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Genetics

Bacillus subtilis σ factor σ^{29} is the product of the sporulation-essential gene *spoIIG*

(bacterial sporulation/monoclonal antibody)

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ABSTRACT **Evidence is presented that the sporulation-essential locus *spoIIG* codes for both σ^{29} and a structurally related protein, P^{31}. This demonstrates that at least one specific *Bacillus subtilis* RNA polymerase binding protein provides a critical function in endospore formation. *spoIIG*-specific RNA is present in *B. subtilis* cultures that are synthesizing P^{31} and σ^{29} and is absent in those that are not. A monoclonal antibody specific for an antigenic determinant on P^{31}/σ^{29} detected crossreacting proteins (P^{25}/P^{21}) but not P^{31} or σ^{29} in a Spo⁻ *B. subtilis* strain with a mutation at the *spoIIG* locus (*spoIIG41*). The appearance of P^{25} and P^{21} occurs in this mutant at a time when P^{31} and σ^{29} would normally appear and suggests that they are homologous proteins. Transformation of the *spoIIG41* strain with plasmid DNA carrying the structural gene for *spoIIG* complements the Spo⁻ phenotype and results in the synthesis of P^{31}, σ^{29}, P^{25}, and P^{21} at the appropriate times during sporulation. In *Escherichia coli*, the cloned *spoIIG* sequence encoded a protein that reacted with the anti-P^{31}/σ^{29} monoclonal antibody and had the electrophoretic mobility of authentic P^{31}.**

The spore-forming bacterium *Bacillus subtilis* synthesizes at least five forms of DNA-dependent RNA polymerase, which are distinguished by the promoter specificity determinant (σ factor) that each carries on a common core enzyme (1–5). One of the σ factors, σ^{29}, is a protein (M_r, 29,000) that is detected only during endospore formation and was therefore predicted to be an important element of spore gene regulation (3, 6, 7). Recently, a protein (P^{31}; M_r, 31,000), structurally related to σ^{29}, was identified in extracts of sporulating *B. subtilis* (8). P^{31} reacts with a monoclonal antibody that is specific for an antigenic site on σ^{29}. In addition, P^{31} and σ^{29} have the same peptide profiles when digested with either *Staphylococcus aureus* V8 or chymotrypsin A4 protease (ref. 9; unpublished results). During sporulation, synthesis of P^{31} precedes that of σ^{29} and occurs in all *Bacillus* species and *B. subtilis* Spo⁻ mutants that have been found capable of synthesizing a σ^{29}-like protein (8). P^{31}, like σ^{29}, is capable of associating with core RNA polymerase. RNA polymerase that carries P^{31} (E-P^{31}) has an elution pattern from DNA-cellulose identical to that of RNA polymerase carrying σ^{29} (E-σ^{29}) (9). P^{31} and σ^{29} differ biochemically in that P^{31} binds relatively poorly to RNA polymerase and the resulting E-P^{31} has no detectable activity on cloned *B. subtilis* DNAs (9). The properties of P^{31} and σ^{29} indicate that they are likely to be the products of the same structural gene, with P^{31} potentially an inactive precursor of σ^{29}.

The genetic locus *spoIIG* is defined by a number of independently isolated mutations that block spore formation at an early stage in development and lie at map position 130 on the *B. subtilis* chromosome (10, 11). This sporulation-essential gene has recently been cloned and sequenced (12). The likely product of *spoIIG*, inferred from DNA sequence data, is a M_r 27,500 protein that carries regions of homology with the major *E. coli* σ factor encoded by *rpoD* (12). Based on its size, time of expression, and homology to *rpoD*, *spoIIG* was suggested to be the structural gene for σ^{29} (12).

We have analyzed the possible relationship between *spoIIG* and σ^{29} with a monoclonal antibody (8) that is specific for an antigenic site exclusively present on P^{31} and σ^{29}. These experiments indicate that the sporulation-essential locus *spoIIG* is the structural gene for both P^{31} and σ^{29}. Thus, one or both of these proteins must play a critical role in endospore formation.

MATERIALS AND METHODS

Bacterial Strains. *B. subtilis* strains BS50 (*spoIIG41*) and BS119 (*spoIIG55*) were obtained from M. Young; SMY was from R. Losick; and NG 17.15, P9, and 26U were from the Bacillus Stock Center (Ohio State University). *E. coli* strain C600 was obtained from B. Eisenstein. The relevant phenotypes of the strains are indicated in the figure legends.

RNA Analysis. *B. subtilis* cells were grown in Sterlini and Mandelstam resuspension medium (13). RNA from vegetative and sporulating [2 hr (T_2) and 4 hr (T_4) into sporulation] cells was prepared as described by Gilman and Chamberlin (4) after disruption of the cells by passage through an Eaton pressure cell (5000 psi; 1 psi = 6.895×10^3 Pa). RNA was spotted onto nitrocellulose and probed with *spoIIG*-specific DNA [1.1-kilobase-pair *Pst* I fragment (12)] labeled by the nick-translation reaction of DNA polymerase I.

Analysis of Cell Extracts with Monoclonal Antibody. The isolation and production of monoclonal antibody, as well as the methods of cell extract preparation and analysis, have been described (8).

RESULTS

P^{31} and σ^{29} appear in *B. subtilis* during an early stage in sporulation and are absent in vegetatively growing cells (8). Thus, if *spoIIG* codes for P^{31} and σ^{29}, RNA homologous to *spoIIG* DNA should be present only in sporulating cells. RNA was extracted from vegetatively growing and sporulating cells (T_2 and T_4), spotted onto nitrocellulose paper, and hybridized with ^{32}P-labeled *spoIIG* DNA. Fig. 1 shows that the *spoIIG* sequence is absent in the RNA obtained from vegetatively growing cells (lane 1), but it is present by 2 hr into sporulation (lane 2). A number of *B. subtilis* Spo⁻ mutants, blocked at the same morphological stage in development (stage II), vary in their ability to synthesize P^{31} and σ^{29} (8). If some of these mutants are probed for *spoIIG*-specific

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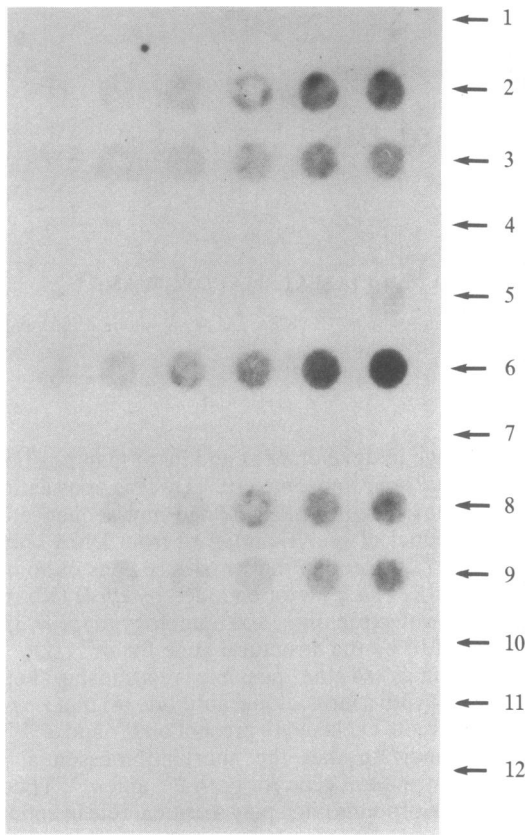

FIG. 1. Dot blot analysis of *B. subtilis* RNA for *spoIIG* sequences. Increasing amounts of RNA (1.25, 2.5, 5, 10, 20, and 40 μg per spot) from vegetative cells and from cells harvested 2 hr (T_2) and 4 hr (T_4) into sporulation were spotted onto nitrocellulose paper and hybridized with 6.7×10^6 cpm of ^{32}P-labeled *spoIIG* DNA. Lanes: 1–3, *B. subtilis* 168M (Spo$^+$) (vegetative, T_2, T_4); 4–6, *B. subtilis* NG 17.15 (*spoIIE64*-σ^{29} absent, P^{31} present) (vegetative, T_2, T_4); 7–9, *B. subtilis* P9 (*spoIIC298*-σ^{29} and P^{31} present) (vegetative, T_2, T_4); 10–12, *B. subtilis* 26u (*spoIIA26*-σ^{29} and P^{31} absent) (vegetative, T_2, T_4).

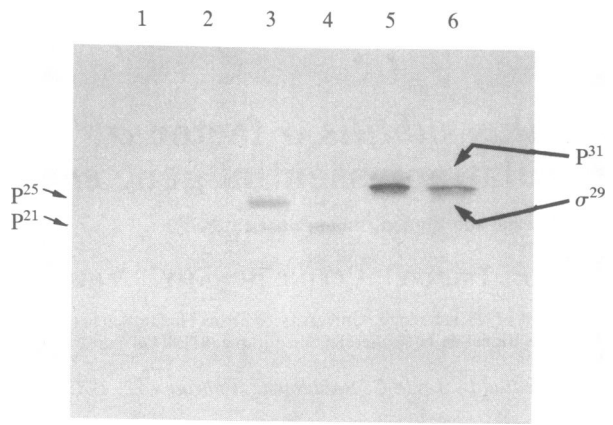

FIG. 2. Immunological analysis of σ^{29}/P^{31} in *spoIIG* mutants. Crude protein extracts were prepared as described (8) from *B. subtilis* cells that were harvested 2 hr (T_2) and 3.5 hr ($T_{3.5}$) after the onset of sporulation in DS medium (15). Portions (100 μg) were fractionated by electrophoresis on 12% $NaDodSO_4$/polyacrylamide gels, transferred electrophoretically to nitrocellulose, and probed with monoclonal antibody (8). Bound antibody was visualized with horseradish peroxidase-conjugated goat IgG against mouse Ig (HyClone). Lanes: 1 and 2, BS119 (*spoIIG55*) T_2 and $T_{3.5}$, respectively; 3 and 4, BS50 (*spoIIG41*) T_2 and $T_{3.5}$, respectively; 5 and 6, SMY (Spo$^+$) T_2 and $T_{3.5}$, respectively.

RNA, we find that *spoIIG* RNA is present after the end of exponential growth in a mutant strain that synthesizes P^{31} and σ^{29} (*spoIIC*; lanes 7–9) or only P^{31} (*spoIIE*; lanes 4–6), but not in a strain that fails to synthesize either of these proteins (*spoIIA*; lanes 10–12). *spoIIG* RNA is thus present in cells that are synthesizing P^{31}/σ^{29} and absent in cells that are not.

We next prepared crude protein extracts from two *B. subtilis* strains that carry mutant alleles of *spoIIG* (*spoIIG41* and *spoIIG55*) and analyzed them by size-fractionation on $NaDodSO_4$/polyacrylamide gels followed by transfer to nitrocellulose paper and probing with an anti-σ^{29}/P^{31} monoclonal antibody. This antibody readily detects P^{31} and σ^{29} in extracts of sporulating *B. subtilis* (Fig. 2, lanes 5 and 6). Neither of the *spoIIG* mutants synthesized detectable amounts of P^{31} or σ^{29} (lanes 1–4), although one of them (*spoIIG41*) produced two proteins [M_r, 25,000 (P^{25}); M_r, 21,000 (P^{21})] that reacted with the σ^{29}/P^{31}-specific antibody (lanes 3 and 4). The presence in this mutant of aberrantly sized proteins that react with the σ^{29}/P^{31}-specific antibody and the absence of P^{31} and σ^{29} suggest that the *spoIIG* defect might directly affect the synthesis of both P^{31} and σ^{29}.

We had previously determined that P^{31} and σ^{29} appear at a precise time in endospore formation (8). If the proteins of the *spoIIG41* strain that are recognized by the P^{31}/σ^{29} antibody were similarly regulated, it would argue that they were, in fact, the counterparts of P^{31} and σ^{29} in this mutant. Fig. 3 shows an analysis of the temporal appearance of the proteins that react with anti-σ^{29} antibody in a Spo$^+$ *B. subtilis* strain (Fig. 3*A*) and the *spoIIG41* mutant (Fig. 3*B*). As expected for homologous proteins, the pattern of synthesis of proteins capable of reacting with the monoclonal antibody appears to be similar in the Spo$^+$ and the mutant strains, with the synthesis of P^{31} and P^{25} preceding the appearance of σ^{29} and P^{21}.

The *spoIIG* cloned DNA was isolated as a sequence that corrected the Spo$^-$ phenotype of *spoIIG* mutations (*spoIIG41* and *spoIIG55*) *in vivo* (12). pGSIIG11 is a *B. subtilis*/*E. coli* shuttle plasmid that carries the entire *spoIIG* structural gene sequence, plus 100 to 200 base pairs of *B. subtilis* DNA that flank each end of this gene, within a cloned 1.1-kilobase-pair DNA fragment (12). When pGSIIG11 is introduced into the *spoIIG41* strain, the transformants synthesize not only P^{25} and P^{21} but also proteins with the mobility of authentic P^{31} and σ^{29} (Fig. 4, lanes 1 and 2). Thus, the plasmid DNA sequence did not affect the synthesis of the defective P^{31}/σ^{29} proteins but instead supplemented their synthesis with that of normal sized P^{31} and σ^{29}. The simplest explanation for these results, given the physical similarities between P^{31} and σ^{29}, is that *spoIIG* defines the structural gene for both P^{31} and σ^{29} and that this gene is defective in the *spoIIG41* mutant. Possibly, *spoIIG41* carries a nonsense mutation leading to the synthesis of a truncated P^{31} (i.e., P^{25}) and σ^{29} (i.e., P^{21}).

We were able to verify directly that the structural gene for P^{31} is carried on pGSIIG11 by introducing the plasmid into *E. coli* and screening extracts of plasmid-containing strains with anti-P^{31}/σ^{29} antibody. *E. coli* that carried the plasmid synthesized a protein that reacted with this antibody and had the electrophoretic properties of authentic P^{31} (Fig. 4, lane 4). The *spoIIG* sequence carried by pGSIIG11 was cloned into the β-lactamase gene of the plasmid vector (12). Expression of P^{31} protein in *E. coli* may therefore be due to transcription initiating from the β-lactamase promoter. A transcriptional fusion between β-lactamase and *spoIIG* may also account for the observation that *spoIIG*-specific RNA is present in vegetative *B. subtilis* if these cells carry pGSIIG11 (unpublished data). Although pGSIIG11 leads to the synthesis of both P^{31} and σ^{29} in the *spoIIG41* strain, we were unable to detect σ^{29} in the pGSIIG11-containing *E. coli* strain. Presum-

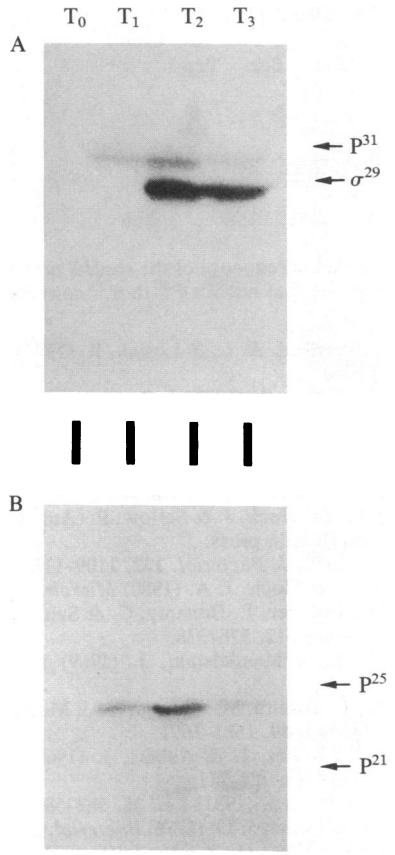

FIG. 3. Time course of P^{31}/σ^{29} accumulation. Crude protein extracts (100 μg) from SMY (Spo^+) (*A*) or BS50 (*spoIIG41*) (*B*), harvested at hourly intervals during sporulation in DS medium (15), were size-fractionated by $NaDodSO_4$/PAGE (15% acrylamide), transferred to nitrocellulose, and probed with anti-σ^{29} antibody as described in Fig. 1. T_0, the end of exponential growth; T_1, 1 hr after T_0, etc.

ably, *E. coli* lacks some necessary factor to synthesize σ^{29} or to process it from P^{31}.

DISCUSSION

It has been suggested that RNA polymerase modification is a critical part of the mechanism(s) activating genes during endospore formation in *B. subtilis* (16). The data presented in this paper support this suggestion. We report here that the RNA polymerase binding proteins P^{31} and σ^{29} are the products of a sporulation essential locus (*spoIIG*).

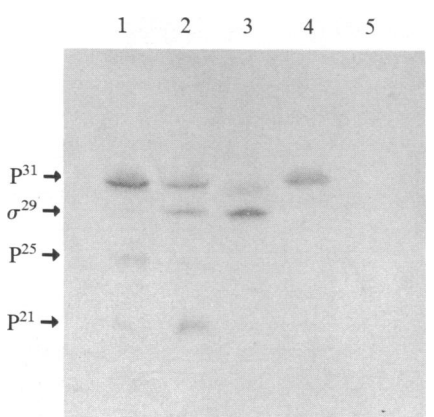

FIG. 4. Complementation of P^{31}/σ^{29} by pGSIIG11. Crude protein extracts were prepared and analyzed as described in Fig. 1. Lanes 1 and 2, BS50/pGSIIG11 in DS medium (15) T_2 and $T_{3.5}$, respectively; lane 3, SMY (Spo^+) T_2 in DS medium; lane 4, *E. coli* strain C600/pGSIIG11 mid-logarithmic phase in LB medium; lane 5, *E. coli* strain C600 mid-logarithmic phase in LB medium (15).

The development of heat-resistant endospores in *B. subtilis* occurs ≈8 hr after the onset of differentiation. Spo^- mutants blocked at stage II of development (e.g., *spoIIG*) are characterized by their ability to divide into forespore and mother cell compartments but are unable to develop beyond this point (17). This asymmetric cell division is normally accomplished by the second hour of development (T_2) (18). T_2 is also the time at which σ^{29} becomes abundant in sporulating cells (8). Thus, a defect in sporulation that does not prevent cells from reaching stage II but arrests development beyond that stage would be the expected phenotype associated with a loss in σ^{29} function (i.e., interruption of sporulation at T_2).

Previous biochemical evidence supported by the present study indicates that P^{31} and σ^{29} are the products of the same structural gene (8, 9). Since the apparent structural gene for these proteins has now been cloned and sequenced, we can survey the primary structure of the *spoIIG* product for characteristics that could bear on the speculation that σ^{29} may be derived from P^{31}. An obvious modification that could yield σ^{29} would be proteolysis at one or both of the ends of the P^{31} molecule. If the "truncated" proteins found in the *spoIIG41* mutant (P^{25} and P^{21}) are the counterparts of P^{31} and σ^{29} and not the result of proteolytic degradation of a mutant protein, it then would be argued that the amino-terminal portion of the molecule is the critical region for processing. Limited digestion of P^{31} with *S. aureus* V8 protease results first in the generation of a protein that still carries the P^{31}/σ^{29} common epitope and has an electrophoretic mobility similar to that of σ^{29} (ref. 9; unpublished results). This protein is more slowly digested to four overlapping peptides, which represent partial digestion products that are still identifiable with the anti-P^{31}/σ^{29} monoclonal antibody. These peptides appear to be identical to the digestion products obtained in a similar reaction from authentic σ^{29} (8, 9). Since the late digestion products of both P^{31} and σ^{29} appear to be indistinguishable, we would therefore assume that the M_r 29,000 initial digestion product of P^{31} is a protein that is very similar to authentic σ^{29}.

S. aureus V8 protease specifically cleaves peptide bonds on the carboxyl-terminal site of either aspartic acid or glutamic acid (19). If the predicted amino acid sequence of the *spoIIG* gene product (12) is examined, a *S. aureus* protease cleavage site can be found 24 amino acids from the amino-terminal end of the molecule (Fig. 5). Cleavage of this site would remove a fragment of M_r ≈3500 from the parent protein. We must assume that this fragment accounts for the estimated M_r 2000 difference between P^{31} and σ^{29} as well as the M_r 4000 difference between P^{25} and P^{21} that was calculated based on their gel migration properties. If our speculations are correct, formation of the active σ factor, σ^{29}, from P^{31} would involve the removal of the equivalent of this 24-amino acid fragment.

Does the hypothetically processed amino-terminal sequence of *spoIIG* (P^{31}) resemble that of other processed proteins? There is a certain similarity between it and prokaryotic signal sequences that are involved in transport across membranes. As with known signal sequences, it has a positively charged amino-terminal sequence (amino acids 1–5), a region relatively rich in hydrophobic amino acids (amino acids 6–21), and an amino acid with a small R group (serine) near the proposed cleavage site (20). This similarity is intriguing given the report that δ^1 (i.e., σ^{29}) is compartmentalized in forespores (14). Perhaps this sequence plays a role in targeting σ^{29} to the developing forespore. Alternatively, the resemblance of the region of P^{31}, suggested to be lost in processing, to prokaryotic signal sequences may

FIG. 5. Amino acid sequence of amino-terminal portion of *spoIIG* protein. The amino-terminal sequence of the *spoIIG* protein predicted by Stragier *et al.* (12) is illustrated, with an arrow indicating the predicted *S. aureus* protease site that mimics P^{31} to σ^{29} conversion.

reflect a structural feature of proteins that are proteolytically processed rather than indicate that this particular protein is transported.

Unraveling the relationship between P^{31} and σ^{29} is likely to reveal mechanisms of gene control that are uniquely suited to orchestrating the program of gene activation that occurs during sporulation. Now that the structural gene for these proteins is available, definitive experiments to probe this relationship will be possible.

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1. Shorenstein, R. G. & Losick, R. (1973) *J. Biol. Chem.* **248,** 6163–6169.
2. Haldenwang, W. G. & Losick, R. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 7000–7004.
3. Haldenwang, W. G., Lang, N. & Losick, R. (1981) *Cell* **23,** 615–624.
4. Gilman, M. Z. & Chamberlin, M. J. (1983) *Cell* **35,** 285–293.
5. Johnson, W. C., Moran, C. P., Jr., and Losick, R. (1983) *Nature (London)* **302,** 800–804.
6. Linn, T., Greenleaf, A. L. & Losick, R. (1975) *J. Biol. Chem.* **250,** 9256–9261.
7. Fukuda, R. & Doi, R. (1977) *J. Bacteriol.* **129,** 422–432.
8. Trempy, J. E., Morrison-Plummer, J. & Haldenwang, W. G. (1985) *J. Bacteriol.* **161,** 340–346.
9. Trempy, J. E., LaBell, T. L., Ray, G. L. & Haldenwang, W. G. (1985) *Proceedings of the Ninth International Spores Conference*, eds. Hoch, J. & Setlow, P. (Am. Soc. Microbiol., Washington, DC), in press.
10. Young, M. (1975) *J. Bacteriol.* **122,** 1109–1116.
11. Henner, D. J. & Hoch, J. A. (1980) *Microb. Rev.* **44,** 57–82.
12. Stragier, P., Bouvier, J., Bonamy, C. & Szulmajster, J. (1984) *Nature (London)* **312,** 376–378.
13. Sterlini, J. M. & Mandelstam, J. (1969) *Biochem. J.* **113,** 29–37.
14. Nakayama, T., Irikura, M., Kurogi, Y. & Matsuo, Y. (1981) *J. Biochem. (Tokyo)* **89,** 1681–1691.
15. Schaeffer, P., Millet, J. & Aubert, J. (1965) *J. Proc. Natl. Acad. Sci. USA* **54,** 704–711.
16. Losick, R. & Pero, J. (1981) *Cell* **25,** 582–584.
17. Piggot, P. J. & Coote, J. G. (1976) *Bacteriol. Rev.* **40,** 908–962.
18. Dawes, J. W., Kay, D. & Mandelstam, J. (1969) *J. Gen. Microbiol.* **56,** 171–179.
19. Drapeau, G. R., Boily, Y. & Houmard, J. (1972) *J. Biol. Chem.* **247,** 6720–6726.
20. Michaelis, S. & Beckwith, J. (1982) *Annu. Rev. Microbiol.* **36,** 435–465.