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BACTERIOPHAGE SP82 INDUCED MODIFICATIONS OF BACILLUS SUBTILIS RNA POLYMERASE RESULT IN THE RECOGNITION OF ADDITIONAL RNA SYNTHESIS INITIATION SITES ON PHAGE DNA

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SUMMARY

The modification of the subunit structure of Bacillus subtilis RNA polymerase which occurs after infection with bacteriophage SP82 alters the transcriptional specificity of the enzyme. The data presented here determine which restriction endonuclease fragments of SP82 are used as initiation sites for RNA synthesis by the modified and unmodified enzymes. The analysis shows that the change in transcriptional specificity is due to the ability of the modified polymerase to initiate RNA synthesis at new sites on SP82 DNA. Reconstitution experiments using subunits isolated from the modified RNA polymerase indicate that the 28,000 molecular weight polypeptide coded by the phage is responsible for the change in initiation specificity.

The infection of Bacillus subtilis by either of the lytic phages SP82 or SP01 results in an ordered appearance of RNAs and proteins coded by the phage DNA (1-4). Among the proteins are several which specifically bind to the host cell RNA polymerase (5-8) and alter the transcriptional specificity of the enzyme on SP82, SP01 and several other phage DNA templates (9-14). The first alteration in polymerase subunit structure takes place between 3 and 5 min after infection and involves the addition of two polypeptides (molecular weights 28,000 and 18,000) and the exclusion of the host σ subunit (6-8). While several mechanisms have been postulated to explain how the addition of the new peptides alters the transcriptional specificity, the methods which have been used to address the question have not allowed an unambiguous discrimination between the models. In this communication, we present evidence that the modification of the polymerase results in the recognition of new promoters on the phage DNA.

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MATERIALS AND METHODS

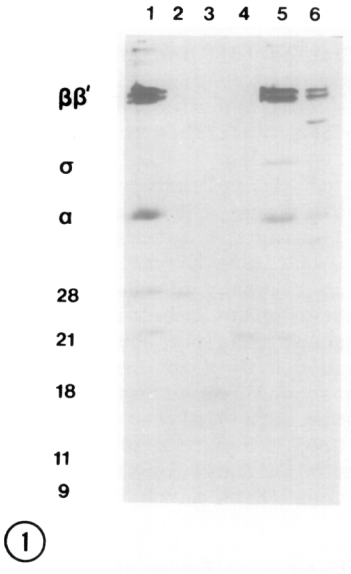
Methods for the growth of bacteria and phage infection (3); isolation of RNA polymerase (15) and phage DNA (3); restriction endonuclease digestion and subsequent analysis by electrophoresis on agarose gels (16) and electrophoresis on sodium dodecyl sulfate polyacrylamide gels (4) have been described previously.

Isolation of the 21,000 molecular weight subunit of RNA polymerase was performed as described earlier (15). Host core RNA polymerase and σ subunits were isolated by the methods of Berg et al. (17) using two successive NaCl gradient elutions from phosphocellulose to purify the core enzyme and substituting DEAE-sephadex for DEAE-cellulose for chromatography of the σ subunit. The peptides which bind to the polymerase after phage infection were isolated by the method of Duffy and Geiduschek (13) except that the salt gradient used for elution from phosphocellulose contained 0.05 M to 0.60 M NaCl. Isolated subunits were rechromatographed on phosphocellulose using appropriate step gradients of NaCl. All enzyme subunits were dialyzed against 0.01 M tris-HCl, pH 7.9, 0.01 M $MgCl_2$, 0.001 M EDTA, 0.05 M β-mercaptoethanol, 0.1 M NaCl, 30% glycerol containing 50 μg/ml phenylmethylsulfonylfluoride and 70 μg/ml L-1-tosylamide-2-phenyl-ethylchloromethyl ketone (both purchased from Sigma Chemical Corp.) and stored at -15°C.

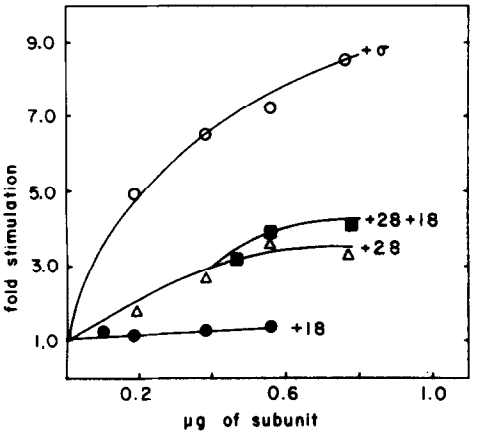
The formation of complexes between RNA polymerase and restriction endonuclease fragments was assayed as described by Jones et al., (18) with the following modifications. DNA-enzyme complexes were formed in 0.5 ml of 0.04 M tris-HCl, pH 7.9, 0.02 M $MgCl_2$, 0.05 M NaCl, 0.01 M β-mercaptoethanol at 37°C. Nucleotide triphosphates, if present, were 0.04 M each guanine, adenine and cytosine. Samples were diluted with 2.0 mls of 0°C 0.04 M tris-HCl, pH 7.9, 0.02 M $MgCl_2$, 0.2 mg/ml bovine serum albumin (dilution buffer) containing 1.0 M NaCl. The samples were gently filtered through nitrocellulose filters, the filters were washed with 2.0 mls of cold dilution buffer and then with 1.5 mls of room temperature dilution buffer without added NaCl. DNA was released by incubating the filters with 0.5 ml of 0.001 M tris-HCl, pH 7.9, 0.001 M $MgCl_2$, 0.1% sodium dodecyl sulfate for 5 min at 55°C, the samples were lyophilized to dryness, and then redissolved in 0.04 ml of agarose gel buffer (16).

RESULTS

Figure 1 shows a sodium dodecyl sulfate polyacrylamide gel of RNA polymerase from uninfected B. subtilis (host holoenzyme, lane 5 and core enzyme, lane 6), polymerase as isolated from cells 8 min after infection (lane 1) and the purified polymerase subunits used in the reconstitution experiments described below (lanes 2, 3, 4). The host core polymerase (i.e., from uninfected cells) is deficient in both the σ and the 21,000 molecular weight (21 K) subunits of the host enzyme. It contains β, β', 2α and two smaller peptides (molecular weights 11,000 and 9,000) (19). The modification of the polymerase which occurs after SP82 infection results in the loss of the σ subunit and the addition of the 28,000 molecular weight (28K) subunit (lane 2). RNA polymerase isolated 8 min after infection (8 min polymerase) also



(1)



(2)

Fig. 1. (Right) SDS-polyacrylamide gel analysis of RNA polymerase and its subunits. SDS polyacrylamide slab gels prepared and run as described by Hiatt and Whiteley (4) were used to analyze the protein components of: uninfected cell RNA polymerase (lane 5, 15.2 μg); uninfected cell core RNA polymerase (lane 6, 8.2 μg); RNA polymerase isolated from cells 8 min after infection (lane 1, 15.0 μg) and isolated RNA polymerase subunits: 28,000 (lane 2, 0.78 μg), 21,000 (lane 3, 0.64 μg) and 18,000 (lane 4, 0.76 μg).

Fig. 2. (Left) Stimulation of core RNA polymerase activity by isolated polymerase subunits. Core RNA polymerase from uninfected cells (2 μg) was assayed using the standard assay described previously (15) with SP82 DNA as the template except that the nucleotide mixture contained 0.8 mM GTP, ATP and CTP and 8 μM ^{3}H UTP (3.2 mCi/mM). The assays contained increasing amounts of isolated 28K peptide (Δ - Δ); 18K peptide (● - ●) and host sigma subunit (0 - 0) or 0.38 μg of 28K peptide and increasing amounts of 18K peptide (■ - ■). All reactions were supplemented with 0.16 μg of 21K peptide.

contains a 18,000 molecular weight peptide (18K peptide). It is not known whether this peptide is specified by the phage DNA, but it is routinely isolated with the 8 min polymerase from SP82-infected cells (4, 15).

Figure 2 shows the effect of adding purified 28K and 18K peptides on the RNA synthesis activity of host core enzyme, using SP82 DNA as a template. In all cases, the core was supplemented with the 21K subunit; a discussion of the effects of this peptide has been presented elsewhere (15). Addition of the 28K peptide results in substantial stimulation of core activity, while addition of the 18K peptide alone has little, if any, effect. On the other

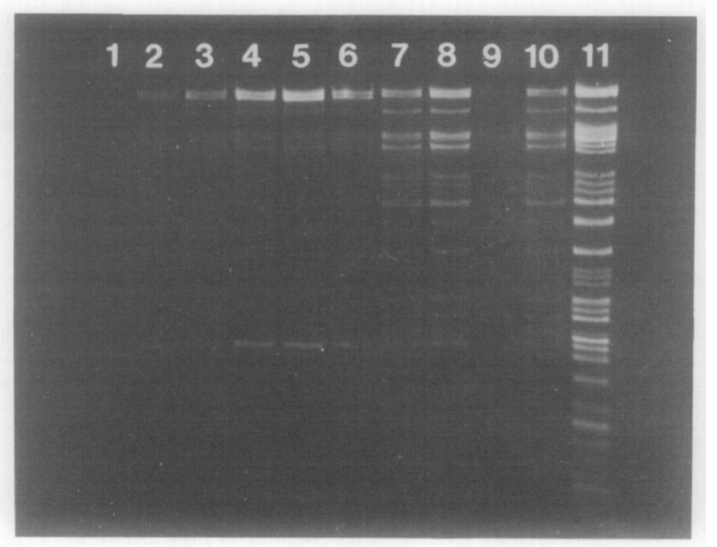


Fig. 3. Agarose gel analysis of restriction endonuclease fragments retained by modified and unmodified RNA polymerase. Enzyme DNA complexes were formed as described in Material and Methods between 1.1 µg of Hpa-II treated DNA and the indicated amounts of enzyme. The fragments retained on nitrocellulose from each incubation were released as described and analyzed by electrophoresis on a 1% agarose gel at a constant voltage of 80 volts. All enzyme-DNA incubations contained nucleotides except those analyzed in lanes 2 and 9. The proteins present in the incubations were: lane 1 and 2, 3.3 µg host RNA polymerase; lane 3, 2.1 µg core RNA polymerase; lane 4, 2.1 µg core RNA polymerase plus 0.7 µg σ subunit; lane 6, 2.1 µg core polymerase plus 0.4 µg 18K peptide; lane 7, 2.1 µg core polymerase plus 0.4 µg 28K peptide; lane 8, 2.1 µg core plus 0.4 µg 28K peptide plus 0.4 µg 18K peptide; lane 9 and 10, 3.3 µg polymerase from cells 8 min after infection. All incubations with core polymerase were supplemented with 0.16 µg of 21K peptide except that analyzed in lane 5. Lane 11 shows a Hpa-II total digest of 0.6 µg of SP82 DNA.

hand, if the 18K peptide is added to core in the presence of the 28K peptide, there is a slight but reproducible stimulation of enzyme activity. The stimulation of core activity indicates that there is a functional interaction between these isolated peptides and the core polymerase.

The effect of the 28K and 18K subunits from the 8 min polymerase on the specificity of transcription was measured using the filter binding technique of Jones et al. (18). As shown by these investigators, retention of complexes between DNA and RNA polymerase does not occur at low temperature and high ionic strength unless the polymerase has initiated synthesis of an RNA chain.

This is illustrated in Figure 3 which shows an agarose gel of the restriction endonuclease fragments of SP82 DNA which are retained on nitrocellulose filters by various enzyme preparations. Also shown is a total restriction endonuclease digest of SP82 DNA (lane 11). When endonuclease DNA fragments are complexed with host cell RNA polymerase and diluted with cold, high salt buffer, the DNA fragments are not retained on the filter (lane 1). If the incubation mixture contains three nucleotide triphosphates (allowing initiation but limiting elongation), the polymerase selectively complexes three of the 37 fragments produced by digestion of SP82 DNA with Hpa-II (lane 2). The parallel experiment using 8 min polymerase is shown in lane 9 (nucleotides omitted) and lane 10 (nucleotides present). Comparison of lanes 10 and 11 demonstrates that the 8 min polymerase uses most, but not all, of the Hpa-II fragments as points of initiation for RNA synthesis. Comparison of lanes 2 and 10 demonstrates that the modification of the RNA polymerase allows the enzyme to use new DNA sites on SP82 DNA for initiation of RNA synthesis.

To examine which of the peptides associated with the 8 min polymerase is responsible for the increased initiation capabilities, the isolated 28K and 18K subunits were added to host core RNA polymerase and the restriction endonuclease fragments bound in the presence of nucleotides were determined. All core preparations were supplemented with 21K peptide except in lane 5. Lane 3 of Figure 3 shows the DNA fragments bound by core polymerase alone. This pattern is identical to that seen with the host holoenzyme, possibly reflecting small levels of contamination of the core with the σ subunit. Addition of the host σ subunit to the core polymerase enhances retention of the same three DNA fragments (lane 4). It is of interest that the 21K subunit has little effect on the formation of initiation complexes between the core plus σ assembly and the Hpa-II DNA fragments (compare lanes 4 and 5). When the 18K peptide is added to core polymerase, no additional restriction fragments are used for RNA synthesis initiation (lane 6). Addition of the 28K peptide, on the other hand, has a dramatic effect since the number of

fragments bound by the filters increases to approximately 20 (lane 7). Addition of the 18K peptide again has no effect on the initiation sites of the core plus the 28K peptide (lane 8) even though it does slightly stimulate total enzyme activity (Figure 2).

DISCUSSION

There have been several demonstrations that the association of the phage specified 28K peptide with the host RNA polymerase increases the total transcriptional capabilities of the enzyme on phage DNA (4, 6-8, 12-14, 20, 21). These experiments have been based on the competition hybridization and *in vitro* translation analyses of RNA synthesized by modified or reconstituted enzymes. In all cases, the modified enzyme continues to transcribe some of the DNA which is transcribed by the uninfected cell polymerase. For example, we have shown previously (21) that the RNA synthesized *in vivo* by unmodified polymerase (0-3 min after infection) and *in vitro* by isolated polymerase binds exclusively to the same three Hpa-II fragments which are retained on the filter by the host enzyme (lanes 2-4, Figure 3). RNA synthesized 5-7 min after infection or *in vitro* by modified polymerase binds to all of the fragments retained by the 8 min polymerase (lane 10, Figure 3). The fragments complexed by the modified enzyme include those used for initiation and transcription by the host cell polymerase. Therefore, the hybridization data do not determine whether the regions of new transcription by the modified enzyme result from new initiation events of from read-through from promoters utilized by the uninfected cell enzyme. Similar arguments can be made about all of the analyses based on competition hybridization or translation of RNAs synthesized *in vitro*. The data in Figure 3, however, measure only initiation points and thus show unambiguously that the modified RNA polymerase is able to form initiation complexes with fragments of SP82 DNA which do not contain initiation sites for the unmodified polymerase (lanes 2 and 10). The reconstitution experiments demonstrate that the crucial modification in altering initiation specificity is the addition of the 28K peptide. The 18K peptide

appears to have no effect on the recognition of initiation sites. Further experiments will be necessary to elucidate what roles, if any, the 18K peptide has in transcription of SP82 DNA. The fact that the restriction fragments bound in Figure 3 by the 8 min and host RNA polymerases are the same as those which hybridize to *in vivo* and *in vitro* RNA products of these two enzymes argues strongly that most, if not all, of the alteration in transcriptional specificity of these two enzymes is due to the change in initiation specificity. Experiments are in progress to investigate the role of the 18K peptide and to examine the promoters for host and modified polymerases.

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