CHARACTERIZATION OF PRECURSORS TO tRNA IN YEAST

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1. Introduction

It is now well established that in procaryotic systems transfer RNA is made through precursor molecules. The first evidence came from the isolation and identification of precursors to $tRNA^{Tyr}_{su_3}$ of *Escherichia coli*, which were amenable to total nucleotide sequence analysis and specific mutant selection [1]. A specific endonuclease activity (endonuclease P) that produces mature tRNA from its precursors was detected in *E. coli* [1] and purified [2].

Also in the phage T4-induced *E. coli* system several phage-specific tRNA precursors were found and analyzed [3, 4]. These precursors and also in vitro transcription products of the whole T4 genome [4] could be specifically cleaved by endonuclease P to yield mature tRNAs.

Very recently, it has been reported [5] that also from *E. coli* unstable RNA species, showing characteristics of precursors to tRNA can be obtained.

In eucaryotic systems, where a more complex situation is encountered, it has also been suggested that tRNA biosynthesis proceeds through precursor molecules, e.g. [6, 7].

In order to study several aspects of tRNA biosynthesis in a eucaryote we chose yeast as a model system. This paper describes precursors to tRNA that were isolated from cells labeled with [^{32}P] phosphate and characterized through kinetic experiments, by nucleotide and oligonucleotide analyses of these rapidly labeled RNA species and by processing them in an in vitro system.

2. Results and discussion

Low molecular weight RNA species can be separated according to chain length by electrophoresis on 10% polyacrylamide gels. Characteristic patterns of RNA bands were obtained, when yeast cells were incubated with [^{32}P]phosphate for different periods of time and the RNA was fractionated by this method (fig. 1).

These patterns were reproduced with high accuracy in a number of experiments. Care was also taken during preparation and fractionation of the RNA to exclude aggregation of low molecular weight RNA species as well as breakdown of high molecular weight RNA.

No significant change in the pattern was observed, when the preparation or the gel electrophoresis of the RNA was modified: for example, extraction of the cells with phenol together with SDS, extraction with hot phenol (55–60°C), preheating of the RNA samples under disaggregating conditions [12], or gel electrophoresis without 7 M urea.

As can be seen from fig. 1b, after long term labeling and applying the standard procedure for isolating the RNA-labeled products with the following characteristics were obtained:

(i) Subfractionated tRNAs (bands 8, 9 and 10): complete digestion with T2-RNAase and two-dimensional thin-layer chromatography [10] gave the nucleotide composition for tRNA including several minor nucleotides. Tl-fingerprints [12] are in agreement with the assumption that the bands are composed of several tRNA species, with the exception of bands 8a and 9a which might represent single tRNA species.

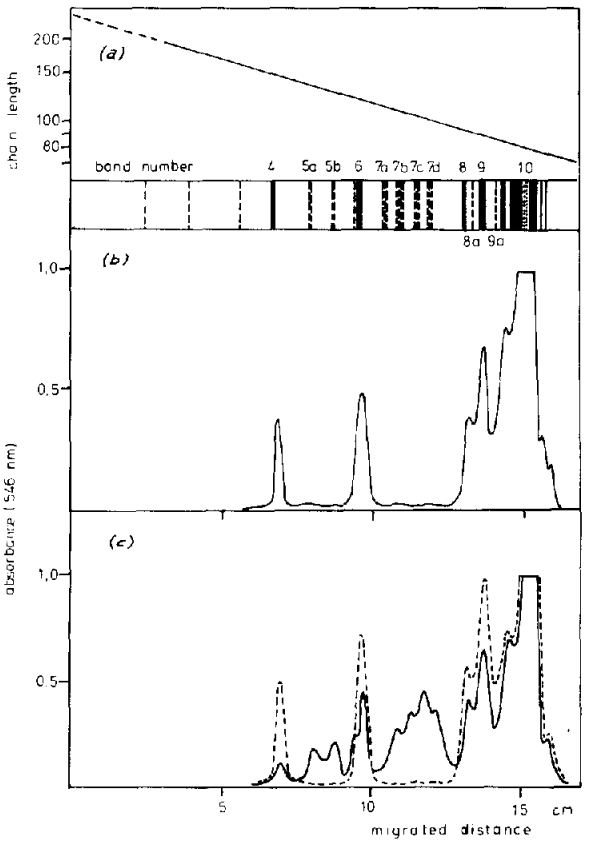

Fig. 1. Electrophoresis of low molecular weight [^{32}P]RNA from yeast on 10% polyacrylamide gel. Yeast cells (*S. cerevisiae* C 836) were cultured at 28°C in the following minimal medium: 2 g citric acid, 10 g glucose, 2.5 g peptone (Merck), 13 mg glyceromonophosphate, 0.5 mg calcium panthotenate, 10 μg biotin, 2.3 g NaCl, 0.3 mg $FeCl_3$, 0.74 g KCl, 0.11 g $CaCl_2$ 0.01 g Na_2SO_4, and 0.102 mg $MgCl_2 \cdot 6\,H_2O$ were dissolved in 1 litre, adjusted to pH 5.5 with NaOH. Extensive labeling with [^{32}P]phosphate (PBS I from the Radiochemical Centre, Amersham) was done by adding 20–50 μCi/ml. For pulse-labeling (0.5 to 20 min), 20 to 200 ml of a culture pregrown for 8–10 hr (middle log phase) were incubated with 0.2–0.5 mCi/ml [^{32}P]phosphate at room temperature, after this immediately brought to 0°C and centrifuged for 2 min to harvest the cells. Routinely, RNA was isolated from yeast cells by the standard phenol method.

Electrophoresis in polyacrylamide gels (preparative and analytical scale) was carried out similar to [8]. In most cases running gels of the following type were employed: 10% acrylamide, 0.4% bismethylenacrylamide, 7 M urea (deionized), in 0.08 M Tris–borate buffer, pH 8.3, 1 mM EDTA. On top of this gel a 5% spacer gel, pH 6.7 (about 1 cm high) was poured [9]. The gels were run for 24–40 hr at 400 V at 4°C in the Tris–borate buffer. [^{32}P]RNA bands in the gels were monitored either by radiography on X-ray film (Kodak RPS 14) or by slicing the gel into 1–2 mm pieces which could be counted in 2 ml H_2O in a liquid scintillation spectrometer (Cerenkov). In some cases photo densitometer traces of the X-ray films were taken [10]. Staining of the gels and photo densitometer tracing was performed as in [9]. (a) Diagrammatic representation of the band pattern and approximate chain lengths of [^{32}P]RNA products. (b) and (c): Intensities of RNA bands after 16 hr and 2 min labeling respectively, (——) distribution of ^{32}P-label (photodensitometer tracing of films), (- - - -) photodensitometer tracing after staining.

(ii) 5 S ribosomal RNA (band 6): Tl-fingerprints and further analyses of oligonucleotides from these were in agreement with the published sequence [13]. Nucleotide analyses by digestion with T2-RNAase showed the absence of minor constituents and gave the correct base ratios.

(iii) '7 S' ribosomal RNA (band 4): Nucleotide analyses showed the absence of minor bases, Tl-fingerprints pointed to a unique species of RNA. Meanwhile, the nucleotide sequence of this species has been established [14].

(iv) In gels that were loaded with high amounts of labeled RNA and over-exposed in autoradiography, additional bands became visible: bands 5a and b that migrate behind 5 S RNA, and bands 7a to 7d that are located between 5 S RNA and tRNA. In long term labeled cells this material represented less than 0.1% of the radioactivity found in the other bands.

In principle, the same [^{32}P]RNA bands were found after pulse-labeling. However, the relative intensities were drastically changed in favor of the label in bands 5 and 7, indicating that these RNA species were rapidly labeled and unstable under in vivo conditions.

A comparison of analytical gel patterns that were obtained by labeling precultured cells for 0.5, 1, 2, 5, 10 or 20 min and loading identical amounts of extracted RNA in terms of A_{260}-units onto the gels gave the following results (one example is presented in fig. 1c): i) The absolute amount of ^{32}P incorporated into low molecular weight RNA increased proportionally with the labeling time; ii) when the gels were stained [9], the patterns were basically the same for all samples, no stained bands were visible in the positions of bands 5 and 7; iii) the amount of ^{32}P-label in bands 5 and 7 obtained after short pulses was considerably higher compared to that obtained from long term labeled cells. The relative proportion of radioactivity in these bands to that in the tRNA bands

Table 1

Nucleotide composition of [^{32}P]RNAs taken from the bands separated by polyacrylamide gel electrophoresis (fig. 1b and 1c). RNA corresponding to single bands was extracted by homogenizing the gel slices in an ultra-turrax for several minutes in an adequate volume (10–20 ml) of 0.1 M NaCl, 0.02 M Na acetate buffer in the presence of some phenol. This extraction was repeated twice. The aqueous phases were diluted three-fold and filtered through DEAE-cellulose filters. From the washed filters the RNA was extracted with 1 M NaCl, 0.02 M Na acetate buffer, pH 5, 0, and precipitated with ethanol. The nucleotide composition was determined by digesting the samples with T2-RNAase and separating the nucleotides by two-dimensional thin-layer chromatography on cellulose plates [10].

Nucleotide	Percent* found in band								
	4	5a+b	6	7a	7b	7c	8	9	10
A	27.3	25.6	27.6	24.3	25.6	24.8	19.3	20.2	17.8
m^1A	–	–	–	>0.2	>0.2	>0.2	0.8	1.1	0.8
i^6A	–	–	–	–	–	–	>0.2	>0.2	0.3
G	24.2	27.4	26.1	23.8	24.8	25.2	28.1	24.2	26.0
m_2^2G	–	–	–	(+)	(+)	(+)	0.2	0.5	0.9
m^7G	–	–	–	(+)	(+)	(+)	(+)	0.8	0.4
m^2G	–	–	–	–	–	(+)	0.3	(+)	0.6
m^1G	–	–	–	(+)	(+)	(+)	(+)	(+)	0.7
C	21.4	18.7	27.4	24.3	23.4	22.7	22.7	24.7	23.5
m^5C	–	–	–	(+)	(+)	(+)	0.8	0.4	0.9
U	26.6	28.3	18.9	23.8	22.9	22.4	19.9	18.9	14.6
T	–	–	–	0.4	0.4	0.8	0.9	1.4	1.3
ψ	0.5	–	–	0.8	0.9	1.2	2.5	2.8	4.1
D	–	–	–	0.9	1.3	1.6	1.8	1.9	3.1

* – means not detectable, (+) traces (0.1–0.2%) found.

was dependent on the labeling time. After labeling for 0.5 min (10 min) the amount of ^{32}P incorporated into the RNAs of band 5 and 7 was about 60% (20%) of that incorporated into tRNA.

The rapidly labeled RNAs were obtained from cells labeled for 2 min in sufficient amounts to do further analyses.

Nucleotide analyses of the RNA bands gave the results shown in table 1. Obviously, the percentages of the major nucleotides in bands 5 and 7 are similar to those in tRNA. The content of minor nucleotides, however, is different. RNAs from bands 5a and 5b do not contain minor bases, in the RNAs from bands 7a to 7d minor nucleotides were found, but in lower amounts compared to mature tRNA.

The relationship of the rapidly labeled RNAs to tRNA was clearly shown by a comparison of the T1-fingerprints that were prepared by the technique of Sanger et al. [12]. The complexity of the oligonucleotide map and the distribution of radioactivity was very similar in all cases. A characteristic spot (approx. 4% of the label) in the oligonucleotide map of mature tRNA is a tetranucleotide spot, the major portion of which represents the tetranucleotide TψCG. This sequence arising from UUCG during maturation is common to nearly all tRNA species (approx. 0.8 moles/mole tRNA on the average). A minor portion of this spot is due to other tetranucleotides of the type (U, U, C) G (approx. 0.17 moles/mole on the average in mature tRNA), which migrate in nearly the same position as TψCG on two-dimensional electrophoresis.

Also the T1-fingerprints of all of the rapidly labeled RNAs showed this characteristic spot, amounting to about 3% of the total radioactivity. Further nucleotide analyses (table 2), however, revealed proportions of U, T, and ψ differing from those in mature tRNA: whereas the tetranucleotide spot from bands 5a and 5b was found not to contain T and ψ, in the spots from bands 7 these modified bases were found in a lower percentage compared to tRNA.

These data are compatible with the assumption that the rapidly labeled RNAs from bands 5 only contain the unmodified sequence UUCG in a proportion similar to that of TψCG in mature tRNA, whereas the RNAs from bands 7 in this sequence are not modified completely.

Table 2
Composition of the 'common' tetranucleotide spot in Tl-fingerprints from the rapidly-labeled [^{32}P]RNAs. Nucleotide analysis was performed as described in the legend to table 1.

Band	Nucleotide composition (%)					Approximate portion of TψCG (%)
	U	T	ψ	C	G	
5a+5b	46.5	0	0	27.0	26.5	0
7a+7b	39.0	5.5	5.0	26.5	24.0	21
7c+7d	32.4	7.8	7.4	27.2	25.2	32
10 (tRNA)	10.2	19.2	18.8	28.0	23.8	78

Final proof that the rapidly labeled RNAs are precursors to tRNA was achieved by processing [1] the RNAs in an in vitro system.

It was found (fig. 2) that upon incubation with yeast extract the rapidly labeled RNAs yielded products that mainly migrated like tRNA in gel electrophoresis giving also a distribution of bands similar to that observed with mature tRNA. This finding together with the complexity of the Tl-fingerprints indicates that each of the rapidly labeled RNAs is a mixture of precursors to tRNA. It was also possible to subfractionate the RNA taken from bands 7a or 7b on acidic gel electrophoresis [15]. The cleavage products of the RNAs from bands 5a and 5b exhibited faint label migrating in the range of bands 7a to 7d in addition to tRNA bands, suggesting that these are intermediate products in the processing of unmodified precursors to mature tRNAs. Samples incubated without extract and run on the same gel exclusively banded in their original position.

These findings suggest that also in yeast a (specific) nucleolytic activity exists that produces tRNA from its precursors. So far, the nature of the nucleolytic activity could not be identified. No bands have been detected among the cleavage products that would correspond to excised segments, thus indicating endonucleolytic activity.

In conclusion, we feel that the methods described in this paper will allow the study of maturation of eucaryotic tRNA in more detail.

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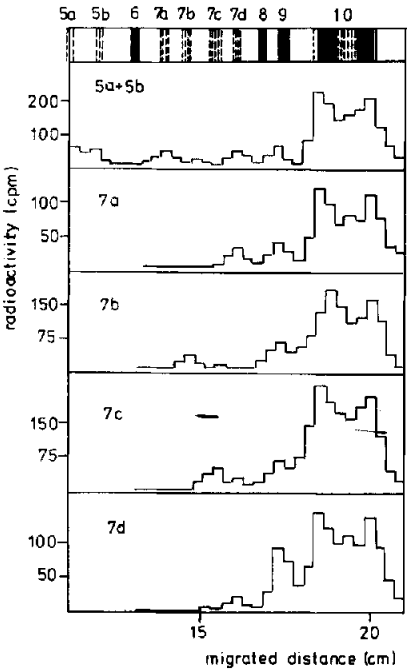

Fig. 2. Polyacrylamide gel electrophoresis of products obtained from tRNA precursors by in vitro cleavage with yeast extract. For the preparation of yeast S 30–supernatant, 20 g of cells frozen in liquid nitrogen were disrupted in a Hughes press and suspended by 30 min stirring in 20 ml of 0.05 M Tris–HCl, pH 7.4, 0.06 M NH_4Cl, 0.01 M $MgCl_2$, 0.006 M mercaptoethanol. The suspension was centrifuged in a Sorvall centrifuge (SS 34) at 15 000 rev/min for 40 min. For precursor cleavage in vitro samples of isolated [^{32}P]RNA from several gel bands (10 000–50 000 cpm) were incubated together with 1 A_{260}-unit cold carrier yeast tRNA and 5 A_{260}-units of yeast 30 000 *g* supernatant. Conditions of incubation (90 min) and treatment of the samples were the same as described in [1], except that they were extracted with phenol before drying. The samples were run in parallel on analytical gels as described above. After exposure to X-ray films the gels were sliced and counted, in order to obtain a better quantitative evaluation of radioactivity. Designation of bands is as in fig. 1.

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