Preparation of Highly Labeled [^{32}P]Nucleic Acids from Yeast; Isolation of "Denaturable" Leucine Acceptor Transfer RNA

Abstract. *Culture conditions were developed that permit efficient incorporation of [^{32}P]phosphate into nucleic acids of baker's yeast. RNA sufficiently labeled for sequence determination was obtained in this way. Pure "denaturable" leucine acceptor transfer RNA ($tRNA^{Leu}_3$) was isolated for this purpose.*

The two-dimensional electrophoretic fractionation procedure devised by Sanger *et al.* (*1*) for the separation of radioactively labeled oligonucleotides is a valuable tool in the sequence determination and rapid characterization of nucleic acids (*2*). Since identification of nucleotides depends only on radioactivity, the method is applicable to microgram quantities of RNA and is, in practice, limited to such amounts. Effective application of the methodology therefore requires highly labeled material. Practical methods to achieve such labeling have been described for *Escherichia coli*, bacteriophages, and mammalian cells (*2*). We report here conditions for efficient incorporation of [^{32}P]phosphate into nucleic acids of baker's yeast (*Saccharomyces cerevisiae*), and a procedure for the isolation of ^{32}P-labeled "denaturable" leucine transfer RNA ($tRNA^{Leu}_3$) (*3*).

A culture of commercial baker's yeast (Standard Brands) was obtained by single-cell isolation. Stocks were then prepared as slants on agar containing 1 percent yeast extract, 1 percent Bactopeptone, and 2 percent glucose.

To obtain labeling throughout log phase and ensure incorporation of [^{32}P]phosphate into the full complement of nucleic acids present under conditions of "normal" growth, a small inoculum of yeast was grown in the presence of label on a medium containing as little phosphate as would give a workable yield of cells. This procedure led to quantitative uptake of label and precluded incorporation primarily into nucleic acid termini and polyphosphate (which accumulates during the stationary phase or "ripening").

The medium described in Table 1 gives a generation time of 2 hours at 28°C and a stationary phase density of 420 Klett units. Typically, cells were grown in 500 ml of medium.

For a starter culture, 50 ml of medium was introduced into a 125-ml side-arm flask, and its KH_2PO_4 concentration doubled to 88 mg/liter by addition of 0.1 ml of stock solution VIII. This phosphate-enriched medium was inoculated with a loop of stock cells and incubated with vigorous shaking at 28°C until the culture was visibly turbid (200 Klett units). This starter culture was now added, along with carrier-free [^{32}P]phosphate (30 mc) to 450 ml of medium (already at temperature) in a heavy-walled 2-liter flask. The complete mixture was incubated at 28°C with vigorous shaking for 12 to 15 hours. The culture was then chilled, and the cells were harvested by centrifugation for 10 minutes at 2000 rev/min. The yield was 5 ml of packed cells, and the uptake of [^{32}P]phosphate was greater than 99 percent.

Low-molecular-weight RNA, consisting primarily of tRNA and 5*S* RNA, was isolated as follows. The packed cells (5 ml) were suspended in 10 ml of buffer (0.01*M* NaCl, 0.01*M* $MgCl_2$, 0.01*M* tris-HCl at *p*H 7.5) in a capped 50-ml plastic tube. Freshly distilled phenol (15 ml) equilibrated with the buffer was added, the mixture was intermittently stirred by a Vortex mixer for 1 hour, and the phases were then separated by a 5-minute centrifugation at low speed. The aqueous layer was retained, and the phenol layer was reextracted with 5 ml of buffer. After centrifugation, the two aqueous extracts were combined and further deproteinized with 5 ml of phenol for 30 minutes. The aqueous phase (~15 ml) was separated, and 0.1 volume of 2*M* potassium acetate (*p*H 5.0) and 2.5 volumes of 95 percent ethanol were

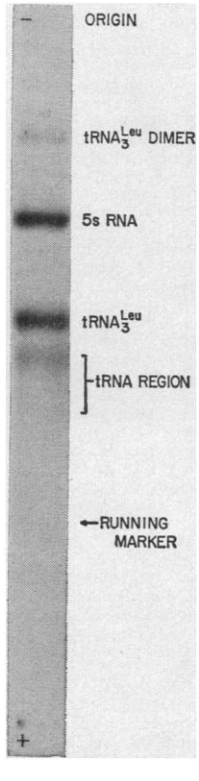

Fig. 1. Electrophoresis of $tRNA^{Leu}_3$-enriched material on 10 pecent polyacrylamide gel. A gel slab was prepared as described by Adams *et al.* (*7*) except that the acrylamide was 9.5 percent, the bis-acrylamide 0.5 percent, and the buffer 0.09*M* tris, 0.09*M* boric acid, 0.0025*M* EDTA, *p*H 8.3. Electrophoresis, 16 hours at 4°C at 400 volts, 300 ma, until the bromophenol blue marker traveled two-thirds of the length of the gel. Radioautography, 5 minutes.

Table 1. Medium limiting in phosphate for incorporation of ^{32}P into growing yeast. The medium was prepared by taking appropriate portions of the concentrated stock solutions and adding distilled water to volume. It was sterilized by passage through a Nalgene filtration unit. Stock solution IV may be substituted for by 20 ml of BME Vitamins, 100× (Flow Labs, Cat. No. 6-124B) per liter of medium.

Stock solution	Times concentrated	Substance	Amount per liter of medium
I	10	$(NH_4)_2SO_4$	3.0 g
		$CaCl_2 \cdot 2H_2O$	0.15 g
		KCl	0.4 g
		$MgSO_4 \cdot 7H_2O$	0.5 g
II	50,000	$FeCl_3 \cdot 6H_2O$	10 mg
III	100	Boric acid	500 μg
		$CuSO_4 \cdot 5H_2O$	60 μg
		KI	100 μg
		$MnSO_4 \cdot H_2O$	450 μg
		$Na_2MoO_4 \cdot 2H_2O$	240 μg
		$ZnSO_4 \cdot 7H_2O$	700 μg
IV	100	Calcium pantothenate	2 mg
		Thiamine-HCl	2 mg
		Pyridoxine	2 mg
		Nicotinic acid	0.5 mg
		Biotin	0.02 mg
V	200	meso-Inositol	20 mg
VI		*d*-Glucose	25 g
VII	10	Citrate (K^+) buffer *p*H 5.4	0.02 mole
VIII	500	KH_2PO_4	44 mg
IX		Carrier-free [^{32}P]phosphate	$H_3{}^{32}PO_4$ in 0.02*M* HCl

Table 2. Distribution of ^{32}P during tRNA isolation.

Fraction	10^3 count/min	Input (%)
Growth medium (input)	550	100
Medium freed of cells	4	1
Crude RNA before DEAE chromatography	140	25
DEAE-column wash	110	20
Crude tRNA eluted from DEAE by 1*M* NaCl	26	5

added. After several hours at −20°C, the precipitate was recovered by centrifugation, dissolved in 10 ml of 0.1*M* NaCl, 0.1*M* tris-HCl (*p*H 7.5), and poured onto a 3-ml column of packed diethylaminoethyl (DEAE)-cellulose equilibrated with the same buffer. The column was washed with 60 ml of 0.3*M* NaCl, 0.1*M* tris (*p*H 7.5); tRNA and 5*S* RNA were then eluted with 1.0*M* NaCl, 0.1*M* tris (*p*H 7.5). Fractions (5 ml) were collected and surveyed with a radioisotope monitor; the three peak tubes, which contain the bulk of the tRNA, were pooled. RNA was precipitated with two volumes of 95 percent ethanol at −20°C over several hours and was recovered by centrifugation. The yield was 60 A_{260} units containing 2.6×10^9 count/min. Table 2 gives the recovery of radioactivity at each step of purification.

By means of appropriate procedures individual tRNA's and 5*S* RNA can be isolated from the "crude" tRNA fraction. The $tRNA^{Leu}_3$ was obtained by the following procedure. The crude tRNA was dissolved in 5 ml of 0.01*M* potassium cacodylate (*p*H 7.0), 0.0005*M* EDTA, passed through a short Chelex column (K^+ form) to remove divalent cations, heated at 60°C for 5 minutes, and cooled to 0°C. By sequentially adding 3*M* KCl to give 0.15*M* K^+ and 1*M* $MgCl_2$ to give 0.01*M* Mg^{2+}, then incubating the solution at 25°C for 30 minutes and cooling again to 0°C, $tRNA^{Leu}_3$ was selectively trapped in the denatured state (*4*). In this form, it was fractionated on a column (2 by 100 cm) of Sephadex G-100 at 4°C (*4*), from which it emerges with 5*S* RNA ahead of the bulk of tRNA. The $tRNA^{Leu}_3$-enriched fractions were concentrated by absorption on a 1-ml DEAE-cellulose column and eluted in a small volume with 1*M* NaCl, 7*M* urea, 0.1*M* tris (*p*H 7.0), from which tRNA was precipitated with two volumes of ethanol at −20°C and washed twice with 95 percent ethanol. The precipitated RNA was dried under vacuum and dissolved in 0.15 ml of gel electrophoresis buffer (see Fig. 1). The density of the solution was then increased by adding 0.05 ml of 40 percent sucrose containing 0.02 percent bromophenol blue as running marker. After carefully layering the sample under buffer atop the polyacrylamide gel, electrophoresis was carried out (Fig. 1). A band of pure $tRNA^{Leu}_3$, well separated from both 5*S* RNA and 4*S* RNA, yielded 40×10^6 count/min after extraction from the gel (*5*). This material was used in the sequence determination of $tRNA^{Leu}_3$ (*6*).

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Nucleotide Sequence of the "Denaturable" Leucine Transfer RNA from Yeast

Abstract. *The nucleotide sequence of "denaturable" leucine acceptor transfer RNA ($tRNA^{Leu}_3$) from baker's yeast was determined on ^{32}P-labeled material. The molecule is 85 nucleotides long and can be folded into the "cloverleaf" model for secondary structure. The basis on which the sequence was deduced from the products of complete enzymatic digestion, prior to its unambiguous determination, is presented.*

We report here the nucleotide sequence of $tRNA^{Leu}_3$ (*1*) from baker's yeast (*Saccharomyces cerevisiae*). This organism contains at least three leucine tRNA's, of which $tRNA^{Leu}_3$ is the major species, comprising some 40 percent of the acceptor activity for this amino acid (*2*). This tRNA can be trapped in a metastable denatured state whose biological and physical properties differ significantly from those of the native form (*2–5*). This denaturability has, in fact, been exploited in its purification (*6, 7*). Because of its unusual conformational properties, the sequence of this tRNA is of particular interest.

We determined the sequence with ^{32}P-labeled RNA (*7*), using techniques developed by Sanger *et al.* (*8*). Complete T_1 and pancreatic ribonuclease digests were fractionated (Figs. 1 and 2), and the separated oligonucleotides were sequenced. Larger oligonucleotides produced by partial T_1 ribonuclease digestion were isolated by homochromatography (*9*) and identified on the basis of the products of their complete hy-

Table 1. General features of tRNA structure.

Primary structure (*10*)	Secondary structure (*11*)
1. $-CCA_{OH}3'$ terminus	1. Invariant arrangement of helical regions relative to each other
2. 5′-Phosphate terminus	2. Constant length of stem helical region
3. GTψC sequence starting 24 residues from 3′ terminus	3. Constant size of anticodon loop and supporting helical region
4. Recurrence of either purines or of pyrimidines at homologous sites	4. Constant size of TψC loop and supporting helical region
5. Recurrence of certain nucleotides or modified analogs at homologous sites	5. Dihydro-U containing loop of variable size supported by helical region of 3 to 4 base pairs
	6. Accommodation of additional residues in segment of variable size and helical content between anticodon and TψC arms