RNase E is a major intracellular endoribonuclease in many bacteria and participates in most aspects of RNA processing and degradation. RNase E requires a divalent metal ion for its activity. We show that only Mg$^{2+}$ and Mn$^{2+}$ will support significant rates of activity in vitro against natural RNAs, with Mn$^{2+}$ being preferred. Both Mg$^{2+}$ and Mn$^{2+}$ also support cleavage of an oligonucleotide substrate with similar kinetic parameters for both ions. Salts of Ni$^{2+}$ and Zn$^{2+}$ permitted low levels of activity, while Ca$^{2+}$, Co$^{3+}$, Cu$^{2+}$, and Fe$^{2+}$ did not. A mutation to one of the residues known to chelate Mg$^{2+}$, D346C, led to almost complete loss of activity dependent on Mg$^{2+}$; however, the activity of the mutant enzyme was fully restored by the presence of Mn$^{2+}$ with kinetic parameters fully equivalent to those of wild-type enzyme. A similar mutation to the other chelating residue, D303C, resulted in nearly full loss of activity regardless of metal ion. The properties of RNase E D346C enabled a test of the ionic requirements of RNase E in vivo. Plasmid shuffling experiments showed that both rneD303C (i.e., the rne gene encoding a D-to-C change at position 303) and rneD346C were inviable whether or not the selection medium was supplied with MnSO$_4$, implying that RNase E relies on Mg$^{2+}$ exclusively in vivo.

RNase E is a 5’-end-dependent endoribonuclease that plays a central role in stable RNA processing and mRNA turnover in *Escherichia coli* (1). RNase E and its paralog, RNase G, are found in many bacteria but not all (1, 2). In common with many intracellular enzymes of nucleic acid metabolism, RNase E requires a divalent metal ion for activity; in addition, it also requires Zn$^{2+}$ to stabilize its quaternary structure (3–5). Pioneering work by Misra and Apirion on RNase E demonstrated that the partially purified enzyme requires divalent metal ions with a preference for Mn$^{2+}$ over Mg$^{2+}$ (5). With 95 pre-RNA as the substrate, these authors reported optimal concentrations of Mn$^{2+}$ and Mg$^{2+}$ as 1 and 5 mM, respectively. Later, Redko et al. used a 3’-fluorescein-labeled decarboxynucleotide (BR10F) as the substrate for the purified catalytic domain of RNase E (residues 1 to 529) and reported the optimal Mg$^{2+}$ concentration as 25 mM (6). Subsequently, the crystal structure of the catalytic domain of RNase E (4) revealed details of how divalent ions contribute to the activity of RNase E. Two conserved residues in the catalytic core, D303 and D346, serve to chelate a Mg$^{2+}$ ion, while N305 donates an H-bond that helps to anchor D303 (4). The hydration shell surrounding the bound Mg$^{2+}$ likely serves as the source of a hydroxyl ion that attacks the scissile phosphate (7). In addition, the bound metal ion likely polarizes the phosphate to enhance its reactivity.

The ability of RNase E to utilize alternative metal ions in vivo has not been explored. The intracellular concentration of Mg$^{2+}$ in *E. coli* can reach almost 200 mM (8, 9); however, most Mg$^{2+}$ ions are chelated (e.g., by ribosomes [10, 11]) and the free concentration is only 1 to 5 mM (8–10, 12). This implies that the activity of RNase E may be limited by the availability of divalent metal ions. However, since RNA binds Mg$^{2+}$, the interaction of substrates with RNase E may increase the local concentration of metal ions (7). The concentration of free Mn$^{2+}$ inside *E. coli* has not been reported to our knowledge, but total Mn$^{2+}$ can range from 15 μM in cells cultured in un-supplemented defined medium to 150 μM in rich medium thanks to active transport mechanisms (13, 14). Most intracellular Mn$^{2+}$ is likely to be chelated resulting in a free pool whose concentration lies in the low micromolar range. Moreover, although Mn$^{2+}$ is a requirement for pathogenesis in related organisms such as *Salmonella enterica* serovar Typhimurium (13), and at least 70 enzymes are reported to be able to utilize Mn$^{2+}$ in *E. coli* (www.ecocyc.org), relatively few enzymes in *E. coli* absolutely require Mn$^{2+}$. Such enzymes include Mn-dependent superoxide dismutase (encoded by *sodA*), several glycolytic enzymes and guanosine 3’-diphosphate 5’-triphosphate 3’-diphosphatase encoded by *spoT* (13).

We have extended previous authors’ work by exploring the ability of other metal ions to support the activity of RNase E in vitro. Only Mn$^{2+}$ will substitute fully for Mg$^{2+}$, while Ni$^{2+}$ and Zn$^{2+}$ exhibit limited but detectable activity. We have also characterized a point mutation, D346C, which eliminates the enzyme’s ability to use Mg$^{2+}$, while permitting full activity in the presence of Mn$^{2+}$. This mutation lowers the $K_m$ of RNase E to 37 nM compared to 80 nM for the wild type but has no significant effect on the maximum rate ($V_{max}$). This Mn$^{2+}$-dependent variant of RNase E has permitted testing whether Mn$^{2+}$ can substitute for Mg$^{2+}$ in vivo. In fact, rneD346C (i.e., the *rne* gene encoding a D-to-C change at position 346) behaves as a null allele even under strongly selective conditions with Mn$^{2+}$ supplementation.

**MATERIALS AND METHODS**

Recombinant plasmids. Mutations were introduced into plasmids containing the catalytic domain of RNase E (residues 1 to 529) in the PET24b backbone using QuikChange mutagenesis as described previously (15, 16). After the induction of the appropriate cultures, the wild-type, D303C, and D346C RNase E, respectively, enzymes were prepared as described previously (16). All contain a C-terminal hexahistidine tag. RNA substrates were prepared by transcription of linear templates in the presence of [α-
$^3$P[CTP as previously described (17). No attempt was made to remove incomplete or truncated transcripts.

Appropriate mutations were introduced into full-length rne$^+$ constructs using the two step strategy outlined in Garrey et al. (15, 16). QuikChange mutagenesis was performed on a derivative of pUC19 containing an ~2.7-kb HindIII-Sall fragment encompassing amino acid residues 184 to 1061 plus the 3' untranslated region (UTR; “fragment 1”) and Sall-cleaved pWSK129 (18). The resultant full-length plasmids were tested to confirm the presence of the desired mutation(s) by allele-specific PCR. Displacement of pSBK1 (rne$^+$ Cmr$^+$) from strain SK9714 (19) was performed as described previously (15, 16). In some experiments, MnSO$_4$ (25 to 50 μM) and ZnSO$_4$ (5 to 10 μM) were included in the growth media.

**Enzyme assays.** Assays of RNase E activity against radiolabeled substates were performed as described previously in 30 μl containing: 25 mM Tris-HCl (pH 7.8), 80 mM KCl, 2.0 mM dithiothreitol (DTT; except where noted), 0.01 mg/ml bovine serum albumin, 5% glycerol, and metal ions as indicated in the figure legends (16, 17). Labeled RNA (20 to 50 nM final concentration) was heated in the assay buffer in the absence of metal ions for 2 min at 50°C and 10 min at 37°C and then chilled. Metal ions and RNase E were added to the annealed RNA on ice and a zero time sample withdrawn. Digestion was then performed at 30°C. Samples were withdrawn at timed intervals, mixed with 3 volumes of buffer containing 90% formamide, denatured by heating to 100°C for 60 s, and chilled quickly prior to separation on 6% (29:1 acrylamide-bisacrylamide) or 15% (24:1 acrylamide-bisacrylamide) polyacrylamide gels containing 8 M urea in Trisborate-EDTA buffer. Gels were fixed, dried, and visualized by phosphorimaging. Quantification was performed using ImageQuant software (Applied Biosystems/GE Healthcare) using exposures in the linear range of the instrument.

Kinetic measurements were made using the substrate BR14FD (20) as described previously (15) with the following modifications. The assay buffer contained 25 mM Tris-HCl (pH 8.1), divalent metal ions as specified (typically, 10 mM MgCl$_2$ or 10 mM MgCl$_2$ + 100 μM MnSO$_4$), 80 mM KCl, 5% glycerol, 10 μg/ml purified bovine serum albumin, 5'-phosphorylated BR14FD (12.5 to 360 nM), and 5 nM RNase E (expressed as monomers). Assays were performed at 30°C in a Cary Eclipse recording fluorospectrophotometer excited at 495 nm with recording at 517 nm.

**RESULTS**

**Activity of RNase E with different metal ions.** Pilot experiments were performed to establish the metal ion optima for several common substrates of RNase E using the catalytic domain of RNase E (RNase E$_{1-529}$ WT) as the source of activity. With 5'-PPP-95 RNA, optimal activity was observed between 300 and 600 μM Mg$^{2+}$ and between 100 and 300 μM Mn$^{2+}$. At the optimal metal ion concentrations, the yield of pre-55 RNA product after 30 min of incubation was roughly 10-fold higher in Mn$^{2+}$ than in Mg$^{2+}$ (data not shown). These findings are in broad agreement with those of Misra and Apirion (5). Both Ni$^{2+}$ and Zn$^{2+}$ supported a low level of conversion (~10 to 15%) of substrate to 113-nt product after 30 min (lanes 9 and 10). Other ions, including Ca$^{2+}$ (lane 5), Co$^{2+}$ (lane 6), Cu$^{2+}$ (lane 7), and Fe$^{2+}$ (lane 8) were unable to support the activity of RNase E$_{1-529}$ at the tested concentrations (~2% conversion, the limit of detection). Higher concentrations led to unacceptable levels of smearing or nonspecific hydrolysis (data not shown).

These data show that several, but not all, transition metal ions are compatible with the activity of RNase E. Crystallographic investigations have shown that two residues in the N-terminal catalytic domain of RNase E, D303 and D346, can chelate Mg$^{2+}$ (4). Moreover, mutation of either residue, for example, as D303A or D346A, results in complete loss of RNase E activity both in vivo and in vitro (4, 15). We thus tested the effects of mutating either D303 or D346 to cysteine or histidine, which are known to coordinate divalent transition metal ions in diverse proteins and en-

![FIG 1](http://jb.asm.org/)

**A.** Schematic of rpsT-332 RNA (17) with the two prominent RNase E cleavage sites indicated by arrowheads. The position of the 33-nt deletion spanning residues 304 to 336 inclusive in the natural rpsT mRNA is indicated by dotted lines. (B) Products of digestion of $^3$P-rpsT-332 RNA (20 nM) with 100 nM (monomer) WT RNase E$_{1-529}$ in the presence of the metal ions indicated in the figure. Mg$^{2+}$ (lane 3) was present at 5 mM; all others were present at 200 μM. Digestion was performed as described in Materials and Methods for 30 min (except lane 4, which was for 7.5 min) and in the absence of DTT to prevent reduction of Co$^{3+}$, Cu$^{2+}$, and Fe$^{2+}$. The sizes of the RNA products in nucleotides are given in the right margin.

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zymes (see chapter 8 in reference 24) and thereby alter the metal ion-dependent activity of RNase E. The appropriate mutations were introduced into plasmid pSG-Rne1-529, encoding residues 1 to 529 of RNase E in a pET24b backbone (15). The activities of the resultant purified recombinant enzymes were assayed against rpsT-332 RNA in the presence of 5 mM Mg²⁺, with or without 200 μM Mn²⁺ (or with Mn²⁺ alone; data not shown). The data in Fig. 2 illustrate the time courses of typical assays, while initial rates are summarized in Table 1. Figure 2A shows that the profile of products obtained with Mg²⁺ alone resembles that obtained with both ions. Additional cleavage products of ~190 nt and <100 nt are clearly visible in lanes 10 to 13 when Mn²⁺ is present. We have not determined whether the additional products are due to relaxation of the specificity of RNase E₁-529 in the presence of Mn²⁺ or to an enhanced rate and/or extent of secondary cleavages. Both RNase E₁-529 D303C and RNase E₁-529 D346C exhibit the normal spectrum of products when assayed in 5 mM Mg²⁺ (Fig. 2B and C, lanes 1 to 7) but in very low yields. In particular, RNase E₁-529 D303C displays only 1% of wild-type activity, while RNase E₁-529 D346C is only marginally more active (Table 1). Mn²⁺ stimulates the activity of both mutant enzymes considerably (Fig. 2B and C, lanes 8 to 13), especially so with RNase E₁-529 D346C. The addition of 200 μM Mn²⁺ to the assay results in a modest (~50%) increase in the activity of WT enzyme, a 3.5-fold increase in the activity of RNase E₁-529 D303C, and a 260-fold increase in the activity of RNase E₁-529 D346C (Table 1). Similar activities were measured with Mn²⁺ alone (data not shown).

Influence of divalent metal ions on enzyme kinetics. To test whether the presence of Mn²⁺ was influencing the affinity of RNase E for its substrate or altering the rate of bond cleavage, we measured kinetic parameters using oligonucleotide substrates, including BR10 (25) and the fluorescent substrate, 5'-P-BR14FD (15, 20). Titration experiments using either 5'-32P-BR10 or 5’-P-BR14FD showed that the optimal ionic conditions for maximal activity of the WT enzyme were 10 mM MgCl₂, similar to the value reported by Redko et al. (6), or 400 μM MnSO₄ (data not shown). Quenching of fluorescence by Mn²⁺ or other transition metal ions is a well-known problem (26). To circumvent this in assays using 5’-P-BR14FD, 80 mM KCl was added to partially mask the backbone charge on the substrate, and divalent ion concentrations were set at 10 mM Mg²⁺ ± 0.1 mM Mn²⁺ (i.e., a 1:10 ratio) despite the latter being suboptimal for Mn²⁺. The resultant kinetic parameters are summarized in Table 2. Under these conditions, the presence of Mn²⁺ does not appreciably change the behavior of the wild-type enzyme. The $K_m$ (76 to 80 nM) and $V_{max}$ (7.3 pmol/min) are essentially identical in the presence or absence of Mn²⁺ (Table 2). The value for the $K_m$ is 2- to 3-fold higher than that reported by Garrey et al. (15). We attribute the difference to the presence of higher KCl concentrations in our assays. RNase E₁-529 D303C was marginally active in the presence of Mg²⁺ alone as expected. The $K_m$ was estimated as 100 nM, while the $V_{max}$ was approximated from rates of cleavage obtained at the highest levels of substrate. By the latter measure, the D303C mutant is only 3% as active as the wild type (compare rows 1 and 2 in Table 2). The additional presence of Mn²⁺ did not significantly change the estimated $V_{max}$ (Table 2). The RNase E₁-529 D346C mutant was also essentially inactive in 10 mM Mg²⁺ alone, with a $V_{max}$ estimated as 0.3 pmol/min. An accurate $K_m$ could not be determined due to the enzyme’s very low activity in Mg²⁺ alone. Remarkably and consistent with the data presented in Fig. 2, RNase E₁-529 D346C exhibited a 2-fold lower $K_m$ than the WT enzyme, 37 nM, and a slightly higher $V_{max}$ 8.1 pmol/min, in the presence of 10 mM Mg²⁺ and 100 μM Mn²⁺. These numbers may be underestimated since 250 μM Mn²⁺ increased the activity of RNase E₁-529 D346C by at least 25% at substrate concentrations below 150 nM (data not shown).
Inviability of mutations at residues 303 and 346. We performed plasmid displacement ("shuffling") to assess whether low-copy-number plasmids containing D303C or D346C alleles of rne could displace pSBK1, an rne− Cmr plasmid in strain SK9714 which harbors rneΔ1018::bla, a chromosomal deletion spanning most of the rne coding sequence (19). Mutant plasmids based on the pWSK129 backbone (11) were constructed as described previously (15, 16). These plasmids contain the promoter region, 5′ UTR, complete rne coding sequence and 3′ UTR, including the transcriptional terminator. Cultures of SK9714 were transformed with the desired plasmid, grown for 20 doublings, and then plated to determine the fraction that retained pSBK1. The resultant data are shown in Table 3. All plasmids tested contained only wild-type sequence at codon 346. Thus, despite application of ostensibly stronger selection, neither pRne-GM125 (rneD346C) nor pRne-GM138 (rneD346H) can displace pSBK1 (Table 3) even when Mn2+ and Zn2+ were added to the media (not shown).

We hypothesized that the failure of the mutant plasmids to displace pSBK1 might be due to their conferring a growth defect. Cells containing the mutant plasmid that also lost pSBK1 would be at a growth disadvantage and would not be recovered after >20 generations. Thus, we repeated the displacement experiments using strain SK9957 which harbors rneΔ1018 on pMOK15 combined with rneΔ1018::bla on the chromosome. The rneΔ1018 deletion removes amino acid residues 428 to 1035 in rne and confers a slow-growth phenotype (19). We also included 50 μM Mn2+ and 10 μM Zn2+ in the selective media. In two independent experiments, Km Cmr transformants were recovered after transformation of SK9957 with pRne-SG1, pRne-GM125, and pRne-GM138. Plasmid DNA was isolated from representative transformants and tested for the presence of D346C or D346H mutations. All transformants so tested contained only wild-type sequence at codon 346. Thus, despite application of ostensibly stronger selection, neither pRne-GM125 (rneD346C) nor pRne-GM138 (rneD346H) can displace pMOK15 from SK9957 (Table 3). In addition, neither the empty vector (pWSK129) nor pRne-GM151 (D346N) is able to displace pMOK15 (Table 3).

**DISCUSSION**

Preference of RNase E for Mn2+ over Mg2+ in vitro. It is well known that Mn2+ will often substitute efficiently for Mg2+ in many enzymes of nucleic acid metabolism in vitro (reviewed in reference 27). Our data and those of Misra and Apirion (5) show that this is also the case for RNase E. Figure 3 shows how binding
of a metal ion to the active site would promote phosphodiester bond cleavage by RNase E. The position of the metal ion, as well as the pucker of the ribose (6), would constrain the phosphodiester bond in an optimal orientation relative to the hydrated metal ion which provides the attacking hydroxyl ion and stabilizes the transition state. These features taken together rationalize why RNase E cleaves only RNA. In support of this model, Redko et al. have shown that 2′-fluoro and 2′-amino substitutions to U5 in the BR10 substrate permit cleavage by RNase E, albeit at lower rates than 2′-OH substrates (6). Both such substitutions maintain normal ribose pucker, as well as the polar character of the 2′ substituent. Moreover, neither a 2′-deoxy-U nor a 2′-fluoroarabinosyl-U substitution at U5 is cleaved, consistent with the model in Fig. 3 (6; GAM, unpublished data). Finally, since RNA is one of the major chelators of intracellular Mg\(^{2+}\), the binding of RNA by RNase E may be integral to the recruitment of the catalytic metal ion (7).

It was surprising, however, that different substrates respond differently to divalent ions. Both 9S and the rpsT-332 RNAs exhibit preferential cleavage by RNase E in the presence of Mn\(^{2+}\) compared to Mg\(^{2+}\). The basis for this preference is not known but could reflect the equilibrium binding constant of each metal ion for RNase E. In this regard, the crystal structure of the RNase E apoenzyme exhibited no density in proximity to D303 and D346 (6). It is even conceivable that the ability to bind metal ions readily dissociate from the enzyme. In addition, the enhanced rate of cleavage in the presence of Mn\(^{2+}\) could also reflect properties of the solvated metal ion itself. Mn\(^{2+}\) is known to display more flexibility in binding to enzymes than Mg\(^{2+}\) (27) and its hydrate exhibits a somewhat lower pKa (28). In contrast to the behavior of 9S or rpsT RNAs, the DB8 oligonucleotide substrate exhibits only modest preference for Mn\(^{2+}\) over Mg\(^{2+}\). In this case it is possible that substrate binding is limiting rather than metal ion binding.

The physiological ion preference of RNase E. As noted in the Introduction, the intracellular concentration of Mg\(^{2+}\) in *E. coli* can exceed 100 mM but almost all (~98%) is chelated by a variety of macromolecules or small molecules (e.g., organic acids, nucleotides, and phospholipids [8–12]). This implies that the activity of RNase E may normally be limited by the availability of divalent metal ions. However, since RNA binds Mg\(^{2+}\) or other divalent metal ions, the interaction of substrates with RNase E may supply the necessary ion (7). It is even conceivable that the ability to bind a metal ion selectively may predispose certain sequences to cleavage by RNase E. As shown in this report, the D346C mutation permits the catalytic domain of RNase E to function on several ribonucleotides, and phospholipids [8–12]). This implies that the activity of RNase E is dependent on Mg\(^{2+}\) or Mn\(^{2+}\) for catalysis by RNase E D346C. In effect, a strain carrying rneD346C alone would behave as an *rne* null. Given the vast excess of Mg\(^{2+}\) over Mn\(^{2+}\), it is unlikely that Mn\(^{2+}\) serves as a cofactor for RNase E in *vivo* except under unusual circumstances. We conclude, therefore, that Mg\(^{2+}\) is the physiological divalent metal ion supporting RNase E’s activity in *vivo*.

Alternatively, rneD346C may somehow be toxic, possibly by conferring relaxed specificity in the presence of Mn\(^{2+}\). Digestion patterns obtained in *in vitro* experiments suggest that RNase E is prone to “overcleave” substrates when Mn\(^{2+}\) is present, as evidenced by greater smearing of the products. Nonetheless, such putative toxicity would require Mn\(^{2+}\) at sufficient concentrations *in vivo* to permit manifestation of the mutant enzyme’s activity. As discussed above, such conditions are unlikely to be met. In addition, we tested RNase E D346C for single-strand DNAse activity against a DNA oligonucleotide but were unable to detect any degradation under conditions where 5% of wild-type activity against RNA would be readily measurable. Thus, although Mn\(^{2+}\)-dependent toxicity of rneD346C seems unlikely, we cannot exclude it completely.

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