Bacteriophage T4 Gene 32 Protein: Modulation of Protein–Nucleic Acid and Protein–Protein Association by Structural Domains†

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ABSTRACT: The cooperative binding of bacteriophage T4 gene 32 protein to single-stranded nucleic acids is dependent on homotypic protein–protein interactions between the N-terminus of a protein monomer with the core domain of an adjacent protein. In a previous report [Casas-Finet *et al.* (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 1050–1054], we demonstrated that synthetic peptides corresponding to various portions of the N-terminal B-domain (residues 1–21) formed a 1:1 complex with core domain and identified a sequence, residues 3–5, Lys-Arg-Lys-Ser-Thr (the *LAST* motif) strongly homologous to a sequence within the central portion of protein (core domain) that was likely to function in nucleic acid binding. On the basis of these observations, we proposed a model where cooperative binding involves an exchange of intramolecular protein–protein interactions involving the internal *LAST* sequence for intermolecular protein–protein interactions utilizing the N-terminal *LAST* sequence. In this paper, we have tested various predictions of the model, and utilizing several proteases, further have defined the domain structure of 32 protein. The interaction of peptides containing *LAST* sequences with 32 protein qualitatively reduces its binding cooperativity, indicating that the peptides bind at the same site within the core domain as the N-terminus of an adjacent intact protein bound to the polynucleotide lattice. As expected, these peptides bind to nucleic acids. The N-terminus of 32 protein is predicted to be largely α-helical, and the circular dichroism spectrum of a peptide corresponding to residues 1–17 is consistent with this prediction. On the basis of the magnitude of protein tryptophan fluorescence quenching, the conformational change in 32 protein brought about by *LAST* peptides may be similar to that effected by oligonucleotides. As predicted by our model, in the presence of interacting peptide, the binding of 32 protein to oligonucleotide becomes salt-dependent. Arg-C endoproteolysis of intact 32 protein indicates that the loss of as few as three or four amino acids from the N-terminus appears to eliminate binding cooperativity, although the remainder of the N-terminal B-domain appears to protect the core from proteolysis. In contrast, this enzyme will catalyze the breakdown of trypsin-generated core domain, which lacks the first 21 residues of the protein. Thus, the presence of residues 4/5–21 attached to core alters its conformation and/or accessibility to protease. Poly(dT) inhibits this digestion, whereas the presence of N-terminal peptide accelerates proteolysis, in agreement with our model. The amino- and carboxy-terminal ends of 32 protein are not readily accessible to exoproteolytic digestion.

Bacteriophage T4 gene 32 protein is a single-stranded nucleic acid binding protein (Alberts & Frey, 1970) required in bacteriophage DNA replication, repair, and recombination [for reviews, see Karpel (1990) and Chase & Williams (1986)]. The protein consists of a single polypeptide chain of 301 amino acid residues (M_r = 33 487) with three distinct functional domains, as demonstrated by limited proteolysis (Moise & Hosoda, 1976; Williams & Konigsberg, 1978). The acidic C-terminal A-domain is required for heterologous binding to the DNA polymerase (and possibly other proteins of the active T4 replisome; Burke *et al.*, 1980), and its presence controls the ability of 32 protein[1] to destabilize natural double-stranded DNAs (Greve *et al.*, 1978). The presence of the basic N-terminal B-domain (residues 1–21) enables 32 protein to bind cooperatively to nucleic acids and accounts for the approximately 1000-fold higher affinity of the intact protein for ss nucleic acid relative to cleavage products lacking this portion of the polypeptide chain (Spicer *et al.*, 1979; Williams *et al.*, 1979; Lonberg *et al.*, 1981; Giedroc *et al.*, 1990). In concert with this observation, the independently determined magnitude of the cooperativity parameter, ω, for intact 32 protein was found to be about 1000 (Kowalczykowski *et al.*, 1981; the overall affinity is equal to $K\omega$, where K is the intrinsic association constant of a 32 protein monomer for an isolated nucleic acid binding site). The loss of as few as nine residues from the N-terminus by V8 protease-catalyzed cleavage (Hosoda, 1983), removes binding cooperativity (Casas-Finet, 1989). The cleavage product lacking the C-terminal A-domain but with an intact N-terminus retains binding cooperativity (Lonberg *et al.*, 1981). These and other results indicate that (1) the intrinsic nucleic acid binding site is located within the core domain, termed *III or 32P-(A+B) and (2) the structural basis of the binding cooperativity of 32 protein monomers interacting with nucleic acid is the association of amino acid

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[1] Abbreviations: *LAST*, $(Lys/Arg)_3(Ser/Thr)_2$; AD, acrylodan (6-acryloyl-2-dimethylaminonaphthalene); ds, double stranded; 32 protein or 32P, bacteriophage T4 gene 32 protein; *I or 32P-A, truncated form of gene 32 protein lacking the C-terminal ("A") domain; *II or 32P-B, truncated form of gene 32 protein lacking the N-terminal ("B") domain; *III, 32P-(A+B) or core domain, truncated form of gene 32 protein lacking both the N-terminal and C-terminal domains; Na_2EDTA, ethylenediaminetetraacetic acid, disodium salt; poly(εA), poly(1,N^6-ethenoadenylic acid); poly(U), poly(uridylic acid); poly(dT), poly(deoxythymidylic acid); ss, single stranded.

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side chains located within the first nine residues of one 32 protein monomer with residues located within the core domain of the adjacent bound monomer (Karpel, 1990). Interaction of the core domain with nucleic acid may not be completely independent of other portions of the protein, and it appears that the presence of the A-domain contributes unfavorably to the intrinsic binding free energy (Giedroc *et al.*, 1990).

In order to identify the residues of the N-terminal domain required for homotypic 32 protein–32 protein interaction and 32 protein–polynucleotide binding cooperativity, in a previous study we determined the 32 protein-interactive properties of a variety of synthetic peptides that spanned different segments of this part of the protein (Casas-Finet *et al.*, 1992). Utilizing fluorescent dye-labeled peptides as well as peptide-induced protein tryptophan fluorescence quenching, we demonstrated that (1) a peptide corresponding to the first 17 residues of 32 protein co-chromatographed with intact protein on a Sephadex G-75 column; (2) upon interaction of the labeled peptide with 32 protein, the fluorescence emission maximum of the dye (acrylodan, 6-acryloyl-2-dimethylaminonaphthalene) blue-shifted from 531 to 517 nm, indicating a change to a less polar environment; and (3) the Trp emission intensity of intact 32 protein and fragments lacking the B-domain was quenched by labeled or unlabeled peptide. Of the two products produced by V8 protease-catalyzed cleavage of the peptide at Glu-9, only the N-terminal product showed binding activity. Proteolysis at Arg-4 with Arg-C protease destroyed binding activity, suggesting that necessary functional residues are found on both sides of this cleavage site. A peptide corresponding to residues 3–8 (Lys-Arg-Lys-Ser-Thr-Ala, peptide 7) induced protein Trp quenching of a magnitude identical to that observed with longer interactive peptides, and titration plots of this peptide with core domain showed tight binding at both 0.015 and 0.44 M Na^+, with a stoichiometry of 1.1 ± 0.2 (Casas-Finet *et al.*, 1992). Thus, based on these experiments with N-terminal peptides, we concluded that the essential residues for protein–protein interaction are located within residues 3–8.

Identification of these essential residues led to the realization that there is a striking homology between residues 3–7 and a sequence within the core domain, residues 110–114: Lys-Arg-Lys-Thr-Ser. This sequence may have a role in nucleic acid binding, since NMR studies with oligonucleotides (Prigodich *et al.*, 1986) and mutagenesis experiments (Shamoo *et al.*, 1989) demonstrated the involvement of an adjacent residue, Tyr-115, a member of the proposed nucleic acid-interactive tyrosine "ladder"; Trp-116 may also be involved in nucleic acid binding (Pan *et al.*, 1989). Moreover, a comparison of the amino acid sequence of 32 protein with other ss DNA binding proteins implicated Arg-111 in nucleic acid interaction (Prasad & Chiu, 1987). We have referred to these sequences, with three basic residues adjacent to two hydroxy-containing amino acids as the *LAST* motif, corresponding to $(Lys/Arg)_3(Ser/Thr)_2$ (Casas-Finet *et al.*, 1992). The existence of this sequence in two locations within 32 protein is likely not accidental, since at the *gene* 32 level there is a striking homology in the corresponding coding sequences: the nucleotides are identical in 15 of 17 positions for the sequences beginning at the second position of the codons for amino acids 2 and 109 and ending with the third position of the codons of residues 7 and 114, respectively. The probability of this happening randomly is 7 in 100 000; for the 15 nucleotides encoding the *LAST* sequences, the probability of randomly obtaining 13 homologies is 8 in 10 000 (Casas-Finet *et al.*, 1992). Either way, we must conclude that this homology occurred *non*randomly.

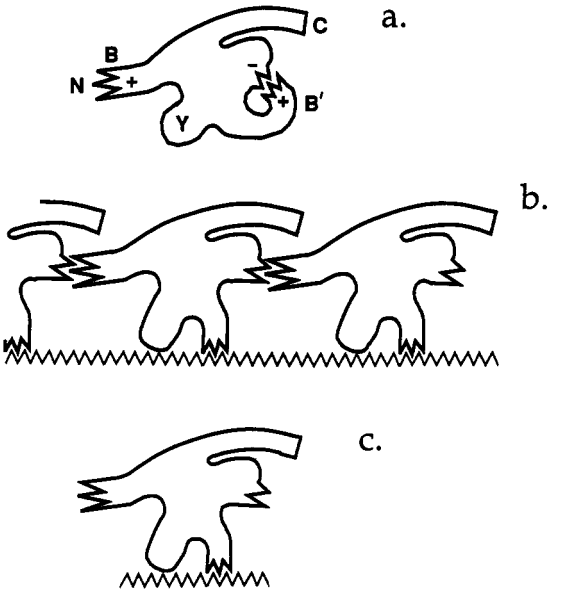

FIGURE 1: Model for the cooperative binding of 32 protein to single-stranded nucleic acid. (a) Representation of unbound 32 protein; N, N-terminal (B) domain; C, C-terminal (A) domain; B, N-terminal *LAST* sequence; B′, core domain *LAST* sequence; Y, tyrosine "ladder". The B and B′ sequences are labeled "+" to indicate their positive charge; the putative acidic surface is labeled "-". (b) Cooperative binding of 32 protein to polynucleotide, denoted as a jagged line. (c) Binding of 32 protein to a short oligonucleotide (absence of cooperative binding).

With these observations, we devised a model for the cooperative binding of 32 protein (Casas-Finet *et al.*, 1992). In this model, we proposed that when 32 protein is not bound to nucleic acid, the internal basic *LAST* sequence (residues 110–114) interacts with an acidic surface within the monomeric protein's core domain (Figure 1a). Upon binding ss nucleic acid in the cooperative mode, a protein conformational change occurs such that the internal *LAST* sequence now interacts with the nucleic acid backbone, freeing the acidic surface to bind to the N-terminal B-domain of an adjacent 32 protein monomer bound to the nucleic acid (Figure 1b). Thus, the internal sequence is capable of interaction with both an acidic protein surface and the acidic nucleic acid surface; the acidic protein surface can bind internally to the core domain *LAST* motif or externally to the N-terminal *LAST* sequence. With the exchange of *intra*molecular protein–protein electrostatic interactions for *inter*molecular interactions, the model provides a qualitative explanation for the salt-independence of the cooperativity parameter (Newport *et al.*, 1981). Moreover, it predicts the observed salt-independence of short oligonucleotide interaction with 32 protein (Kowalczykowski *et al.*, 1981) where, in the absence of intermolecular protein–protein interactions involving the B-domain sequence, there would be no net change in the number of ionic interactions involving *LAST* sequences (*cf.* Figure 1a,c).

In this article, we continue our exploration of the contributory roles of the structural domains of bacteriophage T4 gene 32 protein to its overall cooperative binding mode. One prediction of our proposed model is that *LAST* peptide interaction with the acidic binding surface of intact protein would be competitive with homotypic protein–protein interaction and, therefore, result in a reduction in binding cooperativity. Thus, we have assessed the effect of peptide 7 on the degree of binding cooperativity fluorimetrically. Peptide 7 reduced the sigmoidal character of poly(ethenoadenylic acid) (poly(ϵA)) titrations with 32 protein, indicating a decrease in binding cooperativity. Additional evidence for

the involvement of an exchange mechanism of ionic interactions was obtained by examining the effect of added peptides carrying *LAST* sequences on the salt-dependence of oligonucleotide binding by 32 protein.

The proposed involvement of the internal *LAST* sequence in nucleic acid interaction is probed by observation of the association of ss nucleic acids with a peptide containing the internal *LAST* sequence and the adjacent two aromatic residues (residues 110–116, peptide 8). Both peptide 8 and the aforementioned (N-terminal) peptide 7 display significant binding to ss nucleic acid, as shown by the enhancement of poly(ϵA) fluorescence in the presence of peptide. The limiting enhancement of poly(ϵA) fluorescence induced upon binding peptide 8 was about double that seen with peptide 7, suggesting that the aromatic side chain(s) bring about additional destacking of the ethenoadenine bases, possibly via intercalation.

Proteolytic digestion experiments utilizing high levels of the arginine-specific enzyme, Arg-C endoproteinase, have been used (1) to further define the structural requirements of both the B- and core domains and (2) to examine the effect of interacting peptide on core domain structure. The nucleic acid binding behavior of cleavage products generated by chain scission near the N-terminus is fully consistent with our previous results using N-terminal peptides (Casas-Finet *et al.*, 1992), and indicates that binding cooperativity is lost even with the removal of as few as three or four residues at the N-terminus of the polypeptide. However, the presence of the remainder of the B-domain has a protective effect against proteolysis, suggesting either a direct interaction of this portion of the protein with core domain or a conformational difference in the core that is dependent on residues 4–21 of the B-domain. Assays measuring the activity of exoproteolytic enzymes on the amino- and carboxy-terminal residues of 32 protein revealed protection from cleavage both in the absence and in the presence of nucleic acid. Taken together, these results allow for a refinement of the general model for the nucleic acid binding and homotypic association of gene 32 protein.

MATERIALS AND METHODS

32 Protein and Domains. Gene 32 protein was purified from T4-infected cells as previously described (Bittner *et al.*, 1979) or from the overproducing *Escherichia coli* plasmid, pYS6 (Shamoo *et al.*, 1986), obtained from Dr. Y. Shamoo, Department of Molecular Biophysics and Biochemistry, Yale University School of Medicine (New Haven, CT). The core domain was prepared by trypsin digestion of the protein according to published procedures (Williams & Konigsberg, 1978). Protein aliquots were stored at −80 °C in 20 mM Tris-HCl buffer, pH 8.1, containing 1 mM Na_2EDTA, 1 mM 2-mercaptoethanol, and 20 mM NaCl. All chemicals used were of reagent grade. Buffers were prepared with doubly glass-distilled water or deionized Milli-Q water (conductivity = 18 $m\Omega^{-1}$).

Peptides. Peptides with the sequence KRKSTA (peptide 7), KRKTSYW (peptide 8), and KKKSTA (peptide 9) were synthesized by and purchased from Dr. Tomas Kempe, University of Maryland Protein and Nucleic Acid Laboratory (College Park, MD). The C-terminus of peptide 7 and of peptide 9 was a free -COO^-, whereas it was amidinated (and uncharged) in peptide 8. Peptides 7 and 8 were analyzed (and purified, where required) by HPLC on a Waters 0.5 × 25 cm C-18 μ-Bondapak column equilibrated with 0.05% trifluoroacetic acid (Pierce) in HPLC-grade water (purified utilizing a Millipore Norganic cartridge), solvent A. Elution was performed with 0.05% trifluoroacetic acid in HPLC-grade acetonitrile (EM Analytic), solvent B. HPLC solvents were absorbance-matched at 210 nm. The flow rate was constant at 1 mL min^{-1}. The concentration of solvent B was increased in three linear steps: 0–20% v:v (0–5 min), 20–60% (5–45 min), 60–80% (45–50 min). Peptide 7 eluted at 13% acetonitrile and yielded a single peak on analytical reverse-phase HPLC; it was, therefore, used without further purification. Immediately prior to HPLC purification of peptide 8, the protecting formyl group on its tryptophyl residue was removed by treatment with 10% piperidine and dimethylformamide. The elution properties were similar to those of peptide 7. Peptide 9 was subjected to ion-exchange HPLC on a Phase Sep Spherisorb SCX column and was found to elute as a single peak. Accordingly, it was also used without further purification. Amino acid analysis of HPLC-purified peptides were in excellent agreement with the predicted sequence. The concentrations of peptides 7 and 9 were based on dry weight; peptide 8 concentration was determined spectrophotometrically using an extinction coefficient $\epsilon_{280} = 6.8 \times 10^3$ M^{-1} cm^{-1}. Peptide 1 (residues 1–17 with an additional Cys at the C-terminus) was purified as previously described (Casas-Finet *et al.*, 1992).

Oligonucleotides and Polynucleotides. Poly(dT), poly(ϵA), and $p(dT)_8$ were obtained from Pharmacia-PL Biochemicals. $p(d\epsilon A)_7dT$ was prepared on an oligonucleotide synthesizer using the phosphoramidite precursor of ethenoadenylate (Glen Research). All poly- and oligonucleotides were dissolved in doubly distilled water and used without further purification. Concentrations were determined spectrophotometrically using the following extinction coefficients (in terms of nucleotide residues): $\epsilon_{264} = 8.52 \times 10^3$ M^{-1} cm^{-1} for poly(dT), $\epsilon_{260} = 8.1 \times 10^3$ M^{-1} cm^{-1} for $p(dT)_8$, and $\epsilon_{257} = 3.7 \times 10^3$ M^{-1} cm^{-1} for poly(ϵA). The concentration of $p(d\epsilon A)_7dT$ was determined at its maximum absorbance from a weighted average of its component extinction coefficients ($\epsilon_{258} = 4.2 \times 10^3$ M^{-1} cm^{-1}.

Endoproteolytic Cleavage of 32 Protein with Arg-C Protease. Endoproteinase Arg-C from mouse submaxillary gland (EC 3.4.21.40; type XX-S or protein sequencing grade) was purchased from Sigma as a lyophilized powder. The enzyme was dissolved in 0.01 M Tris-HCl, pH 8.1, and 0.001 M Na_2EDTA. Activity was checked spectrophotometrically using the chromogenic substrate *N*α-*p*-tosyl-L-arginine methyl ester, obtained also from Sigma. When poly(dT) or *LAST* peptides were used in digestion experiments, concentrated stock solutions of these materials were prepared in water. Arg-C protease cleavage products of 32 protein (34 μg) subjected to chromatography on denatured DNA-cellulose were eluted over a ≈100-μL column under conditions established for limited trypsin digestion products (Williams & Konigsberg, 1978). Fractions were concentrated, where necessary, by acetone precipitation prior to electrophoresis. The identity of the major products of digestion of intact 32 protein was established by automated N-terminal Edman degradation on an Applied Biosystems 477A protein sequencer of samples that had been electroblotted to 0.45-μm Millipore Immobilon membranes from SDS–polyacrylamide gels (Benveniste *et al.*, 1988).

Exoproteolytic Cleavage Assays. Yeast carboxypeptidase Y (peptidyl-L-amino acid hydrolase, EC 3.4.16.1) from baker's yeast was purchased from Pierce and from Boehringer Mannheim; *N*-carbobenzoxy-Phe-Ala was from Sigma. Soybean trypsin inhibitor-agarose was obtained from Pierce.

Leu-aminopeptidase (α-aminoacyl-peptide hydrolase, EC 3.4.11.2) from porcine kidney microsomes and L-Leu-amino-*p*-nitroanilide were from Sigma; the enzyme was free of

hydrolytic activity vs sodium benzoylarginine ethyl ester and sodium benzoyltyrosine ethyl ester. Cleavage assays with Leu-aminopeptidase were performed at 25 °C in 10 mM sodium phosphate, pH 7.2. Enzyme activity was measured from spectrophotometric determinations of initial velocity in the presence of the chromogenic substrate L-Leu-amino-*p*-nitroanilide, using a molar extinction coefficient $\epsilon_{405} = 10.6 \times 10^3$ M^{-1} cm^{-1} for the 4-nitroanilide species; protein concentrations ranged from 2 to 7 μM. No endoproteolytic activity on 32 protein was detected electrophoretically in digests performed at 25 or 37 °C for up to 36 h at an enzyme to substrate ratio of 1:100.

Circular Dichroism Spectroscopy. Spectra were collected at 25.0 ± 0.1 °C on an Aviv 60DS CD or a JASCO J-710 spectrometer using strain-free Suprasil quartz cells with 0.2- or 0.005-cm path length. On the Aviv instrument, five spectra were averaged to reduce the noise level, whereas on the JASCO spectrometer, a single spectrum was acquired with a 16-s time constant. Buffer blanks were run for each set of CD spectra and subtracted from the raw data. Smoothing was performed by fitting the data with a third degree polynomial over a sliding 10-point window.

Fluorescence Spectroscopy. Studies were carried out on a SLM 48000S spectrofluorimeter equipped with double monochromators containing holographic gratings with 1500 groove/mm. Excitation was performed with a 450 W Xe arc lamp. Detection was accomplished with cooled red-sensitive photomultiplier tubes offset to null the dark counts. Spectra were collected in the ratio mode using as a quantum counter a triangular quartz cell containing 3 g L^{-1} of rhodamine B solution. Excitation monochromator slits were set at 1-nm bandwidth, where sample photobleaching was undetectable. Emission monochromator slits were set to the appropriate resolution (usually at 4 or 8 nm bandwidth). Samples were held in a dual path length (0.2 × 1 cm) Suprasil quartz cuvette in a thermostated cell holder maintained at 25.0 ± 0.1 °C, with the narrow path length facing the excitation beam. Samples of 200 μL were used in a capped cell. Data acquisition was performed by averaging 16 readings per wavelength. Data were processed by an IBM-PC/XT computer utilizing programs supplied by SLM. Dynamic fluorescence experiments in the frequency domain were performed at the Center for Fluorescence Spectroscopy, University of Maryland at Baltimore, as previously described (Casas-Finet *et al.*, 1991).

Fluorimetric Titrations. Titrations were performed in 1 × 1 or 0.2 × 1 cm Suprasil quartz cells as described earlier (Kumar *et al.*, 1990). In binding experiments utilizing poly(ϵA), aliquots of protein and/or peptide were added to the polynucleotide solution. In titrations employing p(dϵA)$_7$dT, the oligonucleotide was added to solutions of protein in the presence and absence of peptide. Fluorescence readings were accurate to ±2% or less, and volume readings were accurate to ±5% or less.

RESULTS

The N-Terminus of 32 Protein is Largely α-Helical. The Chou-Fasman (1974), Garnier–Osguthorpe–Robson (1978), and Levitt (1978) algorithms predict an α-helical structure for most or all of the first 17 residues of 32 protein. The circular dichroism spectrum of peptide 1 (residues 1–17 with an additional cysteine residue at the C-terminus) is fully consistent with this prediction (Figure 2A). However, peptide 7, residues 3–8, showed little evidence for any secondary structure (Figure 2B). Thus, the existence of secondary structure in the N-terminus is not a prerequisite for its

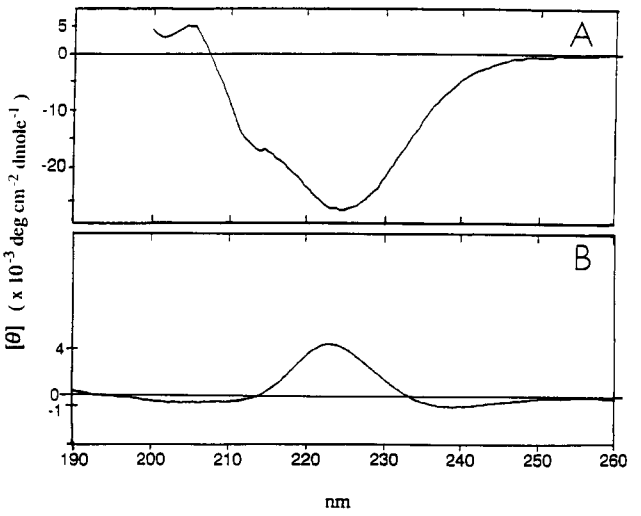

FIGURE 2: Circular dichroism spectrum of (A) peptide 1, 25.6 μM, and (B) peptide 7, 2.0 mM in 1 mM sodium phosphate, pH 7.0 at 25 °C. Note that the vertical scales are different in the two plots.

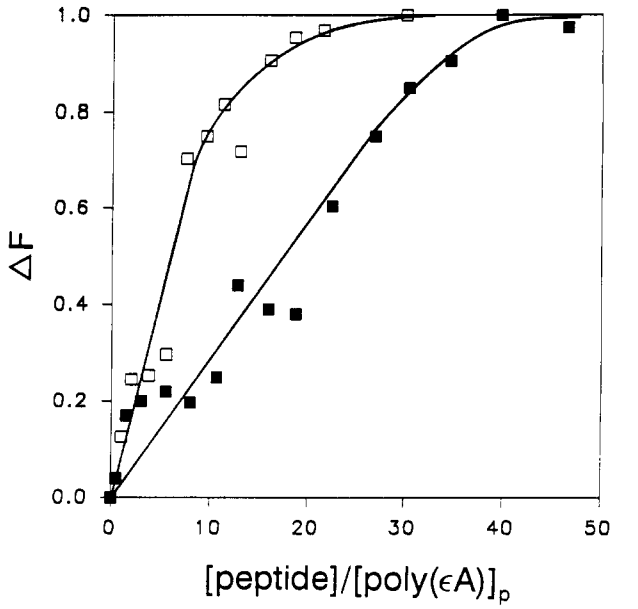

FIGURE 3: Binding of peptide 7 (open squares) and peptide 8 (filled squares) to poly(ethenoadenylic acid), 3 × 10^{-7} M(p) in 0.01 M sodium phosphate, pH 7.2. The maximal ΔF (≡1.0) for peptide 7 was 60% of the poly(ϵA) fluorescence in the absence of added peptide and 100% for peptide 8. The excitation wavelength was 315 nm; the emission wavelength was 410 nm.

intermolecular interaction with protein, although it is conceivable that upon interacting with 32 protein, structure is induced within the peptide.

Peptides Containing LAST Sequences Bind to Nucleic Acids. The nucleic acid binding surface of 32 protein is dependent on its tertiary structure and spans a considerable portion of the core domain (Karpel, 1990). Since we have proposed that the internal *LAST* (B′) sequence is part of this binding surface, it was of interest to assess the polynucleotide binding of a peptide containing this sequence, Lys-Arg-Lys-Thr-Ser-Tyr-Trp, residues 110–116 (peptide 8). At low ionic strength (10 mM sodium phosphate, pH 7.2), addition of peptide 8 to the fluorescent polynucleotide, poly(ϵA), produced a 100% (hyperbolic) increase in the polynucleotide's fluorescence emission at sufficiently high [peptide]:[poly(ϵA)]$_p$ (40:1) (Figure 3). The addition of peptide 7, Lys-Arg-Lys-Ser-Thr-Ala (devoid of aromatic groups) to poly(ϵA) gave a similar plot, although with only a 60% increase (Figure 3).

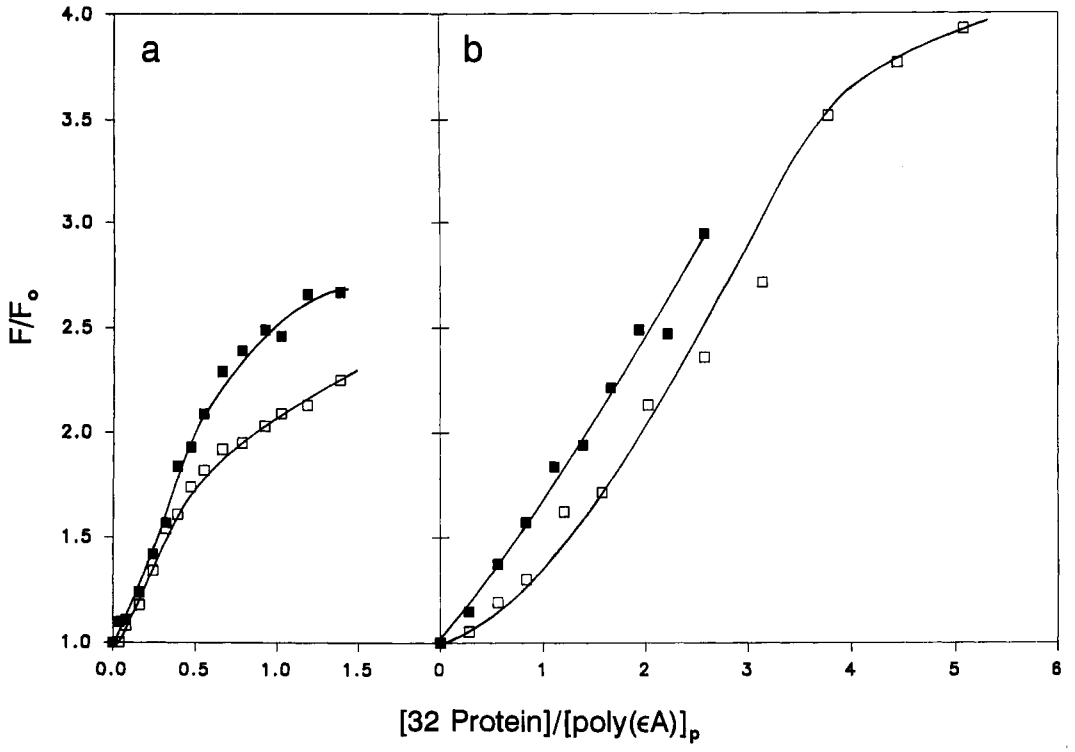

FIGURE 4: Interaction of poly(ϵA) with 32 protein in the presence and absence of peptide 7. Increase in polynucleotide fluorescence (relative to that observed in the absence of added protein, F_o) vs [32 protein]/[poly(ϵA)]$_p$. The excitation wavelength was 315 nm; the emission wavelength was 410 nm. (a) In 0.42 M NaCl, 1 mM sodium phosphate, pH 7.0, and 2×10^{-5} M EDTA, aliquots of 32 protein were added to poly(ϵA) in the presence (filled squares) and absence (open squares) of 2 μM peptide 7. (b) Titration in the same buffer, but with 0.50 M NaCl in the presence (filled squares) and absence (open squares) of peptide 7. The [peptide]:[protein] was kept constant at 30:1 by addition of aliquots of a concentrated stock of peptide 7. For clarity, the *x*-axis scales of the two panels are not identical.

The homologous peptide 9, Lys-Lys-Lys-Ser-Thr-Ala, exhibited binding properties qualitatively similar to those of peptide 7.

The tryptophan fluorescence of peptide 8 also indicated nucleic acid binding. Addition of poly(dT) to a solution of this peptide brought about a small (≈15%) level of quenching. In contrast to the demonstrated binding of peptides to long polynucleotides, a short oligonucleotide capable of interaction with 32 protein [p(dϵA)$_7$dT, see below], showed no evidence of association with *LAST* peptides, even in 1 mM sodium phosphate.

Interaction of Peptides Containing LAST Sequences with 32 Protein Affects Its Cooperativity of Binding to Polynucleotide Lattices. As we have noted, interaction of a *LAST* peptide with intact protein should be competitive with homotypic protein–protein interaction and, therefore, result in a reduction in binding cooperativity. Addition of 32 protein to poly(ϵA) in 0.42 or 0.50 M NaCl yields a sigmoidal titration plot characteristic of highly cooperative binding (Kowalczykowski *et al.*, 1981, 1986), where a lag followed by a steep increase in the polynucleotide's fluorescence is observed as the [protein]:[poly(ϵA)]$_p$ increases (Figure 4a,b). When 32 protein was added to a mixture of poly(ϵA) and peptide 7 at 0.42 M Na^+, the enhancement of fluorescence was distinctively less sigmoidal (Figure 4a). This is most obvious at low levels of [32 protein]:[poly(ϵA)]$_p$, where the [peptide]:[protein] levels were relatively high (for the first three protein additions in the titration shown in Figure 4a, the [peptide]:[protein] values were respectively 25, 12.5, and 6.25). Since *LAST* peptides do not bind *intact* protein stoichiometrically, a significant excess of peptide is required to achieve a high level of peptide–protein binding (Casas-Finet *et al.*, 1992). With this in mind, the experiment was repeated (at 0.50 M Na^+) under conditions of constant [peptide]:[protein] (30:1). In this titration, the presence of peptide produces a more dramatic loss of sigmoidal character (Figure 4b). However, the titration could not be completed, since at [32 protein]:[poly(ϵA)]$_p$ levels above 3 the emission readings became erratic. This is likely due to the time-dependent precipitation of intact protein by peptide (Casas-Finet *et al.*, 1992). Although we have shown that peptides containing *LAST* sequences bind poly(ϵA) at low ionic strength, no interaction was seen at these salt conditions. In contrast, binding of the peptide to protein is relatively salt-independent (Casas-Finet *et al.*, 1992).

The Conformational Change in 32 Protein Brought about by LAST Peptides May Be Similar to That Effected by Oligonucleotides. In a previous study, the average limiting fluorescence quenching of whole or truncated 32 protein effected by various peptides carrying the *LAST* motif was 26 ± 3% (Casas-Finet *et al.*, 1992); the extent of quenching at saturation was comparable for intact 32 protein or fragments lacking the B-domain. Addition of peptide 7 to intact protein produces a quenching of tryptophan fluorescence (integrated emission) of 28% at saturation, achieved with a 7-fold molar ratio of peptide:protein. There was no change in the wavelength maximum (340.5 nm) or in the line width of the emission band (4600 cm^{-1}). As we have noted (Casas-Finet *et al.*, 1992), saturation of core domain is achieved at stoichiometric levels of *LAST* peptides. We have repeated this experiment at 0.43 M Na^+ with peptide 8, which itself contains tryptophan, and the resulting emission spectrum of the complex was best fit with a quenching value of 27% for core domain, and about 16% for the peptide (peptide was equimolar with protein). The identity of protein quenching values seen with these two peptides indicates that upon binding, there is no energy transfer involving the aromatic residues at the C-terminus of peptide 8 (Tyr-Trp), which are not likely involved in the peptide–protein interaction. The extent of

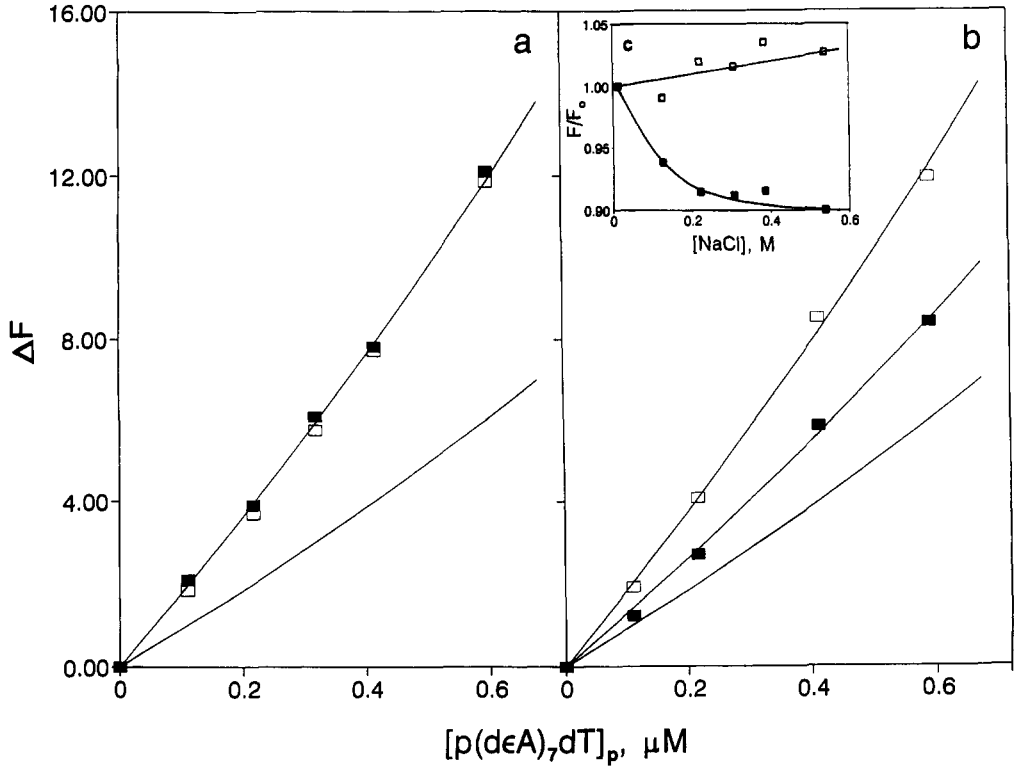

FIGURE 5: Effect of a *LAST* peptide on 32 protein–oligonucleotide interaction, as monitored by oligonucleotide ethenoadenylate fluorescence (arbitrary units). Emission and excitation wavelengths are as given in Figure 2. (a) Addition of p(dεA)$_7$dT to a 32 protein solution (1.5 μM in 10 mM sodium phosphate, (pH 7.0, 13 mM Na^+, 2×10^{-5} M EDTA) in the presence (filled squares) and absence (open squares) of peptide 7 (30 μM). The line without data points was computed for the fluorescence increase seen upon addition of oligonucleotide to buffer, in the absence of protein and peptide. (b) Same as a, but performed in the presence of 0.4 M NaCl. (c) (insert) Reversal of 32 protein (1.5 μM)-induced p(dεA)$_7$dT [0.6 μM(p)] fluorescence enhancement by NaCl in the presence (filled squares) and absence (open squares) of peptide 7 (30 μM). In this plot, F_0 refers to the fluorescence prior to the addition of NaCl.

quenching of core domain or intact protein fluorescence induced by p(dT)$_8$ at saturation was very similar to that seen with *LAST* peptides (about 25–30%). When ternary mixtures of core domain, peptide 8, and p(dT)$_8$ were formulated, the calculated quenching of the protein was of similar magnitude and independent of the order of addition. Since both the peptide and the oligonucleotide affect the intrinsic Trp fluorescence of core domain comparably, it was impossible to use this effect to assess the formation of a ternary complex. However, we note that experiments utilizing the fluorescence of an acrylodan-labeled peptide as a probe suggested that a ternary complex forms, since the addition of a single-stranded nucleic acid to a preformed complex of protein and peptide further blue-shifted the acrylodan fluorescence beyond the protein-induced emission shift (Casas-Finet *et al.*, 1992).

Taken together, these results suggest that binding of core domain to either peptide 7 or p(dT)$_8$ may bring about a similar conformational change in the protein. This conformational change does not affect the oligomerization state of 'III, as frequency-domain dynamic fluorescence experiments indicate that the rotational correlation time(s) of the Trp chromophores are hardly affected upon binding of peptide 7 or p(dT)$_8$ (not shown). The conformational change must be of a local nature, as a saturating amount of peptide 7 did not induce observable effects in the CD spectrum of core domain in the peptidic band region (260–200 nm). We suggest that the observed fluorescence quenching of core domain in the presence of a *LAST* peptide or p(dT)$_8$ stems in both cases from the displacement of a peptidic arm containing the (B′) *LAST* motif present in the protein. This occurs either by interaction of the peptide with the putative acidic surface, displacing the B′ segment, or direct binding of this segment to the oligonucleotide.

LAST Peptides Induce a Salt Dependence in Oligonucleotide–32 Protein Interaction. An earlier model (Kowalczykowski *et al.*, 1981) accounted for the salt-independence of 32 protein–oligonucleotide association by proposing that the basic residues which formed ion pairs with nucleic acid phosphates when the protein was bound to a polynucleotide lattice were covered by a negatively charged flap in the protein when bound to a short oligonucleotide. An alternative explanation is provided by our model, where the electrostatic interactions involving the internal *LAST* sequence and the putative acidic surface within the unbound protein monomer are replaced by intermolecular protein–nucleic acid electrostatic interactions. If peptide were bound to this surface, and remained so after interaction of the protein with the oligonucleotide, there would be a net gain in ionic interactions, and the association with the short nucleic acid should therefore become salt-dependent.

Evidence for such an effect was obtained from binding experiments with the fluorescent oligonucleotide, p(dεA)$_7$dT. When aliquots of this ligand were added to a solution containing 32 protein, the rate of increase in oligonucleotide fluorescence is about 2-fold relative to that seen in the absence of protein (Figure 5). This reflects the enhancement of the oligonucleotide's emission upon binding protein (Kowalczykowski *et al.*, 1981). At low [salt] (0.013 M Na^+), the presence of a 20-fold excess of peptide 7 did not alter this enhancement (Figure 5a); we have noted that the peptide itself does not bind p(dεA)$_7$dT. From this data, and the maximal protein-induced oligonucleotide fluorescence enhancement (F_{rel} = 3.5, determined in a separate experiment),

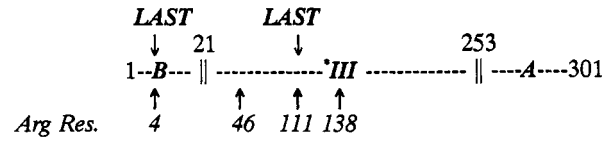

FIGURE 6: Arg-C endoproteinase cleavage sites (arginine residues 4, 46, 111, and 138), trypsin cleavage sites that generate core domain (lysine residues 21 and 253), and location of *LAST* sequences within intact 32 protein.

we calculate that the affinity of 32 protein for the oligonucleotide in the presence or absence of peptide is 5×10^5 M^{-1}. This result is within the range of the values obtained (at 0.1 M Na^+) for octanucleotide interaction with whole or truncated 32 protein, which vary from 2×10^5 to 2×10^6 M^{-1} (Kowalczykowski *et al.*, 1981; Lonberg *et al.*, 1981; Giedroc *et al.*, 1991). The results of similar binding experiments run at 0.4 M Na^+ are instructive: in the absence of peptide, the increase in $p(d\epsilon A)_7dT$ fluorescence is the same as that seen in 0.013 M Na^+, so the affinity is unchanged (Figure 5b). However, in the presence of peptide 7, the rate of oligonucleotide fluorescence increase when added to a 32 protein solution was much lower (only about half as large as in the absence of peptide), thus indicating that oligonucleotide binding affinity was significantly reduced. We calculate that the affinity of the protein for the oligonucleotide is reduced 4-fold, corresponding to a change in the binding free energy of 0.9 kcal mol^{-1}. Therefore, as predicted, binding of peptide 7 by 32 protein induced a salt dependence in 32 protein oligonucleotide interaction.

Another indication that association of peptide with protein induces a salt-dependence in binding can be seen with the effect of added NaCl on protein–oligonucleotide complexes formed at low ionic strength. As seen in Figure 5 (insert), in the presence of a 15-fold excess of peptide, there is a drop of about 10% in the oligonucleotide's fluorescence when the [Na^+] is raised to 0.3 M, whereas in the absence of peptide, there is, if anything, a slight increase in emission. This effect is less dramatic than that seen in Figure 5a,b and might indicate a partial kinetic block to dissociation of the preformed complex in the presence of peptide (see Discussion).

Arg-C Endoproteolysis of Intact 32 Protein and of Core Domain Further Defines the Boundaries of the Latter. Although native, Zn^{2+}-bound 32 protein is relatively refractory to digestion by proteases beyond generation of the core domain, we have found that high levels of proteolytic enzymes bring about further digestion of the polypeptide chain. The results with Arg-C protease are particularly instructive, since there are only four arginines in the protein, at positions 4, 46, 111, and 138 (Figure 6).

At an [Arg-C protease]:[32 protein]$_{wt}$ of 1:100, a ratio where a variety of enzymes bring about cleavage of both A- or B-domains (or similar cleavage products; Williams & Konigsberg, 1978; Moise & Hosoda, 1976; Hosoda & Moise, 1978), no detectable cleavage of 32 protein was observed on SDS–polyacrylamide gels (not shown). However, at higher levels of the enzyme (≥1:20), the protein became susceptible to proteolysis. At very high [protease]:[32 protein] (1:10), SDS–PAGE indicated that full-length 32 protein was more resistant to cleavage than purified core domain (*III, residues 22–253, which had been generated by limited trypsin proteolysis) (Figure 7A). Automated Edman degradation of the cleavage products of intact 32 protein indicated, for *both* bands, an N-terminal sequence of either RKSTAEL (the major run) or KSTAEL (the minor run). Thus, the N-terminus of both polypeptides are identical, each generated from cleavage on the N- and on the C-side of Arg-4. The mobility of the larger band is indistinguishable from that of whole 32 protein, so the C-terminus of that polypeptide is likely intact. From the apparent molecular mass of the smaller band, ≈28 kDa, which ran just above *III (trypsin-generated core domain, 26.2 kDa), it would appear that there was cleavage at or near the trypsin-hypersensitive site at Lys-253 (the polypeptide corresponding to residues 5–253 would have molecular mass of 27.8 kDa). Note that there are no arginines beyond residue 138, so that under the conditions of cleavage the enzyme's specificity is not limited to this side chain. This lower molecular weight species is fairly resistant to further cleavage, so Arg-46, -111, and -138 are protected relative to Arg-4 (or lysine in the vicinity of residue 253).

The two cleavage products resulting from Arg-C protease-directed digestion of full-length protein (residues 4/5–301, 4/5–253) were chromatographed on ss DNA–cellulose. Both products were seen to elute at 0.5 M NaCl, conditions where trypsin-generated core domain chromatographs, but intact protein is retained on the column (Williams & Konigsberg, 1978; Hosoda & Moise, 1978) (Figure 7B). The greater affinity of intact protein for ss DNA–cellulose at 0.5 M NaCl relative to 32 protein fragments is due to the property of binding cooperativity in whole 32 protein, which boosts the overall affinity for nucleic acid by 3 orders of magnitude (Kowalczykowski *et al.*, 1981). Thus, the loss of as few as four residues is sufficient to destroy the functionality of the B-domain. This result is fully in agreement with our data on N-terminal peptides, where peptides cleaved with Arg-C protease lost their ability to bind 32 protein (Casas-Finet *et al.*, 1992).

Unlike the results with intact protein, it appears that all potential Arg-C sites were attacked in *III (Figure 7A). Although not characterized in detail, the major bands seen at 23, 16, 13, and 10.5 kDa could correspond to, respectively, residues 46–253, 112–253, 22–138 or 139–253, and 46–138. As we have noted, under the conditions of cleavage, the enzyme appears not to have absolute specificity for cleavage at arginine positions. Nevertheless, it is clear that the trypsin-generated core domain is much more susceptible to proteolysis than is peptide 4/5–253, even though both peptides bind similarly to ss DNA–cellulose. Thus, the additional stretch of sequence between residues 5 and 21 confers protease resistance to the core domain and raises the possibility that this portion of 32 protein may be in physical contact with *III. Alternatively, the conformation of core domain may be different when residues 5–21 remain attached to it.

In the Presence of Binding ss Nucleic Acid or N-Terminal Peptides, Arg-C Endoproteinolysis of 32 Protein Is Altered. Complexes of 32 protein and trypsin-generated core domain with ss DNA or with N-terminal peptides show interesting cleavage patterns upon treatment with Arg-C protease. When proteolysis was performed in the presence of excess poly(dT), the results indicated that *III cleavage was totally inhibited whereas the cleavage pattern of intact protein is qualitatively unchanged, although generation of the lower molecular weight band (4/5–253) is slightly inhibited (Figure 8A).

In order to determine the effect of the N-terminal peptide on Arg-C endoproteinase digestion, a peptide with lysine in place of arginine but otherwise identical to peptide 7, Lys-Lys-Lys-Ser-Thr-Ala (peptide 9), was synthesized. Although not studied in detail, peptide 9 appeared to have protein-binding properties comparable to those of peptide 7, as assessed by its ability to quench core domain Trp fluorescence. When core domain was subjected to the action of Arg-C protease in the presence of an excess of peptide 9, the rate of protein

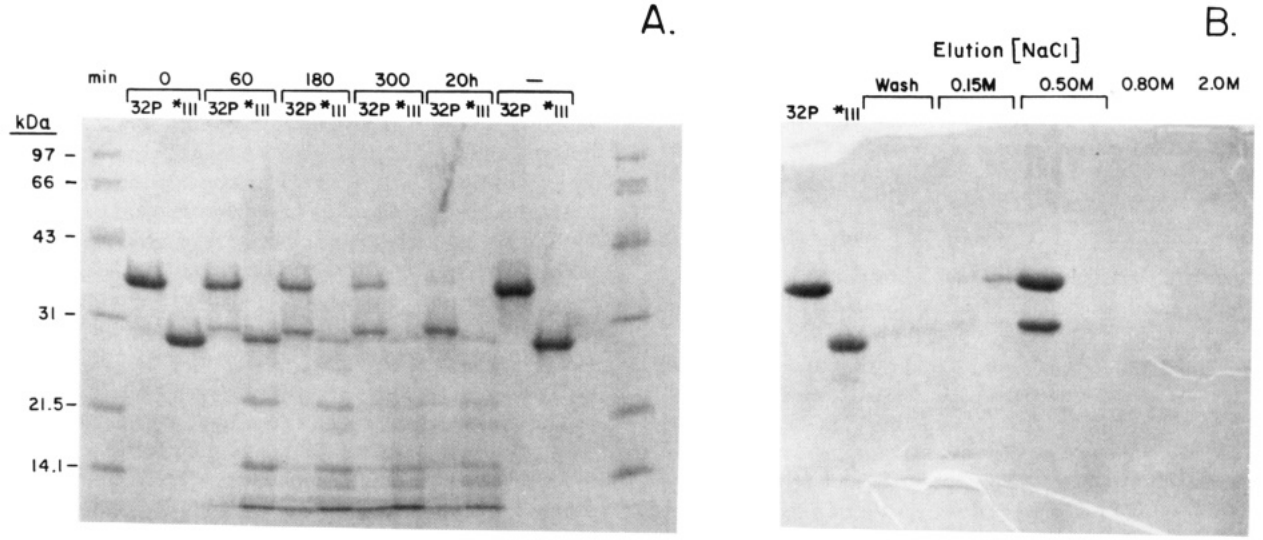

FIGURE 7: (A) Arg-C protease digestion (37 °C) of 32 protein or *III (core domain), as followed by SDS–polyacrylamide gel electrophoresis (16% acrylamide). Lanes 2–11 contained the products of the digestion of 7.1 μg (0.21 nmol) of 32 protein or 7.3 μg (0.27 nmol) of *III by 0.7 μg (≈0.5 unit) of enzyme in 17 mM Tris-HCl buffer, pH 8.1, containing 1 mM Na_2EDTA, 0.7 mM 2-mercaptoethanol, and 17 mM NaCl; digestion durations are indicated. Lanes 1 and 14 contain molecular weight markers (in decreasing molecular weight: rabbit muscle phosphorylase B, bovine serum albumin, hen egg white ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and hen egg white lysozyme). Untreated intact 32 protein and *III are shown in lanes 12 and 13, respectively. (B) SDS–PAGE of salt-eluted fractions of a 32 protein digest with Arg-C protease (3.25 h at 37 °C) chromatographed over a denatured DNA–cellulose column. [NaCl] and markers as indicated.

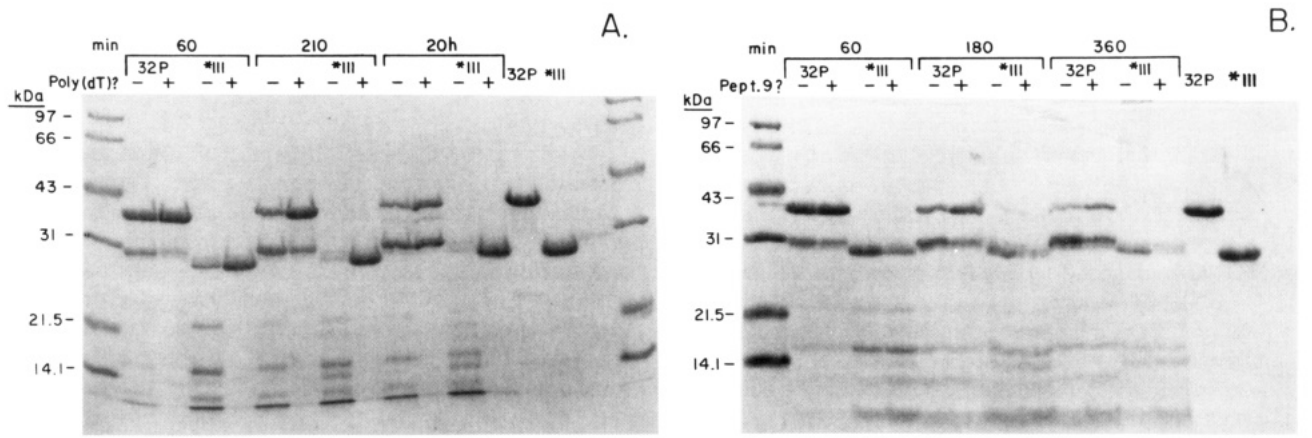

FIGURE 8: (A) Effect of poly(dT) addition on the Arg-C protease pattern of digestion of 32 protein and *III. Digestion was performed as in Figure 7A, except when poly(dT) (3.4 nmol(p), $[poly(dT)]_p$:[protein] ≈ 16) was present. (B) Effect of peptide 9 on Arg-C protease digestion of intact protein and *III. Each well contains the products of the digestion of 0.13 nmol of 32 protein or 0.16 nmol of *III, in the presence or absence of 0.36 nmol of peptide 9 for the durations (at 37 °C) indicated. Undigested intact 32 protein and core domain are also shown. In some of the wells, Arg-C protease can be seen as a faint band, running just above the position of *III.

digestion increased; there appeared to be no obvious change in the pattern of cleavage (Figure 8B). In contrast, the analogous experiment run with intact protein results in a *reduction* in the extent of cleavage (largely limited to chain scission in the vicinity of Lys-253; see above and Figure 8B). This may simply reflect the time-dependent protein-induced precipitation of intact protein (Casas-Finet *et al.*, 1992), which would remove protein from the action of the enzyme. The results with core domain are fully consistent with our model (Casas-Finet *et al.*, 1992), where interaction of the N-terminal domain (or *LAST* peptides) with 32 protein would displace the internal (B′) *LAST* motif from the putative acidic surface, thus leading to increased susceptibility of Arg-111 to proteolysis. The general increase in cleavage we observed is in agreement with this prediction and suggests that the regions around Arg-138 and Arg-46 in 32 protein are also more exposed upon peptide interaction.

The Amino- and Carboxy-Terminal Ends of 32 Protein Are Not Accessible to Exoproteolytic Digestion. The solvent accessibility of the carboxy-terminal end of 32 protein was tested by digestion with yeast carboxypeptidase Y (peptidyl-L-amino acid hydrolase, EC 3.4.16.1). Enzymes from different sources were active as assayed spectrophotometrically (Umetsu *et al.*, 1981) using the chromogenic substrate *N*-carbobenzoxy-Phe-Ala. 32 protein, however, proved resistant to carboxypeptidase activity, either free or bound to poly(dT). On SDS gels, discrete bands appeared which were similar to the pattern seen upon mild tryptic proteolysis of 32 protein and resulted in the production of high molecular weight species of size comparable to *III (not shown). No smear below intact protein or the band attributed to *III was discernible. Overnight digests at variable enzyme concentration produced a similar pattern of bands. If the observed fragments were to originate from carboxypeptidase activity, the average cleavage rate would be ca. 3 residues h^{-1}, a value 10^3–10^4 times lower than that observed from spectrophotometric assays of the enzyme with chromogenic substrates. It is unlikely that the observed digestion products are produced as a result of C-terminal

exoproteolysis of 32 protein, however, as only fragments of defined length without smearing were apparent in sample-overloaded gels. Attempted removal of the putative trypsin-like contaminant by chromatography of carboxypeptidase Y on a soybean trypsin inhibitor-agarose gel yielded an active enzyme solution with no change in the pattern of 32 protein cleavage.

Leu-aminopeptidase from porcine kidney microsomes was active as assayed spectrophotometrically with L-Leu-*p*-nitroanilide. No obvious exoproteolytic activity was detected, even after 36 h at 25 or 37 °C, in digests of 32 protein, 32 protein complexed with poly(dT), or 32 protein complexed with $p(dT)_8$, either electrophoretically (12.5% PAGE) or in spectrophotometric competition experiments with the chromogenic substrate. Conceivably, a small extent of digestion may have occurred without detection via these assays. However, digestion with the enzyme did not generate a product that bound *LAST* peptides with high affinity, as observed with core domain (Casas-Finet *et al.*, 1992). Rather, this material exhibited peptide-induced Trp fluorescence quenching levels comparable to that obtained with intact protein (Casas-Finet *et al.*, 1992). These results indicate that both the N- and the C-terminal residues of 32 protein are refractory to sequential removal by the action of exoproteolytic enzymes.

DISCUSSION

This paper extends our understanding of the domain structure of bacteriophage T4 gene 32 protein and, in particular, of the apparently bifunctional role of the *LAST* motif sequences in both homotypic protein–protein association and protein–nucleic acid interaction. We show that *LAST* peptides are capable of interaction with nucleic acids, although their affinity is significantly lower than the corresponding affinity of these peptides for 32 protein. This is not surprising, since the overall free energy of binding to nucleic acid clearly involves a multitude of interactions, to which the internal *LAST* sequence is likely to contribute only in part. In contrast, our previous report (Casas-Finet *et al.*, 1992) suggests that these small peptides on their own can mimic the involvement of the B-domain in homotypic protein–protein interaction.

Short (6-residue) peptides containing the *LAST* sequence do not possess any obvious secondary structure, whereas, as predicted, the circular dichroism spectrum of the N-terminal 17-mer shows evidence of considerable α-helical structure. Upon binding protein, it is likely that even these short peptides are constrained in a fixed and, as yet, unknown conformation. The results of circular dichroism and dynamic fluorescence experiments described herein indicate that there is no apparent change either in the bulk secondary or gross tertiary structure of the protein upon association with peptide. However, steady-state fluorescence quenching of both intact and truncated protein and proteolysis experiments with core domain indicate that there is a change (conceivably local) in the conformation of this portion of the protein. Consistent with our model, there is an increase in the rate of digestion of core domain by Arg-C endoproteinase when an excess of *LAST* peptide is present. A similar result was not obtained with intact protein, where cleavage under these conditions is largely confined to Arg-4 and the vicinity of Lys-253, locations at or outside the bounds of the core domain. This result is not inconsistent with our model and, furthermore, may simply reflect the tendency of peptide–whole protein complexes to precipitate (Casas-Finet *et al.*, 1992).

Although the use of Arg-C endoproteinase in this study has provided further insights into the domain structure of 32 protein, the enzyme's specificity is obviously not absolute under the conditions we have utilized ([enzyme]:[protein] = 1:10). This is seen in the raggedness of cleavage at both the Lys(3)-Arg(4) and Arg(4)-Lys(5) peptide bonds. Moreover, the hypersensitive tryptic cleavage site at or around Lys(253) [next to another lysine at residue 254 and near Lys(257)] is also attacked by this enzyme. Arg-C endoproteinase-catalyzed cleavage at lysine has been reported previously for a Lys-Lys-Lys cluster within skeletal myosin S-1 (Bertrand *et al.*, 1989).

In contrast to the increased proteolytic sensitivity of core domain upon peptide binding, there is a virtually complete inhibition upon digestion in the presence of single-stranded nucleic acid. This suggests that the cleavage sites within core domain are either in close proximity to the bound nucleic acid or become inaccessible to the enzyme under these conditions. Although all cleavage sites have not been identified, the apparent molecular weights of the major products as seen on SDS–polyacrylamide gels are consistent with the known positions of arginines at residues 46, 111, and 138. In this regard, we note that the set of tyrosine residues that have been identified and/or proposed to have a role in nucleic acid binding span a large portion of the amino acid sequence of the core domain, from position 73 to 186 (Karpel, 1990; Shamoo *et al.*, 1989).

Cleavage at Arg-4 produced a 32 protein fragment with significantly diminished affinity for ss DNA–cellulose. Its elution properties were similar to those of trypsin-generated core domain, suggesting that binding cooperativity is lost upon chain scission at this residue. This would be fully consistent with Arg-C endoproteinase treatment of *LAST* peptides, which abolished their interaction with 32 protein (Casas-Finet *et al.*, 1992). However, loss of the first three or four residues did not produce a truncated protein with functionality identical to that of core domain, since the resulting product was clearly more resistant than trypsin-generated core to further degradation by Arg-C protease. This observation, plus the resistance of the N-terminus to *exo*proteolytic digestion, suggests that the B-domain may bind to the remainder of the polypeptide chain by noncovalent interactions in addition to its attachment via a peptide bond.

As proposed earlier (Casas-Finet *et al.*, 1992), when peptides containing *LAST* sequences interact with 32 protein, the magnitude of its polynucleotide binding cooperativity should be diminished. This would result from peptide–protein association interfering with homotypic protein–protein interactions. Although the effect of peptide on the binding of 32 protein to poly(ϵA) is somewhat subtle, there is a reproducible diminution in the sigmoidal nature of binding isotherms (Figure 4). This is most obvious at the beginning of forward titrations (at relatively low [protein]:$[\text{poly}(\epsilon A)]_p$). Since intact 32 protein tends to slowly precipitate in the presence of peptide, the data points obtained at relatively high [protein]:$[\text{poly}(\epsilon A)]_p$) are less reliable.

Addition of *LAST* peptides has a striking effect on 32 protein–oligonucleotide interactions (Figure 5). We have explained the salt independence of 32 protein–oligonucleotide binding as arising from the substitution of external protein–nucleic acid association involving the internal *LAST* sequence for internal protein–protein interactions. When a *LAST* peptide–32 protein complex binds oligonucleotide, there will necessarily be an *increase* from 1 to 2 in the number of *LAST* sequences involved in ionic interactions. Thus, the observed appearance of an ionic strength dependence in the binding of oligonucleotide to 32 protein complexed with peptide strongly

supports our model. The effect of salt was most strongly seen when the oligonucleotide was added to a mixture of (excess) peptide and protein (cf. Figure 5a,b). Addition of NaCl to a ternary mixture had less dramatic consequences (Figure 5c). We have noted that there might be a kinetic block to salt-induced dissociation of a ternary complex. Alternatively, the effect of NaCl added to the preformed peptide–protein–oligonucleotide complex may reflect, in addition to a peptide-induced salt dependence of oligonucleotide–protein interaction, a comparable oligonucleotide-induced salt dependence in the peptide–protein association.

Although the results presented herein are supportive of our proposed model, further experimentation will be needed to definitively establish the role of the internal (B′) *LAST* sequence in intramolecular protein–protein binding and intermolecular nucleic acid association. In this regard, we are in the process of preparing and characterizing mutant proteins with amino acid changes in this portion of the sequence. The location of the putative acidic surface thought to interact with the B′ segment is at present unknown. Since the C-terminal half of the core domain is distinctively more acidic than the N-terminal half, it is conceivable that the amino acid residues that make up this interactive surface are localized. With its multiplicity of nucleic acid and protein binding properties, in-depth structure–function studies on this model ss-specific nucleic acid binding protein will continue to provide insights of value to other proteins within this class as well as to protein–nucleic acid interactions in general.

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