Functional Expression and Properties of the tRNA<sub>Lys</sub>-specific Core Anticodon Nuclease Encoded by *Escherichia coli* prrC*

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*Escherichia coli* carrying the optional locus prr harbor a latent, tRNA<sub>Lys</sub>-specific anticodon nuclease, activated by the product of phage T4 *stp* gene. Anticodon nuclease latency is ascribed to the masking of prrC, implicated with the enzymatic activity, by flanking, type Ie DNA restriction modification genes (prrA, prrB, prrD, and hsd). Overexpression of plasmid-borne prrC elicited anticodon nuclease activity in uninfected *E. coli*. In vitro, the prrC-coded core activity was indifferent to a synthetic Stp polypeptide, GTP, ATP, and endogenous DNA, effectors that synergistically activate the latent enzyme. Several facts suggest that PrrC is highly labile in the absence of the masking proteins. The core activity decayed with time, below 1 min at 30 °C, and the PrrC portion of a fusion protein was unstable. Moreover, expression of prrC from its own promoter at low plasmid copy number did not allow detection of core activity. Yet, it sufficed for establishment of a latent, T4-inducible enzyme when complemented by the masking Hsd proteins, which were provided by another replicon. Interaction between the antagonistic components of latent anticodon nuclease was also demonstrated immunologically. The coupling of anticodon nuclease with a DNA restriction modification system may serve to ward off its inadvertent toxicity and maintain it as an antiviral contingency.

Phage T4-encoded 3'-phosphatase-polyribonucleotide kinase (encoded by *pseT/pnk*) and RNA ligase (*g63/rlt*) are nesessential for T4 growth on *Escherichia coli* laboratory strains except those restricting T4 *pnk* and/or *rlt* mutants (1-3). The optional locus responsible for restriction, prr (*pnk* and *rlt* restriction), has been mapped on a restrictive host chromosome and transduced into other *E. coli* strains (4). prr restriction is suppressed by mutations in T4 *stp* (1-3).

Mechanisms underlying prr and *stp* activities began to unfold with the discovery of anticodon nuclease (ACNase),¹ whose activity is manifested in T4-infected *E. coli prr* cells (5-7). ACNase cleaves the host tRNA<sub>Lys</sub> near the wobble base, yielding 2′:3′-cyclic P and 5′-OH termini (8). The damaged tRNA<sub>Lys</sub> is normally resuscitated in subsequent repair reactions. Polynucleotide kinase hydrolyzes the 2′:3′-cyclic phosphodiester as well as the resulting 3′-phosphomonoester. It also phosphorylates the 5′-OH end. RNA ligase joins the 3′-OH and 5′-P termini thus formed. However, in T4 *pnk* or *rlt* infections, cleaved intermediates accumulate, and intact tRNA<sub>Lys</sub> is depleted (6, 8). Loss of tRNA<sub>Lys</sub> in the absence of *pnk* and *rlt*-mediated repair can account for cessation of late T4 translation and other lesions seen in the abortive infections (1-3). Suppression of *prr* restriction by *stp* lesions is attributed to a failure to activate ACNase (7).

Molecular cloning and mutational analyses have revealed that prr comprises positive and negative ACNase functions. prrC is implicated with core ACNase activity whereas the flanking genes, prrA and prrD and perhaps also prrB mask prrC's activity (9). Except for a GTP binding motif (to be discussed later), the primary sequence of PrrC resembles no known protein. In contrast, prrA and B&D are homologous with plasmid-borne *hsd* genes encoding type Ic restriction modification systems such as *EcoR124* (10). Genetic linkage of prr with such a system has been suggested previously (4). More recently latent ACNase has been precipitated by antibodies specific to the *EcoR124* enzyme (11) and prr has been shown to encode type Ic restriction modification activity.¹ These facts indicate that Hsd proteins assumed an added role of ACNase masking.

A polypeptide encoded by *stp* (12) may alleviate the masking of PrrC during T4 infection. This conclusion is inferred from activation of ACNase in vitro, by synthetic polypeptides of the deduced Stp sequence (11, 13), or in vivo, by expression of *stp* from a plasmid in uninfected *E. coli* prr+ cells.¹ However, participation of Stp in tRNA<sub>Lys</sub> cleavage as an activator or cofactor of core ACNase has not been excluded.

Here we show that transcriptional induction of prrC in the absence of other prr genes causes translation of the PrrC polypeptide and elicits core ACNase activity. The advent of an *in vitro* core activity permitted us to distinguish between requirements of ACNase activation and tRNA<sub>Lys</sub> cleavage. We also show that PrrC is exceedingly labile in the absence of the cognate Hsd proteins.

**EXPERIMENTAL PROCEDURES**

**Materials**—Synthetic Stp (residues 2-29) was ordered from Multiple Peptide Systems and purified by high pressure liquid chromatography as previously described (13). Rabbit anti-*EcoR124* serum was kindly provided by Dr. Thomas A. Bickle, Biozentrum. Protease inhibitors and immunological reagents were purchased from Sigma. The enhanced chemiluminescence kit and radioactive materials were purchased from Amersham Corp.

**Strains**—Properties and sources of bacterial strains, phage, plasmids, and cosmids used are listed in Table I.

**Plasmid Constructs**—The *prrC* expression plasmid pRRC6 (Fig. 1)²

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¹ The abbreviations used are: ACNase, anticodon nuclease; PCR, polynucleotide chain reaction; GST, glutathione S-transferase; bp, base pair(s); ORF, open reading frame.

² C. Tandy and T. Bickle, personal communication.

¹ I. Morad, M. Penner, and G. Kaufmann, unpublished results.
was derived from the prr insert of plasmid pRR39 (9) using PCR. To place the probable prrC transcript under the T7 promoter, an EcoRI site was created near the theoretical transcription start site, within PCR oligonucleotide 1, ATCGAATTCTGCTATTTAGTTTCCC. A 1.3-kilobase fragment bordered by it and the reverse PCR oligonucleotide 2, CGGTGAACTTCCAGTCT, was amplified. The upstream 196-bp EcoRI-Xmal portion of the product was cloned into plasmid pBluescript SK+ (Stratagene), and its sequence was verified.

The downstream portion of prrC was provided by the XmaI-HpaI fragment from pRRC5 was excised in two steps, first by NdeI cleavage followed by blunting with Klenow polymerase and further cleavage at the ClaI site. The resulting fragment was moved into the corresponding sites of vector pT7-5 to generate pRRC6.

A glutathione S-transferase-prrC fusion was constructed from (i) a prrC containing fragment, (ii) the gst gene, and (iii) a derivative of the expression vector pKK223-3 (Pharmacia LKB Biotechnology Inc.). To fuse the gst and prrC genes, an Ndel site was created at the ATG start codon of prrC in PCR oligonucleotide 3, GGCATCATTGGCAAGACA. A 1.27-kilobase fragment bordered by it and the reverse PCR oligonucleotide 2 was amplified. The upstream 152-bp NdeI-XmaI portion of the product was cloned into the NdeI-XmaI plasmid pBluescript SK' (Stratagene), and its sequence was verified. The downstream portion of prrC was provided by the XmaI-HpaI fragment spanning bp 1801-2968 of the partial prrC insert of plasmid pRR39 (9) and abutted by a 5-bp Smal-BamHI fragment from the pT7-7, -6, and -5 sites, respectively. The combined EcoRI-BamHI fragment was moved into the corresponding sites of vector pACYC184 (18 copies/cell chromosome) (14). pGC1 was generated by moving the prrC insert of pRR10 into the low copy plasmid pGB2 (3-5 copies/cell chromosome) (15), using flanking EcoRI and Sall sites. Inactivation of prrC within the pior locus of cosmid pW16 (9) was achieved by homologous recombination, using a pRR39 derivative in which a BclI fragment of prrC was replaced by a tetracycline resistance cassette derived from plasmid pKK226 (16). This yielded cosmid pW18prrCtet.

**Expression of Anticodon Nuclease Core Gene prrC**

| Strain | Genotype and relevant phenotype | Source |
|-------|---------------------------------|--------|
| E. coli strains | HprC, recA1, lac-pro, endA1, gyrA1, thi-1, hsdR17, supE44, relA1, lacF1, traD36, proAB1, lacI1, ZM15 | S. Tabor |
| JM109 | A. Oppenheim |
| A5039 | Ref. 14 |
| A5039pemB | Ref. 28 |
| 1046 | Ref. 29 |
| XL1-Blue | Ref. 30 |
| C600 | Ref. 31 |
| CTr6X | Ref. 32 |
| Phase T4 strains | pseT7Δ1 | Ref. 33 |
| Cosmids | pW16 | Ref. 34 |
| pW16prrCtet | pW16prrCtet | This work |
| Plasmids | pT7-5 | T7 expression plasmid |
| pT7-6 | T7 expression plasmid |
| pT7-7 | T7 expression plasmid |
| pGP1-2 | T7 RNA polymerase expression plasmid |
| pRR39 | Multicopy partial prr plasmid |
| pRR5C | prrC-core ACNase expression plasmid |
| pRR6C | prrC-core ACNase expression plasmid |
| pGBP | Low copy number vector |
| pACYC184 | Medium copy number vector |
| Bluescript SK+ | High copy number vector |
| pGBC1 | Low copy prrC-plasmid |
| pACC1 | Medium copy prrC-plasmid |
| pPR10 | High copy prrC-plasmid |
| pGC1 | GST-PrrC fusion protein plasmid |
| pGC2 | GST-PrrC fusion protein plasmid |

**Table I**

| Strain | Genotype and relevant phenotype | Source |
|-------|---------------------------------|--------|
| E. coli strains | HprC, recA1, lac-pro, endA1, gyrA1, thi-1, hsdR17, supE44, relA1, lacF1, traD36, proAB1, lacI1, ZM15 | S. Tabor |
| JM109 | A. Oppenheim |
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| Phase T4 strains | pseT7Δ1 | Ref. 33 |
| Cosmids | pW16 | Ref. 34 |
| pW16prrCtet | pW16prrCtet | This work |
| Plasmids | pT7-5 | T7 expression plasmid |
| pT7-6 | T7 expression plasmid |
| pT7-7 | T7 expression plasmid |
| pGP1-2 | T7 RNA polymerase expression plasmid |
| pRR39 | Multicopy partial prr plasmid |
| pRR5C | prrC-core ACNase expression plasmid |
| pRR6C | prrC-core ACNase expression plasmid |
| pGBP | Low copy number vector |
| pACYC184 | Medium copy number vector |
| Bluescript SK+ | High copy number vector |
| pGBC1 | Low copy prrC-plasmid |
| pACC1 | Medium copy prrC-plasmid |
| pPR10 | High copy prrC-plasmid |
| pGC1 | GST-PrrC fusion protein plasmid |
| pGC2 | GST-PrrC fusion protein plasmid |

Anticodon Nuclease Assays—To monitor ACNase in vivo, E. coli cells were pulse-labeled with [32P]P. Low molecular weight RNA was extracted, before infection or at indicated times after infection, with T4 pseT7Δ1 (> prp<). The RNA was separated on denaturing polyacrylamide-urea gel, as previously described (5). The in vivo ACNase assay is based on cleavage of RNA labeled with [32P] in the cleavage-attachment junction. This generates labeled fragment 1-33 carrying a cyclic phosphate end group. The preparation of the substrate was described before (13). Crude core ACNase was derived from thermolysin-induced E. coli K38gpGpG1-2-ppRRC6 cells as follows. All operations were carried out between 0 and 4 °C. The cells were grown in LB medium at 37 °C and harvested at Ano of 1.2. Packed cells (2 g) were washed once in buffer A (0.5 M NaC1, 50 mM Tris-HCl buffer, pH 8.0, 10 mM EDTA, 5 mM β-mercaptoethanol, 10% glycerol, and 10% sucrose) and twice in buffer B (similar to buffer A but containing only 10 mM NaC1). The washed cells were suspended in 2 ml of buffer B containing 0.2 mM diisopropyl fluorophosphate, 10 μg/ml aprotinin, 50 μg/ml leupeptin, and 3.5 mM phenylmethylsulfonyl fluoride. The suspension was passed twice in an Amino pressure cell at 18,000 p.s.i. The cell lysate was centrifuged for 30 min at 20,000 × g, and the supernatant (S-30) was further centrifuged for 4 h at 45,000 rpm
in a 50T1 Beckman rotor. The soluble fraction (S-150) was used as source of enzyme. The standard anticodon nuclease reaction mixture (10 μl) contained 5 μl of core ACNase (the S-150 fraction diluted 2–10-fold in buffer B, as indicated), 5,000 cpm of the 32P-labeled tRNA145 36S (3000 Ci/mmol), 25 mM Tris-HCl (pH 8.0), 5 mM NaCl, 10 mM MgCl2, 5 mM β-mercaptoethanol, 5 mM EDTA, 5% glycerol, and 5% sucrose. It should be noted that the reaction mixtures contained about 10–100 ng of endogenous tRNA145 partly cleaved in vivo. The standard incubation temperature was 10 °C. The reaction was stopped by precipitating the RNA with ethanol. The pellet was washed with 70% ethanol and dissolved in 10 μg/ml RNase A in 7 M urea, 25 mM Tris-borate buffer (pH 8.3), 2.5 mM EDTA. Following autoradiography, intact tRNA and fragment 1–33 bands were excised from the gel and counted in liquid scintillation fluid. Activity is expressed as percent tRNA145 32P label converted into amide in 7 M urea, 25 mM Tris-HCl (pH 8.0), 5 mM NaCl, 10 mM MgCl2, 5 mM β-mercaptoethanol, 5 mM EDTA. An aliquot was separated by electrophoresis on 15% polyacrylamide gel and analyzed by autoradiography. Several prototypic cleavage products of tRNA145 were excised from the gel and sequenced by automated Edman degradation. The 29- and 36-mer fragments were confirmed to be the S150 products described by Smith and Johnson (20) and by purification of the GST-PrrC fusion protein, and GST itself (26 kDa; lane 2). The 45-mer fragment of intermediate size probably arose by proteolysis of the labile PrrC portion of the fusion protein. Because the quantity of the intact fusion protein was very low, we have used for immunization a degraded form that comprises GST fused to about 30-40 N-terminal residues of PrrC (GST-PrrCN). The band containing it was excised from the gel and injected subcutaneously into rabbits. The resulting antiserum was used for immunological detection of free PrrC and the PrrC fusion protein, and GST itself (26 kDa; lane 2). Products of intermediate size probably arose by proteolysis of the labile PrrC portion of the fusion protein. Because the quantity of the intact fusion protein was very low, we have used for immunization a degraded form that comprises GST fused to about 30-40 N-terminal residues of PrrC (GST-PrrCN). The band containing it was excised from the gel and injected subcutaneously into rabbits. The resultant antiserum was used for immunological detection of free PrrC and the PrrC Hsd complex (Fig. 9). Immunization, production of polyclonal antiserum, immunoprecipitation, and Western blotting were carried out as described by Harlow and Lane (17). Detection of secondary antibodies by enhanced chemiluminescence was according to the manufacturer's instructions.

Expression Systems—Thermoinduction and exclusive labeling of the overexpressed protein in the T7 promoter/promoter system were carried out as described by Tabor and Richardson (18). Overexpression and purification of the GST-PrrC fusion protein were carried out as described by Smith and Johnson (19).

**RESULTS**

Induction of prrC Elicits ACNase Activity in Uninfected E. coli—To examine the function of the prrC ORF we investigated the consequences of its transcriptional induction in the absence of other prr genes (prrB background). prrC was placed under control of the T7 promoter in plasmid pRR6 (Fig. 1 and "Experimental Procedures"). It was expressed in the T7 polymerase/promoter system in which transcription of target genes is turned on by thermoinduction of T7 RNA polymerase from the coresident plasmid pGP1-2 (18). The induction elicited ACNase activity, indicated by appearance of typical cleavage products migrating with tRNA145 fragments 34–76 and 1–33 (Fig. 2, lane 5). Such fragments are seen in T4...
pseTΔ1 (pnk)-infected E. coli prr* cells (Fig. 2, lane 1). Manifestation of ACNase activity in the uninfected transformant cells depended on the prrC insert and thermostimulation of T7 RNA polymerase. Cells containing the vector plasmid polyacrylamide gel electrophoresis to monitor the polypeptide manifested times in reaction mixtures containing S-150 fractions of vector plasmid served as a control. This analysis revealed a over the prro background elicits concomitant appearance of a PrrC-like polypeptide and overt ACNase activity.

In Vitro Core ACNase Activity—Core ACNase activity was assayed in vitro, in S-150 fractions derived from the thermoinduced K38 gpGPl-2prRRC6 transformants. It should be noted that the crude S-150 fraction used in this study contained an undetermined quantity of endogenous tRNALy8 and tRNALys fragments generated by ACNase cleavage in vivo. Instability of the core activity (see below) impeded further purification of the enzyme. The endogenous tRNALys was mixed with tracers amounts of [32P]tRNALy8 labeled in the cleavage-ligation junction. ACNase generates from this substrate fragment 1–33 as the only labeled product (13). Due to uncertainties about the effective concentration of the substrate, the activity of core ACNase was expressed as percent tRNALys radioactivity converted into fragment 1–33.

**Table II**

| Reaction mixture | Extent of tRNALys cleavage | Activity of standard reaction mixture |
|------------------|-----------------------------|--------------------------------------|
| Experiment 1     |                             |                                      |
| Standard         | 0.21                        | 100                                  |
| +ATP             | 0.18                        | 86                                   |
| +GTP             | 0.20                        | 95                                   |
| +[ATP, GTP]      | 0.13                        | 62                                   |
| +Stp             | 0.21                        | 100                                  |
| +[ATP, GTP, Stp] | 0.21                        | 100                                  |

**Table I.**

| Preincubation time (min.) | Extent of tRNALys cleavage | Activity of standard reaction mixture |
|---------------------------|----------------------------|--------------------------------------|
| 0                          | 0.21                       | 100                                  |
| 20                         | 0.20                       | 95                                   |
| 30                         | 0.18                       | 86                                   |
| 60                         | 0.13                       | 62                                   |
| 120                        | 0.21                       | 100                                  |

The extent of cleavage under standard conditions was less than half of the maximum attained between 30–60 min, thus simulating agents were expected to increase it.

The DNase I treatment was performed by preincubating the S-150 fraction with 20 units/ml of RNase-free DNase I (Strategene) for 30 min at 0 °C. In this experiment the standard mixture was preincubated similarly but with buffer devoid of DNase I.

**Fig. 5. Temperature dependence and thermal stability of core ACNase.** Core ACNase was assayed at the indicated temperatures, essentially as described in Fig. 4, except that the enzyme was diluted 1:5 in buffer B. Core ACNase was preincubated at 30 °C in the absence of radioactive substrate. Aliquots withdrawn at the indicated times were assayed at 10 °C in the presence of the radioactive substrate.

**Agents that activate latent ACNase do not stimulate the core enzyme**

The S-150 fraction from thermostimulated K38 gpGPl-2prRRC6, containing core ACNase, was diluted 5-fold in buffer B and was assayed for 10 min as described in the legend to Fig. 4.

The specific activity of cleavage was ascertained by hydrolysis of the labeled 2'-3'-cyclic phosphate end group of the product in a reaction catalyzed by 3'-phosphatase-polynucleotide kinase (not shown). Incubation of core ACNase above 10 °C resulted in fast decay of the activity, in a lower extent of tRNALys cleavage and degradation of the reaction product (Fig. 5A). To determine the stability of the core enzyme it was preincubated at 30 °C in the absence of the radioactive substrate, which was added at the onset of the assay at 10 °C. The preincubation
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Core ACNase Is Not Stimulated by Activators of Latent ACNase—ACNase activity in extracts of prr" cells is increased manifold by adding a synthetic Stp polypeptide, ATP, and GTP, while removal of the endogenous DNA abolishes the activity (11). In contrast, the requirements of the prrC-encoded core ACNase were simple. Optimal core activity was obtained in a buffer-salt solution at pH 8.0 and 5-10 mM Mg²⁺. However, about one-quarter of the activity was retained without added Mg²⁺ and with 5 mM EDTA (data not shown). Core ACNase was not stimulated by addition of 2 mM ATP, 0.1 mM GTP, both nucleotides, 33 μM synthetic Stp, or by a combination of all three components (Table II). In contrast, omission of any of these components from the latent enzyme assay mixture drastically reduces ACNase activity (11). Moreover, digestion of endogenous DNA with DNase I did not reduce core ACNase activity (Table II), whereas the same treatment abolishes activation of the latent enzyme (11).

Effect of prrC Gene Dosage on Core ACNase Activity—The prrC ORF is preceded by a sequence resembling the E. coli σ⁷⁰ consensus promoter (9). When prrC was placed in plasmids under control of this sequence and expressed over the prr⁶ background, ACNase activity was seen only at medium to high prrC gene dosage. Three clones containing the same prrC insert embedded in different vectors were compared. pGBC1 was derived from the low copy plasmid pGB2 (3 copies/cell chromosome) (15), pACC1 from pACYC184 (18 copies) (14), and pRR10 from pBluescript KS⁺ (~200 copies) (22). Only pRR10 and pACC1 elicited detectable ACNase activity (Fig. 6A, compare lanes 1 with 2 and 3). When pRR10 was moved to a pcnB⁻ host background that reduces the copy number as much as 15-fold (23), core ACNase activity was abolished (Fig. 6B, compare lanes 4 and 5). However, pRR10 rescued from the pcnB⁻ cells expressed ACNase activity when moved back to a pcnB⁺ background (not shown). If the activity of the indigenous prrC promoter was not altered by vector sequences or by the pcnB lesion (not shown). If the activity of the indigenous prrC promoter was not altered by vector sequences or by the pcnB lesion (not shown). These results are indicative of T4 infection of E. coli irrespective of prr (5).
in prrC due to replacement of an internal BclI fragment with a tetracycline resistance cassette. As shown in Fig. 7, the original cosmid pW16 expressed ACNase activity after T4 infection (lanes 1–3). Cells containing pW16prrC::tet lacked it (lanes 4–6). The combination of pGBC1 and pW16prrC::tet restored a weak but significant level of latent ACNase (lanes 7–9). Namely, tRNA<sup>Lys<sub>1-33</sub></sup> fragments appeared in the double transformants during infection later than fragments of tRNA<sup>Lys<sub>1-48</sub></sup> that characterize T4-infected E. coli, irrespective of prr (5). The reconstitution of latent ACNase was due to complementation in trans rather than restoration of the cosmid's prrC gene by homologous recombination. This conclusion is based on the (i) unaltered restriction pattern of the cosmid and plasmid rescued from the double transformants, (ii) similar level of latent ACNase activity seen with six independent double transformants, and (iii) absence of kan<sup>R</sup>,tet<sup>R</sup> clones among 120 progeny cosmids rescued from three independent double transformants. The reconstitution of latent ACNase by pGBC1/pW16prrC::tet complementation is consistent with a positive effect of the masking genes over prrC's activity, either by augmenting prrC expression or by stabilizing its product(s) or both.

**PrrC Interacts with Hsd Proteins**—Latent ACNase can be precipitated by anti-EcoR124/3 antibodies that cross-react with the homologous masking components of Prr (11). However, the presence of PrrC in this complex has not been investigated. To address this issue we prepared PrrC-specific antibodies. The antigen employed for this purpose was a glutathione S-transferase-PrrC fusion protein (see “Experimental Procedures”). However, only trace amounts of the full-sized 71-kDa fusion protein could be recovered (Fig. 8, lane 1), probably due to instability of the PrrC portion of the fusion protein. An abundant, partial degradation product estimated to contain the N-terminal 30–40 residues of PrrC (GST-PrrC<sup>N</sup>) was used instead.

Immunoprecipitation followed by Western analysis, both performed with GST-PrrC<sup>N</sup> rabbit antiserum, revealed PrrC as a specific band in the thermo-induced, pRRC6-containing cells expressing the core ACNase (Fig. 9A, lane 2, the upper band in the faster migrating doublet). This band was not seen with an extract of control cells containing the vector plasmid pT7-5 (lane 1) or when the extract of the pRRC6-containing cells was treated with preimmune serum (lane 3). This confirmed the existence of the PrrC polypeptide in the core ACNase-expressing cells. A similar analysis revealed a specific band of PrrC in cells transformed with cosmids pW16 expressing the latent ACNase holoenzyme (lane 5) but not in control cells lacking the cosmid (lane 4) or after immunoprecipitation of the holoenzyme-containing extract with non-specific serum (lane 6).

When immunoprecipitations were conducted with the EcoR124/3-specific antiserum and followed by Western analysis with PrrC-specific antiserum, a band corresponding to PrrC was detected in cells expressing the ACNase holoenzyme (Fig. 9A, compare lanes 7 and 8). Control prr<sup>C</sup> cells (lane 9) and cells expressing the prr<sup>C</sup>-encoded core enzyme (lane 10) did not feature this band. Thus, PrrC was precipitated indirectly by the anti-Hsd antibodies, probably by virtue of a physical interaction with the Hsd components of ACNase holoenzyme. Conversely, immunoprecipitation of the holoenzyme with PrrC-specific antiserum followed by Western analysis with EcoR124/3-specific serum detected the masking factor PrrD as a faint but significant band over the background (Fig. 9B, compare lanes 11 and 12). PrrD was lighted up more intensely when the anti-EcoR124/3 antibodies were used both for immunoprecipitation and immunoblotting. Under these conditions a PrrA band was detected as well (compare lanes 14 and 15). These bands were not seen when the extract of holoenzyme-containing cells was treated with preimmune serum (lane 13). The absence of a PrrB band is attributed to the weak homology of PrrB and the EcoR124/3-HsdS counterpart (10) and to the low immunogenicity of the latter (24).

**DISCUSSION**

Is PrrC the Core Anticodon Nuclease Enzyme?—Mutational analysis implicated prr<sup>C</sup> with core ACNase activity (9). This assumption was confirmed by the coincident appearance of the PrrC polypeptide and core ACNase activity upon transcriptional induction of prrC (Figs. 2, 3, and 9). Catalysis of the ACNase reaction by the prr<sup>C</sup> transcript itself is improbable because small C-terminal or internal deletions in the ORF inactivate core ACNase (9).<sup>4</sup> We conclude that the 45-kDa PrrC polypeptide or a derivative is needed for core ACNase activity. However, whether PrrC suffices for core ACNase activity or constitutes only a subunit or auxiliary factor is not known. The pursuit of an answer to this question was impeded by the unusual lability of core ACNase (Figs. 5 and 6).

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<sup>4</sup> D. Chapman-Shimshoni, unpublished results.
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| E. coli strain | prrC status | PrrC | Pre-immune | PrrC | Pre-immune | EcoR124/3 |
|----------------|-------------|------|------------|------|------------|-----------|
| K38-pGP1-2-pT7-5 | +           | +    | +          | +    | +          | +         |
| K38-pGP1-2-pRRC6 | -           | -    | -          | -    | -          | -         |
| K38-pGP1-2-prC6   | +           | +    | +          | +    | +          | +         |
| C600              | +           | +    | +          | +    | +          | +         |
| C600/pW16         | +           | +    | +          | +    | +          | +         |
| C600/pW16         | +           | +    | +          | +    | +          | +         |
| K38-pGP1-2-pT7-5  | +           | +    | +          | +    | +          | +         |
| K38-pGP1-2-prC6   | +           | +    | +          | +    | +          | +         |

**FIG. 9. Immunoprecipitation and Western blotting of core ACNase and ACNase holoenzyme.** S-150 fractions from thermo-induced E. coli K38(pGP1-2-pT7-5) (lanes 1 and 9), K38(pGP1-2-pRRC6) (lanes 2, 3, and 10), or S-30 fractions of strains C600 (lanes 4, 7, 11, and 14) and C600/pW16 (lanes 5, 6, 8, 12, 13, and 15) were treated with PrrC-specific serum (lanes 1, 2, 4, 5, 11, and 12), EcoR124/3-specific antiserum (lanes 7–10, 14, and 15), or preimmune serum (lanes 3, 6, and 13). The immunoprecipitates were separated on SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and analyzed by Western blotting, using as probes the PrrC-specific antiserum (panel A) or EcoR124/3-specific antiserum (panel B).

and 8). Nonetheless, several facts hint that PrrC itself may be the core enzyme. Latent ACNase can be immunoprecipitated by antibodies directed against the Hsd-masking components and then be activated by addition of a prrA-D extract (11), suggesting that all the prr-specific components needed for core activity reside in the immunoprecipitated complex. That PrrC is included in this complex was demonstrated immunologically, using Hsd- and PrrC-specific antibodies (Fig. 9). The probability that another core ACNase component exists in the complex, beside PrrC, is deemed low, because immunoprecipitations of the ACNase holoenzyme with anti-Hsd antibodies failed to detect specific bands of metabolically labeled proteins migrating differently from PrrA-D. Still, PrrC could act upon a common (non-prr) E. coli gene product that is needed for ACNase activity. Against this possibility argues the ability to elicit apparent ACNase activity in human HeLa cells by infection with a recombinant prrC-vaccinia virus. Thus, PrrC may suffice to elicit ACNase activity in the heterologous environment.

Separation of Anticodon Nuclease Activation and tRNA<sup>Lys</sup> Cleavage—A number of cofactors are needed to elicit ACNase activity with the latent holoenzyme, including a synthetic

6 J. Morad, D. Chapman-Shinshoi, M. Amitur, and G. Kaufmann, unpublished results.
6 N. Shterman, O. Elroy-Stein, I. Morad, M. Amitur, and G. Kaufmann, unpublished results.
Expression of Anticodon Nuclease Core Gene prrC

The molecular basis of PrrC's instability is not known, it is conceivable that this property evolved to safeguard ACNase latency. That is, free PrrC may be promptly removed to ward off inadvertent toxicity. However, shielded by the Hsd subunits, it may be kept as an antiviral contingency.

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Fig. 10. A GTP binding motif in PrrC. Residues matching the tripartite consensus GTP binding motif, [A/G]XXX[R/Y][T/S]Xe-N[D/E][K/R]Xe-NKXD (26), within the N-proximal half of PrrC are highlighted by bold type. Invariant residues are double underlined. Deviations from invariance are underlined.

Stp-like polypeptide, ATP, GTP, and endogenous DNA (11, 13). Because these ATPs did not stimulate the prrC-coded core ACNase (Table II), they probably alleviate the masking effect. ATP and DNA, known Hsd-ligands (25), could alter the conformation of Hsd subunits of the latent enzyme (11) and thus unleash the core. The role of GTP is hinted at by a GTP binding motif, albeit imperfect, present in the deduced sequence of PrrC (Fig. 10). Such a motif is found in a diverse array of proteins that are involved in signal transduction and translation, using GTP in conjunction with protein-protein dissociation-association cycles (26). Because GTP has no effect on core ACNase (Table II), the GTP binding motif of PrrC could portend roles in ACNase latency and activation, interfacing the Hsd components of the holoenzyme. By default, a carboxyl domain of PrrC could harbor the tRNA β recognition and cleavage activities. These possibilities can be addressed by mutational analysis aiming to associate PrrC sequences with ACNase functions.

Significance of PrrC's Instability—Core ACNase activity decayed at 30 °C with tₙₐₗ less than 1 min (Fig. 5). The PrrC polypeptide seemed likewise unstable because it was preferentially degraded in a fusion protein (Fig. 8). In contrast, the ACNase holoenzyme is relatively stable both in vitro and in vivo. The in vivo stability is indicated by delayed early schedule of the induction of ACNase activity during T4 infection and its persistence throughout infection (5, 7). Yet, translation of host mRNAs ceases abruptly at the onset of infection (27). Hence, a preexisting ACNase must sustain this time interval. Further credence to a stabilizing effect the Hsd proteins exert over PrrC was lent by reconstitution of latent ACNase from its separate components. The low copy prrC plasmid used in this experiment did not elicit detectable core activity, neither before nor after T4 infection (Fig. 6). Nevertheless, it furnished sufficient PrrC to establish latent ACNase when Hsd components were provided in trans (Fig. 7). A simple explanation of this result is that PrrC is labile in the absence of the cognate masking proteins, which normally interact with it in latent ACNase. In fact, this interaction is indicated by the immunochemical analyses (Fig. 9). An alternative explanation, that prrA,B,D stimulate prrC transcription and/or translation, is refuted by a high level of prrC mRNA expressed from the low copy plasmid pGBC1, relative to prrC-mRNA transcribed from the chromosomal or cosmidsborne prr locus. Furthermore, other prr genes fail to augment the reporter activity of prrC-lacZ gene fusion. Stabilization of PrrC by the masking elements could qualify them as effector subunits of ACNase if the PrrC-Hsd interaction sustains the activation, an issue not addressed yet. Although