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Physical Analysis of *phr* Gene Transcription in *Escherichia coli* K-12

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The *phr* gene of *Escherichia coli* K-12 encodes the light-dependent, DNA repair enzyme photolyase, which removes UV light-induced pyrimidine dimers from cellular DNA. From Southern hybridization analysis of several strains containing successively extended *phr* deletions, we have determined the direction of transcription of the *phr* gene on the *E. coli* K-12 chromosome. Northern (RNA) hybridization analysis suggests that the *phr* gene is cotranscribed with a previously identified gene of unknown function (*orf169*) into two messages of different lengths. S1 nuclease mapping analysis indicates that the two transcripts share a single termination site but initiate at two different sites. Finally, we have determined that the presence of *orf169* is not necessary for *phr* gene activity in vivo.

The exposure of bacterial cells to UV radiation (200 to 300 nm) induces the formation of cyclobutadipyrimidine photoproducts (pyrimidine dimers) in the cellular DNA, which can cause cell death or mutation if left unrepaired. Several repair mechanisms for effective removal of these dimers exist in *Escherichia coli* cells, among them, a direct reversal of damaging reaction to regenerate the normal pyrimidine structure by the photoenzymatic process of photoreactivation. This action is mediated by the DNA repair enzyme deoxyribodipyrimidine photolyase, or photoreactivating enzyme (the product of the *phr* gene), which binds to dimer-containing DNA in the dark and catalyzes splitting of the dimers upon absorption of a photon of photoreactivating light (300 to 500 nm).

E. coli K-12 contains only about 10 to 15 molecules of photoreactivating enzyme per cell in the stationary phase and still less during exponential-phase growth (8). Although it is an effective DNA repair enzyme, its low concentration in the cell limits its ability to repair solar UV-induced damage as rapidly as it is formed under typical midday sunlight (9). The reason for such stringent regulation is not known. The previously determined nucleotide sequence of the *phr* gene (25) suggests that its expression may be dependent upon transcription initiating from another open reading frame of unknown function. This 169-codon reading frame (*orf169*) encodes a gene product which appears to be overproduced in cells containing the *orf169* and *phr* gene coding regions under the control of a strong promoter (26). It is located immediately upstream of *phr* and overlaps the reading frame of *phr* by 4 bp. In the case of a similar overlap of the *umuC* gene by the immediately upstream *umuD* gene, the two loci are organized as an operon (20), suggesting that this may be true of *orf169* and *phr*.

To further elucidate the regulation of *phr* gene expression, we have determined by Southern hybridization analysis the chromosomal orientation of a 21.5-kb *Eco*RI fragment of the *E. coli* K-12 genome which contains the *phr* gene. This has permitted us to determine the gene order and the direction of transcription of *phr* and *orf169* within the *E. coli* K-12 chromosome. From Northern (RNA) hybridization analysis, we have determined that the two genes are cotranscribed into two messages of different lengths, at least under normal growth conditions. S1 nuclease mapping indicated that the two messages initiate approximately 100 and 1,000 bp upstream of the *orf169* initiation codon and terminate approximately 20 bp downstream of the *phr* termination codon. In addition, we have determined that the presence of the *orf169* gene product is not required for *phr* gene activity in vivo.

MATERIALS AND METHODS

Bacterial strains and plasmids. All strains used in this work are listed in Table 1. MCL22, TK3D22, TK3D23, TK3D29, and SR371 were grown in KLB (Luria broth [18] with KCl substituted for NaCl) or KM9 (M9 minimal medium [18] with K_2HPO_4 substituted for Na_2HPO_4). All other strains were grown in Luria broth.

Plasmids were constructed by ligation of restriction fragments with T4 DNA ligase (Bethesda Research Laboratories) according to standard procedures (17) and transformed into $CaCl_2$-treated (4) competent MCL22 cells. Transformed cells were subjected to two UV irradiation photoreactivation cycles (24, 26) to enrich for plasmids carrying the *phr* gene. Plasmids were isolated by the alkaline lysis method (3) and screened by restriction enzyme analysis.

Assay for Phr$^+$ (photoreactivation) phenotype. Following the UV irradiation photoreactivation enrichment cycles, individual colonies were streaked onto duplicate Luria broth agar plates with a sterile toothpick and irradiated with a UV fluence of 1.0 J/m^2, as determined with a Jagger meter (12). One plate was immediately placed in a dark incubator, while the other plate was exposed to photoreactivating light for 45 min. Both plates were incubated overnight at 37°C. Phr$^+$ cells exhibited confluent growth in the streaked patch on the photoreactivated plate, while Phr$^-$ cells showed little or no growth, as did all streaked patches on the nonphotoreactivated plate.

Chromosomal DNA isolation and Southern hybridization analysis. Chromosomal DNA was isolated from overnight cultures by a modification of the method of Davis et al. (5) as follows. Cells were pelleted, washed with 50 mM Tris–50 mM EDTA, pH 8.0, suspended in the same buffer containing 20% sucrose, and incubated in the presence of lysozyme (2.0 mg/ml) at 37°C for 30 min. Sodium dodecyl sulfate was added to a final concentration of 0.6%, and the cells were heated to

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TABLE 1. Bacterial strains

Strain	Genotype	Source or reference
MCL22	*trkA405 trkD1 thi rha* Δ(*kdp-phr*)*214* Δ(*gal-uvrB*) Δ(*srl-recA*)*306*	16
RC62	*thr leu thi*	R. C. Clowes
TK3D22	*trkA405 trkD1 thi rha* Δ(*kdp-phr*)*107* Δ(*gal-bio*)	21
TK3D23	*trkA405 trkD1 thi rha* Δ(*kdp-phr*)*170* Δ(*gal-bio*)	21
TK3D29	*trkA405 trkD1 thi rha* Δ(*kdp-phr*)*256* Δ(*gal-bio*)	21
SR371	*trkA405 trkD1 thi rha* Δ(*kdp-gltA*)*101* Δ(*gal-bio*)	28

70°C for 30 min and then treated with proteinase K (final concentration, 200 μg/ml) for 60 min. Potassium acetate was added to a final concentration of 0.5 M, the solution was heated to 70°C for 15 min, and the cell debris was pelleted by centrifugation. The DNA in the supernatant was precipitated by the addition of polyethylene glycol (molecular weight, 8,000; 10% final concentration), pelleted, washed with 95% ethanol, and suspended in TE (10 mM Tris, 1.0 mM EDTA, pH 8.0 [17]). The DNA solution was treated with RNase A (100 μg/ml), extracted twice with phenol (TE saturated) and once with chloroform (24:1, chloroform-isoamyl alcohol), precipitated twice with ethanol, and resuspended in TE for restriction enzyme cleavage.

Chromosomal DNA (3.0 μg per lane) was cleaved with *Eco*RI, fractionated on a 0.6% agarose gel, and transferred to a nitrocellulose filter according to standard procedures (17). DNA restriction fragment probes were isolated by electroelution and radiolabeled with [α-^{32}P]dCTP (ICN) with a nick-translation kit (Bethesda Research Laboratories). Unincorporated nucleotides were removed by Sephadex G-50 column chromatography, and the probes were denatured by boiling for 5 min followed by rapid cooling in an ice water bath. Hybridization was performed for 18 to 24 h at 42°C, and the filters were washed twice at room temperature for 15 min each time in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate and then subjected to two stringent washes at 65°C for 20 min each wash in 0.1× SSC–0.1% sodium dodecyl sulfate (17).

RNA isolation and Northern hybridization analysis. RNA was isolated from exponentially growing cultures of MCL22 with no plasmid or carrying pCJL10 in KM9 minimal growth medium according to the method of Aiba et al. (1). The RNA (40 μg per lane) was fractionated on a 1.25% agarose–6% formaldehyde gel (14) and transferred to a nitrocellulose filter. Isolated DNA restriction fragments were radiolabeled with [α-^{32}P]dCTP by hexamer extension (7), denatured, and used to probe the RNA blots. Hybridization and wash conditions were as described above.

S1 nuclease mapping. Whole-cell RNA was isolated as described above, hybridized with purified restriction fragment probes in the presence of 80% deionized formamide (6) at 72°C for 15 min, and transferred to 52°C for 3 h. S1 nuclease (87 U) was added to each tube, and the mixture was incubated at 37°C for 30 min. The nucleic acids were recovered by ethanol precipitation, fractionated on a 0.7% agarose–8 M urea gel (15), and transferred to a nitrocellulose filter. Purified DNA restriction fragments were labeled with biotin by nick translation, denatured, and used to prove the filter (27). The hybridized probes were visualized with a streptavidin-alkaline phosphatase conjugate (Bethesda Research Laboratories BluGene kit).

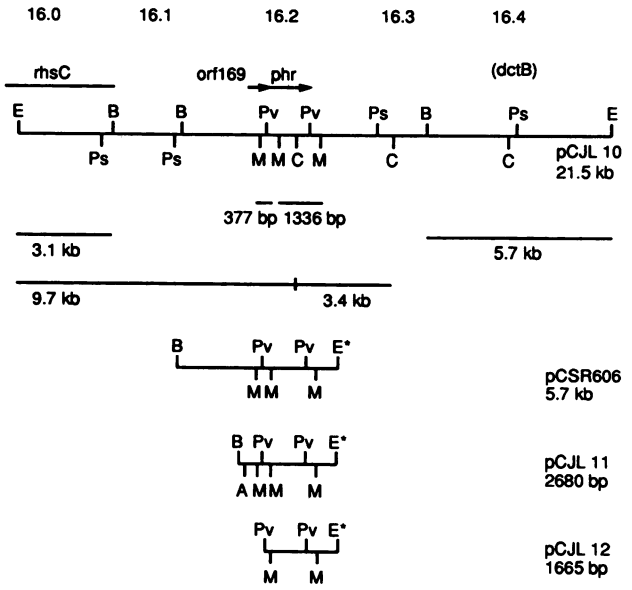

FIG. 1. Physical and genetic map of the 21.5-kb *Eco*RI chromosomal fragment and its derivatives. The *orf169* and *phr* positions are based on restriction mapping and nucleotide sequence data (25). The position of *rhsC* relative to *phr* was determined by others (22) from comparison of published restriction maps. The relative position of *dctB* is from linkage data (2, 23), which only roughly correlates with restriction mapping data. The numbers at the top indicate minutes on the *E. coli* K-12 chromosome. The sizes of the various derivative inserts and the names of the plasmids carrying the inserts are indicated on the right. The restriction fragments used as probes and in S1 mapping are indicated: 3.1- and 5.7-kb *Bam*HI-*Eco*RI fragments (Southern hybridization analysis), 377-bp *Acc*I-*Pvu*II and 1,336-bp *Mlu*I fragments (Northern hybridization analysis), and 3.4-kb *Cla*I and 9.7-kb *Eco*RI-*Cla*I fragments (S1 nuclease mapping analysis). Restriction enzyme site designations: A, *Acc*I; B, *Bam*HI; C, *Cla*I; E, *Eco*RI, E*, *Eco*RI*; M, *Mlu*I; Ps, *Pst*I; Pv, *Pvu*II; S, *Sau*3A. There are additional *Pvu*II sites present in the 21.5-kb fragment which are not indicated.

RESULTS

Subcloning of the *phr* gene. The original *phr* gene clone, pCSR604 (24), contained two *Eco*RI chromosomal fragments of 21.5 and 12 kb. The 21.5-kb fragment carries the gene (26; M. C. Lorence, M.S. thesis, University of Texas at Dallas, 1981). We inserted the 21.5-kb fragment into the *Eco*RI site of pBR322 to give pCJL10. A 5.7-kb *Bam*HI-*Eco*RI* fragment of pCSR604 inserted into *Bam*HI-*Eco*RI-cleaved pBR322 by Sancar et al. (26) gave pCSR606, and a 2,680-bp *Sau*3A partial digestion fragment of this plasmid inserted into *Bam*HI-cleaved pBR322 gave pCJL11. Partial digestion of pCJL11 with *Pvu*II followed by recircularization gave pCJL12, which contains the *phr* gene on a 1,665-bp chromosomal fragment. All of these plasmids fully complemented deletion mutations of the *phr* gene.

A partial restriction map of the *E. coli* chromosomal sequences contained in the above plasmids is in Fig. 1. The direction of transcription of the *phr* gene and *orf169* in these cloned fragments is known from Tn*1000* insertional inactivation of *phr* complementation (26) and was confirmed by DNA sequence analysis (25). The position of *rhsC* relative to

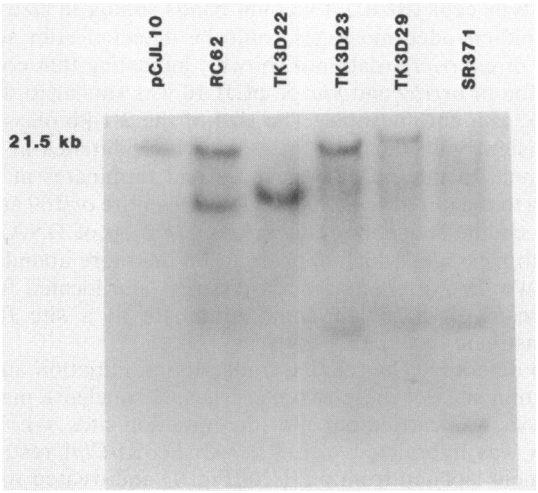

FIG. 2. Southern hybridization analysis of *Eco*RI-digested chromosomal DNA from *kdp* deletion strains (Table 1). Transferred chromosomal DNA was probed with the 21.5-kb *Eco*RI fragment purified from pCJL10. *Eco*RI-cleaved pCJL10 was included as a positive control for hybridization to the 21.5-kb chromosomal fragment. The low-molecular-weight band appears to be a result of cross-hybridization to non-*phr* sequences, since it appears in SR371, in which the *phr* gene (and the 21.5-kb chromosomal fragment) is completely deleted.

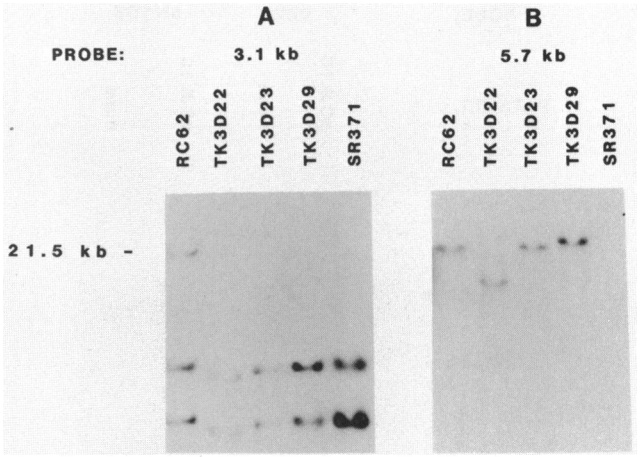

FIG. 3. Southern hybridization analysis of *Eco*RI-digested chromosomal DNA from *kdp* deletion strains (Table 1). Transferred chromosomal DNA was probed with the 3.1-kb (A) or 5.7-kb (B) *Eco*RI-*Bam*HI restriction fragment (Fig. 1). The low-molecular-weight bands seen with the 3.1-kb probe appear to be results of cross-hybridization to non-*phr* sequences, since they were not seen when the 1,336-bp *Mlu*I fragment (*phr* coding sequences) was used as a probe (data not shown).

phr was determined previously (22) by comparison of published restriction enzyme maps, while the approximate position of *dctB* is from linkage data (2).

Chromosomal orientation of the fragment containing the *phr* gene. Availability of a series of *phr* deletion mutations, generated by erroneous excision of bacteriophage lambda integrated in the *kdp* (potassium dependence) operon at 16 min on the *E. coli* linkage map (21), opened the possibility that the *phr* sequences could be oriented to the chromosome. If part, but not all, of the 21.5-kb *Eco*RI chromosomal fragment in pCJL10 and pCSR604 had been deleted in some of these strains, then the deleted region would be the end towards *kdp*.

Purified chromosomal DNAs from wild-type *E. coli* RC62 and deletion mutation strains TK3D22, TK3D23, TK3D29, and SR371 (in which the *phr* gene and the entire 21.5-kb chromosomal fragment are deleted; Table 1) were cleaved with *Eco*RI, resolved on a 0.6% agarose gel, and transferred to a nitrocellulose filter. The blot was probed with the 21.5-kb *Eco*RI chromosomal fragment which had been radiolabeled by nick translation, and an autoradiograph of this hybridization is shown in Fig. 2. Evidently, the deletion mutations in TK3D22, TK3D23, and TK3D29 meet the required criterion: in all three strains, only a portion of the 21.5-kb *Eco*RI chromosomal fragment was deleted (as demonstrated by the alteration in its mobility compared with that of the wild-type strain RC62).

To determine which end of the 21.5-kb *Eco*RI chromosomal fragment had been deleted, a 3.1-kb *Eco*RI-*Bam*HI subfragment (left end of pCJL10 insert in Fig. 1) and a 5.7-kb *Bam*HI-EcoRI subfragment (right end of pCJL10 insert in Fig. 1) were isolated and radiolabeled by nick translation. These fragments were used separately to probe duplicate blots of *Eco*RI-digested chromosomal DNAs from RC62, TK3D22, TK3D23, TK3D29, and SR371. An autoradiograph of the hybridization with the 3.1-kb fragment as the probe is shown in Fig. 3A, while an autoradiograph of the hybridization with the 5.7-kb fragment as the probe is shown in Fig. 3B. Since the 3.1-kb fragment (left end of pCJL10 insert in Fig. 1) did not hybridize to the chromosomal DNAs of the strains carrying *phr* deletion mutations, this end of the 21.5-kb *Eco*RI chromosomal fragment must have been deleted. Conversely, since the 5.7-kb fragment (right end of pCJL10 insert in Fig. 1) did hybridize (exhibiting a pattern similar to that seen in Fig. 2), this end of the 21.5-kb chromosomal fragment must still have been present.

Since the *phr* deletions extend from *kdp* downstream (in the clockwise direction on the *E. coli* K-12 genetic map) to *phr*, the 21.5-kb chromosomal fragment must be oriented as shown in Fig. 1. The end from which the 3.1-kb *Eco*RI-*Bam*HI fragment was derived must be toward *kdp*, while the end from which the 5.7-kb *Bam*HI-*Eco*RI fragment was derived must be toward *dctB*. The direction of transcription of *orf169* and *phr* is towards the end from which the 5.7-kb fragment was derived (Fig. 1), making the direction of transcription of *orf169* and *phr* clockwise on the *E. coli* K-12 linkage map (2). These data confirm the orientation of the 21.5-kb chromosomal fragment determined in the published restriction map of the *E. coli* genome (13) and permit placement of the *phr* gene at approximately 756 kb in the positive orientation on that map.

Transcription of the *phr* gene and *orf169*. Whole-cell RNA was isolated from MCL22 cells containing either pCJL10 or no plasmid, fractionated on an agarose-formaldehyde gel, and transferred to a nitrocellulose filter. A 1,336-bp *Mlu*I restriction fragment of pCJL11, which contains only *phr* gene coding sequences, and a 377-bp *Acc*I-*Pvu*II restriction fragment, which contains only *orf169* sequences, were isolated (Fig. 1), radiolabeled by hexamer extension (7), and used separately to probe duplicate filters of transferred RNA. An autoradiograph of this hybridization is shown in Fig. 4. Both the *orf169* and *phr* gene probes hybridized to two different messages of approximately 2.1 and 3.0 kb in the lanes containing RNA isolated from MCL22 carrying

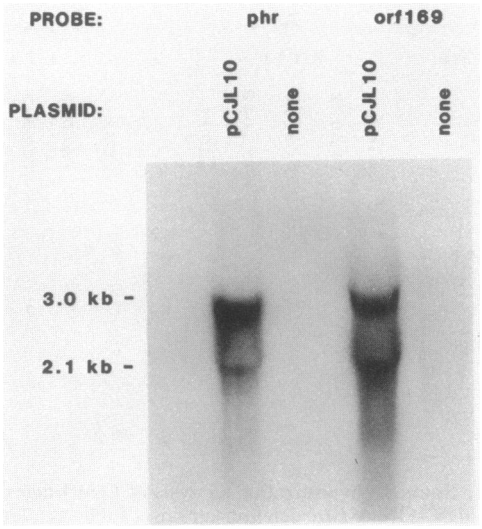

FIG. 4. Northern hybridization analysis of total RNA from MCL22 alone or carrying pCJL10. Transferred RNA was probed with either a 1,336-bp *Mlu*I restriction fragment (*phr* gene probe) or a 377-bp *Acc*I-*Pvu*II restriction fragment (*orf169* probe). See Fig. 1 for derivation of the probes.

pCJL10. No signal was seen in the lanes containing RNA from the host cell alone, indicating that the 2.1- and 3.0-kb messages must be plasmid encoded. When these same Northern analyses were carried out using RNA isolated from wild-type cells (RC62), two faint bands similar in size to the plasmid-encoded messages could be detected after several days of exposure (data not shown), indicating that cotranscription of *orf169* and *phr* on pCJL10 was similar to that on the *E. coli* chromosome. The size of the 2.1-kb message is consistent with that of a message which initiates at a site proximal to the *orf169* sequences and terminates at a site distal to the *phr* gene coding region (the entire *orf169* and *phr* gene coding sequences encompass 1,970 bp of DNA). The 3.0-kb message, which appears to be the more abundant of the two, could initiate from a different site located further upstream of *orf169* or could terminate at a site further downstream from *phr*.

To determine the relative transcription initiation and termination sites of these two messages, S1 nuclease mapping analysis was carried out. For the initiation sites, whole-cell RNA was hybridized with a 9.7-kb *Eco*RI-*Cla*I restriction fragment isolated from pCJL10 (Fig. 1) and treated with S1 nuclease. The resultant fragments were fractionated on a 0.7% agarose–8 M urea gel, transferred to nitrocellulose, and probed with the same 1,336-bp *Mlu*I restriction fragment from pCJL11, which had been biotin labeled by nick translation. Two S1 nuclease-resistant bands of approximately 1.15 and 2.1 kb were generated (Fig. 5A). Since the direction of transcription proceeded towards the *Cla*I site, the sizes of the S1 nuclease-resistant bands suggest the presence of two transcription initiation sites located approximately 100 and 1,000 bp upstream of the *orf169* initiation codon. It is not known whether putative transcription initiation sites are present near the endpoints of the S1 nuclease-resistant

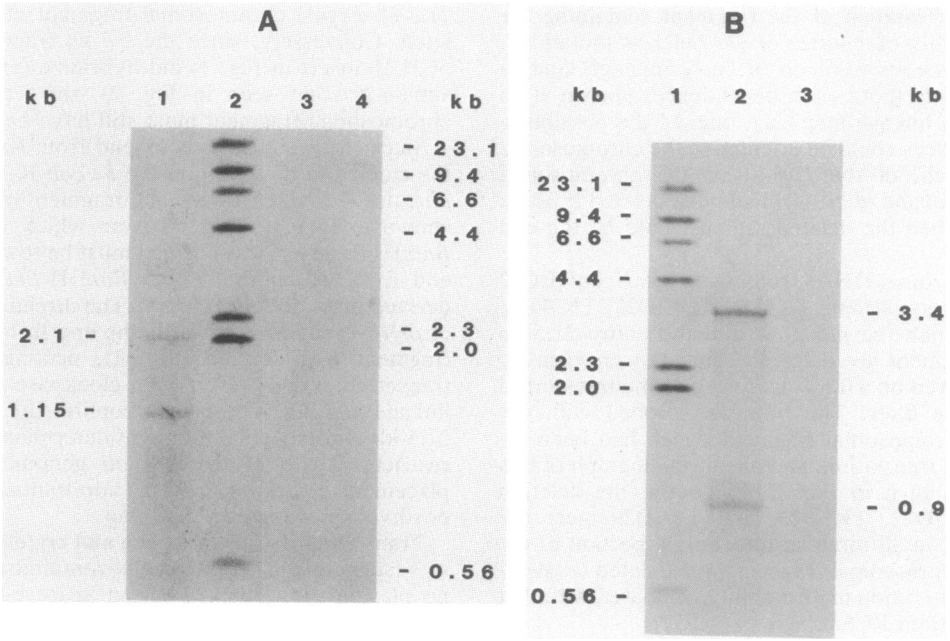

FIG. 5. S1 nuclease mapping of transcription initiation (A) and termination (B) sites. Transferred fragments were probed with a biotin-labeled, 1,336-bp *Mlu*I restriction fragment (*phr* gene probe) and visualized with a streptavidin-alkaline phosphatase conjugate (A) A 9.7-kb *Eco*RI-*Cla*I fragment was used as a probe (Fig. 1). Lanes: 1, S1 nuclease-resistant fragments from the *Eco*RI-*Cla*I fragment hybridized to whole-cell RNA from MCL22 cells carrying pCJL10; 2, *Hin*dIII-digested lambda DNA as a molecular weight standard; 3, the S1 nuclease-treated *Eco*RI-*Cla*I fragment alone (no RNA); 4, the *Eco*RI-*Cla*I fragment hybridized to RNA without S1 nuclease treatment. (B) A 3.4-kb *Cla*I fragment was used as a probe (Fig. 1). Lanes: 1, *Hin*dIII-digested lambda DNA as a molecular weight standard; 2, S1 nuclease-resistant fragments from the *Cla*I fragment hybridized to whole-cell RNA from MCL22 cells carrying pCJL10; 3, the S1 nuclease-treated *Cla*I fragment alone (no RNA). The additional band at 3.4 kb in lane 2 is probably due to the presence of unreacted probe, as evidenced by the probe remaining in lane 3.

fragments, since the nucleotide sequence of this region has not yet been determined.

To identify transcription termination sites, whole-cell RNA was hybridized with a 3.4-kb *ClaI* fragment isolated from pCJL10 (Fig. 1) and treated as described previously. One S1 nuclease-resistant band of approximately 0.9 kb was generated (Fig. 5B), suggesting the presence of a transcription termination site located approximately 20 bp downstream of the *phr* termination codon. This is consistent with the presence of a putative termination site previously identified from DNA sequence analysis (25).

The presence of two transcription initiation sites located approximately 100 and 1,000 bp upstream of *orf169* and a transcription termination site located 20 bp downstream of *phr* leads to deduced sizes of 2.1 and 3.0 kb, respectively, for the two transcripts. These sizes are consistent with those determined by Northern hybridization analysis and indicate that, at least under normal growth conditions, the two genes are cotranscribed into two major transcripts sharing a common termination site but with different initiation sites. The absence of a 0.9-kb band in either the Northern hybridization or the S1 nuclease mapping analysis indicates that the 2.1-kb transcript is not derived from the 3.0-kb transcript by posttranscriptional processing and that these two major transcripts must have distinct origins.

Two putative promoter sequences, identified by DNA sequence analysis (25), are located in the carboxy-terminal coding region of *orf169*. It is possible that minor transcripts, encoding only *phr* coding sequences, are expressed under different physiological growth conditions, a phenomenon observed with other *E. coli* operons (10).

Functional relationship between *phr* and *orf169* gene products. If *orf169* and *phr* are cotranscribed on the same mRNA, a functional relationship might exist between their respective gene products and the ability to repair UV-induced DNA damage through photoreactivation. However, when pCJL12 (Fig. 1), which contains all of the *phr* gene coding region but only the carboxy-terminal two-fifths of *orf169* is transformed into MCL22 (a strain containing a deletion from *kdp* through *phr* which must also delete *orf169*), the *phr* deletion is fully complemented (data not shown). This indicates that whatever the relationship between *orf169* and *phr*, the presence of the *orf169* gene product is not required for *phr* gene activity in vivo.

DISCUSSION

The results outlined above indicate that the regulation of *phr* gene transcription is more complex than our original expectations. The *phr* gene does not appear to have its own promoter, making its transcription dependent upon that of *orf169* or perhaps another, as-yet-unidentified cistron (since the 3.0-kb message is large enough to possibly encode another gene of up to 1.0 kb). Previous studies of *phr* gene expression employed fusions of the *phr* promoter region (contained within a 1.2-kb *PvuII* restriction fragment) to the *lacZ* coding region (11). In wild-type and *uvrA* strains, β-galactosidase activity increased in response to UV irradiation, but in *lexA* and *recA* strains, no increase in activity was observed. In addition, nalidixic acid and mitomycin C appeared to induce β-galactosidase activity, although to a lesser extent than that observed with UV irradiation, suggesting that the *phr* gene may be part of the SOS regulon. The presence of two putative SOS boxes in the carboxy-terminal coding region of *orf169* (11, 25), located approximately 60 and 150 bp upstream of the *phr* initiation codon, appeared to further support that proposition. However, subsequent studies by others (19) indicated that these two putative SOS boxes did not bind purified LexA repressor specifically.

Two putative promoter elements, located from approximately 20 to 50 and 70 to 100 bp upstream of the *phr* initiation codon, are present in the same 1.2-kb *PvuII* fragment employed in the *lacZ* fusion constructs. These putative promoters exhibit significant sequence identity with *E. coli* consensus promoter elements, and a putative ribosome-binding site (AGGAG) is located between the proximal promoter and the *phr* initiation codon (25; M. C. Lorence, Ph.D. dissertation, University of Texas at Dallas, 1984). This putative ribosome-binding site, also located in the *orf169* carboxy-terminal coding region, is presumably required for the translation of photoreactivating enzyme on the cotranscribed message. Although no transcripts appear to initiate from these putative promoter sequences (at least under normal growth conditions), as determined by S1 mapping analysis, these promoters may function in *E. coli* cells grown under different physiological conditions and may be responsible for the *phr* gene promoter region-directed induction of β-galactosidase activity observed following UV irradiation (11).

Further work is needed to determine whether transcription initiates from either of the two putative promoter sequences located in the *orf169* coding region in UV-irradiated cells. Additionally, the sequences upstream of *orf169* encoded in the larger of the two transcripts must be examined to determine whether another open reading frame is present, and the function of *orf169* in the growth of *E. coli* K-12 cells must be elucidated.

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