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Interactions of Bacteriophage T4-coded Gene *32* Protein with Nucleic Acids

III. Binding Properties of Two Specific Proteolytic Digestion Products of the Protein (G32P*I and G32P*III)

NILS LONBERG†, STEPHEN C. KOWALCZYKOWSKI, LELAND S. PAUL AND PETER H. VON HIPPEL

Institute of Molecular Biology and
Department of Chemistry
University of Oregon
Eugene, Ore. 97403, U.S.A.

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Brief treatment of gene *32* protein with proteolytic enzymes produces two specific digestion products in good yield (Moise & Hosoda, 1976). One, representing the native protein with ~60 amino acid residues removed from the C-terminus, is G32P*I. The other, for which ~20 amino acid residues have been removed from the N-terminus *in addition to* the 60 residues from the C-terminus, is G32P*III. Both of these specific "core" fragments of gene *32* protein have been isolated and purified, and their binding properties to single-stranded oligo- and polynucleotides have been studied. We find that the binding properties of G32P*I are relatively little changed from those characteristic of the native gene *32* protein: (1) the apparent binding constants to short ($l=2$ to 8) oligonucleotides are independent of lattice length and essentially independent of base and sugar composition, but do show an increased salt dependence of binding relative to that of the native protein; (2) the intrinsic association constants (K) for polynucleotides binding in the co-operative mode show the same binding specificities as seen with the native protein, but with absolute values increased two to fourfold; (3) the polynucleotide binding co-operativity parameter ($\omega \simeq 2 \times 10^3$) and the binding site size ($n \simeq 7$ nucleotide residues) are the same as for the native protein; (4) essentially the entire salt dependence of the net affinity ($K\omega$) remains in K. However, unlike native gene *32* protein, G32P*I *can* melt native DNA to equilibrium (Hosoda *et al.*, 1974; Greve *et al.*, 1978); this suggests that the kinetic pathways for DNA melting by these two species must differ, since the changes in equilibrium binding parameters measured here are far too small to account for the differences in melting behavior. In contrast to G32P*I, for G32P*III we find that: (1) binding is non-co-operative ($\omega \simeq 1$); (2) the binding site size (n) for the protein has decreased by one to two nucleotide residues relative to that characteristic of the native protein and G32P*I; (3) binding to short ($l=2$ to 8) oligonucleotides is length and salt concentration dependent; (4) while binding to polynucleotides continues to show approximately the same base composition dependence as the native protein, the absolute values of K are

† Present address: Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, MA 02138, U.S.A.

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somewhat different and the salt concentration dependencies of K are less. Polynucleotide ultraviolet light and circular dichroism spectra obtained in the presence of G32P*I and G32P*III are indistinguishable from those measured with the native protein at similar binding densities, indicating that all three protein species distort the polynucleotide lattice to comparable extents.

These results are combined with the equilibrium binding data for native gene *32* protein (Kowalczykowski *et al.*, 1980*a*; Newport *et al.*, 1980) to obtain further insight into the molecular details of the interactions of this protein with its nucleic acid binding substrates.

1. Introduction

Limited proteolysis of bacteriophage T4 coded gene *32* protein produces three defined digestion products: G32P*I, G32P*II and G32P*III (Hosoda *et al.*, 1974; Moise & Hosoda, 1976). G32P*I differs from the native protein in the loss of ~60 amino acid residues from its C-terminus, G32P*II has lost ~20 residues from its N-terminus, and G32P*III has lost *both* the C-terminal *and* the N-terminal peptides. G32P*I has been found to destabilize (lower the melting temperature) of double-helical native DNA (Hosoda *et al.*, 1974; Greve *et al.*, 1978; Hosoda & Moise, 1978), whereas native gene *32* protein (Alberts & Frey, 1970; Jensen *et al.*, 1976) does not. This lack of destabilizing effect of the native protein on native double-stranded DNA has been attributed to a kinetic block (Jensen *et al.*, 1976). Thermodynamic binding parameters are presented here for the interaction of G32P*I and G32P*III with oligo- and polynucleotides, to further extend our understanding of the molecular details of co-operative binding of gene *32* protein to nucleic acids and, in particular, to examine the roles of the N and C-terminal portions of the protein in this interaction. These studies, together with those described in the accompanying papers (Kowalczykowski *et al.*, 1980*a*; Newport *et al.*, 1980), also provide the thermodynamic underpinnings of work in progress on the *kinetics* of the interaction of gene *32* protein, G32P*I and G32P*III with single and double-stranded nucleic acids (see Kowalczykowski *et al.*, 1980*b*; Lohman, 1980).

2. Materials and Methods

(a) *Nucleic acids, polynucleotides and oligonucleotides*

All synthetic polynucleotides were purchased from Miles Biochemicals, and oligonucleotides from P-L Biochemicals or Collaborative Research. Salmon sperm DNA was purchased from Calbiochem. Phage λ DNA was phenol-extracted from purified phage particles. Concentrations were determined using the following extinction coefficients (per mol phosphate) at 25°C in ~0·1 M-NaCl at 260 nm: phage λ DNA, $6{\cdot}6 \times 10^3$ M^{-1} cm^{-1}; salmon sperm DNA, $6{\cdot}5 \times 10^3$ M^{-1} cm^{-1}; poly(dA), $9{\cdot}1 \times 10^3$ M^{-1}; poly[r(A,C)], (83 mol % A), $7{\cdot}9 \times 10^3$ M^{-1} cm^{-1}. See Kowalczykowski *et al.* (1980*a*) for extinction coefficients of the other polynucleotides, as well as for details of the properties and characterization of the oligonucleotides used in this study.

(b) *Preparation and characterization of G32P*I and G32P*III*

Gene *32* protein was prepared and purified as described by Kowalczykowski *et al.* (1980*a*). Tryptic digestion of gene *32* protein was conducted while the protein was bound to single-stranded salmon sperm DNA (prepared from phenol-extracted salmon sperm DNA dissolved in distilled water, heated to 100°C for 10 min and then "quenched" in ice). The digestion

reaction, and subsequent purification steps were carried out in a buffer (hereinafter called prep. buffer) containing 20 mM-Tris, 1 mM-β-mercaptoethanol, 5% (v/v) glycerol, 1 mM-EDTA and NaCl as required (pH 8·0). The digestion mixture contained 0·5 mg gene *32* protein/ml, 25 mM-NaCl and 0·6 μg trypsin/ml; the total single-stranded DNA concentration was 7 mol nucleotide residues/mol gene *32* protein. Digestion was continued for 60 to 80 min at 4°C, a time sufficient to eliminate all the original gene *32* protein as monitored on polyacrylamide gels (see below). The reaction was terminated by the addition of soybean trypsin inhibitor to a final concentration of approx. 1 μg/ml. Mg^{2+} and Ca^{2+} were then added to final concentrations of 10 mM and 4 mM, respectively, and the solution incubated (at 4°C) for 4 h with 20 μg pancreatic DNase I/ml (previously treated with 0·2 μg phenylmethylsulfonyl fluoride/ml to inactivate possible protease contaminants). The solution was then made 0·7 M in NaCl, dialyzed overnight against prep. buffer containing 0·7 M-NaCl, 10 mM-$MgCl_2$ and 4 mM-$CaCl_2$, and then dialyzed into prep. buffer containing 50 mM-NaCl and 10 mM-EDTA. The digest was applied to a single-stranded DNA-cellulose column, and the column was washed with the above (final) dialysis buffer to an emergent A_{280} of approximately zero. The column was eluted in steps with 0·3 M, 0·5 M and 2·0 M-NaCl-containing prep. buffer, washing between steps to ~zero A_{280} each time. The 0·5 M-NaCl eluate was discarded; the 0·3 M eluate contained the G32P*III fraction and the 2·0 M eluate the G32P*I fraction. These fractions were shown to be ~98% pure by sodium dodecyl sulfate/polyacrylamide gel electrophoresis, and were stored at −70°C in 10 mM-HEPES buffer containing 0·1 M-Na_2EDTA, 0·1 M-NaCl and 10% (v/v) glycerol (pH 7·7).

This G32P*I preparation was shown to melt native λ DNA to completion at 20°C (in buffer C (Kowalczykowski *et al.*, 1980*a*) containing 0·1 M-NaCl) at a DNA phosphate to protein ratio of 6:1, with a resultant DNA hyperchromism of 47% at 260 nm.

The extinction coefficient ($\varepsilon_{280} = 3{\cdot}7 \times 10^4$ M^{-1} cm^{-1}) previously determined for native gene *32* protein (Jensen *et al.*, 1976) was also used for G32P*I and G32P*III. This is justified by the fact that no tryptophan or tyrosine residues are removed in either the C-terminal or the N-terminal cleavage reactions. (The N-terminal peptide contains 4 of the 20 phenylalanine residues of the protein, while the C-terminal peptide contains none.)

(c) *Methods*

Ultraviolet light absorbance and fluorescence titrations and calculations were carried out as described by Kowalczykowski *et al.* (1980*a*). Circular dichroism and circular dichroism difference spectra were measured at room temperature (~23°C) in a 2 mm cell using a Cary 60 spectropolarimeter interfaced to a Varian 620*l* computer.

3. Results

(a) *Interactions of G32P*I with short (l=2 to 8 residues) oligonucleotides*

Titrations of G32P*I with oligonucleotides of varying length and composition were performed by monitoring the quenching of intrinsic protein fluorescence and analyzing the resulting titration curves as previously described (Kowalczykowski *et al.*, 1980*a*). The results of these titrations are summarized in Table 1. These data show that the maximum quenching obtained with a given series of oligonucleotides increases with chain length; this increase is especially pronounced for the larger oligomers of dT. Similar results were obtained with native gene *32* protein (Table 1; Kowalczykowski *et al.*, 1980*a*). We again conclude that as the length of the oligonucleotide lattice approaches the protein site size (see below), additional quenching contacts become possible, either *via* direct interaction with fluorescent protein side-chains or indirectly *via* protein conformational changes induced as a consequence of binding.

TABLE 1

*Association constants and quenching parameters for the binding of short ($l=2 \rightarrow 8$) oligonucleotides to G32P*I and G32P*III*

Protein	Oligonucleotide	Q_m (%)†	K_{oligo} (M^{-1})†
G32P*I	$d(pT)_2$	12	$2{\cdot}1(\pm 0{\cdot}5)\times 10^5$
	$d(pT)_3$	9	$4{\cdot}3(\pm 1{\cdot}0)\times 10^5$
	$d(pT)_4$	15	$2{\cdot}8(\pm 0{\cdot}4)\times 10^5$
	$d(pT)_6$	26	$3{\cdot}5(\pm 0{\cdot}4)\times 10^5$
	$d(pT)_7$	33	$5{\cdot}6(\pm 0{\cdot}5)\times 10^5$
	$d(pT)_8$	52	$1{\cdot}2(\pm 0{\cdot}2)\times 10^6$
	$d(pA)_2$	7	$3{\cdot}3(\pm 1{\cdot}5)\times 10^4$
	$d(pA)_3$	5	$1{\cdot}6(\pm 0{\cdot}5)\times 10^6$
	$d(pA)_4$	9	$5{\cdot}0(\pm 0{\cdot}9)\times 10^5$
	$dA(pA)_4$	10	$3{\cdot}7(\pm 0{\cdot}5)\times 10^5$
	$d(pA)_6$	10	$2{\cdot}7(\pm 0{\cdot}2)\times 10^5$
	$d(pA)_8$	18	$5{\cdot}3(\pm 0{\cdot}5)\times 10^5$
	$r(pA)_3$	10	$2{\cdot}8(\pm 1{\cdot}0)\times 10^5$
G32P*III	$d(pT)_3$	31	$7{\cdot}2(\pm 1{\cdot}0)\times 10^4$
	$d(pT)_6$	39	$9{\cdot}0(\pm 1{\cdot}0)\times 10^5$
	$d(pT)_8$	52	—

The titrations were performed in buffer C (Kowalczykowski *et al.*, 1980*a*) containing 0·1 M-NaCl.
† The values of K_{oligo} listed represent the average of several titrations (1 to 4), and the errors reflect uncertainties due both to the scatter of data *within* individual titrations and *between* replicate titrations.

The base specificity of the binding of G32P*I at the oligonucleotide level is very comparable to that of the native protein (Kowalczykowski *et al.*, 1980*a*); i.e. only minimal differences in affinity between oligonucleotides of different base composition are observed (Table 1). Thus the affinity of G32P*I for $d(pT)_8$ is only twofold greater than that of $d(pA)_8$, while for gene *32* protein this difference is $\simeq 1{\cdot}5$-fold. Similarly, both proteins show an approximately twofold relative preference for deoxyribo-oligonucleotides of a given composition over the analogous ribo-oligonucleotides. Again, as demonstrated with the native protein, this specificity behavior contrasts markedly with that observed at the polynucleotide level.

The affinity of G32P*I for oligonucleotides shows an oligonucleotide chain length dependence that is indistinguishable from that of the native protein, up to an oligonucleotide length of six residues (Fig. 1). As before, the expected statistical increase in K_{app} with increasing oligonucleotide length is not observed. The divergence between the native protein and the G32P*I data seen at $l \geq 6$ nucleotide residues in Figure 1 reflects differences in the salt dependence of binding for these longer lattices. Data on the salt dependence of binding of oligonucleotides of $l=6$ and 8 residues to the various protein species are summarized in Figure 2 and Table 2. It is clear that the binding of these oligonucleotides to G32P*I shows a greater salt dependence than does their binding to the native protein. Interpretation of the slopes of the lines for the binding of $d(pT)_6$ and $d(pA)_8$ to G32P*I, using the theory of Record *et al.* (1976), yields values of m' (the number of ionic interactions

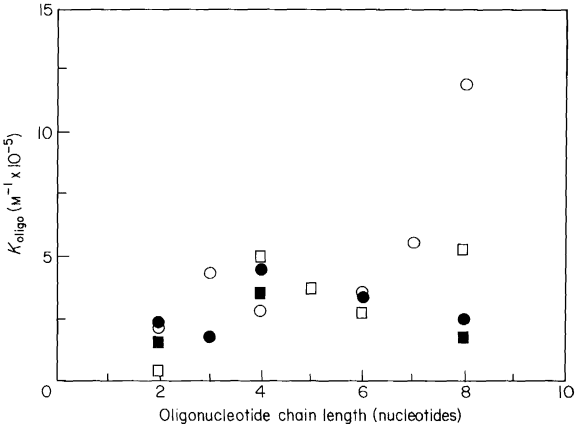

FIG. 1. Chain length dependence of G32P*I–oligonucleotide binding constants, measured at 25(±1)°C in buffer C containing 0·1 M-NaCl. Comparative gene *32* protein binding data, in buffer B containing 0·1 M-NaCl, from Kowalczykowski *et al.* (1980*a*). G32P: (●) $d(pT)_n$; (■) $d(pA)_n$ **G32P*I**: (○) $d(pT)_n$; (□) $d(pA)_n$.

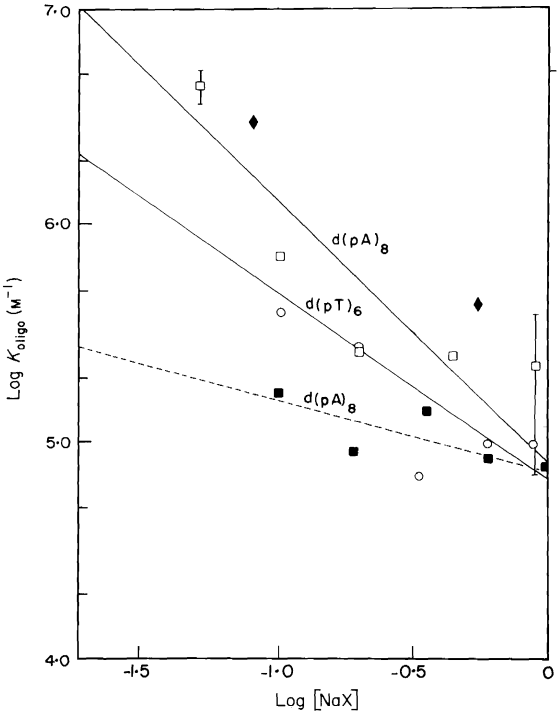

FIG. 2. Salt concentration dependence of G32P*I–oligonucleotide binding constants, measured at 25°C in buffer C, plus added salt. The lines represent the best fit linear least-squares representation of the indicated data. Comparative gene *32* protein binding data, in buffer B plus added salt, from Kowalczykowski *et al.* (1980*a*). (○) $d(pT)_6$ G32P*I; (□) $d(pA)_8$ G32P*I; (◆) $d(pA)_8$ G32P*I (NaF); (■) $d(pA)_8$ gene *32* protein (NaCl).

TABLE 2

*Comparison of the salt dependence of oligonucleotide binding for gene 32 protein, G32P*I and G32P*III*

Protein	Oligonucleotide	$(\partial \log K/\partial \log [Na^+])$	ψ	ψ_{corr}‖	m'
G32P†	$d(pT)_8$	$-0{\cdot}37 \pm 0{\cdot}02$	0·69	0·60	$0{\cdot}6 \pm 0{\cdot}1$
	$d(pA)_8$	$-0{\cdot}28 \pm 0{\cdot}07$	0·78	0·71	$0{\cdot}4 \pm 0{\cdot}1$
G32P*I‡	$d(pT)_6$	$-1{\cdot}25 \pm 0{\cdot}15$	0·69	0·59	$2{\cdot}1 \pm 0{\cdot}3$
	$d(pA)_8$	$-1{\cdot}29 \pm 0{\cdot}1$	0·78	0·71	$1{\cdot}8 \pm 0{\cdot}2$
G32P*III‡	$d(pT)_3$	$-0{\cdot}2 \pm 0{\cdot}1$	0·69	0·49	$0{\cdot}4 \pm 0{\cdot}2$
	$d(pT)_6$	$-1{\cdot}9 \pm 0{\cdot}3$	0·69	0·59	$3{\cdot}2 \pm 0{\cdot}5$
	$d(pT)_6$§	$-1{\cdot}6 \pm 0{\cdot}3$	0·69	0·59	$2{\cdot}7 \pm 0{\cdot}5$

† Data from Kowalczykowski *et al.* (1980*a*); measured in buffer B with added NaCl.
‡ Measured in buffer C (Kowalczykowski *et al.*, 1980*a*) containing added NaCl.
§ Titration in NaF rather than NaCl.
‖ Corrected for oligonucleotide length according to Record & Lohman (1978).

involved in the complex) of 2·1 and 1·8, respectively (these values may be underestimated; see p. 85 of Kowalczykowski *et al.*, 1980*a*). Since the slope of these lines does not change when NaF is substituted for NaCl (Fig. 2), anion binding and release does not appear to be involved in these interactions, and thus the m' value for G32P*I binding to these oligonucleotides can be attributed solely to cationic effects. This implies that about two to three positively charged protein residues are involved in the binding reaction that are not available for oligonucleotide binding interactions with the native protein, presumably because they are covered in some manner by the C-terminal peptide of the protein (see Fig. 13 of Kowalczykowski *et al.*, 1980*a*).

This increased affinity of the longer oligonucleotides for G32P*I is most pronounced at low ionic strengths (e.g. at ~0·1 M-NaCl, the salt concentration used in obtaining the data of Fig. 1). Extrapolation of the data of Figure 2 to 1 M-NaCl shows that the *non*-electrostatic component of binding for the two proteins is identical, and suggests that the increased affinity for G32P*I observed at lower salt concentrations is purely electrostatic in origin.

(b) *Interactions of G32P*I with polynucleotides*

The binding of G32P*I to single-stranded polynucleotides is accompanied by changes in absorbance at 260 nm that are similar to those observed with the native gene *32* protein. Table 3 shows the total change in absorbance for several different polynucleotides on binding to these proteins; both proteins induce similar hyper- or hypochromic shifts on binding to polynucleotides under stoichiometric (tight-binding) conditions.

These absorbance changes have been used to monitor the binding of G32P*I to various polynucleotides. Figure 3 shows a titration curve in which small portions of protein have been added to a fixed amount of poly(rA) in 0·2 M-NaCl; conditions under which binding is essentially stoichiometric. Binding is clearly linear with

TABLE 3

*Hyper- (hypo-)chromicity of polynucleotides binding to G32P*I under low salt (stoichiometric) binding conditions*

Polynucleotide	ΔO.D.$_{260}$ (%)‡
Poly(dA)	+0·12 (+0·12)
Poly(rA)	+0·16 (+0·17)
Poly(dT)	−0·04 (−0·03)
Poly[r(A,C)]†	+0·05 (+0·07)

Measured in buffer C containing 0·1 M-NaCl.

† 0·83 mol A/mol total nucleotide.

‡ ΔO.D.$_{260}$ corresponds to the *fractional* increase (or decrease) in this parameter measured on stoichiometric protein binding. Values marked + correspond to hyperchromic changes; those marked − correspond to hypochromic changes. Figures in parentheses give the values for native gene *32* protein binding to the same polynucleotide lattices (see Newport *et al.*, 1980).

protein concentration until virtually all of the lattice has been saturated. From this experiment we calculate that the site size (n) for the G32P*I monomer is ~7·2 nucleotide residues. Studies with other polynucleotides of different base compositions yield values of n ranging from seven to eight nucleotide residues, close to those determined with the native protein (Kowalczykowski *et al.*, 1980*a*). Similar absorbance titrations can be used to obtain binding constants for G32P*I under conditions in which binding is not stoichiometric; i.e. at higher NaCl concentrations. These binding curves have been analyzed as described previously (Kowalczykowski *et al.*, 1980*a*).

Figure 4 shows a typical titration curve of this type, plotted as fraction lattice saturated *versus free* G32P*I concentration. The unbroken lines represent computer-generated binding curves, obtained as before by inserting various values of ω into equation (15) of McGhee & von Hippel (1974), while holding the product $K\omega$

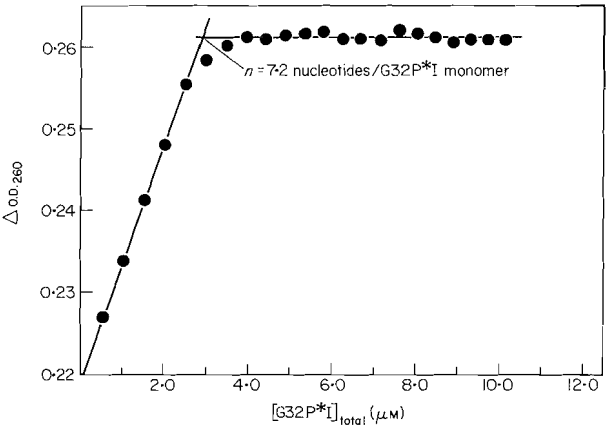

FIG. 3. Stoichiometric (tight-binding) u.v. titration of poly(rA) with G32P*I, in buffer C containing 0·2 M-NaCl, 25°C.

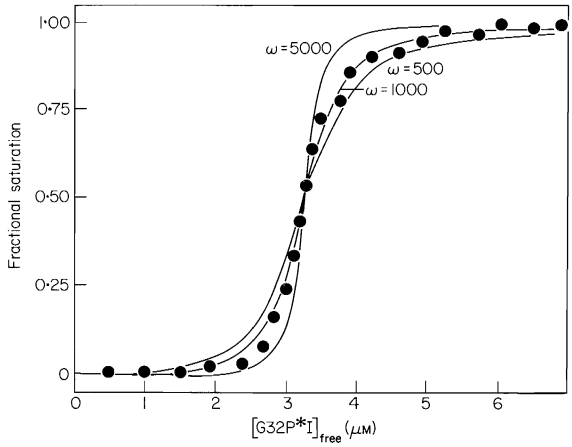

FIG. 4. Ultraviolet titration of poly(rA) (3×10^{-5} M) with G32P*I in buffer C containing 0·5 M-NaCl at 25°C. The points represent the experimental data; the lines correspond to theoretical curves for $n=8$ residues; $K\omega = 3{\cdot}1 \times 10^5$ M^{-1}; $\omega = 500$, 1000 and 5000, respectively.

at the value measured at half-saturation of the polynucleotide. The best fit was obtained with $\omega = 2 \times 10^3$. Values of $\omega < 10^3$ are clearly too small, and values of $\omega > 5 \times 10^3$ too large (Fig. 4). This best fit value of ω is (within experimental error) identical to that measured with native gene *32* protein (Kowalczykowski *et al.*, 1980*a*; Newport *et al.*, 1980).

In Figure 5 we plot values of log $K\omega$ *versus* log [NaCl] for the binding of various polynucleotides to G32P*I and to native gene *32* protein; the $K\omega$ values used were obtained as described above. It can be seen that the salt dependencies (i.e. slopes) and relative nucleotide specificities are similar for both proteins; however, G32P*I binds each polynucleotide approximately two to three times more tightly. Such

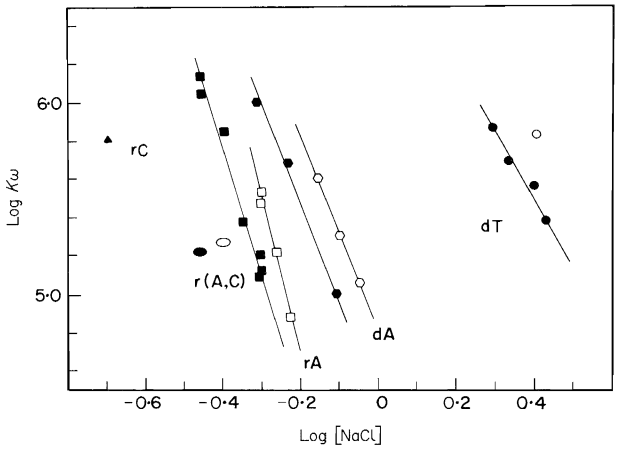

FIG. 5. Plot of log $K\omega$ *versus* log [NaCl] for G32P*I (open symbols) binding to various polynucleotide lattices in buffer C plus added NaCl at 25°C. Comparative gene *32* protein (filled symbols) binding data, in buffer B plus added NaCl; from Newport *et al.* (1980).

titrations were also conducted with polyribo ($1,N^6$-ethanoadenylic acid) (poly(rϵA)), and similar results were obtained: that is $K\omega$ for the binding of poly(rϵA) to G32P*I is approximately two- to threefold greater than $K\omega$ for the gene *32* protein–poly(rϵA) complex under the same conditions.

(c) *Interactions of G32P*III with short oligonucleotides*

Titrations of G32P*III with $d(pT)_3$, $d(pT)_6$ and $d(pT)_8$ were also performed by monitoring the quenching of intrinsic protein fluorescence. As observed with the native protein and G32P*I, the value of Q_m increases with chain length (Table 1). The salt dependence of G32P*III binding to the longer oligonucleotides, however, indicates that ionic interactions are present beyond those seen with the native protein or with G32P*I. Binding of $d(pT)_3$ by G32P*III is fairly salt-insensitive, while binding of $d(pT)_6$ and $d(pT)_8$ shows a salt dependence greater than that for G32P*I; if titrations are conducted in NaF rather than NaCl the log–log slope is slightly decreased and the net affinity somewhat increased (Table 2). This greater salt dependence of the longer oligonucleotides makes it difficult to ascertain whether statistical binding is occurring with G32P*III. In addition, G32P*III was found to bind to $d(pT)_8$ at greater than 1:1 stoichiometry under low salt conditions; this behavior was not observed with the native protein or with G32P*I, and is consistent with our finding of a somewhat smaller site size (n) for G32P*III binding to polynucleotides (see below). This complicates the determination of binding constants for these systems (see Draper & von Hippel, 1978).

(d) *Interactions of G32P*III with polynucleotides*

Polynucleotide–G32P*III interactions were investigated by the same techniques as used with G32P*I. While titration curves obtained with polynucleotide and either native gene *32* protein or G32P*I were found to be very similar to one another and characteristically co-operative, those obtained with G32P*III differed markedly. Titration curves of poly(dT) with G32P*III at various salt concentrations are shown in Figure 6; clearly, the strength of this interaction also decreases with increasing salt concentration, but here we see no sign of the sigmoidal behavior observed with the other protein species.

These titration curves were analyzed as previously described, and the unbroken lines in Figure 6 represent best fit theoretical curves. All titration curves could be fit using $\omega = 1$, indicating quantitatively that binding is non-co-operative. Furthermore, titrations carried out at different total protein concentrations showed no dependence of the apparent binding affinity on this variable, as expected for non-co-operative binding. The best fit site size for these curves corresponds to values of $n = 5{\cdot}5 \pm 0{\cdot}5$ nucleotide residues, a value marginally (but significantly) smaller than the value of about seven residues obtained for polynucleotide binding with the native protein and with G32P*I.

The results of titrations such as those illustrated by Figure 6, obtained with several different polynucleotides, are plotted in Figure 7 as log K (assuming $\omega = 1$) *versus* log [NaX], for experiments conducted in either NaCl or NaF. As before, the log–log plots for salt concentrations greater than $\sim 0{\cdot}1$ M are approximately linear. Figure 7 shows that in general the binding affinities of G32P*III to various

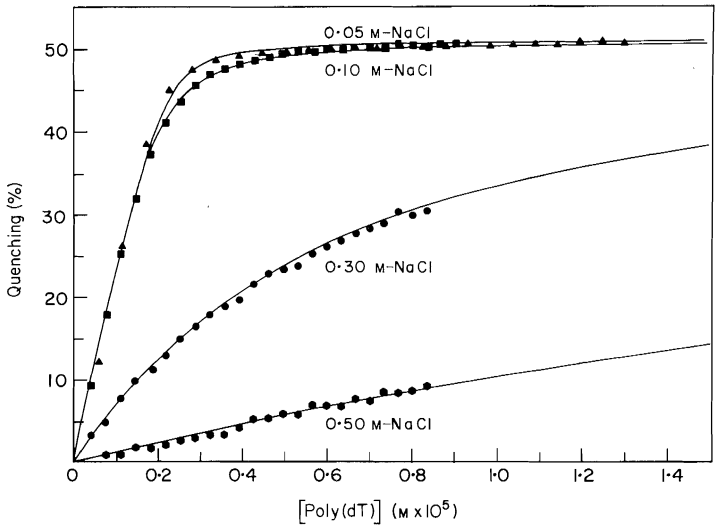

FIG. 6. Fluorescence titration curves for G32P*III with poly(dT) at the indicated concentrations of NaCl in buffer C at 25°C. G32P*III concentrations were $3{\cdot}4 \times 10^{-7}$ M in the 0·05 M-NaCl titration, and $2{\cdot}9 \times 10^{-7}$ M in the others. The points represent experimental data, and the lines the best fit theoretical curves. Parameters used in curve fitting were $n = 5$ residues; $\omega = 1$; and $K = 2{\cdot}5 \times 10^{7}$ M^{-1}, 1×10^{7} M^{-1}, $2{\cdot}3 \times 10^{5}$ and $2{\cdot}7 \times 10^{4}$ M^{-1}, respectively, for the curves from left to right. Q_m for all titrations was taken as 51%.

polynucleotides resemble those of the native protein, though in some cases the *differences* between polynucleotides are less pronounced (e.g. poly(rA) and poly(dA) appear to bind G32P*III with essentially equal affinity).

Figure 7 also shows that some of the log–log plots, particularly that for G32P*III with poly(dT), exhibit curvature at salt concentrations below ~0·1 M-Na^+. This is reminiscent of the data obtained with gene *32* protein and oligonucleotide lattices of intermediate size (Fig. 12 of Kowalczykowski *et al.*, 1980*a*), and may also indicate that G32P*III undergoes a conformational transition to a weaker or non-binding form at low salt concentrations.

The slopes of the log–log plots in Figure 7 are collected in Table 4, and show values of $\partial \log K/\partial \log [\text{salt}]$ which are considerably smaller than those obtained with gene *32* protein or with G32P*I. Data for G32P*III binding to poly(dT) were obtained in both NaCl and NaF; as before, the value of $\partial \log K/\partial \log [\text{salt}]$ was decreased in the latter salt, suggesting that anion release on binding contributes in part to the G32P*III–polynucleotide binding interaction for this system as well. This conclusion is consistent with preliminary titrations in $MgCl_2$, which suggest that a major portion of the salt dependence of the binding of G32P*III to polynucleotides does reflect anion effects (Table 4).

(e) *Deformation of the polynucleotide lattice on binding G32P*I and G32P*III*

Stoichiometric ultraviolet light absorbance titrations of polynucleotides with G32P*I have been described above (Fig. 3). We could not conduct such titrations

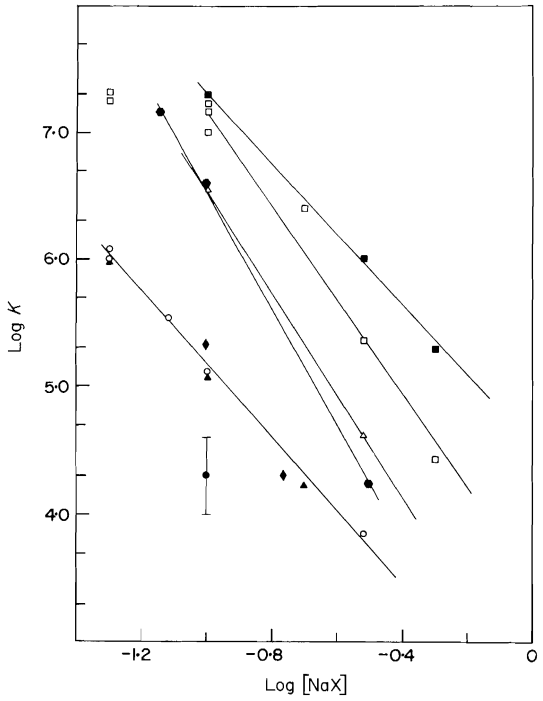

FIG. 7. Plot of log *K versus* log [salt] for G32P*III binding to various polynucleotides. All titrations were done in buffer C plus added NaCl (or NaF, as indicated) at 25°C. (□) poly(dT); (■) poly(dT) (NaF); (△) poly(dU); (○) poly(dA); (▲) poly(rA); (●) poly(rC); (◆) poly(rU); (⬣) poly(reA).

directly with G32P*III, because the net affinity of the protein for the polynucleotides is decreased for this protein (ω has decreased from 10^3 to 1), and thus saturating concentrations of protein are not easily attained. This difficulty is exacerbated with G32P*III because this protein tends to aggregate at high protein ($>10^{-5}$ M) or low salt (<75 mM-NaCl) concentrations. Thus we have measured difference spectra under less than saturating conditions, and *calculated* the values of ΔO.D.$_{260}$ expected at saturation.

Poly(rA) shows a hyperchromicity of ~12·3% at 260 nm on binding G32P*III at a lattice saturation of 65% (calculated from fluorescence quenching data). Assuming that the hyperchromicity measured is proportional to lattice saturation, on stoichiometric binding G32P*III should induce a hyperchromicity in poly(rA) of ~ +0·19; a value very close to that seen for gene *32* protein and G32P*I binding to this polynucleotide (Table 3).

Circular dichroism spectra were obtained on the same samples, and showed a marked reduction of the poly(rA) signal at 260 nm in the presence of G32P*III (Fig. 8). This difference also is similar to that seen with the native protein (Jensen *et al.*, 1976) and with G32P*I binding to poly(rA) (L. Paul, unpublished data), again taking into account the incomplete lattice saturation with G32P*III. We conclude that (within experimental error) all three proteins bring about comparable

TABLE 4

*Salt dependence of polynucleotide binding to G32P*III*

Polynucleotide	Salt	$(\partial \log K/\partial \log [\text{salt}])$
Poly(dA)	NaCl	$-2{\cdot}9 \pm 0{\cdot}4$
Poly(rA)	NaCl	$-2{\cdot}9 \pm 0{\cdot}4$
Poly(rεA)	NaCl	$-4{\cdot}8 \pm 1{\cdot}0$
Poly(dU)	NaCl	$-4{\cdot}0 \pm 0{\cdot}4$
Poly(rU)	NaCl	$-4{\cdot}1 \pm 1{\cdot}0$
Poly(dT)	NaCl	$-3{\cdot}8 \pm 0{\cdot}4$
Poly(dT)	NaF	$-2{\cdot}8 \pm 0{\cdot}3$
Poly(dT)	$MgCl_2$	-3†

Measured in buffer C containing added NaCl; data at [NaCl] < 0·1 M not included in the slope calculations (see the text).

† Estimated limiting slope as a function of $[MgCl_2]$ at 75 mM-Na^+ concentrations.

polynucleotide lattice deformation upon binding as judged by ultraviolet light and circular dichroism spectral differences.

4. Discussion

We have used the analytical methods employed in the preceding papers (Kowalcyzkowski *et al.*, 1980*a*; Newport *et al.*, 1980) to determine thermodynamic parameters for the interaction of nucleic acids with fragments of gene *32* protein produced by specific proteolysis of the native protein. Both fragments investigated retain the "core" of the original protein, and both show strong nucleic acid binding; however, the quantitative differences observed between the binding properties of the two fragments make it possible to extend the model of gene *32* protein and its interaction domains summarized in Figure 13 of Kowalczykowski *et al.* (1980*a*).

We have demonstrated that G32P*I, comprising the original protein shorn of its acidic ~6200 molecular weight COOH-terminal peptide (Hosoda & Moise, 1978; Williams & Konigsberg, 1978), retains essentially unchanged the thermodynamic and spectral interaction parameters of the native molecule. Thus the site size, the co-operativity, the lattice deformation properties and the *relative* polynucleotide binding affinities of G32P*I are all the same (within the limits of error of the measurements) as those of native gene *32* protein. The absolute binding affinities of G32P*I for polynucleotide lattices are increased two to threefold over those characteristic of gene *32* protein, and oligonucleotides of length comparable to the site size ($l=6$ to 8) bind more tightly (and with a larger salt concentration dependence) to G32P*I than to the native protein. These results are consistent with the interpretation that removal of the C-terminal peptide from gene *32* protein removes most or all of the "lump" or "flap" (see Fig. 13(a) of Kowalczykowski *et al.*, 1980*a*) that blocks the "electrostatic" sub-site of the nucleic acid binding site in the oligonucleotide binding conformation of the protein. The values obtained for the slopes of the (salt concentration dependence) log–log plots for the binding of $d(pT)_6$ and $d(pA)_8$ to G32P*I (Table 2), as well as the fact that the total increase in binding

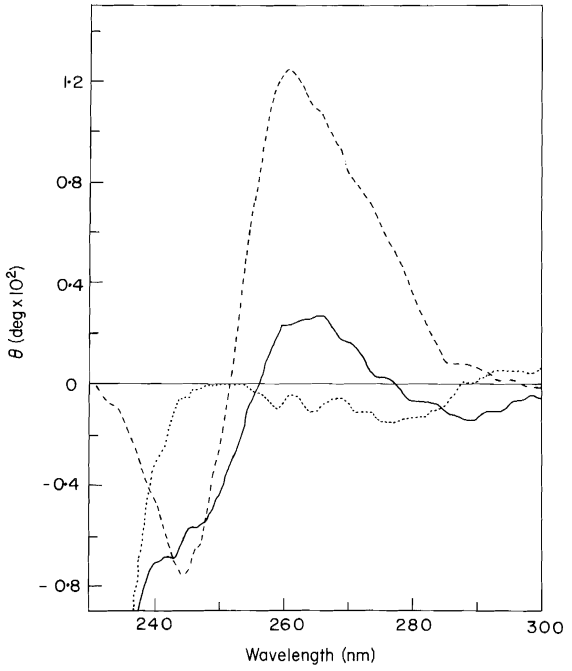

FIG. 8. Circular dichroism spectra of G32P*III (.....), poly(rA) (– – – –), and a mixture of the two (————). Spectra were taken in buffer C containing 0·1 M-NaCl at 23°(±2°)C. The poly(rA) concentration was $1{\cdot}2 \times 10^{-4}$ M (in nucleotide phosphates); the G32P*III concentration was $2{\cdot}5 \times 10^{-5}$ M.

affinity appears to be electrostatic in origin (Fig. 2), are also consistent with this interpretation of the molecular nature of the cleavage responsible for G32P*I generation.

This hypothesis, as well as the model of the oligonucleotide to polynucleotide conformational transition presented in Figure 13 of Kowalczykowski *et al.* (1980*a*), is also nicely supported by the observation reported by Williams & Konigsberg (1978) that the rate of proteolysis of gene *32* protein to form G32P*I is *enhanced* by binding of the protein to polynucleotides, but not by binding to short oligonucleotides.

The observation that the slopes of the *polynucleotide* binding affinity salt dependence log–log plots are unchanged from those characteristic of gene *32* protein suggests that *both* the oligonucleotide and the polynucleotide binding conformations are otherwise unaltered as a consequence of the removal of the C-terminal peptide from gene *32* protein and, in particular, that both the nature *and* the magnitude of the anion release accompanying this protein conformational transition are unchanged. The fact that the ultraviolet light and circular dichroism spectra of G32P*I binding to polynucleotides are virtually identical to those obtained with the native protein (see also Greve *et al.*, 1978) provide additional support for the view that the nucleic acid binding site (including the topographies and distances between the binding sub-sites) is unchanged by the G32P*I modification. These conclusions (as well as those concerning G32P*III, see below)

are also consistent with the results and interpretations presented by Williams *et al.* (1979) and Spicer *et al.* (1979), based on calorimetric and fluorometric measurements of the interaction of gene *32* protein and its proteolytic degradation products with polynucleotides.

The limited nature of the *thermodynamic* (and structural) changes in gene *32* protein binding properties that accompany G32P*I formation should not lead us to forget the major *kinetic* consequences of this modification. As shown by Hosoda & Moise (1978) and Greve *et al.* (1978), the kinetic block to the melting of natural DNAs characteristic of gene *32* protein (Jensen *et al.*, 1976) is relieved for G32P*I, though the rate of denaturation of natural DNAs by this moiety is still relatively slow (N. Lonberg, unpublished observations). As shown by Alberts & Frey (1970) and by Jensen *et al.* (1976), gene *32* protein cannot denature native DNA but can melt poly[d(A-T)]; Greve *et al.* (1978) and Hosoda & Moise (1978) have demonstrated that, in contrast, G32P*I can melt *both* native DNA and poly[d(A-T)]. The melting temperature of poly[d(A-T)] in the presence of G32P*I is $\sim 12°C$ below that for this polynucleotide in the presence of gene *32* protein under comparable conditions (Hosoda & Moise, 1978). We may calculate (see Jensen *et al.*, 1976) that this corresponds to an approximately twofold increase in the net binding affinity of G32P*I for single-stranded poly[d(A-T)], relative to that of the native protein for the same polynucleotide. This result is in good agreement with the differences in net binding affinity for the two proteins measured directly here. Since G32P*I destabilizes native T4 DNA by $\sim 70°C$ in 12 mM-NaCl (Hosoda & Moise, 1978), one would expect that the destabilization of native DNA by gene *32* protein would be $\sim 60°C$, just as predicted by the theoretical analysis presented by Jensen *et al.* (1976). Thus, the fact that gene *32* protein cannot melt native DNA is not due to thermodynamic limitations but must, instead, have a kinetic basis. The nature of this kinetic block and the mechanism of interaction of the native protein and G32P*I with nucleic acid lattices is currently being investigated using rapid reaction methods (work in progress).

The additional removal of the short and relatively basic (Hosoda & Moise, 1978; Williams & Konigsberg, 1978) peptide from the N-terminus of the native protein involved in G32P*III formation changes the binding properties of the protein much more drastically. Thus we have shown here that the co-operativity of binding of gene *32* protein to nucleic acid is entirely lost on the removal of this peptide. In addition, on G32P*III formation (from G32P*I), the polynucleotide binding site size (n) decreases by about one nucleotide residue, the binding affinities (K) for both oligo- and polynucleotides change slightly (Tables 1 and 2), and the salt dependencies of nucleic acid binding are somewhat modified.

As pointed out previously (von Hippel *et al.*, 1977; Kowalczykowski *et al.*, 1980*a*), protein binding co-operativity can arise from direct protein–protein interactions, from distortion of the nucleic acid lattice as a consequence of binding, or from a combination of both types of processes. Williams and Konigsberg (1978) showed that proteolytic attack on the N-terminal peptide of gene *32* protein (but not on the C-terminal peptide) is strongly inhibited by co-operative binding of the protein to single-stranded polynucleotides at stoichiometric binding ratios. This fact, coupled with our observation here that G32P*III brings about approximately the same

distortion of single-stranded nucleic acid lattices on binding as do G32P*I and the native protein, strongly implicate protein–protein interactions between contiguously bound gene *32* protein molecules as the dominant source of binding co-operativity (schematically represented as interlocking units connecting contiguous protein molecules in Fig. 13 of Kowalczykowski *et al.*, 1980*a*).

Finally, the physico-chemical consequences of removing the N-terminal and C-terminal portions of the gene *32* protein polypeptide chain elucidated here, as well as in the studies reported by Hosoda & Moise (1978), Greve *et al.* (1978), Williams & Konigsberg (1978), Williams *et al.* (1979) and Spicer *et al.* (1979), can be compared with studies *in vivo* of the phenotypic effects of mutations in these portions of the chain. Gold *et al.* (1976) and Breschkin & Mosig (1977*a*,*b*), using various amber and temperature-sensitive mutations of gene *32* protein (see summary discussion by Lemaire *et al.*, 1978), suggest that the amino-terminal end of the polypeptide is functionally the most important part of the protein. Mutations within the first (N-terminal) 60 to 100 amino residues of gene *32* protein interfere drastically with initiation of replication and recombination, perhaps by deranging interactions with T4 polymerase and other proteins of the T4 replication apparatus (as well as between gene *32* protein molecules as shown here). Mutations in the C-terminal regions of the chain have little effect on most aspects of biological function, though some modulating interactions of the protein with recombinational nucleases may be altered (Breschkin & Mosig, 1977*a*). These results are compatible with our finding that the removal of the C-terminal peptide from gene *32* protein alters its thermodynamic interactions with nucleic acids relatively little, while removal of the N-terminus destroys binding co-operativity and alters other thermodynamic parameters appreciably; perhaps (at least in part) the *co-operative* binding of gene *32* protein to nucleic acids is central to its biological function.

Very recently, Hosoda *et al.* (1980) have examined the behavior of G32P*I and G32P*III in a T4 *in vitro* replication system. The results provide further detailed information on the role of the terminal domains of the gene *32* protein molecule in interactions with various components of the replication complex. Further integration of physico-chemical, genetic and replication *in vitro* studies should soon lead to a fairly complete understanding of the molecular roles played by the various structural domains of this key protein in the function and control of the T4 DNA replication system.

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