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TRANSCRIPTION OF A SPECIFIC PRODUCT FROM CLONED T_4 tRNA GENES IN XENOPUS GERMINAL VESICLE EXTRACT

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Summary. An extract, prepared from the germinal vesicles of Xenopus oocytes, was capable of transcribing cloned T_4 tRNA genes. The major product was identified as $tRNA^{Ser}$, with some extra nucleotides from neighboring sequences in the tRNA cluster at both termini.

INTRODUCTION

Recent studies have shown that in eukaryotic, as well as prokaryotic, systems the synthesis of several mature RNA species requires the processing of a precursor RNA molecule. In eukaryotes, and certain eukaryotic viruses, intervening sequences within a gene are transcribed into RNA, where the extra nucleotides are probably excised by an endonuclease-RNA ligase system. The implications of this process have been considered in a review by Crick (1). In prokaryotes discontinuous genes probably do not occur, but extra nucleotides are found at the termini of precursor RNA species. In E.coli (2) and T_4 (3) tRNA genes occur in tandem, separated by nucleotide sequences; these are transcribed and subsequently processed into the mature tRNA molecule. The processing of precursor RNA molecules in prokaryotes has also been demonstrated for E.coli 30S rRNA (4), and for B. subtilis 5S RNA (5).

The processing of RNA has also been studied in some in vitro systems. Experiments have been reported about in vitro processing in eukaryotic homologous systems: Xenopus 5S genes were faithfully transcribed in the germinal vesicle (GV) extract of Xenopus oocytes (6), and yeast tRNA precursors were processed in a crude yeast system (7, 8). In vitro heterologous systems have been developed using Xenopus GV extract for the transcription and processing of cloned yeast tRNA genes (9, 10) and for the transcription of Drosophila tRNA (11). Prokaryotic homologous in vitro systems have been primarily used to study transcription and processing of E.coli and T_4 tRNAs, T_7 early mRNA, and processing of E.coli rRNA precursors. These have been extensively reviewed (12, 13, 14, 15, 16).

In this study we report experiments which show that in vitro transcription and perhaps processing of prokaryotic genes is possible in an eukaryotic system.

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Xenopus GV extract can transcribe cloned T_4 DNA, which contains a cluster of seven different tRNA genes, resulting in a complete $tRNA^{Ser}$ molecule with some extra nucleotides at both ends.

MATERIALS AND METHODS

Construction of T_4 tRNA Clones. A clone, λgt tll, was constructed and characterized by Fukada (17). It contains an EcoRI fragment of T_4 (42^-, 56^- den B^-) of about 4000 B.P., including seven tRNA genes, but with a deletion which eliminates the promoter for the tRNA genes. This promoter is located some 1000 base pairs from the tRNA structural genes (17). DNA sequence analysis (26) revealed the order of tRNA genes from the 5' end to be as follows: gln, leu, gly, pro, ser, thr, ileu. DNA nucleotide spacer sequences between tRNA genes were also identified. The following four clones were contructed by Velten (18). The EcoRI fragment of λgt tll was ligated into pBR 322 and transformed into E.coli C600 SF8. A clone was selected and named pTFR 1109. Another clone was independently isolated and called 1501. Both clones contain the seven T_4 tRNAs, but differ in the size of the deletion. Two other clones were constructed by ligating into pBR 322 an AluI fragment of the original λgt t28 clone, which differs from λgt tll in that it does not have a deletion in the T_4 DNA insert. Clones hybridizing to T_4 $tRNA^{Ser}$ were selected. Clones pTFA 314 and pTFA 420 contain an insert of only 170 B.P., starting at nucleotide 11 from the 5' end of $tRNA^{Pro}$ and extending through the spacer sequence and $tRNA^{Ser}$ and through the next spacer sequence and into the first 10 nucleotides of $tRNA^{Thr}$. We repeated our transcription experiments with the four clones pTFR 1109, pTFR 1501, pTFA 314 and pTFA 420 with identical results. We therefore report only the results of experiments with pTFR 1109.

Preparation of Germinal Vesicle (GV) Extract from Xenopus leavis Oocytes. The preparation of GV extract followed the method developed by Birkenmeier, Brown and Jordan (6). An ovarian lobe was removed from an anesthetized Xenopus laevis female, kept one hour at 0° in a priming medium (0.01 M $MgCl_2$, 0.005 M tris-HCl pH 8.0), and then transferred to J buffer (0.07 M NH_4Cl, 0.007 M $MgCl_2$, 0.01 M HEPES pH 7.4, 0.01 mM EDTA, 2.5 mM DTT). GV were dissected (19) out of stage V and VI oocytes (20) and accumulated in J buffer at 0° (1 µl J buffer/GV). The GV were disrupted by pipetting the suspension 5x up and down in an Eppendorf pipette, and were then centrifuged for five min. at 5000 x g. The supernatant, called GV extract, was active after storage at -70° and repeated freezing and thawing.

Conditions for Transcription of T_4 tRNA DNA in Xenopus GV Extract. We essentially used a system described by Birkenmeier et. al. (6) and slightly modified by Schmidt et. al. (11). The incubation mixture contained in a final volume of 15 µl: 10 µl GV extract, 0.3 µg plasmid DNA, 3 nmoles each of 3 unlabeled nucleoside

triphosphates, 25 μCi of one α(^{32}P)-labeled nucleoside triphosphate (approximately 300 Ci/mmole), 5 μg/ml α-amanitin. After two hours of incubation at 23°-24° the reaction was adjusted to 0.015 M NaCl, 0.01 M tris-HCl pH 7.8, 0.005 M EDTA, 0.5% SDS, 0.1 mg/ml Proteinase K, and 300 μg/ml poly(U) in a final volume of 100 μl. After 30 min. incubation at RT the RNA was recovered by two extractions with phenol: chloroform: isoamyl alcohol: 8-hydroxy quinoline (50:48:2:0.05%), back-extraction and ethanol precipitation.

Identification of RNA Products. The ^{32}P-labeled products of the incubation were separated by electrophoresis on a 10% polyacrylamide-4 M urea gel in tris-borate, pH 8.3. Following autoradiography, the appropriate bands were cut out of the gel and the RNA was eluted at 37° with 0.3 M NaCl, 0.2% SDS, 20 mM EDTA and concentrated by ethanol precipitation. The purified RNA was further analyzed by two-dimensional oligonucleotide mapping of the T_1 RNase or pancreatic RNase digestion products, according to the methods of Sanger and his colleagues (21, 22). The identity of oligonucleotides observed in the fingerprint could be confirmed by digesting them further with T_1 or pancreatic RNase, as appropriate, followed by fractionation of the products on DEAE cellulose paper at pH 3.5.

RESULTS

We studied the transcription of T_4 tRNA genes in a system derived from the GV of Xenopus oocytes. A T_4 DNA fragment containing a cluster of seven tRNA genes, but lacking in the transcriptional control region, had been ligated into plasmid pBR 322 (see Materials and Methods). The resulting clone pTFR 1109 was incubated with the GV extract of Xenopus oocytes under conditions developed by Birkenmeier et. al. (6) for the transcription of Xenopus 5S DNA.

The products of the reaction were separated on a 10% polyacylamide-4M urea gel (Fig. 1). The major product, band f, was RNA of approximately 105 ±5 nucleotides, as estimated from its mobility relative to T_4 tRNA markers. No product from the control incubation of the plasmid pBR322 in Xenopus GV extract comigrated with the major band of the experimental clone (Fig. 1).

The following analysis of the major product revealed it to be T_4 $tRNA^{Ser}$ with extra nucleotides at both termini. The RNase T_1 oligonucleotide map of the α(^{32}P)UTP-labeled RNA showed the presence of all the expected products from $tRNA^{Ser}$, as well as three extra oligonucleotides, and the absence of a mature $tRNA^{Ser}$ 5' end. All oligonucleotides were further characterized by pancreatic RNase digestion and the extra oligonucleotides were identified as having the sequences: UAAUU or $UAAUUU_{OH}$, ACUG and AUG.

Figure 2 gives the nucleotide sequence adjacent to $tRNA^{Ser}$ in the T_4 clone. It can be seen that the extra nucleotide sequence, UAAUU or $UAAUUU_{OH}$, coincides with the 3' spacer region between serine and threonine tRNA. The oligonucleotides

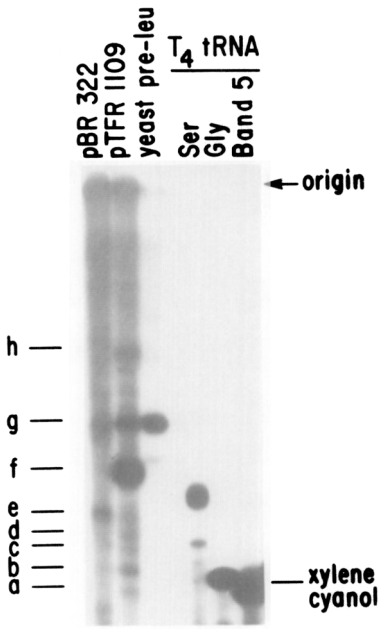

Fig. 1. Polyacrylamide gel profiles of $\alpha(^{32}P)$UTP-labeled transcription products (a-h) of plasmid pTFR 1109 in Xenopus GV extract. In a control experiment the vehicle pBR 322 was incubated with GV extract. Labeled markers were present as indicated: pre-$tRNA_3^{leu}$ is the main incubation products of the cloned yeast $tRNA_3^{leu}$ gene in Xenopus GV extract; T_4 band 5 (23) is a mixture of 5 tRNAs.

ACUG and AUG are derived from the sequence at the 5' end of $tRNA^{Ser}$, extending through the short spacer region CU, into the sequence AUUGGAGA of the neighboring $tRNA^{Pro}$.

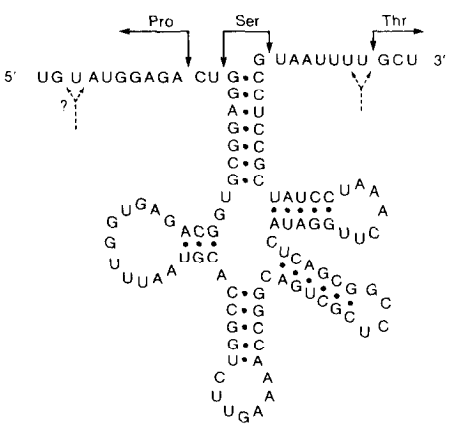

Fig. 2. Nucleotide sequence (without modifications) of T_4 $tRNA^{Ser}$ (24) and neighboring sequences (25 - 27). Nucleotide 35 in T_4 $tRNA^{Ser}$, previously unspecified, was shown to be U by DNA sequence analysis (26). Arrows locate tRNAs. Dotted lines indicate termini found in transcripts from GV extract.

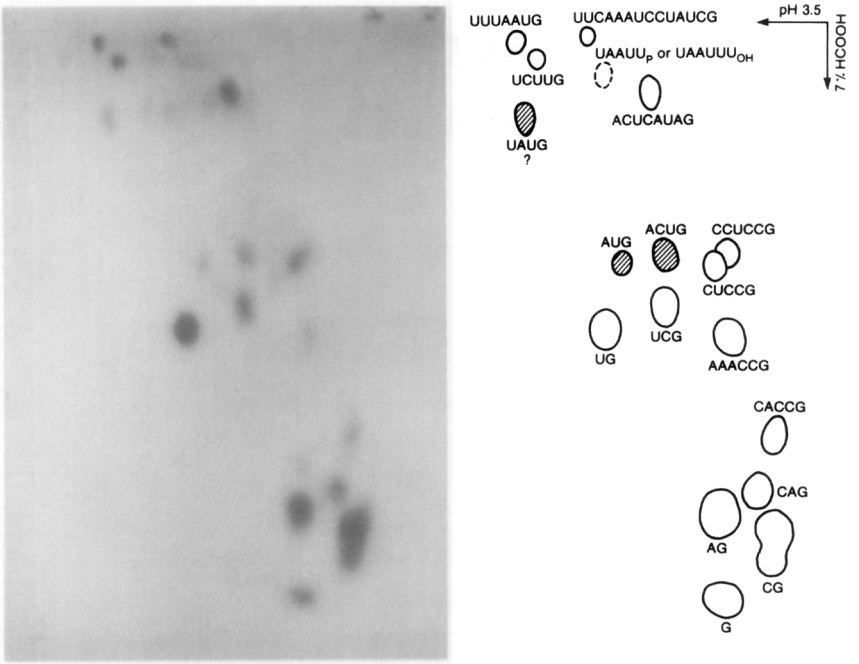

Fig. 3 Autoradiogram and diagram of a T_1RNase fingerprint of band f eluted from a 10% gel (Fig. 1). The RNA was labeled with $\alpha(^{32}P)$GTP. Shaded spots are not T_4 $tRNA^{Ser}$ sequences. The spot indicated with a dotted line is not seen here, but labeled in an analogous T_1RNase fingerprint of $\alpha(^{32}P)$UTP labeled band f.

These observations were confirmed when the $\alpha(^{32}P)$GTP-labeled product was analysed after digestion with T_1 RNase. The oligonucleotide map (Fig. 3) shows that the oligonucleotide UAAUU or $UAAUUU_{OH}$ is absent, as expected, but the oligonucleotides ACUG and AUG are present, as well as all the expected $tRNA^{Ser}$ products. In addition there is one oligonucleotide, which could be UAUG. The low yield of this product did not permit further analysis. The absence of the mature 5' end of $tRNA^{Ser}$ is confirmed.

The $\alpha(^{32}P)$GTP-labeled product was also digested with pancreatic RNase, followed by analysis with T_1 RNase. These experiments again revealed all expected $tRNA^{Ser}$ products. The presence of the extra dinucleotide AU indicated that the extra sequence on the 5' end of the $tRNA^{Ser}$ is at least nine nucleotides long. The extra oligonucleotide GGAGAC, expected after pancreatic RNase cleavage, was unresolved from the $tRNA^{Ser}$ products AGAGU and GGAGGC, but could be accounted for by comparing the molar ratios of the labeled oligonucleotides.

The other 10 transcription products a-e and g-h, separated on the 10% gel (Fig.1), were also analysed after T_1 RNase digestion. They seem to be unique, not random RNA products, but could not be identified as complete T_4 tRNA molecules.

DISCUSSION

We have found that when cloned T_4 DNA, containing seven tRNA genes, was incubated in a GV extract of Xenopus oocytes, T_4 tRNA genes were transcribed, and at least one product, $tRNA^{Ser}$, could be identified. The $tRNA^{Ser}$ produced in this system had four or five extra nucleotides at the 3' end, and 9 or 10 extra nucleotides at the 5' end. These nucleotides could be assigned to the sequences adjacent to $tRNA^{Ser}$, shown in Fig. 2. Sequence studies of the cloned T_4 DNA fragment (26), and of the RNA precursor molecule (27), identified seven nucleotides between the 3' end of $tRNA^{Ser}$ and the adjacent $tRNA^{Thr}$. We found that four or five of these spacer nucleotides were associated with the $tRNA^{Ser}$ product made in the Xenopus GV extract. The extra nucleotides at the 5' end of the $tRNA^{Ser}$ produced in this system extended through the short spacer region CU into the neighboring $tRNA^{Pro}$ sequence AUGGAGA and sometimes perhaps as far as UAUGGAGA.

The cloned T_4 tRNA genes which we studied in the Xenopus system were not expressed in E.coli, when transduced from λgt t11, and this was attributed to the absence of the transcriptional control region (17). However, even λgt t28, in which the control region is present, when tested in E.coli, did not produce $tRNA^{Ser}$, although four other tRNAs out of the seven were made. It is all the more remarkable that in the Xenopus GV system the cloned T_4 DNA was transcribed, and that the major product from the entire T_4 tRNA region was the $tRNA^{Ser}$.

We have obtained identical results with four clones of T_4 DNA. Two of the clones (pTFR 1109 and pTFR 1501) contain an insertion of about 4000 B.P., specifying seven tRNAs. Two other clones (pTFA 314 and pTFA 420) contain an insert of only 170 B.P., which include from the 5' end: most of the $tRNA^{Pro}$ gene, the entire $tRNA^{Ser}$ gene, but only 10 B.P. at the 5' end of the $tRNA^{Thr}$ gene.

Our results, that $tRNA^{Ser}$ is the major product of all the four clones tested, could be explained, if Xenopus RNA polymerase recognized a sequence within the T_4 $tRNA^{Pro}$ as promotor. An alternate explanation would place the promotor site within the plasmid vehicle DNA, dictating for two of the clones a very long transcript up to the $tRNA^{Ser}$ gene (about 3000 nucleotides), and requiring subsequent processing.

We cannot explain why the $tRNA^{Ser}$ should be the only identifiable tRNA product of pTFR 1109 and pTFR 1501 in the Xenopus GV system. Whether this reflects properties of transcription, processing or RNA stability cannot be deduced from our results.

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