Volume 16 Number 22 1988 Nucleic Acids Research

An *in vitro* interaction between the human U3 snRNP and 28S rRNA sequences near the α-sarcin site

Katherine A.Parker*+, James P.Bruzik and Joan A.Steitz

Department of Molecular Biophysics and Biochemistry, The Howard Hughes Medical Institute, Yale University Medical School, 333 Cedar Street, New Haven, CT 06510, USA

Received August 26, 1988; Revised and Accepted October 20, 1988

ABSTRACT

Model transcripts containing mammalian pre-rRNA sequences were incubated with a HeLa cell extract, digested with T1 RNase, and immunoprecipitated with anti-(U3)RNP or control antibodies. Two overlapping fragments derived from the 3' domain of human 28S rRNA were specifically immunoprecipitated although transcripts which spanned the transcription initiation site, the ETS processing site, the 5' end of 18S, and both termini of 5.8S yielded no protected fragments. The sequence of these fragments was determined using a novel technique in which the [^{32}P]-labeled fragment was co-fingerprinted with [^{3}H]-labeled total transcript serving as an internal marker. The fragments immunoprecipitated derive from nucleotides 4570-4590 and 4575-4590 of human 28S and are adjacent to the α-sarcin site. Protection most likely involves the U3 RNA since it is sensitive to pretreatment of the extract with micrococcal nuclease. Complementarity between U3 and this rRNA region is phylogenetically conserved in species ranging from human to S. cerevisiae. The possible significance of this finding is discussed.

INTRODUCTION

Three of the four RNA molecules found in mature ribosomes are synthesized in the nucleolus as a 45-47S precursor (mammalian) of about 13.5 kb, which undergoes several processing reactions to yield the mature 18S, 5.8S and 28S rRNAs [see Fig. 1; reviewed by Hadjiolov (1)]. Because of its nucleolar location (2,3,4,5), U3, a 217 nucleotide [human(6)] small RNA, is presumed to serve some role in the anabolism of rRNA.

An early theory which postulated that U3 acts in the formation of the 3' end of 5.8S rRNA (7,8,9) was based on two facts: 1) a conserved region of rat U3 is complementary to the vertebrate rRNA sequence at this site (7,8,9); and 2) the U3 RNA can be found associated with 28-35S RNA (3,10,11), which is the size of the immediate precursor to 5.8S and 28S (1). This theory now seems unlikely as lower eukaryotes do not maintain this potential base-pairing (12,13,14,15).

More recent models predict that U3 may function at the extreme 5' or 3' end, respectively, of the 45-47S precursor. Stroke and Weiner (personal

communication) have identified in rat an in vivo psoralen crosslink between U3 and pre-rRNA within 350 nucleotides of a processing site in the external transcribed spacer (ETS). This ETS processing site is located about 5 kB upstream of the 5' end of 18S, and 415, 650, or 790 nucleotides downstream of the Pol I initiation site in human (16,17), mouse (18), and rat (Stroke and Weiner, personal communication), respectively. Maser and Calvet (personal communication) have likewise identified a crosslink between U3 and pre-rRNA within 300 nucleotides of the ETS processing site in human. It is as yet unclear precisely where either crosslink occurs in the precursor and whether or not they relate to the function of U3. It is also possible that other base-pairing interactions between U3 and pre-rRNA were not identified with this assay, as psoralen shows a marked preference for crosslinking particular sequences.

More recently, we (19) suggested that U3 might function instead in the maturation of the 3' end of 28S rRNA. This terminus is formed by RNA processing (20,21,22,23) rather than transcription termination, as previously proposed (1). Experimental determination of bases that are single-stranded identified an accessible region of U3 in the RNP with complementarity to conserved pre-rRNA sequences near the 3' end of 28S (19). To date there are no experimental data in support of this theory.

To probe further the elusive function of the U3RNP, we have performed ribonuclease protection-immunoprecipitation experiments. Such analyses have previously been used to define RNA processing signals [such as the 5' splice site (24)] based on their protection from digestion by association with an RNP, followed by immunoprecipitation with antibodies directed against that RNP. Autoimmune patient (5) and monoclonal (25) antibodies which can immunoprecipitate the U3RNP via an associated 34 kD protein were available. When various regions of pre-rRNA were tested, the only region which interacted specifically with the U3RNP in vitro was surprisingly found to derive from mature 28S rRNA near the α-sarcin site.

MATERIALS AND METHODS

Plasmid Constructs

All plasmid constructs were obtained by standard cloning techniques (26) and are depicted in Fig. 1. The 1.2 kb EcoRI/SalI fragment containing the initiation site and 700 nucleotides of ETS sequence (and thus the ETS processing site), and the 2.3 kb SalI/EcoRI fragment containing 1.2 kb of the ETS and 1.1 kb of the 5' end of 18S were obtained from the 18 kb human

EcoRI fragment (27) cloned into λ-phage, which was generously provided by N. Arnheim. These fragments were cloned into the EcoRI and SalI sites of pGEM4 to generate, respectively, pES-ETS and pSE-18S. A 5.3 kb DraIII/EcoRI fragment containing 36 nucleotides of ITS-I, all of 5.8S and ITS-II, and 4.5 kb of 28S was obtained from the rat clone ChR-B4 (28), which was kindly provided by L. Rothblum. This was cloned into the EcoRI and SmaI sites of pGEM4 to generate pDE-5.8S. A 950 nucleotide EcoRI/SalI fragment containing 590 nucleotides of the 3' end of 28S and 360 nucleotides of downstream sequence was obtained from the human clone pAD_{BB} (29), which was kindly donated by R. Schmickel. It was cloned into the EcoRI and SalI sites of pGEM4 to create pES-28S. pNS-28S was generated from pES-28S by cleaving with EcoRI and NcoI, filling in, and religating.

Synthesis of Positive Sense rRNAs

To generate positive sense model rRNA precursors, pES-ETS was cut with SalI and transcribed with SP6 polymerase to generate an RNA of 1200 nucleotides. pSE-18S was cut with EcoRI and pDE-5.8S was cut with BglI (which cuts at position 389 of ITS-II); then each was transcribed with T7 polymerase to generate a 2.3 kb and a 580 nucleotide RNA, respectively. pES-28S and pNS-28S were cut with SalI, Tth111I, or Ava II (see Fig. 1B) and transcribed with SP6 polymerase.

For transcription of the RNAs, the protocol of Melton et al. (30) was followed except that 1 mCi of α-[^{32}P]GTP at specific activity 4000 Ci/mmole (New England Nuclear) was added without unlabeled GTP to a 20 μl reaction with 0.5 mM GpppG cap to generate RNAs of a specific activity of 3.3 x 10^9 cpm/μg.

To synthesize [^{3}H]-labeled transcripts which served as internal standards for fingerprint analyses, 0.25 mCi of [8-^{3}H]GTP at 11 Ci/mmole (Amersham) was added to a 100 μl reaction volume (scaled up proportionately) in the presence of 1,000 cpm of α-[^{32}P]GTP. The trace amount of [^{32}P] enabled the [^{3}H]-labeled RNA to be identified by autoradiography after gel purification. The transcript was eluted from the gel, precipitated with ethanol, resuspended in 100 μl of water and stored at -20°C; 5 μl of this solution was used for each RNA fingerprint.

Antibodies

Mouse monoclonal anti-(U3)RNP antibody 72B9 (25) was generously provided by Eng Tan of the Scripps Foundation. Patient anti-(U1)RNP antibody (AG) was kindly provided by John Hardin and Joe Craft of Yale University,

and the mouse monoclonal anti-Sm antibody Y12 was obtained from ascites fluid as described (31).

Protection-immunoprecipitation reaction

Nuclear extract was prepared from human derived HeLa cells according to the method of Dignam et al. (32) using a 0.6M KCl nuclear extraction buffer and the extract was dialyzed into buffer D (20 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol (DTT), and 20% (v/v) glycerol). Antibodies (20 μl of antiserum or 20 μl of monoclonal antibody ascites fluid per reaction) were pre-bound to 4 mg of protein A sepharose (PAS; Pharmacia) in NET-2 buffer (150 mM NaCl, 40 mM Tris-HCl pH 7.5, .05% Nonidet P-40) as described previously (19), washed, and resuspended in 100 μl of NET-2.

The reaction conditions were based on those of Black et al. (33). For each reaction, a 50 μl volume containing 30 μl of extract with (all final concentrations) 0.4 mM ATP, 10 mM creatine phosphate, 5 mM DTT, 5 mM $MgCl_2$, 100 mM KCl, and 2-5 x 10^7 cpm of α-[^{32}P]-labeled transcript was incubated for 15 min. at 30°C. The sample was placed on ice and 20 μl of freshly diluted RNase T1 (Calbiochem, specific activity 3000 unit/mg) at 30,000 units/ml was added. After 5 min., antibody:PAS conjugate (present in 100 μl of NET-2) was added and the samples nutated at 4°C for 60 min., then the pellets precipitated by spinning for 10 seconds in a microfuge and washed 6 times with 1 ml of cold NET-2. The RNA was phenol:chloroform:isoamylalcohol (50:49:1) extracted, ethanol precipitated, then electrophoresed on a 20% polyacrylamide 8M urea gel with 1/2 X TBE (45 mM Tris base, 45 mM boric acid, 1.25 mM EDTA).

Analysis of RNA Fragments

After electrophoresis, RNA fragments were eluted from the gel in 0.3 M sodium acetate, 1 mM EDTA, 50 mM Tris-HCl pH 7.5, and 0.1% SDS. After ethanol precipitation with 20 μg of yeast RNA as carrier, each [^{32}P]-labeled fragment was mixed with 5 μl of [^{3}H]-labeled total transcript synthesized as described above, dried down, and the sample digested with 2 μl of T1 RNase present at 3,500 units/ml and fingerprinted according to the method of Barrell (34). After the [^{32}P]-labeled spots were visualized by autoradiography, plates were sprayed 3 times with Enhance Spray (New England Nuclear) and the [^{3}H]-spots visualized by a second autoradiography at -70°C.

Micrococal Nuclease Degradation of Nuclear RNA Prior to Incubation

A reaction was prepared as above except that radiolabeled precursor was not added. 1 μl of $CaCl_2$ at 30 mM and/or 1 μl of micrococcal nuclease at 50,000 units/ml were added and incubated for 5 minutes at 30°C. 1 μl of

ethylene glycoltetraacetic acid (EGTA) at 0.1 M was then added to chelate the calcium, and the radiolabeled transcript added and the samples treated as above. One control reaction received calcium and EGTA first, and then micrococcal nuclease for 5 min, and then the radiolabeled RNA. For the add-back experiments, $CaCl_2$ and micrococcal nuclease were added for 5 min., then EGTA added and the samples mixed well. 5 µl of yeast RNA at 10 mg/ml, 5 µl of 16S rRNA (provided by P. Moore, Yale University) at 10 mg/ml, or 5 µl of human nuclear RNA at 1 mg/ml were added and incubated for 5 min. at 30°C, then the radiolabeled RNA added and the reaction performed as described above.

RESULTS

Protection of mature 28S rRNA sequences and immunoprecipitation with anti-(U3)RNP antibodies.

Transcripts corresponding to various regions of the rDNA repeat (Fig. 1) were incubated with a nuclear extract of HeLa cells, digested with RNase T1, then immunoprecipitated. A 1.2 kb RNA that included the human transcription initiation site and ETS processing site, a 2.3 kb RNA that included the 5' end of human 18S, and a 580 nucleotide RNA that included both the 5' and 3' end of rat 5.8S all gave negative results in that no fragments were precipitated more efficiently by an anti-(U3)RNP antibody than an anti-(U1)RNP control (data not shown). In contrast, specific immunoprecipitation of fragments was observed with RNAs transcribed from SalI cut pES-28S, which contained the 3'-most 590 nucleotides of human 28S and 360 nucleotides of downstream sequence. These fragments were mapped by testing progressive deletions of the original pES-28S clone and by RNA fingerprinting. Protection-immunoprecipitation was maintained with a 5' deletion to the NcoI site but lost when extended to the NaeI site (see Fig. 1B). On the 3'-side, protection-immunoprecipitation was maintained when the transcription templates pES-28S or pNS-28S were cleaved with either Tth111I or AvaII.

Fig. 2 shows the results of a protection-immunoprecipitation experiment using a radiolabeled transcript derived from pNS-28S cut with AvaII. Two major fragments of approximately 23 (fragment A) and 18 (fragment B) nucleotides were precipitated by the anti-(U3)RNP antibody (lane 3). The same result was obtained with autoimmune patient antibodies LS and JH, which also immunoprecipitate the U3RNP (19) (data not shown). The anti-(U1)RNP control (lane 4) did not show bands even after a much longer exposure, nor

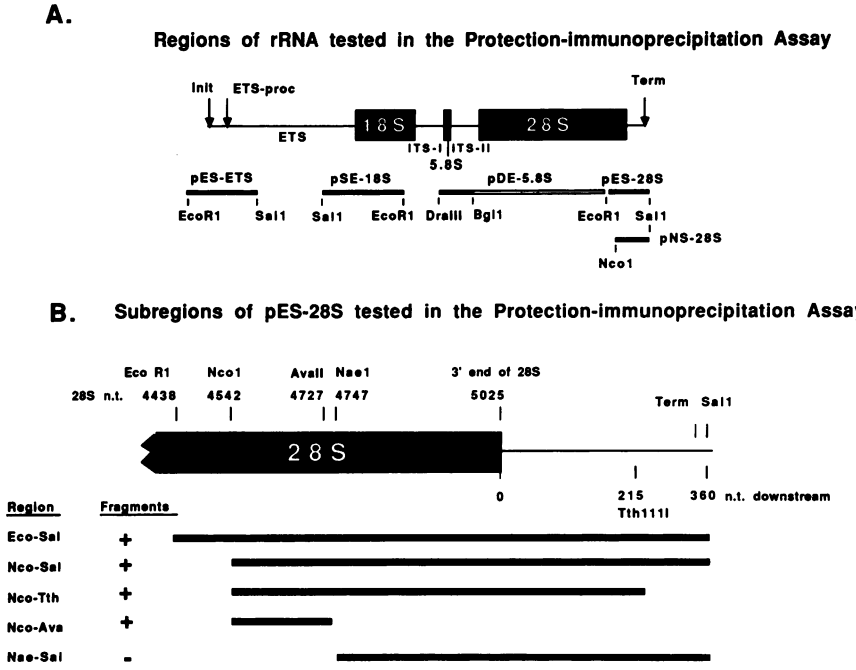

Fig. 1. Plasmid constructs used to synthesize pre-rRNAs for the protection-immunoprecipitation experiments. A partial restriction map of mammalian pre-rRNA and some adjacent sequences is shown. Regions that were cloned downstream of an RNA polymerase promotor are described in detail in the Methods and are identified by boxes below; regions that were actually synthesized for the protection-immunoprecipitation experiments are outlined in black. B. Subregions of pES-28S which were used for further protection-immunoprecipitation experiments are shown. Numbers above indicate the location on 28S of restriction sites or the 3' end; numbers below indicate the distance downstream of the 3' end of 28S. Regions which yielded fragments in the protection-immunoprecipitation assay are indicated by a plus.

did antibodies from a non-immune human or rabbit, or the patient antibody RB (19) which is directed against the 7.2S and 8.2S RNPs (not shown). Although the U3 RNA possesses a trimethyl guanosine (TMG) cap (4), polyclonal rabbit (35) and monoclonal mouse (kindly provided by A. Krainer) anti-TMG antibodies were also unable to immunoprecipitate fragments A and B (data not shown).

U3 differs from other U RNAs in that it is not immunoprecipitable from cell extracts with anti-Sm antibodies, which should therefore serve as a negative control. When protection-immunoprecipitation experiments were performed with an anti-Sm monoclonal antibody (lane 5) or an anti-(U2)RNP

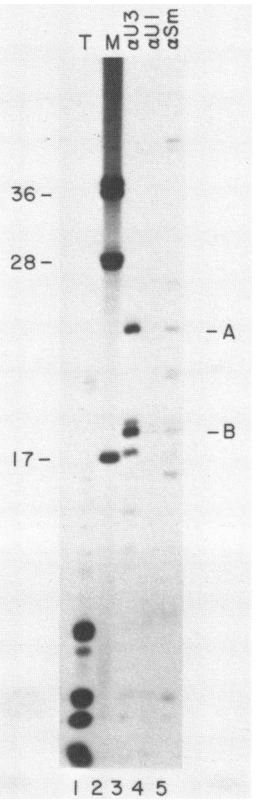

Fig. 2. T1 ribonuclease protection of the α-sarcin site region of human 28S rRNA and immunoprecipitation with anti-(U3)RNP antibodies. pNS-28S was cut with Ava II and pre-rRNA radiolabeled with α-[^{32}P]GTP and incubated with nuclear extract as described in the Methods for 15 minutes. The sample was digested with T1 RNase, and immunoprecipitated with the mouse monoclonal anti-(U3)RNP antibody 72B9 (lane 3) (25), the anti-(U1)RNP patient antibody AG (lane 4), or the mouse anti-Sm monoclonal antibody Y12 (lane 5) (31). The RNA was phenol extracted, ethanol precipitated, and electrophoresed on a 20% polyacrylamide 8M urea gel. Lane 1 shows the RNA fragments present in 1/80 of the total sample of the anti-(U3)RNP antibody at the end of the digestion period, just prior to washing the immunoprecipitate. Lane 2 shows DNA markers obtained by filling in a HpaII digest of pBR322.

patient antibody (not shown), fragments of a similar size to A and B were immunoprecipitated about 5% as efficiently as with the anti-(U3)RNP antibody. In addition, other bands present in the anti-Sm lane could be seen in the anti-(U3)RNP and anti-(U1)RNP lanes after a much longer exposure. Although the significance of the anti-Sm result is therefore unclear, we

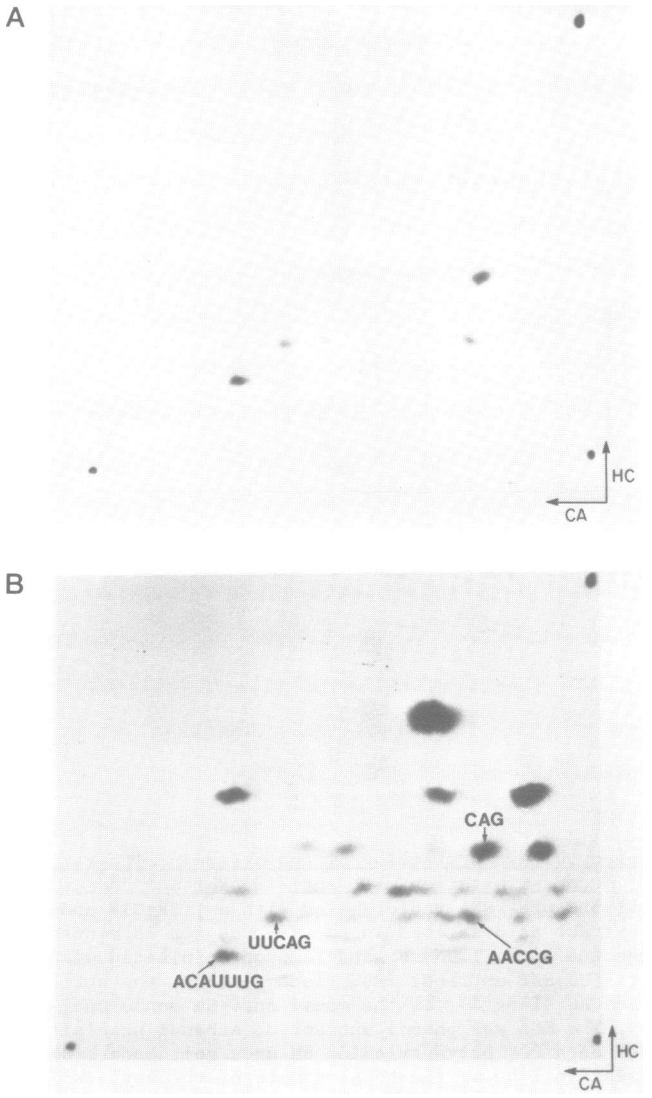

Figure 3. Double-label RNA fingerprint analysis. A. Fragment A shown in Fig. 2 was eluted from the gel, ethanol precipitated and mixed with [^{3}H]GTP-labeled RNAs transcribed from Tth111I cut pNS-28S. The mixture was fingerprinted (34) by electrophoresis on cellulose acetate in the first dimension (CA) followed by homochromatography in the second dimension (HC) and autoradiographed in the absence of a fluor. B. The fingerprint shown in A was sprayed with Enhance and reautoradiographed. The oligonucleotides from A were identified by virtue of which spot they comigrated with in B. The spots in the extreme upper right and both lower corners represent [^{32}P]-labeled markers that were placed on the completed fingerprint prior to the first autoradiography.

conclude that fragments A and B are specifically immunoprecipitated because they are at least 100 fold enriched in the anti-(U3)RNP lane as compared with the other bands in the anti-Sm lane, and are absent in several other controls as well. Furthermore, the fragments seen in the anti-Sm lane that comigrated with bands A and B could not be analyzed because of their low intensity and may not represent the same fragments.

Identification of Fragments A and B using a Double-label RNA Fingerprint Technique

The fragments immunoprecipitated by the anti-(U3)RNP antibodies were identified using a novel variation of the RNA fingerprint technique. Although double-label fingerprinting has been performed, previous analyses of spots consisted of elution from the fingerprint followed by scintillation counting (36). For these experiments, α-[^{32}P]-labeled fragments eluted from the gel were mixed with a total transcript labeled with [^{3}H]GTP, digested with T1 RNase, and the resulting oligonucleotides fractionated in two dimensions as usual (34). Markers were then placed at the corners of this fingerprint by spotting about 10 cpm (Cerenkov) of a [^{32}P]-labeled nucleic acid. The first autoradiogram (Fig. 3A) of the fingerprint identifies [^{32}P]-labeled oligonucleotides from the protected fragment, since [^{3}H] gives no detectable signal in the absence of a fluor. The fingerprint is then sprayed with the fluor Enhance and returned to -70°C for 10 days. The second exposure (Fig. 3B) visualizes the [^{3}H]-labeled oligonucleotides derived from the total transcript which can be identified by their relative positions.

Aligning the two autoradiograms allows the [^{32}P]-labeled spots to be precisely identified by their comigration with [^{3}H]-labeled oligonucleotides. Preliminary experiments (data not shown) had shown that a [^{3}H]-labeled oligonucleotide comigrates with the same [^{32}P]-labeled oligonucleotide; each spot of a fingerprint of a [^{32}P]-labeled total transcript was identified by performing secondary analyses on DEAE paper after digestion of the eluted spot with pancreatic RNase (data not shown). α-thio-[^{35}S]-labeled oligonucleotides migrate considerably slower in the second dimension than the same [^{32}P]-labeled oligonucleotide and thus cannot be used as the second isotope.

Figs. 3A and B show that the protected-immunoprecipitated fragment A contains the oligonucleotides CAG or ACG, AACCG, UUCAG, and ACAUUUG. These can only originate from the sequence AACCG/CAG/G/UUCAG/ACAUUUG at position 4570-4590 of 28S (37). The gel mobility of about 23 nucleotides (see Fig. 2) suggests that it may also have terminal G's; these would not appear on

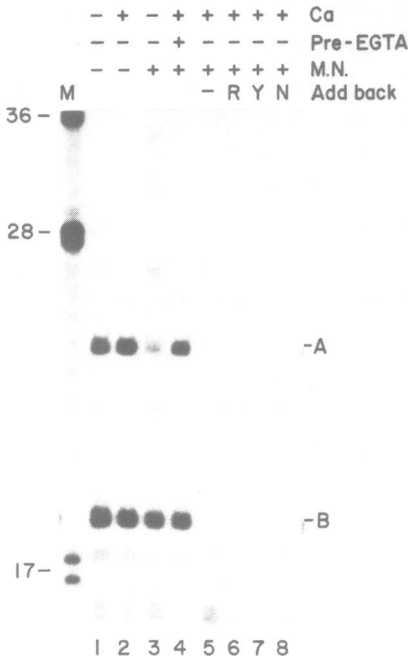

Figure 4. Effect of micrococcal nuclease on the protection-immunoprecipitation of 28S rRNA and immunoprecipitation with anti-(U3)RNP antibodies. Reactions were prepared as described in the Methods except precursor RNA was not added. The samples shown received the following: lane 1: 2 μl of water; lane 2: 1 μl of $CaCl_2$ at 30 mM (final concentration 0.75 mM) and 1 μl of water; lane 3: 1 μl of micrococcal nuclease at 50,000 units/ml (final concentration 1,250 unit/ml) and 1 μl of water; lane 4 (+pre-EGTA): 1 μl of $CaCl_2$, 1 μl of EGTA at 100 mM (final concentration 2.5 mM), and then after mixing, 1 μl of micrococcal nuclease; lanes 5-8: 1 μl of $CaCl_2$ and 1 μl of micrococcal nuclease. The samples were incubated for 5 minutes at 30°C, then 1 μl of EGTA was added to all except sample 4, which had already received it. After chelation of calcium, the sample in lane 6 received 50 μg of 16S rRNA (R), that in lane 7 received 50 μg of yeast small RNAs (Y), and the sample in lane 8 received 6 μg of human nuclear RNA (N). After mixing well, [^{32}P]-labeled RNA transcribed from AvaII cut pNS-28S was added and the protection-immunoprecipitation reaction performed as described previously, using the mouse monoclonal anti-(U3)RNP antibody 72B9 (25) for the immunoprecipitation. Lane M shows markers obtained by filling in a HpaII digest of pBR322.

the fingerprint because the downstream nucleotide (A or U) was not radioactive. Fragment B is CAG/G/UUCAG/ACAUUUG (data not shown), a shorter version of fragment A. One drawback to the double-label fingerprint technique is that short oligonucleotides of identical base content such as CAG and ACG migrate similarly on a fingerprint and may not be distinguished.

Usually, however, the surrounding oligonucleotides will allow the fragment to be precisely identified, as was the case here.

Loss of Protected-immunoprecipitated Fragments Upon Pre-treatment of the extract with Micrococcal Nuclease

The activity of micrococcal nuclease is absolutely dependent upon calcium (38). Thus small RNAs in an extract can be destroyed with this nuclease, then EGTA added to chelate the calcium (but not magnesium) and prevent degradation of the radiolabeled pre-rRNA transcripts that are added subsequently. Fig. 4 shows the effect of micrococcal nuclease on the protection-immunoprecipitation experiment. Although calcium alone (lane 2), micrococcal nuclease alone (lane 3), or micrococcal nuclease added after calcium and EGTA ("pre-EGTA"; lane 4) give results comparable to the control which only received EGTA (lane 1), pretreatment of the extract with calcium and micrococcal nuclease for 5 minutes followed by chelation with EGTA (lane 5) led to a complete loss of fragments. Adding back various RNAs such as E. coli 16S rRNA (R; lane 6), yeast small molecular weight RNAs (Y; lane 7), or human nuclear RNA (N; lane 8) did not restore this activity.

DISCUSSION

Transcripts corresponding to various regions of human or rat pre-rRNA were tested in a protection-immunoprecipitation assay for their specific interaction with the U3RNP. The transcripts depicted in Fig. 1 were radiolabeled and incubated with a nuclear extract of HeLa cells. The mixture was digested with T1 RNase, then immunoprecipitated. Although different models have predicted that U3 may function near the 3′ end of 5.8S (7,8,9), within the external transcribed spacer (Stroke and Weiner, personal communication; Maser and Calvet, personal communication), or near the 3′ end of 28S (19), none of these regions contained fragments which could be specifically immunoprecipitated with anti-(U3)RNP but not control antibodies.

One caveat to these results is that we cannot rule out the possibility that our *in vitro* conditions failed to identify an interaction which occurs *in vivo* because of any of the following reasons: 1) the conditions used were not optimal; 2) only portions of the entire pre-rRNA were present; or 3) the pre-rRNA did not have sufficient time to associate with ribosomal proteins which were necessary for an interaction. To try to address this question, an attempt was made to identify an interaction between intact ribosomes and the U3RNP by performing T1 RNase digestion of nuclear sonicates from cells whose RNA had been labeled *in vivo* for two hours (so as to preferentially

label rRNA precursors), then immunoprecipitating with anti-U3(RNP) antibodies. Unfortunately, the tremendous background in the low molecular weight region made it impossible to identify a fragment specific to the anti-(U3)RNP lane but not the anti-(U1)RNP control (data not shown). A second caveat to these negative results is that one of the regions tested (that including 5.8S rRNA) was derived from rat because the human rRNA region had not yet been sequenced and no convenient restriction sites were known to be available for cloning. However, there is no precedent for species specific RNA processing events among mammalian organisms. Furthermore, if U3 does indeed interact with ITS-II near its junction with 5.8S, as previously proposed (7,8,9), this base-pairing is maintained between human U3 (6) and rat ITS-II (28) [and in fact is slightly better than with human ITS-II (C. Chambers, J. Sylvester, and R. Schmickel, personal communication)].

Surprisingly, the only transcript which gave a positive signal contained sequences from mature 28S rRNA. Two overlapping protected-immunoprecipitated fragments were identified using a novel fingerprinting technique in which a [^{3}H]-labeled total transcript served as an internal standard for [^{32}P]-labeled oligonucleotides derived from the fragment. Fragments A and B (see Fig. 2) correspond to nucleotides 4570-4590 and 4575-4590 of 28S (37), respectively. They are located approximately 400 nucleotides upstream of the 3' end of 28S. Intriguingly, this region lies within 10 nucleotides of the only region of rRNA that can be cleaved by the RNase α-sarcin in the intact ribosome (39,40).

The fact that immunoprecipitation of these protected fragments is lost by pre-treating the nuclear extract with micrococcal nuclease (Fig. 4) suggests that an RNA may be directly involved in the interaction. We therefore looked for sequence complementarity between the U3 RNA and the protected fragments. A portion of a secondary structure model for human 28S rRNA (37) near the α-sarcin site is based on that of Hogan et al. for yeast 26S (41) and is shown in Fig. 5A. The protected region is boxed, and the site of cleavage by α-sarcin (39,40) and the site of modification by ricin (42) indicated. Fig. 5B shows a putative 8 out of 9 base-pair interaction between this region and human U3 RNA [positions 104-112 (6)].

The base-pairing interaction shown in Fig. 5B correlates well with what is known about the structure of the U3RNP. First, this sequence is highly conserved and has been demonstrated to be single stranded and thus available in the human (19) and Xenopus (43) U3RNP. Secondly, RNA fragments from the

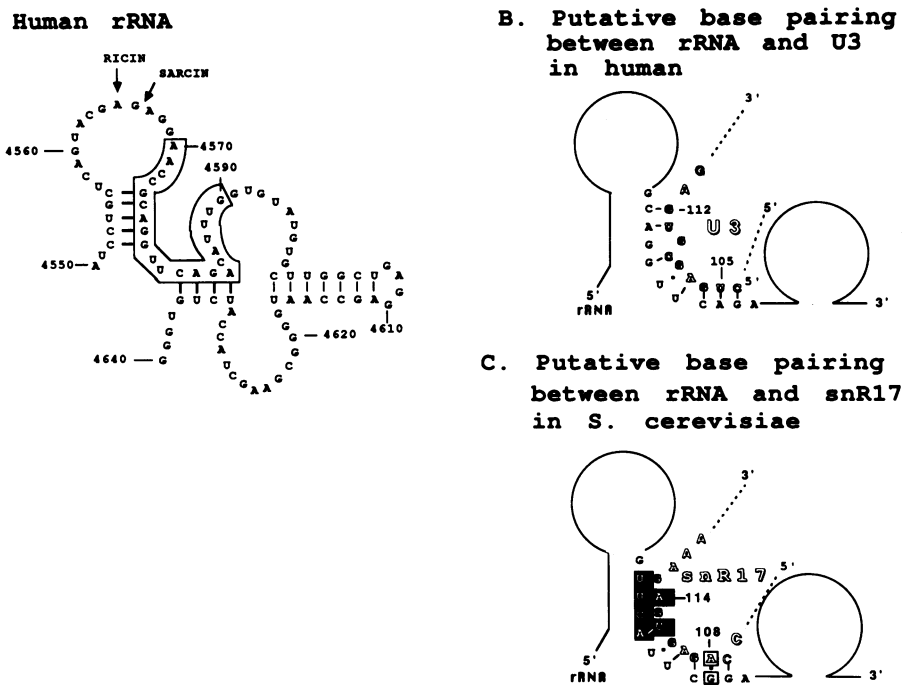

Figure 5. Secondary structure of the protected region and potential base pairing of this sequence with U3. A. A secondary structure model for the region of human 28S (37) immunoprecipitated with anti-(U3)RNP antibodies. The sequence of the fragment (A) is boxed; fragment B includes nucleotides 4575-4590. The site of cleavage by α-sarcin (39,40) and the base that undergoes modification by ricin (42) are indicated by arrows. B. Potential base-pairing between nucleotides 104-112 of human U3 (6) and the protected region. C. Potential base-pairing between nucleotides 107-115 of the S. cerevisiae U3 homolog, snR17 (13), and the homologous region of 26S (40). All differences from the human sequence are boxed; mutations which maintain or improve base-pairing are boxed in black.

3′ domain of human U3 (nucleotides 71-217) were previously shown to be immunoprecipitated after treating the U3RNP with RNase T1 or A (19), while fragments from the 5′ domain (nucleotides 1-64) were absent. This was interpreted to mean that the sequence connecting the two domains (positions 65-70) is highly susceptible to nuclease, and that the antigenic 34 kD protein interacts directly or indirectly with the 3′ domain only. Since the complementary U3 sequence (104-112) lies within the 3′ domain, an explanation as to why anti-TMG antibodies do not immunoprecipitate these protected fragments whereas anti-(U3)RNP antibodies do is provided.

If the putative base-pairing shown in Fig. 5B is of significance, it

should be conserved across species. In rat (44,45,46) and Xenopus (43,47), both the U3 and the rRNA sequence are conserved. In S. cerevisiae (13,40) there are several alterations as shown in Fig. 5C, but a striking conservation of complementarity is maintained.

One argument against the base-pairing model shown in Figs. 5B and C is that it involves one side of what is considered to be a phylogenetically proven stem of 28S rRNA (41). Since the constructs used always included both halves of the stem, however, this would suggest that the region may be accessible for base-pairing in newly synthesized RNA, but that subsequent isomerization would prevent U3 from binding permanently.

The results of our protection-immunoprecipitation experiments were totally unanticipated. It is quite possible that the detected interaction is spurious, having nothing to do with the true function of the U3RNP. For example, this rRNA sequence could resemble a sequence that is normally recognized by the U3RNP, but is only made accessible for binding by these artificial in vitro conditions. Yet, since a total of 4 kb of other regions of the 13.5 kb precursor were negative in this assay, and the putative base-pairing between U3 and this region of 28S is phylogenetically conserved, the very exciting possibility exists that the interaction we have identified in vitro relates to the function of U3.

The region of 28S rRNA which interacts with the U3RNP is cleaved by α-sarcin (39,40) and modified by ricin (42) (see Fig. 5A). This is a highly conserved region which appears to interact with translation elongation factors in intact prokaryotic ribosomes (48). Furthermore, 5.8S rRNA may interact with this region in the 60S subunit (49), even though it originates 5 kB upstream on the rRNA primary transcript and its strongest interaction is probably with the 5′ region of 28S (50). Cleavage of 28S with α-sarcin leads to dissociation of 5.8S from 28S (50) as well as inactivation of the ribosome. Although it is highly unlikely that U3RNPs interact with the mature ribosome, one might envision that this region requires auxiliary factors to fold correctly in the nucleolus and that the U3RNP serves as a sort of catalyst for assembly. The fact that the mammalian U3 RNA has been found associated with a 28-35S RNA (3,10,11) is consistent with this model, as a 32S rRNA precursor exists which contains 5.8S, ITS-II, and 28S sequences (1). The association of the S. cerevisiae U3 homolog (snR17) with the largest (35S) rRNA precursor (51) is not inconsistent with this model if in this species U3 could function more rapidly.

Alternatively, the U3 RNP could still be involved in processing the 3′

end of 28S since the α-sarcin region appears close in terms of secondary structure. An RNA whose structure is so highly conserved as 28S could easily utilize mature sequences distant from the processing site; 5S rRNA recognition by RNase M5 is a known example (52). However, arguing against this possiblity is the fact that the α-sarcin region of S. cerevisiae 26S rRNA can be deleted with no effect on 3' end formation in vivo (22). Likewise, the correct 3' end of Xenopus 28S can be formed with only a very short sequence in vitro or in vivo (23), but the efficiency in this case was extremely low, implying that auxillary sequences may contribute.

In addition to the possibility that the U3 RNP could function in assembly or 3' end processing of 28S, it is possible that recognition of the α-sarcin region could allow it to play some other role, such as nuclear-cytoplasmic transport. Clearly, further experiments will be necessary to determine if the U3RNP-28S rRNA interaction observed here also occurs in vivo and, if so, to delineate what process requires this interaction. One approach underway in our laboratory is to obtain more U3 and rRNA sequence data, and determine if the sequence complementarity is maintained across additional species.

ACKNOWLEDGEMENTS

We would like to thank N. Arnheim, L. Rothblum, and R. Schmickel for generous sharing of clones; E. Tan, J. Hardin, and J. Craft for kindly providing antibodies; I. Stroke and A. Weiner, R. Maser and J. Calvet, and C. Chambers, J. Sylvester and R. Schmickel for sharing unpublished data. We thank I. Stroke, N. Pace, and I. Wool for suggestions on the manuscript. KAP is a recipient of a National Institutes of Health Physician Scientist Award and JAS is supported by GM grant 26154.

*To whom correspondence should be addressed

+Present address: Department of Cell Biology, The Howard Hughes Medical Institute, One Baylor Plaza, Baylor College of Medicine, Houston, TX 77030, USA

REFERENCES

1. Hadjiolov, A.A. (1985) The Nucleolus and Ribosome Biogenesis, Springer-Verlag, New York.
2. Weinberg, R.A., and Penman, S. (1968) J. Molec. Biol. 38, 289-304.
3. Prestayko, A. W., Tonato, M., and Busch, H. (1970) J. Molec. Biol. 47, 505-515.
4. Busch, H., Reddy, R., and Choi, Y.C. (1982) Annu. Rev. Biochem. 51, 617-654.
5. Lischwe, M.A., Ochs, R.L., Reddy, R., Cook, R.G., Yeoman, L.C., Tan,

E.M., Reichlin, M., and Busch, H. (1985) J. Biol. Chem. 260, 14304-14310.
6. Suh, D.,Busch, H., and Reddy, R. (1986) Biochem. Biophys. Res. Comm. 137, 1133-1140.
7. Bachellerie, J.-P., Michot, B., and Raynal, F. (1983) Mol. Biol. Rep. 9, 79-86.
8. Crouch, R.J., Kanaya, S., and Earl, P.L. (1983) Mol. Biol. Rep. 9, 75-78.
9. Tague, B.W. and Gerbi, S.A. (1984) J. Molec. Evol. 20, 362-367.
10. Zieve, G., and Penman, S. (1976) Cell 8, 19-31.
11. Calvet, J.P., and Pederson, T. (1981) Cell 26, 363-370.
12. Wise, J.A., and Weiner, A.M. (1980) Cell 22, 109-118.
13. Hughes, J.M.X., Konings, D.A.M., and Cesareni, G. (1987) EMBO J. 6, 2145-2155.
14. Ozaki, T., Hoshikawa, Y., Iida, Y., and Iwabuchi, M. (1984) Nucl. Acids Res. 12, 4171-4184.
15. Veldman, G.M., Klootwijk, J., von Heerikhuizen, H., and Planta, R.J. (1981) Nucl. Acids Res. 9, 4847-4862.
16. Financsek, I., Mizumoto, K., Mishima, Y., and Muramatsu, M. (1982) Proc. Natl. Acad. Sci. USA 79, 3092-3096.
17. Kass, S., Craig, N., and Sollner-Webb, B. (1987) Mol. Cell. Biol. 7, 2891-2898.
18. Miller, K.G., and Sollner-Webb, B. (1981) Cell 27, 165-174.
19. Parker, K.A. and Steitz, J.A. (1987) Mol. Cell. Biol. 7, 2899-2913.
20. Grummt, I. Maier, U., Ohrlein, A., Hassouna, N. and J.P.-Bachellerie (1985) Cell 43, 801-810.
21. Gurney, T. Jr. (1985) Nucl. Acids Res. 13, 4905-4919.
22. Kempers-Veenstra, A.E., Oliemans, J., Offenberg, H., Dekker, A.F., Piper, P.W., Planta, R.J., and Klootwijk, J. (1986) EMBO J. 5, 2703-2710.
23. Labhart, P. and Reeder, R.H. (1986) Cell 45, 431-443.
24. Mount, S.M., Pettersson, I., Hinterberger, M., Karmas, A., and Steitz, J.A. (1983) Cell 33, 509-518.
25. Reimer, G., Pollard, K.M., Penning, C.A., Ochs, R,L., Lischwe, M.A., Busch, H., and Tan, E.M. (1987) Arth and Rheum. 30, 795-800.
26. Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
27. Miesfeld, R. and Arnheim, N. (1982) Nucl. Acids Res. 10, 3933-3949.
28. Subrahmanyam, C.S., Cassidy, B., Busch, H. and Rothblum, L. (1982) Nucl. Acids Res 10, 3667-3680.
29. Erickson, J.M., Rushford, C.L., Dorney, D.J., Wilson, G.N., and Schmickel, R.D. (1981) Gene 16, 1-9.
30. Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K., and Green, M.R. (1984) Nucl. Acids Res. 12, 7035-7056.
31. Lerner, E., Lerner, M., Janeway, C. Jr., and Steitz, J.A. (1981) Proc. Nat. Acad. Sci. USA 78, 2737-2741.
32. Dignam, J.D., Lebovitz, R.M., and Roeder, R.G. (1983) Nucl. Acids Res. 11, 1475-1489.
33. Black, D.L., Chabot, B., and Steitz, J.A. (1985) Cell 42, 737-750.
34. Barrell, B.G. (1971) Proc. Nucl. Acid Res. 2, 751-779.
35. Chabot, B., Black, D.L., LeMaster, D.M., and Steitz, J.A. (1985) Science 230, 1344-1349.
36. Doolittle, W.F. and Pace, N.R. (1971) Proc. Natl. Acad. Sci. USA 68, 1786-1790.
37. Gonzalez, I.L., Gorski, J.L., Campen, T.J., Dorney, D.J., Erickson,

J.M., Sylvester, J.E., and Schmickel, R.D.(1985) Proc. Natl. Acad. Sci. 82, 7666-7670.
38. Pelham, H.R.B. and Jackson, R.J. (1976) Eur. J. Biochem. 67, 247-256.
39. Endo, Y., and Wool, I.G. (1982) J. Biol. Chem. 257, 9054-9060.
40. Veldman, G.M., Klootwijk, J., deRegt, V.C.H.F., Planta, R., Brantlant, C., Krol, A., and Ebel, J.P. (1981) Nucl. Acids Res. 9, 6935-6952.
41. Hogan, J.J., Gutell, R.R., and Noller, H.F. (1984) Biochem. 23, 3330-3335.
42. Endo, Y., Mitsui, K., Motizuki, M., and Tsurugi, K. (1987) J. Biol. Chem. 262, 5908-5912.
43. Jeppesen, C., Stebbins-Boaz, B., and Gerbi, S.A. (1988) Nucl. Acids Res. 16, 2127-2148.
44. Stroke, I.L., and Weiner, A.M. (1985) J. Mol. Biol. 184, 183-193.
45. Reddy, R., Henning, D., and Busch, H. (1979) J. Biol. Chem. 254, 11097-11105.
46. Chan, Y.L., Olvera, J., and Wool, I.G. (1983) Nucl. Acids Res. 11 7819-7831.
47. Ware, V.C., Tague, B.W., Clark, C.G., Gourse, R.L., Brand, R.C., and Gerbi, S.A. (1983) Nucl. Acids Res. 11, 7795-7817.
48. Moazed, D., Robertson, J.M., and Noller, H.F. (1988) Nature 334, 362-364.
49. Choi, Y.C. (1985) J. Biol. Chem. 260, 12773-12779.
50. Walker, T A., Endo, Y., Wheat, W.H., Wool, I.G., and Pace, N.R. (1983) J. Biol. Chem. 258, 333-338.
51. Tollervey, D. (1987) EMBO J. 6, 4169-4175.
52. Meyhack, B. and Pace, N.R. (1978) Biochem. 17, 5804-5810.