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Template specificity of transcription during sporulation of Bacillus subtilis

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ABSTRACT

Partially purified extracts from sporulating Bacillus subtilis cultures transcribed different natural DNAs with different efficiencies. This template specificity results in an increased or a decreased synthetic activity with respect to extracts from vegetative cells, depending on the template used. With SPP1 DNA a decrease in activity occurs, whereas with T7 DNA an increased activity was observed, which is due to a higher efficiency of initiation. This is not an intrinsic property of RNA polymerase, but is due to some fraction(s) which can be separated from the enzyme. Together with in vivo experiments on transcription and SPP1 phage production during sporulation, these results suggest a possible role of promoter recognition in sporulation.

INTRODUCTION

In recent years a number of papers have reported variations of biochemical properties of extracts from sporulating cells (for a recent collection of articles on this subjects, see ref. [1]). Particular emphasis has been put on transcription [2-7] and, more recently, translation[8], trying to find a biochemical basis for the dramatic alterations in the pattern of gene expression which lead to spore formation.

The main problem encountered in interpreting such results from in vitro systems, in terms of their possible role in the process of sporulation, is twofold. On the one hand, all reported changes in biochemical properties are of the negative type, i.e. loss of some kind of enzymatic activity; this makes difficult to rule out the possibility of artifacts in the extraction procedure, leading to extracellular degradation of the system studied[9]. On the other hand, in vivo transcription and translation studies also show a general decrease in synthetic activity, but until now no clear evidence has been provided that this is a primary event in sporulation and not just a

consequence of the steady state reached by the cells (no comparative study has been reported for the steady states of spore-forming cells in sporulating and non-sporulating media).

In this paper we report in vitro transcription studies in Bacillus subtilis, showing that the activity change, occurring in the transcription machinery at early times of sporulation, is template specific, and can result in a decrease or an increase of synthetic activity (with respect to vegetative extracts) depending on the template used. We also present experiments on the rate of RNA synthesis and on SPP1 phage production in sporulating cells. The results are discussed in terms of a possible alteration in the recognition of promoters in sporulating cells.

MATERIALS AND METHODS

Bacteria-In all experiments with B. subtilis, strain S.B.168 was used. For production of T4 and T7 phages, Escherichia coli B was used as the host. Bacteriophages - SPP1[10] stocks were prepared as described previously[11]. T4 and T7 bacteriophages were the kind gift of Dr. E.N. Brody. High titer T4 lysates were prepared by infecting cultures of E. coli B in M9 medium[12] at an early stage of exponential growth (A_{540}=0.1) with a moi of 0.1[13]. T7 lysates were prepared according to Studier[14]. Lysates were purified by the method of Yamamoto et al.[15]. After CsCl gradient the phages were dialyzed against 10mM Tris-HCl pH 7.5, 10mM $MgCl_2$ and decreasing concentrations (from 2M to 0.1M) of NaCl in order to limit osmotic shock (in the case of T4). The dialyzed suspensions were treated with DNase, centrifuged at low speed and stored at 4 to 4°.

Radiochemicals and chemicals - ^{14}C-Uridine (60 Ci/mole) was purchased from Amersham Radiochemical Center. ^{3}H-UTP (20 Ci/mmole) was obtained from New England Nuclear Co. Non radioactive triphosphates, pancreatic ribonuclease, bovine serum albumin and calf thymus DNA were from Sigma Chemical Co. Electrophoretically purified DNase was from Merck, T1 ribonuclease from Sanyo. Rifampin was a gift from Dr. G. Lancini (Lepetit).

Growth conditions - Schaeffer's nutrient broth[16] was used throught this work for B. subtilis growth and SPP1 infection. Cell concentration was measured in a Zeiss spectrophotometer at a wavelength of 540 nm; samples

were previously diluted to give less than one absorbance unit.

Pulse labeling of RNA in vivo - One ml aliquots from a culture were exposed to 0.5 μCi of ^{14}C-Uridine for 2 min at 37° with shaking. The samples were then treated as described elsewhere[11].

Average burst size of SPP1 infection - Ten ml aliquots from a growing culture of B. subtilis were infected with SPP1 at a multiplicity of 10. After allowing 5 min for phage adsorption (>90%), the infected cultures were chilled in ice, centrifuged, resuspended in 10 ml of medium and immediately diluted 10^4 times. Incubation was continued at 37° and phage production measured after 2 and 3 hours (no significant difference was found between the two values). Average burst size is defined here as the ratio of pfu after 3 hours of infection to the infective centers immediately after resuspension of the infected cells.

Purification of DNAs - Phage DNAs were purified according to the procedure of Bautz and Dunn[17] except that a mixture of redistilled phenol (500g), m-cresol (70g) and 8-hydroxyquinoleine (0.5g) was used for extraction. The final aqueous phase was dialyzed against sterile 10mM Tris-HCl pH 7.9, 5mM EDTA, 1M NaCl and then against 10mM Tris-HCl pH 7.9, 0.1mM EDTA. Dialysis tubes were previously boiled in 1% $NaHCO_3$, then in 0.1M EDTA, kept in 10mM EDTA, 50% ethanol. Just before use, they were washed with the above phenol:m-cresol:8-hydroxyquinoleine mixture, then with ethanol and finally with sterile dialysis buffer. B. subtilis DNA was purified according to the procedure of Saito and Miura[18] and dialyzed as above. Commercial calf thymus DNA was further purified by two phenol extractions and dialyzed as above.

Preparation of partially purified extracts - At various times during growth and sporulation, 500 ml of culture were chilled, centrifuged and extracts prepared as described elsewhere[19].

In vitro RNA synthesis - Standard RNA polymerase assays (250 μl) contained:40mM Tris-HCl, pH 7.9, 10mM $MgCl_2$, 0.1mM EDTA, 1mM DTT, 0.4mM KH_2PO_4, 100 μg bovine serum albumin, 0.2mM ATP, GTP and CTP, 0.2mM ^{3}H-UTP (10 Ci/mole), the quantity of enzyme indicated

for each experiment and saturating amounts of DNA. For each DNA, the amount used was the highest concentration that did not cause a concentration inhibition effect. Incubations were at 37° for 10 min and were terminated by chilling in ice-cold water followed by addition of cold 5% TCA, 1% Na pyrophosphate. After standing in the cold for at least 30 min, the samples were filtered on Millipore membranes, washed with 40 ml of cold 5% TCA, 1% Na pyrophosphate, then with 10 ml cold 10mM HCl, dried and counted in a scintillation counter.

When RNA was to be analysed for self-complementarity, larger volumes (1 ml) of reaction were used and the specific activity of ^{3}H-UTP was increased to 60 Ci/mole. Synthesis was carried out at 37° for 30 min, then rifampin (1 μg) and RNase-free DNase (10 μg) were added and incorporation continued for 10 min. EDTA was then added to a final concentration of 10mM and the RNA extracted twice with phenol and finally precipitated with ethanol.

Assay for self-complementarity of in vitro RNA - Increasing concentrations of RNA in 0.3M NaCl, 30mM Sodium Citrate (final volume 0.4 ml) were incubated at 70° for 3 hours: samples were then treated with 10 μg of pancreatic RNase and 10 units of T1 RNase at 37° for 30 min and finally precipitated with 5% TCA, 1% Na pyrophosphate. After standing in the cold for at least one hour, samples were filtered on Millipore membranes, washed and counted as above.

RESULTS

Rate of RNA synthesis at early times of sporulation - At very early times of sporulation, when the cell mass is still increasing, one can observe a sharp decrease in the rate of RNA synthesis, as shown in Fig. 1. RNA was pulse-labeled for two min at the indicated times, as described in Materials and Methods. Under these conditions (during exponential growth), about 30% of the incorporated label goes into stable RNA, whereas the rest of it is found in material which becomes eventually acid-soluble if RNA synthesis is stopped by addition of actinomycin (data not shown). The results are roughly normalized by dividing the incorporated radioactivity by the absorbance of the culture. As one can see, by the time the cell mass reaches a plateau, the rate of RNA synthesis has dropped to a few percent of the value

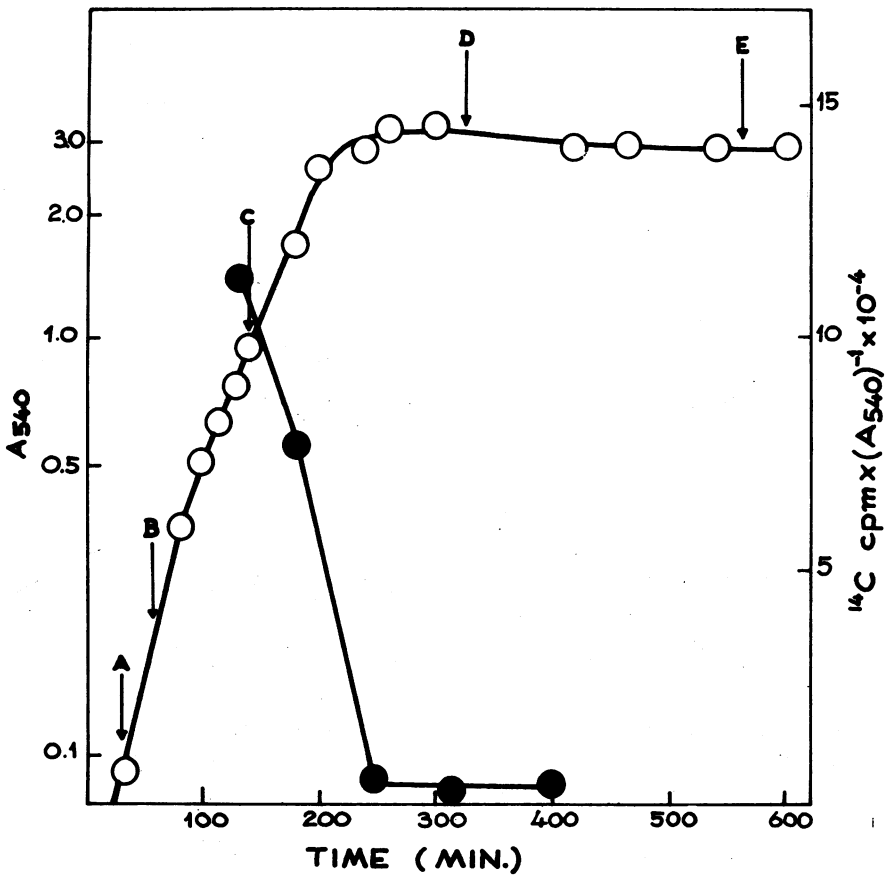

Fig. 1 -
Pulse labeling of RNA at early times of sporulation. Aliquots (1 ml) of the culture were exposed to 0.5 μCi of ^{14}C-Uridine for 2 min at 37° (see Materials and Methods). At the times indicated by arrows, 10 ml aliquots were infected with SPP1 for average burst size determination (see Table I). O—O, A_{540}; ●—● ^{14}C-cpm x $(A_{540})^{-1}$ x 10^{-4}.

in the exponential phase. This finding is consistent with previously reported results, showing that RNA accumulation, measured by continuous incorporation or by longer "pulses" of radioactive precursors, stops almost completely at very early times of sporulation [20-22]. Our results show that the stop in stable RNA accumulation is accompanied by a sharp decrease in overall RNA synthetic activity.

Average burst size of SPP1 infection in the course of sporulation- If SPP1 infection is carried out at various times in the course of sporulation, a dramatic decrease in the average burst size is observed, as has previously been reported in the case of ϕe infection[23]. Table I reports the results of such an experiment.

Table I

Burst size of SPP1 infection in the course of sporulation of B. subtilis

At the times indicated by arrows in Fig. 1, aliquots of the culture were infected with SPP1 (moi=10) and the average burst size determined as described in Materials and Methods. All cells behaved as infective centers after phage adsorption (5 min). Average burst size is defined here as the ratio of pfu after 3 hours of infection to the infective centers after phage adsorption.

Infection time	A	B	C	D	E
Adsorbed phage at 5 min (%)	90	92	91	94	91
Average burst size at 180 min	152	90	7.5	<1	1.2

The same culture used for the experiment reported in Fig. 1 was infected with SPP1 (moi=10) during exponential growth and at the early stages of sporulation.It can be seen that the average burst size (number of plaques formed 3 hours after infection divided by the number of infective centers after phage adsorption) decreases drastically until it reaches about 1 plaque/infected cell.Since the decrease in phage production more or less parallels that of the rate of incorporation of uridine, it is tempting to speculate that

the two facts are strictly correlated, and that the decrease in phage production is the result of a decreased transcription of the viral DNA in the infected cells.

Transcription of SPP1 DNA and other templates by partially purified extracts from vegetative and sporulating cells - Fig. 2 shows relative RNA polymerase activities in partially purified extracts of B. subtilis in the course of sporulation. Parallel cultures were stopped at different times before and after the end of exponential growth and extracts prepared and assayed as described under Materials and Methods. A marked decrease in activity can be seen when SPP1 DNA is used as a template. This result is in agreement with the hypothesis, expressed above, that the reduction in the burst size of SPP1 infection during sporulation is due to a loss of transcription capability of the host RNA polymerase for this viral template, since there is strong evidence[11,24] that the unmodified host RNA polymerase is responsible for all viral transcription in the course of SPP1 infection.

On the other hand, the loss of activity could be due to a trival cause, like an extracellular protease degradation during extraction. In order to investigate this point, a number of different DNA templates were transcribed with the same extracts and RNA polymerase activity was determined for each template in the course of sporulation. Fig. 2 (a and b) shows the results of such experiments. It can be clearly seen that different responses are obtained with different templates. Thus, although T4 DNA behaves more or less like SPP1 DNA (Fig. 2a), T7 DNA gives an opposite response, being transcribed much more efficiently by extracts from sporulating cells than from vegetative ones (Fig. 2a). Calf thymus DNA (Fig. 2b) gives qualitatively the same kind of response as T7 DNA, whereas B. subtilis DNA and the synthetic polymer poly d(AT) give intermediate results and show very little variation with different extracts.

An extract from cells at time t_2, then, transcribes SPP1 DNA with less than one half the efficiency of the corresponding extract from vegetative cells ($t_{-0.5}$); the same t_2 extract, on the other hand, transcribes T7 DNA twice as efficiently as does the $t_{-0.5}$ extract. This implies that the

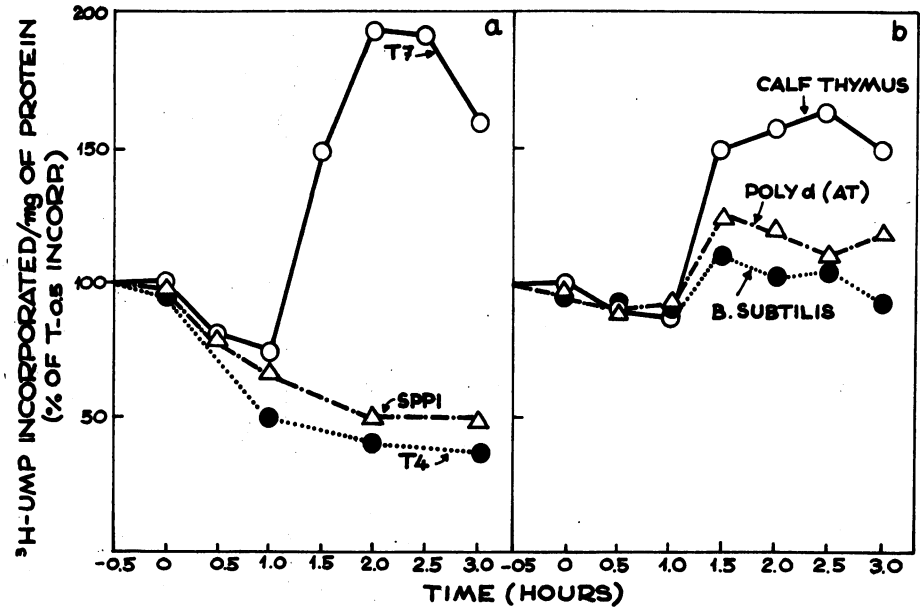

Fig. 2 - Relative RNA polymerase activity in partially purified extracts with different templates.

a) Standard RNA polymerase assays (0.25 ml) were carried out as described in Materials and Methods, with 37 µg/ml of SPP1 DNA, 32 µg/ml of T7 DNA and 16 µg/ml of T4 DNA respectively. About 20 µg were used for each extract. Sporulation times are expressed in hours, counted from the time of arrest of exponential growth (t_0). Results are expressed, for each DNA, as percent of the incorporation with $t_{-0.5}$ extract.

b) Same as a), but with 57 µg/ml B. subtilis DNA, 42 µg/ml calf thymus DNA and 48 µg/ml of poly d(AT) respectively.

loss of activity on SPP1 DNA is not due to a generic loss of RNA polymerase activity, but has some kind of template specificity.

Self complementarity of the RNA made in vitro on SPP1 and T7 DNA - Since, generally speaking, the RNA that can be extracted from bacterial cells is asymmetric, one can consider asymmetry as a rough measure of the fidelity of in vitro transcription. Indeed, strand selection should imply some selectivity in the choice of initiation sites. Table II shows the percentage of self hybridizable material in RNAs made in vitro with partially purified extracts of B. subtilis and SPP1 or T7 DNA.

Table II

Self-complementarity of in vitro RNA

RNAs were synthesized, purified and self-hybridized as described in Materials and Methods, and had specific activities of about 10^5 (^{3}H-UMP) dpm/μg. In experiments 1 to 4, standard conditions were used, whereas in experiments 5 to 8, 2mM $MnCl_2$ was used instead of $MgCl_2$ and NaCl (final conc. 0.1M) was added. The figures reported in the last column are plateau values of curves obtained by increasing the concentration of input RNA in the self annealing reactions, except for experiment 3 in which a plateau was not reached. Blank values (never more than 2%) were obtained from parallel samples which, instead of incubation at 70°, were boiled for 10 min, chilled in ice and immediately treated with RNases as described in Materials and Methods.

Experiment	Extract (time of sporulation)	DNA	Divalent cation	Added NaCl	% of RNA which is RNase resist. after self-anneal.
1	$t_{-0.5}$	SPP1	Mg^{2+}	-	2
2	t_2	SPP1	Mg^{2+}	-	10
3	$t_{-0.5}$	T7	Mg^{2+}	-	>50
4	t_2	T7	Mg^{2+}	-	28
5	$t_{-0.5}$	SPP1	Mn^{2+}	0.1M	4
6	t_2	SPP1	Mn^{2+}	0.1M	16
7	$t_{-0.5}$	T7	Mn^{2+}	0.1M	17
8	t_2	T7	Mn^{2+}	0.1M	23

All the given figures are plateau values of experiments with increasing concentrations of input RNA, as described in Materials and Methods. It can be seen that, when SPP1 DNA is used as the template, a vegetative extract transcribes it in a highly asymmetric fashion; on the other hand, an increase in symmetry is obtained when an extract from sporulating cells t_2 is employed (Table II, experiments 1 and 2). When T7 is used, great precautions have to be taken in order to reduce the amount of symmetry of the RNA obtained[25,26]. We have found that increasing the salt concentration and using Mn^{2+} instead of Mg^{2+} as the divalent cation reduces the amount of symmetry to a reasonable level without affecting the differential efficiency of vegetative and sporulation extracts discussed above (data not shown). As in the case of SPP1 DNA, an increase in self-complementarity can be seen when a sporulation extract is used instead of a vegetative one, but this increase is smaller than the corresponding one obtained with SPP1 DNA and certainly cannot account for the increase in total activity (Table II, experiments 7 and 8). Furthermore, if no precautions are taken to limit the extent of symmetry of the RNA made on T7 DNA and synthesis is carried out under standard conditions in the presence of Mg^{2+}, a vegetative extract gives an RNA which is over 50% symmetric, whereas a sporulation extract gives a much less symmetric RNA (Table II, experiments 3 and 4).

From these results we conclude that the higher activity of a sporulation extract, with respect to a vegetative one, on T7 DNA is not the result of a loss of fidelity of the in vitro transcription, as measured by self complementarity of the RNA.

The gain in activity is not a property of RNA polymerase - The higher activity found in t_2 extracts on T7 DNA is not an intrinsic property of RNA polymerase, since it was lost after DEAE-cellulose purification, as shown in Fig. 3. The fraction eluted from the column at 0.25M KCl (SII), containing the RNA polymerase activity, showed a lower activity on T7 DNA than the corresponding fraction from a $t_{-0.5}$ extract. With SPP1 DNA as a template, a loss of activity is still observed after DEAE-cellulose, as in the case of less purified extracts, when going from a vegetative to a

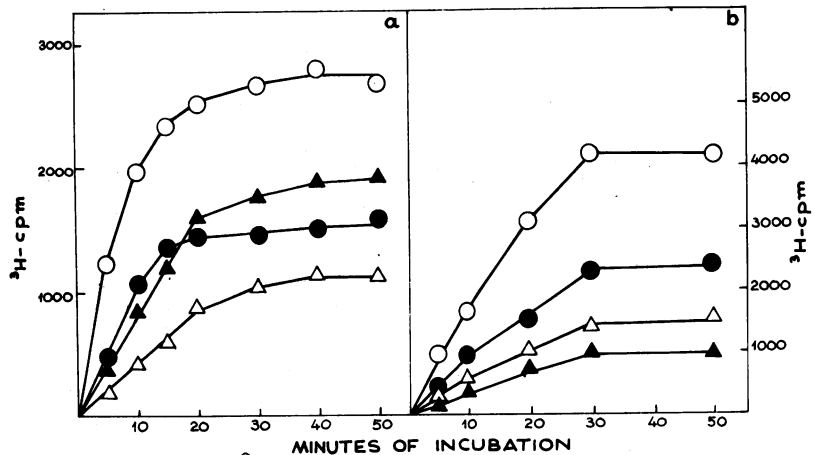

Fig. 3 - Kinetics of ^{3}H-UMP incorporation. One ml standard reaction mixtures (see Materials and Methods) were incubated at 37° with 37 µg of SPP1 DNA or 32 µg of T7 DNA. Reactions were started by additions of enzymes. At the indicated times, 100 µl aliquots were added to ice cold 5% TCA, 1% Na pyrophosphate.

a) Synthesis carried out by 80 µg of partially purified extracts from $t_{-0.5}$ or t_2 cells (same as in Fig. 2).

O—O : SPP1 DNA, $t_{-0.5}$ extract; ●—● : SPP1 DNA, t_2 extract;
△—△: T7 DNA, $t_{-0.5}$ extract; ▲ ▲:T7 DNA, t_2 extract.

b) Synthesis carried out by 15 µg of DEAE-cellulose purified RNA polymerase (SII) from $t_{-0.5}$ or t_2 extracts (see legend to Table III).

O—O: SPP1 DNA, $t_{-0.5}$ SII; ●—● : SPP1 DNA, t_2 SII; △—△:T7 DNA, $t_{-0.5}$ SII;
▲—▲:T7 DNA, t_2 SII.

sporulation fraction.

On the other hand, the fraction eluted from the DEAE-cellulose column at 0.2M KCl, when added back to RNA polymerase, stimulates RNA synthesis with T7 DNA, as shown in Table III (lines 8 to 11). The same fraction has no effect on RNA synthesis if SPP1 is used (Table III, lines 1 to 4). The same table also shows that if the initiation events are confined to a short time by addition of rifampin, the stimulatory activity of the DEAE fraction, eluted at 0.2M KCl, still conserves its template specificity (lines 5 to 7 and 12 to 14). This fact implies that the stimulation observed with T7 DNA is due to an increased efficiency of the initiation mechanism on this template.

DISCUSSION

RNA polymerase activity in extracts from *B. subtilis* shows marked alterations in its template specificity at early times of sporulation. Different natural templates give differential responses, going from 60% inhibition (SPP1 and T4 DNA) to an almost twofold stimulation (T7 and calf thymus DNA).

The fact that the activity is increased with some natural templates makes it difficult to interpret the loss of activity, observed with other natural templates, in terms of possible artifacts due to extracellular degradation. On the other hand, since there is strong evidence that unmodified *B. subtilis* RNA polymerase can completely transcribe SPP1 DNA both *in vitro* and in infected cells[11,24], the loss in activity with SPP1 DNA correlates well with: i) the sharp decrease in total RNA synthetic activity (rate of RNA synthesis) observed in sporulating cells (Fig. 1) and ii) the drop in phage production upon SPP1 infection found in the course of sporulation (Table I), in analogy with the results reported for ϕe[23]. We interpret these findings as suggesting that at early times of sporulation *B. subtilis* RNA polymerase loses affinity for the portion of bacterial chromosome normally transcribed during vegetative growth. The obvious way in which this could occur is through a loss of affinity for initiation sites (promoters). Although we have not measured RNA chain elongation rates, the kinetics in Fig. 3, showing that different plateau levels are reached by vegetative and sporula-

Table III

Template specific stimulation of RNA synthesis by 0.2M KCl DEAE fraction from t_2 extract

Partially purified extract were dialyzed against buffer A of Burgess and then passed on a DEAE-cellulose column (Whatman DE-52). After washing the column with the same buffer, step elution was carried out at 0.2 and 0.25M KCl. Fractions eluted at 0.2M contained no RNA polymerase activity, which was all found in the 0.25M KCl elution step. Fractions were pooled, precipitated with ammonium sulfate and redissolved in storing buffer[27]. Standard RNA polymerase assays were carried out as described in Materials and Methods, with 3.5 µg of fraction SII (0.25M KCl fraction) and 13 µg of 0.2M KCl fraction respectively. Only the 0.2M KCl fraction obtained from t_2 extract was used in these experiments, and it was added either together with RNA polymerase (0 min) or after addition of rifampin (2.5 min).

RNA polymerase	DNA	0.2M KCl DEAE fraction (time of add.)	4 µg/ml Rif (time of addition)	cpm[a]	stimulation[b] %
1 $t_{-0.5}$	SPP1	-	-	6,931	-
2 $t_{-0.5}$	SPP1	0 min	-	6,937	0
3 t_2	SPP1	-	-	3,993	-
4 t_2	SPP1	0 min	-	4,314	+7
5 t_2	SPP1	-	2 min	1,972	-
6 t_2	SPP1	0 min	2 min	1,928	-2
7 t_2	SPP1	2.5 min	2 min	1,617	-18
8 $t_{-0.5}$	T7	-	-	2,375	-
9 $t_{-0.5}$	T7	0 min	-	4,229	+78
10 t_2	T7	-	-	1,670	-
11 t_2	T7	0 min	-	3,389	+102
12 t_2	T7	-	2 min	724	-
13 t_2	T7	0 min	2 min	1,086	+67
14 t_2	T7	2.5 min	2 min	622	-15

a) Data in the presence or in the absence of rifampin are from separate experiments and cannot be directly compared.

b) Stimulation are calculated with respect to the corresponding incorporation in the absence of 0.2M KCl fraction (100%).

tion enzymes with SPP1 DNA, supports the hypothesis that the loss of activity is due to a decreased initiation efficiency.

A twofold increase in RNA polymerase activity is found in extracts from sporulating cells when T7 DNA is used as the template (Fig. 2). As shown in Fig. 3 and Table III, this increased activity is not a property of RNA polymerase itself, but is induced by some factor(s) eluting separately on a DEAE-cellulose column. The increase in activity caused by such factor(s) is due to a higher efficiency in the initiation mechanism. The possibility of an artifact due for example to the opening of new initiation sites by nucleases present in the stimulating fraction is made rather unlikely by parallel experiments with SPP1 DNA, showing no stimulation under the same conditions (Table III). Furthermore, the fidelity of transcription, as measured by self-complementarity of the RNA made, is not too affected by this stimulation and is actually increased if synthesis is carried out in a standard reaction mixture (Table II).

It is tempting to speculate from these results that the increased affinity of extracts from sporulating cells for initiation sites on T7 DNA parallels a higher affinity for promoters of bacterial transcription units which are preferentially transcribed during sporulation. According to this hypothesis, at the beginning of the sporulation process, the transcription machinery of B. subtilis would lose affinity for certain types of promoters (similar to those on SPP1 and T4 DNA) usually recognized in vegetative growth, and acquire a new affinity for other promoters (similar to the ones on T7 DNA), characteristic of sporulation. The fact that B. subtilis DNA shows no important variation in template activity in the course of sporulation could then be explained by the fact that both kinds of promoters are present on it.

We have at present no proof for this hypothesis. What makes it intriguing is perhaps the fact that the alteration we find in template specificity results in both a loss and a gain of activity of the same sporulation extracts, thus limiting to some extent the possibility of artifacts. Further work will be necessary in order to define the biochemical bases of this phenomenon.

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