Proc. Nat. Acad. Sci. USA
Vol. 69, No. 2, pp. 407–411, February 1972

In Vitro Synthesis of Ribosomal RNA by *Bacillus subtilis* RNA Polymerase

(sporulation/hybridization/heavy strand of DNA)

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Communicated by Jack L. Strominger, November 22, 1971

ABSTRACT **Two kinds of hybridization competition experiments show that *Bacillus subtilis* RNA polymerase synthesizes ribosomal RNA (rRNA) *in vitro* with *B. subtilis* DNA as a template. First, RNA synthesized *in vitro* competes with the hybridization of [^{32}P]rRNA synthesized *in vivo* to the heavy strand of *B. subtilis* DNA. Second, unlabeled rRNA synthesized *in vivo* competes with the hybridization of [^{3}H]RNA synthesized *in vitro* to denatured DNA or heavy-strand DNA, but not to light-strand DNA. The ability of RNA polymerase holoenzyme to synthesize rRNA *in vitro* is not lost after extensive purification. RNA polymerase core enzyme, however, which is missing the σ factor, synthesizes little rRNA *in vitro*.**

RNA polymerase purified from wild-type sporulating cells synthesizes little rRNA *in vitro*, while the *in vitro* synthesis of rRNA by RNA polymerase from stationary phase cells of the sporulation-defective mutant *rfr*-10 is apparently unimpaired.

While the ribosomal RNA (rRNA) genes of *Bacillus subtilis* are actively transcribed during logarithmic growth, the synthesis of rRNA is abruptly turned off early during the process of sporulation (1). This turn off is prevented, however, in a mutant known as *rfr* 10, which is resistant to the drug rifampicin (1, 2). *Rfr* 10 cells sporulate with less than 5% the frequency of wild-type cells (2). To test the idea that the turn off of rRNA genes is due to the alteration of RNA polymerase during spore formation (2–5), we have looked for the synthesis of rRNA *in vitro*. Since rRNA accounts for 15–45% of the total RNA synthesized at any given time in rapidly growing bacteria (6–9), it seemed likely that rRNA would comprise a substantial fraction of RNA transcribed *in vitro* from *B. subtilis* DNA. We report here that highly purified RNA polymerase from vegetative cells of *B. subtilis* initiates the synthesis of rRNA *in vitro*. The rRNA is copied from the heavy (H) strand of *B. subtilis* DNA and accounts for at least 8% of the RNA synthesized *in vitro*. We also report experiments on the *in vitro* synthesis of rRNA by RNA polymerase that is purified from wild-type sporulating cells and from stationary phase cells of the oligosporogenous mutant *rfr* 10. Haseltine (manuscript in preparation) has independently discovered that purified *Escherichia coli* RNA polymerase also synthesizes rRNA *in vitro*.

MATERIALS AND METHODS

Bacterial Strains. Wild-type *B. subtilis* is strain NCTC 3610 (ATCC 6051), a Marburg strain. *Rfr* 10 and *Rfr* 3Y are rifampicin-resistant mutants of strain NCTC 3610 that are defective in sporulation (2).

Preparation of DNA. DNA was extracted from *B. subtilis* by the method of Saito and Miura (10). The DNA was precipitated twice with isopropanol to insure the removal of RNA. Alkali-denatured *B. subtilis* DNA was separated into heavy (H)- and light (L)-strand complementary fractions by methylated albumin-Kieselguhr column chromatography according to the method of Rudner *et al.* (11).

Preparation of [^{32}P]rRNA and Unlabeled rRNA. [^{32}P]RNA was extracted from radioactively labeled cells by the method of Solymosy (12). [^{32}P]rRNA was purified by zone centrifugation. Unlabeled rRNA was extracted from purified ribosomes.

In Vitro Synthesis of RNA. The reaction mixtures contained 0.04 M Tris·HCl (pH 7.9); 0.01 M $MgCl_2$; 1 mM EDTA; 1 mM dithiothreitol; 0.5 mg/ml bovine serum albumin; 0.15 mM ATP, 0.15 mM CTP, 0.15 mM GTP, and 0.05 mM [^{3}H]UTP (specific activity as indicated in the *legends*); 0.4 mM potassium phosphate; 16 μg/ml of *B. subtilis* DNA; indicated amounts of RNA polymerase. After incubation at 37°C for 15 min, RNA was extracted with phenol and precipitated with ethanol as described (13).

Hybridizations in Liquid. Hybridization reactions were performed at 69°C for 5 hr with H- and L-strand DNA and 3 hr with alkali-denatured DNA. After hybridization, the reaction mixtures were incubated with 0.8 ml of heat-treated RNase A (Sigma, 5 × crystallized) in 2 × SSC (0.30 M NaCl–0.030 M sodium citrate) (10 μg/ml) for 30 min at 34°C. Hybrids were collected on Schleicher and Schuell B-6 filters and washed with 60 ml of buffer containing 0.01 M Tris·HCl (pH 7.5) and 0.5 M KCl. After drying, the radioactivity retained on the filters was measured in a liquid scintillation counter. For hybrids containing radioactivity from both ^{3}H and ^{32}P, correction was made for the 1% crossover of ^{32}P into the ^{3}H channel.

RESULTS

To test for the synthesis of rRNA *in vitro*, first partially purified RNA polymerase was used to transcribe *B. subtilis* DNA. The enzyme was purified from vegetative cells of *B. subtilis* 3610 after sonication and high speed centrifugation by phase extraction in the polyethyleneglycol–dextran sulfate system of Babinet (15), followed by Agarose gel filtration on a column of Bio-Gel A-1.5m. The A_{280}:A_{260} ratio of the enzyme after this purification procedure is 1.6, which indicates that nucleic acids have been removed. The enzyme has a specific

Abbreviations: SSC, 0.15 M NaCl and 0.015 M sodium citrate; H and L DNA, heavy and light strands of DNA.

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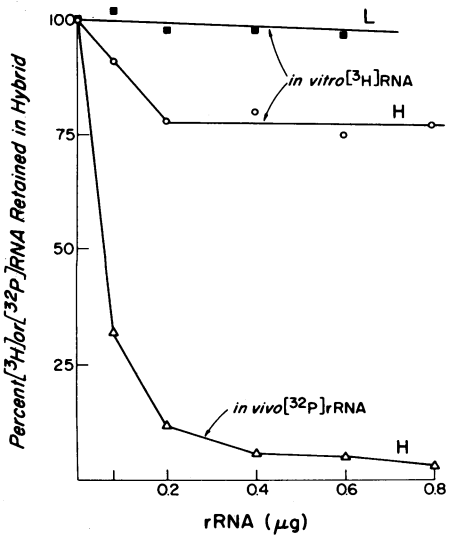

FIG. 1. Competition of the hybridization of *in vitro* synthesized [^{3}H]RNA or *in vivo* synthesized [^{32}P]rRNA to the complementary fractions of *B. subtilis* DNA by unlabeled rRNA. [^{3}H]RNA was synthesized as described in *Methods*. The reaction mixture contained [^{3}H]UTP at a specific activity of 1000 Ci/mol and 34 μg/ml of vegetative Agarose enzyme. Hybridization reactions contained 7100 cpm of *in vitro* synthesized [^{3}H]RNA, 2900 cpm of [^{32}P]rRNA (965 cpm/ng), 2.5 μg of the L- or H-strand DNA, and the indicated amounts of unlabeled rRNA, in a total volume of 170 μl of 2 × SSC. The H-strand DNA used here was actually a late-eluting fraction of the H-strand peak, which was more than three times enriched for rRNA genes, compared to the total H-strand DNA (17). Competition between [^{3}H]RNA and unlabeled rRNA during hybridization with L-strand DNA (■——■). 22% of the input [^{3}H]RNA and less than 0.5% of the input [^{32}P]rRNA hybridized in the absence of competitor. Competition between [^{3}H]RNA (O—O) or [^{32}P]-rRNA (Δ—Δ) and unlabeled rRNA during hybridization with H-strand DNA. 23% of the input [^{3}H]RNA and 66% of the input [^{32}P]rRNA hybridized in the absence of competitor. In each case, the amount of radioactive RNA that hybridized in the absence of competitor is the mean of duplicate samples. A background of 175 cpm was subtracted from all [^{3}H]RNA values and a background of 35 cpm was substracted from all [^{32}P]rRNA values.

activity of 96 units/mg of protein (a unit is 1 nmol of [^{14}C]-AMP incorporated in 10 min at 37°C) with φe DNA as template, 33 units/mg with poly(dAT) as template, and 8 units/mg with *B. subtilis* DNA as template. Transcription from all these templates is completely inhibited by rifampicin. Since transcription of *B. subtilis* DNA is strongly inhibited by salt (unpublished observations), the concentration of KCl in the reaction mixture was less than 40 mM.

To test for rRNA sequences in the RNA synthesized *in vitro* by RNA polymerase, we have taken advantage of the finding that *B. subtilis* DNA can be separated into two complementary fractions known as H- and L-strand DNA by methylated albumin-Kieselguhr chromatography (11). Hybridization to the separated strands of DNA is much more efficient than to denatured DNA. Moreover, Oishi (16) and Margulies *et al.* (17) have shown that rRNA hybridizes exclusively with the H-strand DNA. In the experiment of Fig. 1, *in vitro* synthesized [^{3}H]RNA, which had been mixed with a minute amount of *in vivo* synthesized [^{32}P]rRNA as an internal standard, was hybridized to H-strand DNA in the presence of increasing amounts of unlabeled rRNA. Unlabeled rRNA competes completely with the hybridization of [^{32}P]-rRNA and 23% with the hybridization of *in vitro* synthesized [^{3}H]RNA in the presence (Fig. 1) or absence (not shown) of the [^{32}P]rRNA. As a control, the experiment of Fig. 1 shows that unlabeled rRNA does not cause detectable competition with the hybridization of *in vitro* synthesized [^{3}H]-RNA to L-strand DNA. Moreover, the [^{32}P]rRNA present in the *in vitro* synthesized [^{3}H]RNA did not hybridize to the L strand (not shown). Thus, partially purified RNA polymerase copies rRNA from the H strand of *B. subtilis* DNA.

Since 23% of the *in vitro* synthesized RNA hybridized to the H strand in this experiment and since 23% of this RNA is competed by rRNA, then a minimum of 5.3% of the *in vitro* synthesized product contains rRNA sequences. If the rRNA synthesized *in vitro* hybridizes with the same efficiency as the [^{32}P]rRNA (66%), then about 8% of the *in vitro* product is rRNA.

In vitro synthesized RNA competes with hybridization of [^{32}P]rRNA to H-strand DNA

As an independent test for the *in vitro* synthesis of rRNA, *in vivo* [^{32}P]rRNA was hybridized to H-strand DNA in the presence of increasing amounts of *in vitro* synthesized RNA. If RNA polymerase is synthesizing rRNA *in vitro*, then the *in vitro* product should compete with the hybridization of [^{32}P]rRNA. The experiment of Fig. 2 shows that in fact 180 ng of *in vitro* RNA causes 90% competition. As a control, a mock RNA synthesis reaction was performed in the presence of rifampicin, an inhibitor of RNA polymerase. The experiment of Fig. 2 shows that the extract from the rifampicin re-

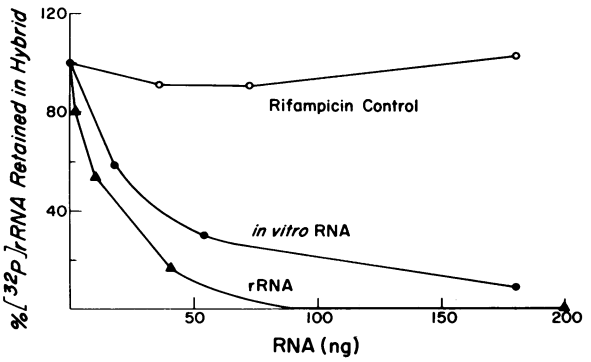

FIG. 2. Hybridization competition of [^{32}P]rRNA by *in vitro* synthesized RNA. Hybridization reaction mixtures contained in 150 μl of 2 × SSC: 2500 cpm of [^{32}P]rRNA (2000 cpm/ng), 0.2 μg of H-strand DNA, and increasing amounts of unlabeled rRNA or *in vitro* RNA. The [^{32}P]rRNA that hybridized in the absence of competitor was 40% of the input. ▲——▲, Competition with unlabeled rRNA. ●——●, Competition with *in vitro* synthesized RNA. RNA was synthesized *in vitro* in a reaction containing 136 μg/ml of vegetative Agarose enzyme and [^{3}H]UTP at a specific activity of 80 Ci/mol. The number of nanograms of *in vitro* synthesized RNA used as competitor was determined from the radioactivity of the *in vitro* product. O——O, Competition with extract from the rifampicin control. A parallel reaction containing 4 μg/ml of rifampicin was used as a control. The amounts of extract from the rifampicin reaction used as competitor correspond to the indicated nanograms of RNA, as determined for the reaction in the absence of rifampicin. A background of 90 cpm has been subtracted from all values.

action does not compete with the hybridization of [^{32}P]rRNA to H-strand DNA. Thus, the competition with [^{32}P]rRNA is not due to rRNA contaminating the enzyme or the template but requires the *in vitro* synthesis of RNA. As we shall see, the competition with the hybridization of [^{32}P]rRNA to H-strand DNA by *in vitro* RNA is specific since RNA synthesized *in vitro* by core polymerase or sporulation polymerase competes poorly with [^{32}P]rRNA.

We calculate from the experiment of Fig. 2 that 30% of the *in vitro* synthesized RNA is rRNA, since about three times as much *in vitro* synthesized RNA is required to give the same amount of competition as caused by *in vivo* synthesized rRNA. We feel, however, that 30% is an overestimate because the unlabeled *in vivo* rRNA, which was extracted from purified ribosomes, is in small pieces and hybridizes with lower efficiency that the *in vitro* synthesized RNA.

Highly purified RNA polymerase synthesizes rRNA *in vitro*

To test whether only the holoenzyme, and not additional factors, is needed for transcription of rRNA genes, RNA synthesized *in vitro* by extensively purified RNA polymerase was tested for the presence of rRNA sequences. Vegetative RNA polymerase was purified from *B. subtilis* 3610 *rfr* 3Y. Cells were disrupted by sonication, and the extract, after high speed centrifugation and ammonium sulfate fractionation, was applied to a DEAE-cellulose column. RNA polymerase was eluted with a 0.1–0.4 M KCl gradient and applied to a phage ϕe DNA–cellulose column (4). After elution from the DNA-cellulose column, RNA polymerase is about 90% pure, as judged by neutral polyacrylamide disc gel electrophoresis and sodium dodecyl sulfate (SDS) gel electrophoresis (4). The enzyme contains small amounts of a 100,000-dalton polypeptide, in addition to β', β, α, and σ. This enzyme has a specific activity of 346 units/mg with ϕe DNA as template, 164 units/mg with poly(dAT) as template, and 17 units/mg with *B. subtilis* DNA as template.

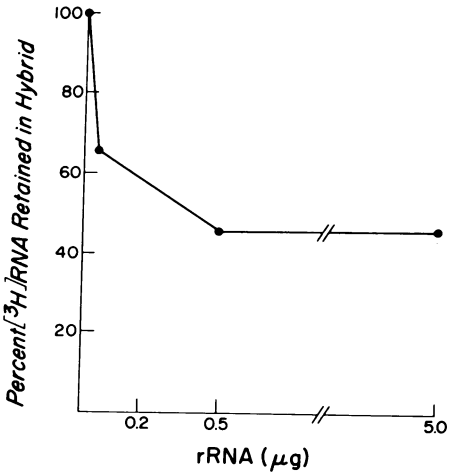

FIG. 3. Competition of the hybridization of *in vitro* synthesized [^{3}H]RNA to DNA immobilized on a filter by rRNA. *In vitro* RNA was synthesized in a 0.5-ml reaction containing 7 μg of vegetative enzyme that was purified by DNA–cellulose chromatography and [^{3}H]UTP at a specific activity of 1000 Ci/mol. Hybridization reactions contained in 200 μl of 2 × SSC: 12,000 cpm of *in vitro* synthesized RNA, increasing amounts of unlabeled rRNA, and a filter sector containing 2.5 μg of denatured DNA prepared as described by Gillespie and Spiegelman (14). Hybridizations were incubated for 24 hr at 67°C. The RNA that hybridized in the absence of competitor was 7% of the input. A background of 50 cpm was determined for filter sectors not containing DNA and subtracted from all values.

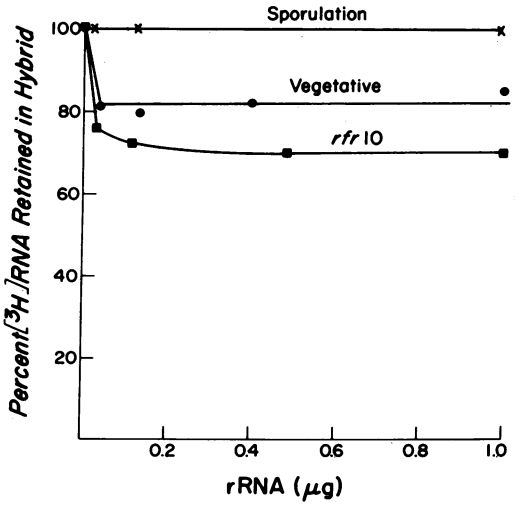

FIG. 4. Competition of the hybridization of *in vitro* [^{3}H]RNA to denatured DNA in liquid by rRNA. *In vitro* RNA was synthesized in 1 ml reactions containing [^{3}H]UTP (2000 Ci/mol) and either 7 μg of the vegetative enzyme that was purified by DNA–cellulose chromatography, 200 μg of $t_{4.5}$ sporulation polymerase, or 200 μg of RNA polymerase from $t_{4.5}$ stationary phase cells of mutant *rfr* 10. Hybridization reactions contained in 140 μl of 2 × SSC: 1.2 μg of alkali-denatured DNA, *in vitro* [^{3}H]RNA, and increasing amounts of unlabeled rRNA. ●——●, Competition of the RNA synthesized *in vitro* by vegetative enzyme that was purified by DNA–cellulose chromatography. The input was 54,000 cpm and 7% of the input hybridized in the absence of competitor. A background of 750 cpm has been subtracted from all values. ×——×, Competition of RNA synthesized *in vitro* by sporulation polymerase. The input was 28,000 cpm and 6% hybridized in the absence of competitor. A background of 750 cpm has been subtracted. ■——■, Competition of RNA synthesized *in vitro* by stationary phase *rfr* 10 polymerase. The input was 33,750 cpm and 4% hybridized in the absence of competitor. A background of 280 cpm has been subtracted. In all cases, addition of unlabeled rRNA did not reduce the background.

To test for the synthesis of rRNA *in vitro*, radioactively labeled RNA synthesized by the enzyme purified on a DNA–cellulose column was annealed in the presence of increasing amounts of unlabeled rRNA, to denatured *B. subtilis* DNA that was either immobilized on a filter or in liquid. The experiment of Fig. 3 shows that rRNA causes about 50% competition with the hybridization of the *in vitro* RNA to denatured DNA that is immobilized on a filter, and 19% competition with the hybridization of *in vitro* synthesized RNA to denatured DNA in liquid (Fig. 4). The competition by rRNA is specific since rRNA did not compete with hybridization to L-strand DNA in the experiment of Fig. 1. Moreover, rRNA does not compete with the hybridization of radioactive RNA transcribed from ϕe DNA to denatured ϕe DNA, and as will be shown below, rRNA does not compete with the hybridization of RNA synthesized by sporulation polymerase to denatured *B. subtilis* DNA (Fig. 4).

Another hybridization method for the detection of rRNA sequences in *in vitro* synthesized RNA is the "blocking" pro-

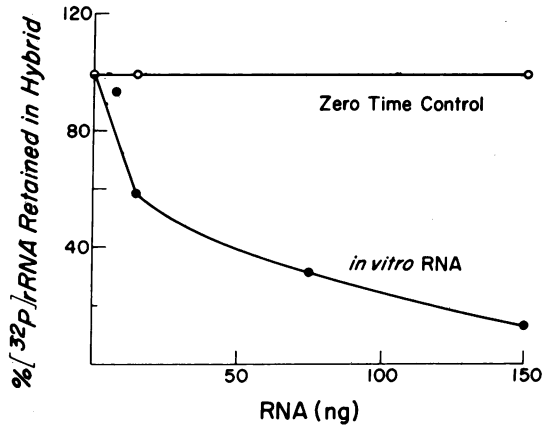

FIG. 5. Hybridization competition of [^{32}P]rRNA by RNA synthesized *in vitro* by vegetative enzyme that was purified by DNA–cellulose chromatography. ●——●, RNA synthesized *in vitro* in a reaction containing 15 μg/ml of vegetative DNA–cellulose enzyme, [^{3}H]UTP (500 Ci/mol), and incubated 15 min at 37°C. The nanograms of *in vitro* RNA used as competitor was determined from the radioactivity of the *in vitro* product. Hybridization reactions contained in 175 μl of 2 × SSC: 0.2 μg of H-strand DNA, 1420 cpm of [^{32}P]rRNA (2300 cpm/ng), and increasing amounts of *in vitro* RNA. In the absence of competitor, 45% of the input [^{32}P]rRNA hybridized. O——O, A parallel reaction mixture that was not incubated at 37°C was used for the zero time control. The amounts of extract from the zero time control used as competitor correspond to the indicated nanograms of *in vitro* synthesized RNA, as determined for the 15-min reactions.

cedure of Gillispie (18). This method ensures that the competition of *in vitro* synthesized [^{3}H]RNA is due to rRNA competitor that has actually hybridized to the DNA. First, DNA filters were preannealed with various amounts of rRNA. Unhybridized competitor was removed by treatment with RNase, followed by extensive washing to remove the RNase. Next, *in vitro* synthesized [^{3}H]RNA was hybridized to the previously annealed filters. We found that 30% less *in vitro* [^{3}H]RNA hybridized to DNA–filters that were previously annealed with rRNA than to control–filters previously annealed in the absence of competitor. Thus, prior "blocking" of DNA by rRNA competes with the hybridization of the *in vitro* product.

As a final test of whether highly purified enzyme synthesizes rRNA, [^{32}P]rRNA was hybridized to H-strand DNA in the presence of increasing amounts RNA synthesized *in vitro* by the enzyme purified by DNA–cellulose chromatography. The experiment of Fig. 5 shows that *in vitro* synthesized RNA completely competes with [^{32}P]rRNA. We calculate that about 15% of the *in vitro* RNA is rRNA. This competition is not due to a contaminant of the enzyme, since extract from a zero time control that was incubated at 0°C instead of 37°C did not cause competition. Thus, vegetative RNA polymerase synthesizes rRNA even after extensive purification.

Vegetative core polymerase synthesizes little rRNA *in vitro*

Since the σ factor of RNA polymerase is needed for correct initiation of transcription from several well defined templates, we expected that the synthesis of rRNA would be dependent on *B. subtilis* σ factor. To test this idea, highly purified core polymerase was prepared from holoenzyme by phosphocellulose chromatography. This procedure dissociates both *E. coli* and *B. subtilis* holoenzyme into core polymerase and σ factor. The experiment of Fig. 6 shows that the *in vitro* synthesized product of core polymerase competes poorly with [^{32}P]rRNA. We calculate that about 1% of the RNA synthesized by core enzyme contains rRNA sequences. Furthermore, preliminary experiments indicate that addition of partially purified σ factor to the core enzyme stimulates rRNA synthesis. These findings suggest that rRNA synthesis is highly dependent on σ factor. It is not excluded, however, that rRNA synthesis requires a protein other than σ that is present as a contaminant in the enzyme purified by the DNA–cellulose chromatography and that is removed by phosphocellulose chromatography.

Sporulation polymerase synthesizes little rRNA *in vitro*

As a first test of the idea that alteration of RNA polymerase is responsible for the turn off of rRNA synthesis during sporulation, polymerase was purified from wild-type and *rfr* 10 cells, 4.5 hr after the end of logarithmic growth. After sonication of the cells and high speed centrifugation, the RNA polymerase was partially purified by phase extraction, a procedure that removes nucleic acids. Next, the enzyme was applied to a DEAE-cellulose column and eluted step-wise between concentrations of 0.13 and 0.24 M KCl. The A_{280}:A_{260} ratio for the DEAE enzyme was 1.6. The ratio of activity with φe DNA as a template to the activity with poly(dAT) as a template was about 0.1 for the sporulation enzyme and 0.9 for the stationary phase *rfr* 10 enzyme.

The experiment of Fig. 4 shows that rRNA does not compete with the hybridization of the RNA synthesized *in vitro* by sporulation polymerase to denatured DNA. Ribosomal RNA does, however, compete with the hybridization of the *in vitro* product of the *rfr* 10 enzyme. The finding that rRNA does not compete with the hybridization of the *in vitro* product of the sporulation enzyme to denatured DNA is not due to a contaminant of the sporulation polymerase that either prevents the synthesis or detection of rRNA, since a mixture of

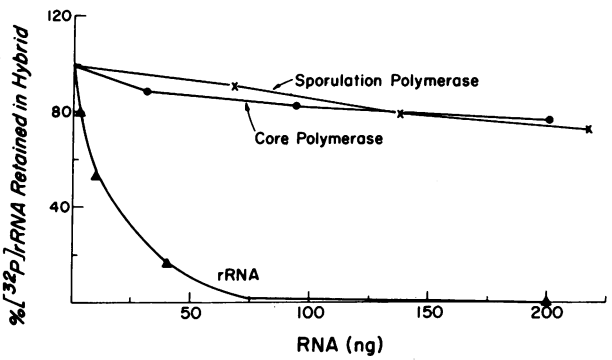

FIG. 6. Hybridization competition of [^{32}P]rRNA by RNA synthesized *in vitro* by vegetative core polymerase and sporulation polymerase. Hybridization reactions contained in 150 μl of 2 × SSC: 1837 cpm of [^{32}P]rRNA (1550 cpm/ng), 0.2 μg H-strand DNA, and increasing amounts of RNA synthesized *in vitro* either by vegetative core polymerase (●——●) or by sporulation polymerase (×——×). The [^{32}P]rRNA that hybridized in the absence of competitor was 37% of the input. A background of 66 cpm has been subtracted from all values. The sporulation polymerase was purified by phase-extraction, Agarose gel filtration, and protamine sulfate precipitation.

sporulation enzyme and *rfr* 10 or vegetative polymerase synthesizes rRNA.

Finally, the experiment of Fig. 6 shows that the *in vitro* synthesized product of the sporulation polymerase competes poorly with the hybridization of [^{32}P]rRNA. We calculate that about 1% of the RNA synthesized by sporulation enzyme is rRNA. Thus, sporulation RNA polymerase synthesizes about the same amount of rRNA as the vegetative enzyme that is purified by phosphocellulose chromatography. Moreover, by using this assay, we find that about 20% of the product of the stationary phase *rfr* 10 enzyme contains rRNA sequences.

DISCUSSION

The rRNA cistrons of *B. subtilis* comprise about 0.4% of the genome (17, 16). Yet rRNA accounts for at least 8% of the product of *in vitro* transcription of *B. subtilis* DNA. This means that RNA polymerase initiates preferentially near the rRNA cistrons. We do not know, however, whether RNA polymerase initiates accurately at the rRNA promoters. It could be imagined, for instance, that RNA polymerase initiates at the promoter for another neighboring cistron and that the *in vitro* synthesis of rRNA is due to read-through from the adjacent cistron. Other experiments, however, based on asymmetry of transcription have indicated that *B. subtilis* RNA polymerase initiates quite accurately on *B. subtilis* DNA *in vitro* (Pero and Losick, manuscript in preparation and discussed in ref. 19).

It should be noted that Pettijohn *et al.* (6) and Travers *et al.* (13) were unable to detect initiation of rRNA synthesis *in vitro* by highly purified *E. coli* RNA polymerase. Travers *et al.* (13) reported that an additional factor, ψ, which does not purify with polymerase, was needed for rRNA synthesis. However, Haseltine (manuscript in preparation) now finds that highly purified *E. coli* polymerase, like the *B. subtilis* enzyme, synthesizes rRNA *in vitro* in the apparent absence of additional factors. Moreover, Haseltine finds that although ψ factor stimulates total RNA synthesis from *E. coli* DNA, ψ factor does not increase the fraction of rRNA synthesized.

We have observed that RNA polymerase purified from sporulating cells of *B. subtilis* fails to synthesize rRNA *in vitro*. The failure to synthesize rRNA could be due to the loss of vegetative template specificity by RNA polymerase early during spore formation (3). Sporulation RNA polymerase is missing the function of the σ factor and contains an altered β subunit (4). Thus, the turn off of the rRNA genes in sporulating cells (1) could be due to the alteration of RNA polymerase. In support of this idea, a rifampicin-resistant, sporulation-deficient mutant of *B. subtilis rfr* 10 continues to synthesize rRNA after the end of logarithmic growth (1). RNA polymerase in the *rfr* 10 mutant retains vegetative template specificity during stationary phase (2) and synthesizes rRNA *in vitro*. Before concluding, however, that alteration of RNA polymerase is the mechanism for turning off rRNA genes in sporulating cells, it will be necessary to demonstrate that sporulation RNA polymerase synthesizes sporulation-specific RNA *in vitro*.

We thank Professors J. L. Strominger and P. Schaeffer for their interest and the use of their facilities, A. L. Sonenshein, J. Legault-Demare, L. Hirschbein, and W. Haseltine for helpful discussions, and A. Greenleaf for purification of the sporulation polymerase used in the experiment of Fig. 6. J. P. is a postdoctoral fellow of the American Cancer Society. R. G. S is a predoctoral fellow of the National Science Foundation, and R. L. was a Junior Fellow of the Harvard Society of Fellows. This work was supported by National Science Foundation grant GB-27610.

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