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Regulation of the in vitro synthesis of the α-peptide of β-galactosidase directed by a restriction fragment of the lactose operon

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SUMMARY

The regulation of the in vitro synthesis of the N-terminal portion of the β-galactosidase molecule (α-peptide) has been investigated using DNA fragments of the lactose operon as template. DNA fragments of about 789 base pairs were isolated after endonuclease (Hin II) digestion of either λplac5, λh80dlacp^{s} or λh80dlacUV5 phage DNA or DNA from the recombinant plasmid PMC3. The regulation of the expression of these fragments is similar to that observed for the synthesis of β-galactosidase using total phage or plasmid DNA as template, indicating that the regulatory regions on the fragments are intact and functional. Thus, the synthesis of the α-peptide required an inducer due to the presence of lac repressor in the E. coli S-30 extract used. In addition a dependency on adenosine 3',5'-cyclic monophosphate (cAMP)[1] for α-peptide synthesis was obtained with the fragments isolated from λplac5 and λh80dlacp^{s} DNAs, whereas little effect of cAMP was seen with the fragment isolated from λh80dlacUV5 phage DNA or PMC3 plasmid DNA containing a UV5 promotor region. However, a significant difference

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[1]The abbreviations used are: IPTG, isopropylthiogalactoside; ONPG, orthonitrophenylgalactoside; ppGpp, guanosine-5'-diphosphate-3'-diphosphate; cAMP, cyclic adenosine 3',5'-monophosphate; CRP, cAMP receptor protein, PEG 6000, polyethylene glycol 6000.

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in the effect of guanosine-3'-diphosphate-5'-diphosphate (ppGpp) was observed. With the total phage DNA as template, ppGpp resulted in a 2-4 fold stimulation whereas with the fragment, or PMC3 plasmid DNA, directed synthesis of the α-peptide no significant stimulation by ppGpp was seen.

INTRODUCTION

For several years our laboratory has been attempting to obtain the DNA-directed *in vitro* synthesis of a bacterial gene in a defined system (1-4); specifically the synthesis of β-galactosidase directed by DNA from a transducing phage carrying the lactose operon. However, with total phage DNA as template, a large number of phage proteins are also synthesized in this *in vitro* system (5). The goal of elucidating all of the factors specifically required for the *in vitro* regulation of the expression of a bacterial gene would be facilitated if a characterized DNA template were available which contained an intact regulatory region and all or part of one structural gene.

The use of restriction endonucleases has made it possible to generate small DNA fragments containing specific promotor and operator regions. These fragments have greatly facilitated studies on the interactions of RNA polymerase and regulatory proteins with DNA (6-11). For example, after incubations of either phage or plasmid DNA, carrying the lactose operon, with endonuclease Hin II, a fragment of about 789 base pairs has been obtained which contains the lac regulatory region plus the N-terminal region of the β-galactosidase gene (10-12). Direct evidence that the regulatory region was intact was obtained from *in vitro* studies with the fragment derived from λplac5 DNA. The synthesis of the α-peptide of β-galactosidase directed by this fragment was stimulated about 2-fold by cAMP and IPTG (10). The present study has investigated this point more carefully. A DNA fragment of about 789 base pairs has been isolated from three different transducing phages and a recombinant plasmid. In all cases the fragment can direct the synthesis of the α-peptide of β-galactosidase *in vitro*. The control

of α-peptide synthesis directed by these fragments is, in general, similar to that observed with the corresponding phage or plasmid DNA as template, i.e., a requirement for IPTG and changes in template efficiency and cAMP dependency due to mutations in the lac promotor region. However, a marked difference in the effect of ppGpp is seen between the phage DNA and fragment directed systems.

MATERIALS AND METHODS

Bacteria and bacterial extracts. E. coli Z19i^q which contains the mutant gene i^q and the M-15 modified β-galactosidase gene on both the chromosome and episome, was kindly provided by Dr. G. Zubay, Columbia University. This strain was used as the source of the S-30 extract as described previously (1-5). E. coli strain DZ291 (kindly provided by Dr. I. Zabin of the University of California, Los Angeles) was used for the preparation of the M15 extract (13) which is required to complement the α-peptide in the β-galactosidase assay (14).

Bacteriophage, plasmid and DNA preparations. Bacteriophages λcI857S7plac5, λh80cI857t68dlacUV5 and λh80cI857t68dlacp^s were prepared by heat induction of the corresponding lysogens. The lysogens were kindly supplied by Dr. G. Zubay, Columbia University, Ms. M. Calos, Harvard University and Dr. I. Pastan, National Institutes of Health respectively. The phages were purified by banding in a cesium chloride density gradient, and phage DNA was extracted by the method of Thomas and Abelson (15).

E. coli δ3 (or PMC3) was constructed and kindly provided to us by Ms. M. Calos of Harvard University. The recombinant plasmid was prepared by ligating a DNA fragment containing the lac control region (a UV5 mutation at the promotor site) and the beginning of the lac Z gene into the Hpa I site of the PMB 9 plasmid. Preparation of the supercoiled plasmid DNA was as described by Tanaka and Weisblum (16).

Enzymes and chemicals. Restriction endonuclease Hin II was purchased from New England Biolabs; agarose from Seakem; chloramphenicol and ethidium bromide

from Sigma. Other chemicals were obtained as described previously (1-5).

Isolation and purification of DNA fragments. The conditions for the complete digestion of phage DNA with Hin II enzyme were the same as described by Landy et al. (17). The DNA fragments were separated by preparative slab gel electrophoresis. The gels (1.85% agarose) were prepared in a buffer containing Tris-Ac, pH 7.5, 20 mM NaAc, 2 mM EDTA and 0.5 µg/ml ethidium bromide, and the electrophoresis was performed in the same buffer. The DNA bands could be visualized under a UV lamp and the gel segment containing the 789 base pair fragment was homogenized with a Dounce homogenizer in 10 ml of a buffer containing 50 mM Tris-Cl, pH 7.5, 300 mM NaCl and 1 mM EDTA. After 16 hr of elution the agarose was sedimented by centrifugation at 10,000 xg for 10 min. The supernatant containing the 789 base pair fragment was collected and the pellet re-extracted as before. 20 ml of distilled phenol were added to the combined supernatants (20 ml) and after shaking and centrifugation the DNA fragment was precipitated from the aqueous phase by the addition of two volumes of cold ethanol. After 16 hrs at -20°C, the solution was centrifuged at 30,000 xg for 1 hr. The pellet was dissolved in a buffer containing 10 mM Tris-Cl, pH 7.5 and 1 mM EDTA.

In vitro protein synthesis. The complete system (35 µl) for protein synthesis contained: 15 mM Tris-Ac (pH 8.2), 11 mM Na^+ dimethylglutarate (pH 6.0); 35 mM NH_4 acetate, 65 mM K acetate, 2.4 mM DTT, 0.93 mM UTP, CTP and GTP, 3 mM ATP, 27 mM phosphoenol pyruvate, 0.05 mM ppGpp, 0.7 mM 3'5'-cAMP, 15 µg calcium leucovorin, 25 µg E. coli B tRNA, 1.25 µg polyethylene glycol 6000, 0.4 mM IPTG, 0.112 mM of each amino acid, 9 mM $MgAc_2$, 0.8 mM spermidine, 0.24 mg S-30 extract, and either 3 µg of phage DNA or 0.1 µg of the 789 base pair restriction fragment. After the mixtures had been incubated for 1 hr at 37°, 5 µg of chloramphenicol (to stop protein synthesis) and 50 µg of M-15 extract protein (to complement any α-peptide formed) were added, and the tubes were incubated for an additional 30 min at 37°. The rate of β-galactosidase activity was then determined by the addition of 1 ml of ONPG reagent and measuring the optical density at 420 nm (1).

RESULTS AND DISCUSSION

In vitro protein synthesis directed by restriction fragments from phage DNA. In the present experiments the procedure of Landy et al. (17) was used to digest the DNA from 3 transducing phages, λplac5, λh80dlacUV5 and λh80dlacp^s. Using Hin II endonuclease more than 50 fragments were produced from each DNA ranging in size from approximately 1.6 x 10^5 to 3 x 10^6 daltons. These results confirm the studies of Landy et al., using λplac5 DNA (17). DNA fragments of 789 base pairs, shown previously to contain the lac regulatory region and a portion of the lac Z gene (10,11) were purified from the DNA digests of the 3 phages by preparative gel electrophoresis as described in the Methods. Each of the fragments gave one major band on agarose gel electrophoresis and these fragments were used as template for the in vitro synthesis of the α-peptide of β-galactosidase using an S-30 extract prepared from E. coli Z19i^q strain. The synthesis of the α-peptide was linear up to 0.15 μg of DNA with each of the fragments. At this concentration, the DNA was not saturating.

Table I compares the dependencies for protein synthesis in this in vitro system using either the fragment or total phage DNA as template. As seen in the Table, the DNA fragment isolated from each of the 3 phages resembles the parent phage DNA in relative template activity. λplac5 DNA contains the wild type lac promotor, whereas λh80dlacp^s DNA (18) and λh80dlacUV5 DNA (19) have mutations in the lac promotor region. It is known that transcription of the lac operon is more efficient in the mutant phages than the wild type phage (7,18), and the fragments isolated from λh80dlacp^s and λh80dlacUV5 DNA were more efficient templates for α-peptide synthesis than the fragment isolated from λplac5 DNA (Table I, line 1). Synthesis of the α-peptide was dependent upon the DNA template (Table I, line 2) and also IPTG because of the presence of lac repressor in the E. coli Z19i^q S-30 extract (Table I, line 3). The UV5 promotor mutation allows transcription of the lac operon to occur in the absence of cAMP and CRP (8,9). As also seen in Table I, line 4, cAMP was required when the fragments isolated

TABLE I

DNA-DIRECTED α-PEPTIDE SYNTHESIS

USING EITHER THE 789 BASE PAIR FRAGMENT OR PHAGE DNA AS TEMPLATE

	789 base pair fragment from			phage DNA		
Reaction Mixture	λplac5 DNA	λh80dlac^s DNA	λh80dlacUV5 DNA	λplac5 DNA	λh80dlac^s DNA	λh80dlacUV5 DNA
1. Complete	0.02	0.24	0.15	0.21	1.10	0.70
2. -DNA	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
3. -IPTG	<0.01	0.01	0.01	0.01	0.02	0.01
4. -Cyclic 3':5'-AMP	<0.01	<0.01	0.11	<0.01	<0.01	0.54
5. -M15 Extract	<0.01	<0.01	<0.01	0.16	0.91	0.60
6. -ppGpp	0.02	0.20	0.14	0.10	0.29	0.38

The experimental details are described in Methods. The numbers represent the A_{420}/hr.

from λplac5 and λh80dlacp^{s} DNA were used as the templates, but cAMP had little effect when the fragment from λh80dlacUV^{5} was used as template. When the 789 base pair fragments were used as templates, M15 extract was required in order to detect β-galactosidase activity (Table I, line 5). In contrast, the M15 extract had little effect on the phage DNA-directed systems indicating that with the phage DNA as template, the complete β-galactosidase molecule was synthesized. ppGpp is known to stimulate the phage DNA directed synthesis of β-galactosidase and is believed to function at the transcription level (20-25). Despite the above findings indicating that the regulatory region of the lactose operon is intact in the DNA fragments, it was observed that ppGpp had little, if any, effect on the synthesis of the α-peptide directed by these fragments (Table 1, line 6). In contrast, a 2-4 fold stimulation by ppGpp of β-galactosidase synthesis was seen when the total phage DNA was used as template.

Restriction fragments from plasmid DNA. The amount of the 789 base pair fragment obtained from the Hin II digest of phage DNA was small and the procedure also required large amounts of the restriction enzyme because more than 50 cleavage sites were present on the phage DNA. In contrast, a good yield of the 789 base pair fragment could be obtained from the DNA of the hybrid plasmid PMC3. After digestion of PMC3 DNA with Hin II nuclease, only three major bands were obtained having approximately 3200, 2300 and 789 base pairs respectively[2]. The 789 base pair fragment from the plasmid DNA could also be isolated by the procedure described in Methods and used as template for the *in vitro* synthesis of α-peptide. As shown in Table II, the PMC3 plasmid DNA and the derived 789 base pair fragment were able to direct α-peptide synthesis. Since the plasmid contained the lac regulatory region with a UV5 mutation, no significant effect of cAMP was obtained with either the plasmid DNA or the derived fragment. Table II also shows that the synthesis of α-peptide directed by either PMC3 plasmid DNA

[2]These results are in agreement with those of Ms. M. Calos, Personal Communication.

TABLE II

α-PEPTIDE SYNTHESIS DIRECTED BY

PLASMID DNA OR A 789 BASE PAIR FRAGMENT

Reaction Mixture	PMC3 DNA	789 Base Pair Fragment
		A_{420}/hr
Complete	0.54	0.26
-DNA	<0.01	<0.01
-IPTG	<0.01	0.02
-Cyclic 3':5'-AMP	0.30	0.22
-M15 Extract	<0.01	<0.01
-ppGpp	0.43	0.25

The experimental details are described in Methods and are similar to the experiments in Table I except that PMC3 plasmid DNA (3 μg) or its 789 base pair fragment (0.1 μg) was used as the template.

or the 789 base pair fragment obtained from it was stimulated less than 25% by the addition of ppGpp.

The reason for the lack of stimulation of α-peptide synthesis by ppGpp is not known. Some possible explanations are 1) ppGpp may affect transcriptional events other than initiation, 2) DNA sequences outside the region of the fragment may have a role in ppGpp function or 3) the tertiary structure of DNA may be important for the ppGpp effect. The latter possibility seemed most attractive although some facts do not support this view. If the tertiary structure were altered, one might expect other regulatory factors to be affected, yet the requirements for cAMP and IPTG were not altered in the fragment directed systems.

In addition, the PMC3 plasmid DNA containing the fragment did not show a significant effect of ppGpp. It could be argued that ppGpp is unable to react efficiently with the lac promotor region present in a plasmid, but this is not so since preliminary in vitro experiments with either PLC20-30 or PLC50 hybrid plasmid DNA[3] (26), which contains the entire E. coli lac operon, have shown that the synthesis of β-galactosidase is stimulated 3-4 fold by ppGpp. Thus, the lack of effect of ppGpp is seen with the fragment whether it is an isolated species or an integrated part of a larger species of DNA. It will be of interest to examine the effect of ppGpp on fragments of different sizes from the lac operon to see whether there is a correlation between the size of the fragment and the effect of ppGpp. In addition, one could investigate the expression of other genes that are affected by ppGpp to see whether restriction fragments show a different response than the intact genome.

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[3]PLC20-30 and PLC50 plasmids were constructed by Dr. J. Carbon (University of California, Santa Barbara) using Col El as the vector. These strains were kindly provided to us by Dr. D. Schlessinger (Washington University, St. Louis).

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