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Maximizing gene expression from plasmid vectors containing the λ P_L promoter: Strategies for overproducing transcription termination factor ρ

(bacterial expression vector/temperature induction/nalidixic acid/transcription factor/ribosome binding site)

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ABSTRACT **We have constructed two plasmids in which transcription of the ρ gene from *Escherichia coli* K-12 is under the control of the λ phage P_L promoter. In p31-356, the normal ρ promoter is deleted, but the remainder of the ρ leader region, including the ribosome binding site, is present. In p39-AS, the ρ leader is completely absent, and the λ cII ribosome binding site replaces that of ρ. Under noninducing conditions, expression of ρ protein from these plasmids is repressed by the λ *cI* protein in hosts carrying λ cryptic prophage. Induction using mitomycin C or nalidixic acid in a cryptic lysogen carrying the *cI*⁺ repressor resulted in the overproduction of ρ protein to levels of 3%–5% of the total cellular protein with p31-356, and to levels of ≈40% with p39-AS. The overproduced protein is functionally indistinguishable from the ρ protein isolated from the K-12 strain W3110, and it can be obtained from cells harboring p39-AS in yields of up to 25 mg of ρ per g of cells. In contrast to chemical induction, heat induction in four cryptic λ lysogens carrying the thermolabile *cI857* repressor failed to yield the same high levels of ρ protein (with either plasmid). Our results show that chemical induction of P_L-containing plasmid expression vectors can serve as a convenient and useful alternative to the commonly used method of heat induction.**

ρ protein was discovered and initially characterized *in vitro* as an *Escherichia coli* factor that enhanced termination of transcription on bacteriophage λ DNA (1). Genetic studies involving ρ mutants confirmed the requirement for ρ protein as a termination factor *in vivo* for both phage and bacterial transcription (2, 3) and demonstrated that it is an essential protein in the cell (3, 4). *In vitro*, ρ protein binds to single-stranded nucleic acids and has an RNA-dependent nucleoside triphosphatase (NTPase) activity that is necessary for ρ-dependent termination of transcription (5). A specific intact region of the RNA transcript also appears necessary for termination *in vitro* (6). *In vivo*, ρ protein is thought to interact directly with the nascent RNA transcript and probably with RNA polymerase as well (7–9).

Although considerable structural and functional characterization of ρ protein has been carried out (for reviews, see refs. 10 and 11), detailed analysis has been hampered by its limited availability. To circumvent this limitation, we have constructed vectors for the overproduction of ρ protein, made possible in part by the recent sequencing of the ρ gene (12). In the process of optimizing conditions to generate large amounts of ρ protein, we have discovered a number of parameters that can influence protein overproduction from plasmid vectors containing the λ phage P_L promoter.

MATERIALS AND METHODS

Plasmids and Bacterial Strains. The ρ-containing plasmids p39 and p356 (13), and the λ P_L-containing plasmid expression vectors pAS-1 and pKC30 (14) have been described. Plasmid pKC31, a derivative of pKC30 constructed by Blair Ferguson, contains a 56-base-pair (bp) insert at the *Hpa* I restriction site in the λ *N* gene. This insert contains a stop codon for *N* translation and an *Xho* I restriction site that is unique in the plasmid. A λ lysogen of GM33 (*dam3*) was made for preparing plasmid DNA unmethylated at the sequence G-A-T-C (15). Strains AR58, AR13, and AR120 were generously provided by Jeff Auerbach. AR58 is a cryptic λ lysogen derived from N99 that is *galE*::Tn*10*, Δ-8 (*chlD–pgl*), Δ-H1 (*cro–chlA*), *N*⁺, and *cI857*. AR13 is a λ cryptic C600 derivative (16) that is Δ-8, Δ-H1, *kil*⁻, *cI857*, *N*⁺, and *galE*::Tn*10*. AR120 is a λ cryptic N99 derivative that is *c*I⁺, Δ-*gal*, and *nadA*::Tn*10*; the left arm of λ distal to the *N* gene is substituted by host DNA carrying *lacZ*; the right arm contains the T11 lesion that inhibits *cro* expression and Δ-(*c*II–*uvrB*). The *cI857* cryptic lysogens M5219 and N4830 have been described (17, 18).

DNA Manipulations and Cloning Procedures. DNA preparation, enzyme reactions, and bacterial transformations were performed as described (19). *Bam*HI-digested mung bean nuclease-treated pAS-1 was generously provided by Allan Shatzman. Restriction endonucleases were obtained from New England Biolabs, and DNA polymerase I Klenow fragment was the gift of Cathy Joyce. T4 DNA ligase was purchased from New England Biolabs or Collaborative Research, Waltham, MA.

Growth and Induction of Bacteria. Maximal induction medium for growing bacteria contained 32 g of tryptone and 20 g of yeast extract per liter, with additions after autoclaving of M9 salts (20) to 1 ×, $MgSO_4$ to 0.1 mM, $FeCl_3$ to 0.001 mM, and ampicillin to 100 μg/ml. After growth at 30°C to OD_{550} = 1.0, plasmid-containing bacteria were heat-induced by the addition of an equal volume of medium preheated to 65°C, with subsequent incubation at 42°C. For chemical inductions, plasmid-containing bacteria were grown at 37°C to OD_{550} = 1.0 and induced by the addition of either mitomycin C (1 mg/ml in H_2O) to 10 μg/ml, or nalidixic acid (2 mg/ml in 0.02 M NaOH) to 40 μg/ml, and incubation was continued at 37°C.

Purification of ρ Protein. ρ was purified from the *E. coli* strain W3110 as described (21) and from our overproducing strain with slight modifications. A 1-liter culture of AR120(p39-AS) was grown at 37°C in maximal induction medium to OD_{550} = 1.0. Nalidixic acid was added to 40 μg/ml, and incubation was continued at 37°C for 4 hr. Bacteria were pelleted and stored at −20°C. Frozen cells (6 g) were thawed, lysed, and treated with DNase and polymin P (21). After removing precipitated nucleic acids by centrifugation,

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0.35 g of $(NH_4)_2SO_4$ was added per ml of polymin P supernatant. The precipitate was washed twice with buffer A [10 mM Tris·HCl (pH 7.6, 25°C)/5% (vol/vol) glycerol/0.1 mM EDTA/0.1 mM dithiothreitol/100 mM NaCl] containing $(NH_4)_2SO_4$ at 0.32 g/ml, resuspended in buffer A, and dialyzed against the same buffer. This sample was chromatographed on a Bio-Rex 70 column (2 × 19 cm; capacity, >300 mg of ρ protein); ρ protein-containing fractions were pooled, dialyzed into storage buffer, and stored at −20°C (21).

Characterization of ρ Protein. The concentration of ρ in solution was determined directly from the absorbance at 280 nm [extinction coefficient, 0.37 $mg^{-1} \cdot cm^2$ (22)] or by quantitative amino acid analysis. The concentration of ρ protein in samples analyzed by gel electrophoresis was estimated by comparing the intensity of the Coomassie blue-stained ρ protein band with that of a ρ protein standard. For NH_2-terminal sequence analysis, ρ protein was coupled to *p*-phenylene diisothiocyanate-activated aminopropyl glass and subjected to solid phase sequencing (23) on a Sequemat Mini-15 sequencer equipped with a P-6 autoconverter. Phenylthiohydantoin derivatives were identified by reversed-phase HPLC (24). Digestion of ρ protein with trypsin in the presence of poly(C) and ATP was performed according to Bear *et al.* (25). Established methods were used to determine gel filtration behavior (26) and poly(C)-dependent ATPase activity (21). The ability of ρ protein to terminate transcription was tested *in vitro* using a DNA template containing the ρ-dependent termination site *trp t′* (27).

Gel Electrophoresis. Proteins were analyzed on 0.1% NaDodSO4/10% polyacrylamide slab gels 0.5 mm thick and 14 cm long (28). Samples were dissolved in sample buffer [0.125 M Tris·HCl, pH 6.8/2% NaDodSO4/10% (vol/vol) glycerol/1% 2-mercaptoethanol/0.001% bromphenol blue], boiled for 1–2 min, loaded onto the gels, and electrophoresed for 2.5 hr at 17.5 mA. After staining with Coomassie blue, as little as 5–10 ng of protein per band could be detected. To estimate the percentage of ρ in a sample, the stained gel was dried down on dialysis membrane and scanned with a Joyce–Loebl microdensitometer.

Source of ρ Protein Antibody. Rabbit antiserum raised against ρ protein purified from an *E. coli* K-12 strain was generously supplied by Stanley Brown. The immunoglobulin G fraction of this serum was partially purified by precipitation with $(NH_4)_2SO_4$ and used for immunoblot analysis of intracellular ρ protein levels.

RESULTS

Construction of the Plasmid Vectors. In the plasmid p31-356 (constructed as shown in Fig. 1*a*), ρ is expressed from the λ P_L promoter; this vector contains most of the ρ leader, the entire ρ protein coding sequence, and the ρ transcription termination signal. To circumvent the possibility that the leader sequence might have a negative regulatory effect on expression, we also constructed a second plasmid, p39-AS (Fig. 1*b*), in which the entire ρ leader region was absent, and the λ *c*II ribosome binding site replaced that of the ρ gene. Both of these plasmids carry the λ *nut* sites required for N protein antitermination function, which may help ensure high levels of ρ expression.

Induction by Heat, Mitomycin C, and Nalidixic Acid. Initial experiments involved attempts to obtain ρ protein overproduction from p31-356 by the standard method of heat induction in a cryptic λ lysogen that carries the thermolabile *cI857* repressor and the *N* gene. Surprisingly, heat induction of p31-356 produced only a slight increase in the observed levels of ρ protein (see below). As an alternative to thermal inactivation of the *cI857* repressor, we added mitomycin C or nalidixic acid to a cryptic lysogen (AR120) that carries the cI^+ repressor and the *N* gene. These compounds induce recA protein-mediated inactivation of the cI^+ repressor at 37°C. A different host was required for this experiment because the *cI857* allele has two distinct lesions: one results in thermal sensitivity, and the other, termed ind^-, renders the repressor resistant to inactivation by recA protein (30). Hence, neither mitomycin C nor nalidixic acid can be used for induction in strains containing the *cI857* repressor. The induction responses of p31-356 to mitomycin C and nalidixic acid in the cI^+ host (AR120) are shown in Figs. 2 and 3. The highest yields from this vector are obtained using nalidixic acid (Fig. 2), with ρ protein levels reaching a maximum of 3%–5% of the total cellular protein at ≈8 hr after induction (Fig. 3, lane 2). For comparison, four *cI857* cryptic lysogens were tested for ρ overproduction using this same vector with heat induction. Surprisingly, AR58, AR13, and N4830 produced <1% ρ protein, while M5219 produced ρ at a level approaching that obtained using nalidixic acid induction in the cI^+ host (data not shown). Upon nalidixic acid induction with a different plasmid, p39-AS, ρ comprises >40% of the total protein (Fig. 3, lane 4). In contrast, with heat induction of p39-AS in the *cI857* hosts, ρ levels reach a maximum 2–3 hr after induction, but they never approach the amounts observed with nalidixic acid induction, even in the best *cI857* host, M5219 (Fig. 3, lanes 5–8).

Heat shifts of AR120(p39-AS) induced with nalidixic acid either before or after the shift had no effects on the high levels of ρ protein produced (data not shown). Therefore, high temperature alone does not appear to be detrimental to ρ overproduction. It is possible that nalidixic acid blocks a host response that prevents ρ overproduction. However, when nalidixic acid was added to heat-induced AR58(p39-AS) cells, ρ levels were still <1% of total cellular protein (data not shown). We infer from these findings that ρ protein overproduction may be less dependent on the method of induction than on the genetic background of the strain.

Purification and Yield of the Overproduced ρ Protein. ρ was purified from AR120(p39-AS) bacteria by treatment of a crude lysate with polymin P and $(NH_4)_2SO_4$, followed by chromatography on Bio-Rex 70. The ρ protein obtained after this procedure is estimated to be >99% pure, as determined by NaDodSO4/polyacrylamide gel electrophoresis (Fig. 4, lane 7). This protein is as pure, or purer, than ρ protein prepared from wild-type bacteria using procedures that require at least three chromatographic columns (21, 22). Several trace protein contaminants are removed by chromatography on heparin agarose or 5′-(4-aminophenyl-phosphoryl)-uridine 2′(3′)-phosphate agarose (21) (Fig. 4, lanes 8 and 9). For the purified ρ, A_{280}/A_{260} = 1.6, indicating that the sample is relatively free of nucleic acid. The yield of ρ protein from AR120(p39-AS) bacteria induced with nalidixic acid is ≈25 mg/g (wet weight) of cells.

Structural and Functional Analysis of the Overproduced Protein. To determine whether the overproduced product of the cloned ρ gene had the intended identity, we examined the purified protein from several aspects. Amino-terminal sequence analysis of the first 16 residues revealed a protein sequence completely consistent with that predicted from the DNA sequence of K-12 ρ (12, 13); since this sequence spans the *Bcl* I site used to reconstruct the ρ gene in the overproducing vector, no alterations can have been introduced in this region. The overproduced protein comigrates on 0.1% NaDodSO4/10% polyacrylamide gels with ρ isolated from W3110, a commonly used *E. coli* K-12 strain. The overproduced protein and W3110 ρ elute at identical positions on gel filtration columns in the presence or absence of poly(C) and have oligomeric sizes consistent with those determined by Finger and Richardson (26). In functional terms, both proteins behave identically in poly(C)-dependent ATPase assays and stimulate termination by RNA polymerase equally well in transcription reactions *in vitro* (data not shown).

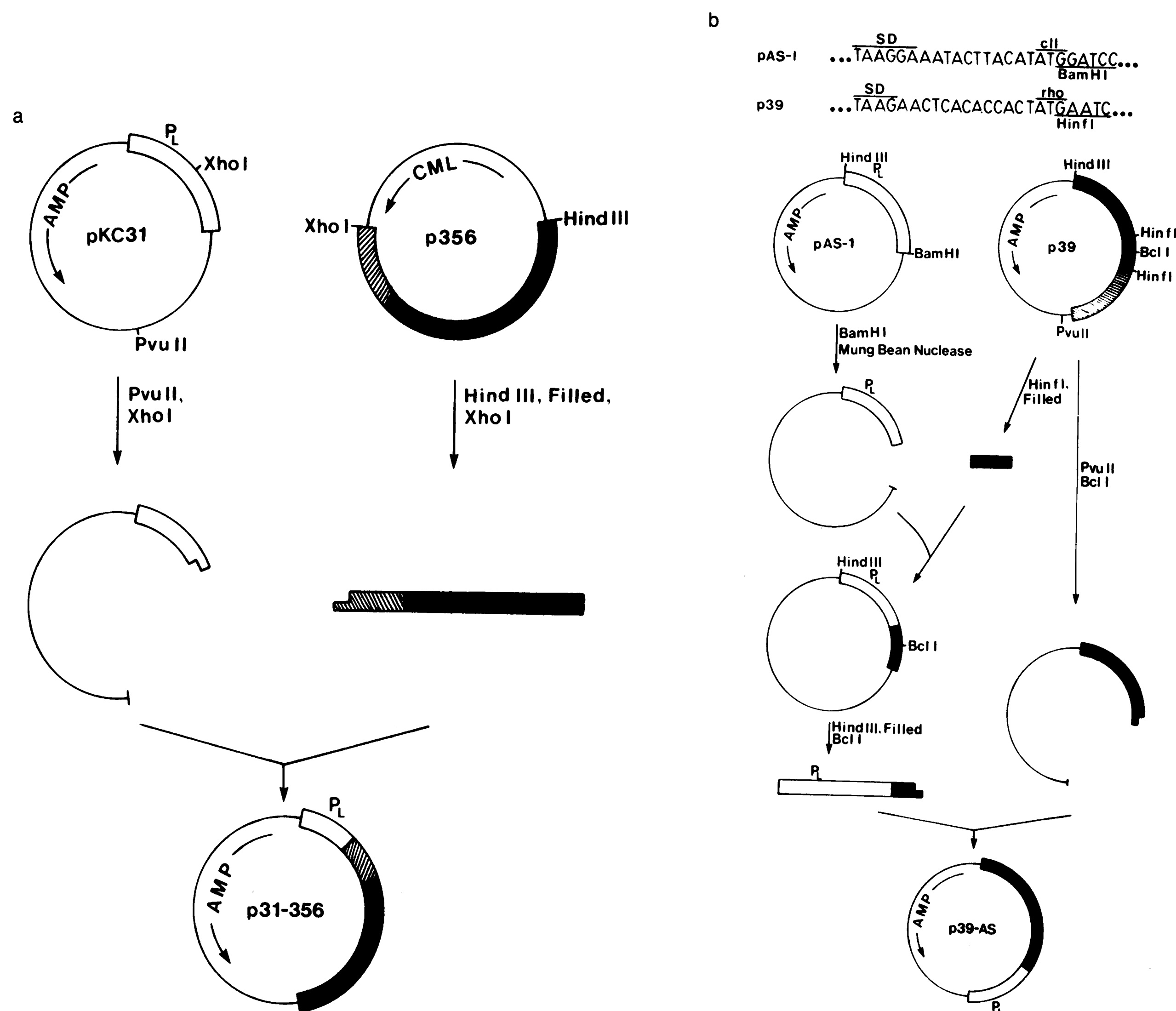

FIG. 1 Plasmid constructions for ρ protein overproduction. (*a*) Expression vector pKC31 is a pBR322 derivative that carries the λ P_L promoter and operator, the *nutL* site, and the proximal portion of the *N* gene, followed by an in-frame stop codon and distal cleavage sites for *Xho* I and *Pvu* II (λ sequences are represented by open block areas). Plasmid p356 (13) lacks the ρ promoter and the first 26 bp of the 256-bp ρ leader region. The distal portion of the ρ leader (hatched area, p356) is followed by the ρ protein coding region and transcription termination site (solid area) (29). Digestion of p356 with *Hin*dIII and filling of the staggered ends with Klenow fragment was followed by digestion with *Xho* I. The resulting fragment was ligated with *Xho* I, *Pvu* II-cut pKC31, as shown. After selection on ampicillin, transformants of AR120 were screened by restriction endonuclease digestion of minilysate DNA to determine the presence and character of the plasmid. (*b*) Vector pAS-1 is a pBR322 derivative that has the λ P_L promoter and operator, the *nutL* and *nutR* sites, the λ cII ribosome binding site, and the unique *Bam*HI site positioned at the ATG initiation codon for cII (14). Thus, digestion of this plasmid with *Bam*HI and mung bean nuclease produced a blunt end at the G·C bp of the cII protein initiation codon. Plasmid p39 (13) contains a 3.6-kilobase insert located between the *Hin*dIII and *Pvu* II sites that includes the ρ promoter and leader region (hatched area, p39) and ρ protein coding sequences and transcription termination site (solid area) (29). After cutting and filling the *Hin*fI site, only the initiating ATG codon is removed from the ρ protein coding sequence, leaving a 299-bp fragment that contains the NH_2-terminal coding region of the ρ gene. This fragment was isolated and cloned into the pAS-1 site generated by *Bam*HI and mung bean nuclease digestion. After transformation, one candidate having the correct orientation of the insert (determined by restriction digests) was sequenced across the fusion junction to confirm restoration of the ATG codon in frame with the ρ protein coding sequence. This intermediate plasmid DNA was then cut with *Hin*dIII, filled, and cut again at the unique *Bcl* I site (in the ρ insert), to generate a P_L–NH_2-terminal ρ fusion fragment that was cloned back into p39, as shown, regenerating the entire ρ gene. Initial screening of the final clones by restriction enzyme analysis was followed by testing for overproduction of ρ protein by various methods.

Based on these criteria, the overproduced ρ protein is identical to that obtained from W3110.

Upon close examination by using high-resolution NaDodSO$_4$/polyacrylamide gel electrophoresis, we have recently detected a very slight mobility difference between ρ protein produced in the strain containing the original ρ clone (13) and that purified from W3110 (unpublished observations). Though partial proteolytic digestion of these proteins in the presence of poly(C) and ATP yields the expected two fragments (25) in both cases, the amino-terminal f1 domain (31 kDa) from the overproduced protein reflects the molecular size difference observed with the intact molecules, migrating marginally slower on NaDodSO$_4$/polyacrylamide gels than the corresponding fragment from W3110 ρ. We believe that this simply reflects an allelic difference between strains that has no functional significance.

DISCUSSION

We have succeeded in producing and purifying exceptional quantities of the *E. coli* ρ protein. Our most successful approach utilizes nalidixic acid to derepress transcription from

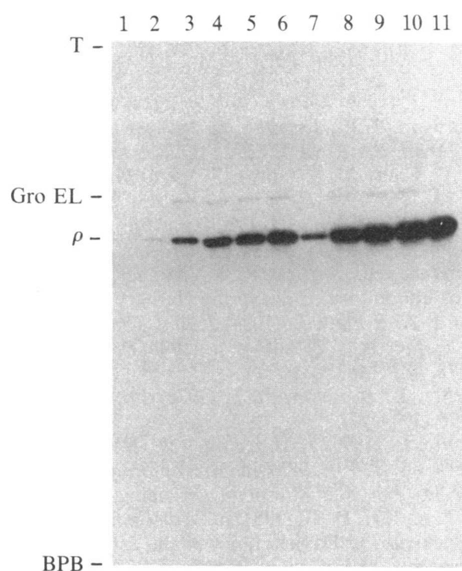

FIG. 2. Comparison of ρ protein induction by different methods. AR120(p31-356) was grown to $OD_{550} = 1.0$ and induced by the addition of mitomycin C (lanes 2–6) or nalidixic acid (lanes 7–11). Cells removed from the cultures at various times after induction were pelleted, washed once in 0.005 M Tris·HCl (pH 6.8), solubilized in sample buffer, and placed in a boiling water bath for 5 min. The total proteins from 5–10 μl of culture were separated on a 0.1% $NaDodSO_4$/10% polyacrylamide gel, electrophoretically transferred to nitrocellulose, probed first with ρ protein antibody and then with ^{125}I-labeled protein A as described (31). The nitrocellulose blot was exposed to x-ray film in the presence of an intensifying screen. In addition to ρ protein, the antibody reacts with a 60,000-Da polypeptide tentatively identified as groEL protein (32, 33), because (i) it comigrates on gels with purified groEL protein, and (ii) purified groEL protein reacts with the antibody. Lane 1 shows uninduced cells; lanes 2–6 show cells taken 1, 3, 5, 8, and 21 hr after mitomycin C addition; and lanes 7–11 show cells taken 1, 3, 5, 8, and 21 hr after nalidixic acid addition. BPB, bromphenol blue; T, top of gel.

the P_L promoter by recA protein inactivation of the λ cI^+ repressor. Although mitomycin C may also be used, nalidixic acid is less expensive, less hazardous (mitomycin C is a potent carcinogen), and also better in terms of ultimate protein yield (Fig. 2). The use of nalidixic acid rather than heat induction has several virtues. First, with large volumes of culture media, a rapid and uniform temperature shift is difficult to achieve. Second, the high temperature may inhibit gene overproduction by destabilizing the desired protein, by enhancing proteolytic degradation, or by altering the regulatory response of genes involved in induction. Third, at least for ρ, nalidixic acid induction results in higher overproduction than with heat (Fig. 3).

In our initial attempts, with expression from the λ P_L promoter using the heat-induction with the λ *cI857* repressor, the increase in the level of ρ protein was unexpectedly low. Since similar vectors have been used previously in *E. coli* to overproduce other prokaryotic and eukaryotic proteins by heat induction (34, 35), it was evident that some other factor(s) must be limiting expression. Of four *cI857* cryptic λ lysogens containing p31-356, only M5219 produced a high level of ρ protein. This level approached that obtained from nalidixic acid inductions of AR120(p31-356). However, with p39-AS, the better overproducing plasmid, ρ expression in M5219 is still significantly lower than with nalidixic acid induction in AR120 (Fig. 3).

The high level of ρ after nalidixic acid induction is especially surprising because plasmid copy number is actually reduced by a factor of ≈ 3, perhaps because of the inhibition of gyrase activity. In some *cI857* (*ind*$^-$) strains induced by heat, the addition of nalidixic acid enhances ρ overproduction (unpublished observations), suggesting that the addition of nalidixic acid may be increasing protein expression. Other ways to cause recA-mediated inactivation of the λ repressor, such as thymine starvation, may be more practical for industrial scale induction.

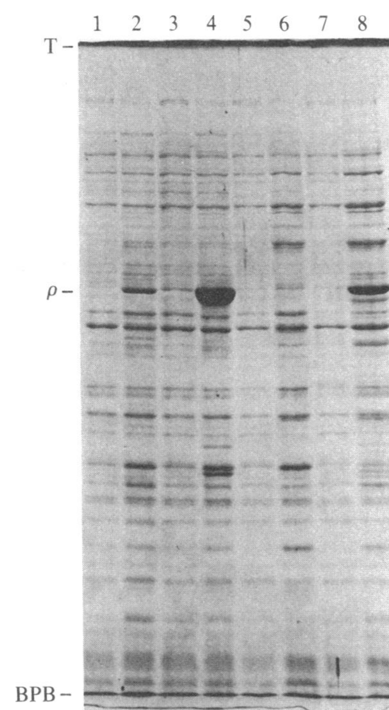

FIG. 3. Inductions of ρ expression by nalidixic acid and heat shifts. The AR120 host, carrying p31-356 or p39-AS, was grown at 37°C in maximal induction medium with 100 μg of ampicillin per ml. Nalidixic acid was added to 40 μg/ml, when cells reached $OD_{550} = 1.0$. Uninduced samples were taken just prior to nalidixic acid addition (lane 1, p31-356; lane 3, p39-AS), and induced samples were taken 7 hr after nalidixic acid addition (lane 2, p31-356; lane 4, p39-AS). For heat induction, the λ *cI857* cryptic lysogens, AR58 and M5219, were grown at 30°C in maximal induction medium with 100 μg of ampicillin per ml. At $OD_{550} = 1.0$, cells were shifted to 42°C and equal volumes of 65°C maximal induction medium were added. Samples were taken 7 hr after heat shift; those from uninduced AR58(p39-AS) and M5219(p39-AS) cells are shown in lanes 5 and 7, and those from heat-induced cells are shown in lanes 6 and 8. BPB, bromphenol blue; T, top of gel.

Specific features of the cloned gene can also affect the final level of gene expression. Particularly curious is the 10-fold difference in ρ protein levels between the vector carrying the ρ leader region (with its natural ribosome binding site), and the one in which this site is replaced by the λ *c*II ribosome binding site. This difference may be entirely due to greater translation efficiency of the *c*II ribosome binding site. Alternatively, it is possible that the ρ leader region has a negative regulatory effect on the expression of ρ, perhaps by an autoregulatory mechanism as suggested by other studies (13, 36). Since transcription from the λ P_L promoter is influenced by the λ antitermination protein N (encoded by the cryptic prophage), autoregulatory effects caused by high levels of ρ protein might be masked in our system.

Construction of overproduction plasmid p39-AS is greatly simplified by the location of the unique *Bcl* I and *Hin*dIII sites in the parent plasmid (Fig. 1). This will allow replacement of a *Bcl* I/*Hin*dIII "cassette" comprising all but the first 16 amino acids of the structural gene and, hence, overproduction of nearly any desired ρ variant.

Under appropriate conditions we can now obtain virtually unlimited amounts of pure ρ protein, in yields of 25 mg per g of cells, after polymin P and ammonium sulfate cuts from the

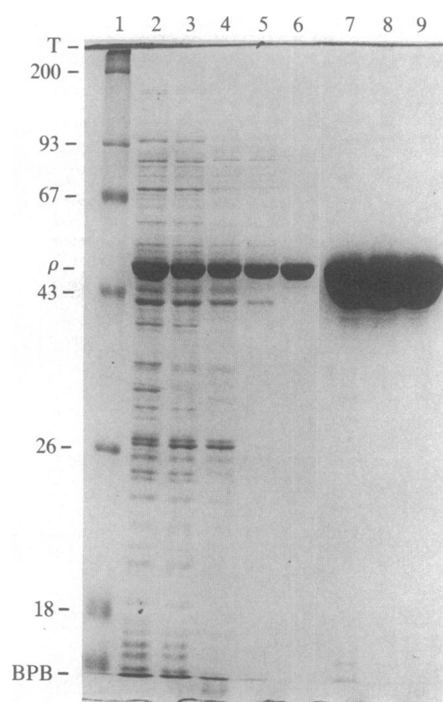

FIG. 4. Purification of ρ protein from the overproducing strain. ρ protein was purified from AR120(p39-AS) bacteria as described. Samples from various stages of the purification were analyzed on a 0.1% $NaDodSO_4$/10% polyacrylamide gel, and proteins were visualized after staining with Coomassie blue. Lane 1, protein standards (in kDa) are indicated on the left; lane 2, intact cells; lane 3, cell lysate after DNase treatment; lane 4, supernatant after polymin P precipitation; lane 5, $(NH_4)_2SO_4$ fraction, dialyzed, before Bio-Rex 70 column; lane 6, pooled fractions from Bio-Rex 70 column, 0.8 μg of protein; lane 7, same as lane 6, but 8 μg of protein; lane 8, ρ protein after purification on Bio-Rex 70 and heparin agarose columns, 8 μg of protein; lane 9, ρ protein after purification on Bio-Rex 70 and 5′-(4-aminophenyl-phosphoryl)-uridine 2′(3′)-phosphate agarose columns, 8 μg of protein. Minor polypeptides in lanes 7–9 having apparent molecular sizes of ≈40 kDa result from some breakdown of ρ protein during boiling of the samples in sample buffer (data not shown). BPB, bromphenol blue; T, top of gel.

crude soluble extract and passage through a single chromatographic column of Bio-Rex 70. Although functional studies in transcription have never required large amounts of ρ, purification has not been trivial. Structural studies that require large amounts of material and very high concentrations, such as spectroscopy, nuclear magnetic resonance, and x-ray crystallography, are now feasible. In concert with continued genetic and biochemical investigations of ρ function, these efforts should lead to an enhanced understanding of the nature of ρ-dependent transcription termination.

Note Added in Proof. After this work was submitted, Shigesada *et al.* (37) also reported overproduction of ρ protein.

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