23. E. Bogenmann and C. Mark, *J. Natl. Cancer Inst.* **70**, 95 (1983); E. Bogenmann, *Int. J. Cancer* **38**, 883 (1986); R. C. McFall, T. W. Sery, M. Makadon, *Cancer Res.* **37**, 1003 (1977).
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Genetic and Crystallographic Studies of the 3′,5′-Exonucleolytic Site of DNA Polymerase I

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Site-directed mutagenesis of the large fragment of DNA polymerase I (Klenow fragment) yielded two mutant proteins lacking 3′,5′-exonuclease activity but having normal polymerase activity. Crystallographic analysis of the mutant proteins showed that neither had any alteration in protein structure other than the expected changes at the mutation sites. These results confirmed the presumed location of the exonuclease active site on the small domain of Klenow fragment and its physical separation from the polymerase active site. An anomalous scattering difference Fourier of a complex of the wild-type enzyme with divalent manganese ion and deoxythymidine monophosphate showed that the exonuclease active site has binding sites for two divalent metal ions. The properties of the mutant proteins suggest that one metal ion plays a role in substrate binding while the other is involved in catalysis of the exonuclease reaction.

THE LARGE PROTEOLYTIC FRAGMENT (Klenow fragment) of *Escherichia coli* DNA polymerase I is the only DNA-synthesizing enzyme for which high-resolution structural information is available (*1*). This makes it an ideal experimental system for a detailed molecular analysis of template-directed DNA synthesis. A large body of evidence (*2*) indicates that the two domains seen in the crystal structure correspond to separate polymerase and 3′,5′-exonuclease functions. The smaller (NH_2-terminal) domain was proposed to contain the exonuclease active site, since it binds deoxynucleoside monophosphate (dNMP) (*1*). A prominent feature of the dNMP complex with Klenow fragment is a divalent metal ion that is coordinated by the carboxylate groups of Asp^{355}, Glu^{357}, and Asp^{501}, together with the phosphate of dNMP (Fig. 1, site A). The binding of dNMP is accompanied by additional electron density that lies between the phosphate of dNMP and the carboxylate of Asp^{424}. The identity of the molecule giving rise to this extra density could not be established with certainty at 3.3 Å resolution, but the possibility that it corresponds to a second metal ion was raised. The proximity of Asp^{424} and the associated small molecule or ion to the phosphate of dNMP (and thus, by inference, to the phosophodiester bond to be cleaved in a DNA substrate) suggested a role for these groups in the exonuclease reaction. To investigate this possibility, we used site-directed mutagenesis to change Asp^{424} to Ala (D424A mutation). Further, to examine the role of the metal ion in site A, we eliminated two of its ligands by a double mutation changing both Asp^{355} and Glu^{357} to Ala (D355A,E357A mutation).

We made the mutational changes by using synthetic oligonucleotides to prime synthesis on uracil-containing M13 templates (*3*) containing portions of the *polA* gene. After characterization in M13, the mutations were transferred to an overproduction plasmid (Fig. 2). In each case the subcloned fragment was sequenced in its entirety to check that there were no additional mutations. Our first purification of the D355A,E357A protein indicated that this protein was substantially exonuclease-deficient. However, the sensitivity of our assay was affected by other cellular nucleases, including wild-type Klenow fragment from the chromosomal copy of *polA*. We therefore constructed a host strain for overproduction that was deficient in exonuclease III and carried the D355A,E357A mutant allele of *polA*. The overproduced proteins were purified to apparent homogeneity as described for wild-type Klenow fragment with one modification (*4*).

While the homogeneous mutant proteins have normal levels of polymerase activity, their exonuclease activity is reduced by about five orders of magnitude when compared with a wild-type preparation of equivalent purity (Table 1). The exonuclease activity present in the mutant protein preparations, although extremely low, is definitely above the background for the exonuclease assay. We do not know whether this residual activity is an intrinsic property of each mutant protein or is due to a very low level of contamination by cellular nucleases. Therefore, the values shown in Table 1 represent an upper limit for the 3′,5′-exonuclease activity of the mutant proteins.

To establish whether the loss of exonuclease activity in these two mutant proteins was due directly to the altered residues or was caused by some more general change in the protein structure, the two mutant deriva-

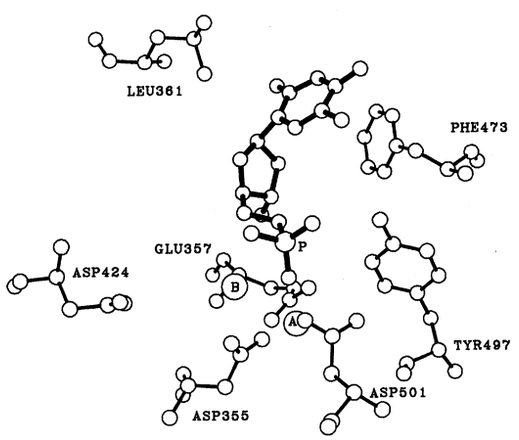

Fig. 1. The 3′,5′-exonuclease active site with the bound product molecule, deoxycytidine monophosphate (dCMP) (shown with thickened bonds). The side chains of residues interacting with the metal ions and dCMP are shown. The two binding sites for divalent metal ions are labeled A and B.

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tives of Klenow fragment were crystallized and their crystal structures were examined. Both the D424A and the D355A,E357A mutant proteins formed crystals under the same conditions as the wild-type protein (*5*); these crystals were isomorphous with those of wild-type Klenow fragment.

A 3.0 Å resolution difference electron density map between the D424A protein complex with deoxythymidine monophosphate (dTMP) (*6*) and the corresponding wild-type complex showed only two significant features (Fig. 3). One negative electron density peak results from the removal of the carboxylate of Asp^{424}. The second, larger, negative electron density peak corresponds to the previously unassigned density associated with Asp^{424}, which is also missing in the mutant protein. These data are consistent with this density being due to a second metal ion (Fig. 1, site B). The absence of other significant positive or negative features establishes that the D424A mutation produced no significant conformational alterations in the protein, and that dTMP and metal ion A bind identically to both the wild-type and D424A proteins.

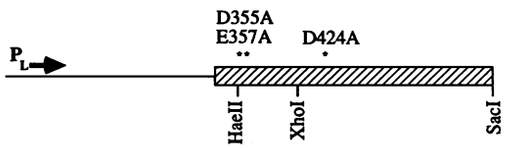

Fig. 2. Relevant portion of the Klenow fragment overproducer plasmid. This plasmid (pCJ122) is derived from the pAS1 vector (*18*) and encodes wild-type Klenow fragment under the control of the λ P_L promoter and the translational start signals of the *c*II gene. The coding region, shown only to the internal Sac I site, is indicated by the hatched area. The approximate positions of the mutations are indicated by asterisks. The D355A,E357A double mutation was made with a 19-base oligonucleotide having the codon changes GAT(Asp) to GCT(Ala) and GAA(Glu) to GCA(Ala). The D424A mutation was made with a 15-base oligonucleotide having the change GAT(Asp) to GCT(Ala). The restriction sites shown are those that were used to transfer the mutations from M13 to pCJ122 (the D355A,E357A mutation on a 160-bp Hae II–Xho I fragment and the D424A mutation on a 470-bp Xho I–Sac I fragment).

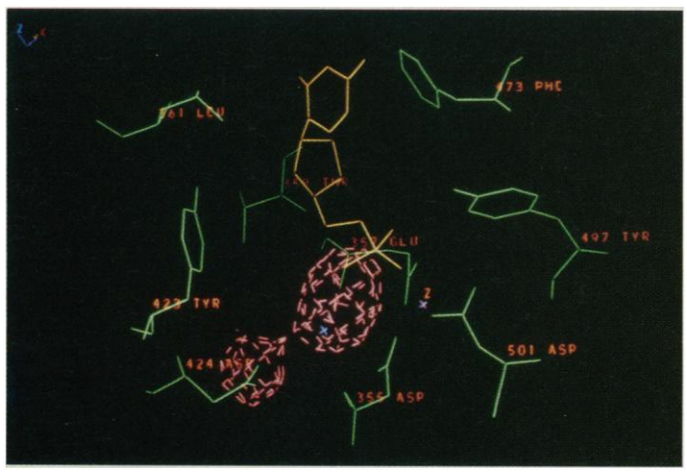

Fig. 3. Difference electron density map at 3.0 Å resolution between the D424A mutant protein and wild-type Klenow fragment (dashed contours in pink are negative). The inhibitor dTMP is shown in yellow, together with the protein residues (green) and metal ions (X) with which it interacts. The difference Fourier was calculated using experimental phases to 8 Å resolution; at higher resolution (3 to 8 Å) phases calculated from the refined coordinates (*19*) were used. The data set for the D424A protein was obtained from one crystal in 2 days with 101,375 reflections measured, giving rise to 18,838 unique reflections. The merging R factor = $(|I_{i,h} - \langle I_h \rangle|)/\langle I_h \rangle$ (where $I_{i,h}$ is the intensity of the ith observation of reflection h) was 0.056. The map is contoured at a level of 10 σ, where σ is the root-mean-square difference electron density throughout the unit cell. The maximum difference electron density was 17 σ at the position of the missing carboxylate of Asp^{424}, and 24 σ at the position of the metal ion site B associated with Asp^{424}.

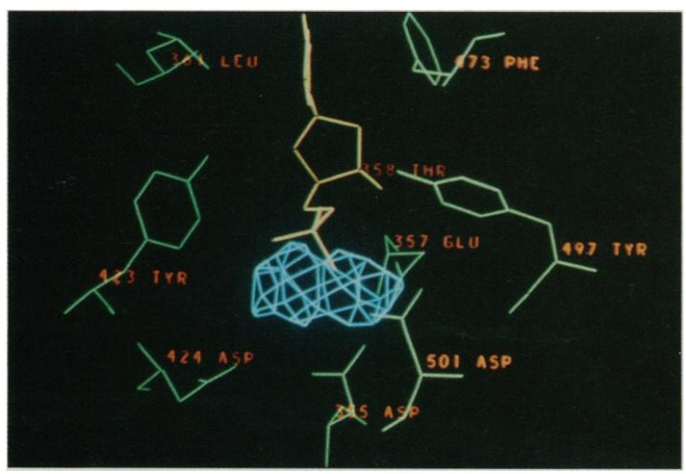

Fig. 4. Electron density map at 4 Å resolution (light blue), showing the presence of two Mn^{2+} ions at the exonuclease active site, superimposed on dCMP (yellow) and the amino acid side chains in the region. Crystals of the wild-type enzyme were soaked in ammonium sulfate solutions containing 14 m*M* dTMP and 50 m*M* $MnSO_4$. X-ray diffraction data to 3 Å resolution were measured with the two-dimensional area detector system of Hamlin-Xuong design (*6*) at Yale University. The map was calculated from the diffraction amplitude differences between Friedel mates due to anomalous scattering; phases used were calculated from the refined coordinates (*19*). The R factor between Friedel mates was 0.051. The maximum electron density is at 6.5 σ and the map is contoured at 4.8 σ.

Since only the added Mn^{2+} ion has a significant anomalous scattering component, this experiment shows two Mn^{2+} ions bound to sites A and B and establishes unambiguously that site B is indeed a binding site for a second divalent metal ion (*7*).

Low resolution difference maps (*8*) comparing the D355A,E357A protein with wild-type Klenow fragment show that this mutant protein does not bind dTMP or either of the metal ions in sites A and B. Like the D424A protein, the D355A,E357A protein exhibits no significant conformational differences from wild-type Klenow fragment.

Since neither mutant enzyme had any alteration in protein structure other than the expected changes in amino acid side chains at the mutation sites, we can conclude that the dNMP binding region on the smaller domain of Klenow fragment is indeed the 3′,5′-exonuclease active site. The lack of any effect of these mutations on the polymerase activity supports our previous conclusion that the active sites for polymerase and exonuclease are located on different domains of the enzyme (*9*). Thus the structural organization of Klenow fragment is analogous to that of *E. coli* DNA polymerase III, which has polymerase and exonuclease activities on separate subunits (*10, 11*).

The properties of the D424A protein identify an important catalytic component, or components, at the exonuclease site. If we assume that the product dTMP mimics the binding of the DNA substrate at the exonu-

Table 1. Assays in vitro of wild-type and mutant Klenow fragments. Assays of the mutant proteins were carried out on peak fractions from the final Sephacryl S-300 column. Polymerase activity was measured by the standard assay (*16*), with the use of poly[d(AT)]. Protein concentration was measured by enzyme-linked immunosorbent assay (ELISA) (*17*), with rabbit antiserum to Klenow fragment. Exonuclease activity was measured as described (*9*). The rate of solubilization of ^{32}P from 3′ end-labeled DNA was divided by the number of polymerase activity units present in the reaction mixture, to give the ratio of exonuclease to polymerase. The 3′ end-labeled DNA substrate was first treated with pyrophosphatase (1.4 unit/ml) for 15 minutes at 37°C to eliminate solubilization due to pyrophosphorolysis. The values reported are the mean (±SEM) of at least four independent determinations.

Protein	Polymerase specific activity (unit/mg)	Ratio of exonuclease to polymerase (arbitrary units)
Wild type	$(1.07 \pm 0.11) \times 10^4$	1 ± 0.3
D424A	$(0.93 \pm 0.16) \times 10^4$	$(1.3 \pm 0.6) \times 10^{-5}$
D355A,E357A	$(1.11 \pm 0.34) \times 10^4$	$(1.4 \pm 0.5) \times 10^{-5}$

clease active site, then the crystallographic studies suggest that the structure of the enzyme-substrate complex is the same for both the D424A and wild-type proteins. Therefore the loss of exonuclease activity in the D424A protein indicates a direct involvement in catalysis of one or both of the components missing in its structure, namely the metal ion at site B and the carboxylate of Asp^{424}. An additional contribution of metal B to the strength of substrate binding is not ruled out by the crystallographic experiment since only a single concentration of dTMP was used.

The failure of the D355A,E357A mutant protein to bind either dTMP or metal ions indicates a role for metal site A in substrate binding. The inability of the D355A,E357A enzyme to bind dTMP was not surprising because previous binding studies in solution (*12*) and in the crystal (*13*) had shown that removal of divalent metal ions with EDTA abolished dNMP binding (*14*). Since the D424A protein binds dTMP in the absence of metal B, then metal site A must be the more important in substrate binding. Additional contacts are important when binding a DNA substrate (as opposed to the inhibitor dNMP), as shown by recent studies on Klenow fragment–DNA crystals in which DNA can bind to the exonuclease active site even in the absence of divalent metal ions (*15*).

The mutagenesis studies described above establish, at the minimum, a role for metal A in substrate binding and for metal B in catalysis of the 3′,5′-exonuclease reaction. They do not exclude additional functions of metal A in catalysis and metal B in substrate binding.

REFERENCES AND NOTES

1. D. L. Ollis, P. Brick, R. Hamlin, N. G. Xuong, T. A. Steitz, *Nature (London)* **313**, 762 (1985).
2. C. M. Joyce and T. A. Steitz, *Trends Biochem. Sci.* **12**, 288 (1987).
3. T. A. Kunkel, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 488 (1985).
4. C. M. Joyce and N. D. F. Grindley, *ibid.* **80**, 1830 (1983). The purification procedure was modified to avoid the use of phosphate buffers because they contain sufficient pyrophosphate to allow a Klenow fragment–catalyzed pyrophosphorolysis reaction which interferes with the 3′,5′-exonuclease assay. Instead of phosphate buffers we used Pipes buffer for the Bio-Rex 70 chromatography and tris-HCl throughout the rest of the purification. As a final purification step we used a Sephacryl S-300 gel filtration column.
5. P. Brick, D. Ollis, T. A. Steitz, *J. Mol. Biol.* **166**, 453 (1983). The crystals were soaked in buffered ammonium sulfate solution containing 14 m*M* dTMP, 1 m*M* $ZnSO_4$, and 20 m*M* $MgSO_4$ (*1*).
6. X-ray diffraction data to 3.0 Å resolution were obtained from crystals of the D424A protein with the use of the Mark II two-dimensional area detector system at La Jolla, CA; N. G. Xuong, S. T. Freer, R. Hamlin, C. Neilsen, W. Vernon, *Acta Crystallogr.* **A34**, 289 (1978).
7. The metal ion in site A is Zn^{2+} in the previously described crystals (*1*) to which 1 m*M* $ZnSO_4$ had been added. The chemical identity of the metal ion in site B has not been established; it could be either Mg^{2+} or a Zn^{2+} ion that is less well ordered.
8. Data to 7.0 Å resolution were measured from crystals of the D355A,E357A protein with a modified Picker diffractometer; H. W. Wyckoff, *Methods Enzymol.* **114**, 330 (1985).
9. P. S. Freemont *et al.*, *Proteins* **1**, 66 (1986).
10. H. Maki and A. Kornberg, *J. Biol. Chem.* **260**, 12987 (1985).
11. R. H. Scheuermann and H. Echols, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 7747 (1984).
12. J. A. Huberman and A. Kornberg, *J. Biol. Chem.* **245**, 5326 (1970).
13. D. L. Ollis and T. A. Steitz, unpublished data.
14. It is clear that the failure to bind the metal ion at site A in the D355A,E357A mutant, is responsible for the loss of dTMP binding (rather than vice versa) since wild-type Klenow fragment can bind the metal at site A in the absence of dNMP (*1*).
15. T. A. Steitz, L. Beese, P. S. Freemont, J. Friedman, M. R. Sanderson, *Cold Spring Harbor Symp. Quant. Biol.*, in press.
16. P. Setlow, *Methods Enzymol.* **29**, 3 (1974).
17. C. O. Thoen, K. Mills, M. Hopkins, *Am. J. Vet. Res.* **41**, 833 (1980).
18. M. Rosenberg, Y.-S. Ho, A. Shatzman, *Methods Enzymol.* **101**, 123 (1983).
19. L. Beese and T. A. Steitz, unpublished data.

20. We thank A. Polesky for help with the ELISA assays and N. Grindley for helpful discussions. Supported by American Cancer Society grant NP-421 (T.A.S.) and by NIH grants GM-22778 (T.A.S.) and GM-28550 (N. D. F. Grindley). High resolution data for the D424A protein complex were collected at the resource for protein crystallography at the University of California at San Diego, supported by NIH grant RR-01644 (N. G. Xuong). J.M.F. acknowledges a postdoctoral fellowship from the Jane Coffin Childs Memorial Fund.

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A Previously Undetected MHC Gene with an Unusual Periodic Structure

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The major histocompatibility complex is a chromosomal segment embodying several gene clusters among which those with immune functions are the best characterized. This region is suspected to host other as yet undetected genes whose characterization may shed light on the population genetics and evolution of the whole gene complex and thus on its unexplained character of marker locus for a number of diseases of nonimmune or unknown pathogenesis. A novel gene was identified that is transcribed in all tissues tested and is located in mouse and man between the *C4* and *Bf* genes of the H-2 and HLA complexes, respectively. From the nucleotide sequence, derived from liver complementary DNA clones, it is predicted that this novel single-copy gene encodes a 42-kilodalton polypeptide that bears no recognizable relation to the protein families known so far, but it displays striking hallmarks of natural selection.

IN ORDER TO SEEK OUT NEW GENES IN the major histocompatibility complex (MHC) (*1*), we set up a collection of genomic DNA probes derived from the mouse H-2S subregion which, on account of its relative length (*2*), is likely to enclose a large number of such undetected transcribed sequences. Because protein coding sequences are subject to stronger evolutionary constraints than noncoding ones, murine DNA segments were selected for their ability to hybridize at high stringency on rat DNA. This strategy, which we have previ-

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