3974–3979 *Nucleic Acids Research, 1995, Vol. 23, No. 19* © 1995 Oxford University Press

The ribonuclease activity of nucleolar protein B23

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Received May 23, 1995; Revised and Accepted August 31, 1995

ABSTRACT

Protein B23 is an abundant nucleolar protein and putative ribosome assembly factor. The protein was analyzed for ribonuclease activity using RNA-embedded gels and perchloric acid precipitation assays. Three purified bacterially expressed forms of the protein, B23.1, B23.2 and an N-terminal polyhistidine tagged B23.1 as well as the natural protein were found to have ribonuclease activity. However, the specific activity of recombinant B23.1 was ~5-fold greater than that of recombinant B23.2. The activity was insensitive to human placental ribonuclease inhibitor, but was inhibited by calf thymus DNA in a dose dependent manner. The enzyme exhibited activity over a broad range of pH with an apparent optimum at pH 7.5. The activity was stimulated by but not dependent on the presence of low concentrations of Ca^{2+}, Mg^{2+} or NaCl. The Ca^{2+} effect was saturable and only stimulatory in nature. In contrast, Mg^{2+} and NaCl exhibited optimal concentrations for stimulation and both inhibited the ribonuclease at concentrations above these optima. These data suggest that protein B23 has intrinsic ribonuclease activity. The location of protein B23 in subcompartments of the nucleolus that contain preribosomal RNA suggests that its ribonuclease activity plays a role in the processing of preribosomal RNA.

INTRODUCTION

The nucleolus is the subnuclear factory for assembly of preribosomal particles (1). Within this structure preribosomal RNA is synthesized, processed and packaged into progenitors of ribosomes (2). The complexity of the ribosome suggests that a broad array of functions are performed within the nucleolus for proper and efficient maturation of the ribosomal precursors. The functional repertoire of the nucleolus must include factors that assemble preribosomal particles and process the 45S rRNA precursor into the final 18, 5.8 and 28S functional forms. The processing machinery likely requires two classes of ribonucleases: those that specifically process the 45S RNA into ribosomal RNAs and those that digest the discarded portions into small fragments and mononucleotides for recycling into *de novo* RNA synthesis.

Protein B23 is an abundant protein in the nucleolus whose properties imply that it plays multiple roles in ribosome biogenesis. In addition to its proposed function in ribosome assembly studies by Borer *et al.* (3) also implicate it in transport of proteins into the nucleus. The transport function is supported by its ability to bind proteins containing nuclear localization signals (4,5) including the HIV-1 Rev protein (6). Protein B23 has also been demonstrated to bind RNA and double- and single-stranded DNA (7–9). The nucleic acid binding properties of the protein have been suggested to be involved in nucleolar localization (9), regulation of nucleolar DNA synthesis (8) and ribosome assembly (7).

Protein B23 has been localized to the dense fibrillar and granular components of the nucleolus (10,11). These components contain nascent preribosomal RNA in various stages of processing and assembly into preribosomal particles. The abundance of B23 at these sites suggests that the protein is involved in the maturation process. Biochemical studies have also demonstrated that B23 is associated with preribosomal RNP particles (12,13). Furthermore, treatment of cells with inhibitors of RNA synthesis (14) or inhibitors of the processing of preribosomal 45S RNA (13) results in a rapid release of protein B23 from the nucleolus and localization to the nucleoplasm. This release might be facilitated by a nuclease present in the immediate vicinity of the protein. Those possibilities led us to search for nuclease activity in protein B23 itself. In this study protein B23 is shown to exhibit intrinsic ribonuclease activity. This novel property of B23 supports the idea of a more direct role for the protein as a factor in ribosomal RNA processing.

MATERIALS AND METHODS

Chemicals, proteins and isotopes

[α-^{32}P]CTP (3000 Ci/mmol) was purchased from New England Nuclear. Ribosomal RNA and human placental ribonuclease inhibitor were obtained from Boehringer Mannheim. Radiolabeled RNA was synthesized *in vitro* from DNA fragments coding for portions of the 5′ end of the 45S ribosomal RNA cloned in plasmid pGEM4Z. The RNA was transcribed using T7 RNA polymerase in the presence of [α-^{32}P]CTP and purified using spun columns (15). Three RNAs were used, two contained segments from the 5′ end of 45S ribosomal RNA and the third was the B23 mRNA. The recombinant forms of the proteins (B23.1, B23.2 and hB23.1) were purified as previously described (16), except that the cDNAs for rat B23.1 and B23.2 were inserted into the pET11c protein expression vector (Novagen) and expressed in *E.coli* strain BL21(DE3). Briefly, the bacterial lysate was initially applied to a column of heparin Sepharose. Fractions enriched in B23 were further purified on a DEAE cellulose column. Protein B23.1 with polyhistidine on its N-terminal end (designated hB23.1) was produced using the pET15b (Novagen)

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vector by the same procedure as for the other two forms except that the final purification step utilized a nickel chelating column. Partially purified natural protein B23 was obtained by chromatography of Novikoff hepatoma (rat) nucleolar extracts (7) on heparin Sepharose as described above.

Ribonuclease gel assay

RNA embedded SDS polyacrylamide gels were used for the initial assays for ribonuclease activity (17). Briefly, discontinuous polyacrylamide gels (18) were used except that 80 μg/ml *E.coli* 16S and 23S rRNA (Boehringer Mannheim) was added to the acrylamide mixture of both the stacking and resolving gels prior to polymerization. The protein samples were diluted 2-fold with loading buffer to yield final concentrations of 62.5 mM Tris–HCl (pH 6.8), 5% 2-mercaptoethanol, 2% SDS and 0.02% Bromophenol blue. The samples were incubated in a boiling water bath for 5 min and loaded onto the gel. Electrophoresis was continued until the dye front reached the bottom of the gel. The gels were then washed and developed at 37°C as in the original method (17) except that the incubation times were adjusted for the percentage of acrylamide. The gels were then stained for undigested RNA using toluidine blue according to the original procedure (17). A clear region in the gel after staining was taken as indicative of ribonuclease activity.

Perchloric acid precipitation ribonuclease assay

The assay was performed as previously described by Eichler and Eales (19). Briefly, 25 μl reaction mixtures containing 50 mM Tris–HCl (pH 7.5), radiolabeled RNA (3 μg/ml) and protein B23.1 or B23.2 were incubated at 37°C for 30 min. In all assays where effectors were added, a protein-free control was performed to ensure that the added effectors had no effect on the efficiency of precipitation. The assays were initiated by the addition of protein. The reactions were stopped by chilling on ice followed by rapid addition of 63 μl 2.5 mg/ml yeast tRNA and 38 μl 25% perchloric acid (PCA). The mixtures were kept on ice for 20 min and then centrifuged for 3 min at 10 000 r.p.m. in a microcentrifuge. Aliquots (100 μl) of the supernatants were removed and 4 ml of scintillation fluid (ScintiVerse BOA, Fisher Biotech) was added. The amount of nonprecipitable CPM (digested RNA) was determined by liquid scintillation counting.

Alternatively, the assay was performed as described above except that fluorescein UTP-labeled RNA (Panvera) at a final concentration of 1 μg/ml was used. The fluorescence (excitation at 488 nm and emission at 530 nm) of the nonprecipitable ribonucleotides was measured in a buffer containing 0.2 M Tris–HCl (pH 9.0), 0.5% SDS and 0.2% sodium azide using an Aminco Bowman series 2 luminescence spectrometer.

HPLC analysis

The purity of the recombinant protein B23.1, as well as the coelution of ribonuclease (RNase) activity with the protein, was verified using HPLC. A Perkin Elmer liquid chromatograph (series 3B) equipped with a UV detector and fraction collector was used for the HPLC analyses. The protein was applied to a reverse phase column (Vydac 214Tp54, C-4, 5 μ, 0.46 × 25 cm) using a 25–40% acetonitrile gradient containing 0.1% TFA. The elution was monitored at 220 nm and fractions (0.5 ml) were collected. Fractions were dried using a Savant Speed Vac and then redissolved in 50 mM Tris–HCl (pH 7.5). The fractions were then assayed for protein composition using 15% SDS polyacrylamide gels and for RNase activity using the previously described fluorescence ribonuclease assay.

Assays using unlabeled RNA were used to determine the specific activity of the protein. These were conducted essentially as above with concentration of 0.8 mg/ml of 16S plus 23S *E.coli* ribosomal RNA. The absorbance at 260 nm of the PCA supernatant was used to calculate the unit of activity. One unit of nuclease activity is defined as the amount of protein which results in an increase in absorbance of 1.0 above the blank sample incubated in the absence of protein.

Analyses of termini of released nucleotides

For use as a substrate, radiolabeled RNA was transcribed from a plasmid containing the 5′ end of rat 45S rRNA using the SP6 RNA polymerase and either [α-^{32}P]CTP, [α-^{32}P]ATP or [α-^{32}P]GTP (NEN DuPont). The RNA was purified using a spun column (15). Aliquots of the radiolabeled RNA were digested for various times with HPLC-purified recombinant protein B23.1. These, along with undigested aliquots were subjected to hydrolysis with 0.3 M KOH at 37°C for 17.5 h and applied to DEAE-Sephadex A25 columns essentially according to the procedure of Eichler and Eales (19). Fractions were collected and aliquots were counted in a scintillation counter after addition of scintillation fluid (Scintiverse BOA, Fisher). The positions of the nucleoside monophosphates and 3′,5′-bisphosphates were determined using AMP and ADP as markers.

RESULTS

Ribonuclease activity of protein B23

As an initial assay for ribonuclease activity, duplicate samples of purified proteins B23.1, B23.2 and a histidine tagged fusion protein of B23.1 (hB23.1) from recombinant bacterial sources were subjected to electrophoresis in gels impregnated with rRNA. After incubation in the assay buffer and staining with toluidine blue, the gels (Fig. 1A, RNA) showed unstained regions produced by each of the recombinant forms of protein B23. Duplicate samples on the same gel stained with Coomassie blue (protein) had bands with mobilities identical to the clear bands. In addition, Western blots using anti-B23 antibody performed on the parts of the gels stained for RNA showed that the B23 bands coincided with the clear bands (not shown), lending further support to the idea that the ribonuclease activity is an intrinsic property of B23.1. It should be noted that all samples were boiled for 5 min in the presence of 2-mercaptoethanol and SDS prior to loading on the gels. This procedure should dissociate all proteins in the mixture and minimize the possibility that contaminating proteins with ribonuclease activity adhere to protein B23. The fact that activity is recovered after this treatment also suggests that the protein can renature after denaturation under harsh conditions. Lane 1 of Figure 1A contains a sample of RNase A run as a positive control. A comparison of the intensities of the clear bands suggests that B23 has a slower rate of RNA hydrolysis than RNase A. In these gels 1.25 μg B23 was loaded compared to 0.25 μg RNase A; nonetheless, the activity of RNase A appeared to be much stronger than that observed for any form of B23. In contrast, bovine serum albumin in lane 2 of Figure 1A, did not show a clear area coinciding with the Coomassie blue-stained band indicating

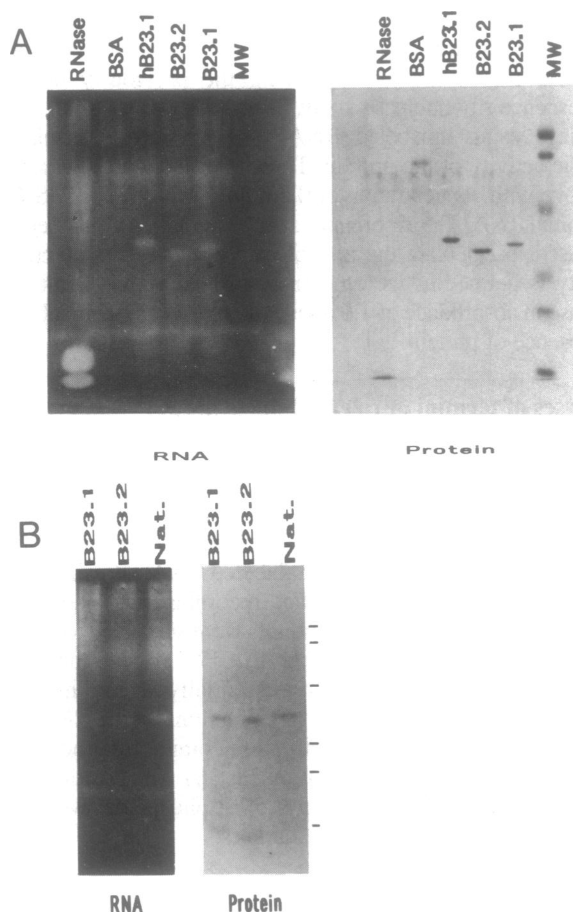

Figure 1. Detection of ribonuclease activity of recombinant and natural protein B23 in polyacrylamide gels. RNA-embedded polyacrylamide gels were used for electrophoretic separation and detection of ribonuclease activity of recombinant B23.1, B23.2, polyhistidine tagged B23 (hB23.1) or rat protein B23 purified from Novikoff hepatoma ascites cells (Nat). For both parts A and B duplicate samples were resolved on the gel; half of the gel was stained for RNA with toluidine blue (RNA) and the other half was stained with Coomassie blue (protein). The samples were prepared as described in the methods section. For both panels: lane MW contains prestained molecular weight markers (106, 80, 49.5, 32.5, 28.7 and 20.5 kDa). Lanes labeled BSA and RNase contain 1.25 μg bovine serum albumin and 0.25 μg ribonuclease A, respectively. The clear areas in the toluidine blue stained gel indicate ribonuclease activity. The lanes with B23 each contained 1.25 μg of protein. The gels in part A contain only the recombinant forms of protein B23. Part B contains the natural form of B23 run with recombinant B23.1 and B23.2. The bars in part B correspond to the molecular weight markers shown in part A.

that ribonuclease activity is not detected in all proteins subjected to this assay.

Since all three forms of protein B23 examined in the initial studies were bacterially produced, it was deemed important to determine whether this activity existed in protein B23 from a natural source. For this, protein B23 was partially purified from an extract of Novikoff hepatoma nucleoli. The toluidine blue- and Coomassie blue-stained gels of this preparation are shown in Figure 1B. As with recombinant protein B23 the natural form (predominantly B23.1) produced a clear band with migration identical to the Coomassie blue-stained band. Thus, ribonuclease activity is also an intrinsic property of the natural form of the protein and not simply an artifact of producing the protein in bacteria.

To verify that the ribonuclease activity behaved like an enzyme several additional studies were performed. First, the activity was monitored as a function of protein concentration using both a PCA precipitation assay and the RNA gel assay. In both cases the ribonuclease activity was dependent on the concentration of B23 (not shown). The PCA precipitation assay also showed that saturation was achieved when increasing concentrations of protein were added to an assay mixture containing constant substrate concentration. The saturation curves provided information on the concentration ranges of protein and substrate in which linearity was seen; this was the basis for initial rate assays used in subsequent studies. The activity assays were performed on several different RNAs: two different fragments from 45S preribosomal RNA, the mRNA for protein B23, tRNA from brewers yeast and *E.coli* 16S plus 23S ribosomal RNA. All RNAs were readily digested, suggesting that the activity was not specific for a given class of RNA (not shown). The ribonuclease activity of protein B23 was also monitored as a function of pH. The profile was relatively broad with an apparent optimum at pH 7.5 (not shown). The remaining studies were performed at pH 7.5.

The specific activities of protein B23.1 and RNase A were compared using unlabeled 16S plus 23S RNA (see unit definition in the Materials and Methods section). Protein B23.1 had a specific activity of ~20 U/mg with this RNA. The assay was found to be linear between 0.3 and 3.5 units of protein. In contrast, the specific activity of purified pancreatic RNase A under the same conditions was 1000 U/mg. Thus, the specific activity of protein B23.1 appears to be ~50-fold lower than pancreatic RNase A.

Additional experiments were done to eliminate the possibility that a contaminating ribonuclease adventitiously associated with protein B23 during the in-gel assay. Purified recombinant protein B23.1 was applied to a reverse phase HPLC column and the ribonuclease activity was measured in the fractions corresponding to the protein B23.1 peak. The location of protein B23.1 was determined by SDS–PAGE of aliquots from the fractions collected during the HPLC run. The ribonuclease activity coincided with the fractions containing protein B23 (Fig. 2). There were no stained bands visible other than protein B23.1 in the fractions containing ribonuclease activity. Conversely, there was little or no activity in fractions not containing protein B23.1. These results provide further evidence that the ribonuclease activity is an intrinsic property of protein B23.

Ribonuclease activities of purified recombinant proteins B23.1 and B23.2

Protein B23.1 is localized to the nucleolus whereas B23.2 is extranucleolar (20). If the ribonuclease activity is involved in preribosomal RNA metabolism, then B23.1 may exhibit a greater activity. The activity was determined at a fixed RNA concentration with varied concentrations of B23.1 and B23.2. The activity of B23.1 was found to be ~5-fold greater than that of B23.2 (data not shown).

Effects of inhibitors on ribonuclease activity

Human placental ribonuclease inhibitor (RNasin) is a specific noncompetitive inhibitor of the RNase A family of nucleases (21). To test the possibility that protein B23 belongs to this class of RNase we used the PCA precipitation assay to examine the effect of RNasin on the activity of B23. Increasing concentrations of RNasin were added to standard reaction mixtures containing

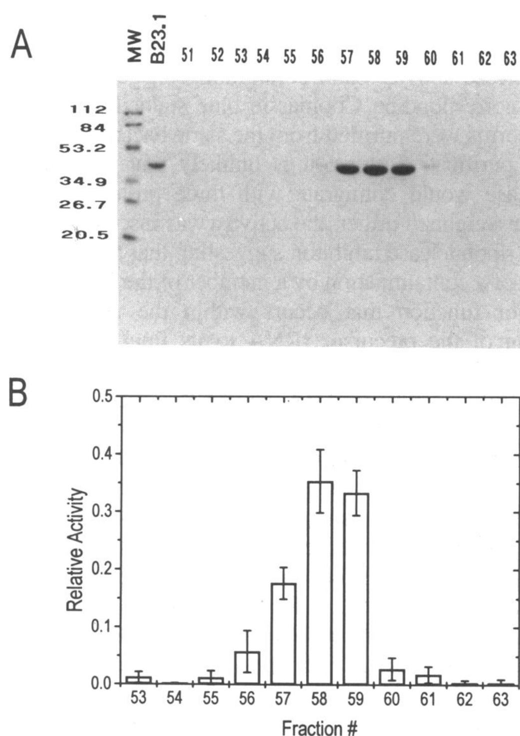

Figure 2. Reverse phase HPLC of purified recombinant protein B23.1. A sample of protein B23.1 (300 μg) was applied to a C4 reverse phase column (0.46 × 25 cm) and eluted with a gradient of 25–40% acetonitrile containing 0.1% TFA. Fractions (0.5 ml) were collected, dried under vacuum and redissolved in 100 μl of 50 mM Tris (pH 7.5). Aliquots (10 μl) from each fraction were taken for SDS–PAGE and for ribonuclease assays using fluorescein-labeled RNA as described in Materials and Methods. (**A**) SDS–PAGE of aliquot fractions containing peak of ribonuclease activity. MW, molecular weight markers as indicated on the left (in kDa); B23.1, purified recombinant B23.1 before application to the HPLC column. Fraction numbers are indicated on top. (**B**) Ribonuclease activity of aliquots from fractions indicated in (A).

saturating concentrations of radiolabeled RNA. These assays were performed in the presence of 5 mM 2-mercaptoethanol because of the requirement of RNasin for reducing agents. In separate experiments it was shown that the addition of the reducing agent had no effect on the activity of B23. In other controls RNasin was shown to inhibit RNase A under the same conditions that were used for the inhibition studies on protein B23.1. Concentrations of RNasin as high as 100 U/ml had no inhibitory effect on the activity (not shown). These results suggest that B23 is not a member of the RNase A family and that the activity is not due to a contaminating RNase A type enzyme. These data further support the idea that the activity is an intrinsic property of B23.

Ribonuclease A is competitively inhibited by high concentrations of DNA (22) and protein B23.1 has been demonstrated to bind, but not digest DNA (8). Therefore, we examined the effect of added DNA on the ribonuclease activity. The addition of increasing concentrations of sonicated calf thymus DNA to the standard assay mixtures resulted in a strong inhibition of the ribonuclease activity with nearly complete inhibition at 20 μM (in base pairs). Essentially the same pattern of inhibition was obtained with protein B23.2 (data not shown).

The effect of ionic strength and divalent metal ions on ribonuclease activity

The ribonuclease activity was monitored with increasing concentrations of NaCl. The activity was strongly dependent on NaCl concentration and showed an optimum at 50 mM where stimulation was ~2-fold above the activity in the absence of salt. Further increases in the NaCl concentration resulted in loss of activity; at 0.2 M the activity was almost completely abolished. Under optimal conditions the assay buffer contained 50 mM Tris–HCl (pH 7.5) and 50 mM NaCl.

Magnesium

Several ribonucleases have been demonstrated to be sensitive to the addition of divalent metals, especially Mg^{2+}. Addition of Mg^{2+} resulted in a 2–3-fold stimulation of activity with an optimum at 2 mM. Further increases in Mg^{2+} caused a gradual inhibition up to 20 mM where the activity was nearly completely abolished. On the other hand, in the absence of added Mg^{2+} or Ca^{2+} there was no inhibition if 5 mM EDTA was added to the assay mixture. This indicates that divalent metals are not required for the ribonuclease activity of protein B23.

Calcium

Increasing concentrations of Ca^{2+} resulted in a 3–5-fold stimulation of activity, with the optimum stimulation observed at 2 mM Ca^{2+}. The effect was saturable with no further increase in activity observed above 5 mM Ca^{2+}. This is in contrast to the effect observed with Mg^{2+}, where increasing the concentration above its optimum resulted in an inhibition of activity. The difference in concentration dependence between the two metals, as well as the saturability of the Ca^{2+} effect suggests that Ca^{2+} may play a more specific role than Mg^{2+}.

Ca^{2+} plus Mg^{2+}

Since Ca^{2+} and Mg^{2+} independently stimulated activity, we examined whether these two ions could act in a synergistic manner. When increasing concentrations of Ca^{2+} were added in the presence of 2 mM Mg^{2+} there was no synergistic effect. Addition of either Ca^{2+} or Mg^{2+} alone resulted in optimal stimulation. The combination of both Mg^{2+} and Ca^{2+} did not result in further increase in activity. These data suggest that both metals may exert their effect via a similar mechanism. The observed inhibition with high Mg^{2+} implies a secondary effect not observed with Ca^{2+}. The differences in activity profiles could arise from several scenarios: one is the presence of a secondary inhibitory Mg^{2+} binding site; secondly, Mg^{2+} could induce a change in RNA structure; thirdly, high concentrations of Mg^{2+} could disrupt the formation of the B23–RNA complex.

Termini of released nucleotides

To test whether the nuclease activity of protein B23 the digestion products of the *in vitro* transcribed ^{32}P-labeled RNA was subjected to further hydrolysis with alkali. Under these conditions each oligonucleotide containing a 5′-terminal phosphate would generate a 3′,5′-nucleoside diphosphate for every terminal nucleotide, whereas termini with 3′ phosphates would result in nucleoside monophosphates. Figure 3 shows that chromatography on DEAE Sephadex A25 of the alkaline hydrolysate of the products from the

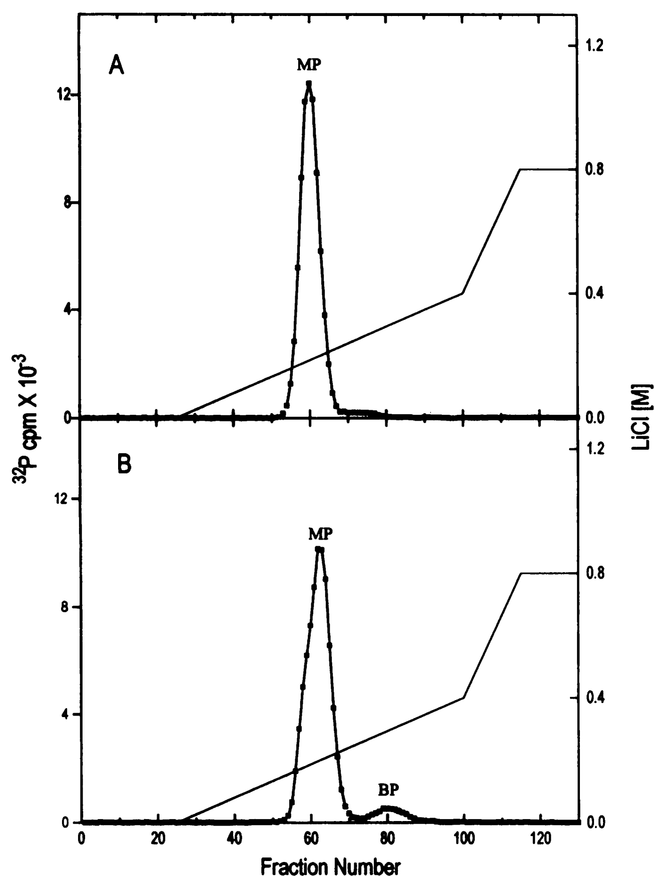

Figure 3. Analyses of termini of products released from RNA after digestion with protein B23.1. Radiolabeled RNA (400 ng in 125 μl) was digested with 1.5 U HPLC-purified recombinant protein B23.1 at 37°C for 30 min and the products were analyzed for the nature of their 5′ termini. The substrate for the nuclease activity was RNA containing a segment of the 5′ end of 45S preribosomal RNA was which was previously labeled by *in vitro* transcription using SP6 RNA polymerase in the presence of [α-^{32}P]ATP. The digestion products were hydrolyzed with KOH as described in the Materials and Methods section and applied to DEAE Sephadex A25 columns for analysis of content of nucleoside monophosphates and 3′,5′-bisphosphates. In typical experiments the acid soluble CPM liberated by digestion was ~80% of the total CPM. An identical sample was taken through the entire procedure without the addition of protein B23.1. The figure shows the DEAE Sephadex profiles of alkaline hydrolysates of undigested (**A**) or B23.1-digested (**B**) radiolabeled RNA. The positions of the nucleoside monophosphate (MP) or bisphosphate (BP) standards are indicated.

[α-^{32}P]ATP-labeled RNA produced two peaks of radioactivity. The first and second peaks correspond to nucleoside monophosphates and diphosphates, respectively. Similar patterns were obtained with RNA labeled with GTP and CTP. However, the diphosphate peak was not seen with undigested labeled RNA or with RNA digested with RNase A, which produces products containing a 3′-phosphate. Thus, the ribonuclease activity of protein B23 cleaves on the 5′ side of the phosphodiester bond.

DISCUSSION

This study presents evidence that protein B23 contains ribonuclease activity. The RNA-gel method strongly supports the idea that the activity is an intrinsic property of the protein. Contaminating ribonuclease activity comigrating with protein B23 seems unlikely since treatment by boiling in SDS should have released any adventitiously associated ribonuclease. Secondly, each of the three forms of the protein, which differ in molecular weight, had a ribonuclease activity band comigrating on gel electrophoresis with the corresponding Coomassie blue stained band. Since all three isoforms were purified from the same bacterial strains using identical purification steps, it is unlikely that a contaminating ribonuclease would comigrate with three proteins of different molecular weights. Further, the activity was insensitive to human placental ribonuclease inhibitor, suggesting that the activity is not the result of a contamination by a member of the RNase A family.

A major function that occurs within the nucleolus is the processing of the precursor rRNA to its final functional form (reviewed in 23–28). The enzymes involved in the processing have yet to be fully identified. As discussed in a recent review (21), several ribonucleases may carry out the same function; therefore, interpretation of genetic deletion studies are difficult and extrapolation of *in vitro* data to *in vivo* function is complex. One approach to elucidating the mechanism of pre-rRNA processing is to identify nucleolar ribonucleases and characterize their activities. Toward this end, several investigators have isolated a variety of nucleolar ribonucleases (19,29–35). All of the nucleases isolated have varying degrees of selectivity for either RNA structure or sequence and have sensitivities to both monovalent and divalent metals different from those of protein B23.

Eichler's laboratory has identified three nucleolar specific ribonucleases. One of these, having a molecular weight of 38.5 kDa and a pI of 9.0, is specific for single-stranded RNA and has preference for digestion of poly(C) (31). A second is an exonuclease that digests both single- and double-stranded RNA as well as the RNA strand of a RNA–DNA hybrid (31). This nuclease requires Mg^{2+} ions and is inhibited by monovalent ions. A third nuclease has a molecular weight of 50–52 kDa and is specific for single-stranded RNA (33). Its activity is stimulated by Mg^{2+} or Mn^{2+} and is inhibited by monovalent cations. This third ribonuclease appears to specifically bind near the +650 processing site of the 45S pre-rRNA (34) and is postulated to be involved in processing of the rRNA (33). In contrast to the previously isolated ribonucleases, B23 is an acidic protein (pI 5.1) with a molecular weight of 32.5 kDa (36). Second, the ribonuclease activity of B23 is stimulated by, but not dependent on, Mg^{2+} ions; unlike those described above it exhibits a discrete optimum followed by inhibition. Third, the B23 activity was stimulated by Na^{+} ions exhibiting a discrete optimum. Finally, the B23 ribonuclease activity was stimulated by Ca^{2+}, a property not reported for any of the other nucleolar ribonucleases. Comparison of the ribonuclease properties of B23 with those previously isolated suggests that the B23 ribonuclease activity is not one of them.

This study presents the first indication that protein B23 possesses intrinsic ribonuclease activity. The protein's localization to the nucleolus (9), the site of rRNA processing, poses the possibility that B23 may be intimately involved in the processing of rRNA. To date, no preference for a particular class of RNAs or for a specific sequence has been found for the B23 nuclease activity. The apparent lack of selectivity for substrate could be interpreted to mean that the B23 ribonuclease activity functions to degrade the discarded portions of the 45S rRNA or to destroy improperly assembled preribosomal particles. Alternatively, the selectivity in cleavage sites may be dictated by interactions of B23 with other nucleolar factors, such as proteins or snoRNAs. Recent studies have implicated a variety of snoRNAs (U3, U8, U13, U14 etc.) in the rRNA processing pathways (37). For example, U3 snoRNP is localized to

the nucleolus (38–40) and is believed to be involved in the first step of pre-ribosomal RNA processing (41). The U3 snoRNA has been demonstrated to bind complementary sequences near the processing site on the 5′ external transcribed spacer (ETS) of pre-rRNA (42–46). This binding is not sufficient for processing, but appears to provide a target for the formation of a processing complex. The existence of these complexes suggests that the processing enzymes are not acting on naked RNA but rather are recognizing complexes of snoRNAs and RNPs. Protein B23 could potentially recognize these complexes and specifically cleave RNA without recognizing specific sites on naked RNA. Another possibility is that the majority of the 45S RNA is bound by proteins and, therefore, protected from digestion by the formation of the RNP particles; therefore, the only available cleavage sites would be unbound portions of the RNA. Protein B23 may readily digest these unprotected sequences without the need for site selectivity residing in the protein itself. This could mean that B23 functions to 'rough cut' the RNA and other factors could subsequently specifically trim the RNA to yield the properly processed rRNA fragments.

It is interesting that the specific activity of protein B23.1 is ~5-fold higher than that of B23.2. The two forms differ only in their carboxyl-terminal ends with B23.1 being 35 residues longer than B23.2. Thus, the C-terminal end seems to modulate the activity of the enzyme. Previous studies have shown that the C-terminal end of protein B23.1 contains its nucleic acid binding activity (9). However, this part of the molecule clearly does not contain the catalytic site because both isoforms have ribonuclease activity. It is also not likely that the C-terminal end of B23.1 is the primary substrate binding site since B23.1 and B23.2 RNase activities were inhibited to a similar extent by DNA. Thus, enhanced activity of B23.1 could be due to the C-terminal end acting as a secondary substrate binding site or by altering the conformation of whole protein. The activity difference is consistent with protein B23.1 being localized to the nucleolus and B23.2 being extranucleolar; i.e., the more active enzyme may be engaged in the processing of preribosomal RNA in the nucleolus.

Protein B23 is a multifunctional protein involved in several aspects of nucleolar function. Identification of this enzymatic activity in the protein strongly suggests a functional role in the processing of pre-rRNA. Elucidating the exact nature of this role will require further characterization, including clarification of the specificity, determination of the mechanism of action and definition of the interactions with other factors in the nucleolus.

ACKNOWLEDGEMENTS

We wish to thank A. Baumann for assistance in preparing transcribed RNA, W. E. Edmonson for preparing recombinant proteins and M. O. Wallace for supplying nucleolar extracts and for HPLC analyses. We also wish to thank Drs D. C. Eichler and S. V. Smith for helpful discussions and Romie Brown for typing the manuscript. This work was supported by NIH grant 5 RO1 GM28349.

REFERENCES

1 Olson, M.O.J. (1991) In *The Eukaryotic Nucleus: Molecular Biochemical and Macromolecular Assemblies*, Stauss, P.R. and Wilson, S.H. (eds), vols 2, pp. 541–546, Telford Press, Caldwell, NJ.
2 Gerbi, S.A., Savino, R., Stebbins-Boaz, B., Jeppesen, C., and Rivera-Leon, R. (1990). In *The Ribosome: Structure, Function and Evolution.*, Chap. 39, pp. 452–469. Hill, W.E., Dahlberg, R.A., Garrett, P.B., Moore, D., Schlessinger, D., and Warner, J.R. (eds), American Society for Microbiology, Washington, DC.
3 Borer, R.A., Lehner, C.F., Eppenberger, H.M., and Nigg, A. (1989) *Cell* **56**, 379–390.
4 Goldfarb, D.S. (1989) *Curr. Opin. Cell Biol.* **1**, 441–446.
5 Szebeni, A., Herrera, J.E. and Olson, M.O.J. (1995) *Biochemistry* **34**, 8037–8042.
6 Fankhauser, C., Izaurralde, E., Adachi, Y., Wingfield, P., and Laemmli, U.K. (1991) *Mol. Cell. Biol.* **11**, 2567–2575.
7 Dumbar, T.S., Gentry, G.A., and Olson, M.O.J. (1989) *Biochemistry* **28**, 9495–9501.
8 Feuerstein, N., Mond, J.J., Kinchington, P.R., Hickey, R., Lindsberg, M.-L. K., Hay, I., and Ruyechan, W.T. (1990) *Bioch. Biophys. Acta* **1087**, 127–136.
9 Wang, D., Baumann, A., Szebeni, A., and Olson, M.O.J. (1994) *J. Biol. Chem.* **269**, 30994–30998.
10 Spector, D.L., Ochs, R.L., and Busch, H. (1984) *Chromosoma* (Berl.) **90**, 139–148.
11 Biggiogera, M., Fakan, S., Kaufmann, S.H., Black, A., Shaper, J.H., and Busch, H. (1989) *J. Histochem. Cytochem.* **37**, 1371–1374.
12 Prestayko, A.W., Klomp, G.R., Schmoll, D.J., and Busch, H. (1974) *Biochemistry* **13**, 1945.
13 Yung, B.Y.M., Busch, H., and Chan. P.K. (1985) *Biochem. Biophys. Acta* **826**, 167–173.
14 Yung, B.Y.M., Busch, R.K., Busch, H., Mauger, A.B., and Chan, P.K. (1985) *Biochem. Pharmacol.* **34**, 4059–4063.
15 Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual,* 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
16 Umekawa, H., Chang, J.-H., Correia, J. J., Wang, D., and Wingfield, P. T. (1993) *Cell. Mol. Biol. Res.* **39**, 635–645.
17 Blank, A., Sugiyama, R.H. and Dekker, C.A. (1982) *Anal. Biochem.* **120**, 267–275.
18 Laemmli, U.K. (1970) *Nature* **227**, 680–681.
19 Eichler, D.C. and Eales, S.J. (1982) *J. Biol. Chem.* **257**, 14384–14389.
20 Wang, D., Umekawa, H. and Olson, M.O.J. (1993) *Cell. Mol. Res.* **39**, 33–42.
21 Deutscher, M.P. (1994) *J. Biol. Chem.* **268**, 13011–13014.
22 Sekine, H., Nakano, E., and Sakaguchi, K. (1969) *Biochim. Biophys. Acta* **174**, 202–210.
23 Perry, R.P. (1976) *Annu. Rev. Biochem.* **45**, 605–629.
24 Perry, R.P. (1981) *J. Cell Biol.* **91**, 28s-38s.
25 Hadjiolov, A.A. and Nikolaev, N. (1976) *Prog. Biophys. Mol. Biol.* **31**, 95–144.
26 Hadjiolov, A.A. (1980) *Biogensis of ribosomes in eukaryotes*, pp. 1–80. In D.B. Roodyn (ed.), *Subcellular Biochemistry*, vol. 7, Plenum publishing corp., NY.
27 Hadjiolov, A.A. (1985) *The nucleolus and Ribosome Biogenesis.* Springer-Verlag KG, Vienna.
28 Crouch, R. (1984) *Ribosomal RNA processing in Eukaryotes*, pp. 214–226. In D. Apirion (ed.), *Processing of RNA*. CRC Press, Inc., Boca Raton, FL.
29 Prestayko, A.W., Lewis, B.C. and Busch, H. (1973) *Biochem. Biophys. Acta* **319**, 323–335.
30 Kwan, C.N. (1976) *J. Biol. Chem.* **251**, 7132–7136.
31 Eichler, D.C. and Tatar, T.F. (1980) *Biochemistry* **19**, 3016–3022.
32 Eichler, D.C. and Eales, S.J. (1985) *Biochemistry* **24**, 686–691.
33 Shumard, C.M., Torres, C. and Eichler, D.C. (1990) *Mol. Cell. Biol.* **10**, 3868–3872.
34 Eichler, D.C., Liberatore, J.A. and Shumard, C.M. (1993) *Nucleic Acids Res.* **21**, 5775–5781.
35 Eichler, D.C. and Eales, S.J. (1983) *J. Biol. Chem.* **258**, 10049–10053.
36 Chang, J.H. and Olson, M.O.J. (1990) *J. Biol. Chem.* **265**, 18227–18233.
37 Fournier, M.J. and Maxwell, E.S. (1993) *Trends Biochem. Sci.* **18**, 131–135.
38 Weinberg, R.A. and Penman, S. (1968) *J. Mol. Biol.* **38**, 289–304.
39 Prestayko, A.W., Tonata, M., and Busch, H. (1970) *J. Mol. Biol.* **47**, 505–515.
40 Ziene, G. and Penman, S. (1976) *Cell* **8**, 19–31.
41 Kass, S., Tyc, K., Steitz, J.A. and Sollner-Webb (1990) *Cell* **60**, 897–908.
42 Maser, R.L. & Calvet, J.P. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 6523–6527.
43 Stroke, I.L. & Weiner, A.M. (1989) *J. Mol. Biol.* **210**, 497–512.
44 Tollervey, D. (1989) *EMBO J.* **6**, 4169–4175.
45 Tyc, K. and Steitz, J.A. (1992) *Nucleic Acids Res.* **20**, 5375–5382.
46 Beltrame, M. and Tollervey, D. (1992) *EMBO J.* **11**, 1531–1542.