Appearance of a Ribonucleic Acid Polymerase-Binding Protein in Asporogenous Mutants of Bacillus subtilis

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A 70,000-dalton protein that is found in sporulating Bacillus subtilis and that binds to ribonucleic acid polymerase is present in asporogenous mutants that proceed to or beyond stage II of sporulation, but is absent from mutants blocked at stage zero.

The loss of vegetative sigma factor activity early during sporulation of Bacillus subtilis causes a change in the template specificity of ribonucleic acid (RNA) polymerase [deoxyribonucleic acid-dependent RNA polymerase (nucleotide triphosphate:RNA nucleotidyldetransferase; EC 2.7.7.6)] (4–6, 9). Brevet and Sonenshein have shown that RNA polymerase in mutants blocked at the earliest stage of sporulation, stage zero, does not undergo this change in template specificity (1). The loss of sigma factor activity occurs normally, however, in asporogenous mutants blocked at stages II, III, or IV of spore formation.

We have reported previously the isolation from sporulating B. subtilis of a 70,000-dalton protein that apparently binds to RNA polymerase (2). This binding protein appears during the first hours of sporulation and persists until late in the sporulation process. This paper reports that the appearance of the binding protein, like the loss of sigma activity, takes place normally during stationary-phase growth in asporogenous mutants blocked at stage II or later, but that the binding protein does not appear in mutants blocked at stage zero.

MATERIALS AND METHODS

Bacterial strains. B. subtilis Marburg strains 168 and SMY are sporulating strains from which the asporogenous mutants were derived (3). All strains were kindly supplied by P. Schaeffer.

Culture medium. The medium used (referred to as DSM) was essentially the nutrient broth medium described by Schaeffer et al. (8). Nutrient broth (Difco) (8 g), KCl (1.0 g), MgSO₄·7 H₂O (0.25 g), and 1 liter of water were mixed, adjusted to pH 7.0 to 7.2, and autoclaved. Then autoclaved solutions of the following were added sterilily: Ca(NO₃)₂, to make 10⁻² M; MnCl₂, to make 10⁻⁴ M; and FeSO₄ to make 10⁻⁴ M.

Growth conditions. Overnight cultures (100 ml) were grown at 30°C; the cells were harvested by centrifugation at room temperature for 2 min at 12,000 g and resuspended in 10 ml of DSM medium at room temperature. A 6-ml amount was used to inoculate a 500-ml final culture grown at 37°C with vigorous rotary agitation. Growth was followed turbidometrically (Klett, green filter). Tₙ of sporulation refers to n hours after the end of exponential growth (1).

Detection of binding protein by antibody precipitation of RNA polymerase. Preparation of a radioactively labeled cell extract, precipitation of RNA polymerase by rabbit anti-RNA polymerase serum, and analysis of the precipitates by electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate (SDS gel electrophoresis) have been described previously (2). The antiserum was prepared as described by Linn et al. (4). In measuring amounts of proteins from densitometer scans of stained gels, we assumed that each protein species binds the same amount of dye per gram of protein.

RESULTS

Appearance of the binding protein in asporogenous mutants. Since the 70,000-dalton binding protein was discovered in B. subtilis strain 3610, we first checked for the presence of the binding protein in the Marburg strains 168 and SMY, parents of the asporogenous mutants used in this study. As previously described (2), an extract was prepared from radioactive tryptophan-labeled cells, and RNA polymerase was precipitated by the addition of rabbit antiserum prepared against purified vegetative core RNA polymerase. The solubilized precipitate was analyzed by SDS gel electrophoresis to determine the presence of the binding protein.

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Antibody added to an extract of radioactively labeled sporulating cells of either strain 168 or strain SMY precipitated not only the β and α subunits characteristic of RNA polymerase from vegetative cells, but, in addition, the 70,000-dalton protein first identified in strain 3610. In strain 168, grown on DSM medium, the binding protein appeared within 1 h after the end of logarithmic growth (T1 of sporulation) (Table 1), more than an hour earlier than in strain 3610 grown on 121B medium (2). The earlier appearance of the binding protein in strain 168 correlates with the observation that, under our growth conditions, refractile spores appeared earlier in strains 168 and SMY (50% refractivity by T1) than in strain 3610 (50% refractivity by T1). The binding protein persisted throughout sporulation and was present in dormant spores (Table 1).

To test for the presence of the binding protein in the asporogenous strains, we precipitated RNA polymerase from extracts of radioactively labeled cells harvested after the end of logarithmic growth. The antibody precipitates of extracts from three different stage zero-blocked mutants harvested at T1 contained essentially only the β and α subunits of RNA polymerase (Fig. 1). The binding protein was also absent from antibody precipitates of extracts from Spo0c cells harvested at T1 or T2 (not shown). In contrast, the precipitates of extracts from mutants that proceed to stage II or beyond contained the 70,000-dalton binding protein (Fig. 1).

The large amount of radioactivity in the binding protein in the precipitates from SpoII, SpoIII, and SpoIV mutants is due to labeling the cells with radioactive tryptophan, since the binding protein is apparently much richer in tryptophan than either the β or α subunits, as illustrated below.

The absence of the binding protein in antibody precipitates of extracts of stage zero-blocked mutants indicates either that the binding protein is not present in these strains or that some component in extracts of the stage zero-blocked mutants interferes with its precipitation by the anti-polymerase serum. To distinguish between these two possibilities, we precipitated polymerase from an extract of a mixture of Spo0c cells radioactively labeled with 3H-tryptophan and Spo+ cells labeled with 14C-tryptophan, both harvested at T1. The binding protein from the Spo+ cells precipitated normally even in the presence of the Spo- cell extract (Fig. 2).

The experiment of Fig. 2 also indicated the presence of a low-molecular-weight species containing radioactivity derived from the wild-type sporulating cells but not from the mutant Spo0c cells. This radioactive species was analyzed further on an SDS gel containing 12.5% acrylamide to determine the number and molecular size of its components. It consisted of a major component of approximately 18,000 daltons which contained only the 14C of the sporulating cells, and a minor, slightly larger component containing both isotopes. From a stained gel, the number of 18,000-dalton molecules present in the precipitate was estimated to be about 1.5 per core polymerase. Whether the presence of this small polypeptide is due to its binding specifically to polymerase is not yet known.

Stoichiometry and radioactive labeling of the binding protein. Gels that were first stained and then sliced indicate that the polymerase-binding protein of strain 168 or strain 3610 (2) is much richer in tryptophan than are the core subunits of polymerase. To investigate further the specific radioactivity of the 70,000-dalton protein, we subjected the antibody precipitates of radioactive sporulation and vegetative polymerases to SDS gel electrophoresis for extended times. Densitometer tracings of stained gels of antibody precipitates of sporulation polymerase indicated that the extended
electrophoresis resolved two protein species of about the same mobility as the radioactive 70,000-dalton binding protein (Fig. 3B). Both of these species were absent in precipitates of polymerase from vegetative cells or mutants blocked at stage zero (Fig. 3A). By slicing between these two proteins, we found that only the higher-molecular-weight species was tryptophan rich and contained about eight times as much radioactivity per gram of protein as did the β subunits. Even though the radioactivity in the 70,000-dalton protein was greater than that in the β subunits, we calculated from the densitometer tracing that the antibody precipitate contained only 0.6 mol of the binding protein for each mole of core enzyme.

The antibody precipitate from sporulating cells (Fig. 3B) contained several polypeptides not present in the precipitate from vegetative (not shown) or stage zero-blocked cells (Fig. 3A) in addition to the tryptophan-rich binding protein. It is not yet known whether these other species are also synthesized de novo during

**Fig. 1.** SDS gel electrophoresis of antibody precipitates of RNA polymerase from radioactively labeled Spo− mutants. Mutant strains were grown in 500-ml cultures as described in Materials and Methods, 1 or 2 mCi of "H-tryptophan was added early during vegetative growth, and the cells were harvested 4 h after the end of logarithmic growth. Antibody precipitates of RNA polymerase from extracts of the cells were analyzed, as described, by electrophoresis on 5% acrylamide gels containing SDS.

**Fig. 2.** SDS gel electrophoresis of an antibody precipitate of RNA polymerase from an extract of a mixture of Spo− and Spo+ cells. Two grams of "H-labeled Spo− cells (strain Spo0c grown and labeled as described in Fig. 1) was mixed with 2 g of "C-labeled Spo+ cells (grown as in Fig. 1 but labeled with 0.1 mCi of "C-tryptophan; 43 mCi/mmol; New England Nuclear Corp.). An extract of the cells was prepared as described, and RNA polymerase was precipitated from the extract with the antiserum and analyzed as for Fig. 1.
Fig. 3. Differential labeling of binding protein and core polymerase with $^3$H-tryptophan. (A) A portion of the antibody precipitate from an extract of Spoob cells, grown as described in the legend to Fig. 1, was subjected to electrophoresis for 13.5 h at 7.5 mA per gel in an SDS gel containing 7.5% acrylamide (0.6 by 9 cm). The gel was stained with Coomassie blue and scanned at 550 nm in a densitometer; the gel was then sliced, and the radioactivity in each slice was measured as described. (B) A portion of the antibody precipitate from an extract of the Spo+ cells (T1 time point) described in Table 1 was analyzed as in (A).

sporulation or are produced in vitro by proteolysis of polymerase (4).

DISCUSSION

A study of several different asporogenous mutants of B. subtilis indicates that the RNA polymerase-binding protein is present during stationary-phase growth in mutants that proceed to stage II, III, or IV of sporulation. The binding protein is not present, however, in antibody precipitates of polymerase from mutants blocked at stage zero of sporulation. Precipitation of the binding protein from an extract of a mixture of wild-type sporulating cells and stage zero-blocked mutant cells separately labeled with two different radioisotopes indicates that the mutant extract does not interfere with antibody precipitation of the binding protein (Fig. 2). Thus, mutants blocked at stage zero apparently do not synthesize the 70,000-dalton species.

Brevet and Sonenshein (1) have recently studied the template specificity of RNA polymerase in a series of asporogenous mutants including those studied in this report. They showed that the specificity change characteristic of RNA polymerase from wild-type cells undergoing sporulation does not occur in mutants blocked at stage zero, but occurs normally in mutants that proceed to stage II or beyond (Table 2). Thus, both the change in template specificity and the appearance of the polymerase-binding protein are under control of events occurring early during the sporulation process.

Previous studies have shown that the binding protein is not derived from in vitro proteolysis of a vegetative protein (2). In addition, the high tryptophan content of the binding protein (Fig. 3) excludes the possibility that it is derived from...
a $\beta$ subunit of polymerase in vivo, since a 70,000-dalton fragment of a $\beta$ subunit (150,000 daltons) could not be eight times as rich in tryptophan as the $\beta$ subunit itself, even if it contained all the tryptophan residues of that $\beta$ subunit.

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LITERATURE CITED