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Specific labelling of the active site of T7 RNA polymerase

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ABSTRACT

We describe a method for specifically labelling T7 RNA polymerase at (or near) the active site. Enzyme molecules that have been modified by covalent attachment of a benzaldehyde nucleotide derivative in the presence of template DNA are subsequently incubated with radioactively labelled nucleoside triphosphates. Labelling of the enzyme occurs as a result of the formation of the first phosphodiester bond. The labelling is template-directed and the expected specificity of initiation at individual T7 promoters is observed. The label has been localized to an 80 kd tryptic fragment that contains the carboxy-terminal portion of the enzyme.

INTRODUCTION

The DNA-dependent RNA polymerase encoded by bacteriophage T7 consists of a single protein species approximately 98 kilodaltons (for review, see ref. 1). The gene encoding this enzyme has been cloned and overexpressed in bacteria, facilitating the production of milligram quantities of purified enzyme (2,3). As the recombinant enzyme is active in vitro and requires no additional transcription factors, the T7 RNA polymerase represents an attractive model system for the study of polymerase structure and function. The phage enzyme also demonstrates an exquisite specificity for its own unique promoter, a property that we have exploited in the current study in order to selectively label the active site of the polymerase.

The use of benzaldehyde esters of nucleotides as affinity labels has been reported for both procaryotic and eucaryotic RNA polymerases (4,5). The enzymes are first modified with a nucleotide ester, then incubated with radioactively labelled substrate in the presence of template. Nucleotide esters that are bound to the polymerase at or near the active site are in a position to participate in phosphodiester bond formation, and the enzyme becomes labelled by the covalent attachment of the incoming substrate nucleotide.

In this work, we present evidence that T7 RNA polymerase can be labelled in

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a promoter directed manner using similar nucleotide esters. Furthermore, we have localized the site of labelling to an 80-kilodalton tryptic fragment of T7 RNA polymerase that represents the carboxy-terminal 80% of the molecule.

MATERIALS AND METHODS

Synthesis of reagents

The benzaldehyde esters of the guanine nucleotides were synthesized using slight modifications (6) of the protocols described for the adenine nucleotide esters (4). ATP and GTP for the syntheses were purchased from Boehringer Mannheim.

Labelling of T7 RNA polymerase

Recombinant T7 RNA polymerase was purified from bacterial cells that carry the cloned polymerase gene (2). Labelling reactions were carried out as previously reported (5), with the following modifications: the volume of incubation was increased to 50 μl; the buffer contained 40 mM Hepes-NaOH pH 7.9, 10 mM $MgCl_2$, 50 mM NaCl, 0.4 mM benzaldehyde derivative, 0.5 μg T7 RNA polymerase and 1.5 μg DNA (unless otherwise indicated). In the experiments described in Fig. 1 the reduction was carried out with 1 mM $NaBH_4$ at 20°C; in Fig. 3 with 0.5 mM $NaBH_4$ at 0°C. These modifications, which involve lower molar ratios of derivative to enzyme and milder reducing conditions than previously employed (5), were found to improve the efficiency of labelling of the T7 enzyme. After addition of 3-5 μCi (α-^{32}P) NTP [(specific radioactivity 600 Ci/mmol (ICN Radiochemicals, Irvine CA) (Fig. 1) or 1200 Ci/mmol (Amersham Buchler, Braunschweig) (Fig. 3)] the incubation was continued for 15 min at 37°C. Electrophoresis was carried out in gels containing 10% acrylamide and 0.1% dodecylsulfate (7).

Trypsin digestion of labelled polymerase

A 55 μl aliquot containing 1.25 μg labelled T7 RNA polymerase, 7.35 μg unlabelled T7 RNA polymerase, 20 mM $MgCl_2$, 20 mM Tris pH 7.9, 5 mM dithiothreitol, 2 mM spermidine-HCl, 100 mM NaCl and 0.05 μg trypsin (TPCK-treated, Sigma) was incubated at 37°C for 0-30 min. Protease digestion was terminated by the addition of 1 μl 40 mM TLCK and 55 μl 2x Laemmli sample buffer (7). Samples were analyzed by polyacrylamide/SDS gel electrophoresis followed by autoradiography.

RESULTS

Labelling of T7 RNA polymerase with nucleotide esters

The compounds used in this study are diagrammed in Table 1. They differ in their attached nucleotides, the number of phosphate residues and by the

TABLE 1. Structures of Nucleotide Derivatives Used in this Study.

	Structure	Dmax in nm[a]
I.	$OHC-C_6H_4-N(CH_3)CH_2CH_2O-P(O)(O)-O-Gua$	1.0
II.	$OHC-C_6H_4-N(CH_3)CH_2CH_2O-P(O)(O)-O-P(O)(O)-O-Gua$	1.3
III.	$OHC-C_6H_4-O-P(O)(O)-O-P(O)(O)-O-P(O)(O)-O-Gua$	1.2
IV.	$C_6H_4(CHO)-O-P(O)(O)-O-P(O)(O)-O-P(O)(O)-O-Gua$	1.0
V.	$OHC-C_6H_4-O-P(O)(O)-O-P(O)(O)-O-P(O)(O)-O-Ade$	1.2
VI.	$OHC-C_6H_4-N(CH_3)CH_2CH_2O-P(O)(O)-O-P(O)(O)-O-Ade$	1.3

[a]Distance from the α-phosphate to the carbon of the reactive aldehyde group (4).

configuration of the reactive group relative to the benzene ring (and thus overall length). A similar set of compounds has been used to label RNA polymerases from E. coli (4) and wheat germ (5). The derivatives have been reported to form Schiff bases with the ε-amino groups of lysine residues, which can be stabilized under mild reducing conditions (4). To test the efficacy of labelling of T7 RNA polymerase with these reagents, the enzyme was incubated with a reactive nucleotide derivative in the presence of T7 DNA. The modified enzyme was then incubated with radioactively labelled substrate (nucleoside triphosphate) in a transcription cocktail. If the nucleotide derivative is correctly positioned within the catalytic site, the formation of a phosphodiester bond between the derivative and the incoming substrate nucleotide will result in the labelling of the enzyme.

The results of such an experiment are shown in Figure 1. Lanes 1-8 demonstrate the labelling of purified T7 RNA polymerase with compound III, a

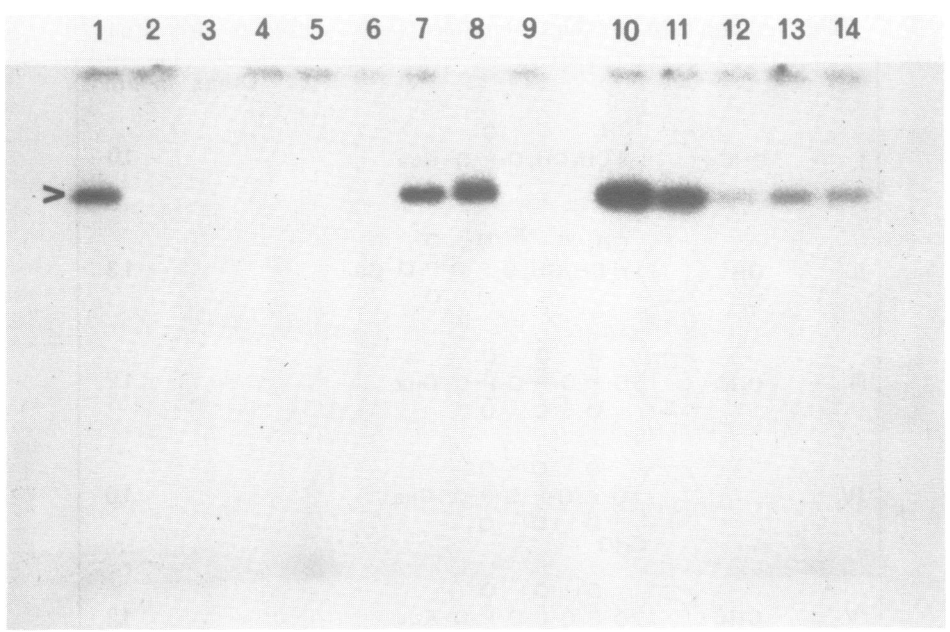

Figure 1. Active site labelling of T7 RNA polymerase with nucleotide esters. Lanes 1-8 contain polymerase labelled in the presence of T7 DNA using derivative III (see Table 1). The reaction in lane 1 contained all components of the mix; lane 2 contained no derivative; lane 3 no DNA; lane 4 no $NaBH_4$. In lane 5 underivatized GTP (0.4 mM) was used in place of the derivative. After the labelling procedure samples in lanes 6-8 were treated with 50 µg/ml proteinase K, 15 µg/ml RNase A, or 50 units ml DNase I, respectively. In lane 9, unlabelled GTP (0.4mM) was added along with the derivative. Lanes 10-14 show results using derivatives II, I, IV, V and VI, respectively. All samples were analyzed by SDS/PAGE. Gels were stained with Coomassie blue prior to autoradiography to visualize the position of the polymerase (marked by an arrow).

p-benzaldehyde derivative of GTP. The labelling is absolutely dependent on the presence of derivative, template DNA, and reducing conditions (lanes 1-5) but is inhibited by molar excess of GTP (lane 9). The labelled enzyme is sensitive to protease treatment, but not to RNAse or DNAse (lanes 6-8).

Other guanosine derivatives were tested, as shown in lanes 10-12. When the p-benzaldehyde derivatives were used, labelling was most efficient with the GDP ester (II). The use of the ortho-derivative of GTP (IV) resulted in a much weaker labelling compared to that obtained with the para-derivative of GTP.

Lanes 13 and 14 show T7 RNA polymerase labelled using the adenosine-containing derivatives V and VI. A low level of labelling is observed.

	-10 +1
T7 consensus	TAATACGACTCACTATAGGGAGA
Ø 10 promoter (Class III)	TAATACGACTCACTATAGGGAGA
Ø 2.5 promoter (Class II)	TAATACGACTCACTATTAGGGAA

Figure 2. Structure of T7 promoters.
The consensus sequence of seventeen T7 promoters is presented in the top line. The sequence of the non-template strand is shown, and initiation is at +1. The sequences of the Ø 10 promoter found in pAR436 and the Ø 2.5 promoter found in pAR219 are also shown (8). Bases that differ from the consensus sequence in the Ø 2.5 promoter are underlined.

Recently, similar observations have been made by Grachev, et al (8). The weak labelling is possibly due to a lower affinity of the T7 RNA polymerase for adenosine derivatives in the acceptor or initiation site. Alternatively, the weaker labelling observed with adenosine-containing derivatives could reflect inefficient initiation of the phage polymerase at promoters that would direct the initiation of RNA chains with ATP. T7 DNA contains both strong (class III) and weak (class II) promoters (9). The class III promoters consist of a 23 basepair consensus sequence that extends from -17 to +6, whereas the class II promoters differ from this consensus at two or more nucleotide positions (Figure 2). All strong promoters, and the majority of the weak promoters, direct a guanosine residue to the +1 position of the nascent RNA chain. However, a subset of the class II promoters can potentially direct an adenosine residue to this position. Utilization of these weak promoters could account for the labelling observed using adenosine-containing derivatives in Figure 1.

Template-directed specificity of labelling

To assess whether or not the labelling of the polymerase is template specific, experiments were carried out with individual phage promoters cloned into pBR322 (10). Results obtained with the Ø10 promoter (plasmid pAR436) and the Ø2.5 promoter (plasmid pAR219) are shown in Figure 3. The Ø10 promoter is expected to direct the synthesis of RNA products that initiate with the sequence pppGpG. In the presence of a template that carries this promoter, efficient labelling was observed only when the enzyme was modified with a derivative of guanosine and the incoming (α-^{32}P)-labelled nucleoside triphosphate was GTP (lanes 6-10). No labelling was observed with template DNA that did not contain a phage promoter (lanes 11-12).

The Ø2.5 promoter is expected to direct the synthesis of RNA chains that

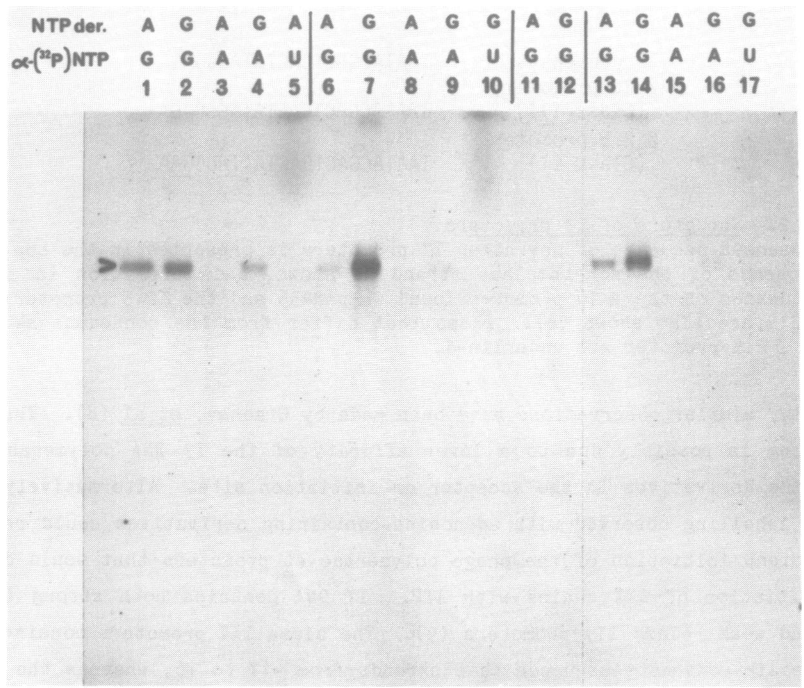

Figure 3. Template directed specificity of labelling
T7 RNA polymerase was modified with compounds III (GTP-derivative) or V (ATP-derivative), as indicated, in the presence of pAR219 (lanes 1-5), pAR436 (lanes 6-10), or a plasmid without a phage promoter (lanes 11-12). Following derivatization, the enzymes were incubated with (α-^{32}P)-GTP, -ATP or -UTP, as indicated. In lanes 13-17 the enzyme was derivatized in the absence of DNA, and template (pAR436) was subsequently added with the substrate. Samples were analyzed by SDS/PAGE and autoradiography. The position of T7 RNA polymerase as determined by staining with Coomassie blue is marked with an arrow.

initiate with ATP (Figure 2). However, it has been observed that RNA chains initiated at this promoter start only 50% of the time with ATP and 50% of the time with GTP (J. Dunn, personal communication) and this ambiguity is reflected in the data of Figure 3 (lanes 1 and 2). The apparent ambiguity in start site selection may be characteristic of certain class II promoters, and could account for the weak labelling seen in Figure 3, lane 4.

Because efficient labelling of the enzyme at both the ø10 and the ø2.5 promoters was observed only when the combination of nucleotide derivative and substrate was consistent with the known initiation specificity of the promoters, we conclude that the labelling of the enzyme by this method is specific, and is promoter-directed.

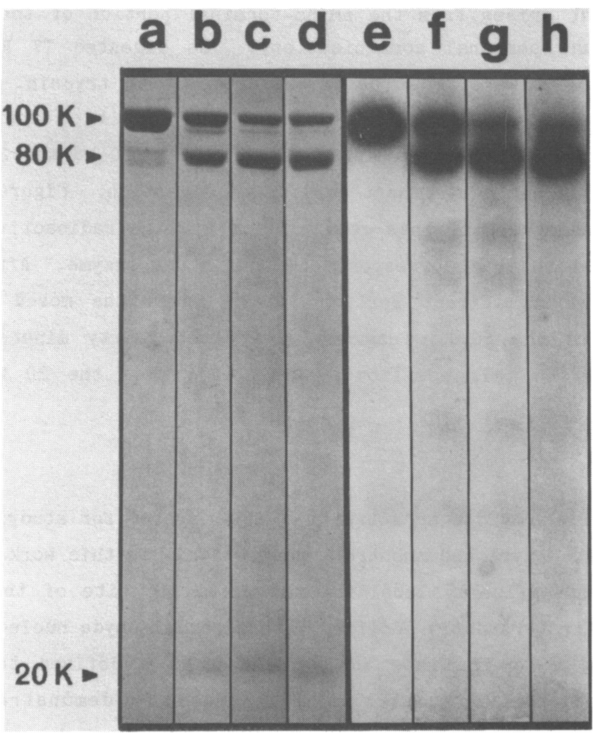

Figure 4. Partial proteolytic cleavage of T7 RNA polymerase labelled at the active site.
Enzyme labelled with compound III and (α-^{32}P)-GTP in the presence of the ø10 promoter was digested with trypsin. The digests were terminated after 0, 2, 10 and 30 minutes (lanes a-d, respectively). Samples were analyzed by SDS/PAGE. Lanes a-d show the gel after Coomassie blue staining; lanes e-h show an autoradiograph of the same gel. The positions of intact T7 RNA polymerase and the primary partial digestion products are indicated.

The attachment of the NTP ester to the enzyme does not require the presence of template DNA. Labelling of enzyme that is modified in the absence of template exhibits template-directed specificity when the polymerase is subsequently incubated with substrates and DNA (Figure 3, lanes 13-17). These results suggest that the binding of the first nucleotide to the initiation (or acceptor) site is not template-directed, but that the subsequent polymerization reaction is absolutely dependent upon the presence of the appropriate nucleotide in the active site.

Localization of the affinity label to an 80kd tryptic fragment

When T7 RNA polymerase is subjected to mild digestion with trypsin the 98 kd intact polymerase is rapidly converted to 80 kd and 20 kd fragments. The

smaller fragment arises from the amino-terminal portion of the protein (ref. 11 and J. Dunn, personal communication). We digested T7 RNA polymerase, labelled at the active site as described above, with trypsin. Aliquots were removed at various times after protease addition and analyzed on SDS/polyacrylamide gels (Figure 4, lanes a-d). The 80 kd and 20 kd digestion products are apparent after short periods of digestion. Figure 4 (lanes e-h) shows an autoradiograph of this gel. Initially, the radioactivity comigrates with the 98 kd band that corresponds to the intact enzyme. After two minutes of digestion, a significant portion of the label has moved to a position corresponding to the 80 kd fragment, and after thirty minutes most of the label has moved to this position. No labelling of the 20 kd fragment is observed.

DISCUSSION

T7 RNA polymerase provides an attractive model system for studying the complex of template DNA, enzyme and substrate nucleotide. In this work, we describe a procedure for specifically labelling the catalytic site of the enzyme. The polymerase is first randomly modified with a benzaldehyde nucleotide ester and is then allowed to initiate RNA synthesis on a defined template in the presence of radioactive substrate NTP. We have demonstrated that the radioactive labelling of the enzyme is template-directed, and observes the expected specificity of initiation at individual T7 promoters.

Our goal is the eventual identification and characterization of the active site of the polymerase. We have tested the ability of various compounds to participate in phosphodiester bond formation when linked to the enzyme. Our results suggest that a particular orientation of the derivatized nucleotide within the active site may be required for the formation of the first phosphodiester bond. For example, the ortho-derivative of GTP produced a distinctly less efficient labelling than the para-derivative. Using similar benzaldehyde esters of adenine nucleotides to label the β subunit of the *E. coli* RNA polymerase, Grachev *et al*. also observed that the efficiency of labelling was much lower with the ortho-derivative than with the para-derivative (4). In addition, Grachev *et al* noted that the extent of labelling of the β subunit increased with the distance between the aldehyde group and the α-phosphorus of the priming nucleotide. Our results suggest that labelling of the T7 enzyme may exhibit a similar dependence upon spacer length (Table 1 and Fig. 1). One must be cautious, however, in interpreting these results as models suggest that the spacer arm of the derivatives is highly

flexible (A. Schäffner, data not shown). Furthermore, interpretation of these data on the basis of the intensity of labelling of the enzyme makes the assumption that all of these compounds have equal access to the active site and are of equal reactivity, and this has not yet been experimentally demonstrated.

Mild trypsin treatment of the T7 RNA polymerase results in the production of 80 kd and 20 kd fragments. Our data indicate that the active site of the enzyme resides within the 80 kd carboxy-terminal fragment. This is consistent with the suggestion of Ikeda and Richardson that the catalytic function of the enzyme resides in this fragment (11). The promoter recognition function of T7 RNA polymerase has also been shown to reside in this part of the molecule, and has been localized to the carboxy terminal third of the protein (12). Experiments designed to identify more precisely the active site of T7 RNA polymerase are in progress.

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