The Binding of Polyribonucleotides to Single-Stranded TP-84 Deoxyribonucleic Acid*

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ABSTRACT: The presence of specific nucleotide clusters on the complementary deoxyribonucleic acid strands of bacteriophage TP-84 has been investigated by the polyribonucleotide binding technique of Szybalski and coworkers. Complex formation between denatured deoxyribonucleic acid and homoribopolymers is detected by a density shift in a CsCl density gradient. The complementary strands of denatured TP-84 deoxyribonucleic acid have been isolated and shown to be distinct in several properties (buoyant density in CsCl, nucleotide base composition, annealing of phage-induced ribonucleic acid exclusively to the heavy strand). The light-strand forms complexes with poly U, and poly C indicating the presence of nucleotide sequences of dA, and dG, respectively. The heavy-strand interacts with poly G, poly (I, G), (suggesting dC sequences), and poly X suggesting dA and/or dT sequences. Normal base-pairing considerations indicate anomalous binding in some cases. Although poly X should complex with both deoxyribonucleic acid strands it does not. Likewise, since poly U binds the light strand then poly A should complex with the complementary deoxyribonucleic acid strand but does not. The accessibility of certain nucleotide clusters to react with homoribopolymers may be a topological feature of the specific deoxyribonucleic acid strand.

Several investigators have shown that the expression of genetic information is subject to temporal control, that is, genetic expression is ordered with respect to time (Jacob *et al.*, 1957; Luria, 1962; Epstein *et al.*, 1963). The biochemical mechanisms responsible for this sequential expression of gene activity are not known; however, some evidence has appeared which suggests that such regulation may occur at the level of gene transcription (Kano-Sueoka and Spiegelman, 1962; Protass and Korn, 1966; Skalka 1966). This regulation may manifest itself in the topological form of the DNA or in specific nucleotide sequences arranged strategically along the chromosome. Here we will report one aspect of our efforts toward obtaining a complete description of the molecular anatomy of the DNA of the thermophilic bacterial virus TP-84. Evidence is presented to show that specific nucleotide clusters which can form complexes with homopolyribonucleotides exist on each complementary TP-84 DNA strand. The fact that some ribopolymers such as poly U do not bind with both DNA strands suggests that the accessibility of certain nucleotide clusters to binding with homoribopolymers may be a topological feature of the specific DNA strand. Szybalski and coworkers have developed an elegant method for the qualitative identification of specific nucleotide clusters in DNA. By virtue of the natural density bias of some *Bacillus* bacteriophages, they have shown a positive correlation between DNA strands bearing deoxycytosine clusters and the ability to hybridize with phage-induced RNA (Sheldrick and Szybalski, 1968).

Bacteriophage TP-84 is specific for certain strains of *Bacillus stearothermophilus* and is routinely grown at 60°. In previous work (Saunders and Campbell, 1965) we have shown that native TP-84 DNA is a duplex molecule of 28 million daltons in molecular weight and its complementary DNA strands are distinct in their physicochemical properties. Denatured TP-84 DNA forms two bands in a CsCl density gradient. These bands can be separated on a methylated albumin kieselguhr column and the two fractions thus obtained are not self-renaturable but when annealed together will again form a duplex structure. Phage-induced RNA is complementary to only one of the DNA strands, the pyrimidine-rich "heavy" strand (G. F. Saunders and L. L. Campbell, to be published).

Materials and Methods

Phage Preparation. TP-84 phage was propagated on *Bacillus stearothermophilus* strain 10 in trypticase medium as described previously (Saunders and Campbell, 1966). The phages were purified by banding in a CsCl density gradient in the preparative ultracentrifuge.

DNA Isolation. TP-84 was isolated by extraction of purified phage particles with phosphate-buffered phenol. The DNA preparations were assayed for purity by analytical CsCl equilibrium centrifugation as described previously (Saunders and Campbell, 1965).

Denaturation and Preparation of DNA–Ribopolymer

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[1] Abbreviations used: poly (IG), copolymer of guanylic and inosinic acids; H, "heavy" pyrimidine-rich DNA strand; L, "light" purine-rich DNA strand.

TABLE I: Base Composition of TP-84 DNA.[a]

	G	A	C	T	Purines/ Pyrimidines	% G + C
Native DNA[b]	22.0	27.7	22.1	28.2	0.99	44.1
H strand	18.4	22.9	23.4	35.3	0.70	41.8
L strand	23.1	33.4	20.3	23.2	1.30	43.6

[a] Nucleotide base analysis of DNase I and venom phosphodiesterase digests was accomplished by elution from Dowex 1-8X-formate columns as described previously (Saunders and Campbell, 1965). H and L strands of ^{32}P-labeled DNA separated by complex formation with poly U from the preparative ultracentrifugation displayed in Figure 4. [b] From Saunders and Campbell (1965).

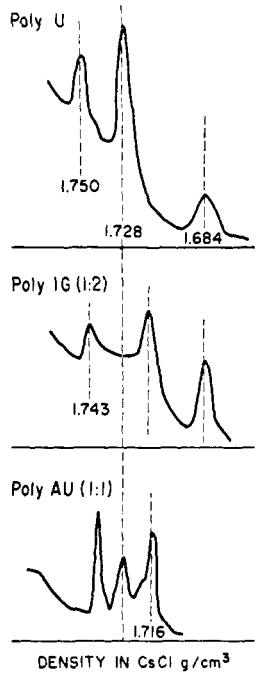

FIGURE 1: Banding pattern of denatured TP-84 DNA–ribopolymer complex in a CsCl density gradient. Samples were prepared as described in Materials and Methods. Centrifugation was carried out in 12 mm, 4° Kel-F cells for 20–24 hr at 42,040 rpm and 25° (top two tracings) or 44,770 rpm (lower tracing). The ultraviolet photographs were scanned with a Joyce-Loebl Mark IIIc double-beam microdensitometer equipped with a cylindrical lens condenser. Buoyant densities were measured using either of two marker native DNAs (Avian PPLO strain 1805 DNA provided by Dr. W. Kelton: peak of ρ 1.684 g/cc on right side in upper and middle tracings, or bacteriophage SP-8 DNA: ρ 1.742 g/cc), peak on left side in lower tracing.

Complexes. The procedure of Sheldrick and Szybalski (1967) for DNA denaturation and the preparation of ribopolymer complex was followed without modification. Briefly, it involves the denaturation of DNA with NaOH at a final concentration of 0.1 M incubation at room temperature for 90 sec, mixing with the appropriate amount of ribopolymer and neutralization with phosphate buffer. The complexes were immediately mixed with optical grade CsCl for analysis. In some cases, as will be noted, samples were stored frozen until analysis in the CsCl density gradient.

Polyribonucleotides. All polyribonucleotides were products of Miles Chemical Co., Elkhart, Ind. The polymers were dissolved in glass-distilled water, analyzed spectrophotometrically at pH 2, 7, and 10, and stored in sterile tubes at −20°.

Chemicals. Sodium *N*-lauroylsarosinate was purchased from K & K Laboratories, Plainview, N. Y. Optical grade CsCl was obtained from Harshaw Chemical Co., Cleveland, Ohio.

Results

Denatured DNA forms complexes with homopolyribonucleotides when the two are mixed under appropriate conditions. The binding detected by a density shift for the denatured DNA as measured in the cesium chloride density gradient is mediated by specific sites on the individual strands which presumably correspond to clusters of the base complementary to the bound homoribopolymer. Szybalski and coworkers have used this technique to show the existence of clusters at various points along the chromosome.

Poly U, Poly (A,U), and Poly (I,G).[1] The CsCl equilibrium patterns of the complexes of poly U, poly (I,G), and poly (A,U), with denatured TP-84 DNA are shown in Figure 1. The DNA strands of TP-84 have buoyant densities in CsCl at ρ 1.714 and 1.727 with native DNA banding at ρ 1.704. Poly (A,U) (1:1) in the lower curve gives a slight shoulder on the dense side of the light strand position and appears not to bind the heavy strand. An input ratio of 3 μg of poly (I,G) (1:1.72)/1 μg of DNA as shown in the middle curve, results in a 15 mg/cc density increase of the H strand. At concentrations of 10 μg of poly U/μg of DNA (upper curve) the L-strand band has disappeared and a new band is found at ρ 1.750. The latter results indicate that either the poly U binds exclusively to the L strand or that both strands react with this ribopolymer with H strand increasing in density by 0.022 g/cc and the L strand by 0.014 g/cc.

To resolve this point, purified L-strand DNA (prepared by methylated albumin kieselguhr column chromatography, Saunders and Campbell, 1965) was titrated with increasing amounts of poly U and the corresponding density shifts determined in the CsCl density gradient.

The data in Figure 2 show that poly U does complex with the L strand and at poly U: DNA = 10:1 the density of the strand is indeed shifted to ρ 1.750. It, therefore, appears that the band observed in Figure 1, upper curve, at ρ 1.750 represents a density crossover of the poly U–L-strand DNA complex. The product with poly U at this input ratio results in a shift in buoyant density of 0.037 g/cc. The high absorption background of free noncomplexed poly U in the ultracentrifuge cell com-

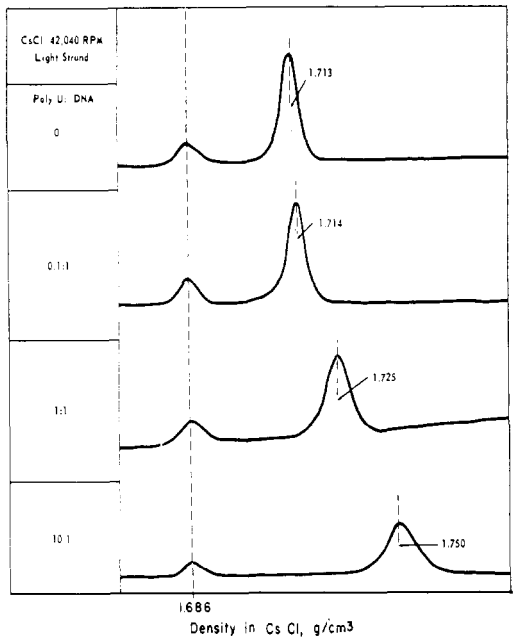

FIGURE 2: Relationship between input ratio of poly U to DNA and buoyant density of the complex formed with the isolated TP-84 L-strand DNA. Photoelectric scans at 265 mμ after 22 hr at 42,040 rpm and 25°. Samples (2–3 μg) were contained in 12 mm, 4° Kel F centerpieces. Reference DNA (0.5 μg) from human PPLO 4330, provided by Dr. W. Kelton, was determined to be 1.686 g/cc with respect to *Escherichia coli* DNA (1.710 g/cc).

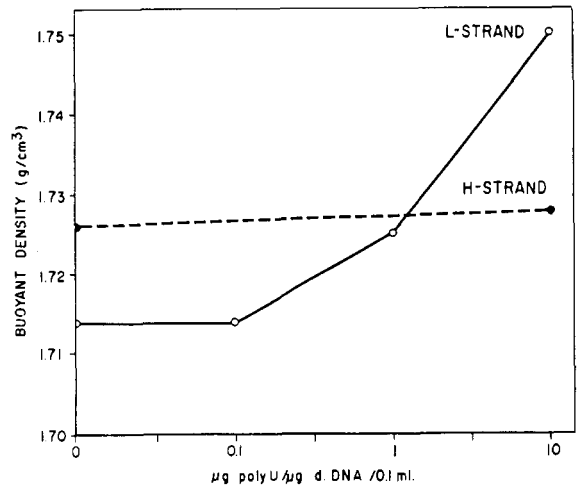

FIGURE 3: Relationship between input poly U concentration and strand density for TP-84 DNA. The buoyant densities are the peak values determined in the experiment presented in Figure 2.

plicates accurate buoyant density measurements in CsCl. For this reason, meaningful data at input poly U: DNA ratios of greater than 10:1 were not obtained. The crossover ratio (that input ratio where both the H strand and the L-strand–poly U complex have the same buoyant density) for TP-84 DNA is 1–2 μg of poly U/μg of DNA (Figure 3). The corresponding crossover ratios for $\phi\mu$-4 and *B. subtilis* phage SP 82 DNAs reported by Sheldrick and Szybalski (1967) were 0.03 and 0.1, respectively.

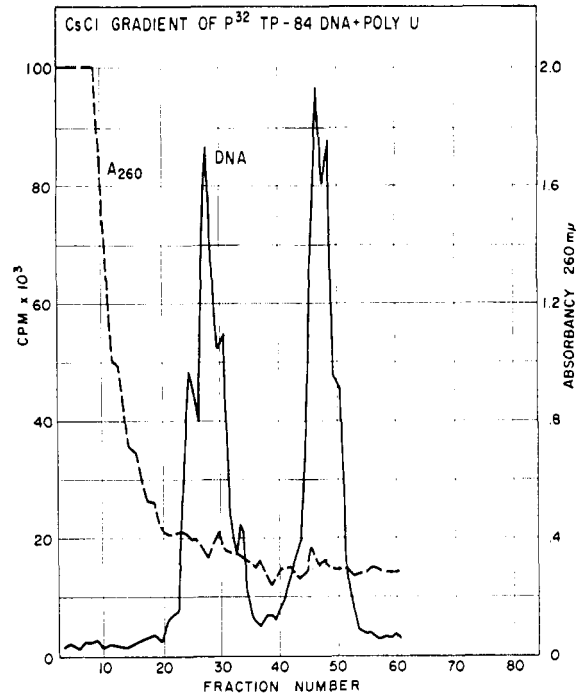

FIGURE 4: Preparative fractionation of the TP-84 [^{32}P]DNA–poly U complex in a CsCl density gradient. Poly U (9 mg in 8 ml of H_2O) was added to 2.5 ml of [^{32}P]DNA (10^6 cpm/ml and 2 μg/ml in 0.15 M NaCl–0.015 M sodium citrate, pH 7). The complex was formed by denaturation of DNA with 0.5 ml of 2 M NaOH for 90 sec at room temperature followed by neutralization with 2 ml of 1 M potassium phosphate (pH 6.7). Sodium *N*-lauroylsarcosinate (26 mg) was added to prevent the selective loss of the heavy strand by absorption onto nitrocellulose centrifuge tubes. The density was adjusted to ρ 1.740 g/cc by adding solid CsCl. The sample was divided, placed in nitrocellulose tubes and centrifuged in the Model L preparative ultracentrifuge at 25° in the type 40 angle head rotor at 33,000 rpm for 72 hr. The tubes were pierced and 14 drop fractions (0.15 ml) were collected. Radioactivity was monitored by pipetting 20-μl portions onto membrane filters and counting in a liquid scintillation system. Absorbancy measurements were accomplished after dilution of each fraction with 1 ml of 0.15 M NaCl–0.015 M sodium citrate (pH 7). Tube 28 in the figure corresponds to the L-strand complex (ρ 1.750 g/cc) and tube 47 to the H strand (ρ 1.728 g/cc).

We have used this technique preparatively to separate the DNA strands. Figure 4 shows a preparative CsCl density gradient of a [^{32}P]DNA complex with poly U; the equivalence in peak areas indicates almost complete strand separation and makes it unlikely that any significant reaction has occurred between poly U and the H strand. The base composition of the separated strands as well as that of native DNA is shown in Table I. The quantities of the component nucleotide bases are complementary between the heavy and light strands; native DNA being essentially the average of the two strands. The strand compositional data are virtually identical with our previous analyses of DNA strands separated on methylated albumin kieselguhr columns.

Since we assume that the poly U is reacting with DNA by complementary base pairing one could, in principle, complex ^{3}H–poly U with DNA, treat with ribonuclease and determine the amount of RNase-resistant poly U re-

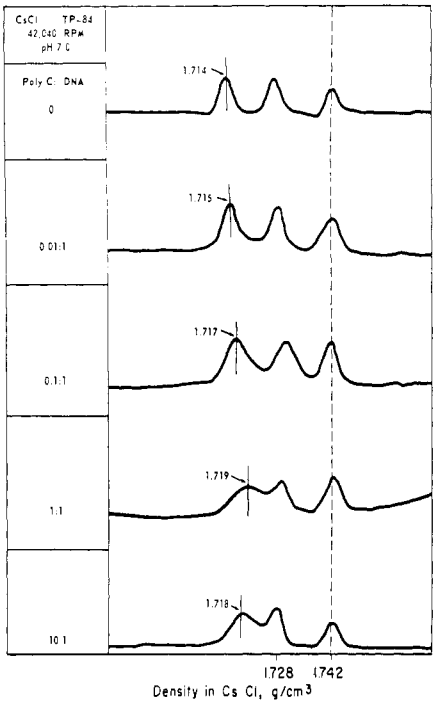

FIGURE 5: Relationship between input ratio of poly C to TP-84 DNA and buoyant density of the complex. The centrifugation conditions were the same as in Figure 2 and the DNA marker was SP8 DNA, ρ 1.742 g/cc.

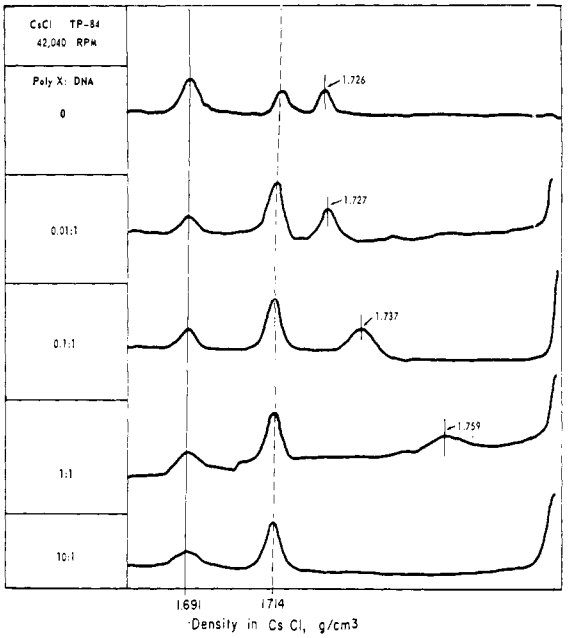

FIGURE 6: Relationship between input ratio of poly X to TP-84 DNA and buoyant density of the complex. The centrifugation conditions were the same as in Figure 2 and the reference DNA was from human PPLO 07, ρ 1.691 g/cc.

maining. In such an experiment an ^{3}H–poly U complex of 1500 cpm was degraded to the background level of 40 cpm by RNase treatment (10 μg/ml of RNase, 15 min at room temperature, two-times standard saline citrate solvent). This result is consistent with either of two possibilities: (1) that complementary DNA clusters are rare and the primary reason for the density shift is due to the intermolecular ribopolymer reaction giving a limited number of aggregate tails of high molecular weight (Sheldrick and Szybalski, 1967); or (2) that this aggregate tail formation does not occur and only one ribopolymer molecule is bound to each deoxynucleotide cluster (Peterson and Guild, 1968).

Poly A and Poly C. Others have shown that poly A and poly C generally give very little if any reaction with denatured DNA at pH 7, but poly C gives a marked reaction at pH 6 (Opara-Kubinska *et al.*, 1965; Sheldrick and Szybalski, 1967). Figure 5 shows that poly C appears to react with TP-84 DNA light strand only. The titration of phage DNA with poly C results in a skewed ribopolymer–DNA band. At neutral pH and an input of poly C:DNA of 10:1 the light strand is displaced by 3–4 mg/cc which is consistent with the 4 mg/cc light strand shift at this pH in *Bacillus* phages $\phi\mu$-4 and SP-82 reported by Sheldrick and Szybalski (1967). The band spreading with a slight increase in buoyant density is as might be expected if no poly C aggregation occurred and only poly C was bound to each dG cluster.

No significant reaction was observed between TP-84 DNA and poly A. In the initial poly A experiments a band was observed at a density midway between the H and L strands. The formation of this band is not dependent on the addition of poly A and can be formed by freezing denatured TP-84 DNA.

Poly X. The titration of TP-84 DNA with poly X is shown in Figure 6. A pronounced reaction with only the H strand is observed. At high input ratios (10 μg of poly X:1 μg of DNA) the H strand has moved to the bottom of the centrifuge cell and its position cannot be measured.

Studies on the physical properties of polyxanthylic acid and its various complexes have shown that X could function in base pairing with A or T (Michelson and Monny, 1967). Stereochemical considerations suggest that the X–T base pair is preferred.

Thermal Stability of the DNA–Ribopolymer Complex. Experiments designed to estimate the chain length of the annealed region, or cluster (that region which is complementary to the DNA), were carried out as renaturation studies in which identical samples of the DNA–ribopolymer complex are incubated at different temperatures for 2 hr. The possibility was entertained that the thermal induced dissociation of the single-strand DNA–ribopolymer complex at a characteristic temperature, would liberate the bound DNA strand thus making it available for renaturation with its original complementary strand. The DNA renaturation can be monitored in a CsCl density gradient since the renatured material has a lower buoyant density.

The band in CsCl of the DNA–ribopolymer complex becomes quite broad and difficult to measure after the 2-hr incubation at 45° or higher. Therefore, the density of the lightest band is presented in Figure 7.

Using this technique to measure the dissociation temperature of the poly G–DNA complex (Figure 7) the

TABLE II: Polynucleotide Interaction Patterns.

Polymer	A	C	U	G	I	X	H Strand	L Strand
A	+ (H^+)	−	+	−	+	+	−	+
C	−	+ (H^+)	−	+	+	−	−	+
U	+	−	+	−	−	+	−	+
G	−	+	−	+	−	−	+	−
I	+	+	−	−	+	+	+	−
X	+	−	+	−	+	+	+	−
H strand	−	−	−	+	+	+	−	+
L strand	+	+	+	−	−	−	+	−

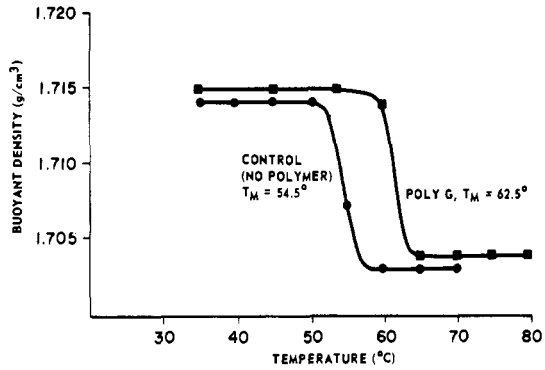

FIGURE 7: Stability of the poly G–DNA complex; 3.6 ml of a poly G–TP-84 DNA complex was prepared as described by Sheldrick and Szybalski (1967) by mixing 100 μg of DNA and 300 μg of poly G in 2.9 ml of 0.015 M NaCl–0.0015 M sodium citrate (pH 7.0). After adjustment to 0.1 M NaOH the solution was neutralized by adding 1 M phosphate buffer (pH 6.8) to final phosphate concentration of 0.2 M. The complex was divided into nine equal samples. Each sample was then incubated at a different temperature for 2 hr and analyzed in the CsCl density gradient. Since the L strand does not react, appreciably with poly G its band position can be easily monitored. The density of the L strand and/or renatured DNA, when it appears, are plotted in the figure. Centrifugation conditions were as given in the legend to Figure 2, densities are relative to the reference DNA of SP8, ρ 1.742.

midpoint of the transition is found at 62.5°. The control curve for the renaturation of alkali-denatured TP-84 DNA is also presented in Figure 7. The control samples were treated identically with respect to denaturation, incubation, and density gradient analysis except that the ribopolymer was omitted. As would be expected, renaturation resulted in the appearance of a band having a density of 1.704 which corresponds to that of native DNA. For a DNA such as TP-84, whose T_m in standard saline citrate is 87.5°, the optimal renaturation temperature in 0.4 M Na^+ should be between 60 and 70°.

When either the poly X–DNA complex or the poly (IG)–DNA complex was examined for thermal-induced dissociation and DNA renaturation a T_m of 54° was observed. This is virtually the same T_m as found for DNA in the absence of ribopolymer (Figure 7). If the data presented can be interpreted in terms of the dissociation of the ribopolymer complex occurring independently of DNA renaturation then the binding sites must be quite short.

Discussion

In order to estimate the chain length of the DNA nucleotide clusters, one should first consider the various base-pairing reactions which were observed. Chamberlin (1965) has investigated the relative stabilities of several synthetic homopolymer pairs. He finds that in 0.001 M sodium phosphate (pH 7.8) containing 10^{-4} M EDTA the ribopolymer deoxyribopolymer heteroduplexes are much more stable than the corresponding deoxyribopolymer duplexes, *i.e.*, rG:dC, T_m = 90°; dG:dC, T_m = 65°; IC polymer pairs, however, have Tm's much closer together, *i.e.*, rI:dC, T_m = 52°; dI:dC, t_m = 46°.

The stability of poly C:oligo G complexes in 0.2 M NaCl–0.002 M cacodylate buffer (pH 6.2) has been studied by Lipsett (1964). She finds that for $(Gp)_n$, when n = 3, T_m = 45°; n = 4, T_m = 58°; and by extrapolation to n = 5, T_m = 68°. By transposing this type of data, one can calculate that the dC clusters on the L strand of TP-84 DNA are a minimum four or five nucleotides long, assuming no marked effect of the difference in ionic strength. However, Lipsett's data are probably not applicable to our study since the dissociation temperature of the oligo G:poly C complex is dependent upon the oligo G concentration.

W. R. Guild (personal communication) has kindly pointed out that the renaturation phenomenon may be a kinetic process, *i.e.*, the rate of displacement of poly G from the binding sites on the TP-84 DNA, rather than a true measurement of the strength of the complex. It may be that the L strand displaces the poly G from the H strand at a lower temperature than the poly G would spontaneously dissociate. Thus, the dC cluster length could be significantly longer than four or five nucleotides.

The dG:dC pair can undergo a simple coil–helix displacement when heated and annealed with rC (Chamberlin, 1965). Although the reciprocal reaction was not carried out, one would expect the rG:dC pair to be favored over the dG:dC pair in annealing reactions. In view of the kinetic arguments stated above, we suspect that the rG:L-strand DNA complex would not be favored over the DNA–DNA duplex at the optimal re-

naturation temperature, and the dissociation temperature of the poly G–DNA complex could only establish a minimum estimate of the length of the complexing chains. The fact that long regions of deoxynucleotides may be in register on either side of the poly G binding cluster may be sufficient to allow the complementary DNA strand to displace the poly G. In the case of the poly (I,G) copolymer, the local base-pairing competition provided by the complementary DNA strands may suffice to eliminate the expected 5° difference in the helical stabilities. These comments are valid only if the dC stretches are of moderate chain length (far less than 20 nucleotides). It would also appear that for the copolymer of poly (I,G), guanylate stretches up to 20 nucleotides long were not complexed with the DNA. Fifty-five degrees appears to be above the dissociation temperature of the presumed rI:dC complex, and the poly X–DNA complex.

A summary of the interactions of several ribopolymers with each other (reviewed by Michelson *et al.*, 1967) as well as with the TP-84 DNA strands is shown in Table II. Thus, we can deduce which types of base pairing reactions are permissible. This table is intended to show the spectrum of polynucleotide complexes which have been reported. One should bear in mind the fact that different counterion concentrations and polymer concentrations are needed to observe several of the ribopolymer interactions. The extents of reaction and stabilities of the complexes are different for each polymer pair. If any significant reaction of TP-84 DNA with the ribopolymers was found, they have been recorded as positive. The analyses of the DNA–ribopolymer complexes were carried out under the conditions described in the Methods section, namely, 5.7 *m* CsCl pH 7, 25° 10 μg of ribopolymer/μg of denatured DNA and centrifuged to equilibrium.

The L-strand forms complexes with poly C and poly U, indicating the presence of nucleotide clusters of dG, and dA, respectively. The H strand interacts with poly G, poly (I,G) (suggesting dC sequences) and poly X suggesting dA and/or dT sequences. These reactions are in agreement with the chemical compositions of the separated strands. The pyrimidine ribopolymers form complexes with the purine-rich (L) strand and the purine ribopolymers complex the pyrimidine-rich (H) strand.

Normal base-pairing considerations indicate anomalous binding in some cases. Although poly X should complex with both DNA strands, it does not. Likewise, since poly U binds the L strand then poly A should complex with the complementary DNA strand but does not. The accessibility of certain nucleotide clusters to react with homoribopolymers may be a topological feature of the specific DNA strand or a function of the relative chemical affinities of the bases involved in the formation of the stable complex.

In summary, we can say that TP-84 DNA contains homogeneous nucleotide stretches or clusters on both strands which may have a marked effect on its secondary structure. This may be of significance in DNA strand selection by RNA polymerase *in vivo*. TP-84 presents another example of a positive correlation between DNA strands bearing deoxycytosine clusters and the ability to hybridize with phage-induced RNA.

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