IMMUNOLOGICAL STUDIES ON THE SIGMA SUBUNIT OF THE RNA POLYMERASE FROM VEGETATIVE AND SPORULATING CELLS OF *BACILLUS SUBTILIS*

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Received 1 October 1974

1. Introduction

The fate of σ subunit of the RNA polymerase in sporulating cells of *Bacillus subtilis* has been the subject of numerous studies leading to ambiguous results [1–3]. It was originally suggested by Sonenshein and Losick [4] that the vegetative RNA polymerase undergoes, during sporulation, structural modifications including alteration or replacement of the σ factor compound. Linn et al. [1] have further suggested the possibility that the loss of σ factor activity during sporulation may either be due to its inactivation or destruction or simply to its removal from the RNA polymerase. On the other hand, Maia et al [5] have demonstrated the presence of σ subunit in purified RNA polymerase isolated from dormant spores of *B. subtilis*, suggesting that σ subunit is also present in the sporulating cells.

Moreover, it was recently reported from this laboratory that the generally observed decrease in the transcribing capacity of ϕe DNA by sporulating RNA polymerase might be due not to loss of sigma factor but rather to an RNase and (or) to an inhibitory complex present in sporulating cells [6].

In the present paper we report the results from immunological studies which provide further evidence for the presence of an active sigma factor in the RNA polymerase of sporulating cells of *B. subtilis*.

2. Materials and methods

Highly purified *B. subtilis* vegetative RNA polymerase holoenzyme was prepared by phase partition chromatography according to Shorenstein et al. [7]. Core enzyme and sigma factor subunit were obtained by phosphocellulose column chromatography. Two passages through this column were necessary to obtain pure core enzyme as shown by polyacrylamide gel electrophoresis using 30 μg of protein. RNA polymerase activity was measured in conditions described previously [8]. Protein concentrations were estimated by the method of Lowry et al. [9] with cristalline bovine albumin as standard.

2.1. *Preparation of antisera*

Two rabbits were immunized for each holoenzyme and core enzyme using the following schedule: a first injection of 25 μg of enzyme into toepads was followed after 50 days by a second intramuscular injection of 12 μg. Ten days later another intramuscular injection of 25 μg of each enzyme preparation was administered. A subcutaneous injection of 10 μg was given ten days later. The volume of each injection was 0.5 ml saline solution mixed with an equal volume of Freunds' complete adjuvant. Rabbits were exsanguinated ten days after the last injection.

2.2. *Partial purification of antisera*

The antiserum fraction obtained by precipitation at 50% saturation with ammonium sulfate was resuspended (1 g/140 ml) in 0.3 M sodium phosphate buffer pH 7.3 and chromatographed on a DEAE-cellulose column previously equilibrated with the same buffer. The effluent containing IgG was collected, precipitated with an equal volume saturated ammonium sulfate neutralized to pH 7.3 and dialyzed against the following buffer (buffer A): Tris–HCl 10 mM; EDTA–Mg (Mg–titriplex) 0.5 mM; KCl 0.15 M; $MgCl_2$ 10 mM; pH 7.9.

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A serum from the same rabbit before immunization was treated in a similar manner.

2.3. *Core enzyme–Sepharose column*

The conjugate of core–enzyme with Sepharose was prepared as follows [10]: 4.5 g of washed Sepharose 4B (Pharmacia) were suspended in 15 ml water and mixed with 0.5 g CNBr. The pH of the suspension was adjusted to 11.0 and was kept at this pH for 8 min by the addition of 2 M NaOH. The suspension was then filtered on glass filter, washed three times with 100 ml cold water.

0.2 g of activated Sepharose was suspended in 0.8 ml of 0.1 M bicarbonate containing 2 mg of dialyzed core enzyme and the mixture stirred for 16 hr at 4°C. After this time the suspension was filtered, extensively washed with 0.1 M bicarbonate until the absorbance at 280 nm of the wash was less than 0.02.

2.4. *Preparation of serum anti-σ*

1.5 ml containing 1.1 mg of dialyzed serum-anti-holoenzyme purified through the DEAE-cellulose step was filtered over the Sepharose-core immunoadsorbent column. The column was washed with buffer A. The effluent and the wash, containing the antibodies anti-σ subunit were concentrated and dialyzed under vacuum.

3. Results and discussion

The present investigation was designed to assess the presence of an active sigma subunit in the RNA polymerase in sporulating cells and in stationary phase cells of a zero stage asporogenic mutant. Fig. 1 shows the immunodiffusion pattern between the 'anti-sigma serum', and cell extracts (ammonium sulfate fractions) prepared from *B. subtilis* 168 exponential cells, and sporulating cells (t_4). It can be seen that the anti-sigma serum makes a continuous precipitation line with these two different extracts. Identical precipitation lines were observed with the extracts from the asporogenic mutants (12A). This figure also shows the precipitation lines obtained between vegetative or stationary phase extracts and the anti-core serum. Here too it appears that the extracts assayed contain an identical core enzyme.

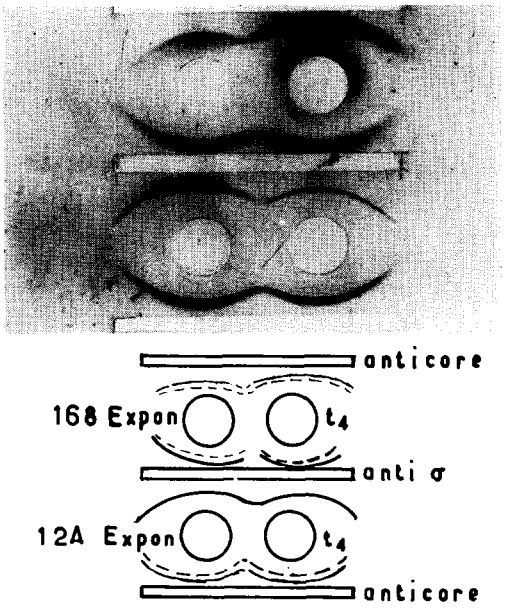

Fig. 1. Immunodiffusion patterns between anti-sigma serum or anti-core serum and extracts from *B.subtilis* 168 (spo$^+$) and 12A (spo$^-$) strains derived from exponential and stationary phase (t_4) cultures.

Fig. 2 was designed to demonstrate the specificity of the anti-sigma serum. For this it was tested against core enzyme and once more against vegetative and sporulating extracts. It can be seen that beside the sharp precipitation line formed between each of the two extracts and the anti-sigma serum, a second line of lower intensity was also observed. Similarly, there is a weak precipitation line between the anti-sigma serum and the core enzyme. This is to be expected since, as already observed by Zillig et al. [11], antisera against each of the purified subunits of the *E. coli* RNA polymerase form precipitation lines with all other subunits.

Fig. 3 shows the inactivation of RNA polymerase activity by increasing concentration of anti-sigma serum. The inactivation curves with RNA polymerase from vegetative cells or from sporulating t_4 cells appear to be very similar. This again shows the presence of an active sigma factor in the sporulating cells.

Table 1 shows that the stimulation of the transcription of φe DNA by purified sigma factor cata-

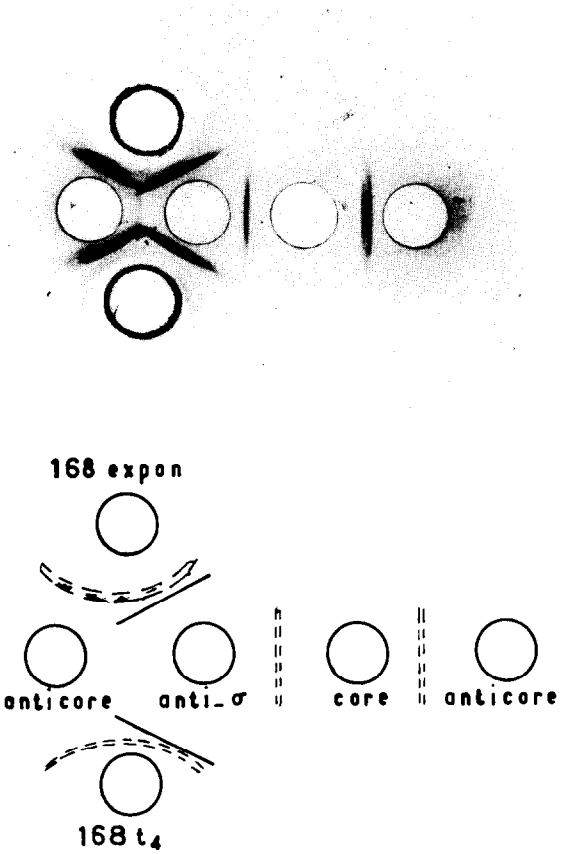

Fig. 2. Immunodiffusion patterns between: a) anti-sigma serum or anti-core serum and extracts from *B. subtilis* 168 (exponential and t_4); b) anti-core serum and core enzyme.

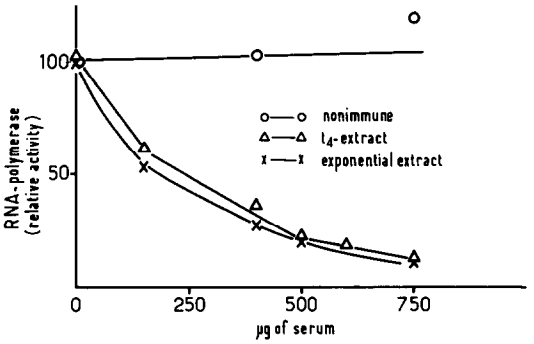

Fig. 3. Inactivation of RNA polymerase activity by anti-sigma serum. RNA polymerase activity was measured as described [8] using φe DNA as template and 60 µg of protein from each extract (ammonium sulfate fraction). The circles represent the RNA polymerase activity of either the exponential or t_4 extracts measured in the presence of non-immune serum.

Table 1
Inactivation of sigma stimulating activity by anti-sigma serum

DNA template	Enzyme	[^{3}H]AMP incorporated (µmole)
φe	Core	2
	Sigma	2
	Core + sigma	42
	Core + sigma + anti-sigma serum	7.3
poly-d(AT)	Core	16.8
	Core + sigma	26.2
	Core + sigma + anti-sigma serum	5.5

The RNA polymerase activity was measured in conditions described previously [8] with 1.3 µg core enzyme, 0.2 µg σ factor and 300 µg anti-σ serum, or serum from the rabbit before immunization.

lyzed by the core enzyme is abolished by the addition of the anti-sigma serum. It can also be seen that the slight stimulation by sigma factor of the transcription of poly-d(AT) [12] is similarly abolished by the anti-sigma serum.

The above results show unequivocally that an active sigma factor is present in the RNA polymerase of sporulating cells and that it is also associated to the enzyme in stationary phase cells of a zero stage asporogenic mutant of *B. subtilis*.

Acknowledgements

This work was supported by grants from the Commissariat à l'Energie Atomique, France and the Fondation pour la Recherche Médicale Française.

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