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Preferential cleavage in pre-ribosomal RNA by protein B23 endoribonuclease

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ABSTRACT

Protein B23 is an abundant nucleolar protein and a putative ribosome assembly factor which possesses an intrinsic ribonuclease activity. In the current work, the effects of RNA sequence and secondary structure on the cleavage preference by protein B23 were studied. Protein B23 ribonuclease preferentially cleaved the single-stranded homopolymers poly(A), poly(U) and poly(C). However, double-stranded co-polymers and poly(G) were resistant to cleavage. No base specificity was observed with an oligoribonucleotide substrate. The action of protein B23 ribonuclease on different regions of pre-rRNA was studied using transcripts synthesized *in vitro* from cloned rDNA segments. Although no specific cleavages were detected in transcripts containing sequences from the 5′ external transcribed spacer or the first internal transcribed spacer, the enzyme preferentially cleaved the second internal transcribed spacer (ITS2) ~250 nt downstream from the 3′-end of 5.8S rRNA. Preferential cleavage was retained when the transcript was extended by 100 nt at the 3′-end, but abolished in a transcript lacking this cleavage site. Furthermore, this site was not susceptible to cleavage by RNase A or RNase T_1. These results, in conjunction with the sub-nucleolar localization of the protein with elements of the processing machinery, suggest that the protein B23 endoribonuclease could play a role in pre-rRNA processing in ITS2.

INTRODUCTION

Assembly of ribosomes in the nucleolus is achieved with the assistance of several non-ribosomal proteins (1). One of the latter is an abundant phosphoprotein called protein B23 (also called nucleophosmin, numatrin or NO38). Using biochemical methods, this protein was found to be associated with maturing pre-ribosomal RNP particles (2–5). Immunofluorescence techniques have also localized protein B23 to the peripheral regions of the dense fibrillar component (DFC) and the granular component (GC) of the nucleolus (6–8), which contain pre-ribosomal precursor particles in various stages of maturation (9). Selective treatment of cells with inhibitors of synthesis of nascent 47S pre-rRNA (10) or processing of 47S pre-rRNA (11,12) results in translocation of protein B23 from the nucleolus to the nucleoplasm, suggesting that it selectively interacts with the processing machinery. Collectively, these data have implicated protein B23 as a factor in pre-rRNA processing and/or ribosome assembly.

Protein B23 exists as two isoforms arising from alternative splicing of a primary transcript of a single gene (13). The predominant nucleolar isoform, B23.1, binds nucleic acids (14) with a preference for single-stranded nucleic acids. It binds them in a cooperative manner and destabilizes the RNA helices (15). The presence of a C-terminal 37 amino acid segment unique to protein B23.1 is essential for its binding to nucleic acids and probably for its localization to the nucleolus (16). In contrast, the minor isoform of the protein, B23.2, lacks the C-terminal tail, does not bind nucleic acids and is localized to the nucleoplasm and the cytoplasm (17).

47S pre-rRNA is synthesized in the nucleolus as a 13.9 kb precursor and processed into mature 18S, 5.8S and 28S RNAs by a complex series of cleavages (18). The initial cleavages within the transcribed spacers are effected by several endoribonucleases (19–21) which appear to be phosphodiesterases (22). Formation of the mature 5′-terminus of 18S rRNA involves an initial endonucleolytic cleavage in the 5′ external transcribed spacer (ETS), upstream of the mature end (23,24), followed by an exonucleolytic trimming process (25). In contrast, the mature 3′-terminus of 18S rRNA is generated by endonucleolytic cleavage alone (26,27).

The 5.8S and 28S rRNAs are released from the transcript by a similar process. Generation of the mature 5′-terminus of 5.8S rRNA involves cleavage in internal transcribed spacer (ITS) 1 followed by exonucleolytic trimming (28). Similarly, the mature 3′-terminus of 5.8S rRNA is formed by endonucleolytic cleavages at several sites in ITS2 followed by the action of exonucleases. The immediate precursor to 5.8S rRNA is 8S RNA (29,30), which contains the 5.8S rRNA and an additional 118 nt downstream of its 3′-end (31). Similar processing intermediates are found in yeast, i.e. 7S pre-rRNA which contains 5.8S rRNA with a 3′ extension of ~140 nt in *Saccharomyces cerevisiae* (32) and ~100 bases in *Schizosaccharomyces pombe* (33). An essential 39 kDa protein exhibiting an exoribonucleolytic activity *in vitro* has been shown to be responsible for generation of the mature 3′-terminus in *S.cerevisiae* (34). The presence of highly conserved homologs of this exonuclease in *S.pombe* and humans strongly suggests that

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the exonucleolytic cleavage mechanism of 5.8S rRNA 3′-terminus formation is conserved throughout eukaryotes. Thus, the processing machinery requires two classes of ribonucleases: endoribonucleases that introduce specific cleavages and exoribonucleases that generate the mature rRNAs and mononucleotides for recycling into *de novo* RNA synthesis.

Recent studies by Herrera *et al*. (35) demonstrated that both isoforms of protein B23 possess an intrinsic ribonuclease activity. Interestingly, the activity of the nucleolar isoform is ~5-fold greater than that of the non-nucleolar isoform. Mechanistically, the enzyme is classified as a phosphodiesterase, the class responsible for introducing endonucleolytic cleavages in pre-rRNA (22). Although several indirect results are suggestive of the association of protein B23 with pre-rRNA in the late stages of processing, there has been no direct evidence demonstrating that protein B23 ribonuclease is capable of cleaving pre-rRNA at specific sites. In the present study, we examine the effect of RNA secondary structure on the cleavage pattern by protein B23 ribonuclease and the possible base preference exhibited by the protein. The action of the protein on different regions of pre-rRNA has been studied using transcripts synthesized *in vitro* from cloned rDNA segments comprising sequences of mature rRNAs and the transcribed spacers. Our results indicate that protein B23 ribonuclease preferentially cleaves pre-rRNA at a specific site in the ITS2 region but not in the 5′ ETS nor ITS1.

MATERIALS AND METHODS

Proteins and nucleic acids

The synthetic homopolymers and co-polymers were purchased from Sigma Chemical Co. The 20 base oligoribonucleotide was synthesized by Cybersyn. All restriction endonucleases, calf intestinal alkaline phosphatase and T4 polynucleotide kinase were purchased from New England Biolabs. Recombinant protein B23 expressed in *Escherichia coli* from a rat cDNA clone was purified by a procedure described previously (35). After the final HPLC purification step, only one band was visible by SDS–PAGE followed by Coomassie blue staining.

Recombinant rat rDNA plasmids

5′ ETS plasmid. A 4.7 kb *Hin*dIII–*Bam*HI insert (pDF15) (36,37), comprising all but 58 bp of the 5′ ETS from the transcription start site (Δ+58) and ~600 bp of the 5′ region of the 18S rRNA sequence, was digested with *Xho*I and *Kpn*I and subcloned into pBluescript SK$^-$ (Stratagene). The resultant rDNA plasmid (pXKDF15) contained 1.3 kb of the 5′ ETS sequence with postions +638 to +1880 from the transcription start site.

ITS1-5.8S-ITS2 plasmid. A 6.2 kb *Hin*dIII–*Eco*RI insert (pDF4) (36,37), containing the 3′-end of 18S rRNA sequence, the entire ITS1, 5.8S rRNA and ITS2 sequence and all but ~500 bp of the DNA coding for 28S rRNA was digested with *Nhe*I and *Bam*HI and subcloned into pBluescript SK$^-$ between the *Xba*I and *Bam*HI sites. The resultant rDNA plasmid (pNBDF4) contained 107 bp from the 3′-end of the 5.8S rRNA sequence, the entire ITS2 and 1.3 kb of the 5′ region of 28S rRNA.

In vitro transcription of rat rDNA plasmids

Plasmids pXKDF15, pDF4 and pNBDF4 were linearized by a restriction endonuclease and *in vitro* transcription of these was performed using a bacteriophage RNA polymerase (38). Unlabeled transcripts synthesized *in vitro* were first dephosphorylated using calf intestinal alkaline phosphatase and then labeled at the 5′-terminus using [γ-^{32}P]ATP (ICN) and T4 polynucleotide kinase (39). Transcripts uniformly labeled with ^{32}P were synthesized with the addition of 50 μCi [α-^{32}P]UTP (New England Nuclear). Transcripts synthesized *in vitro* were treated with DNase I and electrophoretically purified from 5% polyacrylamide–7 M urea gels. Bands containing full-length transcripts were located by autoradiography and excised from the gel. The transcripts were eluted from the excised gel slices in 500 μl buffer containing 0.5 M sodium acetate (pH 6.5), 10 mM magnesium acetate, 0.1% SDS and 1 mM EDTA. After incubation at 25°C for 2 h, the eluate was extracted once with a mixture of phenol:chloroform:isoamyl alcohol (25:24:1) and ethanol precipitated at –70°C. Transcripts were washed twice with ice-cold 70% ethanol, dried under vacuum and resuspended in 10 mM Tris–HCl (pH 7.5).

Protein B23 ribonuclease assays and digestions

Protein B23 ribonuclease reactions were performed at 37°C in a buffer containing 50 mM Tris–HCl (pH 7.5), 50 mM NaCl, 0.5 mM $MgCl_2$ and RNasin at a final concentration of 0.5 U/μl. Reactions were initiated by addition of protein B23 ribonuclease. One unit of nuclease activity is defined as the amount of protein which results in an increase in A_{260} of 1.0 above the blank sample incubated in the absence of protein (35).

The perchloric acid (PCA) precipitation assay was modified from the method described by Eichler and Eales (40). Briefly, 50 μl reaction mixtures containing 100 nmol nucleotides of either the single-stranded polymer or double-stranded co-polymer substrates and 1 U protein B23 ribonuclease were incubated at 37°C. Reactions were terminated at various time intervals by chilling on ice followed by addition of perchloric acid and uranyl acetate to final concentrations of 5 and 0.05%, respectively. The reactions were placed on ice for 20 min and then centrifuged at 13 000 r.p.m. in a microcentrifuge for 5 min. Aliquots of the supernatant were withdrawn and the absorbance of the non-precipitable nucleotides was measured at 260 nm.

For determination of base specificity, a synthetic oligoribonucleotide 20mer with the sequence 5′-CCCCGAUUAGCGGUCAACUG-3′ (41) was labeled at its 5′-terminus using [γ-^{32}P]ATP and T4 polynucleotide kinase. Reaction mixtures (50 μl) containing 1.25 μg oligonucleotide substrate and 0.08 U protein B23 ribonuclease/μg substrate (20mer) were incubated at 37°C. Aliquots (5 μl) of the mixtures were withdrawn at various times and added to equal volumes of stop buffer (95% deionized formamide, 0.025% bromophenol blue, 0.025% xylene cyanol, 0.5 mM EDTA, 0.025% SDS). The products of digestion were analyzed on a 20% polyacrylamide–7 M urea gel.

For *in vitro* transcript digestions, reaction mixtures (80 μl) containing ~1.25 μg transcript RNA and 0.008 U protein B23 ribonuclease/μg substrate were incubated at 37°C. Aliquots (8.5 μl) of the mixture were withdrawn at various times and the reactions were terminated by ethanol precipitation. The precipitated RNA was resuspended in 10 μl 95% formamide–dye mix and analyzed on a 5% polyacrylamide–7 M urea gel.

S1 nuclease protection analyses and DNA sequencing

This assay was performed as described previously (42). A 335 bp uniquely 3′-end-labeled DNA probe (*Sfu*I–*Mlu*I) complementary

to a segment overlapping the 3′-end of 5.8S rRNA and 5′-end of the ITS2 region was generated from pNBDF4. Supercoiled plasmid pNBDF4 linearized by *Sfu*I was labeled at the 3′-terminus using 100 µCi [α-^{32}P]dCTP (New England Nuclear) and Sequenase v.2.0 (US Biochemical Amersham). The 3′-end-labeled plasmid was then digested with *Mlu*I to generate the 335 bp DNA probe. Radiolabeled probes were electrophoretically purified from 5% polyacrylamide gels. Probes, located by autoradiography, were eluted from the excised gel slices, ethanol precipitated and dissolved in DEPC-treated water. Hybridization reaction mixtures (20 µl) were heated at 85°C for 15 min to completely denature the probe. Hybridizations were performed at 60°C for 3 h. The reactions were diluted 10-fold in S1 digestion buffer (50 mM sodium acetate, pH 4.5, 300 mM NaCl and 4.5 mM $ZnCl_2$) containing S1 nuclease at a final concentration of 0.2 U/µl. S1 nuclease digestions were performed at 37°C for 30 min. The S1-resistant hybrids were recovered by ethanol precipitation and resuspended in 10 µl 95% formamide–dye mix and analyzed on a 7% polyacrylamide–7 M urea gel adjacent to the chemical sequencing reactions. Chemical sequencing of the 3′-end-labeled 335 bp *Sfu*I–*Mlu*I DNA probe was performed as described previously (43).

RESULTS

Protein B23 ribonuclease preferentially cleaves single-stranded RNA

The action of a ribonuclease can be affected by the conformation of the macromolecule substrate. The effect of secondary structure on the action of protein B23 ribonuclease was studied by comparing the rates of digestion of synthetic single-stranded and double-stranded polymeric substrates. When single-stranded poly(A) or poly(U) alone was used as the substrate, the amount of non-precipitable nucleotides released increased during the course of the reaction (Fig. 1A). Furthermore, the rates of cleavage of poly(A) and poly(U) by ribonuclease B23 were similar. However, when the double-stranded co-polymer poly(A):poly(U) was used as the substrate, the amount of non-precipitable nucleotides released was essentially negligible. In control experiments, the co-polymer served as an excellent substrate for the double-strand-specific ribonuclease V_1 (44; data not shown). Thus protein B23 ribonuclease could cleave single-stranded poly(A) and poly(U) but did not digest the double-stranded form, suggesting that it is a single-strand-specific nuclease.

To confirm the single-stranded specificity, the PCA precipitation assays were performed on the poly(A) and poly(U) counterparts, i.e. poly(G) and poly(C). When single-stranded poly(C) alone was used as the substrate, the acid-soluble nucleotides increased during the course of the reaction (Fig. 1B). Furthermore, the rate of digestion of poly(C) was similar to that obtained with poly(A) or poly(U). Surprisingly, when poly(G) was incubated with B23 ribonuclease, the release of nucleotides was essentially undetectable, indicating that B23 ribonuclease did not digest poly(G). The double-stranded co-polymer poly(C):poly(G) was also resistant to cleavage by protein B23. Thus, B23 ribonuclease appeared to be a single-strand-specific nuclease with an apparent negative base specificity, whereby it readily cleaved poly(A), poly(U) and poly(C) but it did not digest poly(G).

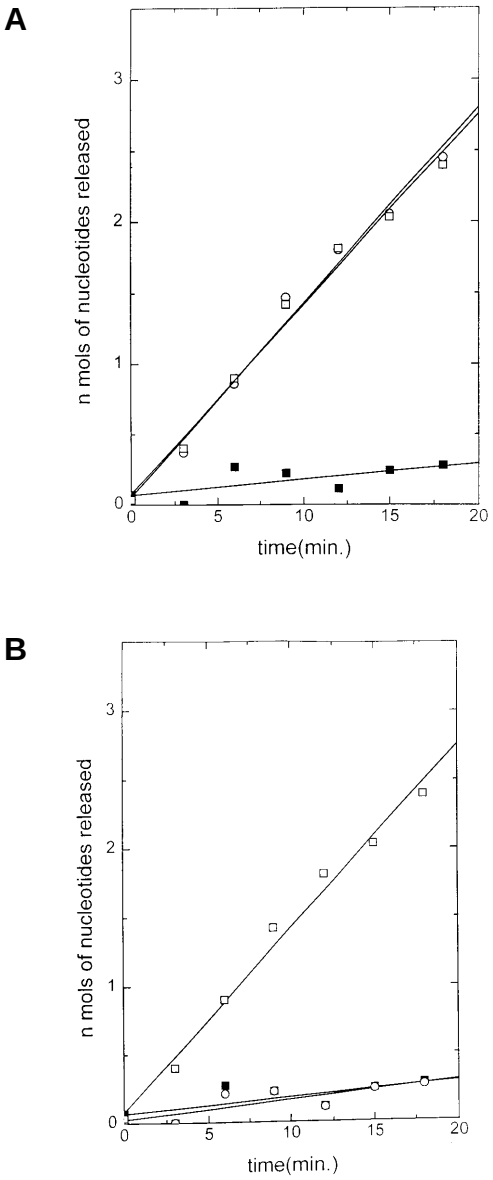


Figure 1. Digestion of single-stranded RNA versus double-stranded RNA by protein B23 ribonuclease. (**A**) Reaction mixtures (50 µl) containing 100 nmol (in nt) of either single-stranded poly(A) (○) or poly(U) (□) or double-stranded poly(A):poly(U) (■) and 1 U protein B23 ribonuclease were incubated at 37°C. Reactions were terminated at various times by addition of perchloric acid and uranyl acetate to final concentrations of 5 and 0.05%, respectively. The reactions were placed on ice for 20 min and centrifuged at 13 000 r.p.m. in a microcentrifuge for 5 min. Aliquots of the supernatant were withdrawn and the absorbance of the non-precipitable nucleotides was measured at 260 nm. (**B**) Reaction mixtures containing either single-stranded poly(C) (□) or poly(G) (○) or double-stranded poly(G):poly(C) (■) and 1 U protein B23 ribonuclease were incubated and processed as described in (A).

Analyses of base specificity of cleavage by protein B23 ribonuclease

To determine if protein B23 ribonuclease exhibited a similar base preference on a mixed arrangement of nucleotides, a synthetic oligoribonucleotide containing all possible dinucleotide combinations (41) was labeled at its 5′-terminus and digested with protein B23

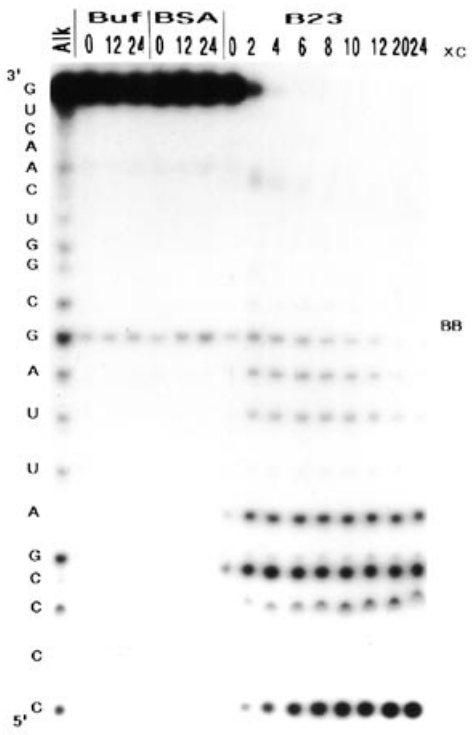


Figure 2. Cleavage of a synthetic oligoribonucleotide by protein B23 ribonuclease. The synthetic 20mer oligoribonucleotide (41) was labeled at its 5′-terminus using [γ-^{32}P]ATP and T4 polynucleotide kinase. Reaction mixtures (50 µl) containing 1.25 µg oligonucleotide substrate and 0.08 U protein B23 ribonuclease/µg substrate were incubated at 37°C. Aliquots (5 µl) were withdrawn at various times (indicated at the top in min) and added to equal volumes of stop buffer (95% deionized formamide, 0.025% bromophenol blue, 0.025% xylene cyanol, 0.5 mM EDTA, 0.025% SDS). The products of digestion were analyzed on a 20% polyacrylamide–7 M urea gel. Alk, partial hydrolysate of the oligonucleotide performed in 50 mM sodium bicarbonate, pH 9.2, at 85°C for 14 min; Buf, buffer; BSA, bovine serum albumin; XC and BB, migration positions of the tracking dyes.

ribonuclease. The 20 base oligonucleotide had a predicted T_m of 12°C and existed in a single-stranded form at the reaction temperature. As seen in Figure 2, when a digest of the oligonucleotide was performed at a relatively high enzyme:substrate ratio, there was cleavage at phosphodiester bonds between nucleotides containing all the bases, with the major products of digestion being mono-, tetra- and pentanucleotides. Interestingly, the G-N and N-G bonds were also cleaved, indicating that the lack of cleavage of poly(G) was not due to the presence of a guanine residue itself. The fact that cleavage occurred at nearly all phosphodiester bonds indicated that the enzyme did not exhibit any base specificity. However, the presence of prominent bands with the digest indicated that although there was no absolute base specificity, the enzyme was sensitive to some conformational aspects of the RNA molecule or to certain sequences which resulted in the non-random cleavage pattern.

Cleavage of pre-rRNA by protein B23 ribonuclease

The above results indicated that although protein B23 endoribonuclease did not exhibit any nucleotide preference, it was sensitive to the structure of the macromolecule, with a marked preference for single-stranded RNA. Eukaryotic rRNA is known to possess a high degree of secondary structure (45). Structural motifs have been shown to play a significant role in recognition by several endonucleases involved in pre-rRNA processing events (46,47). To determine whether nucleolar protein B23 ribonuclease cleaved at specific sites in pre-rRNA, transcripts comprising various regions of pre-rRNA were synthesized *in vitro* using bacteriophage polymerases and used as substrates for digestion by nucleolar protein B23 ribonuclease.

There are no preferential cleavage sites by protein B23 ribonuclease in the 5′ ETS or ITS1 RNAs

Initial studies were performed with transcripts from the 5′ ETS region spanning the primary processing site (+790 in rat) (48) which were uniformly labeled with ^{32}P. Ribonuclease B23 at a low enzyme:substrate ratio of 0.008 U protein B23 ribonuclease/µg transcript was unable to cleave the transcript containing the 5′ ETS region of the pre-rRNA (data not shown). An increase in the enzyme:substrate ratio resulted in non-specific cleavage in the transcript (data not shown).

To detect cleavage in the 18S–ITS1 region of pre-rRNA, uniformly ^{32}P-labeled transcripts were subjected to limited digestion with protein B23 ribonuclease at the same enzyme:substrate ratio (0.008 U protein B23 ribonuclease/µg transcript). Under these conditions, ribonuclease B23 was unable to cleave these transcripts, although higher concentrations of protein resulted in non-specific cleavages (data not shown).

Protein B23 ribonuclease preferentially cleaves at a specific site in ITS2 RNA

As indicated above, at the low enzyme:substrate ratio (0.008 U enzyme/µg transcript), protein B23 was unable to introduce any cleavages either in the 5′ ETS or the 18S–ITS1 RNA transcripts. However, this was not the case with a transcript containing the 3′-end of the 5.8S rRNA and the 5′-region of ITS2. A uniformly ^{32}P-labeled 528 nt *Sac*I–*Ppu*MI transcript was subjected to limited digestion at the same enzyme:substrate ratio used with the previous two transcripts. This transcript was comprised of 107 nt at the 3′-end of 5.8S rRNA and 377 nt at the 5′-end of ITS2 and 44 nt of the polylinker. As seen in Figure 3, after incubation with protein B23 ribonuclease, several distinct RNA fragments >200 nt in length were seen. The most prominent product of the partial digest was a fragment ~400 nt in length. Thus, under conditions of limited digestion, metastable products were formed which were relatively resistant to further cleavage. However, the major band appeared to reach a steady-state level after ~10 min digestion, suggesting that this fragment underwent further cleavage. In experiments using high percentage gels, there was also an accumulation of products ranging from 20 to 100 nt in length, most of which did not appear as discrete bands (not shown). There was little or no increase in acid-soluble material over the time of digestion, indicating that the products were not cleaved to very small fragments. These cleavages accounted for the progressive loss of radioactivity from the parent band.

Although the uniformly labeled transcript permitted detection of preferential cleavage patterns, it did not reveal the location of the cleavages relative to the 5′- or the 3′-end of the transcript. To determine this, the *Sac*I–*Ppu*MI transcript, labeled at its 5′-terminus, was subjected to limited digestion with ribonuclease B23. As seen in Figure 4, the prominent product of the partial digest was the

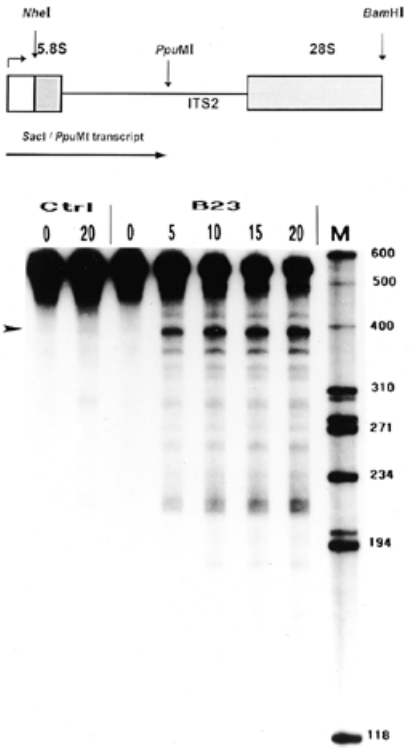


Figure 3. Detection of cleavages introduced in the 5.8S–ITS2 region of pre-rRNA by protein B23 ribonuclease. An *in vitro* synthesized 528 nt *Sac*I–*Ppu*MI transcript comprising 44 nt of the polylinker, 107 nt of the 3′-region of the 5.8S rRNA and 377 nt of ITS2 uniformly labeled with ^{32}P was subjected to limited digestion with protein B23 ribonuclease. Reaction mixtures (80 µl) containing 1.25 µg transcript RNA and 0.008 U protein B23 ribonuclease/µg substrate were incubated at 37°C. Aliquots (8.5 µl) were withdrawn at various times (indicated at the top in min) and the reactions were terminated by ethanol precipitation. The precipitated RNA was resuspended in 10 µl 95% formamide–dye mix and analyzed on a 5% polyacrylamide–7 M urea gel. Ctrl, no enzyme control; M, 5′-^{32}P-labeled denatured 100 bp DNA ladder + *Hae*III digested ϕX174RF DNA. The arrowhead denotes the preferential cleavage site by protein B23.

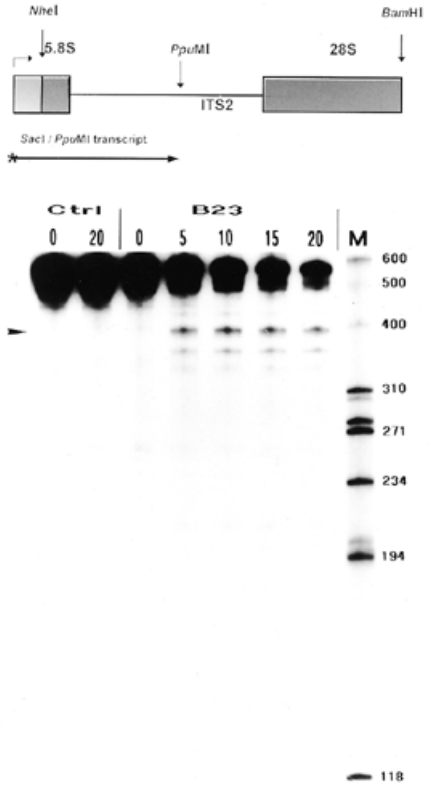


Figure 4. Location of cleavages in the 5.8S–ITS2 region of pre-rRNA by protein B23 ribonuclease. An *in vitro* synthesized 5′-^{32}P-labeled 528 nt *Sac*I–*Ppu*MI transcript was subjected to limited digestion with protein B23 ribonuclease. Reaction mixtures containing 1.25 µg transcript RNA and 0.008 U protein B23 ribonuclease/µg substrate were incubated and processed as described in the legend to Figure 3. Ctrl, no enzyme control; M, 5′-^{32}P-labeled DNA markers as described in Figure 3. The arrowhead denotes the preferential cleavage site by protein B23.

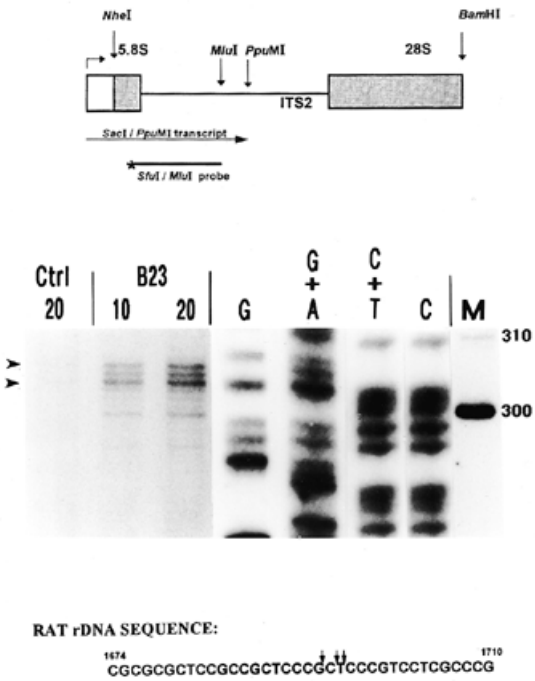
400 nt fragment. Some smaller RNA products were also seen, but their intensities were much weaker in comparison with the 400 nt fragment. The presence of the 400 nt fragment mapped to a distinct cleavage site ~250 nt into the ITS2 region of the unprocessed pre-5.8S rRNA. Secondary structure predictions by Michot *et al*. (49) suggest that this region exists as a single-stranded loop. However, this region is only one of several single-stranded regions within ITS2. With the transcript being 5′-end-labeled, a cleavage 400 nt from the 5′-end should not have masked any preferential cleavages in the upstream regions of the pre-5.8S transcript. Figure 4 shows that minor cleavages also occurred in the ITS2 region, upstream of the preferential cleavage site. Interestingly, no cleavages were detected in the 5.8S region of the pre-5.8S transcript. Thus the cleavage 250 nt into the ITS2 region of pre-5.8S rRNA is the preferential cleavage site for protein B23 ribonuclease.

Precise location of the cleavage site using S1 nuclease protection analyses and DNA sequencing

To precisely locate the preferential cleavage site in the ITS2 region by protein B23 ribonuclease, a S1 nuclease protection assay was performed using the unlabeled 528 nt *Sac*I–*Ppu*MI transcript and a 335 bp *Sfu*I–*Mlu*I probe labeled at the 3′-terminus. The preferential cleavage 250 nt into ITS2 would result in a protected fragment ~300 nt in length. Precise determination of the cleavage site was further achieved by electrophoretic separation of the S1 products in lanes adjacent to the lanes of sequencing reactions obtained with the probe. As seen in Figure 5, the multiple bands arise from three cleavages that are introduced 244–246 nt from the 3′-end of the mature 5.8S rRNA at GpCpT phosphodiester bonds. The fragments resulting from the S1 nuclease digestion do not co-migrate exactly with the chemical sequencing reactions but are offset by 1.5 nt. This is expected, since the chemical sequencing reactions leave a 3′ phosphate group, with loss of a nucleotide, whereas S1 nuclease leaves a 3′ hydroxyl group (50).

Preferential cleavage is retained on a 3′ extended pre-5.8S rRNA transcript

To determine whether protein B23 ribonuclease preference for that specific site was retained in a longer transcript, a 635 nt *Sac*I–*Rsa*I transcript was used as the substrate. The 5′-end-labeled transcript was subjected to limited digestion with nucleolar ribonuclease B23. Cleavage of this transcript at the specific site resulted in a 400 nt fragment (data not shown). Thus the



RAT rDNA SEQUENCE:

1674 CGCGCGCTCCGCCGCTCCCGCTCCCGTCCTCGCCCG 1710

Figure 5. S1 nuclease protection analyses and the nucleotide sequence around the preferential cleavage site in the pre-5.8S rRNA transcript. An unlabeled 528 nt *Sac*I–*Ppu*MI transcript was subjected to limited digestion with protein B23 ribonuclease. Reaction mixtures containing 1.25 µg transcript RNA and 0.008 U protein B23 ribonuclease/µg substrate were incubated at 37°C. Reactions were terminated at various times (in min) and an aliquot (6 µl) was hybridized to a 3′-end-labeled 335 bp *Sfu*I–*Mlu*I DNA probe. Hybridizations were performed at 60°C for 3 h as described in Materials and Methods. Hybridization reactions were diluted 10-fold in S1 digestion buffer containing S1 nuclease at a concentration of 0.2 U/µl. S1-resistant hybrids recovered after ethanol precipitation were resuspended in 10 µl 95% formamide–dye mix. The products of digestion were electrophoretically separated in lanes adjacent to the lanes of the sequencing reactions on a 7% polyacrylamide–7 M urea gel. Nucleotide sequence analyses of the 3′-end-labeled 335 bp *Sfu*I–*Mlu*I DNA probe was performed using the G, G+A, C+T and C reactions as described previously (43). Ctrl, no enzyme control; M, 5′-^{32}P-labeled DNA markers as described in Figure 3. The arrowheads denote the protected fragments after S1 nuclease digestion. The rat rDNA sequence of the ITS2 region from position +1674 to +1710 (64) along with the cleavage sites are shown below.

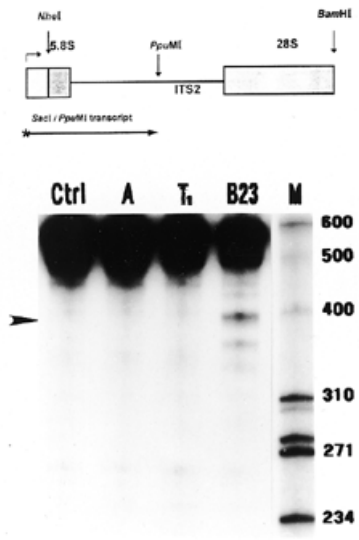


Figure 6. Detection of specific cleavages by bovine pancreatic RNase A and RNase T_1 in the ITS2 region of the pre-5.8S rRNA transcript. An *in vitro* synthesized 5′-^{32}P-labeled 528 nt *Sac*I–*Ppu*MI transcript comprising 44 nt of the polylinker, 107 nt of the 3′-end of 5.8S rRNA and 377 nt of ITS2 was subjected to limited digestion with either pancreatic RNase A, RNase T_1 or protein B23 ribonuclease. Reaction mixtures containing 1.25 µg transcript RNA and 0.008 U either bovine pancreatic RNase A, RNase T_1 or protein B23 ribonuclease/µg substrate were incubated at 37°C. Aliquots were withdrawn after 20 min and the reactions terminated by ethanol precipitation. The precipitated RNA was resuspended in 10 µl 95% formamide–dye mix and analyzed on a 5% polyacrylamide–7 M urea gel. Ctrl, no enzyme control; A, pancreatic RNase A; T_1, RNase T_1; M, 5′-^{32}P-labeled DNA markers as described in Figure 3. The arrowhead denotes the specific cleavage site introduced by protein B23 ribonuclease.

specificity exhibited by the protein for the site was not affected by the addition of 107 nt in the ITS2 region at the 3′-end of the 528 nt transcript. Although additional cleavages of weaker intensities were present upstream of the primary site, no cleavages were introduced in either the 5.8S region or in the ITS2 region immediately downstream of the 5.8S rRNA. Evidently, the conformation of the RNA is maintained in the extended transcript and the protein has the ability to recognize and introduce specific cleavage at this site.

Protein B23 ribonuclease does not cleave a 3′ truncated pre-5.8S rRNA transcript

To test whether the protein could recognize a substrate lacking the cleavage site, a transcript truncated at the 3′-end was subjected to limited digestion using the same enzyme:substrate ratio as described above. A uniformly ^{32}P-labeled 305 nt *Sac*I–*Ava*II transcript was used to detect cleavages introduced by ribonuclease B23 in the upstream regions of ITS2. The transcript, comprised of 44 nt of the polylinker, the 3′ 104 nt of the 5.8S rRNA but only the 5′ 154 nt of ITS2, lacked the preferential cleavage site for the protein. The pre-5.8S transcript lacking the specific cleavage site did not serve as a substrate for protein B23 at the low enzyme:substrate ratio, indicating that no sites sensitive to ribonuclease B23 existed in the ITS2 region abutting the 5.8S rRNA (data not shown). This confirms the idea that protein B23 ribonuclease exhibits preferential cleavage in a short segment of ITS2 RNA.

Other ribonucleases do not preferentially cleave the ITS2 region

To test whether the specific cleavage introduced in the ITS2 region was the result of a site accessible to any single-stranded nuclease, the 5′-end-labeled pre-5.8S RNA transcript was subjected to limited digestion by bovine pancreatic RNase A and RNase T_1. As seen in Figure 6, the two single-strand-specific ribonucleases at the same unit concentration did not introduce any cleavages at the site cleaved by ribonuclease B23. However, the two ribonucleases introduced minor cleavages far upstream of this site. Thus, the cleavage introduced by protein B23 ribonuclease at the site in ITS2 was not merely a result of accessibility of the nuclease to that region of the RNA.

DISCUSSION

The current study shows that protein B23 ribonuclease exhibits a feature common to several ribonucleases, i.e. preferential cleavage due to the three-dimensional structure of the substrate. The first aspect of this sensitivity to structure is the marked preference for single-stranded over double-stranded RNA. The resistance of double-stranded RNA to cleavage was not only seen with the co-polymers poly(A):poly(U) and poly(C):poly(G) but also with poly(G) alone. The inability of protein B23 ribonuclease to cleave poly(G) may be attributed to the monovalent cation-induced secondary structure of the homopolymer (51,52). The intramolecular folding of single G-rich strands in the presence of Na^+ ions to form G-quartets minimizes the proportion of single-stranded regions of the polymer and occludes phosphodiester linkages, thereby preventing access to the cleavage sites. Attempts to inhibit the formation of G-quartets by the elimination of Na^+ ions from the medium resulted in a major decrease in enzymatic activity. Thus it was not possible to determine whether protein B23 ribonuclease was capable of digesting single-stranded poly(G). The apparent resistance to G-quartet structures is a feature characteristic of other single-strand nucleases (53–55). A similar effect was observed with bovine pancreatic RNase A towards poly(C) (56) and spleen acid RNase towards poly(A) (57), which also possess higher ordered structures in the pH range between 5 and 6. Thus, the resistance of poly(G) to cleavage by protein B23 ribonuclease is probably due to its sensitivity towards the secondary structure of the polynucleotide and is not indicative of base specificity.

Secondly, although there was no apparent base specificity, the cleavage pattern seen with the 20mer oligoribonucleotide indicated that cleavage of this single-stranded substrate was not random. This suggests that the B23 ribonuclease recognizes subtle conformational features or possibly even certain sequences, resulting in preferred cleavage sites in a substrate that exists in the single-stranded form.

Finally, the studies with the pre-rRNA transcripts demonstrate that protein B23 ribonuclease preferentially cleaves at a limited number of sites in these RNAs. Protein B23 ribonuclease selectively introduces cleavages after each nucleotide in a specific pGpCpU sequence in the ITS2 region of pre-5.8S rRNA. The GCU sequence is contained in one of the four CGCUCC sequences in the vicinity of the cleavage site; no cleavage was observed in any of the other sequences. This suggests that protein B23 ribonuclease is recognizing features other than the sequence itself. Furthermore, although there are multiple single-stranded regions present in the ITS2 region (49), this site is the only one cleaved by protein B23 ribonuclease. This site is not susceptible to cleavage by other single-strand-specific endoribonucleases. Thus, three-dimensional structural features in the transcript in addition to secondary structure contribute to recognition of this site by protein B23 ribonuclease.

The fact that the cleavage sites are only found downstream of the 3′-end of the 5.8S rRNA sequence suggests that the ITS2 region is particularly sensitive to protein B23 ribonuclease. The introduction of cleavages in the ITS2 region may be attributed to the fact that this region, which is the spacer to be excised and discarded, serves as the natural substrate for nuclease activity in general. In contrast, the 5.8S region of the transcript appears to be resistant to the action of ribonuclease B23. Thus, the pre-5.8S transcript contains two distinct segments, one that functions as a substrate and a second one that does not. A short transcript lacking the cleavage site in the pre-5.8S transcript is resistant to cleavage, whereas the two longer transcripts containing the preferential cleavage site exhibit similar cleavage patterns. This suggests that there is a strong tendency for the RNA to maintain its structure in the vicinity of the cleavage site even when given an opportunity to fold differently. It is also interesting that there were no cleavages in the 5′ ETS and the 18S–ITS1 transcripts under the same conditions. This further supports the idea that a segment of ITS2 is specifically recognized by protein B23 ribonuclease. However, the precise features recognized are not known at this time. Other ribonucleases have also been shown to be sensitive to the secondary and tertiary structure of RNA (44,58,59) and the limited digestion or preferential cleavage in the RNA substrate is due to resistance of large segments to cleavage by the enzymes.

The demonstration that protein B23 ribonuclease preferentially cleaves within a short segment of ITS2 raises the question of the physiological relevance of this finding, i.e. is there any significant role for the enzyme in pre-rRNA processing? Several pieces of evidence indicate that protein B23 is associated with this region of the pre-rRNA transcript. Biochemical studies by Prestayko *et al*. (2), Olson *et al*. (3), Yung *et al*. (4) and Zirwes *et al*. (5) showed that protein B23 is associated with maturing 55S pre-ribosomal RNP particles which contain ITS2 as a part of 32S pre-rRNA (60). Inhibition of rRNA synthesis by actinomycin D in cultured cells causes translocation of this protein from the nucleolus to the nucleoplasm (10). Other components associated with pre-rRNA processing are also translocated from the nucleolus to the nucleoplasm upon inhibition of rRNA synthesis using actinomycin D (61). More importantly, the translocation effect of protein B23 is also observed upon treatment of cells with an inhibitor of 47S pre-rRNA processing, toyocamycin (11,12). Thus, the mere presence of nascent 47S pre-rRNA is not sufficient for nucleolar retention of the protein but it appears to be dependent on the presence of associated pre-rRNA processing complexes. Interaction of protein B23 ribonuclease with the ITS2 region of the pre-rRNA transcript could explain the translocation induced by the drugs. The most probable mechanism is that as the processing intermediates migrate through the different compartments of the nucleolus in a vectorial fashion, the 36S pre-rRNA intermediate which is formed in the dense fibrillar component of the nucleolus (62) recruits protein B23. The absence of either 47S pre-rRNA or the processing intermediates in the presence of the drugs would lead to a loss of the binding site for the protein, causing it to translocate from the nucleolus to the nucleoplasm. It is also interesting that protein B23 is associated with components of the pre-rRNA processing machinery in nucleolus-derived foci during mitosis (63).

Earlier studies mapped cleavages in the rat ITS2 at sites 118 nt downstream of the 3′-end of mature 5.8S rRNA (31) and at 50–65 and 4–20 nt upstream of the 5′-end of mature 28S rRNA (65). The 5′-end of 28S and the 3′-end of 5.8S rRNAs appear to be generated by exonucleases (34). The preferential cleavage sites observed in the current study are found approximately in the center of ITS2, between the previously mapped sites. The sites cleaved by protein B23 have not been observed *in vivo*, however, it is likely that this region of ITS2 is rapidly degraded and that the transiently formed intermediates are difficult to detect. It is also possible that protein B23 participates in this degradation through its endonuclease activity.

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