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The cleavage of transfer RNA by a single strand specific endonuclease from Neurospora crassa

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ABSTRACT

Endonuclease from Neurospora crassa (NcNase), an enzyme with specificity for polynucleotides lacking an ordered structure, was shown to cleave su_3^+ $tRNA^{Tyr}$ from E. coli preferentially in the anticodon region. The enzyme cleaved unfractionated tRNA primarily to 30 - 50 nucleotide size fragments, implying that most or all tRNA species are also cleaved in the anticodon region. The 3' terminal sequence C-A was cleaved as well. The results are discussed with respect to the three dimensional structure of tRNA.

INTRODUCTION

An endonuclease with a high degree of specificity for polynucleotides lacking an ordered structure was discovered by Linn and Lehman is extracts of Neurospora crassa conidia(I). The enzyme lacks an absolute specificity for a particular base but appears to possess preference for diester bonds involving guanosine or deoxyguanosine residues (1,20).

tRNA is known to have high degree of secondary and tertiary structure. Its relative resistance to NcNase, therefore (1), suggested that this enzyme may be useful for structural studies. In this work I show that when tRNA is stabilized by high salt, only the anticodon and the 3'-terminus are cleaved by the enzyme, suggesting that these regions are exposed and in single strand form. These results are in agreement with studies in which chemical reagents and oligonucleotide hybridization were utilized (9-18).

MATERIALS AND METHODS

RNA. ^{32}P-labeled tRNA from ϕ80 psu_3^+ infected cells: Escherichia coli CA274 were grown, infected and labeled according to Landy et al. (2). ^{32}P-labeled su_3^+ $tRNA^{Tyr}$ was purified according to the method of Smith et al. (3).

The preparation of tRNA...N-C-C(^{3}H)C-A and tRNA...N-C-C-(^{3}H)A was described in earlier publication (4).

Enzymes. Neurospora crassa endonuclease with a specific activity of 800 units per mg protein was prepared according to the method of Linn and

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Lehman (1). The enzyme was assayed using native and denatured ^{32}P-DNA from E. coli. The reaction mixture contained 10mM Tris-pH 7.5, 0.03M NaCl, 2mM $MgCl_2$, 3 µg ^{32}P-DNA and 2 enzyme units in a final volume of 300 µl. After 3 hrs at 37°C 94% of the denatured DNA was rendered acid soluble while the native DNA remained 100% acid insoluble. T_1 ribonuclease was purchased from Sankyo, Japan.

Gel Electrophoresis. Acrylamide and N,N'-methylene-bis-acrylamide were purchased from Eastman Organic Chemicals and recrystalized according to Loening (7). Slab gels were prepared between two glass plates using plastic spacers, 2.3 mm wide. Both 10% and 16% gels were prepared according to Peacock and Dingman (5) and electrophoresis was carried out in an apparatus of our design. Electrophoresis buffer contained 0.09M Tris, 2.5mM EDTA and 0.09M boric acid, pH 9.3. The RNA was eluted from the gels by shaking the gel slice in 10 volumes of 0.01M Tris-HCl pH 9.1 containing 0.05M NaCl and 10mM EDTA for 8 hours at room temperature. The buffer was collected and a second similar elution for an additional two hours was carried out. The salt was removed from the RNA by chromatography on a G-10 Sephadex column.

Two dimensional paper electrophoresis ("fingerprint") was carried out according to Sanger et al. (6).

RESULTS

Cleavage of su_3^+ $tRNA^{Tyr}$ by NcNase and by T_1 ribonuclease. Preliminary experiments in which unfractionated, ^{32}P labeled E. coli tRNA was incubated with NcNase in high salt revealed a very slow release of radioactive counts into TCA soluble form. Limited cleavages were then looked for by analyzing the products of a reaction between the enzyme and purified su_3^+ $tRNA^{Tyr}$ on polyacrylamide gels. This particular tRNA was chosen because of the relatively easy procedure that is available for its isolation and purification in amplified quantities (8). Figure I is an autoradiogram from a typical experiment. The apparent contamination of the starting material (time 0') is exaggerated by overexposure of the film: the tRNA was in fact more than 85% pure. The cleavage was rapid in 0.3M NaCl but decreased as the salt concentration increased. Two large fragments appeared as major primary products. From their relative mobility they were estimated to be 50±5 and 35±5 nucleotides in length. Intact su_3^+ $tRNA^{Tyr}$ (85 nucleotides) and its 3'-terminal T_1 fragment (19 nucleotides, obtained by complete digestion in 0.01M EDTA) were used as markers for the size determination.

The autoradiograms were scanned by a recording densitometer for the

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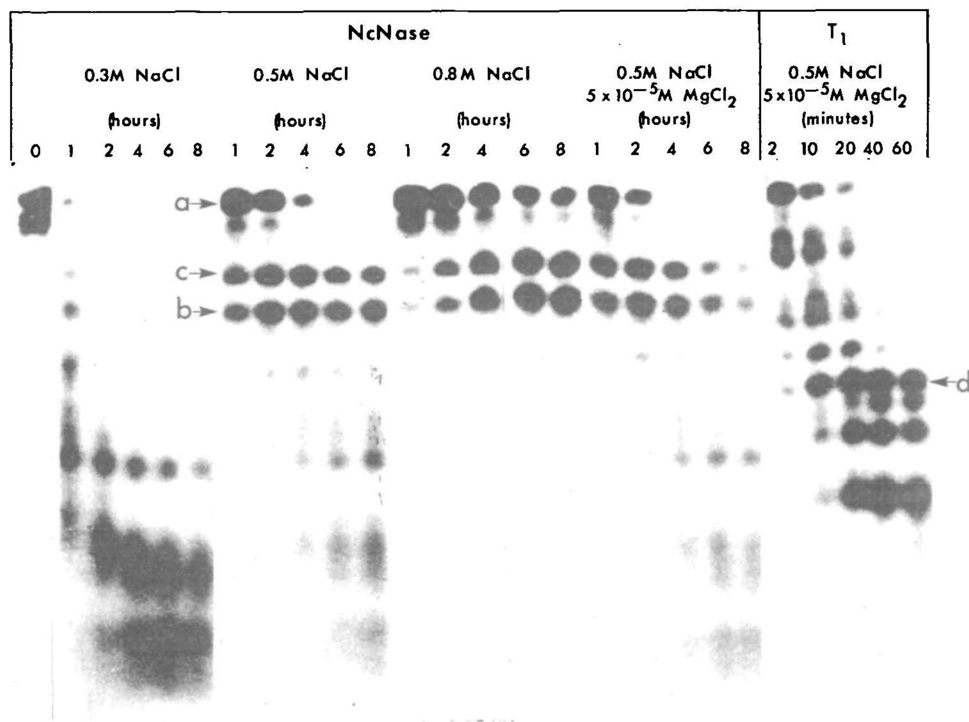

Figure 1. Cleavage of ^{32}P-su^{+} tRNA^{Tyr} by NcNase and by T_1 RNase: Purified ^{32}P-tRNA (10^5 cpm) was incubated at 22°C with NcNase (2.4 units) or T_1 RNase (0.05 units). The reaction mixture contained 0.01M Tris•HCl buffer pH 7.5 and varying NaCl and $MgCl_2$ concentrations in a total volume of 25 μl. At the times indicated 4 μl samples were removed into 10 μl electrophoresis buffer which contained 30% glycerol and a dye marker, and stored in liquid nitrogen until electrophoresis. Electrophoresis was done in 16% acrylamide gel (20 cm long) at 250 V for 14 hrs at room temperature (22°C). The electrophoresis buffer (see Materials and Methods) contained also deionized 7M Urea and 0.2% SDS.
a-Intact tRNA; b,c-large cleavage products; d-3'-end fragment (No. 16 in Table 1).

purpose of quantitative determination of the kinetics of appearance of each fragment at different salt concentration. This analysis revealed two phases of reaction. In the first phase the products were mainly two large fragments; short oligonucleotides as well as mononucleotides accumulated in the second stage. Increasing salt concentrations, although retarding the overall reaction, had a more profound effect on the second stage. Thus, while short oligonucleotides appeared in 0.5M NaCl even before the material at the origin

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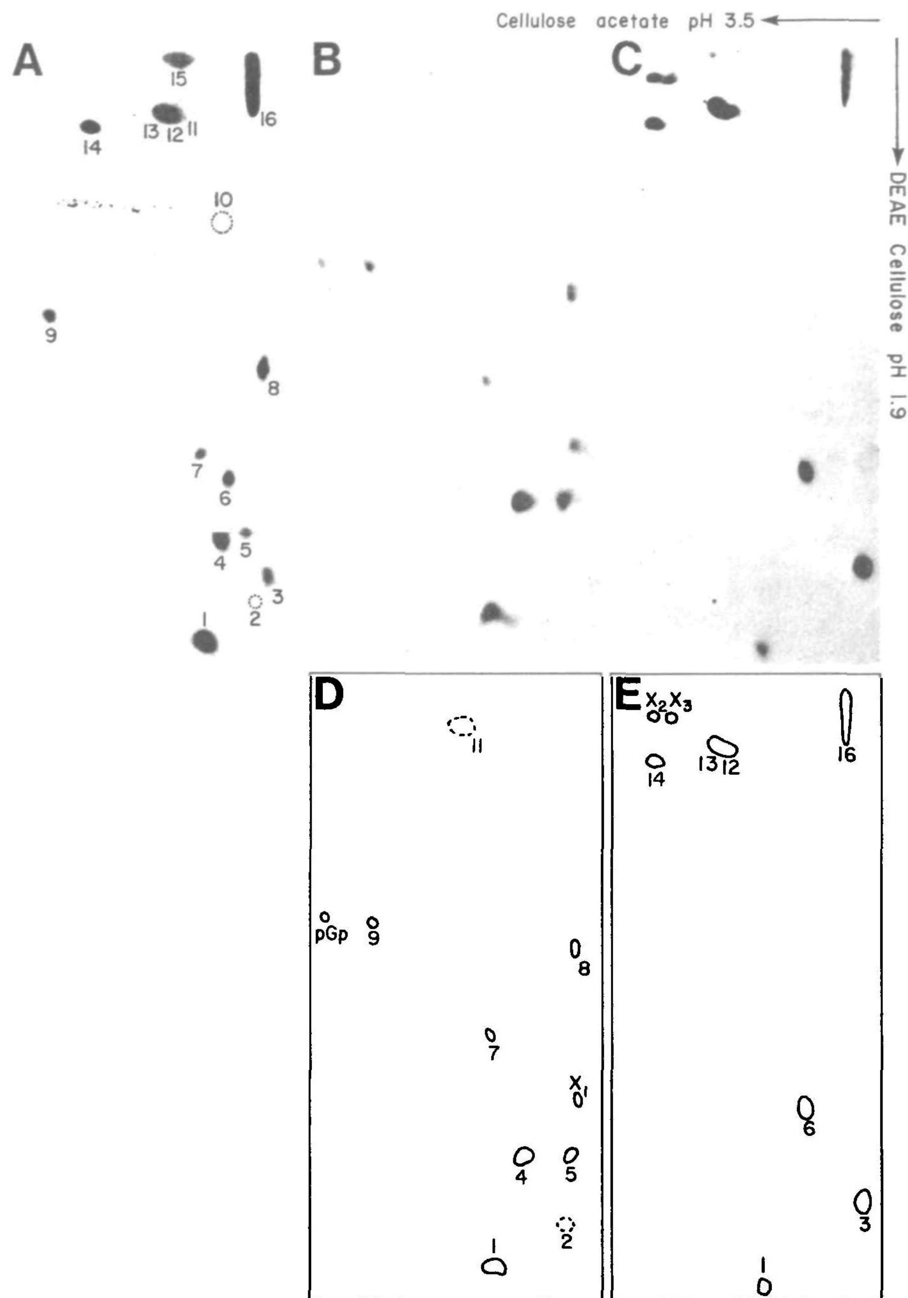

Figure 2. T_1-fingerprint analysis of su$^+$-tRNA^{Tyr} and the NcNase digestion products: A su$^+$-tRNA^{Tyr}; B-5'-fragment; C-3'-fragment; D,E,-diagrammatic explanation of the fingerprints shown in B and C, respectively. The fragments were obtained by a digestion in 0.8M NaCl.

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has completely disappeared, very little secondary cleavage took place in 0.8M NaCl during the first phase. At 5 x 10^{-5}M $MgCl_2$ did not have any detectable effect on the kinetics of the reaction (Fig. 1).

For comparison we followed the degradation of su_3^+ $tRNA^{Tyr}$ by T_1 ribonuclease under the same conditions. T_1 cleaves in a large number of sites, and although it is clear that some sites are cleaved faster than others, the kinetics of appearance of the different fragments appeared to be virtually the same irrespective of ionic strength and temperature. Two phase kinetics was not observed.

NcNase cleaves su_3^+ $tRNA^{Tyr}$ in the anticodon region. In order to detect the major cleavage site at high salt (first phase reaction) the two fragments were eluted from the gel and extensively degraded with T_1 ribonuclease in the presence of EDTA (6). The digests were subjected to two dimensional paper electrophoresis. A T_1 digest of the original tRNA was similarly treated. The results are shown in Fig. 2. Of the spots in Fig. 2A all but one can be detected in either one of the fragments (B and C). The missing oligonucleotide is that of the anticodon (No. 15) (8), thus indicating that the two fragments were formed by the cleavage of the RNA in the anticodon region.

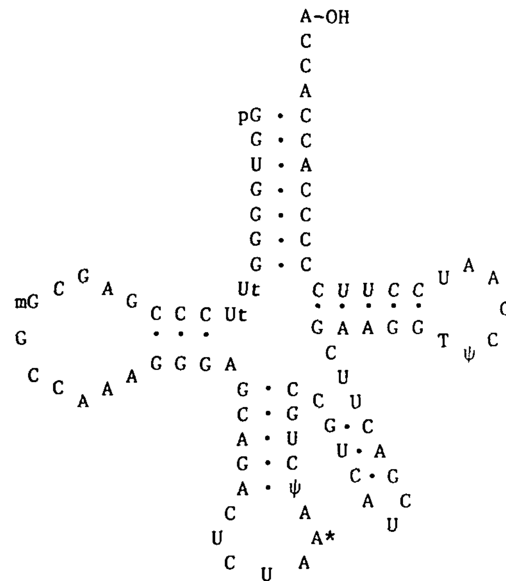

Figure 3. The nucleotide sequence of su_3^+ $tRNA^{Tyr}$ from E. coli. (After Goodman et al., Eur. J. Biochem. 13, (1970), 461.) A* is 2-thiomethyl, 6-isopentenyl adenylic acid. U_t is 4-thiouridylic acid.

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TABLE 1

The Oligonucleotide Composition of T_1 Digest of su_3^+ $tRNA^{Tyr}$.

1.	G	9.	UG
2.	CG(1)	10.	ACUG(2)
3.	CCG	11.	UUCCCG
4.	AG	12.	UCAUCG
5.	CAG	13.	ACUUCG
6.	AAG	14.	TψCG
7.	CGmG	15.	ACUCUAA*AψCUG (Anticodon)
8.	CCAAAG	16.	AAUCCUUCCCCCACCACCA-OH (3'-end)

(1) This sequence is not normally present in $tRNA^{Tyr}$. It is formed in low yields as a result of incomplete methylation of the sequence CGmG (8).

(2) This sequence is formed by T_1 digestion of su^-tRNA^{Tyr} in which the base in position 35 is a modified G. Su^- is present in low yields in su^+tRNA preparations. Due to cell starvation before the infection there is incomplete modification of G_{35}, which results in the formation of small quantities of ACUG upon T_1 digestion (8).

From the sequence of su_3^+ $tRNA^{Tyr}$ (Fig. 3 and Table 1) it is apparent that the spots in Fig. 2B and 2C correspond to the fragments expected from the 5' and 3' parts of the tRNA, respectively. The relative amount of spot No. 11, a hexanucleotide (Fig. 2B) varied in different experiments, probably due to variable efficiency of transfer from cellulose acetate to DEAE paper. Three new spots were generated, one coincident with the 5' fragment (x_1) and the other two with the 3' fragment (x_2, x_3), indicating the presence of multiple cleavage sites in the anticodon region.

Most or all tRNA species are also cleaved in the anticodon region. The cleavage products of ^{32}P labeled unfractionated tRNA were also analyzed. As demonstrated in Fig. 4, the major initial products of enzymatic digestion were 30 to 50 nucleotides in length, a size consistent with cleavage in the proximity of the anticodon. There was a significant accumulation of short oligonucleotides and mononucleotides when the reaction was carried out in 0.3M NaCl (Fig. 4b). It has been shown by Linn and Lehman that a terminal phosphate group was required for the cleavage of short nucleotides to yet smaller products (1). This finding is confirmed in Fig. 4c: when bacterial alkaline phosphatase was included in the reaction mixture the accumulation of short products was largely inhibited, even at 0.3M NaCl. Increasing the salt concentration to 0.5M similarly inhibited this secondary cleavage to a large extent.

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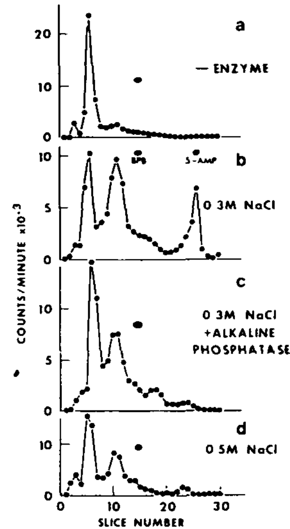

Figure 4. Cleavage of unfractionated, ^{32}P labeled tRNA from E. coli by NcNase : 0.2 Micrograms RNA were incubated with 0.7 units NcNase for 60 minutes, and the reaction products were analyzed on 16% cylindrical gels.
BPB (Bromophenol blue) migrated as a 15-nucleotide fragment, 5'-AMP marker was detected by scanning the gel in UV light.

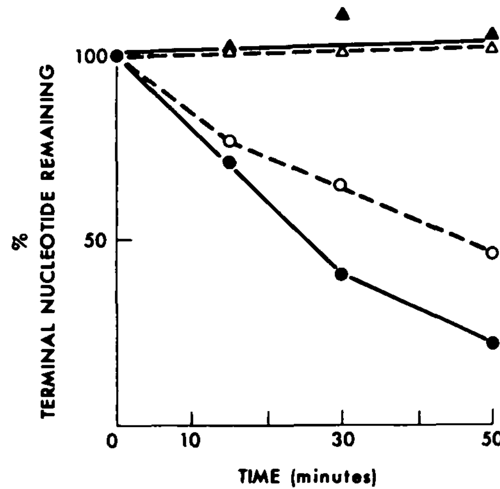

Figure 5. Cleavage of 3'-terminal nucleotides: 2.5 micrograms tRNA...N-C-C-(^{3}H)A (-●—●—) and 1.1 micrograms tRNA...N-C-(^{3}H)C-A (-o-- o--) were incubated each with 10.5 units of NcNase in the presence of 0.3M NaCl at 37°C. Samples taken at the times indicated were analyzed for TCA precipitable counts. ▲ , △ - Control incubations with enzyme omitted.

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The C-C-A region is cleaved by NcNase. In order to test the susceptibility of the C-C-A region to cleavage by the enzyme, two substrates were prepared: unfractionated tRNA....N-C-C-(^{3}H)A and tRNA....N-C-(^{3}H)C-A. When these materials were subjected to the enzyme, radioactivity was rendered acid soluble in both cases, although the terminal labeled adenosine was cleaved at a faster rate than the penultimate labeled cytidine (Fig. 5). These differences were even greater than actually shown in Fig. 5 because the ratio of enzyme to substrate was two-fold smaller in the CMP-labeled tRNA than in the AMP-labeled one.

DISCUSSION

A large number of studies designed to examine the three dimensional structure of tRNA using various chemical reagents (8-13) and enzymes (14,15 18,19) have been reported. The specificity of these enzymes for single stranded regions was not absolute, but at least some of the chemical reagents were highly specific for unpaired nucleotides. The use of the Neurospora crassa enzyme permits specific examination of single-stranded regions in the tRNA molecule, while avoiding the limitations introduced by the unique specificity of most chemical reagents. Although the enzyme has been reported to have preference for bonds involving guanylic acid residue, it cleaved primarily two regions in the su_3^+ $tRNA^{Tyr}$: the anticodon and the 3'-terminus, both of which do not contain G. The specificity toward the anticodon is achieved by increasing the salt concentration. This is consistent with the work of Tenenhouse and Fraser who found that increasing Mg^{++} concentrations inhibited the rates of release of acid soluble material from $tRNA^{Phe}$. Both salt and Mg^{++} stabilize the RNA structure, probably leaving fewer unpaired regions accessible to the enzyme. The possibility of a direct effect of the salt on the enzyme activity, however, cannot be excluded.

The results reported here are in general agreement with studies in which chemical reagents were employed (9-13). Although some of the reagents used also reacted with nucleotides in the dihydrouridine loop (10,12,13), no major cleavage site was detected in this region in high salt concentrations. It should be noted, however, that there are variations in the reactivity of nucleotides in the dihydro-U loop among different tRNA species. Thus, monoperphtalic acid, for example, oxidized adenosine in the dihydro-U region of yeast $tRNA^{Ser}$ but did not react with the two adenosines that occupy the same region of yeast $tRNA^{Phe}$ (9).

The results are in agreement with yet another experimental approach, introduced by Uhlenbeck et al.: When tri- and tetranucleotides

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possessing sequences complementary to those present in several tRNAs were tested for the ability to hybridize to their respective tRNAs, hybridization took place to sections of the anticodon loop and to the C-C-A region in all cases (16,17).

It is interesting also to compare the rate of cleavage of the terminal adenylic acid and the penultimate cytidylic acid. These results are in agreement with a previous finding reported by us that alkaline phosphatase hydrolyzed the exposed phosphate group of tRNA...N-C-Cp at a much faster rate than the phosphate of tRNA...N-Cp (4). A similar observation has been reported for tRNA nucleotidyltransferase (18) and snake venom phosphodiesterase (19). The susceptibility of the terminal C-A to NcNase suggests that this sequence is not involved in the stabilization of the three dimensional structure of the tRNA and that the differences in rate more likely originate from the interference by the tRNA structure with the enzyme's activity.

In summary, it is concluded that an endonuclease from Neurospora crassa cleaves tRNAs mainly in the anticodon and the 3'-terminal regions, which were already shown by other methods to be in a single stranded form. This property of the enzyme makes it valuable for structural studies of RNA. Moreover, the specificity of the enzyme makes it particularly useful for sequence analysis by providing a tool for cleaving tRNAs which lack guanylic acid residues in the proximity of the anticodon.

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During the preparation of this manuscript I learned that similar results were obtained with nuclease S_1 by Harada and Dahlberg (submitted to Nucleic Acid Research).

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