Expression Using the T7 RNA Polymerase/Promoter System

This unit describes the expression of genes by placing them under the control of the bacteriophage T7 RNA polymerase. This approach has a number of advantages compared to approaches that rely on *E. coli* RNA polymerase. First, T7 RNA polymerase is a very active enzyme: it synthesizes RNA at a rate several times that of *E. coli* RNA polymerase and it terminates transcription less frequently; in fact, its transcription can circumnavigate a plasmid, resulting in RNA several times the plasmid length in size. Second, T7 RNA polymerase is highly selective for initiation at its own promoter sequences and it does not initiate transcription from any sequences on *E. coli* DNA. Finally, T7 RNA polymerase is resistant to antibiotics such as rifampicin that inhibit *E. coli* RNA polymerase, and consequently, the addition of rifampicin to cells that are producing T7 RNA polymerase results in the exclusive expression of genes under the control of a T7 RNA polymerase promoter (hereafter referred to as p_{T7}).

To use the two-plasmid p_{T7} system, it is necessary to clone the gene to be expressed into a plasmid containing a promoter recognized by the T7 RNA polymerase. The gene is then expressed by induction of T7 RNA polymerase. The gene for T7 RNA polymerase is present on a second DNA construction. This second construction can either permanently reside within the *E. coli* cell (basic protocol), or can be introduced into the cell at the time of induction by infection with a specialized phage, such as an M13 vector (mGP1-2; Tabor and Richardson, 1987) or a λ vector (CE6; Studier et al., 1990) containing the T7 RNA polymerase gene (second alternate protocol).

In the basic protocol, two plasmids are maintained within the same *E. coli* cell. One (the expression vector) contains p_{T7} upstream of the gene to be expressed. The second contains the T7 RNA polymerase gene under the control of a heat-inducible *E. coli* promoter. Upon heat induction, the T7 RNA polymerase is produced and initiates transcription on the expression vector, resulting in turn in the expression of the gene(s) under the control of p_{T7}. If desired, the gene products can be uniquely labeled by carrying out the procedure in minimal medium, adding rifampicin to inhibit the *E. coli* RNA polymerase, and then labeling the proteins with [^{35}S]methionine (first alternate protocol).

EXPRESSION USING THE TWO-PLASMID SYSTEM

BASIC PROTOCOL

The gene to be induced is subcloned into an expression vector containing p_{T7}. Two series of vectors have been developed for this purpose—the pT7 series (Fig. 16.2.1) and the pET series (Studier et al., 1990); see commentary for discussion of choice of vector. The plasmid containing the introduced gene is then used to transform an *E. coli* strain already containing the plasmid pGP1-2 (Fig. 16.2.2). pGP1-2 contains the gene for T7 RNA polymerase under the control of the λ p_L promoter that is repressed by a temperature-sensitive repressor (*c*I857). pGP1-2 contains a p15A origin of replication that is compatible with the ColE1 origin of replication on the expression vector. The two plasmids are maintained in the same cell by selection with kanamycin (pGP1-2) and ampicillin (the expression vector).

Cells containing the two plasmids are grown for several hours at 30°C and then the gene for T7 RNA polymerase is induced by raising the temperature to 42°C. The production of T7 RNA polymerase in turn induces expression of the genes under the control of p_{T7}. (Rifampicin can be subsequently added to inhibit transcription by *E. coli* RNA polymerase, although this is usually not necessary since T7 RNA polymerase becomes responsible for most of the transcription even in the absence of rifampicin.) After expression

Contributed by Stanley Tabor
Current Protocols in Molecular Biology (1990) 16.2.1-16.2.11

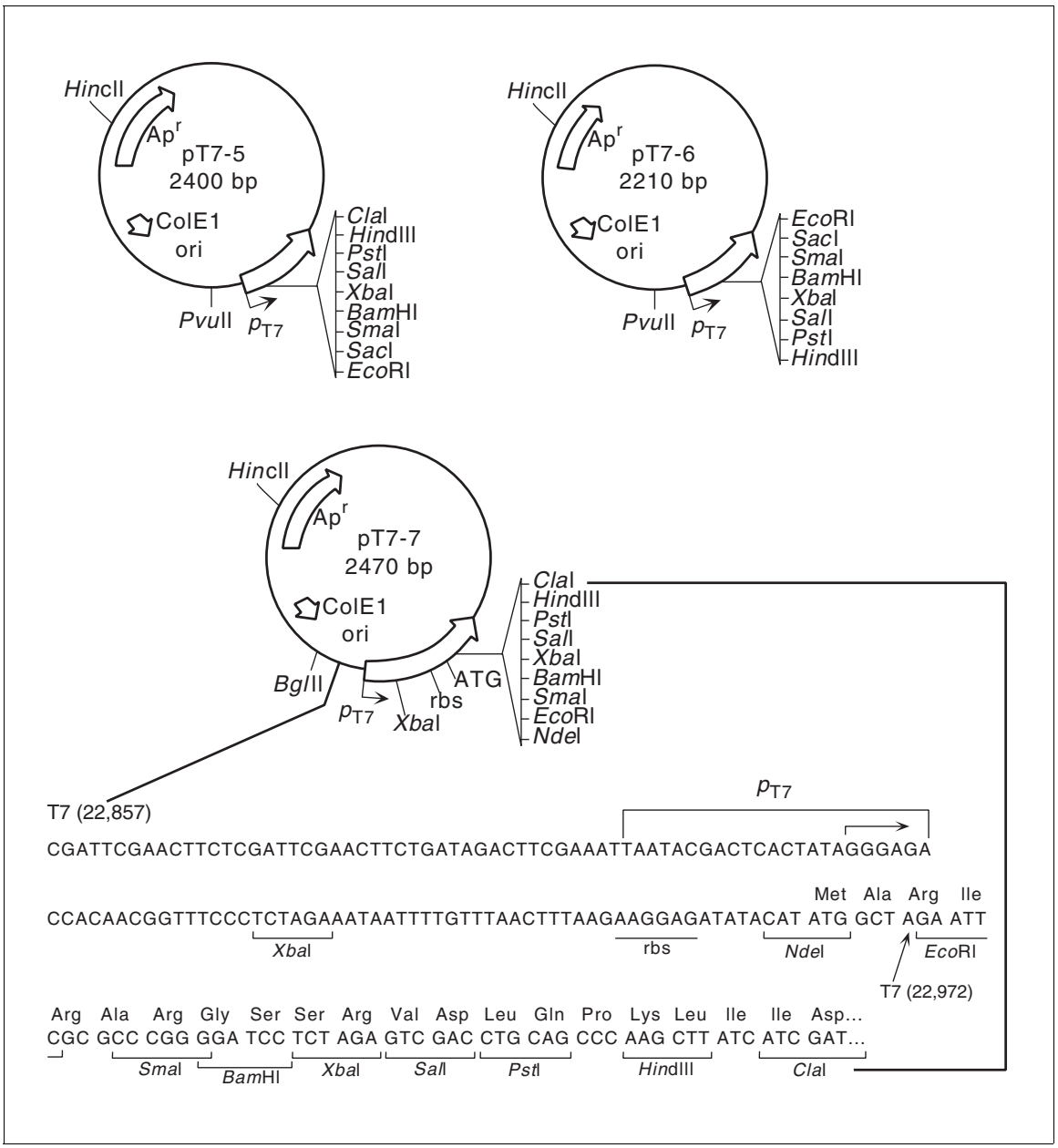

Figure 16.2.1 pT7-5, pT7-6, and pT7-7. pT7-5, pT7-6, and pT7-7 are cloning vectors that contain a T7 promoter and are used to express genes using T7 RNA polymerase. All three vectors contain a T7 RNA polymerase promoter, the gene encoding resistance to the antibiotic ampicillin and the ColE1 origin of replication. pT7-7 has a strong ribosome-binding site (rbs) and start codon (ATG) upstream of the polylinker sequence; the sequence of this region is shown below the map of pT7-7. pT7-5 and pT7-6 lack any ribosome-binding site upstream of the polylinker sequence and consequently are only useful when expressing genes that already contain the proper control sequences. pT7-5, pT7-6, and pT7-7 were constructed by S. Tabor and are derivatives of pT7-1 described in Tabor and Richardson (1985).

of the genes at 37°C, the cells are harvested and the induced proteins are analyzed. An alternative approach is to induce T7 RNA polymerase with IPTG rather than by heat induction. In this method, the expression plasmid containing p_{T7} can be placed into *E. coli* BL21 (DE3), which contains the gene for T7 RNA polymerase on the *E. coli* chromosome under the control of the *lac* promoter (Studier and Moffatt, 1986; Studier et al., 1990).

Materials

pT7-5, pT7-6, or pT7-7 vectors (available from author)
E. coli JM105, DH1, or equivalent (Table 1.4.5)
LB plates and medium containing 60 µg/ml ampicillin (*UNIT 1.1*)
E. coli K38 or equivalent (Table 1.4.5)
pGP1-2 (available from author)
LB plates and medium containing 60 µg/ml kanamycin (*UNIT 1.1*)
LB plates and medium containing 60 µg/ml ampicillin plus 60 µg/ml kanamycin (*UNIT 1.1*)

Cracking buffer
Sorvall SS-34 or GS-3 rotor or equivalent

Additional reagents and equipment for subcloning DNA fragments (*UNITS 1.4 & 3.16*), transformation of competent *E. coli* cells (*UNIT 1.8*), minipreps of plasmid DNA (*UNIT 1.6*), restriction mapping (*UNITS 3.1-3.3*), and SDS-PAGE (*UNIT 10.2*).

1. Subclone the fragment containing the gene to be expressed into pT7-5, pT7-6, or pT7-7. Transform a standard *E. coli* strain (e.g., JM105 or DH1); this strain should *not* carry a plasmid that directs synthesis of T7 RNA polymerase (i.e., pGP1-2). Plate the transformants on LB/ampicillin plates and grow overnight at 37°C.

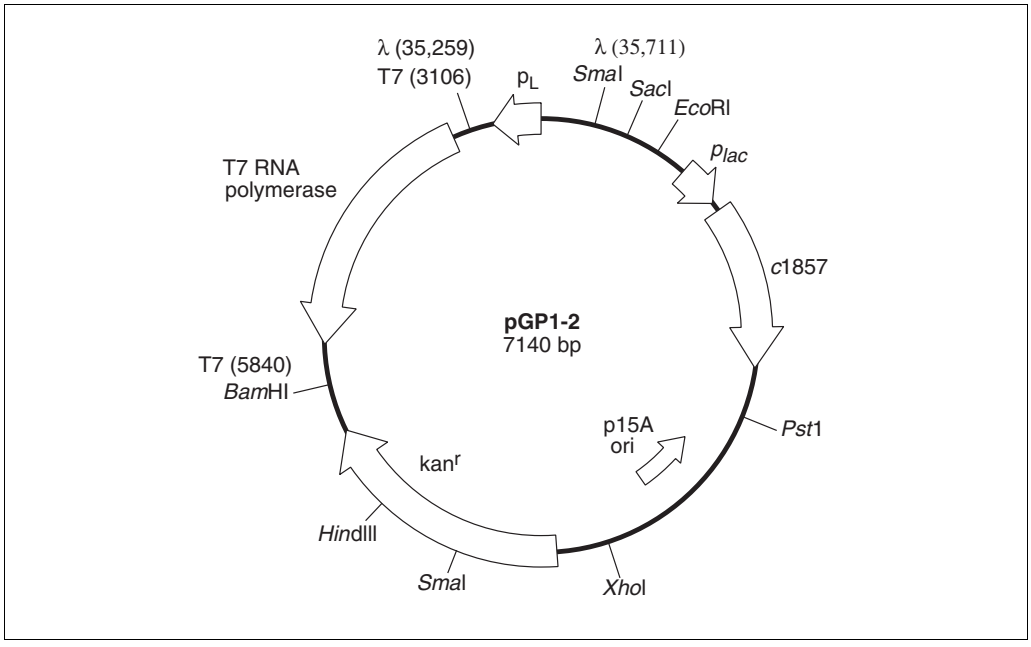

Figure 16.2.2 pGP1-2. pGP1-2 enables T7 RNA polymerase to be produced by heat induction in any *E. coli* host. pGP1-2 contains the gene for T7 RNA polymerase under the control of the λ p_L promoter. It also contains the gene for the λ repressor (*c*I857) that is expressed under the control of *E. coli* p_{lac} promoter. This repressor inhibits transcription from the λ p_L promoter at low temperature (30°C); however, at high temperature (42°C) it is inactivated, resulting in induction of the p_L promoter, that in turn results in induction of the T7 RNA polymerase. pGP1-2 also contains the gene encoding resistance to the antibiotic kanamycin, and the p15A origin of replication. pGP1-2 is described in Tabor and Richardson (1985).

It is important to first transform the plasmid into a strain that contains no T7 RNA polymerase, in case small amounts of the gene product are toxic to the cell (see critical parameters for discussion on toxic genes).

2. Grow individual transformants in LB/ampicillin medium at 37°C and obtain plasmid DNA by a miniprep procedure. Confirm that the gene has been correctly inserted by restriction mapping.

3. Transform *E. coli* K38 with pGP1-2, plate on LB/kanamycin plates, and grow overnight at 30°C. Grow an individual *E. coli* K38/pGP1-2 transformant in LB/kanamycin medium at 30°C.

   Colonies take ~24 hr to appear on plates at 30°C. E. coli K38/pGP1-2 can be stored in the absence of the plasmid containing p_{T7} as a glycerol stock at –80°C (see commentary).

4. Transform the vector containing the gene to be expressed under the control of p_{T7} into *E. coli* K38/pGP1-2 grown in LB/kanamycin medium. Plate the transformants (containing both plasmids) on LB/ampicillin/kanamycin plates and grow overnight at 30°C.

   Cells may be heat-shocked during transformation; the T7 RNA polymerase gene, under the control of a heat-inducible promoter, is not induced by this brief heating step.

   As a control, transform E. coli K38/pGP1-2 with the parent p_{T7} vector (without an insert). If the transformation efficiency of the vector containing the insert is significantly lower (by more than a factor of 50) than that of the parent vector, the gene product may be toxic to E. coli cells. This toxicity arises from background expression of the gene product by basal levels of T7 RNA polymerase. In this situation, the transformants that do arise invariably contain deletions or other mutations in one of the two plasmids, and the desired gene product will not be produced. If the expression of the inserted gene is toxic, it is necessary to use an alternative strategy for the repression and induction of the T7 RNA polymerase gene (see discussion on toxic genes in critical parameters).

5. Pick a single *E. coli* colony that contains the two plasmids with a sterile toothpick or pipet. Inoculate it into 5 ml LB/ampicillin/kanamycin medium and grow overnight at 30°C.

6. Dilute the overnight culture of cells 1:40 into fresh LB/ampicillin/kanamycin medium and grow several hours at 30°C to an $OD_{590} \cong 0.4$.

   The size of the culture will depend on the amount of cells needed. For an analytical preparation, use ~1 ml of cells.

7. Induce the gene for T7 RNA polymerase by raising the temperature to 42°C for 30 min, which in turn induces the genes under the control of p_{T7}.

   To obtain consistent results, raise the temperature relatively quickly. If small cultures (~1 ml) are being induced, place the cultures into a 42°C water bath. For larger cultures (~500 ml), place the flask under hot tap water until the temperature of the media reaches 42°C (measured by inserting a thermometer wiped with ethanol into the flask). Once the cells reach 42°C, continue incubating at 42°C for 30 min.

   The E. coli RNA polymerase can be inhibited by adding rifampicin to a final concentration of 200 μg/ml; when used, it should be added after T7 RNA polymerase has been induced at 42°C for 30 min. Although rifampicin reduces the background of host proteins being expressed, in general it does not significantly increase the final accumulation of gene products, and in some cases it decreases the final yield. Thus, as a general rule, rifampicin is only added to cells when the plasmid-encoded proteins are being uniquely labeled with [^{35}S]methionine (see first alternate protocol).

8. Reduce temperature to 37°C and grow the cells an additional 90 min with shaking.
9. Harvest the cells by centrifuging and discarding the supernatant. For 1-ml cultures, microcentrifuge 20 sec at 10,000 rpm (14,000 × *g*), room temperature. For 2-ml to 100-ml cultures, centrifuge 5 min in a Sorvall SS-34 rotor at 5000 rpm (3000 × *g*), 4°C. For >100-ml cultures, centrifuge 10 min in a Sorvall GS-3 rotor at 5000 rpm (4000 × *g*), 4°C.
10. To analyze the induced proteins by SDS-PAGE, resuspend the equivalent of 1.0 ml of cells in 0.1 ml cracking buffer. Heat at 100°C for 5 min immediately prior to loading a 20-µl aliquot of each sample onto an SDS-polyacrylamide gel (*UNIT 10.2*). To analyze the cells for an induced enzymatic activity, prepare an appropriate cell extract from ~10 ml of cells.

One example of the preparation of an extract for the purification of T7 RNA polymerase is described in Tabor and Richardson (1985).

SELECTIVE LABELING OF PLASMID-ENCODED PROTEINS

ALTERNATE PROTOCOL

Plasmid-encoded proteins under the control of a p_{T7} (see basic protocol) can be exclusively labeled by inducing the T7 RNA polymerase in cells growing in minimal medium, inhibiting the host *E. coli* RNA polymerase with rifampicin, and labeling the newly synthesized proteins with [^{35}S]methionine. This procedure provides an attractive alternative to maxicells or minicells for labeling of plasmid-encoded proteins (Dougan and Sherratt, 1977; Sancar et al., 1981).

Additional Materials

M9 medium (*UNIT 1.1*) without and with 5% (v/v) of 18 amino acid mixture
20 mg/ml rifampicin in methanol (e.g., Sigma #R-3501; store in dark at 4°C for 2 weeks; Table 1.4.1)
10 mCi/ml [^{35}S]methionine (>800 Ci/mmol) diluted 1:10 in M9 medium
Fluorographic enhancing agent (e.g., Enlightning from Du Pont NEN or Amplify from Amersham)

1. Repeat steps 3 to 6 of the basic protocol (using the T7-promoter expression plasmid obtained from steps 1 and 2 of the basic protocol).

An alternative to the use of LB/ampicillin/kanamicin medium for growing cells is M9 medium containing 25 µg/ml ampicillin and 25 µg/ml kanamycin, and any required nutrients. The addition of one part in twenty of the 18 amino acid mixture (0.1% stock, 0.005% final concentration) stimulates the growth of cells in M9 medium without interfering with the subsequent labeling of the proteins with [^{35}S]methionine. Note that to grow in this medium, the E. coli strain must be Cys$^+$ and Met$^+$.

2. When $OD_{590} \cong 0.4$, remove 1 ml of cells, microcentrifuge 10 sec, and discard supernatant.
3. Wash cell pellet with 1 ml M9 medium, microcentrifuge 10 sec at room temperature, and discard supernatant.

Washing the cells after growth in LB medium is very important in order to remove the unlabeled methionine present in LB medium that otherwise dilutes the [^{35}S]methionine during labeling.

4. Resuspend cell pellet in 1 ml M9 medium containing 18 amino acid mixture. Grow cells 60 min at 30°C with shaking.

A time of 30 to 180 min is adequate for adapting cells to M9 medium. Although the OD_{590} may not increase significantly during this step, induction of T7 RNA polymerase and

efficient labeling of the plasmid-encoded proteins will occur even in the absence of apparent cell growth.

5. Induce the gene for T7 RNA polymerase by placing the cells in a 42°C water bath for 20 min.

6. Add 20 mg/ml rifampicin to 200 µg/ml final. Keep cells at 42°C for an additional 10 min after adding rifampicin.

   It is important to incubate the cells at 42°C for an additional 10 min after adding rifampicin, since rifampicin is more effective at inhibiting expression of host proteins at 42°C, possibly because the cells are more permeable to it at this temperature. The temperature of the cells is subsequently reduced for the labeling since in general the labeling is less efficient at 42°C than at 30° or 37°C.

7. Shift cells to a 30°C water bath for an additional 20 min. Remove 0.5 ml of cells for labeling with [^{35}S]methionine.

   The other 0.5 ml can be used to label the cells at a later time point (e.g., after an additional 30 min) in order to follow the duration of protein synthesis.

8. Label newly synthesized proteins by adding 10 µl (10 µCi) diluted [^{35}S]methionine to 0.5 ml of cells and incubating for 5 min at 30°C.

9. Microcentrifuge cells 10 sec and discard supernatant. (*CAUTION*: the supernatant is radioactive; discard properly.) Resuspend cell pellet in 100 µl cracking buffer.

10. Heat samples to 100°C for 5 min. Load a 20-µl aliquot onto an SDS-polyacrylamide gel and electrophorese (*UNIT 10.2*).

11. Treat the gel with a fluorographic-enhancing agent by soaking it in the fluor for 30 min. Dry the gel under vacuum 2 hr at 65°C and autoradiograph (*APPENDIX 3*).

    A 1-hr exposure should be adequate to visualize most proteins induced with this system.

    To determine whether the plasmid-encoded proteins are susceptible to proteases in the E. coli cell, prepare and induce the cells as described above; however, reduce the duration of the labeling step to 1 min (step 8), and follow this with a chase of nonradioactive methionine at 0.5% final concentration. Remove an aliquot for analysis both immediately prior to the chase, and after a chase reaction of 5, 15, and 60 min. After removing each aliquot, immediately pellet the cells by centrifugation, resuspend in cracking buffer, and heat the aliquot to 100°C for 5 min to inactivate the proteases. Analyze as in step 10.

ALTERNATE PROTOCOL

EXPRESSION BY INFECTION WITH M13 PHAGE mGP1-2

Whenever the gene for T7 RNA polymerase is present in *E. coli* cells, low levels of T7 RNA polymerase are constitutively produced. This can be a problem when the gene products under the control of p_{T7} are toxic. One strategy to avoid this is to keep the gene for T7 RNA polymerase out of the cell until the time of induction. In the protocol presented here, T7 RNA polymerase is introduced into the cell by infection with the M13 phage mGP1-2. This phage contains the gene for T7 RNA polymerase under the control of the *lac* promoter (Fig. 16.2.3). Host cells for this phage must carry the F factor so that they are susceptible to M13 infection (e.g., JM101 or K38). The cells are transformed with the single plasmid that contains the gene to be expressed under the control of p_{T7}. The cells are grown at 37°C, and induction occurs by infection with a high multiplicity of mGP1-2 in the presence of IPTG. A λ vector, CE6, that contains the gene for T7 RNA polymerase has also been used to express toxic genes (Studier and Moffatt, 1986; Studier et al., 1990).

Additional Materials

M13 phage mGP1-2 (available from author)
PEG solution (*UNIT 1.7*)
100 mM IPTG (Table 1.4.2)

Additional reagents and equipment for preparing M13 phage (*UNIT 1.15*) and titering phage (*UNIT 1.11*)

1. Prepare a stock of M13 phage mGP1-2 and concentrate the phage by precipitation with PEG solution. (DO NOT proceed to add TE buffer or phenol.) Resuspend phage in M9 medium and titer.

   If the cell proteins are to be labeled, it is important that the phage used to infect the cells are free of unlabeled methionine. In this case, precipitate the phage with PEG twice, each time resuspending the pellet in M9 medium. For long-term storage of the M13 phage mGP1-2, it is best to purify the phage through a CsCl gradient (Nakai and Richardson, 1986).

2. Transform *E. coli* cells susceptible to M13 infection (e.g., JM101 or K38) with the T7-promoter expression plasmid obtained from steps 1 and 2 of the basic protocol. Plate the transformants on LB/ampicillin plates and grow overnight at 37°C.

3. Pick a single colony and grow in LB/ampicillin medium overnight at 37°C.

4. Dilute the overnight culture of cells 1:100 in LB/ampicillin medium and grow several hours at 37°C with gentle shaking to $OD_{590} \cong 0.5$.

   It is very important that only gentle shaking is used when growing cells for M13 infection. Vigorous agitation results in shearing of the pili on the surface of the E. coli cells, resulting in inefficient infection.

5. Infect cells with M13 phage mGP1-2 (from step 1) at a ratio of ~10 phage for each *E. coli* cell. Add 100 mM IPTG to 1 mM final (a 1:100 dilution) to induce production

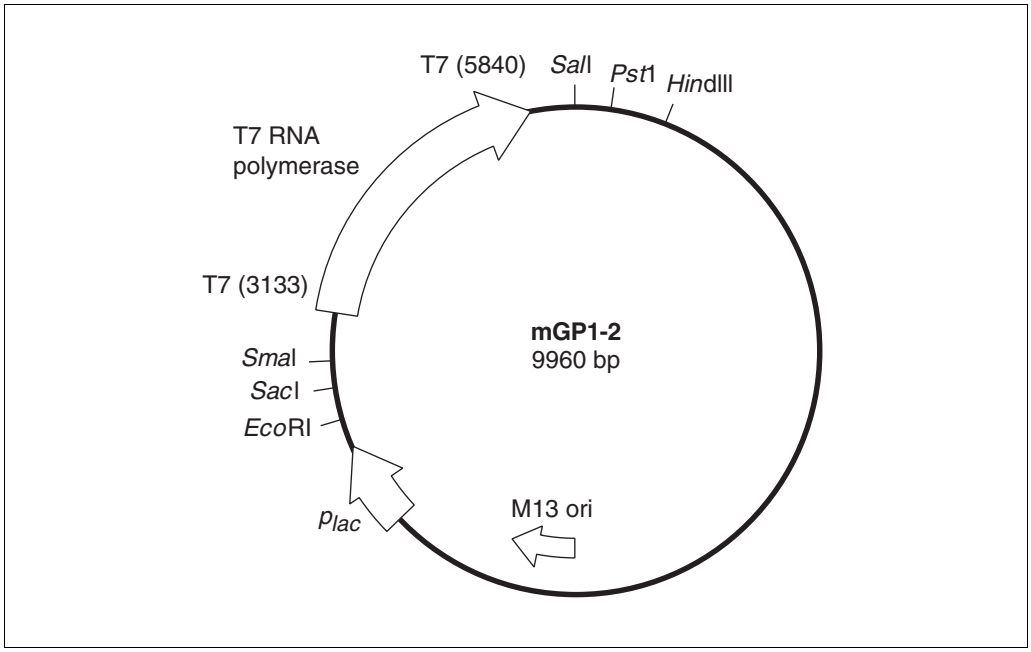

Figure 16.2.3 mGP1-2. M13 phage mGP1-2 contains the gene for T7 RNA polymerase under the control of the *E. coli* p_{lac} promoter. It is especially useful for the production of gene products that are toxic to the *E. coli* cell. When *E. coli* cells are infected with this phage, and IPTG is added to induce the p_{lac} promoter, T7 RNA polymerase is produced. As a result, any genes within the cell under the control of p_{T7} will be induced. mGP1-2 is described in Tabor and Richardson (1987).

of T7 RNA polymerase. Incubate the cells 2 hr at 37°C.

At $OD_{590} \cong 0.5$, the density of E. coli cells will be ~2×10^8 cells/ml. Thus, it is necessary to add M13 mGP1-2 phage at a final concentration of 2×10^9 phage/ml to obtain a multiplicity of infection of 10. Small cultures (~50 ml) can be incubated in a water bath without shaking. Larger cultures should be incubated at 37°C with gentle shaking.

6. Harvest cells and analyze induced proteins as in steps 9 and 10 of the basic protocol.

REAGENTS AND SOLUTIONS

18 amino acid mixture

Prepare a solution containing 0.1% (v/v) of each amino acid except cysteine (minus cysteine) and methionine (minus methionine). Filter sterilize through a 0.2-μm filter. Store at −20°C for several years.

Cracking buffer

60 mM Tris·Cl, pH 6.8
1% 2-mercaptoethanol
1% sodium dodecyl sulfate (SDS)
10% glycerol
0.01% bromphenol blue

COMMENTARY

Background Information

Bacteriophage T7 and T7-related phage (e.g., SP6, T3) encode their own RNA polymerase (see *UNIT 3.8*). Compared to other known RNA polymerases, this RNA polymerase is both relatively simple and highly efficient. T7 RNA polymerase is a single polypeptide of 96,000 kDa. It initiates transcription specifically at a 23-nucleotide promoter sequence, a sequence not present on the *E. coli* genome. Transcription is very processive, producing transcripts that are many thousands of nucleotides in length. Transcription is relatively rapid—five times the rate of *E. coli* RNA polymerase. All of these properties make T7 RNA polymerase and its promoter an attractive system for controlling the expression of foreign genes in *E. coli* and in other organisms. Expression systems in *E. coli* based on the controlled induction of T7 RNA polymerase have been developed by Tabor and Richardson (1985) and Studier and his colleagues (Studier and Moffatt, 1986; Rosenberg et al., 1987; Studier et al., 1990). The vectors described here are those developed by Tabor and Richardson. T7 RNA polymerase/promoter expression systems have also been successfully applied in yeast (Chen et al., 1987) and mammalian cells (Dunn et al., 1988; Fuerst et al., 1986).

Critical Parameters

Choice of vector

Questions that determine what vector to use to express a gene using T7 RNA polymerase include: Is there a ribosome-binding site upstream of the gene? What are the restriction sites available on each end of the gene? Is the gene product toxic to the *E. coli* cell? Examples of three standard vectors (pT7-5, pT7-6, and pT7-7) are shown in Figure 16.2.1. These vectors are derivatives of pBR322. The β-lactamase gene encoding amp^r is in the opposite orientation of p_{T7}; consequently the only plasmid-encoded genes expressed by T7 RNA polymerase are those cloned into the polylinker region. pT7-5 and pT7-6 contain the polylinker region located immediately downstream of pT7 in opposite orientations. There is no ribosome-binding sequence in these two plasmids; they should thus be used either for the production of transcripts without expectation of good translation of the protein, or for the expression of genes that already have strong ribosome-binding sequences. pT7-7 differs from pT7-5 and pT7-6 in that it contains a strong ribosome-binding sequence between p_{T7} and the polylinker region; it is recommended for the expression of genes that lack a strong ribosome binding sequence or for the production of fusion proteins.

An extensive series of additional vectors containing p_{T7}, the pET series, have been described by Studier et al. (1990). These vectors are particularly useful for applications that require a greater selection of restriction endonuclease sites to insert the gene into, or that involve the expression of a gene that is toxic to the cell (see below). Some of these vectors contain other transcriptional regulatory elements (i.e., terminators, operators, RNase III cleavage sites) that could be of use for specific applications.

A large number of commercially available vectors contain a T7 RNA promoter (e.g., pIBI vectors, available from IBI; pSP6/T7-19, available from GIBCO/BRL; pBluescript II vectors, available from Stratagene; and pTZ18R and pTZ19R, available from U.S. Biochemical). These are intended to be used for producing specific transcripts in vitro using T7 RNA polymerase. In principle, they should be useful for the expression of genes using T7 RNA polymerase in vivo as well. In practice, however, the use of some of these vectors can result in some unexpected problems. (1) Most commercial vectors have extremely high copy numbers within the cell; this can accentuate the problems encountered with toxic genes. (2) In most vectors, the β-lactamase gene is oriented in the same direction as p_{T7}, complicating the analysis of radiolabeled proteins. (3) Some commercial vectors have p_{T7} oriented in a potentially deleterious direction. Derivatives of pBR322 that contain p_{T7} oriented clockwise with respect to the standard map are inviable in some *E. coli* strains that contain the gene for T7 RNA polymerase. This is due to the fact that high levels of transcription through the origin region of these plasmids in this orientation interferes with the replication of the plasmids. (4) Most commercial vectors have a *lac* operator sequence within them. This can titrate out the *lac* repressor (*UNIT 1.4*) and cause problems when the p_{lac} is used to control the T7 RNA polymerase gene.

Toxic genes

In some cases the gene to be expressed is toxic to the cells, even when it is not induced. This is due to a low level of constitutive expression present even under uninduced conditions. Although most genes are not toxic when expressed using the two-plasmid p_{T7} system, it is important to recognize the symptoms of toxicity to avoid selecting for mutations and to allow alternate systems for induction to be tried. The degree of toxicity varies greatly with each gene. The symptoms encountered with toxic genes are discussed below, in order of increasing toxicity.

Some genes are mildly toxic to the cells when expressed using the two-plasmid p_{T7} system. In such cases, the cells can be stably transformed with the two plasmids and the gene product is produced at a high level. However, after the cells are several days old, they no longer induce the expected gene product even though they remain resistant to ampicillin and kanamycin. To avoid this problem, it is recommended that the *E. coli* K38/pGP1-2 be stored in the absence of the plasmid containing p_{T7} as a glycerol stock at −80°C (*UNIT 1.3*). The plasmid containing p_{T7} and the gene to be expressed should be stored as DNA at −20°C or −70°C (*UNIT 1.6*). To prepare the strain for induction, streak K38/pGP1-2 on an LB/kanamycin plate at 30°C, grow up a single colony, transform with the plasmid containing p_{T7} and the gene to be expressed, and plate the transformants on LB/ampicillin/kanamycin plates at 30°C. A single colony should then be grown at 30°C and induced as described above. This procedure is not necessary for genes that are not toxic. Strains that do not induce toxic genes can be stored in glycerol at −80°C for many months (*UNIT 1.3*).

A more toxic class of genes consists of those that can be successfully cloned into a plasmid under the control of p_{T7}, but that render the resulting plasmid unable to stably transform a cell that contains the gene for T7 RNA polymerase. Genes that are toxic to the cells only in the presence of pGP1-2 (which expresses the T7 RNA polymerase) are relatively common, occurring on the average ~5% of the time (S. Tabor, unpublished observation). Note that such plasmids will give transformants in *E. coli* cells containing pGP1-2, but that the frequency of transformation will be greatly reduced (>50-fold) compared to the frequency of transformation by the parent vector alone. The cells that do grow in the presence of ampicillin and kanamycin will invariably contain deletions or other mutations in one of the two plasmids, and the desired gene product will not be produced.

When genes are toxic at this level, it is necessary to use an alternative strategy that reduces the expression of the gene under uninduced conditions. One strategy is to remove the gene for T7 RNA polymerase from the cell until induction is desired, and then introduce it by a phage infection. Such an alternate protocol is described using an M13 phage harboring the gene for T7 polymerase, mGP1-2. A lambda

vector, CE6, that contains the gene for T7 RNA polymerase has also been used for this purpose (Studier and Moffatt, 1986; Studier et al., 1990). Another strategy is to retain the gene for T7 RNA polymerase in the cell but reduce the level of transcription by T7 RNA polymerase under uninduced conditions. For example, a system has been developed that expresses an inhibitor of T7 RNA polymerase—the T7 lysozyme—to reduce the activity of T7 RNA polymerase until it is induced (Studier et al., 1990). Another recent modification is the placement of p_{T7} under the control of the *lac* repressor, reducing the activity of T7 RNA polymerase until IPTG has been added (Studier et al., 1990).

Finally, some genes are difficult to clone in multicopy plasmids even in the absence of a known *E. coli* promoter. The difficulty in cloning these genes arises from the fact that their products are extremely toxic and that the residual low level of transcription by *E. coli* RNA polymerase in most plasmids is sufficient to direct the synthesis of small amounts of these proteins. One strategy that can be used to clone such toxic genes is to insert the gene near a strong *E. coli* promoter that is oriented so that transcription by the *E. coli* RNA polymerase results in the accumulation of RNA that is antisense to the toxic gene, reducing the level of its gene product. It is important to remember that the amount of a gene product synthesized is a function not only of the level of transcription but also of the efficiency at which translation is initiated. This is determined primarily by the ribosome-binding sequence located upstream of the start codon. Thus, some toxic genes with relatively weak ribosome-binding sequences can be cloned into multicopy plasmids, but not into a multicopy plasmid that also introduces a strong ribosome-binding sequence (S. Tabor, unpublished observations).

In summary, the first step in using the T7 RNA polymerase/promoter system is to clone the gene into an appropriate vector containing a p_{T7} and be certain it has an efficient ribosome-binding sequence. Once this is accomplished, the next step is determining whether the plasmid can stably transform an *E. coli* cell containing pGP1-2 at an efficiency comparable to that of the parent vector alone. If this is successful, the system is ready to be induced. If unsuccessful, it is necessary to induce the gene either by infection with M13 phage mGP1-2 (see second alternate protocol), or to use one of the more specialized vectors that further reduce the expression of T7 RNA polymerase in the cell under uninduced conditions (Studier et al., 1990).

Troubleshooting

For gene expression, one of the major advantages of the T7 RNA polymerase/promoter system over an *E. coli* RNA polymerase system is the ability to exclusively label the gene products under the control of p_{T7}. If the level of induction of the gene is estimated by inspection of a standard SDS-polyacrylamide gel, and it is difficult to see the expected induced product, then it is recommended that the induced proteins be labeled using [^{35}S]methionine as described in the first alternate protocol. This is a much more sensitive and specific assay for the specific protein production. Be sure that there is at least one methionine codon in the gene other than the one at the start of the protein (which is often removed in *E. coli*; Kirel et al., 1989); if not, then it is necessary to label with a cysteine or some other amino acid.

If it is not possible to detect the expected labeled product, there may be a problem with one of the two plasmids in the cell. One possibility is that the expressed protein is toxic to the cell, and as a result, a mutation has been selected for such that the toxic product is not synthesized. For more information on determining whether a gene is toxic, see the discussion on toxic genes in critical parameters. To determine if the cells and T7 RNA polymerase gene (e.g., pGP1-2) are inducing T7 RNA polymerase, attempt to induce a control protein that has been shown to work well in this system (e.g., the β-lactamase gene in pT7-1; Tabor and Richardson, 1985).

If the expressed protein does not accumulate significantly after induction, determine its stability in *E. coli* cells by pulse labeling with [^{35}S]methionine and chasing for various time periods with unlabeled methionine. If it is rapidly degraded, try to induce the gene in a protease-deficient strain. It should be noted that there are no known mutations that inactivate several very active *E. coli* proteases, and thus there is a strong probability that the mutant strains available (e.g., *lon*$^-$) will have no effect on the stability of the gene product. In addition, such mutant strains generally grow poorly, and as a consequence the gene products are poorly produced upon induction of T7 RNA polymerase.

The most common reason for poor induc-

tion of a gene is that the translation does not initiate efficiently. Therefore, it is very important that there be an efficient ribosome-binding sequence the proper distance upstream of the gene. If a gene product does not induce well, and the problem is not the stability of the product, try a different ribosome-binding sequence—one that is known to work efficiently. The sequence and spacing between the ribosome-binding sequence and the start codon is critical. Because of this, it is recommended that the gene be inserted into a vector such as pT7-7, without altering any of the sequences between the ribosome-binding sequence and the start codon.

Anticipated Results

Under optimal conditions, the gene product expressed by the T7 RNA polymerase/ promoter system can accumulate to >25% of the total cellular protein. However, in most instances the amount of gene product that accumulates is significantly less than this. There are numerous reasons for poor yields of gene product, as discussed in troubleshooting (see above).

Time Considerations

It should take ~1 week to insert the gene of interest into the p_{T7} vector, prepare minipreps of the DNA, and characterize the recombinants for the correct size and orientation of the insert. It should then take 3 days to transform the recombinant plasmid into the *E. coli* strain containing pGP1-2, induce the cells, and test the extracts for the production of the expected gene product.

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Key References

Studier et al., 1990. See above.

Gives extensive list of vectors and protocols for expression using T7 RNA polymerase.

Tabor and Richardson, 1985. See above.

Describes the use of the two-plasmid system for expression of genes using T7 RNA polymerase.

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