DNA binding of polyomavirus large T-antigen: kinetics of interactions with different types of binding sites

Kåre Bondeson[1], Ola Rönn[1], Göran Magnusson*

Department of Medical Immunology and Microbiology, Uppsala University, Biomedical Centre, Box 582, S-751 23 Uppsala, Sweden

Received 20 January 1998

Abstract Polyomavirus large T-antigen binds to GRGGC sites in double-stranded viral DNA, regulating transcription and replication. Using surface plasmon resonance to record interactions of large T-antigen with different types of binding sites, we found that the configuration of recognition motifs influenced both the association and dissociation rates. Particularly, the complex formed at the origin of DNA replication was labile. A comparison of the interactions between large T-antigen and binding sites with one, two and four GRGGC motifs in tandem showed a strong preference for dimer binding, without detectable co-operativity between dimers. Sodium chloride stabilised the complexes, whereas the dissociation increased rapidly by increasing pH above 7.0.

Key words: Biosensor; DNA binding; Large T-antigen; Polyomavirus

1. Introduction

Polyomavirus infection of permissive cells results in the formation of approximately 10^5 DNA molecules. Cellular and viral DNA synthesis take place in parallel and polyomavirus large T-antigen is the only viral protein known to direct the replication of the viral chromosome. This multifunctional protein has a pivotal role in the infection of cells. Interaction with some cellular proteins leads to growth stimulation, and binding of large T-antigen to specific sites in viral DNA regulates its replication and transcription. The protein encoded by simian virus has been studied in great detail. The murine polyomavirus large T-antigen is not as well characterised, but shares many features with the simian virus 40 protein [1].

Large T-antigen has a general affinity for DNA, particularly single-stranded, and binds specifically to sites in double-stranded DNA with the pentanucleotide GRGGC. Contacts are made with guanine bases in the major groove of DNA [2,3]. Binding sites A–C in the regulatory region of polyomavirus DNA (Fig. 1) contain head-to-tail repeats of the pentanucleotide recognition motif separated by 4–6 bp. Occupation of bindings sites B and C decreases transcription of the viral early genes [4–6]. A different configuration of large T-antigen binding motifs is found at the origin of DNA replication (site 1). Here, tandem repeats in opposite orientation form part of a longer base sequence of dyad symmetry (Fig. 1). In polyomavirus DNA these GRGGC motifs are separated by only 2 bp, and the two central motifs in the dyad symmetry overlap. The structure and stability of complexes formed between large T-antigen and the replication origin type of binding site are influenced by ATP [7–9]. In the presence of the nucleotide two hexamers of large T-antigen assemble at the binding sites and then unwind the strands of the DNA molecule [10].

DNA binding of polyomavirus large T-antigen has been analysed by various techniques [2,3,7]. In general, little information on kinetics of the reaction has been provided. Even capture of large T-antigen-SNA complexes on nitrocellulose filters [7] has limited resolution, particularly of the dissociation reaction. Analysis of the simian virus 40 large T-antigen binding kinetics with these methods shows a K_d in the range of $1–5\times10^{-9}$ M [11,12]. This value can be compared with the well characterised Lac repressor-operator interaction, having a K_d of $1–10\times10^{-13}$ M [13].

Protein-DNA interactions can be studied with an instrument that monitors SPR [14,15]. For a review of the use of such systems, see Granzow [16]. Interaction between macromolecules can be observed in real time by recording changes in refractive index near the sensor surface. These changes are proportional to the mass of molecules that bind to the surface and can be monitored continuously. Association and dissociation of protein-DNA complexes can be studied in the same experiment. To study the dissociation reaction, buffer without protein is passed over the protein-DNA complexes on the sensor surface.

We report on results of experiments with large T-antigen. Interaction of the protein with different types of recognition sites in DNA was investigated and differences in both the association and dissociation reactions were observed.

2. Materials and methods

2.1. Instrumentation and chemicals

The BIAcore system, SA and CM5 sensor chips and kit for coupling ligands to the sensor cell surface were from BIAcore AB (Uppsala, Sweden). The coupling kit contained *N*-hydroxysuccinimide, *N*-ethyl-*N*′-(3-diethylaminopropyl)-carbodiimide, and 1.0 M ethanol amine hydrochloride, pH 8.5. Chemical were obtained from the following sources: streptavidin from Boehringer Mannheim GmBH; poly(dI-dC) with an average length of 8600 bp from Pharmacia; aprotinin, leupeptin, PMSF and DTT from Sigma. The F5 peptide, N-Leu-Leu-His-Pro-Asp-Lys-Gly-Gly-Ser-His-Ala-C, corresponding to amino acid residues 40–50 in polyomavirus large T-antigen, was obtained from Dr. Åke Engström, Uppsala University.

2.2. Oligonucleotides, DNA templates and PCR

Oligonucleotides used as primers in PCR reactions correspond to the indicated segments of polyomavirus A2 DNA (GenBank/EMBL accession number J02288). BS1-A, nucleotides 40–17; BS1-B; nucleotides 5268–5294; BSC-A, nucleotides 110–132; BSC-B, nucleotides

*Corresponding author. Fax: (46) (18) 509876.
E-mail: mago@bio.embnet.se

[1]K. Bondeson and O. Rönn contributed equally to the results of this report.

Abbreviations: PCR, polymerase chain reaction; RU, responsive unit; SPR, surface plasmon resonance

PII S0014-5793(98)00111-2

192–196; Py5052-, nucleotides 5052–5078. All these oligonucleotides had a biotin group at the 5′ end. The oligonucleotides BSC-1p, BSC-2p and BSC-3p are all derived from BSCwtp (nucleotides 176–136) with G to A changes as indicated by bold face. BSC-1p, 5′-ATGATGGTGGTG**AA**GCTGAAATGAGGCGGAAGAGGCGGT-3′; BSC2p, 5′-ATGATGGTGGTGA**A**GCTGAAATGA**A**GCGGGAAGAGGCGGT-3′; BSC3p, 5′-ATGATGGTGGTG**AA**GCTGAAATGA**A**GCGGGAGA**A**GCGGTGAAGCTTGA-3′. When possible the PCR primers were selected to give double-stranded polynucleotides with 24–27 bp segments at the ends without large T-antigen binding sites. The BSC-A primer included four nucleotides of non-polyomavirus sequence in its 5′ end, to produce a 24 bp flanking region without a large T-antigen motif. A DNA template without a large T-antigen binding motif (TH.ds.1) was isolated by a PCR-based selection system using thrombin as a selective agent, and then cloned in a bacterial plasmid [17].

To purify DNA for protein binding experiments, PCR products were separated by agarose gel electrophoresis (FMC BioProducts). A strip of agarose with the PCR product was excised, cast in a 1% NuSieve GTG agarose gel and again subjected to electrophoresis. The DNA was extracted from the gel, treated with phenol and finally ethanol precipitated.

2.3. Immobilisation of DNA to the dextran matrix of the biosensor cells

Immobilisation of streptavidin to the dextran matrix via primary amine groups [18] and subsequent binding of biotinylated DNA was performed as described [14]. When sensor chip SA was used, the streptavidin immobilisation step was not necessary. The chip was treated according to instructions from the manufacturer and then conditioned as described.

Each biotinylated double-stranded polydeoxyribonucleotide was dissolved in buffer (20 mM $KHPO_4$, pH 7.0, 150 mM NaCl, 1.0 mM EDTA, 0.05% Tween 20) at 0.01–0.1 ng/ml and was injected into one of the four sensor chip channels with streptavidin-coated surfaces. Thus, four surfaces, each with a different DNA template, were prepared. Binding of the polynucleotide was monitored by the instrument and the injection was interrupted manually. The surface were then washed with buffer A. To obtain the correct amount of immobilised DNA, repeated injections of DNA were done. The amount of immobilised DNA on the four surfaces varied by less than 10 RU.

2.4. Expression and purification of large T-antigen

A recombinant baculovirus, pYMLT, encoding large T-antigen was obtained by using the transfer vector pAcYM.1 [19]. Large T-antigen was prepared and purified essentially as described previously [9]. Growing *Spodoptera frugiperda* Sf9 or *Trichoplusia ni* High Five cells were infected with vAcYMLT at a multiplicity of infection of 1–10 PFU/cell. The cultures were harvested at 42 h post-infection. After extraction, large T-antigen was adsorbed to monoclonal antibody F5 [20] coupled to BioGel P-10 (Bio-Rad). After extensive washing with buffer B (20 mM Tris-HCl, pH 8.5, 500 mM NaCl, 1 mM DTT, 2 U/ml aprotinin), large T-antigen was eluted with buffer B containing 80 μg/ml of F5 peptide [9]. The final protein preparation was dialysed against buffer C (20 mM $KHPO_4$, pH 7.0, 150 mM NaCl, 1.0 mM EDTA, 1.0 mM DTT and 0.05% Tween 20).

2.5. Reaction kinetics of large T-antigen-DNA interactions

Detailed descriptions of the use of SPR in monitoring of protein-protein and protein-DNA interactions have been published [14,15,21–24]. All experiments were carried out at 25°C. In the association phase a flow of buffer A with large T-antigen was passed over the surface with immobilised DNA. At an adequate flow, the protein is constantly replenished and the free concentration is approximately equal to the total concentration. The software of the BIAcore system calculates association and dissociation rate constants from selected parts of the reactions. However, only association reactions that are consistent with the Langmuir binding polynomial can be correctly analysed by the present version of the BIAcore software [25].

The SPR response, measured RU (1 RU of protein $\approx$ 1 pg/mm^2) is directly proportional to the concentration of formed protein-DNA complexes. Therefore, the response values can be used directly without conversion to the number of large T-antigen molecules, and the reaction rate can be recorded as dR/dt. For the dissociation reaction the rate equation can be simplified to $dR/dt = -k_{diss}R$, provided that rebinding of dissociated protein does not occur. This requirement is fulfilled when an adequate liquid flow is maintained.

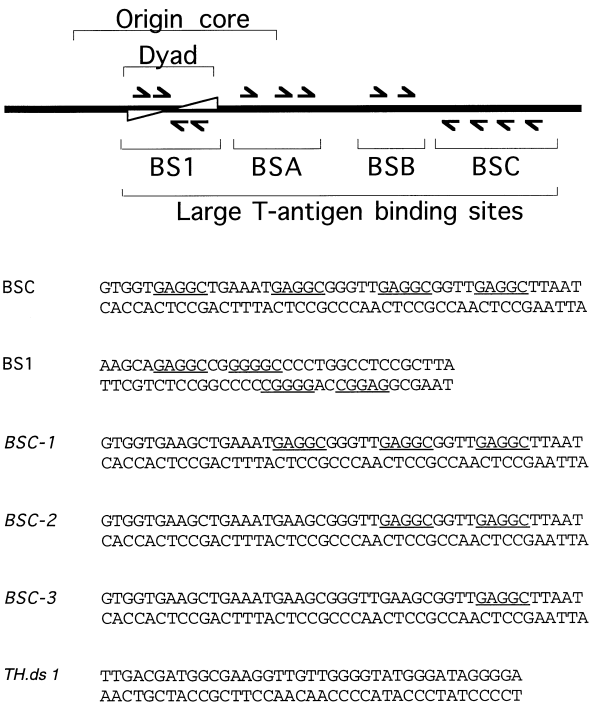

Fig. 1. Map of the replication origin and early promoter of polyomavirus DNA. On the map, arrows indicate the position and orientation of large T-antigen binding motifs (GRGGC). Binding sites with clustered GRGGC motifs are designated according to Cowie and Kamen [2]. The nucleotide sequence of binding sites C and 1 are shown together with the large T-antigen binding segments of the binding site C mutant polynucleotides *BSC-1, BSC-2* and *Bsc3*. The corresponding sequence of the non-binding polynucleotide *TH-ds.1* is also shown. Binding motifs are underlined.

2.6. Solid phase DNase I footprinting

The assay was done by combining two methods previously described [7,26]. DNA fragments containing the polyomavirus origin were prepared as described. Before PCR, the primers BSCwtp, BSC-1p, BSC-2p, and BSC-3p were radioactively labelled with [γ-^{32}P]ATP, using polynucleotide kinase (Fermentas) in a standard reaction. The primer Py5052- contained a biotin group and, thus, the finished radioactive products could be immobilised on streptavidin-coated paramagnetic beads (Dynabeads M280-Streptavidin, Dynal, Oslo). All steps included the use of a magnetic particle concentrator (MPC, Dynal, Oslo). Radioactivity in a portion of the beads was measured in a scintillation counter. Polyomavirus large T-antigen was incubated in 50 μl R buffer (40 mM creatine phosphate di-Tris salt, pH 7.8, 7.0 mM $MgCl_2$, 0.5 mM DTT, 0.2 mg/ml bovine serum albumin) with magnetic beads-DNA complex (1.0 ng) at 37°C for 15 min. An optimised amount of DNase I (Promega) was used as previously described [7]. The beads were then washed with 100 μl 2.0 M NaCl, 0.02 mM EDTA, followed by 100 μl 10 mM Tris-HCl, pH 8.0, 1.0 mM EDTA. After determination of remaining radioactivity, the samples were suspended in loading buffer, denatured and electrophoresed in an 8% polyacrylamide sequencing gel which was then dried and autoradiographed.

3. Results

3.1. Kinetic analysis of large T-antigen binding to two types of naturally occurring binding sites

Double-stranded polyomavirus DNA segments of 70–80 bp, containing binding site C or binding site 1, were made

by PCR. The polynucleotides had a biotin group at the 5′ end of one strand that was used to anchor the molecule to the streptavidin-coated surface of the sensor cell. The two types of polynucleotides were bound to parallel flow cells on a sensor chip. As a negative control we used DNA without large T-antigen binding sites (*TH.ds.1*). On the basis of previous experience we attached relatively low amounts of DNA (60–200 RU) to the surface of the flow cells. In this way, effects of mass transport limitation in the binding reaction and transfer of large T-antigen between polynucleotide molecules during the dissociation phase were kept at a practical minimum.

In the binding experiments buffer A was passed through the detection unit at a constant flow rate. The association reactions were started by injecting large T-antigen diluted in buffer A into one of the sensor cells. Protein binding was recorded as change in SPR over time. The dissociation reaction was then studied by changing back to buffer A without large T-antigen, and the disappearance of protein from the sensor surface was recorded. Addition to and removal of protein from buffer A caused instant resonance responses due to slight changes of buffer composition and pressure. These responses are not included in the calculation of association and dissociation rates.

Fig. 2A shows the changes of SPR when large T-antigen at four different concentrations was passed over a surface containing a polynucleotide with binding site C. The four sensorgrams show the association rate and amount of bound large T-antigen were directly proportional to the protein concentration. When large T-antigen was withdrawn from the flowing buffer, a slow dissociation of the complexes was apparent. Processing of the dissociation data generated a set of dissociation curves (Fig. 2B), showing that the reactions were essentially independent of the amount of bound large T-antigen, but that the superimposed curves were biphasic. This result suggests that at least two different complexes were present. After 300 s of the dissociation phase, most of the labile complex had disappeared. The remaining stable complex dissociated at a much slower rate, making a calculation of the dissociation rate constant inaccurate when measurements were done over short time periods (400 s).

With a polynucleotide containing binding site 1, a set of sensorgrams with a slightly different shape was obtained (Fig. 2C). The association rate was still directly proportional to the protein concentration. However, a plot of the large T-antigen dissociation from binding site 1 (Fig. 2D) showed that the initial dissociation rates were dependent on the amount of bound large T-antigen. Also with site 1 the dissociation rates decreased as a function of time, suggesting that also in this case more than one type of complex had been formed.

Calculation of rate constants was not straightforward, since the binding of large T-antigen to DNA was inconsistent with a Langmuir binding polynomial. To quantify the association and dissociation rates, the amount of protein bound to different types of sites under standardised conditions was determined. In the first experiment 60 RU of double-stranded polydeoxynucleotide, containing binding site 1 or C, was immobilised on the surface. With approximately 250 nM large T-antigen in the flow buffer similar amounts of large T-antigen bound to the two types of polynucleotides with binding

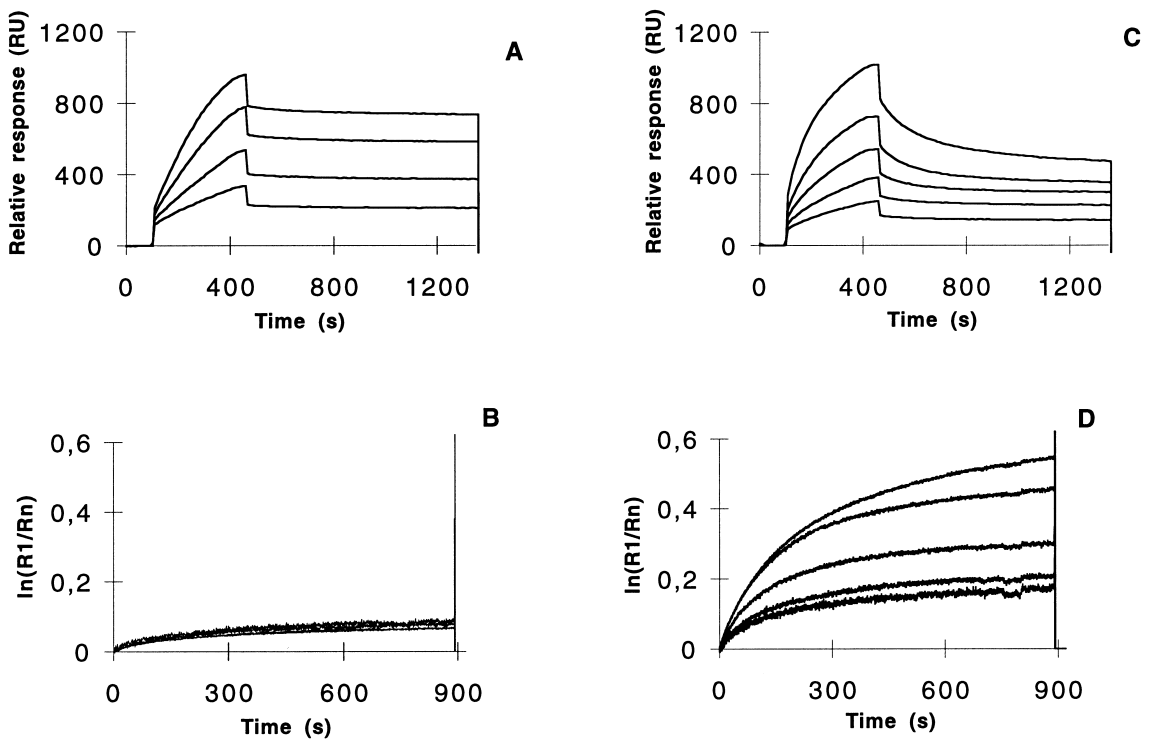

Fig. 2. Superimposed association and dissociation curves of large T-antigen with binding sites C and 1. Large T-antigen at a range of concentrations (100, 150, 200, 250, 300 nM) in buffer A was injected into a cell containing 60 RU of double-stranded polynucleotide with binding site C and 1, respectively. Complete sensorgrams of interactions with site C (A) and site 1 (C) are shown. The curve for protein injected at 300 nM with C was incomplete and, therefore, omitted from the figure. At 460 s the protein was withdrawn from buffer A and dissociation was recorded until 1350 s. Panels B and D display ln(R1/Rn) dissociation curves calculated from the sensorgram data obtained with site C and site 1, respectively. The response (RU) was directly proportional to the concentration.

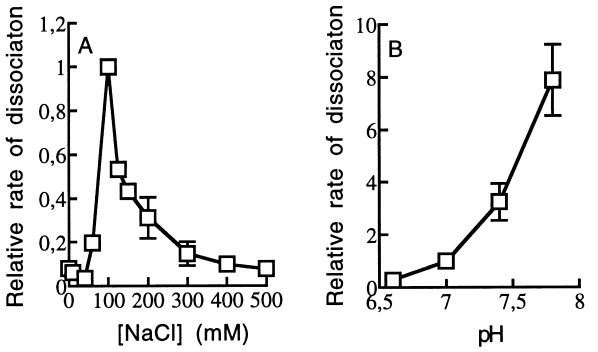

Fig. 3. Analysis of salt and pH dependence of large T-antigen dissociation from site C. Binding of large T-antigen to a polynucleotide with binding site C was performed as described in the legend to Fig. 2. At the shift to the dissociation phase buffer A modified with the indicated concentrations of NaCl (panel A), or to the indicated pH values was used (panel B).

sites (site 1: 550 RU; site C: 784 RU). A polynucleotide of the same length, but without GRGGC motifs, bound only 15% of the amount associated with site C (no site: 115 RU). In the second experiment 100 RU of immobilised polynucleotide was used to bind large T-antigen from a 300 nM solution. Here, 2–3 times more protein was adsorbed to the immobilised polynucleotides, but, again, there was no difference in the amount of protein bound to the two types of polynucleotide containing GRGGC motifs (site 1: 1657 RU; site C: 1659 RU; no site: 220 RU). Since roughly equal amounts of large T-antigen were specifically adsorbed to the two kinds of binding sites, the dissociation curves were directly comparable. Under the experimental conditions, large T-antigen dissociated from binding site 1 at an approximately 6-fold higher rate than from site C (site 1: 30%; site C: 5%). Interestingly, the small amount of large T-antigen complexed with the polynucleotide without a binding site dissociated only at a slightly higher rate than the protein bound to site C (no site: 9%).

The reproducibility of the method was tested by determination of the variability of data derived from five independent experiments (Table 1). The relative association and dissociation rates were calculated using the software of the instrument. Determinations of dissociation rats were based on measurements at 400–800 s after termination of the binding phase, when a large fraction of the protein bound to site 1 already had been released. The small amount of dissociated protein in this time interval made the quantitation inaccurate. This problem is reflected by the high standard deviation of the relative dissociation rates, as determined in different experiments. Nevertheless, the combined data suggest that the relative affinity of large T-antigen for site C and site 1 was determined by differences in both the association and dissociation rates.

3.2. Effect of monovalent salt and pH on large T-antigen binding to site C

We studied the effect of NaCl concentration on the dissociation of large T-antigen from binding site C (Fig. 3A). The protein was bound to DNA in buffer A. During the dissociation phase buffer A, modified by the indicated concentrations of NaCl, was passed through the sensor cell. The results show that the relative dissociation rate was maximal at 0.10 M NaCl. The retention of large T-antigen at lower salt concentration was probably caused by non-specific binding to the negatively charged surface of the cell. At NaCl concentrations above 0.10 M the large T-antigen-DNA complexes were apparently stabilised.

The effect of pH on the dissociation of large T-antigen from site C was also tested. The experiment was performed as described above, with adsorption of the protein done under normal conditions. During the dissociation phase buffer A with the phosphate component adjusted to the indicated pH was used. The relative dissociation rate of large T-antigen increased rapidly when the pH was raised from 6.6 to 7.8 (Fig. 3B). The pH of buffer A, 7.0, is apparently near the optimum for stable DNA binding of large T-antigen.

3.3. Interaction of large T-antigen with polynucleotides containing different numbers of adjacent binding motifs

Large T-antigen binding sites in viral DNA contain at least two adjacent copies of the GRGGC motif, and the related simian virus 40 large T-antigen appears to bind to DNA as a dimer [27]. Thus, we assume that also polyomavirus large T-antigen dimers are the basic DNA binding units. To test this hypothesis for polyomavirus large T-antigen, its interaction with wild-type and mutant binding site C oligonucleotides *BSCwt*, *BSC-1*, *BSC-2* and *BSC-3* containing four, three, two and one GRGGC motifs, respectively, was analysed (Fig. 1). The mutant motifs had the structure GRAGC. To confirm that the mutations abolished large T-antigen binding, DNase I footprinting analyses were done (Fig. 4). The wild-type DNA probe was protected by large T-antigen in a broad area that in the absence of protein contained several hypersensitive DNase I cleavage sites. The footprints agreed with the location of the four GRGGC motifs of binding site C. Two of these DNase I cleavage sites reappeared when GAGGC motif number 1 was mutated. A second hypersensitive site reappeared in DNA with mutation of motifs 1 and 2, and a third when also the sequence of motif 3 was altered. Interestingly, motif 4 of binding site C seemed to be protected by large T-antigen even when the other three sites were mutated.

Table 1
Reproducibility of association and dissociation rate determinations

Binding site[a]	C	1	None
Relative association[b]	1.00 (0.28)	0.64 (0.24)	N.D.
Relative dissociation rate[c]	1.00 (0.84)	2.33 (1.33)	2.33 (0.70)

[a]Polynucleotides with binding site C, and site 1 and the polynucleotide *TH.ds.1* were used.
[b]Association rates were calculated by the BIAcore instrument. The mean was calculated for five experiments and related to the rate of association with binding site C. Values in parentheses show the standard deviations. N.D. signifies not determinable.
[c]Dissociation rates during 400–800 s of the dissociation phase were calculated by the BIAcore instrument. Relative values and standard deviations were calculated as the corresponding association rates.

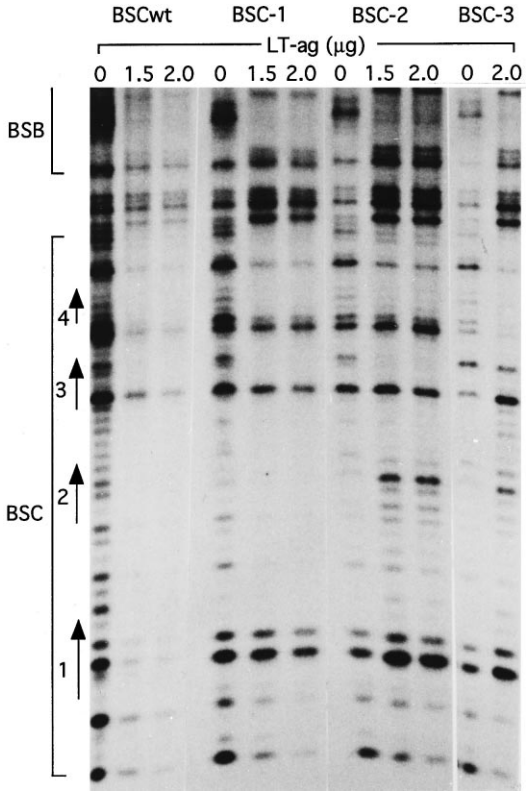

Fig. 4. Footprint analysis of large T-antigen on binding site C wild-type and mutant origins. Large T-antigen (1.5 or 2.0 μg) was incubated with 1 ng of ^{32}P-labelled polynucleotide coupled to paramagnetic beads, at 37°C for 15 min. 1 U of DNase I in 10 μl of buffer was added and the incubation was continued for 60 s. After washing, formamide loading buffer was added and the samples were loaded on a pre-run 8% polyacrylamide sequencing gel. A nucleotide sequence reaction was run in parallel as a marker (not shown). The arrows indicate the location in the autoradiogram, and the direction of the GRGGC binding motifs in site C. The numbers 1–4 correspond to the order in which the motifs are mutated in *BSC-1, BSC-2* and *BSC-3*.

The four double-stranded polynucleotides, containing wild-type and mutant versions of large T-antigen binding site C, were also used in analysis of association and dissociation rates. In this experiment sensorgrams like the ones shown in Fig. 2A were obtained. The shapes of these curves were similar, suggesting that there was no significant co-operative element in the binding. Processing of the data by the BIAcore instrument resulted in a set of relative rates summarised in Table 2. The rate of association to *BSC-3* with one motif was set at 1.00. The association rates for sites with two, three and four motifs were all approximately 2-fold higher. The same trend was also apparent in the dissociation reactions. Again the dissociation rate from *BSC-3* DNA was set at 1.00. The dissociation of large T-antigen from polynucleotides with two, three or four adjacent binding motifs was 1.5–2.0-fold lower (Table 2). Also in this experiment, the high stability of the protein-DNA complexes made the quantitation of the dissociation reactions inaccurate. Still, it is clear that association of large T-antigen was more rapid to DNA with a tandem GRGGC motif than to a single copy, and that dissociation was more rapid from a site with a single motif.

4. Discussion

Binding sites C and 1 in polyomavirus DNA both contain four GRGGC motifs (Fig. 1). However, the orientation and spacing of the sequence motifs in these two sites differ. In binding site 1 the tandem GRGGC pentanucleotides are separated by only 2 bp. Moreover, the two tandem repeats are in opposite orientation and overlap. Due to the spacing of GRGGC a bound protein dimer would not fit the major groove of DNA [2], and the head-to-head orientation of the tandem GRGGC repeats does not allow simultaneous binding of two large T-antigen dimers without distortion of the double helix. Analysis of the contacts made by large T-antigen and the two types of GRGGC binding sites by methylation interference [2], and by protection against DNase I in the absence and presence of ATP [7], confirmed the predicted differences. To analyse the binding kinetics of large T-antigen to the different types of sites in DA, we have used the SPR-based BIAcore system.

DNA binding of the transcription factor Ets has been studied with the SPR technique. These studies showed that the interaction with immobilised DNA was complex [15]. Before binding, the protein had to pass several compartments that were in equilibrium with each other. The individual reactions cannot be resolved by the standard BIAcore computer analysis. There is an ongoing discussion how data obtained from these kinds of instrument shall be processed for determination of absolute rate constants [28–30]. However, in our comparisons of different large T-antigen binding sites the objective was not to obtain absolute values of kinetics constants, but to compare the different types of interactions under standardised conditions.

Large T-antigen bound to site C and site 1 with similar kinetics (Fig. 2A,C). However, there were obvious differences between the dissociation curves, showing that the protein binding – at least to site 1 – must be complex. Thus, the similarity of the net association reactions was probably fortuitous. The clear dependence on protein concentration of large T-antigen binding to site 1 showed that protein-protein interactions must be involved. An interpretation of the data is that initial binding of large T-antigen occurred at a rate that was lower than association with site C. However, once a protein-DNA complex was formed at site 1, formation of a labile complex was facilitated. During the dissociation phase the labile complex disappeared first, but also the remaining pro-

Table 2
Interaction of large T-antigen with polynucleotides with different numbers of pentanucleotide binding motifs

Number of GRGGC motifs[a]	1	2	3	4
Relative association rate[b]	1.00	1.84	2.10	2.03
Relative dissociation rate[c]	1.00 (0.54)	0.66 (0.42)	0.52 (0.09)	0.64 (0.35)

[a]Polynucleotides *BSC-3, BSC-2, BSC-1* and *BSCwt* were used.
[b]Association rates were calculated by the BIAcore instrument.
[c]Dissociation rates were calculated by the BIAcore instrument. Values in parentheses show standard deviations.

tein appeared to be released at a higher rate than the corresponding complex with binding site C. The data do not provide information on the structure of large T-antigen complex at site 1, but the labile complex might consist of hexamers or intermediates in hexamer formation [31].

A compilation of data from several experiments performed with different amounts of immobilised DNA and with different concentrations of large T-antigen showed that the affinity of the protein for site C was higher than for site 1. The difference in binding to the two sites was a result of more rapid association and, in particular, of slower dissociation from site C. Curiously, the dissociation of large T-antigen from a polynucleotide without a GRGGC binding site was as rapid as from DNA containing binding site 1, even when the initial rapid dissociation from site 1 was disregarded. Thus, the specificity of binding to site 1 appeared entirely dependent on the association rate that was at least 10-fold faster than binding to non-specific DNA. This observation may have implications for the understanding of polyomavirus initiation of DNA synthesis. Once the initiation has occurred, the large T-antigen complex bound to site 1 is resolved.

ATP had little effect on the DNA binding of our preparations of large T-antigen, as revealed by the McKay method [32], or in analysis of kinetics using the BIAcore. When binding kinetics of large T-antigen was analysed in the buffer used by Lorimer et al. [7] that contains creatine phosphate, no reproducible binding data were obtained. In the presence of ATP most of the large T-antigen-protein seemed to be converted to a form – probably hexamers – that was inactive in DNA binding. A probable explanation for the different results is that the previous experiments of binding kinetics [7] were done with large T-antigen and DNA present together in solution. Thus, a shift in the equilibrium between monomers and oligomers of the polypeptide could take place as the binding reaction proceeded. In the BIAcore instrument the protein passes a surface with binding sites and the equilibrium between different oligomeric forms of large T-antigen will probably not shift significantly during the short exposure to DNA.

A dimer of large T-antigen is considered to be the primary DNA binding unit [1]. Our data (Table 2) support this observation. We tested binding of polyomavirus large T-antigen to double-stranded polynucleotides with wild-type and mutant forms of binding site C. The mutant polynucleotides had 1–3 adjacent GRGGC motifs changed to GRAGC. Footprint analysis showed that the mutation abolished DNase I protection by large T-antigen (Fig. 4). Analysis of the kinetics of large T-antigen interaction with DNA showed that the association rates to polynucleotides with 2–4 GRGGC motifs were similar, whereas the binding to the polynucleotide with a single intact recognition motif was approximately half as rapid. The dissociation kinetics showed a corresponding dependence on the number of repeated GRGGC motifs, the rate of dissociation being two times higher from the polynucleotide with one motif than from the polynucleotides with two, three or four. The observation that stability of a complex formed with a site containing four GRGGC motifs was no greater than with a two-motif site indicates that adjacent protein dimers did not co-operate in binding. This conclusion is supported by the similarity of the association kinetics.

The BIAcore instrument offers the advantage that dissociation of protein from DNA can be analysed without addition of competitor DNA that traps released polypeptides. Although large T-antigen forms less stable complexes with its cognate binding sites than the Lac repressor [14], the slow dissociation makes accurate rate determinations difficult. We tested whether dissociation could be accelerated by increasing the salt concentration of the binding buffer, or by changing the pH. The data presented in Fig. 3 show that an increase of the NaCl concentration stabilised the large T-antigen complex formed with binding site C. The result suggests that electrostatic forces were not important for the binding. Increasing the pH of the binding buffer did accelerate dissociation of large T-antigen from site C. The stability of the complex appeared to decrease exponentially when pH was raised from 6.6 to 7.8. This effect might be caused by titration of imidazole groups in histidine residues in large T-antigen. NMR studies of the simian virus 40 DNA binding domain indicate that a hydrogen bond involving a histidine residue is important for binding to the GRGGC motif [33]. This region, including the histidine residue, is conserved in polyomavirus large T-antigen.

Acknowledgements: We thank Dr. D. Bishop for the plasmid pAcYM.1. Drs. D. Pallas and C. Prives generously contributed material for the purification of large T-antigen. The work described in this paper was supported financially by the Swedish Cancer Society.

References

[1] Fanning, E. and Knippers, R. (1992) Annu. Rev. Biochem. 61, 55–85.
[2] Cowie, A. and Kamen, R. (1984) J. Virol. 52, 750–760.
[3] Cowie, A. and Kamen, R. (1986) J. Virol. 57, 505–514.
[4] Bergqvist, A., Nilsson, M., Bondeson, K. and Magnusson, G. (1990) Nucleic Acids Res. 18, 2715–2720.
[5] Farmerie, W.G. and Folk, W.R. (1984) Proc. Natl. Acad. Sci. USA 81, 6919–6923.
[6] Rio, D., Robbins, R., Myers, R. and Tjian, R. (1980) Proc. Natl. Acad. Sci. USA 77, 5706–5710.
[7] Lorimer, H.E., Wang, E.H. and Prives, C. (1991) J. Virol. 65, 687–699.
[8] Mastrangelo, I.A., Hough, P.V.C., Wall, J.S., Dodson, M., Dean, F.B. and Hurwitz, J. (1989) Nature 338, 658–662.
[9] Wang, E.H. and Prives, C. (1991) J. Biol. Chem. 266, 12668–12675.
[10] Borowiec, J.A., Dean, F.B., Bullock, P.A. and Hurwitz, J. (1990) Cell 60, 181–184.
[11] Jones, K.A. and Tjian, R. (1984) Cell 36, 155–162.
[12] Schirmbeck, R. and Deppert, W. (1988) Virology 165, 527–538.
[13] Fickert, R. and Müller-Hill, B. (1992) J. Mol. Biol. 226, 59–68.
[14] Bondeson, K., Frostell-Karlsson, Å., Fägerstam, L. and Magnusson, G. (1993) Anal. Biochem. 214, 245–251.
[15] Fishe, R.J., Fivash, M., Casas-Finet, J., Erickson, J.W., Kondoh, A., Bladen, S.V., Fisher, C., Watson, D.K. and Papas, T. (1994) Protein Sci. 257–266.
[16] Granzow, R. (1994) Methods 6, 93–205.
[17] Bondeson, K., Rönn, O. and Magnusson, G. (1995) Eur. J. Biochem. 227, 359–366.
[18] Johnsson, B., Lofas, S. and Lindquist, G. (1991) Anal. Biochem. 198, 268–277.
[19] Matsuura, Y., Possee, R.D., Overton, H.A. and Bishop, D.H.L. (1987) J. Gen. Virol. 68, 1233–1250.
[20] Pallas, D.C., Schley, C., Mahoney, M., Harlow, E., Schaffhausen, B. and Roberts, T.M. (1986) J. Virol. 60, 1075–1084.
[21] Fägerstam, L.G., Frostell-Karlsson, Å., Karlsson, R., Persson, B. and Rönnberg, I. (1992) J. Chromatogr. 597, 397–410.
[22] Karlsson, R., Michaelsson, A. and Mattson, L. (1991) J. Immunol. Methods 145, 229–240.
[23] Malmqvist, M. (1993) Nature 361, 186–187.
[24] O'Shannessy, D.J., Brigham-Burke, M., Soneson, K.K., Hensley, P. and Brools, I. (1993) Anal. Biochem. 212, 457–468.
[25] O'Shannessy, D.J. (1994) Curr. Opin. Biotech. 5, 65–71.

[26] Sandaltzopoulos, R. and Becker, P.B. (1994) Nucleic Acids Res. 22, 1511–1512.
[27] Deb, S.P. and Deb, S. (1989) J. Virol. 63, 2901–2907.
[28] Schuck, P. (1996) Biophys. J. 70, 1230–1249.
[29] Bowles, M.R., Hall, D.R., Pond, S.M. and Winzor, D.J. (1997) Anal. Biochem. 244, 133–143.
[30] Karlsson, R. and Fält, A. (1997) J. Immunol. Methods 200, 121–133.
[31] Wang, E.H. and Prives, C. (1991) Virology 184, 399–403.
[32] McKay, R.D.G. (1981) J. Mol. Biol. 145, 471–488.
[33] Luo, X., Sanford, D.G., Bullock, P.A. and Bachovchin, W.W. (1996) Nature Struct. Biol. 3, 1034–1039.