Volume 12 Number 2 1984 Nucleic Acids Research

Transcription of multimeric tRNA genes

Gennaro Ciliberto[1], Cinzia Traboni[2] and Riccardo Cortese[1]

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

Received 6 September 1983; Revised 30 November 1983; Accepted 7 December 1983

ABSTRACT

We have constructed a set of plasmids carrying a $tRNA^{Pro}$ gene from C. elegans in a head to tail dimeric and trimeric arrangement with virtually no spacer sequence. We show that in two different transcriptional systems, each coding region functions as an internal promoter directing the synthesis of independent transcriptional products. This is in contrast with the property of natural head to tail dimeric arrangements found in yeast, where only one coding region functions as promoter (Mao et al., 1980 Cell 20, 589; Schmidt et al., 1980 Nature 287, 750). The evolutionary significance of our finding is discussed.

INTRODUCTION

tRNA genes in eukaryotes are transcribed by RNA polymerase III (1). In most transcriptional systems it has been shown that the sequences essential for transcription by this polymerase are located within the coding region (1,2) and split in two separate or separable elements (3,4,5,6); as a consequence, initiation of transcription occurs upstream from the promoter signals.

In eukaryotic genomes, tRNA genes are often found in clusters, separated by spacer regions (1), and, in the majority of the cases, they are transcribed as single transcriptional units. In two cases however, two tRNA genes are found to be adjacent in a head to tail (7,8) configuration, with virtually no spacer sequences. In both cases only the first gene contains an active promoter, at least in vitro (7,8) and the primary transcript is a dimeric precursor which is then processed to two mature tRNA species.

We wondered whether the combination of only one promoter for two adjacent genes might not be a necessary requirement for transcription, perhaps because two active promoters in close proximity could exert some kind of reciprocal inhibitory effect. To address this question we artificially constructed a series of dimeric and trimeric $tRNA^{Pro}$ coding region clusters without spacers. In this paper we show that, even in this particular

polymeric arrangement, each monomeric promoter is active, giving rise to its own independent transcript.

MATERIALS AND METHODS

Chemicals and enzymes

Isopropyl-D-thiogalactopyranoside and 5-bromo-4-chloro-3-indolyl-D-galactopyranoside were purchased from Sigma. T4 DNA ligase and endonuclease Eco RI were a gift from V. Pirrotta. DNA polymerase large fragment (Klenow enzyme) was from Boehringer Mannheim. X-ray films were from Fuji. Radioactive compounds were purchased from Amersham.

Bacterial strains and cloning vectors

E.coli K12 strain 71/18 was used for transformation (10). The $tRNA^{Pro}$ coding region was cloned as monomer, dimer or trimer in the M13 mp2 cloning vector (10).

Construction of multimeric $tRNA^{Pro}$ genes

Site directed mutagenesis of $tDNA^{Pro}$ with reverse transcriptase has been described (11). The EcoRI-EcoRI DNA segment from mutant RT11.1 carrying a base pair transversion from C to A at position 56 of the $tDNA^{Pro}$ coding region (12) was isolated according to Cortese et al. (9). The segment was ligated at different ratios with the EcoRI digested M13 mp2 vector. Single, double and triple inserts in the antisense orientation were initially screened by quick hybridization of ssM13 preparations (13) with a ssM13 derivative carrying the $tDNA^{Pro}$ gene cloned in the opposite (sense) orientation (14).

Construction of $1xtDNA^{Pro}$(inv.)

This is the original mutant RT11.1 obtained by site directed mutagenesis with reverse transcriptase of $tDNA^{Pro}$ (12).

Construction of $2xtDNA^{Pro}$(inv.)

The dimeric $tRNA^{Pro}$ gene from $2xtDNA^{Pro}$ (see fig. 3) was isolated by partial digestion with EcoRI and ligated to the EcoRI digested M13 mp9 vector. The inserts in the sense orientation were identified by quick hybridization (12) to the single stranded DNA from $2xDNA^{Pro}$.

The structure of these plasmids was confirmed by DNA sequencing analysis with the dideoxi-chain termination method (15).

Microinjections

Microinjections into the nuclei of X.laevis oocytes were performed as described (14) by using a 40 µl solution of DNA and radioactive RNA precursors (α-^{32}P-GTP 410 Ci/mmole; 10 mCi/ml, 1 Ci = 3.7 x 10^{10} becquerels). Sets of 20 oocytes were injected and incubated for 5 h before RNA extraction (16).

Double stranded M13 DNA containing one, two or three copies of the $tDNA^{Pro}$ gene was injected at the concentration of 200 µg/ml along with plasmid Xbs1 (50 µg/ml), containing one copy of the somatic Xenopus borealis 5S RNA gene (17), as an internal standard for the injection experiment. In vivo processing of $tRNA^{Pro}$ precursors was performed according to Melton and Cortese (18). The RNA corresponding to the $tRNA^{Pro}$ precursor was extracted from the gel and microinjected. Incubation was for 1 h.

In vitro transcriptions

In vitro transcriptions using a cytoplasmic extract from HeLa cells were performed as described by Weil et al. (19).

Polyacrylamide gel electrophoresis

RNA transcripts were separated electrophoretically on Tris-Borate-EDTA, 7M urea, 10% polyacrylamide gels.

RESULTS

In vivo and in vitro transcription of wild-type $tDNA^{Pro}$

In previous studies we have characterized the transcriptional properties of the coding region of a $tRNA^{Pro}$ gene from C. elegans, by microinjection into the nucleus of the Xenopus laevis oocytes (4,5,9). The original $tRNA^{Pro}$ coding region (fig. 1) was cloned with EcoRI linkers at both extremities in the plasmid M13mp2 to give the derivative mpWS* (14). Plasmid mpWS* DNA was microinjected in the nucleus of Xenopus laevis oocytes. Transcriptional analysis shows a 92 bases primary transcript which is processed to mature $tRNA^{Pro}$ (fig. 2, lane 1). Transcription starts four nucleotides upstream from the 5' end of the coding region and terminates at a run of 4 T residues located 17 bp downstream from the 3' end of the coding region in the flanking M13 mp2 sequence (10). A more complete characterization of the structure of these transcripts has been published (20).

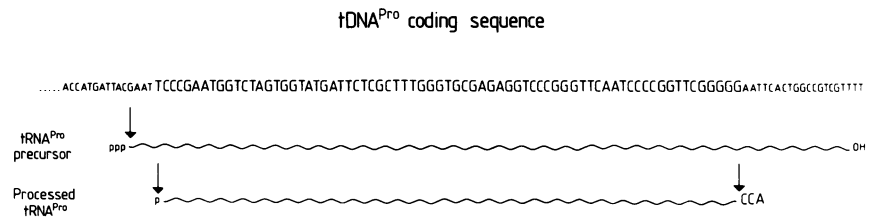

Fig. 1. Sequence of wild-type $tRNA^{Pro}$ coding sequence. Big case letters: $tDNA^{Pro}$ coding sequence. Small letters: flanking M13 sequences.

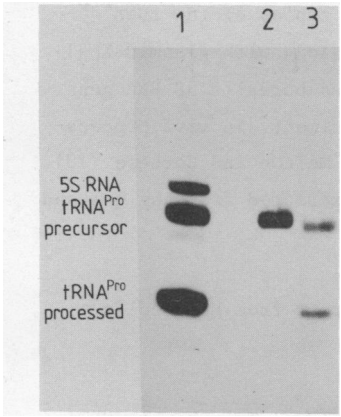

Fig. 2. In vivo and in vitro transcription of wild-type tDNA^{Pro}. Lane 1: In vivo transcription from plasmid mpWS* and plasmid Xbs1 (carrying one copy of a somatic 5S RNA gene). Lane 2: In vitro transcription from plasmid mpWS*. Lane 3: In vivo processing of the 92 bases long tRNA^{Pro} precursor.

In vitro transcription in HeLa cells extract gives only the precursor molecule which is not processed in this system (fig. 2, lane 2).

Construction and transcriptional analysis of tRNA^{Pro} multimeric gene clusters

The segment of DNA coding for tRNA^{Pro} gene has been subjected to extensive site directed mutagenesis (14,18). We had, therefore, a large collection of single base pairs substitution mutants. For the construction of dimeric and trimeric gene clusters we therefore chose to use a mutant tRNA^{Pro} which, as a consequence of a C56 to A56 transversion, is still transcribed, even though at a lower rate, but not processed (12). The multimeric gene clusters shown in fig. 3 were constructed as indicated in the methods section. In all cases the tRNA^{Pro} coding regions are cloned with the same orientation with respect to the vector sequences and are immediately flanked on the 3' side by the same termination signal as in the original mpWS*.

Transcriptional analysis was done by microinjection in the nucleus of the Xenopus laevis oocytes (fig. 4) or by in vitro assays using cytoplasmic HeLa cells extracts (fig. 5). Monomeric, dimeric and trimeric arrangements yield one, two or three transcripts respectively. The size of these transcripts corresponds to that expected if all tRNA^{Pro} promoters, in any

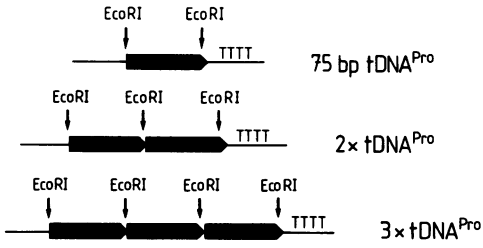

Fig. 3. Structure of the multimeric tRNA^{Pro} genes.

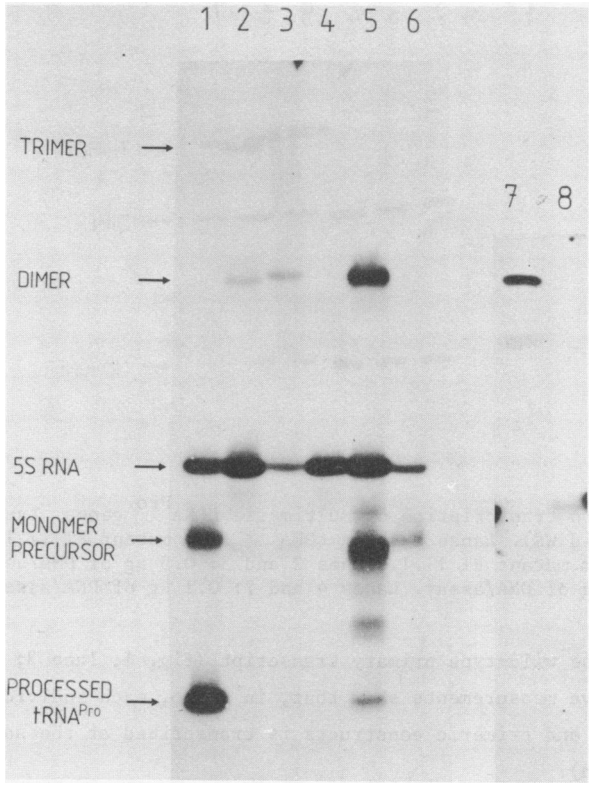

Fig. 4. Microinjection in the nucleus of X. laevis oocytes of multimeric $tRNA^{Pro}$ genes. Lane 1: Wild-type $tDNA^{Pro}$ WS*. Lane 2: 3x $tDNA^{Pro}$ from mutant RT 11.1. Lane 3: 2x $tDNA^{Pro}$ from mutant RT 11.1. Lane 4: Xbs1. Lane 5: 2x $tDNA^{Pro}$ from mutant RT 48.1 carrying a base pair substitution from A to T at position 58 of the $tDNA^{Pro}$ coding region yielding precursors which are partially processed. Lane 6: 1x $tDNA^{Pro}$ from mutant RT 11.1. $tDNA^{Pro}$ monomeric or multimeric constructions were injected at the concentration of 200 µg/ml. In each sample 5S RNA gene (plasmid Xbs1) was coinjected at the concentration of 50 µg/ml as an internal standard. Lane 7: Reinjection in the nucleus of X. laevis oocytes of $tRNA^{Pro}$ dimer from mutant RT 11.1. Lane 8: Reinjection in X. laevis oocytes of $tRNA^{Pro}$ precursor from wild-type WS*.

arrangements, were active. Qualitatively at least, there is complete agreement between the in vivo and in vitro results.

The multiple RNA species obtained with the dimeric or trimeric genes are not generated by processing but are independent transcripts. This is shown by the fact that the longer species are not converted into the smaller ones upon reinjection into the nucleus of the Xenopus oocyte (fig. 4, lane 7).

In this type of experiment a true precursor is processed to its product,

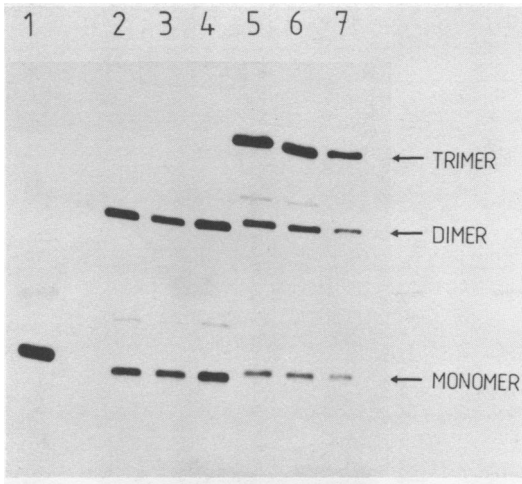

Fig. 5. In vitro transcription of multimeric $tRNA^{Pro}$ genes. Lane 1: Wild-type $tRNA^{Pro}$ (plasmid WS). Lanes 2-4: 2x $tDNA^{Pro}$ from mutant RT 11.1. Lanes 5-7: 3x $tDNA^{Pro}$ from mutant RT 11.1. Lanes 2 and 5: 0.3 µg of DNA/assay. Lanes 3 and 6: 0.2 µg of DNA/assay. Lanes 4 and 7: 0.1 µg of DNA/assay.

as shown for the wild-type primary transcript (fig. 1, lane 3; fig. 4, lane 8). Quantitative measurements show that, in vitro, each individual promoter in the dimeric and trimeric constructs is transcribed at the same efficiency (data not shown).

Of course the transcripts shown in figs. 4 and 5 could in principle derive not from independent initiation events but only from one initiation promoted by the first internal control region and the multiple bands be a consequence of termination occurring at various points within the gene cluster. This is unlikely in view of what is known of RNA pol.III termination signals, invariably constituted by a run of 4 or more T residues in the non-coding strand. The clones $2xDNA^{Pro}$ and $3xDNA^{Pro}$ are constructed, however, in a way that makes it impossible to distinguish between independent initiation and termination between the genes of the cluster, due to the fact that the termination signal is immediately downstream to the tRNA gene cluster.

In order to distinguish between these possibilities we have constructed other clones in which the monomer and dimer $tDNA^{Pro}$ are located very distantly from the nearest termination signal. These constructs are shown in fig. 6A. DNA from $1xDNA^{Pro}$(inv.) and from $2xDNA^{Pro}$(inv.) was digested with PvuII and in vitro run off transcription was done. If termination should sometimes occur between the two $tRNA^{Pro}$ coding regions in $2xtDNA^{Pro}$(inv.), we would

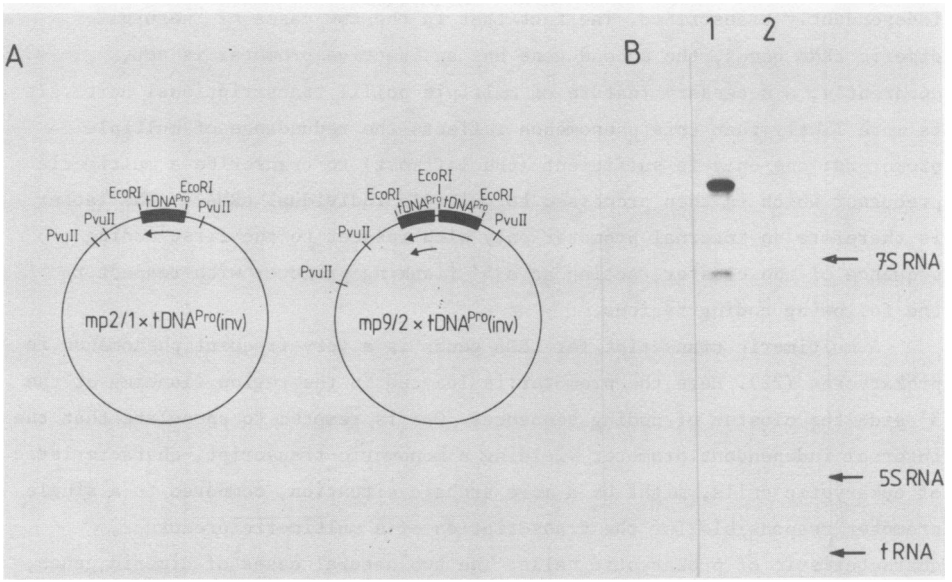

Fig. 6. A) Structure of the plasmids used for in vitro transcription. Note that the monomer $tDNA^{Pro}$ is cloned in the EcoRI site of mp2 (10), whereas the dimer $2xtDNA^{Pro}$ is cloned in the EcoRI site of mp9 (21).
B) Run off in vitro transcription. Lane 1: transcription from mp9/$2xtDNA^{Pro}$ inv. Lane 2: transcription from mp2/$1xtDNA^{Pro}$ inv. Arrows indicate molecular weight markers.

observe a full length transcript starting from the first promoter and going till the end of the PvuII-PvuII segment carrying the $tDNA^{Pro}$ gene cluster and, in addition, a band of about 80 bases from the beginning to the end of the first $tRNA^{Pro}$ coding region. In contrast, if transcription initiates independently at the two promoters, then we expect to see for the dimer two RNA species of about 290 and 370 bases respectively.

This is the result obtained and shown in fig. 6B. Note that the length of the shorter transcript from $2xtDNA^{Pro}$(inv.) is, as expected, 36 bases longer than the unique transcript from $1xDNA^{Pro}$(inv.) because the dimer was cloned in the EcoRI site of M13mp9 vector (at the end of the polylinker sequence) (21), whereas the monomer, in the same orientation, was originally constructed in M13 mp2 (which carries no polylinker) (10).

DISCUSSION

The results presented in this paper indicate that multiple active tRNA gene promoters can coexist in close proximity and are efficiently and

independently transcribed. The fact that in the two cases of "natural" dimeric tRNA genes, the second gene has an inactive promoter is not, apparently, a necessary feature of multiple polIII transcriptional units. It is more likely that this phenomenon reflects the redundance of multiple promoters: one only is sufficient (the leftmost) to transcribe a multimeric precursor which is then processed to multiple individual tRNA's. The latter is therefore an internal promoter only with respect to the first coding sequence of the cluster, acting as a 5' flanking promoter with respect to the following coding regions.

A multimeric transcript for tRNA genes is a very frequent phenomenon in prokaryotes (22). Here the promoter is located in the region flanking at the 5' side the cluster of coding sequences. One is tempted to speculate that the internal independent promoter yielding a monomeric transcript, characteristic of eukaryotic cells, might be a more archaic situation, compared to a single promoter responsible for the transcription of a multimeric precursor, characteristic of prokaryotic cells. The two natural cases of dimeric genes, in which only the first has an active promoter, constitutes an example of a possible intermediate evolutionary step between the eukaryotes (archaic) and the prokaryotes (modern) arrangements. The evolutionary steps could have been: a) separated active tRNA genes, i.e. promoters, were fused together by deletion of useless spacer sequences (our artificial constructs mimic this hypothetical evolutionary intermediate). b) all but the leftmost promoter are made redundant by the existence of processing enzymes: they are therefore exposed to mutational events without loss of their transcriptional product. At the final stage of this process we could have a 5' flanking promoter-coding sequence similar to that found in the "natural" dimeric tRNA genes (7,8). This situation could have evolved into c) a specialized 5' flanking promoter which has lost the function of coding for a tRNA reaching the configuration present in E.coli.

ACKNOWLEDGEMENT

The work done in Italy was supported by a grant from CNR, Progetto Finalizzato Salute dell'Uomo.

REFERENCES

1. Ciliberto,G., Castagnoli,L. and Cortese,R. (1983) Curr. Topics Dev. Biol. 18, 59-88.
2. Hall,B.D., Clarkson,S.G. and Tocchini-Valentini,G. (1982) Cell 29, 3-5.
3. Hofstetter,H., Kressmann,A. and Birnstiel,M.L. (1981) Cell 24, 573-585.

4. Ciliberto,G., Castagnoli,L., Melton,D.A. and Cortese,R. (1982) Proc. Natl. Acad. Sci. USA 79, 1195-1199.
5. Ciliberto,G., Traboni,C. and Cortese,R. (1982) Proc. Natl. Acad. Sci. USA 79, 1921-1925.
6. Ciliberto,G., Raugei,G., Costanzo,F., Dente,L. and Cortese,R. (1983) Cell 32, 725-733.
7. Mao,J., Schmidt,O. and Söll,D. (1980) Cell 2, 509-516.
8. Schmidt,O., Mao,J., Ogden,R., Beckmann,J., Sakano,H., Abelson,J. and Söll,D. (1980) Nature 287, 750-752.
9. Cortese,R., Harland,R. and Melton,D.A. (1980) Proc. Natl. Acad. Sci. USA 77, 4147-4151.
10. Messing,J., Gronenborn,B., Müller-Hill,B. and Hofschneider,P.M. (1977) Proc. Natl. Acad. Sci. USA 74, 3642-3646.
11. Traboni,C., Cortese,R., Ciliberto,G. and Cesareni,G. (1983) Nucl. Acids Res. 12, 4229-4239.
12. Traboni,C., Ciliberto,G. and Cortese,R. (1984) Cell, in press.
13. Barnes,W.M. and Bevan,M. (1983) Nucleic Acids Res. 11, 349-368.
14. Traboni,C., Ciliberto,G. and Cortese,R. (1982) EMBO J. 1, 415-420.
15. Sanger,F., Nicklen,S. and Coulson,A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467.
16. Cortese,R., Melton,D., Tranquilla,T. and Smith,J.D. (1978) Nucl. Acids Res. 5, 4593-4611.
17. Sakonju,S., Bogenhagen,D.F. and Brown,D.D. (1980) Cell 19, 13-25.
18. Melton,D.A. and Cortese,R. (1979) Cell 18, 1165-1172.
19. Weil,P.A., Luse,D.S., Segall,J. and Roeder,R.G. (1979) Cell 18, 469-484.
20. Castagnoli,L., Ciliberto,G. and Cortese,R. (1982) Nucl. Acids Res. 10, 4135-4145.
21. Messing,J. and Vieira,J. (1982) Gene 19, 269-276.
22. Mazzara,G.P. and McClain,W.H. (1980) In "Transfer RNA: Biological Aspects" 3-27. Cold Spring Harbor Mon. Series (Söll, Abelson, Schimmel, eds.).