Articles

DNA Polymerase III of Gram-Positive Eubacteria Is a Zinc Metalloprotein Conserving an Essential Finger-like Domain†

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ABSTRACT: DNA polymerase III (pol III) of Gram-positive eubacteria is a catalytically bifunctional DNA polymerase:3′-5′ exonuclease [Low, R. L., Rashbaum, S. A., and Cozzarelli, N. R. (1976) *J. Biol.Chem. 251*, 1311−1325]. The pol III protein conserves, between its exonuclease and dNTP binding sites, a 35-residue segment of primary structure with the potential to form a zinc finger-like structure [Berg, J. M. (1990) *Ann. Rev. Biochem. 19*, 405−421]. This paper describes results of experiments which probe the capacity of this segment to bind zinc and the role of this segment in enzyme function. The results of metal and mutational analysis of a model pol III derived from *Bacillus subtilis* indicate that (i) the Gram-positive pol III *is* a metalloprotein containing tightly bound zinc in a stoichiometry of 1, (ii) the zinc atom is bound within the 35-residue segment, likely in one of two probable finger-like structures, and (iii) the integrity of the zinc-bound structure is specifically critical to the formation and/or function of the enzyme's polymerase site.

With respect to its primary structure (*1*−*3*) and its capacity to bind dGTP analogues of the HPUra class (*4*), the pol site of pol III of Gr+[1] eubacteria is unique among DNA polymerases. The location of the pol site and other essential features of the primary structural map of a typical Gr+ pol III are summarized schematically in panel A of Figure 1. This structure is based on Bs pol III, the model enzyme of *Bacillus subtilis*, and the comparative analysis of its primary structure with those of seven other Gr+ pol IIIs (*1*, *2*, *5*−*10*).

The Gr+ pol III protein consists of a single polypeptide varying in length from 1434 to 1465 aa residues. In Bs pol III (1437 aa) the segment between aa 400 and 620 ("exo" box) incorporates a strongly conserved 3′-5′ "editing" exo site (*11*)—the only significant block of primary structure which the Gr+ pol III shares with other pol-exos (*3*, *12*). The enzyme's pol domain ("pol" box), including the subdomain responsible for binding the HPUra class of dGTP analogues (right section of pol box; *4*), has been roughly mapped to a region of highly conserved primary structure between aa 1000 and 1300 (*12*).

Comparative analysis of the available Gr+ pol III primary structures reveals, in the region just upstream of the putative pol site, a strongly conserved, 35 residue sequence resembling that of a so-called zinc finger (*13*, *14*; "zf" box in Figure 1). The alignment of these sequences and the consensus of their conserved residues are shown in detail in the panel B of the figure.

The arrangement of the conserved histidine and the four conserved cysteines (i.e., positions H909, C912, C915, C937, and C940 of Bs pol III) suggests that this primary structure has the potential to form at least two distinct finger structures (*13*, *14*). These are depicted schematically in the bottom panel of Figure 1. One, shown on the left, is a 29-residue "cys4" finger (*13*, *14*) in which Zn is tetrahedrally coordinated by the thiolates of the four conserved cysteines. The other, shown to the right, is a 32-residue "his1/cys3" finger with tetrahedral Zn coordination furnished by the imidazole of the conserved histidine (i.e., H909) and the respective thiolates of three of the four conserved cysteines.

The experiments reported in this paper exploit recombinant Bs pol III to address the following two questions concerning this conserved zf sequence: (i) Does it actually bind Zn as expected for a fingerlike structure like those proposed in Figure 1? (ii) If the zf structure does bind Zn, what is the significance of the structure to the function of the enzyme in which it resides? The results of the experiments addressing these questions are described below.

MATERIALS AND METHODS

Materials. Chelex-100 (200−400 mesh, sodium form) was from Bio-Rad. Mutagenic oligodeoxyribonucleotides

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[1] Abbreviations: aa, amino acid(s); Bs, *Bacillus subtilis*; dNTP(s), 2′-deoxyribonucleoside-5′-triphosphate(s); exo, 3′-5′ exonuclease; Gr+, Gram-positive; his6, hexahistidine; HPUra, 6-((*p*-hydroxyphenyl)azo)-uracil; IMAC, iminodiacetic acid; MMTS, methylmethanethiosulfonate; PMSF, phenylmethanesulfonyl fluoride; pol, DNA polymerase; pol C, pol III structural gene; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TMAU, 6-(3′,4′-trimethyleneanilino)-uracil; zf, zinc finger.

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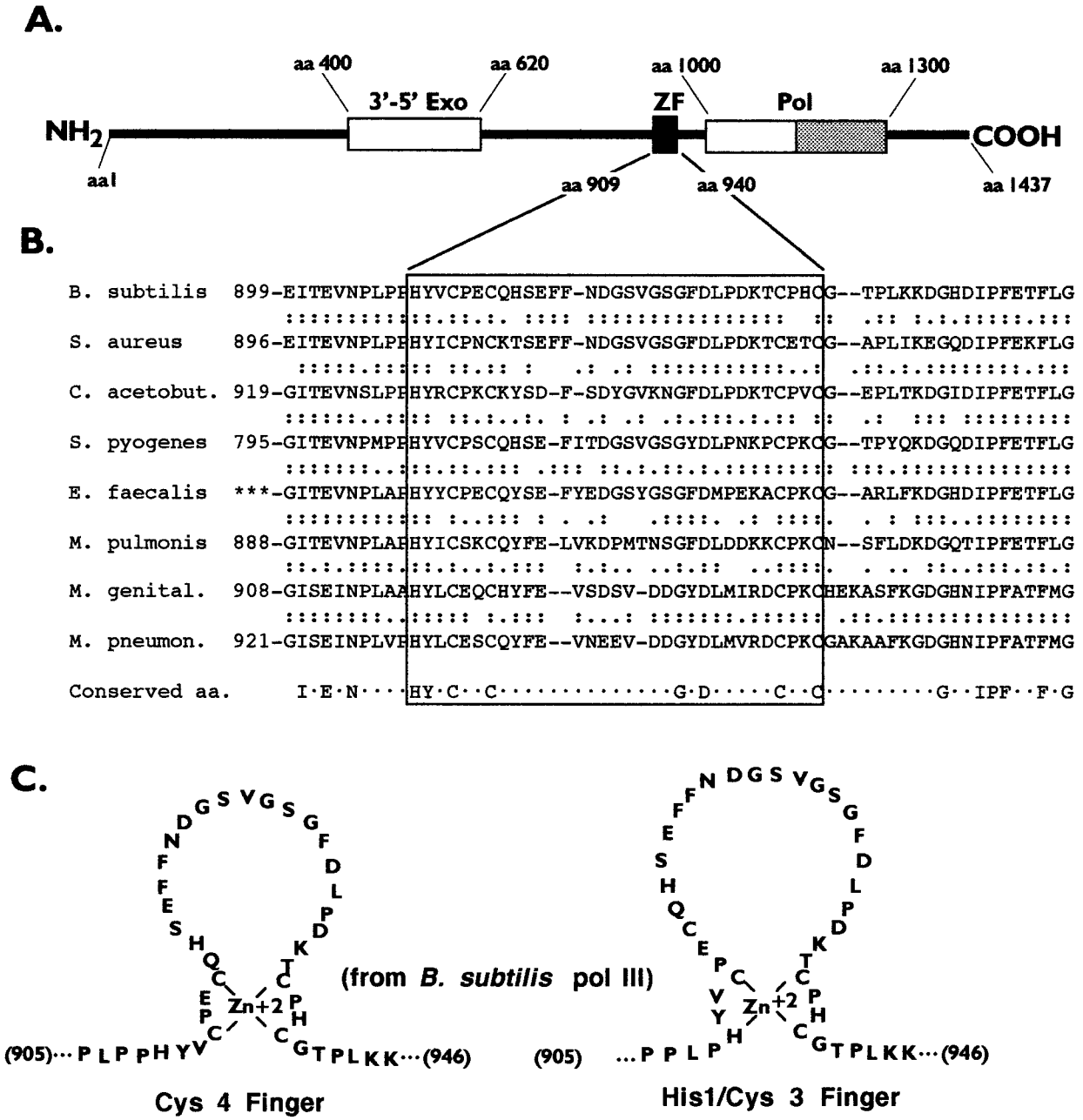

FIGURE 1: Relevant features of the Gr+ eubacterial pol III. (Panel A) Schematic summary of the primary structural map: 3′-5′ exo box, catalytic site of the enzyme's editing exonuclease; zf box, site of putative zinc finger; pol box, DNA polymerase active site; right half of pol box, site of binding of HPUra-type dGTP analogues. (Panel B) Alignment of the primary structures of the zfs of eight Gr+ pol IIIs. *Bacillus subtilis*, ref *1*; *Staphylococcus aureus*, ref *2*; *Clostridium acetobutylicum*, ref *5*; *Streptococcus pyogenes*, ref *6*; *Enterococcus faecalis*, ref *7*; *Mycoplasma pulmonis*, ref *8*; *Mycoplasma genitalium*, ref *9*; *Mycoplasma pneumoniae*, ref *10*. (Panel C) Schematic summary of two hypothetical finger-like structures based on the primary structure of the zf of Bs pol III. left, cys4 structure; right, his1/cys 3 structure.

were purchased from Operon Technology, Inc. Yeast extract and tryptone were obtained from Difco. Calf thymus DNA was obtained from Worthington. $^{[65]}ZnCl_2$ and radioactive DNA precursors were purchased from New England Nuclear.

Recombinant Plasmids. All forms of Bs pol III were generated in *Escherichia coli* by controlled expression of Bs *pol*C from recombinant plasmid vectors. Two different plasmid systems were used for expression. One, based in the plasmid p*K*C30 and *E. coli* AR120, has been described in detail elsewhere (*15*). The other system was based on plasmid pSGA04, an expression plasmid designed to generate recombinant proteins with a removable N-terminal his6 "tag" (*16*). Engineering of Bs *pol*C into pSGA04 required the following five steps: (i) introduction, with PCR, of a new *Hpa*I restriction site at nucleotide 16 of a form of Bs *pol*C which had been engineered previously to contain an *Xho*I site at position 1246 (*17*); (ii) excision of the resulting 1228-bp *Hpa*I-*Xho*I fragment; (iii) recloning of the latter fragment into a fully wild-type Bs*pol*C construct in the vector pKC30 (*15*); (iv) excision of the *pol*C gene lacking the first 15 bases as a *Hpa*I-*Bam*HI fragment; (v) insertion of the latter fragment into pSGA04 which had been digested with *Eco*RI, filled in by treatment with Klenow pol I, and subsequently digested with *Bam*HI. This recombinant *pol*C vector encodes and expresses a form of Bs pol III in which the first 6 aa of the enzyme's amino terminus are replaced with the following 19-residue sequence: NH_2-M G $H_{(6)}$ S G *L F K R* H M S R

I: (italicized residues denote a sequence of cleavage site for protease Kex-2 sequence; *16*).

Production of Mutant pol IIIs. An oligodeoxyribonucleotide-based system (Altered Sites, Promega) was used to introduce mutations into the segment of Bs *pol*C encoding the putative zf subdomain. The mutations were introduced into zf-150, a subcloned fragment encompassing *pol*C nucleotides 2410−2899 and bounded by the unique restriction sites *Sal*I and *Cla*I, the former of which was created by site-directed mutagenesis. Each mutagenic oligodeoxyribonucleotide was designed such that the mutation simultaneously created a unique diagnostic restriction site within the targeted codon. The mutant forms of zf-150 were reincorporated into *pol*C, using the unique *Sal*I and *Cla*I sites, and the respective mutant *pol*Cs were then installed into the pSGA04 his6 expression plasmid in a procedure involving (i) recloning of the relevant *Sal*I/*Cla*I fragment into *Sal*I/*Cla*I-digested wild-type construct in the Bluescript plasmid (Stratagene) and (ii) subcloning of the relevant *Xho*I-*Cla*I Bluescript fragment into an *Xho*I-*Cla*I-digested wild-type clone in pSGA04.

Generation of Homogeneous Forms of Recombinant Bs pol IIIs. Both of the plasmid expression systems noted above were used, and both exploited LB expression medium (0.5% yeast extract, 1% tryptone, 0.5% NaCl, and 0.15 mg/mL ampicillin). The generation of enzyme from each of these systems is summarized below.

(*i*) *"Native" enzyme* was expressed from p*KC*30 *pol*C plasmid transformed into *E. coli* AR120. The structure of the recombinant vector, the induction of expression, and purification of homogeneous enzyme have been described in detail in ref *15*.

(*ii*) *His6-tagged Pol III*. pSGAO4-based clones were introduced into *E. coli* SG101 (*16*) by transformation. Individual transformant colonies were grown at 30 °C to an absorbance (600 nM, 1 cm light path) of approximately 1.0 in LB expression medium containing 15 μg/mL kanamycin. The culture was then chilled to ∼18 °C, IPTG was added to 1 mM, and incubation was continued with shaking at 18 °C for about 18 h. The cells were chilled to 0 °C, centrifuged, washed once in phosphate-buffered saline (0.15 M NaCl:50 mM potassium phosphate, pH 7.6) containing 1 mM PMSF, and finally resuspended, at the rate of 30 mL buffer for each 1 L of culture, in a buffer containing 50 mM potassium phosphate (pH 7.5), 2 mM β-mercaptoethanol, 20% glycerol, and 1 mM PMSF. The following scheme summarizes purification from cells derived from 1 L of induced culture; all steps were performed at 4 °C. Cells were fractured in a French press and centrifuged at ∼27 000g for 2 h. The resulting crude supernatant was loaded on a 12.5 mL column of Ni^{2+}-charged IMAC−agarose (Sigma; prepared according to manufacturer's instructions) equilibrated with IMAC column buffer (50 mM potassium phosphate at pH 7.5:2 mM β-mercaptoethanol:20% glycerol). The column was washed with two volumes of IMAC column buffer and eluted in a 0−200 mM imidazole gradient based in the same buffer but containing 10% glycerol (total gradient volume: 250 mL). Fractions were collected and assayed for pol activity, and the peak fractions were pooled.

The IMAC pool was loaded on a 20 mL MonoQ FPLC column (Pharmacia), washed with 60 mL of a buffer containing 50 mM potassium phosphate at pH 7.5, 5 mM β-mercaptoethanol, and 10% glycerol, and eluted with a 0.1−0.6 M NaCl gradient in the same buffer. The total gradient volume was 240 mL. Fractions of 2 mL were collected and assayed for DNA polymerase activity; homogeneous peak fractions were pooled and used for subsequent analyses. With respect to its specific activity, its K_m for activated DNA and dNTPs, and its affinity for the inhibitory dGTP analogue, TMAU, the his6-tagged pol III is essentially indistinguishable from the native pol III expressed from pKC30 (results not shown). Accordingly, the his6 segment was not removed from any of the his6-tagged pol IIIs prior to their use in experiments.

Labeling of Bs pol III with $^{[65]}Zn$. LB expression medium was depleted of divalent trace metals by stirring it for 24 h at 0 °C with 0.3 vol of Chelex-Na. The depleted medium was then supplemented with 0.1 mM $MgCl_2$, to support a level of *E. coli* growth compatible with pol III expression. *E. coli* AR120 which had been transformed with the pKC30/*pol*C vector (*15*) was grown at 30 °C in this Mg-supplemented medium, and when the culture reached an absorbance of 0.5 (600 nM, 1 cm light path), 10 mL was added to 1 L of the same medium containing $[^{65}Zn]Cl_2$ (specific activity, 2.7 mCi/μmol) at a concentration of 1.1 μCi/ml. Incubation was continued until the culture reached an absorbance of 0.75. The culture was then induced as described (*15*), and the cells were processed for preparation of homogeneous pol III (*15*).

Removal of Adventitiously Bound Zn and Other Divalent Trace Metals from pol III. Large proteins—particularly those with a high cysteine content—can strongly bind Zn and similar trace metals nonspecifically (*18*). To remove this nonspecifically bound Zn, we subjected each enzyme preparation to extensive dialysis against EDTA, using the following dialysis buffer: (100 mM NaCl:10 mM β-mercaptoethanol: 10 mM Na−EDTA:10 mM HEPES−Na, pH 7.5). All operations were carried out at 0 °C and used metal-free plasticware, metal-depleted dialysis tubing, and procedures routinely employed in preparation of metalloproteins for metal analysis (*19*).

Homogeneous enzyme was adjusted to a concentration of 0.5−5 μM with dialysis buffer and dialyzed against 100 vol of the same buffer for 12 h. This regimen was repeated five times against the same buffer and a sixth, and final, time against a buffer containing 0.01 mM β-mercaptoethanol and no EDTA. The samples were then concentrated by centrifugation in Zn-free concentrators (Filtron 10K) to yield solutions containing 20−30 μM enzyme. Both the native and his6-tagged forms of wild-type pol III routinely retain >80% of their original pol and exo activity following this regimen of dialysis and concentration.

Zinc and iron were determined by atomic absorption spectroscopy, using 10−60 μM solutions of EDTA-dialyzed pol III and a Perkin-Elmer 2280 flame instrument. All determinations incorporated determination and subtraction of metal content of appropriate dialysate "blanks". Protein concentrations of samples were determined by amino acid analysis as described in ref *20*.

Assay of Catalytic Activities. *Pol activity* was determined as described (*21*), using activated calf thymus DNA as template:primer and base-labeled [^{3}H]dTTP as the labeled deoxyribonucleotide. One unit of polymerase is defined as

that activity which catalyzes the incorporation of 1 nmol of [^{3}H]dTMP in 10 min at 30 °C (*21*).

For determination of the K_m of the polymerase for DNA, the concentration of activated calf thymus DNA was varied during assay from 0 to 0.8 mg/mL. For determination of the K_m for dGTP, incorporation of [^{3}H]dTMP was followed as a function of dGTP concentration (0−0.5 mM), and the values for incorporation were corrected for dGTP-independent, "background" incorporation. *Exo activity* was assayed as described (*21*), using single-stranded calf thymus DNA labeled at its 3′ end with [^{3}H]dTMP. One unit of exo activity is defined as that activity which catalyzes the release of 1nmol of total nucleotide in 10 min at 30 °C (*21*). For determination of the K_m for the exo substrate, the concentration of single-stranded DNA was varied from 0 to 0.2 mg/mL.

RESULTS

Determination of Zn:Protein Stoichiometry. Simple zf structures such as those proposed in Figure 1C typically bind a single atom of Zn with an affinity constant exceeding that of Zn:EDTA (*13*, *14*, *18*, *19*). We therefore analyzed Bs pol III for the presence of strongly bound, stoichiometric Zn. We examined two forms of the enzyme. One, the so-called wild-type pol III, was identical to the native enzyme found in *B. subtilis* (*15*). The other form incorporated a his6 affinity tag at its N-terminus to facilitate its purification (see methods section for details of construction and properties). Each form of the protein was purified to >95% homogeneity, reduced in volume to a concentration of 30−50 μM, dialyzed extensively against EDTA, and subjected to atomic absorption spectroscopic analysis of Zn (see purification schemes, conditions for concentration and dialysis, and analysis in the methods section).

Five independently derived samples of pol III were analyzed: 4 of the wild-type form and 1 of his6 form. Prior to EDTA treatment, each of the 5 samples contained >4 atoms of Zn/mol of protein—a level not unexpected for a large, undialyzed protein rich in Zn-scavenging thiol groups (Bs pol III has 15 cys residues; see ref *18*). The five-day regimen of dialysis against 10 mM EDTA removed this "loosely" bound Zn, reducing the enzyme's Zn content to a level which was not diminished further by extending the period of dialysis for up to 3 days.

The respective values for the EDTA-resistant Zn content (g atoms/mol of protein) obtained after this exhaustive regimen of dialysis of the four dialyzed wt pol IIIs and the one his-tagged derivative were, respectively, as follows: 1.2, 1.2, 1.0, 0.8, and 1.1. These values strongly suggested that Zn was tightly bound to pol III in a stoichiometry equal to 1.

The Zn Atom Is Removed by Thiol-Specific Electrophilic Reagents. The presence of a single, EDTA-resistant Zn atom in the pol III protein was consistent with its coordination in a finger structure like one of those postulated in Figure 1. Whereas the Zn in both of these hypothetical fingers is coordinated with cysteine, we probed its susceptibility to MMTS, a thiol-specific reagent (*22*) which has been widely used to "eject" Zn from accessible cys-based fingers (*23*). MMTS is a strong electrophile which ejects the Zn by converting the coordinating thiolate to its respective methyl disulfide (i.e., $CH_3-S-S-CH_2-$protein), destroying its capacity for Zn coordination (*22*, *23*).

Approach. Pol III was labeled by expression in the presence of ^{65}Zn and extensively dialyzed against EDTA to remove nonspecifically bound label (see methods section for details of [^{65}Zn]pol III preparation). After further dialysis in HNE buffer [10 mM HEPES−Na (pH 7.6):100 mM NaCl: 0.1mM EDTA] to remove exogenous thiol, the [^{65}Zn]pol III was adjusted with HNE to a concentration of ∼1 μM, and 0.1 mL samples (approximately 2.5 × 10^4 cpm) were incubated at 0 °C in the absence and presence of MMTS at 0.01, 0.03, 0.10, or 0.3 mM. After 60 min, each mixture was applied to a calibrated Sephadex G-25 column capable of separating protein from free $ZnCl_2$ (1.0 mL bed volume; void volume, 0.38 mL; equilibrated with HNE buffer). The column was eluted in 0.05 mL steps, and the fraction eluted with each step was analyzed by liquid scintillation to determine, respectively, protein-bound ^{65}Zn (void volume) vs free ^{65}Zn (included volume).

Results. In the absence of MMTS, all of the ^{65}Zn radioactivity, as expected, remained bound to the protein. In the presence of 0.01 mM MMTS, approximately 50% of the label was released, and at the three higher concentrations release was essentially complete (i.e., >95%).

Replacement of the Zn Atom by Fe and Other Relevant Metal Ions. Tetrahedrally coordinated Zn in zf structures such as those proposed in Figure 1C frequently accommodate metal ions other than Zn^{2+} (*24*). For example, the cys4-based structure of clostridial rubredoxin coordinates either Zn, Co, or Fe with equal affinity (*25*−*27*), and the Zn ion in a variety of other, comparable fingers may be productively replaced with group II metal ions such as Co^{2+}, Ni^{2+}, and Cd^{2+} (*19*, *24*, *27*). To determine whether the Zn-binding site of pol III shared this property, we sought to replace the pol III Zn with at least one these relevant metals.

Approach. Some zfs permit the removal and replacement of Zn directly, in the isolated target protein. Unfortunately, the Zn of pol III is not readily amenable to direct replacement by dialysis-based exchange of the native protein. The Zn readily dissociates from the protein upon denaturation with guanidinium chloride; however, the loss is essentially irreversible (results not shown). Metal also can be introduced into some cys-specific zf proteins by exploiting MMTS treatment (*23*). Following the ejection of the Zn, the thiomethylated finger is reconstituted simply by exposure of the protein to excess thiol and the metal of choice (*23*). Unfortunately, this approach also was not possible with pol III.

In the absence of a better alternative to substitution of the pol III Zn, we took an indirect approach based on expression of the recombinant pol III in medium specifically enriched for the appropriate replacement metal. Exploiting the trace metal-deficient medium in which we generated the ^{65}Zn-labeled enzyme, we expressed the pol III in the absence of added metal and in the presence of the respective chloride salts of Zn^{2+}, Co^{2+}, and Fe^{2+}—each at a concentration of 0.1 mM. The cells from the respective cultures were harvested, and their crude extracts were prepared and analyzed to determine the specific activity of pol III (i.e., units of pol/mg of protein).

The respective specific *pol* activities of each extract were determined and normalized to the specific activity of an

Table 1: Properties of Pol III Mutants

Enzyme	Zn content (g atoms/mol)[a]	relative sp activity		substrate affinity (K_m)			TMAU sensitivity (pol) (IC_{50})[f] (μM)
		exo[b]	pol[c]	exo[d] (mg/mL of DNA)	pol[e] (mg/mL of DNA)	pol[e] (μM dGTP)	
Wild-type	1.1	1	1	0.033	0.065	1.8	4.1
H909A	*h*	0.3	0.01	*h*	*h*	*h*	4.0
C912A	0.07	0.3	<0.001	0.027	*h*	*h*	*h*
C915A	0.4	0.2	0.06	0.021	0.086	1.9	2.9
C937A[g]	*h*	0.6	nd[i]	0.024	*h*	*h*	*h*
C940A	0.1	0.4	0.1	0.032	0.115	0.8	4.0

[a] Determined as described in methods section; values based on a single preparation. [b] Determined with single-stranded DNA using standard exo assay (see methods section). [c] Determined with activated DNA, using standard pol assay (see methods section). [d] Determined with single-stranded DNA, using standard exo assay conditions (see methods section for analysis of K_m determination). [e] Determined on activated DNA (see methods section for analysis of K_m value for dGTP). [f] Determined in the presence of 10 μM dGTP, as described in methods section. [g] Truncated protein missing entire pol domain (i.e., aa 1000−1437). [h]Parameter or value was not determined. [i] nd, not detectable.

identical control extract of cells which were induced to express pol III in conventional LB expression medium. The values for these were as follows: *control*, 1.0; *no trace metal supplement*, 0.20; *plus Zn*, 1.1; *plus Fe*, 1.2; *plus Co*, 1.0.

SDS−PAGE analysis of the above extracts indicated that the Zn-, Fe-, and Co-supplemented cells produced levels of the 160 kD pol III polypeptide equivalent to that of conventionally grown cells (results not shown). We purified and analyzed *one* of these—the Fe-specific enzyme—to determine if Zn had, indeed, been replaced by the substitute metal.

The Fe-expressed enzyme behaved exactly like the conventional Zn enzyme with respect to purification behavior, specific activity of pol and exo, substrate affinity, sensitivity to TMAU, and stability during EDTA dialysis (results not shown). Analysis of a single sample of a homogeneous, EDTA-dialyzed "Fe" pol III (see methods section for details) indicated an Fe content of 1.05 g atom/mol of protein and a Zn content of < 0.2 g atom/mol. This result and the apparently normal catalytic behavior of the Fe-expressed enzyme indicated that the metal binding site had an architecture and coordinating ligands which could accommodate Fe—i.e., it bore features similar to those proposed in the model fingers of Figure 1C.

Susceptibility of Zn Binding to Mutation of Relevant Thiolates. The susceptibility of bound Zn to ejection by MMTS and the capacity of the enzyme to productively incorporate Fe instead of Zn were properties clearly consistent with a finger structure like one of those summarized in Figure 1C. To determine if one of these structures, indeed, comprised the site of metal binding, we exploited a third approach involving site-directed mutagenesis of the five most relevant aa residues of Bs pol III (i.e., H909, C912, C915, C937, and C940) which we postulate to be Zn-binding ligands.

Approach. Each residue was mutated to alanine, replacing the Zn-coordinating thiolate side chains with a "neutral" methyl group. If any of these 5 hypothetical ligands were intimately involved in Zn coordination, its replacement with methyl group would be expected to significantly weaken, if not effectively destroy, the affinity of the enzyme for Zn.

Exploiting the his6 forms of pol III to expedite protein purification, we generated the native pol III and the mutant proteins at >95% homogeneity (see methods section for relevant details). The proteins were then subjected to EDTA-based dialysis as described in the methods section and analyzed for Zn content. The results are summarized in the second column of Table 1.

As noted in the table, two of the five mutant enzymes, H909A and C937A, were not amenable to Zn analysis. The H909A enzyme, although expressed as a full-length protein, was not readily produced in an amount sufficient for metal analysis. The C937A enzyme purified as a truncated protein of ~110 kDa, also in poor yield. Whereas this truncated peptide was likely generated by interruption of the polypeptide structure within the putative Zn binding site, it was not considered a legitimate candidate for comparison with the other, full-length proteins.

Each of the three full-length mutants—C912A, C915A, and C940A—displayed reduced Zn affinity relative to that of the wild-type enzyme. C915A was closest to the wild-type in affinity, retaining approximately 40% of the wild-type Zn complement, while C940A and C912 were significantly more impaired, retaining only 10% and 7%, respectively.

Effect of Relevant Point Mutations on Catalytic Properties. Each of the five purified mutant enzymes of Table 1 was analyzed directly, in its "native", undialyzed form, to assess the impact of the respective mutation on the following: (i) exo and pol activity; (ii) affinity for relevant exo and pol substrates; (iii) sensitivity of the pol activity to TMAU, an inhibitory dGTP analogue to which the Gr+ pol III is exquisitely sensitive (*4*). The results are summarized in the last 6 columns of Table 1.

As indicated in the columns summarizing relative specific activity, each mutation reduced the specific activity of *both* exo and pol. However, the reduction was clearly pol-selective, with the selectivity varying in intensity with each mutation. The lowest exo activity was 20% of wild-type, while pol activity ranged downward from 10 to <0.1% of wild-type for the 4 full-length mutants legitimately comparable to wild-type enzyme (the profound and selective anti-pol effect of C937A, which generates a truncated 110 kDa protein, may result from an indirect effect of the mutation—for example, an effect which renders the region of the protein downstream of the mutation more susceptible to proteolysis).

With respect to exo activity, none of the four mutants which were assayed (including the truncated exo+/pol− C937A) displayed an affinity for single-stranded, substrate DNA which was significantly different from that of wild-type enzyme. The same was true with respect to the substrate affinity and TMAU sensitivity of the two pol-"competent" enzymes, C915A and C940A. The K_ms for DNA and dNTP

(i.e., dGTP) varied little more than 2-fold from wild-type values, and their IC_{50} values for TMAU varied even less.

DISCUSSION

Site and Mode of Zn Binding. Given the characteristics of a conserved segment of primary structure between the pol and exo sites of the Gr+ DNA pol III, we hypothesized (i) that this enzyme is a metalloprotein which strongly binds Zn in a stoichiometry of 1 and (ii) that the Zn is bound within this segment in a fingerlike cluster of 4 cys thiolates or 3 cys thiolates and 1 his imidazole (see Figure 1C). Using Bs pol III as the model protein, we have obtained considerable evidence which, in sum, strongly supports these hypotheses. *First*, direct analysis of the pol III protein with atomic spectroscopy indicates that the enzyme, indeed, contains Zn in a stoichiometry of one. *Second*, the Zn-enzyme complex is highly EDTA-resistant, reflecting an affinity consistent with that observed for Zn finger structures (*13*, *14*, *19*)—particularly those in which Zn is tetrahedrally coordinated as proposed in Figure 1C. *Third*, the Zn−enzyme complex is susceptible to destruction by thiol-specific electrophiles such as MMTS (*22*, *23*). The possibility of thiol coordination of Zn is also supported by the finding that the Zn can be readily substituted by Fe, a metal ligand frequently coordinated by cys thiolates. For example, the Fe^{2+} coordinated by the cys 4 cluster of clostridial rubredoxin (i.e., $-cys[aa]_2cys-[aa]_{29}-cys[aa]_2cys-$) can be replaced by Zn with no significant effect on either the site geometry or the three-dimensional structure of the protein (*25*, *26*). *The fourth* piece of evidence supporting our hypothesis for the location and mode of Zn binding derives from the results of mutagenesis of cys residues proposed to serve as Zn ligands (see Table 1). Substitution of each of these with alanine significantly *decreased* the affinity of the polymerase for Zn, a result consistent with the loss of a coordinating thiolate side chain.

Probable Role of the Zn. In monosubstituted Zn metalloproteins, the Zn ion typically serves one of two roles (*19*). It either participates in catalysis, or it serves to stabilize the structure of a small domain, for example a zf domain (*13*, *14*, *19*). Two pieces of evidence strongly favor a structural, rather than a catalytic role for the Zn of pol III. *The first* is the primary structure of the putative site of Zn binding (Figure 1). It has the characteristics of a classic zf domain and none of those common to the binding site of catalytic zinc (*13*, *14*, *19*). *The second line* of evidence for structural Zn is the response of the enzyme's catalytic activity to the replacement of its Zn with Fe. Substitution of a *catalytic* Zn ion with a redox-active ferric/ferrous ion would be expected to significantly perturb at least one of the enzyme's catalytic activities (*27*). However, the catalytic behaviors of the Fe and Zn forms of the enzyme were equivalent.

Zf-like Domains in Other Template-Dependent DNA Polymerases. Although no other polymerase has yet been identified as a Zn metalloprotein, several have been noted to display zf-like primary structural motifs. Members of this group include the four DNA pols of *S. cerevisiae* (*28*−*31*), human pols *alpha* (*32*), *delta* (*33*), and *epsilon* (*34*), and ad2 pol, the replicative DNA polymerase encoded by adenovirus type 2 (*35*). With the exception of the ad2 pol, all of the latter enzymes display a single finger-like primary structure close to the C-terminus. Ad2 pol, which has *two* finger-like domains (one near each terminus), is the only member of this group in which the function of a putative Zn binding site has been reported. Mutation of specific cys residues of the C-terminal domain significantly depresses DNA synthesis and reduces binding of the enzyme to specific fragments of adenovirus DNA (*35*).

Significance of the Zf Domain for exo vs pol Function. In the absence of information about the tertiary structure of pol III and the position of the zf domain in it, the results of our experiments permit only speculation on its role in enzyme structure and function. Comparison of the relative specific activities of the exo and pol functions of the wild-type and relevant mutant enzymes (Table 1; H909A, C912A, C915A, and C940A) suggests that finger integrity is considerably more important for pol function than it is for exo function. The behavior of C912A, the mutant with the weakest affinity for Zn, most dramatically reflects this selectivity, displaying 30% of exo activity and less than 0.1% of its pol activity.

The intimate relationship of pol activity and the zf structure is also apparent from the results of an experiments examining the effect of MMTS treatment on the enzyme's catalytic activities. The experiment was essentially identical to that which was used to examine ejection of radioactive Zn (see Results section). The enzyme was treated with 0.1 mM MMTS at 0 °C in the absence of thiol for 2 h and then treated for several hours with 50 mM β-mercaptoethanol with Zn to regenerate the metal-coordinating thiols; it retained at least 50% of its exo activity and *no* detectable pol activity. This specific, irreversible loss of pol strongly suggests that the integrity of the finger is critical to the functional integrity of the pol site. It also indicates that, once disrupted, the normal finger−pol relationship is very difficult, if not impossible, to reestablish.

The importance of zf integrity to pol function and the relative lack of its importance to exo function are not unexpected—for at least two reasons. *First*, the zf can be deleted without significant effect on exo activity. For example, the entire 817 residue segment of Bs pol III downstream of aa 620 can be deleted without significantly affecting the exo activity of the resulting truncated peptide (*17*). *Second*, the *position* of the finger structure on the primary structural map of the enzyme is very close to that of the putative pol domain. Given its putative proximity to pol, it is not difficult to envision the zf as a mini domain which is either a structural component of the pol site or somehow involved in the formation of the site and/or the maintenance of its integrity.

Whatever the basis for the interdependence of pol activity and zf integrity, the behavior of relevant finger-specific mutants suggest it is not directly related to the capacity of the pol site to bind its substrates or the HPUra-type inhibitory dNTP analogues. The low polymerase activity of the C912A protein did not permit reliable kinetic analysis of its substrate and inhibitor affinities. However, analysis of the C915A and C940A enzymes, which displayed respectively 6% and 10% of wild-type pol activity was possible. Both of these enzymes, although severely crippled with respect to specific pol activity, displayed affinities for DNA, dNTP, and TMAU (see Table 1) which were close to those of the wild-type pol III.

Given the near-normal substrate affinities of these mutants, the selective reduction in their specific pol activity suggests that zf integrity may be more critical to the *efficacy* of pol action than it is to substrate binding. For example, the mutation of the zf might specifically affect a structure in the pol site which is important for the *turnover* of the pol substrates (*36*) or, perhaps, for the efficient function of a pol-specific DNA binding motif which coordinates "cross-talk" between the exo and pol sites (*37*, *38*). We currently are investigating both of these possibilities.

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