Detection of an epitope, not required for polymerization, that is conserved between *E. coli* DNA polymerases I and III and bacteriophage T4 DNA polymerase

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Received February 29, 1988

ABSTRACT

Monoclonal antibodies directed against the α subunit of the DNA polymerase III holoenzyme (1) of *E. coli* were tested for cross-reactivity with a variety of polymerases. We found that one monoclonal antibody bound to *E. coli* DNA polymerase I as well as to DNA polymerase III. A weaker, but specific, interaction was also detected with T4 DNA polymerase. We exploited the proteolysis procedure developed by Setlow, Brutlag and Kornberg (2) to determine which domain of DNA polymerase I contained the conserved epitope. Contrary to expectations, it was not found in the polymerase domain, but in the 5'→3' exonuclease domain. This reveals a sequence or structure, sufficiently important to be conserved among these polymerases, that is not directly involved in the polymerization reaction.

INTRODUCTION

Through the use of natural replicative assays provided by the single-stranded DNA bacteriophages, M13, G4 and ϕ X174, the components required for the replication of the *E. coli* chromosome have been identified and purified (3). These studies have revealed an exceedingly complex polymerase, the DNA polymerase III holoenzyme, that is required for the synthesis of the majority of the *E. coli* genome (4, 5). DNA polymerase I participates in the removal of the RNA primers required for the synthesis of Okazaki fragments (3, 6). DNA polymerase I exploits the coupled action of its polymerase and 5'→3' exonuclease for this "nick translation" reaction (3, 7). The DNA polymerase III holoenzyme lacks an exonuclease with these capabilities.

DNA polymerase I has served as the prototypical DNA polymerase for the mechanistic studies of many laboratories over the last three decades. The three catalytic activities of DNA polymerase I reside within one polypeptide chain. Treatment with subtilisin results in generation of two fragments of approximately 76,000 and 36,000 daltons (8). The large fragment contains the polymerase and 3'→5' proofreading activity; the smaller fragment contains the 5'→3' exonuclease activity (8). These two fragments, even when separated, still retain the ability to

recognize one another and coordinate polymerization with 5'→3' exonuclease action (8).

Complex bacteriophages, such as λ , T4 and T7, induce many of their own replication proteins. λ directs the synthesis of its own origin-specific proteins, but relies on the *E. coli* DNA polymerase III holoenzyme for its replication (9). Both T4 and T7 induce the synthesis of their own polymerases which act together with other induced replication proteins by mechanisms analogous to the *E. coli* replicative complex (10,11).

The DNA polymerase III holoenzyme of *E. coli* is a complex enzyme that contains at least seven different subunits (3, 5). The catalytic core (α , ϵ and θ subunits) is just as efficient as holoenzyme on unnatural gapped templates. The auxiliary subunits, τ , β , γ , and δ , are required to confer the special properties required for replication of the *E. coli* chromosome. In our studies, we have begun to generate a battery of specific monoclonal antibodies against all of the holoenzyme subunits to facilitate our understanding of the genetic regulation and the biochemical function of these components of the replicative complex (1, 12). In this study, we report the finding that one of three monoclonal antibodies that reacts with the α subunit of the holoenzyme also reacts with two other prokaryotic polymerases.

MATERIALS AND METHODS

Materials

E. coli DNA polymerase III holoenzyme was purified as described (13). *E. coli* DNA polymerase I and T4 DNA polymerase were purchased from PL Biochemicals, Inc. Antibodies were obtained as described (1). We have deposited monoclonal antibodies anti- α_{123-10} and anti- α_{68-1-2} in the the American Type Culture Collection (ATCC.CRL 1707 and ATCC.CRL 1712). Subtilisin and phenylmethylsulfonyl fluoride were purchased from Sigma. The Kodavue electrophoresis visualization kit was purchased from Kodak and used as recommended by the supplier.

Antibody Competition Binding Assay

Polystyrene tubes were coated with DNA polymerase III holoenzyme by incubation with a holoenzyme solution (1 μ g in 0.1 ml of sodium carbonate, 10 mM sodium azide, pH 9.5) for 16 h at 37°C. Tubes were washed three times with 0.05% Tween 20 in PBS buffer (10 mM sodium phosphate, 150 mM sodium chloride, 10 mM sodium azide, pH 7.4) and then three times with PBS alone. Incubation with bovine serum albumin (1% solution in PBS buffer) blocked remaining protein binding sites. Competing antigen (diluted in 1% BSA in PBS buffer) was mixed

Fig. 1. Cross-reactivity of anti- α_{123-10} with DNA polymerase I of *E. coli*. Lanes A and C, DNA polymerase III holoenzyme ($1.8 \mu\text{g}/\text{well}$) and lanes B and D, DNA polymerase I ($250 \text{ ng}/\text{well}$) were subjected to SDS polyacrylamide gel electrophoresis and immunoblotting as described (1). Nitrocellulose strips containing lanes A-B and C-D were cut out and treated with anti- α_{6-1-3} and anti- α_{123-10} , respectively (1600 units of antibody in 5 ml). Peroxidase-labeled goat anti-mouse IgM was used as the detection reagent. As a control (not shown), nitrocellulose strips containing the same polymerases were treated with a non-specific myeloma IgM; no reaction was detected.

with ^{125}I -anti- α_{123-10} and incubated (16 h, 4°C). Tubes containing immobilized holoenzyme were washed as before. The antigen-antibody mixture was added to the holoenzyme-coated tubes and incubated (6 h, 4°C). Tubes were washed extensively (15 times with PBS-Tween 20 buffer) and the bound radioactivity determined. Where indicated, antigen was heat-treated by boiling for five minutes prior to addition to antibody.

Immunological Detection of Proteins Separated by SDS Page

Proteins were subjected to SDS polyacrylamide gel electrophoresis and immunoblotting as described (1). In an effort to reduce background, some procedural steps were modified. Instead of 1% BSA, 5% non-fat milk (Carnation)

Fig. 2. Localization of the conserved epitope in DNA polymerase I. DNA polymerase I (84 μ g) was incubated at 37°C in the presence of 100 ng subtilisin in 63 mM potassium phosphate (pH 6.5). At the indicated time, aliquots (10 μ g) were removed and treated with phenylmethylsulfonyl fluoride (0.5 mM final concentration) to inactivate the subtilisin. Samples were diluted 1:5 in sample buffer (62.5 mM Tris-HCl, pH 6.8, 50% glycerol, 2% SDS, 5% β -mercaptoethanol) and immediately boiled. After all reactions were completed, samples were subjected to SDS polyacrylamide gel electrophoresis. The upper gel was stained by the Kodavue procedure; the lower gel was subjected to immunoblotting. The position of molecular weight markers are indicated in the upper photograph; the position of three holoenzyme subunits, run in a parallel lane, are indicated. Photography exaggerated the low level nonspecific reaction between the large fragment and antibody.

Fig. 3. Competition by DNA polymerases for binding of ^{125}I -anti- α_{123-10} to immobilized DNA polymerase III holoenzyme. Binding of labeled antibody to immobilized holoenzyme in the presence of soluble antigen was performed as described under "Materials and Methods". Antigen competition was with (●), heat-treated DNA polymerase III holoenzyme; (▲), heat-treated DNA polymerase I; (○), native DNA polymerase III holoenzyme and (△), native DNA polymerase I.

was included in the saline solutions used for incubations with antibody and for the blocking step. Incubations with the primary antibody were performed at 4°C for 16 h. Rabbit IgG directed against mouse IgM and ^{125}I -protein A were used as detection reagents. The immunoblot was exposed to Kodak XAR-5 film for 24 h.

RESULTS AND DISCUSSION

We focused our initial screen for cross-reactivity of antibodies directed against the α subunit of the DNA polymerase III holoenzyme upon *E. coli* DNA polymerase I. We subjected holoenzyme and DNA polymerase I to the "Western" immunoblotting procedure. All three monoclonal antibodies, 123-10, 6-1-3 and 68-1-2 (data not shown), indicated strong specific binding with the α subunit of holoenzyme as previously reported (1, Fig. 1). However, antibody 123-10 also reacted strongly with DNA polymerase I. The strength of binding, judged in terms of the intensity of the staining in Western blots performed with varying antibody concentrations, was at least as strong as it was with α .

To further explore the location of this conserved epitope, we subjected DNA polymerase I to mild proteolysis to physically separate the DNA polymerase domain from the small $5'\rightarrow 3'$ exonuclease domain. As previously reported (8), this procedure results in the appearance of a stable fragment of approximately 76,000

Fig. 4. Cross-reactivity of anti- α_{123-10} with T4 DNA polymerase. The indicated quantity of DNA polymerase was subjected to SDS polyacrylamide gel electrophoresis and immunoblotting as described under "Materials and Methods". Lanes A and D, DNA polymerase III holoenzyme (2 μ g); lanes B and E, DNA polymerase I (2 μ g); lane C and F, T4 DNA polymerase (13 μ g). The nitrocellulose sheet resulting from transfer of lanes A-C was treated with antibody 123-10; lanes D-F were treated with a control myeloma mouse IgM.

daltons and a less stable fragment of 36,000 daltons (Fig. 2, upper). When analyzed by the "Western" immunoblotting procedure, only the intact polymerase and the small fragment were found to bind antibody strongly (Fig. 2, lower). This indicates that the epitope recognized by anti- α_{123-10} is located within the small fragment and not the large polymerase-containing domain.

We found the observation that the conserved epitope was not in the polymerase domain of DNA polymerase I to be surprising, since we would not have expected a sequence or structure to be conserved if it was dispensable for function. Perhaps it is important for some other function, such as specific protein-macromolecule or domain-domain interactions, that have not yet been detected. Several years ago, Hübscher and colleagues observed an even broader relationship between polymerases based upon the pattern exhibited upon proteolysis. DNA polymerases from a wide variety of organisms could have a

small domain proteolytically removed from a larger enzyme, generating a smaller active polymerase domain (14).

To add further support to our observation, we examined antibody-polymerase binding by a solution competition assay. Holoenzyme was immobilized onto the sides of a polystyrene tube. When ^{125}I -anti- α_{123-10} was added, it bound to the immobilized holoenzyme. However, this binding could be inhibited if heat-treated holoenzyme or DNA polymerase I was first incubated with the antibody (Fig 3). It is interesting that both DNA polymerase I and III are ineffective competitors if they are not first heated. We interpret this as meaning that the recognized epitope is internal and not exposed to antibody in the native state. This is consistent with our inability to use anti- α_{123-10} to remove native holoenzyme from solution (data not shown). The assay was conducted in the presence of a vast excess of bovine serum albumin in order to avoid non-specific interactions. When the same experiment was performed with antibody anti- α_{68-1-2} , DNA polymerase I did not compete under any condition (data not shown).

We tested other polymerases for cross-reactivity and found that anti- α_{123-10} also bound specifically to T4 DNA polymerase, although not as strongly as to DNA polymerases I or III (Fig 4). We also tested T7 DNA polymerase, T7 Gene 4 protein, T7 RNA polymerase, calf thymus α polymerase, the remaining DNA polymerase III holoenzyme subunits and both *E. coli* *dnaG* primase and RNA polymerase for binding to anti- α_{123-10} . We observed no specific antibody binding under the conditions used with the level of protein available (1-10 μg).

As additional monoclonal antibodies are generated against DNA polymerase III holoenzyme, further insights into the relationship between its subunits and other replicative complexes should result.

ACKNOWLEDGMENTS

This work was supported by grant GM36255 from the National Institutes of Health. We gratefully acknowledge Drs. Ulrich Hübscher and Charles Richardson for the gifts of the calf thymus α polymerase and T7 enzymes, respectively.

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