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Isolation and characterization of a second RNase H (RNase HII) of *Escherichia coli* K-12 encoded by the *rnhB* gene

(RNA·DNA hybrid/gene cloning/protein purification/DNA replication)

MITSUHIRO ITAYA

Laboratory of Microbiology, Department of Molecular Biology, Mitsubishi Kasei Institute of Life Sciences, Machida-shi, Tokyo 194, Japan

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ABSTRACT **An additional RNase H (EC 3.1.26.4), RNase HII, has been isolated from *Escherichia coli* K-12. By screening a library of *E. coli* DNA for clones that suppressed RNase H deficiency of an *E. coli rnh* mutant, a clone was obtained that produced a protein with RNase H activity. The overexpressed RNase HII protein in *E. coli* was purified to near homogeneity and exhibited a strong preference for the ribonucleotide moiety of RNA·DNA hybrid as substrate. The terminal 11 amino acids were determined and were identical to those predicted from the nucleotide sequence. The *rnhB* gene, which encodes RNase HII, was distinct from *rnhA* by its map position (4.5 min on *E. coli* genetic map, between *lpxB* and *dnaE*) and by the lack of significant amino acid sequence similarity. The presence of a second RNase H in *E. coli* indicates that multiple RNase H genes per genome is a general feature of a wide variety of organisms.**

Ribonuclease H (RNase H, EC 3.1.26.4) has been defined as an enzyme that specifically degrades the ribonucleotide moiety on RNA·DNA hybrid molecules. Such activities have been reported in a wide variety of organisms, from bacteria to human cells, and in most cases at least two biochemically distinct RNases H have been described (1).

One of exception was *Escherichia coli*, whose RNase H was first found by Miller *et al.* (2) and whose gene (*rnh*) was located at 5.1 min on the *E. coli* genetic map (3). The *E. coli rnh* gene has been cloned (4, 5) and its amino acid sequence was determined (5). Two reports demonstrated that RNase H activity in certain *rnh* mutants was undetectable, which suggested that there was a single functional RNase H enzyme in *E. coli* (6, 7). From the study of *E. coli rnh* mutants, several roles for RNase H have been suggested: (*i*) a positive role in the creation of RNA primer(s) for the initiation of DNA replication of ColE1-type plasmid (8, 9); (*ii*) suppression of initiation of DNA replication from sites other than *oriC*, the normal origin of replication (6, 7, 10, 11); (*iii*) involvement in degrading RNA primers of Okazaki fragments (12); and (*iv*) involvement in DNA repair and SOS-induced mutagenesis (13). Although several phenotypes can be attributed to mutations in the *rnh* gene of *E. coli* (10–13), it is unclear how these phenotypes would persist if a second RNase H gene were present. In spite of this, it seems odd that eukaryotic cells have more than one RNase H while *E. coli* has only one. I thought that a second RNase H could exist in *E. coli* but that its activity would be below the limits of the biochemical assays used to date.

Recently, *E. coli rnh* mutant strains have been constructed whose temperature-sensitive (ts) growth phenotype can be alleviated by supplying RNase H activity even <1% that of wild-type *E. coli* RNase H. Moreover, genes encoding RNase H from *Salmonella typhimurium* and *Saccharomyces cerevisiae* were cloned because of their ability to complement the ts growth defect of the strains (M.T. and R. J. Crouch, unpublished results). Thus, a search for a second RNase H in *E. coli* was made by the use of the *E. coli* strain. Here, I present evidence that a second, previously unidentified gene (*rnhB*) encodes an RNase H protein (RNase HII) of M_r 23,225. Thus, the number of RNases H in *E. coli* is at least two, similar to the number found in eukaryotic cells (1).

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The *E. coli* strain MIC3037 (*rnh-339*::*cat recC271*) has been constructed (unpublished data). MIC2031 is a derivative of W3110 (from Y. Kohara; ref. 14) in which the *rnhA* gene has been disrupted by the chloramphenicol acetyltransferase gene (*cat*) by P1 transduction of *rnh-339*::*cat* from MIC3001 (unpublished data). *E. coli* BL21(DE3) for overproducing the RnhB product was obtained from Studier (15). JA221 (F⁻ *hsdR hsdM*⁺ *trpE5 leuB6 lacY recA1*) was from K. Nakamura (University of Nagoya, Nagoya, Japan) and has routinely been used in this laboratory (16). pBR322 was used for the construction of an *E. coli* DNA library and for subcloning. pBEST6 [3.74 kilobase pairs (kbp)], which was used for exonuclease III-mediated deletion, was constructed by ligation of the 2057-bp *Ava* I/*Bgl* I fragment from pBR322 and the 1681-bp *Pvu* II/*Bgl* I fragment (including the multiple cloning sites) from pGEM4 (Promega Biotec). pHIG3 was from T. Tanaka (Mitsubishi Kasei Institute of Life Sciences, Tokyo) and was composed of pGEM1 (Promega Biotec) and the *degU* gene of *Bacillus subtilis* (17) located downstream of the T7 promoter. pMIB1025-4B had a 277-bp insertion of a *B. subtilis* DNA fragment between SP6 and the T7 promoter of pGEM4 (unpublished data).

Construction of *E. coli* DNA Library. To avoid recloning the *rnh* gene, the chromosomal DNA was isolated from MIC2031 (*rnh-339*::*cat*) by the method of Marmur (18). DNA fragments (>2 kbp) of a *Sau*3A partial digest were isolated from low melting point agarose gel. They were ligated to the alkaline phosphatase (calf intestine)-treated *Bam*HI site of pBR322 and introduced into MIC3037. Ninety-six tetracycline-sensitive transformants, screened after selection at 30°C on LB plates containing ampicillin (100 μg/ml), were pooled and designated a "club." Forty clubs were obtained that contained a total of 3840 independent colonies.

Enzymes and Substrates. Purified RNase HI protein was a generous gift from S. Kanaya (19). The nick-translation kit, DNase I, RNase A, RNase T1, SP6, and T7 RNA polymerases, was from Boehringer Mannheim, and other enzymes used in this study including type II restriction enzymes were from Toyobo (Tokyo). All of the DNA cloning techniques were according to either Maniatis *et al.* (20) or the suppliers'

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Abbreviations: ts, temperature sensitive; ss, single stranded; ds, double stranded.

manuals. Exonuclease III-mediated deletion analysis was done according to Henikoff (21).

Poly([^{32}P]rA)·poly(dT) was prepared according to Keller and Crouch (22). Nick-translated calf thymus DNA was used as the double-stranded (ds) DNA substrate. Single-stranded (ss) DNA was prepared by heat treatment of the labeled calf thymus DNA (100°C for 10 min followed by rapid cooling on ethanol/dry ice). [^{32}P]dCTP was used as the labeled nucleotide.

Labeled ssRNA was obtained by SP6 RNA polymerase-mediated transcription of pMIB1025-4B plasmid linearized by *Hin*dIII so as to generate run-off transcript (277 bases). dsRNA was obtained by mixing the SP6 and T7 transcripts from pMIB1025-4B followed by nuclease S1 treatment and purified by G-50 gel filtration with 10 mM Tris·HCl, pH 7.5/1 mM EDTA as buffer. [^{32}P]ATP was used as the labeled nucleotide.

The purity of poly([^{32}P]rA)·poly(dT), dsDNA, ssDNA, and ssRNA was confirmed by digestion with RNase HI, DNase I, nuclease S1, and RNase A plus RNase T1, respectively. For ssRNA and dsRNA, purity was checked by PAGE. Greater than 90% was present as a single band of the expected size (data not shown). Assay conditions (50 μl of 40 mM Tris·HCl, pH 7.6/4 mM $MgCl_2$/1 mM dithiothreitol/30 μg of bovine serum albumin at 37°C) were the same as in ref. 3.

Sample Preparation for Renaturation Gel Assay. A 0.5-ml overnight culture of *E. coli* was harvested in a 1.5-ml microcentrifuge tube, suspended in 50 μl of 10 mM Tris·HCl, pH 7.5/1 mM EDTA, sonicated, and centrifuged again. The supernatant was transferred to a fresh tube and adjusted to 40 mM Tris·HCl, pH 6.8/1% SDS/50 mM dithiothreitol/5% (vol/vol) glycerol in 100 μl. Samples, usually 10 μl per lane, were boiled for 3 min immediately before loading on the gel. Renaturation gel assays were carried out according to Carl *et al.* (3) (see legend to Fig. 2*b*), but with a gel of 8 × 10 × 0.1 cm and with the solution volumes decreased accordingly.

Overproduction and Purification of RNase HII. The downstream fragment from the *Bam*HI site, which includes the carboxyl half of the *lpxB* gene (23) and the entire *rnhB* gene, was ligated into the *Bcl* I site of the plasmid pHIG3. The *Bcl* I site in this plasmid is in the *degU* gene of *B. subtilis* (17). The DegU product could be overproduced in *E. coli* strain BL21(DE3) (T. Tanaka, personal communication). Cells carrying the resulting plasmid, pMIC2721 (depicted in Fig. 1), could produce the DegU/lpxB chimeric protein [predicted M_r, 27,683 (17, 23)] and a protein of M_r ≈24,000 (RNase HII) (see Fig. 2*a*).

To obtain a large amount of RNase HII, a single colony of BL21(DE3) carrying the plasmid pMIC2721 was grown at 37°C in 150 ml of LB medium containing ampicillin (100 μg/ml) until absorbance at 590 nm reached 0.6. After 3 hr of incubation at 37°C in the presence of isopropyl β-D-thiogalactopyranoside (final concentration, 1 mM), cells were harvested by centrifugation at 4000 × *g* for 15 min at 4°C. Cells were suspended into 3 ml of solution A (0.02 M Tris·HCl, pH 7.5/0.1 M NaCl) and were broken by sonication with a model B-30 sonifier (Branson). The pellet obtained after centrifugation at 10,000 × *g* for 10 min was dissolved in 3 ml of solution A containing 7 M urea. Half of the clear lysate (1.5 ml) was loaded onto a Sephacryl S-200 column (1.2 × 70 cm; Pharmacia) equilibrated with 0.02 M Tris·HCl, pH 7.9/4 M urea and eluted at 5 ml/hr. The first 12 ml was collected and concentrated to 2.5 ml with Centricon 10 (Amicon). After mixing with 10.5 ml of 0.02 M Tris·HCl, pH 8.5/4 M urea, 10 ml was loaded onto a DEAE-Sephacel column (0.9 × 12 cm; Pharmacia), washed with 10 ml of the same buffer, and eluted with 50 ml of 0.02 M Tris·HCl, pH 8.5/4 M urea by linear gradient from 0.0 to 0.2 M NaCl at 10 ml/hr. Fractions were analyzed by SDS/PAGE stained with Coomassie brilliant blue. Fractions that had the highest ratio of RNase HII to any other bands were pooled and concentrated with Centricon 10. All purification procedures were carried out at 4°C. Protein concentrations were determined by the Bio-Rad protein assay system. The N-terminal amino acid sequence was determined by using an Applied Biosystems model 477A automated amino acid analyzer.

RESULTS AND DISCUSSION

Cloning of the *rnhB* Gene. A combination of *rnh-339*::*cat* and *recC271* mutations produced a ts growth phenotype (M.I. and R. I. Crouch, unpublished results). Such a phenotype is a sensitive tool for cloning RNase H genes, even those derived from other species.

Two screens were adopted to test for a second *rnh* gene of *E. coli*. One was to measure the RNase H activity of each club by the renaturation gel assay. The other was to isolate colonies formed at 42°C when plated after appropriate dilutions of each club on LB plates containing ampicillin (100 μg/ml). Use of the first screen on all 40 clubs gave no indication of an increase in RNase H activity (data not shown). However, by the second method, eight independent clones were isolated and classified into four groups, based on comparison of their restriction enzyme maps. A representative clone from each group was analyzed by the renaturation gel assay. From only one of these four clones could a new RNase H activity band be detected. The plasmid pMIC27, the only representative of its group, was examined further.

Mapping *rnhB* on the *E. coli* Chromosome. A restriction enzyme map of the cloned DNA in pMIC27 was constructed (≈6.5-kbp insertion; details not shown) to localize the fragment on the *E. coli* chromosome map constructed by Kohara *et al.* (14). It was found that pMIC27 was composed of two fragments, one (≈3 kbp) derived from *lpxA-lpxB-ORF$_{23}$* at 4.5 min of the *E. coli* genetic map (corresponding to position 211 kbp on the Kohara map) (14, 23–25), and a second DNA fragment (≈3.5 kbp) from an as yet unidentified region. The 7.15-kbp *Eco*RI/*Sal* I fragment carrying the 6.5-kbp insertion and 0.65 kbp of pBR322 from pMIC27 was subcloned at the *Eco*RI and *Sal* I sites of pBEST6. The copy number of pBEST6 was estimated to be similar to that found for pBR322. The resulting plasmid, pMIC2710, was digested with *Sal* I and *Pst* I, followed by exonuclease III digestion, S1 nuclease treatment, religation, and transformation into MIC3037 (21). One resulting plasmid, pMIC2711, retained the ability to complement the ts phenotype of MIC3037 and had a deletion of ≈3.5 kbp (Fig. 1). Subsequent deletion analyses (summarized in Fig. 2) indicated that *rnhB* corresponds to ORF$_{23}$ (23).

Purification and Characterization of RNase HII Product. As described in *Materials and Methods*, RNase HII could be overexpressed in *E. coli* when cloned downstream of the *lpxB* gene. About 80% of the product remained insoluble after sonification and could be solubilized in the presence of 7 M urea (compare lanes 1 and 2 in Fig. 2*b*). Furthermore, RNase HII had a tendency to precipitate when urea was removed but remained in a soluble form in the presence of glycerol (50%) and Triton X-100 (0.1%). RNase HII activity was difficult to detect in crude lysates since RNase HI was present in the expression strain BL21(DE3) (15). Therefore, activity was monitored during purification by the renaturation gel assay in which both protein size and activity were directly determined (see Fig. 2*b*). The 11 amino acids of the N terminus of RNase HII were identical to the sequence deduced from the nucleotide sequence of ORF$_{23}$ (ref. 23; see Fig. 4). In addition, the molecular weight of RNase HII (estimated on SDS/PAGE as 24,000 in Fig. 2) was similar to the deduced M_r of 23,225 for ORF$_{23}$. Thus, RNase HII appeared to be identical to ORF$_{23}$ (23). The final preparation of RNase HII (0.025 mg/ml) was

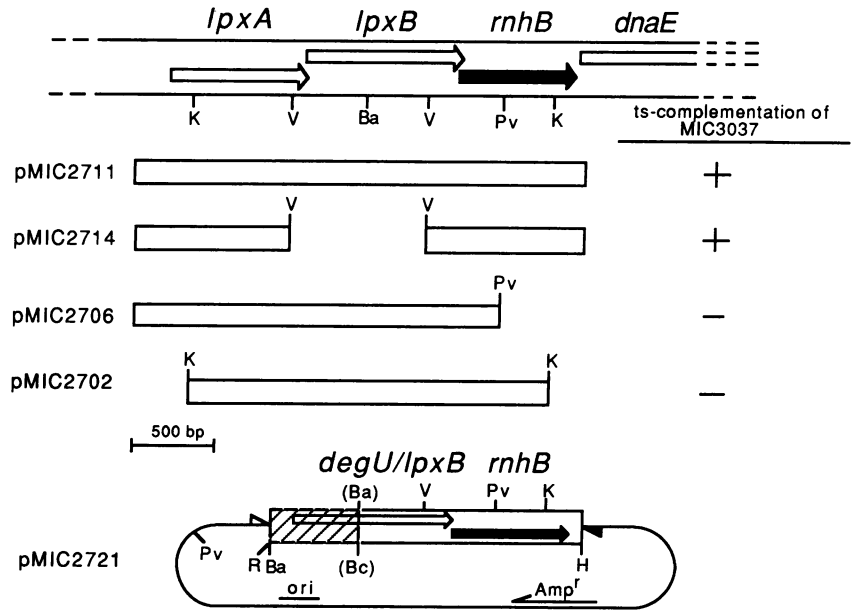

FIG. 1. Localization of *rnhB*. Gene location and orientation of 4.5 min of *E. coli* chromosome map shown at the top are based on refs. 23 and 25. Solid arrow indicates *rnhB* (corresponding to ORF_{23}) (23). Only restriction enzyme sites used for deletion analyses are included. K, *Kpn* I; V, *Eco*RV; Ba, *Bam*HI; Pv, *Pvu* II. In pMIC2721, the shaded box, the open box, and the vector were derived from the *degU* gene of *B. subtilis* (17), pMIC2711, and pGEM4, respectively (see text for details). Bc, *Bcl* I; R, *Eco*RI; H, *Hin*dIII. □ and ■, T7 and SP6 promoters, respectively.

stored in 0.1 M Tris·HCl, pH 7.5/0.01 M $MgCl_2$/20 mM urea/1 mM dithiothreitol/0.1% Triton X-100/50% (vol/vol) glycerol, and it was stable for several weeks when kept at −20°C.

Preference of RNase HII for RNA·DNA Hybrid Substrate. The purity of RNase HII at each step of purification was checked by SDS/PAGE [Fig. 2*b* (*Left*)]. Other activities that degrade the substrate in the renaturation gel assay were eliminated by purification [Fig. 2*b* (*Right*)]. The gel shown in Fig. 2 was run in such a manner that small molecular weight proteins would have migrated completely through the gel. However, no additional RNase H activity was observed even in a renaturation gel assay in which proteins of M_r <14,000 could be detected (data not shown). Therefore, it can be concluded that the purified protein showed RNase H activity and had no other contaminating RNase H-like activity.

To measure the specificity of the purified RNase HII, various RNA or DNA substrates were prepared (Table 1). The time course of the enzyme reaction is shown in Fig. 3, and the specific activities for various substrates are summarized in Table 1. It is clear that RNase HII showed a strong preference for degradation of poly(rA)·poly(dT), although a slight increase of acid-soluble nucleotides was observed when ssRNA and ssDNA were used as substrates (Fig. 3). It

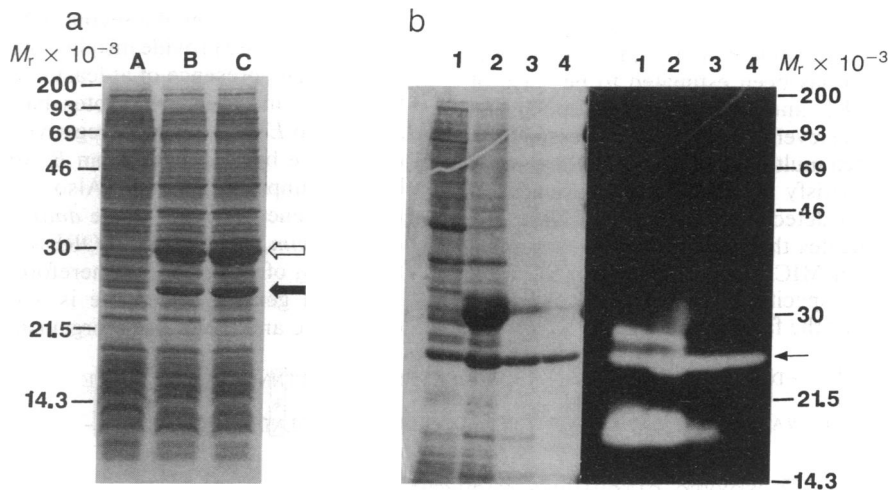

FIG. 2. (*a*) Induced production of RNase HII. Lysates made from BL21 (DE3) harboring pMIC2721 grown at 37°C, were loaded on SDS/polyacrylamide gel stained by Coomassie brilliant blue, uninduced (lane A), and induced with 1 mM isopropyl β-D-thiogalactopyranoside for 70 (lane B) and 110 (lane C) min. Solid arrow indicates RNase HII; open arrow indicates a DegU/lpxB chimeric protein. (*b*) Purification of RNase HII. (*Left*) Coomassie brilliant blue staining. (*Right*) Renaturation gel assay showing RNase H activities as white bands. Arrow indicates RNase HII. The bands larger and smaller than RNase HII are exonuclease III and RNase HI as previously identified (3). Renaturation gel assay: poly([^{32}P]rA)·poly(dT) was embedded in SDS/polyacrylamide gel. After electrophoresis, SDS was removed and the enzymes were renatured. Degraded small RNA products were diffused from the gel. When the gel was exposed to x-ray film (Kodak), white bands could be observed where RNase H or proteins having RNase H-like activity (e.g., exonuclease III) were located. Lanes: 1, supernatant of sonicated cells after centrifugation; 2, cell pellet after centrifugation and solubilization by addition of urea (final concentration, 7 M); 3, after gel filtration on Sephacryl S-200; 4, after DEAE-Sephacel chromatography (0.95 μg of protein).

Table 1. Activity of RNase HII-catalyzed reaction on various substrates

Substrate	Acid-soluble nucleotide, nmol	Relative activity, %
Poly(rA)·poly(dT)	0.43	100
dsDNA	0.0058	1.3
dsRNA	0.0084	2.0
ssDNA	0.035	8.0
ssRNA	0.076	17.6

The acid-soluble nucleotide values were calculated based on both the initial concentration of each substrate (described in the legend of Fig. 3) and the result for the 3-hr time point shown in Fig. 3. One unit is defined as generating 1 nmol of acid-soluble nucleotide per 15 min at 37°C. The calculated specific activity of RNase HII using poly(rA)·poly(dT) as a substrate is 0.57 unit per μg of protein.

could not be determined whether the slow degradation of ssRNA and ssDNA (Fig. 3 and Table 1) was caused by contamination with an unidentified nuclease(s) or resulted from an intrinsic property of RNase HII.

By two separate criteria, complementation of the *rnh-339*::*cat recC271* mutant (MIC3037) and the strong preference for RNA of RNA·DNA hybrids as substrates, it seems reasonable to conclude that RNase HII is, indeed, an RNase H.

The assay condition was the same as that for RNase HI (3) so that the specific RNase H activity could be directly compared. The specific activity of RNase HII was determined to be 0.57 unit per μg of protein using poly(rA)·poly(dT) as substrate—<0.4% that of RNase HI (150 units/μg) (22). The low specific activity might have been caused by something in the purification procedure, particularly the presence of urea. However, (*i*) the failure to detect a measurable increase of total RNase H activity, except by gel renaturation assay, from the lysate of MIC3037 harboring pMIC27, (*ii*) the failure to detect an additional RNase H activity band from the club, including the pMIC27 transformant, by gel renaturation assay as mentioned in *Cloning of the rnhB Gene*, and (*iii*) the activity of RNase HII (seen by renaturation gel assay) appeared to remain constant throughout the purification steps (Fig. 2*b*), all indicated that the specific activity is unchanged during purification and is simply low. The minimum requirement of RNase H activity for ts complementation of MIC3037 (*rnhA-339*::*cat rnhB*$^+$ *recC271*) has been estimated to be <1% of wild-type RNase HI (M.I. and R. J. Crouch, unpublished results). It appears that the level of RNase HII expressed from the *rnhB*$^+$ gene only when multiplied on the pBR322 plasmid (pMIC27) is enough to satisfy the minimum requirement of MIC3037. The failure to detect an increase of RNase HII activity by pMIC27 indicates that the minimum requirement for ts complementation of MIC3037 is still <1% of the wild-type RNase HI. The low specific activity, <0.4% of RNase HI, might also account for the failure to detect the RNase HI

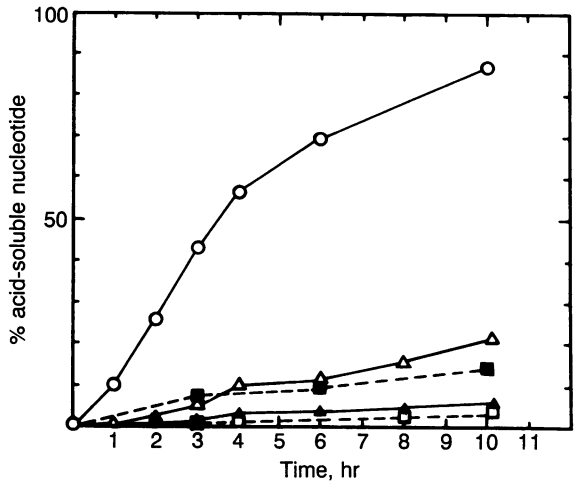

FIG. 3. Time course of RNase HII-catalyzed reaction at 37°C. Reaction conditions and measurement of acid-soluble nucleotide were according to Carl *et al.* (3). The following substrates were used: ○, 40 μM poly(rA)·poly(dT); △, 20 μM ssDNA; ■, 28 μM ssRNA; ▲, 24 μM dsDNA; □, 28.8 μM dsRNA (concentrations are on the basis of nucleotide). Concentration of RNase HII was 0.00625 μg/ml.

activity of RNase HI mutants (*rnhA*) (6, 7). The genetic relationship of RNase HI and RNase HII to genes coding for DNA polymerase III subunits is certainly intriguing. Transcription of the *dnaQ* (the gene encoding the ε subunit of DNA polymerase III) initiates in the coding region of *rnhA* (6). The gene (*dnaE*) for the α subunit of DNA polymerase III is immediately downstream of the *rnhB* gene (ref. 23; Fig. 1).

The amino acid sequences of both RNases H were compared by a homology search program (GENETYX). When the first 155 amino acids of both proteins were compared (as shown in Fig. 4), only 17% similarity was found. The poor similarity between RNase HI and RNase HII supports the idea that RNase H activity, not an interaction between RNase H and another factor(s) (e.g., specific protein–RNase H interaction), is sufficient for complementation of the ts growth defect of MIC3037.

The demonstration of a second RNase H as both a protein and a gene should provide insights into the direction of RNase H study. The presence of at least two biochemically distinct RNases H in many eukaryotes has been reported (1). In addition to *E. coli*, a Gram-negative bacteria, two RNase H genes have been isolated from *B. subtilis*, a Gram-positive bacteria (unpublished data). Also, Lancy *et al.* (26) reported that the gene upstream of the *dnaE* gene is conserved in *S. typhimurium*, suggesting an $ORF_{22.5}$, which corresponds to the ORF_{23} of *E. coli* (23). Therefore, it seems that multiple RNase H genes per genome is a general feature of both eukaryotic and prokaryotic organisms.

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RNase HI   MLKQVEIFT- --DGSCLGNP GPGGYGAILR YRGREKTFSA GYTRTTNNRM ELMAAIVALE
RNase HII  MIEFVYPHTQ LVAGVDEVGR GP-LVGA--V VTAAVILDPA RPIAGLNDSK KL-SEKRRL-

           AL-KEHCEVI LSTDSQYVRQ GITQWIHNWK KRGWKTADKK PVKNVDLWQR LDAALGQHQI
           ALYEEIKEKA LS-WS-LGRA EPHEIDELNI LHATMLAMQR AVAGLHIAPE YVLIDGNRCP

           K-----WEWV KGHAGHPENE RCDELA---R AAAMNPTLED TGYQVEV
           KLPMPAMAVV KGDSRVPEIS AASILAKVTR DAEM-AALLD ---IVFPQYGFAQHKGY PTAFHLEKLA EHGATEHHRR
                                                       SFGPVKRALG TCVLILVSRL SKPESEDV
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FIG. 4. Alignment of amino acid sequences of RNase HI and RNase HII. The 155-amino acid sequence of RNase HI (4, 5) and the N-terminal 155-amino acid sequence of RNase HII (213 amino acids) are shown. Identical amino acids in each sequence are boxed.

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