SUPPLEMENTARY DATA

The DNA polymerase III holoenzyme contains γ and is not a trimeric polymerase

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SUPPLEMENTARY RESULTS

Pol III* from strain PDEC106 (dnaX−γ) contains only the τ form of dnaX.

To confirm that Pol III* from strain PDEC106 (dnaX−γ) expressed only the τ form of dnaX and that the expressed τ was not proteolyzed to a protein near the size of γ, purified Pol III* fraction V were run on a 4-20% SDS-PAGE gel, transferred to nitrocellulose and probed with monoclonal antibody AB251 that recognizes both τ and γ. As a control, fraction V from strain MGC1030 (WT relative to dnaX) were run in parallel. The monoclonal antibody detected only the τ form from PDEC106 (Figure S1B). The monoclonal detected both forms of DnaX from the control strain MGC1030 (Figure S1A).

Figure S1. Immunoblots of DnaX subunits from PDEC106 and MGC1030. A. Immunoblot of Fr. V S300 Pol III* fractions from MGC1030. B. Immunoblot of Fr. V S300 Pol III* fractions from PDEC106. See legends for Figures 4-6 for additional details.

Identification of a protein that co-purifies with Pol III* from strain PDEC105 (dnaXγ− + pA1-γ-C_tag) as inosine monophosphate dehydrogenase

SDS-PAGE analysis of S300 (Fr. V) Pol III* fractions from PDEC105 (dnaXγ− + pA1-γ-C_tag) showed a protein that migrated just above the band expected to be γ (Figure 4B). Given the close association and molecular weight, we thought it prudent to identify both the protein we believed to be γ and the higher molecular weight protein. Fr V from PDEC105 was subjected to SDS gel electrophoresis, the bands of interest were cut out, trypsinized and subjected to mass spectrometry for identification (Figure S2). This analysis confirmed the identity of band 1 as γ-C_tag (82% coverage of expected tryptic peptides) and
identified the band immediately above (band 2) as inosine monophosphate dehydrogenase (90% coverage of expected peptide fragments).

**Figure S2.** Coomassie stained gel bands of purified Pol III* from strain PDEC105 (dnaXγ+ pA1-γ-Ctag) Fraction V (peak fraction #28) used for identification by mass spectrometry. Two regions of the gel, as indicated by the red boxes designated 1 and 2 were cut out, treated with trypsin, the gel slices extracted, and the proteins submitted for analysis. Lane 1 contains 2.5 µg protein. Lanes 2, 3, and 4 contain 15 µg protein.

γ-Ctag yields two forms that can be resolved on SDS gels, one biotinylated and the other non-biotinylated.

Purified fraction V Pol III* from PDEC105 (dnaXγ+ pA1-γ-Ctag), exhibited two forms γ-Ctag when probed with an antibody against DnaX (Figure S3). Comparison with Figure 4 indicated only the upper γ band is biotinylated. The cause of this is most likely proteolysis of a portion of the tag appended to the C-terminus of γ. Quantification of the relative levels of the two bands indicates that 31% of γ contains the full-length tag and is capable of becoming biotinylated. This represents the maximal amount of γ-Ctag in Pol III* from PDEC105 that could be biotinylated.
Figure S3 DnaX immunoblot of Pol III* S300 fractions purified from strain PDEC105 (dnaXγ− pA1-γ-Ctag) using monoclonal antibody AB251 as probe. AB251 detects both forms of the dnaX-expressed proteins τ and γ-Ctag, seen here as a doublet with both non-biotinylated and biotinylated forms. Lane 1 Pol III* markers. Bands were scanned and quantitated. The biotinylated form of γ-Ctag was calculated to be an average of 31% (+/- 7%) of the total γ protein.

SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Buffers

The following buffers were used: Reduction Solution, 4 mM dithiothreitol, 50 mM ammonium bicarbonate; Alkylation Solution, 55 mM iodoacetamide, 50 mM ammonium bicarbonate; Trypsin Solution, trypsin 13 ng/ml (Promega sequencing grade), 25 mM ammonium bicarbonate, 10% (v/v) acetonitrile; Extraction Buffer, 1.7% formic acid, 67% acetonitrile.

Preparing gel slices for in gel digestion with trypsin followed by extraction for mass spectrometry analysis

The procedure is adapted from (68). Protein samples (2.5 -15 µg of Pol III*) were run on a 4-20% SDS-Page gel; the gel was stained with Coomassie Blue, and destained with 10% (v/v) methanol, 10% (v/v) glacial acetic acid to visualize gel bands. Individual gel bands were excised from the gel with a clean scalpel. Individual bands were sliced into smaller cubes less than 1 mm in size, placed in a 1.5 ml Eppendorf tube, and destained with three treatments of 1 ml 50 mM ammonium bicarbonate, 50% (v/v) acetonitrile by agitating the tube (10 min per treatment). Tubes were centrifuged (16,000 x g for 1 min) after each treatment. After the last treatment, 500 µl of acetonitrile was added to shrink gel slices. The tubes were centrifuged and supernatant was discarded.

Protein was reduced and alkylated by addition of 50 µl of Reduction Solution (20 min at 60 °C). The tubes were cooled to room temperature and 500 µl of acetonitrile was added to each and incubated for 10 min. Tubes were centrifuged (16,000 x g, 1 min) and supernatant discarded. 50 µl of Alkylatation Solution was added to each tube, and tubes were incubated (ambient temperature in the dark for 20 min). The supernatant was discarded, and 500 µl of 100 mM ammonium bicarbonate was added and incubated for 10 min. The supernatant was discarded and 500 µl of acetonitrile was added to dehydrate the gel slices. To dry the gel slices, acetonitrile was discarded and the open tubes were dried in a speedvac for approximately 1 h.

Trypsin Solution was made just prior to addition. Sufficient solution (ca. 50-100 µl) was added to each tube to cover the dry gel pieces. The tubes were incubated (30 min, 4 °C). Gel slices were checked to see if all of the solution was absorbed. More trypsin solution was added, as needed. After an additional 90 min on ice, excess Trypsin Solution was removed, and 10-20 µl of 100 mM ammonium bicarbonate was added to cover slices to keep them wet during overnight enzymatic cleavage (37 °C).

Tryptic peptide fragment were extracted from gel slices and desalted prior to LC-MS analysis. Extraction buffer (50-100 µl) was added (37 °C for 15 min). The tubes were centrifuged at (16,000 x g, 1 min) and the supernatant was saved. The extraction step was repeated 2 times more. The supernatants
were collected and dried in a speedvac. For desalting, the dried peptides were dissolved in 0.1% formic acid and desalted with Pierce C18 spin columns according to manufacturer’s instructions.

The final samples were analyzed by the LC-MS core facility at the University of Colorado, Boulder. Tryptic polypeptides were compared to either an *E. coli* Uni-prot protein database, or the sequences of γ, or γ-C_{tag} proteins.

SUPPLEMENTARY REFERENCE

68. Shevchenko, A., Tomas, H., Havlis, J., Olsen, J. V., and Mann, M. (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat. Protoc.* 1, 2856-2860.