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**Chemical synthesis and biochemical reactivity of bacteriophage lambda P<sub>R</sub> promoter**

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**ABSTRACT**

By a combination of chemical and enzymatic methods, a 75 base pair DNA duplex containing the sequence of the lambda P<sub>R</sub> promoter including the O<sub>R</sub><sup>1</sup> and O<sub>R</sub><sup>2</sup> cI repressor binding sites was synthesized. The solid support phosphite triester procedure (Caruthers, M. H. et al., Cold Spring Harbor Symposium on Quantitative Biology XLVII, in press) was used for the synthesis of oligonucleotides comprising the sequence. We report here an adaptation of the method to DNA synthesis in test tubes. Assembly of the oligonucleotides involved the use of T4 polynucleotide kinase and T4 DNA ligase. We show that the synthetic DNA is recognized by RNA polymerase and cI repressor in a manner identical to the same control region contained on a restriction fragment isolated from bacteriophage lambda DNA. Our synthetic approach using chemically synthesized promoter variants is thus suitable for studies probing the function of promoters.

**INTRODUCTION**

The regions of an *E. coli* promoter that are in contact with *E. coli* RNA polymerase in an open or functional complex have been determined (1,2). Considerable additional information has been obtained on how genetic mutations within promoters alter their transcriptional activity (3). This information is necessary but insufficient for developing theories on the recognition and activity of different promoters in complex with *E. coli* RNA polymerase. Clearly of utmost importance is to define this interaction at the base pair and functional group level. We want to know precisely which regions of a DNA base pair and *E. coli* RNA polymerase recognize one another and how this interaction leads to a transcriptionally active complex. Perhaps as a consequence of these studies, we will understand how different levels of transcriptional activity relate to promoter sequences. For the *lac* repressor-*lac* operator interaction, a fruitful approach to this problem has been the site specific introduction of base analogs lacking certain functional groups followed by measurement of the affinity of the repressor for the altered substrates (4). As the first step toward developing an analogous approach for

studying *E. coli* RNA polymerase-promoter interactions, we completed the synthesis of a 75 base pair DNA duplex containing the  $P_R$  promoter of bacteriophage lambda and the  $O_R^1$  and  $O_R^2$  binding sites (5) for the lambda cI repressor (see Figure 1). The chemical synthesis of consensus promoter sequences having biological activity when cloned has previously been reported (6,7). One of our goals is the introduction *in vitro* of base analogs at selected positions in the synthetic  $P_R$  promoter and a study of their effect on the activity of the promoter. Thus it was imperative to verify that the synthetic DNA itself had full activity. To this end, the biochemical activity of this synthetic duplex and a natural restriction fragment from phage lambda containing the  $P_R$  promoter were compared. The results are outlined in this manuscript and demonstrate that the synthetic promoter is fully active *in vitro* as defined by several biochemical criteria.

The synthetic lambda promoter was prepared using a new phosphite triester chemistry that has been outlined previously (8,9). This chemistry can be adapted to several different operational modes ranging from a completely manual approach to either automatic or semi-automatic machines. In this manuscript we also describe for the first time how we have adapted this chemistry to the manual synthesis of deoxyoligonucleotides where test tubes are used as the reaction vessels.

### MATERIALS AND METHODS

#### Materials

Tetrazole, zinc bromide, dimethylaminopyridine, 2,6-lutidine and nitromethane were purchased from Aldrich. Silica was either Vydac A (Separations Group) or Fractosil 500 (Merck). Nucleosides, ribonucleoside triphosphates and CpA were purchased from Sigma. ATP and UTP used for abortive initiation assays (see below) were purified as described by McClure et al. (10) or purchased as HPLC purified samples from ICN. All other materials were reagent grade.

#### Biochemical Procedures

Polynucleotide kinase was purified from *E. coli* cells infected with phage T4 (11). Preparative enzymatic phosphorylation of the 5'-hydroxyl groups on synthetic deoxyoligonucleotides was carried out as described (12). Synthetic DNA duplexes were enzymatically phosphorylated following denaturation of the duplexes (13). Exchange kination of restriction fragments containing a 5'-terminal phosphate was according to the procedure of Berkner and Folk (14) and followed heat denaturation of the DNA. DNA ligase was pur-

chased from NEN. RNA polymerase was purified as described (15). The enzyme was 30-50% active when assayed by the method of Chamberlin and co-workers (16). Gel isolation of an 890 bp *Hae* III restriction fragment of bacteriophage lambda DNA, containing the entire rightward control region, followed a published procedure (10).

Run-off assays were carried out as described (17) but with minor modifications. The reactions were run in buffer containing 30 mM Tris, pH 8.0, 100 mM KCl, 3 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 0.2 mM DTT, and 50 µg/ml BSA. Promoter DNA (4 nM) and RNA polymerase (100 nM) were preincubated for 30 minutes in transcription buffer. Upon addition of heparin to 100 µg/ml, incubation was continued for another 10 minutes. The reaction was initiated by the addition of ATP, GTP and CTP to 200 µM, and labeled UTP (specific activity 10-20 Ci/µmol) to 4 µM. After 10 minutes at 37°C, the reaction was terminated by addition of an equal volume of 6 M urea, 2 mM UTP, 0.2% xylene cyanol and 0.2% bromophenol blue. Aliquots were then analyzed on gels containing 20% polyacrylamide and 7 M urea (18). Experiments exploring the effect of cI repressor on transcription of the synthetic DNA were carried out under conditions chosen to avoid end-binding of RNA polymerase (19; see below) which might interfere with repressor binding. Therefore the RNA polymerase concentration was lowered to 10 nM for these experiments. Analyses of RNA transcription assays were simplified by removing radioactive impurities (found in commercial, labeled UTP) from assay solutions by phenol extraction and ethanol precipitation of RNA prior to loading on polyacrylamide gels.

Functional complex formation between RNA polymerase and promoters was followed by the abortive initiation reaction (20). The standard buffer for these assays was 40 mM Tris-HCl, pH 7.9, 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol; KCl was added as indicated. In experiments where the kinetics of association of RNA polymerase with the P<sub>R</sub> promoter was followed, the reaction was initiated by the addition (at zero time) of RNA polymerase to a prewarmed (37°C) reaction mixture. The final concentrations of the components were as follows: 1 nM DNA, 10 nM RNA polymerase, 1 mM CpA, 40 µM α-<sup>32</sup>P labeled UTP (2-3 X 10<sup>3</sup> cpm/pmol) in 50 µl standard buffer containing 80 mM KCl. At indicated times aliquots were analyzed on Whatman 3 MM paper for the production of CpApU (21). The initial rate of decay of RNA polymerase-promoter complexes was measured as described (22). DNA (15 nM) and RNA polymerase (200 nM) were preincubated for 30 minutes in standard buffer with various concentrations of KCl. At zero time the mixture was diluted two-fold by the addition of heparin in the same buffer to a final concentration of 250 µg/ml. Dissociation

of the complex was followed by removing aliquots at indicated times and assaying for the production of pppApU during a 10 minute incubation at 37°C in standard buffer containing 80 mM KCl, 1 mM ATP and 40  $\mu$ M  $\alpha$ -<sup>32</sup>P labeled UTP.

The cI binding assay followed the procedure of Johnson et al. (23). DNA duplexes, end-labeled with <sup>32</sup>P, were purified on polyacrylamide gels as outlined below. After ethanol precipitation they were dissolved in 10 mM Tris-HCl, pH 7.9, 100 mM KCl and 0.1 mM Na<sub>2</sub>EDTA, heated for 2 minutes in a boiling water bath and cooled slowly (1 h) to 37°C to renature the DNA. Each binding mixture (0.5 ml) contained 1-2 X 10<sup>3</sup> cpm of end-labeled DNA (final concentrations: 10<sup>-11</sup> M for the *Hae* III restriction fragment and the synthetic 67 bp fragment, and 3 X 10<sup>-12</sup> M for the synthetic 75 bp fragment) and various amounts of cI protein (a gift from C. Pabo) in binding buffer: 10 mM Tris-HCl, pH 7.6, 50 mM KCl, 2 mM CaCl<sub>2</sub>, 0.1 mM Na<sub>2</sub>EDTA, 5% (CH<sub>3</sub>)<sub>2</sub>SO<sub>4</sub> and 100  $\mu$ g/ml BSA. After incubation for 20 min at 22°C the mixture was filtered through a Schleicher and Schuell nitrocellulose filter and washed twice with a 0.5 ml aliquot of 10 mM Tris-HCl, pH 7.6, 50 mM KCl, 2 mM CaCl<sub>2</sub> and 0.1 mM Na<sub>2</sub>EDTA. The filters were dried and counted in a toluene based scintillation cocktail. All retention values were corrected for a background of 100-200 cpm found in the absence of added protein. Maximal retention at high protein concentrations was 35-50% of the input radioactivity with the synthetic DNAs and 10% with the *Hae* III fragment.

### Chemical Synthesis in Test Tubes

Two lambda P<sub>R</sub> promoters as described in Figure 1 were synthesized. The 67 bp version lacks a complete O<sub>R</sub><sup>2</sup> site whereas the 75 bp promoter extends five base pairs beyond the O<sub>R</sub><sup>2</sup> binding site. Deoxyoligonucleotides as defined by the brackets were chemically synthesized. Except for segment 4 which was synthesized by a diester procedure (24), the phosphite coupling method utilizing a silica gel support was used for synthesizing deoxyoligonucleotides. The procedure is outlined in Figure 2. The synthesis begins with silica gel derivatized to contain an appropriately protected deoxynucleoside (compounds 1a-d). A cyclical procedure involving removal of the dimethoxytrityl ether to form 2a-d, condensation with 3a-d, capping of unreactive deoxynucleoside with acetic anhydride, and oxidation with iodine complete the steps necessary for adding one nucleotide. A recent more complete outline of this procedure has been published elsewhere (25). For many of the deoxyoligonucleotides that are part of the lambda P<sub>R</sub> promoter, syntheses were completed in 15 ml conical centrifuge tubes. Although the synthesis of deoxyoligonucleotides in centrifuge tubes is not as rapid as some of the continuous flow

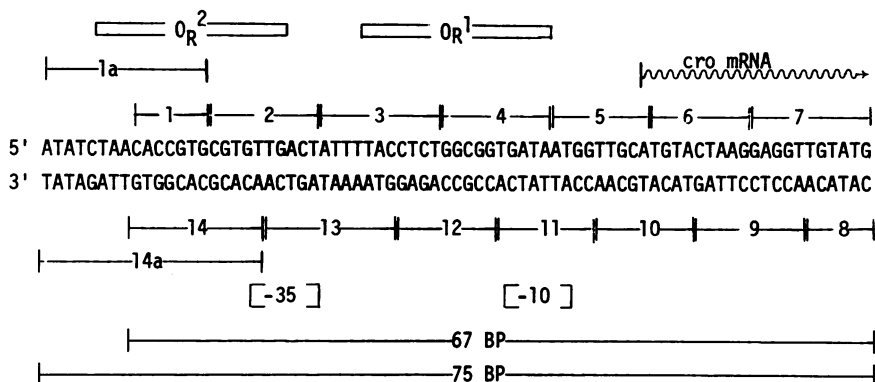


Fig. 1. Synthetic plan for  $P_R$  promoters. The brackets indicate individual, chemically synthesized deoxyoligonucleotides which were enzymatically assembled to give the 67 and 75 base pair duplexes shown. The position of the canonical sequences in the -10 and -35 regions are indicated as well as those of  $OR^1$ ,  $OR^2$  and the presumed start site of transcription.

methods described recently (8,9,26), the method does not require any special equipment. Silica gel is removed from suspension after each step by centrifuging and decanting the supernatant. One full cycle requires approximately 2.5 h and the yields, as measured by the amount of trityl protecting group released during each cycle, averaged greater than 90% per cycle.

Generally eight deoxyoligonucleotides are synthesized simultaneously in eight test tubes. A summary of the synthesis cycle is presented in Table 1.

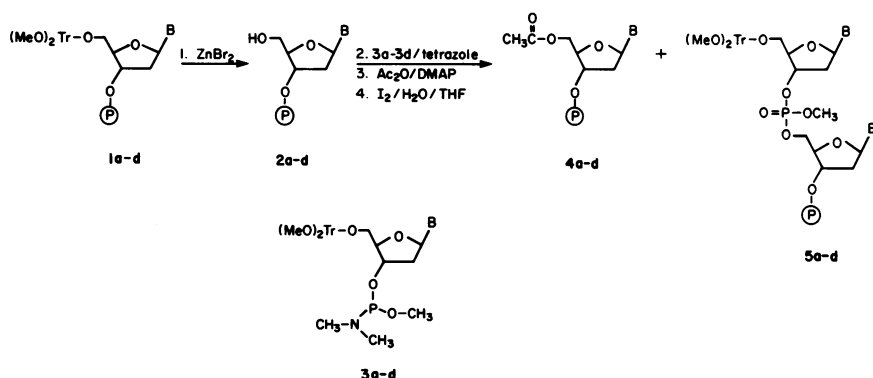


Fig. 2. Steps in the synthesis of a dinucleotide. B. refers to thymine, N-benzoylcytosine, N-benzoyladenine and N-isobutyrylguanine in 1a-d, 2a-d, 3a-d, 4a-d, and 5a-d. See text.

The first cycle starts with step 9 and in the last cycle, steps 5-10 are omitted. Derivatized silica (100 mg) containing approximately 5  $\mu$ moles of compound 1a, 1b, 1c or 1d is added to each test tube. For each coupling reaction (step 1), 540  $\mu$ l of a 0.4 M solution of tetrazole in acetonitrile is added to 40 mg (approximately 50  $\mu$ mole) of compound 3a, 3b, 3c or 3d. This solution is immediately added via syringe to the test tube containing the derivatized silica. Both test tubes and reaction vessels containing the coupling solutions are protected from the air with serum caps and a nitrogen atmosphere. The coupling step is therefore completed under a positive nitrogen pressure and care is taken to ensure that the centrifuge tubes, syringes and coupling solutions are dry. After a five minute incubation of the activated phosphoramidite and derivatized silica, the serum caps are removed from the centrifuge tubes and the remainder of the cycle is completed in open tubes. The capping solution (step 6) was prepared by mixing two reagents (27,28). Dimethylaminopyridine in THF (2 ml; 6.5%, w/v) was first added to the silica gel followed by addition of acetic anhydride:lutidine (1:1; 0.4 ml). The remaining steps are adequately outlined in Table 1. Cleavage of the deoxyoligonucleotide from the silica support, removal of the phosphate and amino protecting groups, and purification of the product on a reverse phase HPLC column are completed as described previously (8).

### Characterization of Deoxyoligonucleotides

The trityl ether is removed from deoxyoligonucleotides after HPLC purification by treatment with 80% acetic acid for 45 minutes. The acetic acid is removed by repeated coevaporation with ethanol. Deoxyoligonucleotides purified in this manner are now ready for enzymatic reactions. The first step is labeling the 5'-end with [ $^{32}$ P] phosphate using [ $\gamma$ - $^{32}$ P]ATP and T4-polynucleotide kinase. Deoxyoligonucleotides are characterized by analytical gel electrophoresis on 20% polyacrylamide containing 7 M urea (18) and by two dimension sequence analysis (29). Our criteria for an acceptable oligomer are: (1) migration as one band on gels; (2) complete digestion to monomers by snake venom phosphodiesterase; (3) absence of "shadow" spots following two dimension analysis; and (4) the correct sequence by the two dimension technique. Oligomers that do not conform to these criteria are further purified by preparative electrophoresis on denaturing 20% polyacrylamide gels. This procedure invariably leads to an acceptable oligomer. All lambda P<sub>R</sub> deoxyoligonucleotides were characterized by analytical gel electrophoresis and enzymatic degradation. Additionally all segments except 1a and 14a were analyzed by the two dimension technique.

Table 1. Cycle for Chemical Synthesis of Oligonucleotides in Test Tubes

Step No.	Solvent or Solution	Function	Volume	Reaction Time
1	0.4 M tetrazole and 0.1 M nucleoside phosphoramidite in freshly distilled acetonitrile	Phosphite coupling	0.6 ml	5 min
2	THF, H <sub>2</sub> O, lutidine (2:2:1)	Wash	2 X 10 ml	
3	0.2 M iodine in THF, H <sub>2</sub> O, Lutidine (2:2:1)	Oxidation	2 ml	1 min
4	Acetonitrile	Wash	3 X 10 ml	
5	THF	Wash	1 X 10 ml	
6	5.4% (w/v) dimethylaminopyridine, 8.3% (v/v) acetic anhydride, 8.3% (v/v) lutidine in THF	Capping	2.4 ml	4 min
7	Methanol	Wash	2 X 10 ml	
8	Nitromethane	Wash	10 ml	
9	Nitromethane, methanol (19:1) saturated with zinc bromide	Detritylation	3 X 5 ml	2 min ea
10	Nitromethane	Wash	10 ml	
11	Methanol	Wash	2 X 10 ml	
12	THF	Wash	2 X 10 ml	
13	Freshly distilled acetonitrile	Wash	3 X 10 ml	

#### Enzymatic Assembly of Promoters

Half-promoters consisting of segments 1a, 2, 3, 12, 13, 14a, and 4, 5, 6, 7, 8, 9, 10, 11 were synthesized, purified, and then combined to yield the final product. For each promoter, 100 pmoles of deoxyoligonucleotide was used. All segments, except the 5'-end segments 1a and 8, were phosphorylated at their 5'-hydroxyl ends to approximately 0.005  $\mu$ Ci per pmole using T4 polynucleotide kinase and [ $\gamma$ -<sup>32</sup>P]ATP. Following heat inactivation of the kinase, the appropriate segments were pooled in stoichiometric amounts and the mixture was ethanol precipitated. The pellets were taken up in 15  $\mu$ l ligase buffer (10 mM MgCl<sub>2</sub>, 40 mM hepes, pH 7.8), warmed to 70°C, cooled slowly to room temperature (1 h), and then transferred to an ice bath. Ligase (1 unit per 100 pmoles of potential ligation sites), ATP (150  $\mu$ M) and DTT (10 mM) were then added, and the reaction was allowed to proceed at 0°C for 16 h. Analytical gel electrophoresis indicated that 30-50% of the radioactivity originally present in the deoxyoligonucleotides was in slow moving band(s) corresponding to the ligated half molecules. The half-promoter

duplexes were isolated free of intermediates and unligated starting materials by chromatography on a 10 ml Sephadex G 100 column using 0.01 M triethylammonium bicarbonate as eluent.

The half-molecules were joined using essentially the same procedure. Purification of the synthetic lambda P<sub>R</sub> promoter was on a non-denaturing, 12% polyacrylamide gel (30). The DNA duplex corresponding to the promoter migrated with a mobility of 0.4 (relative to xylene cyanol blue). The polyacrylamide containing this promoter was cut from the gel. The promoter was extracted from the gel slice (13), purified (10) on a DE 52 column (0.1 ml), ethanol precipitated, and dissolved in a solution containing 0.01 M Tris (pH 8.0), 0.1 M KCl, and 0.1 mM EDTA. The final concentration of promoter was 100 nM. Assembly of the 67 bp promoter followed the same general procedure with the half-promoter duplexes being composed of segments 1, 2, 3, 4, 8, 12, 13, 14 and 5, 6, 7, 8, 9, 10, 11. Generally the yield of a promoter duplex was 5-10 pmole when 100 pmole of each segment was used to form the half-promoters.

The 67 and 75 bp duplexes were characterized by electrophoresis on polyacrylamide gels using denaturing conditions. The gel pattern is shown as Figure 3. The analysis indicates that the individual strands of each double helix migrate with slightly different mobilities and that essentially all (greater than 90%) of the radioactivity for both samples is present in these deoxyoligonucleotides. Based on the mobility of restriction fragments having known lengths as markers (lane a), these deoxyoligonucleotides migrate at the

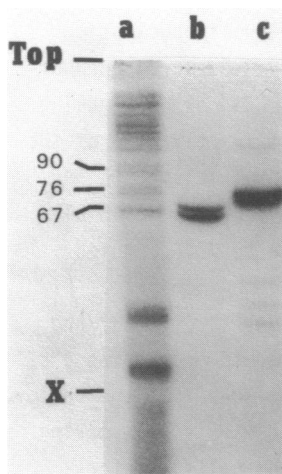


Fig. 3. Gel analysis of assembled DNA duplexes. After gel purification the 67 and 75 base pair DNAs (see Fig. 1) were labeled with <sup>32</sup>P at their 5'-hydroxyl groups as described in Materials and Methods, and the labeled DNA was analyzed on a 20% polyacrylamide, 7 M urea gel (18). On the autoradiograph of the gel, X indicates the position of the xylene cyanol dye marker. Lane a: *Hpa* II digest of pBR322, radiolabeled by exchange kination (14), as source of size markers. The length of some fragments (32) are indicated. Note that the DNA strands for the 76 and 90 base pair duplexes were separated. Lane b and c contain the 67 and 75 base pair promoters respectively.



size expected for the appropriate synthetic promoter segments. Thus the synthetic duplexes consist of double helical DNA having intact strands.

Analysis using the dideoxy sequencing procedure (31) confirmed that these duplexes had DNA sequences as shown in Figure 1.

#### RESULTS AND DISCUSSION

The synthetic lambda  $P_R$  promoters are transcribed by *E. coli* RNA polymerase. The results of a transcription assay using all four triphosphates is shown in Figure 4. The major transcription product is a series of bands corresponding to the RNA product expected if initiation of transcription on the synthetic promoter is at the same position (Figure 1) as has been found for the  $P_R$  promoter carried on lambda phage (33). Size heterogeneity of the runoff RNA product is also observed for transcription of an 890 bp *Hae* III fragment of phage  $\lambda$  containing the entire rightward control region of the phage, including the  $P_R$  promoter (34; our unpublished results). From the observation that both the 67 bp and the 75 bp promoters direct transcription of a runoff RNA that has the size predicted from initiation at the correct position, we draw two important conclusions: Transcription initiates at the same position on both promoters and proceeds in the proper direction.

The kinetics of formation and dissociation of open complexes were meas-

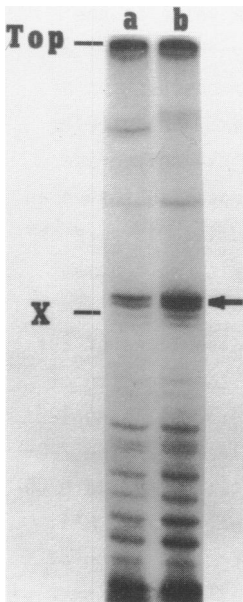


Fig. 4. Transcription of the 67 and 75 base pair DNA duplexes with *E. coli* RNA polymerase. Analysis of the radiolabeled RNA products was by electrophoresis on a 20% polyacrylamide, 7 M urea gel (18). X indicates the xylene cyanol dye marker. The RNA product presumed to originate at the normal start site of the  $P_R$  promoter is indicated by an arrow. The templates were: Lane a: the synthetic 67 base pair promoter. Lane b: the synthetic 75 base pair promoter.

ured in parallel experiments for both the chemically synthesized 75 bp fragment and the *Hae* III restriction fragment. Detection of open complexes was by the abortive initiation assay (22,35), in which promoter occupancy was determined from a quantification of the reiterative synthesis of RNA oligoribonucleotides by promoter bound RNA polymerase. In reactions where the association reaction is monitored, a lag is typically seen, which, although dependent on the conditions of the experiment, is usually longer for weak promoters than for strong promoters. Notably, the observed lag time is generally inversely related to the concentration of RNA polymerase (35). However with the small synthetic fragments but not the 890 base pair restriction fragment the reverse was found to be true for RNA polymerase concentrations between 20 and 67 nM; above 67 nM, the lag times reached a plateau of about 12 minutes. Consequently, at RNA polymerase concentrations above 20 nM, much larger lags were observed with the synthetic DNA fragments than with the restriction fragment. We propose that at these concentrations progressively more RNA polymerase molecules bound to the ends of the DNA fragments as the concentration of the enzyme increased. The binding of RNA polymerase to flush ends of restriction fragments of T7 DNA lacking known promoters has been invoked by Melançon et al. (19) to explain RNA polymerase mediated retention of these fragments on nitrocellulose filters. These workers estimated a dissociation constant of 2-30 nM for this interaction. Our observations on the synthetic 75 bp fragment are consistent with a value at the upper limit of this range. Due to the small size of the synthetic fragments, an end-bound RNA polymerase would interfere with the proper positioning of another polymerase molecule for formation of an open complex. The problem was circumvented by working at a very low concentration of RNA polymerase (10 nM) in order to minimize binding to DNA termini. The results of such an experiment are shown in Figure 5. Lag times of 1.2 min and 1.3 min were obtained for the chemically synthesized DNA and for the restriction fragment indicating that the synthetic DNA functioned as well as the natural fragment. A larger number of similar experiments has yielded average lag times of  $2 \pm 1$  min for both DNAs. This value is in good agreement with the lag time of 110 seconds obtained with the  $P_R$  promoter in standard buffer containing 120 mM KCl (21). The turnover rates with the two DNAs, as reflected by the slopes of the curves, differ by approximately a factor of two. This is probably due to an uncertainty in the relative concentrations of the two DNAs, rather than to an intrinsic difference between them. The concentration of the restriction fragment was determined spectrophotometrically, while that of

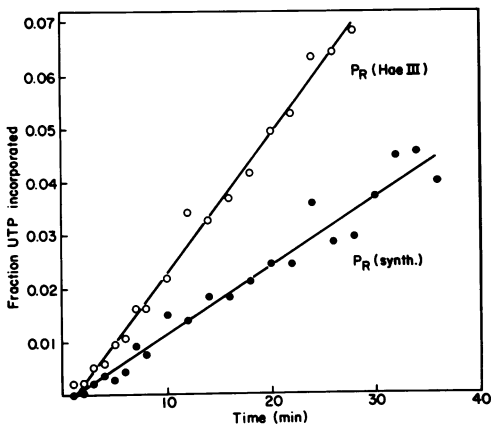


Fig. 5. Kinetics of association of *E. coli* RNA polymerase with the synthetic 75 base pair duplex and with a restriction fragment bearing the  $P_R$  promoter sequence. Abortive initiation reactions were run at 37°C as described in Materials and Methods. (●) Synthetic 75 base pair promoter; (○) *Hae* III fragment.

the synthetic 75 base pair fragment derived from the specific activity of the purified radiolabeled DNA.

The kinetics of dissociation of RNA polymerase from the two different DNAs were also indistinguishable, with half-lives of  $3.4 \pm 1$  h for the experiment shown in Figure 6. This is in reasonable agreement with the published (21) half-life of 4.8 h for the  $P_R$  promoter on the *Hae* III restriction fragment under similar experimental conditions. The dissociation kinetics of RNA polymerase from the synthetic 75 bp fragment and the *Hae* III fragment were also similar at 160 and 80 mM KCl in standard buffer, with half-lives of one and ten hours (160 and 80 mM KCl respectively, data not shown).

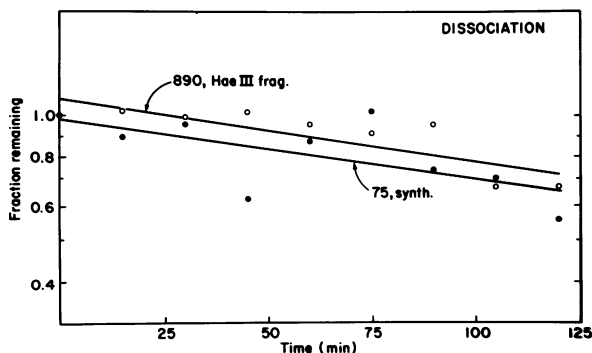


Fig. 6. Kinetics of dissociation of *E. coli* RNA polymerase from the synthetic 75 base pair duplex and from a restriction fragment bearing the  $P_R$  promoter sequence. The reactions were run at 37°C in standard buffer containing 0.12 M KCl. (●) Synthetic 75 base pair promoter; (○) *Hae* III fragment.

The interaction between the synthetic DNA duplexes and cI repressor was measured in equilibrium binding experiments employing a nitrocellulose filter assay to monitor the formation of a complex (see Figure 7). The amounts of protein required to effect half maximal retention of DNA on a filter were very similar for the 67 base pair duplex, the 75 base pair duplex and the *Hae* III restriction fragment, indicating similar cI binding affinities for these DNAs. The only intact repressor binding site that all three DNAs have in common is  $O_R^1$ . The results are thus in general agreement with the finding (5), that of the three operators  $O_R^1$  has by far the greatest affinity for cI, and that the additional presence of  $O_R^2$  (and  $O_R^3$ ) is of minor significance for the overall affinity of the protein for the DNA. The smooth curve drawn in Figure 7 was calculated assuming that cI monomers are in equilibrium with dimers and that only the latter bind to the DNA (23). The best fit to the data points was obtained with half maximal DNA retention occurring at 12 ng/ml cI protein. In a similar buffer, but at pH 7.0, 1.5 ng/ml of cI protein was found to be sufficient for half maximal retention of DNA containing  $O_R^1$  (23). After correction for the observed effect of pH on the binding affinity (23) our value is within a factor of 3 from the published value. The remaining difference is well within a range that can be explained by a combination of experimental error and the use of two different preparations of cI protein.

The binding assay employed is meaningful only when repressor dimers are in excess over the DNA throughout the relevant range of protein concentrations. When this condition is satisfied, the protein concentration giving half maximal DNA retention is independent of the DNA concentration in the assay mixture. Indeed, with the 890 base pair *Hae* III restriction fragment,

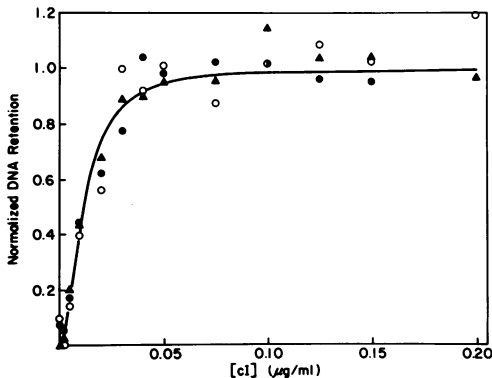


Fig. 7. Repressor binding assays with the synthetic 67 and 75 base pair duplexes and the *Hae* III restriction fragment. The values plotted on the abscissa have been normalized to the plateau values for each DNA. (▲): Synthetic 67 base pair promoter; (●) Synthetic 75 base pair promoter; (○) *Hae* III fragment.

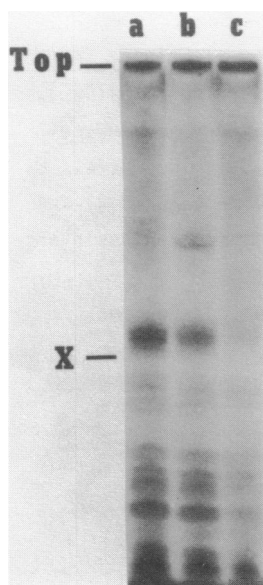


Fig. 8. Effect of cI repressor on the transcription of the synthetic 75 base pair promoter by *E. coli* RNA polymerase. Analysis of the transcripts by electrophoresis on a 20% polyacrylamide, 7 M urea gel (18). Each lane contained  $0.95 \times 10^6 \pm 10\%$  cpm. X indicates the position of the xylene cyanol dye marker. Prior to simultaneous addition of ribonucleoside triphosphates and heparin, the reactions were preincubated at  $37^\circ\text{C}$  as follows: Lane a: RNA polymerase, 5 min. Lane b: RNA polymerase, 5 min followed by repressor (20  $\mu\text{g}/\text{ml}$ ), 5 min. Lane c: Repressor (20  $\mu\text{g}/\text{ml}$ ), 5 min, RNA polymerase, 5 min.

altering the DNA concentration by a factor of 2 (when compared to the concentration of DNA used for the experiments reported in Figure 7) had no effect on the titration curve (data not shown).

The presence of a functional binding site on the synthetic DNA is also inferred from the effect of repressor on the transcription of the synthetic 75 base pair promoter by *E. coli* RNA polymerase. From the autoradiograph shown in Figure 8 it can be seen that cI protein inhibits the synthesis of the putative runoff RNA if incubation of the template with repressor precedes the addition of RNA polymerase. Only a slight inhibition is seen when the two proteins are added in reverse order. Thus the effect is specific and not a result of the presence of contaminants in the cI repressor preparation.

### CONCLUSION

We have described an adaptation of the solid support, phosphite triester DNA synthesis method where test tubes are used as reaction vessels. The synthesized DNAs (67 and 75 base pair lambda  $P_R$  promoters) were specifically recognized by two different proteins, RNA polymerase and cI repressor, demonstrating the feasibility of using selective base analog substitutions in our synthetic DNA to probe for the functional groups which are recognized by these proteins.

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