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Requirements for the high-level expression of murine interleukin-3 cDNA in *Escherichia coli*

Robert Greenberg[a], Keith M. Gewain[a,*], Karen Joy Shaw[a], Beth Frommer[a], James A. Anagnost[a], Sandra Zurawski[b], Gerard Zurawski[b], Satwant K. Narula[a] and Paul J. Leibowitz[a]

[a]*Schering Corporation, Bloomfield, NJ and* [b]*DNAX Research Institute of Molecular and Cellular Biology, Inc., Palo Alto, CA, U.S.A.*

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SUMMARY

Murine interleukin-3 (Mu IL-3) cDNA was previously expressed in *Escherichia coli* using a *tac* promoter and a constitutive high copy number plasmid vector. We found that significant increases in expression levels could be realized by using the *tac* promoter for the expression of Mu IL-3 in a plasmid vector possessing a temperature-inducible runaway-replicon. In contrast, use of an *lpp* promoter under similar conditions did not result in an increase in the Mu IL-3 expression level. Significant differences were observed when the expression levels of IL-3 were monitored in various *E. coli* hosts having different genetic backgrounds. A mutant of *E. coli* which lacks the protease La was found to increase the level of IL-3 produced. This report describes the effect of a specific protease-deficient *E. coli* host strain, as well as the effect of different promoters and plasmid replicons on the expression levels and stability of a heterologous gene product.

INTRODUCTION

Interleukin-3 (IL-3) is induced in T lymphocytes by activated antigen-presenting cells or by concanavalin A. Several different biological properties are attributed to this molecule. These include the stimulation of growth of mast cell lines [24,31]; histamine-producing cells [9] and multi-lineage colonies in vitro from bone marrow cells [1]. IL-3 cDNA was cloned and identified from a pcD cDNA library derived from an inducer T cell line based on a functional assay [10,29]. Subsequent expression in *E. coli* was obtained using a *tac* promoter [23]. However, since the level of expression was low, a study was begun to investigate the effect of parameters such as promoters, host strains and vector systems on expression levels. We report here that a substantial increase in expression of murine (Mu) IL-3 can be realized through the use of a runaway-replication vector system. Furthermore, the choice of an appropriate host strain was observed to play a significant role in achieving a high expression level of Mu IL-3.

* Present address: Merck, Sharpe & Dohme Research Laboratories, Rahway, NJ 07065-0900, U.S.A.
Correspondence: Satwant K. Narula, Schering Corporation, 60 Orange Street, Bloomfield, NJ 07003, U.S.A.

MATERIALS AND METHODS

Host strains

Escherichia coli RB791 and AB1899 were obtained from Dr. Bachman at the *E. coli* Genetic Stock Exchange Center at Yale University. *E. coli* C1-31 is a strain that was derived in our laboratory from a minicell producer DS410. Table 1 contains a description of the strains used in this study.

Plasmids

pKG-2 is a derivative of pVU208, a temperature-inducible runaway-replication plasmid [13]. T270-1 is a plasmid that provides convenient restriction sites for expressing genes under *lpp* control. Both derivatives were constructed in our laboratory. Plasmid extractions were carried out according to published procedures [18]. pBR322 has been described previously [26].

Enzymes

All restriction enzymes, T4 DNA ligase and Klenow were obtained from New England Biolabs and used according to the manufacturer's recommendations.

Table 1

E. coli host strains

Strain	Genotype	Ref.
294	*end, hsdR, thi, pro*	2
W3110	F^-, *hsdR*, $HsdM^+$	5
JM103	*Δ lacpro, supE, thi, strA, sbcB15, endA, hspR4, F' traD36, proAB, lacI*^{q} *ZΔM15*	21
HB101	F^-, *hsdS20*(r_B^-, m_B^-), *recA13, ara-14, proA2*, λ^- *lacY1, galK2, rpsL20*(Sm^r), *xyl-5, mtl-1, supE44*	2
AB1899	*thr-1, leuB6, thi-1, argE3, hisG4, proA2, lon-1, lacY1, galK2, mtl-1, xyl-5, ara-14, strA31, tsx-33.* λ^- · *supE44*	14
RB791	*lacI* p4000 (*lacI*^{q}), *lacZ* p4008 (*lacL8*), λ^-, IN(*rrnD-rrnE*)1	3
DS410	*miniA, minB, rspL, sup*$^+$	8
C1-31[a]	derivative of DS410	
TDH-19[a]	*recA* derivative of DS410	

[a] Isolated in our laboratory.

Nick translations

These were carried out using the nick translation kit provided by New England Nuclear. The [α-^{32}P]dCTP was obtained from New England Nuclear or Amersham.

Growth media

20/10/5 TYE broth contains 20 g tryptone, 10 g yeast extract and 5 g sodium chloride, per liter. GC medium contains 2 g yeast extract, 30 g casamino acids, 5 g potassium phosphate (dibasic), 1 g magnesium sulfate (7 H_2O) and 20 g glycerol, per liter. The tryptone, yeast extract and casamino acids were obtained from Difco Laboratories. All other chemicals were either from Fisher Scientific or Mallinckrodt. Additionally, some of the 20/10/5 TYE broth was obtained as a premixed powder from GIBCO Laboratories.

Evaluation of Mu IL-3 expression in various E. coli strains

All strains were grown overnight in 700 ml of 20/10/5 TYE broth supplemented with the appropriate antibiotics (ampicillin at 100 μg/ml or tetracycline at 20 μg/ml). In the morning, all the strains were diluted 1:20 into 500 ml of GC media. Following dilution, all expression plasmids that carried the pKG-2 replicon were grown at 30°C until the culture reached an optical density of 1 at 660 nm, measured in a 1 cm path length cell. The cultures were then shifted to 42°C for a period of 3 h. Isopropyl β-D-thiogalactoside (IPTG) at a final concentration of 0.4 mM was added and the cultures were returned to 30°C for 2.5 h. All other strains were grown for 2 h at 37°C. IPTG was added to a final concentration of 0.4 mM and the incubation was carried on for another 4.5 h at 37°C. Samples were removed at appropriate time intervals for Western analysis.

SDS-PAGE electrophoresis

This was carried out according to published methods [16].

Western analysis

The monoclonal antibody used in the Western

analysis of Mu IL-3 was obtained from the DNAX Research Institute, Palo Alto, CA. The Western blotting procedure was carried out according to published methods [28].

Northern analysis

Cultures were induced as described above. Four millimeter aliquots were removed from each 500 ml culture, centrifuged, and RNA was extracted from the cell pellets as previously described [24]. RNA was loaded at a concentration of 15 μg/lane, electrophoresed on 1% agarose-formaldehyde denaturing gel [16], transferred to nitrocellulose and hybridized at 65°C in 10 ml of a solution containing the probe labeled at a specific activity of 2×10^8 cpm/μg, 50 μg/ml sheared salmon sperm DNA, 10 μg/ml poly rA and 0.1% SDS in 3 × SSC/5 × Denhardt's solution [27]. The probe used was a 465 bp fragment containing the coding region of Mu IL-3 cDNA.

Primer repair

This was carried out according to published procedures [12].

DNA sequencing

This was performed using the published method of Maxam and Gilbert [20].

Oligonucleotide synthesis

This was carried out on an Applied Biosystems Model 380A DNA Synthesizer. Samples were purified by electrophoresis on urea polyacrylamide gels [20].

RESULTS

Construction of a temperature-inducible high copy number expression vector for Mu IL-3

The plasmid pTAC-RBS Mu IL-3 [23] was digested with *Hin*dIII. The staggered end so generated was blunted using Klenow fragment. This was followed by an *Eco*RI digestion yielding a 700 bp fragment containing the *tac* promoter and the Mu IL-3 cDNA. This fragment was ligated to the 3.2 kb *Eco*RI-*Sca*I fragment from pKG-2 containing the temperature-inducible origin of replication and the tetracycline-resistance gene (Fig. 1). This ligation mix was used to transform *E. coli* RB791. Tetracycline-resistant colonies were picked and plasmid DNA was prepared from these cultures. The construction pKGT 105-11 was confirmed by restriction analysis. Western analysis indicated that upon thermal and chemical induction the expression of Mu IL-3 in this strain was at least five-fold higher than in the starting strain (Fig. 2). At the higher temperatures used for induction, both pKGT 105-11- and Lpt-11-containing strains reveal additional smaller molecular weight bands on the Western blot. These are probably the degradation products of Mu IL-3. The reason for the appearance of these bands may be the activation of a proteolytic activity or activities at these high temperatures. Our comparison of the strains, however, is based exclusively on the main band which coincides with the published molecular weight of Mu IL-3.

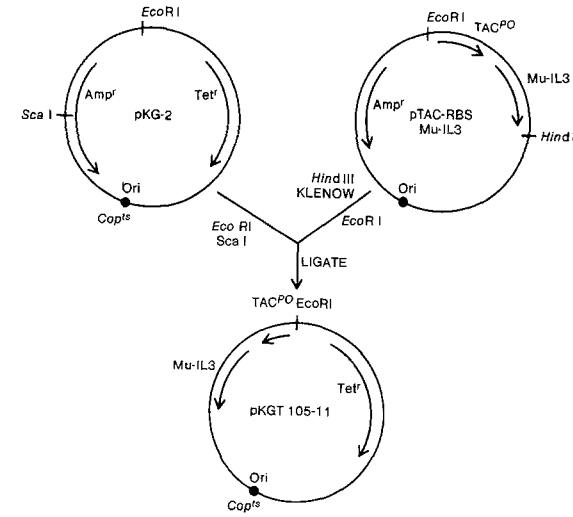

Fig. 1. Construction of the *tac*-Mu IL-3 expression vector, pKGT105-11. The DNA fragment carrying the *tac* promoter and the Mu IL-3 coding region was isolated from the *tac*-RBS Mu IL-3 vector by digestion with *Hin*dIII, treatment with Klenow, and subsequent digestion with *Eco*RI. This fragment was ligated with the large *Eco*RI-*Sca*I fragment of pKG-2 carrying the thermoinducible origin of replication. The ligation was used to transform *E. coli* RB791. The resultant thermoinducible high copy number vector, pKGT105-11, was isolated from one of the tetracycline-resistant transformants. Additional details of the construction are described in the text.

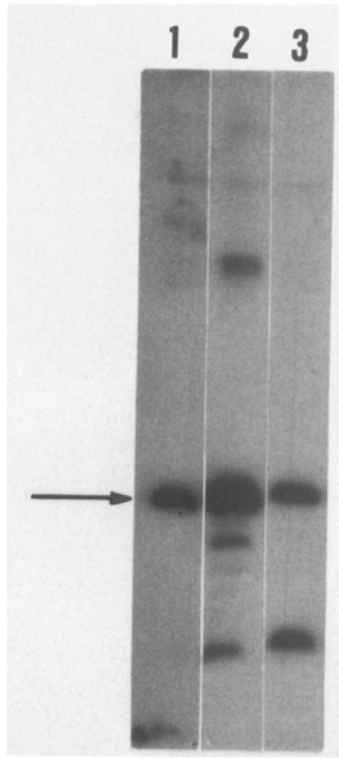

Fig. 2. Western analysis of Mu IL-3 vectors comparing the effect of different promoters on Mu IL-3 expression levels. Mu IL-3 strains were grown and samples prepared for Western analysis as described in the text. Samples, each containing 20 μg of total cell protein, were applied to a 15% SDS-polyacrylamide gel as follows: lane 1, pTAC-RBS Mu IL-3; lane 2, pKGT 105-11; lane 3, pLPT-11. Following electrophoresis, Western analysis was performed as described in the text.

Construction of an expression vector placing Mu IL-3 under the control of the lpp promoter

The mature coding sequence of Mu IL-3 had been cloned abutting the *Cla*I site of vector TrpC11 [7]. A 465 bp *Cla*I/*Hin*dIII fragment containing the Mu IL-3 sequence was isolated. In order to convert the *Cla*I site to a *Nco*I site, the fragment was denatured, and reannealed to the synthetic oligonucleotide AB140: CCATGGATACCCACCGTTTAACCAGAAC (the underlined sequence is the *Nco*I recognition site). After primer extension and cleavage with *Nco*I, the resulting 455 bp fragment was ligated into the plasmid T270-1 cut with *Nco*I. This DNA was then used to transform *E. coli* 294 and the analysis of transformants indicated that *lpp*10 contained Mu IL-3 fused to the *lpp* promoter. DNA sequence analysis of this clone confirmed that the *Nco*I site abutted the mature sequence of Mu IL-3. DNA from *lpp*10 was digested with *Aat*II, followed by *Nco*I partial digestion. Both ends were

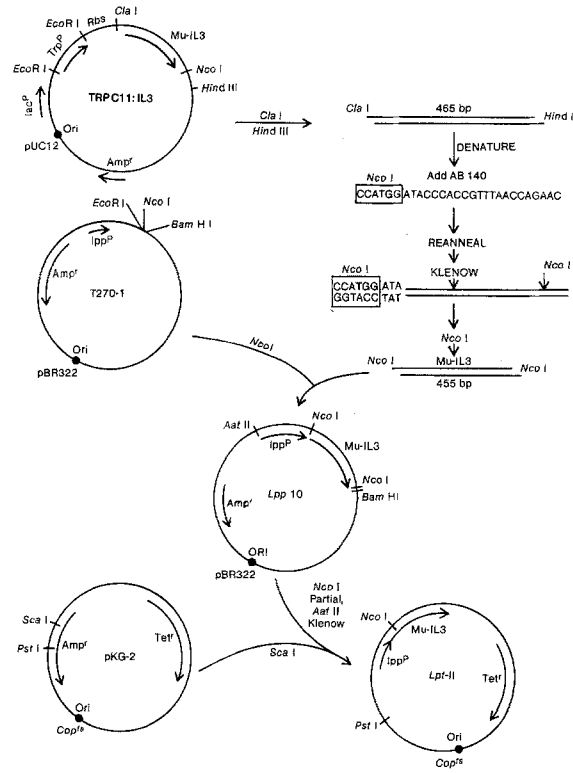

Fig. 3. Construction of the *lpp*-Mu IL-3 expression vector, Lpt-11. The Mu IL-3 coding region was isolated from the plasmid TRPC11 Mu IL-3 by digestion with *Cla*I and *Hin*dIII. Primer extension was carried out in the presence of the synthetic oligonucleotide, AB 140, to place an *Nco*I site at the 5′ end of the fragment, and simultaneously place an ATG start codon just 5′ of the mature coding sequence. The primer-extended fragment was then restricted with *Nco*I and ligated into the *lpp* promoter plasmid, T270-1, following linearization of the vector with *Nco*I. This ligation was then used to transform *E. coli* 294. One of the ampicillin-resistant transformants carried the plasmid, *lpp*10. The Mu IL-3 coding region was isolated from *lpp*10 by digestion of the plasmid with *Aat*II, followed by partial digestion with *Nco*I and treatment with Klenow to fill in the staggered ends. pKG-2 was restricted with *Cla*I, the staggered ends filled in with Klenow, and the linearized plasmid was ligated to the Mu IL-3 coding region fragment. The ligation mixture was then used to transform *E. coli* 294. One of the resulting tetracycline-resistant transformants carried the thermoinducible high copy number Mu IL-3 expression vector, Lpt-11.

blunted using the Klenow fragment. The 798 bp fragment was cloned into the *Sca*I site in the Amp^r gene of plasmid pKG-2. The final plasmid, Lpt-11, was tetracycline-resistant (Fig. 3). In this case, however, the level of expression remained comparable to that of the original strain (Fig. 2).

Evaluation of various host strains for maximizing Mu IL-3 expression

The Mu IL-3 expression plasmids, i.e., pKGT 105-11 and Lpt-11, were used to transform the *E. coli* strains described in Table 1. All of the strains were fermented as described in the Materials and Methods section. The level of expression in these strains was evaluated by Western analysis. As shown in Fig. 4, it is clear that the host background has a significant effect on the levels of Mu IL-3 expression. Various levels of expression were observed depending on the strain used, e.g., *E. coli* strains such as JM103, HB101 and W3110 showed lower levels of expression. With both pKGT105-11 and Lpt-11, the highest levels were observed in the strain AB1899 which carries the *lon* mutation. This effect was more dramatic with Lpt-11, in which the level of expression in almost every other strain was very low (Fig. 4).

Stability of Mu IL-3 in various host backgrounds

The results with the *E. coli* strain AB1899 prompted us to look at the longevity of the Mu IL-3 protein in two different host backgrounds to ascertain whether it was indeed the enhanced stability of the Mu IL-3 protein that was responsible for the higher levels of expression observed in the strain AB1899. Therefore, *E. coli* strains 294 and AB1899 containing the plasmid Lpt-11 were grown in 500 ml cultures and induced as described earlier. Fol-

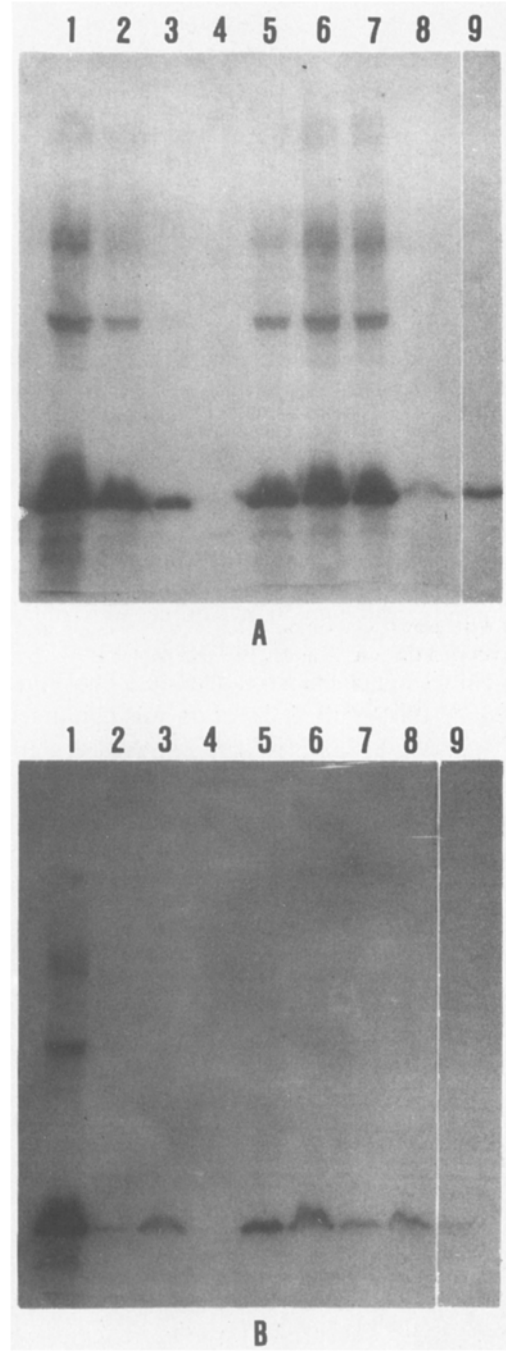

Fig. 4. Western analysis showing the effect of various *E. coli* host strains on the expression levels of Mu IL-3 produced by the Mu IL-3 expression vectors, pKGT 105-11 and Lpt-11. Strains were grown and samples were prepared for Western analysis as described in the text. Samples, each containing 20 μg of total cell protein, were applied to 15% SDS-polyacrylamide gels. Following electrophoresis, the gels were transferred to nitrocellulose and Western-blotted as indicated in the text. The autoradiographs demonstrate the effect of the *E. coli* host background on the expression of pKGT 105-11 (A) and Lpt-11 (B). The gels were loaded as follows, with each host strain carrying either pKGT 105-11 (A) or Lpt-11 (B): lane 1, AB1899; lane 2, HB 101; lane 3, JM 103; lane 4, non-radioactive molecular weight standards; lane 5, W3110; lane 6, Cl-31; lane 7, TDH19; lane 8, C600; lane 9, Mu IL-3 standard.

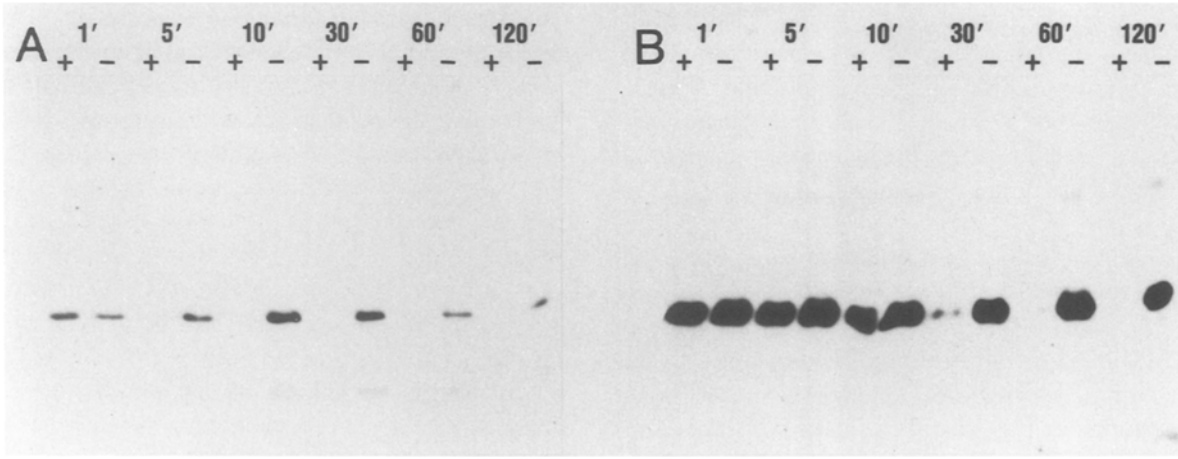

Fig. 5. Western analysis showing the effect of *E. coli* host background on the in vivo stability of Mu IL-3 expressed by the vector Lpt-11 in *E. coli* 294 (A) and AB1899 (B). Following growth and induction of each strain as described in the text, two 50 ml samples were removed from each culture and transferred into 250 ml flasks. To one of each pair, chloramphenicol was added to a final concentration of 100 μg/ml. Incubation was continued at 30°C, and 200 μl aliquots were removed and frozen in a dry ice/ethanol bath at the time points indicated in the figure. Aliquots were thawed and centrifuged to pellet the cells. Cell pellets were resuspended in an appropriate amount of gel sample buffer such that all samples were normalized based on the final A_{660} of the culture. 10 μl (approx. 50 μg) aliquots of each sample were applied to a 15% SDS-polyacrylamide gel. Following electrophoresis, Western analysis was carried out as indicated in the text.

lowing induction, two 50 ml samples were removed and transferred into 250 ml flasks. To one of each pair, chloramphenicol was added to a final concentration of 100 μg/ml. Incubation was continued at 30°C and 200 μl samples were removed at various time points. Mu IL-3 expression levels were determined using western analysis. This method provides a measure of the in vivo stability of Mu IL-3.

In the presence of chloramphenicol all further protein synthesis comes to a halt. Therefore, the intensity of the Mu IL-3 band in these Western blots is indicative of the stability of Mu IL-3 over time. It is clear from Fig. 5 that Mu IL-3 is significantly more stable in *E. coli* AB1899 (panel B) than in *E. coli* 294 (panel A). After 5 min of incubation in the presence of chloramphenicol, almost all of the Mu IL-3 is degraded in *E. coli* 294. In contrast, in *E. coli* AB1899, this effect is apparent only after 10 min of incubation, and significant levels of Mu IL-3 are detectable even after 30 min of incubation. Since this strain carries a *lon* mutation and therefore lacks the protease La, it is very likely that the stability of Mu IL-3 in this strain is directly related to the absence of this proteolytic activity.

Northern analysis

In order to rule out the possibility that the variation seen in the levels of Mu IL-3 expression in various strains was due to different levels of Mu IL-3 mRNA in these strains, a Northern blot analysis was performed on Mu IL-3-expressing strains. The results indicate that the level of mRNA in all these strains is essentially comparable (Fig. 6). Therefore, the differences observed in the expression of the protein in the various backgrounds are not due to differences in mRNA levels. The enhanced stability of the protein, in addition to the Northern blot data, favors the view that in AB1899, the high levels of Mu IL-3 protein observed are due to the *lon* mutation.

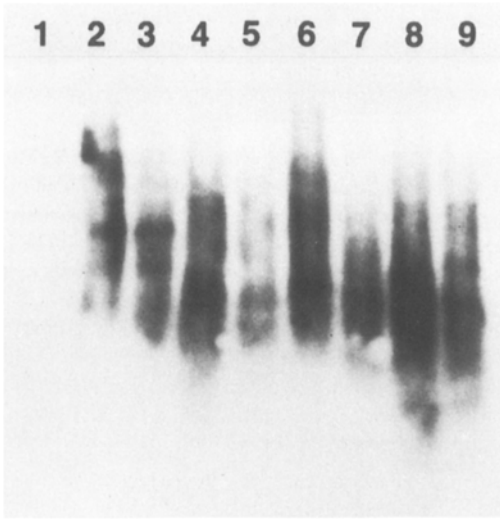

Fig. 6. Northern analysis of RNA samples isolated from various *E. coli* host strains carrying the Mu IL-3 expression vectors, pKGT 105-11 and Lpt-11. Cultures were grown and induced, and RNA was isolated from cell pellets. The samples were electrophoresed on a 1% agarose-formaldehyde gel as follows: lane 1, pKG-2/294; lane 2, pKGT 105-11/294; lane 3, pKGT 105-11/AB1899; lane 4, Lpt-11/294; lane 5, Lpt-11/AB1899; lane 6, Lpt-11/HB101; lane 7, Lpt-11/W3110; lane 8, Lpt-11/C1-31; lane 9, Lpt-11/C600. The RNA was transferred to nitrocellulose and hybridized to a Mu IL-3 coding region probe as described in the text.

DISCUSSION

Expression of heterologous proteins in *E. coli* has been achieved at very high levels [7,15]. The levels of expression are closely linked to the promoter used. However, it is not unusual to find that very strong promoters at times fail to yield desirable levels of the heterologous proteins. This could be due to instability of the mRNA, secondary structure of the mRNA that results in poor translatability, or alternately, instability of the heterologous protein.

We have found a similar situation with the expression of Mu IL-3 in *E. coli*. Strong promoters, such as *tac*, that have previously yielded high levels of heterologous proteins in our hands (data not shown) did not demonstrate similar capabilities in the case of Mu IL-3 expression. However, we found that replacing the pUC-type origin of replication of the pTAC RBS Mu IL-3 with a temperature-inducible high copy number replicon, in the case of the pKGT 105-11 construction, gave at least a five-fold improvement over the original strain (Fig. 2).

This, however, was not true in the case of the *lpp* construction, Lpt-11, where the level of expression remained very low. We are uncertain as to the reason for the differing results observed with the *tac* and *lpp* promoter systems. The Northern analysis indicates that this discrepancy is not due to the levels of mRNA present. A significantly greater mRNA level in one system than the other could result in a higher production level of IL-3 protein. This might increase the steady-state level of the protein, making the effect of the protease deficiency less dramatic. Indeed, the only difference in mRNA levels indicated by the Northern analysis is that the sample isolated from Lpt-11/*E. coli* AB1899 hybridizes to a lesser degree, indicating the presence of less IL-3 mRNA, than samples derived from strains carrying the same plasmid in the other host strains examined. This serves to emphasize the fact that the increase in expression levels observed with the *lon* strain, AB1899, can not be the result of an increased level of the IL-3 mRNA. A possible explanation for the differing results observed with the *tac* and *lpp* promoters may be related to the fact that the IL-3 genes in these vectors are oriented in opposite directions with different untranslated regions up- and downstream of them. Indeed, the presence of different DNA sequences surrounding the gene might result in the more efficient translation of the mRNA expressed under the *tac* promoter. Furthermore, the mRNA expressed under the *tac* promoter may be more efficiently translated due to the presence of an additional consensus ribosome binding site in this vector [23]. In both cases, however, and especially so in Lpt-11, significant increases in expression were obtained by changing the host backgrounds. The most dramatic results could be seen when the plasmid Lpt-11 was transformed into AB1899. This strain is deficient in the production of La protease, the product of the *lon* gene [6]. It has been shown that protease La is an ATP-dependent enzyme that catalyzes the rate-limiting step in the breakdown of

abnormal proteins. For example, mutations in *lon* lead to a decrease in the initial endoproteolytic cleavage of abnormal polypeptides [11,22]. However, not all 'foreign' or abnormal proteins are degraded [11,19], suggesting that other bacterial proteases are also important. The enhanced expression levels of Mu IL-3 in this strain may be directly related to this mutant phenotype. This argument is especially relevant in light of the stability data obtained from the chloramphenicol addition studies. It is clear from these studies that the Mu IL-3 protein is more stable in strain AB1899 carrying the *lon* mutation than in the other strains examined. It has been shown that the *lon* mutation can have a stabilizing effect on the degradation of cloned hybrid phage proteins [30] and at least one small eukaryotic protein, somatomedin-C [4].

We demonstrate here that in addition to the regulatory signals, other parameters such as the choice of the vector or an appropriate host background can significantly increase expression levels of a heterologous protein. In our experience, use of a temperature-inducible high copy number vector has generally resulted in 5–10-fold improvements in expression levels of several other heterologous proteins with further increases of 3–5-fold achieved merely by changing the host backgrounds (data not shown). These methods provide a quick way of achieving expression levels which are feasible for carrying out large-scale purification of interesting proteins to provide material for biological investigations.

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