Bacteriophage T4 gene 32 protein, first isolated and characterized 30 years ago (1), has been a model for single-strand specific nucleic acid-binding proteins (2). 32 protein binds cooperatively to single-stranded nucleic acids, a property essential to its roles in DNA replication, recombination, and repair as well as to its ability to regulate its own expression at the transcriptional level. The protein consists of three domains, each domain are independent of the others, there are indications that there are functional linkages among them. We have observed that the residues within the N-terminal domain most essential for homotypic protein-protein interaction, Lys (3)-Arg (4)-Lys (5)-Ser (6)-Thr (7), are homologous to the aforementioned LAST residues within the core domain, Lys (110)-Arg (111)-Lys (112)-Thr (113)-Ser (114). On the basis of this and other observations, we devised a model for the cooperative binding of 32 protein (8, 9). In the absence of bound nucleic acid, the C-terminal (A) domain has a major role in gene 32 protein interaction with other T4 proteins (10, 11), a property that is the basis of viewing this protein as a “candidate organizing factor” for protein-protein interactions (12). This domain has another very interesting property: it modulates the ability of the protein to lower the melting temperature of natural double-stranded DNA helices. On the basis of its selective binding affinity for single strands, the protein should possess significant helix-stabilizing activity. However, the intact protein fails to lower the Tm of natural dsDNA, suggesting that there is a kinetic block to protein-induced melting (1, 13). Curiously, proteolytic excision of the A domain from the C terminus of 32 protein yields a product (*I) that lowers the Tm of natural dsDNA by ~60 °C, consistent with the removal of the kinetic block. *I promotes rapid renaturation of single-stranded DNA at temperatures below Tm (14). Under certain conditions, full-length 32 protein will renature DNA, but it does not lower Tm under any of the tested conditions (13). Thus, in 32 protein, it appears that the presence of the C-terminal domain establishes the kinetic barrier to DNA helix destabilization. The presence of this domain has a small negative effect on intrinsic nucleic acid binding affinity (3). Although to a first approximation the binding properties of each domain are independent of the others, there are indications that there are functional linkages among them. We have observed that the residues within the N-terminal domain most essential for homotypic protein-protein interaction, Lys (3)-Arg (4)-Lys (5)-Ser (6)-Thr (7), are homologous to the aforementioned LAST residues within the core domain, Lys (110)-Arg (111)-Lys (112)-Thr (113)-Ser (114). On the basis of this and other observations, we devised a model for the cooperative binding of 32 protein (8, 9). In the absence of bound nucleic acid, the intrinsic nucleic acid binding site (4–7). In the crystal structure of this domain complexed to a single-stranded oligonucleotide, the substrate is located within a positively charged cleft (6). Within this cleft is the internal (Lys/Arg)3(Ser/Thr)2 (LAST) motif, which we had previously predicted to be involved directly in nucleic acid binding (8, 9). Although the oligonucleotide is disordered within the crystal, modeling of the substrate clearly showed the basic side chains of Lys (110), Arg (111), and Lys (112) in contact with the nucleic acid phosphates and plausibly positioned the heterocyclic bases in contact with hydrophobic pockets (6). A large number of biophysical experiments indicates that both electrostatic and nonpolar interactions contribute to the overall binding free energy (2). The C-terminal (A) domain has a major role in gene 32 protein interaction with other T4 proteins (10, 11), a property that is the basis of viewing this protein as a “candidate organizing factor” for protein-protein interactions (12). This domain has another very interesting property: it modulates the ability of the protein to lower the melting temperature of natural double-stranded DNA helices. On the basis of its selective binding affinity for single strands, the protein should possess significant helix-stabilizing activity. 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Lisa A. Waidner, Elizabeth K. Flynn, Min Wu, Xing Li, and Richard L. Karpel‡

From the Department of Chemistry and Biochemistry, University of Maryland, Baltimore County, Baltimore, Maryland 21250

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acid, we proposed that there is an internal interaction between core domain LAST residues and an acidic surface located elsewhere on the protein. Upon cooperatively binding single-stranded nucleic acid, these LAST residues now interact with the acidic nucleic acid backbone, and a conformational change is effected whereby the N-terminal B domain is positioned to interact with the acidic surface of the adjacent 32 protein. Thus, these residues are proposed to interact with both an acidic protein surface and the acidic nucleic acid surface; the acidic protein surface is capable of interaction with both N-terminal and core domain LAST sequences. The model links the functionality of nearly identical sequences within the N-terminal and core domains, and as the acidic surface is associated with the C-terminal domain (see below), then an additional function becomes more linked.

Additional evidence that the domains are linked to each other comes from the nucleic acid binding properties of full-length 32 protein with mutations in the N-terminal LAST sequence at positions 3 and 4 (15, 16). Were the domains fully independent, these mutations would be expected to affect only the cooperativity parameter, $\omega$, a measure of homotypic protein-protein interaction. However, several mutants, e.g. Arg (4) $\Rightarrow$ Glu, affect both $\omega$ and $K_{int}$, the affinity for the protein for an isolated binding site on the nucleic acid. Since the nucleic acid-interactive surface within 32 protein is clearly within the core domain, the alteration in $K_{int}$ suggests that there was a change of some sort in this surface brought about by a change in the homotypic protein-protein interaction.

In our ongoing effort to understand how the protein modulates its various functionalities through its structural domains, we provide in this report answers to the following questions. 1) Do the N- or C-terminal domains have an effect on the conformation of the core domain? 2) Is the helix-destabilizing activity of *I a consequence of its nucleic acid binding cooperativity? 3) Is the helix-destabilizing activity of *I a direct consequence of its stoichiometric binding to ssDNA? 4) Can different forms of 32 protein coordinate associate with ssDNA, and can the binding of one form potentiate the binding of another?

We find that the presence of the C-terminal domain decreases the proteolytic susceptibility of residues within the core domain. However, the quenching of the core domain trypophan residue fluorescence by iodide is unaltered by the presence or absence of the terminal domains. These results further support the notion that the C domain modulates accessibility of large substrates to the nucleic acid binding surface, but the overall conformation of the core domain remains largely independent of the flanking regions.

We have studied in detail the helix denaturation and renaturation activities of the three truncated products of gene 32 protein. In addition, we have evaluated the nucleic acid helixdestabilizing activities of gene 32 protein lacking its C terminus (*I) in the presence of full-length protein or core domain (*III, lacking both N and C domains). Our results indicate that under conditions (0.05 M NaCl) where *III shows no measurable effect on $T_m$, the addition of this adduct increases the amount of DNA melting in the presence of a fixed amount of *I. Similar results are observed for the effect of intact protein on *I-effected DNA melting. In both cases, although the hyperchromic change is enhanced, the actual melting temperature increases. In general, the enhancement of hyperchromicity and increase in $T_m$ is monotonic with increasing amounts of intact protein or *III. These results reflect binding of full-length protein or core domain to single-stranded regions created by the action of *I and may be indicative of protein-protein interactions between *I and intact protein or *III.

**EXPERIMENTAL PROCEDURES**

**Cloning, Expression, and Purification of Proteins—**pYS6 (17) and pYS55 (6), expression plasmids encoding the 301-residue whole protein and core domain (*III, residues 22-253), respectively, were digested with EcoRI and NcoI. After gel purification, the EcoRI-NcoI fragment of pYS6, which spans codons 1–81 of the structural gene, was ligated with the NcoI-EcoRI fragment of pYS55, which spans codons 81 to 253. This produced plasmid pEKF1 (encoding *I, residues 1–253), and conversely, the corresponding fragments of pYS6 and pYS55 were ligated to produce plasmid pEKF2 (encoding *II, residues 22–251). The scheme is shown in Fig. 1. Restriction analysis and sequencing of the plasmids confirmed the identity of the clones.

The plasmids were transformed into the AR120 Escherichia coli cell line. Expression was induced with 0.1 mg/ml nalidixic acid when the cells reached an $A_{660}$ of 0.8–0.9, and the proteins were isolated on denatured DNA-cellulose as described previously (7, 17, 18). The chromatographic properties of *I were similar to that of whole protein; peak elution was at $\sim 0.8$ M NaCl. Likewise, both *II and *III eluted at $\sim 0.5$ M NaCl. In all cases, the yield of purified protein was $>30$ mg/liter of cell culture. SDS-polyacrylamide gel electrophoresis indicated that the proteins were of $>95$% purity and had the expected molecular weights. Protein concentrations were determined spectrophotometrically, using $\varepsilon_{280}=3.7 \times 10^4$ M$^{-1}$ cm$^{-1}$ (13). Note that all the Trp and Tyr residues of 32 protein are located within the core domain.

**Other Materials—**Calf thymus DNA (Sigma) and poly(dA-T) (Amersham Pharmacal Biotech) were dissolved in the appropriate buffer, stored at $-20 \degree$C, and used without further purification. Endoproteinase Arg-C from mouse submaxillary gland (EC 3.4.21.35; type XX-S) was obtained from Sigma as a lyophilized powder. Specific activities were typically 570 units/mg of protein (1 unit hydrolyzes $1 \times 10^{-5}$ mol of $N$-$\text{p}$-$\text{tosyl}$-$l$-arginine methyl ester min$^{-1}$ at pH 8.0 and 25 $\degree$C). The enzyme was dissolved in H$_2$O and stored at $-20 \degree$C.

**Proteolysis Experiments—**Proteolysis experiments utilized endoproteinase Arg-C from mouse submaxillary gland and were conducted at 37 $\degree$C with an enzyme/protein mass ratio of 1:10 (7) in 8 mM Tris-HCl, pH 8.1, 28 or 39 mM NaCl (as indicated in the captions to Figs. 2 and 7 and Table III), 0.4 mM EDTA, 4% glycerol, 0.4 mM $\beta$-mercaptoethanol. Part of the NaCl concentration is contributed by the salt content of the commercial enzyme preparation. After incubation for the indicated period of time at 37 $\degree$C, the reaction was terminated with SDS. Each reaction mixture or aliquot was applied to a 10–20% polyacrylamide Tris/Tricine SDS gels (Novex) or, for kinetic experiments, to a standard 15% Tris/glycine SDS gel and then electrophoresed. The gel was stained in 0.1% Coomassie Blue R in 40% methanol, 10% acetic acid and destained in the same solvent. The 15% Tris/glycine gel easily resolved *III from *I.

**Fluorescence Experiments—**Iodide quenching experiments were performed as described by Kelly and von Hippel (19). Fluorescence measurements were performed at 25.0 $\degree$C using 10-mm × 10-mm cells in a
Domain Effects of Gene 32 Protein

SPEX Fluoromax II spectrophotometer. Aliquots of a 1.0 M KI solution treated with $1 \times 10^{-4} \text{ mol Na}_{2}SO_{4}$ to prevent formation of I$_{2}$ (20), were added to 1000-μl solutions containing 1.0 μg intact gene 32 protein or core domain (**III) in 50 mM Na$_2$HPO$_4$, 1 mM Na$_2$EDTA, 1 mM β-mercaptoethanol, pH 7.7. The results were analyzed according to the Stern-Volmer equation, $F/F_0 = 1 + KQ[Q]$, where $F_0$ = emission of unquenched protein, $F$ = emission of quenched protein, [Q] = concentration of quencher, and $K_Q$ is the quenching constant. Plots of $F/F_0$ were subjected to linear regression analysis; the uncertainties in the calculated values of $K_Q$ were obtained from the S.D. of the slopes of these plots.

**DNA Melting and Renaturation Monitored Electrophoretically—**A 75-base pair HindIII restriction fragment of pBR322 (36% G+C) from a 1-kilobase DNA ladder (Life Technologies, Inc.) was isolated by electrophoresis from a dialysis bag and labeled with [$\alpha$-32P]ATP using calf intestinal alkaline phosphatase and T4 polynucleotide kinase. The denatured form of this fragment was prepared by heating the DNA to 95 °C followed by rapid quenching. Reactions were performed in either 5 mM Tris-HCl, pH 7.5, 0.5 mM EDTA (low salt buffer) or in 5 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 50 mM NaCl, and 10 mM MgCl$_2$ (high salt buffer) at 37 °C for 30 min. 1 pmol of DNA (residue) and 15 pmol of protein (except where noted) were used in each reaction. Unless otherwise noted, reactions were stopped by the addition of buffer to create final concentrations of 0.33% SDS, 15% glycerol, and 0.15% bromphenol blue. Electrophoresis was performed on 15 × 15-cm 10% polyacrylamide/1% acrylamide/bisacrylamide in 1 × TAE buffer (21).

**Absorbance-Temperature Profiles—**Teflon-stoppered micro quartz cuvettes containing 100 μl of test solutions were placed in a Gilford 2400–2 spectrophotometer designed to raise the temperature at a constant rate of 25 °C/h. Generally an absorbance-temperature experiment uses a total of 4 cuvettes (a semimicro reference cuvette containing ~1 ml of buffer plus 3 micro cuvettes containing samples of 100 μl). In formulating these solutions, the protein components were mixed before the addition of nucleic acid so as to prevent any order of addition problems. Temperature was continually monitored by means of a calibrated thermistor (Yellow Springs Instruments) inserted through a narrow hole in the stopper of the reference cuvette; absorbance was measured at 260 nm. Absorbance-temperature profiles were graphically differentiated, and $T_{m}$ values were determined two ways, as the inflection point of the transition and as the temperature at which half the DNA was melted. Although the melting profiles were not always symmetric, $T_{m}$ values obtained by either method were within the stated uncertainties. The reproducibility was about ±1 °C, except where otherwise noted.

**RESULTS**

**The Presence of the C-terminal Domain Reduces the Susceptibility to Proteolysis by Endoproteinase Arg-C—**We previously showed that although the core domain of gene 32 protein was susceptible to the action of mammalian endoproteinase Arg-C, the intact protein was refractory to digestion (7, 9). To determine whether the presence of the N- or the C-terminal domain is responsible for this protection, we compared the endo Arg-C digestion behavior of all three truncated products. As seen in Fig. 2, the digestion pattern of *I is similar to that of **III, although the core domain is somewhat more susceptible to the action of the enzyme. The 17- and 11-kDa bands produced by digestion of both **III and *I were previously identified as being generated by cleavage at, respectively, Arg-111 (corresponding to residues 112–253/4) and Arg-138 (corresponding to residues 139–253/4) (7). Both these residues are located within the nucleic acid binding cleft of core domain (6). In contrast, virtually no low molecular weight products are seen when *II is digested, where, as is the case with full-length protein, only small amounts of a band corresponding to *III are observed (9). The interacting oligonucleotide, p(dT)$_5$, inhibits digestion at these residues both in intact and in core protein (Fig. 2). These results indicate that the presence of the C-terminal domain either blocks access to the arginines in the DNA binding cleft or alters the conformation of the protein in the vicinity of these residues.

**Quenching Experiments Indicate That the Local Conformation of Tryptophan Residues within the Core Domain Is Unaffected by the Presence of the N- and C-terminal Domains—**To further probe the influence of the flanking regions on the conformation of core domain, we have determined the effect of the collisional quencher, KI, on the tryptophan fluorescence of intact protein and of core domain. All five Trp residues are located within the core domain. Previous quenching experiments suggest that binding of oligonucleotides exposes a tryptophan side chain to the solvent environment (19). Plots of the ratio of unquenched ($F_0$) to quenched ($F$) fluorescence emission versus quencher concentration (Stern-Volmer) plots are linear and are of identical slope for both forms of the protein (Fig. 3; $K_Q = 5.03 ± 0.09$ and 4.95 ± 0.10 for intact protein and core domain, respectively). These results suggest that the iodide-induced quenching is entirely due to a collisional mechanism, as noted previously for intact protein by Kelly and von Hippel (19) and that the accessibility of the Trp
residues that are quenched is identical for both forms of the protein. Although one or two of the Trp residues in (folded) 32 protein are “dark” (2) and therefore do not serve as reporters for KI quenching experiments, the identical slopes of the Stern-Volmer plots strongly indicates that the local conformation of the remaining tryptophans are equivalent. These results also suggest that the overall conformation of the core domain is essentially independent of the flanking N- and C-terminal domains, a conclusion consistent with a wide body of data (2). Thus, the effect of the C domain on endoproteasease Arg-C digestion is likely due to a reduction in accessibility of Arg-110 and Arg-138 to the active site of the protease.

Intact Gene 32 Protein and Its Three Truncated Forms Differ in Their DNA Helix-destabilizing and -renaturing Activities—As we noted in the Introduction, whole gene 32 protein is unable to lower the $T_m$ of long chain double-stranded DNA, and removal of the C-terminal domain removes this inhibition. Initially, we wished to assess and compare both the nucleic acid helix-destabilizing and renaturing activities of intact and truncated gene 32 protein. To do this, we incubated a $^{32}$P-labeled 75-base pair restriction fragment, either double-stranded or heat-denatured, with each protein for 30 min at 37 °C in either a very low ionic strength buffer (5 mM Tris-HCl, pH 7.5, 0.5 mM EDTA) or in the same buffer with 50 mM NaCl and 10 mM MgCl$_2$. The amount of protein used (15 pmol) was sufficient to fully saturate ssDNA under conditions of high affinity. The two ionic conditions were chosen so that in the absence of protein, the $T_m$ of DNA is above 37 °C in both buffers. Likewise, based on the literature (14) and preliminary melting experiments with calf thymus DNA, we predicted that in the presence of *I, the $T_m$ is below 37 °C at low salt concentrations and above this temperature at high salt concentrations. The results are shown in Fig. 4.

Intact 32 protein is known to have no effect on the melting temperature of natural double-stranded DNA (13). As shown in Fig. 4, panel A, *II also failed to lower the $T_m$ since at low ionic conditions it had no effect on either ss- or dsDNA. At high salt conditions, both intact 32 protein and *II renatured single-stranded DNA to varying degrees (panel A). *I, however, produced dramatic results under both low and high ionic conditions. ssDNA was completely renatured at high salt, and dsDNA was completely denatured at low salt conditions (panel B). Thus, in the low ionic strength buffer, conditions where the temperature is above the $T_m$ of DNA in the presence of *I, this protein stabilizes single-stranded DNA. When incubated in the higher ionic strength buffer with either ssDNA or dsDNA, *I stabilizes dsDNA. Thus, under conditions where intact protein or *II fail to lower $T_m$ or under conditions where these two proteins only partially renature DNA, *I has the property of efficiently bringing DNA to its equilibrium state. The helix-destabilizing activity is largely lost when *I is pre-incubated with proteasease K (not shown). The activity is clearly protein concentration-dependent, since decreasing amounts of *I are less effective in denaturing dsDNA under low salt conditions (panel C). Denaturing activity correlates with site size; with a site size of about 7 (2, 22), saturation of ssDNA should occur at a [*I]/[DNA]$_0$ = 0.14.

The C-terminal Domain Alone Is Responsible for the Kinetic Barrier to DNA Helix Destabilization—To localize the barrier to helix destabilization, a spectrophotometric examination of DNA melting activities of the truncated forms of DNA was undertaken. Under a variety of conditions summarized in Table I, *I displayed helix-destabilizing activity with calf thymus DNA. *II was observed to lower $T_m$ only at very low salt concentrations, ~4 mM NaCl (Table I); only partial melting was observed. No measurable effect on $T_m$ was observed at 50 mM NaCl. Even at very low NaCl concentrations (4.4 mM), no DNA melting was observed with *II up to the point where the protein denatures (50 to 55 °C). Note that the affinities of *II and *III for single-stranded DNA are similar, as are the affinities of intact protein and *I for ssDNA (3, 22). Thus, analogous to intact protein, the likely reason for the failure of *II to lower $T_m$ is the presence of the C-terminal domain.

Intact Gene 32 Protein, Core Domain, and *I Alter the DNA Helix-destabilizing Properties of the *I-truncated Protein: Evidence for Coordinated Function—Although neither intact protein nor *II lowers the $T_m$ of natural dsDNA and *III only partially melts DNA, we have observed that all three proteins modulate the melting activity of *I. Both $T_m$ and the extent of melting (hypermicrometry) are affected. Typical profiles of *I-effected melting of calf thymus DNA

![FIG. 4. DNA helix-destabilizing and renaturing activities of full-length gene 32 protein and of truncated forms lacking the C domain (*I) and the N domain (*II). A $^{32}$P-labeled 75-base pair DNA fragment (1 pmol), double-stranded or heat-denatured, was incubated with protein (15 pmol, except where noted) at 37 °C for 30 min in either low salt buffer (5 mM Tris-HCl, pH 7.5, 0.5 mM EDTA) or high salt buffer (5 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 50 mM NaCl, 10 mM MgCl$_2$). Panel A, in odd-numbered lanes, reactions were begun with single-stranded DNA; in even-numbered lanes, double-stranded DNA was initially present. The proteins and buffers were as follows. Lanes 1 and 2, intact gene 32 protein, high salt buffer. Lanes 3 and 4, *II, low salt buffer. Lanes 5 and 6, *II, high salt buffer. Lanes 7 and 8, no protein present. Panel B, DNA as in panel A. Lanes 1 and 2, *I, low salt buffer. Lanes 3 and 4, high salt buffer. Lanes 5 and 6, no protein present. Panel C, all lanes, DNA was initially double-stranded and was incubated in low salt buffer with the following pmol levels of *I. Lanes 1–6, 11, 5.5, 2.8, 1.4, 0.7, 0.35, respectively. This corresponds to [*I]/[DNA]$_0$ = 0.16, 0.08, 0.04, 0.02, 0.01, 0.005.

| [NaCl] (mM) | Protein | $T_m$ (°C) |
|------------|---------|-----------|
| 50         | *I, 4.4 | 23 ± 2°C  |
| 25         | *I, 4.4 | No DNA melting |
| 5.3        | *I, 4.4 | No DNA melting |
| 4.5        | *III, 4.4 | ~28° |
| 4.0        | *III, 4.4 | No DNA melting |
| 3.3        | *III, 3.3 | ~24° |

- In the absence of protein, $T_m$ = 78 ± 1 °C.
- In the absence of protein, $T_m$ = 67 ± 1 °C.

\[
\text{NaCl Cl}_2\text{NaCl} \quad \text{NaCl} \quad \text{NaCl}_2\text{NaCl} \quad \text{NaCl}_2\text{NaCl}_2
\]
with the additional presence of intact protein or *III are shown in Fig. 5. At 50 mM NaCl, *I decreased the melting temperature of calf thymus DNA from 78 to 23 °C. Under these conditions, none of the other forms of 32 protein has any measurable effect on Tm. However, in the presence of intact protein, *III, or *II, the *I-effected melting profile yielded higher Tm values and increased the hyperchromic change seen upon melting (Table II).

Typical melting profiles of calf thymus DNA with *I and increasing amounts of *III or intact protein are shown respectively in Fig. 5, A and B. With [*I] held constant at 4.4 µM, the increase in Tm and hyperchromicity was monotonic with increasing concentrations of the other protein up to a limiting value (Table II). Similar results were observed with other concentrations of *I (data not shown).

A comparison of the data in Fig. 5B suggests that intact 32 protein is somewhat more efficient than *III in raising the melting temperature and hyperchromicity. For example, with 4.4 µM *I, the Tm was raised to 30 °C in the presence of 2.2 mM whole 32 protein, whereas 4.4 µM *III had to be used to reach this point. Although high levels (8.8 µM) of *I effect essentially complete melting of the DNA (A260 = 0.079 or 40% of the initial DNA absorbance), this was not achieved with 4.4 µM *I and equal or greater concentrations of intact protein or *III.

Although whole 32 protein on its own cannot lower the Tm of natural double-stranded DNA, it can destabilize the double-helix of poly[d(A-T)]. Analogous to the effect of *III on *I-effected calf thymus Tm depression, increasing amounts of the core domain brought about an increase in poly[d(A-T)] Tm and hyperchromicity when added to a fixed level of 32 protein and poly[d(A-T)] under conditions where *III alone does not melt the DNA (Fig. 6).

**Proteolysis Experiments Suggest That the Affinity of *III for Single-stranded DNA Is Increased in the Presence of *I—Endo-proteinase Arg-C digestion experiments were performed to ex-

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**FIG. 5.** Effect of core domain (*III, panel A) and intact protein (panel B) on *I-effected melting of calf thymus DNA. A, [*I] = 4.4 µM and [DNA] = 30 µM (p) in 50 mM NaCl, 7.3 mM Tris-HCl, pH 8.1, 0.37 mM EDTA, 0.37 mM β-mercaptoethanol, and 3.7% glycerol. ■ no *III; ▽, 2.2 µM *III; ▲, 3.3 µM *III. B, [*I] = 2.2 µM and [CT DNA] = 30 µM (p) in 50 mM NaCl, 5.8 mM Tris-HCl, pH 8.1, 0.3 mM EDTA, 0.3 mM β-mercaptoethanol, and 3.0% glycerol. ■ no 32 protein; ▽, 2.2 µM 32 protein; ▲, 4.4 µM 32 protein.

**FIG. 6.** Effect of increasing amounts of *III on 32 protein-effected poly[d(A-T)] Tm and hyperchromicity. [32 protein] = 3.3 mM and [poly[d(A-T)]] = 31 µM (p) in 0.15 M NaCl, 6.1 mM Tris-HCl, pH 8.1, 0.31 mM EDTA, 0.31 mM β-mercaptoethanol, and 3.1% glycerol. A, Tm, B, hyperchromicity.

**TABLE II**

| [*III] (µM) | 0   | 2.2 | 3.3 | 4.4 | 5.5 | 6.6 |
| Tm (°C)     | 23b | 25  | 25  | 32  | 30  | 34  |
| ΔA260       | 0.044 | 0.041 | 0.042 | 0.052 | 0.052 | 0.054 |
| [32 protein] (µM) | 0   | 1.1 | 2.2 | 3.3 | 4.4 | 5.5 |
| Tm (°C)     | 23b | 28  | 30  | 33  | 35  | 33  |
| ΔA260       | 0.040 | 0.045 | 0.062 | 0.045 | 0.057 | 0.050 |
| [*III] (µM) | 0   | 3.4 | 5.1 | 6.7 | 7.1 | 8.7 |
| Tm (°C)     | 26b | 32  | 32  | 32  | 32  | 32  |
| ΔA260       | 0.041 | 0.054 | 0.053 | 0.054 | 0.054 | 0.054 |

a [*I] = 4.4 µM and [CT DNA] = 30 µM (p) in 50 mM NaCl, 7.3 mM Tris-HCl, pH 8.1, 0.37 mM EDTA, 0.37 mM β-mercaptoethanol, and 3.7% glycerol.

b We report Tm of calf thymus DNA in the presence of 4.4 µM *I to be 23 ± 2 °C (Table I). The values listed in this table are those obtained in experiments run simultaneously with the data shown in the same row.

c [*I] = 4.4 µM and [CT DNA] = 30 µM (p) in 50 mM NaCl, 8.8 mM Tris-HCl, pH 8.1, 0.44 mM EDTA, 0.44 mM β-mercaptoethanol, and 4.4% glycerol. A similar trend was seen with 2.2 µM *I.

d [*I] = 4.4 µM and [CT DNA] = 30 µM (p) in 50 mM NaCl, 5.3 mM Tris-HCl, pH 8.1, 0.26 mM EDTA, 0.26% β-mercaptoethanol, and 2.6% glycerol.
The protease Arg-C digestion as seen on the SDS gel was similar to DNA when *I was present. The pattern of products generated are consistent with the binding of *III to single-stranded CT DNA. No protection of *III. These results with a 1:1 ratio of *I and *III also showed very strong proteolysis. Experiments using the same buffer conditions but with *III was further slowed down. Under these conditions, the digestion of *III and at a [*I]:[*III] ratio of 1.5:1, the digestion rate is a general inhibitory effect of double-stranded DNA on the presence of *I significantly reduces the digestion of *III. There were a few proteolysis experiments was lower (39 mM NaCl; Tm = 28 °C), so that after the 30-min incubation at 37 °C the melting process would have been complete.

As shown in Table III and Fig. 7, in the absence of DNA the presence of *I significantly reduces the digestion of *III. There is a general inhibitory effect of double-stranded DNA on the digestion of *III, and at a [*I]:[*III] ratio of 1.5:1, the digestion of *III was further slowed down. Under these conditions, the digestion of *I was almost totally inhibited. These results suggest that *I promoted the binding to DNA of some of the core domain molecules, resulting in their further protection against proteolysis. Experiments using the same buffer conditions but with a 1:1 ratio of *I and *III also showed very strong protection by DNA of *I but almost no protection of *III. These results are consistent with the binding of *III to single-stranded CT DNA when *I was present. The pattern of products generated by endo Arg-C digestion as seen on the SDS gel was similar to those observed in proteolysis experiments with oligonucleotides (7), indicating that the interactions between protein and nucleic acid were probably the same in both cases.

### DISCUSSION

In this paper, we have further explored the effects of the C and N domains on the nucleic acid-interactive properties of gene 32 protein. The susceptibility of *III and *I to the action of mammalian endoprotease Arg-C indicates that the presence of the C domain inhibits cleavage at Arg-111 and Arg-138, both located within the nucleic acid binding cleft of core domain (6). Residue 111, which is protected to a greater degree, is located within the internal LAST motif (7). Likewise, the protective effect against digestion by a binding oligonucleotide, p(dT)5, is observed for both *III and *I. Thus, the presence of the N domain does not bring about any changes in the accessibility of the nucleic acid binding site to the action of the enzyme.

On the other hand, when the C domain is part of the polypeptide chain, there is a major effect on proteolysis. We previously demonstrated that the core domain within whole protein is refractory to digestion by endo Arg-C (9). We have now observed the same result with *III. Thus, in this truncated form as well as in the full-length protein, the C domain clearly protects the core against the action of this protease.

To further probe the effect of the C-terminal domain on the core, we conducted tryptophan quenching experiments with iodide ion. All the tryptophan residues are located within the core domain, and one, residue 144, is located close to the ssDNA binding groove (6). In this study, we have obtained identical Stern-Volmer quenching plots for full-length protein and core domain. Thus, the local conformation of the reporting tryptophan residues is unaffected by the C domain. Conceivably, there could be a difference in the environment of a dark tryptophan (where the fluorescence is completely quenched upon protein folding).

The C-terminal third of gene 32 protein (residues 201 through 301), corresponding to 50 residues of core domain and the proteolytically defined C-terminal domain is very acidic and is a potential mimic of single-stranded DNA. In this regard, Gold and co-workers (10) demonstrate that this portion of gene 32 protein is particularly immunogenic, and they isolated several monoclonal antibodies with epitopes located within the C-terminal 100 amino acids. These antibodies strongly cross-reacted with single-stranded DNA. Conceivably, the C-terminal third of the protein or portions of it could mimic ssDNA and interact with core domain at the ssDNA binding groove. The binding clef can easily accommodate a polypeptide chain. This putative interaction is consistent with the protection against proteolysis at Arg-111 and Arg-138 seen in full-length protein.

### TABLE III

| Effect of *I on *III proteolysis and vice versa |
|-----------------------------------------------|
| Without CT DNA | With CT DNA | Rate with CT DNA |
|----------------|-------------|-----------------|
| [*I]* | [*III]* | [*I]/[*III]* | Without CT DNA | With CT DNA | Rate with CT DNA |
| *I* absent | -0.0112 ± 0.0007 | -0.0093 ± 0.0008 | 0.533 ± 0.089 |
| [*I] 1.0 | -0.0059 ± 0.0006 | -0.0052 ± 0.0005 | 0.575 ± 0.122 |
| [*I] 1.5 | -0.0027 ± 0.0006 | -0.0024 ± 0.0004 | 0.695 ± 0.127 |

*a *III = 25 μM (CT DNA), 108 μM (if present), [Arg-C] = 0.06 μg/ml, in 39 mM NaCl, 18 mM Tris-HCl, pH 8.1, 0.9 mM EDTA, 0.9 mM β-mercaptoethanol, and 9% glycerol.

*b Uncertainties are based on the S.D. of the calculated slopes of the individual decay plots.

*c [*III] = 12 μM, [*I] = 12 μM, (CT-DNA) = 108 μM (if present), [Arg-C] = 0.06 μg/ml, in 39 mM NaCl, 18 mM Tris-HCl, pH 8.1, 0.9 mM EDTA, 0.9 mM β-mercaptoethanol, and 9% glycerol.

d [*III] = 10 μM, [*I] = 15 μM, (CT DNA) = 108 μM (if present), [Arg-C] = 0.06 μg/ml, in 39 mM NaCl, 18 mM Tris-HCl, pH 8.1, 0.9 mM EDTA, 0.9 mM β-mercaptoethanol, and 9% glycerol.
and *II as well as the slightly greater intrinsic binding affinity ($K_{\text{int}}$, for isolated binding sites) of *I and *III relative to *II and intact protein (2, 3, 22).

As we noted in the Introduction, we have proposed that the N-terminal and core domain LAST sequences could alternate binding the same acidic surface, corresponding, respectively, to intermolecular and intramolecular protein-protein interactions (8). The N-terminal LAST sequence of a cooperatively bound gene 32 protein monomer would be bound to the acidic surface of the adjacent DNA-bound monomer. When not bound to nucleic acid, the core domain LAST sequence was envisaged to be of the adjacent DNA-bound monomer. When not bound to nu-
gene 32 protein monomer would be bound to the acidic surface (8). The N-terminal LAST sequence of a cooperatively bound binding the same acidic surface, corresponding, respectively, to intact protein (2, 3, 22).

Our results suggest that this inhibitory activity can be mediated by protein-protein interactions. In experiments where there was enough *I to bring about the melting of only a portion of calf thymus DNA, the addition of increasing amounts of either full-length or *II or *III protein monotonically increased the fraction of DNA undergoing denaturation. Since neither intact protein nor *II is capable of melting natural dsDNA, the kinetic block is at least partially overcome. In the case of *III, there is no kinetic block, but under the conditions employed (0.05 M NaCl, temperatures below 45 °C), the relatively low affinity of the protein for ssDNA generates no $T_m$ depression. In addition to overcoming the kinetic block, there is an apparent increase in the noncooperative binding affinity of *III (and *II) for single-stranded DNA. Along with the effect on hyperchromicity, the observed $T_m$ was seen to increase with increasing levels of full-length, *II, or *III protein.

We can think of two explanations for these effects. One possibility is that as *I brings about the melting of the double helix, the newly formed single strands are now free to bind the other forms of the protein. The *I monomers can rearrange themselves such that there is available free single-stranded DNA to which the other protein can bind. In a sense, *I serves as the "engine" for strand separation. This explanation, however, does not account for the potentiation of melting activity by *II and *III, which bind noncooperatively and more weakly (by three or more orders of magnitude) than intact protein. Alternatively, *I may directly interact with any of the other three forms of the protein, in effect achieving a mingling of (at least two) different forms of the protein while bound to ssDNA. This would be energetically more favorable than *I alone binding to ssDNA, since there will be additional protein-DNA and protein-protein interactions resulting from the mingling. The inhibitory effect of double-stranded DNA on the proteolysis of *III in the presence of *I can be explained by the additional binding of core domain to ssDNA formed upon melting of the double helix. The overall affinity of the mixed protein-ssDNA complex might be lower than for a pure *I-DNA complex, thus reducing the $T_m$ depression (as was observed).

Although this is the first report of cooccupancy on ssDNA of different forms of gene 32 protein, it is known that intact 32 protein and *I can each bind ssDNA simultaneously with T4 gene 59 protein (24). In the case of intact protein, the C-terminal domain affects both the binding of gene 59 protein to ssDNA and the morphology of 32 protein-59 protein complexes (24). In the present work, although the C-terminal domain might modulate the affinity for DNA, the protein-protein interactions that occur are clearly dependent on the N-terminal domain.

Given the involvement of the C domain in both heterotypic protein-protein association and modulation of DNA melting, it is interesting to speculate about a relationship between these two activities. Conceivably, binding of the C domain to another protein, e.g. T4 DNA polymerase, might prevent interaction of residues in the C-terminal third of the protein with the DNA binding site, and induce a conformational change that mimics the removal of this portion of the protein, thus promoting melting activity. With the mingling effect that we have demonstrated, this activity would be applied not only to the gene 32 protein directly in contact with the polymerase but also to adjacent 32 proteins. A large number of heterotypic protein-protein contacts have been demonstrated for 32 protein (11, 12, 25), so the consequences of such an effect would be profound.

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