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Binding studies of SV40 T-antigen to SV40 binding site II

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ABSTRACT

SV40 T-Antigen binding site II was synthesized, cloned and analyzed for its ability to bind purified SV40 T-antigen. We report the binding constant of T-antigen for isolated site II. Using a filter binding assay the calculated binding constant was 6-8 fold less efficient than site I previously reported. Binding constants were calculated using two methods. The first was a direct calculation using a protein titration curve (K_D). The second was by the ratio of measured association and dissociation rates. Both methods gave similar constants. Protection studies with SV40 T-antigen on the T-antigen binding sites in the wild-type array demonstrated that the binding constants of site I and site II are similar to those calculated for the individual sites. These results demonstrate that SV40 T-antigen does not bind cooperatively to sites one and two as earlier believed and are in agreement with recent observations emanating from several laboratories.

INTRODUCTION

T-antigen is a viral gene protein produced when SV40 infects a permissive host. In a lytic cycle the large T-antigen is transcribed from the early promoter and is autoregulatory (1-5). T-antigen is not only responsible for shutting down its own production but also stimulates late gene transcription (6,7).

Initial investigation of the DNA locus involved in binding of T-antigen protein were made possible by the use of an adeno-virus-SV40 T-antigen hybrid gene (8,9) that overproduces the hybrid protein ($AD2^{+}D2$). The T-antigen binding domain on the SV40 genome was determined by analyzing the DNA region protected by this protein from digestion with pancreatic deoxyribonuclease (8,12). Three binding sites which exhibited different affinities for T-antigen hybrid-protein were identified (2). Site I (TI) was

protected from nuclease digestion at the lowest concentration. This protected fragment is approximately 30 bp long. Increasing concentration of protein afforded sequential protection of sites II (TII) and III. The three sites are adjacent to one another and contain the viral origin of replication. Subsequent analysis using T-antigen yielded similar results. Both proteins appear to be interchangeable by most assays (1,9, 12,14) although there is a difference in TI binding affinity for these two proteins (15). In addition to the variation of affinities, T-antigen binding was thought to be cooperative (2). Our initial intent was to investigate the nature of this cooperativity by introducing various spacer mutations between TI and TII and studying their effect on binding and transcription regulation. We have synthesized and cloned TII. Binding studies on TII in the absence of TI revealed only a six fold difference in binding affinity and indicated that cooperativity as previously described was unlikely.

This result agrees with recent reports demonstrating that T-antigen binding affinity for TII remains unaffected by various mutations which cause a loss in binding affinity for TI (9,16, 17). We have checked for cooperativity when sites I and II were in the wildtype configuration as opposed to the isolated sites. We could not detect any change in the affinity for any sites. This indicates that T-antigen binding to its specific DNA binding sites is not cooperative.

MATERIALS AND METHODS

Deoxynucleotide Synthesis and Enzymatic Ligations.

Deoxynucleotides were synthesized as previously described (18,19). Purification was carried out by reverse phase HPLC using a μ-Bondapak C_{18} column from Waters Associates or by polyacrylamide gel electrophoresis. The chain length of each purified deoxyoligonucleotide was verified by partial exonucleolytic cleavage with snake venom phosphodiesterase. The digestion products were analyzed on a 20% polyacrylamide/8 M urea gel. Enzymatic ligation of synthetic deoxyoligonucleotides were completed as described (20).

Molecular Cloning

Isolation of plasmid, DNA cleavage with restriction endo-

nucleases, isolation of DNA fragments, ligation with T4 DNA ligase (Bethesda Research Laboratories) and transformation of E. coli were carried out as described (15).

Colony Screening-Miniprep Analysis

Colonies were initially screened through phenotype selection and then subjected to a miniprep analysis following the procedure of Birnboim (21). 3 ml of culture was lysed by lysozyme followed by alkaline treatment. Chromosomal DNA and plasmid were separated by ammonium acetate precipitation. The plasmid was acid precipitated and protein removed by phenol extraction. The plasmid was initially checked for proper restriction map and then the TII insert sequenced using the method of Maxam and Gilbert (22).

Nitrocellulose Filter Binding Assay

The EcoRI-BamHI fragment of plasmid pVF207 containing TI and the EcoRI-BamHI fragment of pSV110 containing TII were labelled by repair synthesis with Klenow fragment in the presence of [α-^{32}P]dATP and isolated by polyacrylamide gel electrophoresis. Prior to assays the duplexes were further purified through a Gelman No. 4129 sterilization unit. Binding buffer was 20 mM phosphate pH 6.8, 3 mM dithiothreitol, 0.1 mM EDTA, 3% dimethylsulfoxide, 50 µg/ml bovine serum albumin and 100 mM NaCl. The wash buffer contained 20 mM sodium phosphate, 0.1 EDTA and 0.1 dithiothreitol.

Dissociation equilibrium studies were performed using the following procedure. Labelled DNA at a fixed concentration (8-10 x 10^{-12} M) was incubated at room temperature for 30 min with varying amounts of T-antigen protein. The range used is indicated in the figures and is based on the tetrameric form of T-antigen. All points sampled are the mean average of duplicates. The DNA-Protein complex is separated from the free DNA by by nitrocellulose filters. 20-30 µl solution is applied to the filter and washed with 300 µl of wash buffer. Non-specific bound DNA was determined by using the above assay in the absence of any protein. All figures and tables represent the specific bound DNA only. Throughout all these assays the efficiency of binding (bound *vs* total counts) is 50-60%.

Association and dissociation assays were assessed using the

following procedure. A constant amount of DNA (12-14 x 10^{-12} M) and T-antigen (4 x 10^{-12} M) were used. Association kinetics were determined by incubating the protein and the DNA together and aliquots of 25 µl were removed at the indicated time points. Dissociation was determined by the addition of excess cold competitor (100 fold) of plasmid containing TI binding site after the duplex of DNA and protein had formed. Nonspecific DNA binding activity was determined as above.

To determine stability of the DNA-protein complex a parallel assay in the absence of competitor was performed. No dissociation or loss of protein activity over the time range used was apparent.

DNase I Footprinting.

Plasmid pSVOD (23) was cut with the EcoRI and HindIII and labelled using an appropriate [α-^{32}P]deoxynucleotide triphosphate and the Klenow fragment of E.coli DNA polymerase I. The [α-^{32}P]-deoxynucleotide triphosphate was chosen so that only one strand would be labelled. The procedure for protection followed that of Schmitz and Galas (22). Buffer used for binding was 20 mM PIPES with 100 mM NaCl at pH 6.8. DNAase I in binding buffer with 10 mM Mg++ and 5 mM Ca++ was added for 45 sec at a concentration of 5 ng in 20 µl volume. The reaction was stopped using 25 µl of 3 M ammonium acetate and 250 mM EDTA and carrier DNA (3 µg per reaction).

Protein Purification

T-antigen was purified as previously described (25). Protein activity was assessed using methods detailed previously (15).

RESULTS

TII was synthesized using phosphite chemistry (18,19) and enzymatic ligation (20). Because of our previous experience with palindromic and inverted repeat sequences in TI (15), we tried to minimize complementary sequences in the same strand of TII. Therefore small fragments ranging from 8-12mers were synthesized and ligated enzymatically to form a complete site II having blunt ends. Figure 1 displays the fragments used and the ligation strategy. The synthesis of TII proceeded through two intermedi-

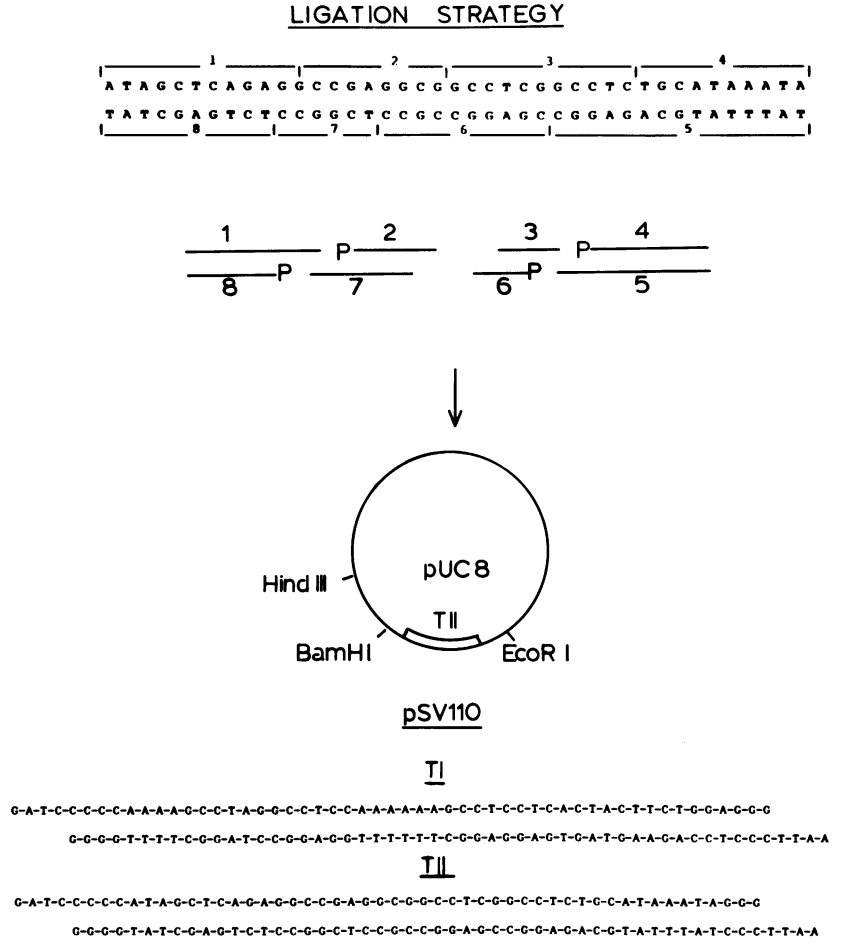

Figure 1. Ligation strategy for assembling T-antigen binding site II. Construction proceeded by first preparing the two halves of the binding site. These duplexes were ligated to form binding site II. Synthetic site II was blunt-end ligated into the Sma I site of pUC8. The sequences of of excised site II from pUC8 and the corresponding site TI are shown at the bottom part of the figure. Duplexes TI and TII were used for the studies reported in this paper.

ates where each corresponded to about one-half of the binding site. Fragments 3, 4, 5, 6 were preannealed and ligated in the normal fashion. Fragments 4 and 6 were phosphorylated with [γ-^{32}P]ATP and T4-Kinase, preannealed with 3 and 5 and ligated to form the right half of TII. Ligation of fragments 1, 2, 7, 8

proved to be more difficult. In this case prior annealing of fragments 1,8 separately from 2,7 allowed for correct ligation. Each product was purified on a native polyacrylamide gel. These duplexes were then phosphorylated using [γ-^{32}P]ATP and T4 kinase, joined together using T4 ligase and the final duplex purified by polyacrylamide gel electrophoresis. The duplex corresponding to TII was ligated into the SmaI site of plasmid pUC8 and the plasmid transformed as described previously (15). After excision of TII using EcoRI and BamHI, the DNA duplex had 5' and 3' ends identical to the TI duplex prepared previously (15). Comparison of these fragments reveals that only sequences characteristic of TI and TII differentiate these duplexes (Fig. 1). The isolated plasmid after characterization by sequencing was called pSV110.

In order to obtain a dissociation constant for site II we used a filter binding assay. Site I previously assayed (13) was used in parallel as a control. The binding of T-antigen protein (P) to either TI or TII is a bimolecular equilibrium reaction which can be described using equation 1:

$$P + Ti \underset{k_d}{\overset{k_a}{\rightleftharpoons}} P^*Ti;\ K_D = k_d/k_a = \frac{[P][Ti]}{[P^*Ti]} \qquad (1)$$

P*Ti represents the DNA-protein complex , k_a is the association rate constant, k_d is the dissociation rate constant, K_D is the dissociation equilibrium constant and Ti indicates either TI or TII. We have assayed and plotted the dissociation equilibrium for TI and TII (Figure 2). The K_D values calculated from these plots are 2.3 x 10^{-12}M (TI) and 16.5 x 10^{-12}M (TII).

The equilibrium constant can be verified by experimentally determining the dissociation and association rate constants separately and then using equation 1. The association rate constant can be calculated from equation 2:

$$k_a t = \frac{1}{[P]_o - [Ti]_o} \ln \frac{[P_o]([Ti]_o - [P^*Ti])}{[Ti]_o([P]_o - [P^*Ti])} \qquad (2)$$

$[P]_o$ is the concentration of T-antigen and Ti is the concentration of binding site. The association constant for TI and TII

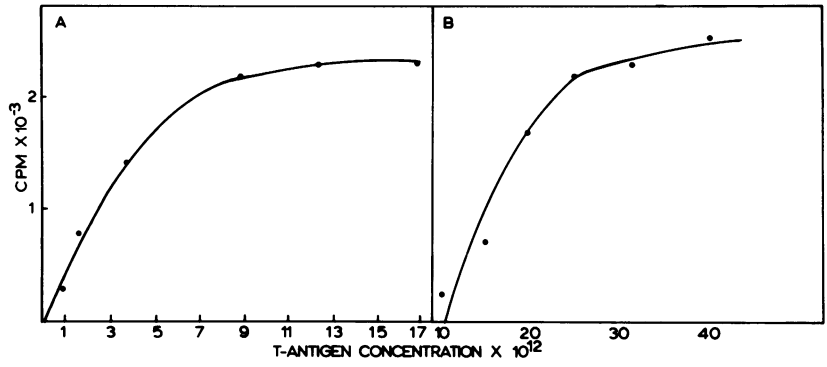

Figure 2. Filter binding assay of kinetic equilibrium plots: (A) binding site I using the EcoRI/BamHI fragment of pVF207 and (B) binding site II using the same restriction fragment from pSV110. Concentration of T-antigen is indicated. The flanking sequences of TII were identical to the flanking sequences of TI.

are virtually identical (Fig.3). Association was complete within the first two minutes with a rate constant of $3.2 \times 10^{8} M^{-1} s^{-1}$. These results are essentially identical to earlier observations on the association of *lac* repressor with *lac* operator in the presence of nonoperator DNA and similar ionic strength (26). The dissociation rate constant was measured by first forming the DNA-

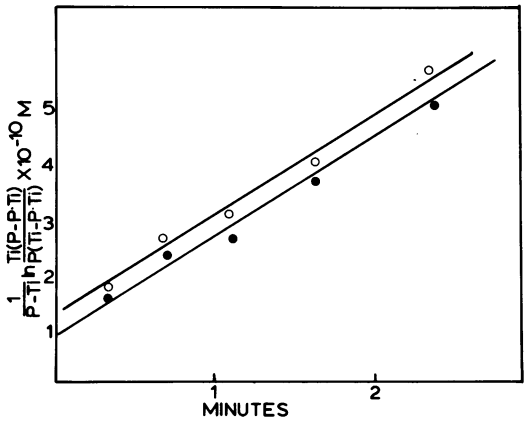

Figure 3. Rate of T-antigen association to binding site I (●--●) and site II (o--o). A constant amount of T-antigen was incubated with labelled TI or TII. Association was measured over the indicated period of time. The bound DNA was calculated from nitrocellulose filter binding assay.

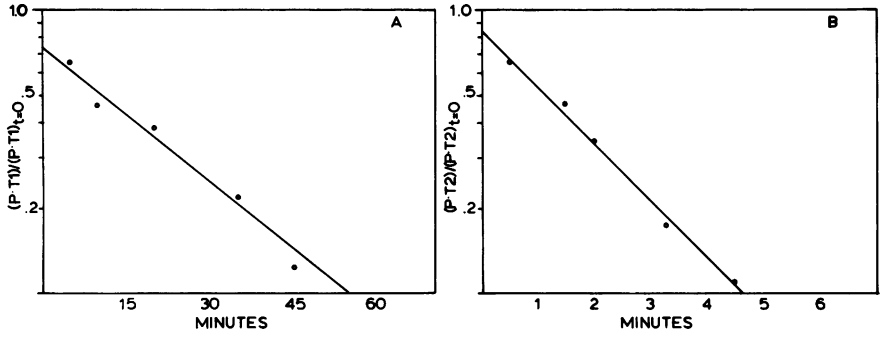

Figure 4. Rate of T-antigen dissociation from either binding site I (A) or binding site II (B). For both experiments, unlabelled plasmid containing T-antigen site was used as cold competitor. Protein-DNA is allowed to equilibrate for 30 min and aliquots of 25 µl are assayed at the indicated times.

protein complex and then adding a 100 fold excess of unlabelled plasmid containing TI. In order to insure pseudo first order kinetics, T-antigen was added to a final concentration that would permit 80% of labelled binding site to form a complex with protein. The dissociation rate can then be calculated from the following equation:

$$\ln \frac{[P*Ti]_t}{[P*Ti]_0} = -k_d t + C \qquad (3)$$

Plots of the data obtained for the dissociation of TI and TII with T-antigen are shown in Figure 4. The dissociation rate constants determined were $6.8 \times 10^{-4} s^{-1}$ (TI) and $44.2 \times 10^{-4} s^{-1}$ (TII). The dissociation equilibrium constant derived from the ratio of dissociation to association rate constants were $2.0 \times 10^{-12} M$ (TI) and $13.8 \times 10^{-12} M$ (TII). These values were similar to those determined by direct measurement. The values obtained through these filter binding studies are summarized in Table I.

Tandem binding TI and TII in the wild-type configuration were then analyzed for T-antigen affinity using the footprinting method. Plasmid pSVOD (23) containing the SV40 origin of replication including the three T-antigen binding domains was used. Figure 5 shows the protection pattern for the tandem DNA binding sites. The gel was scanned as indicated in Figure 5 and the

Table I. Kinetic and Dissociation Equilibrium Measurement of SV-40 T-antigen with Binding Sites I and II

Assay	[T-antigen] ($\times 10^{-12}$M)	[TI] ($\times 10^{-12}$M)	[TII] ($\times 10^{-12}$M)	Parameters Evaluated	Value
Filter Binding Kinetic Parameters:					
Protein Titration	0-15	8.8	----	K_D(TI)	2.3×10^{-12}M
Protein Titration	0-40	----	9.5	K_D(TII)	16.5×10^{-12}M
Calculated Ratio				K_D(TII/TI)	7.12
Association Kinetics	4.0	14.6	----	k_a(TI)	3.4×10^{8}M^{-1}s^{-1}
	4.0	----	12.6	k_a(TII)	3.4×10^{8}M^{-1}s^{-1}
Dissociation Kinetics	4.0	14.6	----	k_d(TI)	6.8×10^{-4}s^{-1}
	24.0	----	12.6	k_d(TII)	44.2×10^{-4}s^{-1}
Calculated K_D				K_D(TI)	2.0×10^{-12}M
$K_D = \frac{k_d}{k_a}$				K_D(TII)	13.8×10^{-12}M
Calculated Ratio				K_D(TII/TI)	6.5
Footprinting Kinetic Parameters:					
DNAase I Protection	0-40	4.4	4.4	K_D(TI)	2.4×10^{-12}M
DNAase I Protection	0-40	4.4	4.4	K_D(TII)	13.6×10^{-12}M
Calculated Ratio				K_D(TII/TI)	5.7

densities were plotted as a function of protein concentration. Figure 6 shows the equilibrium binding properties obtained from the gel. Equilibrium binding constants obtained from this plot were 2.4×10^{-12}M for binding site I and 13.6×10^{-12}M for TII. These constants are equivalent to the binding constants obtained using the filter binding method with isolated TI and TII. Similar results were obtained using other bands in either TI or TII.

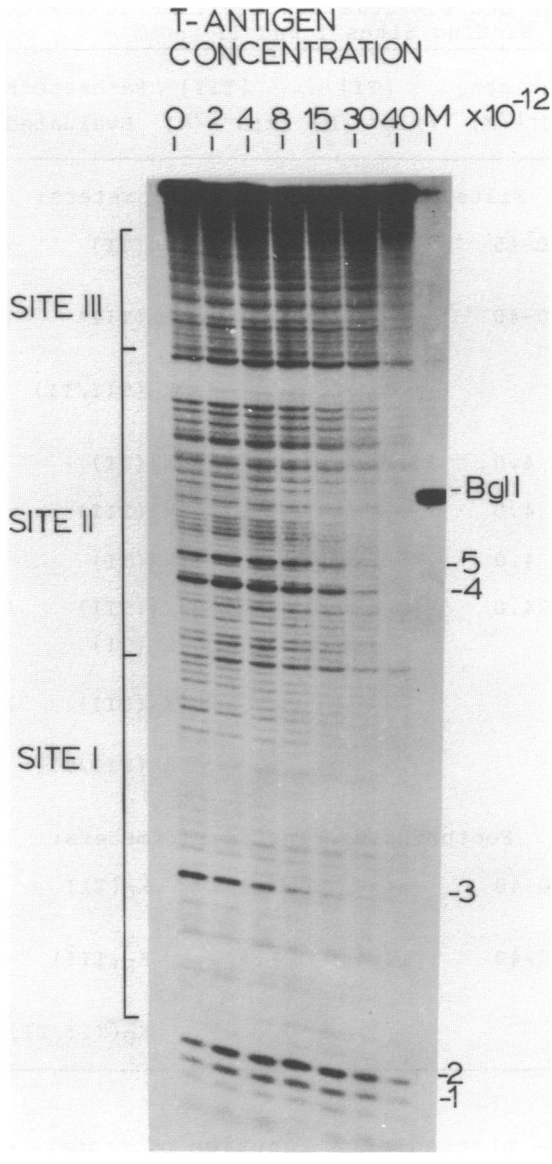

Figure 5. T-antigen protection of binding sites I and II from DNAase I. Protein concentrations are indicated. An 8% denaturing polyacrylamide gel was used to display the results. Numbers 1 through 5 were the bands used in densitometry scans. Numbers 1 and 2 are bands outside of the binding sites and were used to normalize each lane. Band number 3 was used to define the pattern for TI. Band 5 was used for measuring the pattern of TII The results of the densitometry measurements are plotted in Figure 6.

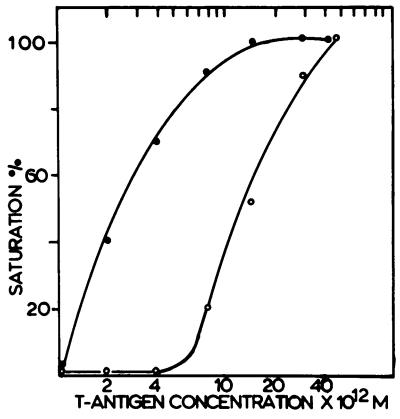

Figure 6. A plot of the densitometry readings obtained from the gel shown in Figure 5. Site I is plotted from band number 3 (●--●) and site II is plotted from band 5 (o--o). The densities are normalized using band 2.

DISCUSSION

We embarked on a study of T-antigen in order to analyze the cooperativity of T-antigen binding to TI and TII (2). Our intent was to introduce base pairs between TI and TII and study the effect of these insertions on binding, transcription, and replication. This approach has been used effectively to study catabolite activation by catabolite activator protein in the *lac* operon (19, 27-29). The first step was preparation of TII. This was accomplished by synthesis. From previous experience (15) the synthesis and construction sequences containing palindromes present difficulties owing to their ability to self-anneal. The use of short nucleotide strands circumvents these problems. In the construction of TII, problems which occurred in the ligation of fragments 1,2,7,8 were overcome by annealing 1 and 8 separately from 7 and 8. On other occasions we have observed this phenomenon. Presumably short stretches of deoxynucleotides can form alternate contacts from those expected by normal Watson-Crick base pairing. These uncharacterized structures appear to be quite stable.

Our results demonstrate that T-antigen does not bind cooperatively to sites I and II. These sites were first titrated individually with T-antigen in order to experimentally determine

their respective dissociation equilibrium constants. The dissociation equilibrium constants for binding TI and TII interacting with T-antigen were also calculated from the experimentally determined association and dissociation rate constants. In both cases the difference in dissociation equilbrium constants was 6-8 fold indicating that TII binds T-antigen somewhat less efficiently than does TI.

The recent work of Jones, Myers and Tjian (17) indicates that TII can not be assayed using a filter binding method although the addition of antibodies to the reaction mixture does allow for specific filter binding. Our results demonstrate that such an assay is possible. The reason for such a disparity in binding capabilities is not immediately apparent. One possibility is purification methods which may differentially alter the protein structure and hence its capacity to bind to either filters or DNA.

T-antigen was used to protect binding TI and TII as found in the wild-type configuration from digestion with DNAase I (Figure 5). Dissociation equilbrium constants obtained from an analysis of the footprinting patterns (Figure 6) were identical to those determined for the individual sites (Table I). The ratio of dissociation equilibrium constants for binding TI and TII with T-antigen (5.7) in tandem was the same as when measurements were completed on the individual sites (6.5 and 7.2). T-antigen therefore appears to recognize binding sites I and II independently.

These observations thus rule out the possibility of increased stability *via* protein-protein interactions and hence cooperativity which would increase the affinity of T-antigen for TII in the natural configuration. These results are in complete agreement with recent reports of T-antigen binding in the absence of a functional site I (9,16,17).

Repression of SV40 early transcription is modulated by the specific interaction of T-antigen with its binding sites. Intact sites I and II are necessary for autoregulation of T-antigen and the spacing between sites I and II as well as the proximity of the promoter region must be preserved (2,17,18,30). Recently, Jones et al. have demonstrated that T-antigen binds to the DNA face through interactions located in successive major grooves

(31). The research described in this manuscript as well as other published results (9,16,17) clearly show that T-antigen does not bind cooperatively to binding sites I and II *in vitro*. If protein contacts do not exist (i.e. cooperativity) for stabilizing and blocking early transcription, then the possibility of other factors involved in regulating transcription should be explored. One possibility is the involvement of host factors such as the 54 K dalton protein (32-34) which interacts with T-antigen. This protein interacting with T-antigen could form a complex having precise structural features in contact with TI and TII binding domains and therefore increase the stability of T-antigen on the DNA.

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REFERENCES

1. Rio, D. Robbins, A., Myers, R.M. and Tjian, R. (1980) Proc. Natl. Acad. Sci. USA 77, 5706-5710.
2. Myers, R.M., Rio, D., Robbins, A.K. and Tjian, R. (1980) Cell 25, 373-384.
3. Hansen, U., Tenen, D.G., Livingston, D.M. and Sharp, P.A. (1981) Cell 27, 603-612.
4. Tegtmeyer, P., Schwartz, M., Collins, J.K. and Rundell, R. (1975) J.Virol. 16 168-178.
5. Alwine, J.C., Reed, S.I., Fergusen, J. and Stark, G.R. (1975) Cell 6, 529-533.
6. Brady, J., Boler, J.B., Radonovich, M., Salzman, N. and Khoury, G. (1984) Proc. Natl. Acad. Sci. USA 81, 2040-2044.
7. Keller, J.M. and Alwine, J.C. (1984) Cell 36, 381-389.
8. Tjian, R. (1978) Cell 13, 165-179.
9. DiMiao, D. and Nathans, D. (1982) J.Mol. Biol. 156, 531-548.
10. Myers, R.M. and Tjian, R. (1980) Proc. Natl. Acad. Sci. USA 77, 6491-6495.
11. Tegtmeyer, P., Anderson, B., Shaw, S.B. and Wilson, V.G. (1981) Virology 115, 75-87.

12. Tenen, D.G., Haines, L.L. and Livingston, D.M. (1982) J. Mol. Biol. 157, 473-492.
13. McKay, R. and DiMiao, D. (1981) Nature 289, 810-813.
14. Tegtmeyer, P. Lewton, B.A., Delucia, A.L., Wilson, V.G., Ryder, K. (1983) J. Vir. 46, 151-161.
15. Fisher,E.F., Fiest, P.L., Beaucage, S.L., Myers, R.M., Tjian, R. and Caruthers, M.H. (1984) Biochem. 23, 5938-5944.
16. Tenen, D.G., Taylor, T.S., Haines, L.L., Brady, M.K., Martin, R.G. and Livingston, D.M. (1983) J. Mol. Biol. 168, 791-808.
17. Jones, K., Myers, R.M. and Tjian, R. (1984) EMBO J 13, 3247-3255.
18. Beaucage, S.L. and Caruthers, M.H. (1981) Tet. Lett. 22, 1859-1862.
19. Caruthers, M.H., Beaucage, S.L., Efcavitch, J.W., Fisher, E.F., Goldman, R.A., deHaseth, P.L., Mandecki, W., Matteucci, M.D., Rosendahl, M.S. and Stabinsky, Y. (1982) Cold Spring Harbor Symp. Quant. Biol. 47, 411-418.
20. Yansura, D.G, Goeddel, D.V. and Caruthers, M.H. (1977) Biochem.16, 1772-1780.
21. Birnboim, H.C. and Doly, J. (1979) Nucleic Acids Res.7, 1513-1523.
22. Maxam, A.M. and Gilbert, W. (1977) Proc. Natl. Acad. Sci. USA 74, 560-564.
23. Mellon, P., Parker, V., Gluzman, Y. and Maniatis, T. (1981) Cell 27, 279-288.
24. Schmitz, A. and Galas, D.G. (1979) Nucleic Acids Res. 6, 111-137.
25. Tegtmeyer, P. and Anderson, B. (1981) Virology 115, 67-74.
26. Goeddel, D.V., Yansura, D.G. and Caruthers, M.H. (1977) Proc. Natl. Acad. Sci. USA 74, 3292-3296.
27. Mandecki, W. and Caruthers, M.H. (1984), Gene 31, 263-267.
28. Caruthers, M.H., Barone, A.D., Bracco, L.P., Dodds, D.R., Eisenbeis, S.J., Goldman, R.A., Mandecki, W., McBride, L.J., Nasoff, M.S., Noble, S.A. and Tang, J-Y. (1984) Proceedings of 16th Meeting of FEBS, Moscow, in press.
29. Yu, X-M. and Reznikoff, W.S. Nucleic Acids Res. 12, 5449-5464.
30. Rio, D. and Tjian, R. (1983) Cell 32, 1227-1240.
31. Jones, K. Rio, D. and Tjian, R. (1984) Cell 36, 155-162.
32. Linzer, D.H. and Levine, A.J. (1979) Cell 17, 43-52.
33. McCormick, F. and Harlow, E. (1980) J. Virol. 34, 213-224.
34. Lane, D.P. and Crawford, L.V. (1979) Nature 279, 262-263.
35. Caruthers, M.H., Barone, A.D., Beltman, J., Bracco, L.P., Dodds, D.R., Dubendorf, J.W., Eisenbeiss, S.J., Gayle, R.B., Prosser, K., Rosendahl, M.S., Sutton, J. and Tang, J-Y. (1985) Proceedings of the UCLA Symposium on Protein Structure, Folding and Design, in press.