Isolation and Characterization of *Escherichia coli dnaA* Amber Mutants

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Specialized transducing phage λ i^{21} *dnaA*-2 was mutagenized, and two derivatives designated λ i^{21} *dnaA17*(Am) and λ i^{21} *dnaA452*(Am) were obtained. They did not transduce such mutations as *dnaA46*, *dnaA167*, and *dnaA5* when an amber suppressor was absent, but they did so in the presence of an amber suppressor. By contrast, they transduced the *dna-806* and *tna-2* mutations in the absence of an active amber suppressor. The *dna-806* and *tna-2* mutations are known to be located very close to the *dnaA* gene, but in separate cistrons. When ultraviolet light-irradiated *uvrB* cells were infected with the derivative phages and proteins specified by them were analyzed by gel electrophoresis, a 50,000-dalton protein was found to be specifically missing if an amber suppressor was absent. This protein was synthesized when an amber suppressor was present. The *dnaA17*(Am) mutation on the transducing phage genome was then transferred by genetic recombination onto the chromosome of an *Escherichia coli* strain carrying a temperature-sensitive amber suppressor *supF6*(Ts), yielding a strain which was temperature sensitive for growth and deoxyribonucleic acid replication. The temperature-sensitive trait was suppressed by *supD*, *supE*, or *supF*. We conclude that, most likely, the derivative phages acquired amber mutations in the *dnaA* gene whose product is a 50,000-dalton protein as identified by gel electrophoretic analysis.

We reported previously the isolation and characterization of a specialized transducing phage, λ i^{21} *dnaA-2* (11, 13, 14), and of *Escherichia coli* mutants which acquired conditionally lethal amber mutations affecting chromosomal replication and which were located very close to, but not within, the *dnaA* gene (11). One of the aims of the studies was to identify the prospective protein which was expected to be coded for by the *dnaA* gene. It is well known that this gene is essential for initiation of *E. coli* chromosome replication (9), but little is known about its function in the initiation process. Indirect evidence, such as isolation of conditionally lethal (temperature-dependent) *dnaA* mutants (9, 10, 17), suggests that the *dnaA* gene may code for a protein which seems to act in a defined step of the initiation process (12, 18) and possibly interacts with other cellular proteins such as DNA-dependent RNA polymerase (3). Synthesis of the *dnaA* protein may be autogenously regulated (7), and there may be a relationship between regulation of *dnaA* product synthesis and regulation of replication initiation (10).

These ideas all predicted that the *dnaA* gene should suffer from acquisition of a nonsense mutation therein. Our previous effort for isolation of a *dnaA* amber mutant by using a localized mutagenesis technique failed, but it led

unexpectedly to the discovery of a new gene which turned out to be essential for chromosome replication and which was located adjacent to *dnaA* (11).

We report in this paper the isolation and some characterization of two derivatives of λ i^{21} *dnaA-2*, each with an amber mutation which most probably resides in the *dnaA* gene. These derivative phages were found to transduce *dnaA* mutations only in the presence of an active amber suppressor. They transduce other mutations near *dnaA* tested in the absence of an active amber suppressor. When UV-irradiated cells were infected with these phages and proteins specified by them were analyzed, a 50,000-dalton protein was identified as the one commonly and solely affected by the amber mutations.

One of these amber mutations on the transducing phage genome was then transferred onto the *E. coli* chromosome by genetic recombination using phage P1-mediated transduction. The *E. coli* strain thus constructed carried a temperature-sensitive amber suppressor *supF6*(Ts) (15). Upon acquisition of the amber mutation, the strain became temperature sensitive in chromosome replication and cellular growth, providing additional evidence, beside the transduction data mentioned above, strongly supporting our conclusion that the new amber mutations oc-

curred in the *dnaA* gene whose product seems to be the 50,000-dalton protein identified by the analysis with UV-irradiated cells. This protein may be identical to a 54,000-dalton protein (8), a 49,000-dalton protein (Murakami, Ozeki, and Yamagishi, personal communication), and a 48,000-dalton protein (Sakakibara, personal communication) which have been proposed to be candidates for the *dnaA* gene product.

MATERIALS AND METHODS

Bacteria and phages. The bacterial strains used were all derivatives of *E. coli* K-12 and are listed in Table 1. The phage strains used were described previously (11), except for λ i^{21} *dnaA167*, which was constructed in this study. It was isolated from a lysate obtained by induction of prophage λ i^{21} *dnaA-2*, which was integrated in the *tna-dnaA* region of *E. coli* strain N167 carrying the *dnaA167* mutation. The other new derivatives of λ i^{21} *dnaA-2* will be described below.

Media. Media were as described previously (11).

Radioactive chemicals. The following products of the Radiochemical Centre, Amersham, England, were used: [2-^{14}C]thymine (52 mCi/mmol) and L-[4,5-^{3}H]leucine (59.8 Ci/mmol).

DNA and protein synthesis. Kinetics of DNA and protein synthesis were measured by a radioactive labeling procedure as described previously (11).

Analysis of phage-coded proteins. The method for the analysis of phage-coded proteins was the same as described previously (11) except that [^{3}H]leucine was pulse labeled as indicated in the legend to Fig. 1.

RESULTS

Isolation of *dnaA* amber mutants. Plaque-forming transducing phage λ i^{21} *dnaA-2* was mutagenized by growing it on *E. coli* strain

KD1087 with a mutator *mutD5*. The mutagenized progeny phage lysate (ca. 10^9 plaque-forming units per ml) was plated after appropriate dilutions on N167 cells; the plates were incubated at 30°C until plaques were formed. The experiment was repeated three times; each time, about 1,000 plaques were picked and screened for those capable of transducing KY8319 cells but incapable of transducing N167 cells. KY8319 (*dnaA46 supE supF*) and N167 (*dnaA167*) grow at 30°C but not at 42°C (Ts). The transductants grew at both temperatures (Ts$^+$). KY8319 carried amber suppressors (Su$^+$), but N167 did not (Su$^-$). The parental phage λ i^{21} *dnaA-2* is capable of transducing both KY8319 and N167 to temperature resistance (TS$^+$) (irrespective of the presence or absence of amber suppressors). Of the total of about 3,000 mutageneized plaques screened, two were found to be capable of transducing KY8319 but incapable of transducing N167; i.e., they were capable of transducing a *dnaA* mutant only in the presence of amber suppressors. The two derivatives of λ i^{21} *dnaA-2* thus obtained arose from two independent series of the experiment and were considered to be candidates for transducing phage strains carrying *dnaA* amber mutations; they were designated λ i^{21} *dnaA17*(Am) and λ i^{21} *dnaA452*(Am).

As shown in Table 2, the two new derivatives were confirmed to be capable of transducing the *dnaA46*, *dnaA167*, and *dnaA5* mutations only in the presence of an amber suppressor. By contrast, such phages as λ i^{21} *dnaA46* and λ i^{21} *dnaA167* lacked transducing activity against these mutations irrespective of whether the re-

TABLE 1. *Bacterial strains*

| Strain	Relevant genotype[a]	Derivation, source, or reference
KD1087	*mutD5*	E. C. Cox (5)
KY8344	*dnaA46*	(11)
KH693	*dnaA46 supE trp-1*	(11)
KY8319	*dnaA46 supE supF*	KH693 *trp$^+$ supF*; this work
N167	*dnaA167*	(1, 11)
N167(φ80 pSu3$^+$)	*dnaA167 supF*	N167 was lysogenized
PC5	*dnaA5*	(4, 11)
PC5(φ80 pSu3$^+$)	*dnaA5 supF*	PC5 was lysogenized
KY936	*supE*	C600 *tonA$^+$*; H. Ozeki
KH5402-1	*ilv supF6*(Ts) *thy*(Low)	A low-thymine-requiring derivative of KH5402 (11)
KY8421	*supF6*(Ts) *thy*(Low)	KH5402-1 *ilv$^+$* (P1-mediated transduction); this work
KY8422	*supF6*(Ts) *thy*(Low) *dnaA17*(Am)	KH5402-1 *ilv$^+$ dnaA17*(Am) (P1-mediated transduction); this work
KY8322	*dna-806*(Am) *supF6*(Ts)	(11)
KY7227	*tna-2*	(11)
N3-1(λ i^{21})	*uvrB* (λ i^{21})	(11)
N3-1(λ i^{21}) *supF*	*uvrB supF* (λ i^{21})	(11)

[a] For gene symbols, see Bachmann and Low (2). Am, Amber mutation.
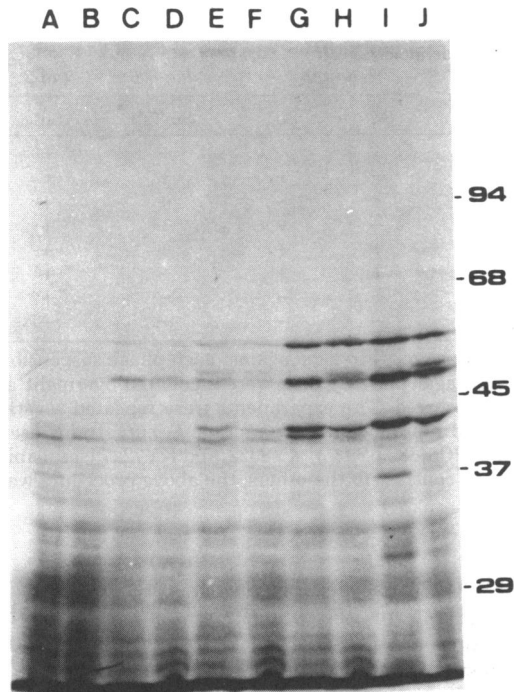
FIG. 1. *Proteins specified by various transducing phages. (A, B) Phage 540; (C, D) λ i²¹ tna; (E, F) λ i²¹ dnaA-2; (G, H) λ i²¹ dnaA17(Am); (I, J) λ i²¹ dnaA452(Am). The host bacteria were N3-1(λ i²¹) without an amber suppressor (A, C, E, G, and I), and N3-1(λ i²¹)supF with an amber suppressor (B, D, F, H, and J). Phage particles were adsorbed to the UV-irradiated host cells at 0°C for 10 min. The infected cell culture was incubated at 37°C for 15 min and was then pulse-labeled for 3 min with 10 μCi of [³H]leucine per ml. The labeling was terminated by the addition of 50 μg of isoleucine per ml and 200 μg of leucine per ml to the culture and incubation for an additional 2 min. Proteins from the cells were subjected to polyacrylamide (9%) gel electrophoresis and fluorography. The numbers indicate molecular weights (×10⁻³) as determined by simultaneous electrophoresis and staining of standard reference proteins.*

cipient carried amber suppressors or not. These four derivatives, however, all retained the transducing activity of the parental phage λ i²¹ dnaA-2 against the tna-2 and dna-806 mutations. Phage 540 (the nontransducing ancestor of λ i²¹ dnaA-2) had none, and λ i²¹ tna (the parent of λ i²¹ dnaA-2) had only the tna-transducing activity, as already reported (11). The dna-806 mutation was also reported previously (11). It is an amber mutation and was shown to affect E. coli chromosomal replication and growth and to belong to a complementation group different from that of the dnaA46, dnaA167, and dnaA5 mutation. Thus, it was considered to reside very

closely to, but outside of, the dnaA gene. The new derivative phages λ i²¹ dnaA17 and λ i²¹ dnaA452 therefore seemed to retain the integrity of the genome structure of their parental phage λ i²¹ dnaA-2, but they also seem to have acquired point mutations of the amber type in the dnaA gene carried by the phages.

Proteins specified by λ i²¹ dnaA17(Am) and λ i²¹ dnaA452(Am). As reported previously (11), at least four proteins with molecular weights of approximately 56,000, 50,000, 48,000, and 43,000 were found to be coded for by the tna-dnaA-transducing segment carried by λ i²¹ dnaA-2 DNA. Of these, the 50,000- and 43,000-dalton proteins were specific to λ i²¹ dnaA-2, and the other two were also encoded by λ i²¹ tna, which did not carry the dnaA-transducing segment. The nontransducing phage 540, the parent of λ i²¹ tna, produced none of the four proteins. These observations were confirmed and were, moreover, demonstrated to be reproducible irrespective of an amber suppressor being present or absent, as shown in Fig. 1, lanes A to F.

The two new amber derivatives λ i²¹ dnaA17 and λ i²¹ dnaA452 were then subjected to a similar analysis, and the result is presented in Fig. 1, lanes G to J. It was found that in both phages, the 50,000-dalton protein band was the only one missing when an amber suppressor was absent, whereas a band with almost the same intensity as in the wild-type λ i²¹ dnaA-2 could be observed when an amber suppressor was present. The other derivative phages, λ i²¹ dnaA46 and λ i²¹ dnaA167, gave exactly the same result as λ i²¹ dnaA-2 did (data not shown).

In the case of λ i²¹ dnaA17, the missing band seemed to be replaced by a new band of ca. 42,000 daltons (Fig. 1, lane G). This probably is an incomplete polypeptide fragment produced as a consequence of the amber mutation, i.e., an "amber fragment." There are a number of candidate bands for the amber fragment in the case of λ i²¹ dnaA452, but the most intensely labeled new band of ca. 37,000 daltons could be the one resulting from the dnaA452 mutation (Fig. 1, lane I). It is noteworthy that the 43,000-dalton protein was not affected by either of the new amber mutations. This protein was previously recognized as being specifically affected by an amber mutation dna-806, which is closely linked to, but is not located within, the dnaA gene (see reference 11 and Table 2). Thus, dnaA17 and dnaA452 seem to be located in the dnaA gene that may code for a 50,000-dalton protein, and dna-806 seems to be located in a new gene (adjacent to dnaA) encoding a 43,000-dalton protein.

An E. coli strain carrying the dnaA17(Am) mutation. Physiological effects

TABLE 2. Transduction analysis by cross-streak test[a]

Phage	Transduction of E. coli							
	dnaA46		dnaA167		dnaA5		dna-806	tna-2
	Su⁻	Su⁺	Su⁻	Su⁺	Su⁻	Su⁺	Su Ts[b]	Su⁻
λ i²¹ dnaA17(Am)	−	+	−	+	−	+	+	+
λ i²¹ dnaA452(Am)	−	+	−	+	−	+	+	+
λ i²¹ dnaA46	−	−	−	−	−	−	+	+
λ i²¹ dnaA167	−	−	−	−	−	−	+	+
λ i²¹ dnaA-2	+	+	+	+	+	+	+	+
λ i²¹ dnaA-2(dna-806)	+	+	+	+	+	+	−	+
λ i²¹ tna	−	−	−	−	−	−	−	+
540	−	−	−	−	−	−	−	−

[a] A loopful of each cell suspension (10⁸ to 10⁹ cells per ml) was cross-streaked against each phage suspension (10⁸ to 10⁹ plaque-forming units per ml) on peptone-nutrient agar plates, which were incubated overnight at 42°C. +, Growth occurred at the cross area; −, no growth occurred. The experiments were repeated several times, and the results were confirmed to be unambiguous and reproducible. The mutant E. coli strains used were KY8344, KH693, KY8319, N167, N167(ϕ80 pSu3⁺), PC5, PC5(ϕ80 pSu3⁺), KY8322, and KY7227 (see Table 1). Since the tna-2 mutation was not temperature sensitive, in contrast to the others, the above procedure was not applicable, and that described by Miki et al. (13) was used.

[b] A temperature-sensitive amber suppressor supF6(Ts) (15) was used.

of the dnaA17(Am) mutation on E. coli cells were investigated by genetically transferring the amber mutation from λ i²¹ dnaA17(Am) onto the E. coli chromosome to examine whether or not the new mutation would express a phenotype comparable to that of a well-known dnaA mutation such as dnaA46 (9). The procedure for construction of an E. coli strain carrying the dnaA17 mutation was as follows. First, KY936 (dnaA⁺ supE) was lysogenized with λ i²¹ dnaA17 at 42°C. Site-specific integration of λ at the bacterial attλ site is known to be thermosensitive (6); thus, we expected to obtain a lysogen which integrated λ i²¹ dnaA17 at the dnaA region rather than at the attλ site. Five independently isolated lysogens were then used as donors for phage P1-mediated transduction. Strain KH5402-1, carrying ilv and supF6(Ts), was used as recipient, and Ilv⁺ transductants were selected. Since the ilv gene is known to be linked to dnaA, the dnaA17 mutation was expected to be cotransduced with ilv. Thus, one lysogen used as the donor yielded an Ilv⁺ transductant that was temperature sensitive for cellular growth, i.e., nongrowing at 42°C. This transductant was purified and established as strain KY8422. It was characterized further and was considered a strain that acquired the amber mutation dnaA17 for the following reasons.

First, the temperature-sensitive trait (Ts) of KY8422 can be accounted for by the presence of a conditionally lethal amber mutation in the strain that carries a temperature-sensitive amber suppressor supF6(Ts) (15). The dnaA gene is known to be essential for E. coli chromosome replication (9), and its amber mutation is expected to be conditionally lethal. Thus, when

KY8422 was lysogenized with either λ i²¹ pSu1⁺, λ pSu2⁺, or ϕ80 pSu3⁺, it was converted to temperature insensitive (growing at 42°C) due to suppression of the amber mutation by supD, supE, or supF, respectively. The temperature sensitivity trait of KY8422 was found to spontaneously revert to temperature insensitivity at a frequency of ca. 10⁻⁶; about two-thirds of the temperature-insensitive revertants arose by reversion of supF6(Ts) to temperature insensitivity.

Second, the amber mutant allele in KY8422 was confirmed to be contransduced with ilv by phage P1 at a frequency of ca. 5%, which corresponds to the well-established map distance between ilv and dnaA (2, 14). Third, the Ts phenotype of KY8422 was found to be complemented by λ i²¹ dnaA-2 or λ i²¹ dnaA-2(dna-806), but not by λ i²¹ tna (carrying no dnaA-transducing segment), λ i²¹ dnaA46, λ i²¹ dnaA167, λ i²¹ dnaA17, or λ i²¹ dnaA452. The amber mutation in KY8422 can therefore be considered to reside in a cistron where the mutation dnaA46, dnaA167, dnaA17, or dnaA452 is located (the amber allele most probably being identical to dnaA17), and this cistron seems to be the dnaA gene. Experiments with deletion derivatives of λ i²¹ dnaA-2 (11, 16) also gave results consistent with this conclusion and indicated that dnaA could be located within a segment of ca. 5 kilobases.

If KY8422 carried the dnaA17 mutation, DNA synthesis of the cell at 42°C would be affected. This was found to be indeed the case. As Fig. 2A shows, DNA synthesis of KY8422 was strongly arrested, but protein synthesis was not, when the cells were cultured at 42°C. They were syn-
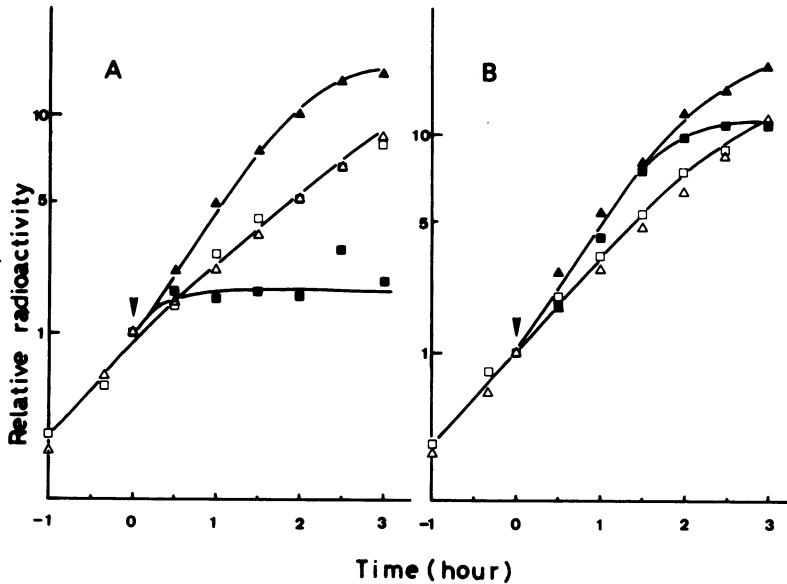
FIG. 2. *Incorporation of [^{14}C]thymine and [^{3}H]leucine during growth of cells at 30 and at 42°C. (A) KY8422 with an amber mutation dnaA17; (B) KY8421, an isogenic wild-type strain. Cells growing exponentially (from 1 × 10^7/ml to ca. 8 × 10^7/ml) at 30°C in medium ECA containing 1.25 µCi of [^{14}C]thymine (■) per ml and 10 µCi of [^{3}H]- leucine (▲) per ml were transferred to 42°C at time zero (arrowhead), and the incubation was continued. Half of each culture was kept at 30°C as a control (□, thymine; △, leucine). At various times, samples were withdrawn and washed with chilled 5% trichloroacetic acid, and insoluble materials were collected on filter disks. They were dried, and radioactivity was counted with a scintillation counter. The radioactive counts were normalized to the count at time zero.*

thesized normally at 30°C. DNA synthesis stopped within 60 min after the shift to the higher temperature, and the final increase was about 50% of the amount of DNA present at the time of the shift. The isogenic wild-type strain synthesized DNA and protein normally at both temperatures (Fig. 2B). The mutant cell was found to be three to five times longer than the wild-type cell after 6 h of incubation at 42°C. During this period, the number of viable cells of the mutant did not increase; rather, it slightly decreased (data not shown).

DISCUSSION

By mutagenizing a specialized transducing phage strain λ i^{21} dnaA-2, two amber mutant derivatives, λ i^{21} dnaA17 and λ i^{21} dnaA452, were isolated. They did not transduce such mutations as dnaA46, dnaA167, and dnaA5 unless an amber suppressor was present. In the presence of an amber suppressor they transduced the dnaA mutations which were themselves suppressed by none of the amber suppressors tested. The amber derivative transducing phages were, moreover, found to transduce other mutations, such as dna-806 and tna-2, under the conditions where no active amber suppressor was available.

The *dna-806* and *tna-2* mutations are known to be transduced likewise by λ i^{21} dnaA-2; they reside very close to the *dnaA* gene, but in separate cistrons clearly distinct from *dnaA* and from each other (11).

These data suggest that the new amber mutations *dnaA17* and *dnaA452* occurred in the *dnaA* gene. This conclusion was strengthened by the finding that, when the *dnaA17* mutation was transferred by genetic recombination with phage P1-mediated transduction from λ i^{21} *dnaA17* onto the chromosome of an *E. coli* strain carrying a temperature-sensitive amber suppressor, the strain acquired the phenotype characteristic of a typical *dnaA* mutation, namely, being conditionally lethal and defective in chromosome replication. The map position of *dnaA17* on the *E. coli* chromosome, which was determined by cotransduction frequency with phage P1 and by complementation analysis with various specialized transducing phages, was consistent with the notion that this mutation resided in the *dnaA* gene, which was located within a segment of ca. 5 kilobases.

Another possibility, however, cannot entirely be ruled out. If the amber mutations have occurred in a cistron adjacent to, and transcrip-

tionally upstream of, the *dnaA* gene, and if the amber mutations have caused a polar effect, then the same result as mentioned above should be expected. Were this the case, at least two protein species, one coded for by the hypothetical upstream cistron and the other by the *dnaA* gene, should be affected by the amber mutations.

As shown in Fig. 1, the gel electrophoretic analysis of proteins specified by λ i^{21} *dnaA17* and λ i^{21} *dnaA452* demonstrated that a band corresponding to a 50,000-dalton protein was commonly missing when an amber suppressor was not available, and this was the sole band detected as missing. The band was normally observed in the presence of an amber suppressor. If this band indeed represented one protein species of 50,000 daltons, and if at the same time the present system indeed detected all the proteins specified by the transducing segment carried by the phage, then the amber mutations could be concluded to have occurred in the *dnaA* gene, which could be considered to code for the 50,000-dalton protein.

This is the most likely interpretation, since a two-dimensional gel electrophoretic analysis demonstrated that only one spot was detected as corresponding to the 50,000-dalton protein band (Kimura, unpublished data). It is also noteworthy that the amber mutations *dnaA17* and *dnaA452* were isolated independently; hence, the chance of them being the same polar-type mutation would be small. There might be, however, a possibility that the *dnaA* protein was not detected as a band under the present experimental conditions. In that case, the uncertainty as to the amber mutations residing in the hypothetical upstream cistron would still remain, but it is expected to be resolved by an experiment now under way. It is aimed at deciphering the nucleotide sequence of the *dnaA* region. The new amber mutations isolated here, *dnaA17* and *dnaA452*, together with the standard *dnaA46* mutation (9) and the previously obtained amber mutations such as *dna-806* (11), will facilitate identification of the coding frames of *dnaA* and the adjacent cistron(s).

Hansen and von Meyenburg (8) have reported that a 54,000-dalton protein appeared to be coded for by the *dnaA* gene. It was based on determination of proteins specified by the *E. coli* chromosome segments of various lengths carried by transducing phages. Molecular weights of the proteins estimated by them are consistently a little higher than those estimated by us. Thus, their 45,000-, 50,000-, 54,000-, and 60,000-dalton proteins seem to correspond to our 43,000-, 48,000-, 50,000-, and 56,000-dalton pro-

teins, respectively. The reason for this difference is unknown, but it could be due to such differences as sample preparations, gel conditions, and molecular-weight markers used. Murakami, Ozeki, and Yamagishi (personal communication) and Sakakibara (personal communication) have also performed a similar analysis. They concluded that a 49,000- and a 48,000-dalton protein, respectively, were candidates for the *dnaA* gene product. Perhaps the same protein has been identified, and its molecular weight has been estimated, with a slight difference, as 50,000 by us, and as 54,000, 49,000 or 48,000 by the others.

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