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Spencer J. Greenwood · Murray N. Schnare
Michael W. Gray

Molecular characterization of U3 small nucleolar RNA from the early diverging protist, *Euglena gracilis*

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Abstract U3 small nucleolar RNA (snoRNA) has been isolated from *Euglena gracilis*, an early diverging protist, and its primary sequence determined. Although this 180-nucleotide-long RNA is considerably smaller than its homolog in vertebrate animals, it contains the conserved sequence blocks (boxes A, A°, B, C and D) characteristic of U3 snoRNAs from other organisms. A secondary structure can be modelled that displays many of the salient features found in published core structures of vertebrate, yeast and trypanosome U3 snoRNAs. The functional significance of this proposed secondary structure is discussed in relation to the role *E. gracilis* U3 snoRNA may have in pre-rRNA processing in this organism. Multiple expressed species of *E. gracilis* U3 snoRNA were found to differ in nucleotide sequence at a number of positions; some of these differences alter pairing in the proposed secondary structure. Analysis of *E. gracilis* genomic DNA revealed a complex pattern of U3-hybridizing sequences that parallels the multiplicity of expressed species of U3 snoRNA revealed by transcript analysis.

Key words U3 snoRNA · Pre-rRNA processing · *Euglena gracilis* · Protist

Introduction

Small nucleolar RNAs (snoRNAs) constitute a class of uridine-rich, metabolically stable and relatively abundant RNAs that are found within the nucleolus (Weinberg and Penman 1968; Prestayko et al. 1970; Zieve and Penman 1976). These RNA species are distinct from the small nuclear RNAs (snRNAs) that function in eukaryotic pre-mRNA processing (Busch et al. 1982; Fournier and Maxwell 1993; Mattaj et al. 1993; Maxwell and Fournier 1995). Early studies implicated snoRNAs in ribosome biogenesis (Prestayko et al. 1970), a role since confirmed by the observations that: (1) snoRNAs are localized in the nucleolus, (2) there are regions of sequence complementarity between snoRNAs and precursor ribosomal RNAs (pre-rRNAs), (3) snoRNAs co-sediment with ribosomal precursors, (4) hydrogen bonding occurs between snoRNAs and both precursor and mature rRNAs, and (5) snoRNAs can be chemically crosslinked to pre-rRNAs (Filipowicz and Kiss 1993; Fournier and Maxwell 1993; Bachellerie et al. 1995; Maxwell and Fournier 1995). A specific role for snoRNAs in rRNA processing is supported by recent in vivo and in vitro studies showing that processing is disrupted when specific snoRNAs or their associated proteins are lost or inactivated (Fournier and Maxwell 1993; Maxwell and Fournier 1995). To-date six snoRNAs (U3, U8, U14, U22, snR10 and snR30) have been shown to have either a direct or indirect role in normal rRNA processing (Tollervey 1987; Kass et al. 1990; Li et al. 1990; Hughes and Ares 1991; Morrissey and Tollervey 1993; Peculis and Steitz 1993; Tycowski et al. 1994; Beltrame and Tollervey 1995).

The most extensively characterized member of this family is U3 snoRNA, which functions in the form of a small nucleolar ribonucleoprotein (snoRNP) particle in the initial processing event within the external transcribed spacer (ETS) of the pre-rRNA (Kass and Sollner-Webb 1990). Evidence supporting a role for U3 snoRNA in rRNA processing has come from studies in mammals (Maser and Calvet 1989; Stroke and Weiner 1989; Kass et al. 1990), *Xenopus* (Savino and Gerbi 1990, 1991; Mougey et al. 1993) and the yeast *Saccharomyces cerevisiae* (Hughes and Ares 1991; Beltrame and Tollervey 1992). U3 snoRNA has been crosslinked within the 5′-ETS, implying a requirement for direct contact near the respective processing sites in mouse (Tyc and Steitz 1992), rat (Stroke and Weiner 1989), human (Maser and Calvet 1989), and yeast (Beltrame and Tollervey 1992). Processing within the 5′-ETS can be disrupted by depletion of U3 snoRNA either by transcriptional

S. J. Greenwood · M. N. Schnare · M. W. Gray (✉)
Department of Biochemistry, Dalhousie University, Halifax, Nova Scotia B3H 4H7, Canada

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repression (yeast), RNase H cleavage (mouse and *X. laevis*) or immunoprecipitation (mouse), further strengthening the inference of a direct interaction between U3 snoRNA and the pre-rRNA (Kass et al. 1990; Hughes and Ares 1991; Mougey et al. 1993).

From an evolutionary perspective, little is known about the extent of conservation of the 5′-ETS processing site and the requirement for U3 snoRNP participation in this processing. However, it has been suggested that U3 snoRNP should be active at this initial processing site in all eukaryotes (Mougey et al. 1993). In the kinetoplastid protist, *Trypanosoma brucei*, an unusually small U3 snoRNA and the associated protein fibrillarin have recently been described (Hartshorne and Agabian 1993). In accordance with the prediction of Mougey et al. (1993), the trypanosome U3 snoRNA is also believed to form an association with the 5′-ETS of trypanosome pre-rRNA (Hartshorne and Agabian 1993). The recent report of an archaeal U3 snoRNA homolog (Potter et al. 1995) suggests that some aspects of eukaryotic pre-rRNA processing may actually pre-date the emergence of the eukaryotic lineage itself.

Phylogenetic trees based on comparisons of small subunit (SSU) rRNA sequences suggest that, together with the kinetoplastid protists, the euglenoid protists diverged from the main line of eukaryotes very early in their evolution (Sogin 1991). To further explore the suggested evolutionary conservation of the U3 snoRNA/pre-rRNA association, we have characterized the U3 snoRNA of the euglenoid protist, *Euglena gracilis*. In this paper we show that *E. gracilis* U3 snoRNA is intermediate in size between its vertebrate and trypanosome homologs and contains all of the recognized conserved sequence blocks (A, A^o, B, C and D) found in the U3 snoRNAs of other organisms (Wise and Weiner 1980; Hughes et al. 1987; Marshallsay et al. 1992). The presence of these conserved boxes permits the derivation of a secondary structure model that displays many of the salient features found in the U3 core structures proposed for other organisms (Parker and Steitz 1987; Ségault et al. 1992; Hartshorne and Agabian 1994). We assess here the functional significance of this secondary structure model in relation to the role U3 snoRNA may play in pre-rRNA processing in *E. gracilis*. We also present data that indicate the existence of multiple copies of U3-hybridizing sequences in the *E. gracilis* nuclear genome and the presence of several distinct species of U3 snoRNA.

Materials and methods

Preparation of RNA from E. gracilis. One-litre cultures of a streptomycin-bleached mutant of *E. gracilis* (Cook and Roxby 1985) were grown at 28°C in a modified salts medium at pH 3.5, with 30 mM ethanol as a carbon source (Cramer and Myers 1952). Cells were harvested when cultures reached mid-log phase and total RNA was extracted after cell disruption in a French pressure cell at 15 000 lb in^{-2} (Schnare and Gray 1990). Nuclear RNA was isolated from mid-log phase cells by a procedure adapted from Bertaux et al. (1985) with the following changes: (1) nuclear isolation buffers contained 10 mM Tris-HCl (pH 7.4), 6 mM $MgCl_2$, 0.13 mM $MnCl_2$; (2) cells were treated with Pronase to generate spheroplasts prior to disruption in a French pressure cell at 1500 lb in^{-2}. Nuclear RNA was extracted by the detergent/phenol-cresol method (Parish and Kirby 1966).

Purification of E. gracilis U3 snoRNA. Total RNA (100–300 µg) was fractionated in a 6% polyacrylamide/7 M urea gel (20 cm × 20 cm × 0.15 cm) containing TBE (50 mM Tris, 50 mM boric acid, 1 mM EDTA, pH 8.3) at 350 V for about 2 h. RNA was visualized either by UV shadowing (Hassur and Whitlock 1974) or ethidium bromide staining. The U3 snoRNA-containing region of the gel was excised and individual RNA species were eluted (Rubin 1973). Linear polyacrylamide carrier (20 µg) was added to the sample in order to facilitate precipitation of small amounts of RNA (Gaillard and Strauss 1990). To enrich for RNAs containing N^2,N^2,7-trimethylguanosine (TMG) cap structures, immunoprecipitation with an anti-TMG monoclonal antibody (Oncogene Science) was carried out according to the procedure of Mottram et al. (1989).

Sequencing of end-labelled U3 snoRNA. Isolated RNA was 3′-end-labelled with [5′-^{32}P]pCp and phage T4 RNA ligase (Peattie 1979), then re-purified in either a 10% or 6% polyacrylamide/7 M urea sequencing gel. Enzymatic (Donis-Keller et al. 1977; Donis-Keller 1980) and chemical (Peattie 1979) sequencing of gel-purified RNA were performed as described (Schnare and Gray 1990); 3′-terminal nucleotide analysis followed the procedure of MacKay et al. (1980). In an effort to determine the 5′-terminal nucleotide, RNA (with or without prior treatment with tobacco acid pyrophosphatase; Perry et al. 1987) was de-phosphorylated and 5′-end-labelled using [γ-^{32}P]ATP and polynucleotide kinase. An attempt was also made to analyze the 5′-end of U3 snoRNA using the terminal deoxynucleotidyl transferase tailing method of Ørum et al. (1991).

Reverse transcriptase sequencing of U3 snoRNA. Total *E. gracilis* RNA (10–20 µg) was annealed with 5′-^{32}P-end-labelled primers (General Synthesis and Diagnostics, Toronto, Canada). Reverse transcriptase (RT) sequencing was performed according to a published protocol (Geliebter 1987) but without actinomycin D. The following oligonucleotides specific for *E. gracilis* U3 snoRNA were used as primers: 5′-CCACTCAAATTGCTGACCTCTCATC-3′ (#1), complementary to positions 155–179; 5′-CTCTGTGAATCGGACTGATACTTC-3′ (#2), complementary to positions 33–56 (see Fig. 3).

Preparation and sequencing of an amplification product of the U3 snoRNA gene. PCR amplification of the gene encoding U3 snoRNA was carried out (Ausubel et al. 1992) in an Ericomp thermal cycler. The 3′ primer (#1 above) was used in conjunction with a 5′ primer, 5′- CTCCACAAGGATCATTTCTTGAGG-3′ (#3, corresponding to nucleotide positions 10–33 of the U3 snoRNA sequence; University Core DNA Services, Calgary, Canada). The amplification reactions contained 10–100 ng of *E. gracilis* nuclear DNA, reaction buffer [25 mM glycine-KOH (pH 9.3), 2.5 mM $MgCl_2$, 25 mM KCl], 10 µg of gelatin (microwaved), 0.1 mM of a dNTP mix (comprising all four dNTPs), 40–100 pmol of the 5′ and 3′ primers, and 2.5 units of *Thermus aquaticus* (*Taq*) DNA polymerase. Amplification cycles were as follows: 96°C/3 min (1x); 55°C/30 s, 72°C/1.5 min, 96°C/15 s (30x); 55°C/30 s, 72°C/5 min (1x). Amplification products were assessed by electrophoresis in a 2% agarose gel that included size markers. Samples of the correct size were then pooled and extracted with phenol/cresol and the supernatant was concentrated by ethanol precipitation at –20°C. To remove primers used in amplification, PCR products were precipitated with polyethylene glycol followed by a final purification in 2% low-melting-temperature agarose (Ausubel et al. 1992). The sequence of the U3 amplification product was determined using the protocol for double-stranded DNA as outlined in the manufacturer's instructions provided with the Sequenase Version 2.0 Kit (U.S. Biochemicals), with the following modifications: (1) denaturation of the double-stranded U3 amplification product was at 96°C for 5 min in the presence of primer #3 (0.5–1.0 pmol); (2) 5 µCi of [α-^{32}P]dATP (3000 Ci/mmol) was included; (3) normal dilutions of dITP labelling mix and Sequenase enzyme were used. In another experiment the amplification was performed in the presence of 5′-end-labelled primer #3 and the resulting gel-purified prod-

uct was subjected to chemical sequence analysis (Maxam and Gilbert 1980) using a modified protocol (D.F. Spencer, personal communication).

Southern-hybridization analysis. *E. gracilis* genomic DNA (10 µg/reaction) was digested with restriction endonucleases (2–3 units/µg) overnight at 37°C. DNA restriction fragments were resolved by electrophoresis in a 0.7% agarose gel in the presence of TBE running buffer at approximately 1V/cm for 18 h (Sambrook et al. 1989). The DNA fragments in the gel were then subjected to de-purination, denaturation and neutralization prior to capillary transfer according to standard techniques (Sambrook et al. 1989). Blots were baked at 80°C for 2 h under vacuum. Southern hybridizations (Sambrook et al. 1989) were performed using the double-stranded U3 amplification product as a probe, labelled by the method of Feinberg and Vogelstein (1983).

Sequence alignment and modelling of the potential secondary structure of E. gracilis U3 snoRNA. The *E. gracilis* U3 snoRNA sequence was aligned with published U3 snoRNA sequences (Gu and Reddy 1994) using the alignment programs Clustal V (Higgins et al. 1992) and ESEE (Cabot and Beckenbach 1989). Published information on phylogenetic comparisons and structure probing of U3 snoRNAs from other organisms (Parker and Steitz 1987; Baserga et al. 1991, 1992; Ségault et al. 1992; Hartshorne and Agabian 1994) was incorporated into a secondary structure model for the *E. gracilis* U3 snoRNA using the secondary structure programs PCFOLD (Zuker and Stiegler 1981) and loopDloop (Gilbert 1992). Regions known to be predominantly single-stranded (boxes B and C) were constrained from base pairing, while helix 2, which forms the conserved 3′-end, was required to base pair. A search for complementarity between U3 snoRNA and the pre-rRNA of *E. gracilis* (Gunderson and Sogin 1986; Schnare et al. 1990; S.J. Greenwood, J.R. Cook, M.N. Schnare and M.W. Gray, in preparation) was carried out using the COMPARE function of the Beckman MicroGenie program (Queen and Korn 1984). The *E. gracilis* U3 snoRNA sequence has been submitted to GenBank, National Center for Biotechnology Information (accession number U27297).

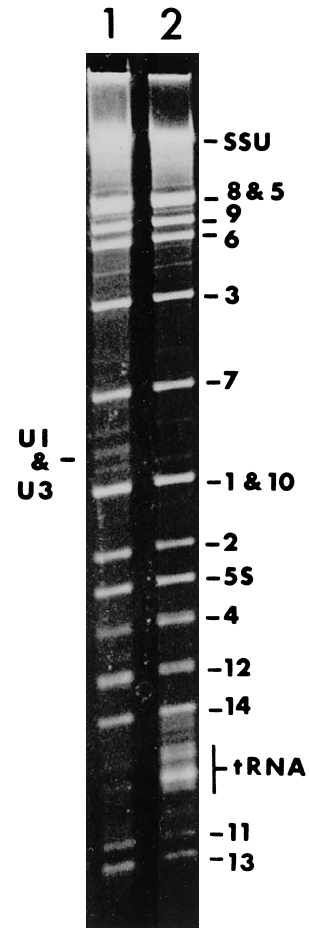

Fig. 1 Profiles of *E. gracilis* RNA separated by electrophoresis in a 6% polyacrylamide/7 M urea gel and stained with ethidium bromide. *Lane 1*, nuclear RNA; *lane 2*, total cellular RNA (3.5 µg RNA per lane). Positions of the mature SSU rRNA, the 14 species comprising the LSU rRNA, 5*S* rRNA and tRNAs are indicated, together with those of the small RNAs U1 and U3

Results

Identification and analysis of *E. gracilis* U3 snoRNA

When total cellular and nuclear RNA preparations from *E. gracilis* were separated in 6% polyacrylamide/7 M urea gels, we observed the previously described complex pattern attributed to the SSU rRNA, the 14 fragments of the mature large subunit (LSU) rRNA, 5*S* rRNA and tRNAs (Schnare and Gray 1990) (Fig. 1). Comparison of the gel profiles revealed additional, low-abundance RNA species that are more prominent in the nuclear RNA profiles. One such band, whose position is indicated in Fig. 1, proved on further analysis to be a mixture of U1 snRNA and U3 snoRNA.

RNAs from the indicated region of a 6% gel (Fig. 1) were recovered and 3′-end-labelled with [5′-^{32}P]pCp, and the products were separated by electrophoresis in a 10% polyacrylamide/7 M urea gel. In addition to labelled bands representing U1 snRNA and contaminating LSU rRNA species 1 and 10, a major (A) and three minor (B–D) labelled bands of U3 snoRNA were resolved by this procedure (Fig. 2).

Most of the U3 snoRNA sequence was obtained through a combination of direct chemical and enzymatic sequencing of the end-labelled RNA and oligonucleotide-primed reverse transcriptase sequencing; however, secondary structure compressions in sequencing gels obscured the ladder corresponding to nucleotide positions 117–128. The sequence of this region was confirmed by analysis of a PCR amplification product corresponding to part of the U3 snoRNA gene (see below). The sequence of this PCR product (C at positions 117 and 128) corresponded to that of the major variant of band U3-A (see below).

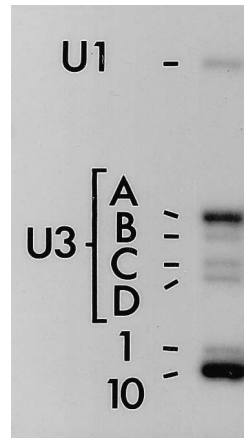

Fig. 2 Resolution in a 10% polyacrylamide/7 M urea gel of 3′-end-labelled RNAs from a fraction enriched in U1 and U3 RNAs (see Fig. 1). Positions of U1 snRNA and the four U3 snoRNA bands (A–D; see text) are indicated, along with the positions of minor amounts of mature LSU rRNA species 1 and 10 that co-isolate with U1 and U3 RNAs

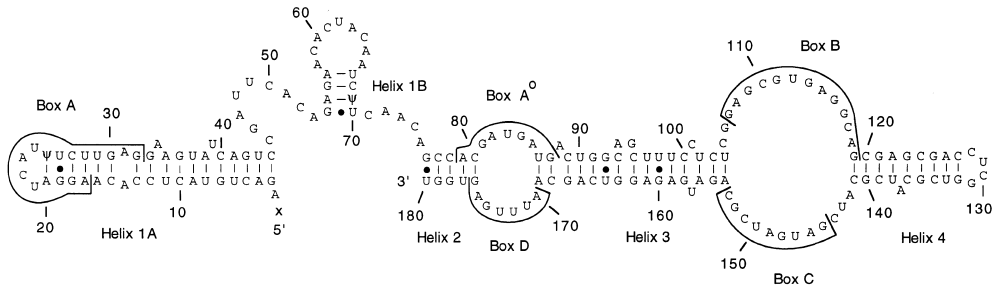

Fig. 3 Proposed secondary structure of *E. gracilis* U3-A snoRNA. Conserved boxes A, A°, B, C and D are indicated. Helices are labelled as in the text. The undetermined 5′-nucleotide is represented by a *lowercase* 'x'. Ψ = pseudo-'uridine (5-ribosyluracil)

The 5′-end-labelling of the mixture of U1 and U3 RNAs did not produce the discrete pattern of bands seen when the same RNA sample was 3′-end-labelled; this was the case whether or not the sample was pre-treated with tobacco acid pyrophosphatase to remove potential 5′-cap structures. Instead, a heterogeneous collection of labelled bands (data not shown) was generated, indicating that the sample was contaminated with other nucleic acid species that were substrates for 5′- but not 3′-end-labeling. Thus, we were unable to determine the identity of the 5′-terminal nucleotide using standard 5′-end-labelling techniques. We therefore resorted to the procedure of Ørum et al. (1991), which has proven useful for 5′-end determination in certain cases. This approach involves: (1) isolation of full-length products of reverse transcriptase sequencing reactions, (2) further extension by terminal deoxynucleotidyl-transferase of those chains that are not terminated by a dideoxynucleotide, and (3) re-electrophoresis of the extended products. However, using this method we were still unable to positively identify the 5′-terminal nucleotide of *E. gracilis* U3 snoRNA; this residue, which is A in all other characterized U3 snoRNAs, is designated 'x' in Fig. 3.

Primary sequence and potential secondary structure of *E. gracilis* U3 snoRNA

The *E. gracilis* U3-A snoRNA sequence (180 nt in length) contains all of the conserved sequence boxes (A, A°, B, C and D; Fig. 3) recognized in U3 snoRNAs from other organisms (Wise and Weiner 1980; Hughes et al. 1987; Marshallsay et al. 1992), with the *E. gracilis* motifs displaying varying degrees of sequence similarity with their counterparts in other eukaryotes. Within the five conserved boxes, *E. gracilis* U3 snoRNA shows highest overall similarity with the mammalian U3 snoRNAs (81.5% identity for both human and rat) and lowest overall identity with the U3 snoRNAs of the protists *Tetrahymena thermophila* (57.4%) and *T. brucei* (62.7%). However, *E. gracilis* U3 snoRNA (22.2% U) is not as uridine-rich as its homolog in vertebrate animals (about 30% U). Not surprisingly in view of their length variation, U3 snoRNAs from different organisms share very little primary sequence identity outside of the conserved boxes.

In our proposed secondary structure for U3 snoRNA (Fig. 3), the 5′-end region can form two helical structures; these are labelled according to the nomenclature used for the secondary structure of *S. cerevisiae* U3 snoRNA (Ségault et al. 1992). The first 5′-terminal helix (1A) forms a stem-loop structure containing box A, with the closing loop containing four nucleotides. Helix 1B is considerably smaller than in other organisms and is bordered by two single-stranded regions. The loop that closes helix 1B contains nine nucleotides. Box A° and box D constitute an internal loop between helices 2 and 3, with both sequences contributing to the base pairs of these helices. Boxes B and C encompass single-stranded regions of a large internal loop located between helices 3 and 4. Helix 4 is a hairpin with a closing loop of four nucleotides. We consider helix 4 experimentally supported because it displayed resistance to RNase cleavage and induced band compressions in sequencing gels (see below).

Sequence heterogeneity and modified nucleosides in *E. gracilis* U3 snoRNA

Chemical sequence determination of the four electrophoretically separated, 3′-end-labelled U3 snoRNA bands (designated U3-A, U3-B, U3-C and U3-D; see Fig. 2) revealed nucleotide substitutions among the different bands (Fig. 4 and Table 1). Because some of the bands of the U3 snoRNA contain sequence heterogeneities that cannot be explained by cross contamination with the other three bands, it is likely that these bands contain several distinct but co-migrating species of U3 snoRNA. There is some uncertainty regarding the exact identity/location of heterogeneities associated with the band compression (indicated in Fig. 4) encompassing nucleotide positions 117–128.

The nucleotide substitutions present in the U3 snoRNA species have varying effects on the proposed secondary structure. Compensatory changes that maintain base pairing in helix 4 are found in U3-C at positions 124 (C to U) and 135 (G to A) (Table 1 and Fig. 3). Three of the nucleotide changes (positions 44, 136, and 141 in Fig. 3) do not alter the secondary structure; they are simple C to U transitions that would maintain base pairing with G within the specified version of U3 snoRNA. However, two substitutions, at positions 42 and 154, would disrupt base pairing in proposed helices (Table 1 and Fig. 3). Five changes (positions 52, 117, 118, 128 and 145) occur in single-stranded regions; two of these substitutions (117 and 118) occur in conserved box B and one (145) in box C. The sequence heterogeneities detected here presumably have an effect on

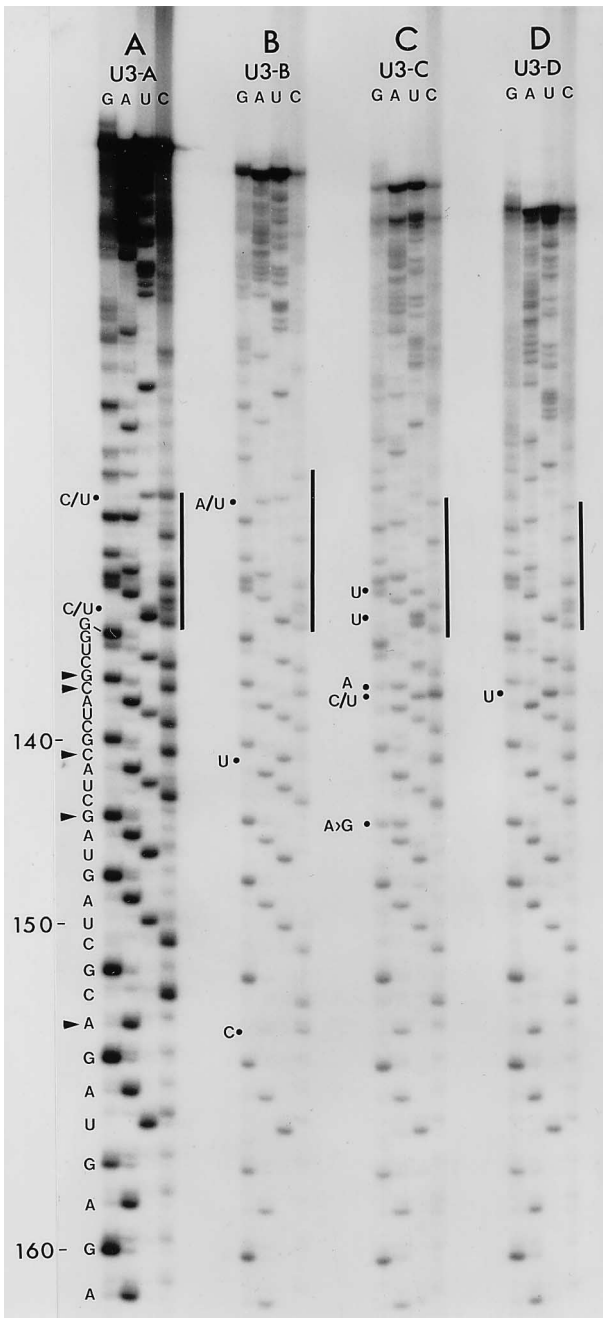

Fig. 4 Chemical sequence analysis of U3 snoRNAs from *E. gracilis*. The 3′-end-labelled U3 snoRNA bands (U3-A to U3-D) are designated according to their migration in 10% polyacrylamide/7 M urea gels (see Fig. 2 and text). *Lanes G, A, U and C* represent the products of chemical sequencing reactions specific for the indicated nucleosides. Some of the nucleoside substitutions between the four U3 snoRNA bands are indicated (all such differences are listed in Table 1). The *line* to the right of the lanes delineates an area of sequence compression (the same number of nucleotides is marked in each case)

Table 1 Sequence variation and positions of modified nucleoside residues in the four sequenced bands of *E. gracilis* U3 snoRNA

Position[a]	U3-A[b]	U3-B	U3-C	U3-D
25	Ψ [c]	Ψ	Ψ	Ψ
42	G	G	G/A	G
44	C	C	C/U	C
52	C	C	C>U	C>U
69	Ψ	Ψ	Ψ	Ψ
117	C/U	C	C	C
118	A	A/U	A	A
124	C	C	U	C
128	C/U	C	U	C
135	G	G	A	G
136	C	C	C/U	U
141	C	U	C	C
145	G	G	A>G	G
154	A	C	A	A

[a] Numbers correspond to positions in the secondary structure model of U3 snoRNA (Fig. 3)
[b] Letters designating each band of U3 snoRNA are based on their individual mobilities in a 10% polyacrylamide/7 M urea gel (U3-A migrating most slowly and U3-D most rapidly; see Fig. 2)
[c] Pseudouridine (5-ribosyluracil)

the overall conformation of the U3 snoRNAs containing them because the various species separated into four discrete bands during electrophoresis in a 10% polyacrylamide/7 M urea gel (Fig. 2). These four U3 snoRNA bands give rise to varying degrees of band compression in helix 4 (see Fig. 4).

Some information on modified nucleosides was obtained from a combination of the chemical and enzymatic sequence data (Table 1). Pseudouridine (Ψ, 5-ribosyluracil) residues are not cleaved by the hydrazine reaction used to determine uridine nucleotides in chemical sequence analysis and therefore the corresponding positions appear blank in chemical sequencing gels. However, RNase Phy M does hydrolyze after Ψ residues, thereby producing U-specific bands in enzymatic sequencing gels. One of the Ψ residues detected (position 25) occurs within box A as part of the base pair that closes the loop of helix 1A, while the other occurs within the stem of helix 1B (Fig. 3). $O^{2'}$-methylnucleoside (Nm) residues can be identified by their resistance to enzymatic/alkali cleavage. Due to secondary structure effects noted above, we were unable to obtain enzymatic sequencing data for positions 117–128 (Fig. 4); consequently, we could not assess the Nm content within this region. However, there are clearly no Nm residues in the rest of the U3 snoRNA sequence.

Anti-TMG immunoprecipitation experiments, combined with sequence analysis, indicated that all four U3 bands (A–D) contain U3 snoRNAs that have TMG cap structures (data not shown), although this result does not rule out the possibility that some U3 transcripts may not be capped.

Genomic organization of U3 snoRNA from *E. gracilis*

Total genomic DNA was digested with several restriction endonucleases that do not have restriction sites within the *E. gracilis* U3 sequence, and the products were separated in a 0.7% agarose gel prior to transfer to nylon membrane.

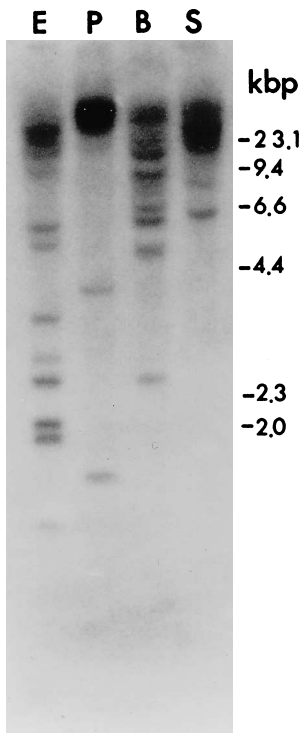

Fig. 5 Southern-blot analysis of U3-hybridizing sequences in the *E. gracilis* nuclear genome (details in Materials and methods). Nuclear DNA was hydrolyzed with the indicated enzymes (*E Eco*RI; *P Pst*I; *B Bam*HI; *S Sal*I), and the resulting blot was hybridized to probes generated by random-primed oligonucleotide-labelling of the U3 amplification product. Sizes of the *Hin*dIII restriction fragments of λ DNA used as markers are indicated

With a labelled probe generated by random priming of the U3 amplification product, a complex but reproducible hybridization pattern was generated (Fig. 5), indicative of multiple U3-hybridizing sequences within the *E. gracilis* genome. When one of the membranes was re-hybridized with an LSU rRNA gene-specific probe, we observed the expected hybridization pattern (Schnare et al. 1990), indicating that the pattern obtained with the U3-specific probe was not an artefact of incomplete restriction endonuclease digestion. The U3 probing results parallel the evidence from chemical sequencing that more than four expressed species of U3 snoRNA are present in *E. gracilis* (Table 1 and Fig. 4).

Discussion

Potential secondary structure of *E. gracilis* U3 snoRNA

The potential secondary structure of the *E. gracilis* U3 snoRNA (Fig. 3) is consistent with information derived from a phylogenetic comparison with, and structure probing of U3 snoRNA from, other organisms. As predicted for non-vertebrates, the 5′-end of U3 snoRNA can be paired to contain two helical structures. The first 5′-helix (1A) forms a stem-loop structure containing box A, similar to the structure proposed for plants (Kiss and Solymosy 1990; Leader et al. 1994), yeasts (Porter et al. 1988; Ségault et al. 1992) and trypanosomes (Hartshorne and Agabian 1994). The terminal loop contains the same box A nucleotide sequence as the U3 snoRNA from *S. cerevisiae*; these nucleotides were shown to be cross-linked by psoralen to the 5′-ETS of yeast pre-rRNA (Beltrame and Tollervey 1992). In *E. gracilis* U3 snoRNA, helix 1A contains a Ψ residue (position 25, Fig. 3), but it is not known whether this residue plays any specific role in binding to the 5′-ETS or in the cleavage of the pre-rRNA. Available information on Ψ content and distribution in U3 snoRNAs (Reddy et al. 1979; Busch et al. 1982; Porter et al. 1988) indicates that any function that may be attributed to *E. gracilis* Ψ25 cannot be phylogenetically conserved.

Helix 1B is considerably smaller than helix 1A and is bordered by two single-stranded regions (Fig. 3). This portion of the structure appears to be quite variable in size, shape and sequence among known non-vertebrate U3 snoRNAs. Accordingly, if the proposed *Euglena* helix 1B does in fact exist, it may not actually be homologous to its counterpart in U3 snoRNA from other organisms. The helix 1B region contains nucleotides that in other organisms can be base-paired with the 5′-ETS of the pre-rRNA; for this reason, it would be expected to be quite divergent (Beltrame and Tollervey 1992). This variation most likely reflects a structural constraint on the U3 snoRNA due to co-evolution with cognate binding sites within the pre-rRNA.

Based on early evidence, all U3 snoRNA conserved boxes were thought to be single-stranded, and it was postulated that they interact with specific regions of the pre-rRNA (Bachellerie et al. 1983; Crouch et al. 1983; Tague and Gerbi 1984). Realization that U3 snoRNA interacts with specific proteins to form a functionally active snoRNP substantially altered our view of the role these conserved regions may play in the association of snoRNAs with high-molecular-weight RNP complexes (Fournier and Maxwell 1993). In all secondary structure models of U3 snoRNA, box C is depicted as being single-stranded; this sequence is known to be required for the binding of fibrillarin and is found in all snoRNAs that are selectively precipitated with anti-fibrillarin antibodies (Baserga et al. 1991). Box C is therefore represented in a single-stranded state in the secondary structure presented for *E. gracilis* U3 snoRNA (Fig. 3). Initial structural data from chemical and enzymatic probing revealed that box B is single-stranded (Parker and Steitz 1987; Ségault et al. 1992). For this reason the *E. gracilis* U3 model depicts the box B sequence forming part of an internal loop with box C (Fig. 3). A possible function for the single-stranded box B has only recently been elucidated (Lübben et al., 1993): in human and rat U3 snoRNAs, a sequence containing boxes B and C is required to stabilize the specific binding of a 55-kDa core protein. The role of this protein-RNA interaction within the U3 snRNP and its relation to fibrillarin binding have not yet been established (Lübben et al. 1993). Further characterization will be required to determine whether homologs of the specific proteins known to interact with the U3 snoRNAs from other organisms also associate with U3 snoRNA in *E. gracilis*.

A portion of the box A^o sequence and the last base of the box D sequence form part of the 5-bp helix 2, with the remainder of the box A^o and box D sequences constituting an internal loop between helices 2 and 3 (Fig. 3). An ab-

Table 2 Regions of complementarity between U3 snoRNA and pre-rRNA in *E. gracilis*

U3 snoRNA	75-ACAACUΨCU**AACAUCACA**AGAGACACUUAGC-45[a]	
Pre-rRNA[b]		
5′-ETS	AACC	**GUUGUGGUGU** GAGA[c]
LSU 1	U***A***CCU	**GUUGUGGUG** GAUG[d]
ITS 2/3	GCCU	**GGGUUGUGGUG** CAU***C***[e]
ITS 1	CGGU	**GUUGUUGUG** CUGG[f]
LSU 9	GCCC	**UUGUGGUG** GGAU
ITS 9/10	GCUG	**UUGUUGUG** GGCC[f]
ITS 4/5	UGGUC	**UUGUUGUGU** GGCU[f]
ITS 1	UCCG	**UUGCAGUGUUCU** GAUC[f]
LSU 12	CGGA	**UGUGGUGU** GCAG
ITS 2	GGUG	**GUGGUGUU** GCCU

[a] The single-stranded region of U3 snoRNA including helix 1B. Nucleotides in bold are found in the loop region, while the doubly underlined nucleotides form the helix. Numbers represent corresponding nucleotide positions in the U3 snoRNA secondary structure model (Fig. 3)
[b] Regions of pre-rRNA complementary to regions of the U3 snoRNA are set in bold, with flanking nucleotides in normal type. ITS 1 and ITS 2 flank the 5.8S (LSU 1) sequence. Additional ITSs are numbered according to the LSU rRNA coding regions that they separate; e.g., ITS 2/3 is located between coding regions for LSU rRNA species 2 and 3
[c] 5′-ETS, corresponding to nucleotides 141–150 upstream of the 5′-end of the SSU rRNA
[d] The bold/italic A is the 5′-nucleotide of LSU 1 RNA (5.8S rRNA)
[e] The bold/italic C is the 5′-nucleotide of LSU 3 RNA
[f] Underlined nucleotides indicate mismatched base pairs with U3 snoRNA at these positions

solute requirement for the 3′-terminal stem in nuclear import and cap trimethylation has been demonstrated for U3 snoRNA (Baserga et al. 1992). Therefore, box A^o most likely functions in maintaining the correct 3′-end structure. However, because of the internal loop it forms with box D, box A^o still has the potential to interact with snoRNP proteins or with the pre-rRNA.

Organization of *E. gracilis* U3 snoRNA genes

U3 snoRNA genes are organized in a variety of ways in eukaryotic genomes. Vertebrates tend to have U3 multigene families that are either dispersed in the genome, as in the 14–20 U3 snoRNA gene copies in *Xenopus* (Savino et al. 1992), or tandemly linked, as in two of the four copies of the mouse U3B gene (Mazan and Bachellerie 1988). Unicellular eukaryotes typically have a reduced number of U3 genes, with a single gene for each expressed U3 snoRNA (Hughes et al. 1987; Selinger et al. 1992; Ørum et al. 1993). However, the slime mold *Dictyostelium discoideum* appears to have multiple dispersed U3 snoRNA genes, similar to the situation in some vertebrate genomes (Wise and Weiner 1980).

Southern hybridization indicates that the *E. gracilis* nuclear genome contains many U3-hybridizing sequences (Fig. 5). It is possible that each of these represents a gene that encodes a different member of the > 4 expressed U3 snoRNAs reported here. At present we cannot rule out the possibility that some of the *E. gracilis* U3-hybridizing signals may represent U3 pseudogenes, which have been found within the genomes of some (human, rat, and tomato) multicellular eukaryotes (Bernstein et al. 1983; Reddy et al. 1985; Stroke and Weiner 1985; Kiss and Solymosy 1990).

The differential expression of multiple U3 snoRNAs has been explored only superficially in rodents, where the three U3 copies are found in a 3:6:1 ratio (Reddy et al. 1979). The functional significance of differentially expressed U3 snoRNAs is unknown, but it is possible that they may alter the rRNA processing pathway to favour specific products, or may serve specific functions at different processing sites. The isolation and characterization of genomic clones should yield further insight into the organization and expression of U3 snoRNA genes in *E. gracilis*.

Possible functions of *E. gracilis* U3 snoRNA in rRNA processing

A common theme to emerge in both mRNA and rRNA maturation is that base pairing occurs between the precursor RNA and small RNAs involved in the processing (Beltrame and Tollervey 1995). To assess the possibility that U3 snoRNA may play a role in rRNA processing in *E. gracilis* that is mediated by base pairing with the pre-rRNA, a search was conducted for complementarities of seven or more nucleotides between the *E. gracilis* pre-rRNA sequence (Gunderson and Sogin 1986; Schnare et al. 1990; S.J. Greenwood, J.R. Cook, M.N. Schnare and M.W. Gray, in preparation) and the accessible single-stranded regions bounded by helix 1A and helix 2 of the U3 snoRNA model, including the stem-loop of helix 1B (Fig. 3). The most striking result is an exact 10-nt complementarity between the U3 snoRNA and the 5′-ETS (Table 2). In all other eukaryotes studied to-date, a U3 snoRNA/5′-ETS interaction required for rRNA processing occurs downstream from the 5’-ETS processing site (Maser and Calvet 1989; Stroke and Weiner 1989; Beltrame and Tollervey 1992; Mougey et al. 1993). In *S. cerevisiae* a 10-nt complementary sequence

located between helix 1A and 1B of U3 snoRNA can be crosslinked to the 5′-ETS; mutational analysis has shown that a U3 snoRNA/5′-ETS interaction at this site is absolutely required for maturation of the yeast SSU rRNA (Beltrame and Tollervey 1992, 1995). In *E. gracilis* the region of complementarity within the U3 snoRNA is the single-stranded loop of helix 1B (plus one nucleotide from the 3′-end of the helix) (Fig. 3). The complementary region of the *E. gracilis* 5′-ETS comprises the sequence between nucleotides 141 and 150 upstream of the 5′-end of the SSU rRNA (S.J. Greenwood, J.R. Cook, M.N. Schnare and M.W. Gray, in preparation), resembling the situation in yeast. Further studies will be required to assess the biological significance of the proposed U3 snoRNA/5′-ETS interaction in *E. gracilis*, and its functional correspondence to the analogous interaction in other eukaryotes.

In *E. gracilis*, non-conventional processing takes place within the LSU rRNA coding region at novel ITSs, whose post-transcriptional removal divides the LSU rRNA into 14 separate pieces (Schnare and Gray 1990). This idiosyncratic processing must involve either normal components of the pre-rRNA processing machinery that have acquired additional functions, or new components (e.g., novel snoRNAs). Schnare et al. (1990) noted that the *E. gracilis* ITSs constitute simple sequence patterns rich in pairs of nucleotides, such as $(T_{1-2}\ G_{1-2})_n$. A characteristic feature of all of the U3 snoRNA-complementary sequences listed in Table 2 is that they are composed of G and U stretches and are mostly found within spacer regions of the pre-rRNA. These similarities raise the intriguing possibility that in *E. gracilis*, U3 snoRNA may participate not only in 5′-ETS processing but in multiple interactions with the novel ITSs during the processing that leads to liberation of the mature SSU rRNA and the highly fragmented LSU rRNA. The results reported here provide a basis for further investigation of this possibility.

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