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ISOLATION AND CHARACTERIZATION OF POLYADENYLATED RNA SPECIES FROM SPORULATING CELLS OF BACILLUS SUBTILIS

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SUMMARY

The appearance of a population of polyadenylated RNA during sporulation of B. subtilis is reported. This poly(A) RNA was characterized by hybridization to [^{3}H]poly(U), by its ability to bind oligo(dT)-cellulose and by its resistance to ribonucleases A and T_1. Most of the poly(A) sequences are located at the 3' terminus of the RNA chains and are of about 160-180 nucleotides length. Little or no poly(A) RNA was found in vegetative cells or in stationary stage cells of an asporogenic mutant blocked at zero stage of sporulation process. These results strongly suggest that the polyadenylated RNA might play a role in the regulation of gene expression during sporulation.

INTRODUCTION

Evidence has been gathered during the last few years indicating the existence of poly(A) sequences in the RNA of prokaryotic cells including bacillus species (1,2,3,4,5,6). It was thus shown that 3 to 5 % of rapidly labeled RNA extracted from B. subtilis cells was bound to oligo(dT)-cellulose and that bound RNA stimulated the incorporation of radioactively labeled amino acids into an in vitro B. subtilis protein synthesizing system (5). Similarly pulse-labeled RNA containing polyadenylated sequences was isolated from exponentially growing cells of B. brevis (4). The size of these poly(A) tracts obtained after digestion by ribonucleases A and T_1 was about 60 nucleotides and were found to be mostly located near the 3' ends of the RNA chains. However a correlation between the appearance of poly(A) RNA species and sporulation was until now only observed in B. polymyxa (6).

In order to generalize the latter observation to other spore forming bacillii and to investigate the function of poly(A) RNA in the developmental process of sporulation we initiated a similar study with

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B. subtilis. The advantage of this organism is that it has been the subject of extensive genetic studies, providing thus a comprehensive map of the chromosomal location of genes affecting sporulation (7).

MATERIAL AND METHODS

Bacterial strains, growth and labeling. B. subtilis 168 M (trp C2) and its spo⁻ derivative 12 A, protease negative (8) were used. Cells were grown at 37°C in the Sterlini and Mandelstam resuspension medium (9). Pulse labeling of the RNA was carried out by treating 100 ml of culture with 200 µCi of [2,8^{3}H]adenosine (sp.act. 35 Ci/mmol) for 3 min of mid-log cells and for 30 min of stationary phase cells (three and six hours after the end of exponential growth, designated as t_3 and t_6 cells). The cultures were immediately chilled in ice, treated with NaN_3, KCl and chloramphenicol (final concentration 30 mM, 0,5 M and 100 µg/ml respectively) centrifuged for 5 min at 5000 xg and frozen in dry-ice ethanol.

Isolation of labeled RNA. The labeled cells were suspended in 2,5 ml of 10 mM Tris-HCl, pH 7,5 containing 10 mM KCl, 5 mM $MgCl_2$ followed by addition of 0,3 ml sodium dodecyl sulfate (10 % solution) and 0,1 ml of heparin (1 mg/ml solution), frozen in an Eaton pressure cell under CO_2 and disrupted (4000 lb/in^2). After thawing 2,5 ml of freshly distilled phenol (saturated with water) was added to the suspension, agitated vigorously for 5 min and the suspension was centrifuged in a sterile glass tube at 6000 xg for 5 min at 20°C. The aqueous layer was repeatedly extracted with phenol and the RNA was precipitated by addition of two volumes of 95 % ethanol in presence of 0,1 M NaCl and the solution was left overnight at -20°C. After centrifugation the precipitate was dissolved in 3,5 ml of 0,1 M Na-acetate buffer, pH 5,0 and was reprecipitated with ethanol. The precipitate was washed extensively with 70 % ethanol containing 10 mM Tris-HCl pH 7,5 and 10 mM NaCl followed by washing in 70 % ethanol then dissolved in 1 ml sterile distilled water and stored at -20°C.

Isolation of poly(A) RNA and poly(A) tracts. Poly(A) RNA species were separated from total RNA by adsorbing the labeled RNA on oligo(dT)-cellulose at high ionic strength and eluting the poly(A) RNA by water according to the procedure of Aviv and Leder (10). Poly(A) stretches of the RNA were separated by virtue of their resistance to ribonucleases A and T_1. The labeled RNA was treated with pancreatic RNase (200 µg/ml) and T_1 (100 u/ml) at 37°C for 2 hrs and the digested mixture was adsorbed on oligo(dT)-cellulose as outlined earlier.

Sucrose gradient centrifugation. Samples of purified RNA were dialysed against 10 mM acetate buffer, pH 8.5 containing 50 mM NaCl, layered onto a linear sucrose gradient (5-20 %) made up in the same buffer and centrifuged for 18 hrs at 76,000 g at 4°C. Fractions of 1,5 ml were collected in a recording fraction collector and the poly(A) sequences were detected by binding to [^{3}H]poly(U) filters according to Sala-Trepat et al. (11).

Tritium post-labeling of polyA RNA. The procedure of Randerath and Randerath (12) was used for tritium labeling of the 3' OH terminal. The vacuum dried samples of poly(A) RNA were dissolved in 50 µl of distilled water and pipetted into 25 µl of a 0,75 mM solution periodate in presence of 10 µl of 100 mM bicine-NaOH buffer, pH 8,3 and incubated for 3 hrs in the dark according to Chen and Roe (13). The tritium label was introduced into the 3'-terminus by the addition of 280 µCi of [^{3}H]$NaBH_4$ (24 mCi/ml) per assay and the incubation was continued for 3 hrs at 23°C in the dark. The excess of borohydride was decomposed by the addition of

acetic acid (final concentration 0,3 N). The samples were dried overnight under nitrogen and the residue was dissolved in 100 µl of high salt buffer containing 10 mM Tris-HCl, pH 7,5 500 mM NaCl and 2 mM EDTA, and precipitated twice by ethanol.

The terminally labeled poly(A) RNA was dissolved in a small volume of high salt buffer and samples were mixed with oligo(dT)-cellulose and left for 1 hr at 37°C. After centrifugation the pellet was washed extensively with high salt buffer until no counts were detected in the wash. The poly(A) RNA was eluted by distilled water and concentrated.

Polyacrylamide gel electrophoresis. Electrophoresis of poly(A) tracts was carried out on slab gels (15x17x0,1 mm) containing 10 % acrylamide, 7 M urea in Tris-borate buffer pH 8,3 as described by Donis-Keller et al. (14). The electrophoretic mobility of the poly(A) tracts was compared with that of synthetic poly(A) markers with different chain length runed in parallel chanels.

RESULTS

Appearance of poly(A) RNA. Pulse labeled RNA isolated from wild type cells of B. subtilis at their vegetative and sporulating stages revealed that during vegetative growth only about 0.6 % of the RNA was bound to the oligo(dT)-cellulose. However, the RNA isolated from the cells at t_3 and t_6 stages of sporulation contained respectively 12 % and 20,5 % poly(A) RNA species bound to oligo(dT)-cellulose (Table 1). This sharp increase of oligo(dT) binding RNA appears to be sporulation specific, since the RNA extracted from stationary phase cells of the asporogenic mutant 12 A contains no such RNA species (Table 1).

Table 1 : Binding of poly(A) containing RNA to oligo(dT)-cellulose

Strain	time of harvest	RNA samples (cpm)	Radioactivity bound to oligo(dT)-cellulose	%
168 M	vegetative	950,000	6,000	0,6
	t_3	675,000	81,000	12
	t_6	735,000	152,000	20,5
12 A	vegetative	755,000	$\pm$ 0	-
	t_3	868,000	$\pm$ 0	-
	t_6	900,000	$\pm$ 0	-

Cells were grown and labeled with [2.8 ^{3}H] adenosine as described in Material and Methods. The time of labeling was 3 min for vegetative cells and 30 min for sporulating or stationary phase cells (t_3 and t_6 cells) RNA was extracted at the end of each labeling, samples of RNA were adsorbed on oligo(dT)-cellulose at high salt concentration and eluted by water.

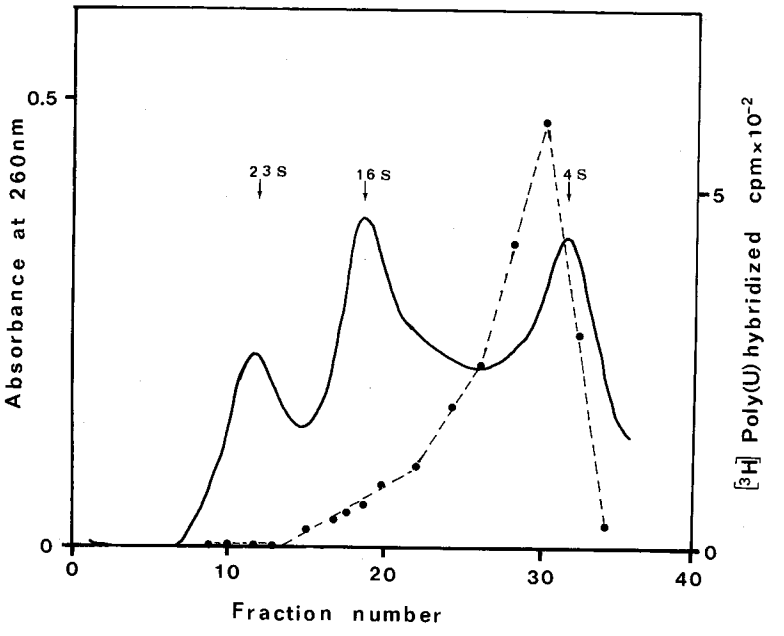

Fig. 1 : Sucrose gradient centrifugation profile of [^{3}H]adenosine labelled RNA isolated from t_3 sporulating cells. For conditions see Material and Methods.

The poly(A) RNA from sporulating cells was further analysed by sucrose gradient centrifugation followed by hybridization of consecutive fractions with [^{3}H]poly(U). It can be seen in Fig. 1 that the poly A RNAs sediment with a peak at 6 S with a broad shoulder, reflecting a heterogenous poly(A) RNA populations of larger sizes up to about 13-14s.

Location of the poly(A) sequences in the RNA chains as determined by post-labeling of the 3'OH terminal with [^{3}H] Na-borohydride.

The procedure involves oxidation of the ribose moiety with sodium metaperiodate and reduction of the resulting dialdehyde with tritiated borohydride (12). By combining this procedure with the action of RNases A and T_1 and alkaline phosphatase we have been able to determine the relative amounts of the poly(A) stretches located at the 3' terminus and those located inside the RNA chains. The results obtained are summarized in table 2 (for details of the procedure, see Material and Methods and legend of this table).

Table 2 : Localisation of poly(A) tracts in the RNA chains determined by 3' terminal post-labeling by [^{3}H] Na-borohydride.

Time of harvest	RNA samples (cpm) (a)	(b)	% (c)	Radioactivity bound to oligo(dT)-cellulose after treatment by RNase A + T1 + Alkp (d)	% (e)	(e-c) %
vegetative	343,000	7,900	2.3	12,048	3.5	1.2
t_3	180,000	32,860	18.2	63,016	35.0	16.8
t_6	170,000	44,078	25.9	65,320	38.4	12.8

RNA was extracted from cells harvested at vegetative growth, t_3 and t_6 and samples of 1.2 mg, 0.66 mg and 0.5 mg respectively, were treated with [^{3}H] Na-borohydride as described in Material and Methods. The terminally labeled samples derived from vegetative, t_3 and t_6 cells were dissolved in 200 µl of high salt buffer and aliquots of 50 µl containing respectively 343,000, 180,000 and 170,000 TCA precipitable counts/min were diluted to 400 µl in the same buffer and mixed with 60 mg of oligo(dT)-cellulose and further treated as described in Material and Methods. To each sample 10 mM Tris-HCl pH 7,5 plus 0.5 M NaCl (final concentration) was added and incubated 2 hrs at 37° in presence of pancreatic RNase (200 µg/ml) and RNase T_1 (100 u/ml). After addition of alkaline phosphatase (8 µg/ml) the incubation was continued for another 1 hr. The poly(A) tracts were again adsorbed on an oligo(dT)-cellulose and eluted under conditions described. The samples were lyophylised, redissolved in 50 µl of water and treated by [^{3}H] Na-borohydride as above. The dried poly(A) samples were dissolved in 100 µl of high salt buffer and precipitated by ethanol in presence of 30 µg of tRNA as carrier. The precipitate was washed by 70 % ethanol containing 10 mM Tris HCl pH 7,5 and 10 mM NaCl until no more counts left in the wash. The final precipitate, dissolved in Tris-borate buffer, pH 8.3 containing 1 mM EDTA was used for determination of the radioactivity (a) and for electrophoresis of the poly(A) tracts (see fig. 2).

Samples of total RNA were submitted to treatment of [^{3}H] Na-borohydride (a) followed by adsorption on oligo(dT)-cellulose. The radioactivity bound measures thus the amount of the poly(A) tracts located at 3' terminal (b and c). After treatment by ribonucleases A and T_1 only the poly(A) sequences which are resistant to these enzymes remain intact. The rationale for the further treatment by alkaline phosphatase is to remove the phosphate groups from the internal poly(A) stretches freed by the RNases action, making these sequences also accessible to [^{3}H]-borohydride treatment (d). Thus the increase in the radioactivity bound to oligo(dT)-cellulose observed afterwards (e) corresponds to the internal poly(A) sequences expressed as percentage of total present in the poly(A) RNA.

Size of poly(A) sequences. The length of the poly(A) sequences was estimated by polyacrylamide gel electrophoresis after digestion of the

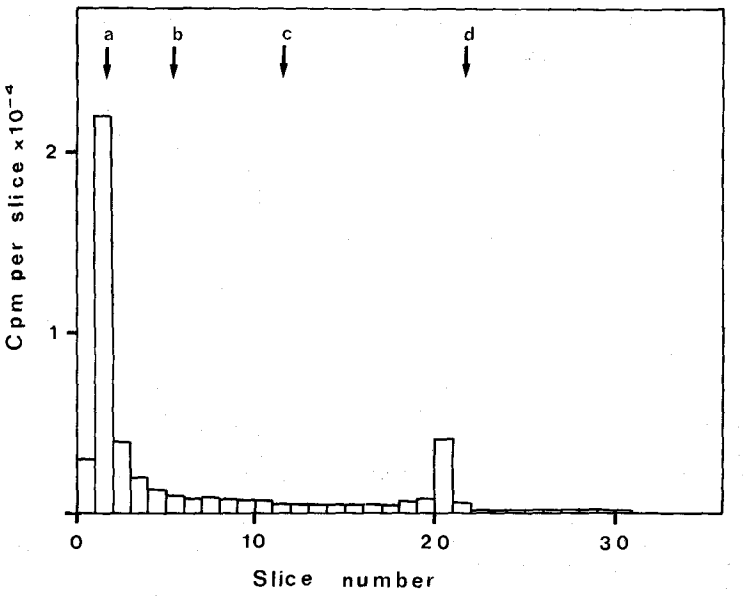

Fig. 2 : Polyacrylamide gel electrophoresis of poly(A) tracts isolated from t_6 sporulating cells after treatment of RNases A and T_1 and post-labeling by [^{3}H]-borohydride. Samples of 60 µl (35.000 cpm) in 30 % glycerol containing traces of bromophenol blue were submitted to electrophoresis for 15 hrs at 75 v. Slices of 5 mm each were mixed with 3 % protosol (MEN) in toluene-omnifluor (v/v) and incubated for 15 hrs at 37°C under shaking. After cooling the samples at 4°C the radioactivity was measured in an Intertechnique scintillation counter. The arrows indicate the position of the poly(A) markers added to each sample before electrophoresis a, b, c, d correspond respectively to 160, 90, 33 and 16 nucleotides length.

poly(A) RNA by ribonucleases A and T_1. The [^{3}H] poly(A) sample was mixed with synthetic poly(A) marker(Miles) containing an heterogenous population with a maximum length of 160 nucleotides. The poly(A) markers (marked by arrows on the figure)used are described in the legend of fig. 2. From the electrophoretic mobility of the radioactively labeled poly(A) sample we concluded that the maximum length is in the range of 160-180 nucleotides.

DISCUSSION

The results reported here show the appearance, during sporulation of *B. subtilis* of a population of polyadenylated RNA, characterized by hybridization to [^{3}H]poly(U), its ability to bind to oligo(dT)-cellulose and its resistance to RNases A and T_1. That this is a sporulation specific event is evidenced by the fact that no poly(A)-RNA was found in vegetative

cells or in stationary stage cells of the asporogenic mutant of B. subtilis 12A blocked at stage zero of sporulation process.

By labeling the cultures with [^{3}H]-adenosine or by post-labeling the RNA with [^{3}H] Na-borohydride we have shown that about 12 and 26 % of the total RNA present respectively in t_3 and t_6 cells are polyadenylated and this corresponds to a 15-20 fold increase compared to that observed in vegetative cells. We have observed that this increase is highly dependent on the growth medium of the culture. The results reported here have been obtained by growing the cells in Sterlini-Mandelstam sporulation medium, whereas growing the cells in nutrient broth (Difco) the increase in poly(A) RNA content during sporulation was only about 2-3 fold (results not reported here).

We have further shown that about 66 % of the total poly(A) sequences are localised at the 3' terminus of the RNA chains whereas about 34 % are in the internal region of the RNA chains. The size of the terminal poly(A) segments, estimated by polyacrylamide gel electrophoresis with RNA samples either post-labeled by [^{3}H] Na-borohydride or pulse labeled with [^{3}H]-adenosine followed by digestion with RNases A and T_1 revealed that about 75-80 % of the label was migrating with the synthetic poly(A) marker of 160-180 nucleotides length. Only 20-25 % are of about 10-15 nucleotides length and probably not retained on oligo(dT)-cellulose.

Although at present little is known about the synthesis, turnover and function of poly(A) RNA in prokaryotic cells the drastic increase of polyadenylated RNA which appears to be specifically associated with sporulating cells, might have an interesting future for a better understanding of the regulation of gene expression during spore development.

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