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Studies on Gene Control Regions. 2. Enzymatic Joining of Chemically Synthesized Lactose Operator Deoxyribonucleic Acid Segments†

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ABSTRACT: The T4 polynucleotide ligase catalyzed joining of six chemically synthesized deoxypolynucleotides corresponding to lactose operator DNA has been investigated. Joining was studied using various combinations of segments. Joining reactions involving multiple sites and the formation of duplex operator DNA were complete in a few hours. Joining reactions involving a single site and the formation of only one strand of operator DNA required several days and repeated annealing in order to go to completion. These studies have permitted the synthesis on a preparative scale (several nanomoles) of operator duplexes and operator single strands.

For investigations on how *lac* operator interacts with *lac* repressor, the DNA that binds repressor tightly has been chemically synthesized (Goeddel et al., 1977). The plan involved the chemical synthesis of seven deoxyoligonucleotides ranging in size from a pentanucleotide to a dodecanucleotide. The present paper describes investigations on the enzymatic joining of these segments or partial segments in various combinations to form polydeoxyoligonucleotides corresponding to *lac* operator DNA.

The plan for synthesis of *lac* operator DNA[1] is shown in Figure 1. The rationale for this particular plan is outlined in the previous paper (Goeddel et al., 1977). The purpose of the work presented in this paper was to investigate possible pathways for preparing operator DNA. Previous work had indicated that in general each ligase joining reaction must be examined individually (Khorana et al., 1972, 1976; Harvey et al., 1975) in order to maximize yield. For reasons which are not yet well defined, some ligations go better than others. Furthermore one set of reaction conditions is usually not satisfactory for all ligase joining reactions. Therefore of major

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[1] Abbreviations used: DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; hyphenated numbers within brackets represent chemically synthesized deoxyoligonucleotides as defined in Figure 1 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 1. The symbol [/] is used to indicate that the segments within brackets form a base-paired duplex.

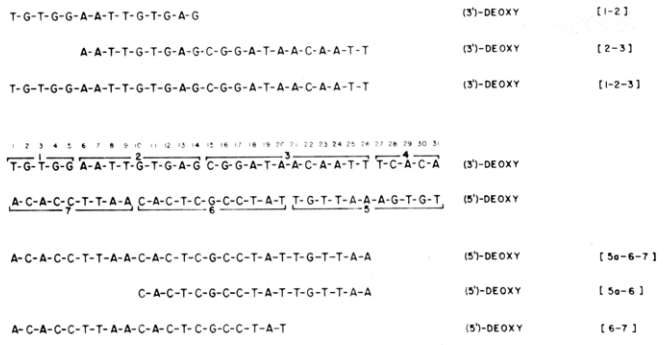

FIGURE 1: A summation of deoxyoligonucleotides that have been enzymatically joined to form *lac* operator DNA segments. The center of the figure displays the plan for chemical synthesis. Segments chemically synthesized are partitioned and numbered. Above the total plan are partial operator sequences corresponding to the top DNA strand and written 5′ to 3′. Below the total plan are partial operator sequences corresponding to the bottom DNA strand and written 3′ to 5′. The numbers in brackets to the right of each partial sequence represent an abbreviated name for each sequence. The numbers separated by a hyphen delineate the chemical fragments used to synthesize the partial operator sequence. Numbers followed by a lower case letter refer to a deoxyoligonucleotide that is only part of the segment referred to by the number.

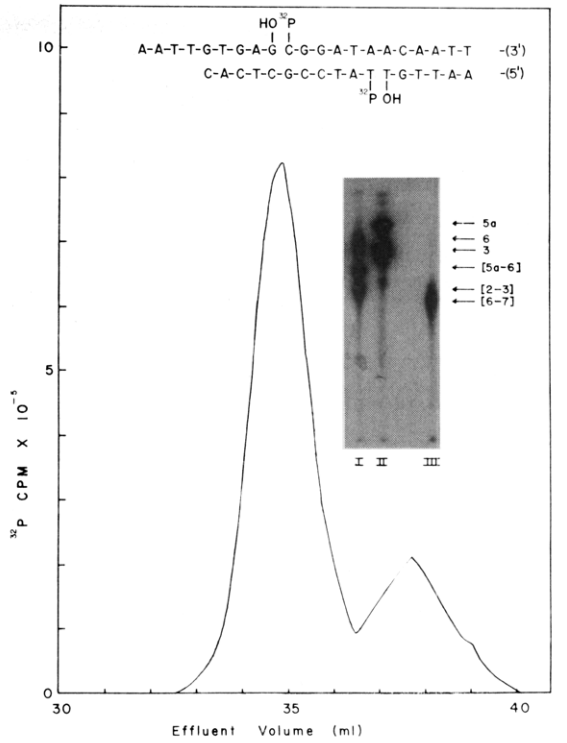

FIGURE 2: Synthesis and purification of duplex [2-3]/[5a-6]. The reaction mixture (140 μL) contained 1 nmol each of segments 2, 3, 5a, and 6, 9 mM $MgCl_2$, 16 μM ATP, and 20 mM Tris-HCl (pH 7.5). Segments 3 and 6 contained [5′-^{32}P]phosphate of the same specific activity. The solution was warmed at 70 °C for 2 min and slowly cooled (1 h) to 0 °C. Dithiothreitol (10 mM) and 350 units/mL of T4 ligase were added. After 26 h, EDTA was added (threefold excess over magnesium chloride) and the solution applied to a Bio-Gel A-0.5m column (1.0 × 90 cm) and the column eluted with 0.01 M triethylammonium bicarbonate at 4 °C. Fractions of 150 μL were collected every 8 min. The inset shows a DEAE-cellulose thin-layer homochromatography analysis of the reaction mixture before fractionation. The deoxyoligonucleotides are: channel I, reaction mixture; channel II, ^{32}P-labeled segment 5a, ^{32}P-labeled segment 3, ^{32}P-labeled segment 6, and an unknown, slow moving compound (this channel contained the assay of a ligation reaction not discussed in the paper); channel III, ^{32}P-labeled segment [6-7].

significance for the present work was an investigation of reaction conditions for synthesizing potentially useful *lac* operator segments and to prepare the appropriate joined segments in quantities sufficient for future studies.

A major objective of this work is to study how *lac* repressor interacts with operator DNA. The strength of the approach outlined here is that specific nucleotides can be modified by chemical or enzymatic procedures and therefore introduce a defined change in operator DNA. Already 5-bromodeoxyuridine and deoxyuridine have been inserted in place of thymidine at most sites in the sequence 6–26 of the operator shown in Figure 1 (Yansura et al., 1976). By using DNA polymerase with appropriate operators, 5-methyldeoxycytidine, 5-bromodeoxycytidine, deoxyinosine, and deoxydiaminopurine will also be inserted in place of specific bases. The purpose of these experiments will be to test the effect of these modifications on the repressor–operator interaction. Such experiments could define if important interaction sites exist in the major or minor groove of the operator. At the present time, we have also chemically inserted deoxycytidine and deoxyadenosine at site 13 of segment 6 (Loder et al., 1976). The change of deoxycytidine will allow us to prepare a known operator constitutive mutant (see Figure 2 of Goeddel et al., 1977). Insertion of adenosine will give an operator with the A-T base pair inverted relative to the *lac* operator. The relative binding of repressor to these modified operators should provide important information about the nature of recognition at this site. In order to carry out these experiments, considerable manipulative ability with operator segments was desirable. These objectives can best be achieved by synthesizing separately the strands of operator DNA. Then these single stranded segments can be mixed in appropriate combinations to give the correct duplex. Therefore many of the reactions outlined in this paper primarily concern the synthesis of single-stranded operator DNAs of variable length.

Materials and Methods

Materials. The deoxyoligonucleotides used in the present work (segments 1, 2, 3, 5a, 6, 7) were chemically synthesized as described in the preceding paper (Goeddel et al., 1977). The segments 1, 3, and 6 contained OH groups at both ends. The segments 2, 5a, and 7 contained 5′-phosphate groups and 3′-hydroxyl groups. The 5′-phosphate was removed using bacterial alkaline phosphatase and conditions described previously (RajBhandary, 1968). ATP labeled in the γ position with [^{32}P]phosphate was prepared as described previously (Glynn and Chappel, 1964). T4 polynucleotide kinase and T4 polynucleotide ligase were prepared according to a published procedure (Panet et al., 1973). The second DEAE-cellulose column of the T4 polynucleotide ligase preparation, however, was run as published by Weiss (Weiss et al., 1968). The primary nuclease contaminant in our T4 kinase and T4 ligase preparations was a 3′-exonuclease. Therefore all columns were monitored carefully for this nuclease and peaks of ligase and kinase activity pooled so as to eliminate this exonuclease. In some cases almost half the kinase or ligase activity was discarded. Snake venom phosphodiesterase, pancreatic deoxyribonuclease, spleen phosphodiesterase, micrococcal deoxyribonuclease, and bacterial alkaline phosphatase were commercial samples.

Methods. Phosphorylation of deoxyoligonucleotides containing [5′-^{32}P]phosphate groups was carried out as described previously (Sgaramella and Khorana, 1972; van de Sande et al., 1972). Reaction conditions for T4 ligase catalyzed joining reactions varied from system to system. Each set of conditions

TABLE I: Characterization of Synthetic Polynucleotides:[a] Multiple Joining Experiments.

Segments Analyzed	3′-Nucleotide Anal. (cpm)				5′-Nucleotide Anal. (cpm)			
	dAp	dGp	dTp	dCp	pdA	pdG	pdT	pdC
[2-3]/[5a-6]	30	11 562 (1.0)	11 452 (1.0)	56	11	23	515 (1.0)	514 (1.0)
[1-2-3]/[5a-6-7]	47	6 705 (2.0)	2 233 (0.7)	397 (1.0)[b]	2 374 (2.0)[b]	91	1482 (0.7)	2328 (1.1)
[1-7]/[1-7]	5	845	10	20	15 241	18	25	2

[a] The numbers in parentheses after the counts per minute (cpm) are the experimental molar ratios. [b] The specific activity of ^{32}P in [6-7] was 0.12 times the specific activity of the ^{32}P in segments 2, 3, and 6.

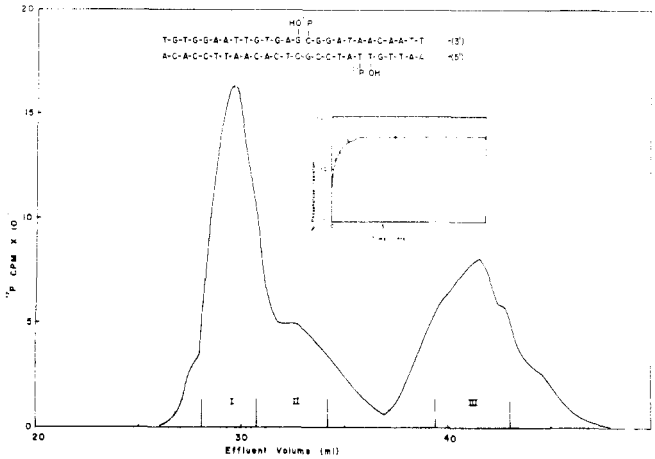

FIGURE 3: Synthesis and purification of duplex [1-2-3]/[5a-6-7]. The reaction mixture (10 μL) contained 32 pmol each of segments [1-2] and [6-7], 38 pmol of segment 3, 46 pmol of segment 5a, 10 mM $MgCl_2$, 27 μM ATP, and 20 mM Tris-HCl (pH 7.5). Segments 3 and [6-7] contained [5′-^{32}P]phosphate of the same specific activity. The solution was warmed to 70 °C for 2 min and slowly cooled (1 h) to 0 °C. Dithiothreitol (10 mM) and 5 units of T4 ligase were added. After 17 h the reaction appeared to plateau at 70% yield based on resistance of [^{32}P]phosphate to alkaline phosphatase. The reaction mixture was applied to Bio-Gel A-0.5m column (1.0 × 90 cm) and the column eluted with 0.01 M triethylammonium bicarbonate at 4 °C. Fractions of 10 drops were collected and monitored for radioactivity.

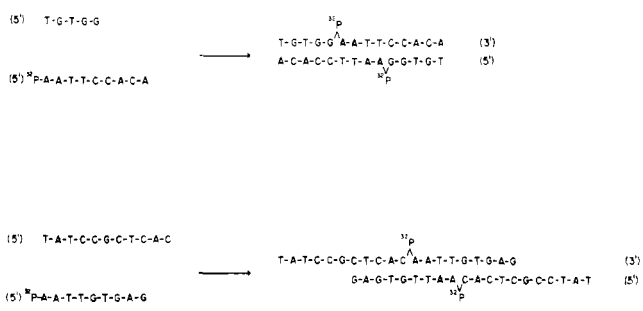

FIGURE 4: Formation and structure of "dimers" formed from segments 1 and 7 or segments 2 and 6. In order for each dimer to form with T4 ligase, two conditions must be present: (1) segments 2 or 7 must be phosphorylated at the 5′ end; (2) the complementary segment 6 or 1 respectively must be present in the appropriate reaction mixture.

is outlined in the appropriate figure legend. The measurement of reaction kinetics was as described previously (Sgaramella and Khorana, 1972). Characterization by degradation of T4 ligase joined segments to 3′-mononucleotides with spleen phosphodiesterase and micrococcal deoxyribonuclease and 5′-mononucleotides with snake venom phosphodiesterase and pancreatic deoxyribonuclease was according to published procedures (Sgaramella and Khorana, 1972). Characterization of joined products on the basis of size was by homochromatography on DEAE-cellulose thin-layer plates by the method described by Brownlee and Sanger (1969).

Results

Multiple Joining Experiments. Various *lac* operator duplexes were prepared containing different combinations of chemically synthesized segments. Duplex [2-3]/[5a-6] was prepared as outlined in Figure 2. Segments 3 and 6 carried [5′-^{32}P]phosphate of the same specific activity. As monitored by phosphatase resistance, the yield was 75%. The inset shows the reaction mixture profile when analyzed by homochromatography on DEAE-cellulose thin-layer plates. This system clearly separates segments [2-3], [5a-6], and unreacted starting materials into three well-defined spots. The elution pattern indicated one major peak which corresponded to the product in duplex form and a second peak of unreacted deoxyoligonucleotides. The characterization is in Table I. The 5′ and 3′ analyses verified that the main product corresponded to the expected duplex. Equivalent counts were found in pdT and pdC for the 5′ and in dTp and dGp for the 3′ analysis. Analysis of peak I by homochromatography gave two spots as expected from the size of the deoxyoligonucleotides in the duplex (data not shown).

The synthesis of [1-2-3]/[5a-6-7] was attempted using preformed [1-2] and [6-7]. The elution profile and reaction conditions are presented in Figure 3. As measured by resistance to alkaline phosphatase, the reaction was complete in 2 h. The yield was 75%. Peak I was analyzed as the duplex [1-2-3]/[5a-6-7]. However, the ligation did not go to completion. Although an excess of 5a was added and the ligation allowed to proceed until no further reaction could be measured, segment 5a did not completely join to the duplex. Both 5′-mononucleotide and 3′-mononucleotide analysis indicated that approximately 30% of the duplex sample does not contain 5a (Table I).

Segments 1 and 7 form a stable duplex which can be joined with T4 ligase (Figure 4). After 69 h, segment 7 was found to join segment 1 with 49% yield. The elution profile on Sephadex G-75 is shown in Figure 5. Peak I was [1-7]/[1-7] and peak II starting material. The 5′ and 3′ analyses shown in Table I indicated that all radioactivity in peak I was in [^{32}P]pdA and dGp, respectively. This duplex was further characterized by sizing on a DEAE-cellulose thin-layer plate. The results are included in the data displayed in Figure 5. Channel I contained the duplex isolated from peak I. The duplex moved as a single deoxyoligonucleotide as expected for [1-7]/[1-7]. When compared with partially degraded [5a-6] in channel III, the segment [1-7] has mobility corresponding to a deoxyoligonucleotide composed of 14 mononucleotides. The calculated size of [1-7] is 14 mononucleotides. Recently the duplex [1-7]/[1-7] has been joined to duplex [1-2-3]/[5a-6-7] to form an operator containing DNA sequences susceptible to cleavage by Eco RI restriction nuclease (Goeddel et al., 1976a). Studies

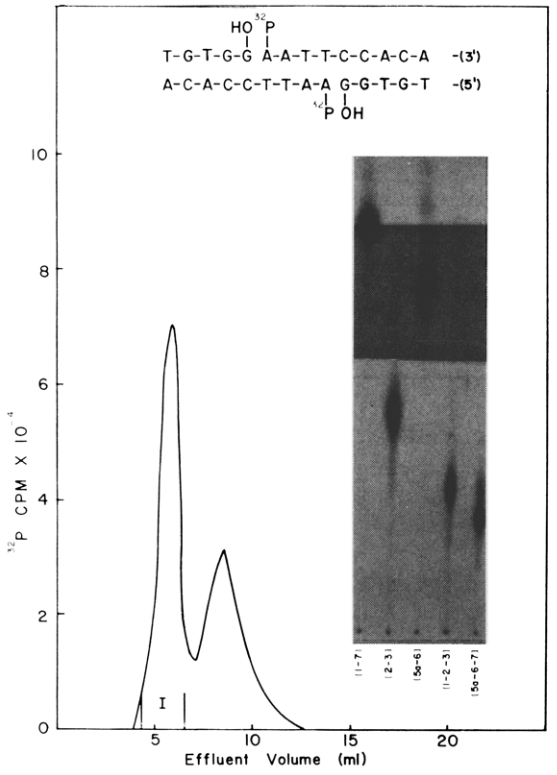

FIGURE 5: Synthesis and purification of duplex [1-7]/[1-7]. The reaction mixture (100 μL) contained 1.1 nmol of segment 2, 1.2 nmol of segment 1, 10 mM $MgCl_2$, 53 μM ATP, and 20 mM Tris-HCl (pH 7.4). Segment 2 contained [5′-^{32}P]phosphate. The solution was warmed to 70 °C for 2 min and slowly cooled (1 h) to 0 °C. Dithiothreitol (10 mM) and 400 units/mL of T4 ligase were added. After 69 h, the reaction was terminated by diluting to 250 μL and warming at 70 °C. The hot reaction mixture was applied to a Sephadex G-75-40 column (45 cm × 0.8 cm) and the column eluted with 0.01 M triethylammonium bicarbonate at 4 °C. Fractions of 10 drops were collected and analyzed for radioactivity. The inset shows the mobility of segment [1-7] (peak I) as compared with several other segments: channel I, segment [1-7]; channel II, segment [2-3]; channel III, partially degraded segment [5a-6]; channel IV, segment [1-2-3]; channel V, segment [5a-6-7].

presently underway include incorporation of this operator into plasmids for cloning experiments.

Single-Strand Joining Studies. The synthesis of [6-7] was examined extensively since it was a template for the preparation of [1-2] and an intermediate in the synthesis of [5a-6-7]. A variety of conditions was investigated in attempts to increase the yield of [6-7]. These are reported in Table II. Results from systems 1, 2, 3 indicated that a fourfold excess of ATP was sufficient for maximum yield whereas slightly more than an equivalent was not adequate. Systems 3, 4, 5, and 6 illustrate that this ligase reaction was very sensitive to magnesium ion. The yield at 5 mM $MgCl_2$ was considerably higher than that found at 15 or 20 mM. At 5 and 10 mM $MgCl_2$, the yields were very similar. Addition of excess segment 2 (system 7) failed to significantly increase the yield, whereas excess segment 6 (system 8) appeared to decrease the yield of [6-7]. Two experiments were attempted which utilized additional segments in order to stabilize single-stranded regions of segments 6 or 7 during the joining reaction. System 9 shows a reaction where segment 3 was added in an attempt to stabilize the 5′ end of segment 6 in duplex form. No change in the extent of reaction was observed. Addition of segment 1 (system 10) permitted synthesis of [1-7]/[1-7] which competed effectively for segment 7 with reduced overall yield for [6-7]. These studies therefore defined how to best synthesize [6-7]. The preparative

TABLE II: Studies of the Synthesis of Segment [6-7].[a]

System	Segments	ATP (μM)	$MgCl_2$ (mM)	Extent Joining (%)
1	2, 6, 7	55	10	48
2	2, 6, 7	2.75	10	35
3	2, 6, 7	11	10	49
4	2, 6, 7	11	5	54
5	2, 6, 7	11	15	34
6	2, 6, 7	11	20	31
7	2,[b] 6, 7	11	10	60
8	2, 6,[b] 7	11	10	33
9	2, 3, 6, 7	11	10	56
10	1, 2, 6, 7	55	10	76[c]

[a] The reaction mixtures contained ATP and magnesium chloride as indicated, 20 mM Tris-HCl (pH 7.6), and 2.5 μM individual oligonucleotides. In all experiments, segment 7 was the only deoxyoligonucleotide containing a [5′-^{32}P]phosphate group. Reaction solutions were first warmed to 70 °C and slow cooled (1 h) to 0 °C. Dithiothreitol (10 mM) and T4 ligase (500 units/mL) were added. The extent of joining is defined as phosphatase resistant counts after 60 h. [b] A twofold molar excess of this segment was present in the reaction. [c] A major product of this reaction was [1-7]/[1-7].

synthesis using conditions for maximum yield and purification of [6-7] are outlined in Figure 6. Peak I was [6-7] and peak II was starting material. The isolated yield of [6-7] was 18%. As shown in Table III, analysis by degradation to 3′-mononucleotides transferred all radioactivity to dCp as expected for [6-7]. The inset to Figure 2 shows in channel III the mobility and purity of segment [6-7] isolated from this preparation.

Several experiments were carried out in attempts to prepare [1-2]. The results recorded in Table IV illustrate that this ligation reaction was very sensitive to stabilization of the correct duplex. When segment 7 was used as the template, the extent of joining was approximately 33% (systems 1–4). However, in the presence of [6-7] as the template, the yield was 84% (system 5). Preparative synthesis of [1-2] on a 12.5-nmol scale was therefore with [6-7]. An outline of this reaction and the elution profile are shown in Figure 7. Kinetic analysis by measuring phosphatase resistance indicated that the yield of [1-2] was 68%. The inset shows the elution profile when analyzed on DEAE-cellulose thin-layer plates. Fraction III has the mobility expected for [1-2], whereas fraction I was primarily [6-7]. Fraction IV was unreacted segment 2. Fraction II contained [1-2], [6-7], and some unidentified products with intermediate mobilities. The isolated yield of [1-2], including material obtained by rechromatography of fraction II, was 50%. The characterization of [1-2] (fraction III) is given in Table III. Degradation to 3′-mononucleotides gave radioactivity in dGp. Analysis by digestion to 5′-mononucleotides gave primarily radioactivity in pdA.

Deoxyoligonucleotides [1-2-3] and [2-3] were prepared using only segment 6 as template. Initial experiments at 55 μM ATP, 10 mM $MgCl_2$, and 2.5 μM deoxyoligonucleotids indicated that the joining of segment 2 to 3 using 6 as template gave a very satisfactory yield (79%) without extensive investigation. A preparative scale synthesis of [2-3] is outlined in Figure 8. Approximately 76 h was required for the reaction to go to completion (63% isolated yield). Peak I was characterized as [2-3] and peak II was unreacted starting material. The figure inset shows the DEAE-cellulose thin-layer chromatography profile for peak I. The product is homogeneous and has less mobility than segment [5a-6]. Results in Table III indicated that degradation to 3′-mononucleotides gave ra-

TABLE III: Characterization of Synthetic Polynucleotides:[a] Single-Strand Joining Experiments.

Segments Analyzed	3′-Nucleotide Anal. (cpm)				5′-Nucleotide Anal. (cpm)			
	dAp	dGp	dTp	dCp	pdA	pdG	pdT	pdC
[1-2]	95	8531	136	426	7083	39	347	10
[6-7]	66	67	68	2241				
[2-3]	92	7097	150	45	59	41	154	5996
[1-2-3]					550 (1)	2	23	644 (1.17)
[5a-6]	16	63	4601	138	106	304	8024	265
[5a-6-7][b]					13	34	926	35

[a] The numbers in parentheses after the counts per minute are the experimental molar ratios. [b] The ^{32}P label in the internucleotide bond joining segments 6 and 7 was less than 1% of the ^{32}P label in the internucleotide bond joining segments 5a and 6.

TABLE IV: Studies on the Synthesis of Segment [1-2].[a]

System	Segments	ATP	$MgCl_2$	Extent of Joining (%)
1	1, 2, 7	11	5	34
2	1, 2, 7	11	10	32
3	1, 2, 7	55	5	30
4	1, 2, 7	55	10	33
5	1, 2, [6-7]	14	10	84

[a] The reaction mixtures for systems 1–4 contained ATP and $MgCl_2$ as indicated, 20 mM Tris-HCl (pH 7.4), and 20 μM individual oligonucleotides. System 5 was 7.5 μM in oligonucleotide. Prior to addition of ligase, the reactions were incubated at 70 °C for 2 min and cooled to 0 °C. Dithiothreitol (10 mM) and ligase (500 units/mL) were added to each sample. The extent of joining was defined as phosphatase resistant counts after 60 h. Segment 2 contained [5′-^{32}P]phosphate.

TABLE V: Studies on the Synthesis of Segment [5a-6].[a]

System	Segments	Concn Segments (μM)	ATP (μM)	$MgCl_2$ (mM)	Extent of Joining (%)
1	3, 5a, 6	1	55	10	43
2	3, 5a, 6	1	55	20	24
3	3, 5a, 6	8	55	10	84
4	3, 5a, 6	19	85	10	90
5	2, 3, 5a, 6	2.5	55	10	92

[a] The reaction mixtures contained ATP, magnesium chloride, and individual oligonucleotides as indicated. All experiments contained 20 mM Tris-HCl (pH 7.6) and segment 6 as the only deoxyoligonucleotide with a [5′-^{32}P]phosphate group. Reaction solutions were first warmed to 70 °C and slow cooled (1 h) to 0 °C. Dithiothreitol (10 mM) and T4 ligase (500 units/mL) were added. The extent of joining was defined as phosphatase resistant counts after 60 h.

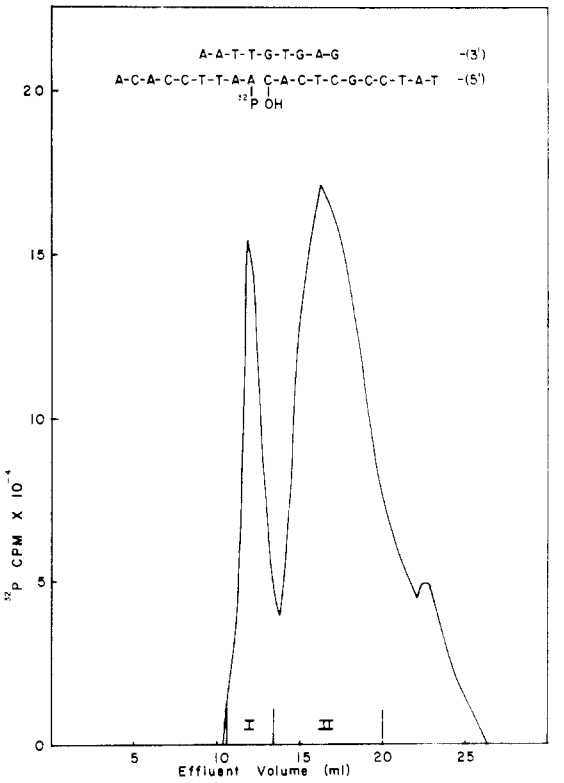

FIGURE 6: Synthesis and purification of segment [6-7]. The reaction mixture (1 mL) contained 25 nmol each of segments 2, 6, and 7 (25 μM), 10 mM $MgCl_2$, and 20 mM Tris-HCl (pH 7.6). Only segment 7 contained [5′-^{32}P]phosphate. The solution was warmed at 70 °C for 2 min and slowly cooled (1 h) to 0 °C. Dithiothreitol (10 mM), ATP (55 μM), and 250 units/mL of T4 ligase were added. After 70 h, the reaction was warmed at 70 °C for 2 min, and cooled to 0 °C. Dithiothreitol (100 μL of 0.1 M solution) and T4 ligase (150 units) were added. The reaction was allowed to continue for an additional 120 h and then stopped by addition of excess EDTA. After warming at 90 °C for 2 min, the solution was applied to a Sephadex G-100-40 column (0.8 × 48 cm) and the column eluted with 0.01 M triethylammonium bicarbonate at room temperature. Fractions of 9 drops were collected and analyzed for radioactivity.

dioactivity only in dGp. Analysis by digestion to 5′-mononucleotides gave radioactivity only in pdC. The synthesis of [1-2-3] using only segment 6 as template is summarized in Figure 9. Again a very long time (132 h) was required for the reaction to plateau (51% yield). The elution profile on Sephadex G-100-40 was very clean with two well-defined peaks. Profiles such as these emphasize that the T4 ligase used in these syntheses was free of exonuclease activity. The inset compares the mobility of the product (peak I or segment [1-2-3]) with the labeled starting material (peak II or segments [1-2] and 3). Peak I was characterized as [1-2-3] as shown in Table III. Digestion to 5′-mononucleotides gave radioactivity in pdA and pdC as expected for [1-2-3].

The joining of segment 6 to 5a in the presence of segment 3 went essentially to completion. The yield after 24 h was 90% at 19 μM deoxyoligonucleotides, 10 mM $MgCl_2$, and excess ATP (Table V). This table also shows that the reaction was sensitive to the concentration of deoxyoligonucleotides (compare systems 1 and 3) and $MgCl_2$ (compare systems 1 and 2). As shown by system 5, the yield of [5a-6] at low concentration of oligonucleotide (~2 μM) can be increased by addition of segment 2. The preparative synthesis of [5a-6] was carried out using segment 3 as template and conditions for maximum reaction. The elution profile and kinetics of joining

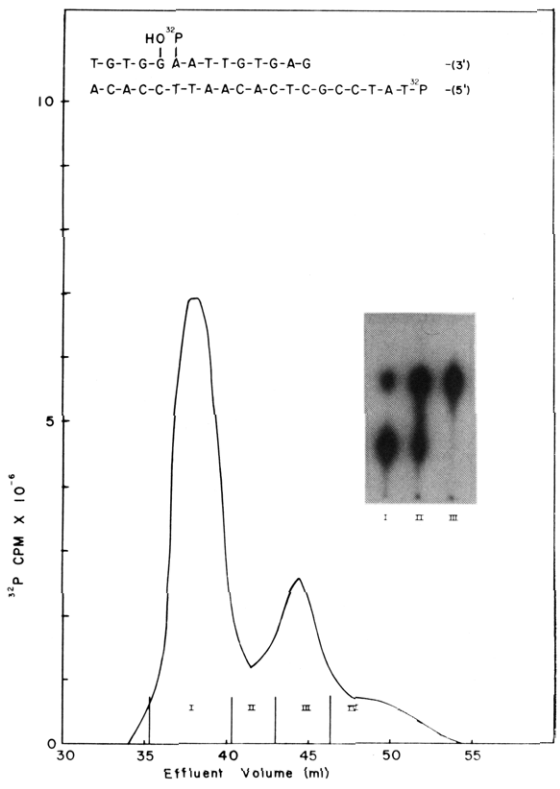

FIGURE 7: Synthesis and purification of segment [1-2]. The specific activities of the [5′-^{32}P]phosphate groups present in segments 2 and [6-7] were identical. The specific activity of ^{32}P internal phosphate in segment [6-7] was 5% the 5′-phosphate. The reaction mixture (1.2 mL) contained 9.6 nmol of segment [6-7], 10 nmol of segments 1 and 2, 10 mM $MgCl_2$, 20 mM Tris-HCl (pH 7.6), and ATP (22 μM). The solution was heated to 70 °C and slowly cooled (1 h) to 0 °C. Dithiothreitol (10 mM) and 167 units/mL of T4 ligase were added. The joining reaction was allowed to proceed 51 h at 0 °C. The ligase reaction was then heated to 70 °C and slowly cooled (1 h) to 0 °C. The pH was adjusted to 7.3. Dithiothreitol (150 μL of a 0.1 M solution) and 200 units T4 ligase were added. The reaction was continued for an additional 39 h and then lyophilized to 0.5 mL. EDTA was then added (10 mM) and the solution warmed at 90 °C for 2 min. The reaction mixture was applied to a Bio-Gel A-0.5m column (180 × 1 cm) and the column eluted with 0.01 M triethylammonium bicarbonate at room temperature. Fractions of 10 drops were collected and analyzed for radioactivity. The inset shows analysis of various fractions by thin-layer homochromatography on DEAE-cellulose. Deoxyoligonucleotides were: channel I, fraction I; channel II, fraction II; channel III, fraction III.

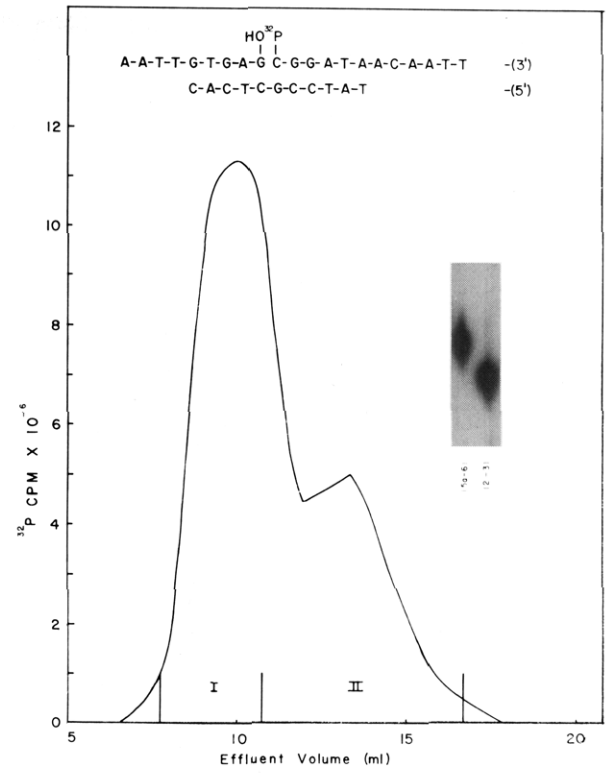

FIGURE 8: Synthesis and purification of segment [2-3]. The reaction mixture (1 mL) contained 12.5 nmol each of segments 2, 3, and 6 (12.5 μM), 10 mM $MgCl_2$, 53 μM ATP, and 20 mM Tris-HCl (pH 7.2). Only segment 3 contained [5′-^{32}P]phosphate. The solution was warmed to 70 °C for 2 min and slowly cooled (1 h) to 0 °C. Dithiothreitol (10 mM) and 250 units/mL of T4 ligase were added. After 31 h, the reaction was warmed to 70 °C for 2 min and cooled to 0 °C. Dithiothreitol (100 μL of 0.1 M solution) and T4 ligase (100 units) were added. The reaction was allowed to continue for an additional 45 h and then stopped by addition of excess EDTA. After warming at 90 °C, the solution was immediately applied to a Sephadex G-75-40 column (0.8 × 45 cm) and the column eluted with 0.01 M triethylammonium bicarbonate at room temperature. Fractions of 8 drops were collected and analyzed for radioactivity. The inset compares the mobility of peak I (segment [2-3]) with segment [5a-6] by thin-layer homochromatography on DEAE-cellulose.

are shown in Figure 10. The reaction was stopped after 69 h when analysis with alkaline phosphatase indicated 83% reaction. Kinetic data shown in Figure 10 suggest that the reaction had not gone to completion. The inset displays an analysis of various peak fractions using DEAE-cellulose on thin-layer plates. Sephadex G-75-40 failed to completely resolve segment [5a-6] from unreacted segment 6. Fractions were pooled as indicated in the figure with impure fractions (II) being rechromatographed. The isolated yield of [5a-6] was 71%. Characterization of the product by degradation to 3′- and 5′-mononucleotides was as expected (Table III). Digestion to 3′-mononucleotides transferred all radioactivity to dTp, whereas analysis for radioactive 5′-mononucleotides showed pdT as the only labeled nucleotide.

The synthesis of [5a-6-7] was studied using several different combinations of segments (Table VI). System 1 used segments 6 and 7 phosphorylated and 5a, 2, and 3 unphosphorylated. Although the phosphatase resistance indicated 59% yield, the characterization of isolated product was inconsistent with results expected for [5a-6-7]. Digestion to 3′-mononucleotides gave [^{32}P]dTp and [^{32}P]dCp with the molar ratio being 3.3. Digestion to 5′-mononucleotides gave [^{32}P]pdT and [^{32}P]pdA with a mole ratio of 2.7. In both cases, the theoretical ratio was 1. This product has not been further characterized. An alternative approach was the joining of segment 5a to phosphorylated, preformed [6-7] in the presence of segment 3. The

TABLE VI: Studies on the Synthesis of Segment [5a-6-7].

System	Segments	Concn Segments (μM)	ATP (μM)	$MgCl_2$ (mM)	Extent of Joining (%)
1[a]	2, 3, 5a, 6, 7	2.5	55	10	59
2[b]	3, 5a, [6-7]	9	26	10	7
3[b]	2, 3, 5a, [6-7]	9	26	10	37
4[a]	[1-2], 3, [5a-6], 7	11	35	10	27

[a] Reaction mixtures contained ATP, magnesium chloride, and individual oligonucleotides as indicated. System 1 contained only segments 6 and 7 with [5′-^{32}P]phosphate. System 4 contained only segment 7 with [5′-^{32}P]phosphate. Reaction solutions 20 mM in Tris-HCl (pH 7.6) were warmed at 70 °C and slow cooled (1 h) to 0 °C. Dithiotheitol (10 mM) and T4 ligase (500 units/mL) were added. The extent joining was defined as phosphatase resistant counts. [b] For details of these reactions, see Figure 10.

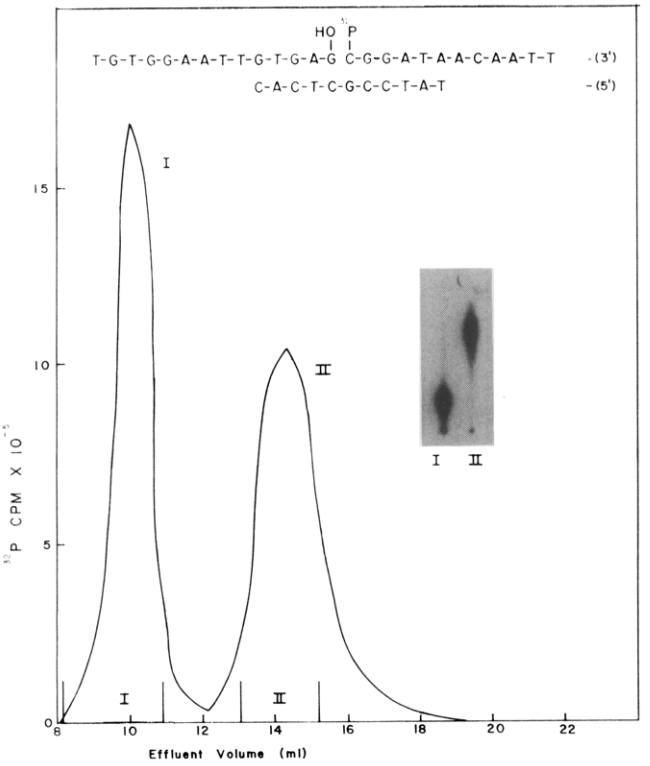

FIGURE 9: Synthesis and purification of segment [1-2-3]. The reaction mixture (200 μL) contained 2 nmol each of segments [1-2], 3, and 6 (10 μM), 10 mM $MgCl_2$, 55 μM ATP, and 20 mM Tris-HCl (pH 7.6). Segments [1-2] and 3 carried [^{32}P]phosphate of the same specific activity. The 5′-phosphate of segment 3 was labeled whereas the internucleotide bond joining segment 1 to 2 in [1-2] was labeled. The solution was warmed to 70 °C for 2 min and slowly cooled (1 h) to 0 °C. Dithiothreitol (10 mM) and 300 units/mL of T4 ligase were added. After 29 h, the reaction was warmed to 70 °C for 2 min and cooled to 0 °C. Dithiothreitol (20 μL of 0.1 M solution) and T4 ligase (50 units) were added. The reaction was allowed to continue for 63 h. Again the reaction was warmed to 70 °C, cooled to 0 °C, and the pH adjusted to 7.3. Dithiothreitol (20 μL of a 0.1 M solution) and T4 ligase (50 units) were added. After 40 h, the reaction was stopped by addition of excess EDTA and warming at 70 °C for 2 min. The hot solution was immediately applied to a Sephadex G-100-40 column (45 × 0.8 cm) and the column eluted with 0.01 M triethylammonium bicarbonate at room temperature. Fractions of 10 drops were collected and analyzed for radioactivity. The inset compares the mobility of peak I (segment [1-2-3]) with peak II (segments [1-2] and 3) on thin-layer DEAE-cellulose.

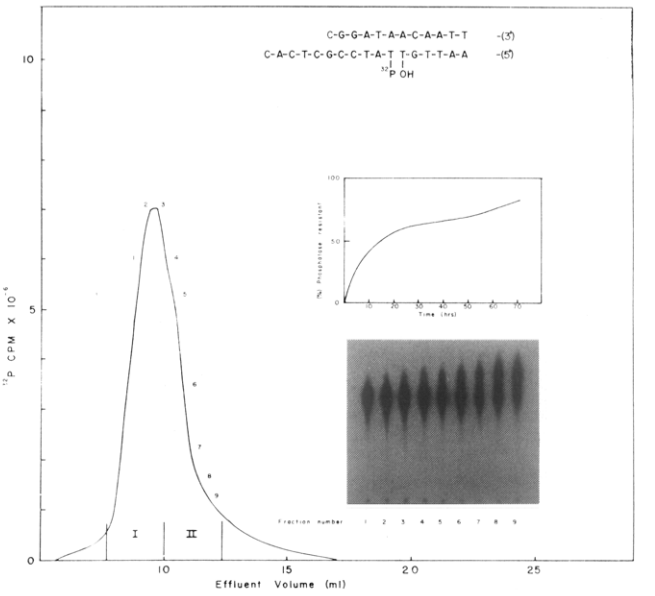

FIGURE 10: Synthesis and purification of segment [5a-6]. The reaction mixture (500 μL) contained 3.8 nmol each of segments 3, 5a, and 6 (7.6 μM), 10 mM $MgCl_2$, 32 μM ATP, and 20 mM Tris-HCl (pH 7.6). Only segment 6 contained [5′-^{32}P]phosphate. The solution was warmed at 70 °C for 2 min and slowly cooled (1 h) to 0 °C. Dithiothreitol (10 mM) and 200 units/mL of T4 ligase were added. After 48 h, the reaction was warmed at 90 °C for 2 min and cooled to 0 °C. Dithiothreitol (50 μL of a 0.1 M solution) and T4 ligase (100 units) were added. The reaction was allowed to continue for 21 h and then stopped by addition of excess EDTA. After warming at 90 °C for 2 min, the solution was immediately applied to a Sephadex G-75-40 column (0.8 × 45 cm) and the column eluted with 0.01 M triethylammonium bicarbonate at room temperature. Fractions of 9 drops were collected and analyzed for radioactivity. The inset shows the analysis of column fractions on thin-layer DEAE-cellulose. Fractions (I and II) were pooled as shown in the figure.

extent of joining after 23 h was only 7% (system 2). However, when segment 2 was added to stabilize [6-7] in duplex form, the yield increased to 37%. The elution profile for this synthesis is shown in Figure 11. One peak is observed for this reaction. However, the thin-layer chromatography data displayed on the inset indicated that the peak was heterogeneous and fairly well resolved. Fractions I and II were segment [5a-6-7], whereas fractions III and IV were primarily segment [6-7]. Degradation to 5′-mononucleotides indicated all the radioactivity was in pdT as expected for [5a-6-7]. The synthesis of [5a-6-7] was also examined using segment 7 phosphorylated and segments [1-2], 3, and [5a-6] not phosphorylated at the 5′ position (system 4). The yield after 95 h was 27%. Again the isolated product gave a 3′- and 5′-mononucleotide analysis consistent with results expected for [5a-6-7] (data not shown).

Discussion

A major objective was to construct *lac* operator DNA in such a way so as to maximize our ability to utilize this DNA for studies on repressor–operator interactions. This work therefore was directed primarily, although not exclusively, toward the synthesis of *lac* operator sequences in single-strand form rather than a series of duplexes. The single-strand segments could then be combined in whatever manner was desirable for various modification experiments. For example, single strands corresponding to specific parts of the operator could be modified by mild chemical or enzymatic methods, annealed with the complementary strand, and tested for repressor binding. Also contributing to this decision was the knowledge that in some cases one strand did not join as completely as the other in multiple site joinings. For example, 5a did not join quantitatively during the synthesis of duplex [1-2-3]/[5a-6-7] as discussed in the Results section. Therefore in order to use this duplex for binding studies, the total duplex would have to be separated from partial operators. For some systems this may not be an easy task. A more practical approach would be to separately synthesize each strand and then combine in the correct ratio. A third factor that also influenced our decision was the difficulty sometimes encountered in the quantitative phosphorylation of DNA duplexes with T4 kinase (Sekiya et al., 1976; Loewen et al., 1976). Conversely this enzyme appeared to phosphorylate the 5′-hydroxyl of single-stranded DNA with little difficulty. The plan here was to prepare *lac* operator in modified form, label this operator quantitatively on the 5′-hydroxyl groups with ^{32}P of high specific activity, and then immediately test repressor binding. Therefore, although the problem now appears to be solved (Lillehaug et al., 1976), earlier work indicated that the method of choice was to synthesize single strands, phosphorylate with T4 kinase, anneal, and then to test for repressor binding.

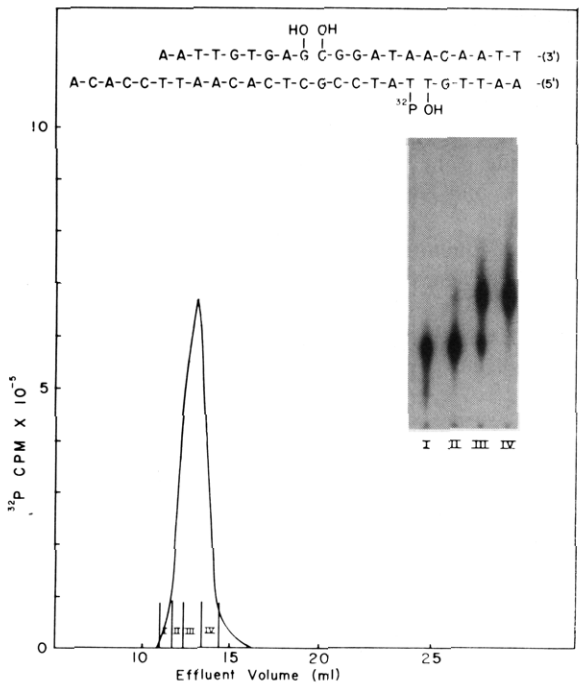

FIGURE 11: Synthesis and purification of [5a-6-7]. The reaction mixture (200 μL) contained 1.7 nmol of segment [6-7], 1.9 nmol of segment 5a, 1.9 nmol of segment 3, 10 mM $MgCl_2$, 26 μM ATP, and 20 mM Tris-HCl (pH 7.6). Only segment [6-7] contained [5′-^{32}P]phosphate. The solution was warmed at 70 °C for 2 min and slowly cooled (1 h) to 0 °C. Dithiothreitol (10 mM) and 250 units/mL of T4 ligase were added. After 23 h, only 7% reaction was observed and therefore segment 2 (1.7 nmol) was added. After a total of 65 h, the reaction mixture was warmed at 90 °C for 2 min and cooled to 0 °C. Dithiothreitol (20 μL of 0.1 M solution) and T4 ligase (50 units) were added. The reaction was allowed to continue for 114 h and then stopped by addition of excess EDTA. After boiling for 2 min, the solution was immediately applied to a Sephadex G-75-40 column (0.8 × 45 cm) and the column eluted with 0.01 M triethylammonium bicarbonate at room temperature. Fractions of 7 drops were collected and analyzed for radioactivity. The inset shows the analysis of pooled fractions on thin-layer DEAE-cellulose.

In order to synthesize either strand, an important problem had to be solved. Segments 2 and 7 have symmetrical 5′ ends. Therefore if reaction conditions are not carefully controlled, side products can form as illustrated in Figure 4. A similar problem has been observed previously (Sgaramella and Khorana, 1972). For the synthesis of segment [6-7], the problem was solved by using only segment 2 unphosphorylated as template. Segment 1 was excluded from the reaction. Although the kinetic yield was not exceptionally high (54%) and the preparative yield much less (18%), the reaction proceeded cleanly to segment [6-7]. Two factors could contribute to the low yield. The protruding single-strand ends on 6 and 7 could destabilize the desirable base pairing. Furthermore both segments 2 and 7 will form self-complementary base pairs (Figure 12). This pairing could reduce the amount of segments 6 and 7 available for the correct reaction. The synthesis of segment [1-2], the complementary problem, was examined using both segment 7 and segment [6-7] as template. Segment [6-7] was by far the superior template. The main reason probably was that segment 2 can form nine stable base pairs with [6-7] whereas only four can be formed with segment 7 (the same number as can be formed with segment 2 base pairing with itself). Of interest was the joining of segments 1 and 7 to form the duplex [1-7]/[1-7]. This duplex contains an RI endonuclease sensitive site and is being used for joining *lac* operator to plasmids at an RI site.

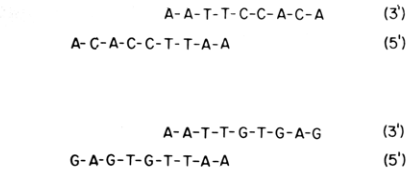

FIGURE 12: Duplex formation in segments 2 or 7 by self-complementary base pairing.

Segments [1-2] and [6-7] can be extended to give [1-2-3] and [5a-6-7], respectively. Both reactions were very sluggish. The yield of [1-2-3] was 51% after 132 h and the yield of [5a-6-7] was 37% after 179 h. The primary reason for these low yields probably was the large percentage of single strands present during the reactions. For example, the joining of segment 3 to [1-2] was carried out with segment 6 as template. The system therefore contained 11 base pairs and 15 nucleotides not involved in base pairing—a very unfavorable circumstance. The joining of 5a to segment [6-7] was unsatisfactory (7% yield) when only segment 3 was the template. Segment 2 had to be added in order to stabilize the duplex and give a satisfactory yield (37%). These low yields are not due to impure segments. For example, segment 5a can be joined to segment 6 in over 90% yield using segment 3 as template. In this case, 12 base pairs are formed and the protruding single strand is only 5 nucleotides. Another example is the multiple joining of segments 2 to 3 and 5a to 6. This reaction proceeded to 75% yield. The single-stranded region here was 4 nucleotides.

The total synthesis of the 31-base-pair duplex has been reported previously (Goeddel et al., 1976b). The synthesis was carried out by using T4 ligase to prepare the duplex [1-2-3]/[5-6-7] and DNA polymerase I to repair through the region corresponding to segment 4. The duplex [2-3]/[5a-6] has been repaired with DNA polymerase I to give a 21-base-pair duplex (Goeddel et al., 1976b). More recently this 21-base-paired duplex and duplex [1-2-3]/[5a-6-7] have been shown to bind repressor specifically with lifetimes of 37 and 46 s, respectively (Goeddel et al., 1976a). The synthetic DNAs reported in this paper are presently being used to prepare modified operators for studies on operator–repressor interactions.

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Scope and Mechanism of Carbohydrase Action: Stereospecific Hydration of D-Glucal Catalyzed by α- and β-Glucosidase†

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ABSTRACT: A unique demonstration is presented of the capacity of glycosidases to create anomeric configuration de novo. Purified *Candida tropicalis* α-glucosidase and sweet almond β-glucosidase have been found to attack the same substrate, D-glucal, and to convert this unusual glycosyl substrate (which lacks α or β anomeric configuration) to 2-deoxy-α- (or β-) D-glucose, respectively. The stereospecificity of the hydration reaction catalyzed by each enzyme in D_2O was revealed by the use of high-resolution (270 MHz) ^1H magnetic resonance spectroscopy. The α-glucosidase caused a specific axial protonation (deuteration) of D-glucal at C-2, and formation of 2-deoxy-α-D-[2(a)-^2H]glucose. The β-glucosidase catalyzed an oppositely directed axial protonation at C-2 and formation of 2-deoxy-β-D-[2(e)-^2H]glucose. These results are not accounted for by the generally accepted mechanisms of carbohydrase action derived from studies with glycosidically linked substrates alone. D-Glucal apparently binds to the enzymes with essentially the same overall orientation as the D-glucosyl moiety of glycosidically linked substrates (with the double bond of D-glucal lying essentially in the plane of the similarly bound D-glucosyl group). Thus, the α-glucosidase evidently protonates D-glucal from above the double bond and α-D-glucosidic substrates from below the glycosidic oxygen; β-glucosidase apparently protonates D-glucal from below the double bond and β-D-glucosides from above the glycosidic oxygen. A detailed mechanism is proposed for the hydration of D-glucal by each enzyme, involving an incipient glycosyl carbonium ion and assuming the presence at the active site of two carboxyl groups arranged to account for catalysis of glycosylations from glycosidically linked substrates. That D-glucal serves as a glycosyl substrate for these enzymes strongly supports the concept that glycosidases and glycosyltransferases are catalysts of glycosylation (i.e., glycosylases), since this concept does not make the usual assumption that carbohydrases are restricted to acting on substrates having a glycosidic bond and either α- or β-anomeric configuration.

The investigation of enzymatic glycosylation reactions that take place without glycosidic bond cleavage appears to hold considerable promise as an approach to gaining a more complete understanding of the catalytic actions of carbohydrases. Previous studies with glycosyl fluorides (Hehre et al., 1973, and cited references), for example, have yielded results that are incompatible with the long-held assumptions that glycoside hydrolases (EC 3.2) cause overwhelming hydrolysis of all their substrates, and that glycosyltransferases (EC 2.4) (transglycosylases, Hehre (1951)) require preexisting glycosidic bonds for their actions in synthesizing glycosidic linkages. These findings strongly support the thesis that a uniform chemical change, glycosyl-X + H-X′ $\rightleftarrows$ glycosyl-X′ + H-X, is effected in all reactions catalyzed by glycosidases (EC 3.2) and glycosyltransferases, including the formation and breakdown of glycosyl-enzyme intermediates. Unlike the hydrolase and transferase models, this concept carries no assumption restricting the nature of the glycosyl donor or the proton source that may take part in a reaction, and none restricting the mechanism(s) whereby the former becomes protonated and the latter glycosylated. Since currently accepted mechanisms of carbohydrase action assume stereospecific protonation of a glycosidic bridge atom, the study of reactions involving substrates without a glycosidic bond may be seen as potentially

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