Presence of RNase A Causes Aberrant DNA Band Shifts

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ABSTRACT

RNase A, which is routinely added during DNA purification to reduce contaminating RNA, causes shifting of DNA bands in agarose gels. DNA band sizes on agarose gels increase as much as 10%–20% when RNase A is present. The low concentrations of RNase A typically used to purify DNA cause shifting of select DNA bands, in contrast to higher concentrations of RNase A where all bands are shifted and smeared. The binding of RNase A to the DNA is specific and the degree of the shift varies; not all DNA bands are retarded, and the deviation is more pronounced in certain buffers. Other proteins, such as bovine serum albumin or proteinase K, do not induce DNA band shift, suggesting the interaction is specific. The binding of RNase A to DNA is reversible. The formation of RNase:DNA complexes may affect experiments involving DNA:protein interactions such as gel shift, footprinting and filter binding assays. Researchers performing DNA characterization from miniprep protocols should be aware that RNase may cause the apparent sizes of DNA fragments to be altered and obscure the presence of very small cloned fragments.

INTRODUCTION

Investigations of DNA:protein interactions require highly purified DNA to observe binding of proteins to a specific sequence. Most DNA purification procedures require a step to remove contaminating RNA, either by centrifugation, salt precipitation or enzymatic digestion using RNase. Using bovine pancreatic RNase (RNase A) to digest RNA is especially common in the rapid protocols used to quickly purify plasmid DNA (10). In many procedures, the RNase A enzyme is subsequently removed by polyethylene glycol (PEG) precipitation, organic extraction or ultracentrifugation. However, in miniprep DNA purification protocols, RNase A is added in the final step to degrade RNA, and the enzyme remains in the solution with the DNA (4,10). It has been assumed by most researchers that the presence of RNase A does not interfere with characterization of the DNA. RNase A is a well-characterized endonuclease. Its small size and stability against temperature and pH fluctuations, renders it the choice of many protein studies, including the work that won Christian Anfinsen a Nobel prize (1). The function of RNase A in the cell is to degrade RNA. In vitro it has also been shown to bind to and unwind DNA, melting the DNA double-stranded helical structure and trapping single-stranded DNA structures (7,9). While it is not yet clear if there is a cellular function for RNase A binding to DNA, the interaction is intriguing. Other RNase molecules have also been shown to bind to DNA or bear sequence homology to DNA regulatory proteins (3,5,8,9,11). For example, bovine seminal ribonuclease causes helix destabilization of DNA (9). RNase A, E. coli RNase and mouse Moloney leukemia virus RNase all have sequence homology with ARGRII, a DNA regulatory protein from Saccharomyces cerevisiae (8).

We have observed that the presence of RNase A in plasmid and viral DNA preparations can cause retardation of DNA bands in agarose gels. In this paper, we characterize RNase A-induced band shift of plasmid and lambda phage DNA under various conditions.

MATERIALS AND METHODS

RNase A from two sources was used in the experiments to demonstrate that the results are not caused by faulty RNase A preparation. RNase A from Qiagen (Chatsworth, CA, USA) was used in the proteinase K experiments. This RNase sample was purchased as part of the QIAprep® Plasmid DNA Purification Kit (Qiagen). RNase A, purchased from Sigma Chemical (Catalog No. R-4642, lot 24H6703; St. Louis, MO, USA), was used in all other experiments. RNase was used according to the manufacturer protocol and stored frozen in a frost-free freezer. Sigma Chemical notes that the product does not require boiling before use, and the enzyme shows no nicking or degradation of DNA in functional tests performed by the manufacturer. Since we were using the enzymes as most researchers do, without additional purification, further purification of the RNase A was not done.

Shift of Plasmid DNA Fragments

pBluescript® (Stratagene, La Jolla, CA, USA) was purified from XL-1

Figure 1. Band shift of plasmid digest fragments by RNase A. (A and B) Lanes 1 and 2 contain pBluescript plasmid, lanes 3 and 4 contain Bluescript digested with KpnI and SacI. Lanes 2 and 4 also contain RNase A. Marker 1 (M1) is lambda-digested with HindIII; Marker 2 (M2) is HaeIII-digested pBR322. (A) Electrophoresis was performed on a 1% agarose gel in 1x TAE at 75 V for 1 h. (B) Electrophoresis was performed on a 3% MetaPhor gel in 1x TBE to visualize the small fragments.

Figure 2. Effect of RNase concentration on band shift. Lambda-digested with HindIII was incubated with RNase A. Lanes 1–6 contained 0, 25, 50, 125, 250, 500 µg RNase/mL of TE buffer. Electrophoresis was performed on a 1% agarose gel in 1x TAE at 75 V for 1 h.
Blue E. coli by the method of Benore-Parsons and Wiland (2), which is a modification of the Yeung and Lau method (12) without the addition of RNase A and with the elimination of the final PEG precipitation step (2,12). In this procedure, cells are broken by alkali lysis in the presence of lauryl sulfate, and plasmid DNA purified from chromosomal DNA and proteins by paper-filtration in potassium acetate buffer, followed by separate plasmid DNA precipitations in isopropanol and ethanol. Thus, RNA was present in the DNA sample (Figure 1). Approximately 10–20 µg plasmid were digested to completion with restriction enzymes KpnI and SacI (Boehringer Mannheim, Indianapolis, IN, USA) in the absence or presence of 20 µg RNase A in 40 µL of the appropriate restriction enzyme buffer. Undigested control plasmid DNA samples were also incubated under the same conditions in the absence or presence of RNase A. Control and digested samples were separated on two gels to characterize large and small digested samples were separated on the same conditions in the absence or presence of RNase A and with the elimination of the final PEG precipitation step (2,12).

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**RNase A Concentration Necessary to Observe Band Retardation**

*HindIII*-digested lambda DNA (7.5 µg; New England Biolabs, Beverly, MA, USA) was incubated in TE (10 mM Tris, pH 8.0, 1 mM EDTA) for 120 min at 37°C with the following concentrations of RNase A: 0, 25, 50, 125, 250 and 500 µg per mL buffer. The samples were loaded onto 1% agarose gels in 1× TAE, and electrophoresis carried out as described above (Figure 2).

**Effect of pH and Salt on RNase A-Induced Band Shift**

To determine the effects of pH and ionic conditions on RNase A-induced band shift, DNA samples were incubated with excess RNase A in the presence of several restriction enzyme buffers (Boehringer Mannheim). *HaeIII* (2.5 µg)-digested pBR322 (mol wt Marker V; Boehringer Mannheim) was incubated in 20 µL buffer in the presence or absence of 50 µg RNase A for 1 h at 37°C. Buffer conditions were: tubes 1, 2 and 11, TE; tubes 3 and 4, buffer A (33 mM Tris-acetate, 10 mM magnesium acetate, 66 mM potassium acetate, 0.5 mM diithiothreitol [DTT], pH 7.9); tubes 5 and 6, buffer H (50 mM Tris-HCl, 10 mM magnesium chloride, 1 mM DTT, pH 7.5); tubes 7 and 8, buffer M (10 mM Tris-HCl, 10 mM magnesium chloride, 50 mM sodium chloride, 1 mM DTT, pH 7.5). RNase A was added to tubes 2, 4, 6, 8 and 10. After incubation, the samples were applied to a 3% MetaPhor gel in 1× TAE and run at 75 V for 1–2 h (Figure 3).

**Effect of Proteinase K on RNase A-Induced Band Shift**

To ensure that the shift observed was specific for RNase A and not simply due to the presence of excess protein, proteinase K and bovine serum albumin (BSA) were tested to determine if they complex with DNA. Tubes containing 2.5 µg of *HaeIII*-digested pBR322 (Boehringer Mannheim) were first incubated with or without RNase A (10 or 20 µg, respectively) for 8 h at 37°C in 20 µL 1× buffer H. Next, 80 µg of proteinase K were added to two tubes containing RNase A and to a control tube without RNase A, and 6 µg BSA (protein standard from Bio-Rad, Hercules, CA, USA) were added to a control tube containing only DNA. The samples were incubated at 37°C for 2 h, loaded onto a chilled 3% MetaPhor gel and run in 1× TAE at 75 V for 1 h (Figure 4). In other experiments using Sigma Chemical-brand RNase A, the proteinase K and RNase A were added simultaneously before incubation.

**RESULTS**

**Shift of Plasmid Bands by RNase A**

The addition of RNase A caused shifting of both supercoiled and linear plasmid DNA bands (Figure 1). The size of the small digest fragment is 102 bp, but in the presence of RNase A, it migrated with the 120-bp band of the *HaeIII* ladder (Boehringer Mannheim). The net effect of the shift was a 10%–20% increase in apparent size. The RNA was digested in the samples containing RNase A.

**RNase A Concentration Necessary to Induce DNA Shift**

Retardation of the 23130-, 2322- and 2027-bp fragments were observed at 25 µg/mL (Figure 2). The other lambda bands were not significantly retarded except at higher RNase A concentrations. Smearing and blurring were apparent at 250 µg/mL and were severe at 500 µg/mL.

**Effect of Buffers on Band Shifting**

The RNase A-induced band shift occurred in all buffers (Figure 3). Maximum retardation occurred in TE and buffers M and L, and maximum smearing was observed in TE and in buffers M and A.

![Figure 3. Effect of buffer on band shift. pBR322 fragments were incubated with or without RNase in various buffers. Lanes 1, 2 and 11, TE; lanes 3 and 4, buffer M; lanes 5 and 6, buffer H; lanes 7 and 8, buffer A; lanes 9 and 10, buffer L. RNase was added to samples in lanes 2, 4, 6, 8 and 10. Electrophoresis was performed on a 3% MetaPhor gel in 1× TAE at 75 V for 1 h.](image)
Effect of Proteinase K on RNase A Binding to DNA

Neither proteinase K nor BSA caused band retardation of any DNA fragment (Figure 4). Addition of proteinase K to DNA samples containing RNase A eliminates the band shift. The results using Sigma Chemical-brand RNase A were similar to those described (data not shown).

DISCUSSION

The results clearly demonstrate that, under certain conditions, RNase A binds to DNA, causing band shifts. The band shifts can be observed at the low RNase A concentrations typically used to digest RNA in DNA preparations (25–50 µg RNase A per mL of buffer containing DNA) (10). At 25 µg/mL, the binding of RNase A to DNA is selective, shifting certain bands without causing retardation of all the DNA fragments (Figure 2). This suggests that the DNA:RNase A interaction is specific, and that certain DNA sequences bind the RNase A with greater affinity than other sequences. At high concentrations, the enzyme causes smearing of all the bands. There are several possible explanations for this effect. One is that the RNase A binds to all of the bands. Alternatively, large DNA:RNase A complexes might obstruct electrophoretic separation of the other DNA fragments, causing smearing. It is not possible to determine from these experiments if the RNase A is simply binding to DNA or causing unwinding of the double-helix, which would increase retardation and smearing of the DNA bands.

The amount of band shift varies with the incubation conditions (Figure 3). This is consistent with reports that ionic conditions affect the degree of DNA unwinding by RNase A (7). It is also well known that the presence of salts affect the migration of DNA in agarose gels. Thus, the variations in RNase A-induced band shifting observed in different buffers may be the result of increased binding of RNase A in the presence of certain ions, or they may simply be an amplification of the RNase A-induced shift.

It has been our experience that the band shift is not always observed. It is not clear why the band shifts do not occur or are more apparent in some DNA preparations that others. The conditions of each preparation may vary enough to induce or reduce the observed band shift. The pH, ionic conditions, DNA:RNase A molar ratio, amount of RNA and preferential binding of RNase A to RNA or single-stranded DNA structures, would determine if DNA:RNase A complexes are formed.

However, the formation of a DNA:RNase A complex is not simply a result of excess protein, as neither proteinase K nor BSA induce shifts of DNA (Figure 4). These proteins were selected because both are used in molecular biology manipulations of DNA. BSA is added to some restriction enzyme buffers to enhance enzyme activity, and proteinase K is used to digest contaminating proteins in eukaryotic DNA preparations (10). Neither of these proteins bind to the DNA or cause shifting or broadening of the DNA bands, even at very high concentrations, confirming that the RNase A-induced band retardation is due to a specific interaction. Furthermore, the RNase A can be shifted by DNA. When DNA is added to RNase A, and polyacrylamide gel electrophoresis is performed followed by Coomassie® blue staining of proteins, the RNase A band is shifted slightly upward compared to a control of RNase A alone (data not shown).

The DNA shift is not permanent and is reversible if the RNase A is digested with proteinase K (Figure 4). Thus, it is possible to digest the DNA using RNase A and then remove the enzyme from the preparation by digestion, centrifugal filtration, organic extraction or PEG precipitation (4,5,10). (Another method to remove large RNA molecules, which is easily incorporated into rapid protocols, is precipitation of large RNA molecules by the addition of an equal volume of 5 M ammonium acetate. The DNA is then recovered by adding two volumes of ethanol to the supernatant.)

The band retardation observed in these experiments was not as large as the retardation typically observed in DNA:protein studies. It is possible that effects are minimized in agarose gels; most gel shift experiments use polyacrylamide gels or special agarose gels.

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Figure 4. Elimination of band retardation. pBR322 fragments were incubated with buffer H. Lanes 2–5 contained 10, 20, 10 and 20 µg RNase, respectively. Lanes 4–6 each contained 80 µg of proteinase K. Lane 7 contained 6 µg of BSA. Electrophoresis was performed on a 3% MetaPhor gel in 1× TAE at 75 V for 1 h.

The small migration differences could also be due to the very large size of the DNA fragments used in these experiments. Most gel shift experiments incorporate small (30–200 bp) DNA fragments, and binding of protein causes a much larger increase in mass and greater retardation on a gel.

These results have serious implications for researchers characterizing and purifying DNA fragments. Screening of miniprep plasmid preparations to identify bands of interest by size should incorporate controls if the bands will not be further scrutinized by sequencing or other characterizations. The binding of RNase A to DNA should also be considered by researchers who study protein-DNA interactions. If RNase A is not properly removed, it could interfere with binding by other proteins. Ribonuclease has been shown to inhibit transformation of the acyl hydrocarbon DNA regulatory protein and decreases the ability of the protein complex to bind to calf thymus DNA (6). RNase A could inhibit labeling or replication of DNA, and anomalous bands in gel-retardation and footprinting experiments might occur.

While studies have documented the binding to and unwinding of DNA by RNase A, a similar cellular role has not yet been demonstrated. The functional significance of the binding of the various RNases to DNA and their homology to DNA-binding proteins is not known. Future experiments on RNase binding to DNA should provide valuable information to the general understanding of DNA:protein binding as well as determine if this complex serves in a natural cellular capacity.

REFERENCES


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