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Synthesis and Biological Activity of a λ Pseudo Operator†

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ABSTRACT: The chemical and enzymatic syntheses of bacteriophage λ pseudo operator DNA are described. The 17 base-paired duplex contains the DNA which has been proposed as the binding site for cI repressor protein. The synthetic duplex is twofold symmetric and represents the best possible nucleotide summation of the six proposed operator sites in the leftward and rightward operators. However, it does not correspond exactly to any single proposed operator sequence. The chemical synthesis includes the deoxyoligonucleotides d(T-A-T-C-A-C), d(C-G-C-C-G-G-T-G-A-T-A), d(T-A-T-C-A-C-C), and d(G-G-C-G-G-T-G-A-T-A). These deoxyoligonucleotides were joined with T4 DNA ligase to form d(T-A-T-C-A-C-C-G-C-C-G-G-T-G-A-T-A) and d(T-A-T-C-A-C-C-G-G-C-G-G-T-G-A-T-A). The cI repressor protein was found to bind to the duplex formed from these two segments.

An important unsolved problem at the gene level of regulation is how various proteins interact with DNA control regions to regulate transcription. The major leftward and rightward control regions of bacteriophage λ could prove to be very useful for studying this problem. These regions are illustrated in the simplified drawing shown in Figure 1. The cI repressor binds at multiple sites (operators) within these control regions (Maniatis and Ptashne, 1973a,b). *Escherichia coli* RNA polymerase transcribes from the leftward promoter P_L toward gene N (Hershey, 1971) and in opposite directions from the rightward promoters P_R and P_{RM} toward cro and cI, respectively (Meyer et al., 1975). Accumulated evidence also indicates that sequences within the operators are recognized by *E. coli* RNA polymerase as part of the promoter sites (Maniatis et al., 1973; Maurer et al., 1974; Allet et al., 1974). Finally, the cro protein appears to bind to the same or similar sequences as does the cI repressor (Folkmanis et al., 1976). Therefore, within these closely linked control regions can be found three promoters and multiple operator sites. These interact with cI repressor, *E. coli* RNA polymerase, and cro protein to regulate the lysogenic and lytic responses of bacteriophage λ (Ptashne et al., 1976; Folkmanis et al., 1976). We would like to contribute toward an understanding of how these various functional units interact to attenuate and direct transcription. The first steps in this study—the chemical and enzymatic syntheses of a pseudo λ operator DNA and a demonstration of its biological activity—are presented in this paper.

Our initial objective is to define the cI repressor binding sites. The approach involves the chemical and enzymatic syntheses of λ control DNA. Although cI repressor binding sequences remain to be established, considerable evidence suggests that six partially symmetric regions in both leftward and rightward control DNA define the sites (Maniatis et al., 1975). Basic assumptions for research reported in this paper are that these partially symmetric sequences constitute the λ repressor binding sites and that the completely symmetric operator se-

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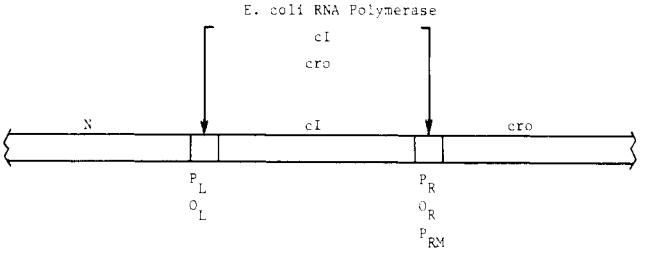

FIGURE 1: Bacteriophage λ major rightward and leftward control regions (over-simplified). The major leftward (P_L) and rightward (P_R) promoters, the cI gene promoter (P_{RM}) and the leftward (O_L) and rightward (O_R) operators are drawn so as to emphasize that these functional genetic units overlap as to DNA sequence. The interaction sites for cro protein, cI repressor, and *E. coli* RNA polymerase are shown in a similar manner. The initial gene products from the leftward promoter (N), the P_{RM} promoter (cI) and the rightward promoter (cro) are also shown.

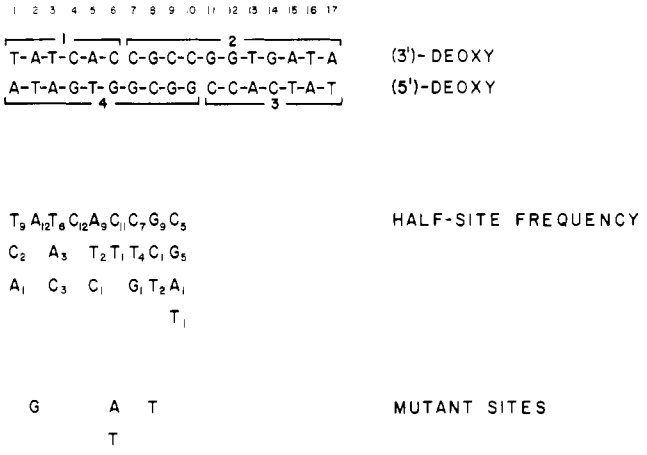

FIGURE 2: The plan for synthesis of λ pseudo operator DNA. The chemically synthesized deoxyribooligonucleotide segments are shown within the brackets, the serial number of the segment being inserted into the brackets.

quence will bind at least as tightly as any natural, partially symmetric sequences to cI repressor. The symmetric sequence, the pseudo operator, is shown in Figure 2. The choice of pseudo operator sequence is based on the most frequent base pair appearing at each position of the assumed 17 base pair λ repressor binding sites. This frequency has been derived (Maniatis et al., 1975; Ptashne et al., 1976) by defining one-half the proposed operator base pairs (nine base pairs) as a half-site and then summarizing the individual nucleotides read 5′ to 3′ for each of the 12 half-sites. This summation is shown in Figure 2 along with the derived pseudo operator.

The plan for chemical synthesis of λ pseudo operator as shown in Figure 2 is based on the following considerations. The approach chosen for the present work involved synthesis of four deoxyoligonucleotides ranging in size from 6 to 11 mononucleotides each. The brackets with enclosed numbers indicate segments that were chemically synthesized. These segments were then joined enzymatically with T4 DNA ligase (Khorana et al., 1972). This plan subdivides each heptadecanucleotide into two segments for chemical synthesis. As a consequence of this plan, several sites of interest can be readily modified. Among these sites are the three most highly conserved positions: 2, 4, and 6 (or 12, 14, and 16). Sequence conservation may be important for repressor binding since three of four mutations that decrease cI repressor binding (Maniatis et al., 1975; Kleid et al., 1976) have been sequenced as base changes at positions 2 and 6. Finally a careful inspection of the total leftward and rightward control DNA sequence (Ptashne et al., 1976) reveals that this pseudo operator can be easily converted to the O_L1 or O_R1 operators simply by changing base pairs within the region defined by segment 3. The plan outlined in Figure 2 isolates all of these sites in easily resynthesized small segments (1 and 3) and the 3′ ends of the larger segments (2 and 4). The 3′ ends of segments 2 and 4 can be readily altered utilizing intermediates from the original synthesis. Alternative synthetic plans including those where these potentially interesting sites are located near the 5′ ends of large segments are less flexible for sequence modification experiments.

Materials and Methods

Materials. Nucleotides were purchased from Papierwerke Waldhof-Aschaffenburg. Nucleosides and some nucleotides were purchased from Sigma Chemical. The protected mononucleotides *N*-benzoyldeoxyadenosine (dpbzA),[1] *N*-anisoyldeoxycytidine (dpanC), *N*-2-isobutyryldeoxyguanosine (dpibG), and their 5′-cyanoethylphosphoryl, 3′-*o*-acetyl, and 3′-*o*-isobutyryl derivatives were prepared as described elsewhere (Khorana et al., 1961; Büchi and Khorana, 1972). Di-, tri-, and other oligonucleotide blocks containing a 5′-phosphate were prepared as previously described (Weber and Khorana, 1972). The protected nucleosides, d(MeOTr)T and d(MeOTr)anC, were prepared as published previously (Schaller et al., 1963). Blocks containing *N*-isobutyryldeoxyguanosine were reisobutyrylated before use. MS and TPS were purchased from Aldrich Chemical Co. and recrystallized from anhydrous pentane before use. Pyridine (reagent grade, J. T. Baker Co.) was purified as described previously (Kössel et al., 1967). Diisopropylethylamine (Aldrich Chemical Co.) was distilled from toluenesulfonyl chloride before use. Triethylamine and hydracrylonitrile were purchased from Eastman and used without further purification. DCC was purchased from Aldrich. Bacterial alkaline phosphatase, snake venom phosphodiesterase, pancreatic deoxyribonuclease, spleen phosphodiesterase, and micrococcal deoxyribonuclease were purchased from Sigma or Worthington. T4 polynucleotide kinase (Panet et al., 1973) and T4 DNA ligase were prepared as described elsewhere (Yansura et al., 1977a). λ cI repressor was isolated from a bacterial strain carrying the repressor over-producing plasmid, pKB 252 (Backman et al., 1976). The repressor was generously provided by Mr. Carl Pabo and Professor Mark Ptashne. The cI repressor was approximately 15–20% active. [γ-^{32}P]ATP was prepared from carrier-free [^{32}P]orthophosphate (Glynn and Chappell, 1964). Materials such as cellulose acetate strips (Schleicher and Schuell), Cellulosepulver MN 300 HR and MN 300 DEAE-cellulose for thin-layer chromatography (Brinkman), crude yeast RNA (Gallard-Schlesinger), precoated silica gel plates (Eastman-6064), Eastman RP-royal blue X-OMAT x-ray film and Schleicher and Schuell nitrocellulose filters (BA 85) were purchased from commercial sources.

[1] Abbreviations used: TPS, triisopropylbenzenesulfonyl chloride; CE, cyanoethyl; TEAB, triethylammonium bicarbonate; MeOTr, monomethoxytrityl; ib, isobutyryl; an, *p*-anisoyl; bz, benzoyl; MS, mesitylenesulfonyl chloride; DCC, dicyclohexylcarbodiimide. The one letter symbols for nucleotides and the symbols for polynucleotides are according to the IUPAC-IUB Commission on Biochemical Nomenclature Recommendations (1970), *Biochemistry 9*, 4022. Hyphenated numbers within brackets represent chemically synthesized deoxyoligonucleotides as defined in Figure 2 that have been enzymatically joined. The numbers left to right within brackets refer to the DNA sequence 5′ to 3′. The actual sequence of each deoxyoligonucleotide defined by the numbers is given in Figure 2. The symbol [/] is used to indicate that the segments within brackets form a base paired duplex. The symbol B refers to 5-bromodeoxyuridine.

TABLE I: Summary of Reaction Conditions, Recoveries, and Yields for Segments 1 and 3.

Starting chain (3′-OH component)	Amount in mmol (*A*)	Oligonucleotide block (5′-phosphate end)	Amount in mmol (*B*)	(*B*/*A*)	TPS[a] (eq/P)	Reaction time (h)	Block recovered[b] (%)	Product	Yield (%)
d(MeOTr)T	2.95	d[pbzA-T(Ac)]	1.85	0.63	1.0	7.5	19	d(MeOTr)T-bzA-T	51
d(MeOTr)T-bzA-T	0.70	d[panC-bzA(Ac)]	2.7	3.9	1.0	6.0	60	d(MeOTr)T-bzA-T-anC-bzA	30
d(MeOTr)T-bzA-T-anC-bzA	0.07	d[panC(Ac)]	1.1	15	1.0	5.0		d(MeOTr)T-bzA-T-anC-bzA-anC	38
d(MeOTr)T-bzA-T-anC-bzA	0.07	d[panC-anC(Ac)]	0.44	6.4	1.0	5.0	35	d(MeOTr)T-bzA-T-anC-bzA-anC-anC	60

[a] Equivalents per phosphate group dissociation in the two components. [b] % of the original amount for further condensation without additional purification.

Methods. General methods used in this laboratory for the chemical synthesis of oligodeoxynucleotides (Goeddel et al., 1977) and enzymatic joining of these chemically synthesized segments (Yansura et al., 1977a) have been outlined previously. The basic procedure for chemical synthesis involved condensation of appropriately protected oligodeoxynucleotides using TPS in anhydrous pyridine (Khorana, 1968). Fractionation was on DEAE-cellulose using gradients of TEAB and ethanol (Khorana et al., 1972). Phosphorylation of deoxyoligonucleotides containing 5′-hydroxyl groups was carried out as described previously (Sgaramella and Khorana, 1972; van de Sande et al., 1972). Characterization by degradation of enzymatically joined segments to 3′-mononucleotides with spleen phosphodiesterase and micrococcal deoxyribonuclease and to 5′-mononucleotides with snake venom phosphodiesterase and pancreatic deoxyribonuclease was according to published procedures (Sgaramella and Khorana, 1972). Base sequence characterization of chemically synthesized segments was by the two-dimensional sequencing technique (Sanger et al., 1973). Gel electrophoresis of deoxyoligonucleotides was completed according to a published procedure (Maniatis et al., 1974).

The binding assay was similar to a published procedure (Chadwick et al., 1970). 5′-^{32}P-labeled λ pseudo operator [1-2/3-4] at 8.9×10^{-2} mM in 5 mM $MgCl_2$ and 20 mM Tris-HCl (pH 7.6) was warmed in a boiling water bath for 3 min and cooled slowly (1 h) to 0 °C. The λ pseudo operator duplex [1-2/3-4] was used directly following column chromatography to remove single strands. The binding mixture (2 mL) contained 10 mM Tris-HCl (pH 7.0), 50 mM KCl, 1 mM $CaCl_2$, 0.1 mM EDTA, 5% Me_2SO, 100 μg/mL salmon sperm DNA, 100 μg/mL bovine serum albumin, and 5′-^{32}P-labeled λ pseudo operator at various concentrations. Each sample was divided into eight 250-μL aliquots and various amounts of repressor were added to each aliquot. After incubation for 1 h at 0 °C, the aliquots were filtered through Schleicher and Schuell nitrocellulose filters, washed once with 0.2 mL of wash buffer, dried, and counted. The composition of wash buffer was 10 mM Tris-HCl (pH 7.0), 50 mM KCl, 1 mM $CaCl_2$, 0.1 mM EDTA, and 5% Me_2SO.

Results

Chemical Synthesis. The plan outlined in Figure 2 required the synthesis of four deoxyoligonucleotides ranging in size from hexanucleotide to undecanucleotide. These syntheses are summarized in Tables I and II. Segments 1 and 3 were prepared from the same pentanucleotide. The first synthetic step was condensation of the nucleoside, d(MeOTr)T, with the dinucleotide d[pbzA-T(Ac)] using TPS as a condensing agent. The product was isolated free of starting materials using a partition method as described previously (Caruthers and Khorana, 1972). The yield (51%) was approximately the same as that observed for many earlier nucleoside plus dinucleotide condensations (van de Sande et al., 1976; Minamoto et al., 1976). The successful use of the partition method was a pleasant surprise because two of three bases in the product are thymine. This pyrimidine contains no hydrophobic blocking groups which would help partition the trinucleotide with two phosphate anions into the organic phase. The next condensation was d[(MeOTr)T-bzA-T] with an excess of the dinucleotide d[panC-bzA(Ac)]. The yield of homogeneous pentanucleotide following purification by anion-exchange chromatography was low (30%). However, the high recovery of dinucleotide (60%) indicated that the low yield probably was due to a poor condensation rather than excessive degradation of the synthesized products. To complete the synthesis of segment 1, the pentanucleotide was condensed with the mononucleotide d[panC(Ac)]. In a similar fashion, the pentanucleotide was condensed with d[panC-anC(Ac)] to form segment 3. Both syntheses yielded essentially homogeneous products after chromatography on DEAE-cellulose.

Syntheses of segments 2 and 4 are summarized in Table II. The first step in the synthesis of segment 2 was condensation of the nucleoside, d(MeOTr)anC, with the dinucleotide d[pibG-anC(ib)]. Again the trinucleotide was isolated by a partition method (52% yield). The synthesis then involved in successive steps the condensation of the dinucleotides d[panC-ibG(ib)] and d[pibG-T(Ac)]. The final condensation utilized the tetranucleotide d[pibG-bzA-T-bzA(ib)]. Isolated yields were comparable to previous results using this synthetic approach. Yields reported here do not include rather significant amounts (10–15%) of product present in impure fractions. Segment 4 was a decanucleotide containing 50% deoxyguanylic acid. As demonstrated by previous syntheses (Jay et al., 1976), guanine rich sequences are difficult to prepare. The condensations usually proceed to a lower extent than with other nucleotides. Furthermore, the isobutyryl blocking group is somewhat unstable to purification procedures involving TEAB on DEAE-cellulose (Jay et al., 1976). In order to minimize the latter problem, dinucleotide blocks which were used to synthesize this sequence were reisobutyrylated immediately before use. Furthermore, both the dinucleotide and tetranucleotide were isolated by an extraction procedure (Caruthers and Khorana, 1972). Therefore this synthesis scheme did not require a TEAB gradient separation on DEAE-cellulose until the hexanucleotide purification step. As a result, the completed product was exposed to these mild deisobutyrylation procedures only three times. As can be seen in Table II, yields for

TABLE II: Summary of Reaction Conditions, Recoveries, and Yields for Segments 2 and 4.

Starting chain (3′-OH component)	Amount in mmol (*A*)	Oligonucleotide block (5′-phosphate end)	Amount in mmol (*B*)	(*B*/*A*)	TPS[a] (eq/P)	Reaction time (h)	Block recovered[b] (%)	Product	Yield (%)
Segment 2									
d(MeOTr)anC	3.80	d[pibG-anC(ib)]	2.4	0.63	1.0	5.5		d(MeOTr)anC-ibG-anC	52
d(MeOTr)anC-ibG-anC	0.64	d[panC-ibG(ib)]	1.7	2.7	1.0	5.0	39	d(MeOTr)anC-ibG-anC-anC-ibG	38
d(MeOTr)anC-ibG-anC-anC-ibG	0.24	d[pibG-T(ib)]	2.6	10.5	1.0	5.0	44	d(MeOTr)anC-ibG-anC-anC-ibG-ibG-T	38
dCEpibG-bzA	1.88	d[pT-bzA(Ac)]	2.7	1.4	1.0	5.0		dpibG-bzA-T-bzA	32
d(MeOTr)anC-ibG-anC-anC-ibG-ibG-T	0.09	d[pibG-bzA-T-bzA(ib)]	0.6	6.7	1.0	5.0	43	d(MeOTr)anC-ibG-anC-anC-ibG-ibG-T-ibG-bzA-T-bzA	13
Segment 4									
d(MeOTr)ibG	2.5	d[pibG(ib)]	3.6	1.4	1.7[c]	120		d(MeOTr)ibG-ibG	80
d(MeOTr)ibG-ibG	2.0	d[panC-ibG(ib)]	2.8	1.4	1.0	5.45	30	d(MeOTr)ibG-ibG-anC-ibG	40
d(MeOTr)ibG-ibG-anC-ibG	0.8	d[pibG-T(ib)]	1.8	2.2	1.0	5.4	48	d(MeOTr)ibG-ibG-anC-ibG-ibG-T	22
d(MeOTr)ibG-ibG-anC-ibG-ibG-T	0.17	d[ibG-bzA(ib)]	0.9	5.1	1.0	5.5		d(MeOTr)ibG-ibG-anC-ibG-ibG-T-ibG-bzA	19
d(MeOTr)ibG-ibG-anC-ibG-ibG-T-ibG-bzA	0.004	d[pT-bzA(Ac)]	0.04	10	1.0	6.0		d(G-G-C-G-G-T-G-A-T-A)[d]	40

[a] Equivalents per phosphate group dissociation in the two components. [b] % of the original amount for further condensation without additional purification. [c] The dinucleotide utilized DCC rather than TPS as condensing agent. [d] The decanucleotide was isolated in the completely deprotected form.

the individual steps ranged from 19 to 40% for dinucleotide additions to the growing segment. As before, these yields reflect only homogeneous product as analyzed by paper chromatography and do not include fractions contaminated with impurities. Because only 4 μmol of the octanucleotide was committed to the final condensation and hydrolysis with aqueous pyridine, the product was isolated by a modified procedure. The reaction mixture was first treated with concentrated ammonium hydroxide and then 80% acetic acid to remove blocking groups. The completely deprotected decanucleotide was isolated by (1) passage through a Sephadex G-50-80 column to remove sulfonic acid and the dinucleotide block and (2) anion-exchange chromatography on DEAE-cellulose in 7 M urea. The isolated yield was 40%.

The synthetic intermediates and final products were characterized by a variety of techniques. During the synthesis, each intermediate was monitored by paper or thin-layer chromatography at the fully protected stage, after removal of base labile protecting groups and after removal of all protecting groups. Only those fractions that appeared homogeneous by these criteria were used for the next synthetic step. Following completion of the synthesis, each segment was purified at the completely deprotected stage by column chromatography on DEAE-cellulose packed in 7 M urea. Elution was with a sodium chloride gradient (Khorana et al., 1972). For each sample, only one major, sharp peak was obtained. The 5′ ends of the synthetic segments were then phosphorylated using [γ-^{32}P]ATP and polynucleotide kinase. These 5′-^{32}P segments were characterized by polyacrylamide gel electrophoresis, homochromatography on DEAE-cellulose, and analysis of the nucleotide sequence by two-dimensional techniques. The two-dimensional sequence analyses for segments 1, 2, 3, and 4 are shown in Figure 3. All compounds displayed a major set of spots that confirmed the nucleotide sequence of the synthetic products. Only trace amounts of unidentified compounds could be detected after prolonged exposure of the x-ray film.

Analysis by gel electrophoresis is shown in Figure 4. Channels e, f, and j contained segments 2, 4, and 1, respectively. Channel b contained segment [3-4] and channel c contained a mixture of segments [1-2] and 2. Other channels contained markers unrelated to the present work. The sequences for these markers are listed in the legend to Figure 4. All these deoxyoligonucleotides were essentially homogeneous and form very sharp, well-defined bands. The second, faint band observed in channel b was at the position expected for the λ duplex. Therefore this band probably represents a duplex of segment [3-4]. Such a duplex is structure a as drawn in Figure 5. This duplex derived from segment [3-4] would have a noncomplementary guanosine-guanosine interaction at position 9. Of particular interest were the relative mobilities of various segments. Observe that segments [3-4] and [1-2] in channels b and c had much greater mobility than the corresponding heptadecanucleotide marker in channel d. The sequences of segments [1-2] and [3-4] have been confirmed by two-dimensional sequence analysis (data not shown) so there was no question as to chain length. A similar variation in mobility was observed when segment 4 in channel f and [5′-^{32}P]d(pC-G-C-C-A-C-A-C-G-C) in channel g were compared. Both were decanucleotides but had different mobilities. It is possible that these mobility differences for small segments are a function of sequence. However, these observations cannot be explained without further studies on a large library of sequence defined deoxyoligonucleotides.

Enzymatic Synthesis. The T4 DNA ligase catalyzed joining of appropriate chemically synthesized segments was the second phase of the λ pseudo operator preparation. Segment [1-2] was prepared by joining 5′-^{32}P-labeled segment 2 to segment 1 (Figure 6). Segment 4 was the template for this joining reaction. Initial experiments indicated that this system was relatively insensitive to $MgCl_2$ and ATP concentration. However, the reaction was very temperature sensitive with 0 °C being superior to higher temperatures. A preparative scale synthesis

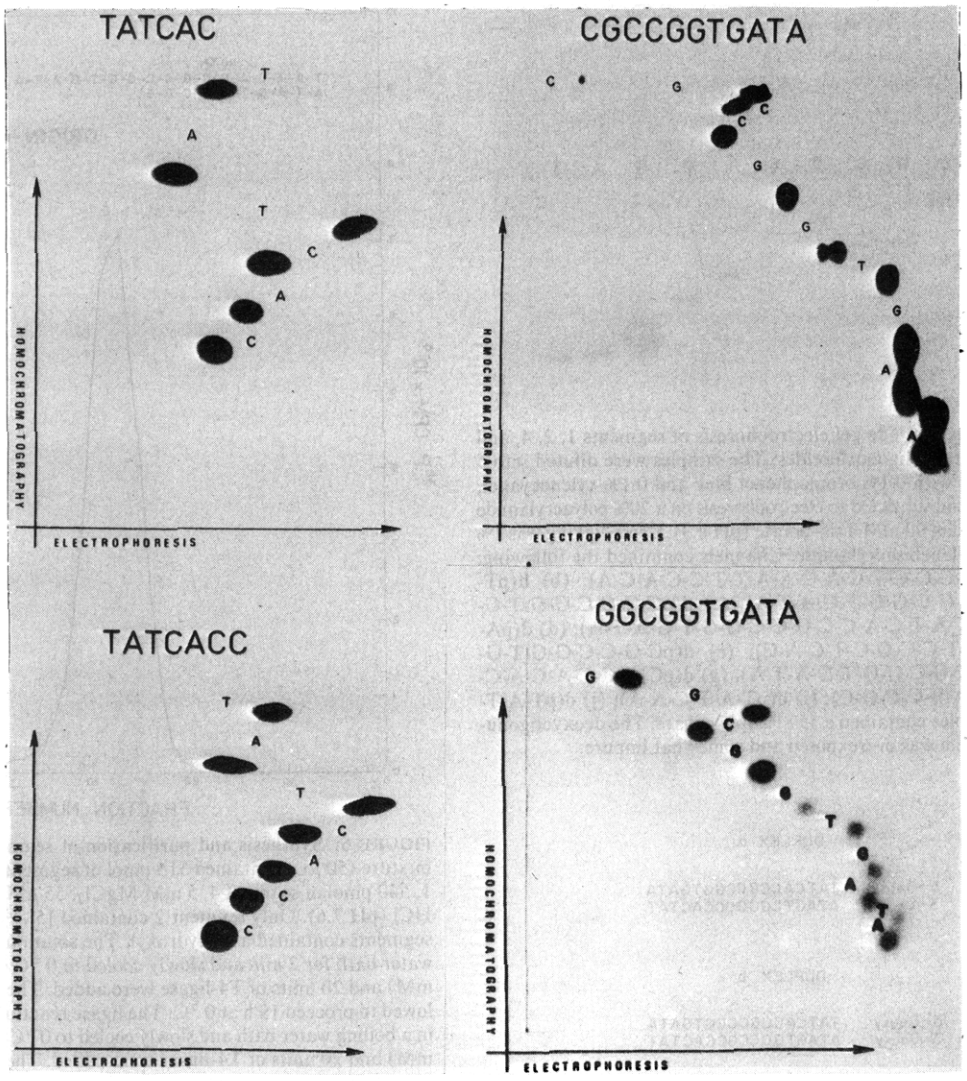

FIGURE 3: Two-dimensional analysis of segments d(T-A-T-C-A-C), d(T-A-T-C-A-C-C), d(C-G-C-C-G-G-T-G-A-T-A), and d(G-G-C-G-G-T-G-A-T-A). Each segment contained [5′-^{32}P]phosphate. Electrophoresis was along the longitudinal axis and homochromatography was along the vertical axis. Nucleotide losses are recorded between the appropriate spots.

TABLE III: Characterization of Synthetic Polynucleotides.

Segments analyzed[a]	3′-Nucleotide analysis (cpm)				5′-Nucleotide analysis (cpm)			
	dAp	dGp	dTp	dCp	pdA	pdG	pdT	pdC
[1–2]	120	130	240	17000	170	150	120	14700
[3–4]	100	75	75	7800	570	28700	580	130
[1–2]/[3–4]	20	90	30	3000	50	7700	60	7100 (1.09)[b]

[a] All radioactive [^{32}P]phosphate was within the duplex [1–2]/[3–4] or the single strands as analyzed by resistance to alkaline phosphatase. [b] The number in parentheses is the experimental molar ratio of d^{32}pG to d^{32}pC.

of [1–2] is described in Figure 6. Approximately 49 h was required for the reaction to go to completion (84% isolated yield). The major peak was characterized as [1–2]. The figure inset shows the gel electrophoresis pattern. The sample appeared homogeneous both before (channel b) and after (channel a) phosphorylation of the 5′-hydroxyl. Therefore the isolation procedure as outlined in Figure 6, including the heat denaturation step before column chromatography, successfully fractionated segment [1–2] from the template, segment 4. If the separation had been incomplete, a second band corresponding to 5′-^{32}P-labeled segment 4 would have been observed after kinasing the isolated product. Of interest is the relative mobility of 5′-^{32}P-labeled segment [1–2] and segment [1–2] containing a 5′-hydroxyl. The 5′-phosphorylated segment has reduced electrophoretic mobility. Characterization of segment [1–2] by degradation to 3′-mononucleotides transferred all radioactivity to dCp, whereas analysis by degradation to 5′-mononucleotides showed pdC as the only labeled nucleotide. These data are presented in Table III.

Segment [3–4] was synthesized by the reaction scheme shown in Figure 7. 5′-^{32}P-labeled segment 4 was joined to segment 3 using segment 2 as template. The procedure for this synthesis is outlined in the legend to Figure 7. As with segment [1–2], a very good yield was observed based upon phosphatase resistance (88%). The elution profile for this preparation is shown in the same figure. The major peak isolated by column

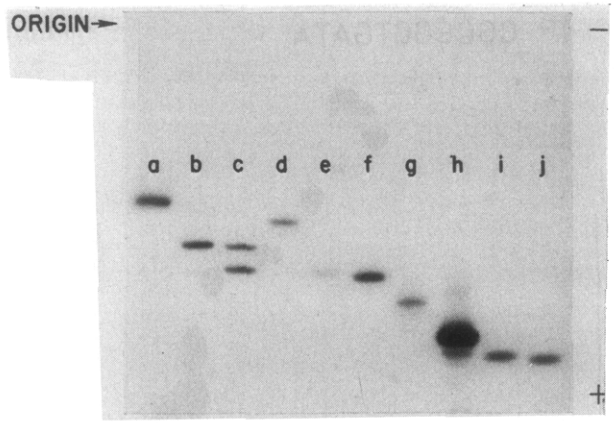

FIGURE 4: Polyacrylamide gel electrophoresis of segments 1, 2, 4, and several marker deoxyoligonucleotides. The samples were diluted into 5 μL of formamide with 0.1% bromophenol blue and 0.1% xylenecyanol, heated to 70 °C, and subjected to electrophoresis on a 20% polyacrylamide gel in 7 M urea using 90 mM Tris–borate (pH 8.3). Cross-linking was 1% with *N,N'*-methylenebisacrylamide. Channels contained the following: (a) d(pT-A-T-C-C-G-C-T-C-A-C-A-A-T-T-C-C-A-C-A); (b) d(pT-A-T-C-A-C-C-G-G-C-G-G-T-G-A-T-A); (c) d(pC-G-C-C-G-G-T-G-A-T-A) and d(pT-A-T-C-A-C-C-G-C-C-G-G-T-G-A-T-A); (d) d(pA-A-T-T-G-T-T-A-T-C-C-G-C-T-C-A-C); (e) d(pC-G-C-C-G-G-T-G-A-T-A); (f) d(pG-G-C-G-G-T-G-A-T-A); (g) d(pC-G-C-C-A-C-A-C-G-C); (h) d(pT-A-B-C-C-G-C); (i) d(pT-A-T-C-A-A); (j) d(pT-A-T-C-A-C). All samples contained a [5'-^{32}P]phosphate. The deoxyoligonucleotide in sample h was overexposed and somewhat impure.

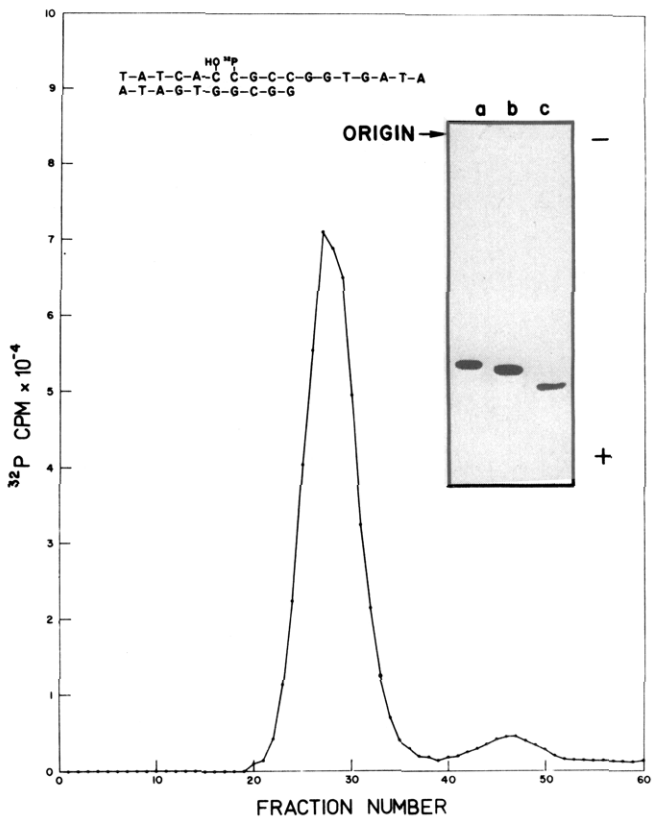

FIGURE 6: Synthesis and purification of segment [1–2]. The reaction mixture (50 μL) contained 515 pmol of segment 2, 930 pmol of segment 1, 540 pmol of segment 4, 5 mM $MgCl_2$, 55 μM ATP, and 20 mM Tris-HCl (pH 7.6). Only segment 2 contained [5'-^{32}P]phosphate. The other segments contained a 5'-hydroxyl. The solution was heated in a boiling water bath for 2 min and slowly cooled to 0 °C (1 h). Dithiothreitol (10 mM) and 20 units of T4-ligase were added. The joining reaction was allowed to proceed 19 h at 0 °C. The ligase reaction was then heated again in a boiling water bath and slowly cooled to 0 °C (1 h). Dithiothreitol (10 mM) and 20 units of T4-ligase were added. The reaction was continued an additional 20 h and then terminated by heating in a boiling water bath for 5 min. The reaction mixture was applied to a Sephadex G-75-40 column (0.8 × 110 cm) and the column eluted with 0.01 M TEAB at room temperature. Fractions of 8 drops were collected and analyzed for radioactivity. The inset shows an analysis of the major peak by polyacrylamide gel electrophoresis. The gel was prepared as outlined in the legend to Figure 4. Channels contained the following: (a) 5'-^{32}P-labeled deoxyoligonucleotide from the major peak; (b) the deoxyoligonucleotide from the major peak; (c) 5'-^{32}P segment 2.

DUPLEX a

5'-Deoxy TATCACCGGCGGTGATA
3'-Deoxy ATAGTGGCGGCCACTAT

DUPLEX b

5'-Deoxy TATCACCGCCGGTGATA
3'-Deoxy ATAGTGGCCGCCACTAT

DUPLEX c

5'-Deoxy TATCAC^{CG}
3'-Deoxy ATAGTG$_{GC}^{C}$

DUPLEX d

5'-Deoxy TATCAC^{CG}
3'-Deoxy ATAGTG$_{GC}^{G}$

FIGURE 5: Mismatched duplexes and hairpin loop duplexes that can be generated from the λ pseudo operator segments.

chromatography was characterized as segment [3–4]. The isolated yield was 73%. The minor peak eluted early from the column was probably the duplex formed from two strands of segment [3–4]. However, this peak was not characterized. The inset shows the gel electrophoresis pattern for the major peak before (channel a) and after (channel c) phosphorylation with [γ-^{32}P]ATP using T4-kinase. The marker was 5'-^{32}P-labeled segment [1–2] in channel b. Therefore this sample was free of segment 2 which was used as a template for the joining reaction. The 5'- and 3'-mononucleotide characterization is shown in Table III. Digestion to 5'-mononucleotides showed pdG as the only labeled nucleotide. Digestion to 3'-mononucleotides indicated that all the [^{32}P]phosphate had been transferred to dCp. These data were consistent with results expected for segment [3–4]. Finally as shown previously in relation to the data displayed in Figure 3, segments [1–2] and [3–4] have much less mobility than segments 2 and 4 as expected for heptadecanucleotides.

cI Repressor–Pseudo Operator Binding Studies. The first step for testing the λ pseudo operator for biological activity was preparation of the duplex (Figure 8). Segments [1–2] and [3–4] were annealed to form duplex [1–2]/[3–4] by the procedure outlined in the legend to Figure 8. The duplex peak was characterized by degradation to 3'- and 5'-mononucleotides. Degradation to 5'-mononucleotides gave [^{32}P]pdG and [^{32}P]pdC with a mole ratio of 1.09. 3'-Mononucleotide analysis indicated all radioactivity had been transferred to dCp. These results are all consistent with those expected for duplex [1–2]/[3–4].

In order to investigate the binding of cI repressor to the pseudo operator, the duplex [1–2]/[3–4] was phosphorylated to high specific activity using [γ-^{32}P]ATP and T4-kinase (Lillehaug et al., 1976). This procedure kinases the 5'-hydroxyl of each segment with a [5'-^{32}P]phosphate. The resulting deoxyoligonucleotides with specific activity of 680 Ci/mmol duplex were annealed and the duplex was fractionated from

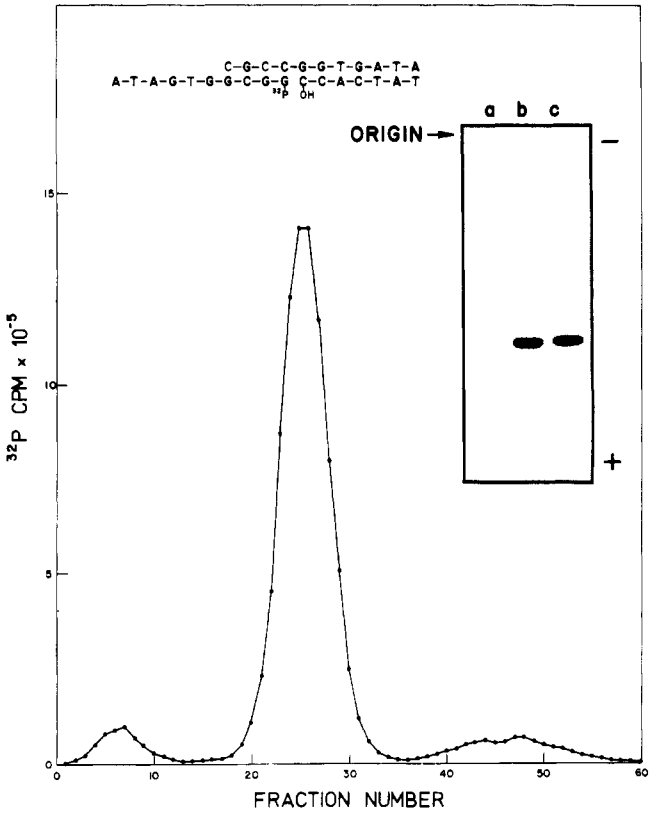

FIGURE 7: Synthesis and purification of segment [3-4]. The reaction mixture (100 μL) contained 1530 pmol of segment 4, 2800 pmol of segment 3, 1560 pmol of segment 2, 5 mM $MgCl_2$, 55 μM ATP, and 20 mM Tris-HCl (pH 7.6). Only segment 4 contained [5′-^{32}P]phosphate. The other segments contained a 5′-hydroxyl. The solution was heated in a boiling water bath for 2 min and slowly cooled to 0 °C (1 h). Dithiothreitol (10 mM) and 30 units of T4-ligase were added. The joining reaction was allowed to proceed for 19.5 h and then terminated by heating in a boiling water bath for 5 min. Phosphatase resistance indicated an 88% yield of the expected product. The reaction mixture was applied to a Sephadex G-75-40 column (0.8 × 110 cm) and the column eluted with 0.01 M TEAB at room temperature. Fractions of 8 drops were collected and analyzed for radioactivity. The inset shows an analysis of the major peak by polyacrylamide gel electrophoresis. The gel was prepared as outlined in the legend to Figure 4. Channels contained the following: (a) deoxyoligonucleotide from the major peak; (b) 5′-^{32}P segment [1-2]; (c) 5′-^{32}P-labeled deoxyoligonucleotide from the major peak.

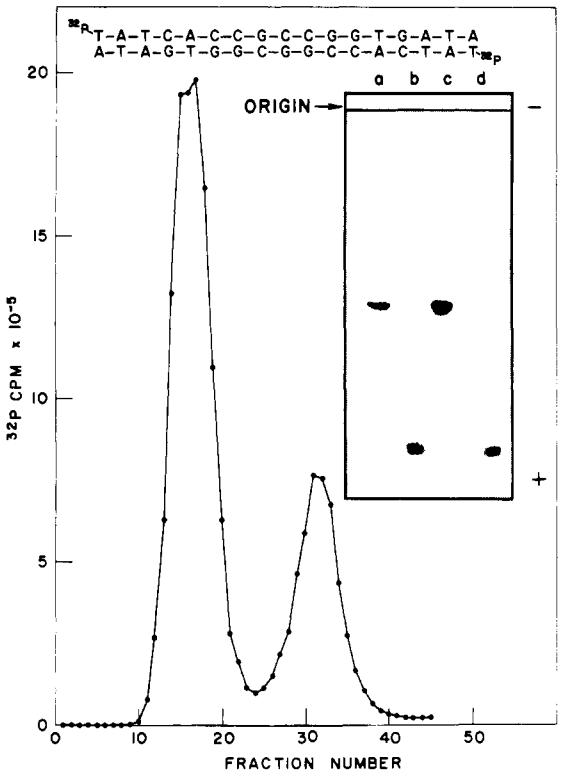

FIGURE 8: Experiments on the stability of duplex [1-2]/[3-4]. The 5′-^{32}P segment [1-2] and 5′-^{32}P segment [3-4], at 5 μM each, 5 mM $MgCl_2$, and 20 mM Tris-HCl (pH 7.6) were heated in a boiling water bath for 2 min at 100 °C and cooled slowly to 0 °C (1.5 h). The annealed duplex was diluted tenfold with cold 10 mM TEAB, loaded on a G-75-40 column (0.8 × 120 cm) preequilibrated in 10 mM TEAB at 0 °C, and fractionated from single strand segments using 10 mM TEAB as eluent. Fractions of 8 drops were collected, analyzed for radioactivity, adjusted to 5 mM $MgCl_2$, and examined by polyacrylamide gel electrophoresis. The inset displays the gel electrophoresis results. Samples from each fraction (2 × 10^{-9} M) in 0.09 M Tris-borate (pH 8.3), 5 mM $MgCl_2$, 0.1% bromophenol blue, and 0.1% xylenecyanol were loaded on the gel. Channels a and c display the electrophoresis pattern obtained from fractions 14 and 15, respectively. Samples from fractions 14 and 15 (2 × 10^{-9} M) in the same buffer were heated in a boiling water bath for 2 min, cooled quickly with ice, and loaded on the gel. Channels b and d display the respective electrophoresis patterns obtained after heat denaturation. Other fractions from the main peak displayed identical results (data not shown). The buffer for polyacrylamide gel electrophoresis was 90 mM Tris-borate (pH 8.3) and 5 mM $MgCl_2$. Cross-linking was 1% with *N,N′*-methylenebisacrylamide.

single strands. This procedure and the resulting fractionation pattern are shown in Figure 8. The major peak was the 5′-^{32}P-labeled pseudo operator. This duplex appeared quite stable when stored in 5 mM $MgCl_2$ at 0 °C without lyophilization. The gel electrophoresis pattern shown in the inset to Figure 8 displays the results of an experiment that tests the stability of the pseudo operator duplex. Channels a and c show the electrophoretic mobility for two aliquots of pseudo operator duplex. The same gel pattern was observed after several weeks storage without freezing. A heat and quick chill cycle completely destroyed the duplex structure. These data are displayed in channels b and d. Observe that the pseudo operator strands now have increased mobility. The mobility was now identical with that of segment [1-2] or [3-4].

The λ pseudo operator forms a complex with λ cI repressor. The assay was filtration of samples through nitrocellulose filters. The complex was retained on the filter whereas the uncomplexed λ DNA passes through the filter. The results are shown in Figure 9. As increasing amounts of repressor were added to a constant amount of operator, a plateau was observed. At saturation no additional pseudo operator formed a complex with cI repressor even at very large excesses of repressor. For all these experiments, 13-15% of the λ pseudo operator was retained on the filter at saturation. The specificity of the complex was also investigated. When the pseudo operator was heat denatured before testing with repressor, only 1 to 7% of the maximum counts retained at saturation were observed to form a complex with cI repressor. The pseudo operator was also challenged with unlabeled λ DNA. In the presence of a 10.6-fold molar excess of whole, unlabeled λ DNA, essentially no radioactivity was retained by the filters. The calculation of excess λ DNA was based on the molecular weight of total λ DNA rather than the number of proposed cI repressor binding sites (Maniatis et al., 1975).

Discussion

The λ pseudo operator duplex, prepared by a combination of chemical and enzymatic procedures, was biologically active. This was shown by the formation of a pseudo operator–cI repressor complex that could be effectively inhibited by a large excess of λ DNA. The heat denaturation experiments sug-

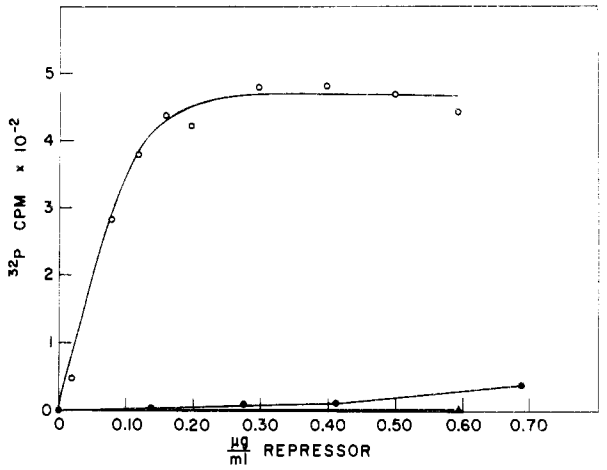

FIGURE 9: Binding studies with λ pseudo operator DNA and cI repressor. The pseudo operator was the sample whose preparation is described in the legend to Figure 8. The sample was completely in the duplex form. The binding to cI repressor at 9.0×10^{-11} M pseudo operator was examined in the absence (○) or presence (●) of a 10.6-fold molar excess of unlabeled λ DNA. Pseudo-operator at 9×10^{-10} M was heated to 100 °C for 2 min and quick chilled. Binding to repressor was then tested at a repressor concentration of 0.59 μg/mL (▲).

gested that only the duplex form of the pseudo operator was recognized by cI repressor and that single-stranded or hairpin structures shown in Figure 5 will not bind repressor. This result was consistent with previous conclusions that cI repressor recognizes duplex DNA rather than a hairpin type DNA structure (Maniatis et al., 1975). Partially symmetric sequences within the major rightward and leftward control regions of bacteriophage λ have been proposed as the cI repressor binding sites (Maniatis et al., 1975). The binding studies reported here strongly support this hypothesis. The pseudo operator sequence was derived from these partially symmetric sequences. However, our research so far does not tell us whether this 17 base-pair duplex represents the total cI repressor binding site. The pseudo operator duplex used for these studies might have been a mixture of several duplexes. One would be the pseudo operator prepared from segments [1-2] and [3-4]. Two other operator duplexes are possible. These are shown in Figure 5 as duplexes a and b and are formed from either two [1-2] or two [3-4] segments. The 5'-mononucleotide analysis of the pseudo operator, however, suggested that duplex [1-2]/[3-4] is the major if not exclusive sample present. Results reported here do not unequivocably eliminate the possibility that duplexes prepared from only segment [1-2] or segment [3-4] contribute toward repressor binding. However, the base ratios of radioactive pdG to pdC suggest that this is a highly unlikely possibility since equivalent amounts of these duplexes would have to be present (duplexes a and b, Figure 5) in order to obtain the correct nearest neighbor analysis. Attempts to reanneal and isolate the duplexes 1-2/1-2 and 3-4/3-4 under conditions identical with those used to attain the 1-2/3-4 duplex have given no 1-2/1-2 duplex and less than 10% 3-4/3-4 duplex (Kawashima and Caruthers, unpublished results). Future experiments will involve half-life studies of the pseudo operator in competition with natural bacteriophage λ for cI repressor DNA. These experiments should tell whether the pseudo operator binds repressor more tightly than any natural sequence. Presently the synthesis of the proposed O_L1 site and the mutant operator V003 are being completed (Drotar and Caruthers). A direct comparison of the pseudo operator with the O_L1 operator site and a weak binding operator mutant will then be possible. Finally, cro protein is also a likely candidate for binding studies with this pseudo operator (Folkmanis et al., 1976). We will want to investigate this system as well.

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Electrophoresis of DNA in Agarose Gels. Optimizing Separations of Conformational Isomers of Double- and Single-Stranded DNAs†

Paul H. Johnson* and Lawrence I. Grossman

ABSTRACT: The electrophoretic behavior of closed circular, nicked circular, and linear duplex forms of bacteriophages ϕX174 and PM2, plasmid ColEl, and rat mitochondrial DNAs has been analyzed by agarose gel electrophoresis as a function of gel concentration, electric-field strength, and ionic conditions. The logarithm of electrophoretic mobility (μ) is a linear function of gel concentration (T) at all agarose concentrations and field strengths tested. The retardation coefficient ($d\mu/dT \equiv K_R$) is characteristic of DNA conformation under controlled conditions. Alterations in electric-field strength and ionic conditions lead to predictable changes in the relative migration rates of the various DNA forms, thus defining conditions under which their separation can be optimized. The alkaline titration of a mixture of duplex conformational isomers was carried out on gels at neutral pH. Under conditions where closed circular DNA is stable to denaturation, separation of the linear and circular complementary strands of ϕX174, ColEl, and PM2 was observed. Electrophoresis of closed circular DNA in the presence of the intercalating dye ethidium bromide provided an estimate of superhelix density for PM2 DNA in good agreement with values determined by other methods. In addition, the use of ethidium bromide offers additional control over conditions for optimizing separations of DNA conformational forms. At ethidium bromide concentrations above that necessary to remove all negative superhelical turns, the relative change in the electrophoretic mobility with increasing dye concentration appears to be distinguishably different for the closed circular, nicked circular, and linear duplex forms of PM2 DNA. The combined results of this investigation define several experimental conditions and strategies for the application of agarose gel electrophoresis to studies of DNA structure and function.

Agarose gel electrophoresis is a technique of high resolving power for the analysis of DNA structure and function. The electrophoretic migration rate of a macromolecule moving through a gel matrix under the influence of an applied electric field is dependent on molecular size, conformation, and net charge. This technique has found important application in nucleic acid research for the separation and molecular weight determination of specific double- and single-stranded DNA fragments and in the analysis of DNA conformation (Thorne, 1966; Hayward, 1972; Hayward and Smith, 1972; Aaij and Borst, 1972; Helling et al., 1974; Tibbets et al., 1974; Flint et al., 1975; Keller, 1975; Pulleyblank et al., 1975; Depew and Wang, 1975; Bailey and Davidson, 1976; DeLeys and Jackson, 1976; Espejo and Lebowitz, 1976; Meyers et al., 1976; Shure and Vinograd, 1976).

In the present investigation, we have examined the effects of electric-field strength, gel concentration, and ionic conditions on the electrophoretic mobility of various molecular weight and conformational forms of DNA. The results define a set of generalizations which may be used to improve conditions for (a) separation of the major conformational isomers of duplex DNA molecules in the molecular weight range of

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