EXPRESSION OF BACTERIOPHAGE T4 MINOR BASEPLATE PROTEINS IN THE BACTERIOPHAGE T7 PROMOTER/RNA POLYMERASE EXPRESSION SYSTEM

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The central part of bacteriophage T4 baseplate is built of several proteins which are present in only a few copies per phage particle. Only some of these minor baseplate components have been identified previously as distinct protein species by biochemical analysis. We have used the bacteriophage T7 RNA polymerase expression system to identify and overexpress the minor baseplate proteins. The products of genes 25, 26 and 51 were identified on the autoradiographs after selective labelling with $^{35}$S methionine.

The overexpression of gene 25 and 51 products was high enough to make possible undertaking their purification and studies of their properties.

The most complicated substructure of a bacteriophage T4 particle is its baseplate which is formed by joining six wedge-like structures around the central hub. Genes which code for baseplate proteins are grouped in two separate clusters on the bacteriophage T4 genome. The first cluster is located between coordinates 76 and 91 of phage T4 genomic map and
comprises genes 53, 5, 6, 7, 8, 9, 10, 11 and 12. The second cluster consists of genes 25, 26, 51, 27, 28, 29, 48 and 54, and maps between coordinates 114 - 121.5. Most of the gene products of the T4 baseplate were identified by Kikuchi & King [1 - 3] but some of the baseplate proteins evaded identification. Kikuchi & King suggested that these gene products need not necessarily be structural fragments of the baseplate but that they rather could act as the enzymes involved in the baseplate assembly.

DNA of the wild type bacteriophage T4 contains 5-hydroxymethyl cytosine which is in the glucosylated form [4]. This modified cytosine makes T4 DNA resistant to most of the restriction enzymes. Mutants which contain non-modified cytosine are available and they are commonly used in the restriction analysis and cloning procedures [5]. One of the fragments from the EcoRI digest of bacteriophage T4 DNA was found (by a marker rescue experiment) to contain the following baseplate genes: 25, 26, 51, 27, 28 and 29. Since this fragment contained most of the genes which Kikuchi & King defined as the minor baseplate components, we have chosen it for construction of hybrid plasmids containing particular baseplate genes. The baseplate gene products were identified and expressed in the bacteriophage T7 promoter/RNA polymerase system. In this efficient system for expression, the bacteriophage T7 RNA polymerase gene is located either on the plasmid [6] or in the bacterial genome [7]. Plasmid vectors to which the foreign DNA is cloned contain a highly specific bacteriophage T7 promoter located closely to a multiple cloning site. Once the synthesis of T7 RNA polymerase is induced, either thermally or by the addition of isopropylthiogalactopyranoside, then very efficient and selective transcription of the insert DNA begins to proceed. E. coli RNA polymerase can be completely eliminated by the addition of rifampicin, so that only the products of the genes cloned behind the T7 promoter are synthesized.

MATERIALS AND METHODS

Bacteria. Escherichia coli CR63 (supD), a permissive host for bacteriophage T4 amber mutants and E. coli Bb, a non-permissive host for amber mutants were used for phage growth and plating. E. coli HB101 was used for transformation after ligations, while E. coli BL21/DE3 which
contains bacteriophage T7 RNA polymerase gene on the chromosome was used for protein expression experiments.

Phages. Amber mutants of bacteriophage T4 used in this study are listed in Table 1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Amber mutants received from</th>
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<tbody>
<tr>
<td></td>
<td>W. Wood</td>
</tr>
<tr>
<td>25</td>
<td>S 52, N 67</td>
</tr>
<tr>
<td>26</td>
<td>N 131</td>
</tr>
<tr>
<td>51</td>
<td>S 29</td>
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Plasmid vectors. pUC18 and pUC19 vectors were from New England Biolabs, Beverly, MA, U.S.A. pT7-5, pT7-6 and pT7-3 were from S. Tabor, Harvard Medical School, Boston, MA, U.S.A. All of the three latter plasmids contain the T7 RNA polymerase φ 10 promoter and the β-lactamase gene. This gene is inserted in the opposite direction to the direction of transcription by T7 RNA polymerase in pT7-5 and pT7-6, and in the correct orientation in pT7-3. pT7-5 and pT7-6 differ only in the orientation of the multiple cloning site.

Cloning procedures. Most of the cloning procedures were adapted from Maniatis et al. [8]. After ligation, the E. coli HB101 strain was used for transformation experiments. The transformants containing the required inserts were grown on a larger scale to obtain plasmid DNA. DNA was purified either by CsCl gradient ultracentrifugation or by the glass beads method.

Complementation test. The synthesis of a particular phage-coded protein in bacteria harbouring plasmids was ascertained by the in vivo complementation. In this test E. coli Bb (a non-permissive strain) containing a plasmid with the T4 DNA fragment was infected with the appropriate T4 amber mutant, and the increase in bacteriophage titer was determined. Plasmids of either the pUC or pT series carrying phage T4 inserts could be used because under natural conditions the cognate phage T4 promoters are utilized for transcription in E. coli Bb.

Selective labelling of proteins coded for by phage T4 DNA fragments cloned into T7 promoter plasmids. Plasmid DNA purified as described earlier was used for transformation of E. coli BL21/DE3. M9 minimal
medium (25ml) was inoculated with a few colonies taken directly from transformation plates and the bacteria were grown at 37°C with agitation until A575 = 0.2 was reached. 0.1 ml of this culture was transferred to an Eppendorf tube, IPTG was added to 0.5 mM concentration and the tube was placed in a water bath shaker at 37°C. After about 30 min, rifampicin was added to a final concentration 200 µg/ml and incubation was continued for 20 min. About 10 µCi of [35S]methionine was then added and labelling was carried out for about 10 min. An equal volume of the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer was added to the tube, the sample was placed in a boiling water bath for 5 min and then a part of the sample (20-50 µl) was applied to an SDS-PAGE gel prepared according to Laemmli [9]. After running the electrophoresis, the gel was fixed and dried, and finally exposed to an X-ray film. Time of exposure ranged from a few hours to two days.

Estimation of the level of the overexpressed proteins. This was done either in a rich medium- like LB broth or in M9 minimal medium. The level of the overexpressed proteins is sometimes higher when bacteria are grown in a minimal medium but this is not a rule. The medium (25 ml) was inoculated with a few colonies from the plates of E. coli BL21/DE3 freshly transformed with plasmid DNA. When A575 = 0.2 was reached, the production of the protein coded for by the cloned DNA was induced by the addition of IPTG to a final concentration of 0.5 mM; 1 ml samples were collected at 0, 1, 2, 3, 4 h after addition of the inducer. Each sample was subjected to a short centrifugation to pellet bacteria. The bacteria were then frozen at -20°C in portions corresponding to about 200 µl of the original bacterial culture. Such portions were mixed with the SDS-PAGE sample buffer, placed in a boiling water bath for 5 min, shortly centrifuged (2-3 min in a microfuge) to remove insoluble debris and the supernatants were applied to an SDS-PAGE gel. After electrophoresis, the gel was stained in Coomassie Brilliant Blue solution (0.1% in methanol/water/acetic acid; 5:5:1, by vol.) for 1 h and destained in the methanol/water/acetic acid (2:8:1, by vol.) solution.

RESULTS AND DISCUSSION

Construction of plasmids and the results of the complementation test. Figure 1 summarizes the constructs derived from the original EcoRI piece
Fig. 1. The positions of fragments containing cloned baseplate genes on the bacteriophage T4 genomic map. The apparent direction of transcription of baseplate genes from the T4 genome is shown at the top of the Figure. The positions of multiple cloning sites in pT7 vectors with respect to bacteriophage T7 \( \phi 10 \) promoter are shown at the bottom of the Figure. The positions of some of the restriction sites on the large EcoRI fragment are marked with symbols (H - HindIII, P - PstI, X - XbaI).

of the T4 DNA containing some of the baseplate genes. The complete sequence of this phage T4 DNA fragment is not yet known, therefore the smaller fragments were obtained by cutting the original EcoRI fragment with restriction enzymes whose approximate restriction sites on this DNA
are known; then the presence of a particular gene was confirmed by the complementation test (Table 2). First, all the fragments shown in the Figure were introduced into pUC vectors. The resulting plasmids were used to transform E. coli Bb, a non-permissive host for bacteriophage T4. After infection with an appropriate amber mutant of bacteriophage T4, the increase in titer relative to the titer of a plasmid-free E. coli Bb infected with the same mutant was determined. A large increase in the titer indicated that the synthesis of a protein absent in a particular phage mutant took place. It has to be pointed out that E. coli Bb is not a bacterial strain in which overproduction of a protein coded for by a plasmid was to be expected. The amounts of proteins needed for complementation are low as the cloned genes code for minor baseplate components.

As the data of Table 2 indicate, the complementation test gives in most cases convincing evidence for the presence of a particular gene in a cloned insert. Two notable exceptions are plasmids with fragments I and II where there is only a slight increase in the titer when bacteriophage T4 26− mutant

Table 2

The results of titration in the complementation test of different T4 amber mutants on the E. coli Bb containing plasmids with T4 DNA fragments presented in Fig. 1

<table>
<thead>
<tr>
<th>Amber mutants</th>
<th>E. coli Bb</th>
<th>E. coli CR63</th>
<th>E. coli Bb transformed with plasmids</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>pUC19 fragment I</td>
</tr>
<tr>
<td>25</td>
<td>2 x 10^5</td>
<td>6 x 10^10</td>
<td>1 x 10^10</td>
</tr>
<tr>
<td>26</td>
<td>1 x 10^5</td>
<td>3 x 10^9</td>
<td>6 x 10^7</td>
</tr>
<tr>
<td>51</td>
<td>1 x 10^7</td>
<td>5 x 10^11</td>
<td>1 x 10^7</td>
</tr>
</tbody>
</table>

is used in complementation experiments. These results are explained in the later part of this paper.

For identification of gene products and overexpression experiments, each of the described phage T4 fragments was cloned into vectors with a promoter for bacteriophage T7 polymerase. In most cases vectors pT7-5 and pT7-6 were used. This allowed for transcription from both DNA strands when the same fragment was cloned into this pair of vectors.
Fig. 2. Synthesis of $^{35}$S-labelled gene 25 and gene 26 products in the T7 RNA polymerase expression system. The autoradiograph of an SDS-PAGE gel (15% acrylamide concentration): 1, BL21/DE3 with plasmid pT7-5-fragment I, non-induced with IPTG; 2, the same as 1, but induced with IPTG; 3, BL21/DE3 with plasmid pT7-5-fragment II, non-induced with IPTG; 4, the same as 3 but induced with IPTG; 5, BL21/DE3 with plasmid pT7-5-fragment IV, non-induced with IPTG; 6, the same as 5 but induced with IPTG. All bacterial cultures were treated with rifampicin before labelling. The positions of the protein molecular mass markers (kDa) are shown on the left hand side.
Fig. 3. Synthesis of $^{35}$S-labelled gene 5I product in the T7 RNA polymerase system. The autoradiograph of an SDS-PAGE gel (10% acrylamide concentration): 1, BL21/DE3 with plasmid pT7-6-fragment III (reverse orientation), non-induced with IPTG; 2, the same as 1 but induced with IPTG; 3, BL21/DE3 with plasmid pT7-5-fragment III (correct orientation), non-induced with IPTG; 4, the same as 3 but induced with IPTG. All bacterial cultures were treated with rifampicin before labelling. The positions of the molecular mass markers (kDa) are shown on the left hand side.
Fig. 4. Overexpression of the products of gene 25 and gene 5/ cloned into plasmids with bacteriophage T7 promoter. SDS-PAGE gels were stained with Coomassie Brilliant Blue. The positions of protein molecular mass markers (kDa) are shown on the left hand side of photographs. A. BL21/DE3 cells containing plasmid pT7-3-fragment I: 1, non-induced; 2, two hours post-induction with IPTG. Gene 25 product is indicated with an arrow. Fragment I was cloned into pT7-3 plasmid which contains β-lactamase gene in the correct position with respect to the direction of transcription by T7 RNA polymerase. Hence the induction of synthesis of β-lactamase and its precursors (marked with dots) is also observed. B. BL21/DE3 cells containing plasmid pT7-5-fragment III: 1, non-induced; 2, one hour post-induction with IPTG. Gene 5/ product of molecular mass about 30 kDa is indicated with an arrow. The synthesis of β-lactamase and its precursors is not observed as the β-lactamase gene in pT7-5 plasmid is in the opposite direction to the direction of transcription by bacteriophage T7 RNA polymerase.
Identification of gene 25 and gene 26 products. For this purpose the following plasmids were used: pT7-5-fragment I (XbaI-PstI), pT7-5-fragment II (XbaI-HindIII) and pT7-5-fragment IV (PstI-HindIII). The complementation test indicated that the third plasmid contained gene 26 and gene 5I. However, when vector pT7-5 is used, T7 polymerase transcribes only gene 26 because gene 5I is transcribed from the complementary strand [10, 11]. Figure 2 represents the results of selective isotope labelling of the proteins for which mRNA was synthesized by T7 RNA polymerase. In the case of pT7-5-fragment I two isotope labelled products were synthesized; one of molecular mass about 16 kDa, the second about 11 kDa. The protein of 16 kDa disappeared when the whole gene 25 was removed (plasmid pT7-5-fragment II) which indicates that this protein is the gene 25 product.

Two strong protein bands are visible in the case of $^{35}$S labelling of bacteria harbouring plasmid pT7-5-fragment IV, which contains the full length gene 26 located in the right orientation with respect to the direction of transcription by T7 RNA polymerase. One of the protein bands (molecular mass about 11 kDa) is in identical position as the band appearing during labelling of bacteria carrying plasmid pT7-5-fragment II or plasmid pT7-5-fragment I. Molecular mass of the second band is about 25 kDa. On the basis of these results we conclude that gene 26 codes for two proteins of these molecular masses. The start codon of the smaller polypeptide must be closer to gene 25 than that of the larger polypeptide because the smaller polypeptide is synthesized even when the beginning of gene 26 is truncated (plasmids with fragment I and fragment II). The synthesis of two polypeptides of these molecular masses in bacteria with plasmids containing gene 26 was also recently observed by Nivinski and al. [10]. As our complementation data show (Table 2), the incorporation of the smaller polypeptide into the 26- defective phage particles leads to some increase in phage titer on a non-permissive strain, but both polypeptides are necessary for full complementation.

Identification of gene 5I product. Identification of this gene product was possible by isotope labelling of bacteria carrying plasmid pT7-5-fragment III. Alternatively, plasmid pT7-6-fragment IV could be used. The main protein band labelled with $[^{35}]$S methionine was of molecular mass about 30 kDa (Fig.3). This is in agreement with the data obtained from sequencing of gene 5I [11]. Three more protein bands of lower intensity and of lower molecular mass are also visible on
autoradiograms. There are several possible explanations of this pattern of protein synthesis. It is possible but not very likely that gene 51 codes for all four polypeptides, and in this case more than one reading frame would be used. It is however, more rational to suppose that the largest polypeptide is unstable and the three other polypeptides are its degradation products. It cannot be excluded, either, that the original precursor protein undergoes post-translational processing which results in polypeptides of required biological activity but of lower molecular mass. This mechanism is well established for other proteins taking part in bacteriophage T4 morphogenesis. One of the approaches to get some insight into this problem (which we plan to try in future) is to obtain a monospecific antiserum against the largest polypeptide and compare by immunoblotting the protein electrophoretic pattern of host cells infected with the wild type and 51− mutant of bacteriophage T4.

Level of expression of recombinant proteins. Figure 4 represents the Coomassie Brilliant Blue electrophoretic gels on which the samples of induced bacteria carrying different pT7 plasmids were applied. The level of gene 25 product is very high and it is the dominant protein in the induced bacteria. The largest polypeptide synthesized in bacteria with the plasmid containing gene 51 is clearly visible but it is present in smaller quantities as compared with the gene 25 product. The full length product of gene 26 (not shown) though visible on the gel, is produced at a level lower than that of the gene 51 product. In our opinion, the gene 25 product and gene 51 product are synthesized in amounts sufficient for their purification, which is now in progress in our laboratory.

REFERENCES


