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Modulation of Deoxyribonucleic Acid Polymerase III Level During the Life Cycle of *Bacillus Subtilis*

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Deoxyribonucleic acid (DNA) polymerase III is not detectable in *Bacillus subtilis* spores; the enzyme activity appears 20 to 30 min after spore activation and rapidly increases just before the onset of the first round of DNA replication (30 min later); the level of polymerase III further increases and reaches its maximum (on a per-genome basis) when the cells enter the vegetative phase of growth; this level is six- to eightfold higher than the one observed during germination. In the stationary phase, the polymerase III drops to levels comparable to those found in germinating spores at the first round of replication. On the contrary, DNA polymerase I is present at appreciable levels in the dormant spore; it increases during vegetative growth by a factor of three and, during the stationary phase, reaches its maximum level which is sixfold higher than that observed in the spores. The block of protein synthesis during vegetative growth does not cause an appreciable reduction of the two enzymes (in absolute terms), showing that the regulation of their levels is probably not due to a balance between synthesis and breakdown. These results indicate that polymerase III is probably one of the factors controlling the initiation of DNA synthesis during spore germination.

Germination of the bacterial spore allows study of the regulation of deoxyribonucleic acid (DNA) synthesis under conditions in which the initiating events take place in a synchronous fashion and at a definite time. In fact, during germination, the macromolecular syntheses start in an ordered progression at reproducible time intervals (2, 11, 21); ribonucleic acid synthesis and protein synthesis begin in *Bacillus subtilis* a few minutes after spore activation, whereas DNA synthesis starts 50 to 60 min after ribonucleic acid and protein synthesis. A knowledge of the factor(s) limiting this initiation would give information on, at least, this particular type of regulation of DNA of synthesis. Two extreme hypotheses can be advanced to explain the delay in the initiation of DNA synthesis (2, 8): (i) the whole machinery for DNA replication must be synthesized in the germinating spore; and (ii) the machinery is in fact present and ready in the spore, but some initiation protein(s) must be synthesized for the replication apparatus to act. Several experiments performed in the past seem to support the latter hypothesis; some enzymes presumed to be involved in DNA replication are in fact present in the spore (7, 8, 22). Furthermore, inhibition of protein synthesis during germination blocks the initiation of DNA synthesis; however, if protein synthesis is inhibited at various times during germination, DNA initiation can occur provided the inhibitor is added at late times (2, 22); this fits with the idea that the synthesis of an initiation protein is the limiting factor. The analogous behavior of mutants temperature sensitive for initiation, shifted at intermediate times after germination, strengthens this conclusion (10, 13, 20).

On the other hand, these results can be interpreted also in the opposite way, namely, that the proteins forming the replication apparatus are absent in the spore and are synthesized at definite times during germination. The presence of DNA polymerase I (Pol I) in spores, demonstrated in the past (7, 8, 22), cannot be held as evidence against this hypothesis.

The availability of a procedure that allows the quantitative measurement of DNA polymerase III (Pol III) in crude extracts of *polA*$^+$ strains (4) encouraged us to determine whether this enzyme, which is an essential piece of the replicative machinery, is in fact present in the spores, and, if not, at what time it is synthesized.

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MATERIALS AND METHODS

Strain. *B. subtilis* SB202 *hisB2 tyrA1 trpC2 aroB2* was used for all the experiments.

Media. The media used were: nutrient broth (NB; Difco Laboratories, Detroit, Mich.); nutrient agar (NA; nutrient broth + 2% Difco agar); sporulation agar (SA; nutrient agar + 10^{-4} M $Ca(NO_3)_2$ + 10^{-5} M $MnCl_2$ + 10^{-6} M $FeSO_4$); Penassay medium (PY; Difco); Bouillon nutritif complet (BNC) (Bio Kar); the minimal medium of Spizizen (MT) (19) enriched with a combination of 0.2% Casamino Acids (Difco), 0.05% yeast extract (Difco), and 50 μg of tryptophan per ml; and the minimal medium of Davis and Mingioli (5) supplemented with the appropriate requirements was used for marker control.

Spore preparation. Spores were prepared by spreading 2 ml of a log culture in PY into a layer of SA (50 ml of SA in a 500-ml flat bottle) and incubating 5 days at 30°C. The spores were collected and purified as described by Yoshikawa (22), subjected to sonic treatment at 0°C (four 30-s bursts) with the microtip of a B-12 sonifier (Branson Instruments Co., Stamford, Conn.), centrifuged at 10,000 × *g* for 10 min, suspended in sterile water at a final concentration of about 0.3 g/ml (about 10^{11} spores/ml), and stored at 0°C.

Sporulation in liquid medium. An overnight culture in PY was diluted 1:20 into prewarmed NB medium containing a solution of 10^{-4} M $Ca(NO_3)_2$, 10^{-5} M $MnCl_2$, 10^{-6} M $FeSO_4$ and grown at 33°C in a Microferm fermentor (New Brunswick Scientific Co. New Brunswick, N. J.) with vigorous aeration.

Log-phase cells. The cells were grown as described above, except that the medium was BNC without salts and the growth was stopped at an A_{560} of 1.5.

Spore germination. Spore viability was checked by plating on NA. Spores were activated by heating for 15 min at 80°C in water, and germination was obtained by diluting the activated spores into prewarmed MT or BNC media (T_0 of germination being the time of dilution) at 33°C in a Microferm fermentor with vigorous aeration. The final concentration was either 8×10^7 or 1.6×10^8 spores per ml corresponding to an A_{560} of 0.4 or 0.8, respectively.

Harvesting of cells. The liquid cultures of germinating log-phase or sporulating cells were collected in the following way: 0.5 liter of frozen medium equilibrated at −40° was added to 2 liters of culture. In this way the temperature drops to 10°C in about 2 min. The cells were then centrifuged at 10,000 × *g* for 10 min, washed with cold saline solution, frozen with liquid N_2, and stored at −20°C.

DNA synthesis. The amount of DNA in the bacterial suspension at various times was determined chemically. Duplicate 10-ml aliquots of culture were centrifuged at 10,000 × *g* for 10 min; the cells were washed with cold saline solution and then incubated at 37°C for 30 min with 1 ml of 0.2 N NaOH; the macromolecules were then precipitated by the addition of 1 ml of cold 10% trichloracetic acid, and, after 10 min at 0°C, they were centrifuged at 10,000 × *g* for 10 min. The DNA was assayed on the precipitate according to Burton (3).

Protein determination. Proteins were assayed by the procedure of Lowry et al. (16).

Preparation of enzyme extracts. To approximately 1 g of spores or frozen cells we added 4 ml of extraction buffer (0.05 M glycyl-glycine-NaOH buffer, pH 7.5,-10 mM dithiothreitol), 0.6 mg of *p*-toluenesulfonyl fluoride per ml, and 4 g of glass beads (0.11-mm diameter). The suspension was then sonically treated for 15 min (30-s bursts at 30-s intervals) at 0°C with a Branson B-12 sonifier large tip at 50 W of power. The extracts were then centrifuged at 10,000 × *g* for 10 min, and the supernatant fraction was collected and fractionated by addition of a 10% (wt/vol) streptomycin sulfate solution to obtain a final concentration of 0.61%. After 30 min of stirring at 0°C, this suspension was centrifuged and the precipitate was suspended in a buffer containing a solution of 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.5, 1 mM dithiothreitol, 1 mM $CaCl_2$, 0.5 M KCl, and 20% glycerol (vol/vol). The streptomycin supernatant fraction and the precipitate were assayed to determine the levels of DNA polymerase activities.

DNA polymerase assays. The DNA polymerases were assayed according to Gass and Cozzarelli (9). Pol III was determined quantitatively as a 6-(*p*-hydroxyphenylazo)-uracil-sensitive fraction; Pol I was determined as the *p*-chloromercuribenzoate-insensitive fraction (4).

RESULTS

Levels of Pol I and Pol III in spores and vegetative cells. A total of 0.6 g (wet weight) of log-phase cells or spores or of a 1:1 mixture of the two cell forms were extracted under conditions suitable for spore breakage (see above). As reported in Table 1, Pol III is almost absent in the spores: there is less than 1% of this enzyme (in terms of specific activity), as in the vegetative cells, and less than 0.3% in terms of total activity. Extraction of a mixture of cells and spores rules out the possibility of inhibitors present in the spore extract. The values of Pol III in the spore-cell mixture extract are in fact slightly higher than the exact average of the values in the separate extracts, as if some sort of activator were present in the spore extract; this could be due to the action of some nuclease

TABLE 1. *Levels of Pol I and Pol III in spores and vegetative cells*[a]

Cell type	Pol I		Pol III	
	U/g of cells	U/mg of protein	U/g of cells	U/mg of protein
Log phase	5.67	0.052	13.7	0.126
Spores	1.05	0.028	<0.04	<0.001
Log phase + spores	2.73	0.037	10.5	0.142

[a] A total of 0.6 g of log cells, 0.6 g of spores, and a mixture of 0.3 g of cells plus 0.3 g of spores were extracted and assayed as described in the text.

enhancing the template efficiency. We have not inquired further into this point.

Since the extraction procedure entails sonic treatment for 15 min in the presence of glass beads, the possibility exists that the enzyme might be inactivated in the spore extract. It appears however that this is not the case: in fact, when vegetative cells are extracted with the same technique for times varying between 2 min (assuring about 90% breakage) and 30 min of sonic treatment, no difference was found in the level of Pol III or Pol I at the different times. Thus, lack of observation of Pol III activity is neither due to the presence of inhibitors, to inactivation, nor to inadequate extraction; in fact, the level of Pol I observed in the spores with this procedure is comparable to the values observed in the past that used milder extraction techniques (7, 8); furthermore, the spore extraction procedure is certainly adequate to give total breakage (over 90% by microscopic observation) and to allow quantitative extraction of a number of other enzymes (6). It must be remembered that extraction by sonic treatment gives a complete suspension of cell material, without macroscopic debris to be removed; it is therefore reasonable to assume that, if the enzyme were bound to a subcellular structure such as membrane, it should be detectable in our assay, unless attached in an inactive form.

The observed absence of Pol III activity has been repeated in different spore preparations; in some instances, some Pol III activity was observed in the spore extract (of the order of 1% total units with respect to vegetative cells); we think however that this is due to a small contamination with nonspore material; in fact, following 80°C activation of the spores, this residue of activity disappears completely, suggesting a location outside the spore.

Thus, the spore seems essentially void of Pol III, whereas it contains significant levels of Pol I. We can thus wonder at what time during germination the Pol III appears, and whether this time is correlated with the onset of DNA replication.

Variations of DNA polymerase levels during germination. Cultures of germinating spores were assayed for the two polymerases and for DNA content at different times during germination. Figures 1 and 2 report the results obtained with a complete medium (BNC) and a minimal medium (MT). Data for the enzymes are reported both as it referred to the total content of the culture (upper panel) and to the DNA content, expressed as genome equivalents. The patterns of variations are similar in the two experiments and have been reproduced several times. DNA replication starts at 60 min

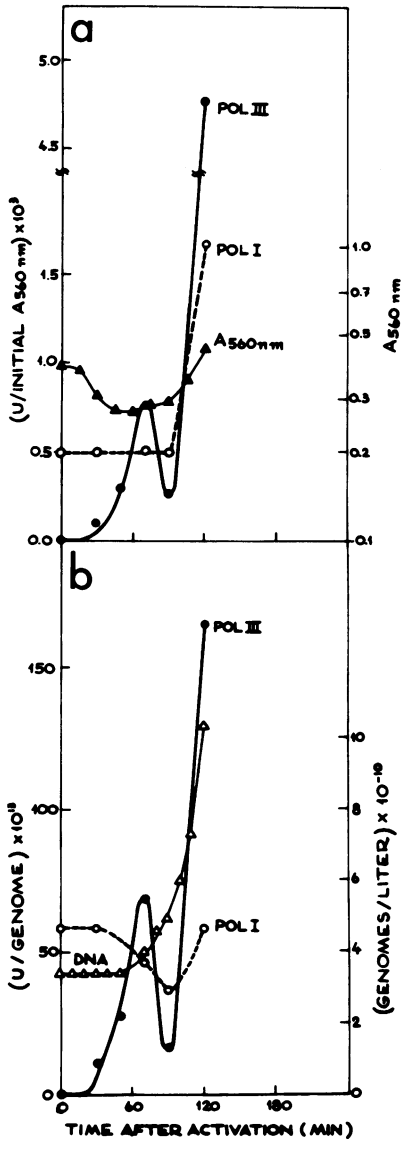

FIG. 1. *Levels of Pol I and Pol III during germination in BNC. The T_0 value was determined by extracting 0.6 g of activated spores and measuring the enzyme activity as indicated in the text. The values for different germination times were obtained in two separate experiments; in each case, a 10-liter culture was inoculated with 1.2 g of the same spore preparation after activation; in one case, 5-liter samples were removed at 30 min and 50 min. In the other, 5 liters was removed at 70 min, 2.5 liters at 90 min, and 2 liters at 120 min. The cultures were also assayed for A_{560} and DNA content. (a) Enzyme activities (determined as above) are reported normalized to the A_{560} of the inoculum; (b) enzyme data are normalized to DNA content of the culture, expressed as genome equivalents. As DNA content of one genome, the value of 1.51 × 10^{-8} nmol of P was used.*

and proceeds at a constant rate up to 90 min, a time sufficient to accomplish 50% of the first replication; the rate of DNA synthesis increases after this time and probably becomes exponential. The abrupt variation in DNA content between 60 and 120 min indicates a satisfactory synchrony of the cells. The level of Pol I remains constant throughout the germination period, including the onset of DNA replication. As the cells enter the log phase, the Pol I level per genome begins to increase. This latter phenomenon is more evident in Fig. 2. The difference in absolute levels of enzymes between experiments 1 and 2 may be attributed in part to the different medium and in part to the different spore preparation. In fact, different spore preparations, obtained by the same procedure, were found to have Pol I levels, in terms of units per milligram, or units per genome, varying by as much as a factor of 3; on the other hand, in exponential phase, the values of Pol I or Pol III activities, expressed as units per milligram, were equal in the two media used in this work. All subsequent experiments were prepared with the same spore preparation used for the data reported in Fig. 1. In any case, the basic pattern of variation is repeated in both media, and a subsequent increase in Pol I level during log phase was observed in other experiments also in NB.

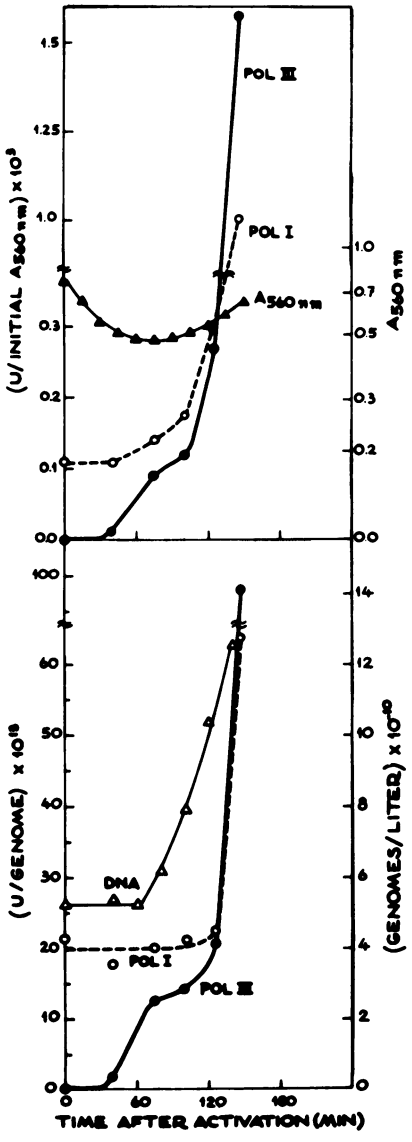

FIG. 2. *Levels of Pol I and Pol III during germination in MT. Medium was enriched as reported in the text. Activated spores (2.4 g) were used to inoculate 10 liters of medium. A total of 2.5 liters was withdrawn for the 40-min and 75-min samples, 1.5 liters for the 110-min sample, and 1 liter for the 125- and 145-min samples. All other details as in Fig. 1.*

On the contrary the level of Pol III undergoes more dramatic variations: the activity, which is still absent after 20 min of germination (data not shown), begins to appear approximately 30 min after activation, and then it increases for a further 30 to 40 min until the onset of DNA replication (about 60 min). At this time the enzyme reaches a kind of plateau or even drops as shown in Fig. 1. If germination is continued, the Pol III activity further increases and reaches the levels found in logarithmically growing cells. The drop in absolute level of Pol III activity observed around 90 min is surprising but has been observed in several other experiments.

DNA polymerase levels during log phase. As the culture goes into the vegetative phase of growth, the level of Pol I, referred to DNA content, raises to a higher value; this can be seen in the experiment of Fig. 2 and is reported for other independent experiments in Table 2. The increase of Pol I does not show any obvious time correlation with the onset of DNA synthesis. As already outlined, Pol III seems to remain at a definite level during the late phase of germination, but when the DNA replication rate becomes exponential, the level of Pol III per genome further increases (about fourfold). As shown in Table 2, this level remains virtually constant during the vegetative growth. These results seem to indicate that the first round of DNA replication during germination occurs in the presence of Pol III levels (referred to DNA content) lower than those observed in exponentially growing cells.

Variations during stationary phase and sporulation. The observed correlation between

TABLE 2. *Levels of DNA polymerases during different germination and vegetative growth stages in BNC medium*

Growth stage	Pol I			Pol III		
	U/g of cells	U/mg of protein	U/10^{13} genomes	U/g of cells	U/mg of protein	U/10^{13} genomes
Spore germination						
T = 0 min	1.05	0.028	42.8	<0.04	<0.001	<1.5
T = 70 min	1.25	0.085	45.9	1.88	0.13	69.3
Vegetative growth						
$A_{560}^{9.4}$	2.44	0.054	78	7.81	0.172	250
$A_{560}^{9.8}$	3.34	0.044	121	9.0	0.117	322

Pol III level and onset of DNA replication encourages study of this enzyme's fate during stationary phase and sporulation when the replication comes to a complete halt (Fig. 3). It can be seen that the Pol III level, which remains constant throughout the log phase, drops drastically during stationary phase to a level comparable to that observed at 60 min of germination.

In contrast, Pol I reaches its highest value during stationary phase; Pol I and Pol III do not seem to undergo a further drop during sporulation, which in our experiments occurs between the 9th and the 20th h of incubation (Fig. 3).

Since our extraction procedure disrupts all cell forms, including the spore, the question arises whether the observed enzyme activities are located outside or inside the spore structure. We have thus extracted an aliquot of the cells after 12 h of growth under conditions that allow the breakage of vegetative cells only; at this time over 80% of the cell forms contain one refractile endospore; the extract was assayed for activity, and the residual spore material was further extracted, as described above (Table 3). Pol III is found only in the outlying portion of the sporangium, and the spore again proves to be void of this enzyme. Although Pol I is also present mainly outside the spores, these contain still a significant fraction of that enzyme. The specific activity of the spore extract is a factor of 2 or 3 lower than that of the total cell extract.

Effect of protein synthesis inhibition on polymerase levels. The pattern of Pol III of variation in relation to onset of DNA synthesis during germination and the properties of some Pol III mutants (1, 14, 18) suggests a possible role of this enzyme in regulation of initiation. In this respect, one could imagine the level of this enzyme being coupled to DNA synthesis, for example, through a balance between degradation and resynthesis of the enzyme during the replicative cycle. To determine whether the

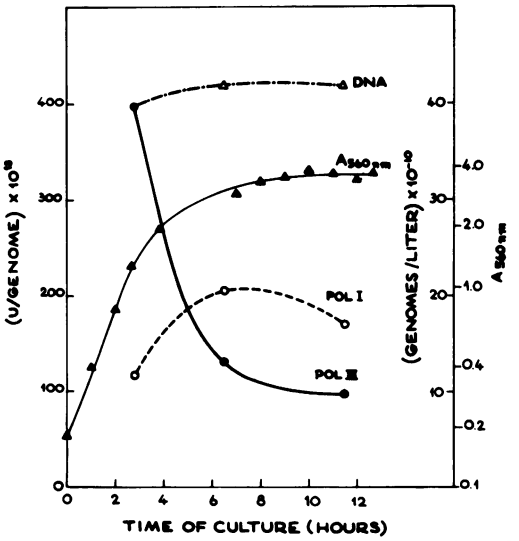

FIG. 3. *Pol III levels during sporulation in liquid medium. A 7-liter culture of NB (plus salt) in the Microferm fermentor was inoculated with 400 ml of an overnight culture in PY; at the indicated times, 1-liter samples were removed, and the cells were washed and extracted for the enzyme assay as reported in the text. Also, the DNA content was measured.*

DNA polymerases are subject to such a turnover, we measured variations of their levels during vegetative growth after a block in protein synthesis (Fig. 4). As expected, the protein synthesis block causes a delayed arrest of DNA replication (Fig. 4). However, during this time the absolute levels of Pol III and Pol I decrease only by a small factor, certainly not sufficient to account for arrest of DNA replication. The enzyme levels, corrected for DNA content, seem to settle around the same values observed during the early germination phase (T = 60 min).

DISCUSSION

The most prominent observation reported in

TABLE 3. *Levels and location of Pol I and Pol III in sporulating cells*[a]

Cell structure	Pol I		Pol III	
	U/liter of culture	% of total	U/liter of culture	% of total
External portion	20.0	98.4	8.48	≥99.5
Endospore	0.32	1.6	≤0.042	≤0.5

[a] A 4-liter culture in NB (plus salts) was incubated until over 80% of the cells were transformed in sporangia. A 1-liter portion was removed; the cells (4.0 g [wet weight]) were washed with saline and extracted in 6 ml of extraction buffer containing 40 mg of lysozyme and 2.4 mg of *p*-toluenesulfonyl fluoride. After 30 min at 37°C, the suspension was sonically treated for 1 min and the unbroken spores were collected; the supernatant fluid was assayed, and the data are reported as external portion. The spores were washed with water, extracted as usual, and assayed for DNA polymerase.

this paper is the absence of any detectable Pol III activity in the spore extracts or at early times after spore activation. At longer incubation times after activation, the Pol III appears and increases rapidly just before the onset of DNA synthesis; the Pol III level remains thereafter constant (of the order of 70 U/10^{13} genomes in BNC) or undergoes a slight drop for about 30 min and finally increases again as the cells enter the exponential rate of DNA replication (Fig. 1 and 2). In exponentially growing cells, the Pol III remains constant at a level that is five- to eightfold greater (per genome) than that observed during the first round of replication after germination. It is not clear why a different level is observed during germination; one can postulate a correlation with the number of growing points, which are two at the first replication wave and increase to six at the second one when DNA replication becomes dichotomous (17). On the other hand, the increase of Pol III in the second part of the growth curve is of the order of five- to eightfold; thus, the number of growing points cannot be the only factor that accounts for the higher level of Pol III in log-phase cells. The time correlation between attainment of the first maximum in Pol III level and onset of DNA replication is quite good, and we propose that, with all probability, this enzyme is one of the factors limiting initiation of DNA synthesis during germination. It can thus be concluded that, besides a hypothetical initiation signal, at least one essential piece of the replicative machinery is also missing in the germinating spores.

As the cell goes into stationary phase, the decrease to zero of the DNA replication rate is paralleled by a decrease of Pol III level, but only to a definite and still significant value (Fig. 3). So, this enzyme remains present at appreciable levels in the cells also when the DNA replication rate is nil. All the Pol III

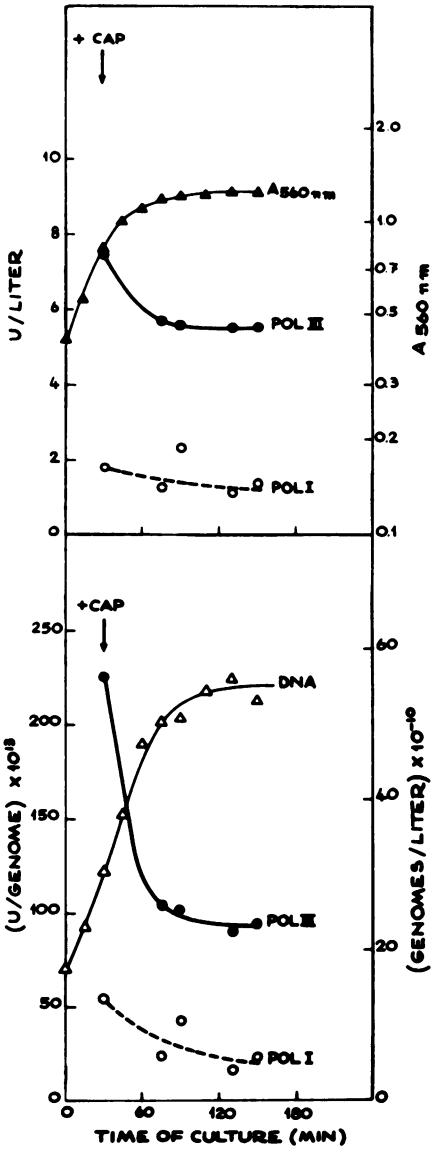

FIG. 4. *Effect of chloramphenicol on DNA polymerase level. A 400-ml overnight culture in PY was diluted into 10 liters of prewarmed BNC medium at 33°C with vigorous aeration in a Microferm fermentor. Chloramphenicol (Serva Corp., Heidelberg) was added at a final concentration of 150 μg/ml. One-liter samples were removed and treated as reported in Fig. 1. CAP, Chloramphenicol.*

activity, however, is found outside the forming spore envelope.

The variations of Pol I are less spectacular. As reported previously (7), the spores contain a significant amount of this enzyme, on the order of one-third of the specific activity (units per milligram) in the vegetative cells. Expressed as units per genome, the activity remains constant throughout the first replication wave during germination and begins to increase when the cells enter the exponential phase of growth (and probably dichotomous replication). The maximum level, on the other hand, is not reached during log phase, but in the stationary phase, including the sporulation period. Figure 5 summarizes the patterns of Pol I and Pol III variation throughout the cell cycle.

The interpretation of these changes is not univocal, but a few considerations are allowed. In the first place, since this enzyme seems absent in the dormant spore, we can say that regulation of at least the first DNA synthesis wave, after germination, occurs also through a modulation of the Pol III level. Other independent observations point to a role for this enzyme in regulation of DNA replication as well as in the elongation process: two independent temperature-sensitive mutants (*dnaF133* and *dnaF69* [1, 14]), which map on one end of the *polC* gene, behave as initiation mutants; these mutants have significantly lower levels of Pol III also at the permissive temperature, and, after an in vivo shift to 48°C, lose this activity completely. The Pol III of these mutants is not temperature-sensitive in vitro, and, therefore, it is not clear whether the mutations concern the structural gene of Pol III or some regulatory gene closely linked to the structural one. In any case, it is not apparent from our data whether the modulation of Pol III level is important also in the normal replicative cycles since synchrony of DNA replication is lost soon after the first replication in the germinating cells; the only indication in this sense comes from the small drop of Pol III observed repeatedly in BNC between the first replication wave and the following one (Fig. 1). It seems unlikely that this drop is due to a selective loss of Pol III occurring in the extract at this particular time; one could surmise that a specific inactivating factor may be active only at this time, but this again would point to a programmed regulation of the Pol III level correlated with the replication cycle. If such a modulation occurs in log phase, it is not due, as one could assume in an oversimplified model, to an alternation of breakdown and resynthesis of Pol III; in fact, as shown in the experiment of Fig. 4, the block in protein synthesis followed by the arrest of DNA synthesis does not lead to parallel disappearance of Pol III in the cell. Further work on this possible regulatory role of Pol III in vegetative growth is required, which will take advantage of the properties of *dnaF* mutants. It must be borne in mind that the observed variations in quantity of the Pol III may be too rough an estimate of its function in the replication apparatus; the purified Pol III of *B. subtilis* has a molecular weight of 166,000 (15), but the cells also contain forms with a higher molecular weight (4), one of which could be analogous to the holoenzyme described in *Escherichia coli* and might have a specific role in the elongation of Okazaki pieces (12). More important variations in molecular structure, which would be more relevant to the replicative process may be underlying a small drop in overall activity.

The different behaviors of Pol I and Pol III may be ascribed to their different main roles in DNA repair and replication, respectively.

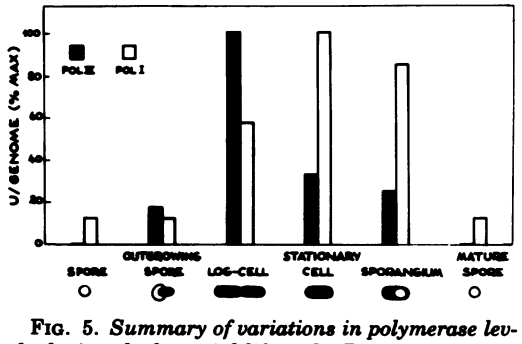

FIG. 5. *Summary of variations in polymerase levels during the bacterial life cycle. Data are averages of several independent experiments. A sketch of the microscopic appearance of the bacterial cells at each stage is reported.*

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