DNA-DEPENDENT RNA POLYMERASE FROM VEGETATIVE CELLS AND FROM SPORES OF *BACILLUS SUBTILIS*

IV. Subunit composition

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1. Introduction

The isolation of a sigma-like factor (σ_s) from *B. subtilis* vegetative RNA polymerase was previously reported from this laboratory [1]. This factor was found to restore the transcription of T_4 DNA template by the vegetative core enzyme, inactive with the same DNA. It was further shown that the 'core enzyme' derived from the dormant spore RNA polymerase retained the same activity with the three DNAs used as templates (thymus, T_4 and *B. subtilis*) as that observed with the total enzyme before treatment with phosphocellulose (PC). In this case no stimulation of activity was observed by the addition of the phosphocellulose eluate fraction which normally contains the stimulating factor. However, when the vegetative σ_s factor was added to the spore core polymerase a considerable stimulation of activity was observed with the above three DNA templates and particularly with the *B. subtilis* DNA. Several possibilities were then envisaged for explaining the behavior of the spore RNA polymerase: 1) destruction of the σ_s factor during the disruption of the spores; 2) partial inactivation of this factor by chromatography on PC, or 3) the absence of a dissociable factor in the spore polymerase. Such a factor could possibly be synthesized during early stages of germination. In order to test all these possibilities, the RNA polymerase from the two sources, vegetative cells and dormant spores, was highly purified and its subunit composition examined. In the present communication we report the results of these studies. We also show that in fact the RNA polymerase of the dormant spores apparently consists of the same major components as those present in the vegetative enzyme but that one of the β polypeptides seems to be altered. We also conclude that the failure of the spore sigma-like factor to stimulate the activity of the spore core enzyme, as the vegetative σ_s factor does, might be due to its inactivation by chromatography on PC*.

2. Materials and methods

RNA polymerase from exponentially growing cells of *B. subtilis*, Marburg strain 12A [3] was purified by two procedures. One procedure was essentially based on that described by Burgess et al. [4] using the following steps: ammonium sulphate fractionation DEAE-cellulose chromatography, agarose gel filtration and chromatography on PC. In the alternative method, the DEAE-cellulose step was followed by thymus DNA-cellulose chromatography and PC treatment. The peak fractions eluted from the PC column by 0.05 M KCl were further purified by glycerol gradient (5–30%) centrifugation. At the last stage of purification, before PC chromatography, the enzyme was 90–95%

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* Part of this work was reported at the Int. Symp. on Genetics of Industrial Microorganisms, Prague, 1970 [2].

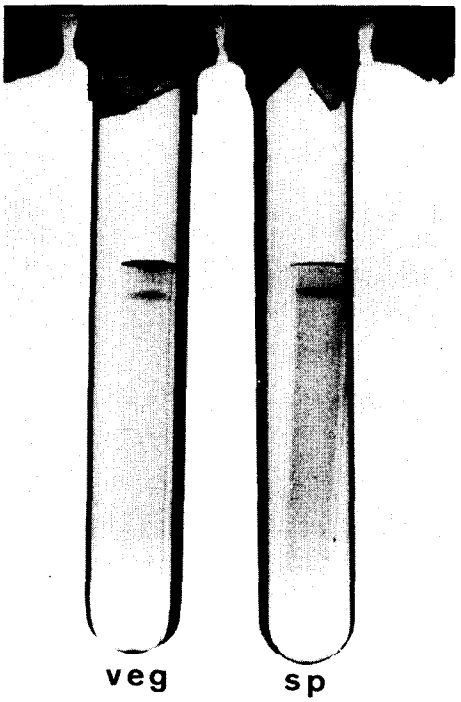

Fig. 1. Polyacrylamide gel electrophoresis of vegetative (left) and spore (right) RNA polymerase. The samples (15 μg) mixed with 5 μl of bromphenol blue were layered on 7.5% polyacrylamide gels according to Lee Huang and Warner [5]. The gels were stained with 0.2% Commassie brillant blue in methano–acetic acid–water (5:1:5) for 30 min. The gels were destained electrophoretically in 7.5% acetic acid.

pure as shown by electrophoresis on polyacrylamide gels and the yield was about 18–20%.

The RNA polymerase from cleaned dormant spores of *B. subtilis*, Marburg, 168 (Fallek Products Co., USA) was purified by ammonium sulphate fractionation, DEAE-cellulose chromatography followed by glycerol gradient centrifugation (10–30%). For some experiments, the peak fractions from the glycerol gradient containing the polymerase activity were pooled and chromatographed on PC. To inhibit proteolytic activity present in the spore extracts *p*-toluene sulphonyl fluoride (pTSF) (Aldrich Chem. Co.) was added throughout all the stages of purification. To analyse the subunit composition of the spore RNA polymerase at certain intermediary stages of purification, an aliquot of the enzyme was subjected to polyacrylamide gel electrophoresis and the single protein band containing the activity was separated from the rest of the gel. This gel fraction was immediately homogenized, frozen at −20° for 10–12 hr and then incubated in 0.04 M tris buffer, pH 7.8 for 3 hr at 0°. The supernatant, freed from the gel granules, containing the pure enzyme, was used for further tests.

Table 1
DNA template specificity in fresh and aged *B. subtilis* vegetative RNA polymerase.

	DNA template (units/mg)			
	Thymus (50)	T_4 (49)	*B. subtilis* (52)	PBS-1 (48)
Fresh				
Total enzyme	219	268	202	40
Core enzyme	271	53	182	–
σ_s	23	12	9	
Core enzyme + σ_s	320	325	171	
Aged				
Total enzyme	240	40	210	24
Core enzyme	132	62	36	12.5
σ_s	0	0	0	0
Core enzyme + σ_s	190	51	140	24

Purified RNA polymerase was chromatographed on phosphocellulose [4]. The aged enzyme was stored 45 days at 0° in 50% glycerol. Core enzyme designes the fraction of RNA polymerase eluted by 0.4M KCl from the PC column and σ_s designes the fraction eluted by 0.05 M KCl. The highest stimulation of activity was observed with 12 μg of protein of σ_s from fresh enzyme and with 38 μg of σ_s from aged enzyme. 1 unit of activity is the amount of enzyme which incorporated 0.1 nmole of ^{14}C-AMP in 20 min. The values in parentheses indicate the amounts in μg of DNA added to the standard incubation mixture. PBS-1 DNA was a gift from Dr. I. Takahashi.

The assay mixture for RNA polymerase contained (μmoles/0.5 ml): 20 tris-HCl, pH 7.8; 10 $MgCl_2$; 2 2-mercaptoethanol; 0.2 phosphate buffer; 25 KCl; 250 μg bovine serum albumin; 0.125 GTP, CTP and UTP; 125 ^{14}C-ATP (2500 cpm/nmole); DNA and enzyme as indicated.

3. Results and discussion

3.1. *Vegetative RNA polymerase*

Fig. 1 shows that at the last stage of purification, the vegetative RNA polymerase presents a single protein band on acrylamide gel electrophoresis. This peak

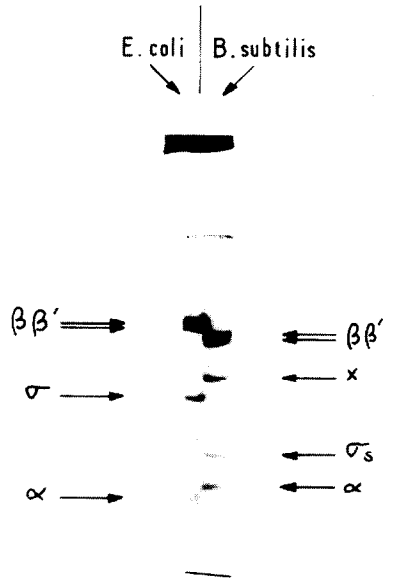

Fig. 2. Polyacrylamide gel electrophoresis on 0.1% SDS split gels of vegetative *B. subtilis* and *E. coli* RNA polymerase. Sample of 8 μg (*B. subtilis*) and 5 μg (*E. coli*) of enzyme were layered on 5% acrylamide gel containing 0.1% SDS and run at 8 mA per tube for 4 hr, stained for 1 hr and destained as described in fig. 1.

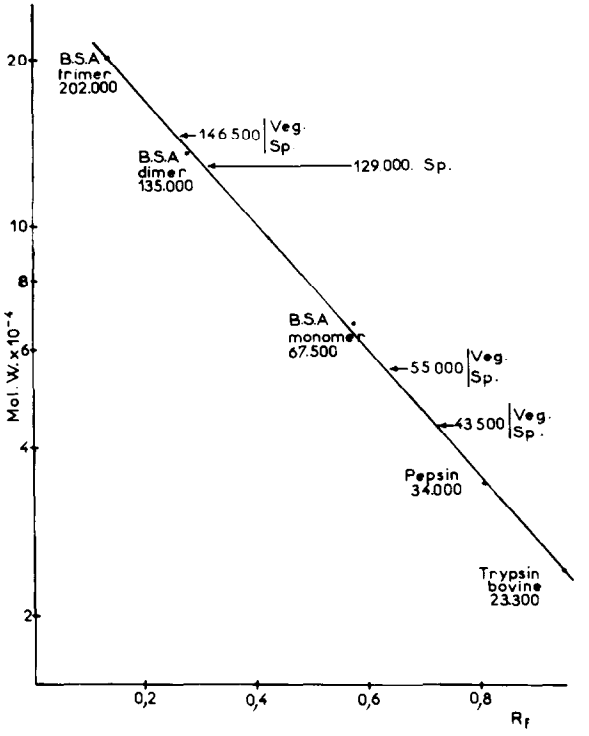

Fig. 3. Molecular weight estimation by SDS polyacrylamide gels. Molecular weights were determined on 5% polyacrylamide gels containing 0.1% SDS by the method of Shapiro et al. [6]. The mobilities of the markers of known molecular weights are shown in the figure.

coincides with the enzymatic activity tested with thymus, *B. subtilis* and T_4 DNA as template (table 1). By treatment of the *B. subtilis* vegetative RNA polymerase with sodium dodecylsulfate (SDS) followed by acrylamide gel electrophoresis in the presence of SDS, the dissociation of the enzyme into four subunits was obtained. In order to compare the number and size of these subunits to those found in the *E. coli* RNA polymerase [4], the two purified preparations were subjected to polyacrylamide split gel electrophoresis and the results are shown in fig. 2. The molecular weights of the subunits were determined on gel electrophoresis by the method of Shapiro et al. [6] using suitable markers, and their respective values were: 146,000; 94,000; 55,000 and 43,000 daltons (fig. 3). It can be seen (fig. 2) that the subunit composition of the two enzymes is similar but a net difference was observed on comparing their size with the *E. coli* RNA polymerase (a gift from Dr. A. Santenac). The difference resides in the presence in the *B. subtilis* RNA polymerase (fig. 2) of a protein band with a molecular weight of 55,000 daltons, which seems to replace the 96,000 dalton σ subunit in the *E. coli* enzyme. Using the nomenclature accepted for *E. coli* polymerase subunits, the protein bands will be designed as ββ′, X, σ_s and α, respectively. In the four polypeptides shown, about 90–95% of the total protein applied to the gel was recovered in the respective proportions: 52, 2–4, 22 and 17%. The molar ratio of the β to the α subunit was calculated from the gel scans (fig. 4) and was found to be equal to 1.0–1.1, in agreement with the values found for the *E. coli* [4] and the RNA polymerase from the *B. subtilis* strain 3610 [8]. By chromatography on phosphocellulose we found that the two polypeptides with the molecular weights of 94,000 and 55,000 daltons were eluted by 0.05 M KCl. They were further separated by glycerol gradient centrifugation (5–30%) and only the 55,000 dalton subunit was found to be active in stimulating the transcription of T_4 DNA by

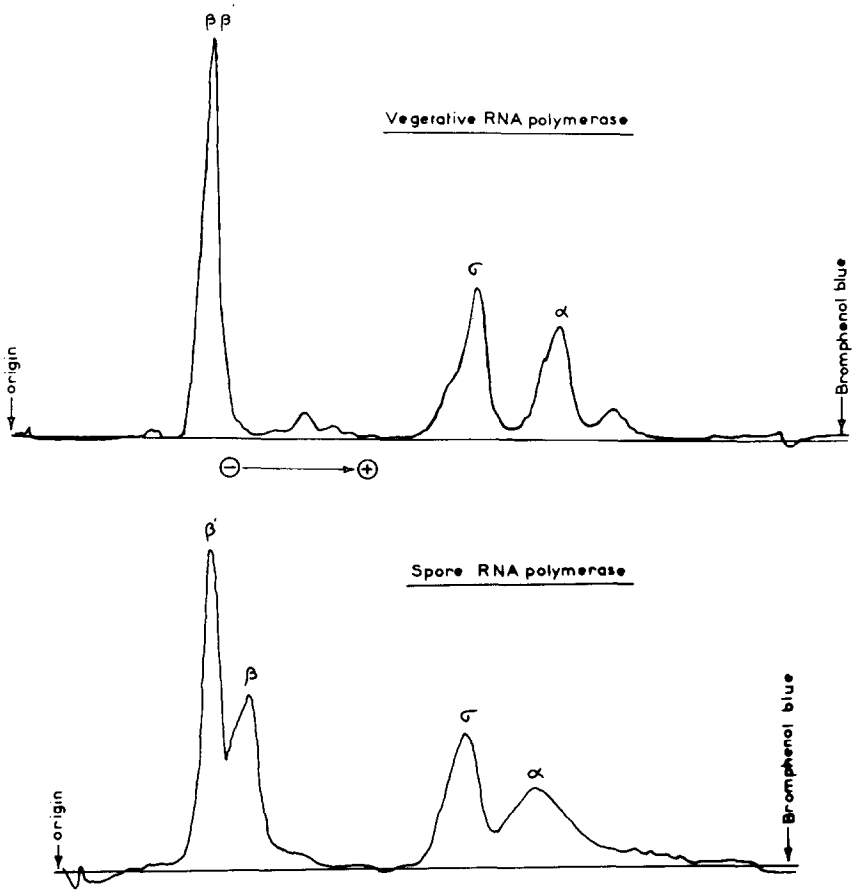

Fig. 4. Subunit composition of *B. subtilis* vegetative and spore RNA polymerase. Electrophoresis in 0.1% SDS polyacrylamide gels. The gels after destaining were scanned in a Gilford scanner at 515 nm.

the core enzyme (table 1). It thus appears that the stimulating factor associated with the *B. subtilis* RNA polymerase (σ_s) has a M.W. of 55,000 daltons and is about half the size of the *E. coli* sigma factor [4]. The role, if any, of the 94,000 dalton polypeptide always found in our preparations remains unknown.

Using an aged partially purified vegetative RNA polymerase of *B. subtilis*, we observed that the enzyme had lost the capacity to transcribe T_4 DNA, while it remained active with thymus or *B. subtilis* DNA as template. Furthermore, after chromatography on PC of this aged preparation, the core enzyme obtained also lost the activity with *B. subtilis* DNA (table 1). The addition of the σ_s factor released from the same aged preparation was able tc stimulate about 4–5 fold the transcription of *B. subtilis* DNA, but the enzyme remained inactive when T_4 DNA was used as template. These surprising results would suggest that in the aged enzyme the σ_s was modified in such a way that it is no longer able, after its release by PC, to reassociate in the proper complex required to promote the transcription of T_4 DNA. Moreover, the enzyme had now acquired a new specificity which enables it to regain the activity with *B. subtilis* DNA. It can also be assumed that in the aged enzyme a subtle alteration occurred in the β or α subunits. This altered core enzyme might have lost the capacity to transcribe *B. subtilis* DNA but is still able to form a new conformational complex with σ_s factor which could now direct the transcription of *B. subtilis* DNA but not of the T_4 DNA.

3.2. *Spore RNA polymerase*

The RNA polymerase from the dormant spores of *B. subtilis* was purified in the presence of an inhibitor of proteases (pTSF) and showed a single protein band on polyacrylamide gel electrophoresis (fig. 1, right).

The subunit structure of the spore polymerase was also analysed in SDS polyacrylamide gels and the electrophorogram is shown on fig. 4. It can be seen that with the exception of one polypeptide band, the spore RNA polymerase consists of subunits having the same electrophoretic mobilities as those found in the vegetative enzyme. The molecular weights of these four subunits were determined in parallel runs with the vegetative polymerase (fig. 3) and their values in daltons are: 146,000 (β'); 129,000; 55,000 (σ_s) and 43,000 (α). Thus the only difference in the molecular weights between these subunits and those obtained from the vegetative RNA polymerase resides in the 129,000 daltons polypeptide. The 94,000 daltons polypeptide always found in the vegetative enzyme has never been found in the spore polymerase. The molar ratio of the 43,000 daltons protein band to the 146,000 and to the 129,000 daltons polypeptides calculated from the electrophorograms was 1:0.5:0.47 (average from three experiments). Furthermore, on polyacrylamide gel (6.5%) electrophoresis of the vegetative polymerase, in 8 M urea, one of the β bands (probably β') remains on the top of the gel while the other moves into the gel (4 mm in 4 hr). With the spore RNA polymerase in the same conditions the migration (1 cm in 4 hr) of the 129,000 dalton band alone was observed, the other remaining on the top of the gel. In SDS polyacrylamide gel electrophoresis in the conditions used by Pringle [9] the β' band and the 129,000 dalton band of the spore RNA polymerase were 3 mm apart in a run of 3–4 hr. No separation of the $\beta\beta'$ bands of our vegetative RNA polymerase was observed in the same conditions even after 8 hr. It seems therefore that in the spore RNA polymerase about 50% of the β subunits present in the vegetative enzyme were degraded giving the 129,000 daltons polypeptide. Similar results, namely the replacement of one β subunit by a smaller polypeptide (M.W. = 110,000) during sporulation of *B. subtilis*, strain 3610 were reported by Losick et al. [8].

Chromatography on PC of the spore RNA polymerase followed by polyacrylamide electrophoresis of the eluates have shown that the fraction eluted by 0.4 M KCl contained, in addition to the core enzyme, a small amount of σ_s factor. Similarly, the fraction eluted by 0.05 M KCl contained, in addition to the σ_s factor a weak band corresponding to the β polypeptides. It thus appears that the spore RNA polymerase molecule has fewer positive charges available for its retention on PC than are present in the other polymerases tested. The fact that some σ_s factor still remains attached to the spore core polymerase after chromatography on PC explains our earlier results showing that this core enzyme is still active with T_4 DNA. On the other hand, since the activity of the spore core enzyme can be stimulated by the vegetative σ_s factor but not by the spore σ_s factor, this implies that the spore σ_s factor was completely inactivated during chromatography on PC.

It is noteworthy that when the spore RNA polymerase was isolated without being protected against proteolytic degradation by pTSF during the preparation, the pure enzyme still contained the same subunit species but the amount of the β polypeptides was decreased to about 20% of that observed when the inhibitor was present at all stages of purification. This means that a protease present in the dormant spores [10] might be activated during the disruption of the spores and be specific in degrading only the β polypeptides of the RNA polymerase.

In conclusion, the question now is to know if the modification observed in the spore polymerase reflects the physiological changes occurring in vivo as a result of the action of a specific protease during the sporulation process. This attractive hypothesis based mainly on the studies of Sadoff et al. [10] on the proteolytic conversion of vegetative cell aldolase to spore aldolase was also suggested for the modification of the RNA polymerase observed during sporulation [8]. The fact that during the process of spore development, as in the dormant spore itself, the only modification observed in the RNA polymerase of two different strains resides in one of the β polypeptides would argue that this is an in vivo process of biological importance. A structural modification in one of the subunits of 'T_4-enzyme' after infection of *E. coli* by T_4 was already reported by Walter et al. [11]. It may be a general feature of complex differentiating systems, like bacterial sporulation or phage infection, that, beside the changes in template specificity directed by specific sigma-like factors, a modulation in the different sub-

units of the core polymerase itself might occur. That such changes might also control the template specifity is suggested by the experiments with the aged RNA polymerase described here. This and other arguments lead to the obvious assumption that, in addition to the function of sigma-like factors, more complex modifications are involved in gene expression in developmental systems.

Acknowledgements

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