containing excess EDTA is an effective reaction quench. Polypropylene (0.65 ml) microcentrifuge tubes are convenient reaction vessels that provide reproducible results.

Immediately before the assay, an appropriate amount of ^{32}P-labeled RNA (either internally or 5' labeled) is briefly heated (100°, 30 sec) in TE buffer, and then cooled on ice. This step removes intermolecular complexes that can form during storage at −20°. Aliquots are combined with RNase III in a buffer containing 30 mM Tris-HCl (pH 8), 250 mM potassium glutamate (or 160 mM NaCl), 5 mM spermidine (optional), tRNA (0.01 μg/μl), 0.1 mM EDTA, and 0.1 mM DTT. Samples are preincubated at 37° for 5 min, and MgCl$_2$ (prewarmed at 37°) is added to initiate cleavage (10 mM final concentration). Reactions are stopped by addition of EDTA–dye mix. Samples are loaded on a denaturing 15% (w/v) polyacrylamide gel containing TBE buffer and 7 M urea, and electrophoresed for 1–2 hr at 350 V. The top gel plate is removed, the excess buffer is removed, and the gel is wrapped in plastic. The cleavage reaction is followed by the rate of appearance of a specific cleavage product, and is quantitated by phosphorimaging (Storm 860 Phosphorimager system; Molecular Dynamics, Sunnyvale, CA) or by radioanalytic imaging (Ambis, San Diego, CA). To ensure linear kinetics the reaction times are kept short (0.5–3 min), such that only a small fraction of substrate is converted to product (typically <30%). For determination of steady state kinetic parameters, the substrate concentration is varied at a fixed enzyme concentration (usually ~10 nM), and saturation of initial velocity is determined by graphic analysis. The K_m and V_{max} values are determined by nonlinear least-squares curve fitting (Kaleidagraph; Synergy Software, Reading, PA), and the k_{cat} is determined from the V_{max}. For the k_{cat} determination it is necessary to have accurate determination of the protein concentration, and of the amount of [^{32}P]RNA recovered in the gel lanes.

Gel Mobility Shift Assay for Substrate Binding

The application of the gel mobility shift assay to monitor $E.\ coli$ RNase III binding to substrate has been described.[22,44] Since then additional changes have been instituted. In the absence of divalent metal ion, some RNase III–substrate complexes cannot be directly observed in a nondenaturing polyacrylamide gel.[22] We have shown that Ca^{2+} can stabilize RNase III–substrate complexes during electrophoresis, while disallowing cleavage.[22] The gel shift assay also can be performed in the presence of Mg^{2+}, and using a catalytically inactive mutant of RNase III (e.g., the Glu117Lys or Glu117Ala mutants).[22] The gel shift protocol described below may not necessarily be applicable to the analysis of RNase III

[44] B. S. Chelladurai, H.-L. Li, K. Zhang, and A. W. Nicholson, $Biochemistry$ 32, 7549 (1993).

orthologs. The reader is referred to [14] and [15] in this volume[44a] for a description of gel shift conditions for two yeast RNase III homologs.

Immediately before the assay, $5'$-^{32}P-labeled RNA is heated in TE buffer at $100°$ for 30 sec, and then placed on ice. This step removes intermolecular complexes that form during storage at $-20°$, which would otherwise be observed in a nondenaturing gel as slower moving species. RNA ($\sim$8000 cpm) is combined with RNase III in binding buffer [160 mM NaCl, 30 mM Tris-HCl (pH 8), 5–10 mM CaCl$_2$ (or MgCl$_2$ for catalytically inactive RNase III mutants), 0.1 mM EDTA, 0.1 mM DTT, 5% (v/v) glycerol, and tRNA at 0.01 μg/μl (250 mM potassium glutamate can be used in place of NaCl)]. Samples are incubated at 37° for 10 min, and then placed on ice for 20 min before loading on a 6% (w/v) polyacrylamide gel (acrylamide:bisacrylamide ratio, 80:1) containing 0.5× TBE buffer supplemented with 5–10 mM CaCl$_2$ (or MgCl$_2$). The binding reactions do not contain dyes, but the side lanes of the gel are loaded with aliquots of the bromphenol blue–xylene cyanol dye mix. The running buffer is also 0.5× TBE, supplemented with 5–10 mM CaCl$_2$ (or MgCl$_2$). The gel is preelectrophoresed ($\sim$7 V/cm) for 20 min, the samples are loaded, and the gel is electrophoresed (7.5 V/cm) at 4° for $\sim$3–5 hr. After electrophoresis, the top plate is removed and the excess buffer is removed. The gel is covered in plastic wrap and the reactions are analyzed by phosphorimaging.

To determine the apparent dissociation constants (K'_D values) for the protein–RNA complex, protein titration experiments are performed in which increasing amounts of protein are added to a fixed amount of RNA. The amount of RNA in the complex is determined, and the fraction of substrate that is bound is plotted as a function of the reciprocal of the enzyme concentration. This yields a linear relation, with the slope equal to the K'_D.[45] In some instances the protein–RNA complex is unstable during gel electrophoresis, and instead of observing a specific complex, a "smear" of radioactivity occurs, which reflects dissociation of the complex during electrophoresis. In this case, quantitation can be carried out by measuring the amount of free (unbound) RNA, which is used to calculate the fraction of substrate bound.[46]

We have attempted to apply the nitrocellulose (NC) filter binding assay[47] to detect RNase III–substrate complexes. However, these complexes are not retained on an NC membrane under a wide variety of conditions (our unpublished observations, 2000). However, a protein–RNA complex involving a catalytically inactive RNase III mutant (see above) can be stably bound to NC in the presence of Mg^{2+}.

[44a] B. Lamontagne and S. Abou Elela, *Methods Enzymol.* **342**, [14] 2001 (this volume); and G. Rotondo and D. Frendewey, *Methods Enzymol.* **342**, [15] 2001 (this volume).

[45] J. Carey, V. Cameron, P. L. deHaseth, and O. C. Uhlenbeck, *Biochemistry* **22**, 2601 (1983).

[46] J. Carey, *Methods Enzymol.* **208**, 103 (1991).

[47] M. Yarus and P. Berg, *Anal. Biochem.* **35**, 450 (1970).

We are currently investigating the experimental parameters that influence the stability of RNase III–substrate complexes on NC membranes.

Summary

It is now evident that members of the RNase III family of nucleases have central roles in prokaryotic and eukaryotic RNA maturation and decay pathways. Ongoing research is uncovering new roles for RNase III homologs. For example, the phenomena of RNA interference (RNAi) and posttranscriptional gene silencing (PTGS) involve dsRNA processing,[48,49] carried out by an RNase III homolog. We anticipate an increased focus on the mechanism, regulation, and biological roles of RNase III orthologs. Although the differences in the physicochemical properties of RNase III orthologs, and distinct substrate reactivity epitopes and ionic requirements for optimal activity, may mean that the protocols describe here are not strictly transferrable, the affinity purification methodology, and substrate preparation and use should be generally applicable

Acknowledgments

[48] T. Tuschl, P. D. Zamore, R. Lehmann, D. P. Bartel, and P. A. Sharp, Genes Dev. 13, 3191 (1999).
[49] E. Bernstein, A. A. Caudy, S. M. Hammond, and G. J. Hannon, Nature (London) 409, 363 (2001).