RESEARCH ARTICLE

Unexpected DNA Loss Mediated by the DNA Binding Activity of Ribonuclease A

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Abstract

Ribonuclease A (RNase A) is widely used in molecular biology research both for analytical assays and for nucleic acid preparation. The catalytic mechanism of RNase A is well understood and absolutely precludes activity on DNA; however anecdotal reports of DNA degradation by RNase A are not uncommon. Here we describe a mechanism by which RNase A treatment can lead to apparent DNA degradation. This results from the surprising finding that RNase A remains functional in a phenol:chloroform mixture, to our knowledge the only enzyme that

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many biochemical techniques, reviewed in [1, 2]. RNase A was the subject of landmark studies in protein chemistry including Anfinsen's Nobel Prize-winning

Fig. 1. RNase and DNase analysis of major satellite species. Total RNA from NIH/3T3 cells was treated with indicated enzymes for 30 min at 37, phenol:chloroform extracted, ethanol precipitated and glyoxylated prior to electrophoresis, then blotted and probed for major satellite sequences, ethidium staining of ribosomal RNA is shown as a control. A: Samples treated in water without and with RNase A. B: Samples treated in NEBuffer 3 with indicated ribonucleases. C: Samples treated in RQ1 DNase buffer without and with DNase I. For quantification, error bars represent ±1 s.d., y-axes in arbitrary units, p values calculated using a one-way ANOVA (n=4) for B and Student•s *t*-test (n=3) for C.

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were variable but on average the major satellite signals were not reduced compared to the control despite complete degradation of the ribosomal RNA (Fig. 1B lane 4). These results were not consistent with RNA, and we therefore performed digestions with RNase-free DNase I, resulting in a complete loss of the major satellite signal while the ribosomal RNA remained in-tact (Fig. 1C; equivalent results were obtained using DNase I from another manufacturer – data not shown). These results suggested that the major satellite signals originate from DNA that is aberrantly targeted by RNase A and RNase I.

To rule out DNase contamination in the RNase A, we prepared RNase A from a different manufacturer exactly as described [12], and also obtained certified DNase-free RNase A from a third manufacturer. All three preparations efficiently removed not only the major satellite signal from total RNA but also a spike of major satellite PCR product (Fig. 2A). This activity was buffer-dependent as PCR-

product removal was complete in water but only partial in PBS or DNase I buffer (Fig. 2B). These surprising results clearly demonstrate that RNase A treatment can remove major satellite DNA sequences from RNA samples.

To test the generality of this phenomenon, we asked whether RNase A could also remove DNA molecular weight marker from a sample. Indeed, 50 ng of molecular weight marker mixed with 1 μg total RNA was completely removed on RNase A treatment, showing that the effect is not a peculiarity of major satellite sequences (Fig. 2C). An unreported DNase activity of RNase A seemed unlikely, and we therefore suspected that RNase A treatment instead leads to the loss of

Fig. 3. Inhibition of DNA removal by RNase A. A: 0.5 ng major satellite PCR product was mixed with 1 μg NIH/3T3 RNA (lanes 1, 2) or with 1 μg DNA molecular weight marker (lanes 3, 4), and treated without or with RNase A followed by phenol:chloroform extraction and analysis as in Fig. 1. B: Identical to A, except that samples were incubated with 20 μg proteinase K for 15 min at 37 after RNase A treatment and phenol:chloroform extraction was omitted. For quantification, error bars represent ± 1 s.d., y-axes in arbitrary units, analysis by one-way ANOVA (n=3), differences in B are not significant.

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treatment while the radioactivity in the phenol phase dramatically increased. This clearly demonstrates that RNase A can mediate the partitioning of DNA to the interphase or the phenol phase during extraction, explaining the ability of RNase A to remove DNA during extraction.

Partitioning of DNA to the interphase during phenol:chloroform extraction is pH dependent and all these experiments were performed at pH 7, a pH that is appropriate for RNA and small DNA fragments. However, phenol:chloroform extraction for genomic DNA is more routinely performed at pH 8. To ensure that our observations were not simply due to pH, we tested extraction with phenol:chloroform pH 7 and pH 8; complete loss of major satellite PCR product in RNase A treated samples was observed in both cases (Fig. 4C).

The clear retention of the DNA binding activity by RNase A in phenol:-chloroform lead us to ask whether the enzyme also remains active. To test this, we added RNase A to phenol:chloroform, vortexed to ensure that the RNase A dissolved into the phenol phase, then added 1 μg RNA. This mixture (RNase A and RNA in 98% water-saturated phenol:chloroform) was incubated at 37°C before extraction with water to separate the RNA. Analysis by gel electrophoresis

demonstrated that the RNase A successfully degraded the RNA in a reaction

Of course, some loss of DNA is often acceptable for downstream applications but only if this loss does not introduce bias into the sample. It is currently unclear whether RNase A could preferentially partition DNA to the phenol phase depending on sequence, but this is likely to be the case. RNase A binds more efficiently to single than double stranded DNA and 'fixes' single stranded regions that form through breathing of duplex DNA; this is the basis of the known activity of RNase A as a DNA melting protein [25, 27]. Given that breathing of DNA duplexes is dependent on AT content, it is very likely that RNase A would bind more strongly to AT-rich sequences.

Overall, our data suggests that care is required during routine treatment of DNA samples with RNase A, and that a proteinase K step and/or high salt should always be employed to remove the enzyme prior to phenol chloroform extraction.

Materials and Methods

NIH/3T3 cells were cultured at 37°C, 5% CO_2 in DMEM (Sigma) with 10% calf serum. RNA was extracted using 1 ml TRI Reagent (Sigma) per 10 cm culture dish according to manufacturer's instructions after washing cells twice with PBS. Major satellite PCR product was amplified using Taq polymerase (NEB) and primers T7 and T3 from pCR4-Maj9-2 [30], digested with *Eco*RI and gel purified using a QIAQuick column (QIAGEN).

RNase A (Sigma R4875) for most experiments was dissolved at 20 mg/ml and boiled for 15 min before dilution to a 1 mg/ml stock solution in Tris pH 7.5 and 50% glycerol. RNase A (Roche 10109142001) was dissolved at 10 mg/ml in

alwayspH 7.5a

hand-held Geiger counter (Mini Series 900 Mini-monitor) by briefly vortexing the sample and placing directly against the detector, counts per second readings were converted to Bq on the assumption that at this distance approximately half the emitted β-particles would be detected.

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Author Contributions

Conceived and designed the experiments: FD JH. Performed the experiments: FD. Analyzed the data: FD JH. Contributed to the writing of the manuscript: JH.

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