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DNA Replication in *Escherichia coli* Mutants That Lack Protein HU

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DNA replication in *Escherichia coli* cells lacking protein HU was studied. HU has been suggested to be involved in the initiation of replication from in vitro studies. The isolated HU mutants, however, are viable under normal growth conditions (M. Wada, Y. Kano, T. Ogawa, T. Okazaki, and F. Imamoto, J. Mol. Biol. 204:581–591, 1988). Chromosomal replication in the mutants appeared to be normal with respect to bidirectional replication from *oriC* and to its dependence on *dnaA* and some other *dna* gene products. No significant defect was observed in DNA synthesis in vitro with a crude enzyme fraction prepared from the mutant cells. These results, along with the earlier in vitro studies, suggest that other histonelike protein(s) may substitute for HU in the initiation of replication in the mutant cells. Minichromosomes were more unstable in the mutants. In the absence of either the *mioC* promoter, from which transcription enters *oriC*, or the DnaA box (DnaA protein-binding site) just upstream of the *mioC* promoter, the minichromosomes were especially unstable in the HU mutant and were integrated into the chromosomal *oriC* region under conditions selective for the plasmid-harboring cells.

Protein HU of *Escherichia coli* is an abundant small basic protein that binds both single- and double-stranded DNA. In solution, HU is a heterotypic dimer of two homologous 90-amino-acid polypeptides. It is a major protein component associated with the bacterial nucleoid (for a recent review, see reference 16). It has the ability to compact DNA and is assumed to be one of the bacterial histonelike proteins which wrap DNA into nucleosomelike structures (7, 43). Several in vitro studies have been performed with HU which suggest that it has additional biological functions in DNA replication (15), transcription (19, 44), and site-specific DNA recombination (11, 24, 54).

Involvement of HU in DNA replication was first suggested by findings that it stimulates enzymatic replication of duplex DNAs of recombinant bacteriophages and plasmids bearing the *E. coli* origin of replication (*oriC*) (15). In a reconstituted system for replication, a prepriming complex is formed before the synthesis of primers and DNA at *oriC* on a supercoiled template by the action of the products of *dnaA*, *dnaB*, and *dnaC* genes and HU (4, 22, 47). DnaA protein binds cooperatively to the four 9-base-pair (bp) recognition sequences (DnaA boxes) within *oriC* (20). With ATP tightly bound, it then opens the superhelical DNA duplex at the left boundary of *oriC* to form an open complex (3, 6, 46). Introduction of DnaB helicase to the open complex is effected by DnaC protein, resulting in the formation of the prepriming complex. Stimulation of replication by HU is observed at low levels (5 to 20 ng/200 ng of template) and it appears to occur by stimulating the specific duplex opening by DnaA protein (6). On the other hand, higher levels of HU (>100 ng/200 ng of template) inhibit replication when RNA polymerase is not added to the reaction (3, 41). This inhibition is attributed to the titration of negative superhelical density by HU binding, making the template unavailable for duplex opening by DnaA protein (3). Transcription by RNA polymerase at or near *oriC* of such template molecules facilitates duplex opening by DnaA protein (transcriptional activation). Similar inhibition by HU and counteraction of the inhibition by RNA polymerase have been reported in the initiation of replication of bacteriophage λ DNA in vitro (36).

No clear picture, however, has been reached on transcriptional activation in the initiation of replication in vivo. The involvement of RNA polymerase in the initiation of replication has been suggested from the sensitivity to rifampin and from genetic studies (for a recent review, see reference 53). Transcription of the *mioC* gene, which is located adjacent to *oriC* and encodes a 16-kilodalton protein of unknown function, proceeds toward *oriC*, with the majority of the transcripts entering it. Deletion of the promoter sequence of the gene decreases the stability and copy number of minichromosomes (31, 48, 49). Transcription from the *mioC* promoter is negatively regulated by DnaA protein bound to the DnaA box just upstream of the promoter (32, 40, 48, 55). Deletion of the DnaA box also decreases the stability and copy number of minichromosomes (48). These results suggest a control of the initiation of replication by transcripts from the *mioC* promoter. Two possible functions have been proposed for the transcripts which require experimental support: transcriptional activation and supply of the primer RNA for DNA synthesis (25, 53). Whatever the function might be, dispensability of the transcripts may suggest involvement of other RNA species in the initiation of replication. Promoters have been reported in or near the *oriC* sequence (25, 33, 40, 45), but their importance remains uncertain.

To gain an insight into the physiological significance of nucleoid structure and the biological functions of HU, insertion and deletion mutations of the *hupA* and *hupB* genes, encoding the subunits HU-2 and HU-1, respectively, were introduced into the *hup* loci of the bacterial chromosome (54). Physiological characterization of the *hupA hupB* double mutants revealed that, although the mutant cells grow at a rate comparable with that of the wild-type cells at 37°C, the mutations caused significant alterations in the phenotypes.

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TABLE 1. Bacterial strains

Strain	Genotype	Source or reference
MW14	*pur trp lys leu thi rpsL argE*::Tn*10*	54
MW17	MW14 *hupA16 hupB11*	54
YK1100	W3110[a] *trpC9941*	54
YK1340	YK1100 *hupA16 hupB11*	54
ON211	YK1100 *recA56 srl*::Tn*10*	This work
ON216	YK1340 *recA56 srl*::Tn*10*	This work
YS1 *recA*	*thr leu minA str thi endA recA56 srl*::Tn*10*	1
PC5	F⁻ *leu-6 thyA47 dra-3 str-153 dnaA5*	10, 56
MIC1002	AB1157[a] *dnaA508 zib*::Tn*10*	M. Itaya
W3110 *rnh*	W3110 *rnh*::Tn*3*	T. Horiuchi
AQ1249	W3110 F⁻ *metB1 metD88 his-29 trpA9605 argH rpoB lacΔ3 thyA deoBorC rnh-224 dnaA850*::Tn*10*	T. Kogoma

[a] Described previously (2).

The reduced growth rate of mutant cells at temperatures below 30°C as well as the severe decrease in viability upon exposure to cold or heat shock suggest that HU plays an important role for cell survival under these conditions of stress. Filamentation, anucleate cell production, and unfolding of the nucleoid are remarkable characteristics of the mutants. Viable cell number and DNA content per A_{650} unit are 15 to 20% and 30 to 60%, respectively, of the normal value. These observations imply that HU functions in folding the chromosome into the compact structure and in its absence, chromosome partitioning and cell division are disturbed.

In this paper, we examined several aspects of DNA replication in the *hupA hupB* double mutants. Apparently normal initiation of replication in the mutant cells suggests involvement of other histonelike protein(s) in the initiation of replication in place of HU. Extreme instability of minichromosomes in the mutant cells lacking either the *mioC* promoter or the DnaA box upstream from the *mioC* promoter suggests that transcription from this region is still required for the minichromosome maintenance in these strains.

MATERIALS AND METHODS

Bacterial strains and growth media. The *E. coli* K-12 strains used in this study are shown in Table 1. In the *hupA16* mutation, the entire *hupA* gene is replaced by a kanamycin resistance gene. In the *hupB11* mutation, the entire *hupB* gene, except for the 45 bp encoding the N-terminal peptide of the HU-1 subunit, is replaced by a chloramphenicol resistance gene. ON211 and ON216 are P1 transductants of YK1100 and YK1340, respectively, made with YS1 *recA* as the donor strain. YS1 *recA* was provided by A. Kornberg, and PC5 was provided by S. Hiraga. Bacteria were grown in LB medium (38) supplemented with 0.2% glucose. For growing cells harboring minichromosomes, ampicillin was added to the medium at a concentration of 20 μg/ml.

Minichromosomes. The minichromosome pTOA5 (2.73 kilobases [kb]) containing *oriC* as the sole origin of replication is composed of the *Aat*II-*Hae*II fragment of pCM959 (base positions −114 to 1046 spanning the *oriC* sequence [Fig. 1]) (8) ligated with the *Aat*II-*Hae*II fragment of pBR322 (5) carrying the ampicillin resistance gene. pTOA26 (= pTOA5 p16k ΔPribnow box) and pTOA27 (= pTOA5 p16k ΔDnaA box) were constructed by replacing the small *Hin*dIII-*Hae*II fragment of pTOA5 with the *Hin*dIII-*Hae*II fragment of pEM289 (= pCM959 p16k ΔPribnow box) or pEM300 (= pCM959 p16k ΔDnaA box). pEM289 and pEM300 have a 13-bp deletion covering the Pribnow box of the *mioC* (16-kilodalton protein) gene and a 6-bp deletion removing most of the DnaA box (R5) located just upstream of the *mioC* promoter, respectively (48). pOC24 (37) carries the *oriC Hin*dIII-*Xho*I fragment (base positions −1889 to 417) ligated to a 3.25-kb DNA fragment containing the ampicillin resistance gene derived from the *Staphylococcus aureus* plasmid pI258. The *mioC* gene is deleted in pOC24, except for a short stretch encoding the C-terminal portion of the gene product. pCM959, pEM289, pEM300, and pOC24 were generous gifts of M. Meijer. pTOA43 was constructed by ligating the *oriC Sma*I-*Bal*I fragment (base positions −46 to 2385) to the *Aat*II-*Hae*II fragment of pBR322 encoding the ampicillin resistance via an *Eco*RI linker.

Determination of origin and direction of replication. The relative frequencies of genetic markers in exponentially growing cells were measured by DNA-DNA hybridization (13). Cellular DNA was prepared from a culture of MW14 (wild type) or MW17 (*hupA16 hupB11*) grown to an optical density at 650 nm of 0.5 as described below. A brief sonication was carried out after the pronase treatment to fragment the DNA. Reference DNA was prepared from an overnight stationary culture of the wild-type cells. These DNA preparations were uniformly labeled to about 5×10^8 cpm/μg with [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.), using the large fragment of DNA polymerase I and oligonucleotide primers by the method of Feinberg and Vogelstein (18). Probes were unlabeled DNAs of λ phage clones containing segments of the *E. coli* chromosome (28). Clone numbers used (and their map positions and sizes of their chromosomal segments) were 4E11 (2.8 min, 19 kb), 2D12 (13.3 min, 17 kb), 9E9 (21.8 min, 18 kb), 9H4 (42.7 min, 16 kb), 6C10 (52.3 min, 16 kb), 10B6 (60.6 min, 17 kb), 5B8 (73.5 min, 17 kb), 6G5 (81.7 min, 18 kb), and

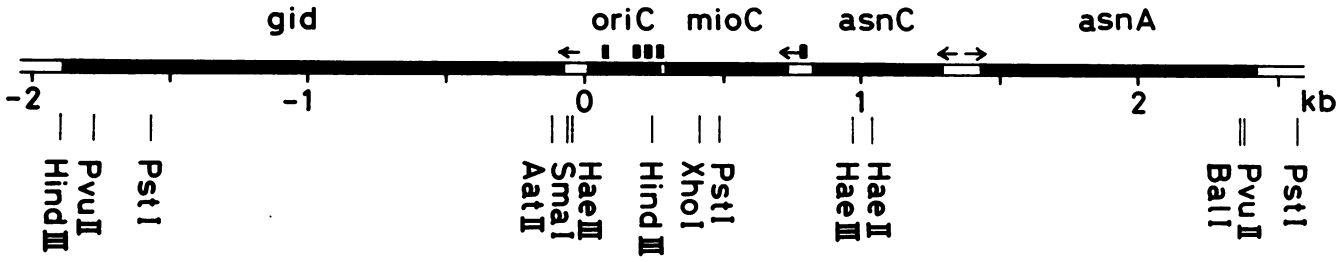

FIG. 1. Map of *E. coli* chromosome around *oriC*. Arrows indicate promoter signals and direction of transcription. Four DnaA boxes in *oriC* and one DnaA box located just upstream of the *mioC* promoter are indicated (▮). Only relevant restriction sites are shown.

1FB (91.8 min, 18 kb). They were provided by Y. Kohara (Department of Molecular Biology, Nagoya University, Nagoya, Japan). Phages were propagated on *E. coli* Q358 and purified by equilibrium centrifugation in CsCl, and their DNAs were extracted by a standard procedure (34). Hybridizations were carried out in duplicate for 48 h as previously described (13), except that reference DNA was hybridized in separate vials under the same conditions.

Stability of minichromosomes. A fresh colony from a transformation plate was grown at 37°C under selective conditions. At an early exponential stage of growth, the culture was diluted with nonselective medium and incubated at 37°C. At intervals, samples were spread on nonselective agar plates, while the culture was kept in exponential phase by diluting with prewarmed medium. The plates were incubated at 37°C, and 200 to 600 colonies from each sample were tested for the presence of the ampicillin resistance marker by transferring the colonies to selective plates. The percentage of the ampicillin-resistant colonies at each time point was plotted on a semilogarithmic section paper, and the half-life was estimated.

Preparation of total cellular DNA. DNA was extracted from harvested cells by a modification of a previously published procedure (52). In brief, suspended cells were lysed by lysozyme-sodium dodecyl sulfate treatment, followed by pronase E (Kaken Seiyaku, Tokyo, Japan) digestion. After phenol extraction and ethanol precipitation, the preparation was treated with pancreatic RNase I and the DNA was recovered by phenol extraction and ethanol precipitation. DNA concentrations were determined by the method of Burton (9).

Copy number estimation. Total cellular DNA was prepared from exponentially growing MW14 cells harboring pTOA5, pTOA26, or pTOA27. The DNA was treated with *Pvu*II and analyzed by Southern hybridization as described (see the legend to Fig. 5). Two portions of each *Pvu*II digest (0.4 and 0.8 μg) were loaded on the gel. Intensities of the plasmid bands and chromosomal *oriC* fragment bands on an autoradiogram were quantitated with a densitometer (Toyo DMU-33C). Plasmid copy number per cell was calculated from the ratio of intensity of the plasmid band to the chromosomal *oriC* band and the number of origins per cell, which was assumed to be four since the generation time of these cells was 30 min (31). The fraction of cells which had lost the plasmids was measured as described above at the time of harvest, and copy numbers were calculated only for plasmid-carrying cells.

Other methods. DNA replication reactions in vitro were carried out, using a crude enzyme fraction of *E. coli* as previously described (21). P1kc phage-mediated generalized transduction was performed as previously described (38). Procedures for constructing minichromosomes, including restriction endonuclease digestion, ligation, gel electrophoresis, and purification of plasmid DNA, were according to Maniatis et al. (34). Enzymes used in these experiments were products of Takara Shuzo, Toyobo, or New England BioLabs, Inc., Beverly, Mass. Southern hybridization was performed using Zeta-probe membranes (Bio-Rad Laboratories, Richmond, Calif.) by the recommendations of the manufacturer. DNA probes were labeled, using oligonucleotide primers (18).

RESULTS

DNA replication in vivo. Origin and direction of DNA replication in MW14 (wild type) and MW17 (*hupA16 hupB11*) were examined by marker frequency analysis (50). If replication was initiated at a fixed origin and proceeded sequentially, the relative gene dosage in exponentially growing cells should be higher at and near the origin and decrease gradually toward the terminus. Experiments with the mutant and the wild type essentially showed the same results (Fig. 2). The relative gene dosage was highest near *oriC* (84 min) and decreased in both directions toward the replication terminus (*terC*, 32 min), as expected for normal replication. Therefore, it is less likely that another mechanism is responsible for replication in the mutant cells, such as constitutive stable DNA replication initiated at multiple sites outside of *oriC* by a mechanism independent of the DnaA protein (12, 27).

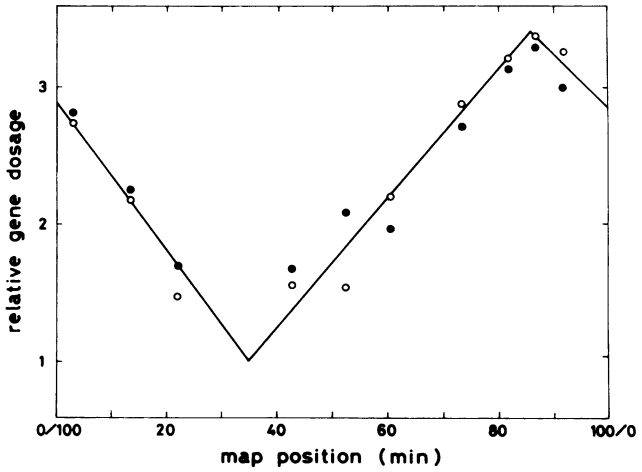

FIG. 2. Origin and direction of replication. Experimental procedures are described in Materials and Methods. The relative gene dosage is the ratio of the amount of sample DNA from MW14 (●) or MW17 (○) to the amount of reference DNA that hybridized to each of the λ phage DNA clones containing the chromosomal segments.

Requirement of the *dnaA* gene product for the growth of *hupA hupB* mutants was confirmed by the temperature-sensitive phenotype conserved in strains PC5 (*dnaA5*) and MIC1002 (*dnaA508*) to which the *hupA16 hupB11* mutations were introduced by P1 transduction. Similar experiments revealed requirements of the gene products of *dnaB*, *dnaC*, and *gyrB* for the viability of *hupA hupB* mutants. In these experiments, lack of the HU protein in the transductants was confirmed by immunoblotting (Western blotting) experiments using antibody raised against HU (data not shown). RecA protein, which is required for stable DNA replication and SOS functions but not for normal replication, appeared to be unessential for DNA replication in *hupA hupB* mutants inasmuch as strain ON216 (*hupA16 hupB11 recA56*) was successfully isolated. On the other hand, YK1340 (*hupA16 hupB11*) permitted introduction of the *rnh*::Tn*3* plus *dnaA850*::Tn*10* mutations, suggesting that HU protein is not essential for stable DNA replication occurring in the absence of RNase H and DnaA protein.

DNA replication in vitro. Inasmuch as the involvement of HU in DNA replication has been clearly demonstrated in an in vitro system in which replication of a supercoiled duplex DNA of recombinant phages and plasmids bearing *oriC* occurs in the presence of a crude enzyme fraction (fraction II) (15), the activity of DNA synthesis was examined with fraction II prepared from mutant cells. Results in Fig. 3 and Fig. 4 show that mutant fraction II supported DNA synthesis

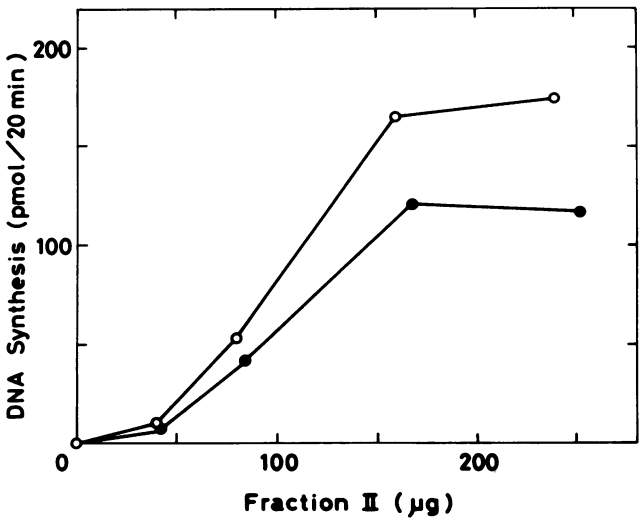

FIG. 3. DNA replication in vitro. Reactions were carried out as previously described (21), with the indicated amounts of fraction II prepared from MW14 (○) or MW17 (●). *oriC* templates used were 0.2 μg of the replicative form I DNA of M13H7 (40).

to a level comparable with the wild-type activity. DNA synthesis was specific to the plasmid templates which carry *oriC*. DNA products synthesized by mutant fraction II during various incubation times were indistinguishable from the wild-type products as analyzed by agarose gel electrophoresis (data not shown).

Since the involvement of HU in the initiation of *oriC* replication appears to be evident from in vitro studies (3, 15, 41; see Discussion), these results may suggest that another histonelike protein(s) functions for HU in its absence.

Behavior of minichromosomes. The *hupA hupB* mutants were able to be transformed with minichromosomes, which carry *oriC* as the sole replication origin (Table 2). The ratio of transformation efficiency of the mutant (MW17) to that of the wild type (MW14) with minichromosomes ranged between 0.16 and 0.33, similar to the value (0.23) obtained with

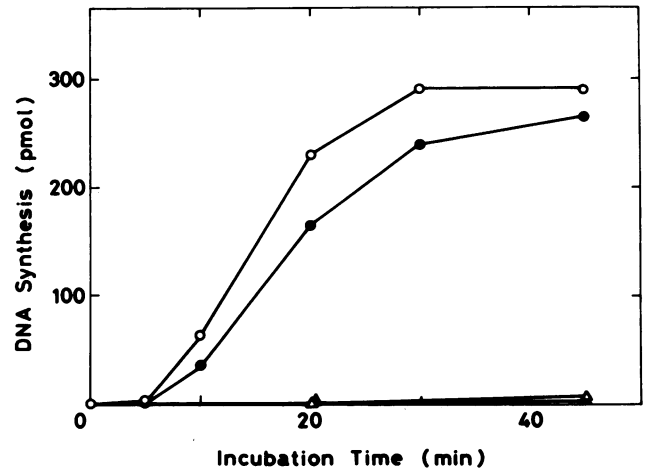

FIG. 4. Time course of DNA replication in vitro. Reactions were carried out with 207 (MW14; ○, △) or 218 (MW17; ●, ▲) μg of fraction II. Templates (0.2 μg) were the replicative form I of M13H7 carrying *oriC* (○, ●) or the replicative form I of vector M13 mp11 (△, ▲).

TABLE 2. Transformation efficiency[a]

Recipient strains	Transformation efficiency					
	pBR322	pOC24	pTOA5	pTOA26	pTOA27	pTOA43
MW14	14.2	8.7	5.8	5.9	6.1	4.5
MW17	3.2	1.4	1.2	1.1	1.3	1.5

[a] Transformation efficiency is expressed by the number of Ap^r transformants per 10^{-3} fmol of DNA molecule. The ratios of mutant (MW17) transformants to wild-type (MW14) transformants for pBR322, pOC24, pTOA5, pTOA26, pTOA27, and pTOA43 were 0.23, 0.16, 0.21, 0.19, 0.21, and 0.33.

pBR322. The efficiency of transformation of the mutant with minichromosomes was 30 to 50% of that with pBR322 and was comparable with the value obtained with the wild-type strain (30 to 60%).

Upon examining the presence of minichromosomes in the transformed mutants, we encountered difficulty in recovering a minichromosome pOC24 by certain procedures, including alkaline lysis or lysozyme-sodium dodecyl sulfate lysis (34). Inasmuch as pOC24 lacks most of the *mioC* gene, including its promoter region, which has been suggested to be involved in the regulation of initiation of replication at *oriC* (31, 40, 48, 49, 51), we examined the effect of disruption of the *mioC* promoter region on the behavior of the minichromosomes (Fig. 5). Two plasmids, pTOA26 and pTOA27, are derived from pTOA5 and have a 13-bp deletion covering the Pribnow box of the *mioC* promoter and a 6-bp deletion removing most of the DnaA box located just upstream of the *mioC* promoter, respectively. The approximate copy numbers of pTOA5, pTOA26, and pTOA27 in exponentially growing wild-type cells (MW14) in LB medium were seven, four, and three per cell, respectively (data not shown). The total cellular DNA was extracted after cultivating the colonies formed on transformation plates for about 25 generations in the selective liquid medium, digested with restriction enzymes, and then analyzed by Southern hybridization. Restriction enzymes *Hin*dIII and *Pst*I cleave the minichromosomes pTOA5, pTOA26, and pTOA27 once and twice, respectively. These enzymes produced *oriC*-containing DNA bands at the positions of the plasmid *oriC* fragment, as well as the chromosomal *oriC* fragment from all DNA preparations. The three minichromosomes gave the same results, and no difference was observed between the wild type and the mutant transformants. In contrast, *Pvu*II, which does not cleave these plasmids, gave different results. After treatment with this enzyme, uncut plasmid, as well as the chromosomal *oriC* fragment of an expected chain length, was detected in DNA from the wild-type cells transformed with any of these three minichromosomes. DNAs from the mutant cells transformed with pTOA5 also gave the same results. However, in the *Pvu*II digest of DNA from the mutant cells transformed with either pTOA26 or pTOA27, both the uncleaved plasmid and the chromosomal *oriC* fragment were missing at the expected positions, and instead, a slow-migrating DNA band appeared in the agarose gel. The size of this band was 6.89 kb as estimated from the mobilities of the molecular weight standards (*Hin*dIII fragments of λ DNA [results not shown]) and was equal to the sum of sizes of the chromosomal *Pvu*II fragment spanning *oriC* (4.16 kb) and the plasmid DNA (2.73 kb). These results indicate that lack of transcription from the *mioC* promoter in pTOA26 as well as too much transcription in pTOA27, caused by the absence of control by DnaA protein, lead to the loss of monomer minichromosomes in the mutant and

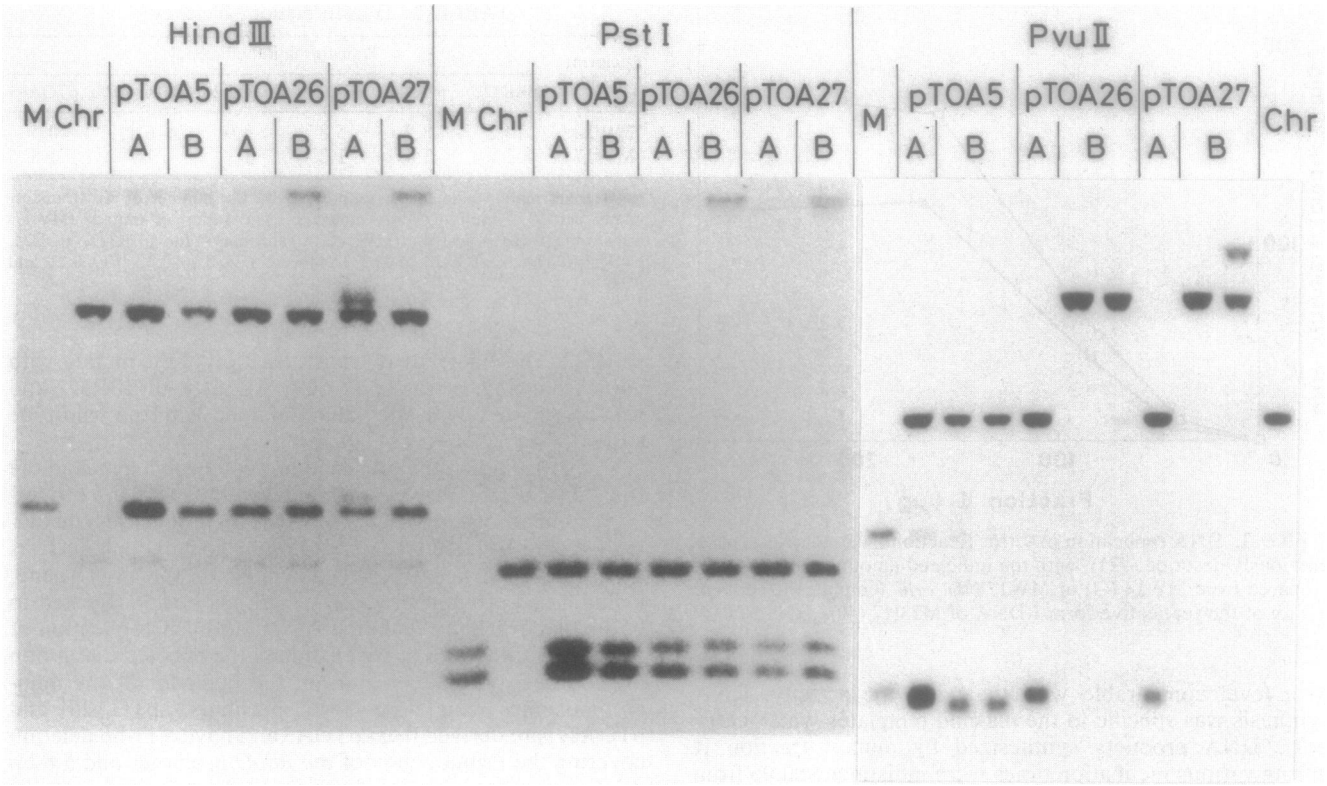

FIG. 5. Restriction analysis of the transformant DNA. Total cellular DNA was prepared from MW14 (A) or MW17 (B) transformed with the indicated plasmids after cultivating a fresh colony on a transformation plate for about 25 generations in liquid medium under selective conditions. One-microgram portions of the DNA were digested with indicated restriction enzymes and subjected to electrophoresis in a 0.7% agarose gel. Southern hybridization was performed with the ^{32}P-labeled *Hae*III fragment (base positions −41 to 974) encompassing *oriC* as a probe. In lanes M and Chr, pTOA5 and DNA from the untransformed MW14 cells were analyzed, respectively. Two independent transformants were analyzed for the *Pvu*II digest of the mutant DNA. The two bands in the lane of *Pvu*II-treated marker DNA are form I and form II of the monomer minichromosome from the bottom to the top, respectively.

that they had been integrated in tandem with respect to the *oriC* sequence into the chromosomal *oriC* region in the mutant. The upper band in a lane containing the pTOA27-transformed mutant could represent integration of two minichromosome molecules at the chromosomal *oriC* region. Experiments with strain ON216 (*hupA16 hupB11 recA56*) essentially gave the same results as for MW17 in Fig. 5 (data not shown), suggesting that this site-specific recombination is independent of the RecA protein. In an early stage of culture of the mutant transformants, a significant portion of these two minichromosomes were present as monomer molecules (Fig. 6).

Stability values of the ampicillin resistance marker carried by the minichromosomes are shown in Table 3. pTOA5 was more unstable in the mutant (MW17) than in the wild-type (MW14) cells in the absence of ampicillin in the medium. Deletion of either the *mioC* promoter or the DnaA box at its upstream location greatly reduced the stability of the minichromosome not only in the wild type as has been previously reported (48) but also in the mutant. When the segregation kinetics was followed after about 25 generations of growth in the selective medium, both pTOA26 and pTOA27 were stably maintained in the mutant (data not shown), in accordance with the integration of these plasmids into the chromosomal *oriC* region (Fig. 5).

DISCUSSION

Construction of the chromosomal deletion mutant of the *hup* genes enabled us to investigate the functions of the HU protein in vivo. Nucleoids of the *hupA hupB* mutants have a dispersed structure and the DNA content per A_{650} unit is reduced by 40 to 70% (54), which might suggest disturbed DNA replication. Production of filamentous as well as anucleate cells by mutants independent of the SOS functions is a phenotype shared by some other *dna* temperature-sensitive mutants under restrictive or semirestrictive conditions for replication (17, 23, 39, 42). Involvement of HU in the initiation of DNA replication has been based on in vitro studies with a crude enzyme fraction (fraction II) (15) or with purified enzymes (3, 6, 15, 41). Depending on the experimental approach, 2- to 10-fold stimulation of DNA synthesis by HU was observed in a reconstituted system. Duplex opening by DnaA protein at the 13-mer repeats in *oriC* is stimulated by a low level of HU (6). On the other hand, the present in vivo study suggested normal operation of the bidirectional replication mechanism from *oriC* and showed dependence on replication proteins, including the DnaA protein, in the *hupA hupB* mutants. Replication in vitro by mutant fraction II was essentially similar to the reaction catalyzed by wild-type fraction II. Thus, the normal replication mechanism seems to operate in the absence of HU, although the efficiency of replication could be impaired. The stimulatory function of HU in the initiation of replication seems to be carried out by other histonelike protein(s). Recently, Kornberg's laboratory has shown that another histonelike protein, IHF (16), can substitute for HU at a similar level in a reconstituted initiation of replication of *oriC* templates (6).

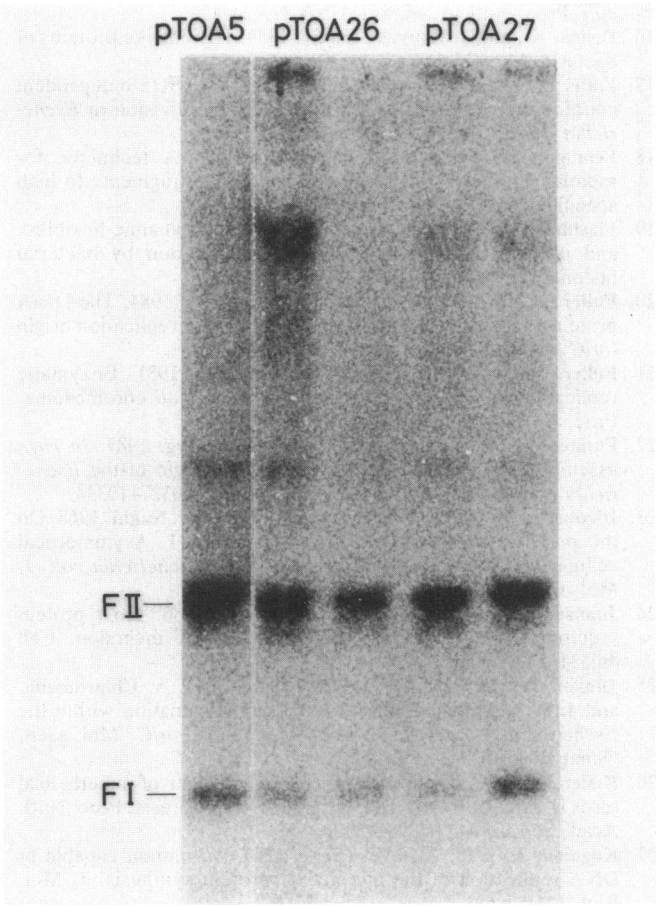

FIG. 6. Presence of minichromosomes in MW17. DNA was extracted from the transformed MW17 cells after cultivating a colony on a transformation plate for about 10 generations in liquid medium under selective conditions and directly subjected to electrophoresis in a 0.7% agarose gel (1 μg per lane). Southern hybridization was performed by using the ampicillin resistance gene of pBR322 (Materials and Methods) as the probe. Two independent transformants were analyzed for pTOA26 and pTOA27. Positions of form I (FI) and form II (FII) of the minichromosome DNA are indicated at the left.

According to an earlier report (15), the *oriC* replication activity in wild-type fraction II was nearly completely inhibited by antibody directed against HU and activity was recovered upon addition of purified HU protein. These results may be explained by the absence of the factor(s) which substitutes for HU from the fraction II used in the experiments. Another possibility is that the HU antibody could inhibit the substituting factor(s).

Examination of the involvement of transcription in initiation of the chromosomal replication in the *hupA hupB* mutants has been hampered by impaired cell growth upon a change of culture conditions. Attempts to synchronize the culture by amino acid starvation or by temperature shift using the *dnaA* or the *dnaC* derivatives have been unsuccessful.

TABLE 3. Stability of the ampicillin resistance marker

Bacterial strain	Half-life (no. of generations)		
	pTOA5	pTOA26	pTOA27
MW14	12.5	4.5	4.5
MW17	6.0	2.2	2.0

Transcription from the *mioC* promoter is involved in control of the initiation of minichromosome and chromosome replication (14; also see above). An attractive hypothesis for the role of this transcription is transcriptional activation. In a reconstituted in vitro system for replication of minichromosomes, transcriptional activation by RNA polymerase is needed to help DnaA protein open the duplex when the negative superhelicity of the template is titrated by higher levels of HU or when a relaxed DNA is used as the template (3). Transcription is not required on a supercoiled template when HU is present at lower levels. From these in vitro results, one might expect that minichromosomes lacking the *mioC* transcription might restore stability in the *hupA hupB* mutants. The present in vivo study, however, showed that minichromosomes pTOA26 and pTOA27 are more unstable in the mutants than in the wild-type cells (Table 3). Thus, *mioC* transcription regulated by the DnaA protein is still required for minichromosome maintenance in the absence of HU. The physiological significance of the effects observed in vitro at higher levels of HU is not certain. Some other histonelike protein(s) could suppress the RNA polymerase-independent initiation, although HU is estimated to represent about 80% of the nucleoid proteins in wild-type cells (57). Another possibility is that a perturbed minichromosome structure could necessitate the transcriptional activation for replication in the mutants. The lack of HU in the *hupA hupB* cells could contribute to the reduced number of negative superhelical turns of plasmids extracted from the mutant cells (7). In addition, the lack of HU could affect transcription, leading to the modulation of the state of plasmid supercoiling (30). In fact, superhelical densities of minichromosomes purified from the mutant cells were 5 to 15% less negative than those from the wild-type cells, as analyzed by the method of Keller (26), and a broader distribution of the topoisomers was observed in agarose gels with plasmids from the mutant cells (data not shown). The importance of DNA superhelicity in minichromosome maintenance has been previously suggested (29). Since HU constrains superhelical turns at a DNA locus where it binds by wrapping the DNA around itself (7), alteration of the intrinsic superhelical tension of plasmids caused by the loss of HU may not be significant in vivo. However, the topological state around *oriC* in the initiation of replication could be altered in the mutant. Furthermore, HU protein could also be involved in other processes of replication, such as elongation, termination, and segregation.

The present study demonstrated that unstable minichromosomes harbored in the *hupA hupB* mutants are integrated into the chromosomal *oriC* region upon cultivation under selective conditions by a homologous recombination mechanism independent of RecA protein. The frequency of integration appeared to increase upon elongation of the chromosomal sequence on the minichromosome (unpublished observation). Integration of *oriC* plasmids by a *recA*-independent mechanism at or near the chromosomal *oriC* has been previously reported (35). Involvement of replication proteins in this reaction is suggested by the researchers.

Negative control of minichromosome replication by transcription has been proposed on the basis of the in vivo observation that minichromosome replication is inhibited by the frequent transcription from the *lac* promoter or from the promoter of the chloramphenicol resistance gene (51). The instability and low copy number of pTOA27 in vivo are best explained by this model, since transcription from the *mioC*

promoter is not sensitive to negative control by DnaA protein in this minichromosome. The requirement of a fine tuning of transcription suggested from these results for the initiation of replication are not obvious from in vitro studies. The minichromosome pEM300 (= pCM959 p16k ΔDnaA box) appears to be as good a template as pCM959 in an in vitro replication system using fraction II (unpublished observation). Transcription from the promoter of phage T3 or T7 by the phage RNA polymerase effectively activates initiation in a reconstituted system (3). Studies bridging the gaps between these in vivo and in vitro results are required for understanding of the regulation of the initiation of replication by transcription.

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LITERATURE CITED

1. **Arai, K., S. Yasuda, and A. Kornberg.** 1981. Mechanism of DnaB protein action. I. Crystallization and properties of DnaB protein, an essential replication protein in *Escherichia coli*. J. Biol. Chem. **256**:5247–5252.
2. **Bachmann, B. J.** 1972. Pedigrees of some mutant strains of *Escherichia coli* K-12. Bacteriol. Rev. **36**:525–557.
3. **Baker, T. A., and A. Kornberg.** 1988. Transcriptional activation of initiation of replication from the *E. coli* chromosomal origin: an RNA-DNA hybrid near *oriC*. Cell **55**:113–123.
4. **Baker, T. A., K. Sekimizu, B. E. Funnell, and A. Kornberg.** 1986. Extensive unwinding of the plasmid template during staged enzymatic initiation of DNA replication from the origin of the *E. coli* chromosome. Cell **45**:53–64.
5. **Boliver, F., R. L. Rodrigues, P. L. Green, M. C. Betlach, H. L. Heyneker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2**:95–113.
6. **Bramhill, D., and A. Kornberg.** 1988. Duplex opening by DnaA protein at novel sequences in initiation of replication at the origin of the *E. coli* chromosome. Cell **52**:743–755.
7. **Broyles, S. S., and D. E. Pettijohn.** 1986. Interaction of the *Escherichia coli* HU protein with DNA: evidence for formation of nucleosome-like structures with altered DNA helical pitch. J. Mol. Biol. **187**:47–60.
8. **Burke, H.-J., and W. Messer.** 1983. The replication origin region of *Escherichia coli*: nucleotide sequence and functional units. Gene **24**:265–279.
9. **Burton, K.** 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. **62**:315–323.
10. **Carl, P. L.** 1970. *Escherichia coli* mutants with temperature-sensitive synthesis of DNA. Mol. Gen. Genet. **109**:107–122.
11. **Craigie, R., D. Arndt-Jovin, and K. Mizuuchi.** 1985. A defined system for the DNA strand-transfer reaction at the initiation of bacteriophage Mu transposition: protein and DNA substrate requirements. Proc. Natl. Acad. Sci. USA **82**:7570–7574.
12. **de Massy, B., O. Fayet, and T. Kogoma.** 1984. Multiple origin usage for replication in *sdrA* (*rnh*) mutants of *Escherichia coli* K12. J. Mol. Biol. **178**:227–236.
13. **de Massy, B., J. Patte, J.-M. Louarn, and J.-P. Bouché.** 1984. *oriX*: a new replication origin in *E. coli*. Cell **36**:221–227.
14. **de Wind, N., P. Parren, A. R. Stuitje, and M. Meijer.** 1987. Evidence for the involvement of the 16kD gene promoter in initiation of chromosomal replication of *Escherichia coli* strains carrying a B/r-derived replication origin. Nucleic Acids Res. **15**:4901–4914.
15. **Dixon, N. E., and A. Kornberg.** 1984. Protein HU in the enzymatic replication of the chromosomal origin of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **81**:424–428.
16. **Drlica, K., and J. Rouviere-Yaniv.** 1987. Histonelike proteins of bacteria. Microbiol. Rev. **51**:301–319.
17. **Faffé, A., R. D'Ari, and V. Norris.** 1986. SOS-independent coupling between DNA replication and cell division in *Escherichia coli*. J. Bacteriol. **165**:66–71.
18. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6–13.
19. **Flashner, Y., and J. D. Gralla.** 1988. DNA dynamic flexibility and protein recognition: differential stimulation by bacterial histone-like protein HU. Cell **54**:713–721.
20. **Fuller, R. S., B. E. Funnell, and A. Kornberg.** 1984. The DnaA protein complex with the *E. coli* chromosomal replication origin (*oriC*) and other DNA sites. Cell **38**:889–900.
21. **Fuller, R. S., J. Kaguni, and A. Kornberg.** 1981. Enzymatic replication of the origin of the *Escherichia coli* chromosome. Proc. Natl. Acad. Sci. USA **78**:7370–7374.
22. **Funnell, B. E., T. A. Baker, and A. Kornberg.** 1987. *In vitro* assembly of a prepriming complex at the origin of the *Escherichia coli* chromosome. J. Biol. Chem. **262**:10327–10334.
23. **Hirota, Y., F. Jacob, A. Ryter, G. Buttin, and T. Nakai.** 1968. On the process of cellular division in bacteria. I. Asymmetrical cellular division and DNA-less bacteria in *Escherichia coli*. J. Mol. Biol. **35**:175–192.
24. **Johnson, R., M. Bruist, and M. Simon.** 1986. Host protein requirements for *in vitro* site-specific DNA inversion. Cell **46**:531–539.
25. **Junker, D. E., Jr., L. A. Rokeach, D. Ganea, A. Chiaramello, and J. W. Zyskind.** 1986. Transcription termination within the *Escherichia coli* origin of DNA replication, *oriC*. Mol. Gen. Genet. **203**:101–109.
26. **Keller, W.** 1975. Determination of the number of superhelical turns in simian virus 40 DNA by gel electrophoresis. Proc. Natl. Acad. Sci. USA **72**:4876–4880.
27. **Kogoma, T.** 1978. A novel *Escherichia coli* mutant capable of DNA replication in the absence of protein synthesis. J. Mol. Biol. **121**:55–69.
28. **Kohara, Y., K. Akiyama, and K. Isono.** 1987. The physical map of the whole *E. coli* chromosome: application of a new strategy for rapid analysis and sorting of a large genomic library. Cell **50**:495–508.
29. **Leonard, A. C., W. G. Whitford, and C. E. Helmstetter.** 1985. Involvement of DNA superhelicity in minichromosome maintenance in *Escherichia coli*. J. Bacteriol. **161**:687–695.
30. **Liu, L. F., and J. C. Wang.** 1987. Supercoiling of the DNA template during transcription. Proc. Natl. Acad. Sci. USA **84**:7024–7027.
31. **Løbner-Olesen, A., T. Atlung, and K. V. Rasmussen.** 1987. Stability and replication control of *Escherichia coli* minichromosomes. J. Bacteriol. **169**:2835–2842.
32. **Lother, H., R. Kölling, C. Kücherer, and M. Schauzu.** 1985. DnaA protein-regulated transcription: effects on the *in vitro* replication of *Escherichia coli* minichromosomes. EMBO J. **4**:555–560.
33. **Lother, H., and W. Messer.** 1981. Promoters in the *E. coli* replication origin. Nature (London) **294**:376–378.
34. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
35. **Masters, M., V. Andresdottir, and H. Wolf-Watz.** 1978. Plasmids carrying *oriC* can integrate at or near the chromosomal origin of *Escherichia coli* in the absence of a functional *recA* product. Cold Spring Harbor Symp. Quant. Biol. **43**:1069–1072.
36. **McMacken, R., C. Alfano, B. Gomes, J. H. LeBowitz, K. Mensa-Wilmot, J. D. Roberts, and M. Wold.** 1987. Biochemical mechanisms in the initiation of bacteriophage λ DNA replication, p. 227–245. *In* R. McMacken and T. J. Kelly (ed.), DNA replication and recombination. Alan R. Liss, Inc., New York.
37. **Messer, W., M. Meijer, H. E. N. Bergmans, F. G. Hansen, K. von Meyenburg, E. Beck, and H. Schaller.** 1979. Origin of replication, *oriC*, of the *Escherichia coli* K12 chromosome: nucleotide sequence. Cold Spring Harbor Symp. Quant. Biol.

43:139–145.
38. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
39. **Norris, V., T. Alliotte, A. Jaffé, and R. D'Ari.** 1986. DNA replication termination in *Escherichia coli parB* (a *dnaG* allele), *parA*, and *gyrB* mutants affected in DNA distribution. J. Bacteriol. **168**:494–504.
40. **Nozaki, N., T. Okazaki, and T. Ogawa.** 1988. *In vitro* transcription of the origin region of replication of the *Escherichia coil* chromosome. J. Biol. Chem. **263**:14176–14183.
41. **Ogawa, T., T. A. Baker, A. van der Ende, and A. Kornberg.** 1985. Initiation of enzymatic replication at the origin of the *Escherichia coli* chromosome: contributions of RNA polymerase and primase. Proc. Natl. Acad. Sci. USA **82**:3562–3566.
42. **Orr, E., N. F. Fairweather, I. B. Holland, and R. H. Pritchard.** 1979. Isolation and characterization of a strain carrying a conditional lethal mutation in the *cou* gene of *Escherichia coli* K12. Mol. Gen. Genet. **177**:103–112.
43. **Rouviere-Yaniv, J., J. Germond, and M. Yaniv.** 1979. *E. coli* DNA binding protein HU forms nucleosome-like structure with circular double-stranded DNA. Cell **17**:265–274.
44. **Rouviere-Yaniv, J., and F. Gros.** 1975. Characterization of a novel, low molecular weight DNA-binding protein from *Escherichia coli*. Proc. Natl. Acad. Sci. USA **72**:3428–3432.
45. **Schauzu, M.-A., C. Kücherer, R. Kölling, W. Messer, and H. Lother.** 1987. Transcripts within the replication origin, *oriC*, of *Escherichia coli*. Nucleic Acids Res. **15**:2479–2497.
46. **Sekimizu, K., D. Bramhill, and A. Kornberg.** 1987. ATP activates DnaA protein in initiating replication of plasmids bearing the origin of the *E. coli* chromosome. Cell **50**:259–265.
47. **Sekimizu, K., D. Bramhill, and A. Kornberg.** 1988. Sequential early stages in the *in vitro* initiation of replication at the origin of the *Escherichia coli* chromosome. J. Biol. Chem. **263**:7124–7130.
48. **Stuitje, A. R., N. de Wind, J. C. van der Spek, T. H. Pors, and M. Meijer.** 1986. Dissection of promoter sequences involved in transcriptional activation of the *Escherichia coli* replication origin. Nucleic Acids Res. **14**:2333–2344.
49. **Stuitje, A. R., and M. Meijer.** 1983. Maintenance and incompatibility of plasmids carrying the replication origin of the *Escherichia coli* chromosome: evidence for a control region of replication between *oriC* and *asnA*. Nucleic Acids Res. **11**:5775–5791.
50. **Sueoka, N., and H. Yoshikawa.** 1965. The chromosome of *Bacillus subtilis*. I. Theory of marker frequency analysis. Genetics **52**:747–757.
51. **Tanaka, M., and S. Hiraga.** 1985. Negative control of *oriC* plasmid replication by transcription of the *oriC* region. Mol. Gen. Genet. **200**:21–26.
52. **Thomas, C. A., Jr., K. I. Berns, and T. J. Kelley, Jr.** 1966. Isolation of high molecular DNA from bacteria and cell nuclei, p. 535–540. *In* G. L. Cantoni and D. R. Davies (ed.), Procedures in nucleic acid research. Harper & Row, Publishers, Inc., New York.
53. **von Meyenburg, K., and F. Hansen.** 1987. Regulation of chromosome replication, p. 1555–1577. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 2. American Society for Microbiology, Washington, D.C.
54. **Wada, M., Y. Kano, T. Ogawa, T. Okazaki, and F. Imamoto.** 1988. Construction and characterization of the deletion mutant of *hupA* and *hupB* genes in *Escherichia coli*. J. Mol. Biol. **204**:581–591.
55. **Wang, Q., and J. Kaguni.** 1987. Transcriptional repression of the *dnaA* gene of *Escherichia coli* by DnaA protein. Mol. Gen. Genet. **209**:518–525.
56. **Wechsler, J. A., and J. D. Gross.** 1971. *Escherichia coli* mutants temperature-sensitive for DNA synthesis. Mol. Gen. Genet. **113**:273–284.
57. **Yamazaki, K., A. Nagata, Y. Kano, and F. Imamoto.** 1984. Isolation and characterization of nucleoid proteins from *Escherichia coli*. Mol. Gen. Genet. **196**:217–224.