INCREASED RIBONUCLEASE II ACTIVITY IN A TEMPERATURE SENSITIVE MUTANT OF *ESCHERICHIA COLI*

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Received 27 November 1972

1. Introduction

In previous reports we described a temperature sensitive mutant (*ts*) of *Escherichia coli* which exhibited in cell free extracts an augmented ribonuclease (RNAase) activity that was dependent upon growth of the mutant at the nonpermissive temperature [1, 2]. In this mutant the functional [2] and chemical [3] half lives of *lac* mRNA, and other messenger [4], compared to that of the parental strain were also shortened. The increase of RNAase activity was shown to be caused by a single-point mutation mapping near minute 74 on the *E. coli* map [1]. Since these observations suggested that there might be a relationship between the increased RNAase activity in cell-free extracts of the mutant strain, and the decreased lifetimes of mRNA, we proceeded to characterize the nature of this altered RNAase activity.

2. Results and discussion

When cell-free extracts (S-12) from both the parental 112-130 and the mutant strains (N4752) were assayed under RNAase II conditions up to 4-fold differences in RNAase II specific activity were observed between the two strains [1, 2, 5]. In order to find out if this increased RNAase activity is dependent upon the presence of ribosomes, cell-free extracts were prepared from both strains and were separated into ribosomes and ribosome-free supernatants. The supernatants were then assayed for RNAase II activity both in the presence and absence of ribosomes from each of the two strains. As can be seen in fig. 1, a 4-fold difference in RNAase specific activity between the two ribosome-free supernatants was found, and it was unaffected by the presence of ribosomes from either of the two strains tested. It therefore seems that the RNAase activity affected by the mutation in strain N4752 is a supernatant factor.

The ionic requirements of the RNAase activities in both strains were determined by assaying the RNAase activity in dialyzed ribosome-free supernatants in absence or presence of one or both of the cations used in the above experiments (i.e., magnesium and potassium). The data in table 1 indicate that the RNAase activities of both strains require both magnesium and potassium for optimal function.

To identify the reaction products, ribosome-free supernatants from both strains were used in RNAase II assays and at the end of the reaction portions of the assay mixes were spotted on Whatmann 3 mM paper and chromatographed with 95% ethanol, 1 M ammonium acetate (1:1, v/v) saturated with sodium borate. The resultant chromatograms repeatedly exhibited a single product when either poly (A) or poly (U) were used as substrates. With poly (U), the product exhibited an R_f (0.45) identical to 5'-UMP (0.44), but different from the R_f values of markers that were chromatographed on the same paper as the reaction mixture (uracil, 0.77; uridine, 0.74; UDP, 0.38; UTP, 0.36; 3'-UMP, 0.60; and 2', 3'-cUMP, 0.80). Similarly, the product of reactions with poly (A) as substrate exhibited the same R_f as a 5'-AMP marker.

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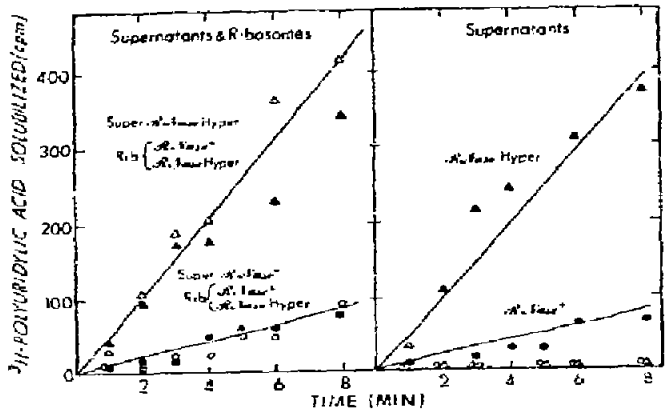

Fig. 1. RNAase activity in supernatants, and in supernatants supplemented with ribosomes. Extracts of cells from both strains 112–130 and N4752 were prepared by alumina grinding [13] treated with DNAase and extracted with buffer (Tris-HCl 10 mM, pH 7.5; magnesium acetate 10 mM; reduced glutathione 10 mM). Ribosome-free supernatants were prepared and stored at −60° for not more than one week, in 20% v/v glycerol containing GSH (10 mM). The ribosomes used were washed four times in a buffer containing 1 M NH_4Cl.

The reaction mixtures contained, per ml: 200 μg of the supernatant protein; and/or 40 μg ribosomes; [^{3}H]polyuridylic acid (10.6 μg, 100,000 cpm); and were 10 mM in Mg^{2+}, 100 mM in K^+ and 6 mM in GSH. Reactions were carried out at 30°. At the specified times, 50 μl aliquots were withdrawn and the assay was terminated. Right panel ○—○ 112-130 ribosomes; △—△ N4752 ribosomes. Left panel open symbols 112-130 ribosomes; closed symbols N4752 ribosomes.

There were at least four factors that influenced the variation of RNAase II activity in extracts: age of extracts, nature of substrates, sulphydryl reagents, and treatment of extracts with DNAase. We observed that the RNAase activity, especially of the parental extracts, tended to increase somewhat upon storage. Poly (A) was a better substrate than poly (U) by a factor of 2 to 3 [9] and DTE and GSH were superior to 2-mercaptoethanol in preserving the activity of the extracts. When extracts were treated with DNAase, their specific activity was lowered; this is probably due to the formation of inhibitory deoxyoligonucleotides (see [5]).

In summary, the experiments described thus far indicate that the activity we are investigating i) is primarily a supernatant factor, ii) requires both magnesium and potassium ions for optimal activity, and iii) can degrade polynucleotide to 5′-mononucleotides. Of all the *E. coli* RNAases I–IV and PNPase only the characteristics of the RNAase II are compatible with the characteristics of the RNAase activity described here.

Table 1
Ion requirements of RNAase activity.

Ions	Strain 112-130	Strain N4752
Complete mix	214 ± 8	767 ± 18
$-K^+$	22 ± 8	39 ± 15
$-Mg^{2+}$	31 ± 12	201 ± 30
$-Mg^{2+}$, $-K^+$	0	0

Strains 112-130 and N4752 were grown as described in [1 and 2] and assayed as described in [10]. Ribosome-free supernatants were prepared from cell extracts made by sonication, and were not treated with DNAase. The supernatants were dialyzed against 10 mM Tris buffer (pH 7.8, 600 ml buffer/ml supernate) containing 2 mM dithioerythritol with frequent changes of buffer (3 hr intervals; 4 buffer changes). The assay mixtures contained 60 μg supernatant protein and [^{3}H]polyadenylic acid (20 μg poly (A) 79,000 cpm per ml, as well as the other necessary components. The reaction mixtures (65 μl) were incubated at 37° for 20 min. The data represent the average of triplicate determinations and are corrected for alcohol soluble counts in the absence of supernatants (110 cpm). The specific activities [9] of the supernatants were 0.5 and 1.9.

Since the assay used was suited to detection of RNAase II activity the above data do not rule out the possibility that other RNAase activities may also be affected by the mutation. To test this we determined the levels of RNAase III and PNPase and RNAase II in cell-free extracts derived from both strains.

The remaining RNAases were not tested because: i) in plate assays for RNAase I colonies of N4752 were as negative for RNAase I as colonies from the parental strain and other RNAase I⁻ strains, ii) there is no simple assay for RNAase IV activity, suitable for cell-extracts [6], and iii) a unique RNAase V enzyme does not seem to exist [7, 8]. The data in table 2 clearly indicate that only the level of RNAase II is affected by the mutation, while the levels of RNAase III and PNPase are not. It can therefore be concluded that from the RNAase tested only the level of RNAase II is affected in the mutant strain at the nonpermissive temperature.

Since the *ts* mutation augments the RNAase II activity, expression of the mutation at the nonpermissive temperature could lead to a reduction in the synthesis of an RNAase II specific inhibitor, synthesis of an RNAase II specific activator, result in an increased synthesis of RNAase II in the mutant strain or lead

directly or indirectly to the production of a structurally altered, hyperactive RNAase II enzyme.

Previous experiments showed that oligonucleotides of DNA inhibit RNAase II of *E. coli*, and that most of the inhibitory capacity of extracts is probably due to DNA [5].

Since the difference in RNAase II activity between the two strains could be demonstrated in supernatants from which nucleic acids were removed, and since mixing such supernatants resulted in additive RNAase activity we conclude that neither an inhibitor nor an activator of RNAase II is affected by the *ts* mutation. Moreover in a specific search, neither an activator nor an inhibitor of RNAase II in extracts of wild type *E. coli* strains, was found.

To further investigate the difference in RNAase II activity between these two strains, the RNAase II enzymes from both strains, grown at the elevated temperature, were isolated and purified according to the procedure of Singer and Tolbert [9]. The specific activity of RNAase II in the mutant extract was 3-times greater than in the parental extract; this difference in specific activity not only persisted throughout purification, but actually increased to an 8-fold difference in the most highly purified samples compared. Gel electrophoresis (pH 7.6) of the purest samples revealed however two bands in the parental enzyme fraction – a major and a minor – and four bands in the mutant enzyme. Moreover, the major band found in the parental strain was a major protein component in the crude extract (S-30) which is carried through all the purification steps and which apparently does not possess RNAase II activity. This was determined by slicing and assaying the gels for RNAase activity and by correlating the increase in specific activity of the enzyme during purification and the relative enrichment of the bands as determined by densitometry.

Therefore it was not possible to determine whether the increase in the level of RNAase II in the mutant strain is due to increased synthesis or due to modification of the enzyme.

At present the major difficulty in pinpointing the cause of increased RNAase II activity in strain N4752 when it is grown at 43° seems to be the lack of an appropriate technique for purification of RNAase II. It is clear, however, that among the known ribonucleases only RNAase II is affected in strain N4752 at the elevated temperature.

Table 2

RNAase activities in cell extracts of strains 112-130 and N4752.

Strain	RNAase II (cpm)	RNAase III (cpm)	PNPase (cpm)
112-130	1250	3500	3660
N4752	5550	4400	3290

Strains 112-130 and N4752 were grown at 30°, transferred to 43° and harvested after growth for 60 min at the elevated temperature. Cell extracts (S-12) were prepared by sonication and were DNAase treated.

The assay mixture for RNAase II contained, per ml: 400 μg of S-12 protein; 20 μg (192,400 cpm) [^{3}H]polyadenylic acid, 1 mM magnesium acetate, and 10 mM potassium chloride. The reaction mixtures (50 μl) were incubated at 43° for 10 min. The reactions were stopped and the alcohol-soluble counts released determined.

The assay mixture for RNAase III [11] contained, per ml: 400 μg of S-12 protein; 20 μg [^{3}H]polycytidylic acid (192,400 cpm); 20 μg polyinosinic acid; Tris-HCl (pH 7.6; 10 mM); and sodium chloride (0.2 M). The reaction mixtures (50 μl) were incubated at 43° for 10 min, the reactions stopped by the addition of 0.1 ml carrier RNA and 1 ml of 66% alcohol, and the alcohol-soluble counts liberated were determined.

The assay mixtures for PNPase [12] activity contained, per ml, 360 μg of S-12 protein. The reaction mixtures (100 μl) were incubated at 37° for 30 min. All these reactions proceed linearly during the length of time tested here. The data represent the averages of duplicate determinations and are corrected for backgrounds, no extracts, 210, 230 and 250 cpm, RNAase II, III and PNPase, respectively. The data are expressed in cpm per 20 μg S-12 protein.

Acknowledgements

We are indebted to Dr. R. Krishna for helping us with the experiments in table 2 and to Dr. T. S. Ko for helping us with the identification of the reaction products. The work was supported by National Science Foundation grant, GB-34158.

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