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Identification of Polypeptides Encoded by an *Escherichia coli* Locus (*hflA*) That Governs the Lysis-Lysogeny Decision of Bacteriophage λ

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We report the cloning of the *Escherichia coli hflA* locus, which governs stability of phage λ cII protein and which has been proposed to encode or regulate a cII-specific protease. The *hflA* locus was cloned on an 18-kilobase DNA fragment by selecting for plasmids that carry the neighboring *purA* gene. The boundaries of *hflA* were delimited by analysis of deletions and insertions constructed in vitro and by use of transposon Tn*1000*. Maxicell analysis of the proteins encoded by the *hflA*-containing fragment shows that *hflA* consists of at least two nonoverlapping genes, *hflC* and *hflK*, encoding polypeptides of 37,000 (C) and 46,000 (K) daltons. We observe that insertions into one gene eliminate the corresponding polypeptide and greatly reduce synthesis of the other. We suggest that these two polypeptides (K and C) interact to form a multimeric complex and that free subunits are unstable. We have constructed two types of fusions between *hflA* and *lacZ*. One is an *hflC-lacZ* protein fusion constructed in vitro; the other is an *hfl-lacZ* operon fusion in which a Mu dX(Ap^r *lac*) has inserted into the *hflK* gene. We have used the operon fusion to infer the direction of transcription of the *hflK* gene—toward *hflC* and in the same direction as *hflC*. Last, we describe evidence that *hflA* contains an additional gene, *hflX*, encoding a 50,000-dalton polypeptide.

The *hflA* gene of *Escherichia coli* governs the lysis-lysogeny growth decision of bacteriophage λ (reviewed in references 10, 16, and 17). In *E. coli* mutants defective in *hfl*, lambda lysogenization is very efficient (the Hfl phenotype, high frequency of lysogenization) (3). Wulff and co-workers isolated the first *hfl* mutants (3, 13) and observed that six of seven contain mutations very tightly linked to, but not within, the *purA* locus at 94.5 min on the *E. coli* chromosome. These six *hfl* mutations were shown to be recessive and to constitute a single complementation group (13); they define the *hflA* locus. The exceptional mutant defines the *hflB* locus, which was described by Banuett et al. (2).

The increased efficiency of lysogenization in *hfl* mutants results from an increased level of the phage cII protein (20; C. Epp, Ph.D. thesis, University of Toronto, Toronto, Ontario, Canada, 1978). cII promotes lysogeny by stimulating production of the phage repressor (cI) and integrase (Int) as well as by inhibiting lytic functions (reviewed in references 16 and 38; see also reference 19). It has been argued that the level of cII plays a key role in the lysis-lysogeny decision of the phage: when the level is high, lysogeny is favored; when the level is low, lysis is preferred. The increased level of cII in *hfl* mutants apparently results because cII stability is increased in these hosts, from approximately 1.5 min in wild-type hosts to 3 min in the mutant (2, 20; Epp, Ph.D. thesis). The *hflA* mutation examined did not affect stability of another unstable lambda protein, O (Epp, Ph.D. thesis).

Other factors that influence the efficiency of lysogenization appear to work via effects on Hfl (5, 20, 22; Epp, Ph.D. thesis) (Fig. 1). Gautsch and Wulff (13) proposed that the phage cIII protein stimulates lysogenization by antagonizing the *hfl* product. This hypothesis was motivated by the finding that the phage cIII protein is not needed for efficient lysogenization in the absence of *hfl* product. Furthermore, Belfort and Wulff proposed that the host catabolite gene activation system (catabolite activator protein-cyclic AMP) antagonizes the action of HflA based on their finding that catabolite activator protein-cyclic AMP is not needed for efficient lysogenization in the absence of Hfl activity (5).

The above observations support the proposal that the level of the cII protein is a key determinant in the lysis-lysogeny decision of phage λ and that *hfl* is crucial in governing the level of cII. Given that the *hflA* product affects stability of the phage cII protein, the simplest view is that HflA is itself a protease. It is, of course, possible that the link between *hflA* and cII is less direct and that *hflA* might control the activity of the actual cII protease. Complex pathways of proteolysis, as found in blood clotting (21), are well known. As a first step in determining the biochemical basis for the interaction between HflA and cII, we have cloned the *hflA* locus and identified its products. We present evidence in this report that the *hflA* locus is complex, consisting of at least two genes, *hflC* and *hflK*, encoding two polypeptides of 37 and 46 kilodaltons (kDa), respectively, that may interact with each other.

MATERIALS AND METHODS

Strains used. The genotypes and origins of bacterial strains are shown in Table 1. Phage strains λ$^+$, λ *c*I60, λ *c*II2002, λ *c*III67, λ *c*III611, λ *c*17, and P1 *vir* are from our collection. Plasmids used are described in Table 2.

Media. λ broth, LB broth, and SM medium were as described by Herskowitz and Signer (18). All media used for growing plasmid-containing strains contained 250 μg of carbenicillin per ml. M63 and M9 were as described by Miller (25).

EMB plates contain EMB agar supplemented with 0.05% maltose (14). LB-Ca is LB broth containing 2.5×10^{-3} M $CaCl_2$. LB-citrate is LB broth supplemented with 0.25%

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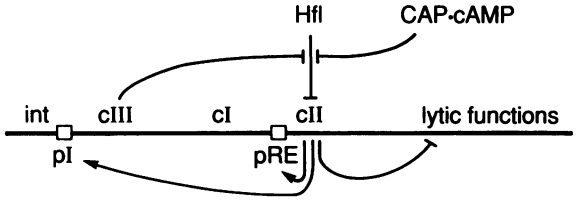

FIG. 1. Central role of Hfl and *c*II in the lysis-lysogeny decision of bacteriophage λ. Arrowheads indicate stimulatory interactions; bars indicate inhibitory interactions. (Modified from Herskowitz and Hagen [17].)

(wt/vol) sodium citrate. SC buffer is a solution of 0.85% (wt/vol) sodium chloride and 0.25% (wt/vol) sodium citrate.

OMBG plates (2) containing the appropriate antibiotic were used for P1 transductions. Antibiotics were used at the following concentrations: kanamycin sulfate, 40 μg/ml; carbenicillin, 250 μg/ml; streptomycin sulfate, 50 μg/ml; tetracycline hydrochloride, 10 μg/ml; ampicillin, 50 μg/ml.

Cb^r transformants were screened on LB-tetracycline plates. Screening for Pur^+ transformants was on M63-carbenicillin-kanamycin medium containing Casamino Acids (Difco Laboratories) and tryptophan. Transconjugants from Tn*1000* mutagenesis were selected on M63-carbenicillin-streptomycin. The Mu dX(Ap^r *lac*) bank was isolated on LB-ampicillin plates. X-gal plates contain 40 μg of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) per ml.

P1 transductions. Cells were grown in LB-Ca to 2×10^8 cells per ml. P1 phage were added at a multiplicity of infection of 0.1 and allowed to adsorb for 30 min at 37°C. Infected cells were washed with SC buffer, and when necessary cells were grown in LB-citrate for 1 h to allow phenotypic expression.

Hfl phenotype test. For quick screening of colonies, 0.05 ml of λ *c*17 at a titer of 10^6 to 10^7 phage per ml were streaked on EMB plates. Colonies to be tested were streaked across the line of phage with a toothpick. *hfl*$^+$ colonies are easily distinguishable from *hfl* mutants because λ *c*17 does not lyse *hfl* bacteria but does lyse *hfl*$^+$ bacteria (4). More accurate determinations were done on lawns of strains to be tested. For this purpose, cells were grown in λ broth to saturation, and 2.5 ml of top agar was added to 0.2 ml of cells and distributed on a λ plate. λ$^+$, λ *c*II$^-$, λ *c*III$^-$, and λ *c*17 in SM buffer at a titer of 10^6 to 10^7 phage per ml were streaked on the lawns. Plaque morphology was scored after overnight incubation at 37°C.

Isolation of *hfl*K::Mu dX(Ap^r *lac*). A Mu dX lysate was prepared by growing strain CAG5050 in LB containing 20 mM $MgSO_4$ at 30°C to 2×10^8 cells per ml (1). The culture was diluted with prewarmed (55°C) medium to 10^8 cells/ml, shifted to 43°C for 30 min, and incubated at 37°C until lysis. Cell debris was removed by centrifugation. The titer of the stocks obtained was $>10^{10}$ PFU/ml. This lysate was used to infect strain X9301 as follows. Cells were grown in LB containing 2.5 mM $CaCl_2$ and 5 mM $MgSO_4$ to 2×10^8 cells per ml and concentrated fivefold in the same medium. Phage were added at a multiplicity of infection of 0.1 to 0.5 and allowed to adsorb at 30°C for 30 min. The infected cells were diluted 1:10 with LB and grown for 30 min at 30°C to allow phenotypic expression. Samples were plated on LB-ampicillin plates (1); 36,000 Ap^r colonies were obtained. The colonies were suspended in LB-Ca, and P1 was grown on the mixed population of Ap^r colonies. The P1 lysate was used to transduce a *purA* strain (X9209) to Pur^+. 2698 Pur^+ colonies were screened for Ap^r. Ten Ap^r colonies were obtained and screened for loss of Hfl activity. Two of them were Hfl^-. Both are blue on X-gal plates. DNA from one of them was used for Southern blot analysis.

Tn*1000* mutagenesis. Tn*1000* (γδ) insertions in pR34 were isolated as described by Guyer (15) and Sancar and Rupp (32). The donor (strain E8038 containing F'128 and pR34) and the recipient (strain WA8065, which carries an *hflA1* mutation) were grown to 2×10^8 cells per ml. Recipient cells were concentrated four- to fivefold. Equal volumes of donor and recipient were mixed and incubated at 37°C for 120 min; 0.1 ml of a 100- and 1,000-fold dilution of the mating mix was plated on M63-carbenicillin-streptomycin plates to select for Cb^r Sm^r transconjugants. These were screened for loss of Hfl complementing activity. Plasmid DNA was isolated from Hfl^- transconjugants, and the position and orientation of the Tn*1000* inserts were determined by restriction enzyme analysis.

Maxicells and protein electrophoresis. Labeling of plasmid proteins in maxicells was as described by Sancar et al. (29, 33) and T. Ogawa (personal communication). Cells were grown to a density of approximately 2×10^8 cells per ml in M9 medium supplemented with Casamino Acids and tryptophan and containing 250 μg of carbenicillin per ml. Cells (5 ml) were irradiated with a germicidal UV lamp at a height of 45 cm, approximately 10 times the λ induction dose. D-Cycloserine (200 μg/ml) was added 1 h after UV irradiation, and the irradiated cells were incubated overnight at 37°C. Cells were suspended in 1/2 volume of M9 without $MgSO_4$ and without Casamino Acids but containing $MgCl_2$. Incubation was continued for 1 h at 37°C. Then 25 to 100 μCi of [^{35}S]methionine (>800 Ci/mmol; New England Nuclear Corp.) per ml was added, and the cells were incubated for 1 h at 37°C. Labeled cells were washed and suspended in 0.2 ml of 50 mM Tris hydrochloride (pH 8)–15 mM EDTA. Then 30 μl of lysozyme (5 mg/ml) in 50 mM Tris hydrochloride (pH 8) was added, and the cells were subjected to freezing and thawing in a dry ice-ethanol bath and then directly suspended in lysing solution (10% glycerol, 5% β-mercaptoethanol, 2% sodium dodecyl sulfate, 0.001% bromophenol blue, 0.0625 M Tris hydrochloride [pH 6.8]). Proteins were denatured by boiling for 2 min at 100°C. Samples of 20 μl (10^4 to 10^5 cpm) were run in 12.5% polyacrylamide–sodium dodecyl sulfate gels (23). The gels were stained with Coomassie blue R250, treated with En^{3}Hance (New England Nuclear) for fluorography, dried, and exposed to Kodak X-Omat AR2 film.

DNA preparation. Chromosomal DNA was prepared (24) by suspending 100 ml of a saturated culture in 4 ml of 10 mM Tris hydrochloride (pH 8)–1 mM EDTA, followed by the addition of 0.5 ml of 10% sodium dodecyl sulfate–0.5 ml of 5 M NaCl and then overnight treatment with proteinase K (500 μg/ml) at 37°C. The mixture was extracted three times with phenol-chloroform-isoamyl alcohol, followed by extensive dialysis against 10 mM Tris hydrochloride (pH 8)–1 mM EDTA. RNase (100 μg/ml) was added, and the solution was incubated at 37°C for 1 h and then phenol extracted once and dialyzed against the same buffer as above. Sodium acetate (0.1 volume, 3 M) was added, and the DNA was spooled on a glass rod and suspended in 10 mM Tris hydrochloride (pH 8)–1 mM EDTA. For Southern blot analysis, after proteinase K treatment, the solution was adjusted to a density of 1.7 g/ml with cesium chloride and centrifuged overnight in a Beckman L-8 ultracentrifuge in a VTi50 rotor at 45,000 rpm. The DNA was collected from the gradient by puncturing the

TABLE 1. *E. coli* strains

Strain	Genotype and relevant markers	Source or reference
CAG5050	F′ *pro lacZ8305*::Mu cts62/Mu dX (*pro lac*)	C. Gross (1)
E8037	*pro-82 thi-1 endA1 hsdR17 supE*/F′128	MM294A of Elledge and Walker (12)
E8038	E8037(pR34)	This study
QR9257	*recA99*(Am) *trp uvrA rpsL*	N1790 of T. Ogawa
QR9259	QR9257(pS8)	This study
QR9260	QR9257(pR34	This study
QR9261	QR9257(pFB509)	This study
QR9262	QR9257(pFB510)	This study
QR9263	QR9257(pR34 *hflC3*::Tn*1000*)	This study
QR9264	QR9257(pR34 *hflC6*::Tn*1000*)	This study
QR9265	QR9257(pR34 *hflK7*::Tn*1000*)	This study
QR9266	QR9257(pR34 *hflK10*::Tn*1000*)	This study
QR9267	QR9257(pR34 *hflK11*::Tn*1000*)	This study
QR9268	QR9257(pR34 *hflC15*::Tn*1000*)	This study
QR9269	QR9257(pR34 *hflC17*::Tn*1000*)	This study
QR9270	QR9257(pR34 *hflC1*::Tn*1000*)	This study
QR9271	QR9257(pBR322)	This study
QR9272	QR9257(pR34 *hflA12*::Tn*1000*)	This study
QR9273	QR9257(pR34 *hflK13*::Tn*1000*)	This study
QR9274	QR9257(pR34 *hflK16*::Tn*1000*)	This study
QR9278	QR9257(pFB40)	This study
WA8067	F⁻ *ser lys trp ilv leu rpsL*	UC4185 of Belfort and Wulff (3)
WA8065	F⁻ *ser lys trp ilv leu rpsL hflA1*	UC2014 of Belfort and Wulff (3)
WA8131	F⁻ *thy purA*::Tn*5*	CBK192 of C. Berg
WA8136	F⁻ *proB thy lacZ*::Tn*5*	1162-43 of D. Berg
WA8211	WA8065(pR34 *hflC1*::Tn*1000*)	This study
WA8212	WA8065(pR34 *hflC2*::Tn*1000*)	This study
WA8213	WA8065(pR34 *hflC3*::Tn*1000*)	This study
WA8214	WA8065(pR34 *hflC4*::Tn*1000*)	This study
WA8215	WA8065(pR34 *hflC5*::Tn*1000*)	This study
WA8216	WA8065(pR34 *hflC6*::Tn*1000*)	This study
WA8217	WA8065(pR34 *hflK7*::Tn*1000*)	This study
WA8218	WA8065(pR34 *hflK8*::Tn*1000*)	This study
WA8219	WA8065(pR34 *hflK9*::Tn*1000*)	This study
WA8220	WA8065(pR34 *hflK10*::Tn*1000*)	This study
WA8221	WA8065(pR34 *hflK11*::Tn*1000*)	This study
WA8222	WA8065(pR34 *hflK12*::Tn*1000*)	This study
WA8223	WA8065(pR34 *hflK13*::Tn*1000*)	This study
WA8224	WA8065(pR34 *hflC14*::Tn*1000*)	This study
WA8225	WA8065(pR34 *hflC15*::Tn*1000*)	This study
WA8226	WA8065(pR34 *hflK16*::Tn*1000*)	This study
WA8227	WA8065(pR34 *hflC18*::Tn*1000*)	This study
WA8228	WA8065(pR34 *hflC17*::Tn*1000*)	This study
X9204	*purA45*	ES4 of E. Siegel (4)
X9205	*hflA1*	Pur⁺ transductant of X9204 with P1 grown on WA8065
X9209	*purA45 lacZ*::Tn*5*	Km^r transductant of X9204 with P1 grown on WA8136
X9246	*hflA1 purA*::Tn*5*	Km^r transductant of X9205 with P1 grown on WA8131
X9283	X9246(pFB500)	This study
X9284	X9246(pFB501)	This study
X9285	X9246(pFB502)	This study
X9286	X9246(pFB503)	This study
X9287	X9246(pFB504)	This study
X9288	X9246(pFB505)	This study
X9290	X9246(pR34)	This study
X9292	X9246(pFB40)	This study
X9301	*lacΔX74* (*lacIPOZY*)	M182 of M. Casadaban
X9304	*araD* Δ(*lac*)*U169 proC*::Tn*5*	SM103 of J. Beckwith
X9313	X9246(pS8)	This study
X9314	X9304(pFB509)	This study
X9315	X9304(pFB510)	This study
X9474	X9209 *hflK*::Mu dX	This study
X9520	X9301 *hflK*::Mu dX	Ap^r transductant of X9301 with P1 grown on X9474

side of the tube with a syringe and was dialyzed against 10 mM Tris hydrochloride (pH 8)–1 mM EDTA.

Plasmid DNA for cloning was isolated as described previously (36, 37). Quick screening of plasmid DNA for restriction enzyme analysis was performed by the procedure of So and Heffron (M. So and F. Heffron, personal communication).

Plasmid construction. Chromosomal DNA from strain WA8067 was partially digested with restriction endonuclease *Bam*HI and ligated into *Bam*HI-digested pBR322 in a mass

TABLE 2. Plasmids used in these studies

Plasmid	Comments	Hfl[a]	PurA[b]
pFB501	18-kb *Bam*HI fragment	+	+
pFB503	6.0-kb *Bam*HI-*Hin*dIII fragment	+	−
pFB504	2.0-kb *Sal*I-*Eco*RI fragment	−	−
pFB505	4.5-kb *Pvu*II-*Eco*RI fragment	+	−
pFB40	5.0-kb *Bam*HI-*Eco*RI fragment	+	−
pR34	3.5-kb *Pvu*II-*Eco*RI fragment from pFB503	+	−
pS8	Inactivation of *Sal*I site of pR34	−	−
pFB509	Insertion of 3-kb *lacZ* fragment at *Sal*I site of pR34 (*hflC'-lacZ-'hflC*)	−	−
pFB510	Same as in pFB509, but in the opposite orientation	−	−

[a] *hflA1* complementing activity of the plasmids was determined in strain X9246 or WA8065.

[b] *purA* complementing activity was determined in strain X9246. For further details see Fig. 2.

ratio of 5:1 to 10:1 with T4 DNA ligase (New England BioLabs) at 16°C for 12 h. The clone bank (containing >90% inserts) was used to transform an *hflA1 purA*::Tn*5* strain (X9246) by the procedure of Cohen et al. (8). Cb^r transformants were selected and screened for Tc^s as well as for the ability to complement the *purA*::Tn*5* allele of strain X9246. Pur$^+$ colonies were then screened for the ability to complement the *hflA1* mutation of strain X9246 (see above). Plasmid DNA from 14 Pur$^+$ Hfl$^+$ colonies was digested with *Bam*HI. All except one contained an identical 18-kilobase [kb] fragment; the exceptional plasmid had an additional 4-kb fragment. Two plasmids, pFB500 and pFB501, containing the 18-kb fragment in opposite orientation (Fig. 2A) were subjected to further analysis (Table 2). Deletion of a *Hin*dIII fragment from each of these plasmids gave rise to pFB502 and pFB503, respectively. Since only pFB503 contains *hflA1* complementing activity, it was used to generate the following plasmids (Fig. 2B; Table 2): a *Sal*I deletion, pFB504; a *Pvu*II deletion, pFB505; an *Eco*RI deletion, pFB40. pFB505 was then deleted with *Eco*RI to generate pR34.

The *Sal*I site of pR34 was inactivated by digestion with *Sal*I, filling in the staggered ends with the Klenow fragment of DNA polymerase (New England Biolabs) at 37°C, and ligation with T4 DNA ligase. The mixture was used to transform strain WA8065, and Cb^r transformants were screened for loss of Hfl complementing activity. Plasmid DNA from candidates was screened for loss of the *Sal*I site. One such plasmid was designated pS8 (Table 2). The mutation it contains is designated *hflA-S8*. Inactivation of the *Sal*I site in this way resulted in generation of a *Pvu*I site as expected (data not shown).

pFB509 and pFB510, which contain inserts of *lacZ* into *hflC*, were constructed by digestion of pR34 and pMC1871 (7) with *Sal*I, ligation of the digests, and transformation of strain WA8065 with selection for blue Cb^r transformants on LB–carbenicillin–X-gal plates. Plasmid DNA from blue Cb^r transformants was screened for the presence of the 3-kb *Sal*I *lacZ*-containing fragment. A plasmid with the same fragment but in the opposite orientation was isolated from a pale blue Cb^r transformant. WA8065 containing either of these two plasmids is phenotypically Hfl$^-$ (Table 2). The *lacZ* open reading frame is read from left to right in pFB509 and from right to left in pFB510 (as the *hfl* locus is drawn in Fig. 2B). The mutations in these two plasmids are designated *hflA509* and *hflA510*, respectively.

Electrophoresis of DNA and Southern blotting. Approximately 1 μg of DNA to be analyzed was digested with the appropriate restriction enzyme in a total volume of 50 μl at 37°C for several hours and subject to electrophoresis on a horizontal (25- by 13-cm) 0.7% agarose gel in Tris-borate buffer, at 50 V for 14 to 16 h. λ DNA digested with *Hin*dIII was used as a molecular weight standard. Gels were stained with 1 μg of ethidium bromide per ml and photographed. The DNA in the gels was denatured and blotted onto nitrocellulose paper (Schleicher & Schuell Co.; BA85) by the method of Southern (35). Filters were washed with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and then vacuum dried for 2 h at 80°C. They were then soaked in hybridization buffer for a few minutes, and the solution was replaced with fresh hybridization buffer containing 10^5 to 10^6 cpm of labeled denatured probe, sealed in a Seal-a-Meal bag, and incubated at 65°C overnight with gentle shaking. Filters were then rinsed with 2× SSC–1% sodium dodecyl sulfate at

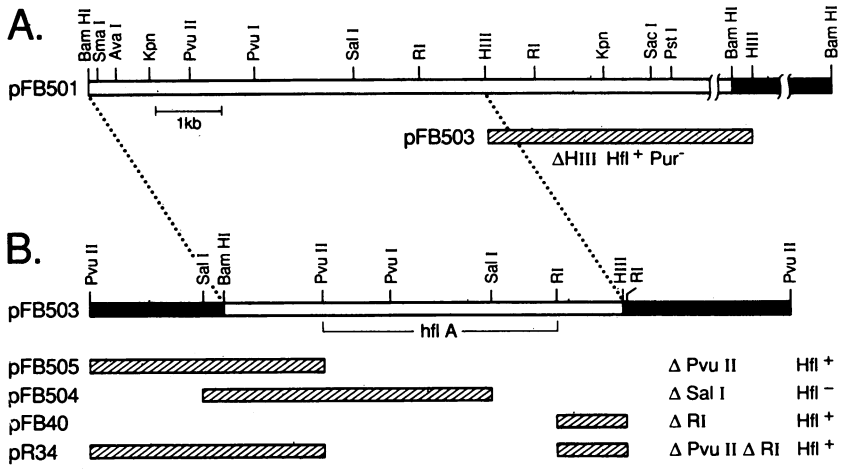

FIG. 2. Restriction endonuclease map of the *hflA* region of *E. coli*. (A) Partial restriction map of the 18-kb *Bam*HI DNA insert in pFB501. pFB500 contains the same fragment in the opposite orientation (data not shown). The 18-kb fragment contains both *purA*::Tn*5* and *hflA1* complementing activities. A *Hin*dIII deletion generates pFB503. This plasmid contains only *hflA* complementing activity. (B) Different plasmids obtained by deletion of different segments of pFB503. Their *hflA* complementing activity is shown to the right. Symbols: ▨, deleted portion in each of the plasmids; ▬, vector sequences.

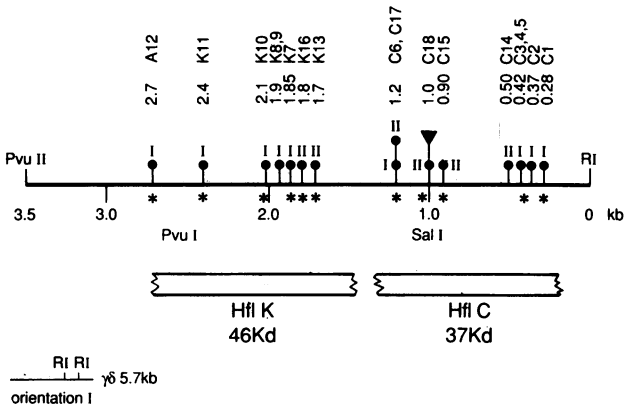

FIG. 3. Physical position and orientation of Tn*1000* inserts in the *hflA* region of pR34 and boundaries of the *hflA* gene products. The line represents the 3.5-kb *Pvu*II-*Eco*RI fragment in pR34. The position and orientation of the Tn*1000* inserts, obtained as described in the text, were determined by digestion with *Eco*RI. (●) Represents the position of the Tn*1000* insertions in pR34. I and II represent the two possible orientations of Tn*1000*. I is shown in the inset. The numbers above the position of the Tn*1000* indicate the point of insertion (in kilobases) with respect to the *Eco*RI site (0 kb) in pR34. The allele number is indicated above the coordinates for each insertion. (▼) represents the 3-kb *Sal*I *lacZ*-containing fragment in pFB509 and pFB510 (see Materials and Methods). (*) shows plasmids containing the indicated insert whose proteins were analyzed by maxicells. The blocks represent the region inferred to encode the two polypeptides, C and K, as determined from maxicell analysis (Fig. 4 and 5). The boundaries of these regions are set by the Tn*1000* insertions.

45°C and then with 3 mM Tris hydrochloride (pH 8) at room temperature. Dried filters were autoradiographed for 24 to 48 h with Kodak XR-5 film with a Du Pont 1 Lightning-Plus intensifying screen.

Preparation of ^{32}P-labeled DNA. The probe was prepared by nick translation of pFB40 with a New England Nuclear nick translation kit. The nick-translated DNA was separated from unincorporated deoxynucleoside triphosphates by chromatography through a P-6 column equilibrated with 0.1 M NaCl–10 mM Tris hydrochloride (pH 8)–1 mM EDTA. The labeled DNA was denatured by heating for 10 min at 90 to 100°C followed by rapid cooling in an ice-water bath.

RESULTS

Isolation of plasmids containing the *hflA* locus. *hflA* and *purA* are very tightly linked by P1 transduction (13). The average frequency of cotransduction with different *hflA* alleles is 92% (13). Thus, we expected that if we isolated DNA fragments containing *purA* complementing activity, some of them might also contain *hflA*. Total chromosomal DNA from an *hflA*$^+$ *purA*$^+$ strain (WA8067) was partially digested with *Bam*HI and ligated to *Bam*HI-digested pBR322. The clone bank (containing >90% inserts) was used to transform an *hflA1 purA*::Tn*5* strain (X9246). Cb^r transformants were screened for the ability to complement the *purA*::Tn*5* mutation by replica plating onto minimal medium lacking adenine (see Materials and Methods). Fourteen Pur$^+$ colonies were obtained and examined for *hflA1* complementing activity as described in Materials and Methods and previously (4). All 14 Pur$^+$ colonies were phenotypically Hfl$^+$. Plasmid DNA was extracted from these colonies. Restriction enzyme digestion showed that 13 of the 14 plasmids contained an identical 18-kb *Bam*HI insert present in either of two possible orientations; the remaining plasmid carried an additional 4-kb *Bam*HI fragment. For further analysis, we chose two plasmids with 18-kb inserts, pFB500 and pFB501, which have the insert in opposite orientations.

Subcloning the *hflA* region. A restriction map of the 18-kb *Bam*HI fragment in pFB501 is shown in Fig. 2A. Deletion of a *Hin*dIII fragment from pFB500 and pFB501 provided a convenient way of delimiting the *hflA* complementing activity. The deleted plasmids were used to transform an *hflA1 purA*::Tn*5* strain. Cb^r transformants obtained after transformation with the pFB501 derivative (pFB503) were Pur$^-$ and Hfl$^+$ (Table 2), and those from the pFB500 derivative (pFB502) were Pur$^-$ Hfl$^-$. The structure of these plasmids was confirmed by restriction enzyme analysis after isolating plasmid DNA from both kinds of transformants (data not shown). These observations indicate that *hflA* is located to the left of the *Hin*dIII site of the insert in pFB501 (Fig. 2A) and that *purA* is most likely interrupted by this site, since in both deletions PurA activity is lost.

pFB503 was used for further deletion analysis. The smallest fragment capable of complementing the *hflA1* mutation was obtained by a series of deletions of pFB503 (Fig. 2B) with restriction enzyme sites (see Materials and Methods). Neither a *Pvu*II deletion (pFB505) nor an *Eco*RI deletion (pFB40) abolished Hfl complementing activity (Fig. 2B). A plasmid carrying both such deletions (pR34) likewise was Hfl$^+$. A *Sal*I deletion (pFB504), on the other hand, resulted in loss of this activity. Moreover, in vitro inactivation of the *Sal*I site (in plasmid pS8; see Materials and Methods) or insertion of a 3-kb *Sal*I *lacZ*-containing fragment at this site (to form plasmids pFB509 and pFB510) resulted in loss of *hflA1* complementing activity. (For a summary of these plasmids see Table 2.) Thus, *hflA* most likely spans the *Sal*I site and lies somewhere in the *Pvu*II-*Eco*RI fragment of pR34 (Fig. 2B). Scarcity of restriction sites precluded further delimitation of the *hflA1* complementing activity by this approach. More precise location of *hflA* was obtained by use of Tn*1000* transposon mutagenesis.

Tn*1000* mutagenesis. The boundaries of *hflA* were determined by Tn*1000* (γδ) (15) transposon mutagenesis (32) of pR34. The source of the Tn*1000* was F'*lac* factor F'128, and the target *hflA* plasmid was pR34.

A strain containing F'128 was transformed with pR34 as described in Materials and Methods. The resulting strain [F'128(pR34)] was mated to an *hflA1* strain (WA8065), and Cb^r Sm^r transconjugants were selected. The recipient strain carried an *hflA* mutation to determine whether the *hflA* gene on the transferred plasmid had been disrupted by an insert. Of 90 Cb^r Sm^r transconjugants screened for loss of *hflA* activity by the cross-streak test with λ *c*17 (see Materials and Methods), 19 were phenotypically Hfl$^-$. These Hfl$^-$ transconjugants contained plasmids that had acquired the 5.7-kb DNA segment corresponding to Tn*1000*. The position and orientation of the Tn*1000* insert in each of them were determined by digestion with *Eco*RI. Tn*1000* contained two *Eco*RI sites positioned near one end (inset in Fig. 3), which allowed mapping of the insert within a plasmid. The 19 inserts were distributed over the *Pvu*II-*Eco*RI fragment and occurred in both orientations (Fig. 3). Since all 19 inserts conferred an Hfl$^-$ phenotype and since they spanned a 2.4-kb region, we concluded that the minimum size of the *hflA* locus is 2.4 kb.

Identification of HflA polypeptides. Identification of the HflA polypeptides was carried out with maxicells (29, 33; T. Ogawa, personal communication). pR34 and its derivatives,

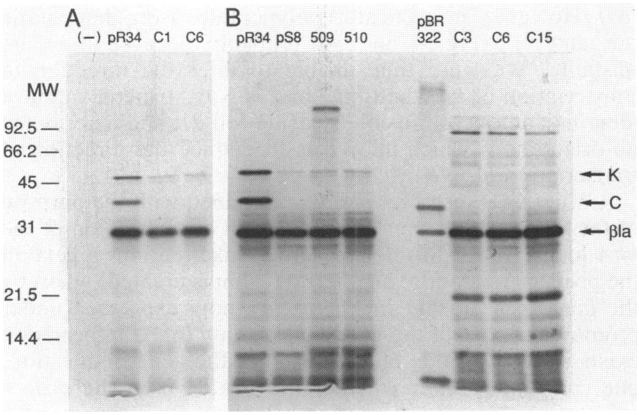

FIG. 4. Identification of the *hflA* polypeptides and phenotype of mutations that define the *hflC* region. The *hflA* plasmid pR34 and its derivatives were introduced into a *recA*(Am) *uvrA* strain (QR9257). The polypeptides produced by these plasmids were labeled with [^{35}S]methionine in maxicells as described in Materials and Methods and analyzed on a 12.5% sodium dodecyl sulfate–polyacrylamide gel. Gels were treated with En^{3}Hance for fluorography, dried, and autoradiographed. (A) Lanes (left to right): 1, cells without a plasmid; 2, pR34; 3, insert C1 (*hflC1*::Tn*1000*); 4, insert C6 (*hflC6*::Tn*1000*). The lanes shown in this panel are all from the same autoradiograph. (B) Lanes (left to right): 1, pR34; 2, pS8; 3, pFB509; 4, pFB510; 5, pBR322; 6, insert C3 (*hflC3*::Tn*1000*); 7, insert C6 (*hflC6*::Tn*1000*); 8, insert C15 (*hflC15*::Tn*1000*). Molecular mass standards (Bio-Rad Laboratories) are shown on the left margin: phosphorylase B (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

which carry Tn*1000* or the *Sal*I fill-in mutation (*hflA-S8*) or the 3 kb *Sal*I *lacZ* insertions (*hflA509* and *hflA510*), were introduced into a maxicell strain (QR9257) as described in Materials and Methods. Labeling of plasmid-encoded proteins, fractionation on sodium dodecyl sulfate-polyacrylamide gels, and autoradiography of the gels are described in Materials and Methods. Figures 4 and 5 show the results of our analysis. Three major polypeptides of 28, 37, and 46 kDa were labeled in cells containing pR34 (Fig. 4A). The 28-kDa band was produced by pBR322 (Fig. 4B) and corresponds to β-lactamase (30). The other two polypeptides must be encoded by the *hflA* locus, which spans most of the *Pvu*II-*Eco*RI region as described above. We designated the 46-kDa band as K and the 37-kDa band as C. That these two polypeptides are encoded by *hflA* was confirmed by analysis of the polypeptides produced by plasmids containing γδ inserts and by plasmids with mutations generated in vitro (Fig. 4 and 5). We first discuss the effects of γδ insertions on synthesis of K and C.

Inserts that abolish synthesis of C. Analysis of the polypeptides produced from plasmids containing Tn*1000* inserts C1, C3, C6, C15 (Fig. 4), and C17 (Fig. 5A) indicates that these inserts result in loss of the 37-kDa C polypeptide. These inserts span the region from 0.28 kb (the position of insert C1) to 1.2 kb (insert C6) of pR34 (Fig. 3). In addition to eliminating the 37-kDa band, inserts C1, C6, C15, and C17 resulted in a marked reduction in the intensity of the 46-kDa band; insert C3 completely eliminated the 46-kDa band (Fig. 4B). Inserts C6 and C17 defined the left boundary of the C region and insert C1 defined the right boundary.

Inserts that abolish synthesis of K. Tn*1000* inserts K10, K11, K13, and K16 (Fig. 5A) resulted in disappearance of the 46-kDa K polypeptide. Similar observations have been made for insert K7 (data not shown). These inserts span the region from 1.7 to 2.4 kb of pR34 (Fig. 3). In addition to eliminating the 46-kDa band, these inserts also caused a marked reduction in the intensity of the 37-kDa band: inserts K13 and K16 resulted in greater reduction than inserts K10 and K11 (Fig. 5A). Overloading the gel with extracts of strains containing plasmids with inserts K13 and K16 showed that the 37 kd band was indeed present, albeit at a low level (Fig. 5B). Insert K11 defined the left boundary of the K region, and insert K13 defined the right boundary.

We thus conclude that the Tn*1000* inserts fall into two major classes: those that inactivate the K polypeptide and reduce C and those that inactivate the C polypeptide and reduce K. In other words, inserts in region K exert a polar effect on C and vice versa. Explanations are considered in the Discussion.

Because mutations other than Tn*1000* inserts exist for the 0.28 to 1.2-kb interval of pR34 (the C region), we asked whether these other mutations lead to the same polypeptide pattern or whether the observed pattern (absence of C, reduction of K) is a property of the γδ inserts. Therefore, we also examined the polypeptides produced from plasmids containing a *Sal*I fill-in mutation (*hflA-S8* in plasmid pS8) or a *lacZ* insertion at the *Sal*I site of pR34 (mutations *hflA509* and *hflA510* in plasmids pFB509 and pFB510, respectively). The *Sal*I fill-in mutation (*hflA-S8* in pS8 led to a polypeptide pattern similar to that of Tn*1000* inserts C6 and C15 (Fig. 4B), which flank the *Sal*I site. The same was true for strains containing plasmids with a *lacZ* insert at the *Sal*I site (Fig. 4B).

The protein pattern observed for insert A12 (Fig. 5A) is puzzling. Even though it was chosen because it leads to a defect in HflA activity, it produced both C and K polypeptides in what appeared to be normal levels. This insert was located to the left of insert K11 (Fig. 3), that is, outside the group of five inserts (K10, K11, K13, K16, K7) that eliminated the K polypeptide band and thus clearly defined the K region. One possible explanation of why insert A12 leads to an Hfl$^-$ phenotype even though normal amounts of K and C

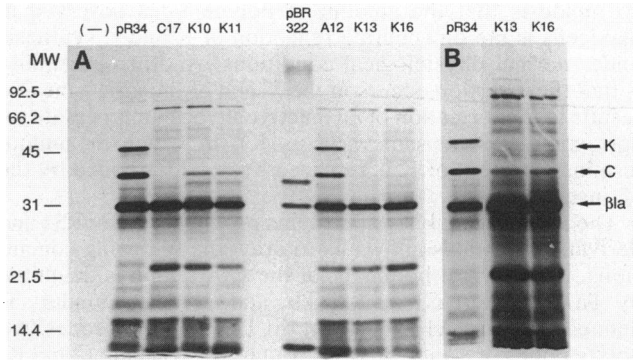

FIG. 5. HflA polypeptides and phenotype of mutations that define the *hflK* region. The *hflA* plasmid pR34 and its derivatives were introduced into a *recA*(Am) *uvrA* strain (QR9257). The polypeptides produced by these plasmids were labeled, fractionated, and autoradiographed as for Fig. 4. (A) Lanes (left to right): 1, no plasmid; 2, pR34; 3, insert C17 (*hflC17*::Tn*1000*); 4, insert K10 (*hflK10*::Tn*1000*); 5, insert K11 (*hflK11*::Tn*1000*); 6, pBR322; 7, insert A12 (*hflA12*::Tn*1000*); 8, insert K13 (*hflK13*::Tn*1000*); 9, insert K16 (*hflK16*::Tn*1000*). (B) Lanes (left to right): 1, pR34; 2, insert K13 (*hflK13*::Tn*1000*); 3, insert K16 (*hflK16*::Tn*1000*). The lanes shown in this panel are all from the same autoradiograph. Molecular mass markers were as for Fig. 4.

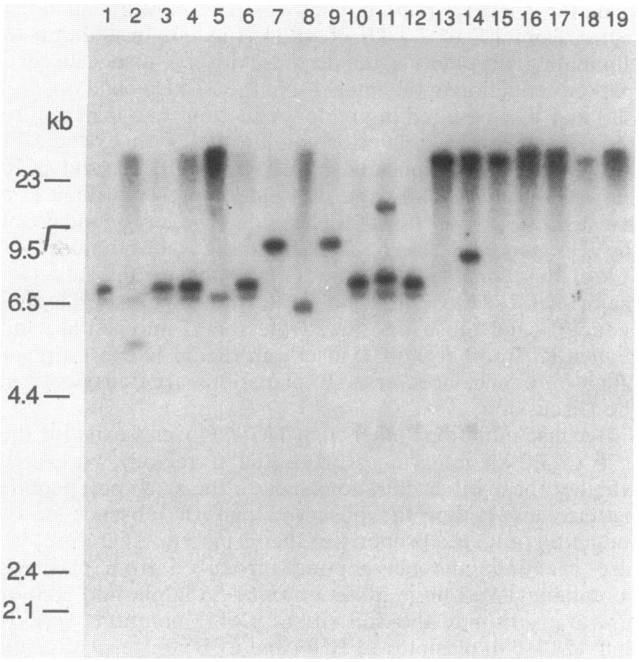

FIG. 6. Determination of physical position and orientation of *hflK*::Mu dX by Southern hybridization. Chromosomal DNA from strains without (X9301, WA8067) and with Mu dX (X9520) was cleaved with the restriction enzymes indicated below, fractionated on a 0.7% agarose gel, transferred to nitrocellulose paper as described in Materials and Methods, and hybridized with ^{32}P-labeled pFB40. Lanes: 1, 4, 7, 10, 13, 16, X9301 (*hflA*$^+$); 3, 6, 9, 12, 15, 18, WA8067 (*hflA*$^+$); 2, 5, 8, 11, 14, 17, 19, X9520 (*hflK*::Mu dX). Restriction enzymes in lanes: 4 through 6, *Kpn*I; 1 through 3, *Kpn*I-*Sac*I; 7 through 9, *Sma*I-*Sac*I; 10 through 12, *Kpn*I-*Pst*I; 13 through 15, *Pst*I; 16, 17, *Sac*I; 18, 19, *Sma*I. The fainter invariant band below the 6.7-kb band in lanes 1 through 6 and 10 through 12 is most likely due to hybridization of the probe with a *Kpn*I fragment extending from the *Kpn*I site (near the *Bam*HI site) leftward, beyond the *Bam*HI site (see Fig. 7). The size markers shown on the left are λ fragments generated by digestion with *Hin*dIII.

are made is that the amount of polypeptides observed in maxicells is not an accurate reflection of K and C synthesis under normal physiological conditions. Another possibility is that the insertion occurs in the 5′ end of the *hflK* gene and results in the formation of an inactive, hybrid polypeptide (of approximately the same size as K), in which an amino-terminal segment of K is replaced by residues coded by the γδ insert element.

The maxicell analysis of proteins produced from pR34 and derivative plasmids allows us to draw the following conclusions. (i) The right boundary of the *hflA* region is delimited by Tn*1000* insert C1 at 0.28 kb, and the left boundary is defined by insert A12 at 2.7 kb. (ii) This region encodes two polypeptides of 37 and 46 kDa, which we designate C and K, respectively. Since the inserts (with the exception of A12) fall into two classes (that inactivate either C or K) and since all the inserts have an Hfl$^-$ phenotype, we conclude that the *hflA* locus consists of two genes, *hflC* and *hflK*, encoding polypeptides C and K, respectively.

Physical mapping of *hfl*K::Mu dX and direction of transcription of *hflK* and *hflC*. We had hoped that the protein analysis of plasmids with Tn*1000* inserts would allow us to infer the direction of transcription of *hflA* because some inserts were expected to lead to production of truncated polypeptides. Such observations have been made by others (31). However, no truncated polypeptides were detected in our analysis (Fig. 4 and 5), probably because they are unstable. We were thus unable to infer the direction of transcription of *hflK* and *hflC* in this way. Instead we have identified an operon fusion by using Mu dX and determined its orientation, which allows us to deduce the direction of transcription of *hflK*.

A Mu dX (1) insert in *hflK* was obtained with the purpose of facilitating studies on the regulation of expression of the *hflA* locus. When Mu dl(Ap^r *lac*) is inserted into a gene in the proper orientation, an operon fusion is created, whereby the *lac* genes present in Mu dl are now expressed under promoter control of the gene under study (6). If information exists as to the mode of regulation of the gene in question, one can readily infer whether the Mu dl is in the proper orientation. If such information is lacking (as is the case for *hflA*), the correct orientation of the insert can be determined by Southern blotting.

A Mu dX insert that is 70% cotransducible with *purA* by P1 and confers an Hfl$^-$ phenotype was isolated as described in Materials and Methods. Because this strain is Hfl$^-$ and forms blue colonies on X-gal plates, we assumed that the insert was within *hflA* and in the correct orientation to place the *lac* genes under control of some promoter, presumably the promoter for *hflA*.

The orientation and location of the insert were determined as follows. Total chromosomal DNA from strains with and without the insert was isolated (see Materials and Methods) and digested with various restriction endonucleases. The fractionated DNA was probed with ^{32}P-labeled nick-translated pFB40 (Table 2) DNA (Fig. 6). The restriction map in Fig. 7 shows that *hflA* is in a 6.7-kb *Kpn*I fragment. Since there are no *Kpn*I sites in Mu dX (26), a Mu dX insert in this fragment is expected to eliminate the 6.7-kb fragment and produce a high-molecular-weight DNA band. Indeed, this is what we observed (Fig. 6, compare lane 5 with lanes 4 and 6), suggesting that Mu dX is in the 6.7-kb *Kpn*I fragment. A *Kpn*I-*Sac*I double digest of DNA from strains without the insert produced a 6.7-kb fragment (Fig. 6, lanes 1 and 3), which was absent in strains with the insert; in its place appeared a 5-kb fragment (Fig. 6, lanes 2). Mu dX had a single *Sac*I site approximately 3.0 kb from one of its ends (Fig. 7). This result indicates that Mu dX is located approximately 2.1 kb to the right of the *Kpn*I site (as shown in Fig. 7), with the *lac* genes nearest this site. A *Kpn*I-*Pst*I double digest supports this conclusion. A single 6.7-kb fragment was generated in the DNA without the insert (Fig. 6, lanes 10 and 12), which was replaced by two fragments, 11.5 and 6.8 kb, in the DNA with the insert (Fig. 6, lane 11). Mu dX had two *Pst*I sites (Fig. 7): one approximately 9.5 kb from the left end and the other approximately 1.7 kb from the right end. The 11.5-kb fragment indicates that Mu dX is approximately 2 kb to the right of the *Kpn*I site (as shown in Fig. 7), with the *lac* genes near the *Kpn*I site. The 6.8-kb fragment indicates that the insert is to the left of the *Eco*RI site, with the *lac* genes farthest away from this site. Were Mu dX in the opposite orientation, fragments other than 11.5 and 6.8 kb would have been generated. Other single and double digests (Fig. 6) confirmed that Mu dX is located within a 550-base-pair region to the right of the *Pvu*I site (Fig. 7), that is, within *hflK*, and that the orientation is such that the *lac* genes are transcribed rightward (Fig. 7). Therefore, we infer that *hflK* is transcribed rightward.

The direction of transcription of *hflC* was inferred after subcloning the *Pvu*II-*Eco*RI *hflA* containing fragment of pR34 into a p_L plasmid vector. Synthesis of both HflK and

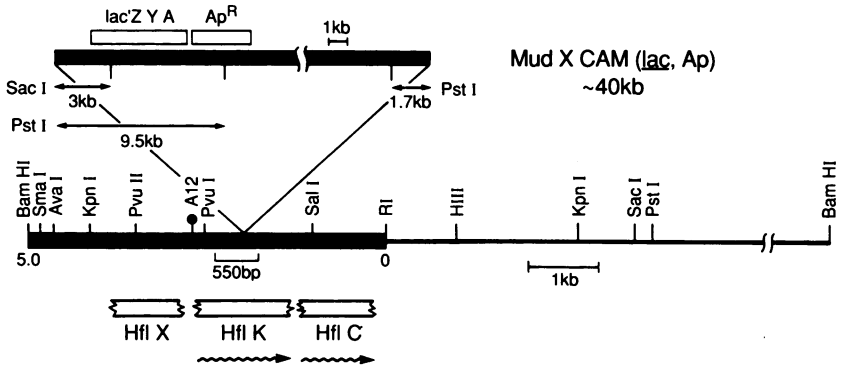

FIG. 7. Physical position of Mu dX in *hflK* and direction of transcription of *hflK*. The lower line represents a restriction endonuclease map of the *hflA* region obtained by Southern blot analysis of restriction enzyme-cleaved chromosomal DNA and by restriction enzyme analysis of pFB501. The probe used was nick-translated pFB40, which contains the *Bam*HI-*Eco*RI fragment shown in the heavy line. The large triangle represents the Mu dX insert in *hflK* as determined by the analysis shown in Fig. 6. The restriction sites within Mu dX that were utilized in this analysis are indicated. (●) represents the leftmost Tn*1000* obtained in pR34 as indicated in Fig. 3. The blocks represent the polypeptides encoded by *hflK*, *hflC*, and *hflX* (see Fig. 8). The wavy arrows indicate the inferred direction of transcription of *hflK* and *hflC*. Whether they are cotranscribed is not known.

HflC was induced when the fragment was oriented with the *Pvu*II site nearest the p_L promoter (A. Hoyt and F. Banuett, unpublished observations). Thus, both *hflK* and *hflC* are transcribed in the same direction, rightward, and most likely constitute a single transcriptional unit.

Identification of *hflX*. The Tn*1000* insertion mutations described above were obtained on plasmid pR34 and define the boundaries of the *hflA1* complementing activity present on this plasmid. Other *hfl* mutations described by Gautsch and Wulff (13) are also complemented by pR34 (F. Banuett, unpublished observation), suggesting that such mutations lie within the *hflA* interval defined thus far. Thus Tn*1000* insertional analysis defines the minimum size of *hflA*.

Work by K. Thomas and I. Herskowitz (unpublished data) suggests that the *hflA* region is larger than that defined by the Tn*1000* inserts. Tn*5* insertions in the bacterial chromosome, which are tightly linked to *purA* and confer an Hfl⁻ phenotype, have been obtained. By Southern blot mapping, it was determined that some of them were inserts in *hflC*, and others were inserts in *hflK*. Surprisingly, some inserts occurred in a region 1 kb upstream of *hflK*, indicating either the existence of another *hfl* gene or the presence of regulatory components important for *hflA* expression in this interval. Therefore, we analyzed by maxicells the polypeptides produced from a plasmid (pFB40; Fig. 1B) containing, in addition to the *Pvu*II-*Eco*RI fragment of pR34, a 1.5-kb region upstream of *hflK*. In addition to the C and K polypeptides, pFB40 also produced a third polypeptide of approximately 50 kDa, which we designate X (Fig. 8). Since Tn*5* insertions in the region upstream of *hflK* conferred an Hfl⁻ phenotype, our finding indicates that the *hflA* locus contains an additional gene designated *hflX*.

DISCUSSION

We have isolated an 18-kb DNA fragment from *E. coli* which complements *hflA* and *purA* mutations (*hflA1*, *purA*::Tn*5*). Deletion analysis of this large fragment led to the identification of a 3.5-kb *Pvu*II-*Eco*RI fragment capable of complementing the *hflA1* mutation. Tn*1000* mutagenesis of this fragment led to a more precise delimitation of the boundaries of *hflA*: Tn*1000* insertions inactivating *hflA* define a 2.4-kb region necessary for *hflA* activity (Fig. 3). Two polypeptides of 37 (C) and 46 (K) kDa are produced from the region delimited by the Tn*1000* inserts. This region contains sufficient informational capacity to encode these two polypeptides. The inserts fall into two main groups that are physically contiguous: those between positions 0.28 and 1.2 kb on pR34 that inactivate the C polypeptide and reduce K, and those between 1.7 and 2.4 kb that inactivate K and reduce C. Thus, we conclude that *hflA* consists of two nonoverlapping genes, *hflC* and *hflK*, coding for the C and K polypeptides, respectively.

hflC and *hflK* are transcribed in the same direction, as inferred from the orientation of a Mu dX insert in *hflK* (Fig. 7) and from the protein pattern obtained after thermal induction of *hflC* and *hflK* when placed under p_L promoter control in either of two possible orientations (A. Hoyt and F. Banuett, unpublished data).

Last, evidence was presented that indicates that the *hflA* locus contains, in addition to *hflC* and *hflK*, a third gene,

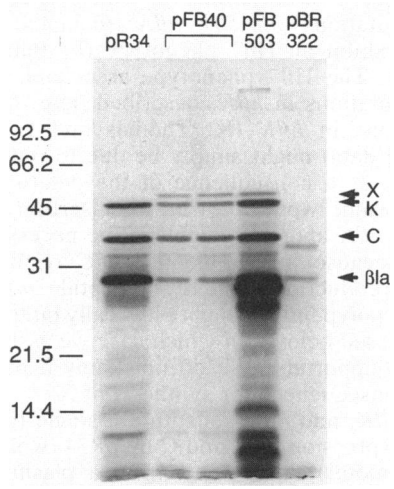

FIG. 8. Identification of the X polypeptide produced from the region upstream of *hflK* in plasmid pFB40. Plasmids pR34, pFB40, and pFB503 were introduced into a *recA*(Am) *uvrA* strain (QR9257). The polypeptides produced by these plasmids were labeled, fractionated, and autoradiographed as for Fig. 4 and 5. Lanes (left to right): 1, pR34; 2, 3, pFB40; 4, pFB503 (Fig. 2B); 6, pBR322. Molecular mass standards are shown on the left.

hflX, coding for a 50-kDa polypeptide. The *hflX* gene lies upstream of *hflK* (Fig. 7).

Nucleotide sequence analysis of the *Bam*HI-*Eco*RI fragment of pFB40 (J. N. Van Arsdell, M. A. Innis, F. Banuett, and I. Herskowitz, unpublished data) shows the presence of three open reading frames which in physical location and coding capacity correspond precisely to the genes defined in the present analysis.

Our studies reaffirm the view that *hflA* and *purA* are close but distinct genes. We have identified a restriction endonuclease site (*Hin*dIII) that inactivates *purA* and is approximately 1.28 kb from the nearest Tn*1000* insert in *hflA*. Thus these two genes lie within 1.28 kb. Insertions into *hflA* (such as *hflA509* and *hflA510*) do not inactivate the *purA* gene; likewise, insertions and deletions in *purA* do not inactivate *hflA* (13).

Complexity of the *hflA* locus. The original studies by Gautsch and Wulff (13) indicated that the *hflA* locus is comprised of a single complementation group; merodiploids containing a mutant *hfl* allele on the episome and different *hfl* alleles on the chromsome resulted in most cases in an Hfl$^-$ phenotype. Some instances of weak complementation were observed, and they were interpreted as intragenic complementation.

Our results demonstrate that the *hflA* locus contains at least two genes, *hflK* and *hflC*, and likely a third as well, *hflX*. Are all of these genes essential for HflA activity? Because our mutants contain insertion mutations, we must consider different possible transcriptional arrangements for the *hflX*, *hflK*, and *hflC* genes. Work described here and the nucleotide sequence of the *hflA* locus (J. N. Van Arsdell, M. A. Innis, F. Banuett, and I. Herskowtiz, unpublished data) show that these three genes are all transcribed in the same direction. If the *hflX*, *hflK*, and *hflC* genes are separately transcribed, we can conclude that all of these genes are essential for HflA function; the product of one gene might be required for expression or stabilization of the others.

If *hflX*, *hflK*, and *hflC* comprise a single transcriptional unit (in the order X-K-C), then we can only decisively conclude that C is essential for HflA function, because insertion mutations (*hflA509*, *hflA510*) in the chromsomal *hflC* gene exhibit an Hfl$^-$ phenotype (F. Banuett, unpublished data). The Hfl$^-$ phenotype associated with Tn*1000* insertion mutations in *hflK*, described here, and also with Tn*5* insertions in *hflK* (K. Thomas and I. Herskowitz, unpublished data) might simply be due to reduced expression of *hflC* as a consequence of the polarity (12) of the insert. The same would be true for insertion mutations in *hflX*. Thus, *hflK* and *hflX* might not be necessary for HflA function. Because insertions into *hflC* result in a great decrease in production of the K polypeptide, we suggest that the K and C polypeptides might physically interact with each other (discussed below), in which case we assume that K is functionally important. We do not know if inserts in *hflC* have any consequences for synthesis of X.

If *hflX*, *hflK*, and *hflC* constitute a single transcriptional unit, then expression of K and C by pR34 (which lacks *hflX* and its promoter) must occur from a plasmid promoter. Another possibility is that there is an additional promoter for *hflK* and *hflC* located between *hflX* and *hflK*.

Polarity and antipolarity: explanations and implications. A striking finding is that insertions in the *hflK* region abolish synthesis of the K polypeptide and greatly reduce synthesis of the C polypeptide, whereas insertions in the *hflC* region abolish synthesis of C and greatly reduce synthesis of K. As noted above, *hflK* and *hflC* are transcribed in the same direction. Whether they are cotranscribed or separately transcribed cannot be determined from our analysis. Assuming for the sake of argument that they are cotranscribed, then, as noted above, the behavior of insertions in *hflK* can be explained as due to polarity on *hflC*. If we accept this view, then the effect of insertions in *hflC* on production of the K polypeptide can be viewed formally as antipolarity. This phenomenon, in which insertions in a downstream gene affect the expression of an upstream gene, has also been observed in some operons of the *Klebsiella pneumoniae nif* regulon (28; reviewed in reference 27). We can imagine several different molecular explanations for this antipolarity. (i) The C polypeptide might be necessary for synthesis of K, for example, for translation of the K mRNA. (ii) The presence of Tn*1000* sequences within the *hflK-hflC* mRNA might destabilize the transcript. Our observation that a small insertion within the *hflC* gene (the mutation created by filling in a *Sal*I site) also exhibits antipolarity provides an argument against the hypothesis that it is Tn*1000* sequences that are responsible for destabilizing the transcript. It is of course possible to invoke more complex models in which translation of the distal region of *hflC* mRNA is necessary to protect against an RNase (a variation on the theme of retroregulation [11]). (iii) Reduction in K caused by the absence of the C polypeptide might reflect physical interaction between K and C polypeptides; individual polypeptides are unstable because they are unable to form a multimeric complex. There is direct evidence in the *nif* regulon of *K. pneumoniae* indicating that the stability of some polypeptides is dependent on the presence of others with which these polypeptides interact (27). Another precedent for dependence of stability of a polypeptide on formation of a multimeric complex with another polypeptide is provided by the fatty acid synthetase multimeric complex of *Saccharomyces cerevisiae* (9, 34).

Functional role of *hflA*. Because cII protein is more stable in the absence of HflA activity, we anticipated that overproduction of the HflA proteins might have the opposite consequences. In other words, λ lysogenization might be reduced in strains carrying an HflA-producing plasmid. In the extreme, wild-type λ might form plaques as clear as a λ *c*II$^-$ mutant. We observe, however, little if any effect of the *hflA*-containing plasmids described in this work on the plaque morphology of wild-type λ. Assuming that these plasmids do, in fact, overproduce HflA polypeptides, these observations suggest that some component other than HflA is limiting for degradation of cII. We anticipate that further study of the *hflX*, *hflK*, and *hflC* genes and the proteins they encode will reveal the way in which *hflA* governs the lysis-lysogeny decision of the phage.

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