Insect Molecular Biology (2005) **14**(5), 523–536

Heterologous rRNA gene expression: internal fragmentation of *Sciara coprophila* 28S rRNA within microinjected *Xenopus laevis* oocytes

A. E. Basile-Borgia, D. A. Dunbar[1] and V. C. Ware

Department of Biological Sciences, Lehigh University, Bethlehem, PA, USA

Abstract

Species-specific pre-rRNA processing variations may result in fragmented 18S, 5.8S and 28S rRNAs. Some insect 5.8S and 28S rRNAs are further cleaved, creating within a 'hidden break' or 'gap'. We investigated the specificity of the processing mechanism by microinjecting *Sciara coprophila* (fungus fly) rDNA into *Xenopus laevis* oocytes to examine insect rRNA maturation within a cell where endogenous rRNAs are not cleaved at homologous sites. Results confirm insect rDNA transcription and pre-28S rRNA fragmentation, demonstrating that fly-specific processing machinery is not required. Instead, oocytes may provide required accessory factors, suggesting that the insect gap processing mechanism is served by an evolutionarily conserved apparatus. Alternatively, these results may suggest that processing in some lineages is an autocatalytic property of the rRNA.

Keywords: rRNA, processing, hidden break, fragmentation, gap, insect.

Introduction

Eukaryotic 18S, 5.8S and 28S rRNAs are transcribed as a single 45S pre-rRNA that is subsequently processed by cleavage and degradation of external- and internal transcribed spacer (ETS and ITS) sequences within the nucleolus. Additional excision events have been demonstrated in a wide variety of organisms, including prokaryotes and eukaryotes, and may include one or several internal cleavages within an rRNA species. The biological significance of differential rRNA processing has not been explored experimentally; thus it is not known how split rRNAs may impact ribosome assembly and/or function.

Two different patterns of rRNA processing outcomes may be distinguished where additional excision events have been described. In one case, the excised nucleotides are 'introns' which are removed and exons are subsequently ligated to create a contiguous rRNA molecule. The well studied autocatalytic group I introns, located within the coding region of pre-rRNA in the ciliate, *Tetrahymena thermophila* (reviewed by Cech, 2002), and the slime mold, *Physarum polycephalum* (e.g. Rocheleau & Woodson, 1994), are examples of excised sequences in the intron category. An extensive array of more than 1200 introns, including those of the Group I type, are dispersed at several conserved locations within 18S and 28S rRNAs, often found within tRNA binding sites or within regions known to be at the subunit interface (reviewed by Jackson *et al.*, 2002). In other cases, the excised nucleotides are treated as 'spacers' with no subsequent covalent ligation of the retained rRNA coding segments. One of the more remarkable fragmentation patterns in this latter category involves *Euglena* 28S rRNA, which is a collage of 13 RNAs, resulting from the removal of 12 segments of sequence (Schnare & Gray, 1990). In some species, additional spacers interrupt both the 5.8S and 28S rRNA coding regions, so that each of these rRNAs is further split (Pavlakis *et al.*, 1979; Jordan *et al.*, 1980; Shimada, 1992).

Additional processing of the central domain of 28S rRNA, creating two nearly equally sized rRNAs (e.g. Applebaum *et al.*, 1966; Balazs & Agosin, 1968; Greenberg, 1969; Ishikawa & Newburgh, 1972; Ishikawa, 1975a,b; DeLanversin & Jacq, 1989), designated 28Sα and 28Sβ (Ishikawa & Newburgh, 1972), has also been referred to as 'gap' processing (Ware *et al.*, 1985). The central 'hidden break' in 28S rRNA has primarily been described in insects, other protostomes, and protozoa (Ishikawa, 1973, 1975a,b), and is masked through non-covalent RNA interactions including hydrogen bonding at regions close to the cleavage site (Wollenzien *et al.*, 1978) and probably through other long-range RNA–RNA interactions.

doi: 10.1111/j.1365-2583.2005.00583.x

Received 6 January 2005; accepted after revision 6 May 2005. Correspondence: V. C. Ware, Department of Biological Sciences, Lehigh University, Bethlehem, PA 18015, USA. Tel.: +1 610 758 3690; fax: +1 610 758 4004; e-mail: vcw0@lehigh.edu

[1]Present Address: Science Department, Cabrini College, Radnor, PA 19087–3698, USA.

© 2005 The Royal Entomological Society

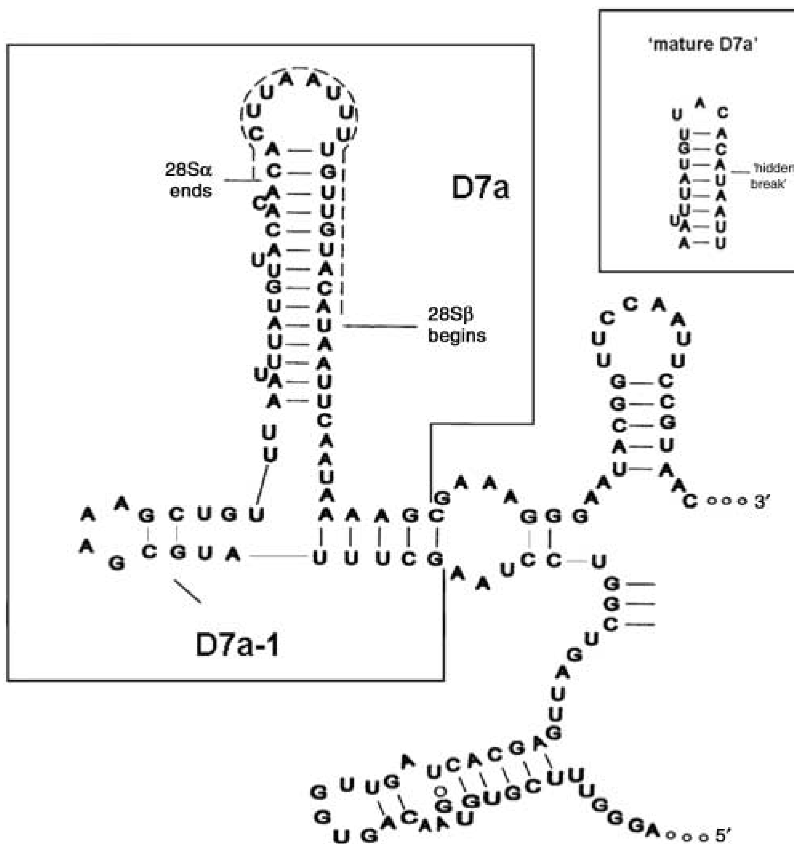

Figure 1. Secondary structure in the gap region of S. coprophila 28S rRNA. A portion of Domain III of *Sciara* 28S rRNA is shown based on consensus models (Cannone *et al*., 2002). The D7a expansion segment is shown within the large box. The dotted line indicates the nucleotides that are removed in gap processing (Ware *et al*., 1985), leaving the mature ends of 28Sα and 28Sβ rRNAs as shown. The small box inset (upper right) shows a portion of the 'mature D7a' expansion segment after gap processing. Note that the nucleotides at the position of the 'hidden break' are not covalently joined; 28Sα and 28Sβ rRNAs remain as separate entities.

In secondary structure models of eukaryotic 28S rRNA, the gap processing site maps to expansion segment D7a (DeLanversin & Jacq, 1989) in the central domain. The excision event removes a variable number of nucleotides from the D7a expansion segment, depending on the species in question. In some instances the exact sites for nucleotide excision have been mapped, allowing investigators to determine the exact numbers of nucleotides that are removed from the gap region. For example, in the fungus fly, *Sciara coprophila*, 19 nucleotides are removed (Ware *et al*., 1985; see Fig. 1 based on Cannone *et al*., 2002); 30 nucleotides from the 28S rRNA of the silk moth, *Bombyx mori* (Fujiwara & Ishikawa, 1986), 54 nucleotides from the 28S rRNA of the blood fluke *Schistosoma mansoni* (Van Keulen *et al*., 1991), approximately 60 (Ware *et al*., 1985) to 75 nucleotides (DeLanversin & Jacq, 1983, 1989) from the 26S rRNA of the fruitfly, *Drosophila melanogaster*, are excised. The gap region in 28S rRNA of the nematode worm, *Trichinella spiralis*, is 86 nucleotides long (Zarlenga & Dame, 1992). The gap region in one species of *Trichinella* may be as large as 202 nucleotides, based on structural comparisons of D7a expansion segments and the 28S rRNA gap boundary as defined in *T. spiralis*.

The gap processing site also interrupts an evolutionarily conserved binding site for ribosomal protein L23 (L25 in yeast), present within all known 23S–28S rRNAs (e.g. Raué *et al*., 1985). Expansion segment D7a in the yeast, *Saccharomyces cerevisiae*, resides in the middle of the site to which ribosomal protein L25 binds (El-Baradi *et al*., 1984; Raué *et al*., 1985). Ribosomal protein L23/25 is an essential RNA-binding protein, among the proteins that bind early to pre-rRNA, and is necessary (along with other ribosomal proteins and *trans*-acting factors (Kressler *et al*., 1999; Venema & Tollervey, 1999) for ITS2 processing and 60S subunit assembly (van Beekvelt *et al*., 2001). Thus, the stability of the fragmented rRNAs is likely to be augmented by RNA–protein interactions as well. It is noted that the length of expansion segment D7a is quite variable, even in organisms wherein the gap processing pathway is not present. Is there a structural size limitation for D7a beyond which L23 binding to 28S rRNA is affected? It is unclear in organisms wherein the gap processing pathway does exist if D7a processing is a necessary step for L23 binding to 28S rRNA. Alternatively, L23 may be a component of the gap processing apparatus or may be a scaffold required for the assembly of gap processing machinery.

In the dipterans *D. melanogaster* and *S. coprophila* a second 'gap' within pre-rRNA has been documented, whereby the pre-5.8S rRNA is cleaved near its 3′ end to yield the '5.8S' and 2S rRNAs (Jordan *et al*., 1976, 1980;

Table 1. Comparison of putative processing features in several lineages. NA, information not available. +, presence; –, absence

Organism	Gap processing	UAAU	D7a base composition	References
D. melanogaster (fruitfly)	+	+	AU rich	DeLanversin & Jacq, 1983; Ware *et al*., 1985
B. mori (silk moth)	+	+	AU rich	Fujiwara & Ishikawa, 1986
S. coprophila (fungus fly)	+	+	AU rich	Ware *et al*., 1985
S. mansoni (blood fluke)	+	+	Less AU rich	Van Keulen *et al*., 1991
T. thermophila (ciliate)	+	–	AU rich	Engberg & Nielsen, 1990
A. albopictus (mosquito)	+	–	AU rich	Kjer *et al*., 1994
T. spiralis (nematode worm)	+	–	AU rich	Zarlenga & Dame, 1992
Simulium santipauli (black fly)	+	NA	AU rich	Morales-Hojas *et al*., 2002
Acyrthosiphon pisum (pea aphid)	–	–	GC rich	Ogino *et al*., 1990; Amako *et al*., 1996
X. laevis (frog)	–	–	GC rich	Clark *et al*., 1984
S. cerevisiae (baker's yeast)	–	–	AU rich	Veldman *et al*., 1981
P. polycephalum (slime mold)	–	–	AU rich	Otsuka *et al*., 1983
M. musculus (mouse)	–	–	GC rich	Michot *et al*., 1984

Pavlakis *et al*., 1979). Likewise, hydrogen bonding at regions near the cleavage site is credited with maintaining the association of these small rRNAs (Pavlakis *et al*., 1979), just as has been described for the 28S moieties.

It has been proposed that in organisms where gap processing occurs, the D7a expansion segment may form an AU-rich loop at the apex of a stem with a conserved sequence just upstream of the 5′ end of 28Sβ rRNA (Fujiwara & Ishikawa, 1986). Within the AU-rich loop, the sequence UAAU has been proposed as a putative processing signal (Fujiwara & Ishikawa, 1986). Other conserved sequence features have been proposed as well, including the presence of base duplications around the gap region (Ware *et al*., 1985). In contrast, the comparable D7a expansion segment from organisms (e.g. *Xenopus*, Clark *et al*., 1984; *Saccharomyces*, Veldman *et al*., 1981; *Physarum*, Otsuka *et al*., 1983; *Mus*, Michot *et al*., 1984) that lack the gap processing pathway is GC-rich throughout. Interestingly, the aphid group is an enigma among the insect groups; aphid 28S rRNA is not subject to gap processing. The aphid D7a expansion segment contains a GC rich stem-loop region and lacks the putative UAAU signal (Ogino *et al*., 1990); as such, the aphid D7a expansion segment has structural features more commonly associated with D7a expansion segments in organisms that do not have the gap processing pathway.

In a survey of several organisms that sponsor gap processing, it is apparent that some of the proposed processing signals are not universally conserved among all groups (see Table 1). The putative processing signal UAAU is absent from the 28S rRNA D7a expansion segment within the ciliate *T. thermophila* (Engberg & Nielsen, 1990; Ware, unpublished results), the nematode worm *T. spiralis* (Zarlenga & Dame, 1992), and the mosquito *Aedes albopictus* (Kjer *et al*., 1994). It is also noteworthy that the proposed UAAU signal is not unique to dipteran D7a expansion segments that undergo excision, as it is present within the D7a expansion segment of *S. mansoni*. The UAAU signal is repeated twice within the *D. melanogaster* '5.8S'/2S region, but is absent from the *S. coprophila* '5.8S'/2S region (Pavlakis *et al*., 1979). Thus, no consensus sequence for excision is readily apparent for 5.8S and 28S rRNA processing, although the excision occurs in both instances within an AU-rich stem-loop region (Fujiwara & Ishikawa, 1986). As more sequences and gap region boundaries are determined, comparative analyses may offer opportunities to refine proposals for specific sequences involved in specifying gap processing. Whether or not the current proposed sequence elements in the D7a expansion segment constitute any portion of the relevant signals for the gap processing machinery remains to be tested formally.

Although the end products of the eukaryotic ribosomal processing pathway are well defined, the enzymatic machinery and putative accessory components that mediate rRNA processing are not. Several important steps in general pre-rRNA cleavage or modification require interactions of small nucleolar RNPs (snoRNPs) (reviewed by Eliceiri, 1999; Venema & Tollervey, 1999; Bachellerie *et al*., 2002; Terns & Terns, 2002). For example, in mouse *in vitro* extracts, the U3, U14, E1/U17 and E3 snoRNAs are necessary for the first cleavage step of pre-rRNA within the 5′ external transcribed spacer (ETS) (Kass *et al*., 1990; Enright *et al*., 1996; see Fig. 2 for layout of rDNA complex). The U3 snoRNA is required for ETS cleavage in frog *in vitro* extracts (Mougey *et al*., 1993). Disruption of U3, U22, E1/U17 and E2 snoRNAs function inhibits cleavage steps necessary for 18S rRNA maturation (Savino & Gerbi, 1990; Tycowski *et al*., 1994; Mishra & Eliceiri, 1997). In *Xenopus* oocytes, U3, E3 and U8 snoRNAs are among the machinery required for processing of large subunit rRNA precursors. All three snoRNAs affect 5.8S rRNA processing (Savino & Gerbi, 1990; Peculis & Steitz, 1993; Mishra & Eliceiri, 1997). The U8 snoRNA is also required for 28S rRNA production (Peculis & Steitz, 1993). Thus far, the majority of snoRNAs appear to be required for rRNA modifications, including 2′-O-ribose methylation (e.g. Kiss-Laszlo *et al*.,

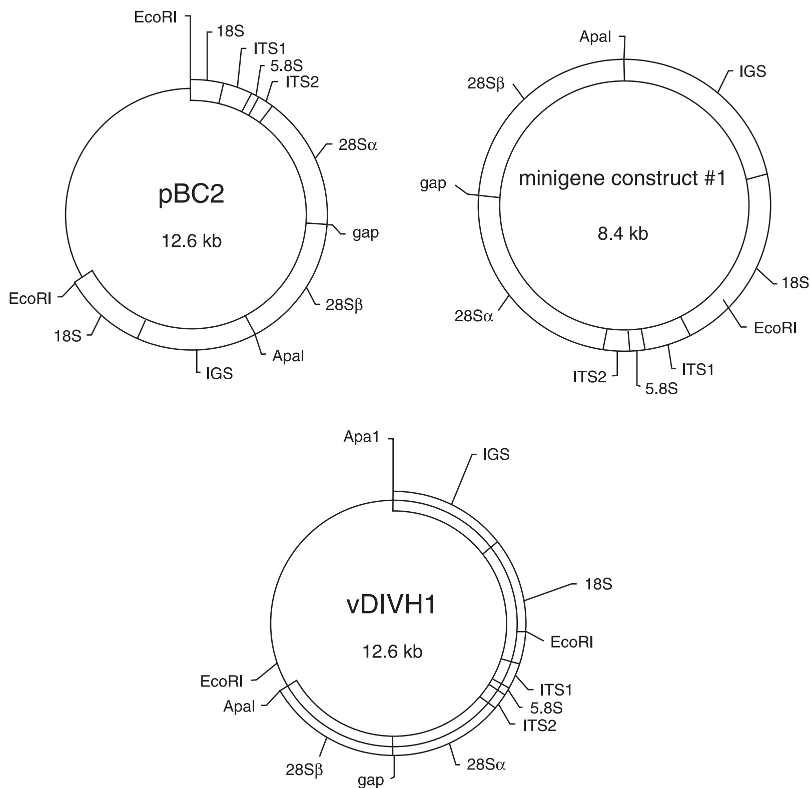

Figure 2. Map of *Sciara* rDNA constructs. Plasmid pBC2 contains a complete rDNA unit cloned into the *Eco*RI site of pBR322 (Renkawitz *et al.*, 1979). Intergenic spacer (IGS), 18S, internal transcribed spacer 1 (ITS1), 5.8S, ITS2 and 28S coding regions are shown. A 'gap' of 19 nucleotides separates 28Sα and 28Sβ coding segments (Ware *et al.*, 1985). Digestion of pBC2 with *Eco*RI and subsequent intramolecular ligation of the 8.4 kbp insert generated minigene construct #1. Digestion of the 8.4 kbp intramolecular ligation product with *Apa*I and subsequent ligation into the *Apa*I site of pRS304 (GenBank Accession Number U03436) generated plasmid vDIVH1. Plasmids are not drawn to scale.

Table 2. Primer pairs used to generate PCR products in RT-PCR experiments

Product	Complementary primer	RNA-like primer
111 bp	β_1: 5′-TATGGTCGTTCCTGTTGCC-3′	β_3: 5′-TTCAATAAAAGCGAAAGGG-3′
162 bp	β_1: 5′-TATGGTCGTTCCTGTTGCC-3′	α_3: 5′-TGCGAAAGCTGTTTAATTT-3′

1996; Nicoloso *et al.*, 1996) or for conversion of uridine nucleotides to pseudouridine at specific sites (Ganot *et al.*, 1997; Ni *et al.*, 1997). Many interactions, required for general rRNA cleavage and modification, involving snoRNPs and rRNA are evolutionarily conserved between lineages (see Bachellerie *et al.*, 2002). In contrast, the conservation of rRNA structural elements within the D7a expansion segment is not as remarkable; considerable length and sequence variation exists within D7a expansion segments even between closely related organisms (Schnare *et al.*, 1996). Thus, it is unclear if a conserved set of accessory components (if required at all) contributes to the gap processing mechanism. Alternatively, the lack of conservation of excision signals may suggest that accessory components have coevolved with the variable D7a expansion segment in some lineages.

Multiple 3′ → 5′ exoribonucleases are contained within the exosome, a processing complex required for the maturation and degradation of multiple RNA species, including 5.8S rRNA (e.g. Allmang *et al.*, 2000). Studies in yeast have yielded the most information about the functions of *trans*-acting factors involved in rRNA processing and ribosome assembly, yet many of the identified components are not defined functionally (e.g. Venema & Tollervery, 1999).

Although progress has been made in identifying components of the general pre-rRNA processing pathways (e.g. Dragon *et al.*, 2002; Grandi *et al.*, 2002; Nissan *et al.*, 2002; reviewed in Granneman & Baserga, 2004), virtually nothing is known about the machinery required for any extra cleavage events that have been described in some organisms. At least two groups (Lava-Sanchez & Puppo, 1975; Burgin *et al.*, 1990) have demonstrated that cleavages within large subunit rRNA can be mimicked *in vitro* using RNases, suggesting that internal fragmentation of 28S rRNA (including gap processing) is dependent on enzyme activities. It is not known if specialized processing machinery is required for gap processing or if the general processing apparatus is utilized in these special cases. A corollary of this is that it is not known if organisms that lack the gap processing

pathway have the required enzymatic machinery for that pathway, but lack the appropriate signals within rRNA for substrate recognition. It is possible that the machinery responsible for these species-specific cleavages has other functions in a wider range of organisms. Therefore, it is of interest to determine the extent of conservation of the processing machinery between organisms that carry out differential rRNA processing.

In this regard, an approach to understanding the degree of conservation of the processing machinery in eukaryotes is described in this study, whereby rDNAs from an insect species were microinjected into the germinal vesicle of amphibian oocytes. S1 nuclease protection and RNA blot experiments suggest that *S. coprophila* rDNA is transcribed to some extent in *X. laevis* oocytes (Supplementary Fig. S1). These data, coupled with results from other studies where the RNA polymerase I transcription specificity question has been re-examined (Trendelenburg *et al*., 1978; Culotta *et al*., 1987), suggest that a heterologous gene expression approach may provide a viable strategy to ask specific questions about the conservation and specificity of the rRNA processing apparatus. DNA constructs containing a complete rDNA repeat from *S. coprophila* were used in this study. We report that microinjected *S. coprophila* rDNA is transcribed within *X. laevis* oocytes and that insect 28S rRNA transcripts are apparently fragmented in the gap region.

Results

Sciara *rDNA is expressed in microinjected* Xenopus *oocytes*

Two types of rDNA constructs containing *Sciara* rDNA are shown in Fig. 2. Construction of rDNAs was confirmed by restriction analysis (Supplementary Fig. S3). Preliminary S1 nuclease protection experiments, coupled with RNA blot analysis and primer extension suggested that the *Sciara* rDNA minigene construct #1 is transcribed, albeit at low levels within oocytes (Supplementary Figs S1 and S2). In order to standardize the construction of an insect rDNA, eliminating the necessity to perform multiple rounds of DNA isolation and ligation to construct the minigene for multiple experiments, we constructed plasmid vDIVH1 (Fig. 2).

Our previous experience indicated that the level of insect transcription within microinjected oocytes was generally low; therefore, we used RT-PCR as a more sensitive assay to detect the presence of insect transcripts within oocytes microinjected with clone vDIVH1 or the minigene construct #1. Two different sized RT-PCR products were possible depending on the sets of 28S-specific primers used in accordance with the sequence of *Sciara* 28S rDNA in this region (Ware *et al*., 1985; Fig. 3, Table 2). In one case a 111 bp product would be indicative of the presence of 28Sβ rRNA sequences, but is not intended to confirm the presence or absence of gap processing. A 162 bp product would indicate the presence of insect-specific transcripts that span the gap region, indicating the presence of precursor 28S rRNAs. Both RT-PCR products could be generated in most experiments with the addition of the appropriate combinations of 28S-specific primers (e.g. Fig. 3). The 111 bp and 162 bp products were generated from *Sciara* total RNA (lane 4) as well as from RNA isolated from oocytes microinjected with the vDIVH1 clone (lanes 5 and 6). No products were detected from *Xenopus* RNA, confirming primer specificity (lane 3), or the no RNA control (lane 2).

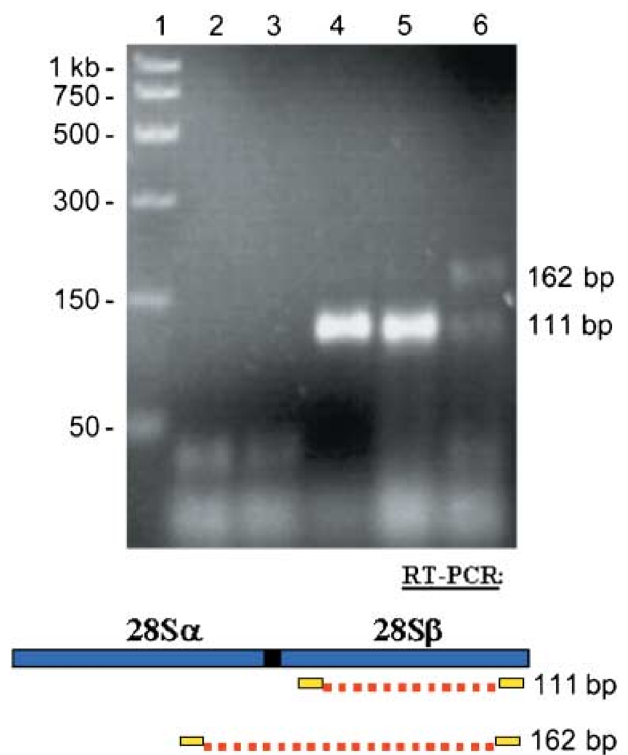

Figure 3. Detection of *Sciara* 28S rRNAs within microinjected oocytes by RT-PCR analysis. PCR products were generated from DNase-treated RNA templates. A PCR product of 111 bp was generated using primers β_1 and β_3. A PCR product of 162 bp was generated using primers β_1 and α_3 (Table 2). The 162 bp product is generated from precursor rRNA templates that contain the gap region. RNA templates used to generate PCR products are as follows. Lane 2: no RNA template added; Lane 3: *Xenopus* total RNA template; Lane 4: *Sciara* total RNA template; Lanes 5 and 6: RNA from vDIVH1-injected oocytes. DNA marker fragments, with sizes indicated on the left, are shown in Lane 1.

In separate experiments, the presence of insect-specific transcripts was confirmed in oocytes microinjected with the minigene construct #1 (Fig. 4, lanes 1, 2) or vDIVH1 (Fig. 4, lanes 5, 7). It is important to note that there was variability in the expression of the insect constructs, as shown in lane 3 for the minigene construct, where products appear to be degraded. Further, the vDIVH1 injection of oocytes in lane 6 did not yield evidence of transcription, as no 111 bp RT-PCR product is visible. The reduced product signal in lane

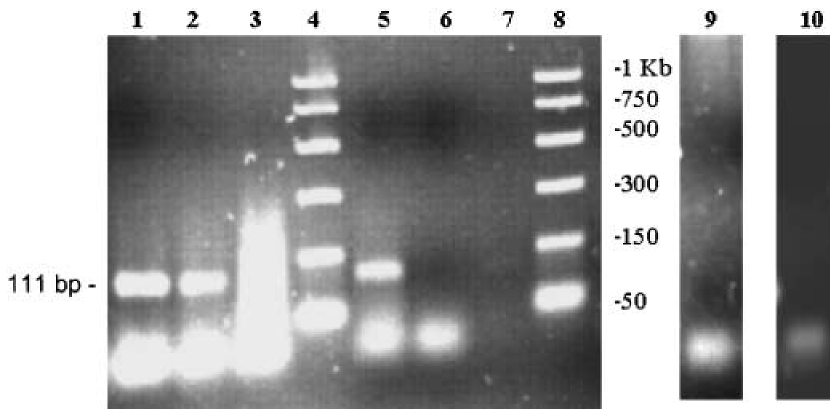

Figure 4. RT-PCR analysis of RNAs from vDIVH1- and minigene construct #1-injected oocytes. PCR products were synthesized from DNase-treated RNA templates using primers as in Fig. 2 to generate the 111 bp product, indicative of the presence of rRNAs containing 28Sβ sequences. RNA templates are as follows. Lanes 1–3: RNA from minigene construct #1-injected oocytes; Lanes 5–7: RNA from vDIVH1-injected oocytes; Lane 9: no RNA template added; Lane 10: *Xenopus* total RNA template. Marker DNAs, with fragment sizes indicated, are shown in lanes 4 and 8.

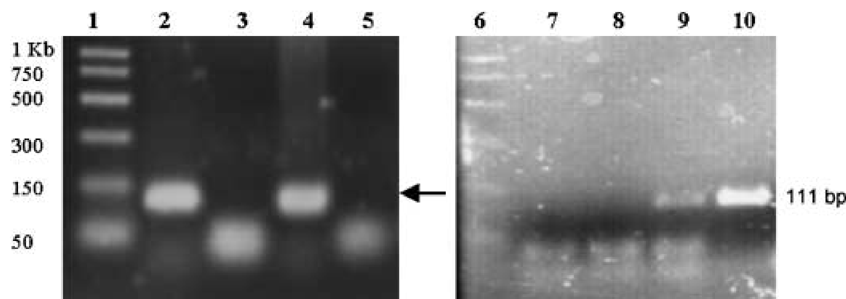

Figure 5. Template- and time-dependent appearance of *Sciara*-specific PCR products. The effectiveness of DNase treatment of samples used for RT-PCR analyses was determined by microinjecting plasmid pBC2 or vDIVH1 into oocytes and immediately isolating total RNA at time = t_0. Subsequently RNAs were subdivided into fractions for DNase treatment or no treatment and then used in RT-PCR reactions. Lane 2: untreated RNA from pBC2-injected oocytes; Lane 3: DNase-treated RNA from pBC2-injected oocytes; Lane 4: untreated RNA from vDIVH1-injected oocytes; Lane 5: DNase-treated RNA from vDIVH1-injected oocytes. The arrow indicates the position of the 111 bp PCR product, present only in samples that were not subjected to DNase treatment (lanes 2 and 4), indicating that a product could be generated from a DNA template at time t_0. DNase treatment effectively degraded the injected constructs, as no PCR product is visible in treated samples. In another experiment, RNAs were isolated at various time points after vDIVH1 injection, subjected to standard DNase treatment, and used in RT-PCR analysis. Lanes 7 and 8: RNA isolated immediately after microinjection at time t_0; Lane 9: RNA isolated at 6 h after microinjection; Lane 10: RNA isolated at 12 h after microinjection. PCR markers with sizes indicated on the left, are shown in lanes 1 and 6.

7 for another vDIVH1 microinjection experiment is probably a loading artifact, as the primer concentration is diminished as well compared to other samples. Importantly, *Sciara*-specific RT-PCR products were detected for each construct in multiple experiments.

In order to confirm that the RT-PCR signal was attributable to insect RNA templates (synthesized from injected DNA templates) and not to the microinjected DNA template itself, several controls were undertaken. In one experiment, we analysed the effectiveness of our standard DNase treatment of all RNA samples used for expression analysis. RNA was isolated from pBC2- or vDIVH1-injected oocytes immediately after construct injection and subjected to DNase treatment or no treatment. It is reasonable to assume that under these experimental conditions, no substantial DNA-dependent RNA transcription from either template would occur. Previously, S1 nuclease protection experiments had confirmed the inability of pBC2 to support a protection pattern consistent with stable heterologous transcription (Supplementary Fig. S1). The orientation of the promoter (within the IGS) and the 28S rRNA coding region within pBC2 probably precludes transcription downstream through vector sequences into the relevant 28S rRNA region (Fig. 2). Figure 5 shows that in the absence of DNase treatment, the 111 bp product can be synthesized using the microinjected DNA template (see lanes 2, 4, where pBC2 or vDIVH1 were microinjected, respectively, and RNAs isolated). However, the observation that upon DNase treatment, no detectable 111 bp product was observed (lanes 3, 5) is important. Thus, we conclude that our DNase treatment was sufficient to remove the template following RNA isolation, eliminating DNA as a potential template source for PCR production.

Additionally, a time-dependent study was performed to confirm that insect transcripts appear in a time-dependent manner post-injection within microinjected oocytes. RNAs were isolated at various time points after microinjection of vDIVH1 and subjected to the standard DNase treatment. Immediately after injection at t = 0, no 111 bp product is detected (Fig. 5, lanes 7, 8). This is consistent with results

seen above in the DNase control experiment (lanes 3 and 5). At 6 h after injection (lane 9), the product is visible. More product is visible at 12 h post-injection (lane 10), suggesting an increase in the amount of insect-specific RNA template over time. Thus, in support of an RNA template-dependent requirement for the production of the 111 bp species are the combined observations that DNase effectively degrades potential DNA templates in isolated RNA samples and that the appearance of the RT-PCR product is time-dependent post-injection.

Sciara *28S rRNA is fragmented in the gap region within* Xenopus *oocytes*

As our experiments were originally undertaken to examine processing in the gap region, we were primarily concerned with determining the internal processing pattern for *Sciara* 28S rRNA. Preliminary experiments using the minigene construct #1 (RNA blot data, Supplementary Fig. S2) suggested that *Sciara* pre-28S rRNA transcripts were fragmented within microinjected oocytes. We sought additional evidence for this possibility. Using a radiolabelled probe of approximately 320 bp, containing α and β sequences which span the gap region, we performed S1 nuclease protection experiments. If complementary RNAs are intact and devoid of internal gaps, then we would expect approximately 270 nucleotides of the full length probe to be protected from nuclease digestion, as approximately 50 nucleotides of the probe are vector-specific. The protection pattern for *Sciara* control total RNA shows two bands of approximately 130 and 110 nucleotides each, as would be expected for native *Sciara* 28S rRNA which is fragmented (Fig. 6). The protection pattern for the *Sciara* rDNA minigene construct #1 is indistinguishable from that of the *Sciara* control. Although a gross analysis of the 28S rRNA termini in the gap region is represented in this experiment, the evidence supports the conclusion that within the *Xenopus* oocyte, *Sciara* transcripts are fragmented within the gap region into α and β domains as would occur within the fly itself.

In order to gain a more detailed understanding of the extent of processing at the gap region within heterologous

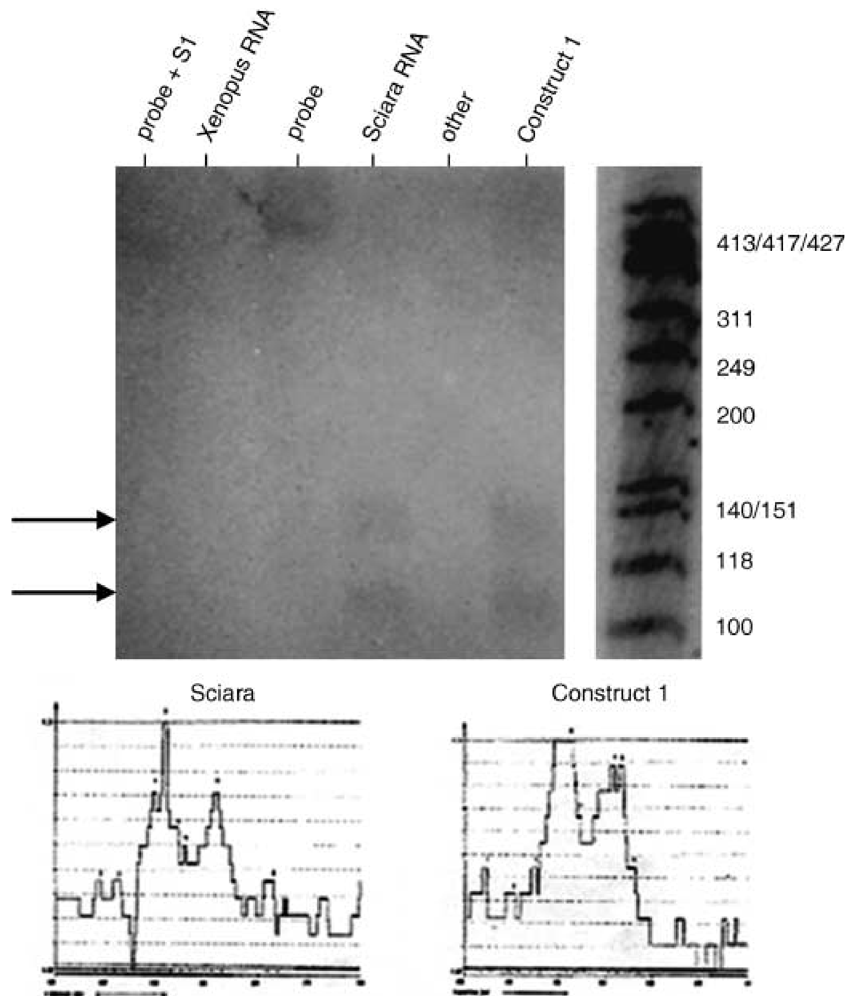

Figure 6. S1 nuclease protection analysis using a radiolabelled PCR-derived probe and construct #1-derived RNA. A radiolabelled 320 bp probe, generated from a pGEM subclone using β_1 and T7 primers and [α^{32}P]-dCTP was used in these experiments. RNAs were hybridized and treated with S1 nuclease, as outlined in Experimental procedures. Protected products were fractionated on a 10% polyacrylamide/8 M urea gel and subjected to autoradiography. Specific samples are indicated above the gel lanes in the figure. A ϕX174 *Hin*fI DNA marker, with sizes indicated, is shown in the lane on the right. Radiolabelled, denatured probe was incubated with 50 ng of *Sciara* RNA, or RNA from 30 oocytes for the *Xenopus* control and microinjected minigene construct #1 samples. The lane labelled 'other' contained probe and RNA from another type of *Xenopus/Sciara* chimeric rDNA construct not described in this paper. Protected fragments of approximately 130 and 110 bases are apparent in the *Sciara* control and construct #1 samples. Densitometric scans of the *Sciara* control and construct #1 lanes are shown in the lower portion of the figure.

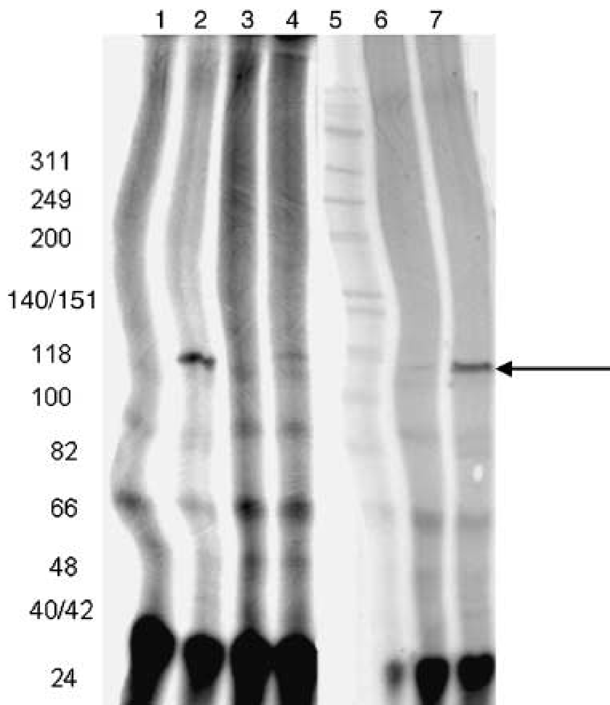

Figure 7. Primer extension analysis to determine 5′ end of vDIVHI-derived 28Sβ rRNAs. RNAs were hybridized to a molar excess of 5′-end labelled *Sciara* 28Sβ_1-specific primer (19-mer) and subjected to primer extension analysis in the presence of AMV reverse transcriptase. The 3′ end of the primer is 95 nucleotides downstream from the mature end of 28Sβ rRNA (Ware *et al.*, 1985). Extension products were analysed on a 10% polyacrylamide/8 M urea gel and subjected to phosphorimaging. Only the segment of the gel where cDNAs were present is shown. Lane 1: primer alone, no RNA added; Lane 2: *Sciara* total RNA; Lane 3: *Xenopus* total RNA; Lane 4: RNA from vDIVH1-injected oocytes; Lane 5: ϕX174 *Hin*fI marker, with sizes indicated on the left; Lane 6: RNA from vDIVH1-injected oocytes (separate experiment from lane 4); Lane 7: *Sciara* total RNA (separate experiment from lane 2). The arrow marks the position of the largest discernible cDNA extension product, coincident with the cDNA product of approximately 114 nucleotides generated from *Sciara* RNA.

transcripts, we determined the 5′ end of heterologous 28Sβ transcripts by primer extension analysis using a *Sciara*-specific β_1 oligonucleotide 19-mer primer (Fig. 7). Note that the β_1 primer used in these primer extension experiments is the same primer used to produce specific RT-PCR products that confirmed heterologous transcription of the vDIVH1 template discussed above. The 3′ end of this primer is approximately 95 nucleotides downstream from the mature 5′ end of *Sciara* 28Sβ rRNA, based on the published sequence of the gap region (Ware *et al.*, 1985). For the experiments shown (lanes 5–7 are from a separate experiment), primer extension analysis was performed using RNA samples that previously generated *Sciara*-specific RT-PCR products, as previously shown (Figs 3 and 4). The primer extension product generated using *Sciara* control RNA as a template is of the expected size of approximately 114 nucleotides (lanes 2, 7). The primer extension species produced using vDIVH1-derived RNA is comparable in size to that of *Sciara* (lanes 4, 6). Specific extension products were not generated using *Xenopus* RNA (lane 3), confirming the specificity of the primer and that the presence of extension products in the *Sciara* and vDIVH1 lanes is dependent on the presence of specific RNA templates. In some experiments, it was not always possible to detect the *Sciara*-specific extension product, probably due to variability in the levels (which were generally low) of *Sciara* RNA transcription within different oocytes. However, in each case where a specific extension product was confirmed, *Sciara* control and heterologous products were indistinguishable. Taken together, the nuclease protection and primer extension experiments show a fragmentation pattern for heterologous transcripts that is consistent with processing of pre-28S rRNA at the gap site within microinjected oocytes.

Discussion

Investigations were aimed at determining the processing fate of heterologous transcripts synthesized from *S. coprophila* rDNA constructs microinjected into *Xenopus* oocytes. These studies were undertaken in order to gain insight into the evolutionary conservation of processing machinery that may direct cleavage events in the gap region within 28S rRNA in some species. Expressed heterologous transcripts containing 28S rRNA sequences were internally cleaved similarly to endogenous *Sciara* RNAs.

Expression of Sciara *rDNA within microinjected* Xenopus *oocytes*

The presence of *Sciara* transcripts within microinjected oocytes was demonstrated by several types of analyses, suggesting that insect rDNA constructs are recognized by the *Xenopus* transcriptional machinery. It is presently unclear where transcription of *Sciara* rDNAs is initiated; transcription may be initiated at the *bona fide* insect promoter or elsewhere within the IGS or within the coding region itself. More rigorous analysis of transcription initiation sites, beyond the scope of this study, is necessary to address this specific issue. The efficiency of *Sciara* rDNA transcription within the frog oocyte system is considerably less than endogenous transcription from frog amplified rDNA units where as much as 5 μg of rRNA can be recovered from a single mature oocyte (Browder *et al.*, 1991). While there are many possible explanations for the reduced expression overall, two possibilities appear to be the most likely. The reduced expression may be due to a lack of sufficient quantities of transcription factors available for the injected fly gene. There are 500–2500 nucleoli in a single mature oocyte (Thiebaud, 1979) which may provide a 'sink' for RNA polymerase I transcription factors. Alternatively, the reduced expression may be due to inefficient interactions

between the fly template and frog transcription factors, thereby reducing the rate of transcription and the quantity of fly transcripts. It is apparent, however, that the *Xenopus* transcriptional factor complexes must recognize some feature(s) of the *Sciara* template to initiate transcription, although perhaps inefficiently so, producing stable transcripts that are competent for processing within the oocyte. Nonetheless, these experiments provide another example of some degree of relaxation of species-specificity requirements associated with RNA polymerase I transcription, as has been shown in several other cases (Trendelenburg *et al*., 1978; Culotta *et al*., 1987).

Reeder (1984) has suggested that different promoter domains of rDNA cistrons are required under different transcription conditions. Sollner-Webb & McKnight (1982) demonstrated that only a small DNA region immediately surrounding the *X. laevis* initiation site is needed in micro-injected *X. borealis* oocytes, whereas a larger upstream region is required for efficient initiation *in vitro*. An interesting possibility is that the *Xenopus* RNA polymerase I transcriptional apparatus recognizes the *Sciara* proximal promoter sequence leading to transcription from the correct initiation site within *Sciara* rDNA constructs. The absence of a complete primary sequence of *Sciara* rDNA precludes a direct comparison of the insect and frog promoter regions to identify regions of conservation.

The Xenopus *oocyte environment supports internal fragmentation of* Sciara *pre-28S rRNAs*

In several organisms, processing of 28S rRNA is characterized by a central break, creating two halves of the rRNA. Processing sites have been mapped to the D7a expansion segment within 28S rRNA. *Xenopus* is among the group of organisms where additional processing of endogenous 28S rRNA in the D7a expansion segment does not occur. A comparison of 28S rRNA primary and secondary structures in the D7a expansion segment and associated elements of core rRNA shows that conserved sequence elements are present in some dipteran insects which are absent in those organisms that do not undergo this processing event (Fujiwara & Ishikawa, 1986). Extensive secondary structure conservation is noted in the core region of eukaryotic rRNAs (Cannone *et al*., 2002). These observations support the hypothesis that specific signals within and/or surrounding the D7a expansion segment of 28S rRNA direct processing at this site, although no formal test of this hypothesis has been reported. Within the resolution of the techniques used in this study to probe the termini of 28Sα and 28Sβ rRNAs, heterologous rRNAs synthesized within microinjected oocytes and native *Sciara* 28S rRNA are virtually indistinguishable, suggesting that the mechanism for nucleotide excision from the gap region can be sustained within the frog heterologous environment.

Several possibilities are considered here which may account for the observed fragmentation results. It is possible that the gap processing event is an intrinsic property of the RNA so that nucleotide excision in the D7a expansion segment is autocatalytic. While the self-splicing capability of several rRNA introns is well documented (reviewed by Cech, 2002), no precedent has yet been established whereby nucleotides are removed from introns without subsequent RNA ligation. Certainly the lack of evidence for this does not, however, preclude the possibility of this as a mechanism.

If the event is autocatalytic, then we must reconcile at least two observations that do not immediately support this conclusion. First, Jordan *et al*. (1976) showed that gap processing is a late event in nuclear processing, unlike the splicing event that removes the intron from *Tetrahymena* precursor 28S rRNA early in the processing pathway (reviewed by Cech, 2002). Why a delay in processing would occur is unclear unless one considers that a specific rRNA conformation required for autocatalysis is only generated late in the processing pathway. Not all Group I introns are self-splicing *in vitro*, for example (Wollenzien *et al*., 1983). Instead, it is likely that many introns do not fold into the correct structure for self-splicing in the absence of proteins. This is the case for the *Neurospora* large rRNA intron which is spliced by guanine addition *in vivo* but does not undergo self-splicing *in vitro* (Wollenzien *et al*., 1983). These investigators have shown that ribosomal proteins bind to 35S pre-rRNA despite the presence of the 2.3 kb intron. This is consistent with the possibility that binding of ribosomal proteins precedes RNA splicing. If gap processing is an autocatalytic event that proceeds late in rRNA maturation, then a conformation conducive for processing may evolve only after binding of specific ribosomal or non-ribosomal proteins, or small RNAs. Secondly, preliminary experiments using conditions which support *Tetrahymena in vitro* intron splicing (Kruger *et al*., 1982) have not shown processing of an *in vitro* transcribed *Sciara* rRNA containing the D7a expansion segment (L. Roginsky and V. Ware, unpublished data), although conformational deficiencies might be equally likely here as well, possibly due to the lack of accessory components required for proper folding *in vitro* of the rRNA. Together, these observations do not eliminate the possibility that gap processing is autocatalytic, but other possibilities appear more favorable.

In the absence of self-excision as an explanation, one must consider that the *Xenopus* cell has biochemical machinery that sponsors *Sciara*-specific 28S rRNA internal cleavage. No information is currently available on the mechanism of gap processing in organisms such as *Sciara*. Lava-Sanchez & Puppo (1975) proposed that non-specific endonucleases may be involved, as they found that the *in vivo* fragmentation pattern in the insect *Musca carnaria* 28S rRNA could be reproduced by treating newly assembled ribosomes with pancreatic RNase. It is possible

that the 19 nucleotides removed from *Sciara* 28S rRNA are exposed late in rRNA maturation to non-specific endonucleases residing in both *Xenopus* and *Sciara* systems. Yet, in our view the *in vitro* results of Lava-Sanchez & Puppo (1975) do not exclude the possibility that specific recognition signals and excision machinery operate *in vivo* to generate internal site-specific cleavages of *Musca* 28S rRNA or other 28S rRNAs. Since the publication of this report (Lava-Sanchez & Puppo, 1975), much has been learned about the functions of specific endonucleases and the involvement of several snoRNPs in cleavage events that remove transcribed spacers from rRNA precursors, particularly in yeast and vertebrate systems. Generally, with few exceptions (Lava-Sanchez & Puppo, 1975), the proposed interactions between rRNAs and *trans*-acting factors favor the interpretation that specificity between interacting components is a key feature in orchestrating complex pathways that produce mature rRNAs.

The specific enzymatic machinery and putative accessory factors that generate 28S and 5.8S rRNA internal fragmentation are unknown. Nucleotides that are removed from internal sites within 28S and 5.8S rRNAs are essentially internal transcribed spacers found within these rRNAs. Are these species-specific internal transcribed spacers removed by the same processing machinery that excises the commonly held ITS regions in all eukaryotic pre-rRNAs or is unique machinery required for species-specific cleavage at these internal sites?

Based on the ability of *Xenopus* oocytes to promote the *Sciara* site-specific cleavage and excision of 28S rRNA, we propose that if extrinsic processing factors are required for gap processing, then these factors must be evolutionarily conserved and are likely to function in the general rRNA processing pathway as well. It is interesting that the capacity of the *Xenopus* machinery to function in gap processing must be dependent on its ability to recognize evolutionarily conserved signals within the foreign substrates; *Sciara* rRNA transcripts must contain features that are common to some class of *Xenopus* substrates. What these presumed structural equivalents are remains to be determined; however, it is conceivable that ITS and/or ETS sequences within *Xenopus* pre-rRNA transcripts share some type of primary and/or secondary structural features with the *Sciara* D7a expansion segment.

Several snoRNAs, known to associate with pre-rRNA during rRNA maturation, are candidates for components of the proposed conserved machinery that functions in gap processing. A role for U8 snoRNP in the biogenesis of 5.8S and 28S rRNAs from 32S pre-rRNA in *Xenopus* oocytes is well documented (e.g. Peculis & Steitz, 1993). There are no data, however, that would support a role for this snoRNP in 28S rRNA fragmentation. In fact, to our knowledge, no U8-like homologue has as yet been identified in a dipteran, although most of the roles of newly identified small RNAs in *Drosophila* have yet to be determined (Yuan *et al*., 2003). Michot *et al*. (1999) have proposed RNA interactions between the 5.8S-ITS2–28S pre-rRNA and U8 snRNA that may facilitate the proper folding of pre-rRNA for ITS2 excision in vertebrates. Interestingly, the E20 stem (De Rijk *et al*., 1996) of 26S–28S rRNA, forming the base of *S. cerevisiae* expansion segment V13 and previously identified as a requirement for ITS2 processing (Jeeninga *et al*., 1997), is brought into close proximity to the ITS cleavage site through the proposed U8–28S RNA–RNA interaction. Although clearly speculative, the proposed interaction of U8 with an internal site within 28S rRNA through complementary base pairing provides a theoretical framework for considering how *trans*-acting factors (yet to be identified) might function later in rRNA maturation as chaperones to assist in the folding of 28S rRNA, providing a stage on which a specific endonuclease might function to facilitate gap processing.

Ribosomal protein interactions in the gap region

The D7a expansion segment is located in the middle of a highly conserved protein binding site in domain III of 23S–28S rRNAs where ribosomal protein EL23/L25 binds (El-Baradi *et al*., 1984; Raué *et al*., 1985). EL23/L25 is an early binding protein required for large ribosomal subunit assembly and function (e.g. Raué *et al*., 1985). Studies have shown that *E. coli* EL23 and yeast L25 proteins can faithfully recognize the equivalent of its cognate binding site in the heterologous rRNA *in vitro* (El-Baradi *et al*., 1984), although there is some indication that the presence of the D7a expansion segment may interfere somewhat with EL23 binding to yeast rRNA. Musters *et al*. (1991) found that replacement of yeast D7a expansion segment with the equivalent element from mouse or *T. thermophila* 28S rRNA (subject to removal of three or four nucleotides in the ciliate (Engberg & Nielsen, 1990; V. C. Ware, unpublished results), does not affect the formation of functional ribosomes *in vivo*, nor does it interfere with the *in vitro* interaction of chimeric rRNA with yeast L25. Thus, the binding site for L25 can tolerate some degree of variability in the D7a expansion segment – the limit of this variability is not clear.

Nascent pre-rRNAs are assembled into RNPs during transcription and post-transcriptional maturation (e.g. Trendelenburg *et al*., 1996). Thus, it is presumed that heterologous rRNAs transcribed from *Sciara* rDNA constructs are assembled into some form of RNP complex during maturation events leading to gap processing. The extent to which *Xenopus* early binding proteins are able to recognize binding sites in *Sciara* rRNA to assemble an RNP complex that is competent for cleavage and modification is unknown, although it must be inferred that at least some protein interactions are stable enough to effect heterologous pre-28S rRNA fragmentation at the gap site. Whatever the

composition of these RNP complexes, it is presumed that their structure(s) supports internal cleavage at the gap site. As ribosomal protein L23 is required for ribosome assembly, it is presumed that *Xenopus* L23 protein does bind the heterologous pre-rRNAs, although this possibility needs further study. A consideration of what machinery is involved in gap processing must include the temporal order of L23 assembly and 28S rRNA gap processing, and what role, if any, L23 may have in the processing mechanism itself. The identification of components and their functions in this system may have general application to understanding reactions involved in eukaryotic rRNA maturation overall.

Experimental procedures

Construction of rDNAs

Two different constructs containing a complete rDNA repeat unit from *S. coprophila* were made for microinjection into *X. laevis* oocyte germinal vesicles. Construct 1 consists of a complete *S. coprophila* rDNA repeat unit derived from the plasmid pBC2, which contains an rDNA unit cloned into the *Eco*RI site of pBR322 (Renkawitz *et al.*, 1979). In this report it is referred to as 'minigene construct #1', or the 'minigene'. The 8.4 kb insert was excised from pBC2 using *Eco*RI and purified from an agarose gel by Qia Ex II extraction (Qiagen, Valencia, CA). The rDNA insert was ligated under conditions which favoured intramolecular ligation (Sambrook *et al.*, 1989) using T4 polynucleotide ligase (Promega, Madison, WI) producing a circular rDNA 'minigene' in which the coding sequences for all rRNAs are contiguous (Fig. 1). The ligation reaction mixture was purified using clean-a-gene protocols, concentrated to 50 μg/ml in TE buffer (10 mM Tris-HCl (pH 7.6), 0.1 mM EDTA), and stored at −20 °C until further use.

The second construct, designated vDIVH1, contains a complete *S. coprophila* rDNA repeat cloned into the *Apa*I site of plasmid pRS304 (kindly provided by Dr Robert Skibbens, Lehigh University, Bethlehem, PA). The circular rDNA minigene (described above) was digested with *Apa*I in order to linearize the rDNA at the 3′ end of the 28S rRNA gene. The linearized rDNA (with all coding sequences contiguous within the construct) was cloned into the *Apa*I site of plasmid pRS304 to create plasmid vDIVH1 (Fig. 1).

Oocyte microinjections

Xenopus laevis females (proven breeders from *Xenopus* I, Dexter, Michigan or Connecticut Valley Supply, Southampton, MA), anaesthetized on ice, were partially ovariectomized to obtain oocytes. The oocytes were immediately placed in a Petri dish containing Holtfreter's medium and manually defolliculated to obtain stage IV or V oocytes for microinjection. While some groups (LaMarca *et al.*, 1973) have utilized stages V and VI oocytes for rDNA injections, we have found that the highest transcriptional levels for microinjected rDNA constructs are demonstrated in stage IV oocytes (comparative data not shown), consistent with the results of Mairy & Denis (1971). Individual oocytes were placed animal pole up on a nylon grid and centrifuged at 1000 ***g*** for 10 min at 15 °C. Approximately 20 nl of a solution containing 50 μg/ml of rDNA construct was microinjected into the germinal vesicle of each oocyte according to Kressman *et al.* (1978). Microinjected oocytes were incubated for 6–24 h at 18 °C prior to total RNA extraction.

RNA extraction

Total RNA was isolated from healthy oocytes by crushing 10–50 oocytes in 500 μl of TENS buffer (0.2 M Tris-HCl pH 8.0, 0.5 M NaCl, 0.01 M EDTA), extracting the mixture with phenol/chloroform/isoamyl alcohol (25 : 25 : 1, v/v/v), vortexing the homogenate for 30 s, and centrifuging at 12 000 ***g*** for 5 min at room temperature. The RNA was ethanol precipitated and pelleted by centrifugation at 4 °C. RNA pellets were washed with 75% ethanol and resuspended in TE buffer, pH 8.0. Endogenous and microinjected DNAs were degraded by incubation with RQ1 RNase-free DNase (Promega) at 37 °C for 30 min. Following phenol/chloroform extraction and ethanol precipitation, RNA pellets were stored at −70 °C.

Total *Sciara* RNA was isolated from frozen flies (a kind gift from Heidi Smith, Brown University, Providence, RI) using a guanidine hydrochloride extraction procedure outlined in Sambrook *et al.* (1989). Following ethanol precipitation, RNA pellets were stored at −70 °C.

Synthesis and labelling of oligonucleotides

DNA oligomers complementary to *S. coprophila* rRNAs were either synthesized on a Biosearch 8600 DNA synthesizer using the phosphoramidite method or purchased from DNA International, Inc. (Lake Oswego, OR) or Oligos, Etc., Inc. (Wilsonville, OR).

The β_1 oligomer was also used for primer extension experiments. Oligomers were 5′ end labelled using T4 polynucleotide kinase (Promega) and [γ^{32}P]-ATP (ICN, Costa Mesa, CA). Unincorporated nucleotides were removed from labelled probes by spun column chromatography (Sambrook *et al.*, 1989) and stored in TE buffer at −20 °C. Labelled oligonucleotides used in primer extension experiments were further purified on 10% polyacrylamide/7 M urea denaturing gels. Gel-purified primers were eluted by the 'crush & soak' method (Sambrook *et al.*, 1989) and stored in TE buffer at −20 °C.

Nuclease protection analysis

Radiolabelled probes for S1 nuclease protection experiments were generated in a polymerase chain reaction using [α^{32}P]-dCTP in a dNTP mix along with β_1 and T7 promoter primers to generate a 320 bp probe from a pGEM3Z (Promega) subclone containing the 3′ half of *S. coprophila* 28S rDNA (containing the gap sequences). Hybridization reactions with the heat-denatured probe were established according to Sambrook *et al.* (1989). The hybridization reaction was preincubated at 85 °C for 10 min and then transferred to a 62 °C water bath for 8 h. S1 nuclease digestion and recovery of nucleic acids were performed according to Berk & Sharp (1977) using 1000 U/ml S1 nuclease. S1-nuclease-resistant fragments were separated on 10% polyacrylamide/8 M urea gels and visualized by autoradiography. Some autoradiographic images with low signals were scanned using a LKB Densitometric Scanner (Pharmacia LKB, Piscataway, NJ).

Primer extension analysis

[γ^{32}P]-5′ end labelled complementary primers were mixed with total RNA from microinjected oocytes and pelleted according to Sambrook *et al.* (1989). The pellet was redissolved in 9 μl of sterile distilled water and 1 μl of 10× reverse transcriptase buffer (340 mM Tris-HCl pH 8.3, 500 mM NaCl, 60 mM $MgCl_2$, 50 mM DTT). The hybridization mixture was incubated at 62 °C for 10 min and then

quickly transferred to a 42 °C water bath for 12 h. AMV reverse transcriptase (9 units) was added, followed by 1 μl of extension buffer (375 μM dNTPs, 0.1 M Tris-HCl pH 8.3, 0.1 M KCl, 10 mM $MgCl_2$, 10 mM DTT). The reaction was incubated at 42 °C for 15 min before the addition of 11 μl of a 10 mM dNTP mix and further incubation for an additional 15 min. Nucleic acids were extracted with phenol/chloroform, precipitated with 95% ethanol, recovered by centrifugation and resuspended in 5 μl of tracking dye containing 80% formamide. Extension products were separated on 10% polyacrylamide/8 M urea gels and visualized by autoradiography or phosphoimaging (Molecular Dynamics, Sunnyvale, CA).

RT-PCR was conducted according to Qiagen (Valencia, CA) specifications, using the OneStep RT-PCR protocol. Specimens were incubated sequentially at three specific temperatures to allow for denaturation, primer annealing, and primer extension via an automated programmable cycling machine (DNA Thermal Cycler, Perkin Elmer, Shelton, CT). Briefly, reactions included 2 μl dNTP mix (10 mM each), 1 μl each primer (final concentration of approximately 0.6 μM each), 2 μl of OneStep RT-PCR Enzyme mix, 10 μl of 5× buffer and nuclease-free dH_20 in 50 μl reactions. Two microliters of RNA template (~0.5 μg) were aliquoted into each PCR tube. All reactions were kept on ice until the cycler reached the appropriate initial starting temperature, and covered with 50 μl of mineral oil. A reverse transcriptase reaction temperature of 50 °C was maintained for 30 min, followed by the initial PCR activation step (15 min at 95 °C), and 10–30 cycles of denaturation (1 min at 94 °C), annealing (1 min at 58 °C), and extension (1 min at 72 °C). Final extension was for 10 min at 72 °C. The last step on the cycler was set for soak at 4 °C. RT-PCR samples were analysed on 3% agarose gels. PCR markers (Promega) were run alongside RT-PCR products.

Acknowledgements

We thank Susan Gerbi, Heidi Smith, Ronald Reeder and Robert Skibbens for providing plasmid pBC2, *Sciara* flies for RNA isolation, plasmid pXlr101A and plasmid pRS304, respectively. We thank Maria Brace for assistance in the preparation of some graphic images. Additionally, we thank Susan Gerbi and Brenda Peculis for their discussions about the work presented in this manuscript. This work was supported by an NSF grant to V.C.W.

References

Allmang, C., Mitchell, P., Petfalski, E. and Tollervey, D. (2000) Degradation of ribosomal RNA precursors by the exosome. *Nucleic Acids Res* **28**: 1684–1691.

Amako, D., Kwon, O.Y. and Ishikawa, H. (1996) Nucleotide sequence and presumed secondary structure of the 28S rRNA of pea aphid: implication for diversification of insect rRNA. *J Mol Evol* **43**: 469–475.

Applebaum, S.W., Ebstein, R.P. and Wyatt, G.R. (1966) Dissociation of ribonucleic acid from silk moth pupae by heat and dimethylsulfoxide: evidence for specific cleavage points. *J Mol Biol* **21**: 29–41.

Bachellerie, J.P., Cavaille, J. and Huttenhofer, A. (2002) The expanding snoRNA world. *Biochimie* **84**: 775–790.

Balazs, I. and Agosin, M. (1968) Isolation and characterization of ribonucleic acid from *Musca domestica* (L.). *Comp Biochem Physiol* **27**: 227–237.

van Beekvelt, C.A., de Graaff-Vincent, M., Faber, A.W., van't Riet, J., Venema, J. and Raué, H.A. (2001) All three functional domains of the large ribosomal subunit protein L25 are required for both early and late pre-rRNA processing steps in *Saccharomyces cerevisiae*. *Nucleic Acids Res* **29**: 5001–5008.

Berk, A.J. and Sharp, P.A. (1977) Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. *Cell* **12**: 721–732.

Browder, L.W., Erickson, C.A. and Jeffery, W.R. (1991) *Developmental Biology*, 3rd edn. Saunders College Publications, PA.

Burgin, A.B., Parodos, K., Lane, D.J. and Pace, N.R. (1990) The excision of intervening sequences from *Salmonella* 23S ribosomal RNA. *Cell* **60**: 405–414.

Cannone, J.J., Subramanian. S., Schnare, M.N., Collett, J.R., Du D'Souza, L.M.Y., Feng, B. *et al.* (2002) The comparative RNA web (CRW) site: an online database of comparative sequence and structure information for ribosomal, intron, and other RNAs. *BMC Bioinformatics* 3: 2; Erratum. *BMC Bioinformatics* **2002**: 3 (1): 15.

Cech, T.R. (2002) Ribozymes, the first 20 years. *Biochem Soc Trans* **30**: 1162–1166.

Clark, C.G., Tague, B.W., Ware, V.C. and Gerbi, S.A. (1984) *Xenopus laevis* 28S ribosomal RNA: a secondary structure model and its evolutionary and functional implications. *Nucleic Acids Res* **12**: 6197–6220.

Culotta, V.C., Wilkinson, J.K. and Sollner-Webb, B. (1987) Mouse and frog violate the paradigm of species-specific transcription of ribosomal RNA genes. *Proc Natl Acad Sci USA* **84**: 7498–7502.

De Rijk, P., Van de Peer, Y. and De Wachter, R. (1996) Database on the structure of large ribosomal subunit RNA. *Nucleic Acids Res* **24**: 92–97.

DeLanversin, G. and Jacq, B. (1983) Sequence of the central break region of the precursor of *Drosophila* 26S ribosomal RNA. *C R Seances Acad Sci* **III**(296): 1041–1044.

DeLanversin, G. and Jacq, B. (1989) Sequence and secondary structure of the central domain of *Drosophila* 26S rRNA: a universal model for the central domain of the large rRNA containing the region in which the central break may happen. *J Mol Evol* **28**: 403–417.

Dragon, F., Gallagher, J.E., Compagnone-Post, P.A., Mitchell, B.M., Porwancher, K.A., Wehner, K.A., *et al.* (2002) A large nucleolar U3 ribonucleoprotein required for 18S ribosomal RNA biogenesis. *Nature* **417**: 967–970.

El-Baradi, T.T., Raué, H.A., De Regt, C.H. and Planta, R.J. (1984) Stepwise dissociation of yeast 60S ribosomal subunits by LiCl and identification of L25 as a primary 26S rRNA binding protein. *Eur J Biochem* **144**: 393–400.

Eliceiri, G.L. (1999) Small nucleolar RNAs. *Cell Mol Life Sci* **56**: 22–31.

Engberg, J. and Nielsen, H. (1990) Complete sequence of the extrachromosomal rDNA molecule from the ciliate *Tetrahymena thermophila* strain B1868VII. *Nucleic Acids Res* **18**: 6915–6919.

Enright, C.A., Maxwell, E.S., Eliceiri, G.L. and Sollner-Webb, B. (1996) 5′ETS rRNA processing facilitated by four small RNAs: U14, E3, U17, and U3. *RNA* **2**: 1094–1099. Erratum in *RNA* **2**: 1318.

Fujiwara, H. and Ishikawa, H. (1986) Molecular mechanism of introduction of the hidden break into the 28S rRNA of insects: implication based on structural studies. *Nucleic Acids Res* **14**: 6393–6401.

Ganot, P., Bortolin, M.L. and Kiss, T. (1997) Site-specific pseudouridine formation in preribosomal RNA is guided by small nucleolar RNAs. *Cell* **89**: 799–809.

Grandi, P., Rybin, V., Bassler, J., Petfalski, E., Strauss, D., Marzioch, M., *et al.* (2002) 90S pre-ribosomes include the 35S pre-rRNA, the U3 snoRNP, and 40S subunit processing factors but predominantly lack 60S synthesis factors. *Mol Cell* **10**: 105–115.

Granneman, S. and Baserga, S.J. (2004) Ribosome biogenesis: of knobs and RNA processing. *Exp Cell Res* **296**: 43–50.

Greenberg, J.R. (1969) Synthesis and properties of ribosomal RNA in *Drosophila*. *J Mol Biol* **46**: 85–98.

Ishikawa, H. (1973) Comparative studies on the thermal stability of animal ribosomal RNAs. *Comp Biochem Physiol B* **46**: 217–227.

Ishikawa, H. (1975a) Comparative studies on the thermal stability of animal ribosomal RNAs. II. Sea-anemones (Coelenterata). *Comp Biochem Physiol B* **50**: 1–4.

Ishikawa, H. (1975b) Polynucleotide fragments from the 28S ribosomal RNA of insects. *Nucleic Acids Res* **2**: 87–100.

Ishikawa, H. and Newburgh, R.W. (1972) Studies of the thermal conversion of 28S RNA of *Galleria mellonella* (L.) to an 18S product. *J Mol Biol* **64**: 135–144.

Jackson, S., Cannone, J., Lee, J., Gutell, R. and Woodson, S. (2002) Distribution of rRNA introns in the three-dimensional structure of the ribosome. *J Mol Biol* **323**: 35–52.

Jeeninga, R.E., Van Delft, Y., de Graaff-Vincent, M., Dirks-Mulder, A., Venema, J. and Raué, H.A. (1997) Variable regions V13 and V3 of *Saccharomyces cerevisiae* contain structural features essential for normal biogenesis and stability of 5.8S and 25S rRNA. *RNA* **3**: 476–488.

Jordan, B.R., Jourdan, R. and Jacq, B. (1976) Late steps in the maturation of *Drosophila* 26S ribosomal RNA: generation of 5.8 S and 2 S RNAs by cleavages occurring in the cytoplasm. *J Mol Biol* **101**: 85–105.

Jordan, B.R., Latil-Damotte, M. and Jourdan, R. (1980) Coding and spacer sequences in the 5.8S–2S region of *Sciara coprophila* ribosomal DNA. *Nucleic Acids Res* **8**: 3565–3573.

Kass, S., Tyc, K., Steitz, J.A. and Sollner-Webb, B. (1990) The U3 small nucleolar ribonucleoprotein functions in the first step of preribosomal RNA processing. *Cell* **60**: 897–908.

Kiss-Laszlo, Z., Henry, Y., Bachellerie, J.P., Caizergues-Ferrer, M. and Kiss, T. (1996) Site-specific ribose methylation of preribosomal RNA: a novel function for small nucleolar RNAs. *Cell* **858**: 1077–1088.

Kjer, K.M., Baldridge, G.D. and Fallon, A.M. (1994) Mosquito large subunit ribosomal RNA: simultaneous alignment of primary and secondary structure. *Biochim Biophys Acta* **1217**: 147–155.

Kressler, D., Linder, P. and de La Cruz, J. (1999) Protein transacting factors involved in ribosome biogenesis in *Saccharomyces cerevisiae*. *Mol Cell Biol* **19**: 7897–7912.

Kressman, A., Clarkson, S.G., Pirrotta, V. and Birnstiel, M.L. (1978) Transcription of cloned tRNA gene fragments and subfragments injected into the oocyte nucleus of *Xenopus laevis*. *Proc Natl Acad Sci USA* **75**: 1176–1180.

Kruger, K., Grabowski, P.J., Zaug, A.J., Sands, J., Gottschling, D.E. and Cech, T.R. (1982) Self-splicing RNA: autoexcision and autocyclization of the ribosomal RNA intervening sequence of *Tetrahymena*. *Cell* **31**: 147–157.

LaMarca, M.J., Smith, L.D. and Strobel, M.C. (1973) Quantitative and qualitative analysis of RNA synthesis in stage 6 and stage 4 oocytes of *Xenopus laevis*. *Dev Biol* **34**: 106–118.

Lava-Sanchez, P.A. and Puppo, S. (1975) Occurrence *in vivo* of 'hidden breaks' at specific sites of 26 S ribosomal RNA of *Musca carnaria*. *J Mol Biol* **95**: 9–20.

Mairy, M. and Denis, H. (1971) Biochemical studies on oogenesis. I. RNA synthesis and accumulation during oogenesis of the South African toad *Xenopus laevis*. *Dev Biol* **24**: 143–165.

Michot, B., Hassouna, N. and Bachellerie, J.P. (1984) Secondary structure of mouse 28S rRNA and general model for the folding of the large rRNA in eukaryotes. *Nucleic Acids Res* **12**: 4259–4279.

Michot, B., Joseph, N., Mazan, S. and Bachellerie, J.P. (1999) Evolutionarily conserved structural features in the ITS2 of mammalian pre–rRNAs and potential interactions with the snoRNA U8 detected by comparative analysis of new mouse sequences. *Nucleic Acids Res* **27**: 2271–2282.

Mishra, R.K. and Eliceiri, G.L. (1997) Three small nucleolar RNAs that are involved in ribosomal RNA precursor processing. *Proc Natl Acad Sci USA* **94**: 4972–4977.

Morales-Hojas, R., Post, R.J., Wilson, M.D. and Cheke, R.A. (2002) Completion of the sequence of the nuclear ribosomal DNA subunit of *Simulium sanctipauli*, with descriptions of the 18S, 28S genes and the IGS. *Med Vet Entomol* **16**: 386–394.

Mougey, E.B., Pape, L.K. and Sollner-Webb, B. (1993) A U3 small nuclear ribonucleoprotein-requiring processing event in the 5′ external transcribed spacer of *Xenopus* precursor rRNA. *Mol Cell Biol* **13**: 5990–5998.

Musters, W., Concalves, P.M., Boon, K., Raué, H.A., van Herrikhuizen, H. and Planta, R.J. (1991) The conserved GTPase center and variable region D7A from *Saccharomyces cerevisiae* 26S rRNA can be replaced by their equivalents from other prokaryotes or eukaryotes without detectable loss of ribosomal function. *Proc Natl Acad Sci USA* **88**: 1469–1473.

Ni, J., Tien, A.L. and Fournier, M.J. (1997) Small nucleolar RNAs direct site-specific synthesis of pseudouridine in ribosomal RNA. *Cell* **89**: 565–573.

Nicoloso, M., Qu, L.H., Michot, B. and Bachellerie, J.P. (1996) Intron-encoded, antisense small nucleolar RNAs: the characterization of nine novel species points to their direct role as guides for the 2′-O-ribose methylation of rRNAs. *J Mol Biol* **260**: 178–195.

Nissan, T.A., Bassler, J., Petfalski, E., Tollervey, D. and Hurt, E. (2002) 60S pre-ribosome formation viewed from assembly in the nucleolus until export to the cytoplasm. *EMBO J* **21**: 5539–5547.

Ogino, K., Eda-Fujiwara, H., Fujiwara, H. and Ishikawa, H. (1990) What causes the aphid 28S rRNA to lack the hidden break? *J Mol Evol* **30**: 509–513.

Otsuka, T., Nomiyama, H., Yoshida, H., Kukita, T., Kuhara, S. and Sakaki, Y. (1983) Complete nucleotide sequence of the 26S rRNA gene of *Physarum polycephalum*: its significance in gene evolution. *Proc Natl Acad Sci USA* **80**: 3163–3167.

Pavlakis, G.N., Jordan, B.R., Wurst, R.M. and Vournakis, J.N. (1979) Sequence and secondary structure of *Drosophila melanogaster* 5.8S and 2S rRNAs and of the processing site between them. *Nucleic Acids Res* **7**: 2213–2238.

Peculis, B.A. and Steitz, J.A. (1993) Disruption of U8 nucleolar snRNA inhibits 5.8S and 28S rRNA processing in the *Xenopus* oocyte. *Cell* **73**: 1233–1245.

Raué, H.A., El-Baradi, T.T. and Planta, R.J. (1985) Evolutionary conservation of protein binding sites on high-molecular-mass ribosomal RNA. *Biochim Biophys Acta* **826**: 1–12.

Reeder, R.H. (1984) Enhancers and ribosomal gene spacers. *Cell* **38**: 349–351.

Renkawitz, R., Gerbi, S.A. and Glätzer, K.H. (1979) Ribosomal DNA of fly *Sciara coprophila* has a very small and homogeneous repeat unit. *Mol General Genet* **173**: 1–13.

Rocheleau, G.A. and Woodson, S.A. (1994) Requirements for self-splicing of a group I introns from *Physarum polycephalum*. *Nucleic Acids Res* **22**: 4315–4320.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Savino, R. and Gerbi, S.A. (1990) In vivo disruption of *Xenopus* U3 snRNA affects ribosomal RNA processing. *EMBO J* **9**: 2299–2308.

Schnare, M.N. and Gray, M.W. (1990) Sixteen discrete RNA components in the cytoplasmic ribosome of *Euglena gracilis*. *J Mol Biol* **215**: 73–83.

Schnare, M.N., Damberger, S.H., Gray, M.W. and Gutell, R.R. (1996) Comprehensive comparison of structural characteristics in eukaryotic cytoplasmic large subunit (23 S-like) ribosomal RNA. *J Mol Biol* **256**: 701–719.

Shimada, T. (1992) Distribution of split 5.8S ribosomal RNA in Diptera. *Insect Mol Biol* **1**: 45–49.

Sollner-Webb, B. and McKnight, S.L. (1982) Accurate transcription of cloned *Xenopus* rRNA genes by RNA polymerase I: demonstration by S1 nuclease mapping. *Nucleic Acids Res* **10**: 3391–3405.

Terns, M.P. and Terns, R.M. (2002) Small nucleolar RNAs: versatile trans-acting molecules of ancient evolutionary origin. *Gene Expr* **10**: 17–39.

Thiebaud, C.H. (1979) Quantitative determination of amplified rDNA and its distribution during oogenesis in *Xenopus laevis*. *Chromosoma* **73**: 37–44.

Trendelenburg, M.F., Zentgraf, H., Franke, W.W. and Gurdon, J.B. (1978) Transcription patterns of amplified *Dytiscus* genes coding for ribosomal RNA after injection into *Xenopus* oocyte nuclei. *Proc Natl Acad Sci USA* **75**: 3791–3795.

Trendelenburg, M.F., Zatsepina, O.V., Waschek, T., Schlegel, W., Troster, H., Rudolph, D., *et al.* (1996) Multiparameter microscopic analysis of nucleolar structure and ribosomal gene transcription. *Histochem Cell Biol* **106**: 167–192.

Tycowski, K.T., Shu, M.D. and Steitz, J.A. (1994) Requirement for intron-encoded U22 small nucleolar RNA in 18S ribosomal RNA maturation. *Science* **266**: 1558–1561.

Van Keulen, H., Mertz, P.M., LoVerde, P.T., Shi, H. and Rekosh, D.M. (1991) Characterization of a 54-nucleotide gap region in the 28S rRNA gene of *Schistosoma mansoni*. *Mol Biochem Parasitol* **45**: 205–214.

Veldman, G.M., Klootwijk, J., de Regt, V.C., Planta, R.J., Branlant, C., Krol, A. and Ebel, J.P. (1981) The primary and secondary structure of yeast 26S rRNA. *Nucleic Acids Res* **9**: 6935–6952.

Venema, J. and Tollervey, D. (1999) Ribosome synthesis in *Saccharomyces cerevisiae*. *Annu Rev Genet* **33**: 261–311.

Ware, V.C., Renkawitz, R. and Gerbi, S.A. (1985) rRNA processing: removal of only nineteen bases at the gap between 28Sα and 28Sβ rRNAs in *Sciara coprophila*. *Nucleic Acids Res* **13**: 3581–3597.

Wollenzien, P.L., Youvan, D.C. and Hearst, J.E. (1978) Structure of psoralen-crosslinked ribosomal RNA from *Drosophila melanogaster*. *Proc Natl Acad Sci USA* **75**: 1642–1646.

Wollenzien, P.L., Cantor, C.R., Grant, D.M. and Lambowitz, A.M. (1983) RNA splicing in *Neurospora* mitochondria: structure of the unspliced 35S precursor ribosomal RNA detected by psoralen cross-linking. *Cell* **32**: 397–407.

Yuan, G., Klambt, C., Bachellerie, J.P., Brosius, J. and Huttenhofer, A. (2003) RNomics in *Drosophila melanogaster*: identification of 66 candidates for novel non-messenger RNAs. *Nucleic Acids Res* **31**: 2495–2507.

Zarlenga, D.S. and Dame, J.B. (1992) The identification and characterization of a break within the large subunit ribosomal RNA of *Trichinella spiralis*: comparison of gap sequences within the genus. *Mol Biochem Parasitol* **51**: 281–289.

Supplementary material

The following material is available for this article online:

Figure S1. Pattern of S1 nuclease protection analysis. (A) Map of plasmid pBC2. (B) S1 nuclease protection analysis.

Figure S2. Northern blot analysis of RNA from microinjected oocytes using a 28Sα probe.

Figure S3. Comparative restriction endonuclease analysis of clones pBC2 and vDIVH1.