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SELECTIVE RESISTANCE OF BACTERIAL POLYADENYLATE-CONTAINING RNA TO HYDROLYSIS BY GUANOSINE 3'-5'-MONOPHOSPHATE-SENSITIVE NUCLEASE OF BACILLUS BREVIS

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SUMMARY

Earlier experiments had shown that the degradation of newly synthesized RNA in permeable cells of *Bacillus brevis* is mediated primarily by a guanosine 3',5'-monophosphate-sensitive 3'-exonuclease [N. Sarkar and H. Paulus (1975) J. Biol. Chem. 250, 684-690]. More recently, we found that a substantial fraction of pulse-labeled RNA in *B. brevis* is polyadenylylated [N. Sarkar, D. Langley, and H. Paulus (1978) Biochemistry 17, 3468-3474], and it was thus of interest to examine the effect of polyadenylylation on the susceptibility of RNA to degradation by the 3'-exonuclease. Purified 3'-exonuclease from *B. brevis* hydrolyzed the unadenylylated fraction of pulse-labeled RNA from *B. brevis* much more rapidly than poly(A)-containing RNA. Similar results were obtained with the pulse-labeled unadenylylated and polyadenylylated RNA fractions from *Bacillus subtilis*. Control experiments showed that the differential hydrolysis of the labeled RNA preparations by 3'-exonuclease was not due to the presence of inhibitors or activators. These results suggest that the stability of mRNA in *Bacillus* species may be regulated by polyadenylylation.

INTRODUCTION

The degradation of newly synthesized RNA in toluene-treated cells of *Bacillus brevis* is primarily due to the action of an exonuclease whose activity is sensitive to inhibition by guanosine 3',5'-monophosphate (cGMP) (1, 2). The observation that this enzyme degrades RNA from the 3'-end and acts very slowly on poly(adenylic acid) (2) suggested that the stability of bacterial mRNA might be modulated by polyadenylylation. The exploration of this possibility led to the discovery of relatively high levels (30-40%) of poly(A)-containing molecules among the rapidly labeled RNA species of *B. brevis* (3). High levels of poly(A)-RNA were also found in *Escherichia*

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coli and *Bacillus subtilis* (4), both of which harbor 3'-exonucleases which resemble the enzyme from *B. brevis* in many respects (5, 6). In this communication, we compare the sensitivities of unadenylylated and polyadenylylated bacterial RNA to hydrolysis by the 3'-exonuclease from *B. brevis*.

MATERIALS AND METHODS

Materials

[2,8-^{3}H]adenosine (25.5 Ci/mmole) and [5-^{3}H]uridine (25 Ci/mmole) were obtained from New England Nuclear and oligo(dT)-cellulose (Type 7) from P-L Biochemicals. Proteinase K (Boehringer) [^{14}C]poly(A) and [^{3}H]poly(U) were made from [^{14}C]ADP (0.1 Ci/mmole and [^{3}H]UDP (0.2 Ci/mmole) respectively as described earlier (2). The sources of all other materials are as published earlier (3, 4).

Bacterial Strains and Growth

Bacillus brevis ATCC 8185 and *Bacillus subtilis* ATCC 6051 were grown in nutrient broth supplemented with salts and glucose (4).

Labeling of RNA and Fractionation

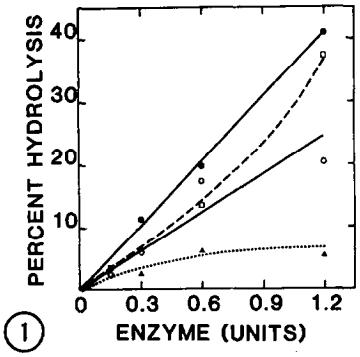
Two ml of exponentially growing cultures were treated with [2,8-^{3}H]adenosine or [5-^{3}H]uridine (20 µCi) for 30 sec and the labeled cells were processed by proteinase K method as described earlier except proteinase K was incubated for 20 min at 37° C (5mg/ml in 10 mM Tris-HCl, pH 7.5) just prior to use (4). Pulse-labeled RNA was fractionated into polyadenylylated and unadenylylated fractions by affinity chromatography on oligo(dT)-cellulose. Unadenylylated RNA was isolated from the unadsorbed fraction by precipitating 5 times with 2.5 volumes of ethanol in the presence of 10 mM $MgCl_2$. Poly(A)-RNA was isolated from oligo(dT)-cellulose bound fraction by repeated ethanol precipitation in the presence of 10 mM $MgCl_2$ without carrier. Labeled rRNA and tRNA were isolated from exponentially growing cultures of *Bacillus brevis* that had been incubated with [^{3}H]uridine for at least 3 generations as described (4).

Nuclease from *Bacillus brevis*

The cGMP-sensitive nuclease was purified from *Bacillus brevis* cells as described earlier (2). Ribonuclease assay was done by incubating labeled substrate in 0.1 M Tris-HCl, pH 7.1, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 10 mM 2-mercaptoethanol and enzyme and measuring the amount of alcohol-soluble product as detailed earlier (2). The assay was done using the conditions where degree of hydrolysis was a linear function of time. A unit of ribonuclease activity was defined as the amount of enzyme that produced 1 nmole of alcohol-soluble product per min from poly(U).

RESULTS AND DISCUSSION

The relative rates of hydrolysis of various polynucleotides by purified 3'-exonuclease from *B. brevis* are compared in Fig. 1. Polyuridylic acid was hydrolyzed considerably more rapidly than polyadenylic acid, whereas rRNA and tRNA were hydrolyzed at intermediate rates. However, since the susceptibility to exonucleolytic degradation is a function of the number of nucleic



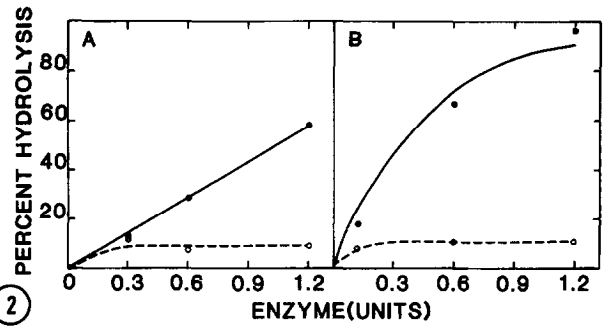


Figure 1: Rate of exonucleolytic degradation of labeled polyribonucleotides. Hydrolysis was measured using standard assay conditions as described in "Materials and Methods," at the enzyme levels indicated. Substrate input per assay was 0.45 mM for [^{3}H]poly(U) (•——•), 0.2 mM for [^{14}C]poly(A) (▲···▲), 0.17 mM for [^{3}H]tRNA (o——o) and 0.17 mM for [^{3}H]rRNA (□---□).

Figure 2: (A) Rate of exonucleolytic degradation of [^{3}H]adenosine-labeled unadenylylated RNA and [^{3}H]adenosine-labeled poly(A)-RNA, isolated from *Bacillus brevis* cells. Hydrolysis was measured using standard assay condition as described in "Materials and Methods" at the enzyme levels indicated. Substrate input per assay was 1500 cpm for [^{3}H]poly(A)-RNA (o---o) and 1400 cpm for [^{3}H]unadenylylated RNA (•——•).

(B) Rate of exonucleolytic degradation of [^{3}H]adenosine-labeled unadenylylated RNA and [^{3}H]adenosine-labeled poly(A) RNA isolated from *Bacillus subtilis* cells. Details are as in (A). Substrate input per assay was 1250 cpm for [^{3}H]poly(A)-RNA (o---o) and 1150 for [^{3}H]unadenylylated RNA (•——•).

acid chains, the relative activity of the nuclease with high molecular weight RNA species such as rRNA may be considerably greater than suggested by these results. One can thus conclude that polyadenylic acid is a relatively poor substrate of the 3'-exonuclease, not only in comparison with synthetic polynucleotides as shown earlier (2) but also in comparison with natural RNAs.

Exponentially growing cultures of *B. brevis* were pulse-labeled with [^{3}H]-adenosine and the RNA was fractionated into an unadenylylated and a polyadenylylated fraction by affinity chromatography on oligo(dT)-cellulose (3). As shown in Fig. 2A, the unadenylylated fraction was hydrolyzed considerably more rapidly than poly(A)-RNA. Similar results were obtained with the corresponding RNA fractions from *B. subtilis* (Fig. 2B), or with RNA fractions pulse-labeled with [^{3}H]uracil (data not shown). The extent of hydrolysis of

polyadenylylated RNA did not exceed 10% even at enzyme concentrations at which unadenylylated RNA was completely hydrolyzed. This behavior suggests that the limited hydrolysis observed may have been due to contaminating unadenylylated RNA in the poly(A)-RNA fraction. Indeed, we had observed earlier that about 15% of this fraction failed to be adsorbed when treated with oligo(dT)-cellulose a second time (3). It is thus possible that pure poly(A)-RNA may be essentially refractory to degradation by the 3'-exonuclease.

On the other hand, experiments of the type described have to be interpreted, with caution, since similar results could be produced by the presence of a nuclease inhibitor or the absence of an activator in the poly(A)-RNA fraction. Accordingly, two types of control experiments were carried out to eliminate such possibilities. One of these involved the simultaneous preparation of pulse-labeled and unlabeled RNA fractions so as to permit appropriate reconstitution experiments. As shown in Table I, the hydrolysis of neither ^{3}H-labeled unadenylylated RNA nor of ^{3}H-labeled poly(A)-RNA was significantly affected by the addition of equivalent amounts of the complementary fraction. In another type of experiment, strict additivity was observed when various mixtures of [^{3}H]polyuridylic acid, [^{3}H]poly(A)-RNA and [^{3}H]-unadenylylated RNA were subjected to hydrolysis by limiting amounts of 3'-exonuclease (Table II). These control experiments show that the differential sensitivities to exonuclease digestion of poly(A)-RNA and unadenylylated RNA are not due to the presence of inhibitors or activators in the various nucleic acid fractions.

Since the discovery of polyadenylylate sequences at the 3'-ends of eucaryotic mRNA (7-9), much attention has been devoted to their potential role in stabilizing mRNA. Experiments to test this possibility have mostly involved the microinjection into frog oocytes of mRNA fractions whose poly(A) content had been modified by *in vitro* treatment with appropriate enzymes. However, the results have been somewhat equivocal, some mRNAs (e.g. globin mRNA) being stabilized by polyadenylylation (10-12) while the stability of others (e.g.

TABLE I

Effect of Addition of Unlabeled RNA Fraction on the Hydrolysis of [^{3}H]Adenosine-labeled RNA by 3' Exonuclease

Radioactive RNA Fraction[a]		Unlabeled RNA Fraction[b]		3'-Exonuclease	Substrate Hydrolyzed[c]
Unadenylylated	Polyadenylylated	Unadenylylated	Polyadenylylated	(Units)	(cpm)
+	-	-	-	0.3	246
+	-	-	-	0.6	422
+	-	-	-	1.2	1110
+	-	-	+	0.3	172
+	-	-	+	0.6	380
+	-	-	+	1.2	1030
-	+	-	-	0.3	20
-	+	-	-	0.6	44
-	+	+	-	0.3	14
-	+	+	-	0.6	46

[a] [^{3}H]Unadenylylated RNA and [^{3}H]poly(A)-RNA were prepared from [^{3}H]adenosine-labeled *B. brevis*, described in "Materials and Methods" and nuclease digestion was done using standard condition. Input for [^{3}H]unadenylylated RNA was 2200 cpm and for [^{3}H]poly(A)-RNA was 630 cpm.

[b] Unlabeled RNA fractions were prepared using same methods as for labeled RNA and amount used was equivalent to the corresponding labeled counterpart.

[c] Alcohol-soluble radioactivity.

TABLE II

3'-Exonuclease Digestion of [^{3}H]Poly(U) in the Presence of [^{3}H]Unadenylylated RNA or [^{3}H]Poly(A)-RNA

[^{3}H]Poly(U) (cpm)	[^{3}H]Poly(A)-RNA (cpm)	[^{3}H]Unadenylylated RNA (cpm)	Substrate hydrolyzed (cpm)
10,000	-	-	1,220
-	1,246	-	140
10,000	1,246	-	1,324
10,000	-	-	1,070
-	-	1,500	1,018
10,000	-	1,500	2,200

Standard nuclease assay was done using 0.24 units of enzyme and either single or combination of labeled substrates as described in "Materials and Methods."

interferon, mengovirus, and α_{2u}-globin mRNAs) was unaffected (13, 14). The resulting uncertainty is due to the fact that the RNA fractions were subjected to *in vitro* modification and thus no longer represented physiological entities, in part to our ignorance of the enzymology of mRNA degradation in eucaryotes. In contrast, the interpretation of the results with bacterial mRNA described in this communication is much simpler. Firstly, we know from experiments with permeable cells of *B. brevis* that the degradation of rapidly labeled RNA is inhibited by cGMP (1, 2), thus implicating the cGMP-sensitive intracellular 3'-exonuclease as a major factor in mRNA degradation. Secondly, we find a nearly all-or-none difference in the sensitivities of total pulse-labeled poly(A)-RNA and unadenylylated RNA to hydrolysis by the 3'-exonuclease It is therefore clear that polyadenylylation of RNA to the extent that occurs naturally in the bacterial cell endows it with almost complete resistance to degradation by the bacterial exonuclease. One of the major functions of RNA polyadenylylation in bacterial cells may thus be the modulation of mRNA stability.

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