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RNase H-defective mutants of *Escherichia coli*: A possible discriminatory role of RNase H in initiation of DNA replication

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Summary. Mutants of *Escherichia coli* completely deficient in RNase H activity were isolated by inserting transposon Tn*3* into the structural gene for RNase H, *rnh*, and its promoter. These *rnh*$^-$ mutants exhibited the following phenotypes; (1) the mutants grew fairly normally, (2) *rnh*$^-$ cells could be transformed with ColE1 derivative plasmids, pBR322 and pML21, though the plasmids were relatively unstable, under non selective conditions, (3) *rnh*$^-$ mutations partially suppressed the temperature-sensitive phenotype of plasmid pSC301, a DNA replication initiation mutant derived from pSC101, (4) *rnh*$^-$ mutations suppressed the temperature-sensitive growth character of *dnaA*^{ts} mutant, (5) *rnh*$^-$ cells showed continued DNA synthesis in the presence of chloramphenicol (stable DNA replication). Based on these findings we propose a model for a role of RNase H in the initiation of chromosomal DNA replication. We suggest that two types of RNA primers for initiation of DNA replication are synthesized in a *dnaA*/*oriC*-dependent and -independent manner and that only the *dnaA*/*oriC*-dependent primer is involved in the normal DNA replication since the *dnaA*/*oriC* independent primer is selectively degraded by RNase H.

Introduction

Ribonuclease H (RNase H) is an enzyme that degrades specifically the RNA strand of the RNA-DNA hybrid molecule and is present in a wide variety of organisms. Although these findings suggested that RNase H may play an important physiological function(s) in vivo, the exact role of the enzyme has not been elucidated except for RNase H encoded in retrovirus genomes (Crouch and Dirksen 1982).

Itoh and Tomizawa (1980) carried out a biochemical analysis of ColE1 DNA replication in vitro and found that RNase H seems to play an important role in initiation of ColE1 replication by processing the RNA primer into a functional form(s). There is no direct evidence that RNase H is involved in DNA replication in vivo. An RNase H mutant has been isolated, but contained 38% of the normal level of activity (Carl et al. 1980)

Kogoma and Lark (1970, 1975) found that when cells were subjected to treatments that inhibit DNA replication (for example, thymine starvation or nalidixic acid treatment) or when the cultures were transferred to a nutritionally rich medium from a poor one, chloramphenicol-insensitive DNA replication was induced. They termed this type of DNA synthesis "stable DNA replication". Subsequently, Kogoma (1978) isolated a mutant, *sdrA*, which exhibited stable DNA replication without inducing treatments. Most recently Kogoma and von Meyenburg (1983) showed that this *sdrA* mutation can suppress both replication origin (*oriC*)-deletion and Tn*10*-inserted *dnaA*-defective mutations. Moreover, Kogoma et al. (1983) reported that the *sdrA* mutation may be identical to *dasF* (Atlung 1981) a suppressor for the Ts growth character of the *dnaA*^{ts} mutant. These mutations are located between the *metD* and *proA* genes on the *E. coli* linkage map. The structural gene for RNase H, *rnh*, has been mapped in this relatively narrow region (Horiuchi et al. 1981). Thus, the possibility that *sdrA* and *dasF* might be alleles of *rnh* had to be considered.

To obtain supportive evidence and to study the role of RNase H in DNA replication, we attempted to isolate *E. coli* mutants completely deficient in RNase H activity. We now report the isolation and properties of such mutants and attention is directed to the possible discriminatory role of RNase H in initiation of DNA replication.

Materials and methods

Bacteria, plasmids and phages. All bacterial strains used in these experiments are derivatives of *E. coli* K12. Strain KH1192 (Cavalli type Hfr) is a *mal*$^+$ derivative of CD4 (Horiuchi et al. 1978). Strain KH692 (Cavalli type Hfr, *dnaA46*) and KH695 (F$^-$, *dnaA46*) are derivatives of LC173 (Nishimura et al. 1971). W3110 was used as the wild-type strain. Plasmid pTT1 was constructed by inserting a 1.6 kb *Eco*RI fragment carrying the *rnh* gene (Horiuchi et al. 1981) into the *Eco*RI site of vector plasmid pACYC184 (Chang and Cohen 1978). Plasmid pSC301 (Kretschmer and Cohen 1977) and phage λcI$^-$ (int)^{d}h^{80} (Miller 1972) were provided by S.N. Cohen and K. Shimada, respectively. Plasmid pML21 (Lovett and Helinsky 1976) and pBR322 (Bolivar et al. 1977) were also used.

Construction of λrnh::Tn3. Plasmid pTT1, which is pACYC184 hybrid plasmid carrying the *rnh*$^+$ gene, was mutagenized randomly by transposition of Tn*3*, according to

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Abbreviations: Ap^r, ampicillin-resistant, kb, kilobase pair(s); NEM, N-ethyl maleimide; Ts, temperature-sensitive

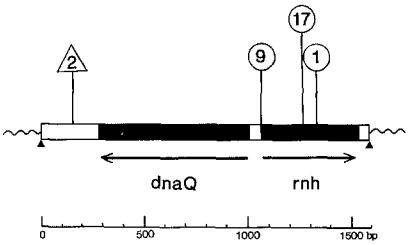

Fig. 1. Tn*3* insertion sites on the 1.6 kb *Eco*RI fragment on which the *rnh* and *dnaQ* genes are located. Circles indicate sites of Tn*3* insertion within the *rnh* gene and the triangle represents outside of the gene. Allele numbers are shown inside the symbols. Arrows indicate direction of transcription of the *dnaQ* and *rnh* genes. △, *Eco*RI site. For more detailed information, see reference Maki et al. (1983)

the method developed by Kretschmer and Cohen (1977). First the Tn*3*-donor plasmid, pSC301::Tn*3* was transferred into strain KH1380 (Maruyama et al. 1983) harboring pTT1. A single colony of the transformant was inoculated in L-broth and incubated at 30° C overnight. An aliquot of the culture was plated on a Penassay broth plate containing ampicillin (50 μg/ml) and tetracycline (10 μg/ml) and incubated at 42° C overnight. A large Ts^+Ap^r colony was selected and purified. The plasmid DNA was extracted and analyzed by agarose gel electrophoresis after *Eco*RI digestion. Among 80 independent Tn*3*-inserted pTT1 derivatives, 15 plasmids were shown to carry Tn*3* within the 1.6 kb *Eco*RI fragment, on which the *rnh* gene is located. By measuring RNase H activity in crude extracts of these Ts^+Ap^r cells, we identified six clones that contained considerably low levels of RNase H activity. Among them #1, 9, 17 (rnh^-) plasmids and as control #2 (rnh^+) plasmid were used for subsequent experiments. Insertion sites of Tn*3* on these plasmids are shown in Fig. 1. After digestion of these plasmids with *Eco*RI, the Tn*3*-inserted DNA fragment was recloned into a λ vector, λgt·λc (Leder et al. 1977).

Isolation of rnh-defective Mutants. The procedure for isolation of λ*rnh*::Tn*3* lysogen was essentially the same as described by Miller (1972). A single colony of KH1192 was inoculated in λ-broth, shaken at 37° C overnight, centrifuged and resuspended in the same volume of 10 mM $MgSO_4$ solution. After standing for 60 min at 37° C λ*rnh*::Tn*3* phages were added at an average multiplicity of ten to 0.1 ml cell suspension and the mixture was left for 10 min at room temperature. λ broth (1 ml) was added and the culture was shaken at 30° C for 2 h. After appropriate dilution, an aliquot of the culture was plated on an Ap-containing EMBO plate seeded with about 10^9 of $\lambda cI^-(int)^d h^{80}$ phages, and the plates were incubated at 30° C. We then isolated stable λcI857 *rnh*::Tn*3* lysogens, which were Ap^r, Ts and λ-immune. After purification they were inoculated in L-broth and grown at 30° C overnight. After appropriate dilution, an aliquot of the culture was put on an L-agar plate containing Ap and incubation carried out at 42° C. We obtained Ap^r Ts^+ clones with about 1×10^{-5} efficiency.

RNase H activity. The procedure for preparation of cell extracts and ammonium sulfate fractions was essentially the same as that described by Wickner et al. (1974). The supernatant solution after streptomycin sulfate precipitation was fractionated by addition of ammonium sulfate to 40% and 55% saturation. The fraction precipitated at 55% saturation was found to contain a most of the RNase H activity. Thus, the 55% fraction, after dialysis against buffer C (Ito and Tomizawa 1978), was applied to a DEAE-cellulose column and the column was washed with buffer C. The eluted RNase H activity was measured according to Berkower et al. (1973).

Other methods. Culturing of bacteria and radioisotopic labeling experiments were performed as described previously (Horiuchi et al. 1978, 1981).

Results

Isolation and characterization of RNase H-deficient mutants

We first attempt to isolate rnh^- mutants from a collection of Ts growth mutants obtained after localized mutagenesis of the *rnh-dnaQ* region of the *E. coli* chromosome. Although we did isolate seven $dnaQ^-$ mutants an rnh^- mutant was not among them. This led to the idea that RNase H might be dispensable for growth of bacteria and thus, as a next step, we prepared to isolate rnh^- mutants by inserting transposon Tn*3* into the *rnh* gene.

The approach used to isolate rnh^- insertion mutants is outlined in Fig. 2. First, *E. coli* strain KH1192 was infected with λ*rnh*::Tn*3* carrying the *c*I857 mutation at 30° C and λ*rnh*::Tn*3* lysogens were isolated by selecting λ-immune, ampicillin-resistant colonies. Because λ*rnh*::Tn*3* lacks normal phage integration capacity, the phage would be integrated into the *rnh* region of the bacterial chromosome by recombination between the homologous regions of the phage and bacterial chromosome. During growth at 30° C these lysogens would spontaneously lose their λ genome due to homologous recombination between the duplicated *rnh* regions, yielding nonlysogenic bacteria that grow at 42° C. We expect that according to sites of recombination, two types of nonlysogenic cells would be produced, one carrying Tn*3* in the *rnh* region (Type 1, shown in Fig. 2) and the other without Tn*3* (Type 2). Thus, we selected ampicillin-resistant colonies among survivors at 42° C.

By using three types of λ*rnh*::Tn*3* phages, which carry Tn*3* at different sites within the *rnh* gene or its promoter (see Fig. 1 and Maki et al. 1983), we obtained three strains of *rnh*::Tn*3* cells (*rnh1*::Tn*3*, *rnh9*::Tn*3* and *rnh17*::Tn*3*). As a control we constructed an rnh^+ strain carrying Tn*3* near but outside the *rnh* gene, which was designated rnh^+::Tn*3* (#2). By P1 phage transduction the Ap^r marker of these strains, conferred by Tn*3*, was mapped. We found that in all cases it was located between *metD* and *proA*, at the location of the *rnh* gene (data not shown).

RNase H activity of *rnh*::Tn*3* strains was next determined. Since interfering nuclease activities are present in a crude extract, the extract was processed to remove such activities. Figure 3A shows an elution profile of RNase H from a DEAE-cellulose column. None or only a small percentage, of the normal level of activity was detected in each of two independent clones of *rnh1*::Tn*3* and *rnh9*::Tn*3* (Fig. 3B and C). Furthermore, the remaining activity found in the mutant samples was NEM-resistant, as reported previously (Berkower et al. 1973). Thus, as expected from the type of mutation, these rnh^-::Tn*3* strains are completely deficient in RNase H activity.

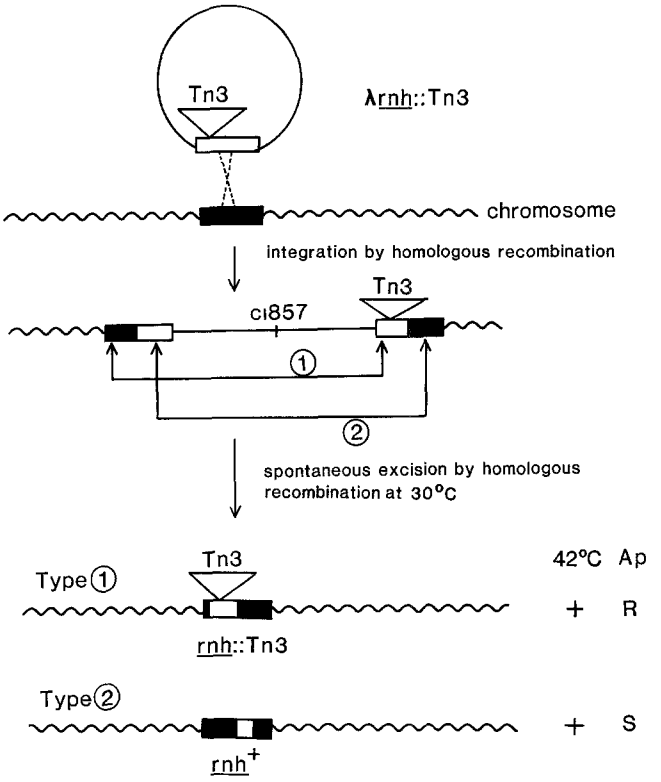

Fig. 2. Schematic diagram illustrating the strategy used for isolation of Tn*3*-inserted *rnh*⁻ mutants. —— λ phage DNA; ■ a part of the *E. coli* chromosome carrying *rnh*⁺; □ the corresponding portion of the *E. coli* chromosome with Tn*3*; ∿∿ remainder of the bacterial chromosome

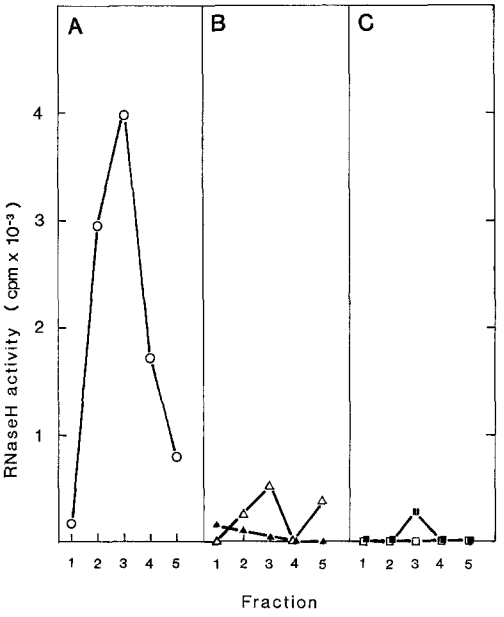

Fig. 3A–C. RNase H activity in bacteria carrying Tn*3* within or outside of the *rnh* gene. Bacteria were grown in L-broth at 37° C to the late exponential phase and then collected. From 2×10^{11} cells, extracts were prepared and processed as described in Materials and methods. A sample of the supernatant (0.2 ml, ca. 2.5 mg protein) was applied to a DEAE-cellulose column (1.5 ml bed volume) and buffer C was used for elution. Fractions of 0.35 ml were collected and RNase H activity in each fraction was determined. **A** KH1608 *rnh*⁺::Tn*3*(#2). **B** KH1594 *rnh1*::Tn*3*; △ clone 1, derived from a small colony; ▲ clone 2, derived from a large colony. **C** KH1600 *rnh9*::Tn*3*, □ clone 1, derived from a small colony, ■ clone 2, derived from a large colony

Effects of rnh mutations on multiplication of bacteria and plasmids

rnh⁻ mutant cells were able to grow at various temperatures in a range between 25° C and 44.5° C. However, the growth rate of these cells was somewhat slower than that of *rnh*⁺ cells, and this character was most remarkable at 30° C in rich medium; average doubling times of *rnh*⁺::Tn*3*(#2) and *rnh1*::Tn*3* cells in nutrient broth at 30° C were 36 and 65 min, respectively. This characteristic was observed with several *rnh*⁻ strains with different genetic backgrounds, including the W3110 wild-type strain to which *rnh1*::Tn*3* allele was transferred by P1 transduction. It seems, therefore, that RNase H is not essential for growth of *E. coli* cells.

Since RNase H is reportedly required for replication of ColE1 DNA in vitro (Ito and Tomizawa 1980) we have examined the effect of *rnh* mutation on the multiplication of ColE1 derivative plasmids, pBR322 and pML21. We found that both *rnh*⁻ and *rnh*⁺ cells could be transformed with these plasmids, at almost the same efficiency, and that there was no difference between sizes of colonies of the rnh⁻ and *rnh*⁺ transformants on the drug-containing plates. However, under nonselective conditions, plasmids in *rnh*⁻ cells were readily lost. As shown in Table 1, *rnh*⁻ transformants yielded plasmid-free cells at certain low frequencies while in *rnh*⁺ cells, both plasmids remained stable. Thus, in the absence of RNase H, plasmids can multiply but there is some interference with replication or segregation of the DNA.

To analyze this, we have used pSC301, a replication initiator mutant of pSC101, which undertakes DNA replication at 30° C but not at 42° C (Kretschmer and Cohen 1977). When *rnh*⁻ and *rnh*⁺ cells harboring pSC301 were grown on drug-containing plates at two temperatures, the results shown in Table 2 were obtained. About 20% of *rnh*⁻ cells harboring pSC301 produced colonies at 42° C, albeit

Table 1. Stability of ColE1 derivative plasmids in Tn*3* inserted *rnh*⁻ mutants

Genotype[a]	Clone	Segregation frequency of plasmid (%)	
		pBR322	pML21
rnh⁺		0	0
rnh⁺::Tn*3*(#2)		0	0
rnh1::Tn*3*	1	0	1.4
	2	17.5	8.4
rnh9::Tn*3*	1	1.1	0.7
	2	0	4.7

[a] Bacteria (*E. coli* KH1192) with various *rnh* genotypes were transformed with pBR322 or pML21 and transformants were isolated on selective plates containing 10 μg/ml tetracycline (for pBR322) or 50 μg/ml kanamycin (for pML21) and then purified on nonselective plates at 37° C. Cells were inoculated into 2 ml L-broth and grown at 37° C overnight. After appropriate dilution, aliquots of culture were put on L-broth plates so as to yield 150–400 colonies per plate, and the plates were incubated at 37° C for 2 days. The plates were then replicated on selective and nonselective plates and the number of colonies formed was determined after a 1 day incubation. Segregation frequency was calculated by dividing the number of cells unable to grow on selective plates by the total number of viable cells and multiplying by 100

Table 2. Suppression of Ts phenotype of pSC301 plasmid by *rnh*$^-$ mutations

Genotype[a]	Colony forming units per ml		Ratio (42°/30° C)
	30° C	42° C	
rnh$^+$::Tn*3*(#2)	7.6×10^9	1.3×10^7	1.7×10^{-3}
rnh1::Tn*3*	4.8×10^9	9.4×10^8	0.20
rnh9::Tn*3*	4.3×10^9	1.2×10^9	0.25

[a] Bacterial strains, KH1192 derivatives carrying plasmid pSC301, were grown to saturation in L-broth containing ampicillin (50 μg/ml). After appropriate dilution, an aliquot of the culture was plated on LB broth (Miller 1972) agar plates containing ampicillin (50 μg/ml) and tetracycline (10 μg/ml). The plates were incubated at indicated temperatures for 2 days and number of colonies formed was counted

Table 3. Suppression of Ts growth character of *dnaA46* mutant by *rnh*$^-$ mutations

Genotype[a]	Colony forming units per ml		Ratio (42°/30° C)
	30° C	42° C	
dnaA46 rnh$^+$::Tn*3*(#2)	3.5×10^9	1.8×10^4	5.2×10^{-6}
dnaA46 rnh1::Tn*3*	2.9×10^9	2.4×10^9	0.82
dnaA46 rnh17::Tn*3*	2.1×10^9	1.8×10^9	0.84

[a] Bacterial strains were grown in medium E containing 0.1% casamino acids, 0.2% glucose, 1 μg/ml thiamine, 20 μg/ml uracil (supplemented E medium) at 30° C overnight. After appropriate dilution, an aliquot of culture was put on supplemented E medium agar plates containing 50 μg/ml ampicillin. The plates were incubated at indicated temperatures for 2 days and the number of colonies formed was counted

of small size. Under equal conditions less than 0.2% of total *rnh*$^+$ cells harboring pSC301 produced colonies. Thus, an *rnh*$^-$ mutation suppresses, at least in part, the temperature-sensitive replication phenotype of pSC301.

Suppression of dnaA mutation by rnh$^-$

Normal function of the *dnaA* gene is required for initiation of replication of the *E. coli* chromosome as well as of pSC101 (Hirota et al. 1970; Hasunuma and Sekiguchi 1977; Frey et al. 1979). The *dasF* that suppresses the *dnaA* mutation has been mapped between *metD* and *proA* genes, a region where the *rnh* gene is located (Atlung 1981; Kogoma et al. 1983). This prompted us to examine the possibility that the *rnh* mutation suppresses *dnaA*^{ts}.

Two *rnh*$^-$ mutations, *rnh1*::Tn*3* and *rnh17*::Tn*3*, were introduced into a *dnaA46* strain, KH692, by P1 transduction. Similarly, a *rnh*$^+$::Tn*3* allele was transduced to the same *dnaA46* strain. Ampicillin-resistant transductants were selected and their Ts growth phenotype was examined. As shown in Table 3, *dnaA*^{ts} *rnh*$^-$ double mutants produced an almost normal number of colonies at 42° C, on minimal medium supplemented with casamino acids. Thus, RNase H-deficient mutants show the Das phenotype. The medium we used here proved more advantageous than the rich medium (L-broth) for observation of the Ts$^+$ phenotype of the double mutants. In this experiment an Hfr strain served as the *dnaA46* strain, but a similar result was obtained when the F$^-$ *dnaA46* strain KH695 was used (data not shown).

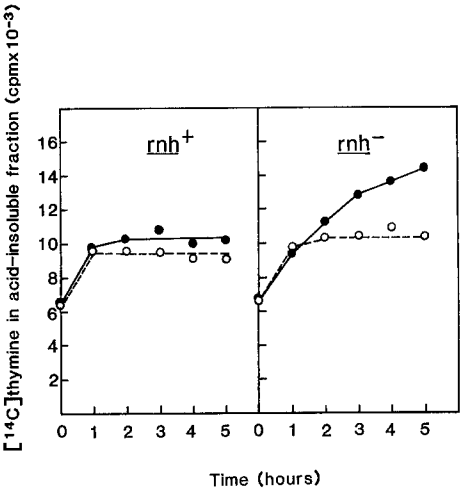

Fig. 4. Effects of chloramphenicol and rifampicin on DNA synthesis in *rnh*$^-$ and *rnh*$^+$ cells. W3110 *rnh*$^+$::Tn*3*(#2) and W3110 *rnh1*::Tn*3* strains were grown in supplemented E medium overnight and an aliquot of culture was inoculated in supplemented E medium containing [^{14}C] thymine (0.19 μmol/ml, 10.5 μCi/μmol). Cultures were shaken at 37° C and when optical density at 660 nm reached about 0.4, chloramphenicol (a final concentration, 150 μg/ml) (●) or rifampicin (100 μg/ml) (○) was added (0 time). Aliquots of the cultures were withdrawn at times indicated and radioactivity of the acid-insoluble fraction was determined

Stable DNA replication in an rnh$^-$ *mutant*

Kogoma (1978) isolated an *sdrA* mutant that is capable of continued DNA synthesis in the presence of chloramphenicol (stable DNA replication). Recently, evidence has been presented that *sdrA* may be identical to *dasF* (Kogoma et al. 1983). Therefore, we looked to see whether stable DNA replication would occur in the *rnh*$^-$ cells.

W3110 *rnh*$^-$ and *rnh*$^+$ cells were grown in medium E supplemented with casamino acids and [^{14}C]thymine after which chloramphenicol or rifampicin was added. As shown in Fig. 4, DNA synthesis of *rnh*$^+$ cells ceased about 2 h after addition of the inhibitors. On the other hand, DNA synthesis of *rnh*$^-$ cells continued for over 5 h in the presence of chloramphenicol, though it was inhibited by rifampicin. This phenotype is similar to that of the *sdrA* mutant (Kogoma 1978).

Discussion

We successfully isolated *E. coli* mutants which are completely deficient in RNase H activity. The mutants grew almost normally under various conditions; therefore, RNase H seems to be dispensable for growth of *E. coli*. However, they exhibited unique phenotypes; *rnh*$^-$ mutations suppressed partially Ts phenotypes of pSC301 plasmid as well as of the *E. coli dnaA*^{ts} mutant and also the mutant was capable of continued DNA synthesis in the presence of chloramphenicol. These phenotypes of *rnh*$^-$ mutants are similar to those of *dasF* and *sdrA* mutants. Since *dasF* and *sdrA* mutations have been mapped in the region of the *E. coli* chromosome where the *rnh* gene is located, it is most probable that the *dasF* and *sdrA* are alleles of *rnh*.

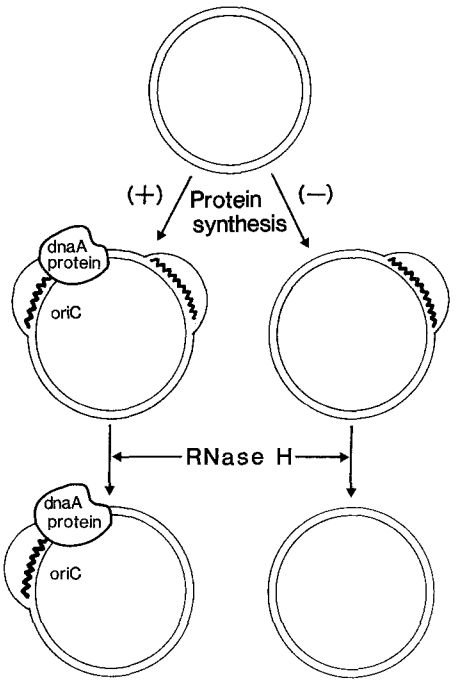

Fig. 5. A model showing the discriminatory role of RNase H in initiation of replication of the *E. coli* chromosomal DNA

How can these phenotypes be attributed to a deficiency in RNase H? By means of explanation, we propose the model outlined in Fig. 5. Let us assume that two types of RNA transcripts serving as initiator for DNA replication are synthesized at the *oriC* and other region(s) of the *E. coli* chromosome and that the latter type of RNA, synthesized at the region(s) other than *oriC*, is preferentially degraded by RNase H. Since de novo protein synthesis is required for normal DNA replication but not for stable DNA replication (Kogoma and Lark 1970), we may further assume that a newly synthesized protein, such as *dnaA* protein, might serve as protector from RNase H digestion. Thus, according to this model, DNA replication would proceed in a *dnaA*/*oriC*-independent manner in the absence of RNase H. This was indeed the case with the *sdrA* mutant, which grew almost normally even though the *oriC* region of the chromosome was deleted or *dnaA* function was defective (Kogoma and von Meyenburg 1983).

There is biochemical evidence to support this model. When RNase H was omitted from the in vitro DNA replication system (for Φx174 and ColE1 DNA) RNA molecules differing from the primary RNA species were used as primer to initiate the reaction (Vicuna et al. 1977; Hillenbrand and Staudenbauer 1982; Ito and Tomizawa 1982). However, it is not certain at present whether RNase H has a role in modification of the primary RNA species at the *oriC* site to a functional form for initiation reaction. Recently, we found that the *oriC* plasmid could transform *dnaA*^{ts} *rnh*$^{-}$ cells at low temperature, thereby suggesting that even in *rnh*$^{-}$ cells, the *dnaA*-dependent initiation system would work (unpublished data). This implies that RNase H may not be essential for initiation of the *dnaA*-dependent DNA replication.

Stable DNA replication can be induced by inhibiting normal DNA replication or treating cells with DNA-damaging agents (Kogoma and Lark 1975; Kogoma 1978). Kogoma et al. (1979) reported that in *recA*$^{-}$ or *lexA*$^{-}$ strains stable DNA replication could not be induced. Thus, induction of stable DNA replication, namely DNA synthesis that uses the secondary initiation system, is under control of the SOS regulon. According to our model, it can be assumed that in induced cells, RNase H might be inactivated by *recA* protease or a new protein might be formed that protects the secondary primer RNA from RNase H digestion. These possibilities are being studied in ongoing investigations.

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