Heterogeneity of RNA polymerase in *Bacillus subtilis*: Evidence for an additional $\sigma$ factor in vegetative cells

(Janey L. Wiggs, Michael Z. Gilman, and Michael J. Chamberlin)

ABSTRACT Preparations of *Bacillus subtilis* RNA polymerase (nucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.7.6) from vegetatively growing cells contain small amounts of an activity (*B. subtilis* RNA polymerase holoenzyme II) that shows a unique promoter specificity with T7 bacteriophage DNA as compared with the normal *B. subtilis* holoenzyme (holoenzyme I) and lacks the normal $\sigma$ subunit [Jaehning, J. A., Wiggs, J. L. & Chamberlin, M. J. (1979) Proc. Natl. Acad. Sci. USA 76, 5470-5474]. By heparin-agarose chromatography we have obtained holoenzyme II fractions that have no detectable holoenzyme I activity as judged by their failure to utilize promoter sites for holoenzyme I on any template we have tested. These fractions are far more active with *B. subtilis* DNA than with T7 DNA or other heterologous templates. This high degree of specificity has allowed identification of plasmids containing cloned fragments of *B. subtilis* DNA that bear strong promoter sites for holoenzyme II. These promoter sites are not utilized at all by *B. subtilis* RNA polymerase holoenzyme I. The specificity of holoenzyme II is dictated by a peptide of M, 28,000 as judged by copurification of the peptide with specific holoenzyme II activity and by reconstitution of the holoenzyme II promoter specificity when the isolated peptide is added to *B. subtilis* core polymerase. Hence the 28,000 M $\sigma$ peptide appears to be a $\sigma$ factor that determines a promoter specificity distinct from that of RNA polymerase holoenzyme I and all other known bacterial RNA polymerases.

A large variety of molecular mechanisms are known by which organisms regulate transcription during growth and development. A dramatic example is the reprogramming of promoter specificity of *Bacillus subtilis* RNA polymerase (nucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.7.6) after infection with phages such as SP01 or SP82 in which a viral protein replaces the normal *B. subtilis* $\sigma$ subunit (1-3). This type of regulatory mechanism has long been considered an attractive possibility in normal cells (4); however, although many "altered" forms of RNA polymerase have been reported (refs. 5-11, see refs. 12-14 for reviews), it is only quite recently that bacterial RNA polymerases have been detected that clearly express novel promoter specificities (15, 16). We have described the development of an *in vitro* test system employing DNA templates from T7 and T3 bacteriophages with which alterations in the promoter (17) or terminator (18-20) recognition properties of bacterial RNA polymerases can be sensitively detected and studied. Using this system, we observed that preparations of RNA polymerase from exponentially growing *B. subtilis* contain an RNA polymerase activity (designated Bs II) that utilizes an *in vitro* promoter site on T7 DNA not used by the normal *B. subtilis* RNA polymerase (16) or any other eubacterial RNA polymerase we have tested (17). Because active Bs II fractions lacking any detectable *B. subtilis* $\sigma$ subunit were obtained, it appeared that the novel promoter specificity of Bs II might well be dictated by another $\sigma$ subunit-like protein. We provide evidence here that this is the case and that strong promoter sites used by this enzyme, but not by the normal *B. subtilis* RNA polymerase, are present in the genome of *B. subtilis*.

MATERIALS AND METHODS

Experimental procedures and materials have been described previously (16) except where noted. Growth of *B. subtilis* W168 in early exponential phase and preparation of fraction 5 RNA polymerase proceeded as before (16) except that: (i) precipitation of RNA polymerase with ammonium sulfate employed 0.4 g/ml after Polymyx fractionation and 0.42 g/ml after DNA cellulose chromatography; (ii) the precipitate from the first ammonium sulfate fractionation was desalted by chromatography on Bio-Gel P-10 (Bio-Rad) in a buffer containing 10 mM Tris-HCl (pH 8), 1 mM EDTA, 1 mM dithiothreitol, 10% (vol/vol) glycerol, and 50 mM NaCl; (iii) RNA polymerase was eluted from the DNA cellulose column with a 4 column volume gradient (0.05-1.0 M NaCl).

*B. subtilis* core RNA polymerase was prepared by chromatography of peak fractions of RNA polymerase holoenzyme I from heparin agarose (fractions HA 116-132) on phosphocellulose (21) followed by rechromatography on poly(rC)-cellulose (22) to remove a contaminating polypeptide of M, 92,000. RNA polymerase from the phosphocellulose column (1.5 mg) in 10 mM Tris-HCl (pH 8)/1 mM EDTA/10 mM 2-mercaptoethanol/10 mM MgCl$_2$/10% (vol/vol) glycerol/0.02 M NaCl was passed through a 2-ml poly(rC)-cellulose column. Flow-through fractions were pooled with those eluted with 0.1 M NaCl and were concentrated by dialysis against 50% (vol/vol) glycerol/10 mM Tris-HCl (pH 8)/1 mM EDTA/1 mM dithiothreitol/10 mM MgCl$_2$/0.1 M NaCl. These fractions contained only the core subunits $\beta$, $\beta'$ and $\alpha$ when analyzed by NaDodSO$_4$ gel electrophoresis.

DNA fractions containing pools of plasmids prepared by inserting endonuclease Pst I fragments of *B. subtilis* DNA into the Pst I site of pHV14 (23) were generously provided by E. Ferrari and J. Hoch (Scripps Institute, La Jolla, CA). Plasmids bearing individual cloned fragments were isolated by transformation (24) of *Escherichia coli* K-12 294 (originally obtained from M. Meselson) followed by purification (25, 26) and transcriptional analysis.

RESULTS

Separation of *B. subtilis* RNA Polymerase Holoenzymes I and II by Chromatography on Heparin-Agarose. Preparations of RNA polymerase from exponentially growing *B. subtilis* contain an RNA polymerase activity that utilizes an *in vitro* promoter site on T7 DNA, designated $J$, that is not used by the

Abbreviation: kb, kilobase(s).

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normal B. subtilis RNA polymerase holoenzyme (16, 17). Chromatography of these fractions (fraction 5 of ref. 16) on phosphocellulose removes B. subtilis α subunit (σ25), but the resulting fractions retain their unique transcriptional specificity (16). This observation suggested that these fractions contain a selective RNA polymerase with a distinct promoter specificity, which we have designated RNA polymerase holoenzyme II to distinguish it from the normal B. subtilis RNA polymerase holoenzyme (holoenzyme I).

Although phosphocellulose chromatography removes σ25 completely from B. subtilis RNA polymerase as judged by NaDodSO4 gel electrophoresis of the proteins, these fractions still utilize the T7 promoters A and C, recognized by holoenzyme I to a minor extent. Our studies and those of several other laboratories indicated that this is because phosphocellulose chromatography does not remove the last traces of σ25 from these fractions. Accordingly we explored other fractionation procedures beginning with fraction 5, to separate B. subtilis holoenzymes I and II. Chromatography on heparin-agarose (27) columns achieves the desired fractionation (Fig. 1). Two peaks of protein result from gradient elution. The first peak has a relatively low level of RNA polymerase activity; however, gel analysis of T7 transcripts indicates that the initial fractions (HA 88-108) read only the T7 J promoter, characteristic of holoenzyme II, while the second peak (HA 116-128) transcribes T7 promoters A and C, characteristic of holoenzyme I. There is no detectable transcription of T7 promoters A and C by the initial fractions, here or in many other experiments, indicating clearly that B. subtilis holoenzyme II does not utilize these promoters, and that these fractions contain no σ25.

Analysis of the polypeptide composition of the fractions obtained after heparin-agarose chromatography (Fig. 1C) shows that both peaks contain predominantly the polypeptides characteristic of B. subtilis core RNA polymerase (BB' and α). However, fractions in the second peak (holoenzyme I) contain B. subtilis σ25, whereas those in the first peak (holoenzyme II) lack σ25 but contain a polypeptide of M, 28,000. There is a direct coincidence between content of the 28,000 M, polypeptide and T7 J transcription. Quantitative estimates of the amount of the 28,000 M, polypeptide relative to the core RNA polymerase subunits suggest that the peaks contain about 0.10–0.20 mol of 28,000 M, peptide per mol of core polymerase; hence there is an excess of core polymerase over holoenzyme II in these fractions. Zone sedimentation of these fractions on 10–30% (vol/vol) glycerol gradients in 0.5 M NaCl shows that sedimentation of 28,000 M, peptide is exactly coincident with RNA polymerase activity having the holoenzyme II specificity measured with T7 DNA as template (data not shown). This led us to the hypothesis that the unique promoter specificity of B. subtilis holoenzyme II is determined by binding of the 28,000 M, peptide to B. subtilis core RNA polymerase.

All of the fractions also contain traces of other contaminating polypeptides, primarily in the 90,000–140,000 M, range. None of these is coincident with σ25 (see also above), and the majority are lost on glycerol gradient sedimentation. These bands are

![Figure 1](image-url)
Table 1. Activities of B. subtilis RNA polymerase holoenzymes I and II, and core RNA polymerase with different DNA templates

<table>
<thead>
<tr>
<th>Template DNA</th>
<th>RNA polymerase activity, units/mg</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Holoenzyme</td>
</tr>
<tr>
<td>A. T7 phage</td>
<td>I</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>5500</td>
</tr>
<tr>
<td>φ29 phage</td>
<td>900</td>
</tr>
<tr>
<td>Poly(d(A-T))</td>
<td>4500</td>
</tr>
<tr>
<td>pBR322</td>
<td>1000</td>
</tr>
<tr>
<td>pHV14 (vector)</td>
<td>1700</td>
</tr>
<tr>
<td>pCD4322 (Bsu insert)</td>
<td>450</td>
</tr>
<tr>
<td>pCD4136 (Bsu insert)</td>
<td>550</td>
</tr>
<tr>
<td>pCD4134 (Bsu insert)</td>
<td>400</td>
</tr>
</tbody>
</table>

B. T7 phage

| B. subtilis | 500 |
| pCD4322 | 1000 |
| pCD4136 | 600 |

Results with two different preparations of holoenzyme II are shown. All plasmid DNAs were linearized by cleavage with Pst I. Data in part A were obtained with holoenzyme II prepared exactly as discussed in *Materials and Methods* and show relative activities of the different RNA polymerases. Assay mixtures (30 μl final volume) contained 0.4 μg of each DNA template and 4 μg of each RNA polymerase fraction. Data in part B are for a second preparation of holoenzyme II in which only 0.3 g/ml of ammonium sulfate was used after DNA cellulose chromatography; this gives improved specific activities of holoenzyme II fractions, probably by reducing the amount of core RNA polymerase present relative to the 28,000 M₉₀, peptide.

**Exaggerated in these gels in comparison to the 28,000 M₉₀, peptide because of its low M₉₀; the gels have been heavily overloaded for the larger peptides to show the 28,000 M₉₀, peptide clearly (up to 60 μg of protein per track).**

**Template Specificity of B. subtilis Holoenzyme II.** The high degree of promoter specificity of holoenzyme II fractions evident in Fig. 1 led us to explore their general template specificity. Comparison of holoenzyme I and II fractions eluted from heparin-agarose, together with B. subtilis core polymerase (Table 1), shows that holoenzyme II fractions are very active with B. subtilis DNA templates and with DNA templates from several plasmids (pCD4322, 4136) containing cloned B. subtilis sequences. However, these fractions show very low activity with heterologous templates, including T7 DNA, which is the template employed for purification, vectors pBR322 and pHV14, and φ29 phage DNA. In contrast, B. subtilis holoenzyme I fractions show similar activities with all templates, and the core polymerase is poorly active on DNA templates but active with poly(d(A-T)). These results strongly suggested that holoenzyme II is highly specific for certain promoter sites contained on B. subtilis DNA.

**The specific activities of the holoenzyme I fractions are low in comparison to the most active B. subtilis RNA polymerase fractions we have studied (18, 27). The specific activities of holoenzyme II fractions are also rather low compared to what one might expect for a highly purified fraction containing 0.1–0.2 equivalent of holoenzyme II. However, all of these fractions contain a substantial amount of the δ subunit, which strongly inhibits transcription (29, 30). Consequently, the actual specific activities we obtain cannot be interpreted directly in terms of active RNA polymerase concentration. It should be possible to develop a direct assay for concentration of active holoenzyme II (18) by using cloned B. subtilis II promoters.**

**Identification of Plasmids Containing B. subtilis DNA Inserts Bearing Promoters for B. subtilis RNA Polymerase II.** Because B. subtilis holoenzyme II fractions showed a high degree of specificity for B. subtilis DNA, we screened a library of plasmids obtained from James Hoch, which contains fragments of B. subtilis DNA inserted into the Pst I site of plasmid pHV14, as templates for holoenzyme II. DNA from plasmid pools was used as template for B. subtilis holoenzyme I and II fractions, and individual DNAs from fractions highly active with holoenzyme II were isolated by transformation. Of the plasmids highly active with holoenzyme II, two were selected for further study. Promoter sites for RNA polymerase II on plasmids were mapped by transcribing plasmids after cleavage with Pst I, EcoR I, and HindIII, respectively. Comparison of the sizes of the resulting transcripts with the restriction map of each plasmid gave the transcriptional maps shown in Fig. 2. Transcription of DNA from pCD4136 after cleavage by restriction endonuclease Pst I using holoenzyme II gives a single transcript of 1700 bases (Fig. 3) corresponding to that diagrammed in Fig. 2. Similarly, transcription of DNA from pCD4322 after cleavage with Pst I using holoenzyme II gives two discrete transcripts, due to transcription from a single promoter site and a partially effective terminator. In neither instance does holoenzyme I give any detectable transcripts of these characteristic mobilities (Fig. 3); run-off transcripts for holoenzyme I with these templates are much larger than 1–2 kb and run at the top of the gel. Furthermore, holoenzyme II gives no discrete transcripts with DNA from either the vector (pHV14) or a clone (pCD4134) that contains a B. subtilis DNA fragment but is not a highly active template (see Table 1). This confirms our previous conclusion that holoenzymes I and II have distinct promoter specificities.

**Reconstitution of RNA Polymerase Holoenzyme II from B. subtilis Core Polymerase and Isolated 28,000 M₉₀, Polypeptide.** The availability of plasmid pCD4136 containing a strong promoter for B. subtilis holoenzyme II made possible a sensitive, specific test of the hypothesis that the 28,000 M₉₀, polypeptide component of holoenzyme II fractions is responsible for its
unique promoter specificity. The constituent polypeptides from a fraction of holoenzyme II (HA100) were resolved by NaDodSO₄ gel electrophoresis and the resulting gel was sliced and proteins in individual fractions were eluted and renatured by using a procedure devised by Hager and Burgess (31). The ability of each fraction to stimulate transcription from the holoenzyme II promoter of plasmid pCD4136 was then tested by adding that fraction to B. subtilis core RNA polymerase prepared from holoenzyme I fractions (Fig. 4). Only the eluate from gel slice 9 restored transcription from the pCD4136 holoenzyme II promoter; this band contains the 28,000 M₉ polypeptide associated with holoenzyme II specificity during purification. In addition, only the eluate from gel slice 9 stimulated transcription from the T7 J promoter in separate experiments (data not shown).

DISCUSSION

Bacteriophage T7 DNA contains a diverse set of in vitro promoter sites that are utilized by purified bacterial RNA polymerases. Although there are differences in the efficiency of utilization of individual T7 promoters by different bacterial RNA polymerases, all of the normal bacterial RNA polymerases we originally tested share the ability to use these different sites (17). In contrast, the specificity of the enzyme we have termed B. subtilis RNA polymerase holoenzyme II is quite distinct. Using the enzyme fractions we describe here, we have not been able to detect any transcription of holoenzyme I promoters on T7 DNA by holoenzyme II fractions, and, conversely, there is no transcription of either T7 promoter J or the cloned B. subtilis holoenzyme II promoters by holoenzyme I fractions. This makes it likely that the promoters for B. subtilis holoenzymes I and II have different sequence recognition elements. This hypothesis can be tested directly by determining the sequence of cloned B. subtilis holoenzyme II promoters.

B. subtilis RNA polymerase holoenzyme II fractions contain a polypeptide of M₉ 28,000. The amount of this polypeptide is consistent with the specific transcriptional properties of the individual fractions, distinguishing it from other associated polypeptides such as the 92,000 M₉ component present in an earlier preparation (16) and 8 polypeptide. Addition of the 28,000 M₉ polypeptide, isolated by gel electrophoresis, to core polymerase derived from holoenzyme I fractions, directs synthesis of transcripts from promoters specific for holoenzyme II; hence we conclude that the 28,000 M₉ polypeptide in combination with core RNA polymerase controls the promoter specificity characteristic of holoenzyme II. Because these fractions contain none of the normal B. subtilis o' subunit, o'5, and lack the promoter specificity characteristic of that subunit, we conclude that the 28,000 M₉ polypeptide has replaced o'5. Thus the 28,000 M₉ polypeptide fills two of the three criteria required if one is to consider it a functional part of the B. subtilis transcriptional apparatus (32), and we will designate it as B. subtilis o'5. Isolation of specific B. subtilis mutants altered in this function will be necessary to confirm this assignment.

The idea that the nature of the o' peptide-like subunit bound to a bacterial RNA polymerase can control the exact promoter identity has been discussed since the original identification of o' in E. coli (33). Binding of E. coli o' has several distinct effects on the properties of that core polymerase, and hence o' is a pleiotropic effector (34). Addition of o' subunits isolated from several of the normal bacterial RNA polymerases to heterologous core RNA polymerases generally leads to utilization of the same promoter sites used by the homologous RNA polymerase holoenzymes (35, 36). This is most easily explained if all o' sub-
units for these enzymes share a common promoter recognition specificity, with core polymerase making important contributions to promoter strength (36).

In contrast, *B. subtilis* RNA polymerases reprogrammed by infection with phages SP01 and SP82 contain polypeptides that replace *B. subtilis* σ29 and dictate a completely altered promoter identity. The promoters read by these altered polymerases differ substantially in sequence from bacterial promoter regions used by the normal RNA polymerase holoenzymes (37). This result is also found for promoters used by two *B. subtilis* RNA polymerases isolated from sporulating *B. subtilis* cells (C. Moran and R. Losick, personal communication). These enzymes also contain *B. subtilis* core polymerase together with distinct polypeptides of M , 37,000 and 29,000, respectively (15, 38, 39), replacing the normal σ subunit. These RNA polymerases (which we suggest designating as *B. subtilis* RNA polymerase holoenzymes III and IV, respectively) have promoter specificities distinct from *B. subtilis* holoenzyme II, as shown by the fact that the plasmid p63 of Haldenwang and Losick (38) does not contain promoters for *B. subtilis* holoenzyme II (unpublished observations). Thus it appears that *B. subtilis* RNA polymerase can interact with at least four distinct cellular σ factors, which control distinct sets of promoter sequences. The role of these multiple specificity factors in the economy of growth of this organism will be of great interest.

The finding of multiple σ factors controlling distinct classes of promoter sites is best explained if the σ subunit interacts directly with the promoter sequence to determine the promoter specificity of RNA polymerase. Two such mechanisms have been discussed (34, 40). In light of current information (37, 41, 42), it now seems most likely that σ binds to the conserved DNA sequence near the start site, possibly effecting DNA strand separation in this region. Determination of the sequences of different promoters controlled by the different σ elements should provide direct information relating to this model.

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