Elevation of RNase R in Response to Multiple Stress Conditions*

Chenglu Chen and Murray P. Deutscher

From the Department of Biochemistry and Molecular Biology, University of Miami School of Medicine, Miami, Florida 33101

Cells respond to adverse environmental conditions by synthesizing new proteins or elevating the levels of pre-existing ones that are needed to cope with the particular stress situation. We show here that *Escherichia coli* RNase R, a processive 3′- to 5′-exoribonuclease, is dramatically increased in response to a variety of different stress conditions. Elevation of RNase R activity by as much as 10-fold was observed in response to entry into stationary phase, starvation, and cold shock, and a ~3-fold increase was seen during growth in minimal medium compared with rich medium. The elevation in RNase R activity was associated primarily with an increase in RNase R protein. RNase R was previously implicated in quality control of rRNA and tRNA and in the decay of mRNAs with extensive secondary structure. Its dramatic increase under multiple stress conditions suggests extensive remodeling of structured RNA in response to the altered environment.

The ability to sense and adapt to environmental change is critical to the survival of bacteria. Thus, upon encountering stress conditions, cells must rapidly alter their gene expression and remodel their RNA complement to deal with the changing environment. As a consequence, both new RNA transcription as well as RNA degradation must take place; however, while considerable information is available regarding changes in transcription in response to stress (reviewed in Ref. 1), very little is known about the RNA degradative machinery that comes into play under these conditions.

The types of stress with which cells must contend include deprivation of nutrients, changes in temperature, and exposure to noxious agents. For each of these conditions, rapid changes in mRNA content, and hence of mRNA stability, might be expected (1). In addition, under certain circumstances, such as starvation, degradation of stable RNA, i.e. rRNA and tRNA, also occurs (reviewed in Ref. 2). Stable RNAs, particularly tRNAs, account for the bulk of cellular RNA (3) and represent a potential storehouse of nutrients that could be tapped to help the cell survive a starvation condition. To do this, a change in ribosome structure and/or in RNase activity must occur that would render the usually stable ribosomes sensitive to degradation. The change in RNase activity could come about by either elevation of a pre-existing RNase or by synthesis of an additional RNase in response to the stress condition.

As part of an ongoing investigation of the functional role of RNases in *Escherichia coli*, we are examining how these enzymes respond to the imposition of a variety of stress conditions. In this paper, we focus on the processive, 3′- to 5′-exoribonuclease, RNase R (4, 5). Our attention was drawn to this RNase because of its ability to digest rRNA and other structured RNAs in vitro (5, 6) and to degrade tRNA fragments and REP sequences in vivo (6, 7). We show here that RNase R levels increase dramatically in response to a variety of altered environmental conditions. A similar response to one of these stresses, cold here that RNase R levels increase dramatically in response to a variety of altered environmental conditions. A similar response to one of these stresses, cold

---

**EXPERIMENTAL PROCEDURES**

**Materials**—[3H]Poly(A) and poly(A) were obtained from Amer sham Biosciences and Sigma, respectively. Antibody against RNase R was prepared from purified protein (5) by Sigma-Genosys. Anti-rabbit IgG horse radish peroxidase conjugate, used as secondary antibody, was from Promega Biotech. All other chemicals were reagent grade.

**Bacterial Strains and Growth Conditions**—The *E. coli* K12 strain CAN20–12E (RNase I′ II′ D′ BN−) and its derivative CAN20–12ER (RNase I′ II′ D′ BN− R′) have been described (4). These served as the RNase R− and R+ cells used in this study.

Cells were grown in liquid culture in either YT (yeast extract/tryptone) or M9/glucose (0.2 or 0.4%) media. Antibiotics, when present, were at the following concentrations: kanamycin, 25 μg/ml; tetracycline, 12.5 μg/ml. Normally, cells were grown at 37 °C and were monitored by absorbance measurements at 550 nm. For experiments under various stress conditions, cells were first grown in YT or M9/glucose medium at 37 °C prior to shifting to the experimental condition. Details are presented in the legends.

Media for starvation were as follows: carbon starvation, M9 without glucose; nitrogen starvation, M9/glucose lacking NH4Cl replaced with additional NaCl; phosphorus starvation, M9/glucose lacking NaH2PO4 and KH2PO4 replaced with additional Na2HPO4 and KCl and with 100 mM Tris–Cl, pH 7.4.

**Preparation of Cell Extracts**—Four mL of cells at ~1 A550 (or the equivalent amount at a different absorbance value) were centrifuged and resuspended in 0.4 mL of solution containing: 20 mM Tris–Cl, pH 7.5, 1 mM dithiothreitol, 300 or 500 mM KCl, and 0.1 mM phenylmethylsulfonfyl fluoride. Extracts were prepared by sonication on ice using two 20-s pulses or five 10-s pulses, followed by centrifugation to remove cell debris. Protein concentration of the extracts was determined by the method of Bradford (9) using reagent purchased from Bio-Rad and bovine serum albumin as the standard.

**Assay of RNase R**—Activity of RNase R was determined by the release of acid-soluble radioactivity from [3H]poly(A) (as described previously (4)). Assays were carried out in 50-μl reaction mixtures containing: 20 mM Tris–Cl, pH 8.0, 0.25 mM MgCl2, 180 mM or 300 mM KCl, 40 μg [3H]poly(A) (50–100 cpm/nmol), and ~15 μg of the indicated cell extract. Reaction mixtures were incubated at 37 °C for the indicated amount of time. After precipitation with trichloroacetic acid, acid-soluble radioactive activity was determined by liquid scintillation counting. One unit of RNase R activity converts 1 nmol of poly(A) to acid-soluble form/min.

**Western Immunoblotting of RNase R**—To determine the amount of RNase R protein present in extracts, proteins were resolved by 8% SDS–PAGE and transferred to a PVDF membrane by electroblotting. After blocking with non–fat milk (5%) in phosphate-buffered saline, RNase R was detected by incubating overnight at 4 °C with 1:10,000 dilution of anti-RNase R serum in phosphate-buffered saline containing 5% milk, 0.5% Triton X-100. Anti-rabbit IgG horse radish peroxidase conjugate was used as the secondary reagent and detection was with p-coumaric acid and Luminol. Undeveloped films were used for quantitation. Titration with varying amounts of purified RNase R indicated that this procedure gave a linear response up to at least 150 ng of protein.

**RESULTS**

**RNase R Levels during Growth**—As an initial assessment of whether RNase R might be subject to regulation, we determined its activity as cells progressed from exponential phase through late stationary phase. The data presented in Fig. 1 show, in fact, that RNase R specific activity in extracts changes dramatically during cell growth. Thus, in the representative experiment shown, RNase R activity (filled triangles), which remains relatively constant during exponential phase, increases ~6-fold as the culture approaches stationary phase and increases an additional 2-fold during an extended stationary phase. In multiple experiments, the initial increase in activity varied from ~4-~6-fold, whereas the increase in the second phase reproducibly was ~2-fold. That the increase in activity is actually all due to RNase R was confirmed by the absence of any activity change in extracts prepared from an RNase R− strain (Fig. 1, empty triangles).

To determine whether the increase in RNase R activity was accompanied by an elevation of RNase R protein, Western immunoblotting with RNase R antibody was carried out on extracts derived from cells at the different growth phases (Fig. 1, filled circles). Quantitation revealed a ~3.5-fold increase (3~4-
fold in multiple experiments) in the amount of RNase R protein as the culture entered stationary phase concomitant with the major increase in RNase R specific activity. In contrast, no increase in RNase R protein was seen in the RNase R− mutant strain (Fig. 1, empty circles). Interestingly, in multiple experiments, no additional increase in RNase R protein was found as the culture progressed through stationary phase, a period in which RNase R specific activity doubles (Fig. 1). These data suggest that the second phase of increase in RNase R activity may occur by a different mechanism, perhaps protein modification. Further work with RNase R purified from stationary phase cells will be necessary to clarify this point.

It should be noted that in all experiments the initial increase in RNase R activity slightly exceeded the increase in RNase R protein. This was due to the fact that in extracts from all phases of growth RNase R activity is somewhat inhibited because of the presence of nucleic acids that compete with the [3H]poly(A) substrate during the assay. The inhibition in extracts from early exponential phase (~50%) was greater than that in extracts from early stationary phase (~25%) resulting in an apparently greater increase in RNase R activity than actually occurred. Upon correcting for this effect, the increase in RNase R protein and increase in specific activity became essentially identical. Thus, these data show that RNase R levels are strongly influenced by growth phase.

**Effect of Growth Rate on RNase R**—Since a major reduction in growth rate accompanies the transition from exponential to stationary phase, it is possible that this slowdown in growth might be related to the observed increase in RNase R. To test this idea, cells growing rapidly in rich (YT) medium were compared with those growing more slowly in minimal (M9/glucose) medium with regard to RNase R specific activity and to levels of RNase R protein. The comparison was carried out with cells early in exponential growth to eliminate any effects due to stationary phase. As can be seen from TABLE ONE, cells growing at a slower rate in minimal medium were found to have ~3-fold higher RNase R activity and RNase R protein compared with those growing in rich medium. Based on these data, it is clear that growth rate does affect cellular levels of RNase R and, therefore, that the slowdown in growth could be related to the initial increase in RNase R seen during the entry into stationary phase.

**Elevation of RNase R in Response to Starvation**—A more extreme stress occurs under conditions of starvation during which cells cease growth. To determine whether starvation might affect the level of RNase R over and above that already seen during growth in minimal medium, cells were initially grown in M9/glucose medium until mid-exponential phase and then transferred after washing into the same medium devoid of either carbon, nitrogen, or phosphorus sources. After incubation at 37 °C for 24 h under these conditions, cells were harvested and extracts assayed for RNase R activity (TABLE TWO). During the starvation period, the A1200 in cultures lacking carbon or nitrogen decreased somewhat, whereas in cultures lacking phosphorus, the A1200 increased slightly (see details in the legend to TABLE TWO). However, in multiple experiments, RNase R specific activity increased under each of the starvation conditions, amounting to ~1.4-fold for carbon starvation, ~2-fold for nitrogen starvation, and ~4-fold in the absence of phosphorus (TABLE TWO) relative to the activity at the initiation of starvation. In RNase R− cells under these conditions, activity increased only slightly indicating that most of the increase observed in RNase R− cells is, in fact, due to RNase R. It should be noted that nitrogen starvation of RNase R− cells led to a significant elevation of RNase R activity presumably due to an effect on another enzyme; however, that increase could not account for the much larger elevation in RNase R− cells.

These data demonstrate that another stress condition, in this case, starvation, leads to an elevation of RNase R activity over that observed in minimal medium. Moreover, the fact that the level of increase in RNase R activity varies with the particular starvation condition (phosphorus starvation was significantly higher) suggests that there may be a specific response to each type of starvation.

**Elevation of RNase R in Response to Cold Shock**—E. coli is known to respond to cold shock by increasing the synthesis of a variety of proteins that help it cope with this particular stress (10, 11). In recent work (8), it was shown that RNase R protein was elevated ~7-fold following transfer of cultures from 37 to 10 °C. To confirm and extend these observations to RNase R activity, cells growing at 37 °C in rich (YT) medium during early exponential phase were rapidly transferred to several lower temperatures. At various times after the temperature downshift, cells were harvested for measurement of RNase R activity and RNase R protein.

The data in Fig. 2 show that upon transfer of cells to 10 °C, RNase R activity is elevated ~10–12-fold. The increase is already apparent at 1 h and reaches a maximum at 3 h after the temperature shift. RNase R activity is maintained near the maximum level to at least 6 h. Cells cease growth under these conditions. No change in activity was observed in extracts from RNase R− cells (Fig. 2) confirming that the elevated activity is due to RNase R. Western analysis revealed that RNase R protein was elevated at 10 °C as well, to a maximum of ~5-fold in multiple experiments (data not shown). It is not yet clear whether the ~2-fold difference between the increase in activity and the increase in protein is meaningful.

Significant, but smaller, increases in RNase R activity also were observed at 15 °C and 25 °C (data not shown). At 15 °C, the increase was still considerable, ~8-fold; however, at 25 °C, only ~2-fold elevation was obtained in multiple experiments during a 6 h time period. It should be noted that cells are able to grow at these latter two temperatures. These data clearly demonstrate that cold shock is an additional stress which leads to dramatic elevation of RNase R protein and activity and that the more severe the cold shock the more pronounced the effect on RNase R.

**Effect of Heat Shock on RNase R**—In contrast to the dramatic elevation of RNase R during cold shock, the effect of heat shock was much more modest. Shifting the growth temperature from 37 °C to 42 °C led to little, if any, increase in RNase R specific activity. However, upon shifting to 45 °C, a reproducible elevation of ~2-fold was observed in multiple experiments after 90 min at the elevated temperature (data not shown). No increase was observed in RNase R− cells. However, since cell growth is slowed somewhat at 45 °C compared with 42 °C, it is not yet clear whether the small elevation of RNase R at 45 °C is a specific response to the heat shock or is due to the reduction in growth rate.

**DISCUSSION**

The data presented here demonstrate that RNase R activity in E. coli is elevated in response to a variety of altered environmental conditions. These include entry into stationary phase, minimal versus rich media, starvation for carbon, nitrogen, or phosphorus, cold shock, and perhaps heat shock. It is not
Effect of starvation on RNase R activity

Cells were grown in M9/glucose (0.4%) medium. At an A_{550} = 0.2, cells were harvested, washed two to three times with the appropriate starvation medium (see "Experimental Procedures"), and then suspended in the starvation medium at the original cell density. After 24 h at 37 °C, cells were harvested for RNase R assays. The A_{550} of the cultures decreased ~50% during carbon starvation, decreased ~20% during nitrogen starvation, and increased ~35% during phosphorus starvation.

| Strain   | RNase R specific activitya |
|----------|----------------------------|
|          | Initial | Carbon | Nitrogen | Phosphorus |
| RNase R+  | 12.2    | 17.1   | 25.0     | 44.6       |
| RNase R−  | 2.3     | 2.4    | 5.6      | 3.7        |

* Values presented are the averages of three or four experiments.

FIGURE 2. Effect of cold shock on RNase R activity. Cells growing in YT medium at 37 °C were rapidly transferred at early exponential phase to 10 °C. At the indicated times, samples were withdrawn for measurement of RNase R activity and RNase R protein (data not shown) as described under "Experimental Procedures." RNase R specific activity is presented relative to the specific activity at the time of the temperature shift, which is set at 1. The actual specific activity at this point was 5.6 units/mg of protein. Depending on the time point, data are from one or are averages of up to three experiments.

---

References:

1. Takayama, K., and Kjelleberg, S. (2000) Environ. Microbiol. 2, 355–365
2. Deutscher, M. P. (2003) J. Biol. Chem. 278, 45041–45044
3. Bremer, H., and Dennis, P. P. (1996) in Escherichia coli and Salmonella (Neidhardt, F. C., ed.) pp. 1553–1569, ASM Press, Washington, D. C.
4. Cheng, Z.-F., Zuo, Y., Li, Z., Rudd, K. E., and Deutscher, M. P. (1998) J. Biol. Chem. 273, 14077–14080
5. Cheng, Z.-F., and Deutscher, M. P. (2002) J. Biol. Chem. 277, 21624–21629
6. Cheng, Z.-F., and Deutscher, M. P. (2005) Mol. Cell. 17, 313–318
7. Cheng, Z.-F., and Deutscher, M. P. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 6388–6393
8. Cairrao, F., Cruz, A., Mori, H., and Arraiano, C. M. (2003) Mol. Microbiol. 50, 1349–1360
9. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254

Acknowledgments—We thank Dr. Chaitanya Jain and members of the laboratory for helpful discussions and comments.

The large increase in RNase R specific activity in response to the various stress conditions raises the question of what functional role elevated RNase R might serve in these situations. RNase R is unique among E. coli exoribonucleases in its ability to digest through extensive secondary structure by itself (4, 6). In this regard, it is complementary to PNPase, which also digests through secondary structure as part of the degradosome (e.g. ref. 13 and 14). In the absence of both of these exoribonucleases, E. coli cells are inviable (4, 7) implying that they are solely responsible for digesting structured RNAs. Interestingly, in a psychrotrophic bacterium, Pseudomonos syringae Lz44W, RNase R is part of the degradosome (15).

RNase R has been shown to play a major role in degradation of REP sequences in mRNA (6) and in quality control of rRNA (7) and of defective tRNA (16). Each of these substrates contains extensive secondary structure. Thus, based on its in vitro catalytic properties (5) and its already known in vivo functions, it is most likely that elevated RNase R would be used to help degrade structured RNAs under stress conditions. It has been suggested that RNase R plays a role in maturation of tmRNA during cold shock (8). However, we consider this unlikely as the processive nature of RNase R makes it ill-suited to function as a maturation RNase. Moreover, in Caulobacter, its action on mature and precursor forms of tmRNA is degradative, and it is not used for maturation (12). In fact, RNase R has not yet been shown to participate in the maturation of any RNA molecule. Rather, all its known functions are in degrading RNAs (6, 7, 16).

Considering this information, and the fact that rRNA, the major cellular RNA, is known to be degraded during stationary phase and starvation (2), we speculate that elevated RNase R is needed for this process. Perhaps, during cold shock, a significant portion of newly made ribosomes are misassembled, and RNase R is required for their destruction as well, akin to its known role in rRNA quality control (7). Inasmuch as PNPase also is known to participate in rRNA quality control (7), it is interesting that this enzyme also is elevated during cold shock (17–19). Whether PNPase is similarly elevated in stationary phase and during starvation is to our knowledge not known. It would be of interest to examine this point.

Based on the information presented here, RNase R appears to be an important stress protein, and consequently, RNA degradation may be an important component of the stress response. Examination of the mechanism of its regulation during stress and of the consequences of its absence under stress conditions should greatly increase our understanding of the stress response and of the importance of RNA turnover for this process.
ACCELERATED PUBLICATION: Up-regulation of E. coli RNase R

10. Thieringer, H. A., Jones, P. G., and Inouye, M. (1998) BioEssays 20, 49–57
11. Yamanaka, K. (1999) J. Mol. Microbiol. Biotechnol. 1, 193–202
12. Hong, S.-J., Tran, Q.-A., and Keiler, K. C. (2005) Mol. Microbiol. 57, 565–575
13. Coburn, G. A., Miao, X., Briant, D. J., and Mackie, G. A. (1999) Genes Dev. 13, 2594–2603
14. Khemici, V., and Carpousis, A. J. (2004) Mol. Microbiol. 51, 777–790
15. Purusharth, R. I., Klein, F., Sultana, S., Jäger, S., Jagannadham, M. V., Evguenieva-Hackenberg, E., Ray, M. K., and Klug, G. (2005) J. Biol. Chem. 280, 14572–14578
16. Li, Z., Reimers, S., Pandit, S., and Deutscher, M. P. (2002) EMBO J. 21, 1132–1138
17. Zangrossi, S., Brianti, F., Ghisotti, D., Regonesi, M. E., Totora, P., and Deho, G. (2000) Mol. Microbiol. 36, 1470–1480
18. Mathy, N., Jarriage, A. C., Robert-Le Meur, M., and Portier, C. (2001) J. Bacteriol. 183, 3848–3854
19. Beran, R. K., and Simons, R. W. (2001) Mol. Microbiol. 39, 112–125
