ABSTRACT A protein–DNA transcription complex, isolated from logarithmic-phase cells of *Bacillus subtilis*, is active in the initiation of new rounds of RNA synthesis *in vitro*. Transcription directed by the endogenous DNA of the complex is sensitive to inhibitors of initiation. In addition, the effect of addition of a competing template indicates that RNA polymerase in the complex is bound to endogenous DNA in a dissociable, preinitiation state. Hybridization-competition analysis of the RNA product obtained from the *in vitro* reaction indicates that it is greatly enriched for ribosomal RNA sequences. This initiating complex should be useful for the study of regulation of expression of ribosomal RNA genes.

The establishment of *in vitro* systems capable of initiating transcription of the genes coding for ribosomal RNA is desirable for the study of regulation of expression of those genes. Recently, Pettijohn’s laboratory (1, 2) reported the isolation of a protein–DNA transcription complex from *Escherichia coli* that is active in the propagation and termination steps of RNA synthesis. This complex is not active in initiation of new rounds of transcription in *in vitro*. The RNA synthesized contains a high proportion of ribosomal RNA (rRNA) sequences. Earlier workers (3, 4) who prepared protein–DNA complexes from *Bacillus subtilis* and *B. megaterium* by alternative procedures had obtained evidence for initiation of transcription with their extracts. This evidence relied on either determination of extent of reaction (3) or sensitivity of the reaction to inhibition by high concentrations of salt (4). We have attempted to substantiate these findings, and to determine whether synthesis of rRNA is obtained with an initiating system.

We report here that RNA synthesis directed by a protein–DNA complex isolated from log-phase cells of *B. subtilis* is sensitive to rifampicin and heparin, inhibitors that specifically affect the initiation step of transcription. In confirmation of the results obtained with initiation inhibitors, we find that in the presence of a competing template, poly(dA-dT), the amount of synthesis directed by the endogenous DNA of the complex is reduced. We conclude from these studies that RNA polymerase exists in the complex in a dissociable, preinitiation state; the synthesis observed represents new rounds of transcription of endogenous DNA initiated *in vitro*. The RNA synthesized by the complex has been characterized by hybridization-competition analysis. The results of this analysis indicate that the product is greatly enriched for rRNA sequences.

**MATERIALS AND METHODS**

*Growth of Cells.* *Bacillus subtilis* strain SB 19 was grown to mid-log phase in the TY medium of Lazzarini (5). Cultures were cooled in ice water, harvested by centrifugation, and washed with buffer containing 10 mM Tris- HCl (pH 7.5)–10 mM MgCl2–0.1 M KCl.

*Preparation of Protein–DNA Complex.* The transcription complex was prepared essentially by the method of Chambon, DuPraw, and Kornberg (4). 2 g (wet weight) of freshly harvested cells were suspended in a total volume of 10 ml with Buffer A [10 mM Tris– HCl (pH 7.8)–10 mM MgCl2–0.1 mM EDTA–10 mM 2-mercaptoethanol] containing 15% sucrose. Lysozyme was added to 500 µg/ml, and the suspension was incubated at 37° for 30 min. The spheroplasts were cooled to 4° and lysed by addition of the detergent Lubrol W-X (final concentration 0.03%). After 20 min at 4°, the viscous lysate was centrifuged at 78,000 × g for 30 min. After decanting the supernatant and washing the surface of the pellet with Buffer A, we suspended the viscous pellet in 10 ml of Buffer A by use of a motor-driven Teflon pestle and glass homogenizer. All the RNA polymerase activity detectable without addition of exogenous DNA was recovered in this membranous fraction. This fraction contained 800 units of enzymatic activity. (1 Unit catalyzes the incorporation of 1 nmol of AMP into acid-insoluble material in 10 min at 37°.) The homogenization procedure freed 70% of the endogenous activity from the fast-sedimenting membranes. A 15-min centrifugation at 78,000 × g removed the membranes; the pellet was discarded. The supernatant was rendered free of ribosomes by a further centrifugation at 164,000 × g for 90 min. This procedure yielded the purified transcription complex that was used in the following studies, unless otherwise stated. The final complex had a specific activity of 29 units/mg of protein. Limited ribonuclease activity is present in the purified complex. An amount of complex containing 32 µg of protein and 5.5 µg of DNA degrades 2 ng of [*H]RNA to trichloroacetic acid-soluble material in 10 min at 28°. Under standard assay conditions, this amount of complex directs the synthesis of 1 µg of RNA product in 10 min. The RNA product obtained is of high molecular weight (see Fig. 1).

Abbreviation: SSC, 0.15 M NaCl–0.015 M sodium citrate.

* To whom inquiries should be addressed.

† Present address, Department of Biological Chemistry, Harvard, Medical School, Boston, Mass.
and it was used at a specific activity of 1 Ci/nmol. Synthesis was allowed to proceed for 30 min at 28°, after which time sodium dodecyl sulfate was added to a final concentration of 0.5%. [3H]RNA was extracted with phenol, precipitated with ethanol, collected by centrifugation, taken up in 2 x SSC, heated at 90° for 5 min, and cooled quickly.

DNA Was Extracted from SB 19 and purified by the method of Marmur (6), with the addition of a Pronase-digestion step in the presence of 2% sodium dodecyl sulfate (7).

Ribosomal RNA from B. subtilis was prepared by sodium dodecyl sulfate–phenol extraction of crude ribosomes, followed by ethanol precipitation, digestion with DNase (RNase-free, Worthington), re-extraction with phenol, precipitation with 1 M NaCl at 4°, and dialysis against water. Sucrose gradient analysis (see Fig. 1) showed the preparation to be free of low molecular weight RNA.

Hybridization was done in solution in 1 ml of 2 x SSC at 63°. Hybrids were collected on nitrocellulose membrane filters according to the procedure of Nygaard and Hall (8), washed with 2 x SSC, and treated with ribonuclease as described by Gillespie (9).

Sucrose Gradient Analysis. Linear gradients of 5–20% sucrose, containing 10 mM sodium acetate (pH 5.0)–0.1 M NaCl, were spun in the Spincow SW65 rotor at 60,000 rpm for 3 hr at 4°. Fractions were collected from the bottom of the tube. The absorbance at 260 nm was determined, and the fractions were precipitated with 5% trichloroacetic acid. The precipitates were collected on glass-fiber filters, and the radioactivity was determined in a liquid scintillation counter.

Protein and DNA Determinations. Protein was assayed by the method of Lowry et al. (10) and DNA was assayed by the method of Burton (11).

Materials. [5-3H]UTP (13 Ci/mmol) was purchased from Schwarz–Mann, [U-14C]GTP (389 Ci/mol) and [5-3H]CTP (28.4 Ci/mmol) from New England Nuclear, and unlabeled ribonucleoside triphosphates from Calbiochem. Polyl(A-dT) was a kind gift of Dr. J. Colbourn of Miles Labs. E. coli RNA

Fig. 1. Sucrose gradient analysis of the in vitro [3H]RNA product. The [3H]RNA product of the reaction directed by the protein–DNA complex was extracted after 30 min of synthesis and subjected to sucrose gradient analysis (Methods). Undegraded ribosomal RNA was included as an absorbance marker. Fractions were collected from the bottom of the tube.

RNA Synthesis Assay. RNA synthesis was measured by the incorporation of [3H]UTP, [3H]CTP (1–4 Ci/mol), or [14C]GTP (1 Ci/mol) into trichloroacetic acid-insoluble material. The final mixture (250 µl) contained 50 mM Tris-HCl (pH 7.8), 4 mM MgCl₂, 1 mM MnCl₂, 10 mM 2-mercaptoethanol, and GTP, CTP, UTP, ATP at 0.15 mM each. The reaction was started by addition of the protein–DNA complex. The standard incubation time was 10 min. The reaction was stopped by addition of 5% trichloroacetic acid–10 mM sodium pyrophosphate. The acid-insoluble material was collected on a glass-fiber filter, and the radioactivity was determined in toluene-based scintillation fluid.

Preparation of [3H]RNA. When the [3H]RNA product of the transcription reaction was desired for hybridization studies and for sucrose gradient analysis, the concentration of the radioactive ribonucleoside triphosphate was reduced to 40 µM.

Fig. 2. Effect of inhibitors of initiation on RNA synthesis directed by the protein–DNA complex. Purified complex containing 52 µg of protein and 5.5 µg of DNA was added to assay mixtures containing rifampicin (A) or heparin (B) at the indicated concentrations, and incubated under standard conditions.

Proc. Nat. Acad. Sci. USA 69 (1972)

Fig. 3. Effect of rifampicin, added at different times of incubation (dotted lines), on RNA synthesis directed by the protein–DNA complex. RNA synthesis was measured under standard conditions. Rifampicin (4 µg/ml) was added at the indicated times of incubation to reaction mixtures containing a membranous fraction of the protein–DNA complex. [14C]GTP was the radioactive substrate.
RESULTS

A protein–DNA transcription complex was isolated from log-phase cells of B. subtilis. The ability of this complex to initiate new rounds of transcription in vitro was determined from the sensitivity of its activity to inhibitors of initiation. Rifampicin (12) and heparin (13) specifically inhibit the initiation step of transcription. Fig. 2 shows the effects of these compounds on RNA synthesis directed by the protein–DNA complex. All the detectable synthesis is sensitive to the initiation inhibitors when they are present at the beginning of the reaction. That RNA synthesis becomes partially resistant to rifampicin after initiation is demonstrated in Fig. 3. That this resistance is not complete (40–50%) is probably due either to heterogeneity in the time of initiation on various transcription units or to recycling of RNA polymerase molecules on the same transcription units. These results indicate that RNA polymerase, as we have isolated it, is associated with the endogenous DNA in a preinitiation state, and that the synthesis we are detecting in vitro represents new rounds of transcription and not propagation of nascent chains that had been initiated in vivo. We observe no propagation of nascent chains, perhaps due to run-off of nascent chains during preparation of the complex.

To further substantiate our conclusion that the protein–DNA complex is initiating new rounds of transcription of endogenous DNA, we studied the effect of addition of a competing template, the double-stranded copolymer poly(dA-dT). The reaction directed by each of the two templates can be studied separately in the presence of the other. The use of [3H]CTP allows transcription of endogenous DNA to be followed without contribution by the poly(A-U) product of the poly(dA-dT)-directed reaction. Alternatively, synthesis directed by endogenous DNA can be prevented either by addition of the inhibitor actinomycin D, which does not bind to poly(dA-dT) (14), or by withholding GTP and CTP; neither procedure interferes with the synthesis of poly(A-U) in the poly(dA-dT)-directed reaction, as measured by the incorporation of [3H]UTP.

Fig. 4 demonstrates that poly(dA-dT) competes with the endogenous DNA for the available enzyme, resulting in diminished incorporation of [3H]CTP. This result is consistent with our observation that the reaction is sensitive to rifampicin and heparin, with the enzyme apparently reversibly bound to endogenous DNA in a preinitiation state. RNA polymerase is known to become tightly bound to its chosen template after initiation of transcription (15, 16). If incorporation of [3H]CTP reflected propagation of nascent chains that had been initiated in vivo, poly(dA-dT) would not exert this effect.

It is apparent from Fig. 4 that a minor fraction of the reaction directed by endogenous DNA is less readily inhibited by increasing amounts of poly(dA-dT). This result may reflect the presence of a second species of RNA polymerase in the complex; this species, having a lower affinity for poly(dA-dT), may be concerned with GTP initiations. Bremer (17) has detected a heterogeneity of E. coli RNA polymerase.

Fig. 4 also shows that an amount of poly(dA-dT) that causes an 80% inhibition of transcription of endogenous DNA is able to stimulate poly(A-U) synthesis at only about 40% of the rate observed with greater amounts of the template. This result indicates that poly(dA-dT) contains a larger number of sites at which it can bind RNA polymerase than does sites at which transcription can be initiated. The single-stranded loops present in poly(dA-dT) (18) may be responsible for this effect.

Since we were able to obtain initiation of new rounds of RNA synthesis in vitro with the protein–DNA complex, we were interested in determining the extent to which RNA was included in the product of the reaction. The [3H]RNA product was isolated for hybridization-competition analysis. This RNA was heterogeneous in size, with an average sedimentation constant of about 12 S (see Fig. 1). As discussed under Methods, limited RNase activity present in the complex has cleaved the product to its final size. This [3H]RNA was hybridized to B. subtilis DNA in the presence of increasing amounts of unlabeled RNA. As shown in Fig. 5, a maximum of 60% of the hybridized [3H]RNA could be competed with by unlabeled RNA. Fig. 5 also presents the results of a control experiment, in which labeled RNA was obtained by transcription of denatured B. subtilis DNA with E. coli RNA polymerase as a generous gift from Dr. J. Leavitt. Nitrocellulose 'B6' filters were purchased from Schleicher and Schuell. Lubrol W-X was obtained from the laboratory of Dr. A. Lehninger. Rifampicin was a gift from Dr. Paul T'so. Heparin was purchased from Sigma.
hybridized DNA the hybridized hybrids. Hybridization species homologous polymerase and that 50% of the input [H]RNA was recovered in RNA-DNA hybrids in the absence of competitor. (b) As a control, denatured B. subtilis DNA was transcribed by E. coli RNA polymerase; the same radioactive label was used as above. The [H]RNA was extracted as described in Methods, except that the final product was heated in a boiling-water bath for 5 min and cooled quickly to eliminate any [H]RNA-DNA hybrids. Hybridization was done with this [H]RNA as in (a). 14% of the input [H]RNA was recovered in hybrids in the absence of competitor. In both (a) and (b), a zero-time background of 15 cpm has been subtracted from the data.

polymerase and analyzed under identical conditions. Little of the hybridized RNA was competed with by rRNA.

The extent to which rRNA is synthesized by the protein-DNA complex cannot be directly ascertained from these experiments, since hybridization efficiencies probably vary for different species of RNA under the particular hybridization conditions we have chosen. However, a minimum value can be obtained on the basis of the available data. When the apparently nonspecific competition observed in the control experiment is corrected for, the results presented in Fig. 5 indicate that 50% of the hybridized [H]RNA consists of sequences homologous to rRNA. Taking into account that 22% of the input [H]RNA was recovered in hybrids in the absence of competitor, we conclude that transcription of rRNA genes accounts for at least 11% of the RNA synthesis obtained with the protein-DNA complex.

**DISCUSSION**

We have shown by three independent criteria—namely, by inhibition of the reaction by rifampicin and heparin, and by depression of synthesis directed by endogenous DNA upon addition of a competing template—that the protein-DNA complex we have isolated from B. subtilis is initiating new rounds of RNA synthesis in vitro. In contrast, the transcription complex isolated by Pettijohn's laboratory was limited in its activity to propagation of chains that had been initiated in vivo. The inability of Pettijohn and coworkers to obtain initiation of transcription was apparently a consequence of the purification procedures they used; crude lysates were subjected to either extraction with 1 M NaCl, or phase extraction with polyethylene glycol-dextran. These procedures yielded a protein-DNA complex that lacked sigma factor (2), a component of RNA polymerase required for specific initiation of new rounds of transcription (19).

 Preferential transcription of rRNA genes occurs in vivo. Genes coding for rRNA represent 0.2-0.4% of the bacterial genome (20-23), while synthesis of rRNA can account for up to 40% of the instantaneous rate of total RNA synthesis in rapidly growing bacteria (23-26). One expects to obtain this selective synthesis of rRNA in a cell-free extract that is faithfully reflecting the situation in vivo. Our hybridization-competition experiments show that the protein-DNA complex we have isolated from log-phase cells directs preferential synthesis of rRNA. A minimum of 11% of the RNA product consists of sequences homologous to rRNA. It will be of interest to characterize the RNA synthesized by a similar complex isolated from sporulating cells of B. subtilis. Synthesis of rRNA ceases abruptly at an early stage of sporulation (27).

The manner in which preferential transcription of the rRNA genes is achieved in vivo is an unresolved question of considerable interest. Travers, Kamen, and Schleif (28) have reported the isolation of a protein factor from E. coli that preferentially stimulates transcription of rRNA genes in a highly purified system containing E. coli DNA and E. coli RNA polymerase. Hybridization-competition analysis of the RNA synthesized in the presence of this factor was performed (28) under conditions that yielded a hybridization efficiency of 10% in the absence of competitor. A maximum of 25% of the hybridized RNA could be competed with by rRNA, indicating that a minimum of 2.5% of the RNA synthesized in the factor-stimulated reaction consists of rRNA sequences. On the basis of more recent experiments, it is estimated that rRNA sequences represent 10% of the in vitro product (A. A. Travers, personal communication).

We are currently attempting to fractionate the DNA-protein complex and identify its active elements. We also intend to use it as an assay system in studies aimed at identifying the effector molecules responsible for stringent control of rRNA synthesis in B. subtilis. An initiating complex has also been isolated from E. coli by the procedures outlined in Methods.

We thank Drs. Donald D. Brown, Andrew A. Travers, and Walter A. Wisten for critically reading a draft of this manuscript. It is a pleasure to acknowledge the capable technical assistance of Miss Pat Kropkowski. Jay Wisten is a predoctoral trainee supported by NIH Training Grant HD-139. This research was supported by NSF Grant GB-8038.