Structural Changes in the T4 Gene 32 Protein Induced by DNA and Polynucleotides*

(Received for publication, August 19, 1977)

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Alterations in the structure of the DNA-binding protein specified by gene 32 of bacteriophage T4 have been detected using partial trypsin digestion as a conformational probe. Limited tryptic hydrolysis of the gene 32 protein removes a fragment ("B" region), of 21 amino acids from the NH terminus and a 6,200-dalton fragment ("A" region) from the COOH terminus. Poly(dT), poly(dC), and single-stranded DNA increase the rate of tryptic hydrolysis of the "A" region but decrease the rate of tryptic hydrolysis of the "B" region. Oligonucleotides, which are too short to permit cooperative binding of the gene 32 protein, do not alter the rate of tryptic hydrolysis of either the "A" or "B" regions. A model which accounts for these findings requires that the "B" region be involved in gene 32 protein:DNA interactions when the gene 32 protein:DNA complex is formed. As a consequence of the gene 32 protein:DNA interaction, the "A" region should be able to participate more effectively in vivo and in vitro with other proteins involved in T4 DNA metabolism.

Proteins which bind single-stranded DNA have been isolated from several organisms and include the Escherichia coli DNA unwinding protein (1), the gene 5 protein of bacteriophage fd (2), and the gene 32 protein of bacteriophage T4 (3). Certain properties of the gene 32 protein distinguish it from the DNA binding proteins in E. coli and bacteriophage fd. For instance, whereas the binding of the gene 32 protein to closed circular single-stranded DNA causes the circle to collapse into a rod (4), binding of the gene 32 protein allows the circle to remain topologically intact (5). The difference may be accounted for by the postulated mode of self-aggregation that these proteins exhibit. At high protein concentrations the gene 32 protein forms long linear aggregates in which the DNA binding sites are thought to be aligned (6). Cavalieri et al. (7) propose that in the gene 5 protein-single-stranded fd DNA complex the DNA is folded into a rod as a result of the symmetrical DNA binding sites. The E. coli DNA unwinding protein is a tetramer (1) and in contrast to the gene 32 protein, the E. coli DNA-unwinding protein contracts the circumference of the circular fd DNA (8).

The DNA binding protein coded by gene 32 of bacteriophage T4 plays a prominent role in normal T4 DNA metabolism. It is required for DNA replication (9), repair (10), and genetic recombination (11). Although the precise function of the gene 32 protein in each of these processes is not understood, it probably depends on the ability of 32P to bind cooperatively to single-stranded DNA (3, 5, 12). The preferential binding to single-stranded nucleic acids results in a decrease of the thermal denaturation temperature of double-stranded polymers such as poly(dA-dT) when mixed with the T4 DNA binding protein (3). For this reason the gene 32 protein is often described as a DNA unwinding protein. It has been suggested that the primary role of the gene 32 protein in T4 DNA replication is to produce a localized unwinding of the DNA in front of the replication fork (3). Support for this concept comes from the observation of Nossal (13) that gene 32 protein will allow some strand displacement synthesis by the T4 DNA polymerase on nicked double-stranded templates, whereas the DNA polymerase otherwise cannot proceed except on denatured DNA strands.

The native T4 DNA unwinding protein contains two regions which are particularly susceptible to hydrolysis with a wide variety of proteinases. These so-called "A" and "B" regions have molecular weights which have been reported to be 8,000 and 1,000, respectively, and are at the opposite ends of the protein (14). Removal of the "A" region enables the gene 32 protein to bind double-stranded DNA cellulose (14) and to denature T4 DNA (15), properties not exhibited by the intact protein. T4 DNA binding protein which lacks the "B" region does not bind to double-stranded DNA cellulose and can be eluted at a lower salt concentration than the native protein from a single-stranded DNA cellulose column. Based on these
in ice water immediately before use. The digestion was allowed to proceed for 60 min at room temperature at which time 2 μl of diisopropylfluorophosphate was added. The DNA was digested by adding 0.09 ml of 1.0 M MgCl₂, 0.18 ml of 0.1 M CaCl₂, and 0.2 ml (2 units) of pancreatic deoxyribonuclease I dissolved by heating at 100°C for 5 min in 0.5 ml NaCl wash and an approximately equal amount of 32P*-A was found in the 0.5 M NaCl wash. The overall yield of 32P*-A was 20 to 25% of theoretical. Higher yields of 32P*-A can probably be attained by decreasing by 50% the amount of DNA added to the reaction mixture.

**Sequence Determination** — Manual NH₂-terminal sequence analysis was performed in sodium dodecyl sulfate as described by Weiner et al. (18). A Beckman model 890C Sequencer was used for the automatic NH₂-terminal sequencing. The thiazolinone derivatives were converted to phenylthiohydantoins by heating in 1.0 N HCl at 80° for 10 min. The phenylthiohydantoin derivative was extracted into ethyl acetate and a portion was chromatographed on polyamide sheets as described by Summers et al. (19). The remainder of the solution was evaporated to dryness and converted back to the free amino acid by heating at 100°C for 5 min with 0.5 M hydroiodic acid. The amino acid was then identified using a Beckman model 121M amino acid analyzer.

The gene 32 protein and the two stable cleavage products derived from it by partial trypsin hydrolysis were oxidized with performic acid using the procedure of Glazer et al. (20). Carboxypeptidase A and B were then used to release amino acids from the COOH terminus of the oxidized gene 32 protein and its cleavage products. The two enzymes were diluted into 0.1 M NaHCO₃, as described by Goldsmith and Konigsberg (21). In the case of the oxidized gene 32 protein, each reaction mixture contained 7.5 μl of protein (0.1 unit of each carboxypeptidase A and B) and was added to a total volume of 0.1 ml of 0.1 M NaHCO₃ and 0.04% sodium dodecyl sulfate. After boiling for 2 min, 10 μl (0.40 unit) each of carboxypeptidase A and B were added and the sample was incubated for varying times at 37°C. The reaction was stopped with 10 μl of 6 N HCl and each sample was alkylated prior to resuspending in the pH 2.2 buffer required for the amino acid analyzer. The oxidized fragments of the gene 32 protein were treated in the same way with the exception that only 0.1 unit of each carboxypeptidase enzyme was used.

**Trypsin Digestion and Polyacrylamide Gel Electrophoresis of Gene 32 Protein** — Reaction mixtures for partial digestion of the T₄ DNA unwinding protein contained 20 μm Tris/HC1, pH 8.0, 0.25 mg/ml of gene 32 protein, 25 μM NaCl, 3% glycerol, and various concentrations of several different oligo- and polynucleotides. After reaction at room temperature the reaction was terminated by the addition of 10 μl of trypsin to give a total volume of 50 μl. After 60 min at room temperature the digestion was stopped by the addition of 25 μl of 188 mM Tris/HC1, pH 6.8, 6% SDS, 30% glycerol, 15% β-mercaptoethanol, and 0.063% bromthymol blue. After boiling for 3 min, samples were then frozen prior to gel electrophoresis. Analytical SDS-slab gel electrophoresis was performed on 15% polyacrylamide gels according to the procedure of Laemmli (22). Samples (75 μl) were carefully layered beneath the electrophoresis buffer in the 1.0 M NaCl wash. The overall yield of 32P*-A was 20 to 25% of theoretical. Higher yields of 32P*-A can probably be attained by decreasing by 50% the amount of DNA added to the reaction mixture.

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**Single-stranded DNA and Polynucleotides** — Bacteriophage fd was prepared using the procedures described by Goldsmith and Konigsberg (21). After differential centrifugation, the phage pellet was resuspended overnight in 0.3 ml of N1 medium. The solution was then layered over a 5 to 20% sucrose gradient in N1 media which had been made in a Beckman centrifuge tube (0.5 x 2.0 inches).
### Results

**Amino Acid Sequence at NH$_2$- and COOH-termini of Gene 32 Protein** – The amino acid composition of 32P was determined and the results in Table I are in good agreement with previously reported compositions of 32P (6, 25). Dansyl-Edman degradation of the purified gene 32 protein gave the NH$_2$-terminal sequence: Met-Phe-Lys-. Automated amino acid sequence analysis confirmed this result and, as shown in Table II, extended the sequence to: Met-Phe-Lys-Arg-Lys-Ala-Thr-Gly-Phe-Ser-. The time course of the carboxypeptidase digestion of the gene 32 protein gave the NH$_2$-terminal sequence as found in the native protein. The 27,500-dalton species lacks a small region, referred to by Moise and Hosoda (14) as the “A” peptide. The conditions described under “Experimental Procedures” allow the isolation of both the 28,800- and 27,500-dalton fragments of 32P in homogeneous form as illustrated by the SDS-polyacrylamide gel in Fig. 2B.

### Table I

| Amino Acid | 32P | 32P*-A | "A" | 32P*(A + B) | "B" |
|------------|-----|--------|-----|------------|-----|
| Cysteine   | 6.4 | 4.7    | 0   | 4.5        | 0   |
| Aspartic   | 53.6| 36.5   | 17.1| 35.5       | 2   |
| Threonine  | 14.0| 10.6   | 3.4 | 9.9        | 1   |
| Serine     | 24.3| 17.3   | 7.0 | 16.3       | 2   |
| Glutamic   | 32.2| 29.6   | 2.6 | 28.4       | 2   |
| Proline    | 10.5| 10.1   | 0.4 | 8.9        | 0   |
| Glycine    | 21.1| 19.3   | 1.8 | 18.1       | 1   |
| Alanine    | 27.0| 22.1   | 4.9 | 18.3       | 5   |
| Valine     | 20.8| 17.2   | 3.6 | 17.8       | 0   |
| Methionine | 9.8 | 6.9    | 2.9 | 6.1        | 1   |
| Isoleucine | 10.8| 9.7    | 1.1 | 10.5       | 0   |
| Leucine    | 21.5| 14.7   | 6.8 | 13.5       | 2   |
| Tyrosine   | 8.5 | 7.9    | 0.6 | 8.3        | 2   |
| Phenylalanine | 18.6| 15.2 | 3.4 | 14.9       | 1   |
| Histidine  | 2.5 | 2.6    | 0   | 2.6        | 0   |
| Lysine     | 35.1| 31.9   | 3.2 | 29.1       | 4   |
| Arginine   | 4.5 | 4.0    | 0.5 | 3.2        | 1   |
| Tryptophan | 5.5 | 5.5    | 0   | 5.5        | 0   |

| Amino Acid | Yields |
|------------|--------|
| Cysteine   | 8.6    |
| Aspartic   | 35.5   |
| Threonine  | 9.9    |
| Serine     | 17.8   |
| Glycine    | 18.1   |
| Alanine    | 18.3   |
| Valine     | 17.8   |
| Methionine | 6.1    |
| Isoleucine | 10.5   |
| Leucine    | 13.3   |
| Tyrosine   | 8.3    |
| Phenylalanine | 9.5   |
| Histidine  | 2.6    |
| Lysine     | 29.1   |
| Arginine   | 3.2    |
| Tryptophan | 5.5    |

### Table II

| Residue | Amino Acid | Yield |
|---------|------------|-------|
| 1       | Methionine | 68    |
| 2       | Phenylalanine | 190 |
| 3       | Lysine     | 93    |
| 4       | Arginine   | 6.5   |
| 5       | Lysine     | 38    |
| 6       | Alanine    | 32    |
| 7       | Threonine  | 35    |
| 8       | Alanine    | 36    |
| 9       | Glx        | 22    |
| 10      | Leucine    | 23    |
| 11      | Alanine    | 39    |
| 12      | Alanine    | 48    |
| 13      | Glx        | 18    |
| 14      | Alanine    | 37    |
| 15      | Lysine     | 17    |
| 16      | Leucine    | 25    |
| 17      | Asx        | 19    |
| 18      | Glycine    | 11    |
| 19      | Asx        | 8.3   |
| 20      | Lysine     | 15    |
| 21      | Glycine    | 10    |
| 22      | Phenylnalanine | 9.5 |
| 23      | Serine     | 16    |
| 24      | Glx        | 15    |
| 25      | Asx        | 12    |
| 26      | Lysine     | 9.4   |

2. By increasing the trypsin concentration (Fig. 2A, Lanes 3 to 5) the amount of the 28,800 product increases and an additional band with a molecular weight of 27,500 appears. All of the 32P can be converted to the 27,500-dalton derivative (Fig. 2A, Lane 7) if enough trypsin is used. The conditions described under "Experimental Procedures" allow the isolation of both the 28,800 and 27,500 trypptic products of 32P in homogeneous form as illustrated by the SDS-polyacrylamide gel in Fig. 2B.

Dansyl-Edman degradation of the 28,800-dalton species gave the same NH$_2$-terminal sequence as found in the native protein (Fig. 3). The time course of carboxypeptidase digestion is identical for both the 28,800- and 27,500-dalton fragments (Fig. 2B) and is consistent with the COOH-terminal sequence: Thr-Ala-Lys-COOH. This differs from the COOH-terminal sequence of the native protein (Fig. 3) so it appears that both the 27,500- and 28,800-dalton fragments lack a 6,200 molecular weight piece which must be at the COOH terminus of the intact protein. This region was referred to as the "B" peptide by Moise and Hosoda (14) and therefore we will refer to it as the "B" peptide. The "A" peptide by Moise and Hosoda as the "B" peptide (14), which is present at the NH$_2$ terminus of the intact protein. The 27,500-dalton fragment, 32P*(A + B), has the sequence Gly-Phe-Ser- at its NH$_2$-terminus. This sequence corresponds to residues 22

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*Serine, asparagine, and glutamine elute very close together off the amino acid analyzer and cannot be differentiated on this basis alone.*
Fig. 1. A, carboxypeptidase digestion of performic acid-oxidized gene 32 protein. The oxidized 32P was digested with 0.4 unit each of carboxypeptidase A and B/7.5 nmol of 32P as described under "Experimental Procedures." The time course for the release of free amino acids shown above is consistent with a COOH terminus of -Thr-Ala-Phe-X-Lys-Val-Leu-COOH for 32P where X is Ser, Gln, or Asn.

B, carboxypeptidase digestion of performic acid-oxidized 32P*-E

FIG. 2. A, partial trypsin digestion of the gene 32 protein. Gene 32 protein at a concentration of 0.24 mg/ml was incubated at 24°C for 60 min with increasing concentrations of trypsin in 20 mM Tris/HCl, pH 8.0, 25 mM NaCl, and 5% glycerol. From left to right, beginning with Lane 1, the following trypsin concentrations were used: 0, 0.10, 0.20, 0.40, 1.0, 5.0, and 20.0 μg/ml. The reaction was stopped as described under "Experimental Procedures" and 50 μl of each digest was run on an SDS-polyacrylamide gel. Standard proteins (bovine serum albumin, ovalbumin, deoxyribonuclease I, chymotrypsinogen, and myoglobin) were run in Lane 8. B, SDS-gel electrophoresis of 32P, 32P*-A, and 32P*-(A + B). Lane 1 standard proteins: (bovine serum albumin (66,000), ovalbumin (43,000), deoxyribonuclease I (31,000), chymotrypsinogen (25,700), myoglobin (17,600) and lysozyme (14,400)); Lane 2, 32P*-(A + B); Lane 3, 32P*-A; Lane 4, 32P.

A and 32P*-(A + B). The oxidized cleavage products of 32P were digested with 0.1 unit each of carboxypeptidase A and B/7.5 nmol of fragment as described under "Experimental Procedures." The time course for the release of free amino acids shown above is consistent with a COOH terminus of (Thr, Ala)-Ala-Lys-COOH for both 32P*-A (□) and 32P*-(A + B) (○).

Through 24 in the native protein (Fig. 3). Thus 32P*-(A + B) is missing the first 21 amino acids ("B" region) from the NH₂ terminus as well as the 6,200-dalton COOH-terminal "A" region. These are the only two regions that can be removed by mild proteolysis of the native protein. 32P*-(A + B) is probably identical with the 32P*-III species described by Moise and Hosoda (14). The amino acid composition of the two cleavage products, 32P*-A and 32P*-(A + B), is shown in Table I. As shown in this table the "A" region contains a high proportion of Asx residues, in agreement with Anderson and Coleman (25). In contrast to the "A" region, the "B" region contains a high proportion of lysine and arginine residues. Isoelectric focusing in polyacrylamide gels (data not shown) reveals that 32P*-(A + B) is more basic than 32P.

Single-stranded DNA Enhances Rate of Proteolysis of "A" Region and Depresses Rate of Proteolysis of "B" Region of 32P. The SDS-polyacrylamide gel densitometry scans shown in Fig. 4A demonstrate that single-stranded DNA increases the rate of trypsin hydrolysis of the COOH terminal "A" region from the gene 32 protein. At low trypsin concentrations (Scan a in Fig. 4A) about 50% of the protein is still intact at the end of the digestion in the absence of DNA while the remainder is in the form of 32P*-A or 32P*-(A + B), the latter appearing as a shoulder on the 32P*-A peak. As shown in Scans b to d in the same figure, the addition of increasing amounts of single-stranded DNA results in an accelerated rate of removal of the "A" region. This effect is most pronounced when the base/gene 32 protein ratio is above 4.6 as shown by the lack of a 32P peak in Scan c (Fig. 4A). If the trypsin concentration is increased 32-fold, then in the course of the 60-min digestion, all of the native protein is converted to 32P*-(A + B) as shown by the top scan in Fig. 4B. In contrast, if single-stranded DNA in a base:32P ratio of 4.6 is included in an otherwise identical reaction mixture (Scan b, Fig. 4B) approximately 70% of the 32P retains the "B" region at the end of the digestion. Fig. 4B (Scans c and d) demonstrates that protection of the "B" region from trypsin digestion be-
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"B" REGION

\[ \text{NH}_2-\text{Met-Phe-Lys} \rightarrow \text{Lys-Gly-Phe-Ser} \rightarrow \text{(Thr, Ala)-Ala-Lys} \rightarrow \text{Lys-Val-Leu-COOH} \]

\[
\begin{array}{c}
32^P - A (28,800) \\
\text{NH}_2-\text{Met-Phe-Lys} \rightarrow \text{Lys-Gly-Phe-Ser} \rightarrow \text{(Thr, Ala)-Ala-Lys-COOH}
\end{array}
\]

\[
\begin{array}{c}
32^P - (A+B) (27,500) \\
\text{NH}_2-\text{Gly-Phe-Ser} \rightarrow \text{(Thr, Ala)-Ala-Lys-COOH}
\end{array}
\]

Fig. 3. Summary of the partial amino acid sequence data on 32P, 32P*-A, and 32P*-(A + B).

"A" REGION

\[
\begin{array}{c}
\text{32P} (35,000) \\
\text{NH}_2-\text{Met-Phe-Lys} \rightarrow \text{Lys-Gly-Phe-Ser} \rightarrow \text{(Thr, Ala)-Ala-Lys} \rightarrow \text{Lys-Val-Leu-COOH}
\end{array}
\]

Fig. 4. A, densitometer tracings of SDS-polyacrylamide gels showing the effect of increasing concentrations of fd DNA on the rate of trypsin hydrolysis of the "A" region of 32P. The gene 32 protein was digested in the absence (Scan a) and the presence of increasing concentrations of fd DNA (Scans b to d) as detailed under "Experimental Procedures." The trypsin concentration was 0.27 µg/ml throughout and the base ratio of fd DNA to 32P in b, c, and d was 4.6, 9.2, and 18.4, respectively. B, densitometer tracings of SDS-polyacrylamide gels showing the effect of increasing concentrations of fd DNA on the rate of trypsin hydrolysis of the "B" region of 32P. The gene 32 protein was digested in the absence (Scan a) and the presence of increasing concentrations of fd DNA (Scans b to d) as described under "Experimental Procedures." The trypsin concentration was 8.6 µg/ml throughout and the base ratio of fd DNA to 32P, in b, c, and d was 4.6, 9.2, and 18.4, respectively.

The homopolynucleotides in Table IV fall into three distinct classes. The difference was observed between ribo- and deoxyribonucleotides. The same experiment was repeated in the presence of a high concentration of trypsin where all of the "A" and most of the "B" region were removed during digestion. The last column in Table IV indicates that, in the absence of added polynucleotides, 94% of the gene 32 protein is converted to 32P*-(A + B). Of the homopolynucleotides tested, only poly(dT) and poly(dC) were able to reduce the rate of conversion of 32P*-(A + B) to 32P*-A and 32P*-(A + B) and thus provide protection of the "B" region from tryptic cleavage. None of the polyribonucleotides, including poly(C), showed this effect.

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Homopolynucleotides Except Those Containing Guanine Facilitate Tryptic Hydrolysis of "A" Region. Only Polydeoxynucleotides Protect "B" Region against Tryptic Digestion – In view of the reported absence of base specificity in the binding of the gene 32 protein to DNA (26), it was assumed that all homopolynucleotides would have the same effect on the rate of trypsin digestion of 32P. As shown in Table IV, this was not the case. With the low concentration of trypsin used in the first experiment, 21% of the "A" region is removed in the absence of any added polynucleotide. Homopolynucleotides, except those containing guanine, increase the rate of proteolysis of the "A" region by 3- to 4-fold as evidenced by the data in the second column of Table IV. No significant

**Table III**

Partial trypsin digestion of 32P in presence of single-stranded DNA

| Base/32P mole ratio | "A" region released with low trypsin | "B" region released with high trypsin |
|---------------------|-------------------------------------|-------------------------------------|
| -fd DNA             | 43                                  | 95                                  |
| fd DNA/32P = 4.6    | 78                                  | 28                                  |
| fd DNA/32P = 9.2    | 100                                 | 63                                  |
| fd DNA/32P = 18.4   | 100                                 | 87                                  |

**Table IV**

Partial trypsin digestion of 32P in presence of homopolynucleotides

| Polynucleotide | "A" region released with low trypsin | "B" region released with high trypsin |
|----------------|-------------------------------------|-------------------------------------|
| Control        | 21                                  | 94                                  |
| Poly(dT)       | 78                                  | 97                                  |
| Poly(dC)       | 85                                  | 91                                  |
| Poly(dA)       | 87                                  | 94                                  |
| Poly(dG)       | 23                                  | 100                                 |
| Poly(U)        | 88                                  | 92                                  |
| Poly(C)        | 79                                  | 92                                  |
| Poly(A)        | 76                                  | 88                                  |
| Poly(G)        | 32                                  | 100                                 |
classes with respect to their effect on the partial trypsin hydrolysis of the gene 32 protein. Poly(dG) and poly(dG) are unique in that they do not appear to affect the rate of trypsin cleavage of either the "A" or "B" regions of the protein. The simplest explanation for this result is that the stability of the tetra-stranded helices assumed by guanine-containing homopolynucleotides in solution (27) prevents poly(dG) and poly(dG) from binding the gene 32 protein. Poly(dA), -(C), -(U), and -(A) all enhance the rate of trypsin digestion of the "A" region without affecting the rate of release of the "B" region. In contrast, poly(dT) and poly(dC) mimic the behavior of fd DNA (Fig. 4, A and B) in that they enhance the rate of proteolysis of the "A" region while they decrease the rate of trypsin digestion of the "B" region.

Optimum Protection of "B" Region by Poly(dT) against Tryptic Hydrolysis Occurs at a Base:32P Ratio near 5 - The results in Table III suggest that optimum protection of the "B" region with single stranded fd DNA occurs at a ratio of 4 to 5 nucleotide bases per 32P monomer. Increasing the ratio of fd DNA/32P resulted in decreased protection. As shown in Fig. 5, the same phenomenon was also observed with poly(dT). Protection of the "B" region of 32P against trypsin digestion reached a maximum of 72% at a poly(dT)/32P base ratio of 4.8. Decreased protection of the "B" region was observed if the base/32P ratio was increased above 4.8. The ratio of 4.8 bases/32P is very close to the estimated binding site size of five bases per protein monomer published by Kelly et al. (26). Various other estimates of the binding site size are 6.7 to 7.5, 10, and 11 bases per gene 32 molecule as reported by Jensen et al. (16), Alberts and Frey (3), and Anderson and Coleman (25), respectively. Aside from the variety of methods used to estimate the size of the binding site there is no apparent explanation for the wide variation reported.

Short Oligonucleotides Do Not Affect Rate of Tryptic Hydrolysis of Gene 32 Protein - If the "B" region of 32P is directly involved in DNA binding then oligonucleotides too short to permit cooperative protein binding would still be expected to decrease the rate of trypsin cleavage of the NH₂ terminus. The fluorescence studies of Kelly et al. (26) suggest that oligonucleotides and even the mononucleotide TMP still have a measurable affinity for the gene 32 protein. The oligonucleotide concentrations in each case were adjusted so that 95% of the 32P present was bound to the respective oligonucleotide (based on the binding constants reported by Kelly et al. (26)). The results shown in the last column of Table V reveal that short dT-containing oligonucleotides do not protect the "B" region from trypsin hydrolysis. In this study all of the 32P was converted to 32P*(A + B) regardless of whether the digestion was done in the presence or absence of d(pT)₆, d(pT)₉, or d(pT)₁₆. The same study was repeated at a lower trypsin concentration to observe the effect of the same oligo(dT) containing nucleotides on the removal of the "A" region of 32P. The results tabulated in the third column in Table V reveal that short oligonucleotides do not enhance the rate of trypsin cleavage of the "A" region.

DISCUSSION

Our results show that cooperative binding of the gene 32 protein to DNA and to some homopolynucleotides increases the rate of trypsin cleavage of the "A" region and decreases the rate of trypsin hydrolysis of the "B" region. Neither of these effects are observed with short oligonucleotides (<8 bases) which still bind 32P. We have interpreted these results in terms of a model for the mode of 32P:DNA interaction (Fig. 6). The essential feature of the model requires that the gene 32 protein exists in at least two different conformations, only one of which is capable of cooperative binding to DNA. According to the work of Carroll et al. (6), gene 32 protein exists mainly as a dimer or higher aggregate forms at concentrations above 0.1 mg/ml even in the absence of DNA. Because of the results reported by Kelly and von Hippel (28) we have depicted the DNA binding sites as being partially occluded in the dimer¹

¹ The binding constant for d(pT)₆ was assumed to be 1.5 x 10⁶ which is the constant reported for d(pT)₂₆ (28). This assumption is probably valid since d(ApA) and d(pA), both have identical association constants (28). The reported constants (26) for d(pT)₂₆ and d(pT)₁₆ were averaged to get an approximate constant for d(pT)₆. d(pT)₁₆, poly(dT), and fd DNA were presumed to have binding constants of at least 10⁶ (26).

² Kelly and von Hippel (28) suggest there may be two kinds of gene 32 protein dimer: one an (heterologous) intermediate in the indefinite aggregation process which should bind oligonucleotides; the other a (isologous) self-limited dimer with very little affinity for oligonucleotides. The isologous 32P dimer is pictured in Fig. 6 although it is not known which of these two possible dimers is predominant under the conditions used in our experiments.
This is, in fact, what is found (Table IV). Bobst and Pan (29) have determined that the gene 32 protein has a greater DNA. The model assumes that 32P binds oligonucleotides (<8) tightly to DNA than Form I. In this conformational state change as proposed in Fig. 6 where Form II would bind more previously suggested that contiguous binding of 32P may be needed to induce a conformational change in the protein which leads to much higher affinity for DNA. We have provided evidence that 32P does indeed undergo a conformational change as proposed in Fig 6 where Form II would bind more tightly to DNA than Form I. In this conformational state (Form II) the "B" region can participate in cooperative 32P:32P interactions whereas it cannot do so in Form I.

We have shown that tight cooperative binding of 32P to DNA and homopolynucleotides can be correlated with a decreased rate of tryptic cleavage of the "B" region. This correlation is supported by two lines of evidence: homopolynucleotides and ssDNA which bind 32P with high affinity can protect the "B" region from trypsin and, secondly, oligonucleotides which are too short to permit cooperative binding, do not decrease the rate of tryptic hydrolysis of the "B" region. The data in Table IV demonstrate that homopolynucleotides differ from each other in their ability to protect the "B" region from trypsin digestion. This phenomenon may be related to the affinity of 32P for each of the homopolynucleotides tested. According to the model shown in Fig. 6 homopolynucleotides which promote interaction of the "W" regions with adjacent 32P molecules, and which therefore bind 32P most tightly, would also be the homopolynucleotides which should give the most protection of the "B" region against trypsin digestion. This is, in fact, what is found (Table IV). Bobst and Pan (29) have determined that the gene 32 protein has a greater affinity for fd DNA and poly(dT) than for poly(A), -(U), and -(dA). They suggest that the association constant of poly(dT) for 32P is at least 3 to 4 orders of magnitude larger than that of poly(U) or poly(dA). In addition, Anderson and Coleman (25) have observed that the 32P-poly A interaction is less stable than the 32P-fd DNA interaction. Our results are consistent with this view. That is fd DNA and poly(dT) provide protection of the "B" region while poly(A), -(U), and -(dA) do not (Tables III and IV).

It is interesting that a similar order of polynucleotide binding of 32P as reported by Bobst and Pan (29) has also been observed with the E. coli unwinding protein. Weiner et al. (30) found that poly(dT), poly(dC), and single-stranded DNA bound tightly to the E. coli DNA unwinding protein while poly(A), poly(U), and poly(dA) exhibited much lower affinity. Results obtained with oligonucleotides (<6) and spermidine (30) in this system suggest that the relative binding affinities for the E. coli DNA unwinding protein may reflect differences in the secondary structures of these polynucleotides rather than differences in base specificity. A similar explanation may also account for the results of the 32P:polynucleotide binding studies.

Maximum protection of the "B" region of 32P-A against trypsin digestion occurs at a base/32P ratio near 5 (Table III and Fig. 5). At the trypsin concentration used in these experiments the "A" region is removed from 32P within the first few minutes of the digestion. Hence, we are actually observing the effect of excess DNA or poly(dT) on the proteolytic removal of the "B" region from 32P*-A. Increasing the base/32P ratio above 5 actually results in decreased protection so that at a base/32P ratio of 15.4 there is only an 8% difference in the rate of proteolysis of the "B" region in the presence and absence of poly(dT) (Fig. 5). Since the gene 32 protein binds cooperatively to DNA (3, 5, 12), then even at a base/32P ratio of 15.4 the protein should still be bound in long clusters along the DNA. We suggest that the decreased "B" region protection at base/32P ratios above 5 results from exchange of 32P-A molecules from these long clusters to exposed sites on the poly(dT) added in excess of the amount required for stoichiometric binding of 32P. If the "B" region is not involved in 32P:32P cooperative interactions it is susceptible to digestion. The exchange of 32P-A from a contiguous to a noncontiguous site on poly(dT) appears to occur rather slowly. With a trypsin concentration of 16 µg/ml and the conditions used in Fig. 5, 90% of the 32P*-A is converted to 32P*-A + B in 10 min (data not shown). In contrast, if poly(dT) is present in a base/32P ratio of 15.4 only 55% of the 32P*-A is converted to 32P*-A + B at this time. At 45 min, however, 88% of the "B" region is removed. Nitrocellulose binding studies are in progress to compare 32P and 32P*-A with respect to their ability to bind DNA cooperatively and to exchange from a contiguous to a noncontiguous binding site on DNA.

Moise and Hosoda (14) showed and we have confirmed that the "B" region is necessary for tight binding of 32P to single-stranded DNA. Our assignment of the "B" region at the N\textsubscript{H\textsubscript{2}} terminus is also consistent with the genetic studies of Breschkin and Mosig (32) and the in vitro studies of Huberman et al. (33). The latter group have found that the P7 mutant has a temperature-sensitive gene 32 protein which is defective in

In contrast to the study by Bobst and Pan (29), Kelly et al. (26) reported the binding of 32P to oligo- and polynucleotides to be nonspecific with respect to base composition. However, Kelly et al. (26) did comment that the apparent discrepancy between the study of Bobst and Pan (29) and their own could be accounted for by binding constant and cooperativity differences for different polynucleotides well within the standard error of their measurements.
stimulating T4 DNA synthesis in vivo and T4 DNA polymerase in vitro above 37°C. They have concluded that it is probably the interaction of the gene 32 protein with DNA that accounts for the temperature sensitivity of the P7 mutant. Breschkin and Mosig (32) have mapped the P7 mutation at a locus near the NH₂ terminus, but not necessarily within the "B" region, of the gene 32 protein which led them to conclude that the NH₂ terminus of the protein is important for DNA binding. If the amino acid substitution in the P7 gene 32 protein is actually within the "B" region, then the P7 protein may be unable to participate in 32P-32P cooperative protein binding interactions at temperatures above 37°C.

Moise and Hosoda (14) further suggested that the interaction of the "A" region with another protein in the replication complex might lead to a conformational change in 32P which increases the ability of 32P to denature dsDNA. This concept formed the basis for their model in which the "A" region localized the unwinding of DNA at a point just in front of the replication complex. Our results which show that the "A" region with another protein in the replication complex might lead to a conformational change in 32P which is required for T4 DNA replication, binds to a single-stranded DNA-gene 32 protein complex but not to 32P or ssDNA alone. This suggests the 44P-62P complex may interact with the COOH-terminal "A" region of the gene 32 protein which we have demonstrated is more accessible (to proteolytic enzymes) after the gene 32 protein is bound to DNA. Since the 44P-62P complex has a DNA-dependent ATPase activity, Alberts et al. (34) suggest the 44P-62P complex along with 32P might serve as a "DNA walking machine," generating the force required to drive the T4 DNA polymerase down its template. Alternatively, the 44P-62P complex may be analogous to the rep E. coli protein which also has an ATPase activity that is stimulated by single-stranded DNA. Together with the E. coli unwinding protein and ATP, the rep protein catalyzes strand separation of double-stranded DNA (35). We are presently trying to determine whether the "A" region of 32P interacts with the 44P-62P complex or other proteins involved in T4 DNA metabolism (34, 36). Such interactions might be expected to protect the "A" region against trypsin digestion in the presence of DNA.

Acknowledgments — We wish to thank Dr. Paul Fletcher for running the Beckman amino acid Sequencer and Dr. Thomas Kempe for operating the amino acid analyzer. We are grateful to Dr. Yasutsuga Nakashima for his assistance with the amino acid sequencing.

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J. Biol. Chem. 1978, 253:2463-2470.

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