DNA Polymerase III Holoenzyme of Escherichia coli

PURIFICATION AND RESOLUTION INTO SUBUNITS

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DNA polymerase III holoenzyme has been purified from Escherichia coli HMS-83, using an assay, the conversion of coliphage G4 single-stranded DNA to the duplex replicative form. The holoenzyme consists of at least four different subunits: α, β, γ, and δ of 140,000, 40,000, 52,000, and 32,000 daltons, respectively. The α subunit is DNA polymerase III, the dnaE gene product. The holoenzyme has been resolved by phosphocellulose chromatography into an α γ δ complex and a subunit β (copolymerase III*); neither possesses detectable activity in the G4 system but together reconstitute holoenzyme-like activity. The α-γ-δ complex has been further resolved to yield a γ-δ complex which reconstitutes α-γ-δ activity when added to DNA polymerase III. The γ-δ complex contains a product of the dnaZ gene and has been purified from a strain which contains a ColE1-dnaZ hybrid plasmid.

In this paper, we report the purification of holoenzyme from E. coli HMS-83 and the identification of its subunits. The holoenzyme contains at least four distinct subunits which range in molecular weight from 32,000 to 140,000 (Fig. 1). The DNA polymerase III holoenzyme thus resembles RNA polymerase in complexity but differs in that its subunits are so easily resolved.

MATERIALS AND METHODS

Bacterial and Phage Strains—Escherichia coli HMS-83 (a recipient of postdoctoral fellowship support from a grant from the Cystic Fibrosis Foundation. Present address, Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305)

DNA polymerase III was partially purified by dialyzing holoenzyme Fraction II (see below; derived from 425 g of E. coli HMS-83; 8 x 10^10 units, 560 mg of protein) overnight against Buffer T (containing 30% glycerol and 25 mM NaCl). This solution was applied to a 110 ml phosphocellulose column equilibrated in Buffer T (containing 30% methylsulfoxide, 10% glycerol, 5 mM dithiothreitol, 1 mM EDTA) and chromatographed on Bio-Rex 70 at pH 7.5 at 4°C. The α, β, γ, and δ subunits were eluted at 100, 70, 40, and 30 mM NaCl, respectively. The α subunit was then further resolved on a hydroxyapatite column at pH 7.5, using a linear gradient of sodium acetate from 50 to 200 mM.

The abbreviations used are: holoenzyme, DNA polymerase III holoenzyme; pol III, DNA polymerase III; pol III*, DNA polymerase III*; copol III*, copolymerase III*; SS DNA, single-stranded circular DNA; RF, phage double-stranded DNA of circular, replicative form; DBP, DNA binding protein; SDS, sodium dodecyl sulfate.

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E. coli HMS-83 and the identification of its subunits. The holoenzyme contains at least four distinct subunits which range in molecular weight from 32,000 to 140,000 (Fig. 1). The DNA polymerase III holoenzyme thus resembles RNA polymerase in complexity but differs in that its subunits are so easily resolved.
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Ammonium Sulfate Fractionation—Ammonium sulfate (0.226 g/ml) was added over a 10-min interval. The solution was stirred for 30 min at 0° and then centrifuged at 20,000 × g, 0°, 40 min). The precipitate was resuspended in 0.20 AS buffer (1/5 of the Fraction I volume) and the insoluble fraction was collected. This procedure was repeated with 0.16 AS buffer (1/6 of the Fraction I volume). The dissolved precipitate was clarified by centrifugation (30,000 × g, 30 min at 0°) and dialyzed for 4 h against Me$_2$SO buffer + 0.1 M NaCl to yield Fraction II (156 ml).

Bio-Rex-70 Chromatography—Fraction II was diluted with Me$_2$SO buffer (to 312 ml) to a conductivity corresponding to Buffer I + 0.2 M NaCl and applied to a column of Bio-Rex-70 (8.5 cm × 20 cm) equilibrated with Me$_2$SO buffer. The column was eluted with 8-column volumes of a decreasing Me$_2$SO gradient (Me$_2$SO buffer + 0.5 M NaCl → Buffer I + 0.5 M NaCl, 30% glycerol). Me$_2$SO stabilizes the holoenzyme subunit structure and appears to increase the affinity of proteins for Bio-Rex-70; in its absence, a separate peak of the β subunit was eluted before the holoenzyme. The activity peak (230 ml) was precipitated by dialysis against Buffer I + 0.1 M NaCl to yield Fraction III (30 ml).

Valyl Sepharose Chromatography—To Fraction III was added 6 ml of valyl Sepharose (in 1.1 M AS buffer) and 39.6 ml of 2% AS buffer. The suspension containing protein adsorbed to valyl Sepharose was applied to a valyl Sepharose column (5 cm × 15 cm) equilibrated with 1.1 M AS buffer. The column was washed with 1-column volume of equilibrium buffer and eluted with a 10-column volume gradient of 1.1 M AS buffer to 0.4 M AS buffer. Fractions containing at least 60% of the activity in the peak (150 ml) were combined and precipitated by dialysis against Buffer I (70% saturated with ammonium sulfate). The precipitated protein was collected and dissolved in Buffer I + 0.1 M NaCl to yield Fraction IV (30 ml).

DEAE-Sephadex Chromatography—Fraction IV was desalted on a Sephadex G-25 column (1.5 cm × 20 cm) equilibrated in Buffer I + 30 mM NaCl. Fractions preceding the salt peak were immediately applied to a DEAE-Sephadex column (2.2 cm × 9 cm) equilibrated in Buffer I. The column was washed with 2-column volumes of Buffer I + 60 mM NaCl and eluted with 5-column volumes of a NaCl gradient (60 to 210 mM). The activity appeared at about 135 mM NaCl to yield Fraction V (25 ml).

The holoenzyme thus obtained was stable for at least 1 month at 0° and for at least 1 year when rapidly frozen and stored in liquid N$_2$.

Physical Properties of Holoenzyme

Glycerol Gradient Sedimentation—Fraction V (6 ml) was precipitated by dialysis overnight against Buffer I (70% saturated in ammonium sulfate). The resulting fraction was dis-
solved in a minimal volume (100 µl of Buffer I which had been diluted with an equal volume of water), layered upon a glycerol gradient (3.7 ml), and sedimented (see "Materials and Methods"). Of the applied holoenzyme activity, 50% was recovered. The sedimentation profile (Fig. 2) relative to standards in the same rotor indicates a sedimentation coefficient of about 11 S for the holoenzyme, the same value as catalase whose molecular weight is 244,000.

**Components of Holoenzyme**

**SDS-Polyacrylamide Gel Electrophoresis of Holoenzyme**—Holoenzyme (0.2 µg) was labeled with diazotized [35S]sulfanilic acid, denatured, and electrophoresed on an SDS-polyacrylamide gel (Fig. 3). Four protein bands were present which have been shown to be components required for elongation of a primed G4 template (see below). The 140,000-dalton protein (α) is presumably pol III, the dnaE gene product. Pol III has been purified by others (4) and was found to contain principally a component of this size. A second subunit of 40,000 daltons, β, may be copol III* previously isolated and identified as a 77,000-dalton polypeptide (3). Two components, γ and δ (52,000 and 32,000 daltons, respectively) have been purified as a complex and are required for the action of α and β (see below). Based upon a densitometer scan of the α-, β-, γ-, and δ-bands, this holoenzyme preparation is at least 60% pure. Also present were two additional proteins of 83,000 and 25,000 daltons, which remained tightly associated with the holoenzyme throughout the purification to this point and sedimented with holoenzyme activity in a glycerol gradient.

**Resolution of Holoenzyme**—Holoenzyme was resolved by phosphocellulose chromatography into the β subunit and an α-γ-δ complex. The β subunit was further purified on DEAE-Sephadex and Sephadex G-150 (Table II) by a procedure similar to that used for resolving copol III* from a holoenzyme complex (1).

Holoenzyme Fraction V (2 ml) was diluted with 10 mM imidazole-HCl (pH 6.8), 20% glycerol, 5 mM dithiothreitol and 1 mM EDTA (to a conductivity equivalent to Buffer I + 30 mM NaCl) and applied to a phosphocellulose column (0.45 ml) equilibrated with Buffer I + 30 mM NaCl. The column was eluted with 0.5-ml aliquots of Buffer I containing NaCl in the following millimolar concentrations: 30 (twice), 60, 100, 150 (twice), 200, 250, 300, and 400. Two fractions were obtained: an unadsorbed Fraction B (p) (9.4 ml) and an eluted Fraction C (α-γ-δ complex) (1.2 ml; 200 to 250 mM NaCl). Fractions B and C were inactive separately, but together reconstituted holoenzyme-like activity (Table III).

The β subunit was further purified as follows. Fraction B (9.4 ml) was diluted with 2 volumes of Buffer T (1 mM diithiothreitol) and applied to a DEAE-Sephadex column (0.35 ml) equilibrated in Buffer T (1 mM diithiothreitol). The column was washed with 1-column volume of Buffer T and eluted with Buffer T + 0.2 mM NaCl. The eluted activity, Fraction D (0.18 ml), was filtered through a Sephadex G-150 column (4 ml, 0.7 cm diameter) equilibrated with Buffer I + 0.1 mM NaCl. The eluted activity was frozen and stored in liquid N₂ to yield Fraction E (0.7 ml).

**Identification of Subunits of Resolved Holoenzyme Components**—Fraction C (0.3 µg) was labeled with diazotized [35S]sulfanilic acid, denatured, and subjected to SDS-polyacrylamide gel analysis. Three distinct protein bands corresponding to the α-, γ-, and δ subunits were seen (Fig. 3). Additionally, the 83,000- and 25,000-dalton proteins observed in the purified holoenzyme persisted in the α-γ-δ complex. Analysis of Fraction E yielded a single band at 40,000 daltons, corresponding to the β subunit of holoenzyme (Figs. 3 and 4).
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TABLE II
Resolution of holoenzyme into the β subunit and α·γ·δ complex

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total units</th>
<th>α·γ·δ complex</th>
<th>Specific activity x 10^3 units/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Holoenzyme V</td>
<td>70</td>
<td>95</td>
<td>620,000</td>
</tr>
<tr>
<td>B. Phosphocellulose, unadsorbed</td>
<td>46</td>
<td>46</td>
<td>880,000</td>
</tr>
<tr>
<td>C. Phosphocellulose, adsorbed</td>
<td>40</td>
<td>40</td>
<td>950,000</td>
</tr>
<tr>
<td>D. DEAE-Sephadex</td>
<td>24</td>
<td>N.D.</td>
<td>700,000</td>
</tr>
<tr>
<td>E. Sephadex G-150</td>
<td>7</td>
<td>7</td>
<td>3,600,000</td>
</tr>
</tbody>
</table>

* Includes 59,000 units of holoenzyme.
* Not determined.

TABLE III
Requirements for reconstitution of holoenzyme activity

Components were added as follows: holoenzyme (14 ng), α·γ·δ complex (15 ng), and β (6 ng); assays are as described under "Materials and Methods."

<table>
<thead>
<tr>
<th>Holoenzyme component added</th>
<th>DNA synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DNA synthesis</td>
</tr>
<tr>
<td></td>
<td>G4 DNA</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>Holoenzyme</td>
<td>133</td>
</tr>
<tr>
<td>α·γ·δ complex</td>
<td>8</td>
</tr>
<tr>
<td>β</td>
<td>98</td>
</tr>
<tr>
<td>α·γ·δ complex + β</td>
<td>98</td>
</tr>
</tbody>
</table>

This band parallels the β activity on a Sephadex G-150 column (Fig. 4). Sedimentation in a glycerol gradient relative to internal standards indicated a sedimentation coefficient of 4.7 S for β (Fig. 5).

Purification of γ·δ Subunit Complex

A complex of the γ·δ subunits of the DNA polymerase III holoenzyme was purified 18,000-fold to 95% homogeneity from E. coli RLM 355 (pLC 6-2) (Table IV). Fractions I to IV were prepared as previously described in the purification of holoenzyme except that the assay for the γ·δ complex (see "Materials and Methods") was used to follow activity.

o-Phenanthroline Treatment—The pol III (α) component of the α·γ·δ complex was selectively inactivated and dissociated by treatment with the Zn^2+ chelator, o-phenanthroline, so that the γ·δ complex could be purified as a distinct molecular species. Fraction IV (3.4 ml) was first desalted on a Sephadex G-25 column (1.5 × 17 cm) equilibrated in Buffer T + 100 mM NaCl. To the activity peak was added 0.34 ml of a solution containing 250 mM o-phenanthroline·HCl, 200 mM Tris base in Buffer T, and 100 mM NaCl. After 1 h at 0°C, the solution was heated for 10 min at 37°C to yield Fraction V (4.3 ml).

Phosphocellulose Chromatography—Fraction V was diluted 4-fold with Buffer T and applied to a phosphocellulose column (1 × 4 cm) equilibrated in Buffer T. The column was eluted by a 1-column-volume gradient (50 mM to 300 mM NaCl in Buffer T) to yield Fraction VI (37 ml).

DEAE-Sephadex Chromatography—Fraction VI was diluted with Buffer T to a conductivity equivalent to Buffer T + 70 mM NaCl and applied to a DEAE-Sephadex column (0.7 × 7 cm) equilibrated in Buffer T. The column was washed with 1-column volume of Buffer T + 50 mM NaCl and eluted with a

FIG. 4. Chromatography of β subunit of holoenzyme on Sephadex G-150 and electrophoretic analysis of the eluted fractions. β activity was assayed as described under "Materials and Methods"; slab gel electrophoresis of eluted G-150 fractions was in a 10% acrylamide gel with 0.1% SDS. Before electrophoresis, fractions were labeled with diazotized [35S]sulfanilic acid (see "Materials and Methods"). Catalase, ovalbumin, and chymotrypsinogen standards were included in the well on the left side.

FIG. 5. Glycerol gradient sedimentation of β subunit of holoenzyme. One unit has the following equivalents: cytochrome c (horse heart), A_280 of 2.7 × 10^-1; alcohol dehydrogenase (yeast), ΔA_340 of 3.4 (assayed by the method of Vallee and Hoch (21)); catalase, ΔA_240 of 3.4 (assayed by the method of Beers and Sizer (22)); and β assayed as described under "Materials and Methods."
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10-column volume gradient (75 to 250 mM NaCl in Buffer T). The eluted activity, Fraction VII (6.75 ml), was rapidly frozen and stored in liquid N₂.

**Requirement for γ-δ Complex to Reconstitute the Holoenzyme Activity**
The γ-δ complex is required to reconstitute holoenzyme-like activity in both the natural G4 assay system and with a long, single-stranded template primed with *E. coli* RNA polymerase (Table V). No activity was observed unless the α, β, and γ-δ subunits were all present.

**TABLE IV**
Purification of γ-δ subunit complex from a strain carrying a ColEl-dnaZ plasmid

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total units</th>
<th>Specific activity</th>
<th>γ-δ</th>
<th>α-γ-δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Lysate supernatant</td>
<td>203,000</td>
<td>27,000</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>II. Ammonium sulfate</td>
<td>140,000</td>
<td>22,000</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>III. Bio-Rex-70</td>
<td>35,000</td>
<td>5,100</td>
<td>320</td>
<td></td>
</tr>
<tr>
<td>IV. Valyl Sepharose</td>
<td>26,300</td>
<td>3,400</td>
<td>1,140</td>
<td></td>
</tr>
<tr>
<td>V. α-Phenanthroline</td>
<td>12,300</td>
<td>0</td>
<td>540</td>
<td></td>
</tr>
<tr>
<td>VI. Phosphocellulose</td>
<td>4,000</td>
<td>0</td>
<td>7,000</td>
<td></td>
</tr>
<tr>
<td>VII. DEAE-Sephadex</td>
<td>1,600</td>
<td>0</td>
<td>27,000</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE V**
Requirement for γ-δ to reconstitute holoenzyme activity

Components were added as follows: holoenzyme (14 ng), β (6 ng), α (55 ng), and γ-δ (1.5 ng); assays are as described under "Materials and Methods."

<table>
<thead>
<tr>
<th>Holoenzyme component added</th>
<th>DNA synthesis</th>
<th>G4 DNA</th>
<th>RNA-primed ΔX DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Holoenzyme</td>
<td>184</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>α</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>β</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>γ-δ</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>α + β</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>α + γ-δ</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>β + γ-δ</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>α + β + γ-δ</td>
<td>142</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>

**Physical Properties of the γ-δ Complex**

**Glycerol Gradient Sedimentation**—Fraction VII (50 μl) was layered on a 25 to 40% glycerol gradient (3.65 ml) as described under "Materials and Methods," except that NaCl replaced (NH₄)₂SO₄. The sedimentation profile (Fig. 6), relative to standards in the same tube, indicates a sedimentation coefficient for the γ-δ complex of 8 S.

**SDS-Polyacrylamide Gel Electrophoresis**—Fraction VII (0.15 μg) was denatured and electrophoresed on an SDS-polyacrylamide gel (Fig. 3; see "Materials and Methods"). Two distinct protein bands were present with molecular weights of 52,000 and 32,000, respectively. Both of these bands paralleled the activity across a DEAE-Sephadex column (data not shown).

The γ-δ Complex Contains a Product of the dnaZ Gene—When holoenzyme was isolated from a temperature-sensitive dnaZ strain, the holoenzyme levels were depressed 10-fold or more (Table VI), as were the levels of the α-γ-δ and γ-δ

**TABLE VI**
Deficiency of γ-δ activity in extracts of a dnaZ mutant

<table>
<thead>
<tr>
<th>Activity assayed</th>
<th>DNA synthesis</th>
<th>HMS-83</th>
<th>AX727 (dnaZ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Holoenzyme</td>
<td>4500</td>
<td>400</td>
<td></td>
</tr>
<tr>
<td>α-γ-δ complex (Fraction C)</td>
<td>4700</td>
<td>600</td>
<td></td>
</tr>
<tr>
<td>β (Fraction III)</td>
<td>4400</td>
<td>2700</td>
<td></td>
</tr>
<tr>
<td>γ-δ (Fraction VII)</td>
<td>4200</td>
<td>400</td>
<td></td>
</tr>
</tbody>
</table>

*Assays of ammonium sulfate functions were performed as described under "Materials and Methods" except that ammonium sulfate precipitation was performed at 0.24 g/ml (instead of 0.226 g/ml as in Table I) and the resulting precipitate was washed with 0.24 AS buffer (1/10 of Fraction I volume).

**TABLE VII**
Complementation of extracts of a dnaZ mutant by purified γ-δ Complex

Assays were performed as described under "Materials and Methods" in the presence of 2 μg of a Fraction II made from AX727 as described in Table II. The amounts of holoenzyme components added are described in Tables III and V.

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>DNA synthesis</th>
<th>HMS-83</th>
<th>AX727 (dnaZ)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol</td>
<td>6,000</td>
<td>6,000</td>
</tr>
<tr>
<td>RLM 365 (pLC-1-21)</td>
<td>CoEl1</td>
<td>8,000</td>
<td>8,000</td>
</tr>
<tr>
<td>RLM 365 (pLC-6-2)*</td>
<td>ColEl1-dnaZ</td>
<td>8,000</td>
<td>20,000</td>
</tr>
<tr>
<td>RLM 360 (pSC-30-3)</td>
<td>ColEl1-dnaZ</td>
<td>24,000</td>
<td></td>
</tr>
</tbody>
</table>

*In a separate experiment in which the cells were grown under optimal conditions on a large scale (100 liters), 12,000 and 88,000 units/g of cell paste were obtained for holoenzyme and γ-δ, respectively.
complexes; β levels were relatively unaffected.

Addition of γ-δ complex to Fraction II of the temperature-sensitive dnaZ strain produced a 3-fold stimulation of activity; additions of α or β were without effect (Table VII).

Overproduction of γ-δ Activity in Strains Carrying ColEI-dnaZ Plasmids – Such strains contained levels of γ-δ activity 3 (or more) times greater than strains which lack this plasmid (Table VIII).

DISCUSSION

DNA polymerase III holoenzyme has been purified 7400-fold from Escherichia coli HMS-83. The complex is at least 60% pure based upon its content of four subunits: α (140,000 daltons), β (40,000), γ (32,000), and δ (32,000). In addition, proteins of 83,000 and 25,000 daltons, closely associated with the holoenzyme during its purification and subsequent glycerol gradient sedimentation, may be part of the holoenzyme complex serving some unknown functions. The very low abundance of the holoenzyme (20 molecules/cell) and its instability have made its isolation and characterization especially difficult.

The holoenzyme has at least four subunits required for conversion of a primed G4 single-stranded circle to the duplex form. The α subunit is pol III, the dnaE gene product (23), estimated to be a large polypeptide near 140,000 daltons (2). Others have recently purified pol III and also found it to contain a 140,000-dalton component (4) (Fig. 1). The β subunit of 40,000 daltons is presumably the previously identified copol III* (3) based upon (a) association with pol III and stimulation of its activity on a long, single-stranded DNA template, (b) resolution from the polymerase component of holoenzyme by phosphocellulose chromatography, (c) resistance to treatment with N-ethylmaleimide and inhibition by antibody directed against copol III* preparations (data not shown), and (d) similarity between cop01 and DNA polymerase III hybrid, even though the dnaZ gene probably codes for only one of these subunits, could be explained in one of the several ways: (a) the plasmid contains the structural genes for both subunits, (b) there is a coordinate regulation of the synthesis of the two subunits, or (c) the non-dnaZ gene product is normally produced in excess.

There is a large discrepancy between the high specific activity estimated for γ-δ (2.7 × 10^7) and that for the holoenzyme (5.2 × 10^6). It could be explained if the bulk of the holoenzyme preparation were inactive or if the catalytic efficiency of a system reconstituted from isolated α, β, and γ-δ subunits was far greater than that of the holoenzyme.

The argument has been made that the DNA polymerase III holoenzyme is not a physical entity and that its activity derives from a mixture of four separate proteins (27). It seems unlikely to us that four proteins related in a common synthetic function were enriched together throughout a 7400-fold purification simply by coincidence. Furthermore, the components of the holoenzyme-α, β, and γ-δ complex-sediment with coefficients of 7 S (4), 4.5 S, and 8 S, respectively, whereas the intact holoenzyme sediments with a coefficient of 11 S.

Isolation of a homogeneous DNA III polymerase holoenzyme in amounts adequate for structural and functional characterization remains an important objective. This multisubunit enzyme is crucial in replication not only for its DNA synthetic function but is very likely the keystone of a larger assembly unit containing multiple elements for initiation, regulation, and termination of the replicative operation.

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C McHenry and A Kornberg


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