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SnoRNA U21 is also intron-encoded in *Drosophila melanogaster* but in a different host-gene as compared to warm-blooded vertebrates

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Abstract U21 is an intron-encoded snoRNA in vertebrates which contains a 13-nt tract of complementarity to an invariant sequence in eukaryotic 28S rRNA. Here, we report the characterization of its *Drosophila melanogaster* homolog which is the first case of an intron-encoded snoRNA in an invertebrate metazoan. In *D. melanogaster*, U21 is encoded within the ARF-1 (ADP ribosylation factor 1) gene, whereas in chicken and mammals it is found in the ribosomal protein L5 gene. In *D. melanogaster*, like in vertebrates, U21 is devoid of a 5′ trimethylguanosine cap, thus, likely resulting from processing of intronic RNA. The only portion of U21 sequence preserved between *D. melanogaster* and vertebrates, in addition to the hallmark box C and box D motifs, corresponds precisely to the 13-nt complementary to rRNA, pointing to an important role of the pairing of U21 to pre-rRNA.

Key words: snoRNA; Intron; U21; rRNA processing; ARF 1 gene; *Drosophila*

1. Introduction

The nucleolus contains a complex population of small RNAs, termed snoRNAs [1–3]. Although their precise functions remain to be precisely established, some of them have already been shown by depletion analysis to be required for ribosome biogenesis while most of the others are implicated in this process by a series of indirect evidence [1–3]. Particularly, a growing family of snoRNAs contain long, phylogenetically conserved sequence complementarities to rRNAs, which must reflect the biological importance of their transient pairing with pre-rRNA in the nucleolus [4]. Moreover, most of the newly identified snoRNAs exhibit a unique biosynthetic pathway [5–18]. Not only they are encoded in introns of protein-coding genes, but they result from a novel form of intronic RNA processing of their host-gene transcript. Remarkably, most of the novel intron-encoded snoRNAs have been found in genes of nucleolar or ribosome-associated proteins [3,4,9,10]. This peculiar mode of gene organization and expression could provide the basis for regulatory linkages in the coordinate production of the protein and RNA components of the ribosome, possibly through couplings between snoRNA processing and splicing of host-gene pre-mRNA.

We have recently identified a novel snoRNA, U21, encoded in an intron of the gene of ribosomal protein L5 in distant vertebrates, chicken, human and mouse [14]. U21 is remarkable by the presence of a 13 nt-long tract of perfect complementarity to a strongly conserved sequence in 28S rRNA. In the present study, we have characterized the U21 homolog of *Drosophila melanogaster*, to test phylogenetically the significance of the rRNA complementarity observed in vertebrate U21. The sequence determined for *D. melanogaster* U21 definitely supports the biological importance of this conserved complementarity. Unexpectedly, the host-gene of intron-encoded U21 differs in *D. melanogaster* as compared to vertebrates, deviating from the pattern observed so far for other intronic snoRNAs since it does not encode a protein directly involved in the biogenesis or activity of the ribosomes.

2. Materials and methods

2.1. Oligonucleotide probes and RNA analysis

Sequences of the chicken U21–3′ and human U3 antisense oligonucleotides have been described previously [14]. Sequences of the oligonucleotides used for amplification of U21 cDNA were as follows: AN1: 5′ AGCTGATATCCCGGGAAGCTTGGATCCAGGCCTGTTAAC 3′. AN2: 5′ AGCTGTTAACAGGCCTGGATCCAAGCTTCCCGGGATATC 3′. Oligonucleotide *D. melanogaster* U21–5′ (sequence: 5′ CACAATGCATAATCATTACAAGC 3′) was selected within the sequence of the 55-nt primer extension of the chicken U21–3′ oligonucleotide, carried out on *D. melanogaster* RNA. Procedures for 5′ end-labeling of the probes, RNA purification, preparation of cellular extracts, immunoprecipitations and RNA analysis were as previously reported [13,14], following classical procedures [19]. The antitrimethylcap antibody (R1131) was a gift of R. Lührmann [20]. The monoclonal 72B9 antifibrillarin antibody was obtained from G. Reimer and E. Tan [21].

Northern hybridization was performed in 5 × SSC, 5 × Denhardt's solution, 1% SDS, 150 μg/ml *E. coli* tRNA carrier, for 16 h at 40°C, followed by two washes in 2 × SSC, 0.1% SDS at the same temperature.

2.2. Synthesis and sequencing of the cDNA. Southern analysis

Sequencing of *D. melanogaster* U21 RNA was completed by cDNA analysis, through a two-step procedure schematized in Fig. 2a.

In a first step, the analysis was restricted to the fifty-five 5′ terminal nucleotides of the RNA. Oligonucleotide chicken U21–3′ was extended by Mu-MLV reverse transcriptase in the presence of 10 μg total cellular *D. melanogaster* RNA (1 h, 37°C). The cDNA was purified by electrophoresis on a 6% acrylamide/7 M urea gel, as reported elsewhere [22], then ligated to 5′ phosphorylated AN2 oligonucleotide in the presence of T4 RNA ligase (16 h, 37°C). The second strand synthesis was performed, after hybridization with oligonucleotide AN1 (complementary to AN2), by a PCR with Taq DNA polymerase (Stratagene) using as second primer oligonucleotide chicken U21–3′. The PCR included a first step at 95°C (5 min), followed by 25 cycles (1 min at 96°C, 1 min at 58°C and 1 min at 72°C) with a final step at 72°C (5 min). Amplified DNA was inserted in the vector pBluescript II KS phagemid (linearized by digestion with *Sma*I) using T4 DNA ligase (BRL). Automated sequencing (ALF) was performed on double-strand plasmid DNA.

The 3′ terminal region of the RNA was sequenced in a second step, as follows. Purified *D. melanogaster* U21 RNA was first ligated at its 3′ end to 5′ phosphorylated AN1 oligonucleotide (T4 RNA ligase,

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24 h, 10°C). The ligation product was then hybridized to AN2 oligonucleotide (complementary to AN1). The cDNA was synthesized with MuMLV reverse transcriptase and amplified by PCR (30 cycles: 1 min at 95°C, 1 min at 55°C, 1 min at 72°C). PCR primers were oligonucleotide AN2 and a 23-mer corresponding to the sequence of segment 15–37 of *D. melanogaster* U21 RNA determined in the first step. The PCR product was cloned and sequenced as described above.

Southern hybridization of *D. melanogaster* DNA, purified according to [19], was carried out in the same medium as the Northern hybridization, for 16 h at 50°C, followed by washes in 2 × SSC, 0.1% SDS (35°C, 30 min) and 0.1 × SSC, 0.1% SDS (37°C, 30 min).

3. Results

3.1. Detection of the D. melanogaster U21 homolog

U21 is a metabolically stable, box C-box D-containing snoRNA encoded in intron 5 of the rpL5 gene in humans, mouse and chicken [14]. Its 3′ terminal region contains, immediately upstream from box D, a 13-nt tract of perfect complementarity to a 28S rRNA sequence which is invariant among eukaryotes. We have previously shown that within the U21 sequence this 3′ terminal region is more particularly conserved among vertebrates: by Northern hybridization with an antisense oligonucleotide, termed U21–3′, spanning the 28S rRNA complementarity and box D in the chicken snoRNA sequence, U21 can be readily detected in amphibians and fish, in relatively stringent conditions of Northern hybridization [14]. In this work, we have analysed in the same conditions *D. melanogaster* and vertebrate RNAs with this probe (Fig. 1a). While vertebrate U21 snoRNA is 92–94 nt long, the strong and unique radioactive band detected for *D. melanogaster* migrates at 78 nt.

A primer extension with the same 5′ end-labeled oligonucleotide was carried out on *D. melanogaster* RNA. In all vertebrates examined so far, the cDNA produced from cellular RNA with this primer has exactly the same size as U21 RNA, since the 5′ terminal nucleotide of the primer matches the 3′ terminal nucleotide of the RNA [14]. As shown in Fig. 1b (lanes 4 and 5), a control primer extension performed on chicken RNA gave rise to a cDNA which is 93 nt long, like chicken U21 RNA [14]. For *D. melanogaster*, an abundant labeled cDNA was also detected (Fig. 1b, lanes 1 and 2). This cDNA is 78 nt long, like the *D. melanogaster* cellular RNA detected with this oligonucleotide by Northern analysis, further supporting the notion that it is reverse-transcribed from the U21 homolog.

In vertebrates, U21 is devoid of 5′ trimethyl-guanosine cap [14], suggesting that its 5′ terminus does not correspond to the 5′ end of a primary transcript. Likewise, the 78 nt-long *D. melanogaster* RNA lacks this typical capped structure. As shown in Fig. 1c, this RNA was not immunoprecipitated by the anti-TMG cap antibody, unlike U3 RNA used as a positive control in that experiment.

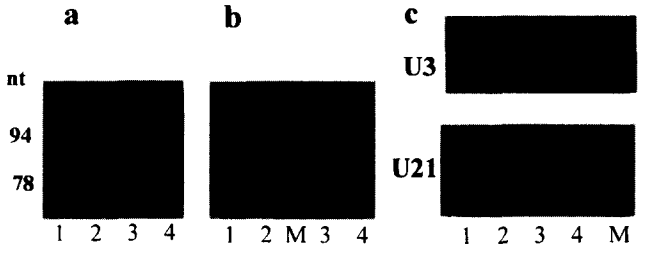

Fig. 1. Characterization of the *D. melanogaster* U21 homolog. (a) Northern analysis of cellular RNA. RNAs (10 μg/lane) were analysed on a 8% acrylamide/7 M urea gel. After electro transfer, the membrane was hybridized with the 5′ end-labeled 23-mer oligodeoxynucleotide complementary to the 3′ terminal region of the chicken U21 sequence [14]. Lane 1: *D. melanogaster*. Lane 2: pigeon. Lane 3: duck. Lane 4: human HeLa cells. (b) Primer extension performed on total cellular RNA with the same 5′ end-labeled oligodeoxynucleotide as in a). Products of the reaction performed on *D. melanogaster* (lanes 1 and 2) and chicken (lanes 3 and 4) RNAs, after a 1-h incubation with reverse transcriptase either at 50°C (lanes 1 and 4) or at 37°C (lanes 2 and 3), were analysed on a 8% acrylamide/7 M urea gel (lane M: DNA size marker). (c) Immunoprecipitation with the antitrimethyl cap antibody. RNAs were analysed on a 8% acrylamide/7 M urea. After electro transfer, the membrane was hybridized with the chicken U21–3′ probe (lower panel) and with a human U3 oligonucleotide probe (upper panel). The analysis was performed in parallel on *D. melanogaster* RNA (lanes 1 and 2) and on hamster RNA (lanes 3 and 4). RNA recovered from the immunoprecipitated fraction was analysed in lanes 1 and 3 (control total RNA is shown in lanes 2 and 4).

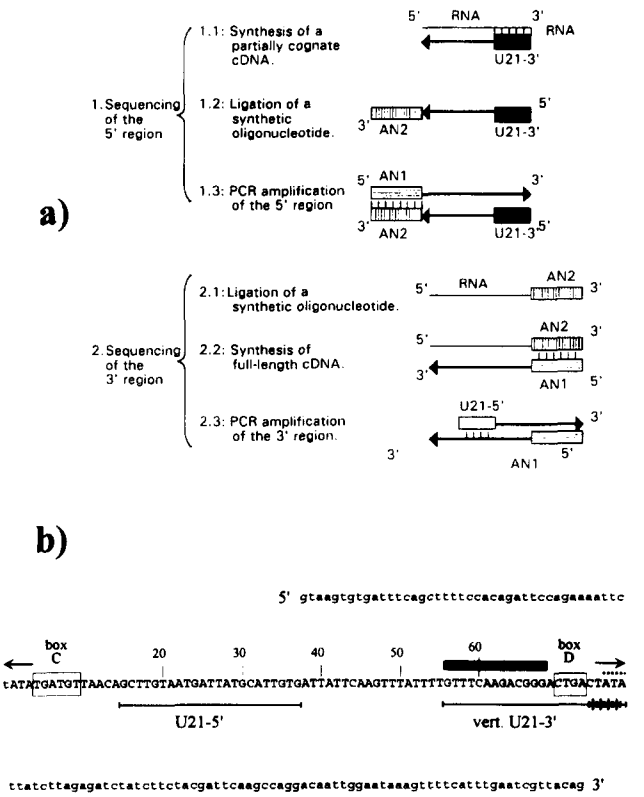

Fig. 2. Sequence of *D. melanogaster* U21 RNA. (a) Strategy for cDNA synthesis and sequencing: The various oligonucleotides (see section 2) are represented by boxes. (b) The *D. melanogaster* U21 RNA-coding sequence. The portion of the ARF1 gene intron [23] which perfectly matches the cDNA sequence is shown in higher case letters (dots overline the three 3′ terminal nucleotides which were not determined by cDNA sequencing, as described in section 3). The remaining parts of the intron are represented in lower case letters. Arrows in opposite orientation denote a potential non-canonical terminal stem (see section 4). The conserved segment of complementarity to an invariant 13-nt sequence in eukaryotic 28S rRNA is overlined by a thick bar. The location of the two antisense oligonucleotides used in this work is denoted by thin bars under the sequence. The only significant sequence similarity with vertebrate U21 snoRNAs, in addition to the box C motif, corresponds precisely to the segment spanned by the vertebrate U21–3′ oligonucleotide (except for the positions denoted by stars, the sequence delineated by the bar is identical in *D. melanogaster*, chicken, mouse and human U21).

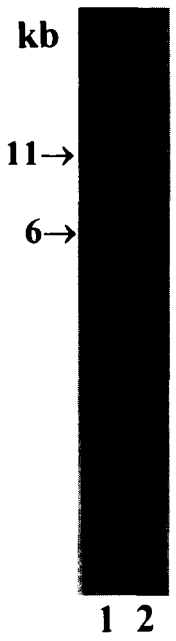

Fig. 3. Southern analysis of *D. melanogaster* genomic DNA. Purified DNA was digested by *Eco*RI (lane 1) or *Hin*dIII (lane 2) and separated on a 1% agarose gel (20 μg/lane). After transfer, the membrane was hybridized with the 5′ ^{32}P-end-labeled chicken U21–3′ probe.

3.2. *Sequencing of the D. melanogaster homolog and identification of its host-gene*

The sequence of the *D. melanogaster* RNA cross-hybridizing with the vertebrate U21–3′ antisense probe was determined as schematized in Fig. 2a. In a first step, a partial determination was carried out for the fifty-five 5′ terminal nucleotides, i.e. the region upstream from the sequence cross-hybridizing to the vertebrate probe. The 78 nt-long cDNA produced by extension of the 23-mer oligonucleotide was amplified, cloned and sequenced. To rule out the possibility that this cDNA resulted from a spurious primer extension, a control Northern hybridization was carried out on total RNA from *D. melanogaster* cells, using oligonucleotide probe U21–5′ the sequence of which had been selected within the 55-nt extension of the cDNA. A unique hybridization signal was observed (result not shown), coincident with the band detected with the U21–3′ probe, at 78 nt, confirming that the reverse transcription product which had been sequenced was the expected cDNA. The sequence of the fifty-five 5′ terminal nucleotides of the *D. melanogaster* RNA does not exhibit any significant homology to the corresponding region of vertebrate U21 sequences, except for the presence of a motif matching the box C sequence, UGAUGU, at positions 4–9 from the 5′ terminus of the RNA sequence, i.e. similar to what is observed for U21 RNA in vertebrates [14]. In that first step of the analysis, the portion of the 3′ region of the *D. melanogaster* 78 nt-long RNA spanned by the reverse transcription primer could not be sequenced. The RNA sequence determination was completed in a second stage as schematized in Fig. 2a. As expected from the efficient and selective primer extension observed with *D. melanogaster* RNA (Fig. 1b), the sequence of the 3′ end of the RNA closely matches that of the chicken U21–3′ primer (Fig. 2b). It is noteworthy that the RNA sequence derived from cDNA analysis was 3 nt shorter than measured by electrophoretic mobility in the Northern hybridization assay suggesting that the three 3′ terminal nucleotides of the RNA have been missed in the cDNA sequence analysis. This probably results from an exonucleolytic trimming of the RNA during its purification, combined with a preferential utilization of the trimmed RNA in the oligonucleotide ligation and PCR.

The complete sequence obtained for the *D. melanogaster* cDNA was compared with the EMBL Sequence Data Library. Remarkably, the 75 nt-long sequence perfectly matches one of the published sequence entries (accession number S62079). The *D. melanogaster* U21 RNA sequence maps within the first intron of the *D. melanogaster* ARF1 (ADP-ribosylation factor 1) gene [23]. It is found within the RNA-like strand of this gene, starting at position 43 within the 193 nt-long intron.

By Southern analysis, we have observed that this sequence is not repeated elsewhere in the *D. melanogaster* genome. Using as probe the U21–3′ oligonucleotide in relatively stringent conditions of hybridization, a single radioactive band was detected after digestion of *D. melanogaster* DNA by *Hin*dIII, *Eco*RI (Fig. 3) or *Bam*HI (not shown). Identical results (not shown) were obtained when the cloned 75 nt-long cDNA product was used as probe.

4. Discussion

Vertebrate and yeast RNAs containing box C, box D and long, phylogenetically conserved sequence complementarities to rRNA represent a growing family of snoRNAs which could function in pre-rRNA folding, nucleotide modification and pre-ribosome assembly, possibly acting in some cases as RNA chaperones [4]. Among these snoRNAs, U18 [12] and U21 are remarkable because of their overlapping 28S rRNA complementarities, which raises the possibility that the two snoRNAs act in a concerted fashion during early stages of ribosome biogenesis [14]. Our previous study has revealed the preferential conservation among distant vertebrates of the 3′ terminal region of U21 including the 13 nt-long complementarity to 28S rRNA and the adjacent, downstream box D motif found in most snoRNAs [1–4]. Functional importance of the box D motif has been directly established in the case of U14 snoRNA, in yeast *S. cerevisiae* [24]. Using an oligonucleotide probe spanning this conserved 3′ terminal region of vertebrate U21, we have identified a 78 nt-long RNA in *D. melanogaster* with the characteristics of a U21 homolog. This is the first direct characterization of a *Drosophila* snoRNA (it is still not known whether the previously reported fragment of *D. melanogaster* genomic DNA [25] showing substantial sequence homology to vertebrate U3 RNA represents a functional gene). Like vertebrate U21 RNA, the *D. melanogaster* homolog exhibits near its 5′ end (at positions 4–9)) the hallmark box C motif, also required for the accumulation of stable yeast U14 in yeast [24] which is preceded by a purine, like in other box C-box D-containing snoRNAs [3,4]. Accordingly, the perfect conservation observed between *D. melanogaster* and vertebrate U21 homologs for the 13-nt tract of complementarity to 28S rRNA strenghtens the view that the pairing to pre-rRNA plays a key role in the function of U21 snoRNA. A large subset of snoRNA containing box C and box D and long complementarities to rRNAs, including vertebrate U21, exhibit a typical 4–5-bp stem involving the 5′ and 3′ terminal nucleotides [3,4]. Experimental evidences indicate that this terminal stem structure, conserved

among vertebrates and yeast for U14 and U24 [24,18], is required for faithful processing of U20 intronic snoRNA [26; Cavaillé, J. and Bachellerie, J.-P., unpubl. results]. The *D. melanogaster* U21 sequence is devoid of an inverted repetition at the appropriate position for a canonical stem/box structure, i.e. with an upstream repeat separated from box C by 2 nucleotides and a downstream repeat adjacent to box D. Interestingly, a 4-nt inverted repetition is located in the immediate vicinity (denoted by arrows in Fig. 2b) which could provide the basis for a sustitute terminal stem. However, the absence of (or substantial deviation from) a canonical terminal structure might reflect significant differences in the biogenesis of U21 snoRNA between vertebrates and the fly. The identification of additional *D. melanogaster* homologs belonging to this snoRNA family should provide further information on this point.

The Southern analysis of *D. melanogaster* genomic DNA unambiguously shows that the fly haploid genome does not contain additional copies of the U21 RNA-coding sequence, besides the one found in the ARF 1 gene intron. U snRNA and U3 snoRNA genes are related by the presence of common transcriptional control elements, the proximal and distal sequence elements, located 50–60 bp and 200–260 bp upstream from the gene, respectively, and the 3′ box, 20–30 nt downstream from the gene [27]. These hallmark motifs cannot be found around the U21-coding sequence in the *D. melanogaster* ARF 1 gene (the U21-containing intron extends over only 42 nt upstream from the snoRNA-coding sequence). Moreover, *D. melanogaster* U21 is devoid of a 5′ trimethyl-guanosine cap, which demarks the 5′ end of a primary transcript, thus, differing from the abundant U3, U8 and U13 snoRNAs or from U snRNAs which are all produced by polymerase II-catalyzed synthesis from autonomous transcription units [27–29]. Accordingly, rather than corresponding to the primary transcript of an independent gene within the intron *D. melanogaster* U21 RNA seems likely to result from intronic RNA processing of its host-gene transcript, like other previously reported intron-encoded snoRNAs [5–8]. It is also noteworthy that we could not identify any U21-related sequence in the homologous intron of the published human ARF 1 gene sequence [30]. Moreover, no U21 variant form was detected by Northern or Southern analyses in the fly or in humans (results not shown).

Host-genes for most intronic snoRNA reported to date code for ribosome-associated or nucleolar proteins. Likewise, U21 is encoded in a ribosomal protein gene in the vertebrate species studied so far. Given the likely involvement of these snoRNAs in pre-rRNA processing [3], it has been proposed that their unusual mode of gene organization and expression might provide the basis for coordinated production of rRNA, r-proteins and proteins directly involved in the biogenesis or activity of ribosomes [3,6–10]. Among the few apparent exceptions to this pattern, host-genes for U14 in vertebrates and for U17 in mammals code for proteins involved in protein and RNA transport into the nucleus [31,32], which could still participate in the control in early stages of ribosome biogenesis. U17, which is encoded in a ribosomal protein gene, rpXS8, in amphibian *Xenopus laevis* [15] has been the first example of a change of host-gene for an intronic snoRNA. Besides U21, two other cases of intronic snoRNAs positioned within different host-genes in different organisms have been reported to date [3] for U18 (located in rpL1 gene in vertebrates and in elongation factor EF-1 β gene in *S. cerevisiae*) and U24. Like for U24 in yeast *S. cerevisiae* [18], the host-gene of *D. melanogaster* U21 codes for a GTP-binding protein without obvious link to ribosome biogenesis or activity. Accordingly, the pattern previously observed for the identity of intronic snoRNAs host-genes might merely reflect the choice of actively transcribed genes, as previously suggested [9].

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