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DNA-Directed Cell-Free Synthesis of Biologically Active Transfer RNA: su^{+}_{III} Tyrosyl-tRNA

(*E. coli*/β-galactosidase/bacteriophage)

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ABSTRACT Biologically active su^{+}_{III} tyrosyl-tRNA has been synthesized in a DNA-directed cell-free system from *Escerichia coli*. Such a system should be most useful for studying the mechanism of tRNA synthesis. This tRNA is capable of suppressing amber mutations in the gene coding for β-galactosidase (EC 3.2.1.23) and, therefore, must be capable of being charged and transferring amino acids. A 4-fold stimulation in the activity of the tRNA formed *de novo* is obtained with isopentenyl pyrophosphate, a compound involved in the post-transcriptional acylation of an adenine base adjacent to the anticodon. It has been suggested elsewhere that formation of RNA subject to stringent control may be inhibited by guanosine tetraphosphate (ppGpp). However, guanosine tetraphosphate did not affect the synthesis of su^{+}_{III} tyrosyl-tRNA, even though the synthesis of this tRNA is subject to stringent control.

DNA-directed cell-free systems are being used with increasing frequency to study mRNA and protein synthesis (1). Recently, attempts have been made to use such systems for the study of rRNA and tRNA synthesis. The rRNA studies have been done with *Escherichia coli* DNA, which contains about 10–12 genes for rRNA, which constitute about 0.5% of the *E. coli* chromosome (2). A protein factor called ψ has been partially purified. This factor is believed to be necessary for initiation of ribosomal gene transcription; this initiation is inhibited by guanosine tetraphosphate (ppGpp) *in vitro* (3, 4). DNA-directed cell-free studies of tRNA synthesis have been done with bacteriophage φ80 psu^{+}_{III} DNA (5), a viral DNA that contains a single bacterial suppressor tRNA that constitutes about 0.2% of the viral chromosome (6). The tRNA synthesized is much larger than mature tRNA, with a sedimentation coefficient of about 8 S. It has been suggested that this is the immediate transcriptional product of the tRNA gene, and that partial degradation of this product in a subsequent maturation reaction is necessary. In the reported RNA synthesis studies, no evidence was presented for the synthesis of biologically competent RNA. The main technique for characterization of the cell-free synthesized RNAs has been hybridization to the transcriptional site on the DNA template.

Possibly a more meaningful approach would be the synthesis of a biologically competent RNA, coupled with analysis of the components essential at the various stages of formation, these stages being initiation, propagation, and termination of transcription, as well as post-transcriptional modification. We have been successful in synthesizing a biologically active su^{+}_{III} tyrosyl-tRNA and defining some of the requirements and conditions for its synthesis.

METHODS

Bacterial and viral strains

λ*dlac* was isolated from a doubly lysogenic strain containing λhφ80 and λhφ80*dlac*.

λ*dlac 545* was isolated from a doubly lysogenic strain containing λhφ80 and λhφ80*dlac* 545. The λCI857t68h80*dlacz*545 used to make the double lysogen was constructed by L. Caine and J. Beckwith, by the following technique: Strain X-7060 (F^{-}, lac-deletion Sm^{S}) was transduced to *lac*$^{+}$ at low multiplicity (about 1 phage particle/200 bacteria) with a lysate containing both λCI 857t68h80 and λCI857t68 h80*dlac*$^{+}$. Lac^{+} transductant clones were purified and tested to see whether they gave rise to transducing lysates without helper phage. A strain (XS-7001) that was presumed to be a defective lysogen by this test was used for further experiments. Strain E-7119 (F'*lac* z545/*lac* z545*trp*$^{-}$) was mated with XS-7001 at 30°C and clones of XS-7001 were picked from glucose–minimal media to detect those that had received the F-*lac* episome. These clones were mated with strain M-7025 (F^{-}*lacz* 90 Sm^{R}), and the mating mixture was spread on lactose minimal agar to look for *lac*$^{+}$ recombinants. Those clones that gave rise to recombinants should have the structure F-*lac* z545/λCI857h80*dlac*$^{+}$. These clones were grown up in culture and aliquots were spread on lactose–tetrazolium agar to look for *lac*$^{-}$/*lac*$^{-}$ homogenotes. A red colony on such medium that still carries phage immunity was presumed to have incorporated the *lac* z545 mutation onto the defective phage. Mating techniques are described by Miller *et al.* (7).

φ80psu^{+}_{III} was grown and isolated as described (6).

Strain 514 was used to make all cell-free extracts. This strain is F^{-}Δ*lac*, *trp*$^{-}$, $T6^{r}$, Sm^{r}.

Preparation of DNA has been described elsewhere (8). The double lysogen is grown to a suitable titer and induced to make viruses. The defective virus is purified and DNA is made from it. The DNA is stored in a solution of 0.01 M Tris acetate, pH 8.2, over chloroform at 5°C.

Conditions for cell-free synthesis and assay

Synthesis and Assay of β-galactosidase (EC 3.2.1.23). Except for slight modifications described herein, all procedures used for synthesis, enzyme assay, and preparation of bacterial extracts and DNA have been described in detail (8). The procedures for synthesis and assay were: the incubation mixture contains per ml: 44 μmol of Tris acetate (pH 8.2); 1.37 μmol dithiothreitol; 55 μmol KAc; 27 μmol NH_4AC; 14.7 μmol

$MgAc_2$; 7.4 μmol $CaCl_2$; 0.22 μmol amino acids; 2.2 μmol ATP; 0.55 μmol (each) of GTP, CTP, and UTP; 21 μmol phosphoenolpyruvic acid; 0.5 μmol cyclic AMP 100 μg of tRNA; 27 μg of pyridoxine·HCl; 27 μg TPN; 27 μg FAD; 11 μg of *p*-aminobenzoic acid. The above ingredients are incubated for 3 min at 37°C with 50 μg/ml of λ*dlac* DNA, with shaking, before 6.5 mg of S-30 extract protein is added. Incubations with shaking are allowed to continue for 60 min at 37°C. After the synthesis has been completed, a 0.2-ml aliquot is removed and mixed with 1.5 ml of assay buffer containing 0.53 mg of *O*-nitrophenyl-β-D-galactoside–0.1 M sodium phosphate (pH 7.3)–0.14 M 2-mercaptoethanol. After a suitable length of time (1–40 hr), the mixture is treated with one drop of glacial acetic acid, chilled, and centrifuged to remove the precipitate. The supernatant is mixed with an equal volume of 1 M Na_2CO_3 and read against water in a 1-cm quartz cell at a wavelength of 420 nm. A zero-time value of 0.035 is subtracted from all readings. Duplicate analyses usually agree within 2%.

Synthesis of $su^3{}_{III}$ Tyrosyl-tRNA. As above, except for the use of $\phi 80psu^+{}_{III}$ DNA instead of λ*dlac* DNA and addition of 10^{-4} M isopentenylpyrophosphate (9).

Partial purification of $su^+{}_{III}$ tyrosyl-RNA made in the cell-free system

After synthesis, the 5-ml incubation mixture contained a precipitate consisting of most of the DNA, presumably in an mRNA–ribosome complex. This precipitate is removed by centrifugation at 1000 × *g* for 5 min. The resulting supernatant is vigorously agitated with an equal volume of 88% phenol for 15 min. The resulting emulsion was centrifuged at 13,000 × *g* for 1 min, and the upper aqueous-layer was collected and precipitated by the addition of 0.1 volumes of 5 M KAc and 2.0 volumes of ethanol. The precipitate is centrifuged as above, redissolved in 3 ml of H_2O, and precipitated again by the addition of 0.3 ml of 5 M KAc and 6 ml of EtOH. The resulting precipitate is redissolved in 1.5 ml of H_2O. Then, 1.6 ml of 2 M NaCl is added and the solution allowed to stand at 0°C for 20 min. The precipitate is removed by centrifugation at 15,000 × *g* for 1 hr. The resulting supernatant is precipitated by the addition of 2 volumes of ethanol. The precipitate is collected by centrifugation at 10,000 × *g* for 1 min, dissolved in 1.5 ml of H_2O, and precipitated by the addition of 0.15 ml of 5 M KAc and 3 ml of EtOH. The precipitate is collected, dissolved, and precipitated as described above; the final precipitate is dissolved in 0.5 ml of H_2O. This solution is stored at −75°C until ready for use.

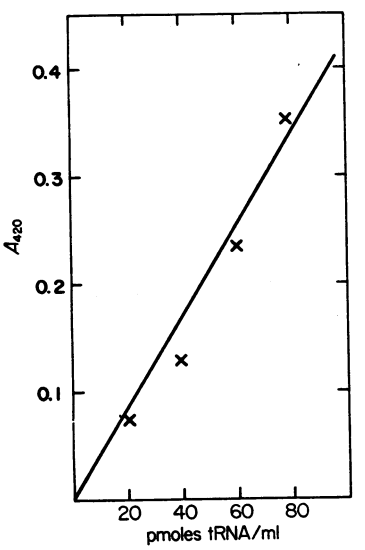

FIG. 1. Synthesis of β-galactosidase as a function of added $su^+{}_{III}$ tyrosyl-tRNA with amber-suppressible *lac* DNA in the cell-free system (see text for explanation).

Preparation of $su^+{}_{III}$ tyrosyl-tRNA made *in vivo*

Suppressor tyrosyl-tRNA was isolated and purified from $\phi 80psu^+{}_{III}$-infected cells essentially as described (6). For these studies, the partially modified tRNA (isopentenyl adenosine adjacent to the anticodon) was used throughout. Moles of tyrosyl-tRNA added are calculated on the basis of tyrosine-acceptor capacity.

RESULTS AND DISCUSSION

The most crucial aspect of our approach was to develop a suitably sensitive and selective assay for a biologically competent tRNA. Advantage was taken of the specific suppression properties of the mutant $su^+{}_{III}$ tyrosyl-tRNA not normally found in *E. coli*. The assay used was the $su^+{}_{III}$ tyrosyl-tRNA stimulation of DNA-directed β-galactosidase synthesis using DNA with an amber triplet in the β-galactosidase gene. Any tRNA competent in such a process must be capable of amino-acid acylation, as well as transfer of amino acid to the correct position on the growing polypeptide chain in protein synthesis. We shall first describe the DNA-directed system for β-galactosidase synthesis, then the suppression assay, and finally the requirements for the cell-free synthesis of the suppressor tRNA.

Comparison of β-galactosidase synthesized in the DNA-directed cell-free system with normal (λdlac) and mutated (λdlac 545) DNA

A DNA-directed cell-free system for β-galactosidase synthesis has been used in gene regulation studies for several years. This system comprises a cell-free extract of *E. coli* (called S-30 extract), DNA from the defective transducing virus λ*dlac*, and the cofactors and substrates necessary for RNA and protein synthesis. The amount of β-galactosidase synthesized in the cell-free system is proportional to the amount of active DNA containing the *lac* operon present.

In order to adapt this system for suppression studies, a mutant DNA (mutant 545) containing an amber triplet in the

TABLE 1. *Amounts of $su^+{}_{III}$ tyrosyl-tRNA synthesized under various conditions*

System	$su^+{}_{III}$ Tyrosyl-tRNA synthesized/ml (pmoles)
Complete	40
+ zero time for synthesis	<1
− $\phi 80psu^+{}_{III}$ DNA	<1
+ $\phi 80$ DNA instead of $\phi 80psu^+{}_{III}$ DNA	<1
+ 2 μg/ml of rifampicin	<1
+ 5×10^{-4} M cyclic AMP	43
+ 50 μM ppGpp	44
− isopentenyl pyrophosphate	10
+ 10^{-4} M *S*-adenosylmethionine	38

Each value is the average of two determinations. The average error of a determination is about ±10%.

early region of the β-galactosidase gene was constructed; this DNA, in the form of λ*dlac* 545, was substituted for the normal λ*dlac* DNA. The effect of the 545 mutation has been described by Zipser (10); very low levels of β-galactosidase are made *in vivo* because of the nontranslatability of the amber triplet. In the cell-free system, this altered DNA gives about 1/4000th of the normal level of β-galactosidase. Under standard assay conditions (see *methods*), this means a ΔA_{420} in a 1-cm tube of 0.005 after 20 hr of incubation. Thus, the single amber triplet introduces a severe block for β-galactosidase synthesis both in whole cells and in the cell-free system.

The amber-mutated β-galactosidase gene can be suppressed by the addition of su^+_{III} tyrosyl-tRNA to the cell-free system

The su^+_{III} tyrosyl-tRNA is a highly effective amber suppressor that inserts tyrosine into the position corresponding to the amber triplet. As high as 70% effective suppression has been obtained when this tRNA is present *in vivo*. When low levels of purified su^+_{III} tyrosyl-tRNA are added to the cell-free system, no effect on the amount of β-galactosidase synthesized is observed as long as normal λ*dlac* DNA is used. However, when the mutant λ*dlac* DNA 545 is used, there is a stimulation of β-galactosidase synthesis that is directly proportional to the amount of tRNA added (see Fig. 1). As little as 10 pmol of tRNA gives a readily detectable increment in enzyme activity.

With minor modifications, the DNA-directed system for β-galactosidase synthesis can be used for tRNA synthesis

The same system used for β-galactosidase synthesis can be used for tRNA synthesis; only minor modifications are necessary. The λ*dlac* DNA is replaced by $\phi 80psu^+_{III}$ DNA and 10^{-4} M isopentenylpyrophosphate is added. After the synthesis step, the resulting tRNA is partially purified, concentrated (see *Methods*), and assayed by its ability to stimulate β-galactosidase synthesis in the suppressible system described above. The amount of tRNA is estimated by comparison with the stimulation produced by a known amount of su^+_{III} tyrosyl-tRNA (see Fig. 1). The estimated amounts of tRNA made in the cell-free system under various conditions are indicated in Table 1. Under optimum conditions, about 40 pmol of active tRNA are synthesized per ml of incubation mixture. This low level can be easily detected because of the sensitive suppression assay and because it can be concentrated before assay. The gross amount of ribopolynucleotide synthesis in the cell-free system (measured by cold-acid-precipitation of [^{14}C]CTP labeled RNA) exceeds that required to account for the biologically mature tRNA by about 20-fold. Since the tRNA gene only comprises about 0.2% of the viral DNA used, it is obvious that the bulk of the RNA made must be viral in origin. It is also likely that more tRNA is transcribed than reaches maturation and, therefore, escapes detection by the suppression assay. Comparison of the complete system (Table 1, line 1) with the controls (lines 2–5) shows that tRNA activity requires incubation with DNA containing the tRNA gene and rifampicin-sensitive initiation of transcription. The compounds adenosine-3′5′-cyclic monophosphate (cAMP) and guanosine tetraphosphate (ppGpp) have no significant effect on the synthesis. Cyclic AMP is a gene coactivator for catabolite-sensitive genes, such as the lactose and arabinose operons, but it has no known effect on other types of genes. ppGpp stimulates the synthesis of catabolite-sensitive gene products, at least *in vitro* (12). Before this fact was known, it was suggested that ppGpp functions as an inhibitor of rRNA synthesis in the stringent response (4). However, under the cell-free conditions used here, ppGpp greatly stimulates catabolite-sensitive genes but has a negligible effect on tRNA synthesis. This is quite interesting, since the tyrosyl-tRNA gene is known to be subject to stringent control (4) and others (2, 3) have suggested that ppGpp inhibits initiation of genes subject to stringent control. The precise role of ppGpp, as well as its mechanism of action, is clearly an important matter for future study.

Post-transcriptional modification is necessary for tRNA activity

Mature tRNA is probably smaller than the immediate transcriptional product (11). It also must be modified by attachment of the trinucleotide pCpCpA to its 3′-hydroxy end and chemical alteration of a number of bases. Some bases are methylated in mature tRNA, and it is known that the active methylating agent is S-adenosylmethionine (AdMet). Addition of AdMet to the cell-free system has no effect on the amount of active tRNA synthesized. However, since this system contains methionine and ATP, it is possible that saturating amounts of AdMet are present endogenously. Alternatively, methylation may not be required for activity of this particular tRNA. Further experiments are necessary to resolve this question.

Isopentenyl pyrophosphate (IPP) functions as a precursor of an acetylated adenine base adjacent to the anticodon. Modification of this base is required for optimum activity of su^+_{III} tyrosyl-tRNA in protein synthesis (9). Consistent with this, it is observed that IPP stimulates accumulation of active tRNA by about 4-fold (see Table 1), but has no direct effect on β-galactosidase synthesis. Although much work remains to be done, we believe that the system described here represents a new, fruitful approach for probing the mechanisms of tRNA synthesis.

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