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Genetics

Escherichia coli mutants suppressing replication-defective mutations of the ColE1 plasmid

(λ-mini-ColE1 hybrid phage/genetic mapping/DNA sequence/RNase H/temperature sensitivity)

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ABSTRACT **Mutants of *Escherichia coli* K-12 have been isolated that suppress *cer* mutants, ColE1 mutants that are unable to replicate as the plasmid. These host suppressors were designated *her*, for *h*ost factor affecting Col*E*1 *r*eplication. Each *her* suppressor showed a characteristic pattern of suppression depending on the *cer* mutation used for selecting the mutant bacteria. One of the suppressors, named *herA*, that suppressed *cer6*, a single-base-pair alteration 160 base pairs upstream of the ColE1 replication origin, was genetically identified as an alteration of the *rnh* gene (RNase H). *HerA* was recessive to its wild-type allele. RNase H activity of *herA* cell extracts was defective. Conversely, *rnh* mutants that were isolated independently of ColE1 replication supported replication of *cer6* DNA. Some *rnh* mutants manifested the HerA phenotype only above a certain transition temperature, and their RNase H activity was found to be temperature sensitive. Therefore, replication of *cer6* DNA *in vivo* is sensitive to RNase H activity. Under the conditions that suppressed *cer6*, the wild-type colE1 replicon replicated normally. Then, ColE1 replication *in vivo* proceeds in the absence of RNase H activity, which has been shown to be required for *in vitro* replication of the DNA.**

Initiation of DNA replication is a complex but controlled process that usually starts from a unique site(s) on a genome. Although recent efforts to understand DNA replication are mainly directed toward establishment followed by biochemical dissection of soluble systems that support DNA replication *in vitro*, possibilities of characterizing elements required *in vivo* have not been extensively explored.

DNA replication of ColE1 plasmid of *Escherichia coli* is initiated *in vitro* by successive actions of *E. coli* RNA polymerase, RNase H, and DNA polymerase I (1, 2). Based on detailed analysis of the reactions, Itoh and Tomizawa proposed the following model to describe the initial reactions of ColE1 DNA replication *in vitro* (1, 2). Starting 555 base pairs (bp) upstream of the replication origin (the *ori* site) (3), RNA polymerase synthesizes a transcript that is cleaved by RNase H (4–6) and is then used as a primer by DNA polymerase I at the *ori* site. Genetic evidence corroborating the validity of the proposed model to describe *in vivo* events is as follows: (*i*) ColE1 DNA replication in wild-type bacteria is sensitive to rifampicin but not in RNA polymerase mutants resistant to the drug. Therefore, RNA polymerase is needed for plasmid replication (7). (*ii*) ColE1 DNA cannot be maintained in *polA1* bacteria, indicating that DNA polymerase I is required for maintenance of the plasmid (8). On the other hand, there is no genetic evidence for the suggested RNase H requirement.

We have previously reported isolation of mini-ColE1 mutants that are defective in DNA replication (9). These mutations were designated *cer*, for *C*ol*E*1 *r*eplication defective. In this communication, we describe a procedure for the isolation of host mutants that allow replication of the defective replicons, intending to identify elements recognizing the *cer* mutant DNA. During the survey, we found that one ColE1 mutation, called *cer6*, was suppressed by the loss of RNase H activity from the host bacteria. In cells defective in the RNase H activity, the wild-type ColE1 replicon replicated.

MATERIALS AND METHODS

Bacteria, Plasmid, and Bacteriophage Strains. The *E. coli* K-12 strains used and their relevant characteristics were as follows: N211(Hfr Cavalli, *recA1 sup*^{0}) (10); KH5401(F$^-$ *thr ilv sup*^{0}), a *sup*^{0} derivative of KH54 (11), was supplied by M. Imai; KL16-99(Hfr *recA1*) (12); KY4644(*leu proA recA1*/KLF4) (13). NB55(*metB metD dapD his proA supE str*^{r}) was prepared by introducing *metB1* and *metD88* of CD4 [CGSC 5096 (14)] and *dapD4* of AT982 (15) into AB1133 (16) (N. Kuwabara, personal communication). ON121 and ON152 were prepared by introducing *rnh59 proA*$^+$ and *rnh91 proA*$^+$ into ON112(*thi proA metE supE endA rna*) (17). The *cer* mutants of pAO3 (18) were as described (9). The position of a particular nucleotide on plasmid pAO3 is designated by the number of nucleotides that separate it from the *ori* site (3), with a plus or minus sign, depending on whether the position is downstream or upstream of *ori*. λVIII has a single *Eco*RI site (srI-2) (19); a hybrid phage, λVIII-pAO3, was called λSN4 (9); λ*psu*$^+$*2* carries *supE* suppressor (20); λ*dnaQ*$^+$ *rnh*$^+$ (21), λ*dnaQ*::Tn*3* *rnh*$^+$ (21), and λ*dnaQ*$^+$ *rnh*::Tn*3* (22) were gifts from T. Horiuchi.

Reagents. Sources of enzymes used were as follows: *Sst* I and *Ava* I (Bethesda Research Laboratories); *Hga* I (New England BioLabs); and *Eco*RI, *Bam*HI, *Hin*fI, T4 DNA polymerase, and T4 ligase (Takara Shuzo, Kyoto, Japan). DNA oligomer d(C-G-G-A-A-T-T-C-C-G) was obtained from Genex, Rockville, MD. Bio-Rad Protein Assay was purchased from Bio-Rad. Ampicillin (Ap) and tetracycline (Tc) were obtained from Banyu Pharmaceutical, Tokyo, Japan, and Sigma, respectively. *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine was obtained from Aldrich. Enzymes and reagents were used as recommended by the supplier. Media and buffers were used as described (9).

Construction of λVIII*Ap and λVIII*Tc Vector Phages. λVIII (19) has a single *Eco*RI site at 54.3% the length of the λ genome (54.3% λ) and retains the *attP* sequence. Circularized λVIII DNA was first partially digested with *Bam*HI. The ends created were filled in by T4 DNA polymerase and ligated to *Eco*RI linker d(C-G-G-A-A-T-T-C-C-G). The mixture was digested completely with *Eco*RI and ligated again. The resultant phage, named λVIII*, has a single *Eco*RI site at 54.3% λ, but the region from 54.3% λ to a *Bam*HI site at 58.1% λ is deleted (N. Inoue, personal communication). Therefore, the phage lacks *attP* and part of the *int* gene. The

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Abbreviations: bp, base pair(s); Ap, ampicillin; Tc, tetracycline; ccc, covalently closed circular.

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β-lactamase gene of pBR322 (23), 1,192 bp long and flanked by *Eco*RI and *Hga*I sites, was purified and cloned into λVIII* DNA between the *Eco*RI site at 54.3% λ and the *Sst* I site at 51.5% λ. The phage was named λVIII*Ap. Similarly, the Tc-resistance gene of pBR322, 1,426 bp long and flanked by *Eco*RI and *Ava* I sites, was cloned into λVIII* at the same position, and the phage obtained was named λVIII*Tc. pAO3 (18) or its mutants were cloned into λVIII*Ap or λVIII*Tc at their unique *Eco*RI sites. The polarity of the cloned fragment was determined by examining *Hin*fI digests, and those clones having leftward direction for RNA I transcription (18) were used.

DNA Sequence Analysis. The DNA sequence of *cer* mutants of pAO3 from position +75 to −662 was determined by the chemical degradation method of Maxam and Gilbert (24).

RNase H Assay. The standard reaction mixture (50 μl) for RNase H assay was 40 mM Tris·HCl, pH 7.8/10 mM $MgCl_2$/0.5 mM dithiothreitol/20 mM NaCl/bovine serum albumin (0.1 mg/ml)/7.6 μM [^{3}H]poly(C) (11.4 cpm/pmol of nucleotide) hybridized to 20 μM poly(dG) and 10 μl of appropriately diluted S-100 extract (17). By definition, 1 unit of the enzyme converts 1 nmol of [^{3}H]poly(C) to 5% trichloroacetic acid-soluble form in 15 min at 30°C. Bio-Rad Protein Assay was used to estimate the protein concentration of the S-100 extract.

RESULTS

Isolation of Bacterial Mutants Allowing Replication of pAO3 Mutant Replicons. The pAO3 is a 1,683-bp segment of the ColE1 plasmid that replicates autonomously in *E. coli* (18). We previously described mutants of pAO3 that are defective in DNA replication (9), and we designated them *cer*. To select for bacterial mutants allowing replication of the *cer* mutant replicons, each *cer* DNA was cloned into λ vectors harboring the Ap- or Tc-resistance gene, and the mutant bacteria were initially isolated as drug-resistant colonies on infection of lysogens with these tester phages. These host mutations were named *her* for *h*ost factor affecting Col*E*1 *r*eplication.

Isogenic tester phages λVIII*Ap-pAO3*cer* and λVIII*-Tc-pAO3*cer* were prepared by *Eco*RI digestion of λVIII-pAO3*cer* DNA (9) and by recloning into λVIII*Ap and λVIII*Tc vectors, respectively. *E. coli* strain N211, a Cavalli-type Hfr harboring *recA1*, was lysogenized with λp*su*$^+$*2* phage, which transduces *supE* suppressor, and was used as the parental strain to isolate host mutants suppressing defective replication of *cer* DNA. Because *cI* of λVIII* sustained an *amber* mutation, the *supE* suppressor was introduced to prevent possible titration of the prophage repressor by replication of superinfecting tester phages.

A two-step selection procedure was used to select *her* mutants; in the first step, N211(λp*su*$^+$*2*) cells mutagenized by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine were infected with λVIII*Tc-pAO3*cer* phages at a multiplicity of about 0.5 phage per cell. Tc-resistant transductants were selected on TY/glc agar containing Tc at 5 μg/ml. In the second step, Tc-resistant colonies were replica-plated onto TY/glc agar containing Ap at 20 μg/ml which had been spread with λVIII*Ap-pA03*cer* harboring the same *cer* mutation at ≈10^7 per plate. In a typical experiment with the *cer6* mutation (9), ≈1.5×10^9 tester phages were used in the first selection, and ≈6,000 Tc-resistant colonies were obtained. Of these, 13 were Ap resistant in the second step. The remaining colonies may have been those harboring revertants of *cer6* that were present among the first tester phages. Of 13 Ap-resistant colonies, 9 were Tc resistant. They may have been revertants of *recA1* among the parental bacteria that integrated the first and the second tester phages. Four Tc-sensitive Ap-resistant clones were those that we sought to find, in which the first tester phage DNA replicating in the plasmid state was excluded by the superinfecting second tester phages because of plasmid incompatibility. The mutations isolated were designated *her* followed by the isolate number. Thus, the four mutations obtained were named *her34*, *her35*, *her38*, and *her39*.

The *cer6* mutation was a single bp alteration, C·G to T·A, at position −160 (9). Other *cer* mutations described previously have since been characterized. Among them, *cer10* was an insertion of one A·T pair within a stretch of seven consecutive A·T's from position −576 to −582; *cer17* was a deletion of one A·T from the same A·T stretch. No other alteration was found for both *cer10* and *cer17*, as well as for *cer6*, between an Ava II site at position −662 and an *Hin*fI site at position +74. [^{3}H]Thymine incorporation of cells infected in the presence of chloramphenicol by the *cer* phages showed that DNA synthesis driven by the plasmid replicon is very much decreased by *cer6*, *cer10*, or *cer17* mutations (data not shown).

Host suppressors for *cer10* and *cer17* were isolated as described above, and they were named *her81*, *her85*, *her87*, and *her89* for *cer10*; *her62* and *her63* for *cer17*. Each *her* mutant was purified by single colony isolation and cured of the tester phages used in its isolation.

Specificity of *her* Mutations. Each *her* mutant was tested for its ability to suppress various *cer* mutations by measuring transduction frequency of the Ap resistance on infection with λVIII*Ap-pAO3*cer* phages. The results (Table 1) showed that every *her* mutant selected using *cer6* phage failed to be transduced by tester phages harboring a *cer10* or *cer17* mutation. On the other hand, those *her* mutants that were selected by suppression of *cer17* were not transduced to Ap resistance by tester phages harboring either a *cer6* or *cer10* mutation. The *her* mutants that were selected by suppression of *cer10* were able to suppress both *cer10* and *cer17* but not *cer6*. The tester phage having the wild-type (*cer*$^+$) pAO3 transduced Ap resistance with high frequencies to every *her* mutant; the vector phage λVIII*Ap that does not contain the pAO3 fragment transduced the resistance to none of the mutant strains (data not shown). Therefore, it appears that each *her* suppressor shows a characteristic pattern of suppression depending on the *cer* mutation used for its selection. The properties of *her* suppressors for the *cer6* mutation will be described in more detail.

Suppression of DNA Replication of pAO3*cer6* Mutant Replicon. Bacteria were infected with λVIII-pAO3 or λVIII-pAO3*cer6* phages in the presence of chloramphenicol, and

Table 1. Frequency of Ap-resistance transduction of *her* mutant bacteria by various *cer* tester phages

Bacteria		Tester phage			
Selection†	Mutant	*cer6*	*cer10*	*cer17*	*cer*$^+$
None	*her*$^+$	8×10^{-7}	7×10^{-5}	2×10^{-5}	0.36
cer6	*her34*	0.30	3×10^{-5}	2×10^{-5}	0.14
	her35	0.39	3×10^{-5}	1×10^{-5}	0.29
	her38	0.22	4×10^{-5}	2×10^{-5}	0.14
	her39	0.19	6×10^{-5}	3×10^{-5}	0.17
cer10	*her81*	6×10^{-6}	0.007	0.034	0.66
	her85	4×10^{-6}	0.084	0.24	0.60
cer17	*her62*	3×10^{-6}	7×10^{-5}	0.32	0.37
	her63	4×10^{-7}	9×10^{-5}	0.20	0.47

λVIII*Ap-pAO3 tester phages harboring various *cer* mutations were added at a multiplicity of 0.05 per cell to bacterial strains that were suspended in TM buffer at 1×10^9 cells per ml, and adsorption was allowed to continue for 20 min at 37°C. Samples were plated on TY/glc agar containing Ap at 20 μg/ml and incubated at 37°C. To detect suppression of the *cer10* mutation, Ap at 10 μg/ml was added to agar. Number of resistants divided by number of tester phages used was designated as the frequency of transduction.

†The *cer* allele of tester phages used for selecting *her* mutant bacteria.

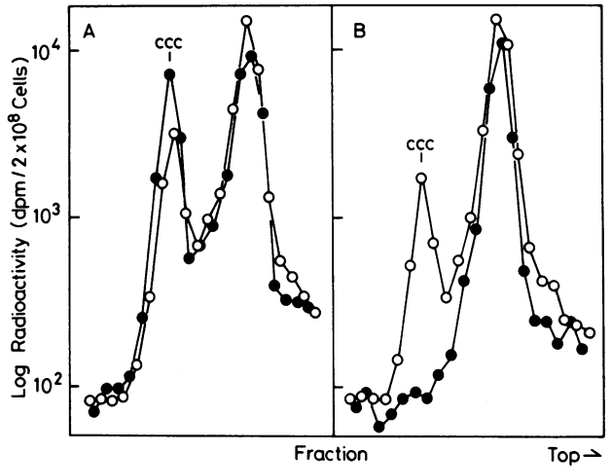

FIG. 1. DNA synthesis in the presence of chloramphenicol. Thymine-requiring cells were grown at 37°C to 5 × 10^8 cells per ml in Casamino acids medium supplemented with thymine (5 μg/ml)/diaminopimelic acid (40 μg/ml)/0.2% maltose. Twenty minutes after addition of chloramphenicol (250 μg/ml), cells were collected by centrifugation and suspended in one-half the original volume of TM buffer (0.2 ml) containing chloramphenicol (250 μg/ml). Phages were added at a multiplicity of 6 phages per cell and were allowed to adsorb for 20 min at 37°C. Phage-infected cells were then diluted 1:10 with prewarmed Casamino acids medium supplemented with thymine (5μg/ml)/[^{3}H]thymine (5 μCi/ml; 1 Ci = 37 GBq)/diaminopimelic acid (40 μg/ml)/0.1% glucose/chloramphenicol (250 μg/ml). After aeration at 37°C for 3 hr, cells were collected by centrifugation and DNA was extracted as described (9) and was fractionated by CsCl/ethidium bromide equilibrium centrifugation of the total cell lysates. Phages used were λVIII-pAO3 (*A*) and λVIII-pAO3*cer6* (*B*). Bacteria used were SN65(λ)*thyA her*$^+$ (●) and SN66(λ)*thyA herA39* (○). They were isogenic thymine-requiring derivatives of SN50(λ) and SN51(λ), respectively (see legend to Table 3). Position of ccc molecules is indicated in the figure.

DNA synthesized after infection was labeled with [^{3}H]thymine. Isogenic bacteria harboring the *her*$^+$ allele were used as a control. After incubation for 3 hr, DNA was extracted and fractionated by CsCl/ethidium bromide equilibrium centrifugation. If infecting phage DNA replicates in a plasmid state, radioactivity will be found at the position expected for the covalently closed circular (ccc) DNA. As shown in Fig. 1, for the sample from *her*$^+$ cells infected with λVIII-pAO3*cer6* phage, essentially no radioactivity was detected in fractions corresponding to ccc molecules. Substantial radioactivity, however, was detected in the fractions in which *her39* cells were used. The wild-type replicon λVIII-pAO3 replicated in a plasmid state in both *her*$^+$ and *her39* cells almost to the same extent. These results indicated that the *her39* mutation suppressed defective DNA replication of the *cer6* mutant replicon and that the suppressor had apparently no effect on replication of the wild-type pAO3.

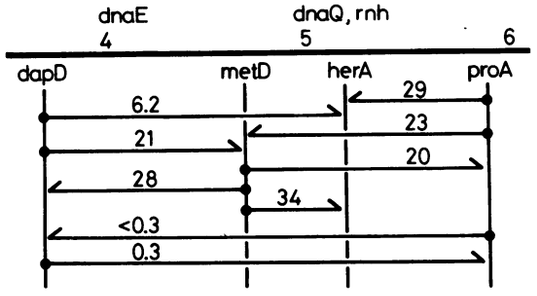

FIG. 2. Transductional mapping of the *herA39* mutation with phage P1. A selected marker is shown at the tail and an unselected marker at the head of an arrow. The number adjacent to an arrow represents the percent cotransduction. The scale, calibrated in minutes, shows a part of the 100-min standard map of *E. coli* K-12 (25).

Table 2. Dominance test for *herA39* mutation

Bacteria	*herA* allele	Frequency of transduction†
SN59(λ) F$^-$	*herA39*	0.21
SN59(λ)/KLF4	*herA39/herA*$^+$	9 × 10^{-6}

SN59(λ) F$^-$ was prepared by P1 cotransduction of *herA39* with *metD*$^+$ into NB55(λ), and then introducing *recA1* by conjugation with KL16-99. SN59(λ)/KLF4 was prepared by sexducing SN59(λ) with KY4644 and selecting for Dap$^+$ merozygotes.
†Phage used was λVIII*Ap-pAO3*cer6*, and frequency of transduction of Ap resistance was measured as in Table 1.

P1 Mapping and Recessiveness of the *her39* Mutation. By conjugation, the *her39* suppressor isolated on a Cavalli-type Hfr strain [N211(λp*su*$^+$*2*)] was transferred to a F$^-$ recipient [KH5401(λp*su*$^+$*2*)] as an early marker (data not shown). One of the transconjugants that received *her39* was used as the donor strain in a P1 transduction experiment using NB55(λ) as a recipient. The results (Fig. 2) indicate that the suppressor is located on the standard map of *E. coli* (25) at 5 min between *metD* and *proA*. Hereafter, we refer to the suppressor gene as *herA*. The locus thus determined is in the region of two closely linked genes, *dnaQ* (14) and *rnh* (5). Other mutations that suppressed *cer6* mapped in the same *metD–proA* region by P1 transduction. On the other hand, suppressors for *cer10* and *cer17* were not located within this region.

A merozygote heterozygous for the *herA* allele was made by sexducing SN59(λ) F$^-$ *herA39 dapD recA1* with KLF4 that has the chromosomal fragment between *thr* and *proB* (12) and selecting for Dap$^+$. The suppression activity of the *herA39* disappeared by introducing the *herA*$^+$ allele (Table 2). When the cell was cured of KLF4 by acridine orange treatment (26), the suppression activity reappeared. Therefore, the *herA39* mutation is recessive to its wild-type allele.

Identification of the *herA* Gene as the Gene for RNase H (*rnh*). Precise mapping of the *herA* gene was possible because the two closely linked genes *dnaQ* and *rnh* have been isolated as a 1.5-kilobase DNA fragment and cloned into a λ phage vector to give a transducing phage coding for two extra proteins of M_r 25,000 and 21,000, corresponding to products of *dnaQ* and *rnh*, respectively (21). Moreover, derivatives of the transducing phage in which each of the two genes was inactivated by insertion of transposon Tn*3* (27) have been prepared (T. Horiuchi, personal communication; ref. 21). Complementation experiments carried out by lysogenization of various transducing phages to the *herA39* bacteria showed that *herA* is an alteration of the *rnh* gene (Table 3). Other mutants, *her34*, *her35*, and *her38*, were examined in the same manner, and they were also found to be mutations of the *rnh* gene. Therefore, all the mutant bacteria allowing replication of the *cer6* mutant replicon were altered in RNase H.

HerA Is RNase H-Defective and Vice Versa. RNase H activity of the *herA39* mutant cells was assayed and was found to

Table 3. Complementation tests of the *herA39* mutation

Bacteria	Frequency of transduction†
SN50(λ)	2.3 × 10^{-3}
SN51(λ)	0.51
SN51(λ)(λ*dnaQ*$^+$ *rnh*$^+$)	5.8 × 10^{-3}
SN51(λ)(λ*dnaQ*::Tn*3* *rnh*$^+$)	6.6 × 10^{-3}
SN51(λ)(λ*dnaQ*$^+$ *rnh*::Tn*3*)	0.57

SN51(λ) was prepared by P1 cotransduction of *metD*$^+$ and *herA39* into NB55(λ). SN50(λ) was an isogenic *metD*$^+$ *herA*$^+$ transductant.
†Phage used was λVIII*Tc-pAO3*cer6*, and Tc-resistant transductants were selected.

Table 4. RNase H activity and suppression of *cer6* replication

Bacteria	Mutation	RNase H activity,† units per mg of protein	Frequency of transduction‡
SN50	*herA*$^+$	23.5 (100%)	7×10^{-5}
SN51	*herA39*	0.05 (0.2%)	0.74
ON112	*rnh*$^+$	19.7 (100%)	3×10^{-4}
ON121	*rnh59*	0.08 (0.4%)	5×10^{-4}
ON152	*rnh91*	ND (<0.02%)	0.75

ND, not detectable.
†Cells were grown at 30°C, and RNase H activity was measured at 30°C.
‡Cells were lysogenized with λ, and frequency of transduction of Ap resistance by λVIII*Ap-pAO3*cer6* was measured at 37°C.

be decreased as compared to that of the parental cells (Table 4). Therefore, the HerA phenotype of *rnh* mutants isolated independently of the ColE1 replication was examined. The strain named ON152 (*rnh91*), which has an undetectable level of RNase H activity, was a typical HerA mutant. On the other hand, ON121 (*rnh59*), which showed trace RNase H activity (0.08 unit per mg of protein) could not support replication of the *cer6* replicon. Because the bacteria used for the enzyme assay were grown and assayed at 30°C, whereas transduction by λVIII*Ap-pAO3*cer6* was carried out at 37°C, possible thermodependency of *rnh59* and *herA39* mutations was tested by using various incubation temperatures. We may infer from the results summarized in Fig. 3 that the mutant cells manifest the HerA phenotype above a certain transition temperature, that the transition temperature is higher for *rnh59* than that for *herA39*, and that the *cer*$^+$ replicon replicated in both mutant strains within the temperature range tested. It is intriguing to observe that the efficiency of transduction was approaching a plateau value as the temperature increased, and it did not decline. The HerA phenotype of ON152 and ON121 disappeared when the bacteria were lysogenized with λ*dnaQ*::Tn*3* *rnh*$^+$ transducing phage but not when lysogenized with λ*dnaQ*$^+$ *rnh*::Tn*3* (data not shown).

RNase H activity of ON121 cells grown at 37°C or at 43°C was assayed at 30°C, and found to be 0.03 units per mg of protein and less than the limit of detection (<0.002 units per mg of protein), respectively. Therefore, at least production of RNase H by ON121 was thermosensitive. The property is consistent with the observation that the *herA* mutation was recessive to its wild-type allele.

DISCUSSION

To correlate functional steps in the initiation of DNA replication with the DNA sequence, we isolated *her* mutants of *E. coli* that suppress replication defects of the *cer* mutations in the *ori* region of the ColE1 plasmid (9). Although only a few *her* mutants were isolated and one group of them, *herA*, has been characterized so far, they proved the present approach to be versatile in the study of ColE1 replication *in vivo*.

First, each *her* suppressor showed a characteristic pattern of suppression depending on the *cer* mutation used for selecting the mutant bacteria (Table 1), suggesting that the DNA sequence altered by each *cer* mutation has a distinct function. Two mutations, *cer10* and *cer17*, occurred at the same locus on the ColE1 DNA; the former was an insertion and the latter was a deletion of one A·T pair within 7 consecutive A·T's located from position −576 to −582. The region separates the −10 and the −35 regions of the promoter for synthesizing the 555-nucleotide-long primer RNA that has been characterized by *in vitro* analysis (2). It is interesting to note that the *her* mutants selected by suppression of *cer10* suppressed both *cer10* and *cer17*, whereas the *her* suppressors isolated by using *cer17* suppressed only *cer17*. On the other hand, they could not suppress *cer6*, which is a single bp alteration at position −160, and suppressors for *cer6* could not suppress *cer10* and *cer17* (Table 1).

Second, *herA*, which suppressed *cer6*, occurred in the *rnh* gene of *E. coli*, and extracts of *herA* cells were defective in RNase H activity. Conversely, mutants defective in RNase H activity that were isolated independently of ColE1 replication supported replication of *cer6* DNA (Table 4; Fig. 3). Under the conditions that suppressed *cer6*, the wild-type ColE1 replicon replicated normally. RNase H is absolutely required for *in vitro* replication of the DNA using purified enzymes (1, 2).

Although the specificity of the RNase H assay was very much increased by using [^{3}H]poly(C)-poly(dG) as substrate (17), we could not discriminate trace activity from no activity. On the other hand, Itoh and Tomizawa (1) observed that addition of excessive amounts of RNase H to their *in vitro* system was detrimental for correct initiation of the wild-type ColE1 replication. Therefore, it may be argued that the presence of low but positive activity of RNase H was still required for replication of the *cer6* DNA that became supersensitive to the enzyme activity. The recessiveness of the *herA* mutation to its wild-type allele (Table 2) is consistent with this interpretation. For thermodependent *herA* mutants, we may consider that the intracellular activity of RNase H decreased monotonically as the incubation temperature increased, and at the lowest temperature manifesting the HerA phenotype, the permissive level of the intracellular enzyme was reached. If this permissive level were low but positive, we may expect an optimal range of incubation temperature as the temperature was further increased. On the contrary, the results for *herA39* cells (Fig. 3) showed that

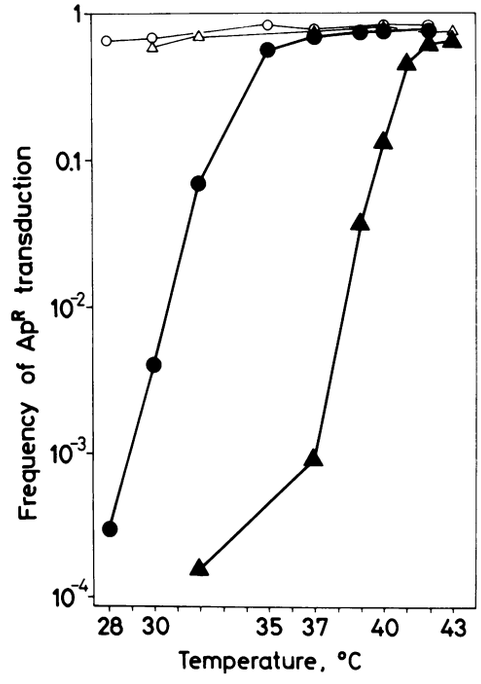

FIG. 3. Temperature dependency of the HerA phenotype. Cells grown at 37°C were infected with tester phages at a multiplicity of ≈0.05 per cell, and adsorption was allowed to continue for 20 min at 37°C. The infected cells were plated on TY/glc agar containing Ap at 20 μg/ml and the number of Ap-resistant colonies was scored after incubation overnight at various temperatures. Transduction frequencies were calculated as in Table 1. Bacteria and tester phages used were SN51(λ)*dap*$^+$ infected with λVIII*Ap-pAO3*cer*$^+$ (○), SN51(λ)*dap*$^+$ infected with λVIII*Ap-pAO3*cer6* (●); ON121(λ) infected with λVIII*Ap-pAO3*cer*$^+$ (△), and ON121(λ) infected with λVIII*Ap-pAO3*cer6* (▲).

once the temperature exceeded a transition point, the HerA phenotype continued to be expressed maximally. The easiest interpretation of the results is to postulate that ColE1 replication *in vivo* proceeds in the absence of RNase H activity.

It was clear that ColE1 replicon replicated in *herA* cells as plasmids. As a step in our isolation of *her* mutants, resident *cer* DNA was eliminated by superinfection of the cell with tester phages having the same *cer* DNA and resistance to different antibiotics. As shown in Fig. 1, *cer* DNA cloned into a λ replicon replicated in *herA* as ccc molecules in the presence of chloramphenicol. Replication of ColE1 DNA has been known to be insensitive to inhibition of protein synthesis in bacteria (28), while λ is not. Furthermore, three ColE1-type plasmids [pBR322 (23), pML21 (29), and pAO43 (18)] were stably maintained in ON152 cells and their copy number was the same as in ON112 (wild-type) cells (17). Preliminary experiments (unpublished results) indicated that ColE1 replication in the wild type as well as mutants defective in RNase H started from the *ori* site; the possibility of initiation from another site was not obvious.

Hillenbrand and Staudenbauer (30) recently reported an *in vitro* study suggesting that RNase H acts as a discrimination factor inhibiting initiation events occurring at sites other than *ori*. According to their model, the primer RNA is protected from degradation by RNase H by forming an extensive secondary structure of the template–primer complex at the *ori* region. Although replication of our *cer6* DNA was sensitive to RNase H activity *in vivo*, its DNA sequence near *ori* was not altered. Therefore, the sensitivity of *cer6* is difficult to explain by this model. Because the *cer6* mutation could be considered to destabilize a possible hairpin structure that starts by hydrogen bonding of bases at positions −160 and −187 (9), the mutation may have introduced a new site sensitive to RNase H by exposing sequences of the primer RNA to become available for hybridization with DNA.

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