An ortholog of the Ro autoantigen functions in 23S rRNA maturation in D. radiodurans

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In both animal cells and the eubacterium Deinococcus radiodurans, the Ro autoantigen, a ring-shaped RNA-binding protein, associates with small RNAs called Y RNAs. In vertebrates, Ro also binds the 3′ ends of misfolded RNAs and is proposed to function in quality control. However, little is known about the function of Ro and the Y RNAs in vivo. Here, we report that the D. radiodurans ortholog Rsr (Ro sixty related) functions with exoribonucleases in 23S rRNA maturation. During normal growth, 23S rRNA maturation is inefficient, resulting in accumulation of precursors containing 5′ and 3′ extensions. During growth at elevated temperature, maturation is efficient and requires Rsr and the exoribonucleases RNase PH and RNase II. Consistent with the hypothesis that Y RNAs inhibit Ro activity, maturation is efficient at all temperatures in cells lacking the Y RNA. In the absence of Rsr, 23S rRNA maturation halts at positions of potential secondary structure. As Rsr exhibits genetic and biochemical interactions with the exoribonuclease polynucleotide phosphorylase, Rsr likely functions in an additional process with this nuclease. We propose that Rsr functions as a processivity factor to assist RNA maturation by exoribonucleases. This is the first demonstration of a role for Ro and a Y RNA in vivo.

[Keywords: Ro ribonucleoprotein; Y RNA; exoribonucleases; rRNA processing; D. radiodurans]

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Many small ribonucleoprotein particles [RNPs] carry out fundamental aspects of gene expression. In eukaryotes, a large number of small RNPs reside in nucleoli, where they modify conserved nucleotides in pre-rRNAs and assist processing. Another nucleolar RNP, RNase P, is required for rRNA maturation. In nuclei, the U1, U2, U4/U6, and U5 small nuclear RNPs [snRNPs] are critical components of the spliceosome, the U7 snRNP is required for histone 3′ end formation, and the telomerase RNP maintains the ends of chromosomes. In the cytoplasm, the signal recognition particle binds the signal sequences of newly synthesized secretory proteins and targets them to the endoplasmic reticulum membrane [Storz et al. 2005; Tycowski et al. 2006].

In addition to these well-characterized RNPs, there are other small RNPs whose functions are far less understood. One of these RNPs, the Ro RNP, was discovered because it is a major autoantigen in patients with systemic lupus erythematosus [Lerner et al. 1981]. The Ro RNP is found in many animal cells and a number of prokaryotes. The major protein component, the Ro 60-kDa protein, is both nuclear and cytoplasmic. In the cytoplasm, the Ro protein binds small RNAs called Y RNAs. The number of distinct Y RNAs varies from four in humans to one in Caenorhabditis elegans and the eubacterium Deinococcus radiodurans. Although they exhibit little primary sequence homology, all Y RNAs fold into structures consisting of a large internal loop and a long stem containing a conserved helix that is the Ro-binding site [Chen and Wolin 2004]. As Y RNAs are greatly reduced in worms and mouse cells lacking Ro, binding by Ro likely stabilizes these RNAs from degradation [Labbe et al. 1999; Chen et al. 2003].

Studies in vertebrate cells have led to the proposal that the Ro protein functions in noncoding RNA quality control. In Xenopus oocyte nuclei, the Ro protein associates with a large class of variant pre-5S rRNAs that contain point mutations that cause them to misfold [O’Brien and Wolin 1994; Shi et al. 1996]. These RNAs are also longer at the 3′ end due to readthrough of the first termination signal. The misfolded RNAs are inefficiently processed to mature 5S rRNAs and are eventually degraded [O’Brien and Wolin 1994]. Further, in mouse embryonic stem cells, the Ro protein associates with variant U2 snRNAs that appear to be misfolded [Chen et al. 2003].

Structural analyses have revealed that the Ro protein...
forms a ring that binds the 3' ends of misfolded RNAs in its central cavity and helical portions of these RNAs on its surface [Stein et al. 2005, Fuchs et al. 2006]. While Ro binding to misfolded pre-5S rRNA requires both a single-stranded 3' end and helices, the sequences of these elements are mostly unimportant, suggesting that Ro can associate with a variety of structured RNAs that contain a 3' tail [Fuchs et al. 2006]. In contrast, the binding of Y RNAs to Ro is sequence specific. The Y RNAs bind on the outer surface of Ro, with invariant amino acids contacting conserved nucleotides [Stein et al. 2005]. Because a bound Y RNA will stericly prevent further RNA binding, Y RNAs were proposed to regulate access of Ro to other RNAs [Stein et al. 2005].

In prokaryotes, the Ro RNP has been characterized only in the radiation-resistant eubacterium *D. radiodurans*. As in animal cells, the *D. radiodurans* Ro protein ortholog Rsr [Ro sixty related] binds and stabilizes an RNA resembling a Y RNA [Chen et al. 2000]. Cells lacking Rsr are more sensitive to ultraviolet irradiation [UV], but not γ-irradiation, than wild-type cells, and both Rsr and the Y RNA are up-regulated following UV [Chen et al. 2000]. Analyses in mammalian cells confirmed that assisting survival after UV was a conserved function of the Ro protein [Chen et al. 2003]. Although the mechanism by which Ro contributes to cell survival after irradiation is unknown, it was proposed that Ro functions in the recognition or degradation of damaged RNAs that misfold or fail to assemble into RNPs [Chen et al. 2003].

A key question concerns the roles of the Ro protein and its associated Y RNA in RNA metabolism in vivo. Although Ro is associated with misfolded RNAs in vertebrates, and contributes to survival after UV in mammals and bacteria, no defects in RNA metabolism have yet been reported in cells lacking Ro. To address this question, we examined the role of Rsr and the Y RNA in *D. radiodurans*. We report that Rsr functions with Y RNAs in assisting survival after γ-irradiation and that Y RNAs inhibit Ro function, maturation is always efficient in cells that either lack the Y RNA or overexpress a mutant Rsr with decreased affinity for Y RNAs. Finally, we provide evidence that Rsr functions with the exoribonuclease polynucleotide phosphorylase (PNPase) in at least one additional process. We propose a model in which the binding of Rsr to the 3' ends of nascent RNAs assists maturation by exoribonucleases.

**Results**

**Rsr is required for efficient 23S rRNA maturation**

To examine the effects of deleting Rsr and the Y RNA on RNA metabolism, total RNA was isolated from wild-type strains and strains lacking either Rsr (Δrsr) or the Y RNA (Δyrm) during growth at 30°C. The RNA was fractionated in formaldehyde agarose gels and transferred to filters for Northern hybridization. Staining of the filter-bound RNA with methylene blue revealed that the 23S rRNA was heterogeneous and migrated slightly slower in wild-type and Δrsr cells than in Δyrm cells [Fig. 1A, lanes 1–3]. Hybridization with oligonucleotides complementary to the 5' and 3' extensions revealed that the heterogeneous, slower-migrating RNA consisted of pre-23S rRNAs with these extensions [Fig. 1A, two bottom panels]. These precursors were undetectable in Δyrm strains [Fig. 1A, lane 3] but were detected when Rsr was also deleted (ΔrsrΔyrm) [Fig. 1A, lane 4]. Thus, the efficient maturation of 23S rRNA in Δyrm cells requires Rsr. Surprisingly, when we examined cells grown at 37°C, pre-23S rRNAs were undetectable in wild-type cells [Fig. 1B, lane 1], indicating that maturation is more efficient at higher temperatures. [The usual growth temperature for *D. radiodurans* is 30°C–32°C [Tanaka et al. 2004]. However, pre-23S rRNAs remained detectable in Δrsr and ΔrsrΔyrm cells [Fig. 1B, lanes 2,4]. To confirm that 23S rRNA maturation becomes more efficient at 37°C, wild-type and Δrsr cells were grown at 30°C and then shifted to 37°C. At intervals, RNA was extracted and subjected to Northern blotting. In wild-type cells, pre-23S rRNAs were undetectable within 4 h at 37°C [Fig. 1C, lanes 1–6]. As *D. radiodurans* doubles in ~90 min at 37°C, this corresponds to two to three doublings. In contrast, pre-23S rRNAs increased two- to threefold in Δrsr cells at 37°C [Fig. 1C, lanes 7–12].

To examine newly synthesized RNA, we performed pulse-labeling experiments. Wild-type and Δrsr cells were grown in low-phosphate medium at 30°C or 37°C and labeled with 32P, for 5 min. Following addition of excess unlabeled phosphate, aliquots were removed at intervals. Although the chase was only partly effective at stopping 32P incorporation, there were clear differences between the strains. After 30 min of chase, phosphorimager quantitation revealed that 88% of the 23S rRNA was matured in wild-type cells at 37°C [Fig. 1D, lane 13]. However, in wild-type cells at 30°C, and in Δrsr cells at both temperatures, only ~28% of the 23S rRNA was matured after 30 min [Fig. 1D, lanes 3,8,18]. We conclude that 23S rRNA maturation is more efficient in wild-type cells at 37°C and that Rsr is required for efficient maturation.

**Increasing the levels of Y RNA-free Rsr results in efficient maturation at 30°C**

We examined whether the levels of Rsr or the Y RNA changed during growth at 37°C. Western and Northern blotting revealed that both Rsr and the Y RNA increased approximately twofold within 4 h at 37°C [Fig. 2A,B, cf. lanes 1 and 4]. To determine whether the association of Rsr with the Y RNA changes at 37°C, we performed immunoprecipitations with anti-Rsr antibodies. Although the Y RNA underwent nicking in extracts [Fig. 2C, lanes 2,3], all of the full-length Y RNA and all of the nicked
Figure 1. Rsr is required for efficient 23S rRNA maturation. (A, B) Wild-type and the indicated mutant strains were grown to OD$_{600}$ = 0.2 at 30°C and shifted to 37°C. Cells were collected at 30°C (A) and after growth for 6 h at 37°C (B). Total RNA from the strains was fractionated in formaldehyde-agarose gels and subjected to Northern analysis. After methylene blue staining (top panels), the filter was probed with oligonucleotides complementary to 23S rRNA internal sequences (second panel), the 5′ leader (third panel), and the 3′ trailer (bottom panel). (C) Wild-type and Δrsr strains were grown at 30°C and shifted to 37°C at time 0. At intervals, RNA was extracted and analyzed by Northern blotting. The filters were stained with methylene blue (top panel) and probed to detect mature 23S rRNA (second panel), the 5′ leader (third panel), and the 3′ trailer (bottom panel). (D) Cells grown at 30°C (lanes 1–10), or for 4 h at 37°C (lanes 11–20) were labeled with $^{32}$P, for 5 min. Following addition of media containing excess unlabeled phosphate, aliquots were removed at intervals and RNA was extracted.

The activity of Rsr was monitored by Northern analysis, showing that Y RNA levels were similar in wild-type cells and cells containing Rsr-H189S (Fig. 2E, lane 2). However, upon addition of media containing excess Rsr, RNA was also present at higher levels (Fig. 2E, lane 2). Thus, Y RNAs may normally be synthesized in excess, compared with Rsr in wild-type cells. However, upon Rsr-H189S overexpression, Y RNA levels were similar to a strain carrying only the vector (Fig. 2E, lane 3), indicating that as in *Xenopus*, this residue is critical for Y RNA binding. Importantly, in the presence of the H189S mutant, maturation of 23S rRNA was efficient at 30°C (Fig. 2F, lanes 2,3).

We attempted to address whether the amount of Y RNA-free Rsr increases at 37°C. Multiple attempts to separate the Y RNA-free Rsr from the Rsr/Y RNA complex by gel filtration were inconclusive, as both Rsr and the Y RNA were detected in most fractions of the Superdex 200 column (data not shown). Nonetheless, our finding that wild-type cells containing increased Y RNA-free Rsr carry out efficient 23S rRNA maturation at 37°C reveals that the presence of Y RNA-free Rsr is sufficient to confer efficient maturation.

**RNases PH and II are required for efficient 23S rRNA maturation at 37°C**

Our result that 3′ and 5′ extended pre-23S rRNAs accumulate in Δrsr strains, coupled with the finding that *Xenopus* Ro protein binds the 3′ single-stranded ends of noncoding RNAs in its central cavity (Fuchs et al. 2006), suggested a model in which Rsr functioned with 3′-to-5′ exonuclease II in maturing the 3′ ends of pre-23S rRNA. We therefore examined whether exoribonucleases were involved in 23S rRNA end maturation. *D. radiodurans* possesses likely orthologs of at least four 3′-to-5′ exoribonucleases previously characterized in *Escherichia coli*: PNPase (*pnp*), RNase PH (*rph*), an RNase R family member (*rph*), and an RNase II family member (*DR0020*; here called RNase II [rnb]). In addition, there are several uncharacterized proteins that contain exonuclease motifs. To examine the involvement of some of these nucleases, we created strains lacking PNPase, RNase PH, and RNase II.

Northern blotting of RNA revealed that, although pre-23S rRNAs are not detected in wild-type strains at 37°C,
these RNAs accumulate in ∆ρph and ∆ρnb strains [Fig. 3B, lanes 1,4,6]. Thus, efficient maturation of 23S rRNA at elevated temperature requires both RNase PH and RNase II. However, pre-23S rRNAs did not accumulate in either ∆yrn∆ρph or ∆yrn∆ρnb cells at 30°C or 37°C [Fig. 3C, data not shown], indicating that ∆yrn cells lacking either RNase PH or RNase II are still able to carry out efficient Rsr-dependent maturation of 23S rRNA. Thus, these exoribonucleases may function redundantly in ∆yrn cells, or another ribonuclease may carry out maturation under these conditions.

Interestingly, in cells lacking PNPase, pre-23S rRNAs were not detected at 30°C or 37°C [Fig. 3D,E, lane 1]. Similar to ∆yrn cells [Fig. 1A,B], efficient 23S rRNA maturation in ∆ρnp cells involves Rsr, as precursors accumulate when Rsr is also deleted [∆ρnp∆rsr] [Fig. 3D,E, lane 2]. One explanation for this result is that the inefficient maturation that normally occurs at 30°C requires PNPase. In the absence of PNPase, the more efficient pathway involving Rsr predominates.

We examined whether the levels of Rsr and the Y RNA change in cells lacking exoribonucleases. Western and Northern blotting revealed that in ∆ρnp and ∆ρph but not ∆ρnb strains, Rsr and Y RNA levels increased at 30°C [Fig. 3F,G]. Quantitation of results from several experiments revealed that both Rsr and the Y RNA increased approximately three- to fivefold in ∆ρnp and ∆ρph strains. These increases are similar to those observed at 37°C in wild-type cells [Fig. 2A,B]. However, while maturation occurs in ∆ρnp strains at 30°C, maturation does not occur at 30°C in ∆ρph strains or in wild-type strains containing increased levels of Rsr and the Y RNA [Fig. 2F].

D. radiodurans lacking Rsr accumulate extended and shortened forms of 23S rRNA

To define the various pre-23S rRNA ends, we performed site-directed cleavage using RNase H and 2′-O-methyl RNA–DNA chimeric oligonucleotides (Linou et al. 1987), followed by Northern analyses of the products. For 3′-end mapping, we used a chimeric oligonucleotide that directs RNase H cleavage 122 nucleotides [nt] from the mature 3′ end. As expected, a band of 122 nt corresponding to the mature end was detected in all strains [Fig. 4A]. In addition, in wild-type cells at 30°C, and ∆rph, ∆rph∆yrn, ∆ρph, and ∆ρnb strains at both 30°C and 37°C, the most prominent bands, other than the mature 3′ ends, contained extensions of 71 and 79 nt [Fig. 4A, lanes 1,2,4,10,12; data not shown]. Interestingly, in ∆rsr∆ρnp and ∆rsr∆yrn∆ρnp strains at both temperatures, these 3′ extended precursors were replaced by slightly larger species with extensions of 81 and 89 nt [Fig. 4A, lanes 6,8,14,16]. In addition, we detected a 3′ shortened species (lacking ~22 nt of the mature RNA) in all strains lacking PNPase [Fig. 4A, lanes 5–8,13–16].

PhosphorImager quantitation revealed that in wild-type, ∆rsr, ∆rsr∆yrn, ∆ρph, and ∆ρnb strains at 30°C, ~60% of the 23S rRNA contained the mature end, while the remainder contained 3′ extensions [Fig. 4A, lanes 1,2,4, data not shown]. In contrast, in ∆yrn, ∆ρnp, and ∆yrn∆ρnp strains, 90%–100% of the 23S rRNA was mature. In ∆rsr∆ρnp and ∆rsr∆yrn∆ρnp strains, only ~50% of the 23S rRNA was mature, ~20% contained 3′ extensions that were longer than those in wild-type and ∆rsr strains, while ~30% was shorter at the 3′ end [Fig. 4A, lanes 6,8]. The levels of mature 23S rRNA varied more...
drastically between the strains after 4 h at 37°C. While nearly all 23S rRNA was mature in wild-type cells [Fig. 4A, lane 9], the fraction of mature RNA in Δrsr, ΔrsrΔyrn, Δphb, and Δnrb cells decreased to ~40%, with a corresponding increase in precursors [Fig. 4A, lanes 10,12; data not shown]. Most strikingly, in ΔrsrΔpnp and ΔrsrΔyrnΔpnp cells at 37°C, the fraction of 23S rRNA containing the mature end decreased to 13% and 20%, respectively, the fraction with a truncated end increased to 60% and 53%, while 27% of the RNA in both strains consisted of 3’ extended precursors [Fig. 4A, lanes 14,16].

Using the same strategy, we determined the 5’ ends. Consistent with the 3’ end analysis, ~60% of the 23S rRNA in wild-type, Δrsr, Δphb, and Δnrb strains at 30°C contained the mature end and ~25% contained a 127 nt 5’ extension. The remaining 15% was truncated by 31 nt [data not shown, but see Fig. 4B]. After 4 h at 37°C, 81% of the 23S rRNA in wild-type cells was mature, the 5’ extended form was not detected, and 19% was the shorter form [Fig. 4B, lane 1]. In contrast, for Δrsr and ΔrsrΔyrn strains, the fraction of mature 23S rRNA declined to 30% at 37°C, while 33% was extended and 37% was shortened [Fig. 4B, lanes 2,4]. Similar to the 3’-end mapping, the most severe declines in 5’ mature 23S rRNA occurred in ΔrsrΔpnp and ΔrsrΔyrnΔpnp strains. In these strains, the fraction of mature 23S rRNA was 31% and 37%, respectively, at 30°C. After 4 h at 37°C, the mature RNA in both strains declined to 9%–10%, ~8%–9% of the RNA contained the 5’ extension, with the remainder split between the form lacking 31 nt and a second species lacking ~3 nt [Fig. 4B, lanes 6,8].

We examined the locations of the precursors on a possible secondary structure of the pre-23S rRNA. In D. radiodurans, 23S rRNA is transcribed separately from 16S rRNA, with the transcription unit consisting of pre-23S rRNA, 5S rRNA, and rRNA5S12 (Fig. 4D). While the immediate flanking regions of most 23S rRNAs fold into stems containing long regions of uninterrupted base pairing that are cleaved by RNase III, a similar stem formed by base pairing the D. radiodurans 23S rRNA flanking regions has not been detected by computational analyses (Saito et al. 2000). However, using the program Mfold (Zuker 2003), we found that a stem containing imperfect base-pairing between parts of the 5’ and 3’ flanks is a feature of all predicted structures for the pre-23S rRNA [Fig. 4C]. Interestingly, the 3’ ends of the +71 and +79 precursors that accumulate in wild-type cells at 30°C and in Δrsr, ΔrsrΔyrn, Δphb, and Δnrb strains at both temperatures are found within predicted double-stranded regions. The formation of these precursors requires FNPase, as these pre-rRNAs are replaced in ΔrsrΔpnp and ΔrsrΔyrnΔpnp cells by RNAs with slightly longer 3’ extensions [Fig. 4C].
Extended and shortened forms of 23S rRNAs are assembled into polyribosomes

To determine whether the various forms of 23S rRNA were incorporated into ribosomes, we subjected cell lysates to sucrose gradient sedimentation. To visualize 23S 3’ ends, RNA was extracted from each fraction and analyzed by site-directed RNase H cleavage and Northern blotting. Comparison of RNA extracted from the lysates lysed by site-directed RNase H cleavage and Northern ends, RNA was extracted from each fraction and also lacking Rsr. Cells lacking RNase II and RNase PH were similar to wild-type strains eliminating Rsr in the strain restores growth, is consistent with the hypothesis that Y RNAs regulate Ro activity.

As the 23S rRNA is mature at both temperatures in E. coli, we examined the growth of cells lacking each nuclease and also lacking Rsr. Cells lacking RNase II and RNase PH were similar to wild-type strains lacking Rsr in the strain restores growth, is consistent with the hypothesis that Y RNAs regulate Ro activity.

Evidence that Rsr and PNPase function in at least one other process

To determine if Rsr exhibits genetic interactions with the exonucleases, we examined the growth of cells lacking each nuclease and also lacking Rsr. Cells lacking RNase II and RNase PH were similar to wild-type strains at all temperatures (data not shown). However, Δppn strains grew slowly at all temperatures, with the growth defect accentuated at 16°C and 37°C (Fig. 6A). Surprisingly, ΔrsrΔppn strains grew nearly as well as wild-type cells, indicating that the growth defect of Δppn strains was caused in part by Rsr. Moreover, while Δyrn strains grew normally, ΔyrnΔppn cells were slightly more cold-sensitive than Δppn cells. However, strains lacking all three genes (ΔrsrΔyrnΔppn) exhibited near wild-type growth (Fig. 6A). The finding that deleting the Y RNA in the Δppn strain worsens the cold sensitivity, while eliminating Rsr in the strain restores growth, is consistent with the hypothesis that Y RNAs regulate Ro activity.

As the 23S rRNA is mature at both temperatures in Δppn and ΔppnΔyrn strains, we note that a defect in rRNA maturation cannot explain either the tempera-
ture-sensitive growth defects or the finding that Rsr and its degradation products (data not shown), the eluate was directly analyzed by multidimensional protein identification technology (Florens and Washburn 2006) to identify less abundant proteins. Interestingly, PNPase was present in the eluate, as peptides covering 29% of its 810 amino acids were recovered. However, RNase II and RNase PH were not detected.

We confirmed the association of Rsr and PNPase by performing immunoprecipitations. Using an anti-PNPase antibody to immunoprecipitate from cells carrying three copies of the Flag epitope fused to Rsr, we detected a small fraction [~2%–5%] of Rsr in the immunoprecipitate (Fig. 6B, lane 3). Rsr was not detected when preimmune serum was used (Fig. 6B, lane 4). Although ribonuclease treatment did not abolish the association, we have not detected an interaction between the purified proteins, either in the presence or absence of the Y RNA (data not shown). Thus, the two proteins may be closely associated on a common RNA, such that ribonuclease is unable to cleave between them, or their interaction may be bridged or stabilized by additional proteins.

Figure 5. Extended and truncated forms of 23S rRNA are present in polyribosomes. Following growth at 30°C, lysates from wild-type [A] and ΔrsrΔpnp [B] cells were fractionated in sucrose gradients. RNA extracted from each fraction was subjected to site-directed cleavage and Northern blotting to visualize 23S rRNA 3' ends. Positions of 30S and 50S subunits, 70S ribosomes, and polysomes were determined by monitoring OD 260. The fractions were analyzed in two gels that are joined at the line. To examine whether pre-23S rRNAs were stable, RNA extracted from lysates [lane 1] was compared with RNA prepared by direct phenol extraction [lane 2]. The prominent precursors with 71 and 79 extra 3' nucleotides are indicated, along with a minor species containing 36 additional nucleotides. [Asterisk] A pre-23S rRNA degradation product.

Figure 6. Rsr interacts with PNPase. [A] Serial fivefold dilutions of the indicated mutant strains were spotted on TGY agar and grown at 16°C, 25°C, 30°C, and 37°C. [B] Lysates from Flag₃-rsr [lanes 1,5,4] or untagged [lanes 2,5,6] strains were incubated with anti-PNPase antibody [lanes 3,5] or preimmune sera [lanes 4,6]. Proteins in immunoprecipitates were subjected to Western blotting with an anti-Flag antibody. [Asterisk] A degradation fragment of Flag₃-Rsr. Although the cells for this experiment were grown at 30°C, similar results were obtained from cells grown at 37°C. [C] Serial fivefold dilutions of the strains were spotted on TGY agar containing either 0 or 0.4 mM hydrogen peroxide and grown at 25°C. [D] Wild-type [solid squares], Δpnp [solid circles], Δrsr [open triangles], and ΔrsrΔpnp [open circles] cells were irradiated with the indicated doses of UV. After irradiation, aliquots were removed and plated on TGY agar, and colonies were counted to determine the fraction of surviving cells.
Since Rsr contributes to cell survival after UV (Chen et al. 2000), we examined the sensitivity of the Δpnp strains to UV and oxidative stress. Cells lacking PNPase were sensitive to UV and hydrogen peroxide [Fig. 6C,D]. Interestingly, ΔrsrΔpnp strains were more resistant to oxidative stress than Δpnp strains and more resistant to UV than either Δrsr or Δpnp strains [Fig. 6C,D]. Our finding that deleting rsr allows D. radiodurans to bypass the requirement for PNPase during growth at temperature extremes, during oxidative stress, and after UV suggests that both Rsr and PNPase may function in these processes.

**Discussion**

Although Ro and the Y RNAs were first described many years ago (Lerner et al. 1981), their function in vivo has been unclear. Our experiments demonstrate that a bacterial Ro and a Y RNA function in 23S rRNA maturation. Our data suggest that 23S rRNA maturation can occur by at least two pathways in D. radiodurans, one of which involves the Ro ortholog Rsr. In one pathway, which occurs in wild-type cells at 30°C and in Δrsr cells at both 30°C and 37°C, 23S rRNA maturation is inefficient and does not require Rsr. However, in wild-type cells at 37°C, maturation is very efficient and involves Rsr and the exo- nuclease RNase PH and RNase II. Since maturation is efficient at 30°C in Δyrn cells and in wild-type cells overexpressing a mutant Rsr with decreased affinity for Y RNAs, the level of Y RNA-free Rsr influences the pathway of maturation.

**A role for a Ro protein in 23S rRNA maturation**

How might Rsr function in 23S rRNA maturation? As the 3′ ends of the precursors that accumulate in Δrsr cells map to regions predicted to be double stranded, one possibility is that Rsr assists RNase II and RNase PH in progressing through regions of RNA structure. This hypothesis incorporates the finding that vertebrate Ro binds the single-stranded 3′ ends of noncoding RNAs in its central cavity and adjacent helices on its surface (Fuchs et al. 2006), as well as the known difficulty of RNase II and RNase PH in digesting structured RNAs (Spickler and Mackie 2000; Wen et al. 2005). Although mechanisms that assist RNase II and RNase PH in degrading through stem–loop structures have not been described, PNPase functions with an RNA helicase, RhlB, either together or as part of the degradosome, which also contains the endonuclease RNase E and the metabolic enzyme enolase [Deutscher 2006]. In addition, PNPase functions with the Sm-like protein Hfq and poly(A) polymerase to degrade unstable mRNAs containing Rho-independent transcription terminators [Mohanty et al. 2004]. In this case, Hfq binding is proposed to transiently destabilize the stem–loop of the terminator, allowing efficient polyadenylation [Mohanty et al. 2004]. As both Hfq and Rsr form rings that bind single-stranded RNA, Rsr could function similarly to Hfq, perhaps by transiently destabilizing adjacent helices. An alternative but not exclusive possibility is that Rsr functions together with RNA helicases and/or other proteins, such as single-stranded RNA-binding proteins, to destabilize regions of base-pairing. Finally, although a small fraction of Rsr and PNPase co-purify, we note that this scenario does not require a direct physical interaction between Rsr and any of the exo- nuclease.

A model that is consistent with our results is shown in Figure 7. At 30°C, 23S rRNA maturation occurs through a pathway that is inefficient and does not require Rsr [Fig. 7A]. Because maturation is efficient at 30°C in Δpnp cells, PNPase may normally be responsible for the inefficient maturation. During growth of wild-type cells at 37°C [Fig. 7B], maturation is efficient and requires Rsr, RNase PH, and RNase II. As the levels of both Rsr and the Y RNA increase at 37°C, one possibility is that the level of Y RNA-free Rsr becomes sufficient to confer efficient maturation at elevated temperature. However,
as the increased Rsr and Y RNA in cells overexpressing wild-type Rsr was not sufficient to allow efficient maturation at 30°C [Fig. 2F], we favor a model in which additional changes at 37°C (such as decreased PNPase activity, decreased affinity of Rsr for the Y RNA, or destabilization of the predicted base-pairing in pre-23S rRNA) also contribute. In cells lacking Rsr [Fig. 7C], or lacking either RNase PH or RNase II, maturation is inefficient at all temperatures. In contrast, in cells lacking the Y RNA [Fig. 7D], the amount of Y RNA-free Rsr is always sufficient for efficient maturation. In Δpnp cells, the Rsr-dependent pathway predominates, allowing efficient maturation by other nuclease[s], such as RNase II and/or RNase PH, under all conditions [Fig. 7E]. Cells lacking both Rsr and PNPase [Fig. 7F] are unable to utilize either the inefficient pathway involving PNPase or the efficient pathway involving Rsr, RNase PH, and RNase II. In these cells, truncated 23S rRNAs accumulate, presumably through the action of other nuclease[s].

As the pre-23S rRNAs that accumulate in wild-type cells at 30°C and in Δsr, Δrib, and Δpnh cells at all temperatures possess both 5’ and 3’ extensions, 5’ maturation also depends on the pathway involving Rsr and these exoribonucleases. Because eubacteria lack 5’-to-3’ exoribonucleases, 5’ maturation of rRNAs occurs via endonucleolytic cleavages [Condon 2003; Deutscher 2006]. For at least one endonuclease, E. coli RNase E, cleavage requires both a single-stranded target site and an unpaired nucleoside monophosphate at the 5’ end [Mackie 1998]. One possibility is that following Rsr-dependent removal of the 3’ extension, the newly unpaired 5’ trailer undergoes cleavage by a similar single-strand-requiring endoribonuclease.

To our knowledge, the increased 23S rRNA maturation that occurs at 37°C is the first description that 23S rRNA maturation can change in response to environmental conditions. What purpose might this serve for cell function? One possibility is that although pre-23S rRNAs are found in polyribosomes, there are subtle increases in activity upon maturation. Consistent with this idea, in E. coli lacking RNase III, most of the 23S rRNA is present as 5’ and 3’ extended precursors [although 16S rRNA is matured], and the cells have a slight growth defect [King et al. 1984]. In this case, 23S rRNA maturation may provide a rapid mechanism for regulating ribosome function. Alternatively, maturation may provide a source of nucleotides during growth at elevated temperature. In this scenario, the role of 23S rRNA maturation in D. radiodurans would be similar to that of ribosome degradation in E. coli, which occurs in response to starvation and other stresses and may serve as a source of nutrients [Deutscher 2006].

Ro and the Y RNAs participate in additional processes

Although our experiments provide the first evidence that a Ro protein and a Y RNA function in RNA metabolism in vivo, we do not know the full range of processes in which they participate in either eukaryotes or bacteria. As the Xenopus laevis Ro can bind diverse RNAs, as long as they contain helices and a single-stranded 3’ end [Fuchs et al. 2006], it is likely that Ro functions in multiple pathways. Moreover, as a critical requirement for Ro binding is a single-stranded tail [Fuchs et al. 2006], the Ro protein may be especially likely to function in processes that involve 3’ extended precursors.

Consistent with additional roles, D. radiodurans lacking PNPase is sensitive to temperature extremes, UV irradiation, and oxidative stress, and deletion of Rsr alleviates the requirement for PNPase. These genetic interactions, while not explained by our model for the role of Rsr in 23S rRNA maturation [Fig. 7], suggest that Rsr functions in at least one other process that also involves PNPase. As both oxidative stress and ultraviolet irradiation can result in RNA damage [Bregeon and Sarasin 2005], one possibility is that PNPase functions in the degradation of certain damaged RNAs. In Δpnp cells, binding by Rsr to these RNAs could alter their pathway of degradation in a way that is harmful to cell function.

It is also possible that, in addition to regulating Ro activity, Y RNAs have other functions. However, as the vast majority of the Y RNA is bound by Ro in both mammalian cells [Wolin and Steitz 1983] and bacteria [Fuchs et al. 2006], and these RNAs are drastically reduced in mice, worms, and bacteria lacking Ro [Labbe et al. 1999; Chen et al. 2000, 2003], it is likely that any additional functions will require the presence of Ro. In this regard, we note that our result that D. radiodurans lacking the Y RNA are viable, coupled with the fact that Y RNAs are unstable in the absence of Ro, is inconsistent with a recent report that these RNAs are required for chromosomal DNA replication [Christov et al. 2006].

How does the role of Rsr in assisting 23S rRNA maturation relate to the proposed role of Ro in noncoding RNA quality control? One possibility is that binding by vertebrate Ro to defective structured RNAs may similarly assist the degradation of these RNAs by exoribonucleases. Whether Ro also functions in normal RNA maturation in mammalian cells remains to be addressed. Similarly, Rsr, as is proposed for the vertebrate Ro protein, may also participate in the targeting of abnormal RNAs for degradation. The identification of additional RNAs whose fate is affected by the presence or absence of Ro RNPs should help to uncover other roles of this abundant and conserved RNP.

Materials and methods

Plasmids, media, and strains

Plasmids pTNK102, pTNK103, and pTNK104 containing pkat-kan, pkat-aadA, and plh-hyg cassettes [Tanaka et al. 2004] were gifts of J. Battista (Louisiana State University, Baton Rouge, LA). Strains (Table 1) were grown in TGY (0.8% tryptone, 0.1% glucose, 0.4% yeast extract) broth or agar (1.5%). For low-phosphate TGY, we modified a yeast procedure (Warner 1991). Briefly, 8 g of tryptone and 4 g of yeast extract were dissolved in 920 mL of water. Inorganic phosphate was precipitated with 10 mL of 1 M MgSO4 and 10 mL of concentrated NH4OH. After 30 min, the precipitate was removed by filtering through Whatman 3MM paper. After adjusting the pH to 7.2, the filtrate was autoclaved and glucose was added. UV
Table 1.  D. radiodurans strains

| Strains | Genotype | Reference |
|---------|----------|-----------|
| R1      | rsr::cat  | Chen et al. 2000 |
| TB2     | rsr::cat  | This study |
| WY4     | yrn::pkat-hyg | This study |
| KY13    | rsr::cat, yrn::pkat-hyg | This study |
| WP6     | pnp::pkat-kan | This study |
| KP5     | rsr::cat, pnp::pkat-kan | This study |
| WPY16   | pnp::pkat-kan, yrn::pkat-hyg | This study |
| KPY2    | rsr::cat, pnp::pkat-kan, yrn::pkat-hyg | This study |
| WH21    | rph::pkat-aadA | This study |
| KH31    | rph::pkat-aadA, rsr::cat | This study |
| YH21    | rph::pkat-aadA, yrn::pkat-hyg | This study |
| WB21    | rnb::pkat-kan | This study |
| KB31    | rnb::pkat-kan, rsr::cat | This study |
| YB11    | rnb::pkat-kan, yrn::pkat-hyg | This study |
| DR122   | proteinA-TEV-Flag-Rsr,cat | This study |
| TFDRB31 | 3XFlag-Rsr,cat | This study |

survival measurements were as described (Chen et al. 2000) except that cells were grown to OD_{600} = 0.3 prior to irradiation. To replace yrn with pkat-hyg, −1 kb of upstream DNA was amplified with 5′-GTGCCACCGGCCGCGTCGAAAG-3′ and 5′-CATGGCCCTTCAGGCCCTCGCATGCAGGTTTCTTCTAGT-3′. Downstream DNA was amplified with 5′-GACAGCCGCCCGGCCGTGACAAAATCTGCGCCCGGCGCC-3′ and 5′-AGAAGCCGGCGGCTGGAGGGG-3′. The pkat-hyg cassette was amplified from pTNK104 with 5′-GCCAGGGGCCTGAGGGCCAT-3′ and 5′-GCAAGGCGGGCGGCTGTGAC-3′. After PCR, the hybrid DNA was cleaved with NcoI and BamHI, and inserted into pTricHisA (Invitrogen) to result in pYNRAko1. After linearization and transformation, integration and gene replacement was confirmed by PCR.

To replace rph with the streptomyacin resistance cassette pkat-aadA, 5′ DNA was amplified with 5′-TTCTGGTGTCTGGAAGAAGGA-3′ and 5′-CATGGCCCTTCAGGCCCTCGCATGCAGGTTTCTTCTAGT-3′. Downstream DNA was amplified with 5′-GACAGCCGCCCGGCCGTGACAAAATCTGCGCCCGGCGCC-3′ and 5′-AGAAGCCGGCGGCTGGAGGGG-3′. The pkat-aadA cassette was amplified from pTNK103 with 5′-GCCAGGGGCCTGAGGGCCAT-3′ and 5′-GCAAGGCGGGCGGCTGTGAC-3′. After PCR, the hybrid DNA was cleaved with NcoI and BamHI and inserted in place of Protein A-TEV-Flag-Rsr in pPFDR12, generating pTFDR2. Transformation resulted in strain TFDRB31. Both the Flag-Rsr and the ProteinA-TEV-Flag-Rsr bound Y RNAs, indicating they were functional in that capacity. However, because a significant fraction of each tag [up to 50%] was removed from Rsr in vivo, we have not determined whether the tagged proteins are functional for 23S rRNA maturation.

Overexpression of wild-type and mutant Rsr

The QuikChange Site-Directed Mutagenesis Kit (Stratagene) was used to mutate Rsr in pTR148. Next, PCR was used to amplify the 120 base pairs (bp) of DNA immediately upstream of D. radiodurans katA and to join this fragment to wild-type Rsr and the H1895 mutant. The resulting DNAs were digested with BamHI and HindIII and inserted into a derivative of pRADI (Meima and Lidstrom 2000). This derivative, pRADI-SPC, contains the streptomyacin resistance cassette pkat-aadA from pTNK103 inserted into the BglII/SacI site of pRAD1. Bac- teria containing plasmids were selected on TGY agar containing 3 µg/mL streptomycin and grown in 0.5 µg/mL streptomycin in liquid culture.

Antibodies, immunoprecipitations, and immunoblotting

The PNPase sequence was amplified with 5′-CGGGATCCGCCGTACCTGGCACTCGCATC-3′ and 5′-GGTACCATGCGGCGGTGCTCGCATC-3′, cleaved with BamHI and KpnI, and inserted into pTrichisA (Invitrogen). The protein was purified under native conditions and injected into rabbits. Other antibodies were anti-Rsr (Chen et al. 2000), anti-SSB [gift of M. Cox, University of Wisconsin, Madison, WI], and anti-Flag [M2] (Sigma). Immunoprecipitations and immunoblotting were as described (Chen et al. 2000) except that cells were lysed using a French press (Thermo IEC) at 10,000 psi.

Temperature shift and pulse-chase experiments

Strains were grown at 30°C to OD_{600} = 0.2 and shifted for 6 h to 37°C. Cells were maintained at OD_{600} between 0.04 and 0.25 by
Cells (three OD$_{600}$) were resuspended in 400 µL of 10 mM Tris-HCl [pH 7.5], 10 mM EDTA, 0.5% SDS, 200 µL of glass beads [0.1-mm, BioSpec], and 400 µL of acid phenol [pH 5]. After incubating for 40 min at 65°C with occasional vortexing, a second phenol extraction and a chloroform extraction were performed, followed by ethanol precipitation. RNAs were fractionated in 1.2% formaldehyde agarose or 5% polyacrylamide/8.3 M urea gels and transferred to ZetaProbe GT membranes (Bio-Rad) or 1.2% formaldehyde agarose or 5% polyacrylamide/8.3 M urea gels and transferred to ZetaProbe GT membranes (Bio-Rad). RNase H cleaves the RNA 5'-end and 3'-end recognition by the Ro protein. Mapping 23S rRNA ends

23S rRNA ends were mapped by site-directed RNase H cleavage [Inoue et al. 1987]. Briefly, 1 µg of RNA and 0.5 µg of 2'-O-methyl RNA–DNA chimeric oligonucleotides were mixed in 5 µL of water. After heating for 5 min to 95°C, annealing for 10 min at 50°C, and cooling to 37°C, 1 U of RNase H [Roche] and 2 U of RNase inhibitor [Promega] were added in 5 µL of 40 mM Tris-HCl [pH 7.5], 40 mM KCl, 20 mM MgCl$_2$, 0.2 mM EDTA, and 0.2 mM DTT. After 2 h at 37°C, reactions were fractionated in 6% polyacrylamide/8.3 M urea gels and analyzed by Northern blotting. RNase H cleaves the RNA 5' to the ribonuclease that base-pairs to the first deoxynucleotide in the RNA–DNA helix [Lapham et al. 1997]. The 2'-O-methyl RNA oligonucleotides were hybridized as described [Tarn et al. 1995]. Oligonucleotides used in Northers were as follows: 23SME3, 5'-CACCCACCTGTGATCAATC-3', 23SME5, 5'-GCAAdGdTdAAUCCCGGCUCU-3', and Oligonucleotide used in Northers were as follows: 23RSR3H3L, 5'-GTCCTTGATGATAAACAGTG-3', 23S5UP, 5'-CAGCCGTTTCCAGTGCCAGGGCATC-3'.

Sucrose gradients

After growing to OD$_{600} = 0.3$, chloramphenicol was added to 0.1 mg/mL and the cells were incubated for 5 min to stabilize polyribosomes. Cells [60 OD$_{600}$] were resuspended in 10 mM Tris-HCl [pH 7.5], 100 mM NaCl, 30 mM MgCl$_2$, 1 mM EDTA, 5 mM vanadyl ribonucleoside complexes, 0.5 U/µL RNAin [Promega], 0.2% diethylpyrocarbonate [DEPC], and 1 x protease inhibitor cocktail [Roche]. After passing through a French press and clearing for 10 min at 18,000g at 4°C, 100 µL were layered on 5%–40% sucrose gradients in 50 mM Tris-HCl [pH 7.5], 50 mM NH$_4$Cl, 10 mM MgCl$_2$, 1 mM EDTA, and 0.1% DEPC, and sedimented at 39,000 rpm in a Beckman SW41 rotor for 2.5 h at 4°C. Fractions were collected with an ISCO density gradient fractionator.

Rsr purification

Strains were grown to OD$_{600} = 0.8$ and lysed with a Microfluidizer [Microfluidics] in buffer A [40 mM Tris-HCl at pH 7.4, 150 mM NaCl, 0.1% NP-40, 2 mM MgCl$_2$, 2 mM MnCl$_2$, 0.5 mM PMSF, 1 mM EGTA, 1 x protease inhibitor cocktail [Roche]]. After clearing for 20 min at 36,000 rpm in a Type 50.2 Ti rotor, the lysate was mixed with IgG-Sepharose [Amersham] for 2 h at 4°C. After washing with 40 mL of buffer A + 20% glycerol and 0.5% NP-40, 30 mL of buffer A + 0.5 mM EGTA, 1.5 mM MgCl$_2$, 1.5 mM MnCl$_2$, 0.1% NP-40, and 20 mL of TEV buffer [10 mM Tris-HCl at pH 7.4, 150 mM NaCl, 0.1% NP-40, 1 mM MgCl$_2$, 1 mM MnCl$_2$, 1 x protease inhibitor cocktail, 0.5 mM PMSF], beads were mixed with 100 U of TEV protease for 16 h at 4°C. The eluate was mixed with anti-Flag agarose (Sigma) for 2 h at 4°C; washed with NET-2 [40 mM Tris-HCl at pH 7.4, 150 mM NaCl, 0.1% NP-40, 1 mM MgCl$_2$, 1 mM MnCl$_2$, and Rsr eluted with 1 mg/mL 3XFlag peptide in NET-2 with 0.02% NP-40. Proteins were directly analyzed by multidimensional protein identification technology [Florens and Washburn 2006].

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