JOURNAL OF BACTERIOLOGY, Apr. 1986, p. 143–147
0021-9193/86/040143-05$02.00/0

Vol. 166, No. 1

RNase H and Replication of ColE1 DNA in *Escherichia coli*

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Received 30 September 1985/Accepted 21 January 1986

Amber mutations within the *rnh* (RNase H) gene of *Escherichia coli* K-12 were isolated by selecting for bacteria capable of replicating in a *sup*$^+$ background replication-defective *cer-6* mutant of the ColE1 replicon. The *cer-6* mutation is an alteration of one base pair located 160 nucleotides upstream of the unique replication origin of this plasmid. Subsequently, we determined the DNA alterations present within these mutants. ColE1 DNA replicated in *rnh*(Am) *recA* cells, indicating that (i) RNase H, which has been shown to be absolutely required for in vitro initiation of ColE1 DNA replication, is dispensable in vivo, and (ii) ColE1 replication in the absence of RNase H is not dependent on "stable DNA replication," which has been reported to be an alternative mode of chromosomal DNA replication. Another class of bacterial mutations was also isolated. These mutations, named *herB*, suppressed *cer-6* replication in *rnh*$^+$ bacteria. *herB* mutations mapped close to the *polA* gene on the *E. coli* chromosome and increased the activity of DNA polymerase I. These findings suggest that when the DNA polymerase I has an opportunity to initiate DNA synthesis before RNase H acts, the replication-defective *cer-6* mutant or the wild-type ColE1 replicates in *E. coli*.

We have previously reported the isolation and characterization of *Escherichia coli* mutants that suppress replication-defective mutations of the ColE1 replicon (19). One of the suppressors, called *herA*, which suppresses the *cer-6* mutation of ColE1 (20), was mapped within the *rnh* gene, the structural gene for RNase H. The *cer-6* mutation is an alteration of one base pair located 160 nucleotides upstream of the unique replication origin of the ColE1 plasmid. The *rnh* mutations isolated as *herA* suppressors behaved either like *ts* or missense mutations, and extracts of these cells showed very low activity for RNase H. Conversely, *E. coli* mutants defective in RNase H isolated independently of the ColE1 replication suppressed the replication of the *cer-6* mutant as well as the wild-type ColE1 replicon (19). These findings indicate that in vivo replication of the ColE1 *cer-6* mutant is inhibited by the activity of RNase H, which has been unequivocally demonstrated to be one of the three proteins absolutely required in the purified system that initiates the DNA replication of ColE1 in vitro (9, 10, 16).

If our postulate is correct, the *cer-6* mutant plasmid may be considered a selective agent to isolate more mutations in *rnh*. These mutations might include stringent mutations such as ambers, which can be verified by determining their DNA sequences. If RNase H were inhibitory for *cer-6* replication, isolation of suppressors for the *cer-6* mutation in *rnh*$^+$ bacteria would disclose beneficial alterations of bacterial gene(s) required for the mutant plasmid to replicate.

In this communication, we report the isolation of *herA*(Am) mutations within the *rnh* gene of *E. coli* and describe a class of dominant suppressors called *herB* that replicate the *cer-6* mutant ColE1 in *rnh*$^+$ bacteria. The *herB* mutation maps on the *E. coli* chromosome closely linked to *polA* and increases the activity of DNA polymerase I.

MATERIALS AND METHODS

Bacterial strains. *E. coli* K-12 strains used and their relevant characters are as follows: N211, Hfr-Cavalli, *recA1 sup*$^+$ (18); NB55, *metB metD dapD proA supE44* (19); KH1234(EQ), *dnaE486 dnaQ49* (8); W3110Thy, *thyA sup*$^+$ (1); and KH5401, F$^-$ *thr ilv sup*$^+$ (20). SN320 [*thyA rnh-136*(Am) *sup*$^+$] and SN321 [*thyA rnh-313*(Am) *sup*$^+$] were constructed by introducing *rnh* mutations into W3110Thy. *recA* derivatives of W3110Thy and SN321 were prepared by introducing first *srl*::Tn*10* (3) and then P1-transducing *recA1* from KL16-99, with selection for Srl$^+$ and UV sensitivity. These derivatives were named SN344 and SN345, respectively. Other strains used were described previously (19) or were prepared in this study.

Media. TY/Glc medium has been described elsewhere (20). M9/casa medium is M9 medium (4) supplemented with 0.5% Casamino Acids (Difco Laboratories), 0.5% glucose, and 1 μg of thiamine per ml. *thyA recA1 rnh*(Am) cells were plated on M9 agar plates containing 0.5% glucose, 1 μg of thiamine per ml, and 20 μg of thymidine per ml.

Tester bacteriophages. Since the tester phages used previously (19) harbor an amber mutation within the *c*I gene, they were made *c*I$^+$ by exchanging the left arm of each phage with that of the wild-type λ phage by cleavage at a unique *Xho*I site (69.4% λ) and ligation. Tester phages thus produced were called λVIII* *c*I$^+$ Ap-pA03 *cer-6* and λVIII* *c*I$^+$ Tc-*cer-6*, depending on whether they harbored the ampicillin resistance gene or the tetracycline resistance gene of pBR322, respectively, to the left of the mutant ColE1 replicon (pA03-*cer-6*) (19). When testing Her phenotypes of *sup*$^+$ nonlysogens, the *P*am3 mutation was introduced into tester phages by crossing them with λ *imm*^{434} *c*$^+$ *P*am3.

Estimation of the copy number of pBR322 plasmid. Cells were grown at 37°C in M9/casa medium (20) supplemented with 5 μg of thymine and 5 μCi of [^{3}H]thymine per ml until the cell density reached 5 × 10^8/ml. Total cellular DNA was extracted, and the copy number of pBR322 was measured by filter hybridization as described previously (25). ^{14}C-labeled pBR322 DNA was used to estimate the efficiency of the DNA-DNA hybridization.

Assay of RNase H activity. Cells were grown at 30°C to 5 × 10^8 cells per ml in M9/casa medium supplemented with 5 μg of thymine per ml. The RNase H activity of S-100 extracts was assayed with [^{3}H]poly(C)-poly(dG) as the substrate (21). One unit of RNase H activity was defined as the amount that

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hydrolyzes 1 nmol of the substrate into acid-soluble form. The substrate was kindly provided by T. Ogawa.

Assay of DNA polymerase I activity. Cells were grown at either 30 or 42°C in TY/Glc medium supplemented with 20 μg of thymine per ml, and cell lysates were prepared as described by DeLucia and Cairns (5). [^{3}H]TTP incorporation was assayed essentially as described by Yamaguchi and Tomizawa (25). KCl (0.1 M) was added to the reaction mixture to inactivate DNA polymerases II and III (23). A 200-μl volume of the reaction mixture contained 50 mM Tris hydrochloride (pH 8.0); 5 mM disodium EDTA; 25 mM $MgSO_4$; 100 mM KCl; 50 μg of activated calf thymus DNA (Pharmacia, Inc.) per ml; 25 μM (each) dATP, dCTP, and dGTP (Takara Shuzo Co.); 25 μM [^{3}H]TTP (108 Ci/mmol; Amersham Corp.); and 50 μl of diluted cell extract (100 μg of protein per ml). The reactions were carried out at 30 or 42°C. Samples (20 μl) were withdrawn from time to time, and the reaction was terminated by the addition of 2 ml of ice-cold 5% trichloroacetic acid–1% Na-PP_i. Protein concentrations of cell extracts were assayed by the Protein Assay (Bio-Rad Laboratories), with bovine serum albumin as the standard.

RESULTS

Isolation of amber mutations in the *rnh* gene. *E. coli* N211 was lysogenized with the wild-type λ phage and used as the parental strain. After mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG), *E. coli* mutants suppressing defective replication of *cer-6* DNA (Her phenotype) were isolated by the two-step selection procedure as described previously (19), except for the use of tester phages containing the *c*I$^+$ gene. Independently isolated *her* mutants were individually conjugated with NB55(λ) harboring *supE44*, and DapD$^+$ transconjugants were tested for the Her phenotype. The *dapD* gene is closely linked to the *herA* (or *rnh*) gene on the chromosome of *E. coli* (19). Out of 76 *her* mutants thus tested, 2 isolates, named N211-136 and N211-313, did not yield Her$^-$ transconjugants (0 of 35 for both of them). When these two strains were conjugated with *sup*$^+$ (*su*$^-$) recipient cells, a substantial fraction of DapD$^+$ transconjugants showed the Her$^-$ phenotype. Moreover, lysogenization of the mutant cells with λ *psu*$^+$*2*, transducing *supE44*, abolished the Her phenotype. Therefore, the HerA phenotype of these mutants is that expected for amber mutations.

Both of these mutations were P1 cotransducible with *metD* or *proA*, suggesting that they map at the *herA* locus. Their HerA phenotype disappeared when the *rnh*$^+$ gene was introduced by lysogenization of the bacteria with λ phage transducing the *rnh*$^+$ gene (19). Therefore, we will hereafter refer to the suppressor mutations in N211-136 and N211-313 as *rnh-136*(Am) and *rnh-313*(Am), respectively.

N211-136 and N211-313 were lysogenized with λ *dnaQ*$^+$ *rnh*$^+$ (7) at 32°C. This transducing phage lacks the *att-int* region but has *c*I857. The lysogens thus obtained were thermoinduced, and phages recovered were lysogenized back to the original bacteria lysogenic with λ *imm*^{434}. Approximately 20% of the recovered phages did not complement the slow growth of *rnh*(Am) *sup*$^+$ cells, judged by the sizes of their colonies on nutrient agar plates. The transducing phages that complemented the temperature sensitivity of KH1234(EQ) cells, indicating the presence of the active *dnaQ* gene, were lysogenized to W3110Thy by using the homology of the *dnaQ-rnh* region. Lysogens thus obtained were subsequently cured of the phage by plating at 40°C. Significant proportions of the cured cells [38% for *rnh-136*(Am) and 29% for *rnh-313*(Am)] showed the Her phenotype. This result verifies that these transducing phages carry the *herA* mutation, and they were named λ* *rnh-136*(Am) and λ* *rnh-313*(Am), respectively. W3110Thy cells harboring *rnh-136*(Am) and *rnh-313*(Am) were named SN320 and SN321, respectively.

Characterization of the amber mutations. Bacterial DNA fragments (1.6 kilobases) were isolated from these *rnh*(Am) transducing phages, and the nucleotide sequence of the region between a *Bam*HI at position 833 and an *Eco*RI at position 1592, according to the numbering used by Maki et al. (15), was determined by the chemical degradation method of Maxam and Gilbert (17). The region sequenced contains the complete *rnh* gene, its promoter sequences, and an amino-terminal part of the *dnaQ* gene. Alterations found for the mutant *rnh* genes were a change from A · T to T · A at position 1407 for *rnh-136* and a change from G · C to A · T at position 1301 for *rnh-313*. Therefore, *rnh-136* and *rnh-313* introduce the amber (TAG) codons at Leu-111 (TTG) and Gln-76 (CAG), respectively. (The following discrepancy from the published sequence [15] was found for the *dnaQ* gene: for both of our transducing phages, the nucleotide at position 938 was A instead of C, yet the transducing phages complemented the *dnaQ49* mutation.)

S-100 extracts of the *rnh*(Am) *sup*$^+$ mutants were prepared, and their RNase H activity was assayed. *rnh*(Am) extracts showed less than 0.1% of the RNase H activity of the extract prepared from the *rnh*$^+$ parental strain (Table 1). The observed values of activity were almost at the limit of detectability by the assay. When the amber cells were lysogenized with λ *psu*$^+$*2* transducing phages, the RNase H activity was restored. However, lysogenization with the wild-type λ phage had no effect (Table 1). Therefore, judged from their DNA sequences, enzyme activity, and suppression by *supE*, *rnh-136* and *rnh-313* are amber mutations.

The plasmid pBR322 transforms the *rnh*(Am) *sup*$^+$ cells with a high frequency, and the copy number of the plasmid DNA per chromosome of growing bacteria was estimated to be 26.0 and 25.9 for SN320 and SN321, respectively. These values are not very different from the corresponding value (23.7) obtained for W3110Thy *rnh*$^+$. pBR322 replicates in these cells for at least 6 h in the presence of chloramphenicol, with the rate of replication no less than that in the wild-type *E. coli* (data not shown), indicating that the replication of the wild-type ColE1 replicon proceeds efficiently in *rnh*(Am) mutants in a *sup*$^+$ background.

ColE1 DNA replication in *rnh* mutants is not due to stable DNA replication. It has been shown that both the *sdrA*

TABLE 1. RNase H activity of S-100 extracts prepared from various *E. coli* strains

E. coli strain	Relevant genotype	RNase H activity (U/mg of protein)	Relative activity
W3110Thy	*rnh*$^+$ *sup*$^+$	19	100
SN320	*rnh-136*(Am) *sup*$^+$	0.007	0.04
SN321	*rnh-313*(Am) *sup*$^+$	0.005	0.03
W3110Thy(λ)		27	100
SN320(λ)		0.016	0.06
SN321(λ)		0.006	0.02
W3110Thy(λ *psu*$^+$*2*)		26	100
SN320(λ *psu*$^+$*2*)		19	72
SN321(λ *psu*$^+$*2*)		11	43
KH5401	*herB*$^+$	39	100
SN282	*herB48*	30	77
SN284	*herB60*	31	80

(stable DNA replication) and *dasF* (*dnaA* suppressor) genes are allelic to the *rnh* gene and that *rnh* mutants can survive the absence of both the *dnaA* function and the *oriC* sequence. The finding was interpreted to suggest that RNase H functions as a specificity protein or a discriminator that prevents aberrant DNA replication starting from sites other than the unique replication origin of the *E. coli* chromosome (6, 22). Cells defective in RNase H continue DNA synthesis in the presence of chloramphenicol, a phenomenon called stable DNA replication, which has been shown to require the function of the $recA^+$ gene and the absence of RNase H (24). Since the parental cells used to isolate our *herA* mutants were *recA*, and since SN345 *rnh*(Am) *recA1* cells support replication of the wild-type ColE1 replicon efficiently, the replication of the ColE1 DNA in *rnh* mutants is not due to the stable DNA replication functional for chromosome replication. In fact, our *rnh*(Am) *recA* cells stopped synthesis of the cellular DNA soon after the addition of chloramphenicol (Fig. 1), confirming that the stable DNA synthesis is not functional in *recA* cells.

Isolation of *herB* mutations that suppress the replication defect of the *cer-6* mutant of ColE1 in the presence of RNase H activity. The HerA phenotype of *rnh* mutants is recessive to its wild-type allele (19). Nevertheless, we have looked for a new suppressor of the *cer-6* mutation that functions in the presence of the rnh^+ gene, hoping to find genes for host factors positively required to replicate the ColE1 replicon. The strategy was again the same as that employed for selecting *herA* mutants, except we selected for suppressors more active at 30 than at 42°C. Screening at a lower incubation temperature helped to minimize the recurrent isolation of temperature-sensitive *rnh* mutants like *herA39* (19). Among 13 suppressor mutations obtained from the strain N211(λ) by NTG mutagenesis and screening at 30°C, 2 mutations did not suppress *cer-6* at 42°C. Both of them were found to map near the *metE* gene at 86 min on the *E. coli* chromosome (2), and they were named *herB48* and *herB60*. One of them, *herB48*, was characterized further; it was found by transduction with P1 phage to map more closely to *metE* (13%) than to *ilv* (<0.4%), and the location almost coincides with that of *polA* at 87 min, which in turn is P1 cotransducible with *metE* at a frequency of 12%. The introduction of F'133 (14) carrying a bacterial DNA fragment encompassing rbs^+ through $argH^+$, containing ilv^+ and $polA^+$, into a *recA herB48* strain did not alter the HerB phenotype. The result indicates that the *herB* suppressor is dominant over its wild-type allele. The temperature dependence of the HerB phenotype of the *herB48* mutation is shown in Fig. 2. The dependency can be contrasted with that of temperature-sensitive *rnh* mutants which suppressed DNA replication of the *cer-6* mutant ColE1 only at temper-

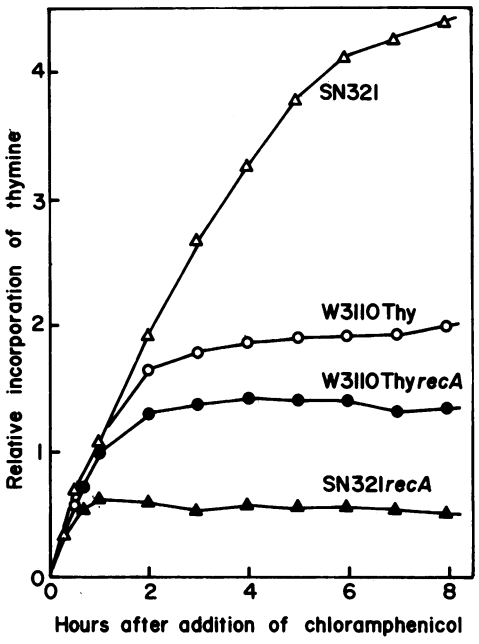

FIG. 1. Stable DNA replication of various strains of *E. coli*. Cells were grown at 37°C in M9/casa medium supplemented with 20 μg of thymine per ml. When cell density reached 3 × 10^8/ml, [^{14}C]thymine (430 μCi/mg) was added at 0.2 μCi/ml. After aeration for 30 min, chloramphenicol (200 μg/ml) and [^{3}H]thymine (357 mCi/mg) were added. Samples (0.1 ml) were taken from time to time, and trichloroacetic acid-insoluble radioactivities were measured. The relative incorporation of thymine was calculated as the amount of thymine incorporated after the addition of chloramphenicol normalized by that incorporated during the 30 min of aeration before addition of the drug. Symbols: ○, W3110Thy; △, SN321; ●, W3110Thy *recA*; ▲, SN321 *recA*.

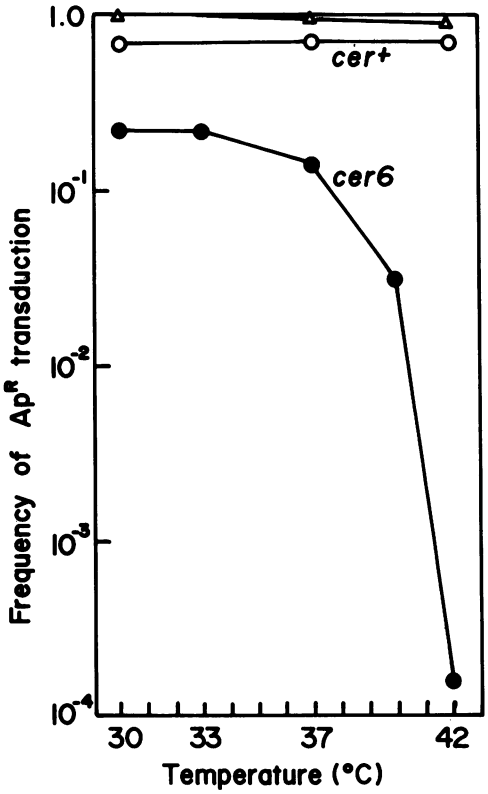

FIG. 2. Temperature dependency of the HerB phenotype. By phenocopy mating, *herB48* was transferred from N211 *herB48*(λ) into CD4 (CGSC 5096), selecting for Met$^+$ Pro$^+$ showing the Her phenotype. Transconjugant cells grown at 37°C were infected with tester phages at a multiplicity of ~0.05 per cell, and the infected cells were plated on TY/Glc agar containing ampicillin at 15 μg/ml. The number of ampicillin-resistant colonies was scored after incubation overnight at various temperatures. The number of resistant colonies divided by the number of tester phages used was designated as the frequency of transduction. Tester phages used: ○, λ *P3* VIII* Ap-pA03 *cer*$^+$; ●, λ *P3* VIII* Ap-pA03 *cer-6*. △, Relative number of colonies.

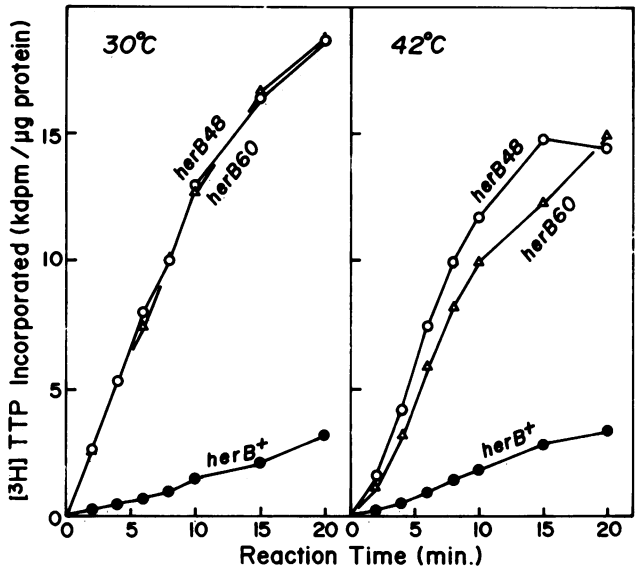

FIG. 3. DNA polymerase I activities of various cell extracts. *E. coli* KH5401 (●) and its isogenic *herB* derivatives, SN282 (○) and SN284 (△), were grown in TY/Glc supplemented with 20 µg of thymine per ml at 30°C (left panel) or at 42°C (right panel). Assays were carried out as described in Materials and Methods, at the temperature at which cells were grown. SN282 and SN284 are *metE*$^+$ transductants of KH5401 and have *herB48* and *herB60* mutations, respectively.

atures higher than a certain threshold (19). The colony-forming ability of the *herB* mutant cells as well as the replication of the wild-type ColE1 replicon in the suppressor mutant is unchanged within the temperature range examined (Fig. 2).

Because of the close linkage of *herB48* to *polA* and the requirement of *polA* function for the initiation of ColE1 DNA replication, the polymerase activity of DNA polymerase I was assayed in extracts prepared from isogenic *herB metE*$^+$ derivatives of strain KH5401 (Fig. 3). The results showed that the enzyme activity of the extracts prepared from *herB* cells grown at 30°C were about nine times higher than that of the *herB*$^+$ cells grown at the same temperature. However, the hyperactivity of DNA polymerase I was also observed for extracts prepared from *herB* bacteria grown at 42°C. The *herB* cells produced more or less the normal activity of RNase H (Table 1).

DISCUSSION

Previously, we have reported that a replication-defective mutant of the ColE1 replicon named *cer-6* replicates if the host bacteria is defective in RNase H (19). The wild-type ColE1 also replicates in the mutant bacteria. Since RNase H has been shown to be one of the three proteins absolutely required in the purified system that initiates the DNA replication of ColE1 in vitro (9, 10), our in vivo result is in apparent contradiction to the results of the in vitro study. In the in vitro system, the addition of 1 U of RNase H per ml has been shown to allow correct initiation of ColE1 DNA synthesis (9). S-100 extracts of wild-type *E. coli* contain about 400 U of RNase H per ml, and the concentration of the enzyme in the cytoplasm of *E. coli* is estimated to be much higher. Therefore, it is possible to argue that the residual activity of RNase H produced by the mutant cells is sufficient to replicate ColE1. Since biochemical assay of a mutant enzyme has inherent limitations as a method to prove no activity, a genetic approach to isolate amber or deletion mutations which are expected to show more stringent defects within the *rnh* gene would be an intriguing method to test the possibility of DNA synthesis without RNase H.

We have shown here that the isolation of amber mutations is possible by selecting for mutant bacteria capable of replicating *cer-6* mutant ColE1. DNA sequencing of two mutant genes isolated independently showed that the *am*136 and *am*313 mutations had in fact introduced amber codons (TAG) at Leu-111 and Gln-76, respectively. The DNA sequencing predicted that the wild-type RNase H protein has 155 amino acid residues (13). Theoretically, it is possible that truncated amber peptides exhibit some enzyme activity or amber codons are sometimes leaky due to occasional readthrough. However, the enzyme assay (Table 1) showed that these amber mutations produce practically no RNase H activity in *sup*$^+$ cells. It may still be argued, however, that the assay of RNase H activity with poly(C)-poly(dG) may not allow accurate measurements of the enzyme activity toward the special substrate, i.e., a hybrid structure at the *ori* site. A complete deletion of the *rnh* gene from the chromosome of *E. coli* may be suggested to secure a clear-cut answer, but the adjacent presence of an essential gene, *dnaQ*, discouraged the attempt, because transcriptional and translational initiation signals for the *dnaQ* gene overlap with the *rnh* gene (15). For the moment, we have to be satisfied with saying that the present results strongly suggest that the in vivo replication of the ColE1 replicon does not require RNase H activity.

Granted that the amber mutations lost RNase H activity completely, we may still surmise that the replication of ColE1 DNA proceeds by alternative pathways depending on whether or not RNase H is available. Manifold phenotypic effects caused by the loss of RNase H in *E. coli* are known to include the suppression of defects in the *dnaA* gene and the *oriC* site, both required to start replication of the bacterial chromosome at a unique site (*oriC*) (13, 22). This suppression has been referred to as stable DNA replication, which functions in *E. coli* having a defective *rnh* and requires the function of the *recA*$^+$ gene. When the concept of stable DNA replication was extended, a model emphasizing the importance of RecA function was proposed to explain the plasmid replication in the absence of RNase H activity (12). However, replication of ColE1 DNA in *recA rnh* double-mutant bacteria shows that the model is not appropriate. In fact, Kogoma (12) is not showing that RNase H-independent replication of pBR322 DNA requires the absence of DNA polymerase I or the presence of RecA function.

Characterization of the secondary-site suppressor mutations within the ColE1 replicon suggested the formation of a hairpin structure that starts by hydrogen bonding of bases at the sites −160 and −187. This structure was broken by the *cer-6* mutation, but the bond can be reformed in one of the partial revertants (20). The in vivo requirement of transcription starting at position −555 was substantiated by isolation of *cer* mutations within the promoter region (19). Replication of *cer-6* DNA in *rnh* mutant bacteria indicates that impairment of the hairpin structure probably makes the mutant ColE1 supersensitive to the action of RNase H. Although in vitro studies supported a model in which RNase H is assumed to function to create a correct primer terminus for DNA synthesis, the addition of excessive RNase H at 100 instead of 1 U/ml is reported to obscure the initiation specificity of wild-type ColE1 (9). Perhaps the processing

activity of RNase H is not needed after all if the transcription of the primer RNA terminates in vivo at the *ori* site.

Of crucial importance is to find factors beneficial for plasmid replication in vivo under a restrictive situation. We have isolated suppressors called *herB* that replicate *cer-6* DNA in *rnh*$^+$ bacteria. These mutants did produce RNase H (Table 1), suggesting that *herB* is not an inhibitor of RNase H. Preliminary characterization of one of them (*herB48*) indicated that the mutation maps close to the *polA* gene and that the mutant produced increased DNA polymerase I activity, which is known to be required for ColE1 replication both in vivo and in vitro (9, 11). If DNA polymerase I is the beneficial factor and if it has been given an opportunity to initiate DNA synthesis before RNase H acts, the *cer-6* DNA may replicate. Overproduction or structural alteration of the DNA polymerase I in *herB* mutant cells is expected to increase the probability of such an event. It is pertinent to note here that Masukata and Tomizawa (16) showed that DNA synthesis in *pri-6*, a mutant similar to *cer-6*, could be observed if they added a fivefold excess of DNA polymerase I to their in vitro system. It may be interesting to see if an increased activity of the wild-type DNA polymerase I by overproduction, for example, rescues the *cer-6* mutant. It is also possible that *herB* mutations alter the properties of DNA polymerase I such that the affected *cer-6* primer RNA can be utilized by the altered DNA polymerase I. These may be some of the formal possibilities which can be tested.

Although *herB* mutants isolated here suppressed *cer-6* only at lower temperatures (Fig. 2), hyperactivity of DNA polymerase I persisted at 42°C (Fig. 3). It may be that the stem-loop structure of the primer RNA sustaining the *cer-6* mutation is more unstable at 42°C than at a lower temperature. Although these assumptions explain our findings, additional biochemical evidence is still needed for substantiation. If our assumptions are proven true, the function of RNase H can be described as merely to eliminate DNA replication of the *cer-6* mutant or certain classes of spontaneous plasmid variants during evolution.

ACKNOWLEDGMENTS

We thank T. Okazaki and K. Yoda for their help in RNase H assay and for valuable discussions. We also thank Pat Lund and Gregg Clark for their assistance in editing the manuscript.

This work was supported in part by grants-in-aid from the Ministry of Education, Science and Culture of the Japanese Government.

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