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Processing of eukaryotic tRNA precursors: secondary structure of the precursor specific sequences affects the rate but not the accuracy of processing reactions

Luisa Castagnoli[1], Gennaro Ciliberto[1,2] and Riccardo Cortese[1,2]

[1]European Molecular Biology Laboratory, Postfach 102209, 6900 Heidelberg, FRG, and [2]Istituto di Chimica Biologica, University of Naples, Naples, Italy

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ABSTRACT

The primary transcriptional product of eukaryotic tRNA genes is a precursor molecule with extranucleotides at the 5' and at the 3' end. We show that the 5' and 3' sequences, uniquely present in the RNA precursor molecule do not play any role in the efficiency and accuracy of processing reactions. If, however, as a consequence of in vitro manipulation, these extranucleotides form a base-paired extension of the aminoacid acceptor stem, the rate of processing is slowed down. The rate of processing is brought back to normal in a single base-pair deletion mutant probably as a consequence of a destabilization of the base-paired extension of the aminoacid acceptor stem.

INTRODUCTION

The primary product of transcription of a tRNA gene, in prokaryotes and in eukaryotes, is a precursor RNA molecule with extra nucleotides both at the 5' and at the 3' extremities; in some eukaryotic tRNAs also introns are present (1-6). The maturation of these tRNA precursors is a multistep ordered process consisting of nucleolytic size-reducing reactions and of nucleoside modifications (7).

All available evidence indicates that the precursor specific nucleotides do not carry any information important for the recognition by maturation enzymes, whose activity depends only on sequences and/or structures within the tRNA part of the precursor; so far both in prokaryoties (8) and in eukaryotes (9) the only class of mutations provoking an alteration of the maturation pathway maps within the tRNA gene coding sequence. Changes in the flanking regions of the tRNA genes, with consequent changes in the sequence of the extra nucleotides at the 5' or the 3' ends of the tRNA precursor molecule do not appear to affect the processing pathway (10).

In this paper we report results showing that the secondary structure of the nucleotides uniquely present in the precursor molecule does influence the efficiency but not the accuracy of the processing reactions.

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MATERIALS AND METHODS

Bacterial strains and plasmids. E.coli K12 strain Hb101 and 71-18 were used for transformation with pBR322 and M13 derivatives respectively, as described (10).

Enzymes and chemicals. Restriction endonuclease BamH I was purchased from Bethesda Research Laboratories (BRL) and was used according to the recommendations of the supplier. Restriction endonucleases EcoR I and Hind III, DNA ligase and T_1 ribonuclease were a gift of Dr. V. Pirrotta. 5 bromo-4-chloro-indolyl-β-D-galactoside and isopropyl-thiogalactoside were purchased from Sigma. Molecular linkers were from Collaborative Research. Radioactive compounds were purchased from Amersham Buchler.

Nucleic acid sequencing and bidimensional oligonucleotide mapping. DNA sequences were determined using the method of Maxam and Gilbert (11) for pBR322 derivative plasmids and with the method of Sanger et al. (12) for the M13 derivatives.

Oocyte injections and analysis of the transcript. Nuclear microinjection of X.laevis oocytes was done as described (10). Double stranded plasmid DNA was injected at the concentration of 200 µg/ml using α-P^{32}-GTP as RNA precursor. Groups of 20 oocytes/sample were injected and incubated for 5 to 6 hours. Reinjection of RNA species into the nucleus or the cytoplasm of the frog oocytes was performed as described (4).

RESULTS AND DISCUSSION

We have previously established that a plasmid (Mcet 1) carrying a DNA segment, 263 base pairs in length, containing the $tRNA^{Pro}$ gene from Caenorhabditis elegans was able to direct the synthesis of mature $tRNA^{Pro}$ when microinjected into the nucleus of Xenopus laevis oocytes (13). Furthermore we showed that all the information necessary to direct the promotion of the transcription resides within the coding sequence of the gene (10).

We have subcloned the $tDNA^{Pro}$ coding sequence (71 bp), which is conveniently flanked at either sides by HaeIII restriction sites, into different plasmid vectors, making use of molecular linkers. We have therefore obtained several subclones in which the $tDNA^{Pro}$ coding region is flanked by different vector derived sequences (fig. 1): pBcet15, where the $tRNA^{Pro}$ coding region flanked by Hind III and Bam HI linkers at the 5' and 3' side respectively, was cloned into pBR322 in substitution of the Hind III-Bam HI segment of the tet gene; pBcet151, where the $tRNA^{Pro}$ coding region flanked by Bam HI and Hind III linkers at the 5' and 3' side respectively was cloned in pBR322 in the

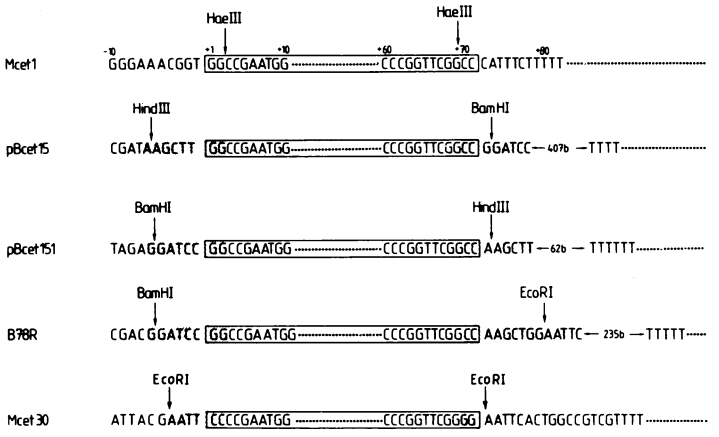

Fig. 1. DNA sequences of the clones containing the coding region of $tRNA^{Pro}$ flanked by various vector derived sequences.
The $tRNA^{Pro}$ coding region is boxed and the dotted line within the box, from nucleotide G_{10} to nucleotide +60 corresponds, for all clones, to the sequence already published (10). The hatched nucleotides correspond to the molecular linkers used for the construction of the various clones. At the 3' side of the coding sequence we have indicated the distance, in bases, to the nearest run of four or more thymidine residues, which function as signal for termination of transcription.

opposite orientation; B78R a derivative of pBcet151 was obtained by adding an EcoRI linker to the filled-in Hind III site and cloning into the mp701 phage vector (as described in ref. 14). These three plasmids contain an identical $tRNA^{Pro}$ coding region which is flanked by different vector derived sequences. Following microinjection into the nucleus of Xenopus laevis

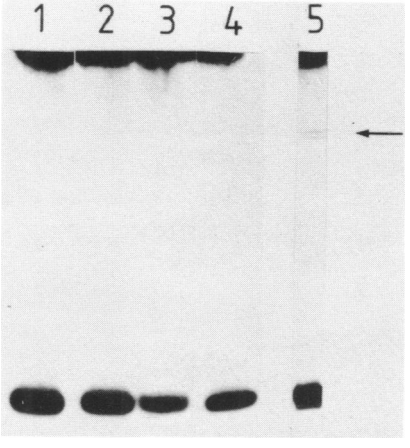

Fig. 2. Autoradiography of a 10% polyacrylamide gel. Plasmid DNA and $\{P^{32}\}$GTP were injected into the nucleus of X.laevis oocytes and after five hours (lane 1,2,3,4) or after one hour (lane 5) the RNA was extracted and electrophoresed in a polyacrylamide gel as described (10).

lane 1 = RNA from Mcet1

lane 2 = RNA from pBcet15

lane 3 = RNA from pBcet151

lane 4 = RNA from B78R

lane 5 = RNA from B78R

oocytes the DNAs from plasmids pBcet15, pBcet151 and B78R are all transcribed (fig. 2, slots 2, 3 and 4) as efficiently as Mcet1 (fig. 2, slot 1). The band visible in every slot corresponds to a mature tRNA^{Pro}, including the added terminal CCA as determined by fingerprint analysis (data not shown). This RNA species, however, is not the primary transcript which, as usual for tRNA, is a molecule longer than the mature product. The transcription of the primary RNA starts somewhere in the 5' flanking region and terminates at the first run of 4 or more thymidine residues in the 3' flanking region (14). When the processing reactions are very efficient, it is difficult to detect the primary transcript, which appears as a faint band only at very short incubation times after microinjection. The band corresponding to the primary transcript of the tRNA^{Pro} gene is shown in slot 5 where, in addition to mature tRNA^{Pro} a related RNA of about 320 nucleotides is present. This length corresponds to that expected for the transcript from B78R, assuming termination at the first run of thymidine residues, in this case located 235 nucleotides downstream from the tRNA^{Pro} coding sequence, in a region of the lac operon, characteristically present in all M13mp vectors (15).
The efficiency of processing of the primary transcriptional products from pBcet15, pBcet151 and B78R is comparable to that observed for Mcet1 (fig. 2, slot 1) where the tDNA^{Pro} coding sequence is flanked by the original nematode sequences. These results imply that the sequence of the extra nucleotides

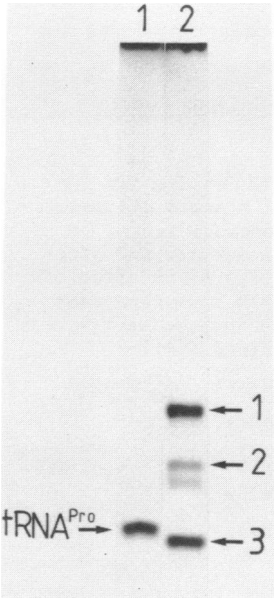

Fig. 3. Autoradiograph of a 10% polyacrylamide gel. Transcriptional products obtained after injection into the nucleus of X.laevis oocytes of the DNAs from pBcet15 (lane 1) and Mcet30 (lane 2).

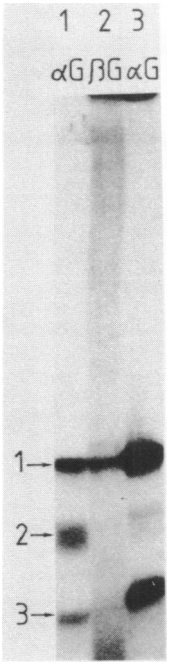

Fig. 4. Transcriptional products from Mcet30 DNA. In lane 1 and 3 {α-P^{32}}GTP was conjected with DNA while in lane 2 {β-P^{32}}GTP (410Ci/μmole) was conjected.

uniquely present in the tRNA precursor molecule is not very important for the efficiency of the processing reactions.

THE SECONDARY STRUCTURE OF THE tRNA PRECURSOR SPECIFIC SEQUENCES AFFECTS THE RATE OF PROCESSING REACTIONS.

One of the plasmids we have constructed (Mcet30) carries the $tRNA^{Pro}$ gene flanked by identical EcoRI linker molecules at the 5' and at the 3' extremities. The DNA sequence of this clone is also shown in fig. 1. The use of identical linkers at the two sides generates the possibility for a longer than usual base-paired region between the 5' and the 3' ends of the primary transcript with a consequent base-paired extension of the aminoacid acceptor stem. When injected into X.laevis oocytes, this DNA directs the synthesis of a tRNA molecule with an altered rate of maturation. In fig. 3, panel A are presented for comparison the transcriptional products of pBcet15 and Mcet30. The RNA produced when pBcet15 DNA is injected (slot 1), is a mature and functional $tRNA^{Pro}$ molecule, as shown by fingerprint analysis (result not shown) and by its capacity to be aminoacylated in vitro with proline (result not shown). In contrast, the transcriptional pattern obtained after injection of Mcet30 DNA consists of three main bands (fig. 3, slot 2, bands 1,2,3). Band 3 is a mature $tRNA^{Pro}$ molecule, which includes CCA at the 3' end as

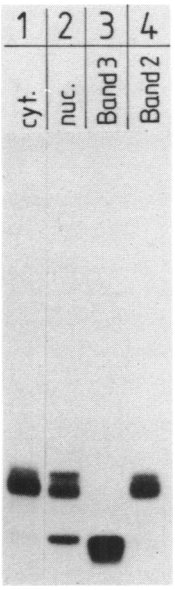

Fig. 5. Autoradiograph of a 10% polyacrylamide gel.
The RNA corresponding to band 2 in fig. 3 was extracted from the gel and microinjected into the nucleus (lane 2) or into the cytoplasm (lane 1) of X.laevis oocytes. A mature $tRNA^{Pro}$ (lane 3) and an RNA extracted from band 2 (lane 4) were run in parallel as controls.

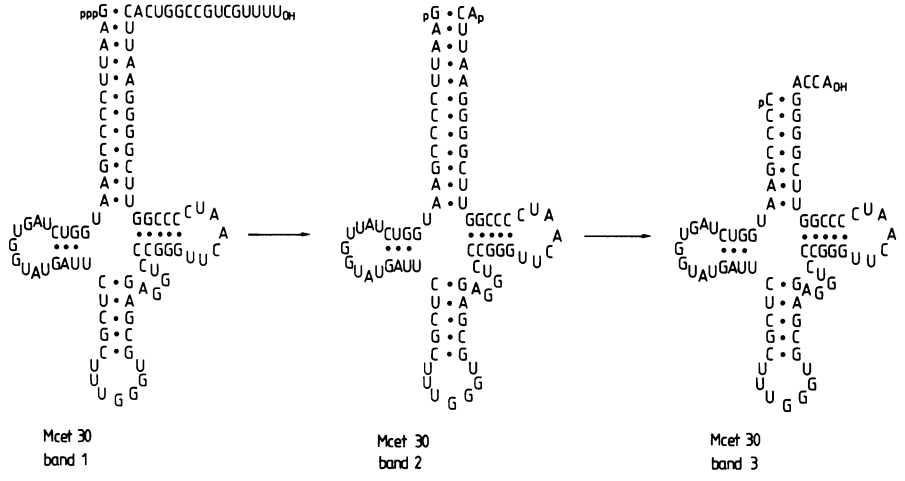

Fig. 6. Sequence of RNA extracted from Band 1, 2 and 3 of fig. 3.
In order to determine the sequences here shown, we have exploited the possibility to separately label the RNA with the four nucleotide triphosphates and then analyse it, after nuclease T_1 digestion and oligonucleotide fractionation, by cellulose acetate electrophoresis and homochromatography on DEAE cellulose plate as described (16).

shown by fingerprint analysis (result not shown).

The mature products of pBcet15 and Mcet30 DNA transcription have the same length as deduced by fingerprint analysis (data not shown); the different migration on polyacrylamide gel can only be due to a two base pairs change in the aminoacid acceptor stem caused by the addition of the EcoRI linker.

Band 1 is the primary transcript as shown by its selective labelling with β-^{32}P-GTP (fig. 4, slot 2).

The intermediate size band 2 is often resolved in multiple RNA species.

Direct evidence that the intermediate size RNA extracted from band 2 can be converted in vivo to mature $tRNA^{Pro}$ was obtained by reinjecting the purified

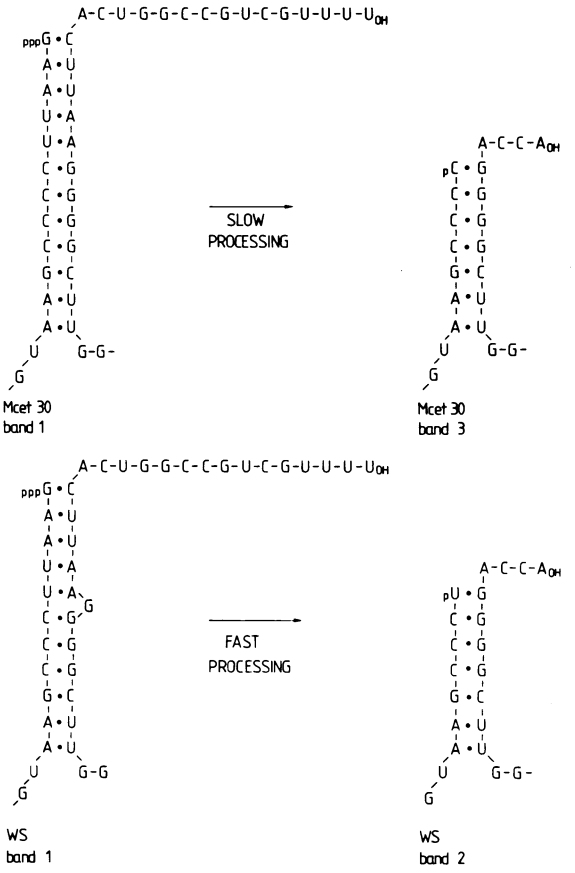

Fig. 7. Aminoacid acceptor stem structures of the Mcet30 and WS primary transcripts, and their maturation products.

intermediate size RNA either into the X.laevis oocyte nucleus or into the cytoplasm. The results of this experiment are shown in fig. 5. In slot 4 is the RNA extracted from band 2, in slot 3 is the purified RNA from band 3 (mature $tRNA^{Pro}$. Slot 2 shows the processing products obtained by injecting band 2 RNA into oocyte nuclei while slot 1 shows that when the same material is injected into the cytoplasm no further maturation takes place.
The sequences of the RNAs corresponding to bands 1,2,3 are shown in fig. 6. Structure 1 is the primary transcript from Mcet30 which, via the formation of the intermediate structure 2 is processed to a mature tRNA (structure 3). The rate of this processing reaction is considerably inferior to the wild type rate (Mcet1) and leads to the accumulation of intermediates. The sequence of the accumulating intermediate 2 reveals a property of the enzyme generally responsible for the maturation at the 3' end of tRNA precursors. Garber and Gage (3) have postulated the existence of an endonuclease which, in oocyte nuclear extract, cleaves a tRNA precursor after the first unpaired base at the 3' end of the aa stem. The enzymatic reaction involved in the processing of structure 1 to structure 2 also cleaves after the first unpaired nucleotide at the 3' site disregarding the unusual length of the aa acceptor stem. This

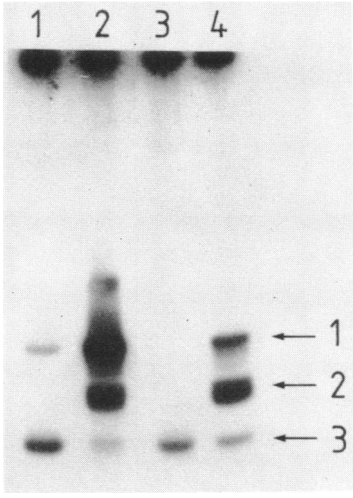

Fig. 8. Autoradiography of 10% polyacrylamide gel electrophoresis.
lane 1: WS DNA, 45 min incubation
lane 2: Mcet30 DNA, 45 min incubation
lane 3: WS DNA, 5 hour incubation
lane 4: Mcet30 DNA, 5 hours incubation

suggests that the main feature recognized by this processing enzyme is near the boundary between double and single stranded RNA structures, regardless of the position of this boundary with respect to the cloverleaf structure.

A SINGLE BASE PAIR DELETION IN THE DNA CODING FOR THE 5' HALF OF THE aa STEM IS SUFFICIENT TO ACCELERATE THE RATE OF MATURATION OF PRECURSOR RNAs.

A derivative of Mcet30 (called WS), constructed as described elsewhere (17), carries a deletion of one of the four cytosine residues present at the 5' end of the aa stem. A consequence of this deletion is a perturbation of the double stranded aa acceptor stem of the primary transcript with the formation of a single base bulge (as shown in fig. 7: WS band 1). When WS DNA is injected into X.laevis oocytes, the primary transcriptional product can only be detected at very short incubation times (fig. 8, lane 1) because it is rapidly processed to a mature tRNA (fig. 8, lane 3) at a rate comparable to the one observed for Mcet1. In contrast incompletely processed Mcet30 transcripts accumulate after short or long incubation time (fig. 8, lanes 2 and 4). Injection of WS DNA and incubation for different lengths of time have never led to the accumulation of RNA intermediates. A reasonable interpretation of this result is that the presence of the single base bulge has a destabilizing effect on the double stranded structure and causes a fraction or all tRNA precursor molecules to have the aa stem extending beyond the single base bulge in a single stranded form. This would create a structure analogous to a normal tRNA precursor which is processed by a cleavage near the boundary between double and single stranded RNA structures. The 3' end maturation of this tRNA would then occur as a single step process and not as a two step process as in Mcet30.

CONCLUSIONS

We have observed that altering the 5' and 3' flanking sequences in tRNA precursors affects neither the accuracy nor rate of the processing reaction. If, however, the 5' and 3' extensions can base pair thereby extending the length of the aa stem, then the processing is slowed down with a consequent accumulation of intermediates, but the accuracy of the reaction is not affected. The slowing down is probably a consequence of the unusual structure of the primary transcript, which is not, apparently, a good substrate for the various processsing enzymes. Even though the structure of precursor 1 does not normally occur in a physiological tRNA precursor, its maturation pathway reveals some properties that could be extended to the normally occurring pathway. It suggests that the cleavage site of a 3' maturating enzyme,

probably the endonuclease postulated by Garber and Gage (3), is at a boundary between double and single stranded structures. Due to the extension of the aa stem in precursor 1 from Mcet30, this transition point is displaced five bases towards the 3' end and so is the cleavage point, which gives rise to the intermediate structure 2.

This intermediate precursor is then processed to a mature tRNA. We do not know which enzymes are responsible for this final maturation step. Very likely, an RNaseP-like activity (2) is involved atthe 5' end, whereas the further processing at the 3' end could be again ascribed to the endonuclease that generates the intermediate precursor 2. Alternatively, or in addition, an enzyme like the prokaryotic exonuclease D (2) might generate the 3' OH terminus necessary for the addition of CCA by terminal nucleotidyl transferase.

A further point deserves comment: The mature tRNA molecule obtained from WS, as a consequence of the deletion of a cytosine residue, shows at the end of the aa stem an unusual $U_1.G_{72}$ base pair, so far not observed in any eukaryotic tRNA (11), which does not seem to interfere with maturation.

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