# ARGUMENTS BASED ON HYBRIDIZATION-COMPETITION EXPERIMENTS IN FAVOR OF THE IN VITRO SYNTHESIS OF SPORULATION-SPECIFIC mRNAs BY THE RNA POLYMERASE OF B. THURINGIENSIS

André F. Klier and Marguerite-M. Lecadet. Institut de Biologie Moléculaire, C. N. R. S., 2, place Jussieu, 75.221 PARIS CEDEX 05 (France)

Received September 13,1976

SUMMARY : Qualitative differences between the RNA transcripts synthesized in-vitro by the vegetative or by the two sporulation RNA-polymerases of B. thuringiensis were demonstrated, using the hybridization-competition technique. On the other hand, the in-vitro RNA preparations obtained with the sporulation Form II enzyme, were shown to present large homologies with "stable" RNA fractions extracted from wild-type sporulating cells.

Comparison of these data with those obtained with in-vivo RNAs from two asporogenous mutants strongly suggests the involvement of this Form II in the synthesis of sporulation-specific RNAs, among them the mRNA coding for the parasporal protein.

The existence of two distinct enzymatic forms of RNA polymerase during the sporulation process in B. thuringiensis was clearly demonstrated (1) (2) (3). Form I and Form II of the sporulation enzyme differ in their subunit composition, but also with regard to their catalytical properties and their specificity of transcription. We have now to consider the nature of the RNA synthezised in-vitro by these enzymes with B. thuringiensis DNA as a template.

The coding capacity of these RNA transcripts in a cell-free system from E. coli was reported in a previous paper (4). The main problem consists to recognize sporulation mRNAs among the in-vitro transcription products. Such an attempt may be considered in B. thuringiensis on account of the existence of a well characterized sporulation-specific protein : the parasporal crystal. So, the question is now to distinguish which of the two enzymatic forms can preferentially transcribe the mRNA coding for such a sporulation protein. Two different approaches have been considered : the

first one consisted in the in-vitro translation of the RNA transcripts and the characterization of the resulting polypeptides (4) ; the second one took advantage of the hybridization-competition technique to compare the different RNA transcripts with in-vivo RNAs extracted from cells throughout the growth cycle.

The present paper deals with the results obtained from such experiments.

MATERIALS AND METHODS : The wild type strain was B. thuringiensis Berliner 1715 (Pasteur Institute Collection). Two asporogenous mutants were isolated after EMS mutagenesis of spores (3), through the selection procedure using vapours of chloroform (5). Mutant C3 was blocked at sporulation stage 0 and as a consequence shows the phenotype $Sp^-Cr^-$. Mutant E2 was characterized as a late mutant (end of stage III) with the phenotype $Sp^-Cr^{++}$ and it synthesizes several large crystals inside the bacterium. Media and culture conditions were described elsewhere (6).

The purification procedure of RNA polymerases from vegetative and sporulating cells at t5 and the extraction of high molecular weight DNA from vegetative cells were previously reported (1).

In-vitro RNAs were synthesized with the different forms of the enzyme and purified as previously described (4) : RNA preparations were used immediately after dialysis against 2xSSC.

Total RNAs were extracted from vegetative and sporulating cells according to Glatron and Rapoport (7). Preparation of the stable RNA enriched fractions was performed after treatment of the sporulating cells with rifampicin (50 µg/ml for 10 min. at 30°C) (8). Ribosomal RNA was extracted from purified ribosomes.

Hybridization experiments with in-vitro ($^3$H)RNAs were carried out according to Gillepsie and Spiegelman (9) using nitrocellulose membrane filters (Schleicher and Schull, 13 mm Ø 0.45 µ pore size), loaded with 4.5 µg of denatured B. thuringiensis DNA, at 66°C, during 20 h in 6xSSC. The actual amount of DNA retained on the filter represented about 72 % of the loaded DNA. For competition experiments increasing amounts of unlabelled RNA were added to the reaction mixture until a ratio of unlabelled RNA/labelled RNA = 20 was reached. The amounts of labelled RNA used in such experiments corresponded to the maximum of hybridization deduced from the saturation curves.

Treatment of the hybridization data according to the method of Bishop et al. (10) (11) gives the theoretical values for saturation and for maximum competition displacement at infinite RNA concentration. The symbols used to calculate the saturation values were defined as follows : $h$ = concentration of labelled RNA ( µg/ml) ; $r$ = hybridization ( µg of RNA hybridized/ µg of DNA) ; $r_s$ = RNA hybridized/ µg of DNA at infinite RNA concentration. Values of $r_s$ were obtained as the reciprocal of the slope of the equation : $h/r = h/r_s + K$.

In a similar manner we can treat the competition data and deduce the $p_c$ value, which is the displacement at infinite unlabelled RNA concentration, from the slope of the equation : $g/C = g/p + K'$, where $g$ = the

---

ABBREVIATIONS : 1xSSC, 0,15 M sodium chloride, 0,015 M trisodium citrate ; EMS, ethyl-methane-sulfonate.

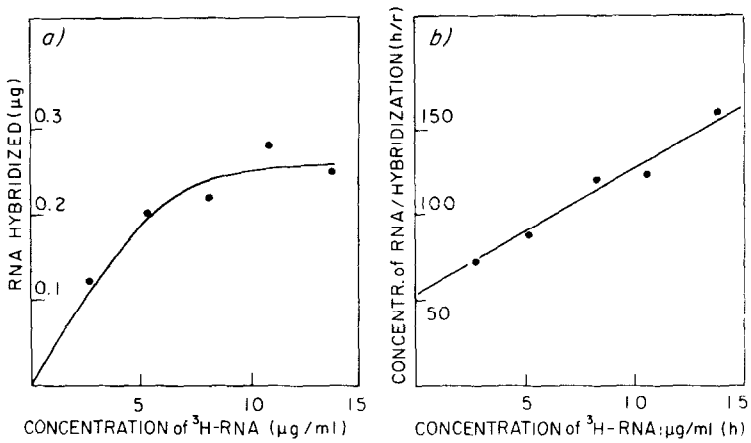


Figure 1 : Saturation of B. thuringiensis DNA with ($^3$H)RNA synthesized by vegetative RNA polymerase.

a) representation giving the amount of hybridized RNA as a function of increasing concentration of labelled RNA ( µ g/ml).
b) representation according to Bishop as defined in the Methods.

concentration of unlabelled RNA and C the competition as a proportion C = $c_0$ - c/$c_0$ ($c_0$ = cpm without competitor, c = cpm in the presence of competitor) (10). When g/C is plotted against g, the slope of the line gives 1/pc. In the results these pc values will be compared with the experimental values of maximum competition displacement (pmax) observed with the higher concentration of unlabelled RNA and given as percentage.

RESULTS : Fig. 1a shows a typical saturation curve obtained with in-vitro ($^3$H)RNA synthesized by vegetative holoenzyme ($\beta'\beta\sigma\alpha_2$), and Fig. 1b corresponds to the same data resulting from the mathematical treatment. The same kind of experiment was performed with RNAs synthesized by sporulation Form I ($\beta\beta'_m\alpha_2$) and Form II ($\beta'\beta\alpha_2$). The resulting saturation values are reported in Table I. We can observe that such values are closely similar for the products of vegetative and sporulation RNA polymerases (the $r_s$ value for the Form I products not given in the table was 0.16). These values appear much lower than those (0.4 to 0.6) observed for the in-vivo RNAs in B. thuringiensis (unpublished results) or in B. subtilis (11) ; high $r_s$ values were also reported for the in-vitro RNA synthesized with systems using phage DNA and homologous RNA-polymerase (12). We must keep in mind that in our system transcription and hybridization were performed with total DNA.

Table I : Competition of the hybridization to B. thuringiensis DNA of in-vitro ($^3$H) transcripts by unlabelled in-vitro RNAs.

| IN-VITRO COMPETITOR RNA SYNTHESIZED WITH | IN-VITRO ($^3$H) RNA SYNTHESIZED WITH | | | |
|---|---|---|---|---|
| | VEGETATIVE ENZYME $r_s$ : 0,15 ± 0,02 | | SPORULATION FORM II $r_s$ : 0,14 ± 0,02 | |
| | pc (%) | pmax (%) | pc (%) | pmax (%) |
| Vegetative enzyme | 100 | 70 | 82 | 60 |
| Sp. Form I | 13 | 2 | 22 | 15 |
| Sp. Form II | 71 | 43 | 100 | 77 |
| Veg. Core-enzyme | - | - | 83 | 62 |

The incubation mixture contained : 15 µg of ($^3$H) in-vitro RNA (specific radioactivity 18000 cpm/ µg) in a final volume of 1 ml. $r_s$, pc and pmax (expressed as percentage) were defined in Material and Methods.

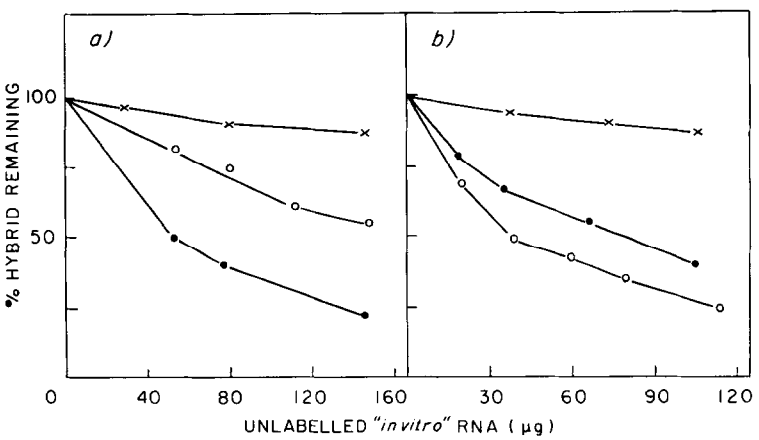


Figure 2 : Hybridization-competition experiments using in-vitro ($^3$H) RNA synthesized with : a) vegetative enzyme ; b) sporulation Form II enzyme.

15 µg of the in-vitro ($^3$H) RNA were competed by unlabelled in-vitro RNA synthesized with : ●—● vegetative enzyme ; x—x sporulation Form I ; o—o sporulation Form II.

Concerning the nature of the in-vitro RNA transcripts, results of hybridization competition experiments were given in Fig. 2a and 2b. We can deduce significative differences between the products of vegetative enzyme and sporulation Form II, thus indicating that both enzymes transcribe homologous but also different sequences on the template. The experimental and calculated values for maximum competition displacement were given in Table I. More precisely, these values indicate : (a) a displacement reaching 100 % at infinite RNA concentration (pc value) when the products of either vegetative enzyme or sporulation Form II were competed by homologous preparations (b) differences included between 15 and 30 % in the reciprocal competitions between the vegetative enzyme and sporulation Form II products (c) a poor competition of these RNA transcripts by the sporulation Form I products (d) small but non negligible differences between the RNA synthesized by the vegetative core enzyme and the Form II products, in spite of the fact that these two enzymatic forms present apparently the same subunit structure.

Taking into account such differences and the preliminary results previously reported (13) (4), we have compared the in-vitro RNA transcripts with total RNAs extracted from cells during vegetative phase and at different stages during sporulation. Results given in table II may be summarized as follows : (a) in-vitro products show large homologies preferentially with RNAs extracted at the stage of growth cycle when RNA polymerases were purified (b) vis-a-vis the Form I products the best in-vivo competitor was total RNA extracted at t5 (c) the products of the Form II were displaced at the higher level by "stable" RNA fractions prepared at t2 or t5, or by t12 RNA which contains initially very large amounts of "stable" species (Rapoport et al., unpublished data). As was previously shown (7) we know that the mRNA coding for the crystal protein belongs to the "stable" fraction. Therefore we may deduce from the results that sporulation Form II is synthesizing preferentially this mRNA.

In order to support such an assumption similar experiments were

Table II : Competition of the hybridization to B. thuringiensis DNA of in-vitro ($^3$H) RNA transcripts by unlabelled in-vivo RNAs.

| IN-VIVO | IN-VITRO ($^3$H) RNA SYNTHESIZED WITH | | | | | |
|---|---|---|---|---|---|---|
| COMPETITOR RNA | VEGETATIVE ENZYME | | Sp. FORM I | | Sp. FORM II | |
| | pc (%) | pmax (%) | pc (%) | pmax (%) | pc (%) | pmax (%) |
| Vegetative RNA | 51 | 47 | 12 | 10 | 21 | 18 |
| Sporulation RNA : | | | | | | |
| t5 RNA | 11 | 13 | 45 | 38 | 36 | 34 |
| t12 RNA | 24 | 16 | 34 | 23 | 100 | 57 |
| t2 "rif" RNA | 28 | 20 | | | 44 | 38 |
| t5 "rif" RNA | 22 | 21 | 28 | 20 | 48 | 36 |
| Ribosomal RNA | 23 | 18 | <10 | <10 | 11 | 7 |

Vegetative RNA-polymerase and the two distinct sporulation enzymes Form I and Form II were used as pure preparations resulting from the DNA-cellulose chromatography (1). t2, t5 and t12 RNA were extracted from sporulating cells respectively 2, 5 and 12 hours after the initiation of sporulation (t0). t2 "rif" and t5 "rif" RNA represented fractions enriched in "stable" mRNA, after treatment with rifampicin. For details, see Material and Methods and legend in Table I.

carried out with the Form II products, using competitor RNAs extracted from two asporogenous mutants. Fig. 3a shows the competitor effect produced by the "stable" fractions from wild type cells ; in Fig. 3b were reported results obtained with "stable" RNAs from t5 cells of the asporogenous mutant C3 ($Sp^-Cr^-$) and with t12 RNAs extracted from the late asporogenous mutant E2 ($Sp^-Cr^{++}$) which is known to synthesize very large amounts of parasporal protein ; these later species exhibit the most important inhibitor effect for the lower concentration of competitor RNA.

It should be noted that at t12 B. thuringiensis cells are just ending the sporulation process (6) while the crystalline inclusion continues

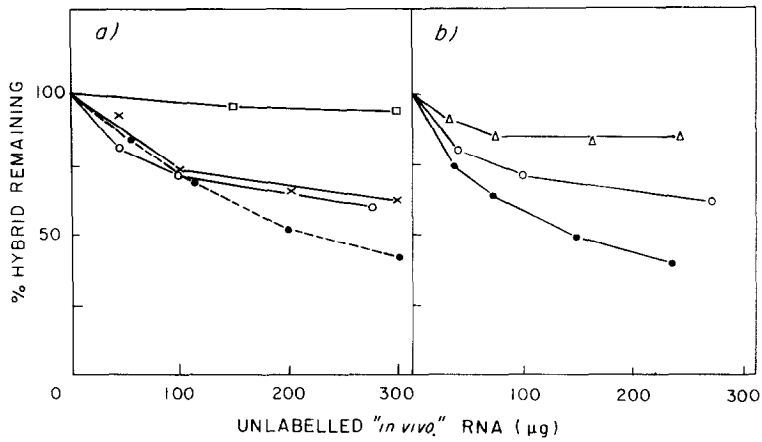


Figure 3 : Competition by in-vivo unlabelled RNA of the hybridization of in-vitro ($^3$H)RNA synthezised by sporulation Form II.

The unlabelled competitors used were : in a) :□—□ ribosomal RNA ; x—x t2 "rif" RNA ; o—o t5 "rif" RNA ; ●--● t12 RNA extracted from wild type sporulating cells ; in b) :Δ—Δ t5 "rif" RNA from the asporogenous mutant C3 ($Sp^-Cr^-$) ; o—o t5 "rif" RNA from wild type strain ; ●—● t12 RNA from the asporogenous mutant E2 ($Sp^-Cr^{++}$). As in table II, t2 "rif" and t5 "rif" represent fractions enriched in "stable" mRNA.

to increase in size ; for the asporogenous mutant E2, crystals accumulate inside the cell from t4 to t13, while the spore coats are not layered onto the prespore. So we may suppose that in both cases a large part of the residual RNA synthesis corresponds to the mRNA for crystal protein.

DISCUSSION : All results reported in that paper clearly demonstrate differences in the transcriptional properties of the sporulation polymerases, specially with regard to the nature of the RNA transcripts synthesized from B. thuringiensis DNA.

One of the important observations consists in the fact that sporulation RNA polymerases synthesize RNA species which exhibit homologies with RNA extracted from the cells during the differentiation process, while vegetative enzyme synthesized preferentially vegetative sequences (in the latter case our results agree with those reported by Pero et al. (14) concerning the in-vitro RNA synthesized by vegetative polymerase).

The second important and more interesting result was related to the preferential synthesis of "stable" mRNA by the sporulation Form II. As a consequence, we can reasonably assume that among such "stable" species, the mRNA for the parasporal crystal (and presumably mRNA for coat proteins) can be synthesized by the Form II of the sporulation enzyme.

In addition, it seems that Form I might transcribe other specific sporulation sequences ; but in this case we do not possess any preliminary indication for an experimental approach.

At present we have not definitely characterized a sporulation-specific mRNA, however the present study provides an indirect evidence for the presence of such specific RNA among the products of the sporulation Form II enzyme.

ACKNOWLEDGMENTS : We are grateful to Professor R. DEDONDER, in whom laboratory this work was conducted, for advice and criticism and for helpful discussions. We wish to thank Mr J. RIBIER for his participation to the isolation and characterization of the mutants. This work was supported by grants from the C. N. R. S. (A. T. P. Differenciation Cellulaire) and by the Commissariat à l'Energie Atomique.

REFERENCES :

1. Klier, A.-F., Lecadet, M.-M. and Dedonder, R. (1973) Europ. J. Biochem., 36, 317-327.
2. Klier, A. and Lecadet, M.-M. (1974) Europ. J. Biochem., 47, 111-119.
3. Lecadet, M.-M., Klier, A.-F. and Ribier, J. (1974) Biochimie, 56, 1471-1479.
4. Rain-Guion, M.-C., Petit-Glatron, M.-F., Klier, A.-F., Lecadet M.-M. and Rapoport, G. (1976) Biochem. Biophys. Res. Comm., 70, 709-716.
5. Millet, J. and Ryter, A. (1972) Ann. Inst. Pasteur, 122, 395-406.
6. Lecadet, M.-M. and Dedonder, R. (1971) Europ. J. Biochem., 23, 282-294.
7. Glatron, M.-F. and Rapoport, G. (1972) Biochimie, 54, 1291-1301.
8. Petit-Glatron, M.-F. and Rapoport, G. (1976) Biochimie, 58, 119-129.
9. Gillepsie D. and Spiegelman, S. (1965) J. Molec. Biol., 12, 829-842.
10. Bishop, J.-O., Robertson, F.-W., Burns, J.-A. and Melli, M.-L. (1969) Biochem. J., 115, 361-370.
11. Bonamy, C., Manca de Nadra, M.-C. and Szulmajster J. (1976) Europ. J. Biochem., 63, 53-63.
12. Pero, J., Nelson, J. and Fox, T.-D. (1975) Proc. Nat. Acad. Sci. U. S. A. 72, 1589-1593.
13. Klier, A.-F. and Lecadet, M.-M. (1975) 10th Meeting F. E. B. S. Paris, Abstr. 288.
14. Pero, J., Nelson, J. and Losick, R. (1975) in Spore VI. American Society for Microbiology, p. 202-211.