# CODING CAPACITY OF THE TRANSCRIPTION PRODUCTS SYNTHESIZED IN VITRO BY THE RNA POLYMERASES FROM BACILLUS THURINGIENSIS

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Received April 1,1976

SUMMARY : RNA transcripts synthesized in vitro from B. thuringiensis DNA by the vegetative RNA polymerase and by the form I and the form II of the sporulation enzyme were shown to contain messenger activities in a cell-free system from E. coli. The MW distribution of the resulting polypeptides was determined by SDS-gel electrophoresis.

Results based on hybridization-competition experiments suggested that the mRNA coding for the parasporal crystal, a specific sporulation protein, might be transcribed preferentially by the form II of the sporulation enzyme.

It has been previously shown that a sequential modification of the RNA polymerase subunits ($\beta$, $\sigma$, $\alpha$) occurred throughout the growth cycle of B. thuringiensis (1, 2). The vegetative enzyme contains the characteristic subunits of RNA polymerase : $\beta'$, $\beta$, $\sigma$, $\alpha 2$, whereas two distinct forms of the enzyme were separated from the sporulating cells at $t_5$ : a form I with a structure $\beta$, $\beta'_{modified}$, $\alpha 2$ is probably present in the prespore compartment while a form II with a structure $\beta'$, $\beta$, $\alpha 2$ is located in the mother cell. Changes in the template specificity were also demonstrated accompanying the structural modification from the vegetative to the sporulation polymerases.

In this paper we describe the properties of the transcription products synthesized in vitro with B. thuringiensis DNA as a template by the different forms of RNA polymerase. By using a cell-free system from E. coli, it is shown that the in vitro transcripts possess messenger activities.

Previous data have also indicated the existence of a class of

Abbreviations : 1xSSC, 0.15 M sodium chloride, 0.015 M trisodium citrate. SDS, sodium dodecylsulfate.

relatively stable mRNAs during the sporulation phase of B. thuringiensis. Furthermore, the mRNA coding for the parasporal inclusion was shown to belong to the long-lived species (3, 4). It was therefore worth to look for the presence of such a specific sporulation mRNA among the in vitro transcription products.

Hybridization-competition experiments presented here suggested that the crystal mRNA is transcribed by the form II of the sporulation RNA polymerase.

MATERIALS AND METHODS : The bacterial strain used was B. thuringiensis Berliner 1715. Media and culture conditions were described elsewhere (5).

The extraction of high MW DNA from vegetative cells and the purification procedure of DNA-dependent RNA polymerases from vegetative and sporulating cells at $t_5$ were previously reported (1).

RNAs were purified from vegetative and sporulating cells at $t_5$ as already mentioned (3). Preparation of the stable mRNA enriched fraction was carried out after treatment of the cells at $t_5$ with rifampicin (50 µg/ml) for 10 min at 30°C (4).

In vitro synthesis of RNA by the different forms of RNA polymerase were performed as follows : 40 µg of B. thuringiensis DNA were incubated at 37°C during 40 min with 40 µg of pure RNA polymerase in 1 ml of an incubation mixture as previously reported(1)but containing the four labeled nucleotides with a total specific radioactivity of 100 µCi/µmole. The reaction was stopped by the action of RNase-free DNase (10 µg/ml) at 0°C during 20 min. RNAs were obtained through a phenolic extraction followed by an ethanolic precipitation. After dialysis during 20 hours against 2xSSC, the resulting RNAs were separated by centrifugation in 5-20 % sucrose gradients (4.8 ml in 10 mM Tris-HCl pH 7.2 - 50mM KCl buffer) for 4 h 30 at 36,000 RPM in a Beckman SW 50 L rotor at 4°C. Fractions of the gradients were diluted to 1 ml with water and the concentration of RNAs was estimated at 260 nm. Fractions were pooled as indicated in fig. 1 (rectangles), dialyzed overnignt against distilled water, lyophilized and used for in vitro translation.

Hybridization-competition was realized according to Gillespie and Spiegelman (6), on nitrocellulose membrane filters (Schleicher and Schull, 13 mm ϕ, 0.45 µ pore size) loaded with 5 µg of denatured B. thuringiensis DNA, at 66°C during 20 h in 6xSSC. Details will be given elsewhere (9).

The preparation of the cell-free system protein synthesizing system from E. coli MRE 600 and the incorporation of (2-3 $^3$H)L-valine (42 Ci/mmole, C.E.A. France) were carried out as mentioned before (4). At the end of the reaction the standard volume (0.1 ml) was incubated at 37°C for 20 min in the presence of RNase (10 µg/ml) and 10 mM unlabeled L-valine. The mixture was brought to 0.5 ml with 50 mM Tris-HCl pH 7.8 and dialyzed overnight against the same buffer.

Electrophoresis on SDS-polyacrylamide gels was done in tubes 10 cm long with 5 % monomer acrylamide in 0.1 M sodium phosphate buffer pH 7.0 containing 0.1 % SDS. Migration was performed at 8 mA per tube at room temperature for 150 min. Gels containing radioactive material were sliced

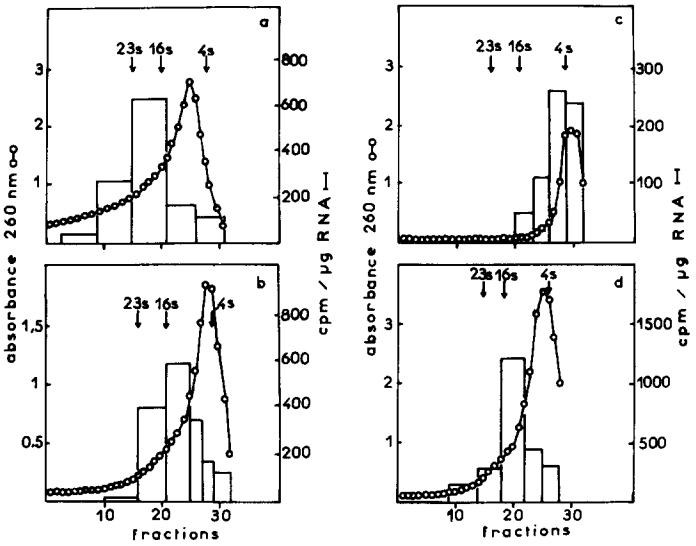


Figure 1 : Sedimentation profiles and specific coding activities of RNAs synthesized in vitro by RNA polymerases from B. thuringiensis.

Products of the vegetative enzyme (a) ; products of the core enzyme (b); products of the sporulation enzyme form I (c) ; products of the sporulation enzyme form II (d). For details see Materials and Methods.

Arrows indicate the location of RNA markers. The coding activity is given as radioactivity of ($^3$H)L-valine incorporated into hot-$CCl_3COOH$-precipitable material per µg of RNA input.

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into 2 mm sections, incubated overnight at room temperature in the presence of 0.3 ml Soluene 350 (Packard) and 10 ml of a toluene base scintillation solvent.Protein markers were run in parallel.

RESULTS : Figure 1 shows the sedimentation profiles in sucrose gradients of the transcription products synthesized in vitro by the different forms of RNA polymerase when DNA from B. thuringiensis was used as a template.

The holoenzyme extracted from vegetative cells (β', β , σ, α2) gave rise to products displaying a wide range of sedimentation constants, from less than 4 s to more than 23 s. Moreover, about 60 % of the material migrated in a region of the gradient corresponding to RNAs with a s value higher than 10 s (Fig. 1a).

The products of the core enzyme (β', β , α2) showed a sedimentation pattern slightly shifted to smaller s values (Fig. 1b) but closely similar to that obtained for the products of the form II of the sporulation polymerase (β', β , α2) (Fig. 1d).

Table 1 : Size distribution and maximal coding capacity of RNAs synthesized *in vitro* by RNA polymerases from *B. thuringiensis*.

| Products synthesized *in vitro* by | Percentage of RNA $\geq$ 10 s | Range of maximal coding capacity ( s values ) |
|---|---|---|
| vegetative enzyme | 60 | 14 - 23 |
| core enzyme | 40 | 10 - 16 |
| sporulation enzyme form I | < 20 | 4 - 7 |
| sporulation enzyme form II | 40 | 10 - 16 |

( Data obtained from figure 1)

In contrast, form I of the enzyme ($\beta$, $\beta'_{mod.}$, $\alpha 2$) synthesized essentially low MW RNAs (Fig. 1c).

The messenger activities of the various RNAs were then estimated. Fractions from the sucrose gradients were pooled and their coding capacity was expressed in a cell-free system from *E. coli* and followed by the incorporation of ($^3$H)L-valine into hot-TCA precipitable material. The specific activity (given as cpm per µg of RNA ) is also reported in Figure 1. A close correlation was established between the maximum of coding capacity and the relative amount of heavy RNAs (> 10 s) among the transcription products (Table 1).

The presence at zero time of chloramphenicol (100 µg/ml) or of ribonuclease (10 µg/ml) inhibited completely the incorporation of labeled valine into the acid-insoluble material, thus confirming the actual RNA dependency for the polypeptide synthesis (data not shown).

The MW of the proteins translated *in vitro* was estimated in each case by polyacrylamide gel electrophoresis in the presence of SDS. The labeled polypeptides were analyzed after extensive dialysis and components with MW less than 10,000 daltons were not recovered after this treatment. RNAs obtained with the holoenzyme from vegetative cells directed the

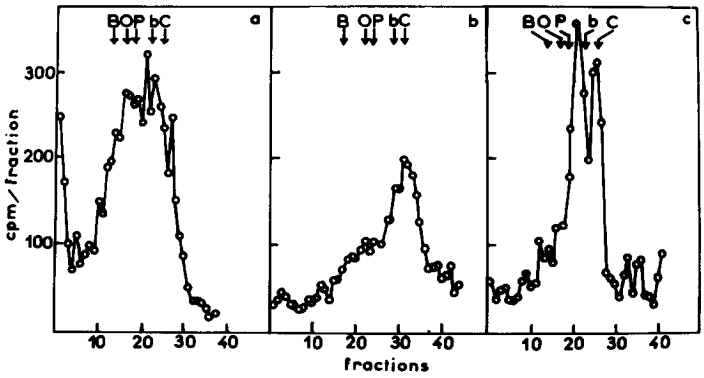


Figure 2 : SDS-gel electrophoresis of polypeptides translated in vitro with the transcription products of RNA polymerases from B. thuringiensis.

Transcription products synthesized by the vegetative enzyme (a), by the sporulation enzyme form I (b) and by the sporulation enzyme form II (c).

The MW estimated was based on the migration of the following protein markers (located by arrows) : B = bovine serum albumin ; O = ovalbumin ; P = pepsin ; C = cytochrome C (b = migration of the bromophenolblue).

synthesis of high MW polypeptides(region of the BSA marker, 65,000 daltons) as well as the synthesis of low MW products (region of the cytochrome C marker, 12,000 daltons)(Fig. 2a). RNAs synthesized with the form II of the enzyme led to a general smaller pattern, with one component of about 30,000 daltons and one located in the region of cytochrome C (Fig. 2c). The core enzyme issued from the vegetative polymerase gave rise to products presenting almost the same size distribution as that obtained with form II (not shown). With the form I products, one peak was essentially detected in the low MW region of the gel (Fig. 2b).

Specific antibodies were used to look for the presence of polypeptides related to the parasporal inclusion among the proteins synthesized in vitro. No significant immunoprecipitate was detected by employing antisera directed against the native crystal protein or against the peptides obtained after CNBr treatment (4). This failure may have been due to the fact that the crystal protein was not completely synthesized with the in vitro mRNA in the E. coli cell-free system, the subunit of the crystal having a MW of about 80,000 daltons (7).

Table 2 : Competition of the hybridization to B. thuringiensis DNA of *in vitro* ($^3H$) transcripts by unlabeled *in vivo* RNAs.

| | Percentage of competition *In vivo* competitor RNA | | | |
|---|---|---|---|---|
| In vitro ($^3H$) RNA synthesized with | vegetative RNA | sporulation ($t_5$) RNA | "stable" fraction of $t_5$ RNA | ribosomal RNA |
| vegetative enzyme | 45 | 19 | 12 | 18 |
| sporulation enzyme form II | 25 | 35 - 40 | 40 | 5 - 10 |
| sporulation enzyme form I | < 10 | 25 | < 10 | 0 |

The incubation mixture contained : 15 µg of ($^3H$) *in vitro* RNA (specific radioactivity, 18,000 cpm/µg) in a final volume of 1 ml. Increasing amounts of unlabeled *in vivo* competitor RNA (until 300 µg) were added to the mixture. Results were reported in percentage of competition given by the higher concentration of competitor RNA used.

Another method based on the hybridization-competition technique was therefore carried out to reveal eventually the existence of the crystal mRNA among the transcription products described above. Different fractions of total RNAs extracted from cells in the vegetative phase and during the sporulation phase at $t_5$ were used as competitors for the *in vitro* products synthesized by the RNA polymerases(Table 2). The competition effect was also estimated with the purified ribosomal RNA. Preliminary results were already reported (8) and details will be given elsewhere (9). Hybridization to *B. thuringiensis* DNA of *in vitro* labeled RNAs was inhibited at the higher level by their homologous unlabeled RNAs, *i. e.* vegetative enzyme products by vegetative *in vivo* RNAs, form II and form I products by the sporulation *in vivo* RNAs.

Ribosomal RNA reacted as a good competitor for the vegetative enzyme products. The percentage of inhibition was found significantly higher than that reported by Hussey *et al.* for the holoenzyme extracted from vegetative cells of *B. subtilis* (10).

During the sporulation phase it appeared that form II of RNA polymerase synthesized preferentially the ribosomal species as compared to the form I enzyme.

A fraction of "stable" mRNAs recovered after treatment of the cells at $t_5$ with rifampicin and known to be enriched in the crystal mRNA (3) was also tested in the hybridization-competition experiment. This fraction competed at the higher level (about 40 %) with regards to labeled RNAs synthesized *in vitro* by form II of the sporulation enzyme. Therefore it is suggested that the crystal mRNA is transcribed *in vivo* by form II of the sporulation RNA polymerase.

DISCUSSION : RNAs synthesized *in vitro* by the different forms of RNA polymerase from *B. thuringiensis* with DNA from the same origin do not share all the same properties. The mean size distribution of the *in vitro* transcripts is in the following decreasing order : MW of the vegetative holoenzyme products > MW of the vegetative core enzyme products $\simeq$ MW of sporulation enzyme form II products > MW of the sporulation enzyme form I products. The different classes of transcripts contain messenger RNA sequences and the size distribution of their maximal coding activity follows rather closely the order mentioned above (Table 1).

It is generally believed that the template specificity of RNA polymerase is dependent upon the presence of $\sigma$ factor. Hussey *et al.* (10) have shown that the products synthesized *in vitro* by the sporulation enzyme from *B. subtilis* compete poorly with ribosomal RNA, suggesting that the loss of $\sigma$ factor was responsible for the turn-off of rRNA synthesis during sporulation. However our results indicated that form II of the sporulation enzyme still transcribes *in vitro* rRNA ; but its efficiency is lower compared to that of the vegetative holoenzyme : this observation is in good agreement with our previous data obtained from *in vivo* experiments which have shown a marked decrease in total RNA synthesis when cells are going from the vegetative phase to the sporulation phase (3). Furthermore it is unlikely that form I of the sporulation enzyme is involved in this process. It has also to be noted that both sporulation enzymes have lost their $\sigma$ factor. Nevertheless, the crystal mRNA which belongs to the specific

sporulation species, seems to be preferentially transcribed in vitro by the form II enzyme, as suggested by the data presented in Table 2. This result implies that the specificity of transcription lies at least for a part in the subunit composition of RNA polymerase.

This represents one of the few attempts to demonstrate the synthesis of a specific mRNA during sporulation in Bacillaceae and seems to be promising for the further study of the regulation mechanism involved in this process.

ACKNOWLEDGMENTS. This work was supported by grants from the CNRS (ATP Différenciation Cellulaire) and by the Commissariat à l'Energie Atomique.

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