Selection of a preribosomal RNA processing site by a nucleolar endoribonuclease involves formation of a stable complex

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ABSTRACT

A nucleolar endoribonuclease from mouse Ehrlich ascites tumor cells, that has been implicated in the endonucleolytic cleavage of mouse precursor ribosomal RNA, specifically and stably binds an *in vitro*-derived rRNA transcript containing the +650 early processing site. The specificity of binding was demonstrated by mobility shift analysis, glycerol gradient velocity sedimentation analysis, and UV-crosslinking studies. Binding did not require Mg^{2+} and therefore was not dependent on cleavage; however, binding was dependent on the presence of the early +650 processing site since a pre-rRNA transcript with the +650 processing site deleted failed to compete in binding. A small nucleolar RNA component was not required for the formation of this stable complex or for the specific cleavage of a processing competent pre-rRNA transcript. UV crosslinking studies using ^{32}P -labeled 5-azidouridine-substituted pre-rRNA with bound nucleolar endoribonuclease identified three closely sized polypeptides of ~50, ~48, and ~45 kDa, respectively, that specifically crosslinked to the processing competent rRNA transcript. These three polypeptides species were identified following ribonuclease digestion and electrophoresis on a SDS-polyacrylamide gel. An identical pattern of labeled polypeptides was also identified from gel mobility shift analysis where the specifically shifted material was U.V. crosslinked. The largest of these polypeptides corresponded to the estimated size of the nucleolar endoribonuclease, while the lower molecular weight species may represent partially proteolyzed enzyme. Overall, these results suggest that the unique specificity of the nucleolar endoribonuclease may, in part, be attributed to the formation of a stable complex at the +650 processing site for mouse preribosomal RNA, and that formation of this unique stable complex affords a means to specifically label the limited amount of available partially purified enzyme for sequence analysis.

INTRODUCTION

We have previously reported the isolation and characterization of a single strand-specific endoribonuclease from Ehrlich ascites tumor cell nucleoli (1) that has been implicated in the processing of mouse precursor ribosomal RNA (2, 3). Evidence supporting the involvement of the nucleolar endoribonuclease in ribosomal RNA processing came from experiments using *in vitro*-synthesized preribosomal RNA transcripts containing specific processing sites as substrates. Cleavages generated by the nucleolar endoribonuclease were localized by S1 nuclease protection analysis, primer extension analysis, and direct analysis of cleavage products using end-labeled transcripts. Results from these studies demonstrated that the nucleolar endoribonuclease specifically recognized and cleaved the early +650 processing site in the external-transcribed-spacer (5'-ETS) region of mouse preribosomal RNA (2). Additional support for the involvement of the nucleolar ribonuclease was obtained using an *in vitro*-derived preribosomal RNA transcript which contained the 3'-region of the 18S rRNA sequence (3). In this case, the pattern of cleavages generated by the highly purified nucleolar endoribonuclease was shown to be essentially identical to the *in vivo* cleavages mapped by Razuiddin *et al.* (6). The major cleavage catalyzed by the nucleolar endoribonuclease mapped precisely to the 3'-end of the mature 18S rRNA sequence. Taken together, these results demonstrated the unique specificity of this nucleolar endoribonuclease relative to defined processing sites in mouse preribosomal RNA.

Recently, Kass and Sollner-Webb (8) demonstrated that factors of a whole cell extract of mouse Ehrlich ascites tumor cells formed a specific and stable complex with pre-rRNA transcripts containing the +650 early processing site. Six polypeptides of the mouse cell extract were identified by UV-crosslinking as contacting a pre-rRNA substrate containing the +650 processing site. One of these polypeptides, a ~52 kDa labeled species, was of a size which was nearly identical to the nucleolar endoribonuclease characterized by our laboratory (1). Since the specificity of an enzyme is determined not only by the catalytic rate but also by substrate binding, we were interested in determining whether the specificity of the nucleolar endoribonuclease was driven, in part, by its ability to specifically recognize and stably

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bind a processing competent rRNA transcript. Moreover, we were also intrigued by the possibility that a stable complex between the enzyme and a processing competent substrate may be used to further characterize the enzyme. In this report, we show that the nucleolar endoribonuclease, in the absence of catalysis, can specifically recognize and stably bind a processing competent transcript containing the +650 early processing site of mouse precursor ribosomal RNA, and as a consequence, the nucleolar endoribonuclease can be photoaffinity labeled using this transcript to identify by SDS-PAGE analysis a ~50 kDa polypeptide.

EXPERIMENTAL PROCEDURES

Materials

T4 polynucleotide kinase, micrococcal nuclease, and the restriction endonuclease *Ava*II were obtained from Boehringer Mannheim Biochemicals. T7 RNA polymerase, and the ribonuclease inhibitor RNasin were obtained from Promega Company. 5-Azidouridine triphosphate (5-Azido UTP) was purchased from Dr Boyd Haley (University of Kentucky). T1 RNase and DNase I were purchased from Pharmacia LKB Biotechnology, Inc. Pancreatic RNase was obtained from Sigma. The nucleolar endoribonuclease was purified from Ehrlich ascites tumor cells by the method of Eichler and Eales (1) and represents fraction V.

rDNA plasmids

The pGEM-Z (Promega Company) constructs carrying mouse rDNA sequences from +546 to +1300 (Δ 546/*Xho*I) and from +669 to +1300 (Δ 669/*Xho*I) were generously provided by Dr Susan Kass and Dr Barbara Sollner-Webb (Johns Hopkins University, Baltimore).

Preparation of RNA substrates

Transcription of the Δ 546/*Xho*I and Δ 669/*Xho*I insert rDNA linearized with *Ava*II was performed by the method of Melton *et al.* (12) using T7 RNA polymerase. RNA transcripts were purified by DNase I treatment and phenol extraction before labeling and gel purification. Uniformly 32 P-labeled transcripts were synthesized in the same manner but with the addition of 50 μ Ci of α - 32 P]CTP. The procedure for 5' end-labeling of *in vitro*-generated RNA transcripts was modified from the method of Maxam and Gilbert (13) for end-labeling DNA. The dephosphorylated transcript was labeled with α - 32 P]ATP (DuPont-New England Nuclear, 2000–3000 Ci/mmol) and T4 polynucleotide kinase. Labeled transcripts were located by autoradiography and extracted from excised gel slices by electroelution. The RNA was recovered from the eluate by ethanol precipitation, dried under vacuum, and resuspended in water treated with diethylpyrocarbonate. Bacteriophage MS2 RNA was prepared essentially by the procedure of Glitz (14).

Micrococcal nuclease treatment of the nucleolar endoribonuclease and extract

Designated amounts of nucleolar endoribonuclease or extract were incubated in reaction mixtures (0.05 ml) containing 20 mM Tris hydrochloride (pH 7.6), 0.1 mg/ml BSA, 1 mM CaCl_2 , and 0.5–1.0 mg of micrococcal nuclease for 15 min at 20 °C. The reactions were terminated by the addition of [ethyl-enebis(oxyethylenenitrilo)]tetraacetic acid (EGTA) to a final concentration of 4 mM. An aliquot (5 μ l) of each treated sample

was incubated in a 25 μ l reaction mixture containing 50 mM Tris hydrochloride (pH 8.0), 100 mM NaCl, 5 mM dithiothreitol, 13 U of RNasin, 600 μ M MgCl_2 , and 1.5 μ g 5' 32 P-labeled Δ 546/*Ava*II rRNA transcript for 30 min at 37 °C. The reaction mixtures were phenol extracted and ethanol precipitated before electrophoresis on a 5% polyacrylamide–7 M urea gel.

Riboprobe analysis for mouse U3B snoRNA

Mouse U3B snoRNA plasmid was kindly provided by Dr Ilana Stroke and Dr Alan Weiner (Yale University). We subsequently subcloned the mouse U3 snoRNA sequence in a pGEM plasmid so that it was under the control of both a T7 and SP6 transcriptional promoter. Using this construct, we were able to make both the riboprobe for mouse U3 snoRNA as well as U3 snoRNA for positive control in our hybridization experiments. Hybridization conditions used were as described by the manufacturer.

Pre-rRNA/nucleolar RNase binding reactions

Reaction mixtures (50 μ l) contained 50mM Tris hydrochloride (pH 8.0), 10% glycerol, 100 mM NaCl, 5 mM dithiothreitol, 10 U of RNasin, 200-fold excess poly(ACI) relative to transcript, and 6 pmol of uniformly-labeled rRNA transcript. The indicated concentration of nucleolar endoribonuclease was added, and the reactions were mixed gently. Binding reactions were incubated at 30°C for 25 min. After incubation, 0.4 μ g of heparin sulfate was added and the reactions were incubated at 30°C for an additional 10 min. For competition reactions, the unlabeled competitor RNA (20 fold-excess) was added to the reaction prior to the addition of the nucleolar endoribonuclease.

Mobility shift analysis

The pre-rRNA/nucleolar RNase binding reactions were performed as previously described. The reactions were analyzed by electrophoresis on a 4% polyacrylamide (4% acrylamide, 0.05% bisacrylamide) gel containing 0.08 M Tris, 0.37 M glycine, and 0.9 mM EDTA, pH 8.5 (Tris–glycine buffer). The gel was electrophoresed at 60 V for 15 hr, and the progress of the gel was followed by the migration of bromophenol blue and xylene cyanol dyes (0.05% each) added to the reaction containing RNA alone. The gel was transferred to Whatman 3MM chromatography paper, dried and autoradiography was carried out at –70°C. Under other conditions, gels were directly exposed without drying so that specific regions could be excised and further analyzed.

Velocity sedimentation analysis

Glycerol gradients (2 ml, 10 to 30%, vol/vol) contained 50 mM Tris hydrochloride (pH 8.0), 100 mM NaCl, 0.1 mM EDTA, and 5 mM dithiothreitol. The pre-rRNA/nucleolar RNase binding reactions were performed as described previously, and were diluted with an equal volume of 50 mM Tris hydrochloride (pH 8.0), 100 mM NaCl, 1 mM EDTA, and 5 mM dithiothreitol just prior to loading onto the gradients. The gradients were centrifuged for 2 h at 4 °C in a Beckman TLS-55 rotor at 55,000 rpm. After centrifugation, 70 μ l fractions were collected from the top of the tube. An aliquot (20 μ l) of each fraction was precipitated with 0.2 ml of 1 mg/ml yeast RNA, 0.01 M $\text{Na}_4\text{P}_2\text{O}_7$, and 0.01 M EDTA, followed by 1 ml of 15% trichloroacetic acid at 0°C. Each sample was filtered through GF/C filters (Whatman), dried, and counted in 4 ml of Cytoscint (ICN) scintillation fluid.

Figure 1. Gel mobility retardation analysis of rRNA-nucleolar RNase binding. Binding reactions containing uniformly ^{32}P -labeled $\Delta 546/\text{AvaII}$ rRNA transcript (0.006 pmol) and 5 μg nucleolar RNase in 50 μl reaction containing 50 mM Tris-HCl (pH 7.5), 10% glycerol, 1 mM EDTA, 5 mM dithiothreitol, 40 U RNasin, 0.01% Nonidet-P40, 100 mM NaCl, and 0.5 mM poly(ACI). After incubation for 25 min at 30°C, 0.4 μg of heparin was added and the reaction was incubated for another 10 min. The binding reactions were analyzed by electrophoresis on a 4% polyacrylamide gel (4% acrylamide, 0.05% bisacrylamide) containing Tris-glycine (pH 8.5) buffer. The gel was electrophoresed at 60 V for 14 hr. The gel was dried, and autoradiography was carried out at -70°C. Lane 1, control (^{32}P -labeled $\Delta 546/\text{AvaII}$ rRNA transcript and no nucleolar RNase); Lane 2, no competitor; Lane 3, 0.1 pmol of competitor $\Delta 546/\text{AvaII}$ rRNA transcript; Lane 4, 0.1 pmol of competitor $\Delta 669/\text{AvaII}$ rRNA transcript; Lane 5, U.V. control (5-azidouridine ^{32}P -labeled $\Delta 546/\text{AvaII}$ rRNA transcript and no nucleolar RNase); Lane 6, U.V. crosslinked; Lane 7, U.V. crosslinked, 0.1 pmol of competitor $\Delta 546/\text{AvaII}$ rRNA transcript; Lane 8, U.V. crosslinked, 0.1 pmol of competitor $\Delta 669/\text{AvaII}$ rRNA transcript. R indicates the position of the uniformly ^{32}P -labeled $\Delta 546/\text{AvaII}$ rRNA transcript without nucleolar RNase; S indicates the position of the ^{32}P -labeled $\Delta 546/\text{AvaII}$ rRNA transcript with decreased mobility due to nucleolar RNase binding.

Nucleolar endoribonuclease and nucleolar extract digestions

The reaction mixture contained 50 mM Tris hydrochloride (pH 8.0), 0.2 mM MgCl_2 , 0.1 M NaCl, 5 mM dithiothreitol, 10 U of RNasin, approximately 0.1 μg of 5' ^{32}P -labeled transcript (20,000–50,000 cpm) and the designated number of units of the nucleolar endoribonuclease or amounts of nucleolar extract, respectively. After 30 min at 37°C, the reaction was terminated by the addition of an equal volume of phenol/chloroform/isoamyl alcohol (22:21:1, vol/vol/vol). The aqueous phase was removed, and the extent of digestion was monitored by electrophoresis on 5% polyacrylamide–7 M urea gels.

UV crosslinking

RNA was synthesized in a total reaction volume of 100 μl containing 40 U RNasin, 500 μM ATP and GTP, 350 μM UTP, 150 μM 5-azido UTP, 11.25 μM CTP, 50 μCi [α - ^{32}P] CTP (800 Ci/mmol), 6 μg plasmid DNA, and 120 U T7 RNA polymerase. The 5-azido-U-substituted radiolabeled RNA was incubated in a 50 μl binding reaction mixture as described previously with 10 μg of dialyzed nucleolar RNase except that β -mercaptoethanol was used instead of dithiothreitol in the binding buffer. The nucleolar RNase (1.25 mg/ml) was dialyzed against 50 mM Tris hydrochloride (pH 7.5), 10% glycerol, 1 mM EDTA, and 0.5 mM β -mercaptoethanol to remove dithiothreitol. The reaction mixtures were incubated at 30°C for 15 min, then on ice for 10 min. Samples were irradiated at a distance of 1 cm using a hand-held UV transilluminator for 5 min on ice, and were then digested with 1 μg pancreatic RNase A, and 0.5 μg T1 RNase for 1 hour at 37°C. Sample buffer for the Laemmli gel was added bringing the sample to 0.0625 M Tris hydrochloride (pH 6.8), 1% sodium dodecyl sulfate, 15% glycerol, 0.1 M β -mercaptoethanol. Each sample was heated to 100°C for 5 min before running on a 10% polyacrylamide/sodium dodecyl sulfate gel. Molecular weights were

Figure 2. Velocity sedimentation analysis of rRNA-nucleolar RNase binding. Binding reactions containing uniformly ^{32}P -labeled $\Delta 546/\text{AvaII}$ rRNA transcript and nucleolar RNase were analyzed by velocity sedimentation analysis on glycerol gradients (10–30%, vol/vol) as described under 'Experimental Procedures'. Bacteriophage MS2 RNA (27S) centrifuged in a parallel gradient was used as a sedimentation marker designated by the open arrow at the top of the figure. A, ^{32}P -labeled $\Delta 546/\text{AvaII}$ rRNA transcript; B, ^{32}P -labeled $\Delta 546/\text{AvaII}$ rRNA transcript and nucleolar RNase; C, unlabeled $\Delta 546/\text{AvaII}$ rRNA transcript, ^{32}P -labeled $\Delta 546/\text{AvaII}$ rRNA transcript and nucleolar RNase; D, unlabeled $\Delta 669/\text{AvaII}$ rRNA transcript, ^{32}P -labeled $\Delta 546/\text{AvaII}$ rRNA transcript and nucleolar RNase.

determined relative to prestained markers run in adjacent lanes. The true molecular weights of the proteins may be slightly less than observed for the UV cross-linked proteins, since one or a few nucleotide residues remain covalently linked to the protein (8).

RESULTS

Electrophoretic stability of the rRNA-nucleolar RNase complex

Based on the findings of Kass *et al.* (8) showing that the primary processing event in the 5'-ETS region of mouse preribosomal RNA involves the formation of stable electrophoretic complex, we were interested as to whether the nucleolar endoribonuclease may demonstrate this same property. To analyze the association of the nucleolar RNase and an rRNA transcript that might form in such a processing reaction, nondenaturing polyacrylamide gel electrophoresis was used. A radiolabeled pre-rRNA transcript, prepared *in vitro* using a template with a bacteriophage T7 promoter cloned upstream of the coding mouse pre-rRNA sequence, was incubated with the nucleolar RNase under conditions which permitted specific binding but did not permit catalysis (absence of Mg^{2+}). As shown in Figure 1, the input pre-rRNA transcript formed a complex which migrated slowly in the gel relative to the rRNA transcript alone.

To determine the specificity of the shifted complex, the binding reaction was challenged by preincubation with unlabeled competitor pre-rRNA transcript. As demonstrated in Figure 1, the shifted band was successfully competed with the same but unlabeled $\Delta 546/AvaII$ transcript (processing competent) (lanes

3 and 7), but not with unlabeled $\Delta 669/AvaII$ transcript (processing incompetent) (lanes 4 and 8) where the +650 site was deleted. Since the $\Delta 669/AvaII$ transcript is similar to the $\Delta 546/AvaII$ transcript except that it lacks the sequence for the +650 site, the inability of the $\Delta 669/AvaII$ transcript to displace the $\Delta 546/AvaII$ transcript in the complex suggested that the shifted band was dependent on the presence of the +650 site sequence.

Sedimentation properties of the rRNA-nucleolar endoribonuclease complex

The rRNA-RNase complex was analyzed by velocity sedimentation analysis to determine the relative size of the complex and to further analyze the stability of binding. Figure 2 shows the sedimentation pattern of labeled transcript with and without the nucleolar endoribonuclease. The distribution of radioactivity in gradients with nucleolar RNase compared to rRNA alone was reproducible under the same conditions of binding, centrifugation, and fractionation. By comparing the location of the complex and of the rRNA in the gradient with that of uniformly 3H -labeled MS2 bacteriophage RNA (27S) centrifuged in a parallel gradient, the sedimentation value of the rRNA was estimated to be ~7S and that of the rRNA-nucleolar RNase protein complex to be ~40S. Proof that this broad peak of material represents the complex was again demonstrated by competition experiments. A processing competent transcript ($\Delta 546$), that is a transcript which contained the +650 processing site, displaced the faster sedimenting label which now sedimented at the position of uncomplexed rRNA alone. Accordingly, a transcript lacking the +650 processing site ($\Delta 669$) did not displace the faster sedimenting label, and the broad peak of complexed rRNA persisted.

The nucleolar endoribonuclease does not contain or require an RNA component for stable binding

U3 small nucleolar RNA (snoRNA) is one member of a family of nucleolar-specific snoRNAs, which also includes U8 snoRNA

Figure 3. Micrococcal nuclease treatment of nucleolar endoribonuclease and nucleolar extract. Nucleolar endoribonuclease (5 μ g) and nucleolar extract (16 μ g) were pretreated with 1 and 2 μ g micrococcal nuclease in 50 μ l reactions containing 20 mM Tris-HCl (pH 7.5), 0.1 μ g/ μ l bovine serum albumin, and 0.2 mM $CaCl_2$ for 15 min at 25°C. The micrococcal nuclease reactions were terminated by the addition of 0.4 mM EGTA. An aliquot (5 μ l) of each treated extract was incubated in 25 μ l reactions containing 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 5 mM dithiothreitol, 13 U of RNasin, 0.6 mM $MgCl_2$, and 1.5 μ g $5'$ - ^{32}P -labeled $\Delta 546/AvaII$ rRNA transcript for 30 min at 37°C. The reactions were phenol extracted and ethanol precipitated before electrophoresis on a 5% polyacrylamide-7 M urea gel. Lanes C, control, rRNA transcript carried through procedure but with no nucleolar RNase or extract, and no micrococcal nuclease pretreatment; Lane 1 and 2, rRNA transcript carried through procedure with no nucleolar RNase, extract, or EGTA to inhibit micrococcal nuclease in the pretreatment step; Lane 3, nucleolar RNase, no pretreatment with micrococcal nuclease; Lane 4 and 5, nucleolar RNase, pretreatment with micrococcal nuclease, 1 and 2 μ g respectively; Lane 6 and 7, nucleolar extract, no pretreatment with micrococcal nuclease; Lane 8 and 9, nucleolar extract, pretreatment micrococcal nuclease, 1 and 2 μ g respectively; Lane M, DNA size markers, the numbers on the right represent DNA marker sizes in base pairs. The cleavage at the +650 site is indicated by the arrow and number.

Figure 4. Hybridization with U3 snRNA riboprobe to determine the effectiveness of micrococcal nuclease pretreatment of nucleolar RNase. Nucleolar extract (16 μ g) and nucleolar endoribonuclease (5 μ g) were pretreated with micrococcal nuclease in 50 μ l reactions containing 20 mM Tris-HCl (pH 7.5), 0.1 μ g/ μ l bovine serum albumin, 0.2 mM $CaCl_2$ for 15 min at 25°C. The micrococcal nuclease reactions were terminated by the addition of 0.4 mM EGTA. The samples were then blotted onto nitrocellulose. Hybridization was carried as described in 'Experimental Procedures' using a ^{32}P -labeled U3B snRNA riboprobe. Nucleolar Extract, Lane 1, no extract; Lane 2, extract not incubated under pretreatment conditions with micrococcal nuclease; Lane 3, extract incubated but no pretreatment with micrococcal nuclease; Lane 4, pretreatment with 1 μ g micrococcal nuclease; Lane 5, pretreatment with 2 μ g micrococcal nuclease. Nucleolar RNase, Lane 1, no nucleolar RNase; Lane 2, nucleolar RNase not incubated under pretreatment conditions with micrococcal nuclease; Lane 3, pretreatment with 1 μ g micrococcal nuclease; Lane 4, pretreatment with 2 μ g micrococcal nuclease. U3B snRNA standards, 8.0, 4.0, 2.0, 1.0, 0.5, 0.25, 0.13, 0.06 ng, respectively.

and U13 snoRNA (15). Because of its nucleolar location, its association with pre-ribosomal particles, and its hydrogen-bonded interactions with rRNA, U3 snoRNA has been thought to function in pre-rRNA processing (10, 16, 17). Moreover, Kass *et al.* (7) have shown that processing at the +650 site by whole cell extracts required U3 snoRNA and was inhibited by micrococcal nuclease pretreatment. For this reason, we examined the effect of micrococcal nuclease pretreatment on the ability of the nucleolar endoribonuclease to specifically cleave the +650 processing site. A parallel experiment was similarly carried out using the nucleolar extract from which the nucleolar RNase was purified as a control to assess the effectiveness of the methodology. Treated extract and nucleolar RNase were compared to untreated samples with regards to processing at the +650 site using an *in vitro*-derived rRNA transcript (Δ 546) As shown in Figure 3, neither the efficiency or specificity of processing was affected by micrococcal nuclease pretreatment for either the nucleolar RNase or the nucleolar extract. To insure that the levels of micrococcal nuclease used in our experiments were sufficient to degrade any snoRNA, we examined the loss of U3 snoRNA from samples treated with micrococcal nuclease using a riboprobe of

mouse U3B snoRNA. These results are shown in Figure 4 and further support our findings that the nucleolar RNase is not a ribonucleoprotein (2), and that the levels of micrococcal nuclease used were sufficient to hydrolyze U3 snoRNA in the nucleolar extract below detectable levels.

Identification of the protein species associated with the rRNA substrate by UV crosslinking

To identify the nucleolar RNase protein specie(s) specifically associated with processing competent rRNA transcript, we labeled the nucleolar RNase using a radiolabeled rRNA transcript and UV crosslinking. For these experiments, the rRNA transcript was synthesized to contain the photoreactive uridine analog, 5-azidouridine-5'-triphosphate, which can substitute for UTP in transcription and can form covalent bonds with associated proteins upon UV irradiation (18). The 5-azido-U-substituted radiolabeled transcript was incubated with the nucleolar RNase to allow formation of the RNase/RNA complex. To demonstrate that nucleolar endoribonuclease could still complex with the 5-azidouridine substituted transcript, mobility shift analysis was carried out as shown in Figure 1, lanes 5 through 8. The 5-azido-uridine substituted transcript behaved identically to the unsubstituted transcript relative to binding.

Bound nucleolar endoribonuclease was irradiated with UV light, and following ribonuclease digestion to destroy the bulk

Figure 5. SDS/Polyacrylamide gel analysis of photocrosslinked rRNA-nucleolar RNase. 5-Azidouridine substituted Δ 546/*Ava*II rRNA transcript (0.009 pmol) containing the +650 site was incubated with 10 μ g nucleolar RNase in a 80 μ l reaction mixture containing 50 mM Tris-HCl (pH 7.5), 10% glycerol, 5 mM EDTA, 0.5 mM β -mercaptoethanol, 100 mM NaCl, and 0.5 mM poly(ACI). Each binding reaction was incubated 15 min at 30°C, then on ice for 10 min, and irradiated for 5 min at 0°C using a UVSL-25 mineralight under short-wave length at a distance of 1 cm. After photocrosslinking, all samples were digested with pancreatic RNase and T1 RNase (0.25 mg/ml and 0.013 mg/ml, respectively) for 1 hr at 37°C. Each sample was brought to 80 μ l with protein sample buffer, and the samples were heated at 100°C for 5 min before loading onto a 10% SDS-containing discontinuous polyacrylamide gel electrophoresis system. (A) Coomassie stained SDS/polyacrylamide gel described above. Lane M, molecular weight standards. Arrows indicate positions of coomassie stained bands of the nucleolar endoribonuclease which corresponded to bands in the autoradiograph. (B) Autoradiograph of the same SDS/polyacrylamide gel. Lane 1, 5-azidouridine substituted 32 P- Δ 546/*Ava*II rRNA transcript and nucleolar RNase; Lane 2, 5-azidouridine substituted 32 P- Δ 546/*Ava*II rRNA transcript and nucleolar RNase, U.V. crosslinked; Lane 3, 5-azidouridine substituted 32 P- Δ 546/*Ava*II rRNA transcript competed for by unlabeled 5-azidouridine substituted Δ 546/*Ava*II rRNA transcript and nucleolar RNase; Lane 4, 5-azidouridine substituted 32 P- Δ 546/*Ava*II rRNA transcript competed for by unlabeled 5-azidouridine substituted Δ 546/*Ava*II rRNA transcript and nucleolar enzyme, U.V. crosslinked.

Figure 6. SDS/Polyacrylamide gel analysis of photocrosslinked rRNA-nucleolar RNase complex from mobility shift gel. After exposure to X-ray film, the mobility shift gel shown in Figure 1 was excised at positions which corresponded to the shifted material in lane 6 and the displaced competed material in Lane 7 of Figure 1. The gel slices were U.V. irradiated, then electroeluted, and the eluted labeled was concentrated by precipitation with ethanol. The pellets were washed twice with ice-cold 70% ethanol, and then digested with pancreatic RNase and T1 RNase before running on a 10% denaturing polyacrylamide gel as described in 'Experimental Procedures'. Lane 1 represents the displaced labeled material from lane 7 of Figure 1; and Lane 2 represents the shifted material from lane 6 of Figure 1. Molecular weight markers are indicated by arrows and their corresponding size.

of the crosslinked rRNA, the samples were resolved on a SDS-polyacrylamide gel. As shown in Figure 5B, three discernable protein bands were observed from the autoradiograph. The specificity of these protein bands was demonstrated by competition with a processing competent transcript (lane 4). The largest band on the autoradiograph (~50 kDa) corresponded to the indicated Coomassie stained band of this gel (Figure 5A).

To further demonstrate that these proteins species were specifically associated with the complex observed by mobility shift analysis, U.V. crosslinked material from the shifted band (Figure 1, lanes 5 and 6) was electroeluted and then subjected to the ribonuclease digestion and analysis on a SDS-polyacrylamide gel. As shown in Figure 6 (lane 2), labeled material from the shifted position of a mobility shifted gel yielded the same three labeled polypeptide species to those observed from the crosslinking study. In contrast, labeled material displaced by competition with cold transcript yielded no distinct or detectable pattern (Figure 6, lane 1). We concluded from these studies that this family of similar sized polypeptides is directly responsible for complex observed by mobility shift analysis.

DISCUSSION

The ability of an enzyme to discriminate between available substrates is a function of both the catalytic rate and substrate binding. Since we previously established that the nucleolar RNase could specifically catalyze the cleavage of a precursor rRNA transcript at a site which mimicked the *in vivo* processing, we were therefore interested as to the basis for this specificity. The work from Kass and Sollner-Webb (8) further suggested that the nucleolar RNase may be a component of a mouse whole cell extract that specifically assembled a stable processing complex with an *in vitro*-derived rRNA transcript containing the +650 early processing site. For these reasons, we were interested in determining whether the nucleolar endoribonuclease alone could specifically and stably bind to an *in vitro*-derived transcript containing the +650 early processing site of mouse precursor rRNA.

Our results showed that this was indeed the case; that the nucleolar endoribonuclease specifically and stably bound a rRNA transcript containing the +650 processing site. Both mobility shift analysis and glycerol gradient sedimentation were used to demonstrate the stability and specificity of this binding. Moreover, the stable association of enzyme with the rRNA substrate occurred in the absence of Mg^{2+} , and in the presence of excess EDTA, demonstrating that cleavage was not required for stable binding (1). We cannot, however, explain why the shift was so large, or why the relative sedimentation size was so broad and sedimented so rapidly. Whether heterogeneity in binding modes contributes to the apparent size of the complex has not been determined. Nevertheless, the specificity and stability of this interaction was clearly and reproducibly established by both mobility shift analysis and sedimentation velocity studies.

The findings of Kass *et al.* (7), demonstrating a requirement for the nucleolar associated U3 snoRNA in the processing of the +650 site suggested that this snoRNA may be an important component of a processing complex. In addition, studies carried by Maser and Calvet (10), involving *in vivo* U.V. crosslinking, demonstrated that U3 snoRNA crosslinked downstream of nucleotide +414, the human equivalent of the +650 site in a region of sequence conserved between human and mouse. In our studies, however, we did not observe a requirement for U3

snoRNA. Micrococcal nuclease pretreatment of the nucleolar endoribonuclease had no apparent effect on specificity or on the efficiency of cleavage at the +650 processing site. Although hybridization experiments demonstrated the presence of U3 snoRNA in the nucleolar extract from which the enzyme was purified, subsequent pretreatment of the nucleolar extract with micrococcal nuclease resulted in complete loss of hybridizable material but not the processing endonucleolytic activity. Consistent with our earlier report (3), no U3 snoRNA was detected with the purified nucleolar RNase.

A possible explanation for the differences in our results compared to those of Kass *et al.* (7) may be related to the more stringent requirements of a whole cell extract. Formation of a stable processing complex using a whole cell extract may require the presence of intact U3 snoRNP if, for example, U3 snoRNP influences the assembly of a multicomponent complex from the whole cell extract. However, in the absence of other factors, including U3 snoRNP, there must still remain sufficient structure in the pre-rRNA for the nucleolar endoribonuclease to recognize, stably bind and then catalyze the correct endonucleolytic cleavage at the +650 processing site. The ability of the nucleolar endoribonuclease to form a stable, specific complex with the +650 processing region, as demonstrated in this report, supports this conclusion and strengthens the proposal for the involvement of this nucleolar endoribonuclease in the processing of preribosomal RNA.

Finally, we took advantage of the formation of this stable complex to specifically label the nucleolar RNase. A 5-azido-U-substituted radiolabeled rRNA transcript containing the +650 processing region was incubated with the nucleolar RNase, and crosslinked using UV light. UV-induced crosslinking using the azido-photoactive group has many advantages over other chemical and UV-induced crosslinking methods, requiring shorter irradiation times and yielding higher levels of incorporation. The efficiency of crosslinking minimizes protein photolysis and results in increased sensitivity of detection. After digestion with pancreatic and T1 ribonucleases to destroy the bulk of the rRNA component, the polypeptides crosslinked to the remaining ^{32}P -labeled rRNA were resolved on a SDS-polyacrylamide gel. The labeled region in the gel was identified by autoradiography and contained three distinct labeled species. The largest species had an estimated size of ~50 kDa, essentially identical to the estimated size of the nucleolar RNase of ~51 kDa (1). The two smaller size bands were ~48 and ~45 kDa, respectively. The largest band directly correlated with a Coomassie stained band of this gel.

One possibility to account for the multiple species of labeled material was that the cross-linked rRNA component was differentially digested by pancreatic and T1 RNases giving rise to three distinct labeled species. However, RNase digestions were carried out at several concentrations and for various times to establish that the heterogeneity did not result from incomplete RNA digestion. Alternatively, it was possible that a post-translational modification (e.g. phosphorylation, adenylation, etc.) leads to differential species of nucleolar endoribonuclease which resolve on the SDS-polyacrylamide gel. At this time, this possibility can not be ruled out. However, the simplest explanation for the three nearly identical size bands is that they result from restricted proteolysis of the ~50 kDa species which may have occurred during the purification of the nucleolar RNase. The limited amount of protein available presently precludes sequence information on all labeled bands; however, we are

currently carrying out amino acid sequence analysis on internal tryptic peptides of the ~50 kDa species for which there was sufficient protein. A blocked amino terminus prevented terminal analysis of the intact polypeptide (data not shown).

In summary, the nucleolar endoribonuclease is able to distinguish, stably bind and process a competent rRNA transcript containing the +650 early processing site. The uniqueness of this interaction was demonstrated by the dependence of binding on the presence of a functional processing site, thus binding was not simply a random association of the RNase with the macromolecular pre-rRNA substrate. Undoubtedly, the ability of this enzyme to recognize and stably bind a pre-rRNA containing a functional processing site contributes to the overall unique specificity of endonucleolytic cleavage demonstrated by this enzyme.

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