Conclusion

With the strategy outlined above, we find that we can successfully express most heterologous proteins directly in *E. coli*. Our definition of success is that an obvious band is visible on a Coomassie brilliant blue stain of an SDS–polyacrylamide gel of the total cell lysate following induction.

[6] Use of T7 RNA Polymerase to Direct Expression of Cloned Genes

By F. WILLIAM STUDIER, ALAN H. ROSENBERG, JOHN J. DUNN, and JOHN W. DUBENDORFF

The RNA polymerase of bacteriophage T7 is very selective for specific promoters that are rarely encountered in DNA unrelated to T7 DNA.[1,2] Efficient termination signals are also rare, so that T7 RNA polymerase is able to make complete transcripts of almost any DNA that is placed under control of a T7 promoter. A very active enzyme, T7 RNA polymerase elongates chains about five times faster than does *Escherichia coli* RNA polymerase.[3,4] These properties, together with the availability of the cloned gene,[5,6] make T7 RNA polymerase attractive as the basis for expression systems in *E. coli*[6,7] and, potentially, in a variety of cell types.[8–10]

In principle, T7 expression systems can be completely selective, if the host cell RNA polymerase and T7 RNA polymerase recognize completely different promoters and if the host cell DNA contains no T7 promoters. In this situation, any DNA that can be cloned in the cell can be placed under control of a T7 promoter, since addition of the T7 promoter will not increase expression by the host RNA polymerase. T7 RNA polymerase introduced into such a cell will transcribe actively and selectively only the

[1] M. Chamberlin, J. McGrath, and L. Waskell, *Nature (London)* **228,** 227 (1970).
[2] J. J. Dunn and F. W. Studier, *J. Mol. Biol.* **166,** 477 (1983).
[3] M. Chamberlin and J. Ring, *J. Biol. Chem.* **248,** 2235 (1973).
[4] M. Golomb and M. Chamberlin, *J. Biol. Chem.* **249,** 2858 (1974).
[5] P. Davanloo, A. H. Rosenberg, J. J. Dunn, and F. W. Studier, *Proc. Natl. Acad. Sci. U.S.A.* **81,** 2035 (1984).
[6] S. Tabor and C. C. Richardson, *Proc. Natl. Acad. Sci. U.S.A.* **82,** 1074 (1985).
[7] F. W. Studier and B. A. Moffatt, *J. Mol. Biol.* **189,** 113 (1986).
[8] T. R. Fuerst, E. G. Niles, F. W. Studier, and B. Moss, *Proc. Natl. Acad. Sci. U.S.A.* **83,** 8122 (1986).
[9] W. Chen, S. Tabor, and K. Struhl, *Cell* **50,** 1047 (1987).
[10] J. J. Dunn, B. Krippl, K. E. Bernstein, H. Westphal, and F. W. Studier, *Gene* **68,** 259 (1988).

DNA under control of the T7 promoter. In at least some cases,[7] transcription by T7 RNA polymerase is so active that transcription by the host RNA polymerase apparently cannot compete, and almost all transcription in the cell rapidly becomes due to T7 RNA polymerase.

This article describes materials and techniques for using T7 RNA polymerase to express cloned DNA in *E. coli*.[7,11] Other configurations have been described[6] and further improvements are being explored, but we concentrate here on the current state of the system we have developed. In this system, T7 RNA polymerase is delivered to the cell from the cloned gene by induction or by infection. Vectors for cloning and expressing target DNA derive from the multicopy plasmid pBR322; all carry a T7 promoter followed by a unique cloning site, and some also carry translation initiation signals, a transcription terminator, or an RNase III cleavage site. This system is capable of expressing a wide variety of DNAs from prokaryotic and eukaryotic sources. Under favorable circumstances, the resources of the cell are devoted almost entirely to the production of target RNAs and proteins: within 1–3 hr, target RNA can accumulate to amounts comparable to ribosomal RNA, and target proteins can constitute the majority of total cell protein (Fig. 1).

Hosts and Vectors for Expression

HMS174 and BL21

The bacterial hosts for cloning and expression are the *E. coli* K12 strain HMS174 (F^- *recA* $r^-_{K12}m^+_{K12}Rif^R$)[12] and the B strain BL21 (F^- *ompT* $r^-_Bm^-_B$).[7,13] HMS174 is used as the host for initial cloning of target DNA into pET vectors and for maintaining plasmids. As an expression strain, BL21 has the potential advantage that, as a B strain, it should be deficient in the *lon* protease, and it also lacks the *ompT* outer membrane protease that can degrade proteins during purification.[13] Thus, at least some target proteins might be expected to be more stable in BL21 than in host strains that contain these proteases. HMS174 has the potential disadvantage that rifampicin cannot be used to inhibit transcription by the host RNA polymerase in cases where a reduction of background synthesis of host RNA and proteins may be desirable.

[11] A. H. Rosenberg, B. N. Lade, D. Chui, S. Lin, J. J. Dunn, and F. W. Studier, *Gene* **56,** 125 (1987).

[12] J. L. Campbell, C. C. Richardson, and F. W. Studier, *Proc. Natl. Acad. Sci. U.S.A.* **75,** 2276 (1978).

[13] J. Grodberg and J. J. Dunn, *J. Bacteriol.* **170,** 1245 (1988).

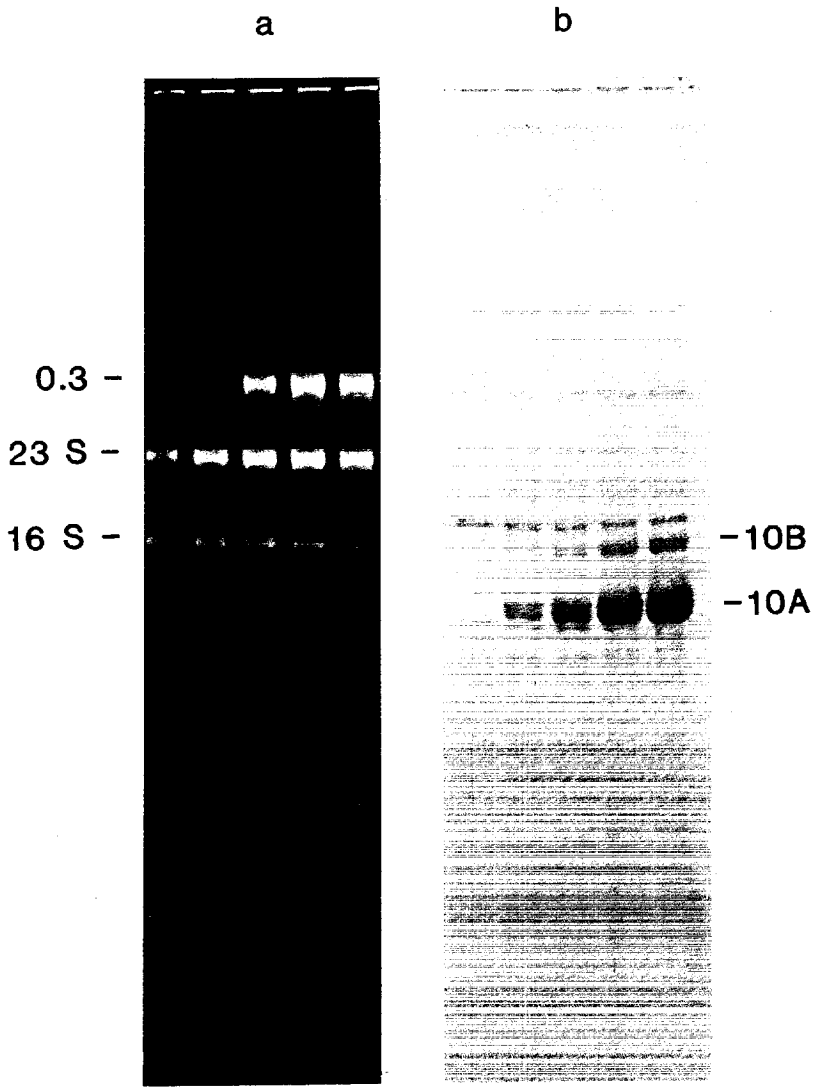

FIG. 1. Accumulation of target RNA and protein. Electrophoresis patterns of total cell RNA [visualized by ethidium bromide fluorescence (a)], and protein [visualized by Coomassie brilliant blue staining (b)] are shown for samples collected immediately before and 0.5, 1, 2, and 3 hr after induction of expression of target DNA (left to right within each set). Cultures of BL21(DE3) containing target plasmid were grown in M9ZB + ampicillin at 37° and expression was induced by addition of 0.4 m*M* IPTG when the OD_{600} reached about 0.7. For both cultures, the plating assay showed that more than 99% of the cells were capable of expressing target DNA at the time of induction. (a) The target plasmid was pAR946, which carries the T7 R*0.3* RNase III cleavage site and gene *0.3* under control of the ϕ*10* promoter (in a configuration equivalent to cloning in pET-1). The position of plasmid-length RNA produced by RNase III cleavage of T7 transcripts that have gone completely around the plasmid is indicated, as are the positions of the 16 and 23S ribosomal RNAs. After samples of culture were centrifuged, the cell pellet was resuspended in 5 volumes of sample buffer, heated for 2 min in a boiling water bath, and 5 μl was subjected to electrophoresis through a 1.4% agarose gel in 40 m*M* Tris-acetate (pH 8.0), 2 m*M* Na_3EDTA. (b) BL21(DE3) contained both pLysS and the target plasmid pAR3625 and was therefore grown in the presence of chloramphenicol as well as ampicillin. This target plasmid contains T7 gene *10* and its flanking ϕ*10* and Tϕ signals (the equivalent of gene *10* cloned in a pET-3 translation vector). The positions of the major gene *10* protein, *10A*, and a minor protein, *10*B, which arises by

BL21(DE3) and HMS174(DE3) Lysogens

Bacteriophage DE3 is a λ derivative that has the immunity region of phage 21 and carries a DNA fragment containing the *lacI* gene, the *lac*UV5 promoter, the beginning of the *lacZ* gene, and the gene for T7 RNA polymerase.[7] This fragment is inserted into the *int* gene, and, because the *int* gene is inactivated, DE3 needs a helper for either integration into or excision from the chromosome. Once a DE3 lysogen is formed, the only promoter known to direct transcription of the T7 RNA polymerase gene is the *lac*UV5 promoter, which is inducible by isopropyl-β-D-thiogalactopyranoside (IPTG). (T7 RNA polymerase is produced from its own translation start and not as a fusion to the beginning of the *lacZ* protein.) Addition of 0.4 m*M* IPTG to a growing culture of either the BL21(DE3) or HMS174(DE3) lysogen induces T7 RNA polymerase, which in turn transcribes the target DNA in the plasmid.

pLysS and pLysE

Target genes whose products are sufficiently toxic cannot be established in BL21(DE3) or HMS174(DE3) because the basal level of T7 RNA polymerase activity will promote some transcription of the target gene in the uninduced cell. One way to reduce this basal activity (and thereby increase the range of target genes that can be maintained and expressed in these cells) is through the use of T7 lysozyme, a natural inhibitor of T7 RNA polymerase.[14,15]

T7 lysozyme is a bifunctional protein: it cuts a specific bond in the peptidoglycan layer of the *E. coli* cell wall,[16] and it binds to T7 RNA polymerase and inhibits transcription.[14] When produced from the cloned gene, relatively high levels of T7 lysozyme can be tolerated by *E. coli,* apparently because the protein is unable to pass through the inner membrane to reach the peptidoglycan layer. Treatments that disrupt the inner membrane but do not normally cause lysis, such as addition of chloroform or mild detergents, induce rapid lysis of cells that contain even small amounts of T7 lysozyme.

[14] B. A. Moffatt and F. W. Studier, *Cell* **49,** 221 (1987).
[15] F. W. Studier, unpublished work, 1986.
[16] M. Inouye, N. Arnheim, and R. Sternglanz, *J. Biol. Chem.* **248,** 7247 (1973).

translational frameshifting, are indicated. Samples of culture (20 μl) were mixed with 3× concentrated sample buffer (10 μl) and heated for 2 min in a boiling water bath, and the entire sample was subjected to electrophoresis through a 10–20% gradient polyacrylamide gel in the presence of sodium dodecyl sulfate essentially as described [F. W. Studier, *J. Mol. Biol.* **79,** 237 (1973)].

T7 lysozyme can be provided to the cell from a clone of the T7 lysozyme gene in the *Bam*HI site of pACYC184.[17] The cloned fragment we have used (bp 10,665–11,296 of T7 DNA[2]) also contains the *ϕ3.8* promoter for T7 RNA polymerase immediately following the lysozyme gene. A plasmid having this fragment oriented so that the lysozyme gene is expressed from the *tet* promoter of pACYC184 is referred to as pLysE; cells carrying this plasmid accumulate substantial levels of lysozyme. A plasmid having the fragment in the opposite orientation is referred to as pLysS; cells carrying this plasmid accumulate much lower levels of lysozyme. These plasmids confer resistance to chloramphenicol and are compatible with the pET vectors for cloning target genes (described below). Neither lysozyme plasmid interferes with transformation of cells that contain it; pLysS has little effect on growth rate but pLysE causes a significant decrease in the growth rate of cells that carry it.

The presence of either pLysS or pLysE increases the tolerance of BL21(DE3) or HMS174(DE3) for toxic target plasmids: unstable plasmids become stable, and plasmids that would not otherwise be established can be maintained and expressed. Some target plasmids are too toxic to be established in the presence of pLysS but are able to be established in the presence of pLysE, and a few are too toxic to be established even in the presence of pLysE.

The low level of lysozyme provided by pLysS usually has little effect on expression of target genes on induction of T7 RNA polymerase, except for a short lag in the appearance of target gene products. Apparently, more T7 RNA polymerase is induced than can be inhibited by the small amount of lysozyme. (The level of lysozyme might be expected to increase somewhat on induction, since T7 RNA polymerase should be able to transcribe completely around the pLysS plasmid from the *ϕ3.8* promoter to make lysozyme mRNA; however, the *ϕ3.8* promoter is relatively weak,[18] and most transcription should be from the much stronger *ϕ10* promoter used in the target plasmids.)

The higher level of lysozyme provided by pLysE can substantially increase the lag and substantially reduce the maximum level of expression of target genes on induction of T7 RNA polymerase. This damping of expression is sufficient that cells containing a target gene whose product is relatively innocuous can continue to grow indefinitely in the presence of IPTG, a property that may be useful in some circumstances. (In contrast, the high level of expression in the absence of lysozyme or in the presence of pLysS almost always prevents continued growth of the cell.) Because of this damping of expression, most target genes will be expressed to higher levels

[17] A. C. Y. Chang and S. N. Cohen, *J. Bacteriol.* **134,** 1141 (1978).

[18] W. T. McAllister, C. Morris, A. H. Rosenberg, and F. W. Studier, *J. Mol. Biol.* **153,** 527 (1981).

by CE6 infection (described below) than by induction in the presence of pLysE.

The presence of pLysS (or pLysE) has the further advantage of facilitating the preparation of cell extracts. After the target protein has accumulated, the cells are collected and suspended in a buffer such as 50 m*M* Tris-Cl, 2 m*M* Na_3EDTA, pH 8.0. Simply freezing and thawing, or adding 0.1% Triton X-100, will allow the resident lysozyme to lyse the cells efficiently. This property can make it advantageous to carry pLysS in the cell even when it is not required for stabilizing the target plasmid.

Bacteriophage CE6

Target plasmids that are too toxic to be established in DE3 lysogens (even in the presence of pLysE) can be expressed by infecting with a bacteriophage that provides T7 RNA polymerase to the cell. No T7 RNA polymerase will be present in the cell before infection, so any target DNA that can be cloned under control of a T7 promoter should be expressible in this way.

A convenient bacteriophage for delivering T7 RNA polymerase to the cell is CE6, a λ derivative that carries the gene for T7 RNA polymerase under control of the phage p_L and p_I promoters and also has the *c*I857 thermolabile repressor and the *Sam*7 lysis mutations.[7] When CE6 infects HMS174, the newly made T7 RNA polymerase transcribes target DNA so actively that normal phage development cannot proceed. Comparable levels of target RNAs and proteins are produced whether T7 RNA polymerase is delivered to the cell by induction or by infection.

pET Vectors

The plasmid vectors we have developed for cloning and expressing target DNAs under control of a T7 promoter are designated pET vectors (*p*lasmid for *e*xpression by *T*7 RNA polymerase).[11] They contain a T7 promoter inserted into the *Bam*HI site of the multicopy plasmid pBR322 in the orientation that transcription is directed counterclockwise, opposite to that from the *tet* promoter (Fig. 2). In the absence of T7 RNA polymerase, transcription of target DNAs by *E. coli* RNA polymerase is low enough that very toxic genes can be cloned in these vectors. However, some expression can be detected, so it is possible that an occasional gene may be too toxic to be cloned in them.

Most of the pET vectors described here confer resistance to ampicillin. In such vectors, the *bla* gene is oriented so that it will be expressed from the T7 promoter along with the target gene (Fig. 2). However, in the pET-9 series of vectors, the *bla* gene has been replaced by the *kan* gene in the opposite orientation (Tables I and II). In these vectors, the only coding

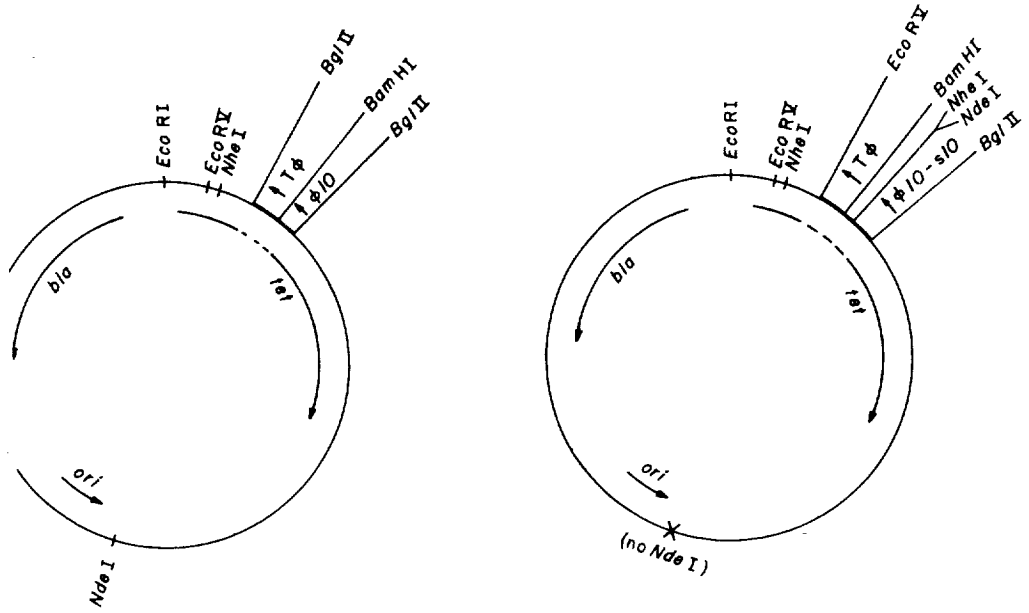

FIG. 2. Transcription vector pET-3 and translation vector pET-3a, b, or c. Fragments of T7 DNA containing the $\phi 10$ promoter (Fig. 3), the $\phi 10$ promoter plus the s*10* translation initiation region for the gene *10* protein (Fig. 7), and the Tϕ transcription termination signal (Fig. 4) were inserted into the *Bam*HI site of pBR322 in the indicated orientations. Relative locations of selected restriction sites, the *bla* and interrupted *tet* genes, and the origin of replication are shown.

sequence transcribed from the T7 promoter will be that of the target gene.

The T7 promoter in the pET vectors is derived from the $\phi 10$ promoter, one of six strong promoters in T7 DNA that have the identical nucleotide sequence from positions -17 to $+6$, where $+1$ is the position of the first nucleotide of the RNA transcribed from the promoter.[2] The $\phi 10$ promoter fragments carried by the vectors all begin at bp -23 and continue to bp $+2$, $+3$, $+26$, and $+96$ or beyond. Some of the vectors also contain a transcription termination signal or an RNase III cleavage site downstream of the cloning site for the target DNA.

Details of the construction of most of the vectors described here are given in Ref. 11. The nucleotide sequence of each vector can be assembled from the known nucleotide sequences of pBR322,[19,20] the *kan*[21] and *lacI*[22,23] genes, and the inserted fragment(s) from T7 DNA,[2] together with the sequences of linkers and any changes introduced in the construction of the vector (given in Tables I and II and Figs. 3–7). The linker sequences in most of the vectors contain restriction sites that allow easy removal of control elements for use in constructing other vectors, or that may be

[19] J. G. Sutcliffe, *Cold Spring Harbor Symp. Quant. Biol.* **43,** 77 (1979).
[20] K. W. C. Peden, *Gene* **22,** 277 (1983).
[21] A. Oka, H. Sugisaki, and M. Takanami, *J. Mol. Biol.* **147,** 217 (1981).
[22] M. P. Calos, *Nature (London)* **274,** 762 (1978).
[23] P. J. Farabaugh, *Nature (London)* **274,** 765 (1978).

TABLE I
TRANSCRIPTION VECTORS[a]

Vector	Selective marker[b]	Upstream site	Promoter	Leader RNA (nt)[c]	Cloning sites	Downstream elements	
pET-1	*bla*	*ClaI*[d]	*φ10*	29	*Bam*HI		
pET-2	*bla*	*Bgl*II	*φ10*	29	*Bam*HI		
pET-3	*bla*	*Bgl*II	*φ10*	29	*Bam*HI	Tφ	*Bgl*II[e]
pET-4	*bla*	*Bgl*II	*φ10*	29	*Bam*HI	R*1.1*	*Bgl*II
pET-5	*bla*	*ClaI*[d]	*φ10*	29	*Bam*HI–*Eco*RI[f]	*tet* deletion	
pET-6	*bla*	*Bam*HI	*φ10*	2	*Stu*I–*Bam*HI[g]		
pET-7	*bla*	*Bgl*II	*φ10*	2	*Stu*I–*Bam*HI[g]		
pET-9	*kan*	*Bgl*II	*φ10*	29	*Bam*HI	Tφ	*Bgl*II[e]
pET-10	*bla*	*Bgl*II	T7*lac*[h]	31	*Bam*HI		
pET-11	*bla*	*Bgl*II	T7*lac*[h]	31	*Bam*HI	Tφ	*Bgl*II[e]

[a] Transcription and RNA processing signals from T7 DNA are directed counterclockwise in the *Bam*HI site of pBR322 (Fig. 2). The nucleotide sequences of these signals and the linkers used in cloning them are given in Figs. 3–6 and in footnote *h* below.

[b] The selective antibiotic resistance marker is the *bla* gene from pBR322 (Fig. 2) or the *kan* gene of Tn*903*, from pUC4KISS [F. Barany, *Gene* **37**, 111 (1985)]. To convert from *bla* to *kan*, a *Bsp*HI–*Eco*RI fragment of pBR322, bp 3196–4361, was replaced by an 867-bp fragment that starts at a *Bsp*HI site 50 bp ahead of the *kan* initiation codon and ends at a newly introduced *Eco*RI site immediately following the termination codon. This places the *kan* coding sequence in the orientation opposite to that of the *bla* coding sequence. Plasmids of the pET-9 series confer resistance to kanamycin concentrations of at least 250 μg/ml.

[c] The length of RNA from the start point for T7 RNA polymerase to the first point of cleavage in the first cloning site is given in nucleotides (nt).

[d] The *Cla*I site will be methylated by the *dam* methylase of *E. coli* and will not be cut unless the plasmid DNA is isolated from a *dam*$^-$ host. The *Cla*I site of pET-5 is unique; pET-1 has a second *Cla*I site at bp 23 of the pBR322 DNA.

[e] We have just discovered that an *Ava*I cleavage site (CPyCGPuG) is also present downstream of Tφ in pET-3, pET-9, and pET-11, presumably because more than one *Bgl*II linker (GAGATCTC) was incorporated when the *Bgl*II site was introduced into the common precursor of these vectors.

[f] In pET-5, the *Bam*HI cloning site is followed immediately by a unique *Eco*RI site in the sequence GGATCCGAATTC; the DNA between the *Bam*HI and *Eco*RI sites of pBR322 (including the *tet* promoter) has been deleted.

[g] When cloning into the *Stu*I site, pET-6 and pET-7 should be isolated from a *dcm*$^-$ host such as BL21 (see text).

[h] The T7*lac* promoter was made by inserting a 25-bp *lac* operator sequence into the *Stu*I site 2 bp downstream of the start site of the T7 promoter in pET-7 (Fig. 6). The nucleotide sequence from the RNA start site to the *Bst*NI–*Bam*HI site immediately beyond the operator sequence is GGGGAATTGTGAGCGGATAACAATTCCCCTGGATCC, where the inserted *lac* operator sequence is underlined. The pET-10 and pET-11 series of vectors also contain a *lacI* fragment (bp −50 to +1152 relative to the first nucleotide of the *lacI* mRNA) cloned into the *Sal*I site so that the *lacI* and T7*lac* promoters diverge. The *lacI* fragment was cloned initially into the *Bam*HI site of pBR322 by use of linkers (CCGGATCCGG upstream and CGGGATCCCG downstream), and was subsequently moved into the *Sal*I site by blunt-end ligation between filled-in *Bam*HI and *Sal*I ends. pET-11 contains the downstream elements of pET-3.

TABLE II

TRANSLATION VECTORS[a]

Vector	Selective marker[b]	Upstream site	Expression signals[c]	ATG cloning site[d]	Fusion cloning aa[e]	Fusion cloning Site	Fusion cloning rf[f]	Downstream elements
pET-1a	*bla*	*Cla*I[g]	*ϕ10–s10*	*Nde*I	12	*Bam*HI	GGA	
pET-1b	*bla*	*Cla*I	*ϕ10–s10*	*Nde*I	12	*Bam*HI	GAT	
pET-1c	*bla*	*Cla*I	*ϕ10–s10*	*Nde*I	12	*Bam*HI	ATC	
pET-2a	*bla*	*Bgl*II	*ϕ10–s10*	*Nde*I	12	*Bam*HI	GGA	
pET-2b	*bla*	*Bgl*II	*ϕ10–s10*	*Nde*I	12	*Bam*HI	GAT	
pET-2c	*bla*	*Bgl*II	*ϕ10–s10*	*Nde*I	12	*Bam*HI	ATC	
pET-3a	*bla*	*Bgl*II	*ϕ10–s10*	*Nde*I	12	*Bam*HI	GGA	Tϕ *Eco*RV[h]
pET-3b	*bla*	*Bgl*II	*ϕ10–s10*	*Nde*I	12	*Bam*HI	GAT	Tϕ *Eco*RV
pET-3c	*bla*	*Bgl*II	*ϕ10–s10*	*Nde*I	12	*Bam*HI	ATC	Tϕ *Eco*RV
pET-3d	*bla*	*Bgl*II	*ϕ10–s10*	*Nco*I	12	*Bam*HI	ATC	Tϕ *Eco*RV
pET-3xa	*bla*	*Bgl*II	*ϕ10–s10*	*Nde*I	260[i]	*Bam*HI	GGA	Tϕ *Eco*RV[h]
pET-3xb	*bla*	*Bgl*II	*ϕ10–s10*	*Nde*I	261	*Bam*HI	GAT	Tϕ *Eco*RV
pET-3xc	*bla*	*Bgl*II	*ϕ10–s10*	*Nde*I	261	*Bam*HI	ATC	Tϕ *Eco*RV
pET-3xd	*bla*	*Bgl*II	*ϕ10–s10*	*Nco*I	261	*Bam*HI	ATC	Tϕ *Eco*RV
pET-4a	*bla*	*Bgl*II	*ϕ10–s10*	*Nde*I	12	*Bam*HI	GGA	R*1.1* *BglII*
pET-4b	*bla*	*Bgl*II	*ϕ10–s10*	*Nde*I	12	*Bam*HI	GAT	R*1.1* *BglII*
pET-4c	*bla*	*Bgl*II	*ϕ10–s10*	*Nde*I	12	*Bam*HI	ATC	R*1.1* *BglII*
pET-5a	*bla*	*Bgl*II	*ϕ10–s10*	*Nde*I	12	*Bam*HI	GGA	*Eco*RI GAA[j]
pET-5b	*bla*	*Bgl*II	*ϕ10–s10*	*Nde*I	12	*Bam*HI	GAT	*Eco*RI AAT
pET-5c	*bla*	*Bgl*II	*ϕ10–s10*	*Nde*I	12	*Bam*HI	ATC	*Eco*RI ATT
pET-8c	Previous designation for pET-3d							
pET-9a	*kan*	*Bgl*II	*ϕ10–s10*	*Nde*I	12	*Bam*HI	GGA	Tϕ *Eco*RV[h]
pET-9b	*kan*	*Bgl*II	*ϕ10–s10*	*Nde*I	12	*Bam*HI	GAT	Tϕ *Eco*RV
pET-9c	*kan*	*Bgl*II	*ϕ10–s10*	*Nde*I	12	*Bam*HI	ATC	Tϕ *Eco*RV
pET-9d	*kan*	*Bgl*II	*ϕ10–s10*	*Nco*I	12	*Bam*HI	ATC	Tϕ *Eco*RV
pET-10a	*bla*	*Bgl*II	T7*lac*–s*10*	*Nde*I	12	*Bam*HI	GGA	
pET-10b	*bla*	*Bgl*II	T7*lac*–s*10*	*Nde*I	12	*Bam*HI	GAT	
pET-10c	*bla*	*Bgl*II	T7*lac*–s*10*	*Nde*I	12	*Bam*HI	ATC	
pET-11a	*bla*	*Bgl*II	T7*lac*–s*10*	*Nde*I	12	*Bam*HI	GGA	Tϕ *Eco*RVΔ[k]
pET-11b	*bla*	*Bgl*II	T7*lac*–s*10*	*Nde*I	12	*Bam*HI	GAT	Tϕ *Eco*RVΔ
pET-11c	*bla*	*Bgl*II	T7*lac*–s*10*	*Nde*I	12	*Bam*HI	ATC	Tϕ *Eco*RVΔ
pET-11d	*bla*	*Bgl*II	T7*lac*–s*10*	*Nco*I	12	*Bam*HI	ATC	Tϕ *Eco*RVΔ

[a] Transcription, translation, and RNA processing signals from T7 DNA are directed counterclockwise in the *Bam*HI site of a derivative of pBR322 in which the *Nde*I site has been eliminated by opening, filling in, and religating (Fig. 2). The nucleotide sequences of these signals and the linkers used in cloning them are given in Figs. 3, 4, 5, and 7 and in the footnotes to the tables.

[b] See footnote *b* of Table I.

[c] The *ϕ10–s10* segment contains the *ϕ10* promoter and the translation initiation region for the gene *10* protein (Fig. 7). The T7*lac* promoter is described in footnote *h* in Table I. The junction between the T7*lac* promoter and the s*10* translation initiation region was made by blunt-end ligation of the filled-in *Bst*NI end downstream of the T7*lac* promoter and the filled-in *Xba*I site upstream of the

useful for moving cloned target DNA linked to the transcription, translation, or processing signals.

Transcription Vectors. Vectors containing a T7 promoter but no potential translation initiation site ahead of the cloning site are referred to as transcription vectors (Table I). Vectors pET-1 to pET-5 all carry the same *ϕ10* promoter fragment (−23 to +26) followed by a unique *Bam*HI cloning site (Fig. 3). This fragment should contain all of the upstream and downstream sequences normally used by T7 RNA polymerase to initiate transcription at the *ϕ10* promoter. RNA initiated at the promoter in these vectors will have 29 nucleotides ahead of the GATC of the *Bam*HI cloning site, the first 21 of which can form an 8-bp stem-and-loop structure (Fig. 3). Retention of this naturally occurring stem-and-loop structure in the RNA made from these vectors may confer some as yet undefined advantage in, for example, stability or translatability.

The pET-1 to pET-5 vectors differ from each other in the restriction sites found in the linkers immediately upstream of the promoter and in the signals found downstream of the *Bam*HI cloning site, as indicated in Table I. pET-1 and pET-2 have no additional signals downstream of the *Bam*HI cloning site, and T7 RNA polymerase is capable of transcribing completely around these plasmid DNAs multiple times.[7,18] pET-3 has a T7 DNA fragment containing the transcription terminator Tϕ (Fig. 4) downstream

translation start (Fig. 7). This ligation recreated a unique *Xba*I site, which is convenient for exchanging target genes between the *ϕ10* and T7*lac* promoters in the pET translation vectors.

[d] Coding sequences can be joined to the gene *10* initiation codon, using a unique *Nde*I site (CA′TATG) or a unique *Nco*I site (C′CATGG) (Fig. 7).

[e] The number of codons before the first in-frame codon of the *Bam*HI fusion cloning site.

[f] The first codon within the *Bam*HI recognition sequence (GGATCC) that is in the same reading frame as the gene *10* initiation codon (see Fig. 7 and footnote *i*).

[g] See footnote *d* of Table I.

[h] Besides the *Eco*RV site in the downstream linker, a second *Eco*RV site is present at bp 185 of the pBR322 DNA.

[i] The pET-3x series is equivalent to the pET-3 series except that the *Bam*HI fusion site is after the two hundred and sixtieth rather than the eleventh codon of gene *10*. The same set of *Bam*HI linkers (Fig. 7) was attached after a filled-in *Asp*718 site (G′GTACC) at codons 259 and 260 (bp 23,740 of T7 DNA). The initial C of the linkers regenerated a unique *Asp*718 site in each vector. In pET-3xa the two hundred and sixty first codon is the GGA of the *Bam*HI site; in pET-3xb the two hundred and sixty first codon specifies alanine and is followed by the GAT of the *Bam*HI site; in pET-3xc and pET-3xd the two hundred and sixty first codon specifies glycine and is followed by the ATC of the *Bam*HI site.

[j] The pET-5 translation vectors have the same *Bam*HI–*Eco*RI sequence and *tet* deletion as in pET-5 itself (Table I). The *Eco*RI cloning site can be used instead of the *Bam*HI site to place coding sequences in the reading frame of the gene *10* initiation codon: the first in-frame codon within the *Eco*RI recognition sequence (GAATTC) is given.

[k] The *Nhe*I site at the second and third codons of gene *10* is unique in pET-11a, b, c, and d, because the *Nhe*I site at bp 229 of pBR322 has been removed from these vectors by deleting the DNA between the *Eco*RV sites immediately downstream of Tϕ and at bp 185 of pBR322.

[6]

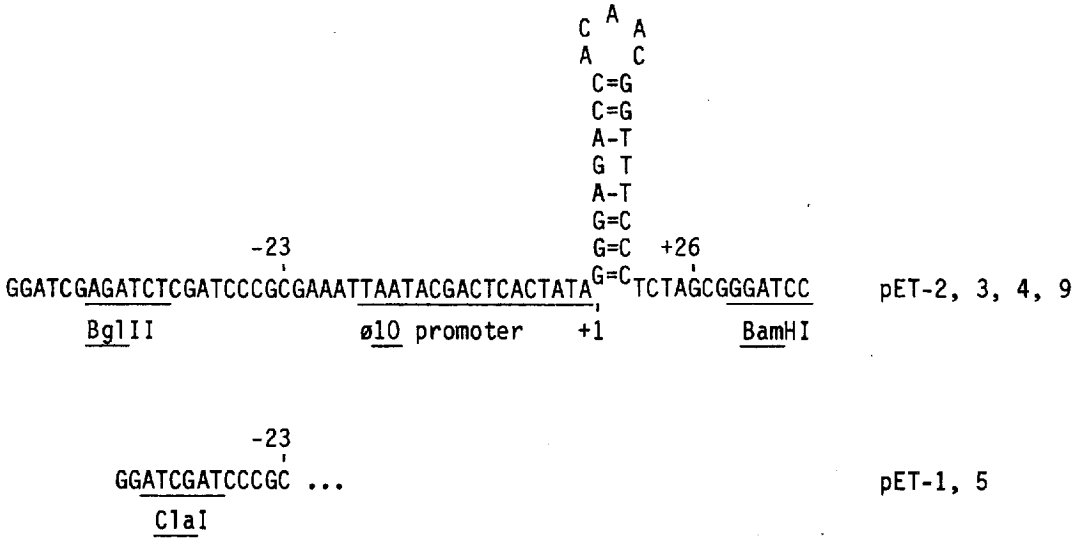

FIG. 3. Nucleotide sequence of the cloned *ϕ10* promoter in pET-1, 2, 3, 4, 5, and 9. The cloned *ϕ10* fragment (bp 22,880–22,928 of T7 DNA) extends from bp −23 to +26 relative to the RNA start at +1. Linker sequences through the original *Bam*HI site of pBR322 are shown on both sides. The upstream conserved T7 promoter sequence is underlined and the potential stem-and-loop structure at the beginning of the RNA is indicated.

of the *Bam*HI cloning site. The T*ϕ* fragment causes efficient termination of transcription by T7 RNA polymerase both *in vivo* and *in vitro.*

pET-4 has a T7 DNA fragment containing the R*1.1* RNase III cleavage site (Fig. 5) downstream of the *Bam*HI cloning site. RNase III is a host nuclease that efficiently cuts RNA within the R*1.1* cleavage site to leave a potential stem-and-loop structure at the 3′ end of the upstream RNA,[2] a structure that appears to stabilize the RNA against degradation.[24] Cleavage at this site reduces to unit length the long RNAs made by transcribing multiple times around the plasmid DNA.[7] Such unit-length RNA can be detected by gel electrophoresis, a simple confirmation that full-length transcripts of target DNA have accumulated (Fig. 1).

In pET-5, the DNA between the unique *Bam*HI and *Eco*RI sites of pET-1 has been deleted. This removes the *tet* promoter of pBR322, so that transcription by *E. coli* RNA polymerase across these two cloning sites is low in both directions. Some target DNA fragments that have coding sequences in both directions can be cloned in pET-5 but not pET-1, apparently because expression of either coding sequence from the *tet* promoter is toxic to the cell.

pET-6 and pET-7 carry a shorter *ϕ10* promoter fragment (−23 to +2) ending in a unique *Stu*I site followed immediately by a *Bam*HI site (Fig. 6). The *Stu*I site (AGG′CCT) is placed so that target DNA can be inserted by

[24] N. Panayotatos and K. Truong, *Nucleic Acids Res.* 7, 2227 (1985).

FIG. 4. Nucleotide sequence of the cloned Tϕ transcription terminator. The cloned Tϕ fragment (bp 24,106–24,228 of T7 DNA) extends from bp −104 to +19 relative to the last nucleotide of the terminated transcript at −1. Linker sequences from the upstream *Bam*HI cloning site through the downstream *Bgl*II site of the pET-3, 9, and 11 transcription vectors or the downstream *Eco*RV site of the pET-3, 3x, 9, and 11 translation vectors are shown. We have just discovered that an *Aval* cleavage site (CPyCGPuG) is also present downstream of Tϕ in pET-3, 9, and 11, presumably because more than one *Bgl*II linker (GAGATCTC) was incorporated when the *Bgl*II site was introduced into the common precursor of these vectors. The DNA between the *Eco*RV site immediately downstream of Tϕ and the one located at bp 185 of pBR322 has been eliminated from the pET-11 translation vectors. A potential stem-and-loop structure just before the last few nucleotides of the terminated transcript is indicated and all stop codons ahead of this structure are underlined: the TAA at nt −51 is the natural stop codon for the gene *10B* protein of T7; the TAG at nt −47 is in a second reading frame; and the TAA at nt −100, the TGA at nt −79, and the TGA at nt −58 are all in the third reading frame.

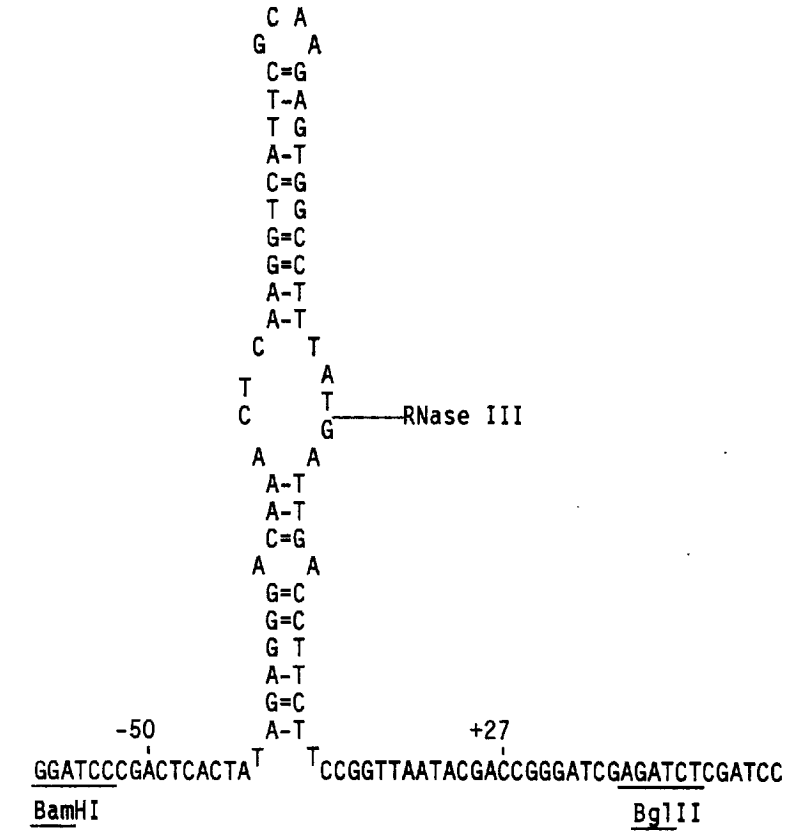

FIG. 5. Nucleotide sequence of the cloned R*1.1* RNase III cleavage site of pET-4. The cloned R*1.1* fragment (bp 5838–5914 of T7 DNA) extends from bp −50 to +27 relative to the cut site. Linker sequences from the upstream *Bam*HI cloning site through the original *Bam*HI site of pBR322 on the downstream side are shown. The potential stem-and-loop structure similar to that found in eight RNase III cleavage sites in T7 RNA is indicated, as is the known position of cleavage in this sequence.

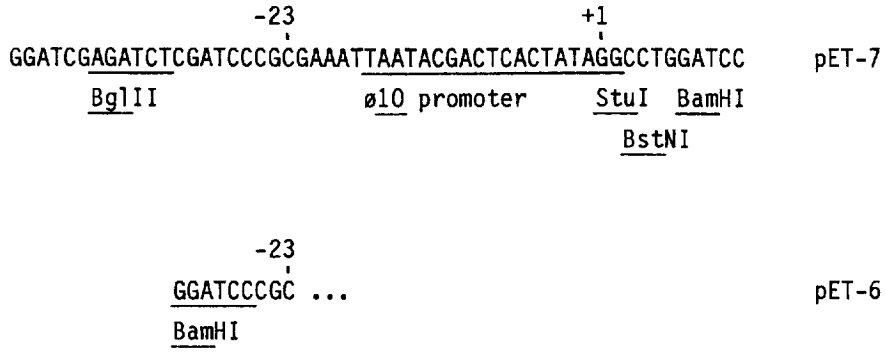

FIG. 6. Nucleotide sequence of the cloned *ϕ10* promoter of pET-7 and pET-6. The cloned *ϕ10* fragment (bp 22,880–22,904 of T7 DNA) extends from bp −23 to +2 relative to the RNA start at +1. Linker sequences through the original *Bam*HI site of pBR322 are shown on both sides. The conserved promoter sequence and the upstream *Bgl*II or *Bam*HI site are underlined, and the positions of the downstream overlapping *Stu*I (AGG'CCT), *Bst*NI (CC'TGG), and *Bam*HI (G'GATCC) sites are indicated.

blunt-end ligation after position +2 of the promoter, and T7 RNA polymerase will transcribe target DNA cloned at this site with only a GG ahead of the cloned sequence. The loss of a few nucleotides of conserved promoter sequence in the transcribed region may reduce promoter strength when certain sequences are cloned into the *Stu*I site, but we expect that almost any DNA cloned into this site will be actively transcribed if no competing T7 promoters are present. pET-6 contains a second *Bam*HI site upstream of the promoter whereas pET-7 is somewhat more versatile in having a unique *Bgl*II site at this position.

The *Stu*I–*Bam*HI sequence of both pET-6 and pET-7 contains the sequence CCTGG, which is both a cleavage site for *Bst*NI and a methylation site for the *dcm* methylase of *E. coli*. Methylation at this site prevents cleavage by *Stu*I, so plasmid DNA must be prepared from a dcm^- strain if it is to be cut efficiently by *Stu*I. *E. coli* B strains are known to be dcm^-, and BL21 is a good host for obtaining pET-6 or pET-7 that can be cut by *Stu*I.

pET-9 is equivalent to pET-3, except that the *bla* gene has been replaced by the *kan* gene in the opposite orientation (Table I, footnote *b*). pET-10 and pET-11 are equivalent to pET-2 and pET-3, except that they carry the T7*lac* promoter and the *lacI* gene (Table I, footnote *h*, and the section Vectors That Contain a *T7lac* Promoter, below).

Translation Vectors. Vectors for placing cloned DNA under control of both $\phi 10$ and the efficient translation initiation signals for gene *10* protein (the major capsid protein of T7) are listed in Table II. Most of these translation vectors carry a fragment (−23 to +96 relative to the RNA start) that includes the first 11 codons for the gene *10* protein; they have been constructed in sets of three, such that cloning a DNA fragment into the unique *Bam*HI site places the cloned nucleotide sequence in a different reading frame relative to the gene *10* initiation codon in each of the three vectors (Fig. 7). The configuration of upstream restriction sites and downstream signals is indicated by the pET number; the three reading frames are identified by the suffixes a, b, and c to indicate whether the GGA, GAT, or ATC triplet of the *Bam*HI site (GGATCC) is in the open reading frame. In the pET-5 translation vectors, the *Bam*HI site is followed immediately by an *Eco*RI site, which can also be used to fuse coding sequences in each of the three reading frames.

The pET-3, pET-5, pET-9, and pET-11 translation vectors are not strictly equivalent to their respective transcription vectors: pET-3, 9, and 11 have a *Bgl*II linker downstream of Tϕ, whereas the corresponding translation vectors have an *Eco*RV linker at this position (Figs. 2 and 4; Tables I and II); pET-5 itself has a *Cla*I linker upstream of $\phi 10$ whereas the pET-5 translation vectors have the *Bgl*II linker of pET-2 at this position (Fig. 3; Tables I and II).

```
C A A
A   C
 C=G
 C=G
 A-T
 G T
 A-T
 G=C                1  2  3  4  5  6  7  8  9 10 11
 G=C +26      +50     +61 Met Ala Ser Met Thr Gly Gly Gln Gln Met Gly Arg Gly Ser
pppG=CTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATG GCT AGC ATG ACT GGT GGA CAG CAA ATG GGT CGC GGA TCC      pET-3a
      XbaI          SD      NdeI   NheI                                      BamHI

                                                    ... Arg Asp
                                                    ... CGG GAT CC            pET-3b
                                                          BamHI

                                                    ... Arg Ile
                                                    ... CGG ATC C             pET-3c
                                                          BamHI

                                  1  2  3
                          +50     +61 Met Ala Ser ...
                          ... AAGGAGATATACC ATG GCT AGC ...                   pET-3d
                                SD      NcoI   NheI
```

FIG. 7. Target mRNA and protein sequences specified by pET translation vectors. The cloned *φ10–s10* fragment (bp 22,880–22,998 of T7 DNA) extends from bp −23 to +96 (relative to the RNA start at +1) for all of the translation vectors in Table II except the pET-3x series, which extends to bp +843 (bp 23,745 of T7 DNA), and the pET-10 and pET-11 series, in which RNA transcribed from the *lac* operator replaces the RNA ahead of the *Xba*I site (footnotes *h* of Table I and *c* of Table II). Nucleotide and amino acid sequences from the start of the mRNA through the *Bam*HI fusion cloning site are shown for the pET-3 series. The potential stem-and-loop structure at the 5′ end of the mRNA is indicated and the Shine–Dalgarno (SD) sequence is underlined. The indicated *Xba*I, *Nde*I, *Nco*I, and *Bam*HI sites are unique in the vectors that contain them; the *Nhe*I site is unique in the pET-5 and pET-11 series, but the other vectors contain a second *Nhe*I site at bp 229 of the pBR322 DNA. The nucleotide sequence AT at bp +62 and +63 in pET-3c was replaced by C in pET-3d, thereby eliminating the *Nde*I site and creating an *Nco*I site; this is the only difference between these two vectors. In the pET-3x series, the gene *10* coding sequence extends through codon 260; the linker amino acids are given in footnote *i* of Table II.

A coding sequence in the proper reading frame in the *Bam*HI site of the pET translation vectors will be translated as a fusion protein linked to the first 11 amino acids of the gene *10* protein plus an arginine specified by the linker sequence (Fig. 7). However, coding sequences can also be joined directly to the gene *10* initiation codon at a unique *Nde*I site (CA'TATG) that includes the initiating ATG. Protein synthesis will be directed by the efficient upstream translation signals of gene *10* and the resulting protein will not contain any foreign amino acids. Potentially, any natural initiation codon (or any internal methionine codon) can be converted to an *Nde*I site and joined to the vector without altering the coding sequence.

ATG is also found in the cleavage sites for *Nco*I (C'CATGG), *Bsp*HI (T'CATGA), and *Afl*III (A'CPuPyGT). Initiation codons (or internal methionine codons) not already part of one of these sites can be converted to one with little or no change in coding sequence. To allow such coding sequences to be joined directly to the upstream gene *10* translation signals, the initiation codon in pET-3c was converted to an *Nco*I site to create pET-3d (formerly pET-8c) (Fig. 7). In our limited experience, we find that the gene *10* initiation site of pET-3d seems to direct protein synthesis about as well as that of pET-3c, indicating that the alteration in nucleotide sequence that created the *Nco*I site has little effect on translation efficiency.

Coding sequences can also be joined at the second and third codons of gene *10*, through the *Nhe*I site at this position (Fig. 7). Ends produced by *Nhe*I (G'CTAGC), *Xba*I (T'CTAGA), *Spe*I (A'CTAGT), and *Avr*II (C'CTAGG) can be joined directly to this site. Most of the pET vectors also contain a second *Nhe*I site at bp 229 of the pBR322 sequence, but this site has been deleted from the pET-5 and pET-11 series.

For some applications, such as making antibodies to relatively small peptides, fusions to a relatively large protein can be useful. Therefore, we have also created the pET-3x set of translation vectors, which is equivalent to the pET-3 set except that the *Bam*HI cloning site has been placed after the two hundred and sixtieth rather than the eleventh codon for the gene *10* protein. The 260-amino acid fragment of the gene *10* protein is itself insoluble, so the fusion proteins will likely be insoluble as well. Such insolubility can be an aid in the purification of protein for making antibodies and may also help to stabilize fused peptides that might otherwise be unstable. The pET-3x vectors are also convenient for placing coding sequences directly behind the gene *10* initiation codon: loss of the 780 bp of gene *10* coding sequence between the *Nde*I (or *Nco*I) and *Bam*HI sites reduces the size of the vector DNA enough that gel electrophoresis readily shows whether both cuts have been made.

Vectors That Reduce Colony Size. Some of the pET vectors, most notably pET-1, pET-1c, and pET-2c, cause a significant reduction in

the pBR322 DNA. The nucleotide sequence AT at bp +62 and +63 in pET-3c was replaced by C in pET-3d, thereby eliminating the *Nde*I site and creating an *Nco*I site; this is the only difference between these two vectors. In the pET-3x series, the gene *10* coding sequence extends through codon 260; the linker amino acids are given in footnote *i* of Table II.

colony size of HMS174 and BL21. The effect is not understood in detail, but the growth rate in log phase cultures seems little affected. This effect does not impair the usefulness of the vectors; in fact, insertion of target sequences has always restored normal colony size, and this can be a useful phenotype for identifying plasmids that have inserts.

Vectors That Contain a T7lac Promoter. We have placed a T7 promoter under control of *lac* repressor by inserting a *lac* operator sequence just downstream of the start site of a T7 promoter.[25] In the presence of appropriate levels of *lac* repressor, transcription from this T7*lac* promoter is greatly reduced, but normal transcription can be restored by addition of IPTG. In order to provide sufficient *lac* repressor to occupy the operator sites on a multicopy plasmid vector, a DNA fragment containing the natural promoter and coding sequence for *lacI* was placed upstream of the T7*lac* promoter, oriented so that the *lacI* and T7*lac* promoters diverge. Vectors analogous to the pET-2 and pET-3 series of vectors but containing the T7*lac* promoter and *lacI* are designated the pET-10 and pET-11 series (Table I and II).

When the pET-10 or pET-11 vectors are used for expressing target genes in BL21(DE3) or HMS174(DE3), the *lac* repressor acts both to repress transcription of the T7 RNA polymerase gene by *E. coli* RNA polymerase and to block transcription of the target gene by any T7 RNA polymerase that is made. This double repression significantly reduces basal expression of the target gene in the uninduced cell but seems to have little effect on the level of expression on induction, relative to the analogous configuration using the $\phi 10$ promoter rather than the T7*lac* promoter.

Only a few target genes have been encountered that are too toxic to be stable in the pET-10 or pET-11 vectors in BL21(DE3) or HMS174(DE3). However, addition of T7 lysozyme, as provided by pLysS, reduces the basal activity of T7 RNA polymerase sufficiently that each of these plasmids can be maintained. The slight delay in expression of target genes on induction in the presence of pLysS is increased when the gene is controlled by the T7*lac* promoter, but substantial levels of expression can nevertheless be attained.

Genes already cloned in any of the pET translation vectors can usually be placed under control of the T7*lac* promoter in the pET-10 or pET-11 translation vectors by using equivalent *Xba*I, *Nde*I, or *Nco*I sites to exchange the appropriate DNA fragments (Fig. 7).

Potential Problems with Other Vectors

Vectors for placing cloned DNA under control of a T7 promoter are widely available from commercial sources. In principle, many such vectors

[25] J. W. Dubendorff and F. W. Studier, unpublished work, 1988.

could be used for expressing target DNA in the expression system described here. However, some vectors are inappropriate, and the reason may not be immediately apparent. For example, many commercial vectors carry a *lac* operator, whose presence is not always obvious in descriptions of the vector. (Vectors that provide a complementing fragment of β-galactosidase for use in a blue–white color test for fragment insertion would carry a *lac* operator.) We have received several reports of plasmid instability in BL21(DE3) that turned out to be due to the use of such vectors. Apparently, the multicopy vector introduces more copies of the *lac* operator than can be titrated by the *lac* repressor present in the cell. As a result, T7 RNA polymerase (itself controlled by *lac* repressor) increases to a level where transcription of the target plasmid becomes too high for stability. Users should be alert to the possibility that this or other types of incompatibility may be encountered when trying to express DNA cloned in vectors that were not designed for use in this system.

Growth Media

BL21 and HMS174 grow on minimal or complex media, and presumably a wide range of growth media would be suitable for growth of these strains and expression of target DNAs. For routine growth of cultures we use ZB medium (10 g of N-Z-amine A and 5 g of NaCl in 1 liter of water). N-Z-amine A is obtained from Sheffield Products (P.O. Box 398, Memphis, TN 38101); Bacto Tryptone (Difco) in place of N-Z-amine A in any of the media described gives essentially equivalent results. Defined media are usually M9 medium, containing 1 g of NH_4Cl, 3 g of KH_2PO_4, 6 g of Na_2HPO_4, 4 g of glucose, and 1 ml of 1 *M* $MgSO_4$ in 1 liter of water, or B2 medium, which is essentially M9 medium in which all but 0.16 m*M* of the phosphate is replaced by salts and bis-Tris buffer.[26] M9mal and B2mal are the equivalent media in which glucose is replaced by maltose. Richer media include M9ZB, which contains the components of both M9 and ZB; ZY [10 g of N-Z-amine A, 5 g of Bacto yeast extract (Difco), and 5 g of NaCl in 1 liter of water]; or ZYG (ZY medium plus 4 g of glucose per liter). Sugars, $MgSO_4$, and phosphate solutions are autoclaved separately and added to the media after cooling.

The many small (1–3 ml) cultures generated in cloning DNA fragments and isolating recombinant plasmids are usually grown in M9ZB + antibiotic in standing 13 × 100 mm culture tubes in a 37° incubator. After overnight incubation of standing cultures, HMS174 remains largely dispersed throughout the culture whereas BL21 cells mostly settle to the bottom of the tube. Larger cultures are grown in shaking flasks at 37°. When cultures are grown overnight in shaking flasks, the growth medium

[26] F. W. Studier, *J. Bacteriol.* **124**, 307 (1975).

is usually ZB, because continued shaking after saturation in rich media containing glucose (such as M9ZB or ZYG) leads to some lysis of BL21.

Dilutions for titering bacteria or phage are made in ZB. Plating is done by mixing samples with 2.5 ml of melted top agar [ZB containing 0.7% (w/v) agar] and spreading on standard 100 × 15 mm plastic petri dishes containing 20 ml of hardened bottom agar (ZB containing 1% agar).

Where antibiotics are added to liquid growth media or bottom agar of petri dishes, ampicillin is typically used at 20 μg/ml and chloramphenicol or kanamycin at 25 μg/ml. It is not necessary to use preformed antibiotic plates: where bottom agar contains no antibiotic, selection can be accomplished by adding 200 μg of ampicillin or 250 μg of chloramphenicol or kanamycin per milliliter of top agar at the time of plating.

For induction of T7 RNA polymerase in BL21(DE3) or HMS174(DE3), a growing culture is made 0.4 m*M* in IPTG. For induction on plates, top agar is made 1 m*M* in IPTG at the time of plating.

Storage of Strains

For long-term storage, 1.5 ml of a growing or saturated culture is placed in a cryovial, mixed with one-tenth volume of 80% (v/v) glycerol, and the tube is stored directly in a −75° freezer. We avoid higher concentrations of glycerol because they become increasingly toxic to cells at room temperature. Plasmid-bearing strains, particularly those having any tendency toward instability, are titered at the time of freezing to be sure that the vast majority of cells in the culture have the intended host–plasmid combination (see Toxic Genes and Plasmid Instability, below). To inoculate a culture from the frozen stock, a few microliters is scraped or melted from the surface, typically with a sterile pipet or plastic culture loop, and the remainder is returned to the freezer without thawing. Cells stored at −75° in this way have remained viable for several years and presumably will remain viable for very long periods. (In our experience, cells survive for many months in a −20° freezer, but survival for longer periods is variable.)

Cloning Target DNAs

Target DNAs are cloned into the pET vectors by standard techniques.[27] We use HMS174 as the host for initial cloning and analysis of plasmids, because plasmid DNAs remain monomers in the *recA* background and expression of the target DNA is minimal in the absence of T7 RNA

[27] T. Maniatis, E. F. Fritsch, and J. Sambrook, "Molecular Cloning, A Laboratory Manual." Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982.

polymerase. Any easily transformable, preferably *recA* strain should be suitable for this purpose. BL21 is not appropriate because it is *recA*$^+$ and it has a somewhat lower transformation efficiency. DE3 lysogens should not be used for initial cloning because of potential problems from expression of the target gene by the small amounts of T7 RNA polymerase present in the uninduced cell.

Once the desired plasmid is obtained, the target DNA can be expressed by infection with CE6, or, if the target plasmid is stable, by induction in BL21(DE3) or HMS174(DE3) or in one of these strains carrying pLysS. To test for stability, transformations with the target plasmid are attempted in a set of four strains: BL21, BL21(DE3), BL21(DE3)pLysS, and BL21(DE3)pLysE, or the equivalent set based on HMS174. Plasmids having no target gene, or whose target gene is relatively innocuous, will give transformants with about equal frequency in all four hosts; at some level of toxicity, plasmids will fail to transform the DE3 lysogen itself (where the basal activity of T7 RNA polymerase is highest); at somewhat higher toxicity the lysogen containing pLysS will also fail to be transformed; and a few target plasmids are so toxic that even the lysogen containing pLysE cannot be transformed, although the host that lacks the gene for T7 RNA polymerase will be transformed at normal frequency.

A few of the target genes we have worked with are more stable in HMS174(DE3) or its derivatives than in the equivalent derivative of BL21(DE3). A possible explanation for this difference (suggested by S. Shuman, personal communication) is that small amounts of some target gene products induce the SOS response of *E. coli*. This in turn induces the prophage and kills BL21(DE3), but the *recA* deficiency of HMS174 prevents induction of the prophage and killing of HMS174(DE3).

Expressing Target DNA by IPTG Induction of BL21(DE3) or HMS174(DE3)

If a target plasmid can be established in BL21(DE3), HMS174(DE3), or in one of these strains containing pLysS, induction of T7 RNA polymerase by IPTG is a convenient way to direct expression of the target DNA. We usually grow the cells in M9 or M9ZB containing the selective antibiotic (and also 25 μg of chloramphenicol/ml if the cells carry pLysS), and make the culture 0.4 m*M* in IPTG when the culture reaches an OD_{600} of 0.6–1.

Immediately before induction, the culture is titered to determine the fraction of cells that carry inducible plasmid. This involves plating on four plates, which differ in the composition of the top agar used in plating. Typically, the culture would be plated at a dilution of 10^5 on plates that

have both IPTG and antibiotic or just IPTG added to the top agar, and at a dilution of 2×10^6 on plates that have just antibiotic or nothing added to the top agar. This test and its interpretation are described more fully in the next section. (We usually do not test for the relatively stable pLysS.)

If appropriate attention is paid to possible problems of plasmid instability, more than 98% of the cells in the culture will usually contain expressible target plasmids. Cells are usually harvested 2–3 hr after induction, enough time for substantial accumulation of target protein but before the culture can be overgrown with cells that have lost plasmid or are otherwise unproductive. However, some target proteins continue to accumulate for much longer times.

Occasional results have suggested that the basal activity of T7 RNA polymerase in uninduced cells may be somewhat lower when the growth medium contains glucose than when it does not. Induction of the *lac*UV5 promoter (which directs transcription of the T7 RNA polymerase gene in the DE3 lysogens) is not subject to catabolite repression,[28] but perhaps the repressed promoter retains some sensitivity. We have not analyzed this effect in detail, but we suspect that media containing glucose (such as M9 and M9ZB) may be more suitable than media without glucose (such as ZY) for growing DE3 lysogens for the induction of target genes.

Toxic Genes and Plasmid Instability

Plasmid pBR322 and many of its derivatives are relatively stable and are retained by a very high fraction of host cells even after growth for many generations in the absence of a selective antibiotic. However, problems of plasmid instability can arise when a gene whose product is toxic to the host cell is cloned in the plasmid. The level of expression may be such that the plasmid can be maintained but growth of the cell is impaired; segregation of cells lacking plasmid may also be increased because of decreased copy number or for other reasons. In such a situation, cells that lack the plasmid can rapidly overgrow the culture whenever selective antibiotic is lacking. If the plasmid is to be maintained in a significant fraction of the cells, the culture must not be allowed to grow in the absence of selection for the plasmid.

Use of ampicillin as a selective antibiotic requires special care, because β-lactamase is made in substantial amounts and is secreted into the medium, where it can destroy all of the ampicillin. This means that a culture whose cells carry an unstable plasmid will be growing under ampicillin selection only until enough β-lactamase has been secreted to destroy the

[28] A. E. Silverstone, R. R. Arditti, and B. Magasanik, *Proc. Natl. Acad. Sci. U.S.A.* **66**, 773 (1970).

ampicillin in the medium; from that point on, cells that lack plasmid will not be killed and will begin to overgrow the culture. For a typical pBR322-based plasmid growing in a medium containing 20 μg of ampicillin per milliliter, this point is reached when the culture is barely becoming turbid, perhaps around 10^7 cells per milliliter. Growth in the presence of 200 μg of ampicillin per milliliter delays this point to a slightly higher cell density, but, given the catalytic activity of β-lactamase, it would not be feasible to add enough ampicillin to the medium to keep the cells under selection all the way to saturation.

A further complication is that certain toxic genes, while having little effect on cells that are growing logarithmically, kill cells at saturation. Almost all cells retain plasmid until saturation, but on continued incubation, fewer and fewer plasmid-containing cells survive and, because no ampicillin remains, cells that lack plasmid overgrow the culture.

A culture grown to saturation from selective conditions will have secreted a considerable amount of β-lactamase into the medium even if it becomes substantially overgrown by cells that lack plasmid. Subcultures might typically be grown from dilutions of 200- to 1000-fold into fresh ampicillin-containing medium. However, enough β-lactamase is typically present in the saturated culture that, even at these dilutions, enough remains to destroy all of the ampicillin before the cells that lack plasmid can be killed. Therefore, the subculture will grow completely in the absence of selection. The inoculum may already have had a substantial fraction of cells lacking plasmid, and by the time the subculture has grown to a density where expression of the target gene is induced, it is quite possible that only a minor fraction of the cells will contain the target plasmid. Failure to appreciate these potential problems can easily lead to the erroneous conclusion that certain target genes are poorly expressed, when in fact only a small fraction of cells in the cultures that were tested contained plasmid.

Most of the pET vectors described here have ampicillin as the selective antibiotic, and simple precautions are advisable to maximize retention of plasmid through the procedures for isolating, maintaining, and expressing target plasmids. We use the following isolation protocol, which usually produces the highest possible fraction of cells containing functional target plasmid. A colony from the transformation plate is inoculated into 2 ml of M9ZB + ampicillin and incubated for a few hours, until the culture becomes lightly turbid, when a sample is streaked on a plate containing ampicillin to obtain a single colony. As soon as the colony develops (usually overnight at 37°), it is inoculated into 2 ml of M9ZB + ampicillin and grown almost to saturation, when 1.5 ml of culture is mixed with 0.15 ml of 80% glycerol in a cryovial and stored in a −75 freezer. If there is any question about the possible stability of the plasmid, the culture is

titered at the time of freezing to determine what fraction of the cells contain functional target plasmid.

For cells that carry a plasmid but no source of T7 RNA polymerase, titering in the presence and absence of ampicillin (200 μg/ml in the top agar) determines the fraction of cells that have plasmid. When the target plasmid is carried in BL21(DE3) or HMS174(DE3), the fraction of cells able to express the target gene can be tested by including 1 m*M* IPTG in the top agar, which will prevent colony formation by any cell that has both the inducible gene for T7 RNA polymerase and a functional target plasmid (but will not prevent growth of cells that lack plasmid or mutants that have lost the ability to express target DNA). In the presence of pLysS, IPTG also prevents colony formation (except in rare cases, including pET-3 itself). In the presence of pLysE, IPTG usually does not prevent colony formation unless the target gene product is toxic.

In practice, DE3 lysogens that carry a target plasmid that confers ampicillin resistance are titered on four plates, which have ampicillin, IPTG, both, or neither added to the top agar: all viable cells will grow on the plate with no additive; only cells that retain plasmid will grow in the presence of ampicillin; only cells that have lost plasmid or mutants that have lost the ability to express target DNA will grow in the presence of IPTG; and only mutants that retain plasmid but have lost the ability to express target DNA will grow in the presence of both ampicillin and IPTG. In a typical culture useful for producing target proteins, almost all cells will form colonies both on plates without additives and on plates containing only ampicillin, less than 2% of the cells will form a colony on plates containing only IPTG, and less than 0.01% will form a colony on plates containing both ampicillin and IPTG. With unstable target plasmids, the fraction of cells that have lost plasmid will be reflected by an increase in colonies on the IPTG plate and a decrease on the ampicillin plate. Mutants that retain plasmid but have lost the ability to express target DNA arise in some cases, but relatively infrequently.

If the plasmid is stable, cultures for expressing the target gene can be grown from the freezer stock without special precautions: even if the ampicillin in the fresh medium is destroyed or if the culture is incubated overnight at saturation, almost all of the cells will retain the target plasmid. However, if the target plasmid is unstable, cultures are grown from a dilution of 10^4 or higher from the freezer stock and grown directly to the density used for expression. Because of the potential for loss of plasmid, we always determine the composition of the cells in the culture by plating immediately before induction. This simple test can be invaluable in interpreting any unusual properties of an induction and in making sure that effort is not wasted on processing cells that had suboptimal levels of expression.

Some of the problems outlined here might be circumvented by using the pET-9 series of vectors, which provide resistance to kanamycin rather than ampicillin. However, the general principles and procedures should be useful in dealing with the problems of cloning toxic genes in any vector.

Expressing Target DNA by Infection with CE6

In principle, any target DNA that can be cloned in HMS174 can be transcribed by T7 RNA polymerase produced during infection with CE6. However, expression by CE6 infection is generally less convenient than expression by induction of DE3 lysogens because active phage stocks must be prepared and specialized growth conditions are needed to ensure good infection. Typically, we use CE6 infection only if a target plasmid is not able to be expressed in a DE3 lysogen, alone or in the presence of pLysS. (Lysogens of CE6 are not a good source of inducible T7 RNA polymerase because constitutive transcription from the p_I promoter in the lysogen provides basal levels that are too high to allow establishment of target plasmids.)

Preparation of CE6 Stocks

General procedures for working with λ have been described.[27,29] We grow CE6 stocks on host strain ED8739 ($r_K^- m_K^-$ *metB supE supF*),[30] which lacks the *Eco*K restriction and modification systems and provides *supF* for suppressing the *Sam7* mutation of CE6. These phage effectively direct the expression of target genes in HMS174, and presumably in other nonrestricting hosts that adsorb λ. Although we have not examined the ability of unmodified CE6 to direct expression of target genes in a restricting host, one might expect such expression to be reduced. Where it may be desirable to express target genes in a host that has an active *Eco*K restriction system, CE6 stocks could be grown on a host that will provide *Eco*K modification, such as the ED8739 derivative ED8654 ($r_K^- m_K^+$ *metB supE supF trpR*)[30,31] or the equivalent LE392.[27] (HMS174 has no amber suppressor and therefore is not a host for growth of CE6.)

CE6 stock lysates are grown by adding a single plaque and 50 μl of a fresh overnight culture of ED8739 to 35 ml of ZY medium in a 125-ml flask and shaking at 37° until lysis. Larger volumes are grown by adding 10 μl of lysate and 1 ml of culture to 500 ml of ZY medium in a 1-liter flask. Lysates are made 0.5 *M* in NaCl and centrifuged for 10 min at

[29] R. W. Hendrix, J. W. Roberts, F. W. Stahl, and R. A. Weisberg, "Lambda II." Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1983.

[30] K. Borck, J. D. Beggs, W. J. Brammar, A. S. Hopkins, and N. E. Murray, *Mol. Gen. Genet.* **146,** 199 (1976).

[31] N. E. Murray, W. J. Brammar, and K. Murray, *Mol. Gen. Genet.* **150,** 53 (1977).

10,000 *g* and 4°. The supernatant typically contains a few times 10^{10} infective phage particles per milliliter and is suitable either for long-term storage or for further purification.

CE6 phage particles are purified by precipitation with polyethylene glycol 8000 followed by rapid isopycnic banding in CsCl step gradients. All solutions used during purification, including CsCl solutions, contain 10 m*M* Tris-Cl (pH 8.0), 10 m*M* $MgSO_4$, and 100 μg of gelatin per milliliter to keep the phage intact. The purified phage are stored refrigerated in the CsCl solution, and dilutions are made in 0.1 *M* NaCl, 50 m*M* Tris-Cl (pH 8.0), 10 m*M* $MgSO_4$, 100 μg of gelatin/ml.

Lysates and purified phage stocks both lose titer on storage, so fresh stocks must be prepared from time to time. We find that either clarified lysates or purified phage in the above diluent can be mixed with one-tenth volume of 80% glycerol and stored frozen at −75° and thawed without significant loss of titer. However, we don't as yet have sufficient experience to know whether this is a better means for long-term storage of phage stocks than simple refrigeration.

Expressing Target Genes

Efficient expression of target genes by CE6 infection requires that the host cell be appropriately receptive to infection: synthesis of the λ receptor is known to be stimulated by maltose but inhibited by glucose. An appropriate ratio of phage particles to receptive cells is also important, since too few phage particles will leave a substantial fraction of the cells uninfected and too many will completely inhibit protein synthesis.[7] The following protocol was developed to optimize expression of target genes in M9 medium, where proteins can be labeled by incorporation of radioactive amino acids. An equivalent protocol can be used in B2 medium, where RNA can also be labeled by $^{32}PO_4$. Efficient expression of target genes can also be obtained by infecting cells that are growing in ZY medium supplemented with 0.04% (w/v) maltose.

HMS174 containing the target plasmid is grown in M9mal + antibiotic in a shaking flask at 37°. When a logarithmically growing culture reaches an OD_{600} of 0.3, glucose is added to give a concentration of 4 mg/ml. After an additional 1–2 hr of growth, when the OD_{600} reaches 0.6–1.0 (a cell concentration around 5×10^8/ml), $MgSO_4$ is added to give a final concentration of 10 m*M*, and purified CE6 phage is added to give a final concentration of 2×10^9 to 4×10^9 infective phage particles per milliliter (usually 1 μl of an $OD_{260} = 6$ phage stock per milliliter of culture). The multiplicity of infection should be around 5–10 phage particles per cell. Immediately before infection, the culture is titered on plates with and without antibiotic

to confirm that essentially all cells carry the target plasmid; the efficiency of infection, typically greater than 95%, is measured by titering surviving colony-forming units 5 min after infection. In this protocol, addition of glucose and $MgSO_4$ is not necessary but seems to give slightly better production of protein from the target genes. Cells are usually harvested 2–3 hr after infection, enough time for substantial accumulation of target protein but before the culture can be overgrown with uninfected cells.

Analyzing Target RNAs and Proteins

Target RNAs and proteins are analyzed by gel electrophoresis,[7] agarose gels being convenient for analyzing large RNAs, and gradient acrylamide gels for analyzing proteins or small RNAs (Fig. 1). The distribution of total RNA can be visualized by ethidium bromide fluorescence, total protein by staining with Coomassie brilliant blue, and radioactively labeled components by autoradiography. The amount of culture needed for analyzing RNAs and proteins depends on a number of factors, such as the host, culture medium, cell density, specific activity of label, and the gel system used. In a typical expression experiment using our standard protocols, 5–50 μl of culture is appropriate for visualizing proteins by Coomassie brilliant blue staining and 1–5 μl for visualizing RNAs by ethidium bromide fluorescence. For labeling proteins, 50 μl of culture labeled for 2–5 min with 1 μCi of [^{35}S]methionine usually gives enough label to visualize proteins after overnight exposure of the autoradiogram.

Total cell contents are analyzed by placing the cells in sample buffer [50 m*M* Tris-Cl (pH 6.8), 2 m*M* Na_3EDTA, 1% (w/v) sodium dodecyl sulfate, 1% (v/v) mercaptoethanol, 8% (v/v) glycerol, 0.025% (w/v) bromophenol blue], heating for 2–3 min in a boiling water bath, and applying directly to the gel. When analyzing proteins, samples of culture can be mixed directly with sample buffer; when analyzing RNAs, the gel patterns seem to be better if the cells are collected by centrifugation and suspended in sample buffer. Where samples are collected over a period of several hours, they can be left in sample buffer at room temperature and all heated together before loading onto a gel; where considerably more time will elapse before electrophoresis, they are kept in a −20° freezer and heated before analysis. Placing sample buffer (itself or the appropriate mixture with growth medium) in the outside wells adjacent to the samples on the gel can help to keep patterns from spreading out in the gel during electrophoresis.

Where a target protein is made in relatively small amounts or is obscured by a host protein having a similar mobility, pulse labeling is a sensitive means of detecting its synthesis. Pulse–chase experiments can

also be used to look for possible instability of target RNAs or proteins. Where a target protein is very difficult to detect, rifampicin can be used to inhibit transcription by host RNA polymerase and thereby reduce any background incorporation of label into host proteins. Typically, 200 μg of rifampicin per milliliter of culture is added 30 min after induction or infection, after which the host mRNAs decay while the target mRNA continues to be produced by the rifampicin-resistant T7 RNA polymerase. Within a few minutes, the only proteins still being made should be those under control of the T7 promoter. This test must of course be done in a host whose RNA polymerase is sensitive to rifampicin (such as BL21, but not HMS174).

A common problem encountered when proteins are expressed in *E. coli* is that the target protein is insoluble.[32] A quick test for this is to pellet a sample of cells (30 sec in an Eppendorf centrifuge at room temperature) and resuspend them in 50 m*M* Tris-Cl, 2 m*M* EDTA, pH 8.0, typically in a volume not much less than the original culture volume. If the cells contain T7 lysozyme, simply freezing and thawing this suspension will usually make a good lysate; otherwise, the suspended cells are first treated with commercial egg white lysozyme (100 μg/ml for 15 min at 30°). A further 2-min centrifugation pellets the insoluble protein. The protein contents of the total lysate, the supernatant, and the resuspended pellet are compared by gel electrophoresis.

Factors That Affect Production of Target Proteins

This T7 expression system has produced substantial amounts of target protein from a wide variety of genes, both prokaryotic and eukaryotic. However, some proteins are made in disappointingly small amounts, for reasons that are obvious in some cases and obscure in others. We here summarize briefly some of the known or likely reasons for obtaining low levels of expression.

The target protein itself may interfere with gene expression or with the integrity of the cell. Sometimes pulse labeling shows a gradual or rapid decrease in the rate of protein synthesis as target protein accumulates, or sometimes all protein synthesis stops before any target protein can be detected. Occasionally, considerable lysis of a culture is observed.

One might expect that instability of target mRNA might limit expression in some cases, although in each case we have examined, substantial amounts of target mRNA seem to accumulate. This apparent stability of target mRNA could be due to the stem-and-loop structures at both ends of RNAs that are initiated at the usual $\phi 10$ promoter and terminated at Tϕ or

[32] F. A. O. Marston, *Biochem. J.* **240,** 1 (1986).

cut at R*1.1*; or the mRNA may be relatively inaccessible to exonucleases by being embedded in the long RNAs produced by T7 RNA polymerase in the absence of Tϕ; or perhaps so much RNA is produced that the normal mRNA degradation system is overloaded.

Instability of certain target proteins might also be expected, although BL21 is apparently deficient in the *lon* and *ompT* proteases and many proteins produced in this strain are quite stable. Some relatively short proteins produced by out-of-frame fusions are also quite stable in this strain, whereas others are so rapidly degraded as to be undetected by pulse labeling.

Many target proteins seem to be made in equivalent amounts whether or not the Tϕ transcription terminator is present in the vector. In some cases, however, having Tϕ behind the target gene increases the production of target protein. In the cases we have encountered, the target mRNA is translated from its own translation initiation signals rather than from the strong T7 gene *10* signals. A possible interpretation is that some translation initiation signals do not compete well against the *bla* mRNA, which is made along with the target mRNA, and that Tϕ, by reducing the amount of this competing mRNA, allows more target protein to be made. In the pET-9 vectors, where the *kan* gene and the target gene have opposite orientations, no competing mRNAs are known to be made along with the target mRNA.

Some target proteins are made in relatively small amounts even though both the mRNA and protein appear to be relatively stable and the coding sequence is joined to the efficient T7 translation initiation signals. The cause of the poor translation of these mRNAs is not well understood but could perhaps be due to factors such as unfavorable distributions of rare codons, relatively high levels of translational frameshifting, or interfering structures in the mRNA.

Potential Improvements

The inducible expression system is convenient to use and can produce high levels of target gene products. The most serious limitation is in its ability to maintain and express genes whose products are toxic to the cell. Addition of pLysS reduces basal T7 RNA polymerase activity enough that most target genes are stable yet still expressible at high level. The presence of T7 lysozyme in the cell has the additional advantage of facilitating the preparation of extracts. The repressible T7*lac* promoter reduces basal expression by a different mechanism, by blocking transcription from the target promoter. The T7*lac* promoter itself appears to stabilize more target genes than even the high level of lysozyme provided by pLysE, and seems to have little if any effect on the level of expression after induction. The

combination of the T7*lac* promoter and pLysS appears to allow the maintenance and expression of almost any gene in BL21(DE3) or HMS174(DE3). Full expression from the T7*lac* promoter takes longer to induce in the presence of pLysS and may not reach as high a level as with the natural $\phi 10$ promoter, but substantial levels of expression have been obtained in most cases tested so far.

Another way to reduce basal expression of target genes might be to place the gene for T7 RNA polymerase under control of inducible promoters that are more tightly repressed than the *lac*UV5 promoter. A high level of induction from the promoter may not be very important, since relatively small amounts of T7 RNA polymerase are capable of saturating the translation apparatus with mRNA. The appropriate way to test other inducible promoters would be to investigate whether they enable the system to tolerate and express ever more toxic genes. Even with more tightly regulated promoters, the system might well need the additional control provided by T7 lysozyme or the T7*lac* promoter.

Antisense promoters[33] may also be useful for reducing basal expression of toxic target genes. We find[34] that a promoter for *E. coli* RNA polymerase in the antisense direction can stabilize toxic target plasmids in BL21(DE3) or HMS174(DE3). However, the level of expression achieved on induction of T7 RNA polymerase seems to depend both on the strength of the antisense promoter and on the particular gene that is being expressed. The yield of target protein can sometimes be increased by adding rifampicin to shut off the synthesis of antisense RNA after T7 RNA polymerase has been induced. An antisense promoter has also permitted the cloning and expression of certain genes we had previously been unable to clone at all, apparently by antagonizing or neutralizing the effects of promoters for *E. coli* RNA polymerase in the target DNA fragment.

Other variations or improvements to the vectors for cloning and expressing target genes could also be considered. Additional antibiotic resistances could be substituted for ampicillin or kanamycin resistance as the selective marker. Equivalent vectors having compatible replicons might be useful for expressing different proteins in the same cell. Vectors with higher or lower copy numbers might also be useful in some applications. It may also be possible to reduce the already low basal expression of target genes in the absence of T7 RNA polymerase, perhaps by inserting transcription terminators ahead of the T7 promoter or by eliminating nonessential regions of the plasmid that may contain weak promoters for *E. coli* RNA polymerase.

The expression system can be expanded to other hosts that might be

[33] P. J. Green, O. Pines, and M. Inouye, *Annu. Rev. Biochem.* **55**, 569 (1986).
[34] L. Lin, A. H. Rosenberg, and F. W. Studier, unpublished work, 1988.

specialized for various purposes. A wide range of host strains can be easily lysogenized with DE3, using helper bacteriophages that both provide *int* function and select for growth of the appropriate lysogen. Derivatives of λ that have other host ranges or derivatives of other bacteriophages that carry no T7 promoters could also be used to deliver the gene for T7 RNA polymerase to the cell. Vehicles to deliver T7 RNA polymerase and vectors to carry target genes could in principle be developed for a wide variety of bacteria besides *E. coli.*

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[7] High-Level Translation Initiation

By LARRY GOLD and GARY D. STORMO

Introduction

Promoters are cassettes; they work in a manner that is independent of the surrounding nucleotide sequences, with some perturbations allowed for the torsional strain or relaxed state of the DNA. Ribosome binding sites (RBS), the translation equivalent of promoters, are not known to be portable. However, we think translation initiation is simple, and portable RBS are easy to imagine.

Before we describe translation initiation in a simple manner, which leads directly to the design of portable RBS, we disclaim the extension of these ideas to explain the behavior of *all* mRNAs. We have studied the initiation activity of hundreds of different mRNAs; many of the RBS of those mRNAs are far more efficiently utilized than we would expect. Some mRNAs must have evolved nonstandard mechanisms for fast binding to ribosomes and the subsequent steps; examples are the RBS of the coat protein genes of the RNA phages of *Escherichia coli:*

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                    * * *     1 1     * * *
QB coat RBS:  GUUGAAACUUUGGGUCAAUUUGAUCAUGGCAAAAUUAGAG
                  > > > > >     < < < < <
                    * * * *     8     * * *
MS2 coat RBS: AGAGCCUCAACC GGAGUUUGAAGCAUGGCUUCUAACUUU
                                > > > > >     < < < < <
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