Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 8452–8456, November 1986
Biochemistry

Gene *32* protein, the single-stranded DNA binding protein from bacteriophage T4, is a zinc metalloprotein

(DNA replication)

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Communicated by Alan Garen, July 9, 1986

ABSTRACT **Gene *32* protein (g32P) isolated from bacteriophage T4-infected *Escherichia coli* and from an overproduction vector derived from the plasmid pKC30 contains 1 mol of tightly incorporated Zn(II) per mol of protein. A linear incorporation of three molar equivalents of *p*-hydroxymercuriphenylsulfonate (PMPS) results in a linear release of 1.1 mol of Zn(II) from the protein. Reversal of formation of the g32P-PMPS complex with thiol in the presence of EDTA results in a zinc-free apo-g32P. Cd(II) and Co(II) can be exchanged with the intrinsic Zn(II) ion. The Cd(II) protein shows a charge-transfer band at ≈250 nm. The Co(II) protein shows a set of absorption bands typical of a tetrahedral Co(II) complex (ε_{max} = 660 $M^{-1}\cdot cm^{-1}$ at 645 nm), and two intense charge-transfer bands are present at 355 nm (ε = 2250 $M^{-1}\cdot cm^{-1}$) and 320 nm (ε = 3175 $M^{-1}\cdot cm^{-1}$). These observations are consistent with three cysteines as ligands to the Zn(II) ion in g32P. Zn(II) g32P undergoes precise limited proteolysis by trypsin to produce the small fragments A and B and the core, g32P−(A+B). Under identical conditions, apo-g32P is hydrolyzed rapidly beyond the g32P−(A+B) stage to produce many proteolyzed fragments. Fluorescence quenching experiments show that at low protein concentration apo-g32P has markedly altered binding affinity for poly(dT) relative to native g32P. Three of the four cysteines of g32P are found in a tyrosine-rich sequence corresponding to residues 72–116 and implicated in DNA binding by ^{1}H NMR investigations. Zn(II) appears to provide a conformational element contributing to DNA binding by coordinating the cysteine and possibly histidine side chains in the sequence -Cys-X_3-His-X_5-Cys-X_2-Cys-, residues 77–90, located in the DNA binding domain of g32P.**

The product of gene *32* of bacteriophage T4 (g32P) is one of a class of proteins that bind to single-stranded (ss) DNA (or ss RNA) in a stoichiometric fashion and are referred to as helix-destablizing proteins or ss DNA binding proteins (SSBs) (1–4). Other widely studied members of this class include gene *5* protein from bacteriophage fd and SSB from *Escherichia coli* (5, 6). g32P is known to play key roles in DNA replication, recombination, and repair (2). g32P is thought to be able to quickly cover those transiently single-stranded regions that arise near the advancing T4 DNA replication forks and in so doing stabilize a particular ss DNA conformation that is most appropriate to serve as a substrate for other catalytic proteins (2). *E. coli* SSB probably functions in much the same way (6). Gene *5* protein, in contrast, acts to prevent replicative DNA synthesis by covering newly synthesized fd ss DNA until packaging into the phage coat can occur (3, 5).

The molecular details of how each of these SSBs interacts with ss DNA has been probed by one- and two-dimensional ^{1}H NMR techniques applied to the oligonucleotide complexes of these proteins (7–11). For both gene *5* protein and the g32P tryptic core, the results support a model involving intercalation of tyrosine and phenylalanine side chains with the bases of the bound oligodeoxynucleotide (10). A tyrosine-rich region of the protein that includes residues 72–116 contains six of the eight tyrosine residues in the molecule, positioned in a regularly spaced fashion suggesting that this domain of the protein forms part of the surface interacting with the nucleic acid lattice (9, 12).

A role for zinc in replicative and transcriptional processes associated with cell division has been indicated by derangements of cell morphology, arrest of cell division, and major distortions in the relative concentrations of DNA and the profile of RNA species in simple eukaryotic cells made zinc deficient (13). Zn(II) is an intrinsic component of bacterial RNA polymerases, in which both ions would appear to play structural roles rather than acting catalytically in the synthesis of RNA (14). Other simple DNA and RNA polymerases from phage and bacterial sources, however, do not contain intrinsic Zn(II) (15). The recent demonstration of significant Zn(II) associated with the eukaryotic transcription factor IIIA from *Xenopus laevis* oocytes (16, 17) has fueled speculation that Zn(II) acts in maintenance of a particular structural motif directly involved in nucleic acid binding and therefore may play a regulatory role rather than a catalytic one.

Kozloff and coworkers (18, 19) have shown that Zn(II) is an essential component of the T4 phage baseplate bound to the short tail fibers, the protein product of T4 gene *12*. These fibers attach to the host membrane and apparently aid in the injection of viral DNA. ^{65}Zn(II) uptake experiments with T4-infected *E. coli* cells showed that most of the ^{65}Zn(II) was associated with g12P; however, infection by T4 g12P$^-$ mutants showed reduced but significant incorporation of ^{65}Zn(II) into other unidentified phage-induced proteins (18). Herein, we report that g32P and its tryptic core g32P−(A+B) contain 1 atom of nondialyzable Zn(II) per molecule, while gene *5* protein from fd and *E. coli* SSB are devoid of the metal.

MATERIALS AND METHODS

Materials. Homogeneous g32P was prepared from T4-infected cells by using standard procedures (20). Cloned wild-type g32P was overproduced by temperature induction (40°C) of *E. coli* M5248 transformed with pYS6, which contains the structural gene *32* DNA sequence inserted into the *Hpa* I site of pKC30. The details of the construction of pYS6 are as outlined by Prigodich *et al.* (10) except that the

Abbreviations: g32P, native gene *32* protein; g32P−(A+B), g32P that has undergone limited proteolysis by trypsin, which removes residues 1–21 (B region) and 254–301 (A region); ss, single-stranded; SSB, ss DNA binding protein; PMPS, *p*-hydroxymercuriphenylsulfonate.

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codon for Tyr-115 was not altered. The tryptic core of g32P, g32P−(A+B), was purified from a limited tryptic digest of g32P as described (21). *E. coli* SSB and gene *5* protein were obtained as previously outlined (22, 23). Trypsin treated with L-tosylamido-2-phenylethyl chloromethyl ketone was from Worthington, while the sodium salts of *p*-hydroxymercuriphenylsulfonate (PMPS) and poly(dT) were Sigma products. All buffers were prepared from stock solutions of 2 M Tris·HCl, pH 8, and 2 M NaCl, both passed through Chelex X-100 (Bio-Rad) to lower free Zn(II) levels.

Atomic Absorption Spectroscopy. Spectroscopy was performed with an Instrumentation Laboratories IL157 atomic absorption spectrometer with an IL555 graphite furnace attachment as described (14).

Preparation of Apo- and Metallo-g32Ps. Apo-g32P was prepared by addition of 4 equivalents of PMPS to native g32P, followed after 10 min by 40 additional equivalents, and then incubation for 15 min at 25°C. EDTA was added to 1 mM and then the formation of the PMPS-g32P complex was reversed with addition of dithiothreitol to 0.2 mM. The solution was immediately dialyzed against 10 mM Tris·HCl, pH 8/0.2 M NaCl/5% (vol/vol) glycerol (TNG) buffer containing 1 mM EDTA and 0.1 mM dithiothreitol with several changes under nitrogen. The resultant protein and others prepared in similar fashion were found to contain 0.03–0.12 mol of Zn(II) per mol of protein. Reconstituted Zn_1 g32P was obtained as described for apo-g32P except the EDTA was omitted prior to the initial treatment with dithiothreitol. Dialysis was not initiated for 36 hr (4°C), after which time the above dialysis protocol was followed. The resulting g32P contained 1.22 mol of Zn(II) by atomic absorption. Cd(II) g32P was obtained by dialysis of g32P against 2 mM cadmium chloride for 24 hr followed by a similar dialysis against metal-free buffer as above. Co(II) g32P was obtained by dialysis against 0.1 M cobalt sulfate in TNG buffer for several days followed by dialysis against metal-free TNG buffer.

Partial Proteolysis of g32P with Trypsin. Proteolysis was carried out according to the previously published protocols (9, 21). Trypsin was added to 240-μg/ml (7.16 μM) g32P in metal-free TNG buffer.

Fluorescence Measurements. Concentrated solutions of apo- and metallo-g32P were diluted to 0.1–2 μM in binding buffer (10 mM Tris·HCl/1 mM EDTA, pH 8) and loaded into 2-ml fluorescence cells. The intrinsic protein fluorescence was determined on a model 8000 SLM spectrofluorometer using an excitation wavelength of 282 nm and an emission wavelength of 347 nm. Poly(dT) (20–200 μM) was then added to the protein solution in small amounts and the remaining fluorescence was determined in triplicate. Fluorescence quenching values were determined at each poly(dT)-to-g32P ratio after correcting for dilution, photobleaching, and absorption of incident light by poly(dT). Best-fit values of site size (n) and apparent binding constants, K_a, were ascertained by using standard procedures (24).

RESULTS

Zn(II) Content of g32P. Several preparations of homogeneous g32P were collected and dialyzed exhaustively against metal-free TNG buffer to remove loosely bound Zn(II) ions. A wide range of concentrations (≈2–50 μM) of protein was employed during dialysis to reduce potential artifacts resulting from a constant low level (≤50 nM) of Zn(II) found in Chelex-treated buffer. Table 1 shows that different preparations of g32P (2–10 μM) all contain ≈1 mol of Zn per mol of protein. These include protein produced from the cloned gene inserted into an overproduction vector as well as the wild-type g32P from T4-infected cells. Two preparations of purified g32P−(A+B) analyzed also contained stoichiometric quantities of Zn(II).

Table 1. Zn(II) content of several SSBs

SSB	Treatment*	Zn(II), mol/mol of protein†
T4 g32P		
Prep. 1	Dialysis	1.00 ± 0.02
Prep. 1	Dialysis + 10 mM EDTA	1.32
Prep. 2	Dialysis	1.03 ± 0.06
Prep. 3	Dialysis	0.90 ± 0.01
Prep. 3	Dialysis + 10 mM EDTA	0.88
Prep. 3	Dialysis + 10 mM DPA	1.06
Prep. 4‡	Dialysis	0.96
Prep. 4‡	Dialysis + 0.1 mM EDTA	0.97 ± 0.02
T4 g32P−(A+B)		
Prep. 1	Dilution	0.98
Prep. 2	Dialysis	0.99
Prep. 2	Dialysis + 10 mM EDTA	0.98
E. coli SSB	Dilution	0.02
	Dialysis	0.03
	Dialysis + 10 mM EDTA	0.14
fd gene *5* protein	Dilution	0.17

Prep., preparation.

*"Dialysis" indicates protein samples (2–50 μM, 0.5–4 ml) were dialyzed against 500 ml of metal-free TNG buffer or TNG buffer with the indicated additions (DPA, dipicolinic acid) with three or four changes of dialysate over 24–72 hr at 4°C. Zn(II) content and protein concentration ($\varepsilon_{280} = 3.7 \times 10^4\ M^{-1}\cdot cm^{-1}$) were determined on these samples diluted to 2–10 μM. "Dilution" indicates Zn(II) analysis was performed on protein diluted 50- to 200-fold into metal-free TNG buffer.

†Where a range of values is given, the indicated preparation was dialyzed and prepared for Zn(II) analysis two to four times. Results are mean ± SD.

‡The g32P was produced by the gene inserted into an overproduction vector.

Table 1 also shows that the Zn(II) associated with both g32P and g32P−(A+B) is resistant to removal during prolonged dialysis against chelators, including EDTA (0.1–10 mM) and dipicolinic acid (10 mM), under conditions that labilize the intrinsic Zn(II) ions in enzymes such as alkaline phosphatase, carbonic anhydrase, and carboxypeptidase, but not from the tetrathiolate sites in aspartate transcarbamoylase and *E. coli* RNA polymerase. Finally, when two other ss DNA binding proteins, *E. coli* SSB and fd gene *5* protein, were subjected to similar dialysis, the resulting Zn(II) content in both proteins was far less than stoichiometric—i.e., 0.02–0.17 mol of Zn (Table 1).

Labilization of the Intrinsic Zn(II) of g32P. 1,10-Phenanthroline (10 mM) can displace the Zn(II) from g32P slowly over several days (Table 2). To more readily remove the Zn(II) from g32P, a strongly dissociating sulfhydryl reagent, PMPS, was employed. PMPS has been studied extensively in its reaction with aspartate transcarbamoylase, in which titration of the 4 sulfhydryl groups responsible for coordination of the Zn(II) ion in each of the six regulatory chains of the c_6r_6 units results in a linear incorporation of 24 mol of the mercurial per mol of enzyme, with concurrent release of the 6 Zn(II) ions (25). We have recently developed a method that employs a similar technique to dissociate one of the two intrinsic Zn(II) ions of *E. coli* RNA polymerase (14).

Fig. 1*A* shows a titration of native g32P with PMPS by following the formation of the PMPS-sulfhydryl chromophore at 250 nm. The data indicate a linear incorporation of PMPS up to ≈3.2 equivalents of added reagent, whereupon no additional incorporation results, up to 10- to 15-fold molar excess, suggesting that only 3 SH groups of the 4 in g32P are titratable by PMPS. Zinc released during the PMPS reaction can be titrated with the Zn(II)-binding dye 4-(2-pyridylazo)-resorcinol (PAR). $Zn(II)PAR_2$ exhibits an absorption maxi-

Table 2. Metal contents of apo- and metallo-g32P

g32P	Treatment	Metal content, mol/mol of protein		
		Zn(II)	Cd(II)	Co(II)
Apo-g32P				
Prep. 2	PMPS/EDTA/thiol	0.10	—	—
Prep. 3	PMPS/EDTA/thiol	0.03	—	—
Prep. 3	Dialysis + 10 mM o-P, 24 hr	0.22	—	—
Prep. 3	Dialysis + 10 mM o-P, 36 hr	0.04	—	
Zn_1 g32P				
Prep. 2	PMPS/thiol	1.22	—	—
Cd g32P				
Prep. 1	Cd(II), 0.02 mM	0.20	0.64	—
Prep. 2	Cd(II), 1.0 mM	0.15	0.74	—
Prep. 3	Cd(II), 2.0 mM	0.04	0.76	
Co g32P				
Prep. 3	Co(II), 100 mM	0.05	—	1.06

Proteins were prepared as described in the text. Prep., preparation. Zn_1 g32P was the reconstituted product. The apo-, Cd, and Co g32Ps derived from native g32P prep. 3 were used for the partial proteolysis and fluorescence studies described in Figs. 3 and 4, respectively. o-P, 1,10-phenanthroline.

mum at 500 nm (25). PMPS titration in the presence of PAR shows a linear release of Zn(II) up to approximately 3 equivalents of PMPS added to the g32P solution, whereupon color development levels off (Fig. 1*B*). The color development corresponds to 1.12 ± 0.04 mol of Zn(II) released per mol of g32P.

Preparation of Metal-Free Apo-, Cd(II), and Co(II) Metallo-g32Ps. Treatment of native g32P with excess PMPS and EDTA with subsequent dialysis gives rise to a protein with ≥90% of the Zn(II) removed, referred to as apo-g32P (Table 2). Reconstituted Zn_1 g32P refers to the product of a slow time-dependent reassociation of a stoichiometric amount of Zn(II) with apo-g32P when the PMPS is removed with dithiothreitol (Table 2). Since Zn(II) has no convenient spectroscopic characteristics, a common technique used to explore the chemical and physical environment about the metal ion is to substitute spectroscopically useful metal ions such as Cd(II) and Co(II). Treatment of zinc g32P with 2 mM Cd(II) through dialysis or direct treatment results in binding of 0.76 mol of Cd(II) and a proportional drop in the amount of protein-bound Zn(II) (Table 2). This protein also exhibits a significant $S^- \rightarrow$ Cd(II) charge-transfer absorption band centered at ≈255 nm, suggestive of cysteinyl coordination about the Cd(II) ion (data not shown).

Co(II) can replace the Zn(II) in gp32P by dialysis of the protein against 0.1 M cobalt sulfate in TNG buffer at pH 8.0 for 5 days, followed by dialysis against metal-free TNG buffer. After dialysis the protein contained 1.06 mol of Co(II) per mol (Table 2). The Co(II) protein is cobalt-blue in color with the following absorption bands (ε is in units of $M^{-1} \cdot cm^{-1}$): 680 nm (ε = 480); 645 nm (ε = 660); 605 nm (ε = 430); 355 nm (ε = 2250); and 320 nm (ε = 3175). The spectrum is shown in Fig. 2 and compared to the rather similar absorption spectrum obtained when the Zn ions of the catalytic sites of horse liver alcohol dehydrogenase are exchanged with Co(II), the so-called "blue hybrid" (26).

Susceptibility of Metallo-g32 Proteins to Limited Proteolysis. Under controlled conditions trypsin can remove two small polypeptides from g32P: the 47-residue A peptide from the carboxyl terminus and the 21-residue B-peptide from the amino terminus. The result, g32P−(A+B) (sometimes called g32P* or core g32P), is highly resistant to further proteolysis. This limited proteolysis abolishes the oligomerization observed for native g32P in solution as well as the cooperativity of binding of g32P to ss DNA (9, 21, 27). The core protein still appears to preserve all its interactions with ss DNA, but the affinity is reduced, reflecting the loss of cooperative binding. The susceptibility of native g32P to proteolysis is markedly enhanced by removal of the Zn(II) ion (Fig. 3). At 25°C, the A and B regions of native g32P are quickly removed to give core g32P, which remains intact. A similar selective proteo-

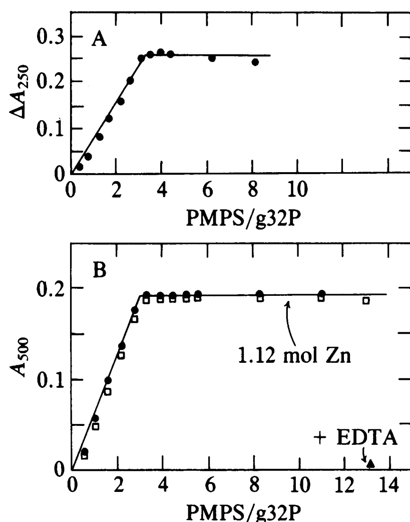

FIG. 1. Titration of native g32P with PMPS. (*A*) Native g32P (27 μM in 0.2 ml of TNG buffer) was treated with successive 1.2-μl portions of a 1 mM PMPS solution in TNG to give the indicated molar ratios of PMPS to g32P. The A_{250}, indicative of $S^- \rightarrow$ Hg(II) charge-transfer absorption, was measured relative to that of the start of the titration (ΔA_{250}). The maximum absorption reached corresponds to 3.21 mol of titrant per mol of protein. (*B*) A parallel titration was performed in duplicate with the same g32P solution as used in *A* (2.6 μM in 0.7 ml of TNG buffer) except that 0.1 mM 4-(2-pyridylazo)resorcinol was added to both protein and buffer cuvettes. The indicated maximum A_{500} corresponds to an average of 1.12 ± 0.04 mol of Zn(II) released per mol of g32P (25). ● and □, two separate experiments; ▲, absorbance after addition of 2 mM EDTA.

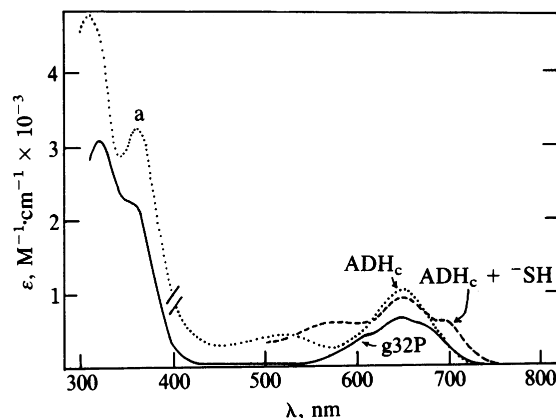

FIG. 2. Optical absorption spectra. —, Spectrum of Co(II)-substituted g32P. Protein concentration was 2 mg/ml and the spectrum was obtained in masked quartz cuvettes with a Cary 219 spectrophotometer. Absorption represents that of the Co(II) chromophore alone, since the UV absorption and scattering of the protein was corrected for by subtracting the spectrum of the same concentration of native Zn(II) protein. ····, Spectrum of horse liver alcohol dehydrogenase with Co(II) substituted at the catalytic site, ADH_c, replotted from ref. 26. The extinction coefficients of the near UV bands of ADH_c should not be referred to the left ordinate, since it is not clear that the corrections are the same as applied to the g32P spectrum; only band positions should be compared. Band a is reported to have $\varepsilon = 7000\ M^{-1} \cdot cm^{-1}$ (26). ---, Spectrum of the ^-SH complex of ADH_c (26).

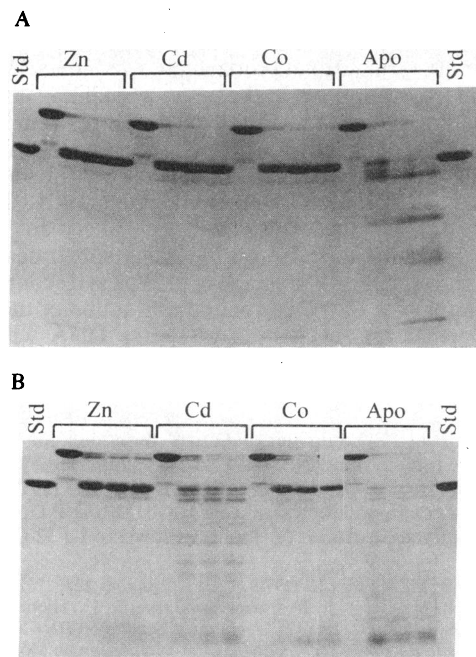

FIG. 3. Limited proteolysis of metallo-g32Ps. (*A*) g32Ps were treated with trypsin at a 25:1 (wt/wt) ratio of g32P to trypsin at 22°C, and aliquots were removed at the times listed below and prepared for electrophoresis. Lanes 1 and 18, g32P−(A+B) standard (Std); lanes 2–5, native g32P digested for 0, 10, 30, and 50 min, respectively; lanes 6–9, Cd g32P treated for 0, 10, 30, and 50 min, respectively; lanes 10–13, Co g32P treated for 0, 10, 30, and 50 min, respectively; lanes 14–17, apo-g32P treated for 0, 10, 30, and 50 min, respectively. (*B*) g32Ps were treated with trypsin at a 100:1 (wt/wt) ratio of g32P to trypsin at 45°C and aliquots were removed and prepared for electrophoresis at 0, 10, 20, and 30 min for native g32P (lanes 2–5), Cd g32P (lanes 6–9), Co g32P (lanes 10–13), and apo-g32P (lanes 14–17). Lanes 1 and 18 represent g32P−(A+B) standard.

lysis is true of Cd and Co g32Ps. Apo-g32P is much more susceptible to proteolysis, since there is no time at which the core protein concentration builds up (Fig. 3*A*). Upon cleavage of the A and B regions, which appears to occur normally, the resulting core is immediately further proteolyzed to smaller peptides. At 45°C, Zn and Co g32Ps are similarly resistant to core proteolysis, while Cd g32P exhibits somewhat enhanced susceptibility; apo-g32P, however, is almost entirely digested to low molecular weight peptides (Fig. 3*B*).

Poly(dT) Binding to Metallo-g32 Proteins. The binding of g32P to ss DNA templates such as poly(dT) can be used to determine approximate binding constants, degree of cooperativity, and site size (n) characterizing complex formation. A convenient method to assess polynucleotide binding by g32P is by titrating a set concentration of g32P with poly(dT), resulting in quenching of the fluorescence of protein tryptophan residues (24).

A typical titration of native g32P with poly(dT) is shown in Fig. 4. Poly(dT) gives a maximum quenching of ≈53% with $n = 6.8$ when a protein concentration of 1 μM is used. In contrast, the apo-g32P at 1 μM gives only ≈33% quenching. As the protein concentration is reduced, the fluorescence quenching by poly(dT) falls off much more rapidly for apo-g32P than for the native protein, which still shows ≈33% quenching at 0.1 μM, while essentially no quenching is detected at this concentration for the apoprotein. The best interpretation would appear to be that the binding constant for poly(dT) has been considerably reduced by the removal of Zn(II). The apparent decrease in site size as concentrations get into the region of the probable dissociation constants is difficult to interpret from the present data alone. More

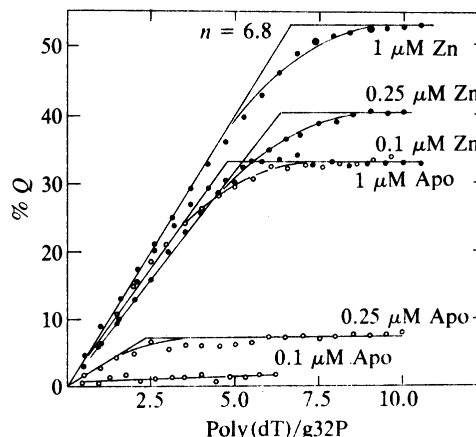

FIG. 4. Fluorescence quenching titration of apo- and metallo-g32Ps with poly(dT). Fluorescence measurements were carried out as described in *Materials and Methods*, with percent quenching (% Q) determined at each poly(dT)-to-g32P ratio. The poly(dT)-to-g32P ratio indicates the moles of TMP residues divided by moles of g32P present. •, Native g32P present at 1, 0.25, and 0.1 μM as indicated. ○, Apo-g32P present at 1, 0.25, and 0.1 μM as indicated.

extensive studies designed to determine K_a values will be required to obtain a more quantitative description of the change in poly(dT) binding induced by Zn(II) removal.

DISCUSSION

In this report we present evidence that T4 g32P is a Zn(II) metalloprotein and that removal of the metal greatly affects the structure and DNA binding capacity of the molecule. Since g32P−(A+B) isolated from limited tryptic digests maintains stoichiometric amounts of Zn(II), it appears that all protein coordination ligands are found in the large core fragment. While typical Zn(II) chelators do not readily remove the metal from the protein, the sulfhydryl reagent PMPS displaces the intrinsic zinc from g32P (Fig. 1). The demonstration that mercurial modification of three of the four cysteine residues results in quantitative Zn(II) release, coupled with the observation that Cd(II), which has a high affinity for S^- ligands, can exchange with the Zn(II) in g32P, provides strong evidence that three cysteine residues provide coordination ligands to the Zn(II) ion in g32P.

Removal of the Zn(II) from native g32P significantly increases the susceptibility of the core fragment, g32P−(A+B), to further proteolysis by trypsin. Co(II), and to a lesser extent Cd(II), can substitute for Zn(II) in this stabilization of the core protein (Fig. 3). Thus, significant structural alterations must have occurred in the molecule as a result of metal ion removal. The detailed relationship between Zn(II) removal and conformational perturbations in the DNA binding domain of g32P requires further investigation. All preparations of the apo-g32P show dramatic changes in poly(dT) binding as assayed by fluorescence quenching (Fig. 4). The relative loss of fluorescence quenching should not be interpreted to mean that affinity for ss DNA has been lost on Zn(II) removal, since when the protein concentration is increased the binding properties of apo-g32P more closely approximate those of native g32P (Fig. 4). Indeed, numerous preparations of apoprotein retain the ability to bind to ss DNA-cellulose columns and are eluted at only marginally lower salt concentrations than is the native protein. Thus, Zn(II) is only one of a number of determinants involved in the DNA–protein interactions of g32P. We have observed that some samples of apoprotein, especially the apo-g32P−(A+B), appear to have lost most of their binding affinity for

DNA-cellulose, suggesting that the Zn-free protein may be susceptible to further unfolding.

^{1}H NMR studies carried out in this laboratory have implicated 5 tyrosine and 2 phenylalanine residues of g32P as involved in DNA binding (9, 10). An amino acid sequence from residue 72 to residue 116 of g32P contains 6 of the 8 tyrosine residues in the molecule, and this region is highly likely to form at least part of the DNA binding domain of the protein (10). Tyrosine residue 115 has been shown by site-directed mutagenesis to be one of those interacting with a bound deoxyoligonucleotide (10). This same sequence also contains 3 cysteine residues, as shown in the amino acid sequence from residue 72 to residue 92 (Fig. 5).

The absorption spectrum of the Co(II)-substituted protein clearly establishes the metal binding site as tetrahedral, probably containing several S^- ligands giving rise to the charge-transfer bands (Fig. 2). The absorption spectrum is most similar to that of the N,S,S,S, site of alcohol dehydrogenase produced when the Zn at the catalytic site is replaced with Co(II) and the water ligand normally present is replaced with the inhibitor $^-$SH (Fig. 2). The amino acid residues Cys-77, His-81, Cys-87, and Cys-90 could easily form such a tetrahedral site in g32P. Its location in the middle of a region believed for other reasons to participate in DNA binding would explain the effects of Zn removal on fluorescence quenching by poly(dT) (Fig. 4).

Transcription factor IIIA from *Xenopus laevis* oocytes, which binds to the gene encoding 5S RNA and is also found in a 7S ribonucleoprotein particle, contains variable amounts of Zn(II) (16, 17). As many as 9–12 atoms of Zn(II) have been found in the intact 7S particle. It has been proposed that there are 9 Zn(II) binding sites in transcription factor IIIA contained within 9 roughly repeated amino acid sequences which occur in this protein, all of which contain Cys-X_{2-5}-Cys-X_{12}-His-X_{2-3}-His. These "metal binding fingers" are proposed to form tetrahedral Zn(II) complexes, which insert into both major and minor grooves of the double-stranded DNA and thereby regulate transcription of the 5S RNA gene (17). ADR1 is another eukaryotic transcription factor that contains a similar Cys–Cys–His–His arrangement of amino acids (29). Other DNA binding proteins closely related to g32P, including several retroviral ss DNA-binding proteins, have some sequence homology to g32P. This includes the potential Zn(II) binding sequence located between residues 72 and 92, which is compared in Fig. 5 to the sequences of the other ss DNA binding proteins containing this potential ligand pattern.

A computer search of cysteine-rich sequences in DNA binding proteins has recently been used to predict that such sequences are metal binding sites (30). It is unknown at present how often these predictions will prove correct, since similar cysteine-rich sequences occur in non-zinc proteins—

		Start	Sequence	End
T4	g32P	72	-W-Y-I-E-T-[C]-S-S-T-[H]-G-D-Y-D-S-[C]-P-V-[C]-Q-Y-	92
BLV	p12	19	-P-P-P-G-P-[C]-Y-R-[C]-L-K-E-G-[H]-W-A-R-D-[C]-P-T-	39
BLV	p12	44	-P-P-P-G-P-[C]-P-I-[C]-K-D-P-S-[H]-W-K-R-D-[C]-P-T-	64
MuLV	p10	21	-L-C-R-C-Q-[C]-A-Y-[C]-K-E-K-G-[H]-W-A-K-D-[C]-P-K-	41
ASV	p12	16	-R-S-R-G-L-[C]-Y-T-[C]-G-S-P-G-[H]-Y-Q-A-Q-[C]-R-K-	36
ASV	p12	42	-N-S-R-E-R-[C]-E-L-[C]-N-G-M-G-[H]-N-A-K-Q-[C]-R-K-	62

FIG. 5. Amino acid sequences of ss DNA binding proteins containing potential zinc binding ligands, cysteine (C) and histidine (H). Line 1, g32P from T4; lines 2 and 3, repeated sequences from the p12 protein from bovine leukemia virus; line 4, p10 protein from murine leukemia virus; lines 5 and 6, repeated sequences from the p12 protein from avian sarcoma virus (28).

e.g., T7 phage RNA polymerase (31). It is more significant to turn the statement around and note that in cases in which Zn(II) sites are turning up in proteins involved in nucleic acid metabolism, they appear to be sites containing multiple cysteine sulfide moieties as ligands. These sites also appear to have at least four protein ligands, suggesting that they do not contain coordination positions open to solvent. The electron-rich nature of sites containing several S^- ligands also suggests that these sites are not likely to readily exchange ligands with those on incoming substrates or on other macromolecules. In the case of g32P, Zn(II) contributes to the maintenance of the correct conformation of the DNA binding domain for recognition of the ss DNA with high affinity in a stoichiometric fashion and thus establishes a basic structural role for Zn(II) in this protein.

We thank Yousif Shamoo and Richard V. Prigodich for preparing several of the protein samples. This work was supported by National Institutes of Health Grants AM09070-21 and GM21919-12 and National Science Foundation Grant PCM8104118. D.P.G. was supported by National Institutes of Health Fellowship 1-F32-GM10972.

1. Alberts, B. M. & Frey, L. (1970) *Nature (London)* **227**, 1313–1318.
2. Alberts, B. M., Barry, J., Bedringer, P., Burke, R. L., Hibner, U., Liu, C.-C. & Sheridan, R. (1980) in *Mechanistic Studies on DNA Replication and Genetic Recombination*, ICN-UCLA Symposium on Molecular and Cell Biology, eds. Alberts, B. M. & Fox, C. F. (Academic, New York), Vol. 19, pp. 449–471.
3. Coleman, J. E. & Oakley, J. L. (1980) *CRC Crit. Rev. Biochem.* **1**, 247–289.
4. Jensen, D. E., Kelly, R. C. & von Hippel, P. H. (1976) *J. Biol. Chem.* **251**, 7215–7228.
5. Alberts, B., Frey, L. & Delius, H. (1972) *J. Mol. Biol.* **68**, 139–152.
6. Molineux, I. J., Friedman, S. & Gefter, M. L. (1974) *J. Biol. Chem.* **249**, 6090–6098.
7. O'Connor, T. P. & Coleman, J. E. (1983) *Biochemistry* **22**, 3375–3381.
8. Alma, N. C. M., Harmsen, B. J. M., Hull, W. E., van der Marcel, G., van Boom, H. H. & Hilbers, C. W. (1981) *Biochemistry* **20**, 4419–4428.
9. Prigodich, R. V., Casas-Finet, J., Williams, K. R., Konigsberg, W. & Coleman, J. E. (1984) *Biochemistry* **23**, 522–529.
10. Prigodich, R. V., Shamoo, Y., Williams, K. R., Chase, J. W., Konigsberg, W. H. & Coleman, J. E. (1986) *Biochemistry* **25**, 3666–3672.
11. Clore, G. M., Gronenborn, A. M., Greipel, J. & Maass, G. (1986) *J. Mol. Biol.* **187**, 119–124.
12. Williams, K. R., LoPresti, M. B. & Setoguchi, M. (1981) *J. Biol. Chem.* **256**, 1754–1756.
13. Falchuk, K. H., Hardy, C., Ulpino, L. & Vallee, B. L. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 4175–4179.
14. Giedroc, D. P. & Coleman, J. E. (1986) *Biochemistry* **25**, 4969–4978.
15. King, G. C., Martin, C. T., Pham, T. T. & Coleman, J. E. (1986) *Biochemistry* **25**, 36–40.
16. Hanas, J. S., Hazuda, D., Bogenhagen, D. F., Wu, F. Y.-H. & Wu, C.-W. (1983) *J. Biol. Chem.* **258**, 14120–14125.
17. Miller, J., McLachlan, A. D. & Klug, A. (1985) *EMBO J.* **4**, 1609–1614.
18. Zorzopulos, J. & Kozloff, L. M. (1978) *J. Biol. Chem.* **253**, 5543–5547.
19. Kozloff, L. M. & Zorzopulos, J. (1978) *J. Biol. Chem.* **253**, 5548–5550.
20. Bittner, M., Burke, R. L. & Alberts, B. M. (1979) *J. Biol. Chem.* **254**, 9565–9572.
21. Hosoda, J. & Moise, H. (1978) *J. Biol. Chem.* **253**, 7547–7555.
22. Weiner, J. H., Bertsch, L. L. & Kornberg, A. (1975) *J. Biol. Chem.* **250**, 1972–1980.
23. Anderson, R. A., Nakashima, Y. & Coleman, J. E. (1975) *Biochemistry* **14**, 907–917.
24. Kowalczykowski, S. C., Paul, L. S., Lonberg, N., Newport, J. W., McSwiggen, J. A. & von Hippel, P. H. (1986) *Biochemistry* **25**, 1226–1240.
25. Hunt, J. B., Neece, S. H., Schachman, H. K. & Ginsberg, A. (1984) *J. Biol. Chem.* **259**, 14793–14803.
26. Maret, W., Andersson, I., Dietrich, H., Schneider-Bernlöhr, H., Einarsson, R. & Zeppezauer, M. (1979) *Eur. J. Biochem.* **98**, 501–509.
27. Williams, K. R. & Konigsberg, W. H. (1978) *J. Biol. Chem.* **253**, 2463–2470.
28. Copeland, T. D., Morgan, M. A. & Oroszlan, S. (1983) *FEBS Lett.* **156**, 37–40.
29. Hartshorne, T. A., Blumberg, H. & Young, E. T. (1986) *Nature (London)* **320**, 283–287.
30. Berg, J. M. (1986) *Science* **232**, 485–487.
31. Davanloo, P., Rosenberg, A. H., Dunn, J. J. & Studier, F. W. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 2035–2039.