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Electron microscopy of SV40 DNA cross-linked by anti-Z DNA IgG

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Electron microscopy has revealed the specific binding of bivalent anti-Z DNA immunoglobulin G (IgG) to different sites on supercoiled Form I SV40 DNA. The anti-Z IgG links together left-handed regions located within individual or on multiple SV40 DNA molecules at the superhelix density obtained upon extraction. Velocity sedimentation, electrophoresis, and electron microscopy all show that two or more Z DNA sites in the SV40 genome can be intermolecularly cross-linked with bivalent IgG into high mol. wt. complexes. The formation and stability of the intermolecular antibody-DNA complexes are dependent on DNA superhelix density, as judged by three criteria: (1) relaxed circular (Form II) DNA does not react; (2) release of torsional stress by intercalation of 0.25 μM ethidium bromide removes the antibody; and (3) linearization with specific restriction endonucleases reverses antibody binding and DNA cross-linking. Non-immune IgG does not bind to negatively supercoiled SV40 Form I DNA, nor are complexes observed in the presence of competitive synthetic polynucleotides constitutively in the left-handed Z conformation; B DNA has no effect. Using various restriction endonucleases, three major sites of anti-Z IgG binding have been mapped by electron microscopy to the 300-bp region containing nucleotide sequences controlling SV40 gene expression. A limited number of minor sites may also exist (at the extracted superhelix density).

Key words: left-handed Z DNA/enhancer/alternating purine-pyrimidine

Introduction

We have previously developed an electrophoretic assay which detected left-handed Z DNA sites in SV40 DNA (and other circular genomes) using anti-Z DNA IgG binding to negatively supercoiled Form I molecules (Zarling *et al.*, 1984a, 1984b). The formation of DNA-IgG complexes depended on external conditions (ionic strength and temperature), nucleotide sequence, the physical state of the DNA (torsional stress) and the anti-DNA immunoglobulin (specificity, valency and concentration). Changes in electrophoretic migration were attributed to the formation of various oligomeric species; the existence of trimers suggested the expression of at least two Z DNA sites per SV40 DNA.

In the present study, SV40 Form I DNA-IgG complexes were preparatively purified by velocity sedimentation using conditions which optimally stabilize natural Z DNA sequences in protein-free supercoiled DNA (low NaCl concentration and low temperature). Populations of IgG-DNA complexes consisting of monomers and cross-linked species were further analyzed by agarose gel electrophoresis. We then sought to visualize the individual IgG-DNA complexes by electron microscopy. Fixation conditions were chosen to preserve IgG binding after linearize-

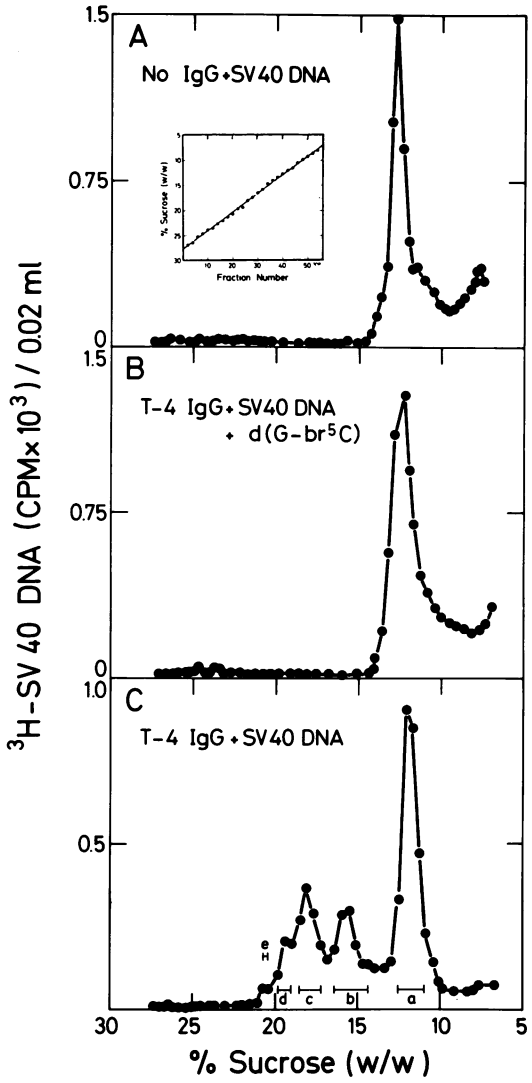

Fig. 1. Velocity sedimentation of T-4 IgG-SV40 DNA complexes. Reaction mixtures were incubated at room temperature for 3 h and centrifuged on linear 5-30% sucrose gradients. Reactions contained either **(A)** 180 ng of SV40 DNA and 0 ng T-4 IgG, or **(B)** 180 ng of SV40 DNA, with a 10x excess (w/w) of poly[d(G-br^{5}C)] and 3.6 μg T-4 IgG. **(C)** shows a preparative gradient that contained 11 μg of SV40 DNA and 217 μg of T-4 IgG. Fractions a-e were pooled and analysed by agarose gel electrophoresis (see Figure 2A) and by electron microscopy (see Figure 3). Fractions labeled [a] contained relaxed monomer, [b] SV40 form I monomer bound to T-4 IgG, [c] crosslinked SV40 dimer, [d] trimers, and [e] tetramers.

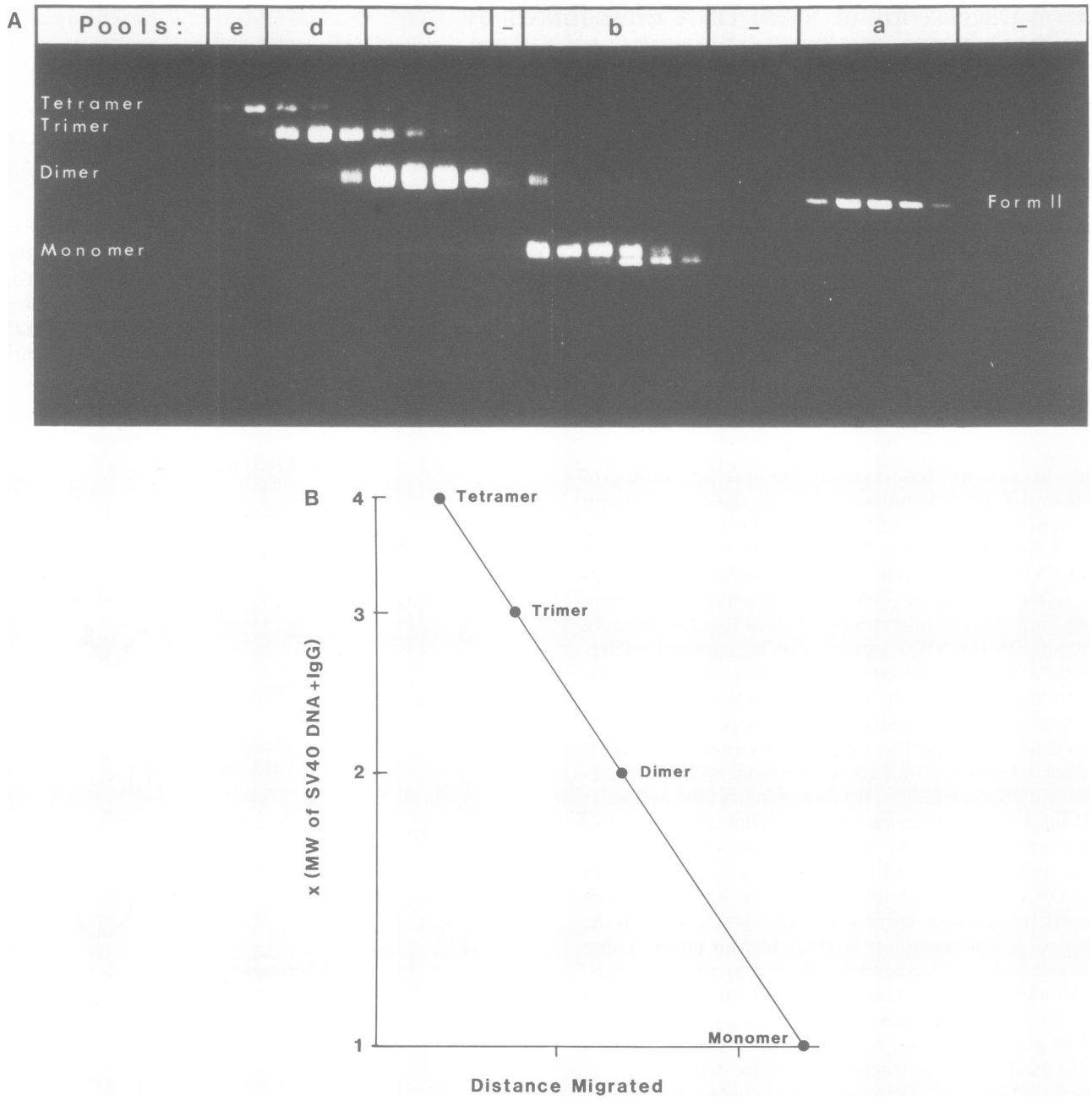

Fig. 2. **(A)** Agarose gel electrophoresis of T-4 IgG-SV40 DNA complexes. Samples obtained from the preparative sucrose gradient shown in Figure 1C were electrophoresed on 0.7% agarose gels. Pools a, b, c, d and e correspond to fractions labeled a–e on the sucrose gradient (Figure 1C). **(B)** The electrophoretic mobilities of the DNA-IgG complexes shown in **A** were plotted against the logarithm of the relative mol. wt. of the presumed DNA-IgG multimer complex. Note the log-linear relationship suggesting that discreet multimeric IgG-SV40 DNA complexes were formed.

tion or relaxation of the supercoiled DNA. Unique endonuclease cleavage sites on SV40 DNA were used to generate defined ends of the molecules, and thereby to map the location and number of specific Z DNA sites on individual SV40 Form I DNA molecules at their extracted superhelix densities.

Three Z DNA sites have been previously identified in SV40 DNA experimentally underwound *in vitro* (Nordheim and Rich, 1983; Rich *et al.*, 1983). These regions were mapped to specific restriction fragments by retention of antibody-DNA complexes on nitrocellulose filters and by specific blockage of restriction endonuclease cleavage sites.

Results

Velocity sedimentation of SV40 DNA bound by anti-Z IgG

SV40 virion Form I DNA was reacted under optimal binding conditions (see Materials and methods) with sequence-independent anti-Z DNA specific IgG (preparation T-4; Zarling *et al.*, 1984b). The complexes were analyzed by velocity sedimentation on sucrose gradients. Figure 1 shows that T-4 IgG complexed to SV40 Form I DNA produced new discrete species with a faster sedimentation rate compared with Form I SV40 DNA. The formation of these higher mol. wt. species was completely and specifically competed by inclusion of 10-fold excess unlabell-

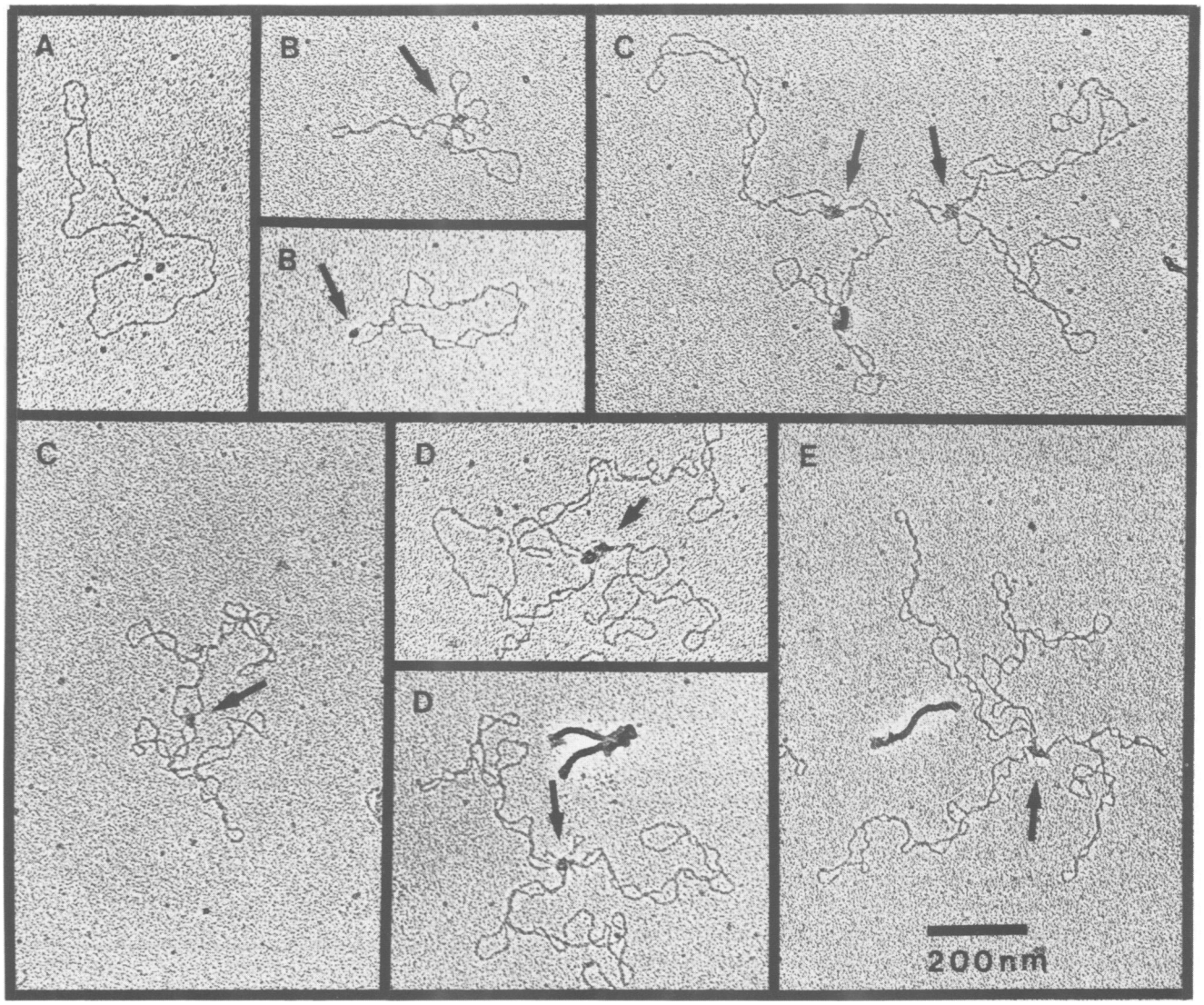

Fig. 3. Electron microscopy of SV40 DNA cross-linked by anti-Z DNA IgG. Micrographs **A**–**E** show representative DNA-IgG complexes from the corresponding pooled and dialyzed fractions labeled a–e in Figure 1C. Arrows indicate the position of antibody cross-linking SV40 DNA molecules. The bar represents 200 nm.

ed Z-poly[d(G-br^{5}C)] into the reaction mixture (Figure 1, panel B). The B form of calf thymus DNA was completely ineffective as a competitor. Thus, Z DNA helices in negatively supercoiled SV40 DNA (at its extracted superhelix density) were bound by anti-Z DNA IgG, as seen by an apparent increase in the sedimentation rate of the molecules. The anti-Z IgG used is bivalent and recognizes all known Z DNA conformations and sequences in the poly[d(G-C)], poly[d(A-C)·d(G-T)] and poly[d(A-T)] families and various mixed sequences of alternating purines and pyrimidines (Zarling *et al.*, 1984b). Thus, the antibody could potentially cross-link various DNA sequences on different molecules. To test this hypothesis, the DNA-antibody complexes were analyzed by electrophoresis and directly examined by electron microscopy.

Agarose gel electrophoresis of SV40 DNA-IgG complexes

SV40 DNA complexed with T-4 anti-Z IgG was separated by velocity sedimentation and pooled fractions were analyzed by electrophoresis in agarose gels. Figure 2A shows that fractions from pool a (Figure 1C) only contain relaxed (form II) DNA. The faster sedimenting molecules from peaks labelled b,c,d and e (Figure 1C) consisted of presumptive monomers, dimers, trimers and tetramers, respectively, of Form I SV40 DNA bound and cross-linked by the bivalent anti-Z IgG. Plots of electrophoretic mobilities *versus* relative mol. wts. (Figure 2B) showed a log-linear relationship (which, however, would not be expected to be the same as that corresponding to a series of supercoiled circular DNA molecules of increasing length). Thus, discrete multimers were apparently generated by antibody cross-linking. Theoretically, a dimer but not a trimer or higher oligomers could be formed in the case of a single Z DNA site (capable of accommodating one antibody combining site) per molecule. Thus, the existence of SV40 DNA trimers in the faster sedimenting complexes in peak d suggested that each of these SV40 DNA molecules had at least two Z DNA sites. In another study, antibody aggregation was shown not to be involved in the oligomerization of SV40 DNA by T-4 IgG (Zarling *et al.*, 1984b). The existence of antibody mediated cross-linking of DNA molecules was assessed further by electron microscopic visualization.

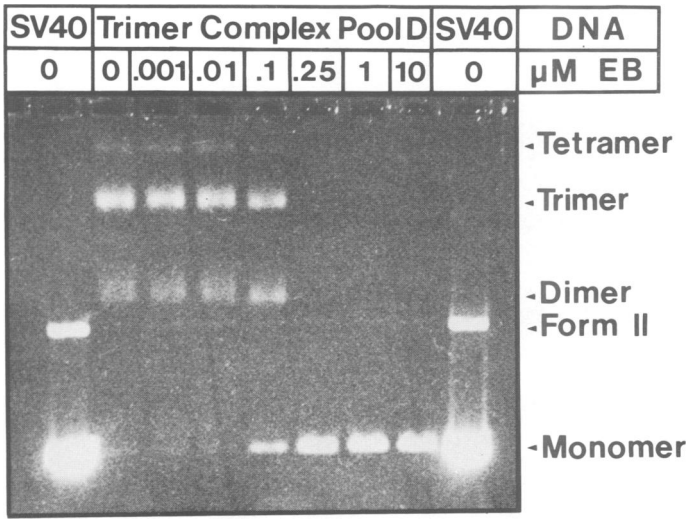

Fig. 4. Reversal of antibody binding by ethidium bromide intercalation. Samples from the trimer fractions (pool d, Figure 1C) from the sucrose gradient were dialyzed and incubated with increasing concentrations of ethidium bromide for 3 h at room temperature. These samples were analyzed by electrophoresis on a 0.7% agarose gel. Form I SV40 DNA was used as a marker. Note that the midpoint of the ethidium bromide induced Z→B transition is between 0.1 and 0.25 μM ethidium bromide.

Electron microscopy of anti-Z IgG cross-linked to SV40 Form I DNA

Individual DNA molecules from the preparative sucrose gradient (Figure 1C) were visualized. Fractions a–e (Figure 1C) were dialyzed to remove the sucrose. Electron micrographs 3A–E show representative molecules from each individual pool.

The electron microscopic data directly supported the interpretation of the gel electrophoresis results. A relaxed SV40 DNA molecule is shown in panel A of Figure 3. In panels B–E appear supercoiled monomers (B), dimers (C), trimers (D) and tetramers (E), respectively. In all of these preparations oligomers were not observed involving linear or relaxed (Forms III or II) DNA. The SV40 DNA molecules comprising the oligomers were linked by the anti-Z DNA IgG (indicated by arrows in the micrographs). Electrophoretic analysis showed that non-immune rabbit IgG did not bind SV40 DNA under the same conditions.

We have consistently seen higher order oligomers in which the antibody binding sites appear to be clustered in a single binding region. At this level of resolution it was not possible to distinguish between 1–3 bound antibody molecules. Chemical fixation with glutaraldehyde and formaldehyde altered neither the morphology nor the frequencies of occurrence of the antibody-DNA complexes. Loops were also observed between two antibody binding sites on individual DNA molecules. In many instances, the loop sizes were quite similar, representing ~10% of the length of the genome (see below).

Reversal of anti-Z IgG binding by ethidium bromide intercalation or restriction endonuclease cleavage

Ethidium bromide induces a reverse Z→B transition in linear and supercoiled DNA molecules (Pohl *et al.*, 1972; DiCapua *et al.*, 1983; Jovin *et al.*, 1983; van de Sande and Jovin, 1981). In closed circular DNA the drug concentration required for effecting Z→B transitions depends on the negative superhelix density (Thomae *et al.*, 1983; DiCapua *et al.*, 1983). The destabilization by ethidium bromide of Z-DNA-IgG complexes is shown in Figure 4. Trimers of SV40 DNA bound to anti-Z IgG (pool D,

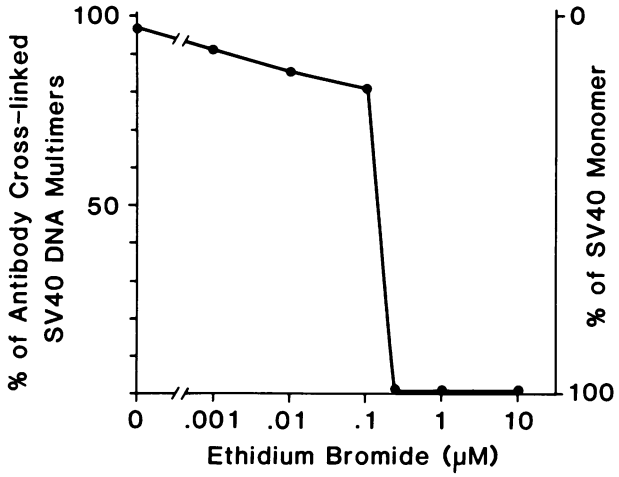

Fig. 5. Analysis of the ethidium-induced reversal of antibody binding to DNA by electron microscopy. Each sample, prepared in Figure 4, was used for electron microscopy and the frequency of multimers and monomers formed was determined. A total of 520 objects were counted. Note that these results are similar to those obtained by electrophoresis and that the Z-B transition midpoint is between ~0.1 and 0.25 μM ethidium bromide.

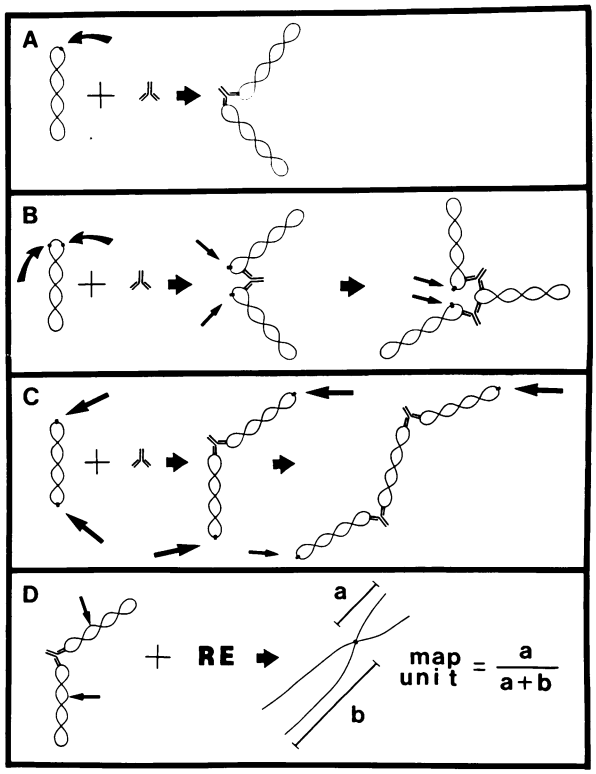

Fig. 6. Mapping procedure. **(A)** Negatively supercoiled DNA with only one Z DNA site plus a bivalent immunoglobulin G can dimerize the DNA. **(B)** Supercoiled DNA with two terminal sites can form radial DNA structures with an antibody binding center. Arrows indicate unoccupied binding sites in diagrams A–C. Compare this with electron micrographs in Figure 3B–E. **(C)** Supercoiled DNA with two Z DNA sites on opposite sides of the molecule which could generate long chains of DNA cross-linked by antibody. **(D)** Mapping of Z DNA sites. Crosslinked DNA dimers were purified and chemically fixed. Dimers were digested with a restriction enzyme at a unique site. The arrow denotes the cleavage site. Molecules from electron micrographs were measured with a digitizer to determine the distance from the cut ends to the antibody center (drawn as a dot), where the separate DNA strands cross. Map distances between the Z DNA site and the restriction site were calculated and expressed as a fraction of the total monomer length. Frequency distributions were calculated.

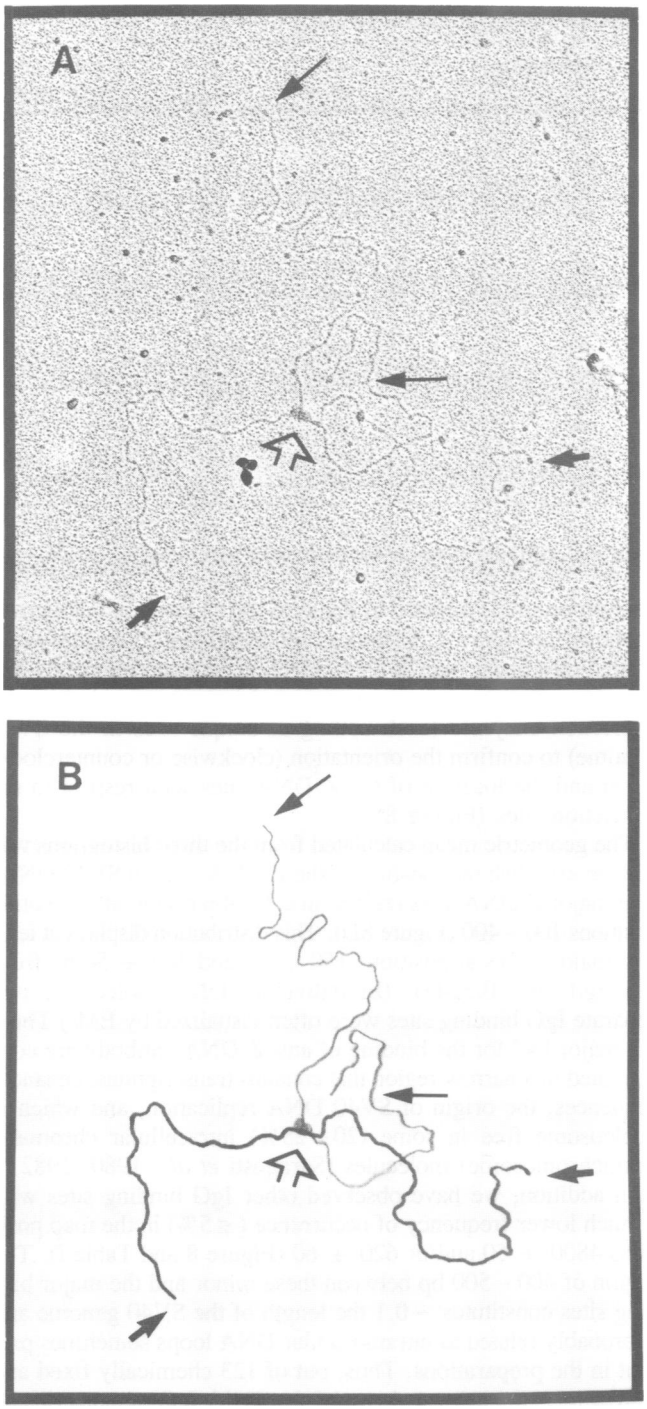

Fig. 7. Electron microscopy of *Bam*HI endonuclease cut DNA-IgG complexes. **(A)** Electron micrograph of SV40 DNA dimers from sucrose gradient fraction c that were aldehyde fixed, dialyzed, cut at a unique restriction site and mounted on grids. The large open arrow indicates the position of the antibody crosslinking the two DNA monomers. The smaller arrow indicates the ends generated by the restriction endonuclease. **(B)** The illustration displays the two linear cross-linked monomers in black and grey shades. The binding sites were mapped to nucleotide positions 168 ± 50 (black molecule designated by thick arrows) and 278 ± 50 (grey molecule designated by thin arrows). These sites lie within the transcriptional enhancer region.

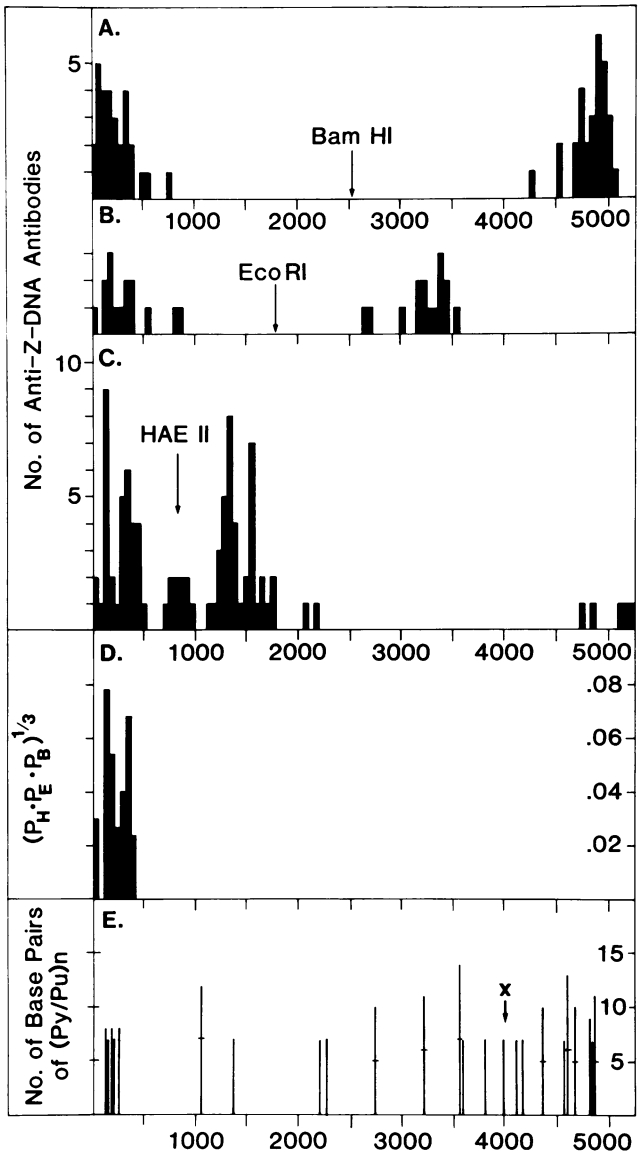

Fig. 8. Anti-Z DNA IgG binding sites on the SV40 genome. Map positions were measured from electron micrographs like those shown in Figure 7, using the mapping method illustrated in Figure 6D. Antibody binding frequencies were plotted using the *BglI* site as position 0/5243. Preparations were treated with **(A)** *Bam*HI, **(B)** *Eco*RI, or **(C)** with *Hae*II. Each antibody binding site was plotted in both directions from the restriction site, generating maps with symmetrical profiles around the restriction site. **(D)** Geometric mean of anti-Z DNA antibody binding sites. Plotted is the cube root of the product of the three individual observed probabilities of occurrence at each map position (p_B, p_E, p_H) displayed in panels **A, B, C,** respectively. A total of 88 observations (29, 15, and 44 in the case of p_B, p_E, p_H, respectively) were used, plotted bidirectionally from the cleavage sites with a resolution of 1% of the genome length. Structures with intramolecular loops were excluded from the analysis. This form of data representation reduces the directional ambiguity associated with the mapping by the individual restriction endonucleases, and also emphasizes the overall concordance within the total data set, although suppressing sites of low frequency. **(E)** Distribution of all alternating pur-pyr sequences that either are seven nucleotides or longer without any interruptions or those 10 nucleotides or longer with one interruption. The vertical lines indicate the length of the pur-pyr stretch and the horizontal tics the positions of interruptions (see also Table I).

Figure 2) were titrated with increasing concentrations of ethidium bromide. After equilibration (3 h) with the drug, the samples were divided in half. One was analyzed by agarose gel electrophoresis and the other by electron microscopy. Both methods gave similar results. With increasing ethidium bromide concentrations a reversal of antibody-DNA cross-linking was observed

Table I. Correlation between anti-Z DNA IgG binding and properties of selected alternating purine-pyrimidine sequences in SV40 DNA[a].

Genome position	Sequence	Length	Interruption	ATA, TAT block	Anti-Z DNA IgG binding
1995	GTGTGT	6	no	no	no
4852	TGCACAC	7	no	no	no
1056	GCGTGTA	7	no	no	no
258	ACACACAT	8	no	no	yes
126	ATGCATGC	8	no	no	yes
198	ATGCATGC	8	no	no	yes
4825	GCATATGCA	9	no	yes	b
4367	ACACA.GCATG	10	yes	no	no
4675	TATGT.CACAC	10	yes	yes	no
3218	GCACAC.TGTAC	11	yes	no	no
4876	GTACAT.TGCAT	11	yes	no	b
1056	GCGTGTA.ATACA	12	yes	yes	no
4606	ACACAT.TATACAC	13	yes	yes	no
3575	TGTACAC.CATGCAT	14	yes	no	no

[a]At the superhelix density achieved upon extraction.
[b]Possible locus for low-frequency sites and for loops comprising ~10% of the genome and originating in one of the three major sites in the region 126–266.

by electrophoresis (Figure 4). The transition midpoint occurred at a concentration of ~0.15 μM ethidium bromide, and complete antibody reversal was observed at 0.25 μM, a concentration very similar to that obtained by Thomae *et al.* (1983) with a plasmid substrate. For electron microscopy, samples were diluted and mounted on grids at identical DNA concentrations to minimize possible overlapping of individual molecules which could artifactually appear as multimers. Titration of the SV40 DNA-antibody complexes with ethidium showed a sharp reversal of the antibody-cross-linked multimers between 0.1 and 0.25 μM (Figure 5).

Ethidium bromide may be able to interact directly with left-handed DNA and induce a reverse Z→B transition as, for example, in the case of linear poly[d(G-C)] (van de Sande and Jovin, 1982). It is also possible that ethidium bromide may not directly influence the Z DNA sequences in SV40 DNA. Indeed, it is most probable that the drug intercalates randomly and thereby releases the torsional strain required to stabilize the Z helix and its complex with anti-Z DNA IgG.

The requirement for the torsional stress of negative supercoiling in maintaining DNA-antibody cross-links was tested further. SV40 DNA-antibody complexes (pools d and e, Figure 2A) consisting predominantly of trimers and tetramers, respectively, were linearized with *Bam*HI endonuclease diluted in EM buffer (containing 150 mM NaCl, see Materials and methods) and examined microscopically. Under these conditions the anti-Z IgG bound trimers and tetramers were completely abolished. In other similar experiments, oligomers were successively fixed with formaldehyde and glutaraldehyde. *Bam*HI endonuclease cutting, following aldehyde fixation, produced no significant removal of antibody; ~95% or more of the antibody remained bound to the DNA. The capability to preserve antibody cross-links after endonuclease cleavage was exploited in the antibody mapping techniques described in the following section.

Immuno-electron microscopic mapping of Z DNA sites on IgG cross-linked multimers of SV40 DNA

Dimer IgG-DNA complexes (pool c, Figure 1) were fixed with glutaraldehyde and formaldehyde, linearized with unique restriction endonuclease cuts and mounted for electron microscopy. The strategy was to measure the distance (in map units) between the cleaved ends of the DNA and the antibody binding site. The mapping procedure is depicted in Figure 6 and an electron micrograph is shown in Figure 7. Distances for individual molecules were plotted on a linear map of SV40 DNA to generate frequency distributions. This procedure was repeated three times with different restriction enzymes (each cutting at unique sites in the SV40 genome) to confirm the orientation (clockwise or counterclockwise) and the location of the Z DNA sites with respect to the restriction sites (Figure 8).

The geometric mean calculated from the three histograms was used to establish the location of the Z DNA sites in SV40 DNA. The major Z DNA sites resided in a 300-bp region at nucleotide positions 100–400 (Figure 8D). This distribution displays at least two major peaks at positions 150 ± 50 and 300 ± 50 bp from the *Bgl*I site (0/5243). (In individual DNA molecules, two separate IgG binding sites were often visualized by EM.) Thus, the major loci for the binding of anti-Z DNA antibody are concentrated in a narrow region that contains transcriptional enhancer sequences, the origin of SV40 DNA replication, and which is nucleosome free in some (20–25%) intracellular chromatin (minichromosome) molecules (Saragosti *et al.*, 1980, 1982).

In addition, we have observed other IgG binding sites with a much lower frequency of occurrence (≤5%) in the map positions 4800 ± 50 and/or 620 ± 60 (Figure 8 and Table I). The region of 400–500 bp between these minor and the major binding sites constitutes ~0.1 the length of the SV40 genome and is probably related to intramolecular DNA loops sometimes present in the preparations. Thus, out of 123 chemically fixed and restriction endonuclease cleaved SV40 DNA-IgG monomeric and multimeric complexes, 35% contained loops. Of these, half originated at the major mapped antibody binding site (position 100–400 in the SV40 genome) and had sizes in the range of 720 ± 390 (standard deviation) base pairs.

Using filter binding assays to isolate chemically cross-linked antibody-SV40 DNA complexes, Nordheim and Rich (1983) mapped three binding sites within this area at nucleotide positions corresponding to the alternating purine-pyrimidine sequence at position 258, and to the 72-bp repeat(s) (two identical repeats in SV40 strain 776) at positions 126 and 198.

Alternating pur-pyr tracts containing seven or greater uninterrupted nucleotides, or 10 nucleotides or greater with one interruption are present in the SV40 genome (24 stretches in all, Figure 8E). Significant antibody binding could be correlated only

with the three pur-pyr blocks which are eight nucleotides long. This anti-Z DNA IgG binding region contains two heptamers (TGCATAC) in addition to the three octamers. However, we suggest that these heptamers were not responsible for binding the antibody, since an identical heptamer located at map position 3987 (labeled with an X in Figure 8E) did not bind anti-Z IgG.

Discussion

Electron microscopy of Z helical regions in SV40 DNA

The existence of at least two Z DNA sites in SV40 DNA was initially suggested from the formation of higher order multimers (e.g., trimers) of SV40 DNA complexed with specific bivalent anti-Z IgG and separated by electrophoresis in agarose gels. Multiple Z DNA sites can be detected by the cross-linking effect of bivalent IgG acting intramolecularly and/or intermolecularly. The anti-Z DNA IgG leads to a decrease in electrophoretic mobility (Figure 2) and to an increase in sedimentation rate (Figure 1). In the present EM study, we were able to interpret clearly the nature of the multimers of supercoiled SV40 DNA with attached IgG.

Multimers were consistently observed in which the bound antibodies connected different DNA molecules *via* major sites on the genomes. No examples of cross-linked DNAs in the form of chains were found. In addition, the multimeric complexes contained apparent loops comprising up to ~10% of the unit length of SV40 DNA. These structures presumably arose due to cross-linking by bivalent IgG of separated Z DNA sites. Revet *et al.* (1984) have seen bivalent IgG bridging two different sites in φX174 DNA with apparent stabilization of supercoiling within the resultant DNA loop even after linearization by restriction endonuclease cleavage elsewhere in the molecule. We did not observe this phenomenon in the case of SV40 DNA under our experimental conditions; release of topological stress by endonuclease action invariably led to the reversal of antibody binding unless the complexes had been stabilized by chemical fixation.

In the case of closed circular DNAs containing one region in the left-handed confirmation capable of accommodating a single anti-Z DNA IgG molecule, oligomerization would be limited to a dimer. However, if the DNA has more than one (contiguous or dispersed) Z DNA site, then linking can be more extensive and generate trimers, tetramers and higher order multimers. Previous electrophoretic screening has shown that negatively supercoiled genomes from various viruses and plasmids contain multiple (two or more) Z DNA sites (Zarling *et al.*, 1984a, 1984b).

In the present study, based on direct examination by electron microscopy, the Z DNA sites in the genome of SV40 were determined at the extracted superhelix density. The sites in the SV40 transcriptional enhancer region mapped by Nordheim and Rich (1983) with biochemical techniques and using experimentally underwound DNA appear to correspond to the major anti-Z DNA antibody binding sites we have observed. However, other minor sites also exist, albeit at a very low frequency (5%). Contrasting results have been obtained by Revet *et al.* (1984) from the quantitative estimation of the major and minor Z DNA sites in the φX174 genome, probably due to the more dispersed distribution of appropriate alternating pur-pyr sequences. It is interesting that the expression of multiple sites in individual φX174 DNA molecules is limited and apparently non-cooperative, a finding attributable at least in part to the relaxation of torsional stress by the initial B→Z transitions (which necessarily would occur more frequently at the favored major sites).

SV40 sequences binding anti-Z IgG

Some sequence requirements for the formation of Z DNA conformations can be suggested by correlating the sites of alternating pur-pyr sequences with those that bind anti-Z antibodies (Table I). This comparison suggests three simple characteristics about natural alternating pur-pyr sequences and their potential to adopt a Z-conformation.

(i) The minimum length for a SV40 Z DNA sequence is about eight uninterrupted alternating pur-pyr nucleotides. None of the 26 pur-pyr tracts which are six nucleotides in length and none of the 16 pur-pyr tracts which are seven nucleotides long bound anti-Z DNA IgG at the isolated superhelix density under our experimental conditions (see also Results). However, all the three $(pur\text{-}pyr)_n$ sequences eight nucleotides long were competent in binding. Two of the Z DNA sites in SV40 DNA consist of a tandem repeat of the d(ATGC) tetranucleotide, and one sequence is almost a perfect d(A-C) repeat (Table I). However, the SV40 genome contains few $d(G\text{-}C)_n$ sequences and as a consequence, our binding data do not permit an assessment of the minimum length requirement for such regions in the Z conformation. This point is discussed further in Revet *et al.* (1984), in Miller *et al.* (in preparation; this study deals with the plasmid pBR322 DNA and phage PM-2 DNA as substrates for anti-Z IgG), and in Konopka *et al.* (1985; a general survey of numerous viral and episomal sequences).

(ii) Interruptions in $(pur\text{-}pyr)_n$ sequences, like adjoining purines (GG, GA, AG, AA) or pyrimidines (CC, CT, TC, TT), would be expected to destabilize the Z configuration in comparison with that of an uninterrupted $(pur\text{-}pyr)_n$ of the same length. This conclusion is based on the inspection of all uninterrupted alternating sequences six or seven nucleotides long that adjoin or abutt neighboring pur-pyr tracts. Figure 8E shows eight alternating pur-pyr segments 10 nucleotides or greater in length which contain one interruption. None of these sequences were found to be correlated with anti-Z antibody binding except possibly as components of infrequent loop structures (Table I). We conclude that a single interruption appears to destabilize the Z-forming potential of any long sequence if neither of the two constituent segments is longer than seven nucleotides in pure pur-pyr alternation. We emphasize that this type of simple analysis does not permit one to predict whether short (<7 bp) pur-pyr sequences are able to adopt the Z conformation if they border on longer left-handed regions in negatively supercoiled molecules.

(iii) Tetranucleotide ATAT (TATA) or trinucleotide ATA (TAT) sequences probably cannot be driven into the Z form under most circumstances, but an AT (TA) dinucleotide embedded in a longer GC-rich region can. For example, the nonamer at position 4825 in SV40 DNA (sequence GCATATGCA) does not bind (at least readily) the anti-Z DNA IgG, whereas the two AT dinucleotides in the octamer ATGCATGC (positions 126 and 198) do not prevent recognition by the antibody.

The properties of naked DNA *in vitro* may reflect poorly the structural features prevailing in chromatin, particularly in view of the dramatic influence of topological state on the expression of the Z conformation. Proteins with specificity for particular helical conformations and/or higher-order topological features will certainly modulate the frequency and function of the structural element considered in this work, namely the left-handed Z double helix. Ample evidence in support of this statement has been presented elsewhere in studies of cytological material

(Robert-Nicoud *et al.*, 1984). However, the present study demonstrates clearly the phenomenon of intermolecular (and intramolecular) associations mediated by a protein specific for an infrequent and locally restricted conformational state. One would surmise that this model system might serve as a prototype for the *in vivo* situation.

Materials and methods

DNAs

SV40 strain 776 Form I DNA was purified from virus-infected BSC-1 or CV-1 monkey cells. The DNA was phenol extracted and contained ~90% superhelical DNA. [^{3}H]thymidine-labeled SV40 Form I DNA was purified from infected CV-1 cells by the Hirt extraction procedure (1967) with the following modifications. The Hirt supernatant DNA was phenol extracted, ethanol precipitated, and dialyzed against 10 mM Tris-HCl, 1 mM EDTA, pH 7.0. The specific activity of the ^{3}H-labeled SV40 DNA was 80–110 c.p.m./ng. Calf-thymus DNA was purchased from ICN. Poly[d(G-br^{5}C)] was prepared as described elsewhere (McIntosh *et al.*, in preparation).

Antibodies

Polyclonal antibody against left-handed Br-poly[d(G-C)] (preparation T-4) was raised in rabbit as described by Zarling *et al.* (1984b). IgG fractions were isolated by precipitation with 30% (w/v) ammonium sulfate, dialysis against 10 mM sodium acetate, pH 5.5, and two extractions with DEAE-A50. The IgG was further purified with protein A-Sepharose (Pharmacia) chromatography, dialysed against 10 mM Tris-HCl, 1 mM EDTA, pH 7.2, and stored frozen.

DNA-IgG complexes

For cross-linking studies, preparative amounts of antibody-DNA complexes were formed by incubating T-4 IgG (217 μg) with unlabeled SV40 DNA (10 μg) plus tracer amounts of [^{3}H]thymidine-labeled SV40 (80 000 c.p.m., 1 μg) in 10 mM triethanolamine-HCl (TEA), 1 mM EDTA, 20 mM NaCl, pH 7. Reactions were at room temperature for 3 h.

Velocity sedimentation and agarose gel electrophoresis

Antibody-DNA complexes were purified by velocity sedimentation on preformed linear 5–30% (w/v) sucrose gradients (in 20 mM NaCl, 10 mM TEA, 1 mM EDTA, pH 8). Centrifugation was in a Beckman SW 50.1 rotor at 30 000 r.p.m. for 340 min at 10°C. In a separate tube, 180 ng of tracer [^{3}H]SV40 DNA alone (110 c.p.m./ng) was analysed as a marker. In competition experiments 180 ng of [^{3}H]SV40 DNA equilibrated with an 11-fold excess (2 μg) of Z-poly[d(G-br^{5}C)] was incubated with 3.6 μg of T-4 IgG. The same amounts of labeled SV40 DNA and T-4 IgG equilibrated with 2 μg of calf thymus were also used.

Sucrose gradient fractions were assayed for radioactivity using Aqualuma-Plus (Baker). Aliquots of each fraction were analysed by agarose gel electrophoresis at 4°C on 0.7% agarose gels containing 0.1 M Tris-HCl, 76 mM boric acid, and 25 mM EDTA, pH 8.5. The DNA was stained with 0.5 μg/ml ethidium bromide and photographed under u.v. illumination using Kodak Tri-X Pan film and a red filter.

Chemical cross-linking of antibody-DNA complexes

The peak fractions from preparative sucrose gradients containing DNA-IgG complexes were pooled and divided. One half was fixed sequentially in 1% formaldehyde and 0.6% glutaraldehyde. Each fixation reaction was performed on ice for 15 min. Fixed and unfixed controls were subsequently dialyzed at 4°C against 500 volumes of 10 mM Tris-HCl, 1 mM EDTA, pH 8, to remove sucrose and excess fixative. These complexes were then used for agarose gel electrophoresis and electron microscopy.

Electron microscopy

Fractions purified by velocity sedimentation were diluted in an EM buffer consisting of 150 mM NaCl, 2 mM spermidine, 1 mM EDTA, pH 7.8, and mounted onto glow-charged carbon-coated grids. Grids were stained with 0.17 mM uranyl acetate, and rotary shadowed with tantalum/tungsten (Stockton *et al.*, 1983).

Restriction endonuclease cleavage of IgG cross-linked DNA complexes

Samples containing 9 μl of antibody cross-linked dimer or trimer complexes were digested with *Bam*HI, *Eco*RI, or *Hae*II restriction endonucleases (B.R.L.) for 2 h at 37°C and mounted and shadowed for electron microscopy. Antibody binding sites were mapped from photographic enlargements using a Micro-Plan II Image Analysis System Digitizer.

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