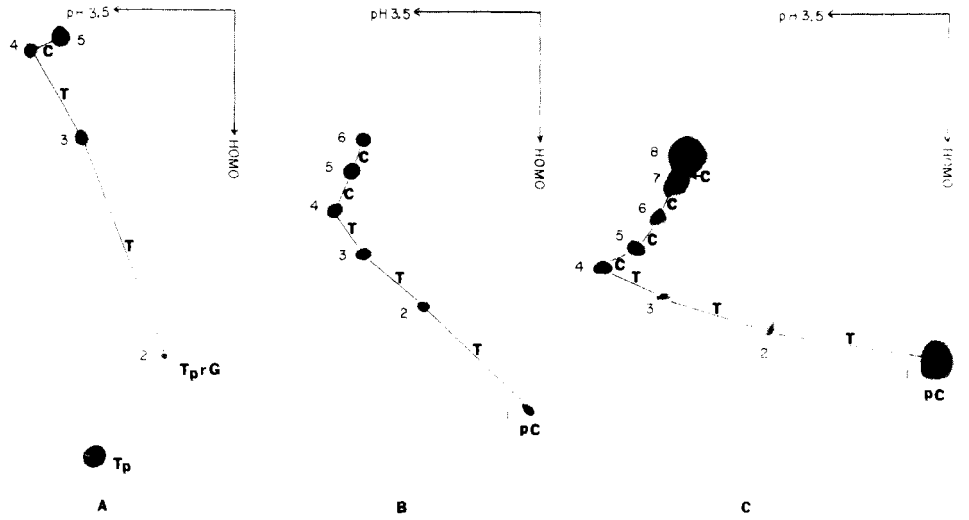

FIGURE 1: (A) Two-dimensional electrophoresis–homochromatography of a partial spleen phosphodiesterase digest of d(C-T-T-T-p*rG-OH). Incubation with spleen phosphodiesterase was carried out at 37 °C in 10 μL containing 4.5 mM potassium phosphate, pH 6.0, 0.4 mM EDTA, 0.01% Tween 80, and 0.01 unit of enzyme (0.03 μg). Samples were taken at 5, 15, and 25 min. (B) Two-dimensional map of a partial snake venom phosphodiesterase digest of d(p*C-T-T-T-C-C-OH). Incubation with snake venom phosphodiesterase was carried out at 37 °C as described (Jay et al., 1974). Samples (2 μL) were taken after 5, 10, 20, and 30 min and blown into 50 μL of 1 M ammonium hydroxide containing 1 mM EDTA. The combined digest was dried in a desiccator before dissolving in 5 μL of water for electrophoresis. (C) Two-dimensional map of a partial snake venom phosphodiesterase digest of d(p*C-T-T-T-C-C-C-C-OH). The conditions used are the same as described in (B). The conditions for fractionation by two-dimensional ionophoresis–homochromatography have been described elsewhere (Jay et al., 1974). The ribonucleotide is denoted by the prefix r. The prefix d for deoxyribonucleotides is omitted in the Figure for simplicity.

TABLE I: Summary of Conditions for the Chemical Synthesis of Octamer and Yields of Condensation Reactions.[a]

3′-OH Containing Comp	(A) Amount (μmol)	5′-P Containing Comp	(B) Amount (μmol)	TPS (μmol)	Py (mL)	Time (h)	Yield (%)	Size of Product
d(mmt-anC-T-OH)	610	d(pT-T-OAc)	550	3300	10	6.5	49	Tetramer
d(mmt-anC-T-T-T-OH)	51	d(panC-anC-OAc)	124	330	5	7	91	Hexamer
d(mmt-anC-T-T-T-anC-anC-OH)	34	d(panC-anC-OAc)	136	408	3	6	41	Octamer

[a] The percent yield of all products is given as yield determined spectroscopically after complete purification by DEAE-cellulose column chromatography followed by precipitation of the oligonucleotides. The value is calculated from the conversion of 3′-OH containing component to the product.

water bath for 10 min in a siliconized glass tube. It was then fractionated by one-dimensional homochromatography on DEAE-cellulose using partially hydrolyzed yeast RNA (Homomix III, Jay et al., 1974) or by Sephadex G-100 column chromatography.

Results

Synthesis and Sequence Analysis of Pyrimidine Octanucleotide. The chemical synthesis of the pyrimidine octanucleotide and the yields of the intermediate products are given in Table I. The nucleotide composition and the sequence of the synthetic products were checked by two methods. First, approximately 2 A_{260} units of the completely deprotected synthetic tetramer and octamer were digested by spleen phosphodiesterase and the resulting nucleotides and nucleosides were fractionated by paper chromatography (Wu, 1970). In each case, the expected ratios of each nucleotide and nucleoside were found (data not shown). The second method is the same as that used for verifying the sequences of the synthetic oligonucleotides complementary to the λ and T4 lysozyme gene (Wu et al., 1973; Padmanabhan et al., 1974a). For sequence analysis, the 3′-labeled tetramer-p*rG-OH (obtained by the addition of prG using calf thymus terminal transferase as described under Methods) was partially digested by spleen phosphodiesterase (Figure 1A). The 5′-labeled hexamer and octamer were partially digested by venom phosphodiesterase (Figures 1B and 1C, respectively). The partial digests were separated by a two-dimensional ionophoresis–homochromatography system (Brownlee and Sanger, 1969; Sanger et al., 1973; Jay et al., 1974). In this system, the addition of a dpC always causes a decrease in mobility, whereas the addition of a dpT causes an increase in the first dimension (electrophoresis on cellulose acetate at pH 3.5). Therefore, in the two-dimensional system, the mobility of the nucleotide extended by a dpC always shifts to the right and that extended by a dpT shifts to the left. As shown in Figure 1A, spot 1 is Tp* by cochromatography on paper (Wu, 1970) which was labeled due to the transfer of ^{32}P from ribo-G residue at its 3′ end. Therefore, spot 2 can be deduced to be the dinucleotide Tp*rG-OH. Spots 3 and 4 resulted from the successive addition of T residues and spot 5 resulted from the addition of a C residue; thus, the sequence deduced from this partial spleen phosphodiesterase digest of tetramer-prG-OH is d(C-T-T-TprG-OH). In Figure 1B, spot 1 is dpC by cochromatography on paper and spots 2–4 resulted due to the successive addition of T residues to spot 1, to yield the sequence of d(pC-T-T-T-OH) to spot 4. Spots 5 and 6 resulted from successive additions of C residues to spot 4 to yield the sequence of d(pC-T-T-T-C-C-OH) to the hex-

TABLE II: Binding of Complementary Octanucleotide to P2 DNA.

Expt	Reaction Comp	% Binding	Size of the Oligonucleotide in the Complex[b]
a	P2 DNA + octamer[a]	0	
b	P2 DNA + octamer + dGTP + Polymerase	39	9
c	P2 DNA + octamer + dGTP +dCTP + Polymerase	57	11
d	P2 DNA + octamer + dGTP +dCTP + dATP +Polymerase	64	11
e	P2 DNA + 5′-[^{32}P]octamer[c]	0	
f	P2 DNA + 5′-[^{32}P]octamer +dGTP + Polymerase	24	9
g	P2 DNA + 5′-[^{32}P]octamer +dGTP + dCTP +Polymerase	33	11
h	P2 DNA + 5′-[^{32}P]octamer +dGTP + dCTP + dATP +Polymerase	44	11

[a] The pyrimidine octanucleotide d(CpTpTpTp*Cp*Cp*Cp*C-OH) obtained by depurination of labeled P2 DNA was used in the binding experiments, a to d. The amount of P2 DNA used was in 12-fold excess over that of the octanucleotide primer. [b] The separation of the octanucleotide in the form of DNA-bound complex from the free form was done by agarose column chromatography (Wu et al., 1974). Recovery of ^{32}P counts from the column was between 70-80%. The % binding was calculated as the fraction of the labeled octamer bound to P2 DNA. [c] Chemically synthesized octanucleotide labeled at its 5′ end with ^{32}P was used as primer (in the experiments e to h) in fourfold excess over the template P2 DNA. The % binding was calculated as the fraction of the P2 DNA which formed hybrid with the labeled octamer.

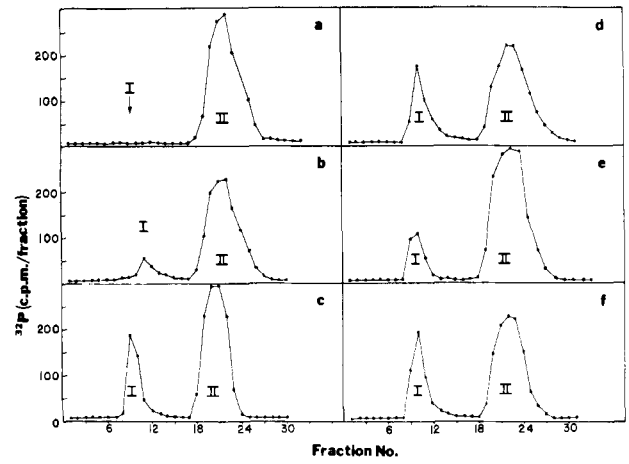

FIGURE 2: Agarose column chromatographic analysis of binding of nonanucleotide to P2 DNA. The nonanucleotide d(C-T-T-T-C-C-C-Cp*rG-OH) was used as a primer at a concentration twice that of the right-hand cohesive end of P2 DNA (0.54 pmol) as template. (a) Nonamer alone; (b) nonamer + polymerase; (c) nonamer dCTP + polymerase; (d) nonamer + dCTP + dTTP + polymerase; (e) nonamer + dCTP + dTTP + dGTP + polymerase; (f) nonamer + dCTP + dTTP + polymerase; incubated for 4 h. Then, dGTP was added and the incubation was continued for an additional 4 h. Peak I represents the DNA-primer duplex and peak II represents the free primer. The fractions of P2 DNA which formed hybrid with the labeled primer, expressed as percent, are 0, 20, 52, 54, 34, and 60, respectively, for experiments a to f. The use of a fourfold excess of primer over P2 DNA in Expts a to e yielded very similar results.

amer. In Figure 1C, spots 1-6 have similar patterns of mobility shifts as the spots 1-6 in Figure 1B, which indicate the presence of hexamer sequence in the 5′-labeled octamer. In addition, the mobility shifts of spots 7 and 8 indicate the successive addition of 2 C residues to the 3′ end of the hexamer to give the sequence of d(pC-T-T-T-C-C-C-C-OH) for the synthetic octamer.

Studies on the Stability of Duplex Formation between Native P2 DNA and Octanucleotide Isolated by the Depurination of Enzymatically Repaired 3′-Labeled P2 DNA or by Chemical Synthesis. The pyrimidine octanucleotide, d(CpTpTpTp*Cp*Cp*Cp*C-OH), was isolated from the enzymatically repaired cohesive ends of P2 DNA as described under Methods. Table II (Expt a) shows that this oligonucleotide did not form a stable duplex with native P2 DNA, in agreement with the fact that the pyrimidine octanucleotide of identical sequence could not form a stable duplex with the complementary sequence at the cohesive end of 186 DNA (Padmanabhan et al., 1972). From the knowledge of the location of binding of this octanucleotide to the right-hand cohesive end of P2 DNA, the feasibility of using this octanucleotide as a primer in the DNA polymerase I catalyzed repair synthesis was examined. If it served as a primer, by using [^{3}H]dGTP alone or dGTP and dCTP in the repair synthesis, one could lengthen the octanucleotide to a nonanucleotide and an undecanucleotide, respectively (see Figure 5a). Subsequently, the stability of the duplex formed is expected to be increased. The results of Experiments b and c in Table II showed that such was indeed the case (see also Harvey et. al. (1973) for a similar approach). In Experiment d, the presence of dGTP, dCTP, and dATP allowed the complete repair of the left-hand cohesive end of P2 DNA (unlike the case of 186 DNA where all 4 dNTP are required for complete repair of both cohesive ends). The complete repair of one of the cohesive ends eliminated the competing reaction of duplex formation between the two 19-nucleotide long cohesive ends and favored the yield of stably bound oligonucleotide-DNA hybrid. The reason the extent of binding was not more than 64% of the input [^{32}P]octanucleotide might be due to the primer being degraded by the 3′ → 5′ exonuclease activity of DNA polymerase I during incubation (Brutlag and Kornberg, 1972) (see Figure 5b).

When the binding studies were carried out using a fourfold excess of 5′-^{32}P-labeled synthetic octanucleotide over P2 DNA concentration, the extent of binding of oligonucleotide to P2 DNA increased as the chain length increased from 8 to 11 and reached a maximum value when the enzymatic repair synthesis at the left-hand cohesive end was complete (Table II, Expts e to h), similar to the results obtained in Expts a to d, where a 12-fold excess of P2 DNA over the primer was used. But the reason for the somewhat lower extent of binding when the synthetic octamer was used as the primer is unknown.

Studies on the Stability of Duplex Formation between Native P2 DNA and the Nonanucleotide d(C-T-T-T-C-C-C-CprG-OH). The nonanucleotide was enzymatically synthesized by adding a ribo-G residue to the 3′ end of the synthetic octanucleotide using deoxynucleotidyl terminal transferase, as described under Methods. The nonanucleotide was tested for its ability to form a stable duplex with P2 DNA. As shown in Figure 2a, the nonamer in the absence of polymerase I did not form a stable duplex under the conditions used: ^{32}P counts appeared as free oligonucleotide (peak II) and none bound to the DNA (peak I), but, in the presence of the enzyme, nonamer was able to bind to P2 DNA to an extent similar to that of the enzymatically extended octanucleotide shown under Expt f in Table II. This stabilizing effect of DNA polymerase

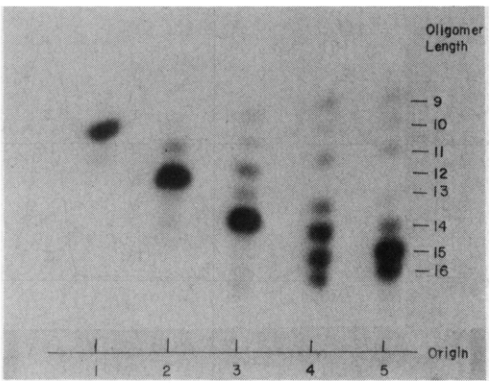

FIGURE 3: Dissociaton of DNA–oligonucleotide duplex by one-dimensional homochromatography. Peak I from Figure 2b–f was desalted and dissociated as described under Methods. The oligonucleotides were separated by one-dimensional homochromatography (Jay et al., 1974) using Homomix III. Columns 1–5 resulted from the experiments b to f in Figure 2. The slanted mobilities of the numbered oligonucleotides in columns 1 to 5 were due to the artifact of the run, as judged from, and can be corrected by, the mobilities of the blue and yellow dye markers.

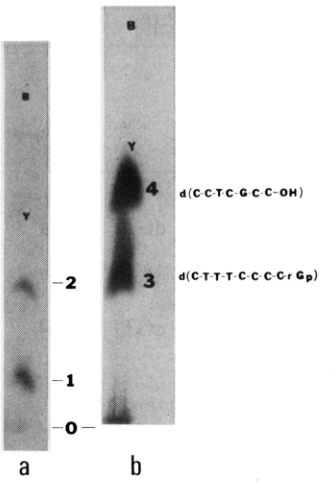

FIGURE 4: (a) Dissociation of DNA–13-mer and DNA–16-mer duplexes by one-dimensional homochromatography. 13-mer with the sequence d(C-T-T-T-C-C-C-Cp*rG-Ċ-Ċ-Ṫ-Ċ-OH) was isolated from a binding experiment containing 1.8 pmol of P2 DNA, a fivefold excess of the nonamer as primer, dĊTP, dṪTP, and DNA polymerase I. DNA–oligomer duplex was separated from the excess-labeled dNTP and the free primer by agarose column (1.1 × 66 cm) chromatography (Wu et al., 1974). After desalting, DNA oligomer duplex was dissociated by heating in a boiling water bath for 10 min and immediately applied to a column of Sephadex G-100 (0.75 × 90 cm). The column was eluted with 0.05 M NH_4HCO_3 and 0.001 M EDTA, and 1.3-mL fractions were collected. DNA and the oligomer (13-mer) were eluted after 10 and 24 mL of eluate had passed through the column, respectively. The 13-mer isolated after desalting was used as a primer in a reaction containing 1.8 pmol of P2 DNA, 0.08 pmol of 13-mer, dĠTP, dĊTP*, and DNA polymerase I. DNA–oligomer duplex isolated as described above was dissociated by one-dimensional homochromatography using a 2.8% Homomix d (Brownlee et al., 1968). B and Y refer to xylenol-FF and orange G dye markers. (1) 16-mer; (2) 13-mer; (0) origin. (b) Cleavage of 16-mer by ribonuclease T_1. Approximately 0.04 pmol of 16-mer isolated as described in Figure 4a was incubated with 125 units of ribonuclease T_1 (Calbiochem) for 2 h. The sample was fractionated by one-dimensional homochromatography using Homomix VI (Jay et al., 1974). The nucleotide sequences of spots 3 and 4 were assigned from the nearest-neighbor and 3′-end-group analysis (data not shown). Due to the differences in the resolution of oligonucleotides by the two different Homomix preparations used in 4a, b, the mobilities of spots 1 and 2 in 4a cannot strictly be compared to spots 3 and 4 in 4b.

I in duplex formation has previously been observed (Padmanabhan et al., 1974a). When the nonamer was extended by the polymerase I in the presence of dCTP to an undecamer by the addition of 2 C residues (see Figure 5a), the stability of the duplex formed increased to 52% (see Figure 2c). The stability of the duplex formed did not increase significantly when the undecamer was lengthened to 13-mer, as shown in Figure 2d.

When the repair synthesis at the 3′ end of the primer was carried out in the presence of 3 dNTP (dCTP, dTTP, and dGTP), the extent of binding was significantly reduced (Figure 2e). The most likely explanation for this observation is that repair synthesis was initiated at the 3′ end of the native P2 DNA at the right-hand end (see Figure 5a) as well as the 3′ end of the primer. As a result, the enzyme molecule during the process of elongation of the natural 3′ end of P2 DNA presumably displaced some of the primer from the primer–DNA duplex. However, when the nonanucleotide primer was first extended to 13-mer by the addition of 3 C and 1 T residues to its 3′ end in the presence of DNA polymerase I, dCTP, and dTTP (Figure 2f), initiation of repair synthesis at the natural 3′ end of P2 DNA by the addition of dGTP no longer affected the binding of the extended primer.

In order to analyze the products formed during the repair synthesis at the 3′ end of the nonanucleotide primer, the DNA–oligonucleotide hybrid was dissociated as described under Methods. The oligonucleotides were separated by one-dimensional homochromatography. As shown in Figure 3, the 9-mer (column 1) was extended to 11-mer by the addition of 2 C residues (column 2) and to a 13-mer by the addition of 3 C and 1 T residues (column 3). When the repair synthesis was carried out in the presence of dCTP, dTTP, and dGTP at the natural 3′ end of P2 DNA as well as at the 3′ end of nonamer, all the intermediate oligonucleotides formed during the conversion of 9-mer to 16-mer can be seen in column 4 of Figure 3. However, the net yield of the DNA–oligonucleotide hybrid decreased. When the nonanucleotide primer was extended to 13-mer prior to the addition of dGTP (Figure 2f), the repair synthesis resulted in the accumulation of 15- and 16-mers, rather than the shorter intermediates (column 5).

In order to circumvent the initiation of repair synthesis at the natural 3′ end of P2 DNA (see Figure 5a) and to increase the yield of 16-mer extended from the 3′ end of primer, a partial repair synthesis in the presence of dCTP and dTTP was carried out at the 3′ end of the nonamer. Under these conditions, nonamer is extended to 13-mer by the addition of 3 C and 1T residues (see Table III, Expt c). DNA–13-mer duplex first purified by agarose column chromatogarphy (Wu et al., 1974) was then dissociated and fractionated on a Sephadex G-100 column as described under Methods. The 13-mer was further used as a primer in a reaction containing P2 DNA, dGTP, dCTP, and polymerase I. Under these conditions, repair synthesis at the natural right-hand 3′ end of P2 DNA stops with the addition of 1 G residue and does not elongate further due to the absence of dTTP in the reaction mixture. In Figure 4a, spot 1 is the 16-mer and spot 2 is the unutilized 13-mer bound to P2 DNA template. Since the nonanucleotide primer had a ribo-G residue at its 3′ end before the addition of 7 nucleotides in the repair synthesis, the 16-mer product should be susceptible to cleavage by ribonuclease T_1 to give rise to a nonamer and a heptamer. These products were indeed formed after ribonuclease T_1 digestion, as shown in Figure 4b (spots 3 and 4, respectively).

Nearest-neighbor analyses of the products formed during the repair synthesis at the 3′ end of nonamer are shown in Table III. The complete digestion of nonanucleotide primer showed the expected transfer of ^{32}P from the rG residue to the terminal

TABLE III: Nearest-Neighbor and 3′-End Group Analysis of Oligonucleotides after Repair Synthesis.

Expt[a]	Primer Used for Repair Synthesis	Products after Enzyme Digestion[b] Nucleoside	Nucleotide	Sequence Deduced
a	Nonamer alone	—	dCp*	Pyrimidine Octamer-p*rG-OH
b	Nonamer + dĊTP + Polymerase	1 dĊ	0.8 dĊp; dCp*	Pyrimidine Octamer-p*rGpĊpĊ-OH
c	Nonamer + dĊTP +dṪTP + Polymerase	1 dĊ	2.1 dĊp 1 dṪp; 0.7 dCp*	Pyrimidine Octamer-p*rGpĊpĊpṪpĊ-OH
d	13-mer primer + dĠTP + dĊTP* + Polymerase	1 dĊ	1.1 dGp*; 1.2 dĠp 1.5 dCp*; 1Ṫp; 4 dĊp	Pyrimidine Octamer- p**rGpĊpĊpṪpĊpĠp*Ċp*Ċ-OH[c]

[a] Expts a–c are the same as Expts b–d described in Figure 2. In Expt d, a mixture of ^{3}H- and ^{32}P-labeled dCTP was used at a final concentration of 2 μM. In addition, the 13-mer isolated by a partial repair synthesis from Expt c was used as a primer for the synthesis of 16-mer (see spot 1 in Figure 4a). [b] After repair synthesis catalyzed by DNA polymerase I, the DNA–oligonucleotide duplex was isolated by agarose column chromatography and dissociated as described under Materials and Methods. The purified oligonucleotide was enzymatically digested as described (Padmanabhan et al., 1974b). The resulting nucleotides and nucleosides were separated by two-dimensional paper chromatography and analyzed (Wu, 1970). [c] The specific activity of [^{32}P]nonamer (indicated as **) was twice as much as that of dCTP used for the repair synthesis (indicated as *).

dC of the pyrimidine octanucleotide (Expt a). When DNA polymerase I and dCTP were added, the primer was extended to an 11-mer by the addition of 2 dC residues (Expt b). When the repair synthesis was carried out in the presence of dCTP and dTTP, 3 dC and 1 T were added to the primer to yield a 13-mer (Expt c).

The sequence of this 13-mer could be unambiguously established from the 3′-end-group analysis alone. The 13-mer isolated in a preparative scale, as mentioned above, was used as a primer in the synthesis of 16-mer (spot 1, Figure 4a). The nearest-neighbor and 3′-end analysis of spot 1 in Figure 4a showed the addition of 1 G and 2 C residues and the presence of GpC, CpC sequences, and a C at the 3′ end (see Table III, Expt d).

The data shown in Table III indicate that the nonamer bound to the expected location at the right-hand cohesive end of P2 DNA (Figure 5a) and that the primer was extended by DNA polymerase I to yield the expected products on the P2 DNA template.

Elimination of Repair Synthesis at the Natural 3′ Ends of P2 DNA. In order to test whether the DNA polymerase I catalyzed repair synthesis can displace bound primer (see Figures 5a and 2c), the following experiment was done. Two ribo-G residues incorporated, one at each 3′ end of P2 DNA using DNA polymerase I and Mn^{2+} ions (Berg et al., 1963; Wu et al., 1972), were oxidized with $NaIO_4$ (0.1 M final concentration) at pH 7.4. Triethylammonium bicarbonate (2 M solution, pH 9.8) was added to adjust the pH of the DNA solution to 8. Final concentration of $Et_3NH_2CO_3$ was 0.2 M. Then, 1 M lysine, pH 9.0, was added to give a final concentration of 0.1 M and the solution was incubated at 45 °C for 3 h. The DNA solution was finally dialyzed against 0.01 M Tris–0.001 M EDTA buffer (pH 7.4). Initiation of repair synthesis at the two 3′ ends of P2 DNA would be expected to be blocked due to the formation of 3′-P, and, hence, the hybridization of the primer and its enzymatic extension would not be affected by displacement.

As shown in Figure 5b, when the repair synthesis was carried out in the presence of 5′-^{32}P-labeled octanucleotide primer, dCTP, dTTP, dGTP, and DNA polymerase I, no products longer than the primer (column 2) were formed. However, when the repair synthesis at the 3′ end of native P2 DNA was blocked, hybrid formation between the octanucleotide and the DNA was facilitated and appreciable amounts of 11-mer and small amounts of 16-mer were formed (column 3). Due to the existence of an equilibrium between the formation and dissociation of the octanucleotide primer and P2 DNA, the 3′ → 5′ exonucleolytic activity of DNA polymerase I degraded part of the octanucleotide to smaller fragments (Figure 5b, columns 2 and 3).

Efficiency of Shorter Fragments as Primers to P2 DNA in the DNA Polymerase I Catalyzed Repair Synthesis. In order to test whether an oligonucleotide shorter than octamer could serve as a primer in the DNA polymerase I catalyzed repair synthesis, the binding experiments were done using d(pC-T-T-T-OH), d(pC-T-T-T-C-C-OH), and the heptanucleotide, d(pC-T-T-T-C-C-C-OH), isolated by partial digestion of octanucleotide with phosphodiesterase. None of these shorter oligonucleotides could serve as a primer for the synthesis of 11-mer in the presence of dGTP, dCTP, and DNA polymerase I, whereas octamer under the same conditions could serve as a primer (data not shown), indicating that an octamer is the minimum length of the primer in the present system.

Discussion

The factors affecting the stability of a duplex formed between an oligonucleotide and a single-stranded DNA include both the length and the base composition of the oligonucleotide. The stability of a duplex is directly related to the number of GC pairs it contains. In addition, the stability of the duplex formed between the cohesive end of a phage DNA and an oligonucleotide is affected by the intra- or intermolecular duplex formation between the two cohesive ends. A nonanucleotide complementary to one of the cohesive ends of λ DNA, capable of forming 8 GC pairs (the GC content of the duplex region is 89%), or a synthetic dodecanucleotide (Harvey et al., 1973) with 10 GC pairs, bound to λ DNA in the absence of polymerase, but d(C-T-T-T-C-C-C-C-C-rG-OH) with 6 GC pairs (and a ribo-G in place of a dG) could not form a stable duplex under similar conditions (see Figure 2a). However, when this nonanucleotide was formed in the partial repair synthesis using pyrimidine octanucleotide as primer, dGTP, and DNA polymerase I, a stable duplex with P2 DNA could be isolated (see Expts b and f in Table II). Therefore, it seems that for a stable duplex formation between an oligonucleotide (at least 9-nucleotides long) primer and a complementary DNA template, a GC content of 83–89% in the duplex form is necessary. DNA polymerase I seems to contribute to the stability of the duplex

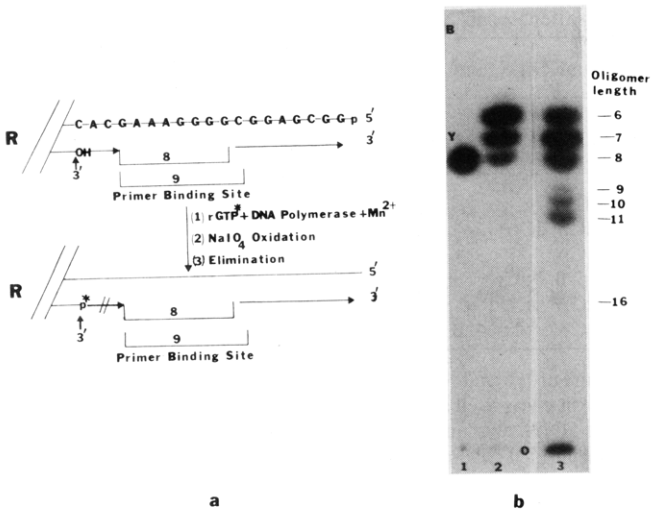

FIGURE 5: (a) Elimination of repair synthesis at the natural 3′ ends of P2 DNA and exclusive synthesis at the 3′ end of primer. The complete nucleotide sequence at the cohesive ends of P2 DNA has already been published elsewhere (Padmanabhan et al., 1974a). R represents the nucleotide sequence at the right-hand cohesive end of P2 DNA; → represents the two possible locations of initiation of DNA polymerase I catalyzed repair synthesis; ⌊⌋ represents the location of pyrimidine octanucleotide or its transferase addition product (9-mer), binding to the cohesive end template. The 3′-phospate-terminated P2 DNA was prepared as described under Methods. (b) One-dimensional homochromatography of the products of binding and extension of [5′-^{32}P]octanucleotide to P2 DNA. P2 DNA (0.4 pmol) and a 4.5-fold excess of primer were used in a final incubation volume of 0.13 mL. After incubation at 5 °C for 10 h in the presence of dĊTP, dĠTP, dṪTP, and DNA polymerase I, the reaction mixture was diluted to 1.5 mL and passed through a small column of DEAE-Sephadex A 50 (0.5 mL volume) in an Eppendorf plastic tip (W. Sarstedt, Inc.). It was then washed with 5 mL of water and 10 mL of 0.3 M $Et_3NH_2CO_3$ to wash off the excess dṄTP. The column was then eluted with 2 M $Et_3NH_2CO_3$ and the eluate was desalted by repeated evaporations under Evapomix. The recovery of the ^{32}P-labeled oligonucleotides was 90% of the input [5′-^{32}P]octanucleotideotide used. One-dimensional homochromatography was run with Homomix VI. Column 1 represents the [5′-^{32}P] marker; columns 2 and 3, oligonucleotides formed in the binding experiment using labeled octanucleotide as primer and native P2 DNA (column 2) or 3′-P terminated P2 DNA (column 3). The symbols B, Y, and 0 refer to the same as mentioned in Figure 4. DNA at origin under column 3 was labeled due to the use of rGTP* prior to periodate oxidation shown in Figure 5a.

form with a lower GC content, as no stable duplex could be isolated in the absence of the enzyme. The present studies point out that the minimum length of a pyrimidine oligonucleotide for a primer function is 8; complementary tetramer, hexamer, and heptamer could not serve as primers for DNA polymerase I catalyzed repair synthesis.

The right-hand cohesive end of P2 DNA offered a unique advantage to study the strand-displacement activity of DNA polymerase I at 5 °C. When the repair synthesis was allowed to take place at the natural 3′ end of P2 DNA as well as the 3′ end of the primer simultaneously, the completion of repair synthesis at the 3′ end of primer was inhibited. It is possible that there is a competition between the enzyme extending the natural 3′ end resulting in the displacement of the nonanucleotide primer and the enzyme extending the primer in the duplex form (see Figure 2e). Once the partial repair synthesis at the 3′ end of the primer was allowed to form a 13-mer, the initiation of repair synthesis at the natural 3′ end of P2 DNA by the addition of the required dGTP did not affect the completion of repair synthesis at the 3′-end of the primer. This could be interpreted to mean that the strand displacement by DNA polymerase I at (5 °C) low temperature and high salt content (180 mM NaCl conductivity equivalent) is not significant when the duplex is at least 13-nucleotides long (5′ → 3′ exonuclease activity of the enzyme is sufficiently suppressed under these conditions; see Wu, 1970).

The addition of a ribonucleotide to the 3′ end of an oligonucleotide primer offers a definite advantage for the DNA sequence analysis of the repaired region beyond the 3′ end of the primer. The primer molecule may be cleaved off from the labeled region of interest by using a specific ribonuclease or alkali, making the sequence analysis easier (see Figure 4b).

Another problem that is encountered in DNA sequence analysis using a primer of defined sequence on a very long linear DNA template (either a single-stranded DNA or a DNA template created by partial exonuclease III digestion) is due to the internal initiation of DNA synthesis at the 3′ termini of the template. This might result in labeling of other regions on the template which would interfere in the subsequent dissociation of DNA–oligonucleotide duplex and the sequence analysis of the labeled region of interest. The present studies have shown that by converting the 3′-OH groups in the template to 3′-phosphate by incorporation of an appropriate ribonucleotide, followed by periodate oxidation and base-catalyzed elimination, repair synthesis could be blocked at these points (see Figure 5a). Such treatment of the template prior to the primer binding and extension might improve the yield of the labeled region of interest.

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Antisera to Poly(A)·Poly(U)·Poly(I) Contain Antibody Subpopulations Specific for Different Aspects of the Triple Helix†

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ABSTRACT: Rabbit antibodies to the triple-helical polynucleotide poly(A)·poly(U)·poly(I) were fractionated into three major antibody populations, each recognizing a different conformational feature of the triple-helical immunogen. Two distinct populations were purified from precipitates made with poly(A)·poly(U)·poly(U) and poly(A)·poly(I)·poly(I). The former reacted with double-stranded poly(A)·poly(U) or poly(I)·poly(C), and similar populations could be purified with either double-stranded form. The second population recognized the poly(A)·poly(I) region of the triple helix, and the third required all three strands for reactivity. These immunochemical studies suggest that the poly(A) and poly(U) have the same orientation in the triple-helical poly(A)·poly(U)·poly(I) as in the double-helical poly(A)·poly(U), in which they have Watson-Crick base pairing.

Several antibodies to nucleic acids have been shown to be specific for conformational features of helical structures. Some can react specifically with double-stranded RNA (Nahon et al., 1967; Schwartz and Stollar, 1969; Stollar, 1970; Plescia et al., 1969; Talal et al., 1971) or RNA-DNA hybrids (Stollar, 1970) and can identify corresponding structures in samples of biological origin (Silverstein and Schur, 1970; Stollar and Stollar, 1970). Anti-triple-helix antibodies can differentiate triple-stranded structures built on poly(A) from those built by poly(dA) (Stollar and Raso, 1974). It has been suggested that triple-helical nucleic acids may be involved in viral replication (Bishop et al., 1965), gene regulation (Miller and Sobell, 1966; Britten and Davidson, 1969), bacterial transcription (Zubay, 1958, 1962), and chromosome structure (Crick, 1971), but the triple-helical forms have not been clearly demonstrable in vivo; therefore, antibodies that recognize such structures could be useful reagents to test their presence in naturally occurring nucleic acids. Additionally, anti-triple helix antibodies are important in defining conformation-dependent antigenic determinants of nucleic acids and in providing a model for specific protein–nucleic acid interactions.

De Clercq et al. (1975) have used a sensitive assay based upon interferon induction to demonstrate the formation of the triple helix poly(A)·poly(U)·poly(I) in displacement reactions involving polynucleotides. They have further demonstrated the existence of this polymer by physical-chemical techniques. We report here that antibodies induced by the triple-stranded polyribonucleotide poly(A)·poly(U)·poly(I) can be separated into subpopulations that are specific for three different aspects of the helix, and that these antibodies may be of help in the characterization of the polymer's structure.

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