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Template Specificity of Ribonucleic Acid Polymerase in Asporogenous Mutants of *Bacillus subtilis*

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Asporogenous mutants of *Bacillus subtilis* were examined for the change in template specificity of ribonucleic acid (RNA) polymerase characteristic of wild-type cells undergoing sporulation. Mutants blocked at stages II, III, and IV showed a changed specificity of the enzyme after the end of growth and were in this respect indistinguishable from the wild type. The RNA polymerase of eight stage-zero mutants (out of nine tested) which possess mutations that map at six distinct loci retained the template specificity of vegetative cells.

Spore formation in *Bacillus subtilis* is accompanied by loss of ability of the bacterial deoxyribonucleic acid (DNA)-dependent ribonucleic acid (RNA) polymerase (nucleoside triphosphate:RNA nucleotydyltransferase; EC 2.7.7.6) to transcribe in vitro the DNA of phage ϕe, a phage that successfully infects vegetative cells of *B. subtilis* but gives rise to no progeny phage after infection of sporulating cells (8, 14). While the DNA of ϕe serves as a template in vitro for the RNA polymerase of growing cells, it is not a template for the enzyme extracted from sporulating cells. The synthetic template poly d(AT) is active with both enzymes. The discovery of this change in template specificity at the transition point between the growth phase and the sporulation phase suggests that this modification of enzyme activity is responsible for the ordered transcription of genes expressed at different times during spore formation (8).

Since transcription of ϕe DNA in vitro by vegetative RNA polymerase is dependent on the activity of a subunit of the enzyme, called the sigma factor (σ), that determines transcriptional specificity (7), the change in template specificity associated with sporulation may be due to the destruction or inactivation of σ or to the loss of affinity of the core polymerase for σ. In support of the latter possibility, Losick, Shorenstein, and Sonenshein (7) have shown that the core polymerase isolated from sporulating cells differs structurally from the vegetative core in that one of its high-molecular-weight subunits (i.e., β' or β) has a significantly altered mobility in gel electrophoresis. This modified core enzyme characteristic of sporulating cells is unable to interact functionally with the vegetative σ factor (7). The possibility remains, however, that the loss of response to vegetative σ might be due to other changes in the core polymerase not visible by gel electrophoresis.

In the present work, we have taken advantage of the isolation in this and other laboratories of a large number of asporogenous mutants blocked at various steps in the sporulation process. The purpose of our study was twofold: to determine the stage of sporulation at which the change in template specificity occurs, and to estimate the number of genetic loci that influence directly or indirectly this phenomenon. One of these loci is already known to be that at which mutations in a subunit of RNA polymerase can occur since a class of mutants resistant to the antibiotic rifampin (Rfm^r) become blocked at stage zero of sporulation (SpoO) and lose the ability to change the template specificity of the enzyme after the end of growth (14). This latter observation reinforces the previous suggestion that the change in template specificity of RNA polymerase plays an essential role in sporulation. It was especially interesting for us to compare these mutants with stage-zero sporulation mutants, bearing mutations that map at many loci distinct from *rfm*, with respect to alterations in

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template specificity of RNA polymerase after the end of growth.

MATERIALS AND METHODS

Chemicals. Dithiothreitol (Cleland reagent, DTT) was purchased from Calbiochem, ethylendiaminetetraacetate (EDTA) from Siegfried, and unlabeled adenosine, guanosine, cytidine, and uridine triphosphates (ATP, GTP, CTP, UTP) from Sigma. ^{3}H-ATP and ^{3}H-UTP were obtained from either the Commissariat à l'Énergie Atomique, Saclay, France, or New England Nuclear Corp. Poly d(AT) was obtained from Miles. Nutrient broth was the Difco product. Inorganic salts were Prolabo or Merck products.

Bacterial strains. Spo$^-$ strains (Table 1) were isolated from the spore-forming strains 168, SMY, and GSY 225, or were obtained from I. Takahashi. Strains 168 and SMY are Marburg strains from our laboratory collection. GSY 225 and GSY 427 were gifts of C. Anagnostopoulos.

Bacterial cultures. Bacteria were cultivated at 37 C with vigorous agitation in 2-liter Fernbach flasks containing 500 ml of a nutrient broth medium permitting spore formation (12). Under these conditions, at T_8 (8 hr after the end of exponential growth) 80% of the cells were resistant to heating for 10 min at 80 C.

Buffers. The buffers used derive from those described by Burgess (1). Buffer G: 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, *p*H 7.5; 0.01 M $MgCl_2$; 0.2 M KCl; 0.1 mM EDTA; 0.1 mM DTT; 10% (v/v) glycerol. Buffer A: 0.01 M Tris-hydrochloride, *p*H 7.9; 0.1 mM EDTA; 0.1 mM DTT; 10% (v/v) glycerol.

Template preparation. DNA was extracted from phage ϕe with 0.5% sodium dodecyl sulfate in 0.1 M Tris-hydrochloride, *p*H 9. Protein was eliminated by extraction with a phenol-cresol mixture. The aqueous phase was dialyzed overnight against 0.1 M Tris-hydrochloride, *p*H 7.9, containing 0.5 mM EDTA. Poly d(AT) was solubilized in the same Tris-EDTA buffer at a concentration of 250 μg/ml.

Enzyme extracts. Cultures (500 ml) were harvested in exponential growth phase and at T_0, T_1, $T_2 \ldots T_{4.5}$ (T_n referring to the time in hours after the end of exponential growth as defined by Schaeffer [10, 11]). Cells were washed with buffer G (40 ml) and frozen at −70 C until use. The pellet was resuspended in 10 ml of buffer G and sonically treated at 4 C for 9 to 12 min. The crude extract obtained was centrifuged at 100,000 × *g* for 1 hr, and the RNA polymerase activity was precipitated from the supernatant fluid between 30 and 60% of saturation with ammonium sulfate (100% saturation corresponds to 700 g added to 1 liter of extract). The precipitate was resuspended in buffer A (2 to 4 ml) containing 0.2 M KCl and 50% (v/v) glycerol and stored at −20 C.

Assays. Protein was measured by the procedure of Lowry et al. (6) with crystalline bovine serum albumin as standard.

RNA polymerase activity was measured with ϕe DNA or poly d(AT) as template. Unless otherwise noted, the assay mixture (0.25 ml) had the following composition: 0.04 M Tris-hydrochloride, *p*H 7.9; 0.01 M $MgCl_2$; 0.15 M KCl; 0.4 mM K-PO_4 buffer; 0.1 mM EDTA; 0.1 mM DTT; 0.5 mg of bovine serum albumin per ml; 0.15 mM GTP, CTP, UTP, and ATP, ^{3}H-ATP or ^{3}H-UTP (3,000 counts per min per nmole); and 15 μg of ϕe DNA or 6 μg of poly d(AT). Between 20 and 40 μg of enzyme was used per assay. After incubation for 10 min at 37 C the reactions were stopped by adding 3 ml of 5% trichloroacetic acid containing 0.01 M sodium pyrophosphate and were chilled in ice. After 10 min the precipitates were collected on cellulose nitrate filters and washed four times with 5% trichloroacetic acid solution. The filters were dried and counted in 10 ml of Toluene-2,5-diphenyloxazole-1,4-bis-(2-4-methyl-5-(phenyloxazolyl)benzene scintillation fluid.

One unit of enzyme activity incorporates 1 nmole of adenosine monophosphate or uridine monophosphate in 10 min. Specific activity is expressed in units per milligram of protein.

TABLE 1. *Spo$^-$ mutants studied*

Strain[a]	Phenotype[b]	Reference
SpoIV-11T	Ab$^-$ Spr$^-$ NarR$^-$ Est$^-$	5
SpoIII-94U	Ab$^-$ Spr$^-$ NarR$^-$ Est$^-$	5
SpoII-4Z	Ab$^-$ Spr$^-$ NarR$^-$ Est$^-$	5
SpoO-4U	Ab$^-$ Spr$^-$ NarR$^+$ Est$^-$	J. F. Guespin-Michel, *personal communication*
SpoO-1U	Ab$^-$ Spr$^\pm$ NarR$^-$ Est$^-$	J. F. Guespin-Michel, *personal communication*
SpoOa 5NA	Ab$^-$ Spr$^-$ NarR$^-$ Est$^-$	5, 9
SpoOb 6Z	Ab$^-$ Spr$^\pm$ NarR$^-$ Est$^-$	5, 9
SpoOc 9V	Ab$^-$ Spr$^\pm$ NarR$^-$ Est$^-$	5, 9
SpoO-1	Ab$^-$ Spr$^\pm$ NarR$^\pm$ Est$^-$	16
SpoO-C14-1	Ab$^-$ Spr$^-$ NarR$^-$ Est$^-$	16
SpoO-98-8	Ab$^-$ Spr$^-$ NarR$^-$ Est$^-$	16
SpoO-170-2	Ab$^-$ Spr$^-$ NarR$^-$ Est$^-$	16

[a] The nomenclature for Spo$^-$ strains is that suggested by Young (17).

[b] Sporulation-related phenotype designations are abbreviated as follows: antibiotic, serine-protease, and esterase productions are Ab$^+$, Spr$^+$, and Est$^-$, respectively; inducibility of nitrate reductase is NarR$^-$, constitutivity is NarR$^-$.

RESULTS

Parent Spo$^+$ strains. Before beginning our mutant study, it was first necessary to verify that the change in RNA polymerase template specificity described for the wild-type strain 3610 grown in glucose-casein hydrolysate medium (medium 121 B, reference 15) existed in the parent strains of our mutants grown in complex medium. Figure 1 shows an example of the results obtained with the strain 168. It can be seen that the specific activity of RNA polymerase preparations assayed with poly d(AT) as template remains practically constant throughout growth and sporulation, but that in vitro transcription of phage ϕe DNA drops rapidly at about T_0 and becomes stabilized at its minimal

value at T_1. The residual activity with ϕe DNA probably reflects the presence of a 20% fraction of the population that fails to sporulate. The simple comparison of the specific activity with ϕe DNA of enzyme extracted from vegetative and sporulating cells may, however, give rise to erroneous conclusions due to lack of uniformity in purification achieved by ammonium sulfate fractionation from preparation to preparation. For this reason we have found it preferable to establish for each time-point a ratio, R Mg, of the activity measured with ϕe DNA to that measured with poly d(AT), a nonselective template. Table 2 shows that R Mg is greater than 2.5 for enzyme extracted from vegetative cells, whereas it is less than 1.0 for enzyme prepared after T_1. For our studies we consider that the change in template specificity has occurred when the value of R Mg is less than 1.0 in an extract of cells harvested at $T_{4.5}$.

The change seen with strain 168 is also found with other wild-type strains (Table 3). In the case of GSY 225, the ratio at $T_{4.5}$ is somewhat higher than normal, a finding consistent with the lower production of spores in this strain by comparison with the other parents.

Late-blocked Spo⁻ strains. Analysis of Table 3 shows that, in mutants blocked at stages II, III, and IV of sporulation, the change in template specificity occurs normally, that is, as in the parent strains. Thus, the decision to modify the specificity of RNA polymerase is made before stage II as suggested by the time-course experiment of Fig. 1. Mutants blocked at stage I were not available in this laboratory.

Strains blocked at stage zero (SpoO). In Table 3 it can be seen that in all SpoO mutants RNA polymerase, even at $T_{4.5}$, is at least twice as active with ϕe DNA as with poly d(AT). These mutants are thus unable to activate whatever functions are necessary to alter template specificity.

Similar data for three additional mutants are presented in Table 4. With these mutants, for reasons which we are unable to explain, little or no activity with poly d(AT) as template was observed in our standard assay system containing 10 mM $MgCl_2$. By replacing the $MgCl_2$ with 1 mM $MnCl_2$, however, we have been able to assay the extracts of these mutants with poly d(AT). Normally extracts active with poly

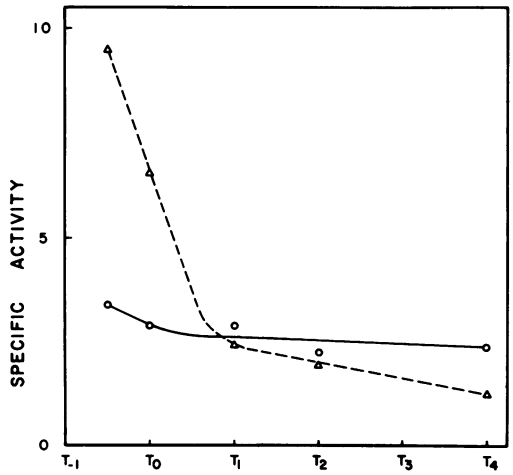

FIG. 1. *Specific activity of RNA polymerase measured with ϕe DNA and poly d(AT) in extracts of cells of B. subtilis 168 harvested at indicated times before and after the end of growth. Symbols: Δ, ϕe DNA; O, poly d(AT).*

TABLE 2. *RNA polymerase in extracts of B. subtilis 168 harvested at different times during growth and sporulation*

Time of cell harvesting	Specific activity with ϕe DNA	Specific activity with poly d (AT)	R Mg[a]
$T_{-0.5}$	9.48	3.42	2.77
T_0	6.55	2.92	2.24
T_1	2.44	2.92	0.84
T_2	1.96	2.26	0.87
T_4	1.26	2.38	0.53

[a] R Mg = [specific activity with ϕe DNA]/[specific activity with poly d (AT)].

TABLE 3. *RNA polymerase activity in Spo⁺ and Spo⁻ strains*

Strains	Specific activity with ϕe DNA		Specific activity with poly d (AT)		R Mg[a]	
	Enz $T_{-0.5}$	Enz $T_{4.5}$	Enz $T_{-0.5}$	Enz $T_{4.5}$	Enz $T_{-0.5}$	Enz $T_{4.5}$
168	6.55	1.26	2.5	2.4	2.52	0.53
SMY	4.1	0.36	1.37	4.0	2.98	0.09
GSY 225	6.3	2.76	2.63	2.36	4.6	1.17
GSY 427	5.0	2.7	1.85	3.2	2.7	0.85
SpoIV-11T	8.3	0.94	2.64	2.06	3.14	0.46
SpoIII-94U	6.7	3.5	2.4	5.4	2.8	0.65
SpoII-4Z	6.2	1.12	1.34	2.4	4.6	0.47
SpoOa 5NA	4.2	4.3	0.87	1.4	4.8	3.1
SpoOb 6Z	5.6	8.0	0.95	1.9	5.8	4.2
SpoOc 9V	10.5	5.8	0.91	1.75	11.5	3.3
SpoO-C14-1	5.0	4.4	0.9	0.79	5.5	5.5
SpoO-98-8	8.1	6.9	2.58	1.85	3.14	3.73
SpoO-170-2	6.25	4.65	2.4	1.66	2.60	2.80

[a] R Mg = [specific activity with ϕe DNA]/(specific activity with poly d (AT)].

d(AT) in the presence of Mg^{2+} as divalent cation are four to five times more active in the presence of Mn^{2+}.

This enzymatic activity stimulated by Mn^{2+} is clearly that of RNA polymerase since it is sensitive to rifampin (*unpublished experiments*). Phage ϕe DNA is poorly active as a template for RNA polymerase when Mn^{2+} is the divalent cation. For this reason, activity measurements with ϕe DNA presented in Table 4 are those obtained with Mg^{2+}. To decide whether a given strain undergoes the change in template specificity when the assays are performed as in Table 4, we define a second ratio of specific activities, R Mn, representing the ratio of activity with poly d(AT) as template assayed with 1 mM $MnCl_2$ to that obtained with ϕe DNA as template assayed with 10 mM $MgCl_2$. Under these conditions, the vegetative enzyme has a ratio, R Mn, less than or equal to 1.5, and the change in template specificity is said to have occurred when R Mn becomes greater than 3.0.

In Table 4, the results for two stage-zero mutants, SpoO-1 and SpoO-1U, indicate a defect in the modification of activity of RNA polymerase, in agreement with the findings for SpoO mutants presented in Table 3. For the last SpoO mutant, SpoO-4U, we are unable to draw any firm conclusions.

TABLE 4. *RNA polymerase activity in Spo⁺ and Spo⁻ strains*

Strain	Specific activity with ϕe DNA		Specific activity with poly d (AT)		R Mn[a]	
	$T_{-0.5}$	$T_{4.5}$	$T_{-0.5}$	$T_{4.5}$	$T_{-0.5}$	$T_{4.5}$
168		3.4		10.2		3.02
GSY 225	7.95	1.21	11.0	4.4	1.38	3.6
SpoO-1	4.75	2.62	7.43	3.63	1.59	1.39
SpoO-1U	6.45	1.98	6.7	2.95	1.04	1.5
SpoO-4U	7.95	2.91	14.8	7.75	1.86	2.66

[a] R Mn = [specific activity with poly d (AT)]/[specific activity with ϕe DNA].

DISCUSSION

The change in specificity of in vitro transcription by DNA-dependent RNA polymerase during sporulation of *B. subtilis* previously described in strain 3610 (8) is observed also in the Spo⁺ strains from which are derived the Spo⁻ mutants studied here; it is thus a general phenomenon. The search for this modification of activity in various Spo⁻ mutants clearly leads to the conclusion that mutants blocked at stage II or later change their RNA polymerase template specificity after T_0, whereas at least eight out of 9 SpoO mutants do not. It therefore seems that this alteration is required to attain stage II of sporulation but that, as expected, it is not sufficient to go beyond this stage. Other functions necessary for successful sporulation are apparently lacking in mutants blocked at stages II, III, and IV.

The observation that none of the nine stage-zero mutants modifies like the wild type the template specificity of RNA polymerase after the end of growth reflects the complexity of the problem. It should be noted that the mutations involved map in at least six distinct chromosomal regions (see Fig. 2). The products of some genes are certainly implicated directly in the change in template specificity. For example, specific functions are presumably required to destroy or inactivate the vegetative sigma factor, or to bring about modification in the core sufficient to eliminate its affinity for vegetative σ (7). In fact, both mechanisms may be involved. That a large number of changes in RNA polymerase may occur during sporulation is suggested by analogy with the changes observed in *Escherichia coli* RNA polymerase after the end of growth (2) or after infection by phage T4 (11). Other functions responsible for the commitment of the cell to sporulation might influence the change in template specificity. For

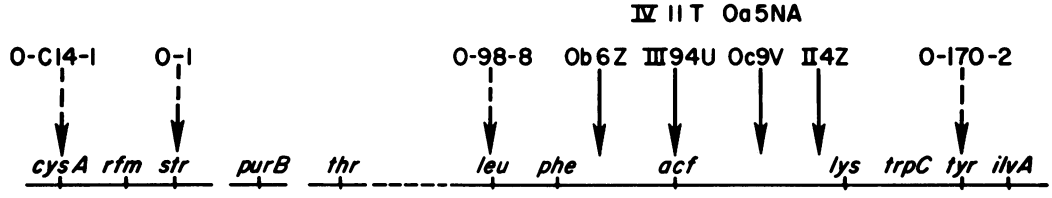

FIG. 2. *Approximate chromosomal location of the Spo⁻ mutations used (redrawn from reference 5). The horizontal line stands for the B. subtilis chromosome, with its auxotrophy and resistance reference markers. Arrows point to the approximate location of Spo⁻ mutations, designated by their Spo phenotype and mutant isolation number. Heavy and dotted arrows refer to mutations located by Ionesco et al. (5) and by Takahashi (16), respectively.*

example the release of catabolite repression at T_0, the mechanism generally thought to be the trigger for spore formation (13), might necessitate the expression of several genes. Furthermore, modifications apparently occurring in the cytoplasmic membrane after T_0 (3) may play an important and early role in the sporulation process. By studying the mutants described here, and others carrying mutations in RNA polymerase which render the enzyme unmodifiable (14), we hope to identify the various functions responsible for the change in template specificity.

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LITERATURE CITED

1. Burgess, R. R. 1969. A new method for the large scale purification of *Escherichia coli* deoxyribonucleic acid-dependent ribonucleic acid polymerase. J. Biol. Chem. **224**:6160–6167.
2. Chelala, C. A., L. Hirschbein, and H. N. Torres. 1971. Interconvertible forms of *Escherichia coli* RNA polymerase. Proc. Nat. Acad. Sci. U.S.A. **68**:152–154.
3. Freese, E., W. Klofat, and E. Galliers. 1970. Commitment to sporulation and induction of glucose-phosphoenolpyruvate-transferase. Biochim. Biophys. Acta **222**:265–289.
4. Goff, C. G., and K. Weber. 1970. A T4-induced RNA polymerase α subunit modification. Cold Spring Harbor Symp. Quant. Biol. **35**:101–107.
5. Ionesco, H., J. Michel, B. Cami, and P. Schaeffer. 1970. Genetics of sporulation in *Bacillus subtilis* Marburg. J. Appl. Bacteriol. **33**:13–24.
6. Layne, E. 1957. Spectrophotometric and turbidimetric methods for measuring proteins, p. 448–449. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 3. Academic Press Inc., New York.
7. Losick, R., R. G. Shorenstein, and A. L. Sonenshein. 1970. Structural alteration of RNA polymerase during sporulation. Nature (London) **227**:910–913.
8. Losick, R., and A. L. Sonenshein. 1969. Change in template specificity of RNA polymerase during sporulation of *Bacillus subtilis*. Nature (London) **224**:35–37.
9. Michel, J. F., and B. Cami. 1969. Sélection de mutants de *Bacillus subtilis* bloqués au début de la sporulation. III. Nature des mutations sélectionnées. Ann. Inst. Pasteur **116**:3–18.
10. Ryter, A., P. Schaeffer, and H. Ionesco. 1966. Classification cytologique par leur stade de blocage des mutants de sporulation de *Bacillus subtilis* Marburg. Ann. Inst. Pasteur **110**:305–315.
11. Schachner, M., and W. Zillig. 1971. Fingerprint maps of tryptic peptides from subunits of *Escherichia coli* and T4 modified DNA-dependent RNA polymerase. Eur. J. Biochem. **22**:513–519.
12. Schaeffer, P., H. Ionesco, A. Ryter, and G. Balassa. 1965. *In* Régulation chez les microorganismes. Colloq. Int. Cent. Nat. Rech. Sci. **124**:553–563.
13. Schaeffer, P., J. Millet, and J. P. Aubert. 1965. Catabolic repression of bacterial sporulation. Proc. Nat. Acad. Sci. U.S.A. **54**:704–711.
14. Sonenshein, A. L., and R. Losick. 1970. RNA polymerase mutants blocked in sporulation. Nature (London) **227**:906–909.
15. Sonenshein, A. L., and D. H. Roscoe. 1969. The course of phage ϕe infection in sporulating cells of *Bacillus subtilis* strain 3610. Virology **29**:265–276.
16. Takahashi, I. 1969. Genes controlling sporulation in *Bacillus subtilis*, p. 102–111. *In* L. L. Campbell (ed.), Spores IV. American Society for Microbiology, Bethesda, Md.
17. Young, F., and G. A. Wilson. 1972. Genetics of *Bacillus subtilis* and other gram-positive sporulating bacilli, p. 77–106. *In* L. L. Campbell (ed.), Spores V. American Society for Microbiology, Washington, D.C.