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Genetics

Increased expression of a eukaryotic gene in *Escherichia coli* through stabilization of its messenger RNA

(*Neurospora crassa*/catabolic dehydroquinase/pVK88/polynucleotide phosphorylase)

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ABSTRACT **The expression of a cloned eukaryotic gene [catabolic dehydroquinase (3-dehydroquinate hydro-lyase, EC 4.2.1.10) (*qa-2*$^+$) from *Neurospora crassa*] is dramatically increased (as much as 100-fold) in *Escherichia coli* strains deficient in polynucleotide phosphorylase (*pnp*) (polynucleotide: orthophosphate nucleotidyltransferase, EC 2.7.7.8) and RNase I (*rna*). The increased expression is controlled primarily by the absence of polynucleotide phosphorylase and appears to be specific for the eukaryotic gene. No increase in the specific activity of either chromosomal or plasmid-borne prokaryotic genes has been observed. In polynucleotide phosphorylase-deficient strains of *E. coli* the half-life of plasmid (pVK88, *amp*^{r} *qa-2*$^+$)-encoded mRNAs increases from 1.0 to 2.8 min. This increase must be due primarily to stabilization of the *qa-2* mRNA because no increase in the half-lives of pBR322 vehicle mRNAs was observed in polynucleotide phosphorylase-deficient strains. These results suggest that there are inherent structural differences between prokaryotic and eukaryotic mRNAs.**

Recently, structural genes from various eukaryotic sources have been shown to be functionally expressed in *Escherichia coli* (1–7). In the case of the catabolic dehydroquinase (3-dehydroquinate hydro-lyase, EC 4.2.1.10) gene (*qa-2*) from *Neurospora crassa*, transcription is efficiently initiated from a promotor within the cloned eukaryotic DNA (8). Moreover, the resulting mRNA is translated to yield a protein identical by various biochemical and immunological criteria to authentic *N. crassa* enzyme (1). Because the *N. crassa* catabolic dehydroquinase activity can be readily quantitated in crude extracts and easily distinguished from the *E. coli* isozyme (1), the *qa-2* gene is an excellent probe for investigating factors that regulate the expression of eukaryotic genes in *E. coli*. Of particular interest are host modifications that lead to increased expression of eukaryotic genes either by enhancing the efficiency of transcription or translation or by increasing the stability of the eukaryotic mRNA.

In this communication it is shown that the *qa-2* mRNA synthesized in *E. coli* strains lacking the enzyme polynucleotide phosphorylase (polynucleotide:orthophosphate nucleotidyltransferase, EC 2.7.7.8) is significantly stabilized. This message stabilization results in a 20- to 100-fold increase in the specific activity of *N. crassa* catabolic dehydroquinase. The effect appears to be unique for the eukaryotic message, because the mRNAs for the pBR322-encoded Ap^r and Tc^r proteins are not stabilized in these mutants nor is the level of expression of the *E. coli* biosynthetic dehydroquinase increased.

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MATERIALS AND METHODS

Materials. Reagents were obtained from the following sources: CsCl (high purity), Penn Rare Metals; Sarkosyl NL-97, Geigy Chemical Co.; T1 ribonuclease, Worthington; agarose, Seakem Laboratories; ethidium bromide, Calbiochem; lysozyme, chloramphenicol, tetracycline, rifampicin, 2-deoxyadenosine, and RNase type 11A, Sigma; ampicillin (Omnipen), Wyeth; Sepharose 4B, Pharmacia; [5,6-^{3}H]uridine (40–50 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) and [6-^{3}H]thymidine (15 Ci/mmol), New England Nuclear.

Bacterial Strains and Media. The genotypes of the bacterial strains are described in Table 1. Plasmid pVK88 contains the intact catabolic dehydroquinase gene (*qa-2*) from *N. crassa* and has been described (1, 8). pDV604 was graciously supplied by N. K. Alton and D. Vapnek. Plasmids were transformed into various recipients by using the method of Kushner (9). Cells were grown in Luria broth (10) or K medium (11), supplemented with required amino acids at a concentration of 50 μg/ml. Cell cultures used for enzyme assays, extraction of RNA, and determination of plasmid copy number were grown in K medium with only 0.1% casamino acids.

Nucleic Acid Procedures. Plasmid DNA was purified according to the method of Alton and Vapnek (12). Isolation of RNA and hybridization of 3-min pulse-labeled [^{3}H]RNA to increasing concentrations of DNA immobilized on nitrocellulose filters were performed as described by Davis and Vapnek (13).

mRNA half-lives were determined by an experimental protocol modeled after Gupta and Schlessinger (14). A 10-ml culture grown at 37°C (10^8 cells per ml) was labeled for 1 min with 0.5 mCi of [^{3}H]uridine and then treated with rifampicin (300 μg/ml) and nalidixic acid (20 μg/ml). After 1 additional min cold uridine (200 μg/ml) was added. The addition of [^{3}H]uridine was taken as zero time and 1.0-ml aliquots were removed at subsequent intervals. Cell lysis, RNA isolation, and hybridization to saturating amounts of DNA on nitrocellulose filters were performed as described by Davis and Vapnek (13).

Plasmid copy numbers were determined by bouyant density equilibrium centrifugation according to the method of Clewell (15). A 10-ml culture was labeled for one doubling time (60–120 Klett units, no. 42 green filter) with 0.5 mCi of [^{3}H]thymidine in the presence of 100 μg of 2-deoxyadenosine per ml. The cell pellet was washed three times with 10 ml of K medium prior to cell lysis. Centrifugation was performed by using 1.0 ml of cleared cell lysate, 7.0 ml of buffer (50 mM Tris·HCl, pH 8.0/5 mM EDTA/50 mM NaCl) containing 3 mg of ethidium bromide, and 7.6 g of cesium chloride.

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Table 1. Bacterial strains

Strain	*arg*	*his*	*leu*	*metB*	*rna*	*pnp*	*aroD*	Other markers	Source
JC1557	*G6*	*1*	*7*	*1*	+	+	+		A. J. Clark
KA604	+	+	−	+	+	+	+	pDV604	D. Vapnek
N7060	+	+	+	*1*	*19*	*13*	+	*rnb-464 tryA451*	D. Apirion
SK107	+	+	*6*	+	*19*	*7*	+	*thr*	S. Kushner
SK692	+	*1*	*7*	*1*	+	+	+		JC1557 × SK107 ArgG$^+$ transductant
SK694	+	*1*	*7*	*1*	+	*7*	+		JC1557 × SK107 ArgG$^+$ transductant
SK698	+	*1*	*7*	*1*	+	*13*	+		JC1557 × N7060 ArgG$^+$ transductant
SK1520	*E3*	*4*	+	+	+	+	*6*		(1)
SK1572	*E3*	*4*	+	+	+	+	*6*	pVK88	(8)
SK2117	+	+	*6*	+	*19*	*7*	+	*thr* pBR322	This paper
SK2121	+	+	*6*	+	*19*	*7*	+	*thr* pDV604	This paper
SK2124	+	+	*6*	+	*19*	*7*	+	*thr* pVK88	This paper
SK2128	+	*1*	*7*	*1*	+	+	+	pVK88	This paper
SK2129	+	*1*	*7*	*1*	+	*7*	+	pVK88	This paper
SK2132	+	*1*	*7*	*1*	+	*13*	+	pVK88	This paper
SK2193	+	*1*	*7*	*1*	+	+	+	pBR322	This paper
SK2194	+	*1*	*7*	*1*	+	*7*	+	pBR322	This paper
SK2262	+	*1*	*7*	*1*	+	+	+	*tet*^{r}	Tn10 insertion near *argG*
SK2266	*G6*	+	+	+	+	+	+	*endA sbcB15 tet*^{r} *hsrD4*	SK1592 × SK2262 Tet^R transductant
SK2284	+	+	+	+	+	*7*	+	*endA sbcB15 hsrD4*	SK2266 × SK107 ArgG$^+$ Tet^S transductant
SK2299	*G6*	*1*	*7*	+	*19*	+	+		*rna-19* derivative of JC1557
SK3408	+	*1*	*7*	*1*	*19*	+	+	pVK88	This paper
SK3410	+	*1*	*7*	*1*	+	*7*	+	pVK88	This paper
SK3423	+	+	+	+	+	*7*	+	*endA sbcB15 hsrR4* pVK88	This paper
SK3425	+	*1*	*7*	*1*	+	+	+	pVK88	This paper
SK3518	+	*1*	*7*	*1*	+	+	+	pVK88	This paper
SK3520	+	*1*	*7*	*1*	+	+	+	pVK88	This paper
SK3525	+	*1*	*7*	*1*	+	*7*	+	pVK88	This paper
SK3527	+	*1*	*7*	*1*	+	*7*	+	pVK88	This paper

Enzyme Analyses. Strains were grown, lyzed, and assayed for dehydroquinase activity as described (1). One unit of activity represents 1 μmol of dehydroshikimate produced per min per mg of protein at 37°C. The *N. crassa* and *E. coli* dehydroquinase isozymes were differentiated by exploiting the heat stability of the *N. crassa* enzyme (16). Chloramphenicol acetyltransferase (EC 2.3.1.28) activity was assayed as described by Davis and Vapnek (13) and β-lactamase (EC 3.5.2.6) activity was measured colorimetrically by the method of Imsande (17). One unit of chloramphenicol acetyltransferase represents 1 μmol of chloramphenicol acetylated per min per mg of protein and one unit of β-lactamase represents 1 μmol of H^+ produced per min per mg of protein. Protein determinations were by the method of Lowry *et al.* (18). The absence of polynucleotide phosphorylase was determined by a modification of the phosphate exchange assay described by Reiner (19).

RESULTS

***N. crassa* Catabolic Dehydroquinase Activity in Various *E. coli* Hosts.** SK107, a ribonuclease I (*rna-19*)- and polynucleotide phosphorylase (*pnp-7*)-deficient strain of *E. coli*, was transformed with pVK88 (*qa-2*$^+$) plasmid DNA and tetracycline-resistant transformants were selected. As shown in Table 2, SK2124, a representative transformant, exhibited a catabolic dehydroquinase specific activity approximately 100-fold greater than that observed in a pVK88-transformed *rna*$^+$ *pnp*$^+$ control strain (SK2128).

In order to establish whether the absence of ribonuclease I or polynucleotide phosphorylase was responsible for the observed effect, *E. coli* strains carrying either the *rna-19* allele or one of two *pnp* alleles (*pnp-7* or *pnp-13*) were transformed with pVK88 plasmid DNA. The level of catabolic dehydroquinase activity in representative tetracycline-resistant transformants of each strain are shown in Table 2. Strains SK2129 (*pnp-7*) and SK2132 (*pnp-13*) each exhibited a 50-fold enhancement of enzyme activity, whereas the *rna-19* single mutant (SK3408) showed only a 2-fold increase. Moreover, the *pnp-7* allele in a different genetic background exerted a similar effect when the cells were transformed with pVK88 plasmid DNA (strain SK3423). The catabolic dehydroquinase activity observed in all of these strains was shown to be biochemically and immunologically identical to authentic *N. crassa* enzyme.

Specificity of Overexpression. In order to determine if the enhanced expression was specific for the eukaryotic *qa-2* gene, the effect of polynucleotide phosphorylase deficiency on the specific activity of three prokaryotic enzymes was determined. As shown in Table 3, the level of the chromosomally encoded *E. coli* dehydroquinase was unaltered in a *rna-19 pnp-7* strain which showed a 100-fold increase in the *N. crassa* activity. Moreover, the specific activities of two plasmid-encoded prokaryotic enzymes, β-lactamase carried by pBR322 and

Table 2. Levels of catabolic dehydroquinase activity in various strains of *E. coli*

Strain	Genotype	Plasmid	Specific activity*
SK1520	*pnp*$^+$ *rna*$^+$	—	<0.0007
SK107	*pnp-7 rna-19*	—	<0.0007
SK2128	*pnp*$^+$ *rna*$^+$	pVK88	0.029
SK2124	*pnp-7 rna-19*	pVK88	3.34
SK2129	*pnp-7 rna*$^+$	pVK88	1.16
SK2134	*pnp-13 rna*$^+$	pVK88	1.01
SK3408	*pnp*$^+$ *rna-19*	pVK88	0.054
SK3423	*pnp-7 rna*$^+$	pVK88	0.51

* Specific activity of *N. crassa* catabolic dehydroquinase synthesized in *E. coli*; μmol/min per mg of protein.

Table 3. Specific activities of various enzymes in *pnp*$^+$ and *pnp-7* strains of *E. coli*

Strain	Plasmid	Genotype	Specific activity*			
			Dehydroquinase			
			N. crassa	*E. coli*	β-Lactamase	Chloramphenicol acetyltransferase
SK2128	pVK88	*pnp*$^+$ *rna*$^+$	0.029	0.061	—	—
SK2124	pVK88	*pnp-7 rna-19*	3.34	0.051	—	—
SK1571	pBR322	*pnp*$^+$ *rna*$^+$	—	—	71	—
SK2117	pBR322	*pnp-7 rna-19*	—	—	114	—
KA604	pDV604	*pnp*$^+$ *rna*$^+$	—	—	—	123
SK2121	pDV604	*pnp-7 rna-19*	—	—	—	116

* Micromoles/min per mg of protein.

chloramphenicol acetyltransferase carried by the recombinant plasmid pDV604, were only slightly affected by the absence of polynucleotide phosphorylase.

Absence of Plasmid Alterations in Phosphorylase-Deficient Strains. Walz *et al.* (20) have reported that increased expression of certain yeast genes in *E. coli* could be obtained after either deletion of specific plasmid sequences or insertion of an IS sequence which permitted more efficient transcription initiation. In order to determine if the increased expression reported here was due to similar plasmid alterations, pVK88 plasmid DNA was isolated from a *pnp-7* strain (SK3410) which showed a high level of catabolic dehydroquinase activity. This DNA was then used to transform both *pnp*$^+$ and *pnp-7* strains of *E. coli* (SK692 and SK694). As shown in Table 4, the level of catabolic dehydroquinase in both *pnp*$^+$ and *pnp-7* strains is unaffected by whether or not the pVK88 plasmid DNA has previously been propagated in a phosphorylase-deficient *E. coli* host. In addition, restriction enzyme analysis of pVK88 plasmid DNA from *pnp*$^+$ and *pnp-7* strains with either four-base or six-base enzymes did not reveal any differences in molecular weight or fragment patterns (data not shown).

Analysis of Plasmid Encoded mRNAs in *pnp*$^+$ and *pnp-7* Strains. Pulse-labeled [^{3}H]RNA was isolated from *pnp*$^+$ and *pnp-7* strains of *E. coli* transformed with either pBR322 or pVK88 plasmid DNA. As shown in Fig. 1 *C* and *D*, the level of pBR322-specific mRNA was similar in *pnp*$^+$ and *pnp-7* strains. In contrast, the level of pVK88-specific mRNA was approximately 7-fold higher in a *pnp-7* host than in an isogenic *pnp*$^+$ strain (Fig. 1*A*). The pVK88 mRNA from a *pnp-7* strain also showed increased hybridization to pBR322 DNA (Fig. 1*C*).

In order to determine whether the observed increase in hybridization was due to increased mRNA stability, the half-lives of pBR322 and pVK88 plasmid-encoded mRNAs from isogenic *pnp*$^+$ and *pnp-7* strains were determined. As shown in Fig. 2*A*, the rate of decay for pVK88-encoded mRNAs in a *pnp-7 E. coli* host is significantly slower than in a *pnp*$^+$ background. The data translate into a half-life for pVK88 mRNA of 2.8 min in a *pnp-7* strain and 1.0 min in a *pnp*$^+$ strain (Table 5). In contrast, pBR322-specific mRNAs show identical decay rates in both *pnp*$^+$ and *pnp-7* strains and have an average half-life of 1.3 min (Fig. 2*B* and Table 5).

Table 4. Catabolic dehydroquinase activity in various polynucleotide phosphorylase-deficient strains

Strain	Genotype	Plasmid	Source of plasmid	Catabolic dehydroquinase*
SK3518	*pnp*$^+$	pVK88	SK1572	0.022
SK3525	*pnp-7*	pVK88	SK1572	1.20
SK3520	*pnp*$^+$	pVK88	SK3410	0.033
SK3527	*pnp-7*	pVK88	SK3410	1.18

* Micromoles/min per mg of protein.

Plasmid Copy Number. The plasmid copy numbers of both pBR322 and pVK88 were determined in *pnp-7* and *pnp*$^+$ strains. As expected, the copy number for the larger recombinant plasmid (pVK88, 11.1 kilobases) was considerably lower than for the pBR322 (4.3 kilobases) vehicle itself (Table 5). Surprisingly, however, both plasmids exhibited a 3- to 4-fold increase in copy number in a polynucleotide phosphorylase-deficient strain compared to a wild-type control (Fig. 3 and Table 5).

Properties of Overexpressing Strains. Polynucleotide phosphorylase-deficient strains of *E. coli* transformed with pVK88 plasmid DNA grow more slowly than wild-type controls, such that generation times often increase by a factor of

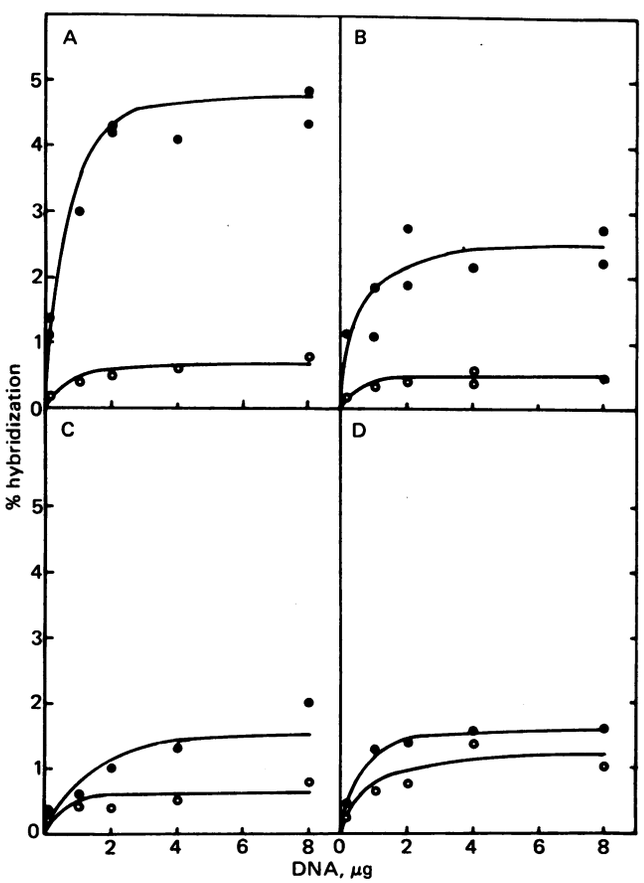

FIG. 1. Hybridization of pulse-labeled [^{3}H]RNA to pBR322 and pVK88 plasmid DNA. (*A*) Hybridization of RNA from pVK88-transformed strains SK3425 (*pnp*$^+$) and SK3410 (*pnp-7*) to pVK88 DNA. (*B*) Hybridization of RNA from strains SK3425 and SK3410 to pBR322 DNA. (*C*) Hybridization of RNA from pBR322-transformed stains SK2193 (*pnp*$^+$) and SK2194 (*pnp-7*) to pVK88 DNA. (*D*) Hybridization of RNA from strains SK2193 and SK2194 to pBR322 DNA. ○, *pnp*$^+$ strains; ●, *pnp-7* strains.

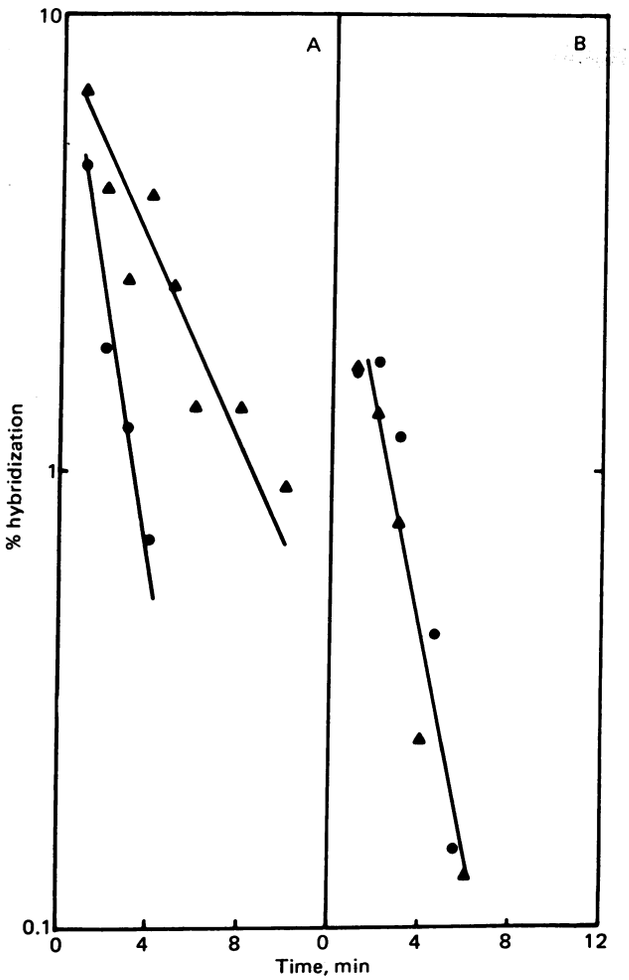

FIG. 2. Decay of pulse-labeled [^{3}H]RNA complementary to plasmid sequences. (*A*) Hybridization of RNA from pVK88-containing strains SK3425 (*pnp*$^+$) and SK3410 (*pnp-7*) to pVK88 DNA. (*B*) Hybridization of RNA from pBR322-containing strains SK2193 (*pnp*$^+$) and SK2194 (*pnp-7*) to pBR322 DNA. ●, *pnp*$^+$ strains; ▲, *pnp-7* strains.

two or more in minimal medium. In the absence of drug selection, pVK88 is rapidly cured from *pnp-7* strains, whereas pBR322 and pDV604 exhibit no such curing under similar circumstances. In minimal medium a decrease in the concentration of casamino acids yields a slower growth rate and a higher specific activity for catabolic dehydroquinase (unpublished data).

DISCUSSION

The results presented in this communication establish that the expression of eukaryotic DNA in *E. coli* can be regulated by

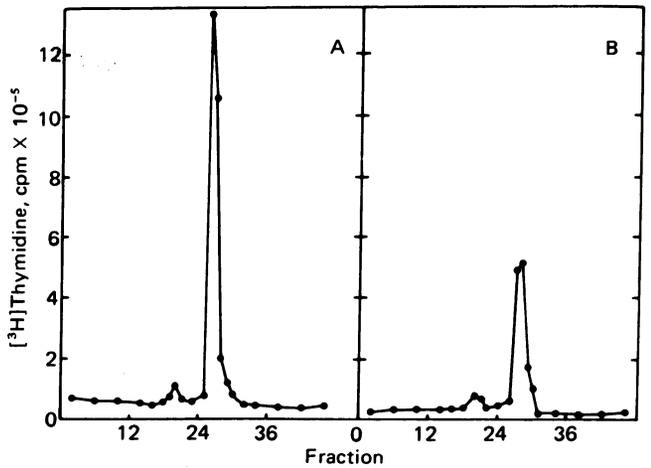

FIG. 3. Cesium chloride/ethidium bromide equilibrium centrifugation of [^{3}H]DNA. (*A*) SK3425 (*pnp*$^+$ pVK88). (*B*) SK3410 (*pnp-7* pVK88). Data were similar but are not shown for pBR322.

certain genetic modifications to the host. In the case of catabolic dehydroquinase from *N. crassa*, a 20- to 50-fold increase in specific activity can probably be attributed to a deficiency in *E. coli* polynucleotide phosphorylase (Table 2). The absence of ribonuclease I also appears to increase expression slightly (*ca.* 2-fold), but the consequences of this deficiency need further investigation. The increased expression appears to be specific for the eukaryotic gene because neither the chromosomally encoded *E. coli* biosynthetic dehydroquinase (*aroD*$^+$) nor the plasmid-encoded prokaryotic enzymes β-lactamase and chloramphenicol acetyltransferase are over-expressed. In agreement with these results Krishna and Apirion (21) have shown that β-galactosidase was not over-expressed in polynucleotide phosphorylase-deficient strains and that the β-galactosidase mRNA was not stabilized.

In contrast, polynucleotide phosphorylase must be involved in the turnover of eukaryotic mRNAs synthesized in *E. coli*, because its absence results in a significant increase in the half-life of *qa-2* mRNA (see Table 5 and Fig. 2). It should be pointed out that the observed increase in half-life is actually a lower limit because the hybridizations were carried out against intact pVK88 plasmid DNA. Thus, the 2.8-min half-life in *pnp-7* strain SK3410 represents a composite of the half-lives of two procaryotic mRNAs (*amp* and *tet*, 1.3 min, Table 5) as well as the *qa-2* mRNA. Similarly, the half-life of 1.0 min for pVK88 mRNAs in *pnp*$^+$ strain SK3425 is an upper limit for the *qa-2* mRNA, again due to the influence of the prokaryotic mRNA half-life of 1.3 min. Therefore, it seems likely that in wild-type *E. coli* strains the half-life of the eukaryotic mRNA is shorter than that of prokaryotic mRNAs and that in phosphorylase-deficient strains, the half-life is even longer than reported here.

Table 5. Catabolic dehydroquinase activity, mRNA half-life, and plasmid copy number in *pnp*$^+$ and *pnp-7* strains

Strain	Genotype	Plasmid	Catabolic dehydroquinase*	Plasmid-encoded mRNA half-life, min	Plasmid copy number
SK3425	*pnp*$^+$	pVK88	0.015	1.0	10
SK3410	*pnp-7*	pVK88	0.924	2.8	35
SK2193	*pnp*$^+$	pBR322	—	1.3	30
SK2194	*pnp-7*	pBR322	—	1.3	140

* Micromoles/min per mg of protein.

This hypothesis is currently being tested by isolating the 6.8 kilobase *N. crassa* fragment from pVK88 (8) and repeating the half-life measurements.

The observed stabilization of the *qa-2* message suggests that eukaryotic mRNAs may possess some unique secondary or tertiary structure not present in prokaryotic mRNAs which can be recognized and processed through the intervention of polynucleotide phosphorylase. Therefore, in the absence of this enzyme, the eukaryotic mRNA may be partially protected from the normal *E. coli* mRNA degradative enzymes.

The increased copy number of both the pBR322 and pVK88 plasmids in polynucleotide phosphorylase-deficient strains is an interesting and somewhat surprising result. It could relate to the involvement of an RNA primer in ColE1 plasmid replication and other ColE1 plasmids are currently being examined in this regard. Surprisingly, however, the increase in copy number of pBR322 does not result in a significant increase in pulse-labeled hybridizable mRNA (see Fig. 1*D*) nor in the level of β-lactamase (see Table 3).

Even if plasmid copy number is involved in overexpression, only a 10-fold increase in specific activity of catabolic dehydroquinase would result from the combined effects of copy number and mRNA stabilization, whereas 20- to 50-fold increases in enzyme activity have been observed. The extreme stability of the catabolic dehydroquinase enzyme (22) may account for the remainder of the increase in that the protein may accumulate in the cell.

The generality of increased expression of eukaryotic genes in polynucleotide phosphorylase-deficient strains of *E. coli* is supported by results with the *his3* gene of yeast (unpublished results) which are similar to those reported above. Taken together, these findings suggest that there are inherent structural differences between eukaryotic and prokaryotic mRNAs.

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