RNase H confers specificity in the dnaA-dependent initiation of replication at the unique origin of the Escherichia coli chromosome in vivo and in vitro

(TORU OGAWA*, GAVIN G. PICKETT†, TOKIO KOGOMA†, and ARTHUR KORNBERG*)

*Department of Biochemistry, Stanford University School of Medicine, Stanford, CA 94305; and †Department of Biology, The University of New Mexico, Albuquerque, NM 87131

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ABSTRACT Escherichia coli rnh mutants defective in RNase H activity in vivo feature the features of previously described sdrA (stable DNA replication) and dasF (dnaA suppressor) mutants: (i) sustained DNA replication in the absence of protein synthesis, (ii) lack of requirement for dnaA protein and the origin of replication (oriC), and (iii) sensitivity of growth to a rich medium. Both the sdrA mutants (selected for continued DNA replication in the absence of protein synthesis) and the dasF mutants (selected as dnaA suppressors) are defective in RNase H activity, measured in vitro. Furthermore, a 760-base-pair fragment containing the rnh* structural gene complements the phenotype of each of the rnh, sdrA, and dasF mutants, indicative of a single gene. One function of RNase H in vivo is in the initiation of a cycle of DNA replication at oriC dependent on dnaA*. In keeping with these results, RNase H contributes to the specificity of dnaA-dependent replication initiated at oriC in a partially purified enzyme system.

Replication of the Escherichia coli chromosome starts at a unique site (oriC) and proceeds bidirectionally (1–3). The DNA fragment containing oriC has been cloned and sequenced (4, 5). Subsequent analyses of the structure and in vivo function of the oriC region have disclosed that a 245-base-pair sequence is required and that within this region certain unique sequences surrounded by defined stretches of spacer sequences must be strictly maintained (6, 7).

The initiation of replication at oriC is dependent on several gene products, particularly dnaA (for review, see ref. 8). The dnaA protein, purified to near homogeneity (9), is an essential component required at an early stage of the initiation reaction in an in vitro system which replicates oriC plasmids (10, 11) and binds a 9-base-pair sequence which appears four times in oriC. The dnaB and dnaC gene products, essential for ongoing replication, are also required during or shortly after initiation (12, 13). RNA polymerase has also been implicated by the inhibitory effect of rifampicin at this stage of replication (14) and by a dnaA suppressor identified with rpoB, the β-subunit gene of RNA polymerase (15).

The initiation of each new round of replication of the E. coli chromosome requires de novo protein synthesis (14, 16, 17). The biochemical nature of this requirement for protein synthesis is still unknown. Mutants, termed constitutive stable DNA replication (Sdr*) mutants, have been isolated that maintain DNA synthesis despite the absence of protein synthesis (stable DNA replication) (18, 19). One of these mutant alleles (sdrA224) maps 5 min between metD and proA (unpublished observations). The sdrA224 mutant tolerates transposon insertional inactivation of the dnaA gene or oriC deletion (20). A group of extragenic suppressor mutations (dasF) of dnaA* mutations has been located near proA (21). The dasF mutants exhibit the Sdr* phenotype (unpublished observations). These several results suggest the presence of an alternative initiation pathway, distinct from the dnaA*– and oriC*–dependent system, and a suppression of this alternative pathway under normal growth conditions by the sdrA* (dasF*) gene product.

In this report, we present evidence that both the sdrA and dasF genes are identical with the rnh gene, which encodes RNase H, and that RNase H is a specificity protein in dnaA*– and oriC*–dependent DNA replication in E. coli.

MATERIALS AND METHODS

The E. coli strains used are listed in Table 1. The levels of RNase H in extracts of ON121 (rnh–59) are 0.1% and in ON152 (rnh–91) less than 0.05% of the wild-type level (22). Plasmid pSK760 (24), a derivative of pBR322 that carries a 760-base-pair sequence of E. coli DNA including the RNase H gene, was obtained from R. Crouch (National Institutes of Health).

Media used were M9 salts/glucose (25) and L broth (LB medium) (26). Depending on the strains used, required supplements, in μg/ml, included: thymine, 8; histidine, 20; tryptophan, 20; arginine, 100; asparagine, 20; thiamin-HCl, 3; and for the plasmid-containing strains, ampicillin, 50.

RNase H activity in extracts was assayed with [3H]poly(C)–poly(dG) as substrate (22, 27). Extracts were prepared by lysing the cells (grown to late logarithmic or stationary phase and washed once with 50 mM Tris-HCl, pH 8.0/10% sucrose) in a solution containing 50 mM Tris-HCl at pH 8.0, 10% sucrose, 300 mM KCl, 2 mM dithiothreitol, 2 mM EDTA, egg lysozyme at 250 μg/ml, phage T4 lysozyme at 0.1 μg/ml, and 6 × 10⁷ to 6 × 10¹⁰ cells/ml for 30 min at 0°C followed by 2 min at 30°C. The debris was removed by centrifugation at 10,000 × g for 10 min. One unit of RNase H activity was defined as the amount that released 1 nmol of trichloroacetic acid-soluble radioactive material in 15 min.

Protein concentration was determined (28) with bovine serum albumin as a standard. Transformation with plasmid DNA was carried out as described (26).

In vitro reactions (25 μl) for DNA replication were for 30 min at 30°C as described (29, 30). Proteins included were: protein HU, topoisomerase I, DNA gyrase, RNA polymerase, single-stranded DNA-binding protein, dnaB and dnaC proteins, primase, DNA polymerase III holoenzyme, and flow-through and 0.5 M KCl eluate fractions obtained by Amicon red-A agarose column chromatography of a crude enzyme fraction. dnaA protein and RNase H were added as specified.

RESULTS

The rnh Mutants Exhibit the Sdr* Phenotype. Observations that RNase H contributes to the specificity of a dnaA and
Table 1. E. coli K-12 strains

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Relevant genotype</th>
<th>Source/ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ON112 (a)</td>
<td>rnh&lt;sup&gt;+&lt;/sup&gt; proA</td>
<td>(22)</td>
</tr>
<tr>
<td>ON121 (a)</td>
<td>rnh-59</td>
<td>(22)</td>
</tr>
<tr>
<td>ON152 (a)</td>
<td>rnh-91</td>
<td>(22)</td>
</tr>
<tr>
<td>AQ634 (b)</td>
<td>sdr&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(23)</td>
</tr>
<tr>
<td>AQ666 (b)</td>
<td>sdrA224 proA&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(23)</td>
</tr>
<tr>
<td>AQ685 (c)</td>
<td>sdr&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(20)</td>
</tr>
<tr>
<td>AQ699 (c)</td>
<td>sdrA224 proA&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(20)</td>
</tr>
<tr>
<td>AQ1355 (d)</td>
<td>dasF373 dnaA5 proA&lt;sup&gt;+&lt;/sup&gt;</td>
<td>P1-Tc373 (21) × DK175, select pro&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>AQ1363 (d)</td>
<td>dasF377 dnaA5 proA&lt;sup&gt;+&lt;/sup&gt;</td>
<td>P1-Tc373 (21) × DK175, select pro&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>AQ1722 (c)</td>
<td>sdrA225 proA&lt;sup&gt;+&lt;/sup&gt; dnaA5</td>
<td>Transformant of DK83</td>
</tr>
<tr>
<td>AQ1724 (d)</td>
<td>dasF373 dnaA5 metD&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Transformant of DK282</td>
</tr>
<tr>
<td>AQ1732 (d)</td>
<td>rnh-91 dnaA5 metD&lt;sup&gt;+&lt;/sup&gt;</td>
<td>P1-ON152 × DK175, select metD&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>AQ1760 (d)</td>
<td>rnh-91 dnaA5 metD&lt;sup&gt;+&lt;/sup&gt; ΔoriC1071</td>
<td>Transductant of AQ1732 with hasn132 ΔoriC1071 (20)</td>
</tr>
<tr>
<td>AQ1773 (d)</td>
<td>rnh-91 dnaA5 metD&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Transformant of AQ1732</td>
</tr>
<tr>
<td>AQ1789 (c)</td>
<td>sdrA225 proA&lt;sup&gt;+&lt;/sup&gt; dnaA5</td>
<td>Transformant of DK83</td>
</tr>
<tr>
<td>AQ1790 (d)</td>
<td>dasF373 dnaA5 metD&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Transformant of DK282</td>
</tr>
<tr>
<td>AQ1791 (d)</td>
<td>rnh-91 dnaA5 metD&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Transformant of AQ1732</td>
</tr>
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<td>DK83 (c)</td>
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<td>P1-Tc743 (20) × AQ699, select tetracycline resistance</td>
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<td>DK175 (d)</td>
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<tr>
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<td>ΔoriC1071</td>
</tr>
<tr>
<td>DK249 (c)</td>
<td>sdrA224 proA&lt;sup&gt;+&lt;/sup&gt; dnaA5</td>
<td>(20)</td>
</tr>
<tr>
<td>DK282 (d)</td>
<td>dasF373 dnaA5 metD&lt;sup&gt;+&lt;/sup&gt;</td>
<td>P1-Tc373 (21) × DK175, select metD&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
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</table>

*Remaining genotypes are as follows: (a) F<sup>+</sup> metE val<sup>+</sup> thi endA rna supE; (b) F<sup>+</sup> argH ilv metB his-29 trpA9605 pro thyA deoB (or C); (c) F<sup>+</sup> argH metB1 his-29 trpA9605 proA<sup>+</sup> metD88 thyA deoB (or C) rpoB; (d) F<sup>+</sup> argH metB1 his-29 trpA9605 proA<sup>+</sup> metD88 thyA deoB (or C) rpoB ilv-v2 sensitive to rich media (Srm<sup>+</sup>), the Sdr<sup>+</sup> phenotype.

Table 3 below; also, unpublished observations and the ability of an sdrA mutant to dispense with dnaA gene function and the oriC sequence (20) prompted us to test rnh mutants for the Sdr<sup>+</sup> phenotype.

The amount of DNA in cultures of two rnh mutants [ON121 (rnh-59) and ON152 (rnh-91)] and of the wild-type strain (ON112) was measured after inhibition of protein synthesis (Fig. 1). In the wild-type strain, DNA synthesis stopped 2 hr after inhibition of protein synthesis. During this period, the DNA content increased by about 40%, as expected from continued replication until the terminus was reached. In contrast, the rnh<sup>-</sup> mutated continued synthesis for at least 7 hr after inhibition of protein synthesis, and the DNA content increased by 150% or more. Introduction of plasmid pSK760, which contains the rnh gene, suppressed the stable DNA replication in the rnh mutants. This plasmid, a derivative of pBR322, contains a 760-base-pair fragment of the E. coli chromosome covering the entire rnh gene and part of the dnaQ gene (24, 32). The vector, pBR322, did not suppress the stable DNA replication of the rnh-91 mutant. These results indicate that the observed Sdr<sup>+</sup> phenotype is caused by rnh mutations. Some increase of the DNA content in plasmid-containing strains may be attributable to replication of the plasmids that occurs even in the presence of chloramphenicol (33).

The rnh Mutant Shares a Common Phenotype with sdrA and dasF Mutants. Since the sdrA and dasF mutations map near rnh (5 min on the E. coli chromosome) (18, 21) and all these mutations cause the Sdr<sup>+</sup> phenotype, we examined the possible identity of these three genes. In addition to constitutive stable DNA replication (Sdr<sup>+</sup>), sdrA and dasF mutants exhibit three other phenotypic features in common: Das (suppression of dnaA), Dos (suppression of ΔoriC), and Srm (sensitivity to rich media) (ref. 20; unpublished observations). The rnh mutants were examined for these additional phenotypic features. As shown in Fig. 2, the rnh-91 mutation suppressed the temperature sensitivity of dnaA5 when transferred into DK175. This Das activity was 13% linked to metD. The rnh-91 mutation also suppressed dnaA850::Tnl0 transferred into ON152. The Das phenotype of rnh-91 mut (AQ1732) was complemented by pSK760 (Fig. 2). The rnh-91 mutation also enabled cells to dispense with the oriC sequence, although growth was significantly perturbed. AQ1760 in Fig. 2 is an example of ΔoriC rnh-91 constructed by the procedure reported previously (20).

ON152 (rnh-91) is not sensitive to rich media (22). However, when the rnh mutation was transferred into DK175 by selection for the Das phenotype, all the Das<sup>-</sup> clones were sensitive to rich media (Srm<sup>-</sup>). It appears that ON152 con-

Fig. 1. Stable DNA replication in rnh mutants. Cells were grown at 37°C in M9/glucose medium. At about 2 × 10<sup>8</sup> cells per ml, chloramphenicol was added at 10 μg/ml. At indicated times, 1-ml portions of the culture were withdrawn and the amount of DNA was estimated (31). (A) ON112 (wild type), (B) ON121 (rnh-59), and (C) ON152 (rnh-91).
contains a suppressor mutation (sls) (unpublished observations) that specifically alleviates the Srm<sup>+</sup> phenotype of sdrA mutants. The Srm<sup>+</sup> phenotype of AQ1732 (rnh-91) was complemented by pSK760 (Fig. 2).

The sdrA and dasF Mutants are Defective in RNase H Activity. Inasmuch as the rnh mutants possess the phenotype of sdrA and dasF mutants, we examined the RNase H activity in extracts of sdrA and dasF mutants. As shown in Table 2, RNase H activity was undetectable in all sdrA and dasF mutants tested. Introduction of pSK760 into two of these mutants, as well as into the wild-type strain, resulted in a 10-fold enhancement over the wild-type level.

The rnh<sup>+</sup> Plasmid Complements all the Phenotypic Features of sdrA and dasF Mutants. The rnh<sup>+</sup>-containing plasmid, pSK760, corrected the Sdr<sup>+</sup> phenotype of sdrA224 and dasF373 mutants (Fig. 3). The vector, pBR322, did not affect the Sdr<sup>+</sup> phenotype of the sdrA224 mutant (data not shown). The Srm<sup>+</sup> phenotype of sdrA and dasF mutants was also suppressed by pSK760 (Fig. 2).

Complementation of the Das phenotype of sdrA and dasF showed certain complexities (Fig. 2): on rich plates, the sdrA dnaA and dasF dnaA double mutants were completely temperature sensitive, although they grew well at 30°C (i.e., Srm<sup>+</sup>); however, they were not clearly temperature sensitive on minimal plates. Measurement of [<sup>3</sup>H]thymine incorporation into DNA revealed that, although the rate of DNA synthesis in these mutants carrying pSK760 is much slower at 42°C than that of the strains not carrying the plasmid, DNA synthesis continued at a slower rate for a considerable period of time (data not shown). Thus, the extent of comple-

**Fig. 2.** Presence or absence of colony formation of rnh, sdrA, and dasF mutants on minimal or broth plates at 30°C or 42°C. Mutant cells were allowed to form colonies on M9/glucose plates at 30°C. Cells of a single colony from these plates were inoculated onto two M9/glucose and two L broth plates and spread with toothpicks. One M9/glucose and one L broth plate were incubated at 30°C (A, C) and the others at 42°C (B, D). The M9/glucose plates (A, B) and L broth plates (C, D) were incubated for 48 and 24 hr, respectively. Spread strains: 1, QA685 (wild type); 2, DK175 (dnaA5); 3, DK83 (sdrA224 dnaA5); 4, DK282 (dasF373 dnaA5); AQ1732 (rnh-91 dnaA5); 6, AQ1722 (sdrA224 dnaA5 (pSK760)); 7, AQ1724 (dasF373 dnaA5 (pSK760)); 8, AQ1773 (rnh-91 dnaA5 (pSK760)); 9, AQ1789 (sdrA224 dnaA5 (pBR322)); 10, AQ1790 (dasF373 dnaA5 (pBR322)); 11, AQ1791 (rnh-91 dnaA5 (pBR322)); and 12, AQ1760 (rnh-91 dnaA5 ΔoriC1077).
mulation of the Das phenotype by pSK760 appears to be allele specific under certain conditions.

**RNase H is a Specificity Factor for Replication of oriC DNA in Vitro.** Ability of rnh (sdrA, dasF) mutants to dispense with dnaA and oriC functions suggests the presence of a second pathway of initiation of replication alternative to the dnaA+ and oriC+-dependent mechanism. It appears that, in wild-type cells, RNase H prevents the use of this alternative pathway. This role of RNase H was directly demonstrated in *vitro* (Table 3). In the absence of RNase H, efficient replication took place on supercoiled replicative form (RF) I DNA of phage ϕX174 as well as on that of phage M13 [2], containing the E. coli oriC+ sequence) both in the presence and absence of the dnaA protein. Addition of RNase H severely inhibited ϕX174 DNA synthesis but not that of the dnaA+ and oriC+-dependent reaction.

**FIG. 3.** Complementation of Sdr+ phenotype of sdrA224 and dasF373 mutants by pSK760. Cells were grown in M9/glucose medium at 37°C (A, B) or 32°C (C) to about 1.5 x 10^6 cells per ml. Chloramphenicol and [3H]thymine were added at final concentrations of 250 µg/ml and 4 µCi/ml, respectively (1 Ci = 37 GBq). At indicated times, a 0.5-ml portion of the culture was withdrawn and radioactivity in the acid-insoluble fraction was measured. (A) AQ634 (wild type); (B) AQ666 (sdrA224); (C) AQ1355 (dasF373 dnaA5).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>Activity, units/mg</th>
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</thead>
<tbody>
<tr>
<td>AQ634 (wild ty)</td>
<td>None</td>
<td>32</td>
</tr>
<tr>
<td>AQ666 (sdrA224)</td>
<td>pSK760</td>
<td>386</td>
</tr>
<tr>
<td>DK241 (sdrA224 ΔoriC1071)</td>
<td>None</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>DK249 (sdrA224 dnaA850::Tn10)</td>
<td>None</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>AQ1355 (dasF373)</td>
<td>None</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>AQ1363 (dasF373 dnaA5)</td>
<td>None</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

**DISCUSSION**

In this report, we have shown that rnh (RNase H) mutants exhibit several distinctive phenotypic features: Sdr+ (constitutive stable DNA replication), Srm (sensitivity to rich media), Das (dnaA+ suppression), and Dos (oriC+ suppression). sdrA mutants, isolated as mutants capable of constitutive stable DNA replication, and dasF mutants selected for their ability to suppress dnaA+ mutations, were defective in RNase H activity (Table 2). Furthermore, the phenotypes due to each of these three independent mutations were complemented by a 760-base-pair segment of E. coli chromosome containing the rnh gene carried by pSK760. This sequenced segment (24) contains no complete gene except that for RNase H. Thus the sdrA, dasF, and rnh genes appear to be identical.

These findings have indicated a role for RNase H in the initiation of replication of the E. coli chromosome. The fact that rnh mutants can survive the absence of the dnaA function and the oriC sequence implies that chromosomal replication can be initiated at other sites in a dnaA-independent mechanism and that, in wild-type cells, RNase H prevents the use of this alternative initiation system. This specificity activity of RNase H was directly demonstrated in *vitro* (Table 3). Discriminatory actions by RNase H have also been reported in *vitro* in replicon-specific replication of fd viral DNA (35) and ColE1 DNA (36, 37). The simplest explanation for the role of RNase H is that it eliminates an RNA transcript hybridized to template DNA that would allow abnormal priming of DNA replication. This explanation does not exclude a more direct involvement of RNase H in dnaA+ and oriC+-dependent initiation. For example, in the creation of a correct primer terminus for DNA synthesis as in the ColE1 system (38). Another possible role for RNase H is in the removal of the 5' terminus of longer primer RNA linked to the nascent short DNA (Okazaki) fragments during discontinuous DNA replication (22).

At present, little is known about the molecular mechanism of initiation of replication in rnh (sdrA) mutants. DNA replication in these mutants is recA+-independent unless protein synthesis is inhibited (39), suggesting the presence of a dnaA-dependent oriC-dependent replication system (see below). Operation of the stable replication pathway would also be possible under recA+ conditions. However, the broth sensitivity of these mutants suggests that replication is not as efficient as in wild-type cells. Under dnaA+ ororiC+ conditions, the rnh mutants depend totally on the stable replication pathway. Slow growth of the rnh ΔoriC mutant suggests the inefficiency of this pathway.

Genetic studies suggest that stable replication is one of the recA+ lexA+-dependent SOS functions (40, 41). In wild-type cells, stable replication can be induced by several treatments that induce SOS functions. Protease-related activity of recA protein may be required to establish stable replication, and maintenance of stable replication may depend on the recombination activity of recA protein (39, 40). A better un-
standing is needed of the events leading to stable replication in wild-type cells and of the relationship of induced stable replication in wild-type cells to constitutive stable replication in rnh (sdrA) mutants.

We thank Robert Crouch for plasmid pSK760, Tove Atlung for strains and Ted Torrey for excellent technical assistance. We also thank Jon Kaguni, Nicholas Dixon, Robert Fuller, and LeRoy Bertsch for the oriC reconstitution assay. This work was supported by grants from the National Institutes of Health to A.K. (GM 07581) and T.K. (GM 22092 and Minority Biomedical Research Support Grant PR08139) and from the National Science Foundation to A.K. (PCM 82-09380A1).