N^6-(Δ^2-ISOPENTENYL) ADENOSINE: A HETEROLOGOUS SYSTEM FOR IN VITRO SYNTHESIS IN E. COLI B TRANSFER RNA

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Received October 6, 1970

Summary: An enzyme extract from yeast which catalyzes the incorporation of mevalonic acid into E. coli B transfer RNA to form N^6-(Δ^2-isopentenyl) adenosine (IPA) has been isolated. A maximum synthesis of 1 molecule of IPA in 10 t-RNA strands has been obtained.

This heterologous system provides a means of studying specificity in the synthesis of IPA and the effect of IPA on the functioning of t-RNA.

Introduction

The role of the minor bases in t-RNA is unclear. Publication of the sequences of a number of t-RNA's (1, 2, 3) has directed interest to the role of the frequently modified base immediately adjacent to the 3' end of the presumed anticodon. A frequent modification is to IPA (1, 2, 3) which may be further modified to 2-thiomethyl IPA (4). The modified base Y whose structure is not known, has also been found in this position in yeast phenylalanyl-t-RNA (5).

It has been found by several workers that removal of the base by chemical methods (6) or the lack of the modifying side chain as obtained in mutants (4) reduces ribosome binding but has no effect on the amino acyl synthetase reaction.

The partial purification of an enzyme from yeast which catalyzes the synthesis of IPA in yeast t-RNA and has some activity with E. coli t-RNA as a substrate has been reported (7, 8). The side chain was previously shown by Peterkofsky (9) to be derived from mevalonic acid (MVA) through the intermediate Δ^2-isopentenyl pyrophosphate.

We report here on the characteristics of the heterologous system for synthesis of IPA in E. coli B t-RNA.

Experimental

Materials: E. coli B t-RNA lot 7001 was obtained from Schwarz BioResearch Company. 2-^{3}H-MVA (DL) (93 mCi/mM) was obtained from the Amersham-Searle Corporation and diluted to a specific activity of 22.5 mCi/mM with cold MVA obtained from Sigma Chemical Company. DEAE-cellulose was Whatman DE32

microgranular obtained from the Reeve Angel Company. Thin layer chromatography was done on Baker Flex cellulose sheets obtained from Fisher Scientific.

Assay of IPA Formation: The optimized reaction mixture contained in 100 μl: 0.75 O.D.$_{260}$ *E. coli* B t-RNA, 40 mμM MVA, 4 mμM ATP, 5 μM Mg^{++}, 15 μM Tris-HCl buffer pH 7.2 and enzyme.

After incubation the reaction mixture was mixed with 0.5 ml of a cold slurry of DEAE-cellulose in 0.1 N NaCl, 0.1 M Tris-HCl pH 7.5, with the volume of settled DEAE-cellulose to the volume of buffer 1:1.

After 15 minutes the DEAE-cellulose was washed four times with 6 ml portions of the NaCl-Tris buffer solution after which there was no O.D.$_{260}$ or radioactivity in the supernatant.

The final pellet was resuspended in 1 ml of 1.0 N NaCl in 0.1 M Tris-HCl buffer pH 7.5 with mixing for 15 minutes. After centrifugation a fraction of the supernatant was withdrawn for determination of radioactivity and optical densities at 260 mμ and 280 mμ.

Preparation of the Enzyme Extract: All operations in the preparation of the enzyme were carried out at 4°C.

25 g of packed yeast (Saccharomyces cerevisiae) were washed two times with 50 ml of distilled water. The pelleted cells were then resuspended in 5 ml of 0.05 M Tris-HCl buffer pH 7.5 containing 0.005 M $MgCl_2$, 0.02 M β-mercaptoethanol and 10% glycerol. This suspension was pipetted into a prechilled pressure cell and thoroughly frozen on dry ice. After approximately 30 minutes the frozen cell suspension was extruded from the cell under high pressure.

The lysate was then thawed and an additional 40 ml of buffer were added. The suspension was centrifuged at 40,000 x g for 10 minutes and the pH of the resulting supernatant was adjusted to 7.5 by the addition of dilute NH_4OH.

4 ml of the crude enzyme solution were then chromatographed on a Bio Gel P10 column (1.5 x 45 cm) with the buffer in which the enzyme was prepared. The fractions containing maximum activity were pooled and concentrated and the resulting mixture was used as the crude enzyme preparation in the following experiments.

Results

Incorporation Conditions: Figure 1 shows the effects of variation of the concentrations of the components of the reaction mixture. The results for each component were obtained with the other components near their optimum concentrations.

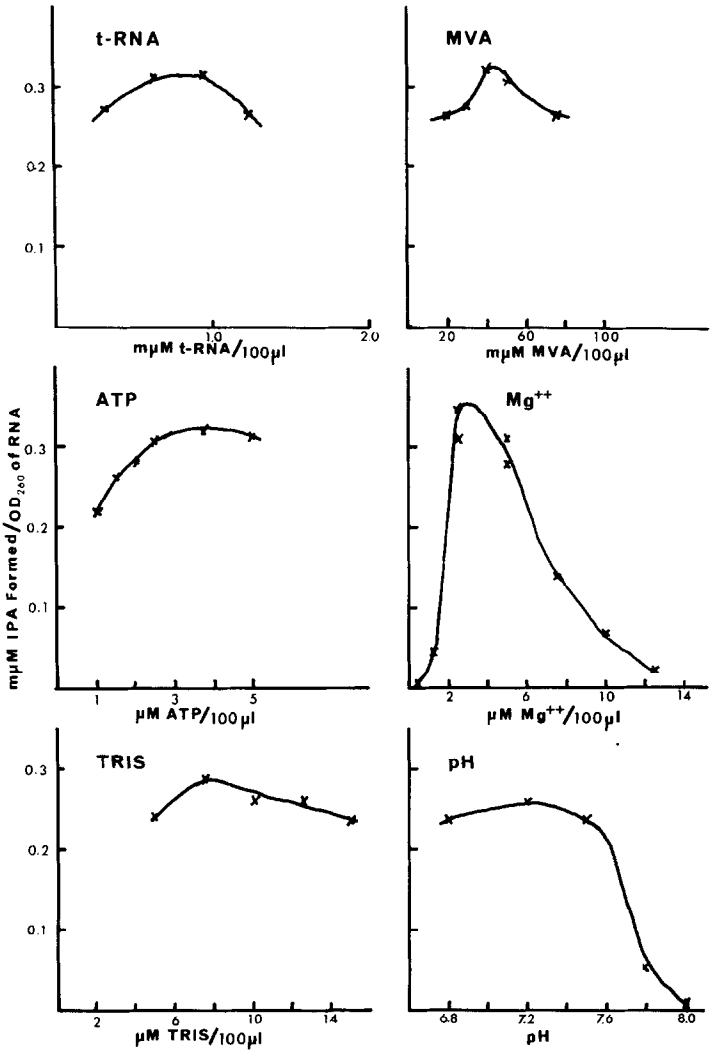

FIG. 1. Effect of variation of the components of the reaction mixture. The results for each component were obtained with the other components near their optimum concentrations.

Figure 2 shows the time course of the formation of IPA as indicated by incorporation of ^{3}H-label into RNA in the presence and absence of added E. coli B t-RNA. In the absence of added t-RNA the maximum incorporation is about 25% of that obtained in the presence of added t-RNA.

Nuclease Activity: The decrease in the amount of label incorporated into the yeast nucleic acid after 60 minutes suggested the presence of nuclease activity. In order to determine whether the E. coli t-RNA had been degraded samples of modified E. coli t-RNA and yeast nucleic acid were

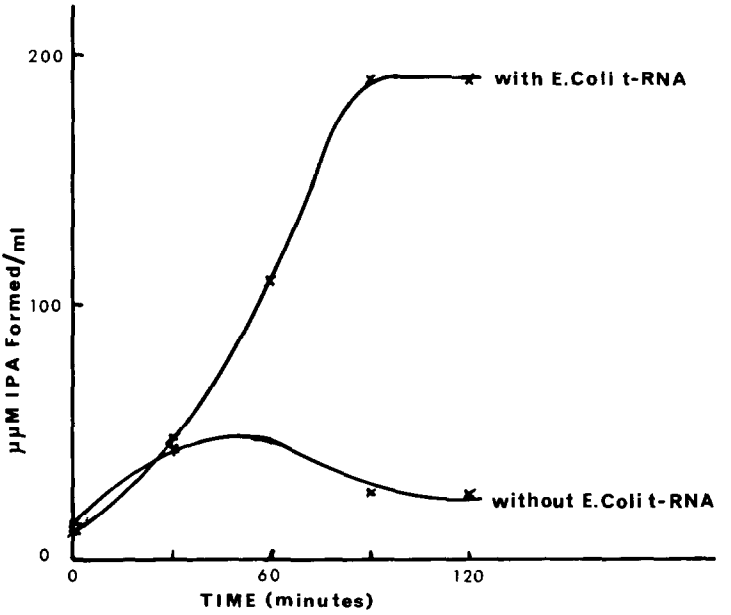

FIG. 2. Time course of formation of ^{3}H-IPA in E. coli B t-RNA. The reaction mixture is the optimized reaction mixture described in the text.

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compared by chromatography on a Bio Gel P30 column (1.0 x 35 cm).

As can be seen in Figure 3A 80% of the $O.D._{260}$ from the E. coli t-RNA sample and all of the radioactivity elutes in a sharp peak at the void volume of the column as would be expected of intact t-RNA. In contrast, in Figure 3B the corresponding peak from the yeast nucleic acid sample is not as sharp suggesting the presence of more heterogeneous material which might be due to degradation. In addition the second peak from the yeast sample which presumably represents degraded material contains as much $O.D._{260}$ as the corresponding peak from the sample supplemented with E. coli t-RNA (3A). These data support the suggestion that only the yeast RNA in the crude extracts is a substrate for the nucleases present in the extract.

The P30 chromatography step was included in the preparation of batch amounts of t-RNA for the following work.

Degradation of Modified E. coli t-RNA: To confirm that the label incorporated into the t-RNA was only present as IPA, labelled E. coli t-RNA containing 1 mole of label per 10 moles of t-RNA was hydrolysed with 0.3 M KOH for 18 hours at 37°C. This material was chromatographed on a Dowex-50 (H^{+}) column (bed volume 2 ml) equilibrated with distilled H_2O eluting first

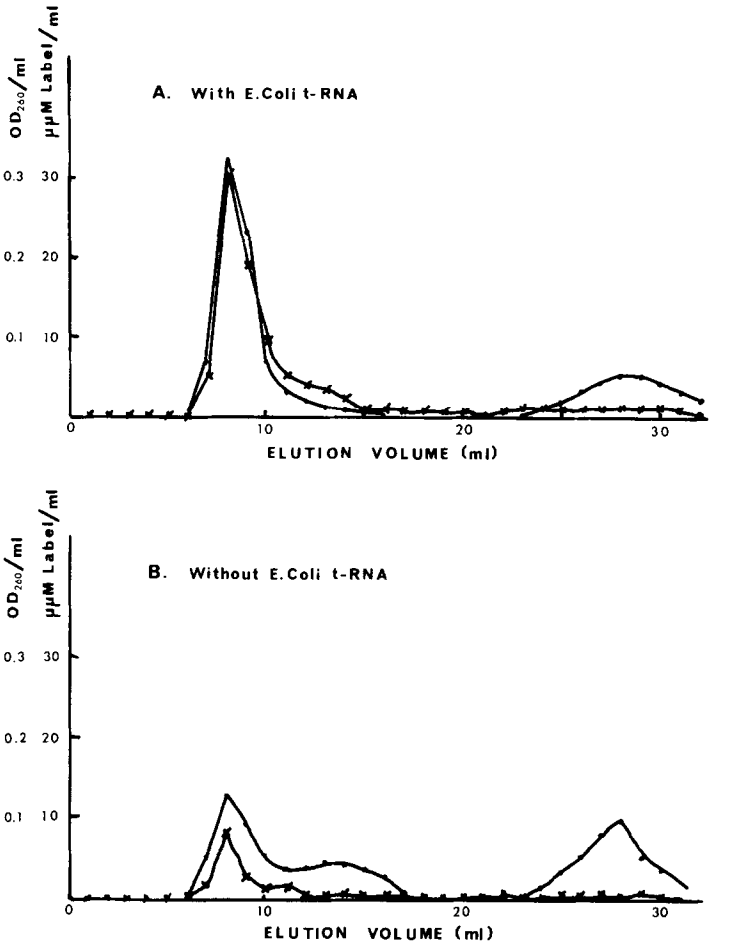

FIG. 3. Chromatography of material from the final step of the DEAE-cellulose batchwise assay on a Bio Gel P30 column (1.0 x 35 cm). Shown are the elution profiles of A) a sample from the experiment in which E. coli t-RNA was included and, B) a sample from the experiment in which E. coli t-RNA was not included. $O.D._{260}$ profile -.-, radioactivity incorporation profile -x-.

with H_2O and then with 1 M NH_4OH. The fractions containing label, which eluted after the eluant change, were then pooled and the pH lowered to approximately 8.3 by the addition of solid CO_2. This solution was treated with bacterial alkaline phosphatase for 18 hours at 37°C and finally lyophilized.

The final product was then chromatographed on thin layer cellulose sheets using authentic IPA as a marker. The solvent systems and results are shown in Table 1. Under all conditions tested the labelled base chromatographs identically to authentic IPA.

TABLE I

Rf's for IPA and other nucleosides in thin layer chromatography of t-RNA digest on Cellulose sheets

	Solvent			
	I	II	III	IV
IPA (marker)	0.92	0.97	0.98	0.57
^{3}H-label	0.94	0.97	0.97	0.55
Adenosine	0.29	0.44	0.69	0.58
Cytidine	0.17	0.23	0.59	0.82
Uridine	0.27	0.45	0.72	0.93
Guanosine	0.15	0.23	0.54	0.64

Solvents

I	1-Butanol/H_2O 86:14
II	Ethyl acetate/1-propanol/H_2O 4:2:1
III	2-propanol/1% aq. $(NH_4)_2SO_4$ 2:1
IV	H_2O

Discussion

Data presented by several workers (4, 8) suggest that the role of IPA in t-RNA may be very important, affecting ribosome binding and thus protein synthesis and thus possibly functioning in translational control. Therefore its synthesis in t-RNA and the recognition of particular t-RNA species by the synthetic enzyme are of considerable interest.

The heterologous system allows us to study both the recognition of t-RNA by the enzyme (10) and the effects of base modification on t-RNA function. In addition it avoids the requirement for the $KMnO_4$ treatment of t-RNA, used in the homologous yeast system, in which case only about 1% of the original IPA is replaced by labelled IPA (7).

While E. coli t-RNA has been found to contain IPA and 2-thiomethyl IPA (11) a considerable number of sites recognized by the yeast enzyme are apparently unmodified. Preliminary results previously reported (10) showed that modification of E. coli t-RNA in the heterologous system was specific and the effects of the modification were similar to those reported by other workers (4, 6, 8).

Acknowledgments

We would like to express thanks to Miss Allison LeDain for assistance running the thin layer chromatograms.

This research was supported by grants from the National Research Council of Canada, the National Cancer Institute of Canada and the Jane Coffin Childs Memorial Fund for Medical Research.

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