Interaction of AD2$^+$D2 Protein and Simian Virus 40 Large T Antigen with the Large Tumor Antigen Binding Site I†

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ABSTRACT: In a lytic infection of a permissive host by SV40, the large tumor antigen (T antigen), which is a product of early transcription of the SV40 A gene, has been previously shown to autoregulate its own transcription by binding to SV40 DNA. The DNA region to which T antigen binds most tightly was synthesized and subsequently introduced into the bacterial plasmid pUC8. The interaction of SV40 T antigen with the DNA duplexes, derived from both chemical synthesis and the recombinant plasmid, were examined by using nitrocellulose filter binding assays. An SV40-adenovirus hybrid protein, AD2$^+$D2 protein, was also tested. The SV40 T antigen was found to bind more tightly than the hybrid protein. Kinetic assays demonstrated that the association rates for the two proteins with the DNA binding site were equivalent; however, once formed, the T antigen–DNA complex dissociated more slowly than the AD2$^+$D2 protein–DNA complex.

Simian virus 40 (SV40) was initially observed as a vacuolating agent in rhesus monkey cell cultures (Sweet & Hillerman, 1960). In addition to lytically infecting these cultures, the virus will transform other mammalian cell lines by lysogenic infection (Sambrook et al., 1968). Shortly after infection, either lytic or lysogenic, the early region of the viral genome is transcribed and matured through splicing to produce two mRNAs that are translated into the large tumor antigen (T antigen) and the small tumor antigen (t antigen). The function of T antigen in either a lytic or lysogenic infection is pleiotropic. In lytic infection, T antigen is autoregulatory since it induces the shift in transcription from the early viral genes to the late viral genes (Reed et al., 1976). Studies with temperature-sensitive mutations of T antigen confirm that it is essential for initiation of transformation (Tegtmeyer, 1975). Whether this protein is also necessary for maintenance of the transformed state is still not clear (Topp et al., 1980). Initial investigations of the DNA locus involved in this interaction were made possible by constructing an adenovirus–SV40 T antigen hybrid gene (Tjian, 1978) that overproduces the hybrid protein (AD2$^+$D2). The region of the SV40 viral genome that binds to the fusion protein was determined by analyzing the DNA region protected by this protein from digestion with pancreatic deoxyribonuclease (Tjian, 1979). Three tandem binding sites which exhibited different affinities for the fusion protein were identified. The first or TI site was protected from nuclease digestion at the lowest concentration of either T antigen or AD2$^+$D2 protein. This protected fragment was approximately 30 base pairs in length and is shown in Figure 1. Studies with T antigen indicated that this protein binds to the same DNA sequences as does the AD2$^+$D2 protein (Meyers et al., 1981).

The objective of this research was to measure the kinetics of interaction between the SV40 T antigen or the AD2$^+$D2 protein and the TI binding site. Because limited quantities of T antigen were available, whereas the hybrid protein could be produced more readily, it was desirable to study the kinetics of both protein interactions with site TI. Comparable kinetics would suggest that further studies with the AD2$^+$D2 protein could be applicable to T antigen. Additionally, TI was synthesized chemically so that these kinetics could be measured independently of any contributions due to the presence of site II or site III sequences. An alternative method of isolating site I by restriction enzyme cutting followed by nuclease digestion and cloning was considered impractical. Additionally, our major interest in this system is to study the recognition of TI by T antigen or AD2$^+$D2 protein. This will be accomplished by chemical insertion of base analogues (uracil and hypoxanthine) into TI followed by kinetic analysis of the effect these analogues have on the recognition process. In this way, contact sites for T antigen or AD2$^+$D2 protein on TI can be mapped in a manner analogous to earlier studies on the interaction of *lac* repressor with *lac* operator (Caruthers, 1980). The chemical and enzymatic synthesis of TI proved to be very challenging because of many direct and inverted repeat sequences. A description of these problems and their resolution is also included. This description may be of benefit to others, who wish to synthesize DNA duplexes having such sequence elements.

Materials and Methods

Materials. Monomethoxytrityl chloride, dimethoxytrityl chloride, and 2,4,6-triisopropylbenzenesulfonyl chloride were obtained from Aldrich Chemical Co. and recrystallized from pentane prior to use. Methanol was distilled from magnesium turnings. 1*H*-Tetrazole, obtained from Aldrich Chemical Co., was sublimed at 110 °C and 0.1 mmHg. Silica gel Vydac A used for polymer-supported deoxyoligonucleotide synthesis was purchased from The Separation Group. Adenosine [γ-^{32}P]-triphosphate was purchased from Amersham. (*N*,*N*-Dimethylamino)trimethylsilane was purchased from Alfa Products. Cellulose acetate strips and nitrocellulose filters (BA-85, 27 mm) were obtained from Schleicher & Schuell, Inc. Phenyl-Sepharose CL-4B and DEAE-Sepharose CL-6B were purchased from Sigma. Snake venom phosphodiesterase was purchased from Sigma and calibrated before use. Urea, ultrapure grade, was obtained from Bethesda Research Lab-

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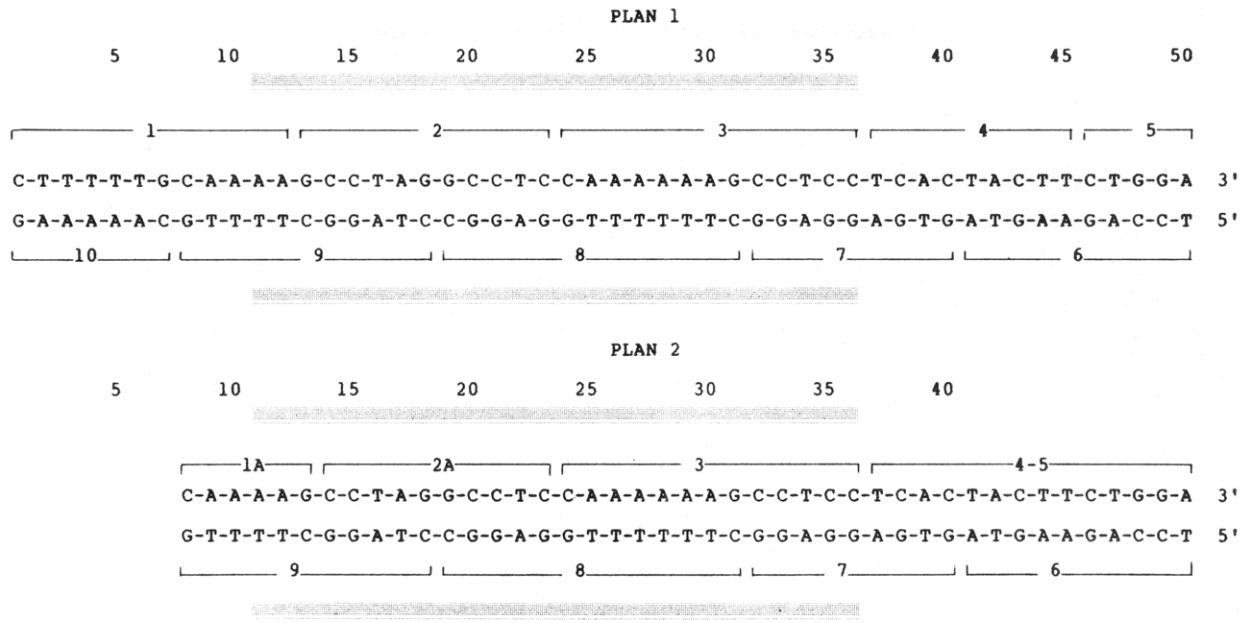

FIGURE 1: Synthetic strategies for preparing the SV40 TI binding site. The deoxyoligonucleotides in the TI binding site region are numbered every five base pairs. Plan 1 and plan 2 are two different synthetic strategies for preparing this binding site. Plan 1 consists of a 50 base pair duplex, whereas plan 2 containing a subset of the sequences in plan 1 was a 43 base pair duplex. Chemically synthesized segments as defined by brackets with inserted numbers are also shown. The heavy overlined region is the approximate limit of the TI binding site protected from nuclease digestion by $AD2^{+}D2$ protein.

oratories. Dulbecco's modified eagle media and fetal calf serum were obtained from Gibco and stored at −20 °C. Monkey cell line CVI and SV40 strain 776 were generous gifts from K. Danna. Plasmid pUC8 and *Escherichia coli* strain JM83 were gifts from G. S. Stiegler.

Preparation of Deoxynucleoside and Deoxynucleotide Derivatives. The exocyclic amino groups of deoxynucleosides and deoxynucleotides were protected by acylation (Schaller et al., 1963; Weber & Khorana, 1972). Protection of the 5′-hydroxyl as a monomethoxytrityl or dimethoxytrityl ether, cyanoethylation of the 5′-phosphate, and acylation of the 3′-hydroxyl were according to published procedures (Schaller et al., 1963). 3′-Benzoyl-*N*-benzoyldeoxyguanosine was prepared by using published procedures (Holý & Souček, 1971). *p*-Chlorophenyl phosphorodichloridate was prepared according to a published procedure (Cramer & Winter, 1959). The 5′-*O*-(dimethoxytrityl)deoxynucleoside-3′ *p*-chlorophenyl β-cyanoethyl phosphates were prepared as described (Stawinski et al., 1977). Dichloromethoxyphosphine (Martin & Pizzolato, 1950), chloro(*N*,*N*-dimethylamino)methoxyphosphine (Beaucage & Caruthers, 1981), and chloromorpholinomethoxyphosphine (McBride & Caruthers, 1983) were prepared according to published procedures. The 3′-phosphorylated derivatives were prepared by published procedures (Matteucci & Caruthers, 1981; Beaucage & Caruthers, 1981). Polymer-supported 5′-*O*-(dimethoxytrityl)deoxynucleosides were prepared according to published procedures (Chow et al., 1981).

Diester Coupling of Protected Deoxynucleotides. The general method for coupling deoxymono- or deoxyoligonucleotides has been described (Goeddel et al., 1977a). 2,4,6-Triisopropylbenzenesulfonyl chloride was used exclusively as the condensing agent. 5′-*O*-(Monomethoxytrityl)deoxytrinucleoside diphosphates and 5′-*O*-(Monomethoxytrityl)-deoxypentanucleoside tetraphosphates were purified by a partition method (van de Sande et al., 1976). β-Cyanoethyl, 3′-*O*-acetyl, and 3′-*O*-isobutyryl groups were removed by alkaline hydrolysis as described (Schaller et al., 1963). Deoxydinucleotide disphosphates, deoxytrinucleotide triphosphates and monomethoxytrityl-containing deoxyoligonucleotides were purified by ion-exchange chromatography on DEAE-cellulose (DE-23). Reactants and products were characterized by paper chromatography. After a deoxyoligonucleotide had been synthesized, 2–5 mg was deprotected as described previously (Weber & Khorana, 1972). The sample was purified by gradient elution on a DE-52 cellulose column (0.8 cm × 200 cm) followed by gel filtration through a Bio-Gel P-2 column (2 cm × 80 cm). This method was used to synthesize two deoxyoligonucleotides as summarized in Table I.

Triester Coupling of Protected Deoxynucleotides. Deoxyoligonucleotide triesters were synthesized according to published procedures (Agarwal & Riftina, 1978). 2,4,6-Triisopropylbenzenesulfonyl tetrazolide was used as the coupling agent. After each coupling, the product was isolated by silica gel chromatography, and the dimethoxytrityl group was removed. After synthesis of a deoxyoligonucleotide triester, the *p*-chlorophenyl protecting groups were removed according to published procedures (Reese et al., 1978). The exocyclic amino protecting groups and the dimethoxytrityl groups were removed as previously described. The deoxyoligonucleotide synthesized when this approach was used is included in Table I.

Polymer-Supported Phosphite Coupling of Protected Deoxynucleotides. The synthetic cycle is diagrammed in Figure 2 and has been described elsewhere (Caruthers et al., 1983). Saturated zinc bromide in nitromethane/methanol (95:5 v/v) was used exclusively to remove the dimethoxytrityl group during the synthesis (Matteucci & Caruthers, 1980). 3′-(Tetrazolylmethoxyphosphino)-5′-*O*-(dimethoxytrityl)deoxynucleoside, 3′-[(*N*,*N*-dimethylamino)methoxyphosphino]-5′-*O*-(dimethoxytrityl)deoxynucleosides, or 3′-(morpholinomethoxyphosphino)-5′-*O*-(dimethoxytrityl)deoxynucleosides were used to synthesize different deoxyoligonucleotides.

After the synthesis of a deoxyoligonucleotide was completed, the polymer-supported, fully protected product was treated with *p*-dioxane/thiophenol/triethylamine (2:1:2 v/v/v) in order to remove the phosphate protecting groups. The deoxyoligonucleotide was hydrolyzed from the support by treatment with ammonium hydroxide at room temperature for 4 h. The

Table I: Deoxyoligonucleotides Synthesized for the SdV40 TI Binding Site

compound	segment	synthetic approach or phosphorylating agent[a]	av yield per condensation[b]
d(CpTpTpTpTpTpGpCpApApApA)	1	C	58
d(CpApApApApG)	1A	D	71
d(GpCpCpTpApGpGpCpCpTpC)	2	C	76
d(CpCpTpApGpGpCpCpTpC)	2A	D	64
d(CpApApApApApApGpCpCpTpCpC)	3	M	82
d(TpCpApCpTpApCpTpT)	4	diester	29
d(TpCpApCpTpApCpTpTpCpTpGpGpA)	4-5	M	85
d(CpTpGpGpA)	5	D	46
d(TpCpCpApGpApApGpTpA)	6	diester	34
d(GpTpGpApGpGpApGpG)	7	C	67
d(CpTpTpTpTpTpTpGpGpApGpGpC)	8	C	70
d(CpTpApGpGpCpTpTpTpTpG)	9	C	69
d(CpApApApApApG)	10	triester	49

[a] The symbols C, D, and M refer to dichloromethoxyphosphine, chloro(*N,N*-dimethylamino)methoxyphosphine, and chloromorpholinomethoxyphosphine, respectively. Fully protected deoxynucleosidephosphine intermediates prepared with these reagents were used to synthesize deoxyoligonucleotides via the phosphite triester approach. Compounds prepared by the phosphate diester or phosphate triester approaches are indicated by diester and triester, respectively. [b] The yields are based on purified deoxyoligonucleotide. Losses due to handling were not considered. Therefore, the actual coupling efficiencies were higher than reported in this table.

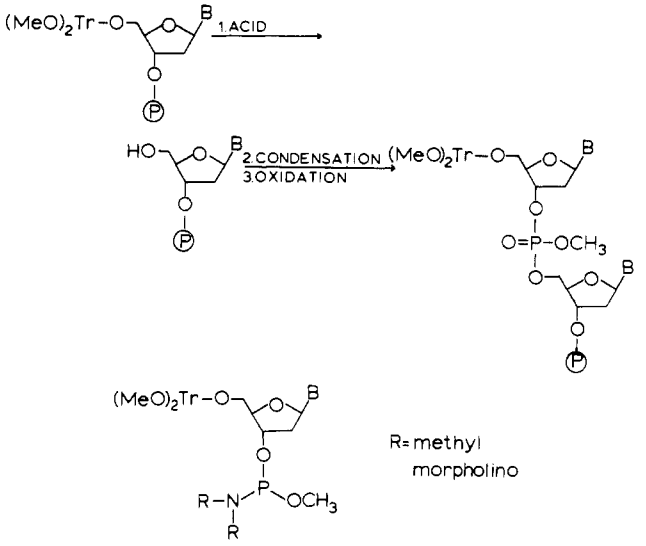

FIGURE 2: Synthesis of a deoxydinucleotide by the polymer-supported phosphite coupling procedure. B represents any properly protected pyrimidine or purine base. Ⓟ represents the insoluble silica gel support.

exocyclic amino protecting groups were then removed. The 5′-*O*-(dimethoxytrityl)deoxyoligonucleotide was purified by reverse-phase high-performance liquid chromatography (HPLC). Separations were accomplished isocratically with 0.1 M triethylammonium acetate (pH 7.0)/acetonitrile (23–26:77–74 v/v). The dimethoxytrityl group was then removed with acetic acid/water (4:1 v/v). A summary of deoxyoligonucleotides synthesized via this approach is included in Table I.

Enzymatic Techniques. Synthetic deoxyoligonucleotides and duplexes to be used in nitrocellulose filter binding assays were phosphorylated by using T4 polynucleotide kinase and adenosine [γ-^{32}P]triphosphate (Maxam & Gilbert, 1977). Two-dimensional sequence analysis of each deoxyoligonucleotide was completed as published (Jay et al., 1974). Deoxyoligonucleotides were enzymatically joined by using T4 ligase as published (Yansura et al., 1977). An alternative buffer system, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.7)/10 mM $MgCl_2$/10 mM dithiothreitol/0.4 mM ATP, was used. Restriction endonuclease digestions were completed according to supplier's recommendations.

The synthetic binding site I was cloned into the *Sma*I restriction site of pUC8. This construct was used to transform *E. coli* strain JM83 according to published procedures (Bolivar & Beckman, 1979). The *Sma*I site was chosen because two restriction endonuclease recognition sites, *Eco*RI and *Bam*HI, are located 5′ proximal and 3′ distal, respectively, within 10 base pairs of the *Sma*I site. After initial histochemical selection and analysis of the restriction endonuclease cleavage pattern, two plasmids, pVF207 and pVF208, were identified. These two plasmids contain a single copy of the TI binding site, having opposite orientations relative to the vector. The sequences of the inserts in pVF207 and pVF208 were confirmed by using the method of Maxam & Gilbert (1977).

Nitrocellulose Filter Binding Assays. The *Eco*RI–*Bam*HI restriction fragment of plasmid pVF207 containing site TI was phosphorylated with adenosine [γ-^{32}P]triphosphate by T4 polynucleotide kinase and isolated by polyacrylamide gel electrophoresis. The duplex was further purified by DEAE-cellulose chromatography followed by filtration through a Gelman no. 4129 sterilization unit. AD2$^+$D2 protein (Tjian, 1978) and SV40 T antigen (Tegtmeyer & Anderson, 1981) were prepared according to published procedures. Filter binding assays were performed as described (Meyers & Tjian, 1980; Riggs et al., 1970). Two binding buffers which differed in ionic strength contained 20 mM sodium phosphate (pH 6.8)/3 mM dithiothreitol/0.1 mM ethylenediaminetetraacetic acid (EDTA)/3% (v/v) dimethyl sulfoxide/50 μg/mL bovine serum albumin. Sodium chloride concentrations of 75 and 150 mM were used. The washing buffer was 20 mM sodium phosphate/0.1 mM EDTA/0.1 mM dithiothreitol.

Results

The deoxyoligonucleotides as defined by the symbols within brackets (Figure 1) were synthesized by using the diester, triester, or phosphite coupling procedures. The project was initiated at a time when the diester approach was used in this laboratory. However, first the phosphate triester approach and then the phosphite triester approaches became the dominant chemistries. As a consequence various deoxyoligonucleotides have been synthesized by using different procedures as summarized in Table I. All compounds, irrespective of the synthetic method, were characterized by two-dimensional sequence analysis. A typical result, as shown in Figure 3, indicated that each compound was homogeneous prior to various enzymatic ligation reactions. Because of the interesting

Table II: Results of Ligation Reactions within Site TI[a]

reaction designation	unphosphorylated segment[b]	5′-phosphorylated segment	[5′-^{32}P]phosphorylated segment	ligation junction analyzed[c]	estimated yield (%)
A	1, 9	10	2	1–2	0
B	2, 8	9	3	2–3	30
C	3, 7	8	4	3–4	30
D	4, 6	7	5	4–5	0
E	1, 9	2	10	9–10	0
F	2, 8	3	9	8–9	50
G	3, 7	4	8	7–8	30
H	3, 6	4	7	6–7	40

[a] Reactions were analyzed by polyacrylamide gel electrophoresis followed by autoradiography. [b] Each segment contained a 5′-hydroxyl group. [c] Ligation junction refers to the T4 ligase catalyzed joining site for two adjacent segments.

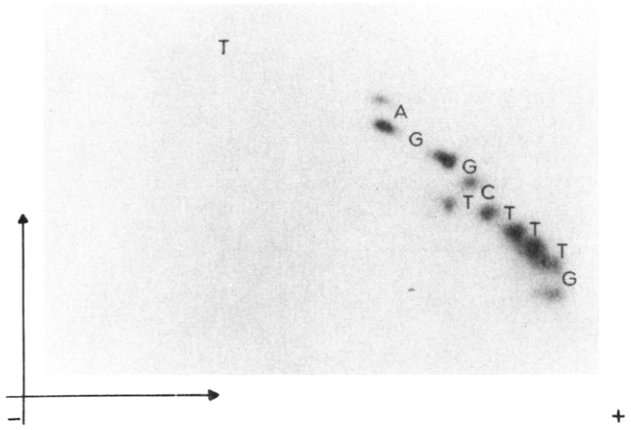

FIGURE 3: Two-dimensional analysis of d(CpTpApGpGp-CpTpTpTpTpG). Electrophoresis on cellulose acetate in pyridinium formate (pH 3.5) was along the longitudinal axis, and homochromatography on DEAE-cellulose was along the verticle axis. Nucleotide losses are recorded between the appropriate spots.

results and problems encountered during various ligation studies, it was important to demonstrate that these synthetic deoxyoligonucleotides were pure and correctly synthesized.

The TI binding site and its immediate flanking region were chemically synthesized in segments as outlined in the first synthetic design (Figure 1). This synthesis plan included external sequences since the precise boundary of site I has not been defined (Tjian, 1979). This duplex contained many palindromes and directly repeated sequences. For example, the first synthetic plan contained ten base pair (3–12), eight base pair (13–20), and six base pair (17–22) palindromes within the left half of the binding site. Direct repeats of eight base pairs (9–16 and 27–34) and six base pairs (19–24 and 31–36) in length and three poly(dA·dT) tracts (2–6, 9–12, and 25–30) were also present. Analytical ligation reactions were designed to test each ligation junction because of the potential problems associated with joining such sequence elements. Each reaction mixture contained four deoxyoligonucleotides which formed a DNA duplex when annealed. Two had 5′-hydroxyl groups and therefore could not participate as 5′-phosphate donors in enzyme-catalyzed joining reactions. One deoxyoligonucleotide contained a 5′-phosphate having no radioactive label and the other contained a [5′-^{32}P]phosphate. Under these conditions, all enzymatic reactions involving a specific deoxyoligonucleotide could be carefully monitored. A summary of these results is included in Table II. Reactions B, C, F, G, and H were observed to produce products having the correct electrophoretic mobility as judged by the method of Frank & Köster (1979). In addition to the expected ^{32}P-labeled product from reaction B, higher molecular weight byproducts were also observed. Since no such products were present in any related reactions, [5′-^{32}P]phosphorylated segment 3 (100 pmol) and segment 8 containing a 5′-hydroxyl group (100 pmol) were tested in a ligation reaction. Analysis of the reaction mixture by polyacrylamide gel electrophoresis indicated that segment 3 had joined to itself forming a 3–3 dimer. These results suggest that a duplex containing eight base pairs and single-stranded regions having only two of five correct base pairs can form stable ligation sites for T4 ligase.

Analysis of reactions A, D, and E indicated that no joining had occurred. Various modifications of reaction conditions did not alter these results substantially. The ligation junction between segments 4 and 5 (reaction D) required a 100-fold molar excess of segment 5 in order to obtain 10% product based on segment 4. Therefore, segment 5, a deoxypentanucleotide, was a very poor 5′-phosphate donor substrate for T4 DNA ligase. The lack of enzymatic joining at the junctions between segments 1 and 2 and segments 9 and 10 was attributed to secondary structure problems. Segments 1 and 2 both contain inverted repeats. Therefore these deoxyoligonucleotides presumably self-annealed or formed hairpin structures. If such structures were stable in reaction mixtures, then the desired duplex involving segments 1, 2, 9, and 10 would not form.

The SV40 binding site was therefore redesigned as shown in the second synthesis plan. Three new deoxyoligonucleotides (segments 1A, 2A, and 4–5) were prepared, and the first seven base pairs 5′ to the TI binding site were eliminated. Deletion of the first seven base pairs (segment 10 and the 5′-terminus of segment 1) eliminated the 10 base pair repeat and yet did not infringe upon the region protected from deoxyribonuclease digestion. Replacement of segments 1 and 2 with 1A and 2A also shifted the ligation junction in this region by one nucleotide. Thus, the inverted repeat within segment 2A (relative to segment 2) was reduced from eight to six deoxynucleotides. Finally, chemical synthesis of segment 4–5 solved the problem associated with T4 ligase catalyzed joining of segment 4 to segment 5.

The redesigned TI site was synthesized via a convergent strategy. All individual joining reactions were checked by using only one ^{32}P–labeled deoxyoligonucleotide per test system. Unlike the results reported for the first plan, all joining sites were observed to react properly. The only difficulty left unresolved by either plan was the self-joining of segment 3. The 5′-hydroxyl of a deoxyoligonucleotide must be phosphorylated in order to be a substrate for T4 DNA ligase. Therefore, segment 3 was not phosphorylated prior to joining with segment 4–5. The total synthesis involved a three-step ligation procedure. Segment 3 (unphosphorylated) was joined to segment 4–5 in the same reaction mixture as segments 6, 7, and 8. The products of this reaction (segment 3–4–5 and segment 6–7–8) were purified and separated as single strands by preparative gel electrophoresis (Figure 4, part a). Segment 3–4–5 was then phosphorylated by using T4 kinase and [γ-

Table III: Kinetic and Equilibrium Measurements of the Interaction of SV40 T Antigen and AD$^+$D2 Protein with the TI Binding Site[a]

assay	protein	[protein] ($\times 10^{-12}$ M)	[DNA] ($\times 10^{-12}$ M)	parameter(s) evaluated	value
protein titration	AD2$^+$D2	0–58	9.1	(1) filter binding efficiency	0.67
				(2) dissociation equilibrium constant	10.3×10^{-12} M
TI binding site titration	AD2$^+$D2	4.0	0.1–100	dissociation equilibrium constant	7.4×10^{-12} M
kinetic association	AD2$^+$D2	7.6	15	association rate constant	4.2×10^{8} M^{-1} s^{-1}
kinetic dissociation	AD2$^+$D2	20	15	(1) dissociation rate constant	4.3×10^{-3} s^{-1}
				(2) dissociation equilibrium constant[b]	9.6×10^{-12} M
protein titration	T antigen	0–160	12	(1) filter binding efficiency	0.71
				(2) dissociation equilibrium constant	3.3×10^{-12} M
TI binding site titration	T antigen	8.3	0.1–150	dissociation equilibrium constant	2.2×10^{-12} M
kinetic association	T antigen	8.0	3.1	association rate constant	3.5×10^{8} M^{-1} s^{-1}
kinetic dissociation	T antigen	4.0	3.6	(1) dissociation rate constant	6.6×10^{-4} s^{-1}
				(2) dissociation equilibrium constant[a]	2.0×10^{-12} M

[a] The TI binding site (Figure 5) was isolated from pVF207. The concentration of sodium chloride was 150 mM. [b] The dissociation equilibrium constant was derived from the ratio k_d/k_a.

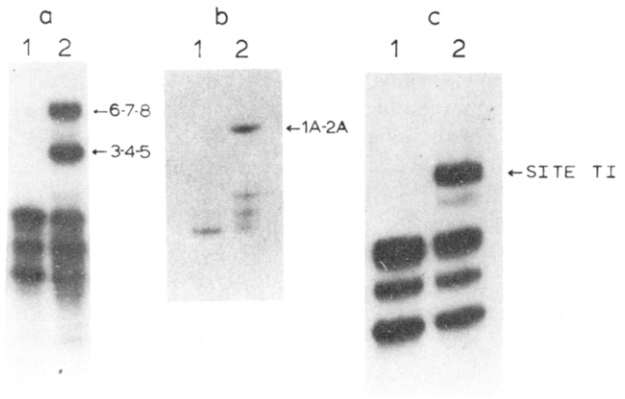

FIGURE 4: Gel electrophoresis results of three joining reactions to form the TI site. Parts a–c show starting materials (lanes numbered 1) and products (lanes numbered 2) formed during the joining reactions to form segments 3–4–5 and 6–7–8 (part a), segment 1A–2A (part b), and the TI site (part c). Arrows designate the positions of these synthesized segments. The two segments corresponding to site TI are partially separated on this gel.

^{32}P]ATP. In a parallel reaction, segment 1A was joined to 2A by using segment 9 as template. An analytical gel showing the results of this ligation is shown in Figure 4 (part b). The DNA strands were separated by preparative gel electrophoresis. Finally, 5′-^{32}P-labeled segments (3–4–5) and 9) and unphosphorylated segments (1A–2A and 6–7–8) were joined by T4 ligase to form site TI. The final duplex was separated from unreacted starting materials by using preparative gel electrophoresis, phosphorylated at the 5′ ends, and cloned into the *Sma*I site of pUC8. Appropriate clones containing the TI duplex in both orientations relative to the adjoining *Bam*HI and *Eco*RI sites were isolated, and the insert was characterized by DNA sequencing. The total sequence of the DNA duplex including the *Eco*RI and *Bam*HI linker sequences is shown in Figure 5.

The binding of either protein (P) to the TI binding site (TI) can be described as a biomolecular equilibrium:

$$P + TI \underset{k_d}{\overset{k_a}{\rightleftharpoons}} P{\cdot}TI \qquad K_D = k_a/k_d = [P][TI]/[P{\cdot}TI] \qquad (1)$$

Table IV: Equilibrium Measurements of the Interaction of AD2$^+$D2 with Synthesized and Recombinant TI Binding Site Duplexes at Differing Ionic Strengths[a]

source of duplex	[protein] ($\times 10^{-12}$ M)	[DNA] ($\times 10^{-12}$ M)	[NaCl] ($\times 10^{-3}$ M)	K_D ($\times 10^{-12}$ M)
synthetic	0–138	10.6	75	13
synthetic	0–138	8.3	150	33
recombinant	0–138	20.0	75	3.8
recombinant	0–138	31.5	150	14

[a] The equilibrium measurements were accomplished by using the protein titration method. The synthetic duplex had the DNA sequence shown in plan 2, Figure 1.

where P·TI represents the protein–DNA complex, k_a is the association rate constant, k_d is the dissociation rate constant, and K_D is the dissociation equilibrium constant. Equation 2

$$[P]_{1/2} = K_D + \tfrac{1}{2}[TI]_0 \qquad (2)$$

relates the concentration of D2 protein required to bind 50% of the TI binding site present, $[P]_{1/2}$, and the total molar concentration of TI binding site, $[TI]_0$, to K_D. Analogously, the relationship between the total concentration of DNA binding protein, $[P]_0$, the concentration of TI binding site at 50% saturation, $[TI]_{1/2}$, and the dissociation equilibrium constant, K_D, is expressed in eq 3. Both protein–DNA interactions were analyzed by these two methods of titration. The equilibrium constants determined are reported in Tables III and IV. The AD2$^+$D2 protein did not form as tight a complex with the TI binding site as did the SV40 T antigen in either assay.

$$[TI]_{1/2} = K_D + \tfrac{1}{2}[P]_0 \qquad (3)$$

The equilibrium constant can also be calculated from the association rate constant and the dissociation rate constant. The differential equation describing the rate of change in [P·TI], the concentration of D2 protein–TI binding site complex, is given in eq 4. In the early portion of the association

$$\frac{d([P{\cdot}TI])}{dt} = k_a([P]_0 - [P{\cdot}TI])([TI]_0 - [P{\cdot}TI]) - k_d([P{\cdot}TI]) \qquad (4)$$

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          5         10        15        20        25        30        35        40
G-A-T-C-C-C-C-C-A-A-A-A-G-C-C-T-A-G-G-C-C-T-C-C-A-A-A-A-A-A-G-C-C-T-C-C-T-C-A-C-T-A-C-T-T-C-T-G-G-A-G-G-G   3'
    G-G-G-G-T-T-T-T-C-G-G-A-T-C-C-G-G-A-G-G-T-T-T-T-T-T-C-G-G-A-G-G-A-G-T-G-A-T-G-A-A-G-A-A-G-A-C-C-C-T-T-A-A  5'
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FIGURE 5: Binding site TI including the *Bam*HI and *Eco*RI sequences derived from pVF207. The numbering is from plan 2, Figure 1.

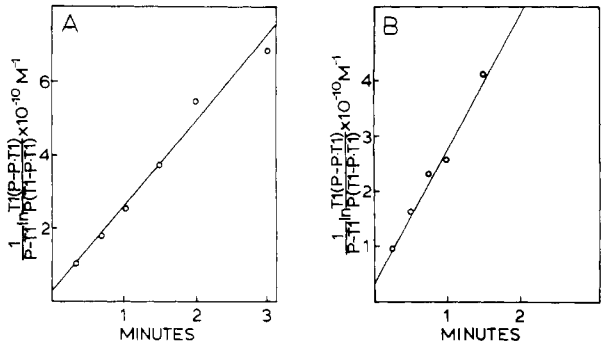

FIGURE 6: Rate of association of AD2$^+$D2 protein (panel A) and T antigen (panel B) with binding site TI from the recombinant plasmid (DNA sequence shown in Figure 5).

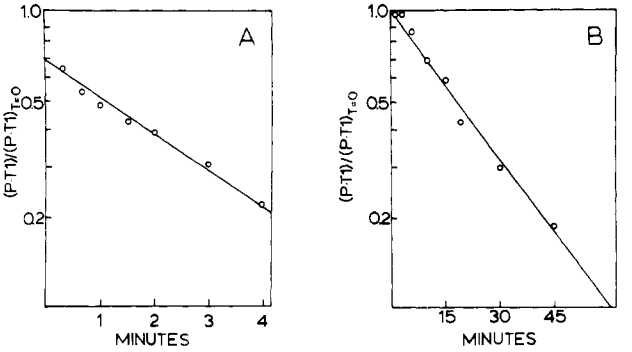

FIGURE 7: Rate of dissociation of the AD2$^+$D2 protein (panel A) and the T antigen (panel B) from binding site TI from the recombinant plasmid (DNA sequence shown in Figure 5).

kinetics, the term $k_d([P{\cdot}TI])$ does not contribute significantly to the change in concentration of complex and therefore can be neglected. The integrated rate equation for the bimolecular reaction is given in eq 5. A plot of the data obtained for the

$$k_a t = \frac{1}{[P]_0 - [TI]_0} \ln \frac{[P]_0([TI]_0 - [P{\cdot}TI])}{[TI]_0([P]_0 - [P{\cdot}TI])} \quad (5)$$

two proteins with binding site I (Figure 5) is shown in Figure 6. The two rate constants, 4.2×10^8 M^{-1} s^{-1} for AD2$^+$D2 protein and 3.5×10^8 M^{-1} s^{-1} for SV40 T antigen, are essentially equivalent and diffusion limited (Goeddel et al., 1977b).

The dissociation rate constant can be easily determined if the association rate constant can be neglected. Dissociation kinetic data were obtained by mixing a 100-fold molar excess of unlabeled TI binding site–protein complex. In order to ensure pseudo-first-order kinetics, AD2$^+$D2 or T antigen was added to a final concentration that would permit only 80% of the labeled TI binding site to be complexed with the protein. Under these conditions the following relates the initial labeled concentration of protein–DNA complex and the kinetic concentration of labeled complex to the dissociation rate constant:

$$\ln \frac{[P{\cdot}TI]}{[P{\cdot}TI]_0} = -k_d t + C \quad (6)$$

A plot of the data obtained for the dissociation of TI binding site–AD2$^+$D2 protein and T antigen complexes is shown in Figure 7. The dissociation rate constants determined were 4.3×10^{-3} s^{-1} and 6.6×10^{-4} s^{-1} for the two interactions, respectively. The dissociation equilibrium constants derived from the ratio of the two rate constants determined were 9.6 $\times 10^{-12}$ M and 2.0×10^{-12} M for the AD2$^+$D2 protein interaction and the SV40 T antigen interaction, respectively. These values are in agreement with the constants measured by equilibrium studies. Therefore, it is apparent that the rates of formation of the two complexes differed only slightly, whereas the rates of dissociation differ by over sixfold.

Discussion

The TI binding site, as well as other gene control regions, contains both inverted and direct repeat elements. These features must be considered in the design of the synthetic deoxyoligonucleotide precursors. Short palindromes within a given fragment must be minimized. This is complicated by the four base pair overlap requirement for efficient T_4 DNA ligase catalyzed joining reactions (Sgaramella & Khorana, 1972). If the palindrome is bisected in one strand, it will be mamximized in the complementary strand. Therefore, part of the inverted repeat has to remain intact in both of the two strands. Directly repeated sequences pose another type of problem. If either the 5′-terminus or the 3′-terminus of two different fragments exhibits extensive homology, they may substitute for one another in the joining reaction leading to undesirable side products. This problem can usually be avoided by analyzing the synthetic design for such homology and shifting the junctions of the deoxyoligonucleotides if necessary. Deoxyoligonucleotides which are to be 5′-phosphate donors in joining reactions should be a minimum of seven nucleotides in length. Compounds that donate 3′-hydroxyls may be shorter but on the basis of a large number of experiments, compounds shorter than deoxyhexanucleotides should be avoided.

The dissociation equilibrium constants obtained for the AD2$^+$D2 protein interaction with the recombinant TI binding site at 150 mM NaCl varied from 7.4×10^{-12} to 14×10^{-12} M, with an average value of 9.1×10^{-12} M. The dissociation equilibrium constants for the T antigen–recombinant TI binding site interaction varied from 2.2×10^{-12} to 3.3×10^{-12} M, with an average value of 2.5×10^{-12} M. The association rate constants for the AD2$^+$D2 protein interaction, 4.2×10^8 M^{-1} s^{-1}, and the T antigen interaction, 3.5×10^8 M^{-1} s^{-1}, are essentially identical. A sixfold difference in the dissociation rate constant was observed for the two different interactions. Since the TI binding site, the buffer system, the competitor DNA, and the other experimental parameters of the kinetic study were held constant, the difference must be attributed to the protein samples. The difference in binding free energy change between the two protein interactions, 4 kJ/mol, was determined. This is equivalent to loss of a single hydrogen bond (Matthews et al., 1982). Alternatively, this difference may result from slightly weakening multiple hydrogen-bond interactions. It may be possible to discriminate between these two explanations by nucleoside analogue studies.

The dissociation equilibrium constant for the T antigen–TI binding site has been estimated to be 1×10^{-12} M at 20 mM $NaHPO_4$, pH 6.8, and 67 mM NaCl (Jessel et al., 1976). The values obtained for the synthetic and the recombinant TI sites were 13×10^{-12} and 3.8×10^{-12} M at 75 mM NaCl, respectively. These differences may be attributed, in part, to length effects. It has been noted in other investigations that shorter DNA duplexes tend to have less stable interactions with DNA binding proteins (Goeddel et al., 1977b). At this ionic strength, interaction of the AD2$^+$D2 protein preparation with nonspecific DNA was significant (data not shown). Therefore, in order to elicit specific interaction between the AD2$^+$D2 protein and the TI binding site, high ionic strength was necessary (Tjian, 1978; Meyers & Tjian, 1980). This high ionic strength buffer system (20 mM sodium phosphate, pH 6.8, 150 mM NaCl) was therefore used for analyzing both the SV40 T antigen and AD2$^+$D2 protein interactions with site TI. The dissociation equilibrium constants obtained were 33

$\times 10^{-12}$ M and 14×10^{-12} M for the synthetic and recombinant TI binding sites, respectively.

The AD2$^+$D2 protein and SV40 T antigen therefore bind specifically to similar but perhaps not identical sequence recognition elements. This conclusion is based primarily on the reproducibly different dissociation rate constants for the two proteins. Additionally, the synthetic duplex formed a less stable complex with AD2$^+$D2 protein than did the same binding site isolated from a recombinant plasmid. The relative binding constants differ by approximately threefold. This difference can easily be attributed to length effects as has been observed previously for synthetic *lac* operators (Goeddel et al., 1977b) and *lac* operators derived from recombinant plasmids (Winter & von Hippel, 1981; Berg et al., 1981). However, a complex having a binding constant of 33×10^{-12} M is sufficiently stable for various studies involving base analogues (Caruthers, 1980). This approach should therefore prove useful for deducing specific binding site contacts with either SV40 T antigen or AD2$^+$D2 protein.

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Registry No. d(CpTpTpTpTpTpGpCpApApApA), 92396-48-0; d(CpApApApApG), 92396-49-1; d(GpCpCpTpApGpGpCpCpTpC), 92396-50-4; d(CpCpTpApGpGpCpCpTpC), 92396-51-5; d(CpApApApApApApGpCpCpTpCpC), 92489-82-2; d(TpCpApCpTpApCpTpT), 92396-52-6; d(TpCpApCpTpApCpTpTpCpTpGpGpA), 92489-84-4; d(CpTpGpGpA), 92396-53-7; d(TpCpCpApGpApApGpTpA), 92420-17-2; d(GpTpGpApGpGpApGpG), 92396-54-8; d(CpTpTpTpTpTpTpGpGpApGpGpC), 92489-83-3; d(CpTpApGpGpCpTpTpTpTpG), 92396-55-9; d(CpApApApApApG), 75567-41-8; plan 2 nucleotide, 92396-47-9.

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