Volume 11 Number 17 1983 Nucleic Acids Research

Gene expression: chemical synthesis and molecular cloning of a bacteriophage T5 (T5P25) early promoter

Johanne Rommens, Douglas MacKnight, Lynn Pomeroy-Cloney and Ernest Jay+

Department of Chemistry, University of New Brunswick, Fredericton, New Brunswick E3B 6E2, Canada

Received 22 June 1983; Revised and Accepted 9 August 1983

ABSTRACT

A sixty base pair DNA duplex containing the nucleotide sequence of the bacteriophage T5 early (T5P25) promoter has been constructed using a combination of chemical synthesis and enzymatic methods. Subsequent to cloning into pBR322, the promoter has been demonstrated to be biologically active being capable of directing the efficient expression of genes under its control. This serves as a prototype for an approach to the study of the in vivo structure-function relationships and efficiency of promoters.

INTRODUCTION

Gene expression is controlled, in part, at the level of transcription. The productivity of transcription, together with the variation in the stabilities of the RNA transcripts determine the level of the mRNA present in the prokaryotic cell. The regulation of transcription is believed to be achieved by modulating the efficiency with which the DNA dependent RNA polymerase (RNA polymerase) recognizes and interacts with the initiation site (promoter) on the DNA template (review: ref. 1,2). Much effort has been devoted in recent years to study the functional activities of prokaryotic promoters and to identify specific signals in the DNA template required for recognition by the RNA polymerase during the initiation process. This would provide a better understanding of the structure-efficiency relationships of different promoters. Two sets of more or less conserved sequences up-stream from the start point of transcription have been identified and are believed to be involved in interaction with RNA polymerase (3). These two domains, referred to as the -35

region and the -10 region (Pribnow box) have the consensus sequences of 5'-T-T-G-A-C-A (4,5) and 5'-T-A-T-A-A-T (6,7), respectively. Despite the extensive sequence information for more than one hundred promoters (8), little is understood about what features determine the efficiency of promoters and how sequence changes in the two conserved domains and other parts of a given promoter affect the promoter efficiency. Attempts to correlate the efficiency of promoters in different transcriptional units with their promoter sequences may not be very meaningful since other factors may indirectly affect their overall transcription. Other approaches, including *in vitro* binding studies, do not necessarily provide real *in vivo* information.

A probable approach to this problem would be to construct a versatile system whereby the promoter of a specific transcriptional unit may be specifically removed without affecting other parts of the DNA template. The promoter can then be replaced by its analogues containing specific base modifications, insertions or deletions, or by other well defined promoters. Assessment of the ability of the different promoter sequences to express the same gene under identical environments *in vivo* would allow meaningful comparison of the strength of the different promoters. This may provide more conclusive insight into the direct contributions of specific structural features to the functional activity of promoters. Such an approach may be accomplished using a combination of chemical synthesis of DNA and molecular cloning. This would initially require the synthesis and construction of a DNA duplex that corresponds to a promoter. It could then be appropriately inserted onto a gene which itself is void of a promoter. After insertion into a suitable vector and subsequent cloning into *E. coli*, expression of this gene may be measured by a number of ways. Once this is successful using the prototype promoter, other promoters and promoter analogues may be synthesized so their biological activities could be assessed and compared.

A large number of DNA fragments containing promoters from various sources have been used for *in vitro* binding studies.

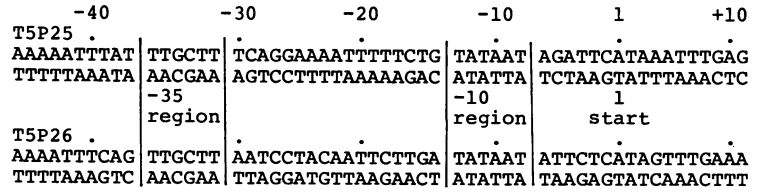

Figure 1. Nucleotide sequence of the bacteriophage T5 (T5P25 and T5P26) early promoter regions.

The bacteriophage T5 early promoters (T5P25 and T5P26) have been shown to far exceed other promoters in the rate of complex formation with RNA polymerase (9,10). Surprisingly, neither of the T5 promoters contains the complete -35 region consensus sequence of 5' T-T-G-A-C-A. Earlier attempts to directly clone DNA fragments carrying the early T5 promoters have not been successful unless a strong termination signal for transcription is simultaneously inserted downstream (11). It thus appears that the chemical synthesis and cloning of the T5 promoter and its analogues could provide an interesting system for studying the relationship of the structural features of promoters and their functional activities. Furthermore, inclusion of an inducible control signal in conjunction with the very strong T5 promoter might provide a valuable 'super promoter' for high level transcription and expression of mammalian genes in *E. coli*.

We report here the construction of a 60 base pair DNA duplex using a combination of chemical and enzymatic methods. The duplex contains the T5P25 promoter sequence which spans the region from position -46 to +10. It has been cloned into pBR322 in which the fragment between the EcoRI ahd Hind III sites had been removed. Thus, the natural promoter for the tetracycline resistance (Tc^r) gene was removed and replaced by the synthetic T5P25 promoter. The biological activity of the synthetic T5P25 promoter has been demonstrated by the ability of *E. coli* cells carrying the recombinant plasmid (pJP_1) to exhibit Tc^r.

MATERIALS AND METHODS

Materials:

CHEMICALS. Deoxynucleosides were purchased from Sigma. Silica gel (Vydak A) used as support for deoxyoligonucleotide synthesis was purchased from Separations Group. Formamide used in the HPLC purification of the oligonucleotides were purchased from BDH Chemicals. All other reagents and solvents were reagent grade and purchased from Fisher Scientific or Aldrich Chemical Co. All other biochemicals were purchased from Sigma. ENZYMES AND RADIOISOTOPES. T4 DNA ligase and restriction enzymes, EcoRI and Hind III and Hae III were purchased from BRL. T4 polynucleotide kinase was purchased from Biogenics. Calf alkaline phosphatase was purchased from Bohringer Mannheim. [γ-32P]-ATP was purchased New England Nuclear Corp. DNA AND BACTERIAL STRAINS. pBR322 and the constructed pJP_1 DNA were isolated according to published procedures (12,13). E. coli K-12 (LE 392) strain were from Dr. G. Jay (NIH).

STRATEGY FOR THE CONSTRUCTION OF THE T5P25 SYNTHETIC PROMOTER.

To ensure inclusion of all the necessary recognition sequences of the T5 promoter, the synthetic duplex included from 10 base pairs upstream of the -35 sequence, through the Pribnow box and up to 9 base pairs downstream from the initiating nucleotide. To demonstrate the feasibility of the approach and the biological activity for the synthetic promoter, it would initially be used to replace the Tc^r promoter in pBR322. The Tc^r promoter conveniently lies between

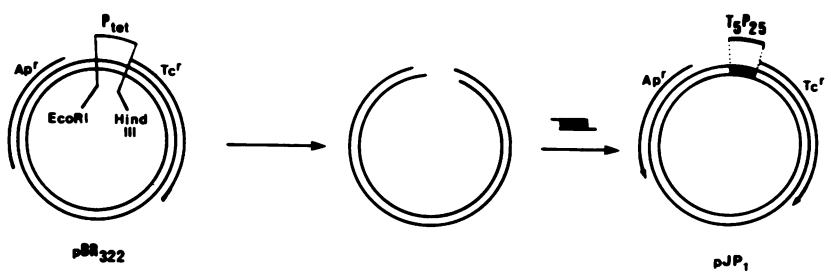

Figure 2. Scheme for the construction of the pJP_1 plasmid containing the synthetic T5P25 promoter and testing of the biological activity for the synthetic promoter.

the EcoRI and the Hind III cleavage sites (14). The synthetic duplex thus contains, in addition to the promoter sequence, also an EcoRI enzyme cohesive sequence and an Hind III enzyme cohesive sequence at the termini as shown in figure 3. This allows its proper insertion into pBR322 in which the small fragment between the EcoRI and the Hind III sites has been removed. E. coli cells harboring this modified plasmid (pJP_1) should exhibit tetracycline resistance if the synthetic promoter is biologically functional. A similar assay to demonstrate biological activity for a modified Tc^r promoter that replaced the natural one has been reported (15).

CHEMICAL SYNTHESIS OF THE PROMOTER FRAGMENTS.

Ten oligonucleotide fragments varying in chain lengths from 10 to 14 nucleotides were chemically synthesized using an improved modified (16) rapid solid phase phosphite approach (17). The promoter duplex was dissected in such a way as, shown in figure 3, to minimize the presence of repetitive, complementary or palindromic sequences between fragments. This would avoid non-specific hybridizations between the fragments and enable their proper ligation to form the complete duplex.

The syntheses were carried out in glass columns (10 mm diameter) in which the properly derivatized silica gel (100 mg) containing 50 - 100 μmole nucleoside per gram polymer was tightly packed. The reagents were sequentially passed through the column at a flow rate of 2-3 mL/min using a HPLC pump

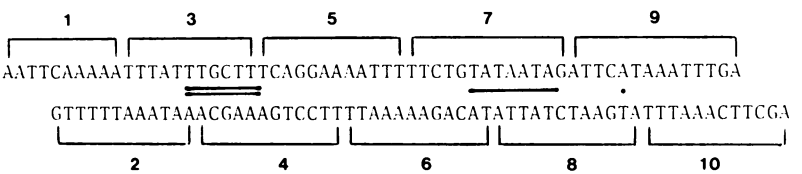

Figure 3. The synthetic plan for the 60 base pair T5P25 promoter duplex. The brackets indicate the ten individual synthetic oligonucleotides. The location of the -35 region is indicated by [=] and the -10 region is indicated by [-]. The initiating nucleotide is indicated by [.]. The EcoRI enzyme cohesive sequence (AATT) is located at the left terminus and the Hind III enzyme cohesive sequence (AGCT) is located at the right terminus of the duplex.

Table 1.

Reaction cycle for chemical synthesis of oligonucleotides

Step	Reagent	Function	Time (min)
1	Chloroform ($CHCl_3$)	Washing	2
2	2% Trichloroacetic acid/$CHCl_3$ at 4°	Detritylation	2
3	10% Pyridine in Acetonitrile	Drying	3
4	Nucleoside phosphite at 4°	Coupling	3
5	Iodine solution	Oxidation	2
6	Phenylisocyanate/Pyridine	Capping/drying	0-2
		Total cycle time	14

Details of the chemical synthesis will be published elsewhere.

according to Table 1.

After completion of the synthesis, the protecting groups were removed similarly as described by published methods (17). The phosphate protecting groups were first removed using thiophenol (18). The oligonucleotides were hydrolyzed from the polymer and the amino protecting groups were simultaneously removed using ammonium hydroxide (19). The purification of the fully deprotected products was carried out with High Pressure Liquid Chromatography (HPLC) on Partisil 10-SAX anion exchange column using a 0-1 M NaCl gradient in 80% formamide. The major peak which contained the correct product was collected and further purified on ODS C_{18} reversed phased column using a 10-15% acetonitrile gradient in water buffered with 100 mM triethylammonium acetate. The major peak which again contained the desired product was lyophillized to remove the solvents and triethylammonium acetate. This invariably gave the products in greater than 90% purity as judged by electrophoretic fractionation in 20% polyacrylamide gel containing 8M urea (20) after labelling the 5' end using [γ-32P]-ATP and polynucleotide kinase (21).

CHARACTERIZATION OF SYNTHETIC DEOXYOLIGONUCLEOTIDES

The oligonucleotides isolated from HPLC were labeled at

the 5' end and analyzed on a 40 cm 20% polyacrylamide gel containing 8M urea for purity and size determination. Each synthetic oligonucleotide has been fully characterized by sequence analysis using a modified (22) Maxam and Gilbert chemical degradation method (23). The conditions for each of the five reactions were as follows: G reaction: dimethylsulfate at 20° for 30 min; A/G reaction: pyridinium formate pH2 at 50° for 80 min; T/C reaction: hydrazine at 50° for 20 min; T reaction: 0.5mM $KMnO_4$ at 20° for 20 min (24); C reaction: hydrazine with 1.8M NaCl at 50° for 20 min. The hydrazine reaction mixtures after the first precipitation were treated with acetylacetone prior to piperidine hydrolysis as previously described (22).

CONSTRUCTION OF THE PROMOTER DUPLEX

The oligonucleotide fragments 1 to 10 were phosphorylated at the 5' termini using [γ-32P]-ATP (20 Ci/mmole) and T4 polynucleotide kinase (21). 5 pmole of each of the phosphorylated oligonucleotide fragments were used for the preparative construction of the promoter duplex. The oligomers were combined in half the volume (25 µL) for ligation containing 100 mM Tris-HCl pH 7.5 and 20 mM $MgCl_2$ and heated at 70° for 5 min. The mixture was allowed to slowly cool to 4° over a period of 4 hours to allow proper hybridization of the fragments. The ligation reactions were carried out in a final volume of 50 µL at 12.5° for 16 hours after adjusting to contain 2 mM oligonucleotide (total), 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP and 50 units/mL T4 polynucleotide ligase. Since the termini of the duplex contain an EcoRI cohesive sequence at one end and a Hind III cohesive sequence at the other, polymerization of the promoter duplex occurred to form large oligomers. Recleavage of the ligation mixture with both EcoRI and Hind III enzymes converted the promoter oligomers into the desired monomer. This has been taken advantage of to allow unambiguous identification of the band corresponding to the correct duplex after separation of the ligation products on a 10% polyacrylamide gel. The DNA band was detected by autoradiography and the gel slice containing the promoter duplex was extracted, desalted on a micro DE 52 column

(50 µL volume) and recovered by precipitation with ethanol. The yield of the recovered promoter was 25-30%.

CONSTRUCTION OF pJP_1 RECOMBINANT PLASMID

pBR322 DNA (5 µg) was digested with Hind III followed by EcoRI. The larger of the two fragments produced was isolated after electrophoresis on 1% agarose gel by electro-elution (25) and recovered by precipitation with ethanol. 0.5 µg (ca. 0.18 pmole) of this large fragment was ligated to one molar equivalent of the purified synthetic promoter under conditions (20 µg/mL) that favors circularization of plasmid DNA (26). After transfection to competent *E. coli* LE 392 cells, clones resistant to ampicillin (Ap) were initially selected as previously described (27). On the assumption that the synthetic promoter would be biologically active and thus capable of restoring the ability of the tetracycline resistance gene present in the pBR322-EcoRI/Hind III large fragment to be expressed, clones were further screened for tetracycline resistance on nutrient agar plates containing the antibiotic (20 µg/mL). Several of these Ap^rTc^r clones were isolated for further characterization by restriction mapping, Southern Blotting hybridization (27) and sequence analysis (21,23).

RESULTS AND DISCUSSION

CHEMICAL SYNTHESIS OF THE PROMOTER FRAGMENTS

The chemical syntheses of the ten promoter fragments 1 to 10 shown in figure 3 were carried out by the rapid solid phase phosphite approach described in the Methods Section. Attempts to purify the oligomer mixtures obtained from the syntheses with HPLC and ion-exchange columns using published elution buffer systems (29,30) did not give satisfactory results in our hands. It has been observed that oligomers containing several G residues in a row could not be separated (28). The purification of all the promoter fragments was achieved using a 0 - 1M NaCl gradient containing 80% formamide and buffered with 50 mM sodium acetate (pH 6.5) at room temperature for elution. A typical elution profile for purification of the synthetic mixtures using Partisil 10-SAX column after removal of all protecting groups is shown in figure 4a for fragment 9. The

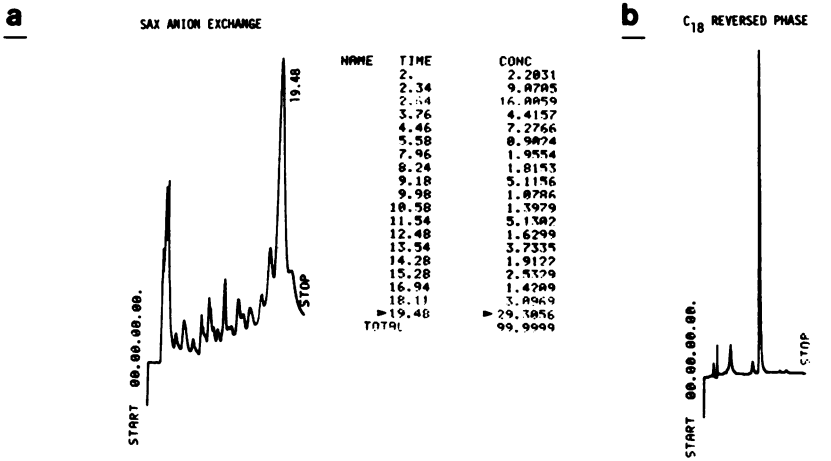

Figure 4. HPLC purification of promoter fragment 9 on (a) Partisil 10-SAX anion exchange column and (b) ODS (Beckman) C_{18} reversed phase column. The major peak from the anion exchange column with the retention time of 19.48 min was collected and further purified on the reversed phase column after desalting. The major peak from the reversed phase contained the desired fragment 9.

major peak with retention time of 19.48 min, which corresponded to 30% of all UV (270 nm) absorbing materials after 14 coupling cycles was further purified on a C_{18} reversed phase column after desalting. The major peak shown in figure 4b, which contained the correct product was collected for further characterization.

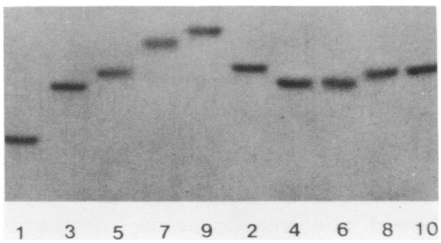

Figure 5. Polyacrylamide gel (20%) electrophoresis analysis of the synthetic oligonucleotides 1 to 10 after labeling their 5' termini using [γ-32P]-ATP and polynucleotide kinase.

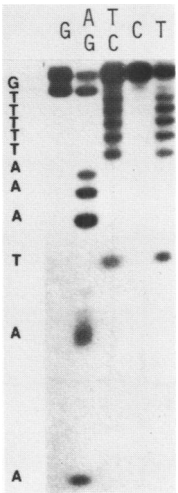

Figure 6. Sequence analysis of promoter fragment 2 using a modified Maxam and Gilbert chemical cleavage method.

The oligomers were terminally labelled using polynucleotide kinase and [γ-32P]-ATP for purity and size analysis by electrophoresis in denaturing polyacrylamide gels containing 8M urea as shown in figure 5. Using this sequence of procedures for the purification of the synthetic products invariable gave oligomers with greater than 95% purity. This demonstrates the efficiency of the synthesis and the purification systems. All ten promoter fragments were fully characterized using the modified (23) Maxam and Gilbert chemical cleavage method (22) as shown in figure 6 for fragment 2.

CONSTRUCTION OF THE PROMOTER

The preparation of the complete promoter was carried out in a single ligation reaction. The proper conditions for the construction of the complete promoter duplex was worked out by carrying out analytical ligation mixtures containing increasing numbers of the oligomer fragments in equimolar amounts (0.5 pmole) to stepwise produce DNA duplexes of increasing chain lengths. This allowed the unambiguous identification, as shown in figure 7a, of the complete duplex (the major band in lane h) after separation by electrophoresis on polyacrylamide gel. It can be seen starting from the four-fragment ligation in lane b, that with the exception of the seven-fragment ligation in

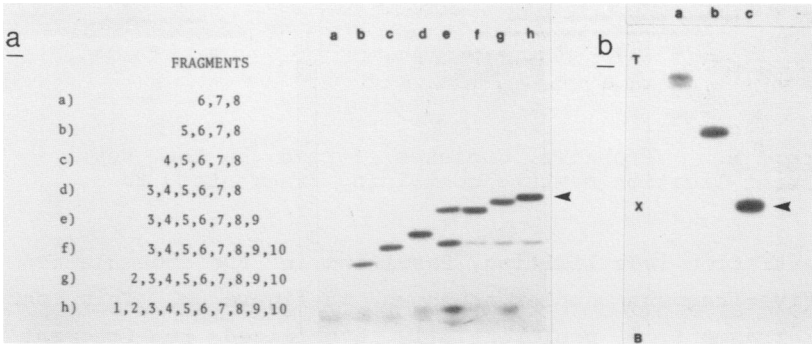

Figure 7. Construction of the T5P25 promoter duplex by ligation of the ten synthetic oligonucleotides using T4 DNA ligase. (a) Analytical ligations using increasing numbers of promoter fragments to sequentially produce larger DNA duplexes. The fragments present in each of these ligation mixtures are indicated on the left. The reaction mixtures were analyzed on a 10-15% gradient polyacrylamide gel. The reaction mixtures in lanes f and g were digested with EcoRI and the reaction mixture in lane h was digested with both EcoRI and Hind III prior to gel electrophoresis. (b). Preparation of the promoter duplex for subsequent cloning. Lane a: Ligation reaction containing all 10 promoter fragments. Lane b: Ligation mixture in lane a cleaved with EcoRI. Lane c: Ligation mixture in lane a cleaved with both EcoRI and Hind III. The complete promoter duplex is indicated by ◄

lane e the expected larger duplexes were progressively formed with the increase in the numbers of fragments present in the ligation mixture. This demonstrated that the proper ligation of the fragments at each step and the correct formation of the ten fragment complete promoter duplex in lane h had occurred. In lane e, instead of the expected seven-fragment duplex, an eight-fragment and a five-fragment duplex were formed. Examination of the nucleotide sequence showed that there is a stretch of six nucleotide homology between the 3' ends of fragments 5 and 10. Thus, in the absence of fragment 10, it appears that fragment 5 also hybridized to fragment 9 and was ligated to fragment 8 to form an eight fragment duplex as shown in figure 8a.

Since fragment 5 was used twice in the formation of the eight-fragment duplex, it, being present in equimolar

Figure 8. Probable duplexes formed in the seven component ligation mixture containing fragments 3 to 9.

concentration was limiting, resulting in the accumulation of the five-fragment duplex as shown in figure 8b. This showed that fragment 5 preferentially hybridized to fragment 9 containing six base pairs, despite the presence of two terminal mismatched bases, rather than to fragment 6 containing five base pairs and without mismatches. When fragment 10 was added to the mixtures in lanes f to h, it preferentially hybridized to fragment 9 and thus prevented most of fragment 5 from hybridizing to fragment 9. This gave the desired eight, nine and ten-fragment duplexes respectively. Small amounts of the undesirable eight component duplex was however still evident in these lanes. This could presumably be totally suppressed by the use of a slight excess of fragment 10. This difficulty was anticipated as a result of the computer analysis of the promoter sequence. It was originally planned to perform the ligation of the promoter by first constructing two intermediate blocks containing fragments 1-6 and 7-10. However, the results obtained indicated that the yield of the complete duplex was sufficiently high in the single step ligation containing all ten fragments.

It should be noted that since the two end fragments, 1 and 10, contained restriction enzyme cohesive sequences, dimers or oligomers of the expected partial and complete promoter duplexes respectively would be formed. Thus all ligation mixtures that contained fragment 1 were digested with EcoRI, and all ligation mixtures that contained fragment 10 were digested with Hind III prior to their analysis on PAGE (fig. 7a). The formation of the oligomers and the requirement to digest the ligation mixtures with the restriction enzymes to provide the monomeric promoter duplexes may be prevented by using hydroxyl instead of phosphoryl ended fragments 1 and 10

for the ligation. However, this gave inferior ligation and the duplex would have to be phosphorylated for subsequent ligation reactions. Furthermore, the oligomerization of the promoter duplex, as shown later, may be advantageously used in the characterization of the ligation reaction since only proper ligation of all ten promoter fragments to form the complete duplex would allow oligomerization into large duplexes.

The preparative construction of the promoter duplex for subsequent cloning was thus carried out using the conditions worked out in the analytical ligation reactions. Equimolar amounts of all ten fragments 1 to 10 were used and the results are shown in figure 7b. Upon ligation, oligomerization of the promoter duplex formed and resulted in large oligomers as seen in lane a. Digestion of the oligomeric products with Hind III (or alternatively with EcoRI) converted the complicated mixture into the dimer as shown in lane b. Upon digestion with both Hind III and EcoRI, they were all converted into the desired monomeric promoter duplex containing the EcoRI and the Hind III cohesive ends for subsequent insertion into the plasmid vector. This, as mentioned above, further verified that the band in lane c in figure 7b, contained the correct duplex. Furthermore, it can be seen that it has the same mobility as that produced in the analytical step ligation in lane h of figure 7a. The duplexed was isolated from the gel for insertion into pBR322. A total of 1.7 pmole of the promoter duplex was recovered starting from 5 pmole each of the synthetic fragments.

CONSTRUCTION AND CHARACTERIZATION OF THE SYNTHETIC T5P25 PROMOTER CONTAINING pJP_1 RECOMBINANT PLASMID

The constructed synthetic promoter duplex (0.1 pmole) was inserted into pBR322 plasmid DNA in which the small fragment between the EcoRI and Hind III sites had previously been removed. The terminal phosphate groups in the plasmid DNA had been removed by treatment with calf alkaline phosphatase to prevent self oligomerization during subsequent ligation to the promoter duplex. Upon transfection of the recombinant pJP_1 plasmid to competent *E. coli* LE 392 cells, clones resistant to both ampicillin and tetracycline were selected. Only those

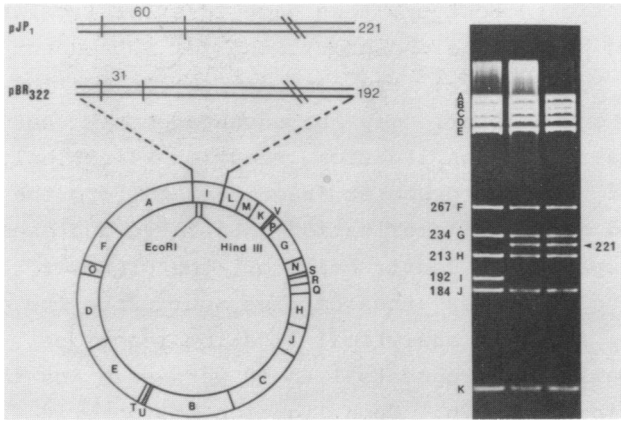

Figure 9. Characterization of pJP_1 recombinant plasmids by restriction enzyme digestion. Lane a: Digestion of pBR322 with Hae III. Lane b: Digestion of pJP_1 clone 105 with Hae III. Lane c: Digestion of pJP_1 clone 103 with Hae III. The 221 base pair fragments from the pJP_1 clones indicated by ◄ contained the synthetic T5P25 promoter.

clones that carry a functional synthetic promoter will exhibit tetracycline resistance. Of the 100 Ap^r clones tested, 97 were Tc^r.

Several of the Ap^rTc^r clones were isolated and their plasmid DNA characterized by restriction mapping. The 31 base pair fragment between the EcoRI and Hind III restriction sites in pBR322 lies within the 192 base pair fragment I of the Hae III restriction digest. Replacement of this 31 base pair fragment with the 60 base pair synthetic promoter in pJP_1 will result in the conversion of this 192 base pair fragment I into a larger fragment. This is clearly shown for two of the clones tested in figure 9. As compared to the Hae III digestion pattern of pBR322 (lane a), the I fragment is totally absent in both of the pJP_1 clones (lanes b and c) and instead, a new fragment migrating between the fragments G and H of the pBR322 digest was generated, in agreement with the expected size of 221 base pairs.

These clones were further characterized by Southern blotting using terminally labeled promoter fragment as probe

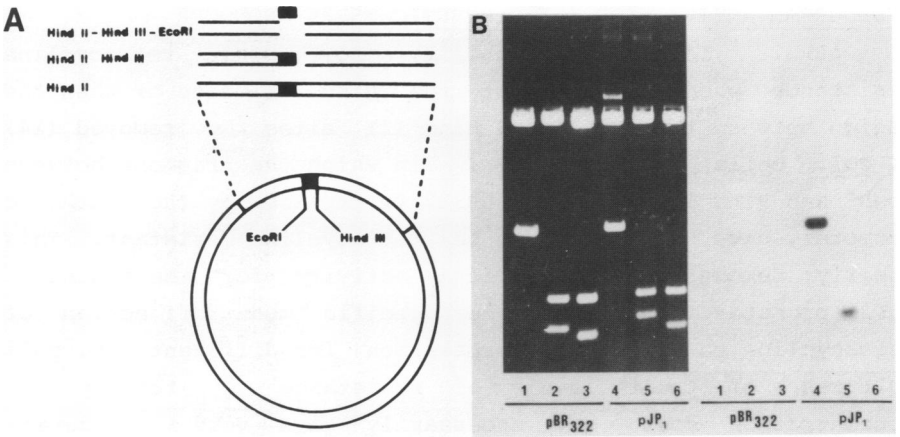

Figure 10. Characterization of the pJP_1 plasmids by Southern Blot Hybridization using labeled fragment 9 as probe. (a) Ethidium Bromide stained (1%) agarose gel. (b) Autoradiogram after hybridization. Lanes 2 and 4: Hinc II digest of pBR322 and pJP_1 respectively. Lane 2 and 5: Hinc II + Hind III digest of pBR322 and pJP_1 respectively. Lanes 3 and 6: Hinc II + Hind III + EcoRI digest of pBR322 and pJP_1 respectively.

(fragment 7 in figure 3), to show the presence of the promoter sequence. As shown in figure 10 for one of these clones, both pBR322 and pJP_1 were digested by Hind II into two fragments A and B in lanes 1 and 4, respectively. The smaller B fragment produced by pJP_1 hybridized to the labeled synthetic probe (lane 4). Upon further cleavage of the B fragments with Hind III, two new fragments were produced. The smaller of these new fragments in pJP_1, which contained the promoter, also hybridized to the labeled probe (lane 5). Upon further cleavage with EcoRI, the complete promoter was removed, and the resultant fragment no longer hybridized to the synthetic probe (lane 6). None of the pBR322 fragments hybridized to the labeled probe. Also note that all the promoter containing fragments in pJP_1 were slightly larger than the corresponding pBR322 fragments. This demonstrated the presence and correct location for the synthetic T5P25 promoter in pJP_1. The insert has subsequently been confirmed by complete sequence analysis.

BIOLOGICAL ACTIVITY OF THE SYNTHETIC T5P25 PROMOTER

pBR322 loses its ability to express tetracycline resistance when modified at the Hind III site or when the region between the EcoRI and Hind III sites is removed (14). E. coli cells harboring pJP_1, in which the fragment between EcoRI and Hind III sites in pBR322 is replaced by the synthetic promoter, have been shown to be tetracycline resistant. This clearly demonstrates biological activity for the synthetic T5P25 promoter. Due to the specific mode of action of tetracycline resistance, determination for different E. coli cultures of their levels of resistance to tetracycline concentration does not necessarily give very accurate correlation with the efficiency of expression of the Tc^r gene. However, E. coli cells harboring pBR322 are resistant up to a tetracycline concentration of 70 µg/mL and do not survive at the concentration of 75 µg/mL while those carrying pJP_1 plasmids are viable even at 100 µg/mL concentration. This suggested that the synthetic T5P25 promoter is more efficient than the promoter for the tetracycline resistance gene in pBR322.

As mentioned earlier, a previous attempt to directly clone restriction fragments containing the T5 early promoters had not been successful unless a strong termination signal for transcription was simultaneously inserted downstream from the promoter (11). The reason for this is not exactly known, especially since the termination sequence had been inserted backwards. The authors had suggested that an alternate type of termination signal had been accidently generated. Thus, a possible explanation for the failure may be due to the following: insertion of the DNA fragment containing the strong T5 early promoters which perhaps also contain strong translational signals resulted in the over production of exogenous polypeptides in E. coli in levels that were lethal to the cells. The presence of the termination signal downstream abolished this difficulty. When the synthetic T5P25 promoter was inserted in front of the Tc^r gene in pJP_1, the ribosome binding site for the Tc^r gene is comparatively weak and thus does not result in an over production of the Tc^r protein and

allowed successful cloning of the synthetic promoter.

In planning the construction and cloning of the synthetic T5P25 promoter, a mutant promoter containing a down mutation was also constructed in case the wild type T5P25 indeed cannot be cloned. It contains a transitional mutation converting the Pribnow box TATAAT sequence into a less conserved AATAAT sequence. E. coli cells carrying the pJP_2 plasmid containing this mutant promoter have been shown to exhibit Tc^r to the same level as those carrying the pBR322 plasmid and as expected, to a lower level than those carrying the pJP_1 plasmid. Thus, this has served as a very simple and convenient assay to demonstrate the functional activity for the synthetic promoters and their application for studying their structure-function relationships. The results of more quantitative comparative studies of these two promoters and others presently being constructed will be published elsewhere.

CONCLUSION

We have described the chemical synthesis and cloning into pBR322 of a 60 base pair duplex containing the bacteriophage T5P25 early promoter sequence. The stepwise ligation of the oligomers unambiguously characterized the construction of the promoter duplex. This, in combination with the rapid solid phase synthesis for deoxyoligonucleotides using the phosphite approach and the improved method for their purification would allow rapid synthesis of a large number of promoter duplexes for functional studies. Using a similar approach, it will allow the construction of complete genes for small and medium sized proteins in relatively short time.

The synthetic T5P25 promoter, as well as a down-shift mutant, when cloned into pBR322 plasmid DNA replaced the natural Tc^r promoter normally found in pBR322. The ability of E. coli cells harboring these recombinant plasmids to express tetracycline resistance demonstrated the functional activity for the synthetic promoters. This demonstrates the feasibility of using the combination of chemical synthesis of promoter mutants and molecular cloning for the study of promoter functioning. We have now succeeded in attaching the synthetic

promoter in front of the chloramphenicol acetyl transferase (CAT) gene originated from the Tn5 transposon (31) after removal of its natural promoter. E. coli cells carrying this recombinant plasmid have been shown to be resistant to very high levels of the drug. These cells have also been shown to produce large amount of the CAT protein which can be very easily quantitated by PAGE analysis of the total cell extract or by a simple enzymatic assay (34). Thus, this should provide an extremely simple in vivo system for studying promoter efficiency and structure-function relationships of promoters. These results will be published in detail elsewhere. Specific mutants of the T5P25 promoters as well as other unrelated promoters are now being synthesized for comparative studies.

Recently, similar approaches of using chemically synthesized lambda P_R promoter for in vitro binding and in vitro transcription studies (32) and the use of constructed hybrids of the natural trp and lac UV-5 promoters for the study of promoter efficiency have been reported (33). We feel that our approach of using chemical synthesis of promoters for directly the in vivo expression of a specific gene offers greater versatility. Many promoter sequences can be very efficiently synthesized and constructed. Their in vivo efficiency may compared using the CAT system we have developed. This, we believe will provide invaluable information towards the understanding of the structure-function relationships of promoters.

ACKNOWLEDGEMENTS

This work has been supported in parts by grants from the National Cancer Institute of Canada and the Natural Sciences and Engineering Research Council of Canada.The authors wish to thank Genelab Inc. for their support and assistance. J.R., D.M. and L.C. are recipients of NSERC scholarships and D.M. is recipient of a scholarship from Imperial Oil of Canada.

+ To whom enquiries should be addressed.

REFERENCES

1. Rosenberg, M. and Court, D. (1979) Ann. Rev. Genet. 13, 319-53.
2. Siebenlist, U., Simpson, R. B. and Gilbert, W. (1980) Cell 20, 269-281.
3. Johnsrud, L. (1980) Proc. Natl. Acad. Sci. USA 75, 5314-18.
4. Gilbert, W. in Starting and Stopping Sequences for RNA Polymerase, Losick, R. and Chamberlin, M. (eds.), Cold Spring Harbor Laboratory (1976).
5. Seeburg, H. P., Nüsslein, Ch. and Schaller, H. (1977) Eur. J. Biochem. 74, 107-13.
6. Pribnow, D. (1975) Proc. Natl. Acad. Sci. USA 72, 784-89.
7. Schaller, H., Gray, C. and Herrmann, K. (1975) Proc. Natl. Acad. Sci. USA 72, 737-41.
8. Hawley, D. K. and McClure, W. R., (1983) Nucl. Acids Res. 11, 2237-2255.
9. von Gabain, A. and Bujard, H. (1977) Mol. Gen. Genet. 157, 301-11.
10. von Gabain, A. and Bujard, H. (1979) Proc. Natl. Acad. Sci. USA 76, 189-93.
11. Gentz, T., Langner, A., Chang, C. Y., Cohen, S. N. and Bujard, H. (1981) Proc. Natl. Acad. Sci. USA 78, 4936-4940.
12. Clewell, D. B. and Helinski, D. R. (1969) Proc. Natl. Acad. Sci. USA 62, 1159-1166.
13. Maniatis, Y., Fritsch, E. F. and Sambrook, J. (eds.), in Molecular Cloning - a Laboratory Manual, Cold Spring Harbor Laboratory, pp. 86-96 (1982).
14. Rodriquez, R. L., West, R. W., Heyneker, H. L., Boliver, F. and Boyer, H. W. (1979) Nucl. Acids Res. 6, 3267-87.
15. Dobrynin, V. N., Korobko, V. G., Severtsova, I.V., Bystrov, N. S., Chuvipo, S. A. and Kolosov, M. N. (1980) Nucl. Acids Res. Symposium Series 7, 365-376.
16. Details to be published elsewhere.
17. Matteucci, M. D. and Caruthers, M. H. (1981) J. Amer. Chem. Soc. 103, 3185-91.
18. Daub, G. W. and van Tamelen, E. E. (1977) J. Amer. Chem. Soc. 99, 3527-28.
19. Khorana, H. G. et al. (1972) J. Mol. Biol. 72, 209-426.
20. Maniatis, T., Jeffrey, A. and Van de Sande, H. (1975) Biochemistry 14, 3787-94.
21. Maxam, L. and Gilbert, W. in Methods in Enzymology, Grossman, L. and Moldave, K., (eds.), Vol. 65, 499-560 (1980).
22. Maxam, A. M. and Gilbert, W. (1977) Proc. Natl. Acad. Sci. USA 74, 560-64.
23. Jay, E., Seth, A. K., Rommens, J., Sood, A. and Jay, G. (1982) Nucl. Acids Res. 10, 6319-29.
24. Rubin, C. M. and Schmid, C. W. (1981) Nucl. Acids Res. 9, 6413-19.
25. Winberg, G. and Hammarskjold, M. L. (1980) Nucl. Acids Res. 8, 253-64.
26. Dugaiczyk, A., Boyer, W. W. and Goodman, H. M. (1975) J. Mol. Biol. 96, 171-184.

27. Jay, G., Khoury, G., Seth, A. K. and Jay, E. (1981) Proc. Natl. Acad. Sci. USA 78, 5543-48.
28. Southern, E. M. (1975) J. Mol. Biol. 98, 503-17.
29. Gait, M. J. and Sheppard, R. C. (1980) Nucl. Acids Res. 8, 1081-96.
30. Miyoshi, K., Miyake, T., Hozumi, T. and Itakura, K. (1980) Nucl. Acids Res. 8, 5473-89.
31. Tu, C. D. and Cohen, S. N. (1980) Cell 19, 151-60.
32. deHaseth, P. L., Goldman, R. A., Cech, C. L. and Caruthers, M. H. (1983) Nucl. Acids Res. 11, 773-87.
33. de Boer, H. A., Comstock, L. J. and Vasser, M. (1983) Proc. Natl. Acad. Sci. USA 80, 21-23.
34. Shaw, W. in Methods in Enzymology, J. H. Hash (ed.), Vol. 43, 737-755 (1975).