Benchmarks


The authors wish to thank Prof. Dr. H. Kössel and Dr. P. Zeltz (Freiburg, Germany) for the gift of the antibody specific for the rpoA gene product and Prof. Dr. R.G. Herrmann and Dr. R. Oelmüller (München, Germany) for the antibodies specific for plastid α-ATPase and β-ATPase. We are also grateful to Dr. Margret Sauter (Hamburg, Germany) for helpful comments on the manuscript. This article is based in part on a doctoral study by R.W.L.S in the Faculty of Biology, University of Hamburg. Address correspondence to Roland W.L. Suck, Botanisches Institut der Universität Köln, Gyrhofstraße 15, 50931 Köln, Germany.

Received 23 October 1995; accepted 20 February 1996.

Roland W.L. Suck and Karin Krupinska
Botanisches Institut der Universität Köln
Köln, Germany

Use of 33P for Ribozyme Assays: The Safe Way

BioTechniques 21:422-424 (September 1996)

Ribozymes or RNA enzymes are promising tools for the specific inhibition of gene expression and virus replication in animal or plant cells. Studies of the in vitro cleavage of synthetic radiolabeled RNA target by labeled or unlabeled ribozymes is a necessary step before in vivo applications. These cleavage assays are usually performed using 32P-labeled transcripts, which imposes the handling of unnecessary large quantities of high-energy radioactivity. The transcripts are not used in excess as probes that have to hybridize to nonradioactive nucleic acids fixed to a membrane, but they are all part of the reaction and detected directly in the dried gel.

To reduce the exposure to high levels of radioactivity, different methods have been tested. Recently, ribozyme self-cleavage assays using nonradioactive labeled RNA was demonstrated (5). Depending on the constructs tested, steric hindrance due to the haptene-labeled nucleotides may prevent or reduce the interaction with the target and

Figure 1. Cleavage of the 33P-labeled 464-nt RNA target by the nonradioactive 243-nt ribozyme. The two transcription products were incubated 90 min at 60°C in 50 mM Tris-HCl, pH 7.5, 10 mM MgCl2. Half of the reaction was denatured for 1 min at 95°C and loaded on a 6% acrylamide gel.
the secondary (hammerhead, hairpin, etc.) and tertiary folding of the target-ribozyme complex and of the ribozyme itself. Therefore, this approach is not only much more time-consuming than radioactive labeling, but also does not achieve the aim of the in vitro assays, which is to be as close as possible to the in vivo conditions. Although some reports on the successful use of $^{35}$S have been published recently (1,8), a phosphorothioate CpA junction has been reported to inhibit the cleavage reaction (2,3). Moreover, potential volatile impurities have been reported for certain $^{35}$S-labeled compounds (4,6). Thus, we decided to assay $^{33}$P-labeled ribozymes and target RNA with our ribozyme system to establish if the substantial difference of price between $^{32}$P and $^{33}$P could be compensated by a noticeable increase in safety and simplification of handling of ribozymes.

We have been studying the cleavage of the genome length transcript (8.2 kb) of cauliflower mosaic virus (CaMV) by an asymmetric antisense hammerhead ribozyme (7). The hammerhead ribozyme is derived from the self-cleaving positive strand of the satellite of the tobacco ring spot virus [(+)sTRSV]. As a first attempt to assay our 243-nucleotide (nt) ribozyme, a short synthetic RNA target (464-nt) has been constructed.

About 1 µg of the two clones pRzala and pTg (the ribozyme and the target, respectively) were transcribed with T7 RNA polymerase using only 1 µL of Easytides™ [$\alpha$-$^{33}$P]UTP (10 mCi/mL, 2000 Ci/mmol) (NEN Life Science Products, Boston, MA, USA) and 2 µL of 1 mM nonradioactive UTP in 20 µL transcription reaction containing 1 µL of ATP, CTP and GTP (at 10 mM each). Nonradioactive transcriptions were carried out in similar conditions. In vitro cleavage reaction (Figure 1) was performed by mixing 50 mM Tris-

Vol. 21, No. 3 (1996)
HCl, pH 7.5, 10 mM MgCl₂, 1 µL of 33P-labeled pTg transcript to 1 µL of nonradioactive pRzala transcript (4 pmol) in a final volume of 20 µL. The reactions were incubated 90 min at 60°C. Half of the reactions was loaded on a 6% acrylamide gel containing 7 M urea, after addition of 2× loading buffer (95% formamide, 20 mM EDTA) and denaturation for 1 min at 95°C. An association kinetic study (Figure 2) was carried out at 37°C with 10 nM 33P-labeled Rzala in 20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 500 mM NaCl in a final volume of 30 µL. The reaction was started with the addition of 20 nM nonradioactive pTg transcript. Aliquots of 3 µL were withdrawn at different times, and each reaction was stopped by the addition of 2× loading buffer. All the samples were loaded on a 6% acrylamide gel containing 7 M urea, after denaturation and running conditions and the samples were loaded on a 6% acrylamide gel containing 7 M urea, after denaturing running conditions and without prior denaturation.

As with 32P, association and dissociation kinetics were performed using 33P-labeled transcripts. Incubation of nonradioactive ribozymes with the target (Figure 1) gave a partial cleavage of the 464-nt RNA into two smaller molecules, the 5′ product (266 nt) and the 3′ product (198 nt) after 90 min. Figure 2 shows the association of labeled ribozyme with the nonradioactive target. The appearance of a lower band, corresponding to the folding of the ribozyme still annealed to the 5′ cleavage product, indicates the activity of a 33P-labeled ribozyme.

The first advantage of 33P over 32P is the higher resolution obtained (data not shown). The second advantage is that a ribozyme reaction needs only a low amount of radioactivity. Thus, in our conditions, only 1 µL of [α-33P]UTP (10 mCi/µL, 2000 Ci/mmol) was used for 20-µL transcriptions, yielding about 10 pmol of RNA per µg of plasmid. A typical ribozyme cleavage or association reaction is performed with about 2 to 5 nM labeled RNA in a 20-µL reaction volume (<0.1 pmol). Therefore, one transcription can be used for more than 100 ribozyme reactions. Indeed, because of the longer half-life of 33P (25 days, compared with 14 days for 32P) and the application of the phosphor-imager technology, the transcription products were used for more than three months. Indeed, the transcripts freeze/thawed often presented some smear after two months, but the transections used occasionally and stored at -20°C did not show any noticeable degradation. The higher price of 33P is partly counterbalanced by its longer half-life. But the most important advantage is the possibility to handle ribozyme reactions without special Plexiglas™ shielding and the reduced high exposure to strong radiations. This is especially significant for the hands, which are usually exposed the most. The radioactivity in the ribozyme reaction mixture (usually <0.1 µCi) in a microcentrifuge tube is not or hardly detected with a sensitive detector. The only problem we encountered was one found with 32P as well. After two months, attempts to carry out new transcriptions with the 33P nucleotide, even stored at -20°C, were unsuccessful. They appeared as a ladder of bands beneath the full-length transcript on a polyacrylamide gel. Although two months is a long period, this problem reduces the advantage of being able to use small quantities of 33P. It would be useful to have this radiochemical available in much smaller quantities, if few people are using it in a laboratory.

To our knowledge, this is the first report on the use of 33P with ribozymes. We have shown that the use of relatively expensive 33P can be justified by the numerous advantages of 33P over 32P with ribozymes as follows: better resolution, longer life and, especially, much safer handling. It is hoped that this work will help to inspire other researchers working on ribozymes to switch to a less hazardous isotope.

REFERENCES

Address correspondence to Patrice R.A. Wolff, Department of Virology, John Innes Centre, Colney Lane, Norwich NR4 7UH, England, UK. Internet: wolff@bbsrc.ac.uk
Received 20 November 1995; accepted 7 February 1996.

Patrice R.A. Wolff and Roger Hull
John Innes Centre
Norwich, England, UK