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Multiple Defects in *Escherichia coli* Mutants Lacking HU Protein†

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The HU protein isolated from *Escherichia coli*, composed of two partially homologous subunits, α and β, shares some of the properties of eucaryotic histones and is a major constituent of the bacterial nucleoid. We report here the construction of double mutants totally lacking both subunits of HU protein. These mutants exhibited poor growth and a pertubation of cell division, resulting in the formation of anucleate cells. In the absence of HU, phage Mu was unable to grow, to lysogenize, or to carry out transposition.

The *Escherichia coli* HU protein is the most abundant DNA-binding protein present in the bacterial cell (38). This small, basic, dimeric protein interacts with double-stranded DNA, single-stranded DNA, and RNA. As with mixtures of the four core eucaryotic histones, HU can introduce negative supercoils into a relaxed closed circular DNA in vitro if topoisomerase I is present, and the resulting condensed structures resemble nucleosomes (5, 40). In addition, just as the histones in eucaryotes are conserved, the HU protein is highly conserved among procaryotic organisms and also in mitochondria and chloroplasts (10). It was shown earlier that HU is associated with the *E. coli* nucleoid (37, 47) (although some cytological observations were interpreted as locating HU on the edge rather than inside of the nucleoid [11]), but whether it has a role in maintaining the structure of the chromosome as a whole is not known.

Biochemical studies have suggested a role for HU in the site-specific recognition of nucleotide sequences by other proteins (for a review, see reference 10). Among these interactions are the formation of protein-DNA complexes involved in the initiation of replication of the bacterial chromosome at *oriC*, in the *hin*-mediated gene inversion, and in the transposition of transposon Tn*10* and bacteriophage Mu. More precisely, replication from *oriC* (9, 13) and the transposition of Tn*10* (29, 34) are moderately stimulated by HU protein, whereas the flagellar phase variation in *Salmonella typhimurium* (21) and the transposition of bacteriophage Mu (7, 8) cannot be detected in vitro in the absence of HU protein.

In order to better understand the role of HU in vivo, we have initiated a study of mutants defective in this protein. Since it is not obvious what phenotype(s) to expect in HU-negative bacteria and because HU is a heterodimer composed of two closely related subunits (39), it was necessary to mutate the genes coding for both subunits. Imamoto et al. have cloned and mapped *hupB*, the gene encoding the HU-1 subunit, and *hupA*, the gene encoding the HU-2 subunit. They are located at 10 and 90 min on the *E. coli* map, respectively (22–24). We constructed, by gene disruption, double mutants that lack both HU subunits. In the present study, we show that the growth, lysogeny, and transposition of bacteriophage Mu are affected in this double mutant. In addition, we observed that the absence of HU perturbs cell division, causing formation of anucleate cells.

After completion of this work, a paper from Wada et al. appeared describing the construction of similar insertion mutations of *hupB* and *hupA* (49). In general, their results are in agreement with ours and will be discussed later.

MATERIALS AND METHODS

Strains and bacteria. The bacterial strains, plasmids, and phages used in this study are listed in Table 1. Bacteria were grown in LB (10 g of Bactotryptone [Difco Laboratories], 5 g of yeast extract, and 5 g of NaCl per liter) and counted on L agar (LA) (28). Minimal medium was M9 supplemented with thiamine (10 μg/ml), amino acids (100 μg/ml) or casamino acids (0.4%, wt/vol), and 0.4% (wt/vol) sugar (glycerol, maltose, lactose, or glucose). Kanamycin (25 μg/ml), chloramphenicol (12.5 μg/ml), streptomycin (200 μg/ml), and spectinomycin (100 μg/ml) were included when appropriate.

Methods for Mu assays. The general methods used to manipulate the phage have been described previously (6, 12).

(i) Mu lysogenization assay. Serial dilutions of Mu *c*ts62pAp1 and Mu *c*ts62pKm7701pAp1 lysates (10 μl) were spotted on lawns of the bacteria to be tested on LA and LA supplemented with ampicillin (20 μg/ml). The plates were incubated overnight at 30°C. The frequency of lysogenization is given by the number of Ap^r colonies divided by the plaque titer of the lysate.

(ii) Mu transposition assay. The frequency of transposition onto the pUZ8 plasmid was measured by a mating-out assay. The Mu *c*ts62pAp1 lysogens (or the bacteria infected with the bacteriophage λ mini-Mu Ap^r) carrying pUZ8 were mated with the MFG625B recipient on a filter. After incubation for 2 h at 42 or 37°C, the bacteria were separated from the filter and diluted. The Km^r Sp^r transconjugants resulting from the transfer of pUZ8 to the recipient were counted at 37°C on LA supplemented with kanamycin and spectinomycin. The Km^r Ap^r Sp^r transconjugants resulting from the transfer of the Mu *c*ts62pAp1 (or mini-Mu Ap^r) by pUZ8 were counted at 37°C on LA supplemented with kanamycin, ampicillin, and spectinomycin. The frequency of transposition of Mu (or mini-Mu) onto pUZ8 was calculated as the ratio between the number of Km^r Ap^r Sp^r transconjugants and the number of Km^r Sp^r transconjugants.

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† This work is dedicated to our friend Olivier Huisman, who died unexpectedly on 23 March 1988.

TABLE 1. Bacterial strains, plasmids, and phages

Strains, plasmid, or phage	Genotype[a] or derivation	Origin or reference
Strains		
JC7623	*thr leu his arg pro gal ara-14* Δ(*gpt-lac*)*14 xyl mtl rpsL supE44 recB21 rec*C22 *sbcB15 sbcC201*	20
JRY1	*thr leuB6 pro lacY1 thi tonA21 supE44 hsdR*	R. Davis
OHP96	Same as JRY1, but *hupB*::Km^r	This work
OHP109	Same as JRY1, but *hupA*::Cm^r	This work
OHP111	Same as OHP96, but *hupA*::Cm^r	This work
C600	*thr leuB6 lacY1 thi tonA21 supE44*	2
OHP250	Same as C600, but *hupB*::Km^r	This work
OHP251	Same as C600, but *hupA*::Cm^r	This work
OHP252	Same as OHP251, but *hupB*::Km^r	This work
OHP176	*leu-6 pro trp-31 his-1 argG6 lacZ* Δ*r1 xyl-17 mtl tonA2 tsx rpsL104 supE44*	Met$^+$ transductant of NK7254 (34)
OMP177	Same as OHP176, but *hupB*::Km^r	This work
OHP178	Same as OHP176, but *hupA*::Cm^r	This work
OHP190	Same as OHP178, but *hupB*::Km^r	This work
OHP191	Same as OHP178, but *hupB*::Km^r	This work
OHP286	OHP176 lysogenized with λ540 c^+ and carrying pUZ8	This work
OHP287	OHP177 lysogenized with λ540 c^+ and carrying pUZ8	This work
OHP288	OHP178 lysogenized with λ540 c^+ and carrying pUZ8	This work
OHP289	OHP190 lysogenized with λ540 c^+ and carrying pUZ8	This work
OHP292	OHP176 lysogenized with Mu *c*ts62pAp1	This work
OHP293	*hupB*::Cm^r transductant of OHP292	This work
OHP294	*hupA*::Km^r transductant of OHP292	This work
OHP295	*hupA*::Km^r transductant of OHP293	This work
MFG625B	Δ(*lac-pro*) *thi galE rpsE recA* Mu^r λ^r (Mu c^+) (λ imm^{21} *h*80)	This work
Plasmids		
pUC4K	Plasmid containing a gene of resistance to kanamycin	48
pMW1	pBR322 derivative with a *Cla*I-*Bam*HI insert encoding the *hupB* gene	24
pMWKP	pMW1 derivative containing the Km^r gene of pUC4K inserted at the *Pst*I site of *hupB*	This work
pMWKR	pMW1 derivative containing the Km^r gene of pUC4K inserted at the *Eco*RV site of *hupB*	This work
pSVCAT	pBR322 derivative containing the *cat* gene	14
p385	pSVCAT with a *Bam*HI site substituted for the *Hin*dIII site	This work
pK01	pUC9 derivative containing the *hupA* gene	22
p355	pK01 with a *Bgl*II site substituted for the *Kpn*I site	This work
p387	p355 derivative with the *Bam*HI fragment of p385 containing the Cm^r gene inserted in the *Bgl*II site	This work
pUZ8	IncP Tra$^+$ Km^r Tc^r Hg^r	16
Phages		
Mu *c*ts62pAp1		25
Mu *c*ts62pKm7701pAp1		45
λ540 c^+		30
λ540 c^+ *int*-Mu18		
λ540 c^+ *int*-Mu18 *A*.1		46
P1 *vir*		Institut Pasteur collection
P1 *clr*100CM		35

[a] Gene symbols are from Bachmann (3).

Analysis of HU synthesis. *E. coli* C600 and its derivatives were grown in M9 medium supplemented with 0.4% glucose and all amino acids (100 μg/ml each) except methionine to an OD_{600} of about 0.3. After cooling, cells were centrifuged and resuspended in 0.5 volume of the same medium. The cells were further incubated at 30°C for the pulse-chase experiment. Pulse labeling was carried out for 45 s with [^{35}S]methionine, and unlabeled methionine (10 mg/ml) was added for the chase. A 2-ml sample was removed (pulse labeled), and the incubation was carried on for the chase experiment (30 min). Labeling was finally stopped by adding 2 mM NaN_3 and by cooling the samples; cells were then centrifuged, washed, and frozen.

A rapid extraction of HU was performed to analyze HU on urea-Triton-polyacrylamide gels. Pellets were suspended in 500 μl of rapid extraction buffer (100 mM Tris hydrochloride [pH 7.5], 400 mM NaCl) and heated for 10 min at 100°C. After centrifugation, the supernatant fraction, which contained all the heat-resistant proteins, including all of the HU, was immunoprecipitated with *E. coli* HU antiserum as described previously (J. Rouvière-Yaniv, A. Jacq, U. Hibner, and E. Brady, submitted for publication).

Preparation of the sera against *E. coli* HU and electrophoresis in urea–Triton X-100–polyacrylamide gels were performed as described before (1, 4).

Analysis of nucleoid segregation. The production of anucleate cells in HU mutants was analyzed by using a fluorescent DNA-staining technique (18). It consists of observing 4,6-diamino-2-phenylindol (DAPI)-stained bacteria through a fluorescent microscope under phase contrast by UV and

visible light simultaneously. Microscopic observations were performed either on freshly inoculated clones without previous overnight cultivation or directly by transfer of colonies with a toothpick in 10 μl of saline medium deposited on the slides. The number of particles and the cell volume distribution were analyzed with a Coulter counter coupled with a C1000 channelizer.

Measurement of CAT activity. Bacteria (10 ml; OD_{600}, 0.3) were suspended in 1 ml of Tris hydrochloride (pH 8.0)–250 mM dithiothreitol–5 mM glycerol (15%) and sonicated four times for 30 s each. Extracts were cleared by centrifugation at 10,000 *g* for 10 min. Protein concentration was determined by the procedure of Lowry et al. (27). Chloramphenicol acetyltransferase (CAT) assays were done by the method of Gorman et al. (14). Briefly, ^{14}C-labeled chloramphenicol was incubated with acetyl coenzyme A (CoA) and bacterial extracts for 30 min at 37°C. The chloramphenicol and its acetylated derivatives were extracted with ethyl acetate, concentrated, fractionated by thin-layer chromatography, and autoradiographed.

RESULTS

Construction of *hup* mutants. HU is one of the major structural proteins of the nucleoid, and because the protein is a heterodimer, it was of interest to know whether *E. coli* could survive with only one form of the protein or indeed if both were essential or if both could be eliminated. Thus we set out to construct null mutations in both *hupB* and *hupA* genes. The results described here are surprising in that they show that *E. coli* can survive in the complete absence of HU protein but its viability is extremely compromised.

Many of the data presented in this paper stem from our initial attempt to construct a *hupAB* double mutant, which therefore will be presented in detail. The *hupB* gene (HU-1) carried by the plasmid pMW1 (24) was inactivated by insertion at the *Pst*I or *Eco*RV site of either a *Pst*I or *Hin*cII fragment of plasmid pUC4K containing the kanamycin resistance gene (48). The resulting plasmids were named pMWKP and pMWKR, respectively. The Km^r cassette was then transferred into the bacterial chromosome by homologous recombination in a *recBC sbcBC* strain (JC7623) (20) after transformation with the DNA of these different plasmids linearized by *Hin*dIII digestion. The Km^r recombinants which had incorporated the mutated *hupB* gene in the chromosome were identified by their Ap^s phenotype. The absence of the HU-1 protein was demonstrated by failure to detect this subunit after immunoprecipitation of ^{35}S-labeled bacterial proteins with anti-HU antibodies, followed by gel electrophoresis in a urea-Triton gel as described below, which separates HU-1 and HU-2 (39). The mutated gene was then transferred to appropriate *E. coli* strains by transduction of the Km^r marker with bacteriophage P1 (28).

Similarly, the *hupA* gene was disrupted by the insertion of a chloramphenicol resistance cassette. To achieve this, the *Hin*dIII site of pSVCAT (14) was converted to a *Bam*HI site by the addition of a synthetic linker (p385). The *Bam*HI-*Bam*HI restriction fragment of this modified pSVCAT containing the Cm^r gene was inserted into a *Bgl*II linker site introduced at the *Kpn*I site of pK01, the plasmid containing *hupA* (22). The resulting plasmid (p387) carrying the *hupA* gene interrupted by the Cm^r cassette was then linearized with *Hin*dIII, and the mutated gene was recombined into the chromosome of a *recBC sbcBC* strain. The localization of the mutation in the *hupA* gene was verified by the absence of HU-2 protein (Fig. 1). The *hupA*::Cm^r mutation was transduced by bacteriophage P1 either into a wild-type *hup*$^+$ strain (JRY1) or into its *hupB* derivative (OHP96). In this first series of mutants, in which the double mutation was constructed by transducing the *hupA*::Cm^r mutation into the *hupB* derivative, the frequency of Cm^r transductants was very low. The reason for this was an abnormal hypersensitivity of the double mutant to chloramphenicol, which will be discussed below. To avoid this problem, the other isogenic set of bacteria used in this work were constructed by introducing the *hupB*::Km^r mutation into the *hupA*::Cm^r mutant, selecting for Km^r. It should be noted that the growth properties of the mutants (e.g., the doubling time and colony morphology) vary depending on the genetic background. Therefore, all experiments presented here were carried out with sets of strains that were isogenic except for the *hup* loci. All strains were checked for the absence of the appropriate subunits by immunoprecipitation and analysis on urea-Triton gels.

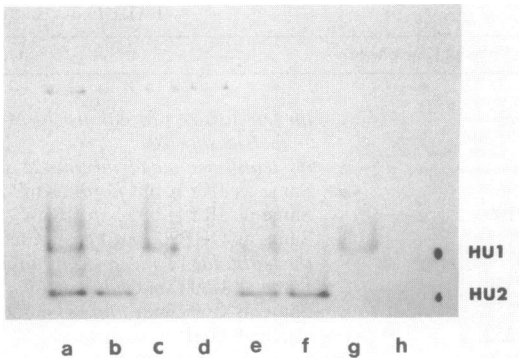

FIG. 1. Presence or absence of the HU subunits in the different mutants. *E. coli* cells were grown to an OD_{600} of 0.3 and labeled with [^{35}S]methionine as described in Materials and Methods. The ^{35}S-labeled proteins were extracted, immunoprecipitated with HU-specific antibodies, and fractionated on urea-Triton-polyacrylamide gels. Bacteria were labeled for 45 s (lanes a to d); this pulse was followed by a 30-min chase with cold methionine (lanes e to h). Lanes: a and e, wild-type strain (C600); b and f, *hupB* strain (OHP250); c and g, *hupA* strain; d and h, *hupAB* strain (OHP252). The positions of the HU-1 and HU-2 subunits are indicated by dots, deduced from their positions on the stained gels.

Figure 1 shows the synthesis or the absence of synthesis of HU in a series of mutants. Strains C600 (hup$^+$), OHP250 (*hupB*::Km^r), OHP251 (*hupA*::Cm^r), and OHP252 (*hupB*::Km^r *hupA*::Cm^r) were grown in M9 glucose supplemented with amino acids at 37°C. At an OD_{600} of about 0.3, the cultures were pulse-labeled with [^{35}S]methionine; HU was extracted and immunoprecipitated as described in Materials and Methods. Immunoprecipitates were analyzed on urea-Triton gels to separate HU-1 from HU-2 (39). Both HU subunits were present in the HU$^+$ strain (C600) and absent in the HU$^-$ strain (OHP252) (Fig. 1). HU-1 and HU-2 were not detected in strains with the *hupB*::Km^r (OHP250) and *hupA*::Cm^r (OHP251) mutations, respectively. The absence of the HU subunits in the double mutants was confirmed by Western immunoblot analysis of total bacterial extracts with highly purified anti-HU sera. This result allowed us to eliminate the possibility that HU proteins from the mutants could have biochemical properties different from those of the wild-type protein, such as altered localization or heat stability, which would have made the extraction procedure ineffective (see Materials and Methods). Furthermore, Fig. 1 shows that the absence of one subunit did not seem to

TABLE 2. Plating and lysogenization efficiencies of Mu in the *hup* mutants[a]

Bacterial strain	Mu cts62pAp1		Mu cts62pKm7701pAp1	
	EOP	Frequency of lysogenization	EOP	Frequency of lysogenization
hup$^+$	1	5.8×10^{-2}	1	4.1×10^{-1}
hupB	1	10^{-1}	1	3.8×10^{-1}
hupA	1.5	1.7×10^{-1}	1.1	4.8×10^{-1}
hupAB	1×10^{-7}	2×10^{-4}	1×10^{-7}	5.6×10^{-4}

[a] The EOP and the frequency of lysogenization were measured in OHP176 (*hup*$^+$), OHP177 (*hupB*), OHP178 (*hupA*), and OHP190 (*hupAB*). The EOP was calculated as the ratio between the titer of the lysate on the strain tested and on OHP176. The frequency of lysogenization was determined as described in the text.

TABLE 3. Induction and transposition of Mu cts62pAp1 and mini-Mu in the *hup* mutants[a]

Bacterial strain	Mu cts62pAp1 phage production (phage/ml)	Frequency of transposition		
		Mu cts62pAp1	λ540-Mu18.1 (A^- B^-)	λ540-Mu18 *A*.1 (A^+ B^-)
hup$^+$	6.5×10^9	3×10^{-4}	2.2×10^{-6}	1.8×10^{-4}
hupB	4.5×10^9	3.2×10^{-4}	1.2×10^{-6}	2.8×10^{-4}
hupA	6×10^9	3.5×10^{-4}	3.3×10^{-6}	3.8×10^{-4}
hupAb	5×10^1	2×10^{-5}	1.4×10^{-6}	2.6×10^{-6}

[a] Phage production was determined after induction of OHP292 (*hup*$^+$), OHP293 (*hupB*), OHP294 (*hupA*), and OHP295 (*hupAB*). The pUZ8 plasmid was introduced in the different lysogens to study the transposition of the Mu cts62pAp1 prophage from the chromosome onto the plasmid by the mating assay (see text).

deregulate the synthesis of the remaining subunit. In this experiment, which involves short pulse-labeling of nascent proteins, the rates of synthesis of HU-1 and HU-2 were approximately the same in each of the single mutants as in the wild-type strain, in which both subunits were synthesized equally. It should be underlined that this pulse-labeling experiment was performed at 30°C. Recently we have shown that at 37°C (or even more noticeably at 42°C) there is appreciable instability of HU-1 in the *hupA* mutant described here (E. Bonnefoy, A. Almeida, and J. Rouvière-Yaniv, submitted for publication.

Growth of bacteriophage Mu on the *hup* mutants. Transposition is crucial for phage Mu development. Upon infection, the Mu genome integrates at random into the host genome by conservative transposition and subsequently replicates by successive rounds of replicative transposition (for a review, see reference 44). In vitro studies have demonstrated that HU is required for an early step in transposition (7). We used the single and double *hup* mutants to verify that HU is indeed essential for Mu transposition in vivo.

Since transposition after infection or induction could follow different routes (15), phage growth was tested by measuring the efficiency of plating (EOP) on lawns of single and double *hup* mutants and phage production upon induction of the same strains lysogenic for a thermoinducible Mu prophage. Two Mu derivatives were used. One, Mu cts62pAp1, expresses all the phage early functions, while the other, Mu cts62pKm7701pAp1, is deleted for several early genes, some of which could encode small basic proteins, leaving open the possibility that one or more of them could substitute for the host HU protein. The EOP was not affected on either the *hupB* or the *hupA* single-mutant strain, but plaques could not be detected on the *hupAB* double mutant (Table 2). Wild-type and *hup* mutant strains lysogenic for either Mu cts62pAp1 or Mc cts62pKm7701 were induced, and phage production was measured on a wild-type strain (Table 3). Phage production was not affected in the single mutants but was severely reduced in the double mutant (10^{-8}-fold as many as by the wild-type strain). Thus Mu did not grow after either infection or induction if the host strain was mutated in both *hupB* and *hupA*. These results show that HU is required in vivo for Mu to grow. However, since growth was observed on both single-mutant host strains, the HU-1 and HU-2 homoproteins must be sufficient for Mu growth and are as efficient as the heterodimeric form normally found in wild-type cells (see Discussion).

The effect of the *hup* mutations on lysogenization was also measured. Table 2 gives the frequencies of Ap^r colonies obtained after infection with Mu cts62pAp1 and Mu cts62pAp1pKm7701 at 32°C. Here again, the single *hup* mutations had no effect, showing once more that the three forms of HU are essentially equivalent, but the double mutant was lysogenized 1,000-fold less efficiently than the parental strain. Whether this reflects a real defect in lysogenization rather than a higher sensitivity of the *hupAB* strain either to phage infection or to ampicillin resistance selection remains to be elucidated.

In order to verify that the observed growth defect was indeed due to a defect in transposition, the frequency of transposition of a Mu cts62pAp1 phage integrated into the chromosome of the *hup* mutant and parental strains onto the conjugative plasmid pUZ8 was measured by the standard mating assay with a *hup*$^+$ strain as a recipient (see Materials and Methods). Transposition of Mu was reduced 10-fold in the double *hup* mutant (Table 3). To eliminate complications due to phage production, it is convenient to use derivatives of Mu (mini-Mu) which do not have the late genes involved in capsid formation and lysis. Transposition from a nonreplicating λ*imm*^{21} phage of such a mini-Mu carrying a functional transposase gene (mini-Mu A^+) onto the same pUZ8 plasmid was measured. This allows easy detection of cointegrate formation between the λ*imm*^{21}::mini-Mu and the pUZ8 plasmid, due to replicative transposition of the mini-Mu. The few Sp^r Km^r Ap^r colonies obtained in the mating with the double *hup* mutant as a donor all contained the λ*imm*^{21} vector, as did about 50% of the colonies obtained with either single *hup* mutation as a donor (Table 3). Thus, most of the mini-Mu transposition events were replicative. A mini-Mu A^- was used as a negative control. In the *hupAB* mutant, no difference in the residual transposition frequency was found between the mini-Mu A^+ and the mini-Mu A^- (Table 3), indicating that those events were not dependent on the Mu transposase. The two mini-Mu used contain a complete IS*1* sequence (33), which could be responsible for that low level of transposition. Thus, in the absence of HU, there was no Mu-dependent transposition, and here again the two homodimeric proteins were as efficient as the heterodimer.

Finally, we tested the fate after induction of the different *hup* mutant strains lysogenic for Mu. Mu induction kills *E. coli* either through expression of a *kil* gene(s) or through replicative transposition (36, 45). To determine which mechanism is operative, we measured survival at 42°C of the single and double *hupAB* mutants lysogenic for either Mu cts62pAp1 (*kil*$^+$) or Mu cts62 pKm7701 (*kil*). All *hup* single mutants were killed by both phages, while the double *hupAB* mutant was only killed by Mu cts62pAp1 (data not shown). This shows that in the absence of HU there is no replication-

TABLE 4. Susceptibility and viability of *hup* mutants[a]

Expt	Bacterial strain	Genotype	Doubling time (min)	No. of viable cells on LA supplemented with:			
				No addition	Kanamycin	Chloramphenicol	Kanamycin + chloramphenicol
A	C600	*hup*$^+$	55	1.6×10^8	—[b]	—	—
	OHP250	*hupB*	55	1.1×10^8	1.1×10^8	—	—
	OHP251	*hupA*	60	5.0×10^7	—	4.5×10^7	—
	OHP252	*hupAB*	75	8.4×10^4	1.3×10^5	1.0×10^3	1.0×10^2
B	C600	*hup*$^+$		1.2×10^7	—	—	—
	OHP250	*hupB*		8.2×10^6	7.3×10^6	—	—
	OHP251	*hupA*		1.2×10^7	—	7.8×10^6	—
	OHP252	*hupAB*		7.3×10^4	8.2×10^3	2.5×10^3	1.4×10^3

[a] In experiment A, the different strains were grown at 37°C to an OD_{600} of about 0.3 in M9-glucose medium supplemented with all the amino acids except methionine. The cultures were diluted with cold M9, and cells were counted at 37°C on solid M9 supplemented or not with kanamycin and/or chloramphenicol. In experiment B, isolated colonies of the different strains were suspended in 0.5 ml of cold LB, and cells were counted after dilution at 37°C on LA and LA supplemented with kanamycin and/or chloramphenicol.

[b] —, Strain not resistant to the antibiotic(s).

dependent killing because replicative transposition is blocked.

Effect of *hup* mutations on transcription. Several bacterial operons sensitive to catabolite repression (*lac*, *malP*, and *lamB*) were shown to be sensitive to inhibitors of DNA gyrase (41). In the presence of nalidixic acid, the transcription of these genes was shown to decrease, whereas biosynthetic operons (*thrA*) were unaffected. Since HU was initially isolated as a factor stimulating the in vitro transcription of a lambda template (38), it was of interest to see whether the absence of HU would modify the activity of such promoters. For this purpose, the *hupA*::Cm^r and *hupB*::Km^r mutations were transduced into a *lac*$^+$ or a *lac* mutant strain carrying a transcriptional fusion of the *lacZ* gene to genes having different responses to gyrase inhibition (*thrA*, *lamB*, and *malP*), and β-galactosidase synthesis was quantified. In our tests, no striking differences between catabolic and biosynthetic operons were detected. The *hup* mutations did not seem to alter transcription from the *lac* and *malP* promoters, slightly stimulated transcription from the *lamB* promoter, and slightly reduced it from the *thrA* promoter (data not shown).

Growth properties of the *hup* double mutant. The doubling time of *hupAB* mutants is significantly longer than that of either single mutant or wild-type strain at 37°C. The generation time was slowed down in LB as well as in M9-glucose supplemented with amino acids. In addition, a lag in growth was frequently observed upon inoculation. To minimize this disturbance, we routinely avoided overnight cultures and started growing cultures from freshly isolated colonies streaked out on LA plates from the stock culture kept at −20°C in glycerol.

The double mutants (as mentioned above) do not show uniform colony morphology: in some backgrounds (C600), the *hupAB* colonies are particularly tiny, and in others (JRY1 or NK7254), they are heterogeneous in size. Nevertheless, all the double mutants exhibited one unusual characteristic, extreme fragility and great sensitivity to chloramphenicol and to a lesser extent to kanamycin. This special sensitivity to chloramphenicol was observed during the construction of the mutants. The poor growth of the *hupAB* mutant on plates containing chloramphenicol could be due simply to low expression of CAT as a consequence of the absence of HU. To check this possibility and to avoid problems due to the poor growth of this double mutant, which, without strict precautions, could lead to enrichment of pseudorevertants or contaminants, three parameters were analyzed in the same culture. (i) The absence or presence of HU subunits was verified by pulse labeling (as described in the legend to Fig. 1). (ii) The efficiency of colony formation was determined on solid medium supplemented or not with antibiotics. (iii) The level of CAT activity was measured in exponentially growing cultures of the mutants. The plating efficiency (Table 4, experiment A) of the *hupAB* double mutants on chloramphenicol-containing medium was considerably lower than on kanamycin-containing plates or on plates without any antibiotic. Addition of kanamycin to chloramphenicol plates further increased this sensitivity. No effect was observed with either of the single mutants, which plated as efficiently as the wild-type strain with or without antibiotics. The CAT levels were measured in sonicated extracts of *hupA* (OHP251) and *hupAB* (OHP252) mutants. As shown in Fig. 2, no difference was observed in the CAT activity between the *hupA* mutant and the *hupAB* double mutant. These results suggest that the severe growth inhibition of the *hupAB* mutant in the presence of chloramphenicol is not caused by a reduction in the activity of the modifying enzyme. For the moment we have no explanation for this special sensitivity to chloramphenicol. However, the data in Table 4 reveal a more general property of the double mutant: very poor viability on plates without any antibiotic (10^4-fold fewer cells compared with the *hup*$^+$ strain). This could in

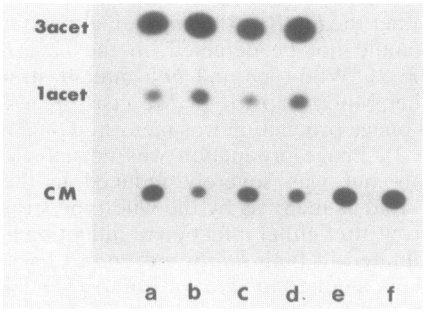

FIG. 2. CAT activity in *hup* mutants. CAT activity was assayed as described in Materials and Methods. Lanes a and b, *hupA* strain (OHP251), 0.75 and 1.5 μg of protein, respectively; lanes c and d, *hupAB* mutant (OHP252), 0.7 and 1.4 μg of protein, respectively; lanes e and f, *hup*$^+$ strain (C600), 0.65 and 1.3 μg of protein, respectively. CM, Chloramphenicol; 3acet and 1acet, tri- and monoacetylated forms, respectively.

fact be due to an extreme sensitivity of these cells after dilution, since lysis was often observed on plates or by microscopy (see next section).

The same result (high sensitivity to chloramphenicol and low EOP) was obtained by measuring directly the number of viable cells contained in a single colony of the *hup* mutants. Appropriate dilutions of the resuspended colony were plated on LA with or without antibiotics. The results of such an experiment are given in Table 4 (experiment B). The number of viable cells contained in single colonies of the double mutant was more than 100-fold lower than in a *hup*$^+$ colony. The initial *hupAB* colonies were, as already mentioned, smaller than the wild-type or single-mutant colonies; however, the difference cannot account for the numbers obtained. This easy test enabled us to check the generality of the phenomenon: (i) quantitatively similar results were found with rich and minimal medium, and (ii) the same fragility or poor viability was observed in all the double mutants described in this work.

With this technique of analyzing individual colonies, the progeny of the double mutant (OHP252) were studied. Ten large and 10 small colonies were picked from LA plates and LA supplemented with both antibiotics; all colonies (derived from an experiment similar to that described in Table 4) had lost the fragility described above and now plated nearly as efficiently as the *hup*$^+$ wild-type strain (data not shown). In addition, the EOP was not at all affected by the presence of antibiotics. Three of these derivatives were analyzed further. They no longer generated anucleate cells (see next section). However, they still did not permit the growth of Mu, and they contained no detectable HU protein (data not shown). The results suggest that strains lacking HU completely are very unstable and that to survive they acquire compensatory mutations suppressing their abnormal fragility in the absence of HU.

Analysis of nucleoid segregation in *hup* mutants. HU double mutants form colonies of heterogeneous size on plates, and this heterogeneity varies from strain to strain. The colonies were analyzed by microscopy: anucleate cells, which can be detected by their absence of fluorescence with DAPI staining (see Materials and Methods), were observed.

The quantitative method used to detect these anucleate cells shows that parental strains as well as the *hupB* single mutants produced less than 0.05% anucleate cells but the *hupA* mutants produced about 0.2%. Surprisingly, exponential-phase cultures of *hupAB* mutants (OHP190 and OHP252) produced variable amounts of anucleate cells depending on the cultures, varying from 0.05 to 11% (Fig. 3). These cultures remained resistant to Mu phage infection and did not contain HU. Cultures producing more than 1% anucleate cells showed a broader cell volume distribution than the parental strain, although the modal volume (analyzed with a Coulter counter) was similar. In addition, the ratio of viable cells to particle number was lower.

To study precisely the heterogeneity observed in the *hupAB* double mutant, large and small colonies from OHP190, a double mutant derived from NK7254, were transferred directly from LA plates to microscope slides and analyzed by DAPI staining. It appeared that large colonies contained less than 0.05% anucleate cells, whereas the microcolonies contained high levels (about 10%). In another experiment, with OHP252, the double mutant from C600, which seemed to have only very small colonies, among seven microcolonies, only one exhibited less than 0.1% anucleate cells; the remaining six contained a high level. When large amounts of anucleate cells were present in the microcolonies, bacterial cell ghosts were detected simultaneously with living cells, showing that part of the double mutant population had lysed.

At this point we wondered whether those cultures with low levels of anucleate cells (0.05%) could be pseudorevertants of the type discussed in the preceding section. To test this assumption, the descendants of one of the microcolonies of OHP252, which contained 11% rod-shaped anucleate cells, was streaked on LA plates. The progeny remained heterogeneous in colony size. Ten clones of different sizes (from micro to large) were analyzed directly by DAPI staining; all contained less than 1% anucleate cells. These results suggest that the double *hupAB* mutant was unstable with respect to the DNA segregation defect and rapidly acquired secondary mutations which usually eliminated the formation of anucleate cells. Furthermore, it should be recalled that the pseudorevertants, which had lost their extreme sensitivity to antibiotics, no longer produced anucleate cells. These observations imply that the same compensatory mutations can correct both defects and suggest that the antibiotic sensitivity and the defect in plating are linked to the abnormality in DNA segregation. Interestingly, a double *hupAB* mutant derived from NK7254 (OHP191) was recently found not to be as sensitive to antibiotics as OHP190, and when examined by DAPI staining it was found to be producing a special class of anucleate cells, namely minicells. We are analyzing this mutant strain further.

DISCUSSION

We used in vitro mutagenesis to derive plasmids carrying *hupB*::Km^r or *hupA*::Cm^r mutations which were subsequently transferred into the bacterial chromosome. Mutants deficient for both HU subunits were obtained by transduction. The *hupB* single mutant does not show any characteristic phenotype and behaves like the parental strain; the *hupA* strain shows a phenotype intermediate between those of the parental strain and the *hupAB* double mutant. Under strict growth conditions and with internal controls, we investigated in vivo the putative phenotypes corresponding to those biological roles for HU suggested by in vitro experiments. Here we report the effect of HU deficiency on the life cycle of phage Mu and show that its physiological behavior fits well with previous biochemical studies. In addition, cell division is perturbed in the mutants, as evidenced by the abnormal production of anucleate cells; this could reflect a defect in the initiation of DNA replication or an effect on the cell division process itself. Finally, our results show clearly an abnormal fragility of the double mutant.

Phage Mu has two modes of transposition. Upon infection, the viral genome integrates at random into the host genome by a conservative transposition event, during which both DNA strands are transposed simultaneously (26, 44). In most infected bacteria, the phage then enters the lytic cycle and amplifies its genome by replicative transposition. A minority of the infected cells (about 1%) become lysogens. Phage Mu does not grow and appears not to lysogenize normally in the double *hupAB* host mutants, consistent with in vitro studies showing that HU is required for the integration of the viral genome in vitro (G. Chaconnas, personal communication). Replicative transposition of Mu, as tested by the ability of the phage to transpose from the bacterial chromosome onto a conjugative plasmid, is reduced in the double *hupAB* mutant, and this reduction is greater when a

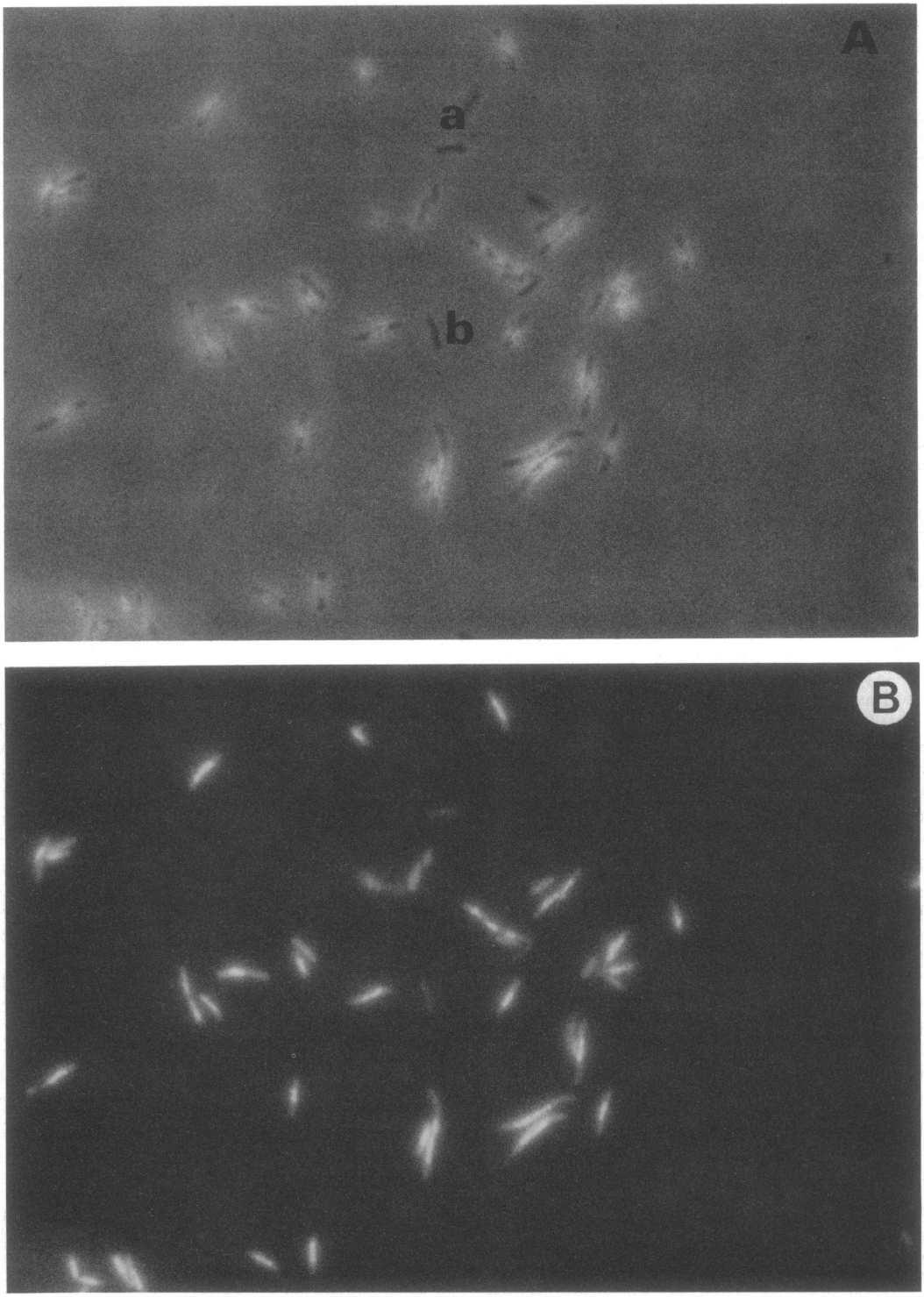

FIG. 3. Formation of anucleate cells in *hup* mutants. A microcolony of strain OHP190 was inoculated in LB medium and grown at 37°C. Panels A and B are pairs and show the same field of cells. Panel A was obtained by the Hiraga fluo-phase combined method, which detects all cells; panel B was obtained by fluorescence with UV light alone. Anucleate cells (a and b) do not have fluorescent bodies.

mini-Mu A^+B^- is used instead of a complete Mu phage, suggesting that the phage may encode a protein, absent from the mini-Mu, which could partially substitute for the HU defect. Upon thermal induction, a *hupAB* (Mu *c*ts62) lysogen is not killed if the prophage is Kil^-, and in such a strain, transposase can be detected by Western blotting analysis of the proteins synthesized (A. Ferhat, personal communication). These observations are consistent with those made in vitro, which show that HU is involved in the assembly of the transposition nucleoprotein complex (8, 43) rather than

being required for the expression of Mu-specific early functions.

The data obtained with the *hupA* and *hupB* single mutants indicate that neither the conservative transposition nor the replicative transposition of Mu is affected by the absence of one of the two HU subunits. Therefore, each of the homomeric forms of the protein can substitute efficiently for the natural heterodimeric form in both modes of transposition used by the phage. The potential for the HU-1 or HU-2 homoproteins to replace the heterodimers for Mu transposition has been reported recently for *S. typhimurium* and *E. coli* (17, 42). Furthermore, we have recently shown that the HU proteins present in the single mutants (*hupB*::Km^r or *hupA*::Cm^r strains) exist as homodimers of HU-1 and HU-2, respectively (Bonnefoy et al., submitted).

Since HU binds to single- and double-stranded DNA and because it has been reported to affect the topological structure of DNA (which has been shown to influence transcription of certain genes), we examined transcription in the different *hupAB* strains. Transcription initiated at the *lac* promoter and at different promoters fused to *lacZ* was measured in *hup*$^+$ and *hup* strains. No strong changes in transcription efficiency were observed. This was somewhat surprising, since the HU protein was initially isolated as a factor stimulating transcription from a lambda template in vitro (38). The weakness of the effects observed may be due to the fact that many other factors modulate transcription so that the absence of HU is not detected in this test.

HU double mutations generate anucleate cells, a feature also associated with DNA gyrase defects (32), with DNA perturbation in the absence of the SOS-dependent division inhibitors (31), and with mutations causing minicell production (19). This phenotype is compatible with the observations that HU is one of the major protein components of the bacterial nucleoid and that it participates with topoisomerases in the maintenance of DNA topology in vitro. Hence, it is not surprising to find that its complete absence in the cell creates perturbations in the partitioning of the DNA during cell division. More experiments are needed to understand the full role of this protein in the important process of cell division.

This work shows that HU is involved in the growth, lysogenization, and transposition of bacteriophage Mu, in DNA partitioning and in bacterial growth. Recently, Wade et al. reported the construction of a viable *hupAB* double mutant (49). They found that the strain is abnormally sensitive to cold and heat shock, which probably correlates with our finding of extreme fragility of the cells. The growth rate of their strain seems to be less affected than those constructed by us in a different genetic background. Moreover, they did not report observing the rapid appearance of a heterogeneous population. In the double mutants described here, in three different genetic backgrounds, we observed the appearance of pseudorevertants, some of which had acquired better growth characteristics and did not form anucleate cells, although they still lacked any detectable HU protein. We interpreted these observations as indicative of the important role of HU in the cell, such that compensatory mutations arise to increase the viability of the cells. It is striking, however, that the *E. coli* mutants totally lacking HU can nevertheless survive. It will be fascinating to elucidate the cell's means of compensating for the absence of HU.

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