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The *dnaN* gene codes for the β subunit of DNA polymerase III holoenzyme of *Escherichia coli*

(DNA synthesis)

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ABSTRACT **An *Escherichia coli* mutant, *dnaN59*, stops DNA synthesis promptly upon a shift to a high temperature; the wild-type *dnaN* gene carried in a transducing phage encodes a polypeptide of about 41,000 daltons [Sakakibara, Y. & Mizukami, T. (1980) *Mol. Gen. Genet.* 178, 541–553; Yuasa, S. & Sakakibara, Y. (1980) *Mol. Gen. Genet.* 180, 267–273]. We now find that the product of *dnaN* gene is the β subunit of DNA polymerase III holoenzyme, the principal DNA synthetic multipolypeptide complex in *E. coli*. The conclusion is based on the following observations: (*i*) Extracts from *dnaN59* cells were defective in phage φX174 and G4 DNA synthesis after the mutant cells had been exposed to the increased temperature. (*ii*) The enzymatic defect was overcome by addition of purified β subunit but not by other subunits of DNA polymerase III holoenzyme or by other replication proteins required for φX174 DNA synthesis. (*iii*) Partially purified β subunit from the *dnaN* mutant, unlike that from the wild type, was inactive in reconstituting the holoenzyme when mixed with the other purified subunits. (*iv*) Increased dosage of the *dnaN* gene provided by a plasmid carrying the gene raised cellular levels of the β subunit 5- to 6-fold.**

DNA polymerase III (pol III) holoenzyme, the principal DNA synthetic enzyme in *Escherichia coli*, is composed of at least eight polypeptides. The complex is readily assayed by its requirement in the conversion of primed, single-stranded, circular phage φX174 or G4 DNA to the duplex form and in the further replication of these duplexes (1, 2). The holoenzyme consists of a tripolypeptide pol III core (α, ε, and θ subunits, which are 140,000, 25,000, and 10,000 daltons, respectively) (3), and the following separable subunits: β, 37,000 daltons (4); γ, 52,000 daltons (5); δ, 32,000 daltons (6); τ, 83,000 daltons (7); and ζ.§

Genetic loci for some of these polypeptides have been identified as *dnaZ* for the γ subunit (5), *dnaX* for δ (6), and most probably *dnaE* (*polC*) for α (8). For lack of knowing the loci for the other polypeptides, their physiologic function and status as essential subunits are uncertain.

The discovery of the temperature-sensitive *dnaN* mutant with a quick-stop phenotype in DNA synthesis at the restrictive temperature (9) and the knowledge that the *dnaN* gene encodes a polypeptide near the known size of the β subunit (10) suggested that *dnaN* might be the structural gene for the β subunit. We report here enzymatic and further genetic studies that prove the *dnaN59* mutant to be defective in the β subunit, and we conclude that the *dnaN* gene encodes the β subunit.

MATERIALS AND METHODS

Bacterial Strains, Buffers, and Enzymes. Sources were *E. coli* HC193 (HfrC, *pyrE*, *metB*, *thy*), HC194 (HfrC, *dnaN59*, *metB*, *thy*) (9), and *E. coli* HMS83 (*polA1*, *polB100*, *thy*, *lac*Zam, *rha*, *lac*, *str*^{r}) (11). The following buffers were used: buffer A, 10 mM Tris·HCl, pH 7.5/15% (vol/vol) glycerol/0.1 mM EDTA/5 mM dithiothreitol; buffer B, 20 mM Tris·HCl, pH 7.5/20% (vol/vol) glycerol/5 mM dithiothreitol/1 mM EDTA. The enzymes used were as described (6). β subunit (5×10^6 units/mg) was prepared essentially as described (4). pol III* (4×10^5 units/mg) was prepared from purified holoenzyme as described below for the crude enzyme fractions.

Assay for β Subunit. The assay measures formation of holoenzyme from β and pol III* by its function in the conversion of single-stranded G4 DNA to the duplex replicative form. The 25-μl reaction mixture contained 20 mM Tris·HCl at pH 7.5, 8 mM dithiothreitol, 80 μg of bovine serum albumin per ml, 4% (wt/vol) sucrose, 10 mM $MgCl_2$, 100 μM each GTP, CTP, and UTP, 2 mM ATP, 48 μM each dATP, dGTP, and dCTP, 18 μM [^{3}H]dTTP (600–1000 cpm/pmol), 67 units of single-stranded DNA binding protein, 225 pmol (as nucleotide) of single-stranded G4 DNA, 25 units of primase, 30 units of pol III*, and the β subunit to be assayed. Incubation was at 30°C for 5 min. The reaction was stopped by addition of 100 μl of 0.1 M sodium pyrophosphate and 1 ml of 10% trichloroacetic acid. The precipitate was collected after 20 min at 0°C on a Whatman GF/C filter, washed three times with 1 M HCl containing 0.1 M sodium pyrophosphate, rinsed with 95% (vol/vol) ethanol, dried, and assayed for radioactivity in a toluene-based scintillation fluid in a liquid scintillation counter. One unit is defined as the incorporation of 1 pmol of total deoxynucleotide per min at 30°C.

Complementation of Mutant Enzyme Fractions in the Conversion of φX174 or G4 Single-Stranded Circular DNA to the Duplex Form. The 25-μl reaction mixture contained 20 mM Tris·HCl at pH 7.5, 8 mM dithiothreitol, 80 μg of bovine serum albumin per ml, 4% (wt/vol) sucrose, 10 mM $MgCl_2$, 100 μM each GTP, CTP, and UTP, 1 mM ATP, 48 μM each dATP, dGTP, and dCTP, 18 μM [^{3}H]dTTP (600–1000 cpm/pmol), 67 units of single-stranded DNA binding protein, 225 pmol (as nucleotide) of single-stranded φX174 or G4 DNA, and purified replication proteins and crude enzyme sources as indicated in the legends to the tables and figures. Incubations were at 30°C for the times indicated. Further operations were as described above.

Preparation of Crude Enzyme Fractions for Replication Assays. Cells were grown in 2 liters of L broth supplemented with 50 μg of thymine per ml at 30°C to $OD_{595} = 1.0$, harvested at 0°C, resuspended to $OD_{595} = 400$ in 50 mM Tris·HCl, pH 7.5, containing 10% sucrose, and frozen in liquid N_2. To obtain

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Abbreviations: pol III, DNA polymerase III; kb, kilobase pairs.

§ The ζ subunit has recently been identified as a protein necessary for *in vitro* replication of G4 DNA.

cells exposed to a restrictive temperature, cells were grown in 1 liter of the broth at 30°C to OD_{595} = 1.0 and then the temperature was shifted to 42.5°C by addition of 1 liter of broth prewarmed to 55°C. After further incubation at 42.5°C for 30 min the cells were harvested as above. For lysis, the thawed cell suspension was brought to 15 mM spermidine·HCl, 100 mM NaCl, and 5 mM dithiothreitol, and the pH was adjusted to 7.5 with 2 M Tris base. The suspension was diluted to OD_{595} = 200 with 50 mM Tris·HCl, pH 7.5, containing 10% sucrose, and freshly dissolved lysozyme (20 mg/ml in 50 mM Tris·HCl, pH 7.5/10% sucrose) was added to a final concentration of 0.2 mg/ml. The suspension was left on ice for 45 min and then placed in a 37°C bath for 1.5 min. After a further 5 min on ice, the mixture was centrifuged in a Beckman JA20 rotor at 14,000 rpm for 60 min at 0°C. To the supernatant (fraction 1, 6–9 ml), solid ammonium sulfate (0.26 g/ml) was added with stirring. After 1 hr at 0°C, the precipitate was collected by centrifugation at 20,000 rpm for 20 min. The pellet was dissolved in buffer A (0.3–0.4 ml) and dialyzed against buffer A until the conductivity had dropped to that of buffer A containing 25 mM ammonium sulfate (fraction 2). Aliquots were frozen in liquid N_2 and stored at −80°C. Protein concentrations were 10–20 mg/ml.

To separate the β subunit, fraction 2 (0.3 ml) was diluted with buffer B (0.5 ml) until a conductivity equivalent to that of buffer B containing 20 mM NaCl was reached and loaded onto a 0.8-ml phosphocellulose column in buffer B/20 mM NaCl. The column was washed with 5 bed volumes of buffer B/20 mM NaCl and eluted with 2 bed volumes of buffer B/300 mM NaCl. The flowthrough and initial wash (1.4 ml) contained β activity and the high-salt eluate, pol III* activity.

Construction of Plasmids Containing the *dnaN* Gene. Phage λ*gt*-HF39 DNA (1 μg) was partially digested with restriction endonuclease *Eco*RI (1 unit) in a 10-μl reaction mixture containing 20 mM Tris·HCl at pH 8.0, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA, 100 mM NaCl, and 0.1 mg of bovine serum albumin per ml at 37°C for 15 min (12). The enzyme was then inactivated by an incubation at 70°C for 5 min. Vector DNA (plasmid pMOB45, 100 ng) was completely digested with *Eco*RI under the above conditions and 5′-terminal phosphates were removed by a 30-min further digestion with calf alkaline phosphatase (Boehringer Mannheim, 0.1 unit) in the same buffer (13, 14). Vector DNA was isolated by phenol extraction and ligated to the partially digested λ*gt*-HF39 DNA by incubation with phage T4 DNA ligase (0.1 unit) at 10°C for 16 hr in a 15-μl reaction mixture used for the *Eco*RI digestion supplemented with 1 mM ATP and 10 mM dithiothreitol. The DNA was introduced into *dnaN59* cells according to Davis *et al.* (15). Ligation for completely digested λ*gt*-F81 DNA (1 μg) and transformation of the DNA were performed in exactly the same way. Colonies were selected for tetracycline resistance, chloramphenicol sensitivity, and ability to grow at 42°C. λ*gt*-HF39 DNA contains *E. coli* 1.0- and 3.3-kilobase pair (kb) *Eco*RI fragments neighboring each other, carrying the *dnaA* and *dnaN* genes. λ*gt*-F81 DNA contains the 3.3-kb fragment only, which lacks part of the *dnaA* gene (12, 16).

RESULTS

A Crude Enzyme Fraction from *dnaN* Mutant Cells Is Defective in Replication of φX174 DNA. An ammonium sulfate fraction (fraction 2) from *E. coli* HC194 *dnaN59* cells grown at 30°C was fully active in the conversion of single-stranded φX174 DNA to the replicative form (Fig. 1). Even after being heated to 49°C for 10 min, fraction 2 was still fully active when the assay mixture was supplemented with the rather thermolabile *dnaB* and *dnaC* proteins (results not shown). However, fraction 2 from *dnaN59* cells, exposed to 42.5°C for 30 min, was severely defective in φX174 DNA replication, whereas the activity of the corresponding fraction from the isogenic, wild-type strain was not affected by this *in vivo* treatment (Fig. 1). Thus, the mutant *dnaN* gene product appears to be thermostable *in vitro* but can be inactivated by heating *in vivo* at the restrictive temperature.

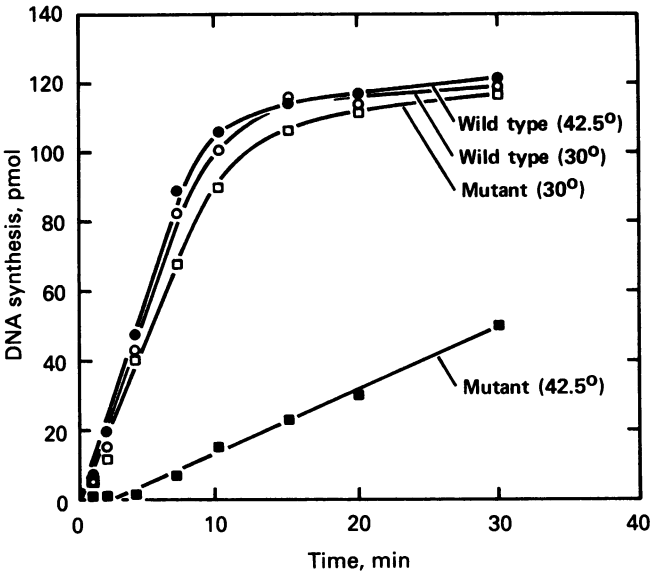

FIG. 1. φX174 DNA synthesis by enzyme fractions from *dnaN59* and wild-type cells grown at 30°C and the effect of culturing the cells at 42.5°C for 30 min. Fraction 2 (62 μg) was added to each 25-μl assay mixture.

The Defective Enzyme Fraction from *dnaN* Mutant Cells Is Complemented by pol III Holoenzyme or the β Subunit. The inactivity of fraction 2 from *dnaN59* cells exposed to 42.5°C cannot be attributed to an inhibitor because a small amount of wild-type fraction 2, displaying a very low activity by itself, restored replication activity of the mutant fraction 2 (Table 1). The restored activity was comparable with that of wild-type fraction 2 added in a similar amount to that of mutant fraction 2 (Fig. 1). The defect was also overcome by addition of purified pol III holoenzyme or the β subunit. The prepriming proteins n, n′, n″, i, *dnaB* and *dnaC* were inactive, as were pol III core and the other holoenzyme subunits. A significant increase in activity by the addition of primase (*dnaG* protein) is due to suboptimal primase levels in extracts of both wild-type and mutant cells. To obviate the use of large amounts of fraction 2, purified primase was added routinely to saturate the need for this enzyme in the assay.

The Crude Enzyme Fraction from *dnaN* Mutant Cells Is Defective in G4 DNA Replication and Is Complemented by the β Subunit. Conversion of single-stranded G4 DNA to the duplex form depends on primase, single-stranded DNA binding protein, pol III core, and subunits β, γ, and ζ (5). This G4 system for measuring β subunit activity is simpler than the φX174 replication system. Only small amounts of fraction 2 from wild-type cells or *dnaN59* cells grown at 30°C were needed to saturate the assay for conversion of single-stranded G4 DNA to the duplex form (Fig. 2). Again, no defectiveness in DNA replication was observed after heating the enzyme fraction from *dnaN59* cells at 52°C for 10 min. However, after exposure of the mutant cells to 42.5°C for 30 min, not even large amounts of mutant fraction 2 could sustain efficient G4 DNA synthesis (Fig. 2). This defect was complementable with purified β subunit but not with pol III core or γ subunit (Fig. 3). These results with both φX174 and G4 replication systems suggest that the

Table 1. Complementation of *dnaN* mutant fraction 2* with purified *E. coli* replication proteins

Protein added	DNA synthesis,† pmol/20 min
None (fraction 2, alone)	(0)
Wild-type fraction 2,‡ 10 μg	122
Protein n	0
Protein n′	0
Protein n″	0
Protein i	5
dnaB protein	0
dnaC protein	5
Primase	14
Holoenzyme	105
pol III core	0
β subunit	111
γ subunit	0
δ subunit	0
τ subunit	0
Holoenzyme heated at 90°C for 10 min	0
β subunit heated at 90°C for 10 min	0

* The *dnaN* mutant fraction (46 μg of protein per assay) was prepared from *dnaN59* cells grown at 30°C and then shifted to 42.5°C.

† Assayed with φX174 DNA under the conditions described in *Materials and Methods* and corrected for background DNA synthesis of *dnaN* mutant fraction alone (28 pmol/20 min).

‡ DNA synthesis with this amount of wild-type fraction 2 alone was 15 pmol; all other proteins were present in twice the amount necessary to saturate the assay.

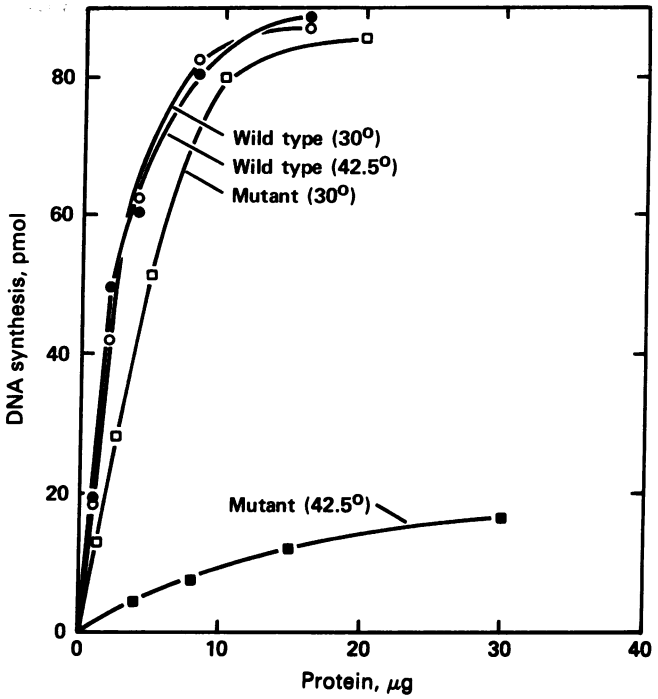

FIG. 2. G4 DNA synthesis by enzyme fractions from *dnaN59* and wild-type cells grown at 30°C and the effect of culturing the cells at 42.5°C for 30 min. Assay mixtures contained the indicated levels of fraction 2, 25 units of primase, and other components as described in *Materials and Methods*. Incubations were at 30°C for 5 min.

defect in the *dnaN* mutant is in the β subunit of pol III holoenzyme.

The Mutant β Subunit Is Deficient in Assembly of the Holoenzyme *in Vitro*. Phosphocellulose chromatography of crude or purified pol III holoenzyme partitions the β subunit into the flowthrough; the remainder of the holoenzyme, termed pol III*, is adsorbed and can be eluted with salt. This chromatography performed with fraction 2 from wild-type and *dnaN59* cells grown at 30°C (*Materials and Methods*) demonstrated β activity in the flowthrough (fraction 3). The high-salt eluate contained pol III* activity without significant β activity (data not shown).

Although pol III holoenzyme activity in fraction 2 from *dnaN59* cells grown at 30°C was still 50–70% of that from wild type (Fig. 2), fraction 3 from *dnaN59* cells had only 15% β activity compared with the corresponding wild-type fraction (Fig. 4). This deficiency can be attributed either to a lower yield of the mutant protein or to its reduced effectiveness in reconstituting pol III* to an active holoenzyme. The latter interpretation appears more likely as judged by the reconstitution of holoenzyme from purified polypeptides. Reconstitution from pol III core, γ, ζ, and partially purified wild-type β (fraction 3) was almost as efficient as that from pol III* with wild-type fraction 3 (Fig. 4). By contrast, the mutant fraction 3 was completely inactive (Fig. 4). In control experiments, the activity of wild-type fraction 3 was not diminished by addition of mutant fraction 3. These results indicate that the inactivity of the mutant fraction 3 is due to an inherent defect in the mutant β subunit.

The β Subunit Is Abundant in Cells and Is Overproduced by Gene Amplification. The number of β subunit dimer molecules per *E. coli* HMS83 cell can be estimated to be about 300 on the basis of the following: a determined value of 220,000 units in fraction 1 derived from 1 g of cell paste containing about 1 × 10^{12} cells, a molecular weight of 74,000 for a β dimer (4), and a specific activity of the pure protein of 5.4 × 10^6 units/mg.

The *dnaN* gene resides within a 3.3-kb *Eco*RI segment of the *E. coli* chromosome, and is transcribed principally from the promoter of the adjacent *dnaA* gene, which lies in a region of the 3.3-kb segment and a 1.0-kb *Eco*RI segment neighboring it (16). A recombinant plasmid of pMOB45 that carried the 1.0-

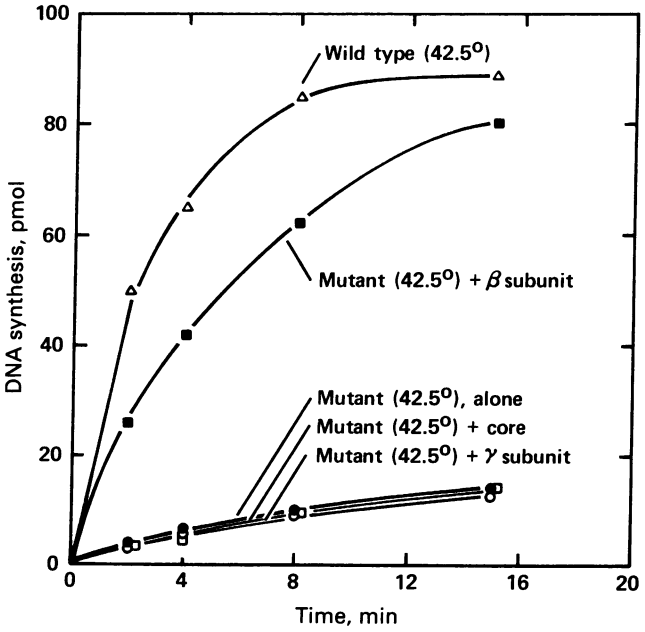

FIG. 3. Complementation of enzyme fractions from *dnaN59* cells exposed to 42.5°C for 30 min. Assay mixtures contained 4 μg of fraction 2 and 25 units of primase. Additions of purified holoenzyme subunits where indicated were: pol III core, 10 units; γ subunit, 60 units; and β subunit, 40 units.

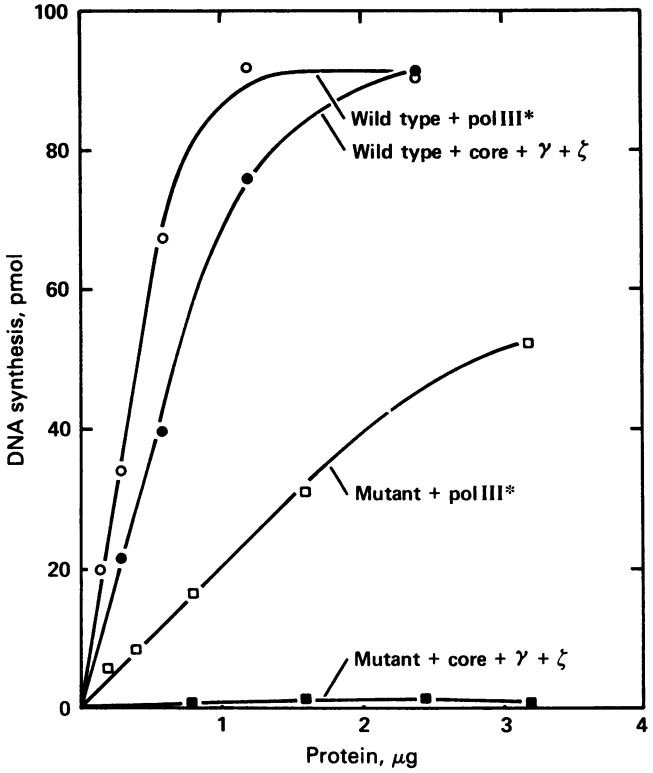

FIG. 4. Activity of the β subunit partially purified from *dnaN59* or wild-type cells in the reconstitution of holoenzyme with either pol III* or with pol III core, γ, and ζ subunits. The assay was with G4 DNA. Additions were: 30 units of pol III* and indicated amounts of wild-type fraction 3 (○); 10 units of pol III core, 60 units of γ, 12 units of ζ, and wild-type fraction 3 (●); 30 units of pol III* and mutant fraction 3 (□); 10 units of pol III core, 60 units of γ, 12 units of ζ and mutant fraction 3 (■). Incubations were at 30°C for 10 min. The observed DNA synthesis was corrected for background DNA synthesis (3.7 pmol in the absence of an added source of β).

and 3.3-kb fragments endowed *dnaN59* cells with a 5- to 6-fold higher β activity. A pMOB45 recombinant carrying only the 3.3-kb fragment was unable to raise the level of β activity in *dnaN59* cells, but the β activity in the plasmid-containing cells was not inactivated by incubation at 42.5°C, in contrast to that in plasmid-free *dnaN59* cells. These results support the conclusion that the *dnaN* gene is the structural gene for the β subunit of pol III holoenzyme.

DISCUSSION

Of the eight or more polypeptides in *E. coli* pol III holoenzyme, the genetic loci for α, β, γ, and δ have now been determined by analyzing conditionally lethal mutants in which DNA synthesis stops promptly when the temperature is raised to the restrictive temperature. In this report the β subunit of the holoenzyme is identified as the product of the *dnaN* gene. The thermolability of the mutant β subunit was not demonstrable by heating either the holoenzyme in cell extracts or the partially purified β subunit. Surprisingly, the thermosensitive defect was observed only upon heating the culture at the restrictive temperature (42.5°C) and then measuring holoenzyme and β subunit activities in extracts of these heated cells (Figs. 1 and 2). The thermal instability of the mutant protein *in vivo* may be due to proteolytic or other effects that are not carried over into the extracts.

The replicative defect in extracts of heated mutant cells was complemented only by holoenzyme or the β subunit (Table 1, Fig. 3). In addition to this evidence, identification of the β subunit as the product of the *dnaN* gene became clear from studies of the reconstitution of the holoenzyme from its subunits: (*i*) the partially purified β subunit from the *dnaN* mutant grown at the permissive temperature was far less active than that from wild-type cells in restoring holoenzyme activity to pol III* (holoenzyme from which only the β subunit had been removed), and (*ii*) the mutant β subunit was inert in reconstituting the holoenzyme from its purified subunits, whereas the wild-type β subunit was active (Fig. 4).

Additional evidence that *dnaN* is the genetic locus of the β subunit is the overproduction of the β subunit by cells that maintain a plasmid carrying the *dnaN* sequence. Because efficient expression of the *dnaN* gene depends on the transcription from the promoter of the adjacent *dnaA* gene, plasmids carrying the *dnaN* gene but lacking the *dnaA* promoter failed to overproduce the β subunit.

A report that a mutation in *dnaA* suppresses a mutation in *dnaZ*, the structural gene for the γ subunit of the holoenzyme, was taken as suggestive evidence for interaction between the products of the *dnaA* and *dnaZ* genes (17). However, it seems more plausible to consider an interaction between the *dnaN* and *dnaZ* gene products (the β and γ subunits of holoenzyme), and additional fine mapping is desirable to establish that the mutation is in the *dnaA* rather than the adjacent *dnaN* gene. Even if the mutation proves to be in *dnaA*, it is still possible that, through polarity in gene expression or interactions between gene products, the effect may result from the function of the *dnaN* gene product rather than of the *dnaA* gene product.

A mutation in the *dnaE* gene, which codes for the α subunit of holoenzyme, is suppressible by a mutation (*sueA77*) in a gene encoding a polypeptide near 40,000 daltons (18). Earlier attempts (unpublished observations) to identify the *sueA77* gene product as the β subunit did not yield definitive results, although there appeared to be a defect in the holoenzyme as judged by the failure of phosphocellulose chromatography to resolve it into an active β subunit and pol III*. Furthermore, genetic studies have revealed the *sueA77* mutation to be in the *dnaN* locus (18). Thus there is genetic evidence for interaction of the α and β subunits of holoenzyme in addition to that presumed from biochemical studies (4).

Although there are only 10–20 molecules of pol III holoenzyme per cell (2), dimers of the β polypeptide are estimated to be 10 times or more numerous. There may be additional functions for the β polypeptide in connection with other DNA polymerases or metabolic events. The abundance of β molecules may explain a leakiness in the *dnaN* mutant sufficient to sustain the initial conversion of a phage φX174 circular DNA to the duplex form at the restrictive temperature but inadequate for further polymerase function to maintain the full replicative cycle of the phage (9).

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