

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

I. Characterization of the Binding Interactions

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(Received 8 April 1980, and in revised form 2 August 1980)

In this paper we examine molecular details of the interaction of bacteriophage T4-coded gene 32 protein with oligo- and polynucleotides. It is shown that the binding affinity (K_{oligo}) of oligonucleotides of length (l) from two to eight nucleotide residues for gene 32 protein is essentially independent of base composition or sugar type. This binding also shows little dependence on salt concentration and on oligonucleotide length; even the expected statistical length factor in K_{oligo} is not observed, suggesting that binding occurs at the end of the oligonucleotide lattice and that the oligonucleotide is not free to move across the binding site. Co-operative (contiguous) or isolated binding of gene 32 protein to polynucleotides is very different; here binding is highly salt dependent ($\partial \log K\omega / \partial \log [\text{NaCl}] \simeq -7$) and essentially stoichiometric at salt concentrations less than ~ 0.2 M (for poly(rA)). Binding becomes much weaker and the binding isotherms appear typically co-operative (sigmoid) in protein concentration at higher salt concentrations. We demonstrate, by fitting the co-operative binding isotherms to theoretical plots at various salt concentrations and also by measuring binding at very low protein binding density (ν), that the entire salt dependence of $K\omega$ is in the intrinsic binding constant (K); the co-operativity parameter (ω) is essentially independent of salt concentration. Furthermore, by determining titration curves in the presence of salts containing a series of different anions and cations, it is shown that the major part of the salt dependence of the gene 32 protein–polynucleotide interaction is due to *anion* (rather than to cation) displacement effects. Binding parameters of oligonucleotides of length sufficient to bind two or more gene 32 protein monomers show behavior intermediate between the oligonucleotide and the polynucleotide binding modes. These different binding modes probably reflect different conformations of the protein; the results are analyzed to produce a preliminary molecular model of the interactions of gene 32 protein with nucleic acids in its different binding modes.

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1. Introduction

The protein encoded by gene 32 of the bacteriophage T4 plays an essential role in the life-cycle of this phage; amber or temperature-sensitive mutations in gene 32 severely inhibit, or prevent altogether, the successful completion of DNA replication, recombination and repair processes (Epstein *et al.*, 1963; Tomizawa *et al.*, 1966; Bernstein, 1968; Berger *et al.*, 1969; Riva *et al.*, 1970; Wu & Yeh, 1973). The function of the protein depends on its tight and co-operative binding to single-stranded DNA sequences formed as intermediates in the above processes. Binding appears to be stoichiometric and complete to these transient single-stranded regions, and is thought to serve (at a minimum) to: (1) protect these sequences from the single-strand-specific nucleases of the cell; (2) remove (weak) double-stranded hairpin loops from these structures; (3) facilitate the utilization of these sequences by T4-specific enzymes and enzyme complexes in carrying out the orderly progression of molecular events involved in phage development.

Recently this situation has become more complex. It has now been demonstrated unambiguously (Krisch *et al.*, 1974; Gold *et al.*, 1976; Russel *et al.*, 1976; Lemaire *et al.*, 1978), using approaches both *in vivo* and *in vitro*, that gene 32 protein titrates the single-stranded DNA structures present in the cell at various parts of the phage life-cycle, and autogenously regulates its own (free) intracellular concentration *via* translational control of synthesis. These workers have shown that this process involves titration of the available single-stranded DNA sites, followed by binding of the protein to its own (stable) messenger RNA in a way that shuts down the translation of this message, but does not affect the utilization of other T4 mRNAs. Furthermore, this shutdown is reversible and appears to be a function of the intracellular gene 32 protein concentration. As required to establish the reality of such an autogenous control system, Lemaire *et al.* (1978) have shown by means of elegant translation studies *in vitro* that when the concentration of single-stranded DNA rises to a critical level (presumably thereby drawing off the excess free protein) the system is derepressed and more gene 32 protein is synthesized. Clearly, a complex balance of net affinities of the protein for DNA and for at least some sequences of RNA is involved, and in this and the accompanying papers (Newport *et al.*, 1980; Lonberg *et al.*, 1980) we report measurements designed to provide further molecular (and functional) insight into the nature of the various gene 32 protein-nucleic acid complexes involved.

In earlier studies from this laboratory it was shown that the binding of gene 32 protein to nucleic acids results in a partial quenching of the intrinsic tryptophan fluorescence of the protein (Kelly & von Hippel, 1976; see also Hélène *et al.*, 1976). This quenching was used to measure the binding constants of oligonucleotides of varying sugar and base composition and residue length to gene 32 protein (Kelly *et al.*, 1976). Binding parameters were also measured by studying changes due to gene 32 protein binding in the optical and melting properties of certain single and double-stranded polynucleotides (Jensen *et al.*, 1976; McGhee, 1976). These studies established the following facts relevant to the nucleic acid binding affinity and specificity of gene 32 protein. (1) The protein binds various short oligonucleotides (1 to 8 residues in length) with an affinity that can be measured by intrinsic tryptophan fluorescence quenching. (2) The maximum quenching obtained depends on the

properties of the oligonucleotides, varying from 2 to 3% for mononucleotides up to ~30 to 40% for large oligo- and polynucleotides. (3) The magnitude of the observed quenching also depends on the presence or absence of terminal phosphate groups; short oligonucleotides quench very differently, depending on whether they carry 3' or 5'-phosphate termini. This demonstrated that binding of the protein to the nucleic acid lattice is *polar*. (4) The apparent binding constants for oligonucleotides, ranging from two to eight nucleotide residues in length, are approximately constant at a value of $K_{\text{oligo}} \simeq 10^5 \text{ M}^{-1}$ in $\sim 0.1 \text{ M-NaCl}$. This suggested that the primary binding site on gene 32 protein is two nucleotides in length, but showed also that the statistical effect on the apparent binding constant (i.e. a trinucleotide should be able to bind in two different ways, a tetranucleotide in three different ways, etc., and thus should increase K_{oligo} proportionately) appeared to be absent, or to be offset by unfavourable binding interactions that increase in magnitude with increasing oligonucleotide chain length. (5) Within the limits of error of the absolute measurements made, the binding affinity to short oligonucleotides was estimated to be *virtually independent* of sugar or base composition. (6) Binding measurements made with polynucleotides showed that the site size for binding of gene 32 protein to nucleic acids is $\sim 7 \pm 1$ nucleotide residues, that binding is co-operative in protein concentration ($\omega \simeq 10^3$), and that $K\omega \simeq 10^8$ to 10^9 M^{-1} (in $\sim 0.01 \text{ M-NaCl}$). Crude estimates from titration curves suggested the co-operative binding affinities of gene 32 protein for poly(dA) and poly(rA) to be of comparable magnitude, in apparent accord with point (5) above; thus it appeared that ω and K might have similar values for the binding of the protein to DNA and to RNA. (7) The observation reported by Alberts & Frey (1970), that native double-strained DNA could not be melted by gene 32 protein, was confirmed. Since under the same conditions poly[d(A-T)] *could* be melted to equilibrium, we concluded that the block to native DNA melting is kinetic, rather than thermodynamic.

Some of these results seemed inconsistent with the findings of Gold and co-workers on the autogenous regulation of this protein, since their findings appeared to require that the *physiologically effective* binding affinities of the protein decrease in the following order: single-stranded DNA > gene 32 protein mRNA > other mRNAs (Lemaire *et al.*, 1978). Bobst & Pan (1975), *via* competition measurements with spin-labelled polynucleotides, had also shown some apparent binding specificity at the polynucleotide level. We therefore undertook a further examination of the system. The results presented here permit the development of a much more detailed molecular picture of the interactions of this genome regulatory protein, and also show that the autogenous regulation of gene 32 protein synthesis can be completely explained and interpreted on the basis of measured parameters and simple physico-chemical principles. In this paper we consider the interaction of the protein with oligo- and polynucleotides, in order to further elucidate the nature of the binding interaction, the binding site(s) on the protein, and the molecular properties of the binding co-operativity. In the second paper (Newport *et al.*, 1980), we examine the specificity of the binding of gene 32 protein to various synthetic and natural polynucleotides, and show how the very small differences in absolute binding affinity described in this paper are increased and modulated in the polynucleotide binding mode, and then further amplified into a control system of remarkable

specificity as a consequence of binding co-operativity. In the third paper (Lonberg *et al.*, 1980), we present data on nucleic acid binding to specific gene 32 protein fragments produced by controlled proteolytic degradation. These results permit us to begin to define the roles played by certain domains of the protein in binding affinity, co-operativity and specificity.

2. Materials and Methods

(a) *Chemicals and buffers*

All chemicals used were reagent grade, and all solutions were made with $2 \times$ glass-distilled water. Three general classes of buffers were used: buffer A (50 mM- Na_2HPO_4 , 1 mM- Na_2EDTA , 1 mM- β -mercaptoethanol, pH 7.7); buffer B (1 mM- Na_2HPO_4 , 0.1 mM- Na_2EDTA , pH 7.7); buffer C (10 mM-HEPES, 10 mM- NaCl , 0.1 mM- Na_2EDTA , pH 7.7). NaCl (and other salts) were added to these buffers as required to adjust the salt concentrations.

(b) *Oligonucleotides*

All oligonucleotides were purchased from either P-L Biochemicals or Collaborative Research, and were used interchangeably with no detectable differences. When necessary, the commercial preparations were checked for purity and/or size heterogeneity by thin-layer chromatography. Concentrations were determined spectrophotometrically, using the extinction coefficients tabulated by Kelly & von Hippel (1976). Extinction coefficients for other oligonucleotides were obtained using these data and the "additive" method of calculation described by Fasman (1975).

(c) *Polynucleotides*

All polynucleotides (except poly(rA)), were purchased from Miles Biochemicals, and were used without further purification. Poly-1, N^6 -ethanoadenylic acid (poly(rA)) was obtained from P-L Biochemicals and fractionated on a Sepharose CL-4B-200 (Sigma) column before use. Concentrations were determined using the following extinction coefficients (per mol phosphate) at $\sim 25^\circ\text{C}$ in ~ 0.1 M- NaCl : poly(dA), $10.0 \times 10^3 \text{ m}^{-1} \text{ cm}^{-1}$ at 257 nm; poly(rA), $10.3 \times 10^3 \text{ m}^{-1} \text{ cm}^{-1}$ at 260 nm; poly(dT), $8.1 \times 10^3 \text{ m}^{-1} \text{ cm}^{-1}$ at 260 nm; poly(dC), $7.2 \times 10^3 \text{ m}^{-1} \text{ cm}^{-1}$ at 270 nm; poly(rC), $6.5 \times 10^3 \text{ m}^{-1} \text{ cm}^{-1}$ at 267 nm; poly(rU) (and poly(dU)), $9.2 \times 10^3 \text{ m}^{-1} \text{ cm}^{-1}$ at 260 nm; poly(dI), $9.4 \times 10^3 \text{ m}^{-1} \text{ cm}^{-1}$ at 260 nm; poly[d(A-T)], $6.65 \times 10^3 \text{ m}^{-1} \text{ cm}^{-1}$ at 262 nm; and poly(rA), $5.11 \times 10^3 \text{ m}^{-1} \text{ cm}^{-1}$ at 260 nm.

(d) *Preparation of gene 32 protein*

Gene 32 protein was prepared from *Escherichia coli* B grown in a 23-l fermenter at 37°C to a final density of $\sim 4 \times 10^9$ cells/ml. In order to grow log phase cells to this density in a 23-l fermenter it was necessary to use very rich growth medium (Super Luria broth), and to aerate the fermenter with pure O_2 at ~ 12 l/min. These cells were then infected (at a multiplicity of infection of 5) with a T4 mutant phage which overproduces gene 32 protein (T4 33⁻, 55⁻, 61⁻). The cells were harvested by centrifugation 1 h after infection. These conditions typically yielded 500 g infected cells, from which we isolated ~ 500 mg of pure gene 32 protein. Protein purification was carried out according to the procedure of Alberts & Frey (1970), as modified by Jensen *et al.* (1976). Protein prepared by these procedures was $>95\%$ pure by polyacrylamide gel electrophoretic analysis, and was free of ribonucleases. These preparations did occasionally contain a DNase contaminant which, however, was inactive in our usual EDTA-containing (no divalent cations) buffers. When necessary, this contaminant was removed by the procedure of Bittner *et al.* (1979). We are very grateful to Dr Larry Gold and his co-workers for providing both the cell growth procedure and the T4 phage mutants used in this protein preparation.

(e) *Fluorescence spectra and titrations*

Fluorescence spectra and fluorescence titration curves were obtained using the computer-controlled Schoeffel Instrument Corp. spectrofluorimeter previously described by Kelly & von Hippel (1976). For some titrations the fluorimeter-computer system was modified by the addition of a computer-controlled syringe apparatus. With this modification, titration data were collected automatically by programming the computer to deliver a prescribed volume of titrant (as small as 1 μ l), stir the solution, stop, wait a fixed time for equilibrium, average the readings of the sample and reference photometers 1000 to 10,000 times to minimize noise, and then repeat the sequence until 20 to 50 data points had been obtained. These data were typically accurate to $\sim \pm 0.5\%$. Sample volumes were generally 1.2 to 1.6 ml. For studies utilizing the quenching of intrinsic protein fluorescence, an excitation wavelength of 290 nm was used with an emission wavelength of 340 nm. Titrations in which we monitored the enhancement of the fluorescence of poly(r₂A) as a consequence of gene 32 protein binding were conducted similarly, except that an excitation wavelength of 309 nm was used and emission was monitored at 406 nm. (A 405 nm band pass filter was added to the emission monochromator in these latter experiments.)

Titrations with oligonucleotides were typically performed by adding the nucleic acid component (*via* a syringe) to a fixed amount of protein in the cuvette. Inner filter effects were avoided by working at total final nucleotide concentrations designed to maintain the A_{290} of the system below 0.05. Poly(r₂A) experiments were conducted by using the syringe system to add portions of protein to a fixed amount of nucleic acid.

Some polynucleotide binding determinations were also carried out by means of a "salt titration" procedure, in which measured portions of concentrated salt solutions were added to bring about progressive salt-induced dissociation of a preformed gene 32 protein-nucleic acid complex. The experimental procedures were similar to those described above, with binding monitored by following the increase in intrinsic protein fluorescence accompanying dissociation of complex (see Fig. 5).

(f) *Ultraviolet absorbance titrations*

Titrations of gene 32 protein with nucleic acids, in which reactions were followed *via* changes in the apparent optical density of the system as a consequence of protein-nucleic acid complex formation, were performed using either a Cary 16 or a Cary 14 u.v.† spectrophotometer interfaced to a Varian 6201 computer.

(g) *Determination of binding parameters by computer-fitting of theoretical binding isotherms to the experimental binding data*

The protein binding (titration) data obtained by monitoring either fluorescence or absorbance changes have been analyzed by fitting them to theoretical curves generated using eqn (15) of McGhee & von Hippel (1974) (see also correction, McGhee & von Hippel, 1976). This equation (which applies to nucleic acid lattices of infinite length) takes into account both overlap of potential protein binding sites and protein binding co-operativity. Best fits were obtained by matching the theoretical curves to the experimental data, though some deviations in the fit to the high binding density (ν) part of the titration curves have to be taken into account when using data obtained with shorter polynucleotide lattices for which the infinite lattice approximation starts to break down (Newport *et al.*, 1980, and unpublished results). In these calculations the raw data were corrected for dilution and for the contribution of the protein to the observed fluorescence or optical density; generally titration curves were plotted as fraction lattice covered (θ) *versus* total protein concentration added.

Theoretical titration curves were generated by inserting the experimentally determined values of n (protein binding site size; ~ 7 nucleotide residues/gene 32 protein monomer; Jensen *et al.*, 1976), and test values of K (the intrinsic association constant, in units of M^{-1}) and ω (the protein co-operativity parameter, unitless; see McGhee & von Hippel, 1974) into

† Abbreviation used: u.v., ultraviolet light.

the above equation with the constraint that the product ($K\omega$) be held equal to the experimentally observed value. Values of L_F (the free ligand concentration) were then calculated at constant increments of ν (binding density, in mol protein bound/mol nucleotide phosphate) ranging from 0 to $1/n$. The amount of protein bound to the polynucleotide (L_B) lattice is added to these calculated values of L_F to obtain the total ligand concentration (L_T). These calculations were performed on a Varian 620i computer, and the results plotted using a Tektronix 4662 digital plotter. Theoretical curves were plotted directly over the experimental data using this arrangement, and best fits were determined by visual inspection.

(h) *Measurements of protein binding constants at low protein binding density using DNA-cellulose columns*

deHaseth *et al.* (1977a) devised a procedure for measuring protein-nucleic acid association constants by measuring the retardation of the protein in a DNA-cellulose column. This procedure was modified to measure binding constants at very low binding densities (ν), in order to permit the determination of K for this co-operatively binding protein system under conditions where virtually all the protein is bound at isolated sites on the DNA lattice.

In these experiments we used small columns containing single-stranded calf thymus DNA-cellulose prepared as described by Jensen *et al.* (1976). Experiments were performed by loading 0.5 ml portions (1.6×10^{-6} M in gene 32 protein in buffer C plus various levels of added NaCl) onto 0.6 ml columns of single-stranded DNA-cellulose previously equilibrated with the same buffer. The column was eluted with the same buffer in each case, and 0.5 ml fractions were collected. The free protein concentration in the first few fractions was assayed on the Hitachi spectrofluorimeter (Jensen & von Hippel, 1976) using the intrinsic protein fluorescence at 340 nm (excitation wavelength = 290 nm) calibrated relative to portions containing 1.6×10^{-6} M-protein concentration in the same buffer and salt concentration. After each set of experiments had been completed, the concentration of (protein-accessible) DNA on the column was determined spectrophotometrically by digesting the column packing with DNase I at 37°C for 3 h in 10 ml of 10 mM-MgCl₂, 20 mM-Tris, 100 mM-NaCl (pH 8.1).

3. Results

(a) *Binding of short oligonucleotides to gene 32 protein*

Titration curves were carried out using automatic procedures and computer averaging as described in Materials and Methods. A typical titration curve (for d(pT)₈ in 0.1 M-NaCl⁻) is shown in Figure 1(a), and the Scatchard plot for this titration is shown in Figure 1(b). At these protein concentrations ($\sim 0.1 \mu\text{M}$) the protein exists almost entirely as monomers (see Carroll *et al.*, 1975; Jensen *et al.*, 1976). Binding constants (K) and maximum quenching parameters (Q_m ; defined as the maximum quenching, as a percentage, for the system extrapolated to infinite oligonucleotide concentration) were obtained as follows.

We initially assumed that each protein binds only one oligonucleotide, and that the total ligand concentration can be taken as approximately equal to the free ligand concentration (a valid assumption for $K < 10^6 \text{ M}^{-1}$ at these protein concentrations). The data were then fit to a linear Scatchard plot (e.g. see Fig. 1(b)) using initially an unweighted linear least-squares procedure, and then a procedure that weights each point in proportion to the amount of quenching obtained. The values of K and Q_m obtained from the slopes and intercepts of these plots by this procedure were then used to calculate a new free ligand concentration which, in turn, was used to recalculate (by means of a least-squares fit) new values of K and Q_m , etc. This iteration procedure was repeated until the difference between successive values of K

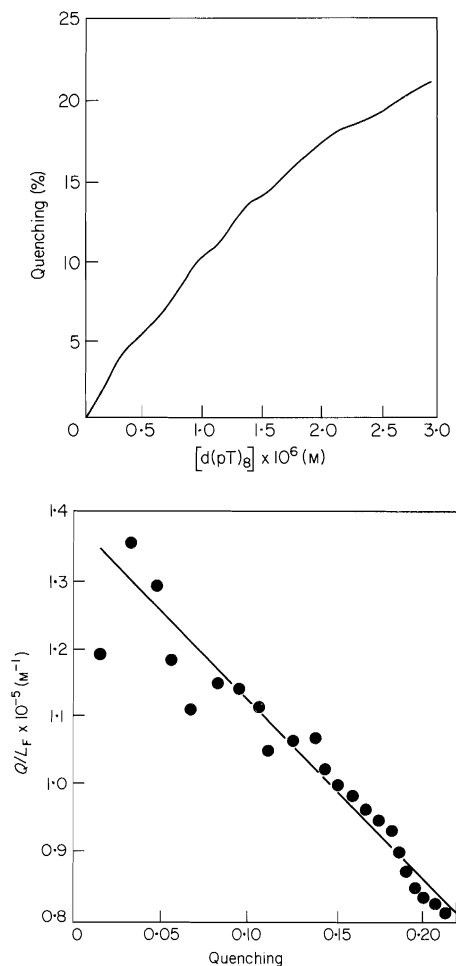


FIG. 1. (a) Fluorescence titration curve of gene 32 protein with $d(pT)_8$ in buffer A at 25°C; protein concentration = 2.6×10^{-7} M.

(b) Scatchard plot for the titration data of (a). Analysis as described in the text yielded a value of $Q_m = 53\%$ and $K_{oligo} = 2.6 \pm 0.1 \times 10^5 \text{ M}^{-1}$, with the first data point discarded (see the text).

was less than 1%. Typically, only two such iterations were required after the initial determination of K .

To minimize the effects of instrumental noise on the initial data points (which typically represent extents of quenching comparable to the average noise level), these points were eliminated from the fit one-by-one, until a minimum in the standard deviation was obtained. (Thereafter the standard deviation began to increase as “good” data points were eliminated.) The final (“best”) values of K and Q_m were typically derived from data sets from which several (~ 5) of the initial data points had been eliminated. The validity of this method of analysis was checked using both artificial (computer-generated) “data”, and experimental data for oligonucleotide–gene 32 protein complexes for which binding constants had been

established by other procedures. The results of such analyses for the various oligonucleotide-protein complexes investigated are presented in Table 1.

Table 1 shows that, for most oligonucleotides, Q_m increases somewhat with chain length. For oligonucleotides longer than $l=6$ this increase is dramatic; thus the $l=8$ oligonucleotides of Table 1, as well as longer oligonucleotides and polynucleotides (see below), quench much more than the short oligomers. This suggests that interactions not available to the short oligonucleotides become important in fluorescence quenching when the entire site size of the protein can be spanned (e.g. for $l=8$), or when binding becomes co-operative (for oligonucleotides with $l \geq 10$; see below).

TABLE 1
Association constants and quenching parameters for the binding of short (1=2 to 8) oligonucleotides to gene 32 protein

Oligonucleotide	Q_m (%)†	K_{oligo} (M^{-1})†
d(pT) ₂	9	$2.3(\pm 0.6) \times 10^5$
d(pT) ₃	15	$1.8(\pm 0.5) \times 10^5$
d(pT) ₄	12	$4.5(\pm 1.5) \times 10^5$
d(pT) ₆	16	$3.5(\pm 0.5) \times 10^5$
d(pT) ₈	57	$2.5(\pm 0.2) \times 10^5$
d(pA) ₂	13	$1.5(\pm 1.0) \times 10^5$
d(pA) ₄	5	$3.5(\pm 2.0) \times 10^5$
d(pA) ₈	28	$1.7(\pm 0.1) \times 10^5$
d(pG) ₂	12	$2.3(\pm 0.4) \times 10^5$
d(pG) ₄	26	$1.3(\pm 0.3) \times 10^5$
d(pG) ₈	44	$2.0(\pm 0.3) \times 10^5$
d(pC) ₂	8	$2.0(\pm 0.3) \times 10^5$
d(pC) ₄	18	$2.0(\pm 0.5) \times 10^5$
d(pC) ₈	33	$1.8(\pm 0.4) \times 10^5$
rC(pC) ₃	40	$5.7(\pm 2.0) \times 10^4$
rA(pA) ₃	20	$4.0(\pm 2.0) \times 10^4$
rA(pA) ₇	19	$5.5(\pm 2.0) \times 10^4$

Titration were performed in buffer A (Materials and Methods).

† The values reported for K_{oligo} and Q_m are average values based on 1 to 7 replicate titrations with each oligonucleotide. The standard errors (in parentheses) for K_{oligo} represent weighted averages, including both replicate titrations and the scatter of the points around the "best fit" Scatchard plots in individual titrations. Percentage standard errors in Q_m are comparable to, or slightly larger than, those listed for the associated value of K_{oligo} . The ribo-oligonucleotide values were obtained by interpolation of the data for the salt dependence of K_{oligo} (and Q_m) to 0.1 M-NaCl.

The results summarized in Table 1 show that there is very little base specificity in the binding of the oligonucleotides by gene 32 protein; certainly the differences are less than a factor of 2 in K . Perhaps, considering particularly the data of Table 1 for the 8-mers (for which Q_m is largest and the standard errors therefore smallest) a *marginal* decrease in K for the oligodeoxyribonucleotides in the order $d(pT)_8 > d(pG)_8 > d(pA)_8 \approx d(pC)_8$ can be detected. However, these differences are

very small, and do not approach those seen for binding to polynucleotides in the cooperative binding mode (see Newport *et al.*, 1980). The results with the oligoribonucleotides suggest that binding to gene 32 protein for these entities, as a group, is somewhat (3 to 6-fold) weaker than for the deoxyribose-containing analogues†.

The invariance of the apparent binding constant (Table 1) of the various oligonucleotides as a function of chain length confirms the previous finding (Kelly *et al.*, 1976) that a dinucleotide (actually a dinucleoside monophosphate) achieves the full level (in terms of free energy) of interaction attained by an oligonucleotide with a single gene 32 protein molecule, suggesting that the *interaction site* for binding in the oligonucleotide binding mode is quite short. In addition, the fact that the apparent binding constant does not even show the increase with chain length expected from purely statistical considerations suggests that the oligonucleotide lattice cannot bind equally to the protein *via* any dinucleotide sequence along its length. If the lattice were free to “shuffle” into any of the presumably equivalent binding positions, and if, as indicated above, the number of residues (m) that actually *interact* with the lattice is 2, then for a trinucleotide the apparent binding constant (K_{oligo}) should be twice that for a dinucleotide (K_m) and, in general: $K_{\text{oligo}} = (l - m + 1) K_m$ (see Kelly *et al.*, 1976). Table 1 shows quite clearly that K_{oligo} does not increase with l , indicating that the protein is *not* free to bind statistically to the oligonucleotide lattice.

We have also characterized the binding of these short oligonucleotides to gene 32 protein as a function of salt concentration, again using quenching of intrinsic protein fluorescence to monitor binding. (Q_m for any particular oligonucleotide lattice appears to be approximately independent of salt concentration.) The results of these measurements are presented in Figure 2 as plots of $\log K_{\text{oligo}}$ versus $\log [\text{NaCl}]$ (Record *et al.*, 1976); the slopes obtained from these plots are summarized in Table 2. Figure 2 shows that the various oligonucleotides tested exhibit a small but measurable linear dependence of $\log K_{\text{oligo}}$ on $\log [\text{NaCl}]$; the binding constant increases $\sim 30\%$ per tenfold decrease in salt concentration.

The slope of plots such as those of Figure 2 can be interpreted, using the approach of Record *et al.* (1976), to obtain an estimate of the number of ionic interactions involved in the binding process‡. Assuming no binding of oligonucleotide-displaceable anions to the protein, and no change in effective water activity (and

† We note that these oligoribonucleotides are not entirely analogous to the oligodeoxyribonucleotides tested, since the former lack a 5'-terminal phosphate. On the other hand (see Kelly *et al.*, 1976) we have previously shown that for $l \geq 2$, the presence of a terminal phosphate does not appear to affect K_{oligo} .

‡ It should be noted that this analysis is based on the interpretation of salt effects on protein-nucleic acid interactions as outlined by Record *et al.* (1976,1978), and that the assumptions underlying this approach underlie the interpretations made here as well. Most of these assumptions have been supported by calibration studies for the interaction of *double*-stranded nucleic acids with proteins and model ligands (Mg^{2+} , oligolysines, *lac* repressor; see Record *et al.*, 1976,1978; deHaseth *et al.*, 1977*b*; Revzin & von Hippel, 1977), and we assume here that this approach is equally applicable to *single*-stranded nucleic acid-protein interactions (using, of course, parameters such as ψ (see below) applicable to this system). As shown in this and the accompanying papers, the results obtained are often supported by (and consistent with) observations on the system that do not depend on the interpretation of salt effects. In addition, current studies on the salt dependence of binding of T4 DNA polymerase to single-stranded DNA appear to serve as partial “calibrating” support for such interpretations (Newport & von Hippel, unpublished results).

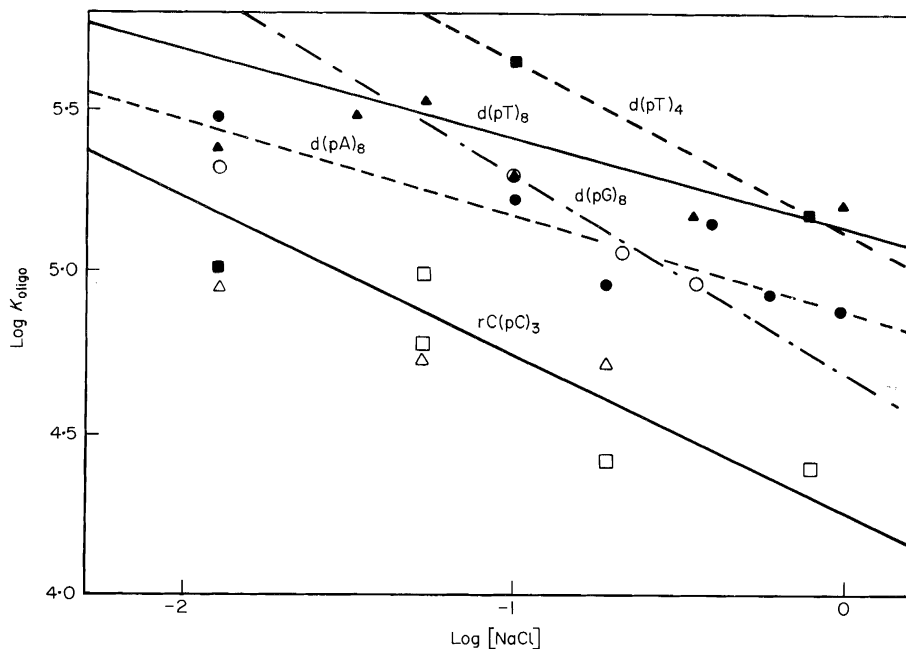


FIG. 2. $\text{Log } K_{\text{oligo}}$ versus $\text{log } [\text{NaCl}]$ for gene 32 protein binding to various oligonucleotides in buffer B plus added NaCl. The lines represent the linear least-squares best fit to the indicated experimental points (data obtained at $[\text{NaCl}] < 50 \text{ mM}$ were not used in the fit). (○) d(pG)_8 ; (●) d(pA)_8 ; (△) rA(pA)_7 ; (▲) d(pT)_8 ; (□) rC(pC)_3 ; (■) d(pT)_4 .

TABLE 2

Effect of varying salt concentration on the binding affinity of short oligonucleotides to gene 32 protein

Oligonucleotide	$(\partial \log K_{\text{oligo}} / \partial \log [\text{NaCl}])^\dagger$	ψ^\ddagger	ψ_{corr}^\S	m'
d(pA)_8	-0.28 ± 0.07	0.78	0.71	0.4 ± 0.1
d(pT)_8	-0.37 ± 0.02	0.69	0.60	0.60 ± 0.03
d(pC)_8	-0.20 ± 0.03	0.69	0.60	0.33 ± 0.05
d(pG)_8	-0.6 ± 0.3	0.78	0.71	0.9 ± 0.4

[†] Based on least-squares fit to data at salt concentrations $\geq 0.05 \text{ M-NaCl}$ (in buffer A).

[‡] Taken from Record *et al.* (1976); see the text.

[§] Corrected as per Record & Lohman (1978); see the text.

thus protein water of hydration) over the salt concentration range tested (see Record *et al.*, 1978), we may write (Record *et al.*, 1976):

$$\frac{\partial \log K_{\text{oligo}}}{\partial \log [\text{NaCl}]} = -m'\psi, \quad (1)$$

where the left-hand side of the equation is the slope of the log-log plot (Fig. 2), m' is the number of ionic interactions formed between nucleotide phosphates and basic

protein residues as a consequence of the interaction, and ψ is the (thermodynamic) fraction of the phosphate charge neutralized in the free nucleic acid by counterion condensation. The assumption of no anion effects for oligonucleotide binding is supported by experiments in which sodium salts with other anions were used in place of NaCl as the prime source of solvent counterions; in marked contrast to the polynucleotide binding situation (see below), changing anions had no effect on the slope of the log-log plots.

The (uncorrected) values of ψ listed for polynucleotides in Table 2 were obtained from Record *et al.* (1976); the poly(rA) value was used for all the purine-containing oligonucleotides and that for poly(rU) for all the pyrimidine-containing moieties. Corrections for the decreased counterion binding (relative to an infinite polynucleotide lattice of the same base composition) per unit lattice due to the decreased electrostatic potential at the ends of the oligonucleotide were made by the semi-empirical procedure of Record & Lohman (1978). The resulting (average) values of ψ^{corr} are also listed in Table 2, and were used to calculate $m'\dagger$.

Only data obtained at salt concentrations greater than 50 mM-NaCl were used to calculate m' ; at lower salt concentration (see especially Fig. 12) we find that K_{oligo} starts to *decrease* with decreasing salt concentration. This may reflect a salt-dependent conformational change to a non-nucleic acid binding form of the protein; similar observations involving decreased polynucleotide binding at low salt have been made by Jensen *et al.* (1976).

Table 2 shows that for short oligonucleotides m' falls between zero and unity. Since anion binding does not appear to contribute to the slope, we conclude that, at most, one ionic interaction is involved in complex formation between gene 32 protein and short oligonucleotides. This result, of course, is in good accord with reasoning based on the chain length dependence of the binding constant (see also Kelly *et al.*, 1976).

(b) Binding of gene 32 protein to polynucleotides

Previous studies of the co-operative binding of gene 32 protein to polynucleotides, primarily based on analysis of the perturbation of poly[d(A-T)] melting curves by this helix-destabilizing protein, had shown that the binding is co-operative in protein concentration, and is characterized by a co-operativity parameter (ω) of $\sim 10^3$. Under the conditions of the poly[d(A-T)] melting analyses (~ 0.01 M- Na^+), the effective co-operative binding constant ($K\omega$) for the protein to this nucleic acid lattice was found to be $\sim 10^9$ M $^{-1}$, suggesting that the polynucleotide binding interaction might, as a first approximation, be viewed simply as the product of the binding constant for a dinucleotide (such as d(ApA); $K_{\text{oligo}} \simeq 10^5$ M $^{-1}$) and the co-operativity parameter ($\omega \simeq 10^3$). The subsequent discovery of the very different salt

\dagger The values of m' obtained by this method may represent an underestimate of the true value, since the oligonucleotide values of ψ^{corr} used in Table 2 were obtained by Record & Lohman (1978) as *average* values for the entire oligonucleotide chain. The *ends* of the oligonucleotide lattice may bind *less* counterions than assumed here. This would result in a lower value for ψ^{corr} than that used here and, consequently, would result in a greater value of m' than reported. However, this should not be a large effect, and for most of the oligonucleotides listed in Table 2 should not invalidate our conclusion that m' falls between zero and unity.

dependencies of short oligonucleotide and polynucleotide binding to gene 32 protein (this paper), together with the enhanced dependence of the binding on the base and sugar-composition of the polynucleotide (Newport *et al.*, 1980), have shown that the co-operative polynucleotide binding interaction of this protein must differ appreciably from its mode of binding to short oligonucleotides, and has led us to examine the polynucleotide binding interaction in detail. In this paper the general features of co-operative binding to polynucleotides will be elucidated; in the following paper (Newport *et al.*, 1980) the nature and consequences of the different binding specificities to various polynucleotides will be considered.

Four different procedures were used to monitor binding; two (the u.v. and poly(rεA) fluorescence titrations) involve the monitoring of changes in nucleic acid spectra that arise as a consequence of the base unstacking that accompanies binding; and two (the protein fluorescence and salt titrations) follow the quenching of the intrinsic fluorescence of the protein as a consequence of nucleic acid binding. Each method has some advantages, and all have been used in a concerted way to measure binding parameters and the perturbation of these parameters by variations in salt concentration.

(c) *Ultraviolet light titrations*

In low salt (e.g. buffer B containing 10 mM-Na⁺), u.v. absorbance titrations of polynucleotides with gene 32 protein are essentially stoichiometric (i.e. virtually all the protein added is bound). Such experiments are useful for establishing site size (n), and confirmed for several polynucleotides (see Newport *et al.*, 1980) that n for gene 32 protein binding co-operatively is $\sim 7 \pm 1$ nucleotide residues. (A typical example of such a "stoichiometric" titration is shown at the left in Fig. 3.) Because binding under these conditions is very tight, such titration curves can only establish *minimum values* of $K\omega$ as calculated from component concentrations. Since these solutions contained $\sim 10 \mu\text{M}$ -polynucleotide binding sites, such experiments established only that the overall binding constant ($K\omega$) in 0.01 M-Na⁺, is $> 10^7 \text{ M}^{-1}$. Figure 3 shows, however, that this binding (for poly(rA)) is very salt sensitive, and that at higher salt concentrations binding becomes less tight so that binding parameters can, in principle, be measured. We note the typical "co-operative" (sigmoid) shape of the binding isotherms at higher salt concentrations (compare Fig. 7, McGhee & von Hippel, 1974), a lag phase at low protein concentration over which no binding is seen, followed by a typically abrupt rise in binding as the protein concentration exceeds the co-operative "threshold" value. Figure 3 shows that this threshold protein concentration increases markedly with increasing salt concentration; from $\sim 0.5 \mu\text{M}$ -gene 32 protein at 0.35 M-NaCl, to $\sim 6 \mu\text{M}$ -protein at 0.50 M-NaCl.

It has been shown elsewhere (e.g. see McGhee & von Hippel, 1974) that for co-operatively binding systems for which $\omega \gg n$, the value of the free ligand (protein) concentration at the midpoint of the titration curve is:

$$L_{F, 0.5} = 1/K\omega. \quad (2)$$

$L_{F, 0.5}$ is calculated by subtracting the amount of bound protein (based on $n = 7$ nucleotide residues) from the total added (remember the abscissa in plots such as

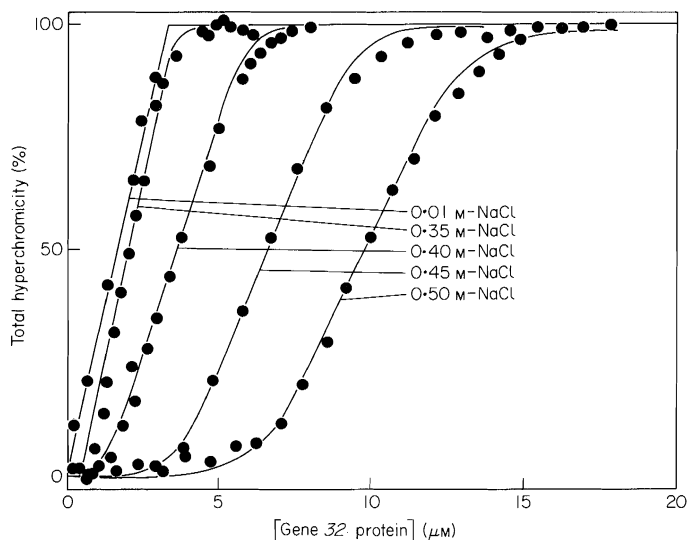


FIG. 3. Salt concentration dependence of the binding of gene 32 protein to poly(rA) in buffer B plus added NaCl, 25°C, measured by change in u.v. absorbance. The concentrations (mol phosphate) of poly(rA) were (from left to right) 2.46, 1.97, 3.58, 3.73 and 3.62×10^{-5} M. The unbroken lines (except for that labelled 0.01 M-NaCl, where binding was stoichiometric) are calculated theoretical curves using the following best fit parameters: $n = 7$ nucleotide residues, $K\omega =$ experimental values (see text) and $\omega = 2.0, 2.0, 2.5$ and 2.0×10^3 for the curves, from left to right, respectively.

Fig. 3 corresponds to *total* protein added). Values of $K\omega$ for poly(rA) calculated in this way using the data of Figure 3 are $1.7 \times 10^6 \text{ M}^{-1}$, $8.2 \times 10^5 \text{ M}^{-1}$, $2.5 \times 10^5 \text{ M}^{-1}$ and $1.6 \times 10^5 \text{ M}^{-1}$, for 0.35 M, 0.40 M, 0.45 M and 0.50 M-NaCl, respectively. This represents a substantial dependence of $K\omega$ on salt; it will be shown below (and by Newport *et al.*, 1980) that for this co-operative system a plot of $\log(K\omega)$ versus $\log[\text{NaCl}]$ is linear with a slope of ~ -6.6 . The curves connecting the data points of Figure 3 (and Fig. 4) represent best fit values of K and ω , calculated with the constraint that the *product* $K\omega$ is held constant at the value established at the midpoint of each titration (eqn (2)). The values of K and ω used in constructing the theoretical curves shown are indicated in the legend to each Figure.

Figure 4 shows data for the binding of gene 32 protein to poly(rA) in 0.5 M-NaCl for three different polynucleotide concentrations. This plot confirms that the binding co-operativity is indeed a function of protein, rather than polynucleotide concentration, since the protein concentration required to *reach* the co-operative threshold concentration is virtually independent of polynucleotide concentration. (The slopes of the rising parts of the titration curves do, of course, depend on polynucleotide concentration, since in this phase more *total* protein is required to reach the various titration levels because more is bound at the higher polynucleotide concentrations. As expected, the *same* curve is obtained for all three polynucleotide concentrations when the data are replotted as a function of *free* protein concentration; Fig. 4.)

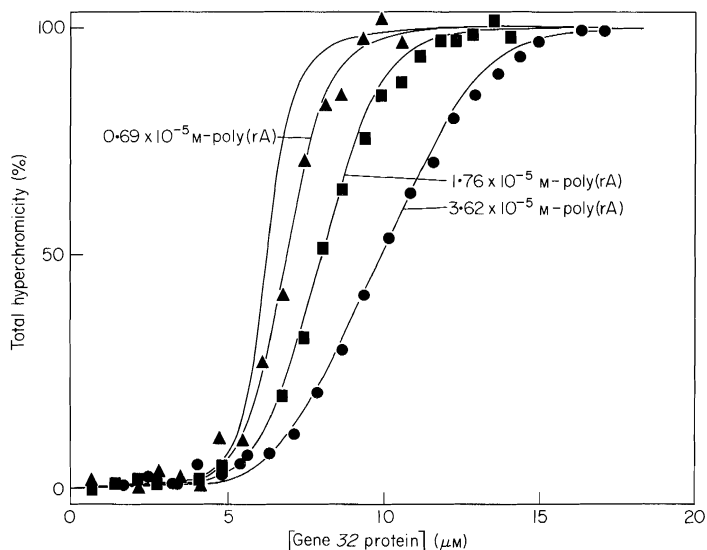


FIG. 4. Effect of increasing poly(rA) concentration on the binding of gene 32 protein in buffer B plus 0.5 M-NaCl, 25°C. The unbroken lines represent the calculated theoretical curves for $n=7$ nucleotide residues, $K\omega = 1.6 \times 10^5 \text{ M}^{-1}$, and $\omega = 1.5, 1.5, 2.0$ and 2.0×10^3 (left to right). The leftmost curve (with no data points) applies to $[\text{poly}(\text{rA})] = 0$; i.e. the expected curve if the x -axis were plotted as concentration of *free*, rather than total, protein for all three sets of data.

(d) Salt titrations

The u.v. absorbance titration procedure outlined above is optimally useful for titrations with polynucleotides (such as poly(rA)) for which base stacking in the uncomplexed form is appreciable. With many other polynucleotides the changes in u.v. absorbance spectra of the system are small, or virtually absent. A convenient way to explore binding (and its salt dependence) for such systems is by monitoring quenching of the intrinsic protein fluorescence as a function of salt concentration. We call this a "salt back-titration" procedure. The principle of this procedure is shown in Figure 5, using the poly(rU)-gene 32 protein interaction as an illustration. Here (extreme left of the Figure) a portion of gene 32 protein is added to a cuvette at low salt (buffer C), the initial (uncomplexed) intrinsic fluorescence of the protein is measured, and portions of poly(rU) are then added (under these low salt-tight binding conditions) until quenching of the protein fluorescence is complete. At this point we begin adding portions of concentrated (5 M) NaCl, resulting eventually in the restoration of the original free protein fluorescence after all of the complex has been dissociated. The salt concentration of the system at which *one-half* the original (uncomplexed) protein fluorescence has been restored represents the point at which one-half the protein is free ($L_{F, 0.5}$), and therefore (eqn (2)) provides a measure of $K\omega$ at this salt concentration.

Data such as those shown schematically for a single salt back-titration in Figure 5 can be represented as conventional binding isotherms as a function of salt concentration. A series of such isotherms, representing increasing concentrations of gene 32 protein (and therefore of poly(rU)), are shown in Figure 6(a). The midpoints

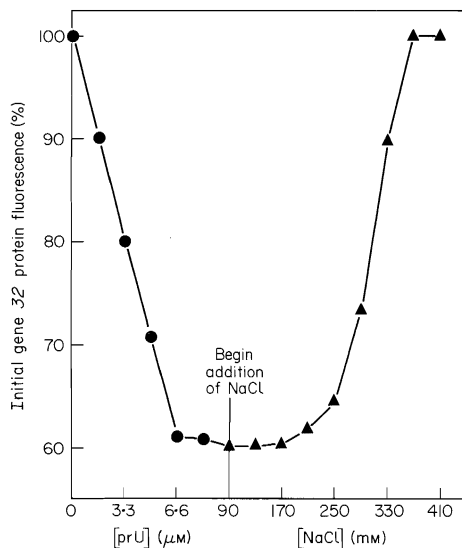


FIG. 5. Forward and reverse salt (fluorescence) titrations of gene 32 protein binding to poly(rU) (prU); initial volume of 1.2 ml containing 10 mM-HEPES (pH 7.8), 0.1 mM-EDTA, 50 mM-NaCl and 9.4×10^{-7} M-gene 32 protein. The first half of the plot shows the decrease in gene 32 protein fluorescence due to the addition of 2- μ l portions of poly(rU) (stock concn = 9.9×10^{-4} M); the second half shows the increase in fluorescence due to the addition of 10- μ l portions of 5 M-NaCl. All measurements have been corrected for dilution effects.

of the binding isotherms fall at progressively higher values of NaCl concentrations, corresponding to higher values of $L_{F, 0.5}$, and thus to lower values of $K\omega$.

Values of $K\omega$ were determined from the midpoints of the titrations of Figure 6(a), and are plotted as $\log K\omega$ versus $\log [\text{NaCl}]$ in Figure 6(b). As expected, a linear plot is obtained, again (as for the poly(rA) data cited above) showing a very large dependence of $K\omega$ on salt concentration. For poly(rU) (Fig. 6(b)) the slope ($\partial \log K\omega / \partial \log [\text{NaCl}]$) is -7.4 ± 0.5 . Thus these data show a very large decrease in $K\omega$ with increasing salt concentration, in marked contrast to the small decrease seen with short oligonucleotides (Table 2). The molecular bases of this salt dependence are considered further below.

(e) *Fluorescent titrations of polyribo (1,N⁶-ethanoadenylic acid)*

Ribo(1,N⁶-ethanoadenylic acid) (r ϵ A) was first synthesized by Secrist *et al.* (1972), and first incorporated into a polynucleotide (poly(r ϵ A)) by Steiner *et al.* (1973) and Janik *et al.* (1973). The extended ring system of the monomer results in a marked fluorescence of this compound in the near ultraviolet; this monomer fluorescence is largely quenched (~ 10 to 25-fold) and slightly blue-shifted in poly(r ϵ A), presumably as a consequence of base-stacking. Since binding of gene 32 protein to single-stranded polynucleotides extends the backbones and unstacks the bases (Delius *et al.*, 1972; Jensen *et al.*, 1976), we have investigated the fluorescent properties of poly(r ϵ A) in the presence and absence of gene 32 protein.

Figure 7(a) shows that when saturating amounts of gene 32 protein are added to

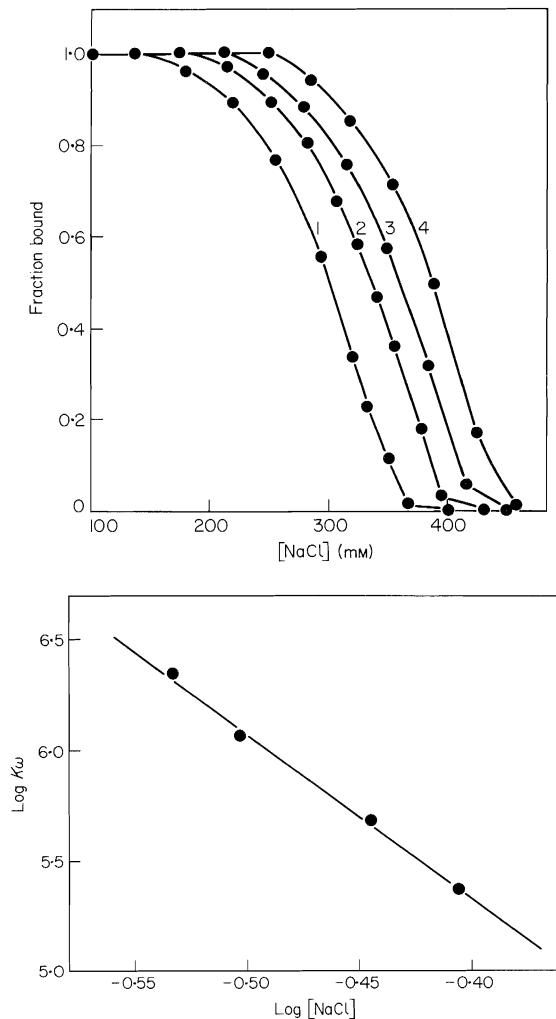


FIG. 6. (a) Poly(rU)-gene 32 protein salt back titrations at various gene 32 protein concentrations under the same conditions as for Fig. 5. The initial gene 32 protein concentrations were: curve 1, 9.3×10^{-7} ; curve 2, 1.8×10^{-6} ; curve 3, 4.4×10^{-6} ; curve 4, 9.1×10^{-6} M. The poly(rU) to gene 32 protein ratio equals 7 nucleotides/protein monomer.

(b) Effect of NaCl concentration on $K\omega$ for gene 32 protein binding to poly(rU), based on the data of (a). Each point was obtained by determining the amount of gene 32 protein free at the midpoint of the relevant salt back titration (see the text).

poly(rεA), the fluorescence of the polynucleotide is enhanced approximately twofold; in addition (Fig. 7(b)), the difference spectrum shows that the fluorescence maximum shifts from ~ 400 to ~ 405 nm. This increase in fluorescence of the analogue is similar to that induced by thermal or solvent denaturation of the polynucleotide (Steiner *et al.*, 1973; Lehrach & Scheit, 1973), indicating that gene 32 protein does bind to and unstack this polynucleotide.

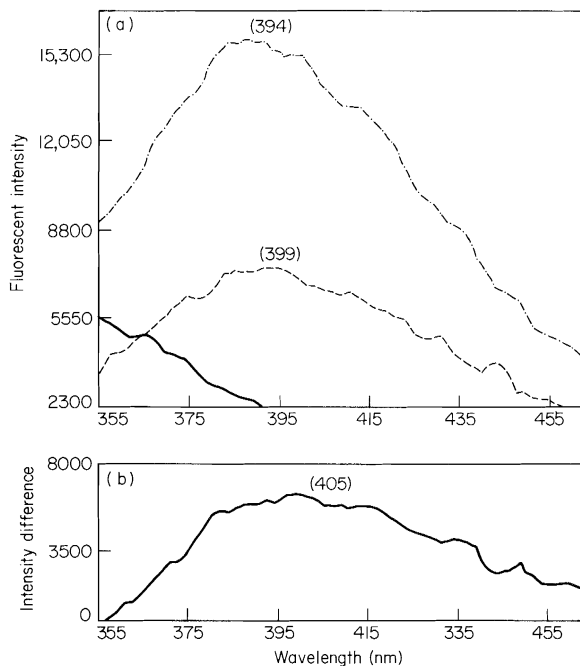


FIG. 7. (a) Fluorescence emission spectra of poly(rεA) alone (-----), gene 32 protein alone (————), and the gene 32 protein–poly(rεA) complex (— · — ·). The excitation wavelength was 308 nm and the concentrations of poly(rεA) and buffer A, 25°C. The wavelengths of maximum intensity are indicated on each spectrum. (See also Toulmé & Hélène, 1980.)

(b) Difference spectrum, calculated from the spectra of (a).

This change in polynucleotide fluorescence at 405 nm has been used to examine further and to quantitate the interaction of gene 32 protein with polynucleotides. Titrations were carried out at low salt (0.10 M), where binding is essentially stoichiometric; a fluorescence enhancement at 405 nm of ~twofold was obtained, and a site size (n) = 6 ± 1 nucleotide residues established (in good agreement with values of n obtained by other procedures; data not shown). At higher salt concentrations the titration curves of poly(rεA) with gene 32 protein have the same form as those obtained with other polynucleotides; i.e. there is a pronounced lag region, the length of which is very salt dependent, followed by a sharp increase in amount bound (e.g. see Figs 3 and 4).

Such poly(rεA) titration curves were used to determine the salt dependence of $K\omega$ for this polynucleotide. As pointed out above, the *free* protein concentration at the midpoint of the titration is equal to $1/K\omega$. Values of $K\omega$ determined by this means for gene 32 protein binding to poly(rεA) as a function of salt concentration are summarized in Table 3. Plotting these values as a log–log plot *versus* [NaCl], a straight line is obtained with a slope of -6.5 ± 0.5 ; very close to the measured value of $(\partial \log K\omega / \partial \log [\text{NaCl}])$ determined with poly(rA) (-6.6) and poly(rU) (-7.4). Such data were also used to determine the temperature dependence (between 18 and 38°C) of the binding of gene 32 protein to poly(rεA) at three different salt

concentrations; the standard enthalpy values calculated from these data at each salt concentration are also summarized in Table 3.

An average (assumed temperature-independent) value of $\Delta H^0 \simeq -22 \pm 2$ kcal/mol is obtained from these data; we have used this ΔH^0 value to calculate values (at 25°C) for ΔG^0 and ΔS^0 (Table 3). The results summarized in

TABLE 3
Thermodynamic parameters for the binding of poly(rεA) to gene 32 protein

[NaCl] (M)	$K\omega$ (M ⁻¹)†	ΔH^0 (kcal/mol)	ΔG^0 (kcal/mol)†	ΔS^0 (cal/mol °K)†
0.35	4.0×10^7	-25	-10.3	-41
0.40	1.4×10^7	-21	-9.8	-42
0.45	7.0×10^6	-22	-9.4	-44

Measured in buffer B plus the indicated concentrations of NaCl.

† Data for 25°C.

Table 3 show that the binding process is primarily *enthalpy* driven, i.e. electrostatic interactions (ion release), which result primarily in favorable *entropies* of binding (Record *et al.*, 1976), do not dominate the binding process. Since (see Newport *et al.*, 1980) the interaction of gene 32 protein with poly(rεA) is very similar to those of the other polynucleotides tested, we assume that these thermodynamic parameters are characteristic of the formation of gene 32 protein complexes with other polynucleotides as well.

(f) *Dependence of K and ω on salt concentration*

To this point we have focused on determinations of the *product* of K and ω , based on determinations of the midpoints of titration curves (eqn (2)). Of course, K and ω are *separable* parameters, and in attempting to define binding mechanisms it is important to see whether the strong salt dependence of the co-operative binding of gene 32 protein to polynucleotides resides in K or in ω . We have taken two approaches to this: one involves fitting the complete titration curves (e.g. Fig. 3 or 4) of various polynucleotides as a function of salt concentration to theoretical curves generated by systematically varying K and ω ; the other involves working at such low protein binding densities (ν) that the average size of the cluster of co-operatively bound proteins on the lattice is essentially unity (i.e. the proteins bind almost exclusively to *isolated* sites; see McGhee & von Hippel, 1974). Both approaches show unambiguously that virtually the entire salt dependence of $K\omega$ is in the intrinsic binding constant K .

(g) *Computer "fit" of theoretical binding isotherms to experimental titration data*

The closed analytical equation for the co-operative binding of large ligands to an infinite lattice (eqn (15) of McGhee & von Hippel, 1974) shows that at all binding densities in a titration curve (other than at the midpoint) K and ω are handled very differently and thus have different effects on the overall curve shape. The major

effect of increasing ω is to make the curves "sharper"; a value of $\omega = \infty$ transforms the titration into a step function. The major effect of increasing K is to shift the entire curve toward lower protein concentrations; i.e. to reduce the length of the lag phase of the titration (e.g. see McGhee & von Hippel, 1974; Fig. 7).

The procedure we have used for fitting theoretical titration curves to experimental data is outlined in Materials and Methods; a typical set of trial theoretical fits to a poly($r\epsilon A$) titration are shown in Figure 8. Clearly, the most discriminating

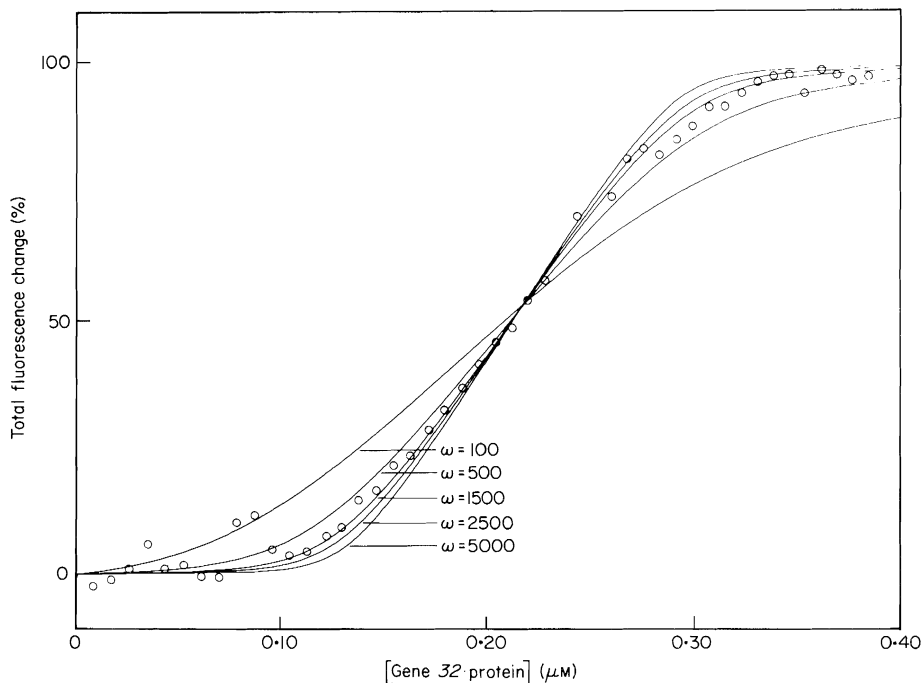


FIG. 8. Titration of poly($r\epsilon A$) (1.90×10^{-6} M) with gene 32 protein in buffer B with 0.45 M-NaCl at 25°C. The unbroken lines are theoretical curves generated for various values of ω (with $K\omega = 7.0 \times 10^6$ M $^{-1}$ and $n = 7$).

parts of the fit are in the curved regions at the foot and at the top of the steeply rising part of the curve. Since the fact that the nucleic acid lattices used are not of infinite length perturbs the fit most at the top of the titration curves (see Newport *et al.*, 1980, and unpublished calculations), we emphasize the fit to the foot region here. A typical set of best-fit parameters of ω and K as a function of salt concentration are collected in Table 4 for poly($r\epsilon A$); and more extensive data for other polynucleotides are summarized in Table 3 of the following paper (Newport *et al.*, 1980). The results are clear-cut; within the limits of error the salt dependence of $K\omega$ is all in K , and ω is essentially salt independent.

(h) Salt dependence of protein binding to isolated sites

By definition (McGhee & von Hippel, 1974) ω is the ratio of the probability that a ligand will bind to a contiguous site (i.e. extend a previously initiated protein cluster

TABLE 4
Temperature dependence of $K\omega$, K , and ω for poly($r\epsilon A$)

[NaCl] (M)	T (°C)	$K\omega$ ($M^{-1} \times 10^{-7}$)	ω range ($\times 10^{-3}$)	Best fit ω ($\times 10^{-3}$)	K ($M^{-1} \times 10^{-3}$)
0.35	25	4.0	2.0-10	4	10
	38	0.7	1.0-2.5	1.5	4.7
0.40	17	3.4	2.0-7	5	6.8
	25	1.4	2.5-5	4	3.5
	35	0.4	1.5-3	2	2.2
0.45	25	0.7	4.5-10	5	1.4
	37	0.2	0.5-2.5	1	1.7

Titration in buffer B, plus added NaCl.

on the lattice) to the probability that a ligand will bind to an isolated site (i.e. initiate a new protein cluster). At relatively high binding densities (ν), binding is predominantly in clusters; as ν is decreased the average cluster size will decrease, approaching unity. Using equation (15) of McGhee & von Hippel (1974), it can be shown that the limiting value of the free protein concentration (L_F) as $\nu \rightarrow 0$ is ν/K ; for practical purposes this limit is attained at $\nu\omega \leq 0.1$. Thus at $\nu > 0.1/\omega$, L_F will be a function of both K and ω ; at $\nu < 0.1/\omega$, L_F will be a function of K only. Therefore, in order to determine which component of $K\omega$ is changing as the salt concentration is being varied, one need only to monitor L_F (while keeping ν constant), as the salt concentration is changed. In the limit of $\nu \rightarrow 0$, one would expect L_F to vary linearly with K , if K is being affected by changes in salt concentration; at the other extreme, if only ω is being affected by alterations in salt concentrations, then *no* change in L_F would be observed as the salt concentration is varied.

The low values of ν required to perform such experiments with a protein characterized by a value of $\omega \simeq 10^3$ preclude the use of spectroscopic methods. On the other hand, chromatographic methods can be applied. To this end we have used small, single-strand DNA-cellulose columns under conditions where the concentration of DNA binding sites far exceeds the amount of bound protein, to maximize binding to isolated sites.

To carry out the necessary measurements, solutions of known total protein concentration at varying salt concentrations were added to the column, and after equilibration the protein was eluted by buffer of the *same* salt concentration in each case. Portions of approximately the same volume as the original column volume were collected and analyzed for protein as described in Materials and Methods. By extrapolation of these data the free protein concentration L_F of each original sample equilibrated with the column could be determined.

Values of L_F measured at various salt concentrations in a set of such experiments are presented in Figure 9. From previous data we know that either K or ω (or both in concert) decrease by six to seven orders of magnitude per tenfold increase in NaCl concentration. The theoretical lines in Figure 9 were obtained by fixing either K or ω at the value shown, and allowing the other variable to decrease by 6.3 orders of

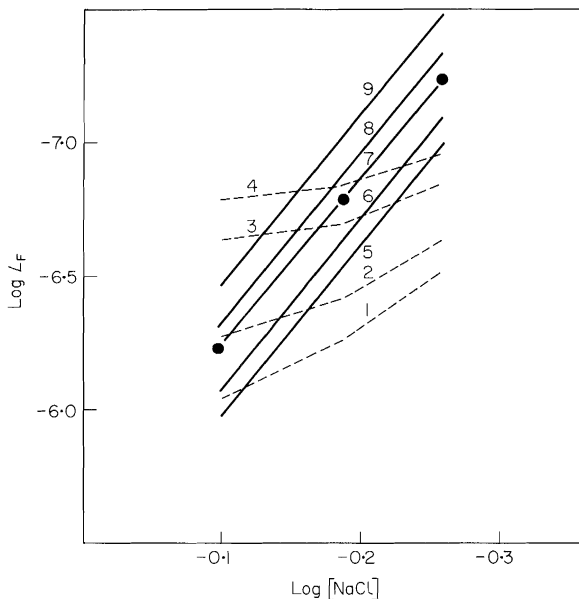


FIG. 9. Plot of "single-plate" DNA-cellulose column determinations of $\log L_F$ as a function of $\log [\text{NaCl}]$, $\sim 23^\circ\text{C}$. The points represent experimental measurements made at $\nu = 1.3 \times 10^{-4}$; the size of the points indicates the magnitude of the standard error. The 2 sets of theoretical lines represent values of L_F (i.e. $1/K\omega$) for fixed values of $K = 100, 200, 500$ and 740 m^{-1} with ω varying with salt (lines 1 through 4), and for fixed values of $\omega = 500, 750, 1000, 1500$ and 2000 with K varying with salt (lines 5 through 9). Experimental values of $K\omega$ were obtained from measurements made on gene 32 protein binding to ϕX174 DNA (see Fig. 4, Newport *et al.*, 1980).

magnitude per $\log [\text{NaCl}]$ over the indicated range of salt concentration. L_F for each salt concentration was then calculated from equation (15) of McGhee & von Hippel (1974) for the values of the *total* protein concentration used in the experiment; the lines for constant K and varying ω show some curvature because at the experimental protein concentration some protein molecules still bind at contiguous sites. Clearly, the measured values of $\log L_F$ show a straight-line dependence on $\log [\text{NaCl}]$ with a good fit to the theoretical line corresponding to a constant value of ω of $\sim 10^3$, while no fit is possible to the theoretical lines generated by varying ω at constant K . Thus these experiments also demonstrate that it is the intrinsic binding constant of the protein monomer to the polynucleotide lattice, and not the protein co-operativity parameter, which varies with salt concentration.

(i) *The salt dependence of K for the binding of gene 32 protein to polynucleotides depends on both cations and anions*

To this point we have used equation (1) to represent the salt dependence of the binding of gene 32 protein to nucleic acid lattices. This equation attributes the entire salt effect upon K to cation displacement; it is assumed that no anions subject to displacement are bound to the free protein. This assumption has been justified experimentally for the binding of Mg^{2+} , oligolysines and *lac* repressor to DNA (see Record *et al.*, 1976).

Based on a slope ($\partial \log K\omega / \partial \log [\text{NaCl}]$) of ~ -7 in the log-log plots (see above and Newport *et al.*, 1980), and assuming equation (1), we calculate a value of $m' = 10$ to 11 ionic interactions for each molecule of gene 32 protein bound to single-stranded polynucleotides. Since the binding site size (n) for gene 32 protein is only six to seven nucleotide residues in length, the above value of m' is obviously far too large, suggesting that anion binding (or perhaps changes in protein hydration) must also be involved. To determine what proportion of the salt dependence of gene 32 protein binding to polynucleotide is due to anion displacement, a series of titrations was carried out using sodium salts with different anions. In Figure 10 it can be seen that changing the anion has an effect on both the slope and position of

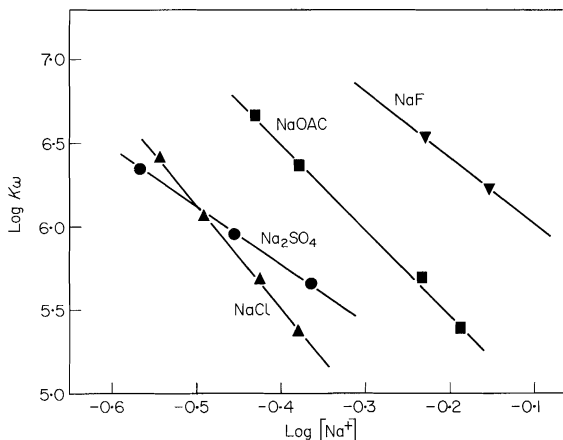


FIG. 10. Effects of various anions on $K\omega$ of gene 32 protein binding to poly(rA), determined by titrations conducted identically to those of Fig. 6(a) and (b). OAc, acetate.

the line defining $K\omega$ as a function of $[\text{Na}^+]$. This strongly suggests that anions are released from the protein when it binds to a polynucleotide.

Anions that bind with relatively higher affinity to proteins, as predicted by the Hofmeister series (see von Hippel & Schleich, 1969), have a larger effect on the binding of gene 32 protein to poly(rA) than do anions that are expected to be less tightly bound. Titrations with poly(rC) (not shown) at low salt concentration show the same change in slope that is observed with poly(rA). For salt concentrations ≥ 150 mM, the $\log K\omega$ versus \log [salt] plots are linear for all polynucleotides. At constant pH (following Record *et al.*, 1976), we may write:

$$-\frac{\partial \log K\omega}{\partial \log [\text{MX}]} = m'\psi + \frac{a K_A[\text{X}^-]}{1 + K_A[\text{X}^-]}, \quad (3)$$

where a is the number of independent anion binding sites per protein, K_A is the binding constant for anions to these sites, and $[\text{X}^-]$ is the concentration of anions.

The lack of curvature in the plots of Figure 10 suggests that $K_A[\text{X}^-] \gg 1$, and therefore equation (3) reduces to:

$$-\frac{\partial \log K\omega}{\partial \log [\text{MX}]} = m'\psi + a. \quad (4)$$

Thus the value of $\partial \log K\omega/\partial \log [\text{MX}]$ must be attributed to *both* cation ($m'\psi$) and anion (a) displacement. The slopes obtained from log-log plots for the various sodium salts tested (Fig. 10) are summarized in Table 5. For these salts ($m'\psi + a$) is smallest for NaF which, based on the Hofmeister series, would be expected to contain the weakest-binding anion tested (F^-). Thus m' can be *no more* than 4.5 for Na^+ and may be less if F^- does bind to the protein.

TABLE 5
Salt dependence of the binding of gene 32 protein to polynucleotides

Polynucleotide	Salt	$-(\partial \log K\omega/\partial \log [\text{MX}])\dagger$	Ions§	$-(\partial \log K\omega/\partial \log [\text{M}^{2+}])\dagger, \parallel$	$a\ddagger, \P$
Poly(rA)	NaCl	6.6	Na^+	~ 3	
	Na_2SO_4	3.5‡	Mg^{2+}	~ 1.5	
	Na(OAc)	5.1			
	NaF	3.5			
Poly(rU)	NaCl	7.4	Cl^-		~ 4
	MgCl_2	6.1	OAc ⁻		~ 2
	Mg(OAc)_2	5	F^-		$\sim 0-1$

Based on data obtained by the salt back-titration method (see Figs 10 and 11). All titrations were carried out in buffer C plus added salt as indicated.

† See eqn (4); all slopes show a standard error of ± 0.5 .

‡ Na_2SO_4 may be incompletely ionized at these concentrations (Robinson & Stokes, 1959).

§ Single-ion values estimated from the slopes of the Na^+ and Mg^{2+} -containing salts, based on eqn (4) and the constraint that $(m'\psi)_{\text{Na}^+} = 2(m'\psi)_{\text{Mg}^{2+}}$.

|| Cation contribution to the overall slope; this term = $m'\psi$.

¶ Anion contribution to the overall slope.

OAc, acetate.

(j) Mg^{2+} binding

To determine the fraction of the salt dependence of the binding of gene 32 protein to polynucleotides that is due to anions, a series of titrations similar to those of Figure 10 was carried out using Mg^{2+} salts (Fig. 11). Mg^{2+} binds much more tightly to polynucleotides than does Na^+ (deHaseth *et al.*, 1977b). Furthermore, because it is divalent, that portion of the slope ($\partial \log K\omega/\partial \log [\text{MgCl}_2]$) defining the gene 32 protein salt dependent binding that is due to cation competition should be one-half of that observed when Na^+ is used as the cation. As can be seen in Figure 11, Mg^{2+} *shifts* the position of line defining the salt dependence of gene 32 protein binding to poly(rU) to much lower salt concentrations, indicating that Mg^{2+} binding and displacement *do* occur. However, there is only a small effect on the *slopes* of these lines relative to those obtained using Na^+ with the same anions. (All titrations in Mg^{2+} were carried out at Mg^{2+} concentrations low enough to avoid any salt dependent changes in polynucleotide conformation.)

Both of these observations are consistent with the notion that $m'\psi$ for Na^+ (and Mg^{2+}) is small, and that the slope is dominated by anion displacement effects. The change in slope is consistent with the single ion values assigned in Table 5, which

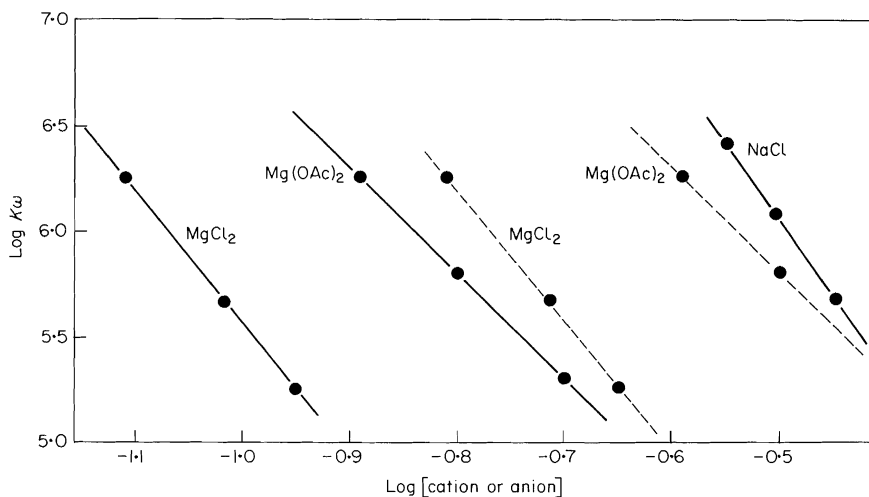


FIG. 11. Effects of Mg^{2+} concentration on $K\omega$ for gene 32 protein binding to poly(rU). The unbroken lines correspond to $\log K\omega$ plotted against the logarithm of the cation concentration; the broken lines represent the same data plotted against the logarithm of the anion concentration. OAc, acetate.

were calculated based on equation (4), with the additional constraint that $(m'\psi)_{Na^+}$ must be twice $(m'\psi)_{Mg^{2+}}$. We note that there is no requirement that $m'\psi$ be an integer; the estimates of Table 5 simply represent first approximation values of these parameters, consistent with the accuracy to which the differences in the slopes of the $\log K\omega$ versus $\log [MX]$ plots can be estimated. We conclude from these results that m' for Na^+ is between two and four; i.e. two to four ionic interactions involving nucleic acid phosphates and basic residues of the protein are formed on complex formation between a gene 32 protein monomer and a nucleic acid lattice. This is larger than the value of m' measured for short oligonucleotide binding to gene 32 protein (we note also that oligonucleotide binding showed no anion dependence). However, the results of the salt dependence for polynucleotide binding suggest that up to four Cl ions may be bound to the protein and displaced by nucleic acid binding in the *polynucleotide mode* (see Discussion).

(k) *Binding of gene 32 protein to oligonucleotides of intermediate length*
($l = 10$ to 12)

A number of semiquantitative experiments was performed to examine the binding of gene 32 protein to oligonucleotides sufficiently long to be capable of binding *two* protein monomers, to obtain some insight into the *transition* region between short oligonucleotide binding to a single protein monomer, and cooperative protein binding to a polynucleotide. Titrations of 10- and 12-mers were carried out as described for the short oligonucleotides, monitoring the quenching of intrinsic protein fluorescence as oligonucleotides were added to a fixed concentration of gene 32 protein. The protein concentrations used were $\sim 2 \times 10^{-7}$ to 3×10^{-7} M, and titrations were performed in buffer B containing appropriate concentrations of added NaCl.

All 10-mers and 12-mers examined ($d(pT)_{10}$, $d(pT)_{12}$ and $d(pA)_{12}$) bound two protein monomers per oligonucleotide. This conclusion is based on the fact that at conditions close to binding saturation (at both high and low salt concentrations), the ratio of bound protein to oligonucleotide always fell above one (and, of course, below 2). Values of binding constants (K_{app}) were estimated as the reciprocal of the values of L_F that apply at the midpoints of the titrations. Clearly, the constants obtained represent "hybrid" values; if we assume that one protein binds essentially in the oligonucleotide binding mode (K_{oligo}) and the other binds (co-operatively) in the polynucleotide mode ($K_{poly\omega}$), then K_{app} should be of the order of magnitude of the sum of K_{oligo} and ($K_{poly\omega}$) under these conditions (see derivation by Draper & von Hippel, 1979).

The salt dependence of K_{app} (measured as above) is shown in Figure 12, and also summarized in Table 6. Figure 12 shows that $\log K_{app}$ is an approximately linear

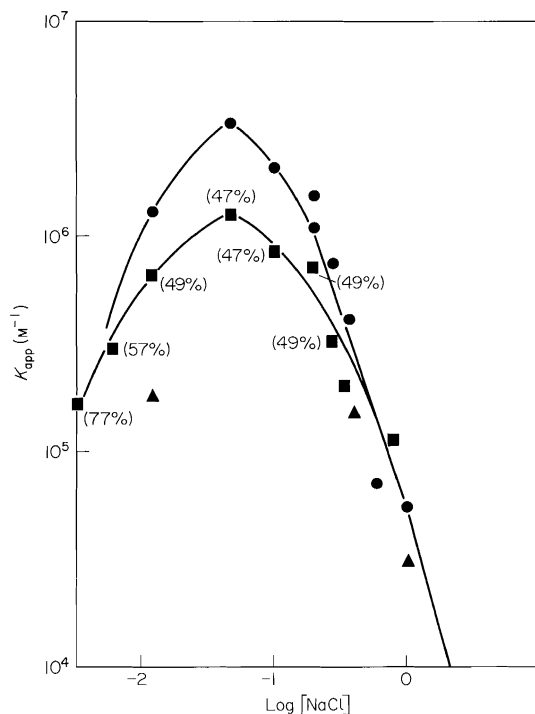


FIG. 12. Salt dependence of K_{app} for various intermediate sized oligonucleotides in buffer B plus added NaCl at 25°C. The numbers in parentheses are the values of Q_m obtained in that particular titration; note that Q_m increases at low salt concentrations. (■) $d(pT)_{10}$; (●) $d(pT)_{12}$; (▲) $d(pA)_{12}$.

function of $\log [NaCl]$ for salt concentrations greater than ~ 0.1 M. The slopes ($\partial \log K_{app} / \partial \log [NaCl]$) listed in Table 6 are obtained from these data. Figure 12 also shows that oligonucleotide binding becomes weaker at low salt concentrations; K_{oligo} dropping precipitously with decreasing salt concentrations below ~ 0.01 M-NaCl. This is in keeping with earlier observations indicating that gene 32 protein

TABLE 6
*Salt dependence of the binding of intermediate length
 oligonucleotides to gene 32 protein*

Oligonucleotide	$(\partial \log K_{\text{app}}/\partial \log [\text{NaCl}])\dagger$	$\psi_{\text{corr}}\ddagger$	$m'\S$
d(pT) ₈	-0.37 ± 0.02	0.60	0.60
d(pT) ₁₀	-1.0 ± 0.1	0.62	1.7
d(pT) ₁₂	-1.8	0.63	2.9
d(pA) ₈	-0.28 ± 0.07	0.71	0.39
d(pA) ₁₂	-1.8	0.73	2.4

K_{app} determined from titrations as described in the text.

† Determined using only the data points of Fig. 12 obtained at $[\text{NaCl}] > 0.1 \text{ M}$.

‡ Polynucleotide values of ψ (Record *et al.*, 1976) for poly(rU) were used for the T-containing oligonucleotides, and ψ values for poly(rA) were used for the A-containing oligonucleotides. End effect corrections to these polynucleotide values of ψ were made as described by Record & Lohman (1978); see the text.

§ Values of m' are determined *assuming* no anion binding effects for gene 32 protein binding to these oligonucleotides. This is probably not entirely correct; see the preceding sections.

becomes denatured at low salt (Jensen *et al.*, 1976), and suggests that the protein undergoes a (reversible) change to a non-binding conformation at low salt concentration. We note that Q_m also changes with salt concentration at low salt (Fig. 12), which is consistent with a conformational change in the protein under these conditions. This non-binding (low salt) form of the protein should not affect binding data obtained at 0.1 M-NaCl or above.

The salt dependencies of the oligonucleotides listed in Table 6 show a marked increase of the salt dependence of K_{app} on oligonucleotide length. (Data for d(pT)₈ and d(pA)₈, taken from Table 2, are included for comparison.) Clearly, as l increases the salt dependence changes toward that characteristic of the polynucleotide binding mode, in keeping with the interpretation that one protein is binding in the short (low-salt dependence) oligonucleotide mode, and the other (co-operatively) in the (high-salt dependence) polynucleotide mode.

4. Discussion

In this paper we have established the following facts concerning the interaction of gene 32 protein with nucleic acids:

(1) Oligonucleotides of length shorter than, or approximately equal to, the protein monomer site size ($n = 7 \pm 1$ nucleotide residues) bind with a binding constant ($K_{\text{oligo}} \simeq 10^5 \text{ M}^{-1}$). Within the limits of error of our (absolute) measurements, K_{oligo} is virtually independent of base composition and chain length (for $2 \geq l \geq 8$), though RNA oligomers may bind somewhat more weakly (3 to 6-fold) than the homologous DNA oligomers. K_{oligo} is also relatively salt insensitive ($\partial \log K_{\text{oligo}}/\partial \log [\text{NaCl}] \leq -1$ for this binding mode), and the binding is anion independent.

(2) Gene 32 protein binds co-operatively to polynucleotides, with an apparent

affinity ($K\omega$) that varies significantly with base composition and sugar type (see Newport *et al.*, 1980). Binding is also strongly dependent on salt concentration ($\partial \log K\omega / \partial \log [\text{NaCl}] \simeq -7$ for this binding mode), and a large part of this effect is due to the displacement of bound anions from the protein as a consequence of complex formation. The experiments in which K and ω have been measured separately also show that the intrinsic binding constant (K) for a protein molecule bound to a *polynucleotide* lattice at either an isolated or a contiguously bound site is the same, and that the salt dependence of the binding is virtually all in K . The co-operativity parameter (ω) appears to be invariant with salt concentration at a value of $\sim 10^3$.

These results suggest that there are two modes of interaction of gene 32 protein with nucleic acid, and that these two modes show quite different binding characteristics to nucleic acid lattices and probably involve different protein conformations. Figure 13 presents models for the binding interactions that are *consistent* with all these facts.

In Figure 13(a) we depict the interaction of gene 32 protein with short oligonucleotides. To account for the absence of the statistical ("shuffling") factor in the binding constant, we surmise that binding can take place only at the end of the

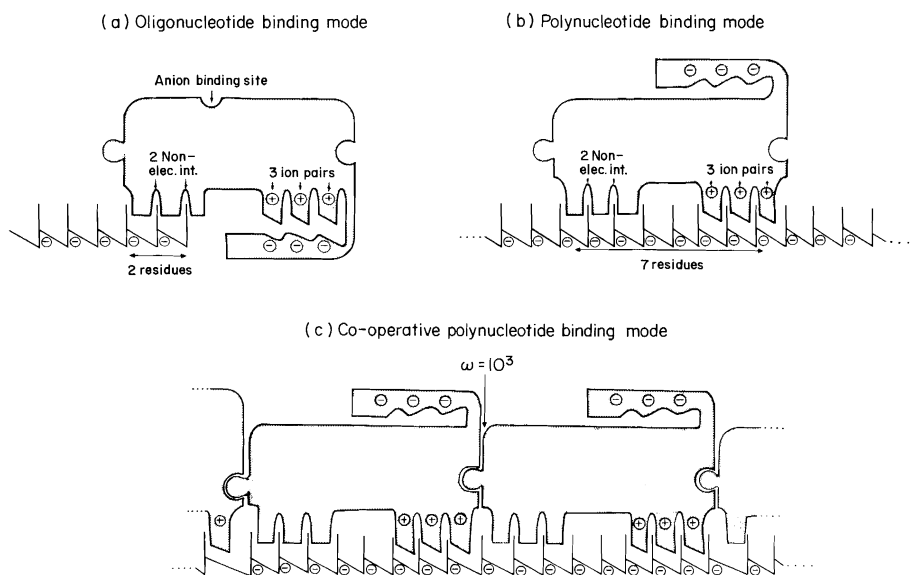


FIG. 13. Schematic models of 3 modes of gene 32 protein binding to nucleic acid lattices.

(a) Binding in the oligonucleotide binding mode; note the presence of the block to statistical shuffling of the oligonucleotide in the binding site, as well as the presence of the anion binding site (see the text).

(b) Isolated binding in the polynucleotide mode; note that the shuffling block has been moved away, exposing the positively charged binding sub-site, and the anion binding site has been disrupted. Also the nucleic acid lattice between the 2 binding sub-sites is somewhat stretched, and the non-electrostatic (XpX) binding sub-site is somewhat altered, indicating that gene 32 protein binding in this mode (conformation) shows somewhat enhanced base compositional specificity (see the text; and Newport *et al.*, 1980).

(c) Contiguous binding in the polynucleotide mode; the gene 32 protein binding conformation is unchanged from that of (b), except for protein-protein interactions and co-operative extension of the nucleic acid lattice between and through contiguously bound protein monomers (see the text).

oligonucleotide lattice (we have no basis, at present, to choose between the 5' and the 3'-end), and involves primarily two nucleoside units and the intervening phosphate. This is consistent with the fact that the full binding affinity is attained with $d(XpX)$ (or $r(XpX)$) and that K_{oligo} is not increased over the XpX value for $pXpX$ or $XpXp$ (Kelly *et al.*, 1976). It is also consistent with the salt dependence of binding, which suggests the involvement of at most one ionic interaction between a phosphate and a basic amino acid residue. The longer oligonucleotide lattices do bring about appreciable additional quenching of the intrinsic (primarily tryptophan; see Kelly & von Hippel, 1976) fluorescence of the protein, suggesting that the chain makes contact with the protein surface, though not in a way that contributes significantly to the binding free energy. The easiest way to think about this binding interaction is to imagine that there is a "block" ("flap"; "lump"; see Fig. 13(a)) in the binding site that prevents statistical binding (by a simple steric effect?) of oligonucleotides longer than $l=2$ when the protein is in its oligonucleotide binding conformation.

In Figure 13(b) we model the interaction of a gene 32 protein monomer bound to a polynucleotide lattice in the "isolated" binding mode. In order to "span" the polynucleotide (i.e. to let it bind in such a way that the lattice runs "through" the protein), the lump blocking lattice shuffling in Figure 13(a) has been removed, permitting binding across the entire protein molecule. Concomitant with the removal of this block, a positively charged site on the protein is "disrupted", with the accompanying displacement of several (~ 4 , if Cl^-) anions from the protein (see also Lonberg *et al.*, 1980). The nature of this anion binding site and its disruption is not clear; obviously it cannot involve simple replacement of the Cl ions bound by additional nucleotide phosphates, since then additional Na^+ (or other cationic) counterions bound to the DNA would have to be displaced from these. Presently, the simplest view is to imagine that the adoption by the protein of the polynucleotide binding mode requires a conformational change that results in the pulling apart of a cluster of positively charged residues on the protein that had previously comprised the tight anion binding site(s); (note that the schematic "depression" in Fig. 13(a), corresponding to the supposed anion binding site, has been removed in Fig. 13(b) and (c)). Two to four basic amino acid residues (three are shown in Fig. 13(b)) that had been shielded (perhaps by the block to oligonucleotide shuffling, see above) from interacting with the nucleic acid simultaneously became available for DNA phosphate binding.

This interpretation of the differences between the short oligonucleotide and polynucleotide binding modes is consistent with the binding properties of intermediate length oligonucleotides that can bind *two* protein molecules. The length dependence of the binding interaction, the magnitude of the apparent binding constant, and the salt dependence of the binding are all consistent with two proteins binding co-operatively; one in the short oligonucleotide and one in the polynucleotide binding mode.

We may ask why the sites of interactions involved in polynucleotide binding are not available to oligonucleotide lattices of sufficient length ($l=6$ to 8)? Presumably the free energy cost of the anion displacement, together with that of the conformational change in the protein (including the physical displacement of the lump of Fig. 13(a)) is such as to make isolated binding at moderate salt

concentrations *less* favorable than oligonucleotide-type end binding (see also discussion by Newport *et al.*, 1980). For a long polynucleotide lattice there are so few ends that effectively only isolated binding in the polynucleotide mode is available to the system, even at very low binding density.

Figure 13(c) shows several gene 32 protein monomers binding contiguously (co-operatively) to the polynucleotide lattice. The major interactions between the protein and the polynucleotide are identical in both isolated and contiguous binding (see above), but co-operativity further stabilizes the interaction by increasing the effective binding affinity by a factor of $\sim 10^3$ (ω) per protein monomer. At this point we know relatively little about the molecular interactions corresponding to this co-operativity. They may involve favorable protein-protein contacts (see Fig. 13(c); also Lonberg *et al.*, 1980); though these interactions cannot be electrostatic in nature, since ω shows virtually no salt dependence. Another possibility is that co-operativity of binding may arise in part from the *relative placement* of the two nucleic acid binding sub-sites (Fig. 13(b)).

One way of imagining this latter possibility is to place the XpX and the salt dependent nucleic acid binding sub-sites on the protein monomer in such a way as to require the lattice to "stretch" (be deformed) in order to bind to both sub-sites simultaneously. This stretched configuration of the backbone might then be stabilized by the next contiguously binding protein monomer, which is shown in Figure 13(c) as being "in register" with the stretched configuration required to span the two sites in the initial protein monomer. This "lattice distortion" model is consistent with the preferential binding of gene 32 protein clusters to DNA relative to RNA (see Newport *et al.*, 1980), though results obtained with specifically proteolytically cleaved fragments of gene 32 protein suggest that direct protein-protein interactions may indeed be responsible for most of the observed co-operativity (see Lonberg *et al.*, 1980).

This research was supported in part by United States Public Health Service research grant GM-15792 and by American Cancer Society post-doctoral fellowship PF-1301 (to S. C. K.). One of us (J. W. N.) was a pre-doctoral trainee on United States Public Health Service training grants GM-00715 and GM-07759; a portion of this work has been submitted (by J. W. N.) to the Graduate School of the University of Oregon in partial fulfillment of the requirements for the Ph.D. degree in Chemistry. We are grateful to Dr Larry Gold of the University of Colorado for helpful discussions throughout this work, and for providing the gene 32 protein-overproducing T4 strains. A preliminary account of some of this work was presented at the FASEB meeting in Dallas, Texas, April 1979; as well as at the FEBS meeting on Protein-Nucleic Acid Interactions in Prague, Czechoslovakia, September 1979.

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