A novel RNA digesting activity from commercial polynucleotide phosphorylase

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RNase A_4 is a new RNase activity found as a contaminant in commercial polynucleotide phosphorylase. This enzyme has the ability of hydrolyzing the phosphodiester bond between pyrimidine-A in both loop and paired regions of RNA.

Polynucleotide phosphorylase *Ribonuclease* *tRNA*

1. INTRODUCTION

The advent and availability of restriction endonucleases was the driving force in the emergence of recombinant DNA technology. The lack of such enzymes is largely responsible for the retarded development in RNA manipulation. Available RNA endonucleases are limited to either base specific or secondary structure specific enzymes; neither of which affords preparative amounts of long oligoribonucleotides.

We have inadvertently discovered a new RNase activity which is present as a contaminant of commercial polynucleotide phosphorylase preparations and which has the unusual property of cutting RNA between pyrimidines and purines. The enzyme shows no discrimination based on secondary structure. Here, we describe experiments characterizing this new enzyme called RNase A_4, which may be a useful tool in RNA research.

2. MATERIALS AND METHODS

Yeast $tRNA^{Phe}$, *E. coli* $tRNA^{Glu}$ and *E. coli* $tRNA^{Tyr}$ preparations as well as $(Ap)_3A$ (lot. 1239506), $(Ap)_9A$ (lot. 1489306), $(Up)_3U$ (lot. 1339104) and polynucleotide phosphorylase from *Micrococcus luteus* (lots 1143220 and 1313520) were purchased from Boehringer Mannheim. $(Ap)_2A$ (lot. 61C-1900), ApApC (lot 35C-0099) and ApCpC were from Sigma. All tRNAs have been labelled with ^{32}P and polyacrylamide gel electrophoresis has been performed as in [1]. The standard buffer was 50 mM Hepes, pH 7.2, 50 mM NaCl and 1 mM EDTA. The RNase activity has been assayed in standard buffer (plus or minus 5 M urea) containing 2.2 μg/10 μl of tRNA, by incubation at 37°C if not stated otherwise. The reactions were terminated by boiling the samples for 5 min, cooling in dry ice and vacuum drying. They were subsequently dissolved in deionized formamide containing tracking dyes and applied onto polyacrylamide gels [1]. Another rapid activity test involved incubation of 3'-labelled $tRNA^{Phe}$ in standard buffer containing 10 mM $MgCl_2$, followed by acid precipitation of the sample on filter paper. Under such conditions the Cp labelled CCA terminus of tRNA is the main target of attack and the acid insoluble radioactivity counts correspond to unreacted tRNA. DEAE-cellulose (3.5 1 column) was eluted with a 10 × column volume gradient of NaCl (0.02–0.5 M) in standard buffer. Sephadex G-100 gel filtration (2.5 ml column) has been performed in standard buffer containing 0.1 M NaCl at room temperature. Aliquots of selected fractions were incubated with ^{32}P-labelled tRNA and analyzed by 15% gel electrophoresis.

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3. RESULTS AND DISCUSSION

We observed that the incubation of yeast $tRNA^{Phe}$ in the presence of a commercial sample of $(Ap)_3A$ led to digestion of the tRNA. As seen in fig.1, the major cuts introduced in $tRNA^{Phe}$ by this nuclease activity are between U_8 and A_9 and between C_{28} and A_{29} regardless of the experimental conditions. Cuts between C_{13}-A_{14} and C_2-G_3 are also clearly seen. The specificity inferred from the cleavage pattern on a polyacrylamide sequencing gel indicated that no RNase currently used in RNA analysis could be responsible for the digestion. The cuts were primarily observed between C and A or U and A in both single and double stranded regions of tRNA. The same putative nuclease activity was present in the trinucleotide $(Ap)_2C$ but not in ApCpC, $(Ap)_2A$, $(Ap)_9A$ or $(Up)_3U$ preparations (see section 2 for the source of oligonucleotide samples). This enzymatic activity, which we call A_4, eluted from a Sephadex G-25 column in the void volume before $(Ap)_3A$, was sensitive to heating at 100°C and could be eliminated from the oligonucleotides by HPLC using a C_{18} reverse phase column.

Subsequently, a commercial preparation of the polynucleotide phosphorylase (PNPase) from *M. luteus*, the enzyme used to synthesize different oligoribonucleotides, was tested for A_4 activity. The enzyme assay was based on the reduction of acid precipitable counts during incubation of extracts with 3′-[^{32}P]pCp labelled $tRNA^{Phe}$. A_4 activity assayed in this manner cannot be confused with the phosphorolysis activity of polynucleotide phosphorylase, since the substrate is 3′-phosphorylated [2]. Within 8 min half of the radioactivity is solubilized when 100 g of the commercial PNPase preparation is incubated with 10 g of 3′-labelled $tRNA^{Phe}$ at 22°C in standard buffer containing 10 mM $MgCl_2$.

DEAE-cellulose chromatography of PNPase allows isolation of the nuclease activity which elutes at about 0.2 M NaCl. A large A_{280} peak elutes later at 0.3 M NaCl which corresponds to the elution position of PNPase [3]. When the 0.2 M fraction is tested with yeast $tRNA^{Phe}$ and analyzed by polyacrylamide gel electrophoresis, two distinct RNase activities seem to be present. In fig.1, the effect of pooled 0.2 M NaCl fractions from DEAE cellulose on yeast $tRNA^{Phe}$ is compared with that of an $(Ap)_3A$ sample at different temperatures. One of the nucleases found in the PNPase preparation corresponds to that present in the $(Ap)_3A$ sample. The other appears to be sen-

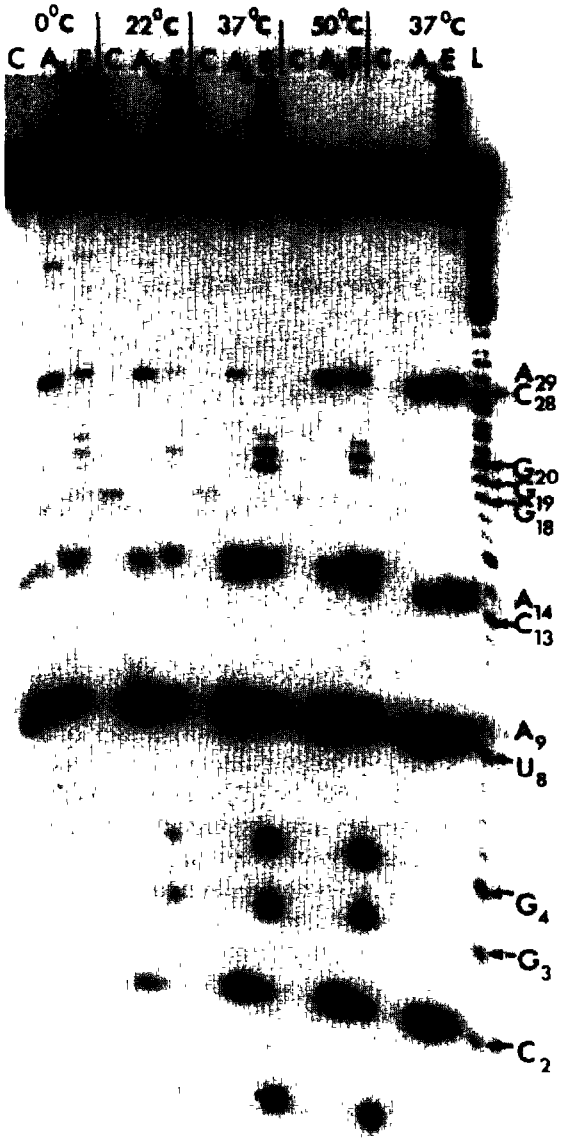

Fig.1. PAGE (15%) of 5′-labelled $tRNA^{Phe}$ incubated with 0.005 A_{280} (per 10 μl) of pooled 0.2 M NaCl fraction from DEAE cellulose (E) or with 1.25 A_{280} of $(Ap)_3A$ preparation (A_4) in the standard buffer. A third control lane (C) is used at each of the various conditions: 0°C (lanes 1–3), 22°C (4–6), 37°C (7–9), 50°C (10–12) and at 37°C (13–15) in the presence of 5 M urea. Lane 16 corresponds to a formamide ladder (L).

sitive to 5 M urea and is G-specific (T_1-like activity).

A_4 and G-specific activities can be separated on Sephadex G-100 chromatography (not shown): A_4 elutes before the T_1-like activity and their elution positions indicate molecular masses in the range of 50 and 30 kDa, respectively. This step does not lead however to a homogenous preparation of A_4, since it is still contaminated with some protein as judged by SDS–PAGE. However, the crude preparation of PNPase can thus be directly used as a source of ribonuclease A_4 provided that incubations are carried out in the presence of 5 M urea.

Fig.2 summarizes the results obtained in the digestion of 5′ and 3′ labelled yeast $tRNA^{Phe}$ by the A_4 RNase. The enzyme attacks the phosphodiester bonds between pyrimidines and purines preferring pyrimidines followed by A rather than G. Especially susceptible are stretches of pyrimidines when followed by a stretch of purines. Both single stranded and double stranded regions are attacked. From comparison of cleavage patterns with 5′- and 3′-labelled tRNA it may be

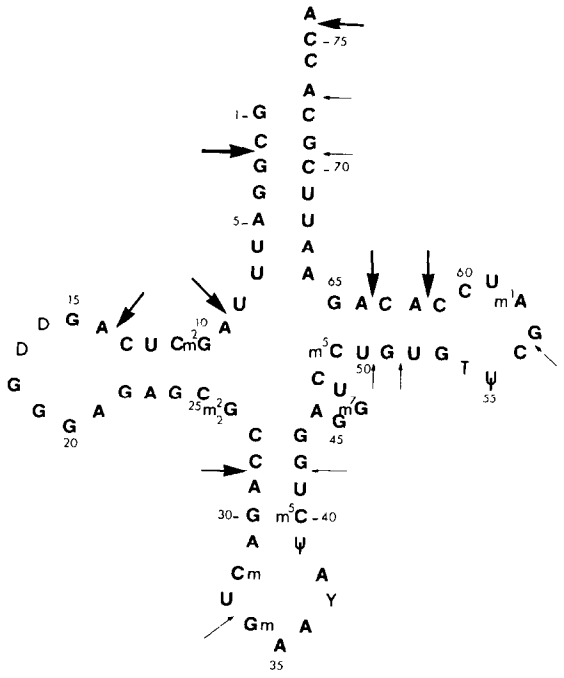

Fig.2. A cloverleaf of yeast $tRNA^{Phe}$ indicating the major (large arrows) and minor cuts (small arrows) obtained in the presence of RNase A_4 as deduced from experiments with both 5′- and 3′-labelled $tRNA^{Phe}$.

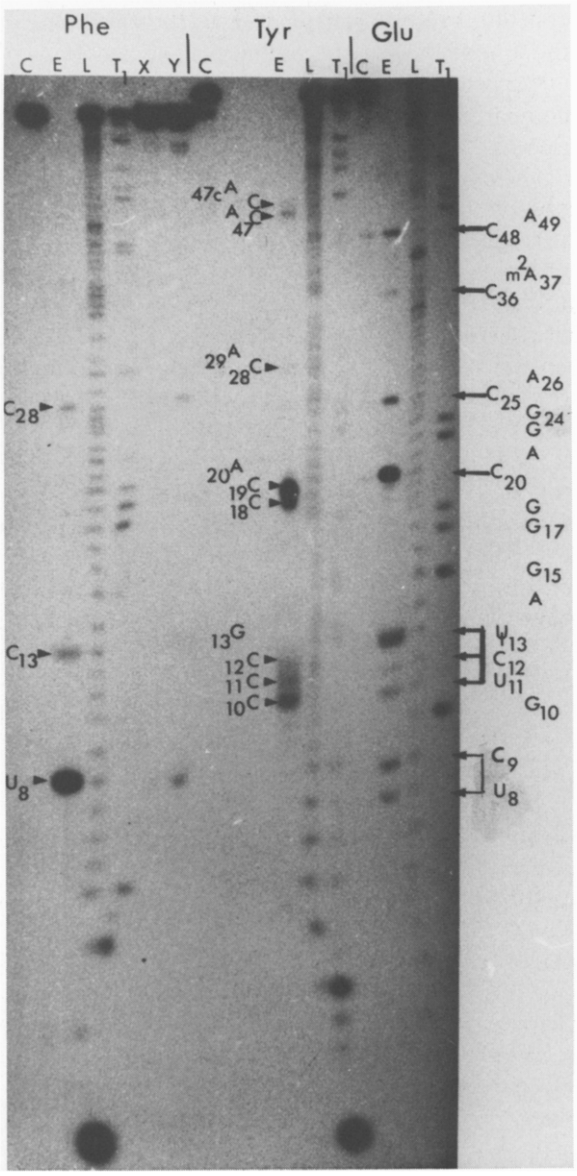

Fig.3. PAGE of 5′-labelled yeast $tRNA^{Phe}$ (lanes 1–6), *E. coli* $tRNA^{Tyr}$ (7–10) and $tRNA^{Glu}$ (11–14). The tRNA samples have been incubated with 0.0015 A_{280} of pooled fractions containing A_4 activity obtained after Sephadex gel filtration, in a standard buffer containing 5 M urea at 37°C for 60 min. Lanes 5 and 6 correspond to incubations in the presence of the same (X) or twice (Y) the amount of the enzyme preparation in the absence of urea and in the presence of 15 mM $MgCl_2$ at 0°C. C indicates control without enzyme, L is the ladder, T_1 is a partial digestion with T_1 and A_4 is the digest of the RNase described here.

concluded that the enzyme leaves the phosphate on the 3′-position of the pyrimidine nucleotide.

Cleavage patterns of yeast $tRNA^{Phe}$ have been compared with those of *E. coli* $tRNA^{Tyr}$ and $tRNA^{Glu}$ (fig.3; and not shown). After an extensive digestion, leaving no intact tRNA molecules, we observe tRNA fragments generated from cuts at the major cleavage sites. The identical enzymatic specificity is observed for all samples; however, in *E. coli* tRNAs, some fragments ending in a stretch of pyrimidines appear to be further degraded at the 3′-end. It is possible that modified nucleotides are responsible for this effect, since the octamer generated from the 5′-end of $tRNA^{Phe}$, which does not contain modifications, has never been further digested in this way. From fig.3, we see that nuclease A_4 produces fragments of RNA larger than those usually obtained by extensive digestions with single base specific RNases such as T_1. It can thus provide a useful tool for producing large fragments of RNA for structural studies and/or for mapping larger RNA molecules.

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