Identification, expression and functional analysis of U3 snoRNA genes from *Neurospora crassa*

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Received 28 March 2008; received in revised form 24 April 2008; accepted 24 April 2008

Abstract

By screening of a cDNA library constructed with small RNAs from *Neurospora crassa* and genome analysis, three U3 snoRNAs of 262, 270 and 275 nt were identified and named as NcU3A, NcU3B and NcU3C, respectively. Expression of the three U3 snoRNA genes was further analyzed by Northern blot hybridization. Meanwhile, two 5′-truncated U3 transcripts with different sizes were also identified, indicating a complex posttranscriptional processing of the U3 snoRNAs. In addition to the known functional elements complementary to 18S rRNA, a novel antisense sequence to 26S rRNA was found in NcU3A. A primer extension assay showed that the antisense sequence did not guide 2′-O-ribose methylation of 26S rRNA but probably functioned as an rRNA chaperon. In the *N. crassa* genome, three U3 snoRNA genes are independently transcribed and each of them contains a small intron. The same intron-inserting site in the U3 snoRNA genes among *N. crassa* and yeasts shows the ancient origin of the intron in these organisms. Our results provide new insights into the structural and functional diversity of U3 snoRNA family.

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Keywords: *Neurospora crassa*; U3 snoRNA; rRNA processing

1. Introduction

In eukaryotes, transcription and processing of ribosomal RNA genes play essential roles in cell life and mainly take place in nucleolus [1]. Posttranscriptional processing and modification events of rRNAs depend on some small nucleolar RNAs (snoRNAs) which function in the form of small nucleolar ribonucleoprotein (snoRNP) [2–4]. Seven snoRNAs, including U3, U8, U14, nR30 (in yeast), U22, 7-2/MRP, and snR10 (in yeast) have been demonstrated to be involved in the processing of pre-rRNA [5]. Moreover, deletion of any of the above snoRNAs can prevent the processing of pre-rRNA, leading to the termination of cell life [6,7].

Among the snoRNAs involved in the cleavage of pre-rRNA, U3 snoRNA is essential to the maturation of 18S rRNA, with U3 interacting with 5′ ETS (external transcribed spacer) [8] and 18S rRNA of the pre-rRNA [9]. U3 snoRNA possesses seven conserved elements and can be also folded to the conserved secondary structure, so it is a good model to study the evolution of structure and function of RNA.

*Neurospora crassa* is a multicellular fungus, which shares more similar biological properties with higher eukaryotes than unicellular yeasts [10]. As a model organism, the genome of *N. crassa* has been completely sequenced [10], and 9826 protein coding genes, 74 5S rRNAs, 424 tRNAs, and 9 snRNAs were predicated; but only 4 snoRNAs (snR39, snR52, snR60, and snR61) were found [http://www.broad.mit.edu/annotation/genome/neurospora/Home.html]. Obviously, the majority of snoRNA genes remain to be identified, especially some snoRNAs involved in the posttranscriptional maturation of rRNAs. Thus, we conducted the research into snoRNAs in *N. crassa*.
Through construction and screening of a snoRNA-enriched cDNA library, a set of snoRNA genes, including three U3 snoRNAs and their processed transcripts, have been identified. The structure and function of the U3 snoRNAs have been further analyzed.

2. Materials and methods

2.1. Strains and medium

The *N. crassa* wild-type strain (As 3.1604, purchased from CGMCC) was used for the construction of the cDNA library and all RNA analyses. The *N. crassa* strain was grown in PSA medium containing 2% sucrose, 20% extract of potato at 30 °C. The *Escherichia coli* strain TG1 grown in 2YT (1.6% Bacto tryptone, 1% yeast extract, 0.5% NaCl) liquid or solid medium was used for cloning procedures.

2.2. Construction and screening of the cDNA library

Preparation and purification of total RNA from *N. crassa* were essentially performed as described previously [11]. The total RNA (~20 µg) was first polyadenylated using poly (A) polymerase (TaKaRa). The A-tailed RNA was subsequently reverse-transcribed using the [γ-32P]-labeled primer dT16CD and M-MLV reverse transcriptase (Promega). The product was size-fractionated on a denaturing 8% polyacrylamide gel (8 M urea, 1 × TBE buffer). Subsequently, cDNA fragments ranging in size from 60 to 120 nucleotides and from 120 to 434 nucleotides were excised and eluted from the gels. The cDNAs were tapered at the 3'-end with dTGP using terminal deoxynucleotidyl transferase (TDT, Takara). The G-tailed cDNAs were then amplified by PCR with primers polyCM and dT23H2. After purification on 8% polyacrylamide gel (8 M urea, 1 × TBE buffer), the amplified products were cloned into pMD-18T vector (TaKaRa) and then transformed into the strain TG1 of *E. coli*. To exclude the abundant and known small RNAs, we performed dot hybridization to screen the library. Membranes (Hybond-N⁺; Amersham) of colony PCR products with P47 and P48 were UV cross-linked and hybridized with oligonucleotide probes that antisense to the sequence repeated in the library.

2.3. Northern blot analysis

The total RNA (~30 µg) was separated on 8% polyacrylamide gel (8 M urea, 1 × TBE buffer) and then was

<table>
<thead>
<tr>
<th>Table 1</th>
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<tr>
<td>The sequences of U3 snoRNAs and their 5'-truncated transcripts identified from the <em>N. crassa</em> cDNA library.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Length/nt</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>262</td>
<td>AUGU[C[AGAGA][ACUC][CGAGA][AAAUCAUCCUCUAU]GUAUUGCUCUCUUUUGUUGUCCCCUAAGG</td>
</tr>
<tr>
<td>270</td>
<td>AUGU[C[AGAGA][ACUC][CGAGA][AAAUCAUCCUCUAU]GUAUUGCUCUCUUUUGUUGUCCCCUAAGG</td>
</tr>
<tr>
<td>183</td>
<td>AUGU[C[AGAGA][ACUC][CGAGA][AAAUCAUCCUCUAU]GUAUUGCUCUCUUUUGUUGUCCCCUAAGG</td>
</tr>
<tr>
<td>192</td>
<td>AUGU[C[AGAGA][ACUC][CGAGA][AAAUCAUCCUCUAU]GUAUUGCUCUCUUUUGUUGUCCCCUAAGG</td>
</tr>
<tr>
<td>83</td>
<td>AUGU[C[AGAGA][ACUC][CGAGA][AAAUCAUCCUCUAU]GUAUUGCUCUCUUUUGUUGUCCCCUAAGG</td>
</tr>
</tbody>
</table>

Lowercase letters indicate that these nucleotides were not cloned by the library construction strategy we used. Conserved motifs are boxed. Box GAC, A', A, C, B, C, D are shown in NcU3A and NcU3B, box C and D are shown in NcU3A-2, NcU3B-2 and NcU3A-3.
2.4. 26S rRNA gene cloning

Primers (Nc26SF/Nc26SR) according to the 26S rRNA sequence in NCBI database were designed. The N. crassa 26S rRNA gene was amplified by PCR with primer pairs Nc26SF/Nc26SR and the N. crassa genomic DNA. PCR products were purified in agarose and then cloned into pMD-18T vector (Takara) and transformed into E. coli. After screening the library by colony PCR, positive clones were subsequently chosen for sequencing.

2.5. Mapping of 2'-O-ribose methylation

Ribose-methylated nucleotides of the N. crassa rRNA were determined by primer extension at low dNTP concentrations as described previously [13]. Briefly, two reverse transcription reactions with a dNTP concentration of either 4 µM or 1.0 mM were performed in parallel using 15 µg of total RNA and 8 ng of 5'-end-labeled primers in a 20 µl reaction mixture. The 26S rDNA was directly sequenced with the same primer used for rRNA methylation mapping and run in parallel with the reverse transcription reactions on 8% polyacrylamide denaturing gels.

2.6. Oligonucleotides and primers

The oligonucleotides used for construction of the cDNA library were as follows: dT16CD: 5'-CTCCCAAAAGCTTTTTTTTTTTTTT-3', polyCM: 5'-GGAATTCCGGATCCCCCCCCCCCCCCCCCCCCC-3'; P47: 5'-GGCCAGGGTTTTCCAGTCAGC-3'; P48: 5'-GCCGGATAAACAATTTTACACAGGA-3'. The primers used for PCR amplification of N. crassa 26S rRNA gene were as follows: NC26S F7: 5'-TCCGATCGGATCCCCCCCCCCCCCCCCC-3'; polyCM: 5'-GGAATTCCGGATCCCCCCCCCCCCCCCCC-3'; P47: 5'-CGCACCTAGT-GGAATACCC-3'; P48: 5'-AGCCGGATAAACAATTTTACACAGGA-3'. The oligonucleotides used for Northern blot analysis were Nc U3A (5'-AGATCTAGGCGAAGGAGGGC-3') and Nc U3B (5'-CGCCAGGGTTTTCCAGTCAGC-3'). The oligonucleotides used for Northern blot analysis were Nc U3A (5'-AGATCTAGGCGAAGGAGGGC-3') and Nc U3B (5'-CGCCAGGGTTTTCCAGTCAGC-3'). The oligonucleotides used for Northern blot analysis were Nc U3A (5'-AGATCTAGGCGAAGGAGGGC-3') and Nc U3B (5'-CGCCAGGGTTTTCCAGTCAGC-3'). The oligonucleotides used for Northern blot analysis were Nc U3A (5'-AGATCTAGGCGAAGGAGGGC-3') and Nc U3B (5'-CGCCAGGGTTTTCCAGTCAGC-3').

2.7. Bioinformatics analysis software


3. Results

3.1. Identification of U3 snoRNAs from N. crassa

Through the screening of the cDNA library of small nuclear RNAs from N. crassa, two novel box C/D snoRNA sequences were identified, with the lengths of 262 and 270 nt, respectively (Table 1). As has been reported, box C/D snoRNAs are mainly ranged in size from 80 to 100 nt, and the one which is longer than 200 nt is probable to take part in the processing of pre-rRNA. Our computer analysis showed that these two novel snoRNAs possessed seven conserved motifs of U3 snoRNA (GAC box, box A', box A, box B, box C', box C and box D) and displayed partial sequence homology to U3 snoRNA in yeast. Thus, they were named NcU3A and NcU3B, respectively. By further analysis of the N. crassa genome, a DNA sequence highly identical to NcU3B was identified and was named as NcU3C.

To investigate the expression of U3 snoRNA genes, we performed Northern blot analysis using specific 5'-end-labeled primers. The result revealed that NcU3A and NcU3B had the same size of segment we got from cDNA clones, but that the size of NcU3C was approximately 275 nt. Moreover, in addition to the three snoRNAs of full size, two U3 transcripts of different sizes were also detected by the Northern blot analysis (Fig. 1). Actually, during the screening of the cDNA library, we also identified three clones of almost the same sizes as the U3 transcripts mentioned above, which were named as NcU3A-2, NcU3A-3 and NcU3B-2 (Table 1). Compared with the intact U3 snoRNAs, these U3 transcripts lacked 5'-terminal sequence, but all had C box, D box and terminal inverted sequence. As we know, the U3 snoRNAs are the characters of typical snoRNAs, and they play an important role in nucleolar localization and stability of snoRNA in cells [14]. Our experimental results and analyses showed that besides the expression of U3 snoRNAs of full size, there were still U3 transcripts of different sizes in N. crassa.
3.2. The structure and function of the N. crassa U3 snoRNAs

With the help of bioinformatics analysis, the secondary structure of the U3 snoRNAs was predicted (Fig. 2). We found that NcU3A, B and C had a similar secondary structure: two stem loops at the 5'-terminal region, and hairpins 2, 3, 4 in the middle. This structure of U3 in N. crassa is inclined to that in yeast and some lower eukaryotes [15,16] rather than higher eukaryotes [17]. Sequence comparisons of the three U3 snoRNA gene copies showed that GAC box, box A, box A, box B, box C, and box D were highly conserved. NcU3B and NcU3C shared 94.1% identity. When compared with NcU3A, a few mutations appeared in the sequences of NcU3B and NcU3C, including the base-compensatory mutations at the pairs 80–61 and 81–60 and the base insertions at the sites of 121, 128, 157, 167, and 205. The rest of the mutations were base alternations, most of which took place at the loop or bulge. Interestingly, most stem regions presented few base alternations or a few base-compensatory mutations (Fig. 2). Taken together with previous results, we concluded that in addition to the seven conserved boxes, the stem structure is important too. It is possible that the stem plays an important role in maintaining the function of U3 snoRNA.

Further, function analysis indicated that U3 snoRNAs have two conserved antisense elements, both of which can form base-pair interaction with 18S rRNA, one with sites from U9 to C25, the other with A1134–A1138. The two functional elements are homologous to that in yeast and other eukaryotes. These sequences complementary to rRNA have been known to guide the cleavage and processing of pre-rRNA 5'-terminal regions, and are essential to cell growth. Surprisingly, NcU3A and its processed transcripts (NcU3A-2, NcU3A-3) have an antisense element especially complementary to 26S rRNA, which has not been reported before (Fig. 3). Generally, about 10 nt base-pair interaction between box C/D snoRNA and rRNA can potentially guide 2'-O-methylation or help folding of rRNA. A primer extension at low dNTP concentrations was carried out to identify the 2'-O-methylation at 26S-A1226. The result showed that there was no stop at the predicted site and adjacent sequence (Fig. 3). Thus,
we concluded that NeU3A does not guide 2’-O-ribose methylation of 26S rRNA but probably functions as an rRNA chaperon.

3.3. Analysis of N. crassa U3 snoRNA genes

NeU3A and NeU3B locate on chromosome I, whereas NeU3C locates on chromosome II. The three U3 snoRNAs are homologous, sharing 90% identity. These genes all contain a typical promoter region TATA box, with a conserved sequence of GCACAGACC upstream of the promoter region. A downstream CT-rich region which may be the binding site of some transcriptional factors was also found. In addition, on the downstream of U3 snoRNA genes exists a poly-(T) region which is similar to the signal for transcription termination in Saccharomyces cerevisiae

Interestingly, each of the three U3 snoRNA genes contains an intron which has a typical splicing signal (5’-GT ... AG-3’) and a branch-point sequence (CTAAC) (Fig. 4). The insertion of the intron locates between Box A’ and Box A, namely between CA and GA (Fig. 2).

Table 2

The length and intron of U3 snoRNA identified from different organisms.

<table>
<thead>
<tr>
<th>Organism</th>
<th>T. bruce</th>
<th>S. cerevisiae</th>
<th>S. pombe</th>
<th>D. melanogaster</th>
<th>X. laevis</th>
<th>A. thaliana</th>
<th>R. norvegicus</th>
<th>H. sapiens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length (nt)</td>
<td>143</td>
<td>333/332</td>
<td>255/254</td>
<td>211</td>
<td>219</td>
<td>220</td>
<td>215</td>
<td>215</td>
</tr>
<tr>
<td>Intron</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
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</table>

“-” represents no intron in U3 snoRNA gene; “+” represents the presence of intron in U3 snoRNA gene (Data from http://www.ncrna.org/frnadb/main_html and http://www.sanger.ac.uk/cgi-bin/Rfam/).
4. Discussion

The secondary structure of U3 snoRNAs is highly conserved among eukaryotes, whereas the primary sequences diverge rapidly. Even in some cases, there can be a great difference of up to 190 nt in the length of U3 snoRNA (Table 2). Therefore, by direct sequence comparison, it is difficult to identify U3 snoRNAs from distant organisms.

In this study, we identified for the first time three U3 snoRNAs and their processed transcripts from the filamentous fungus *Neurospora crassa*. To date, there have been no reports about the different transcripts of U3 snoRNA in higher eukaryotes, but a similar phenomenon was observed in *Trypanosoma brucei* and *Euglena gracilis* [19,20]. For example, 5′-truncated U3 snoRNAs were obtained from the *Euglena gracilis* cDNA library constructed with the immunoprecipitation method. Therefore, we infer that this phenomenon may be widespread among lower eukaryotes. Owing to the conserved motifs and the similar size to typical box C/D snoRNA, these 5′-truncated U3 snoRNAs could be stable in cells like other box C/D snoRNAs. We also found that NcU3A possesses antisense elements complementary to both 18S rRNA and 26S rRNA, so NcU3A may be a dual functional molecule. The findings of different processed transcripts and the new antisense elements of U3 snoRNA in *N. crassa* imply a more complicated structure and function of U3 snoRNA than anticipated.

All U3 snoRNA genes in different organisms are independently transcribed, and in higher eukaryotes there is no intron in U3 snoRNA genes. It was first reported in *S. cerevisiae* and *Hansenula wingei* that U3 snoRNA gene had an intron [21,22]. In the *Schizosaccharomyces pombe* U3 snoRNA, however, no such intron exists [23]. So we wondered whether an intron existed in U3 snoRNA genes in multicellular fungi. Our results showed that each copy of U3 snoRNA genes in *S. pombe* possesses antisense elements complementary to both 18S rRNA and 26S rRNA, so NcU3A may be a dual functional molecule. The findings of different processed transcripts and the new antisense elements of U3 snoRNA in *N. crassa* imply a more complicated structure and function of U3 snoRNA than anticipated.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant Nos. 30470385 and 30570398) and the National Program on Key Basic Research Projects (Grant No. 2005CB724600).