

Immunofluorescence Localization of Z-DNA in Chromosomes: Quantitation by Scanning Microphotometry and Computer-assisted Image Analysis

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ABSTRACT Anti-Z-DNA polyclonal and monoclonal immunoglobulins raised against left-handed polynucleotides show various degrees of specificity for base sequence and substitution. Class 1 IgGs recognize all Z-DNA with equal affinity; class 2 IgGs show a preference for $d(G-C)_n$ sequences and class 3 IgGs for $d(G-C)_n$ sequences with substitutions at the C5 position of the pyrimidine. These antibodies served as probes for the localization of Z-DNA in polytene and metaphase chromosomes and in interphase chromatin by indirect immunofluorescence. A quantitative assessment of the binding of anti-Z-DNA IgGs to polytene chromosomes of *Chironomus* and *Drosophila* was made by scanning microphotometry and by computer-assisted image analysis of double immunofluorescence and DNA-specific dye fluorescence images. The three classes of antibodies bind to most of the bands in acid fixed polytene chromosomes of *C. thummi*; however, preferential binding of one class of antibody over another can be observed in certain regions. These differences can be quantitated by arithmetic division or subtraction of the normalized digital images. If a class 2 antibody is first bound at saturating concentrations the binding of class 1 antibody is reduced throughout most bands by 40–50%. However, the telomeres of the three large chromosomes bind >10 times as much class 1 antibody as class 2 antibody, indicating that the Z-DNA tracts in these regions are comprised largely of alternating sequences containing the A·T basepair, e.g., A-C. High-resolution image analysis of class 1 and class 2 immunofluorescence patterns and the total DNA distribution from polytene chromosomes of *D. melanogaster* show that the two antibody distributions are very similar in a large majority of the bands, but they often deviate from the mean DNA distribution profile. Z-DNA sequences of both G-C and A-C type are detectable at all levels of ploidy from $2n$ to $2^{13}n$ and in species as diverse as insects and man. We conclude that the vast majority of polytene chromosome bands (genes) contain one or a few DNA sequences with potential for undergoing the B→Z transition and contain both alternating purine–pyrimidine G-C and A-C tracts or mixed sequences. Highly heterochromatic bands and telomeres have more Z potential sequences than do other bands.

The first polynucleotide sequence shown by spectroscopic (1) and crystallographic (2, 3) methods to be interconvertible from a right-hand helical (B) to a left-hand helical (Z) conformation was the alternating co-polymer poly[d(G-C)]. Subsequently, the facilitation of the B→Z conformational transition was demonstrated in modified sequences such as poly[d(G-m⁵C)] (4) or in poly[d(G-C)] reacted with 1 N-acetoxy-N-

acetyl-2-aminofluorene (5). More recently, we have studied a number of alternating purine–pyrimidine sequences with different patterns of substitution and determined the ionic and temperature dependence of their respective B-Z transitions (6–8). The hierarchy in potential for adopting the left-handed conformation is $d(G-x^5C)_n > d(G-C)_n > d(A-x^5C) \cdot d(G-T)_n$ (where x is a methyl or halogen substituent on the C5 position

of the pyrimidine base). Using sequences inserted into closed circular plasmids it has also been shown that unmodified $[d(A-C)\cdot d(G-T)]_n$ tracts can form left-handed helices if subjected to torsional stress in the form of underwinding (9, 10).

Linear polynucleotides that exist in the left-handed conformation under physiological ion conditions are highly immunogenic (11, 12). These polynucleotides can be used to generate both polyclonal and monoclonal anti-Z-DNA antibodies with a high degree of sequence specificity (12–14) as well as antibodies of broader specificity to Z-DNA, as demonstrated by their ability to bind to Z conformations of polymers within both the $d(G-C)$ and $d(A-C)\cdot d(G-T)$ sequence families (12, 13, 15).

It follows from the above that the most probable natural DNA sequences capable of undergoing the B→Z transition are tracts of alternating $d(G-C)_n$ and $d(A-C)_n$. Using data analysis of known DNA sequences from eukaryotic viruses, mitochondria, and bacterial phages Konopka et al. (16) have not found pure alternating sequences of either $d(G-C)_n$ or $d(A-C)_n$ with $n > 3$ except in adenovirus type 2 DNA. Note that the sequences used in these analyses were predominantly from coding regions and that non-coding sequences are more likely to contain repetitive elements (e.g., the intron of the human fetal β -globin gene (17) or mammalian telomeric DNA (18). In addition, very long runs (40–100 basepairs [bp])¹ of alternating A-C or G-T tracts have been localized in nonviral DNA libraries. For example, long tracts were found in *Drosophila* genomic libraries (including one derived from the 43A-43C puff) using probes derived from the RNA extracted from third instar stage salivary glands (19). A tract of up to 124 bp of G-T is transcribed in human liver as has been shown by Santoro and Costanzo (20).

The experimental data available at present indicate that Z-DNA is not limited to pure alternating sequences of A-C or G-C. Antibodies made against synthetic Z-polynucleotides can recognize mixed sequences of alternating purine-pyrimidine. For example, the sequences in form I Simian virus 40 DNA (21, 22) or in pBR322 (23, 24) or Φ X174 (25), which bind anti-Z-DNA antibodies, are predominantly mixed alternating purine-pyrimidine tracts. Even procaryotes have these tracts, as has been shown by the selection of sequences from *Escherichia coli* cloned into a vector plasmid using one monoclonal anti-Z-DNA IgG column (14). We have recently shown that numerous extracted plasmid and viral DNAs are recognized by anti-Z-DNA antibodies which have broad specificity for Z-polynucleotides, whereas they are bound to different extents by sequence specific antibodies (12, 13, 15). The antibody binding has an absolute requirement for topological stress in the form of negative supercoiling, is reduced at high salt concentrations, and is only moderately affected by temperature. Thus, different classes of antibodies with sequence specificity for Z polynucleotides can be important probes for the localization of potential Z tracts and may provide insight into the possible roles of these sequences in natural genomes.

In previous studies with eukaryotic systems we have shown that potential Z DNA sequences are distributed throughout the genome in a specific pattern that can be demonstrated in fixed Dipteran polytene chromosomes by immunofluorescence staining. Some of these antibodies with different sequence specificities show qualitatively different immunofluorescence patterns (7, 12, 13, 26–28). In this article we present

data that demonstrate and quantitate the specific and non-homologous binding of these antibodies to Dipteran polytene chromosomes from different species using a scanning microscope photometer and a computer-assisted image analysis system. We find that the appearance of Z-DNA tracts is not dependent upon polytenization and can be demonstrated in interphase and metaphase $2n$ cells.

The conditions and effectors that promote the left-handed conformation of DNA in chromatin have been investigated (7, 27, 29). One important phenomenon is the stabilization of Z-DNA regions by homologous intermolecular association, a process leading to the aggregated state denoted as Z*-DNA (7, 27, 30). In chromosomes, left-handed regions might function as structural organizers through such Z* interactions.

MATERIALS AND METHODS

Antibodies and Reagents: Polyclonal and monoclonal antibodies were raised and purified as described previously (12–15). All antibodies were used as the purified IgG fractions. Specificities and characterization were made by radioimmunoassay or competition radioimmunoassay on defined linear polynucleotide templates (12–14) in both low and high salt and at temperatures from 22–63° as described. Further characterization of the same antibodies was made on cloned plasmids with defined inserts of either $d(G-C)_n$, $d(A-C)\cdot d(G-T)_n$, or other known sequences in supercoiled or relaxed configurations (15) and gave the same sequence specificities found with the linear polynucleotides. Binding and immunoelectron microscopy studies with naturally occurring extracted phage, viral, and plasmid DNA using these antibodies have been published elsewhere (12, 13, 22, 25). All of the antibodies are specific for left-handed polynucleotides and show no activity towards single-stranded DNA, RNA, or any right-handed DNA. Second antibodies were IgG affinity-purified goat or sheep anti-rabbit IgG fractions labeled with fluorescein (Tago, Inc., Burlingame, CA) or Texas red-conjugated anti-mouse F(ab)₂, (New England Nuclear, Boston, MA).

Cytological Preparations: Polytene chromosomes were made by acetic acid squash of explanted salivary glands or Malpighian tubule cells from the fourth instar stage of *Chironomus thummi* larva or from salivary glands of the third instar larva of *Drosophila melanogaster* (26).

Cell lines used were *Drosophila* D2 (Oregon S embryos), the kind gift of Dr. F. Leibenguth, grown in Schneider's medium at 25°C; and primary bovine skin fibroblasts, the kind gift of Dr. K. Wagner, grown in Dulbecco's Modified Eagle's medium. Metaphase chromosome spreads were prepared from tissue culture cells by standard procedures. Interphase tissue culture cells were grown on 10-mm glass coverslips, fixed in methanol for 10 min at –20°C, and either treated in 45% acetic acid for 3 min, treated in 100 mM glycine-HCl buffer at pH 1.8 for 10 min, or heated at 90°C for 5 min in 10 mM Tris-HCl, pH 7.3, 1 mM EDTA. Staining with anti-Z-DNA antibodies was as described before (26). All anti-Z-DNA immunofluorescence could be eliminated by in situ competition with Z-polynucleotides but not with B-polynucleotides (see also references 6, 26, and 27).

Polynucleotides: The synthesis and characterization of the polynucleotides have been described elsewhere (6, 30).

Microscopy: Phase-contrast and immunofluorescence observations were made with a Zeiss Universal epi-illumination microscope. Selective excitation-emission filter combinations (Zeiss 487702, 487717, and 487715) allowed the H-33342, fluorescein, and Texas red images from the same chromosome to be viewed, photographed (Kodak Tri-X, 1,600 ASA), or photometrically measured without spectral overlap.

Quantitative Cytophotometry: A scanning microscope photometer, UMSP 80 (Carl Zeiss Research Laboratories, Oberkochen, Federal Republic of Germany) was equipped with an He-Cd laser (442 nm) or an Hg arc lamp and was used for scanning photometry of fixed *Chironomus* polytene chromosomes labeled by indirect immunofluorescence using anti-Z-DNA antibodies and the DNA dye H-33342. The 100-W Hg arc lamp was focused through the epi-illumination optical train with a 50- μ m aperture focused in the object plane to 2 μ m through a 63 \times , 1.25 numerical aperture oil immersion objective. A high resolution serial scanning stage driven by the MPC 64 electronics (Carl Zeiss) using a Hewlett Packard 16 computer (Hewlett-Packard Co., Palo Alto, CA) and LINESCAN 4 software (Carl Zeiss), was programmed with the chromosome shape, and the scanning and measurement were automatically performed at a speed of 5 μ m/s. Fluorescence intensities were measured for an 0.8- μ m area (80- μ m aperture in the virtual image) with an HTV R928 photomultiplier.

¹ Abbreviation used in this paper: bp, basepairs.

Quantitative Fluorescence Image Analysis System: Fluorescence images were obtained with a Zeiss Universal epi-illumination microscope using a Cohu Inc. (San Diego, CA) series 4410 double-intensified Newicon low light level TV camera. The latter was operated in a mode that permitted manual adjustment of intensifier gain and black level blanking. These settings were kept constant during the acquisition of a set of images. Image acquisition (resolution 512×512 pixels, 256 grey levels), manipulation, and display were carried out with the Incos2 system of Signum (Munich). The latter is configured on a DEC Q-bus (Digital Equipment Corp., Maynard, MA) using video boards (analog processor, frame buffers, arithmetic logic unit) from Imaging Technology (Woburn, MA) and other components. The images were subjected to linear (frame summation, background subtraction, normalization) and nonlinear (thresholding, filtering) operations for the purposes of enhancement and quantitation.

RESULTS

Characterization of Immunoglobulins against Z-DNA with Sequence Specificity

Both polyclonal and monoclonal antibodies can be generated with specificity for the sequence of the Z polynucleotide. In previous publications we have given complete characterizations of the antibody specificities on linear polynucleotides over a large range of temperature and salt concentrations (12–14; see, for example, in reference 12, Table 3, and Figs. 3, 4, 6, and 7); on extracted phage and virus DNA (12, 13, 22, 25; see, for example, in reference 12, Figs. 8–10, and in reference 13, Table 1, Figs. 8, 9, and 11, and discussions therein); and on plasmid DNAs with cloned $d(G-C)_n$, $d(A-C) \cdot d(G-T)_n$, or globin gene inserts under conditions either promoting or inhibiting the Z conformation of the insert (reference 15 and unpublished data of D. Zarlino and T. Jovin, and of F. Pohl). In Fig. 1 is shown an example of the specificities found for members of three classes of antibodies determined as de-

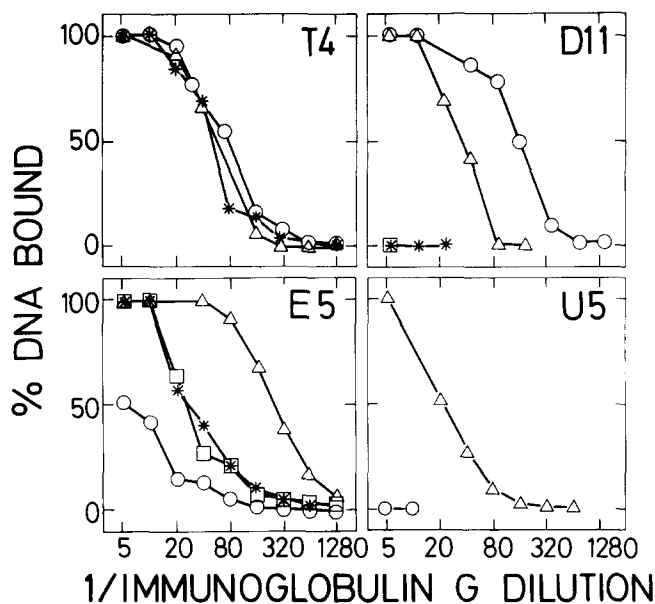


FIGURE 1 Binding specificities of purified IgG fractions of class 1, 2, and 3 anti-Z-DNA antibodies. IgG concentrations were adjusted to 2.8 mg/ml. The percentage of the radioactive polynucleotide precipitated with the immune complex is plotted as a function of IgG dilution. Reactions with poly[d(G-C)] family were carried out in 4 M salt at 22°C and in 5.4 M salt at 56°C with the poly[d(A-C)·d(G-T)] family for 1 h, and then incubated with second antibody (goat anti-rabbit or goat anti-mouse IgG) at the same temperature. Class 1, T4; class 2, D11; class 3, E5 and U5. O, poly[d(G-C)]; Δ , poly[d(G-m⁵C)]; \square , poly[d(G-br⁵C)]; *, poly[d(A-m⁵C)·d(G-T)].

scribed above. Radioimmune titrations were performed with unmodified Z-poly[d(G-C)], Z-poly[d(G-m⁵C)], Z-poly[d(G-br⁵C)], and Z-poly[d(A-m⁵C)·d(G-T)] as substrates for binding to serial dilutions of the given antibodies. Class 1 antibodies will recognize all Z-DNAs with about equal affinity (Fig. 1, panel T4). Since these include both polyclonal (e.g., T4) and monoclonal antibodies (e.g., M63) the haptenic site is probably some universal feature of the backbone or the dinucleotide repeat in Z-DNA. A second class (class 2) preferentially recognizes unmodified Z-poly[d(G-C)], binds significantly less to modified derivatives of this family, and cannot recognize the Z-poly[d(A-C)·d(G-T)] family (e.g., monoclonals D11 and B7, see Fig. 1, panel D11). As stated above and in reference 15, the D11 antibody maintains its strong preference at 22 or 37° in physiological salt concentrations when tested with plasmids carrying either $d(G-C)_n$ or unmodified $d(A-C) \cdot d(G-T)_n$ inserts in the Z conformation. At all concentrations of the class 2 antibody tested it failed to bind to plasmids with Z- $d(A-C) \cdot d(G-T)_n$ sequences. Indeed, the selectivity is greater than 10^4 for Z- $d(G-C)_n$ sequences (Pohl, F., private communication). A third class (class 3) demonstrates a high degree of preference for poly[d(G-C)] sequences that have methyl groups or halogen atoms in the C 5 position of the pyrimidine (e.g., polyclonal E5 or U5, and monoclonal M14; see Fig. 1, panels E5 and U5).

Immunofluorescence Staining of Chromosomes by Sequence Specific Anti-Z-DNA Antibodies

Most of the condensed bands of fixed polytene chromosomes of *C. thummi* bind Z DNA specific antibodies. With all antibody classes the staining could be shown to be specific for Z-DNA as evidenced by competition experiments with synthetic B and Z polynucleotides. However, several significant differences could be discerned in the immunofluorescence patterns produced by classes of antibodies with different sequence specificities, as shown in Fig. 2, A–C for staining of chromosomes II and III. Class 2 antibodies (which cannot recognize Z-poly[d(A-C)·(G-T)] sequences) did not bind to the telomeres of the three large chromosomes in this species. For example, the first band visible in the left end of the chromosome II in Fig. 2B is denoted with a small filled arrowhead but is the penultimate band of the same chromosome in Fig. 2, A or C. The actual telomere (large filled arrowhead) of this chromosome is not stained at all, as seen in the immunofluorescent pattern in Fig. 2B. The staining difference is especially marked in the ectopic fibers connecting the telomeres of different chromosomes seen strongly stained in Fig. 2, A and C and visible only by DNA staining in B (data not shown). The class 3 antibodies, which have an increased affinity for Z-DNA with base modifications in the pyrimidine C 5 position, do not show strong differences in the staining of *Chironomus* chromosomes as compared with the class 1 antibodies. A lack or extremely low occurrence of methyl modification of bases has been found in nonpolytenized chromatin of *Drosophila* (31). Because chromatin from Insecta is usually undermethylated, we would not observe dramatic differences in the staining of Fig. 2, A and C. The class 3 antibodies demonstrate markedly enhanced binding to regions of known methyl cytosine substitution on bovine metaphase chromosomes as shown in Fig. 3A, in which the antibody staining is seen, and in B, in which the R-banded

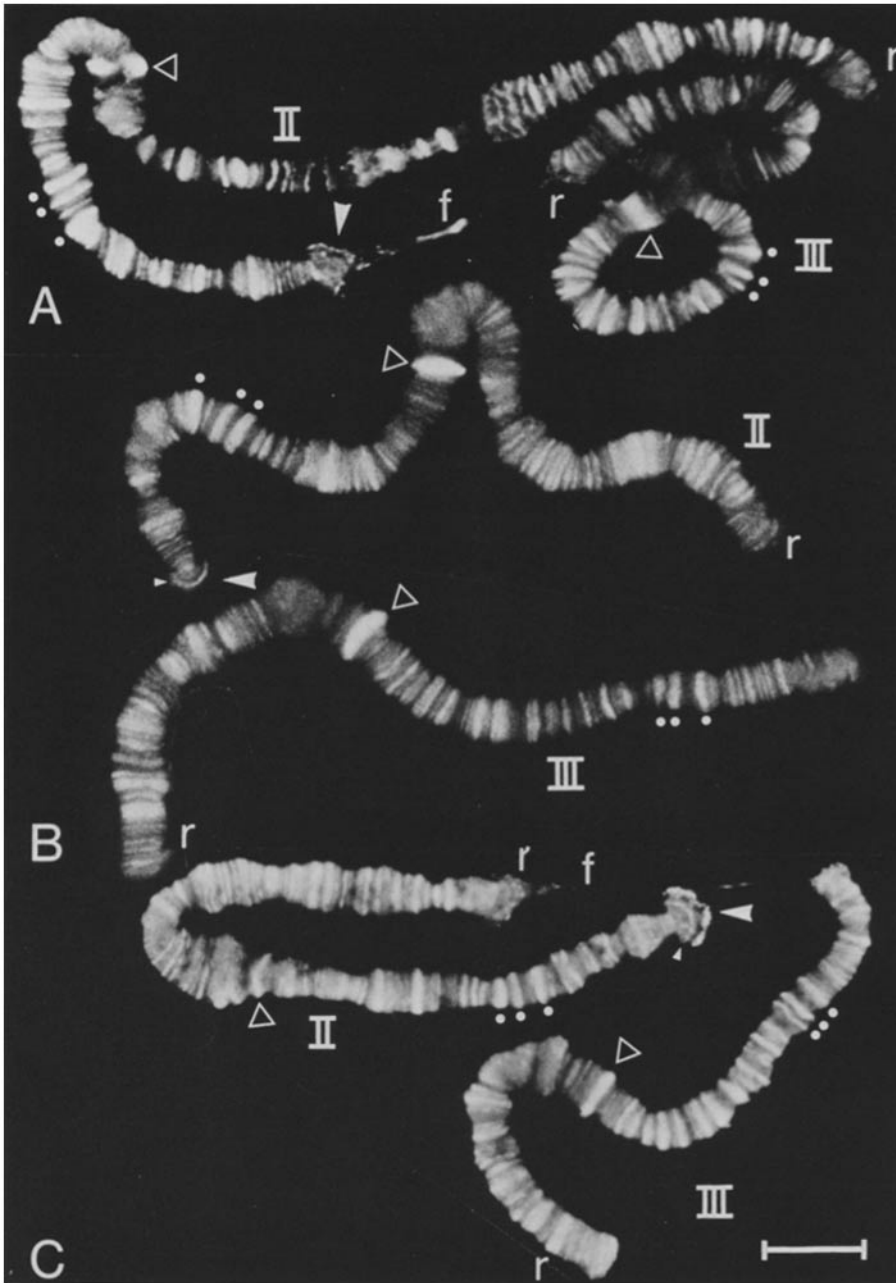


FIGURE 2 Immunofluorescence pattern of anti-Z DNA antibodies bound to acid-fixed polytene chromosomes II and III of *C. thummi*. (A) Class 1 polyclonal antibody, T4, 25 $\mu\text{g/ml}$; (B) class 2 monoclonal antibody, D11, 15 $\mu\text{g/ml}$; (C) class 3 polyclonal antibody, U5, 20 $\mu\text{g/ml}$. Indirect fluorescein-labeled affinity-purified goat anti-rabbit (A and C) or anti-mouse (B) IgG (Tago, Inc.) was used. Roman numerals denote the chromosome numbers; filled large arrowheads, telomeres; filled small arrows, penultimate telomeric band; *f*, the ectopic fibers connecting the chromosomes; open arrowheads, heterochromatic bands in IIc4 and IIIb3; dots, bands in regions IIc4-e1 and IIIc1-c4; *r*, the right-hand end of the chromosomes. Bar, 20 μm . $\times 640$.

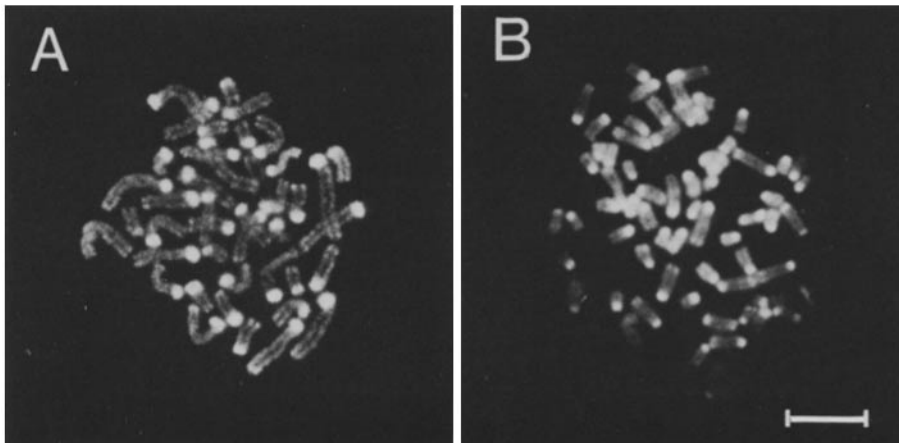


FIGURE 3 Correspondence of Z-DNA localization and GC-rich heterochromatin in metaphase chromosomes from primary bovine skin cells. (A) Binding of class 3 anti-Z-DNA U5 IgG at 50 $\mu\text{g/ml}$, detected by indirect staining with fluorescein-labeled goat anti-rabbit IgG. (B) R-banding by acridine orange (100 $\mu\text{g/ml}$) staining of a similar spread after heat denaturation for 30 min at 87°C. Fluorescence emission from 510 to 550 nm was photographed. Bar, 10 μm . $\times 1,000$.

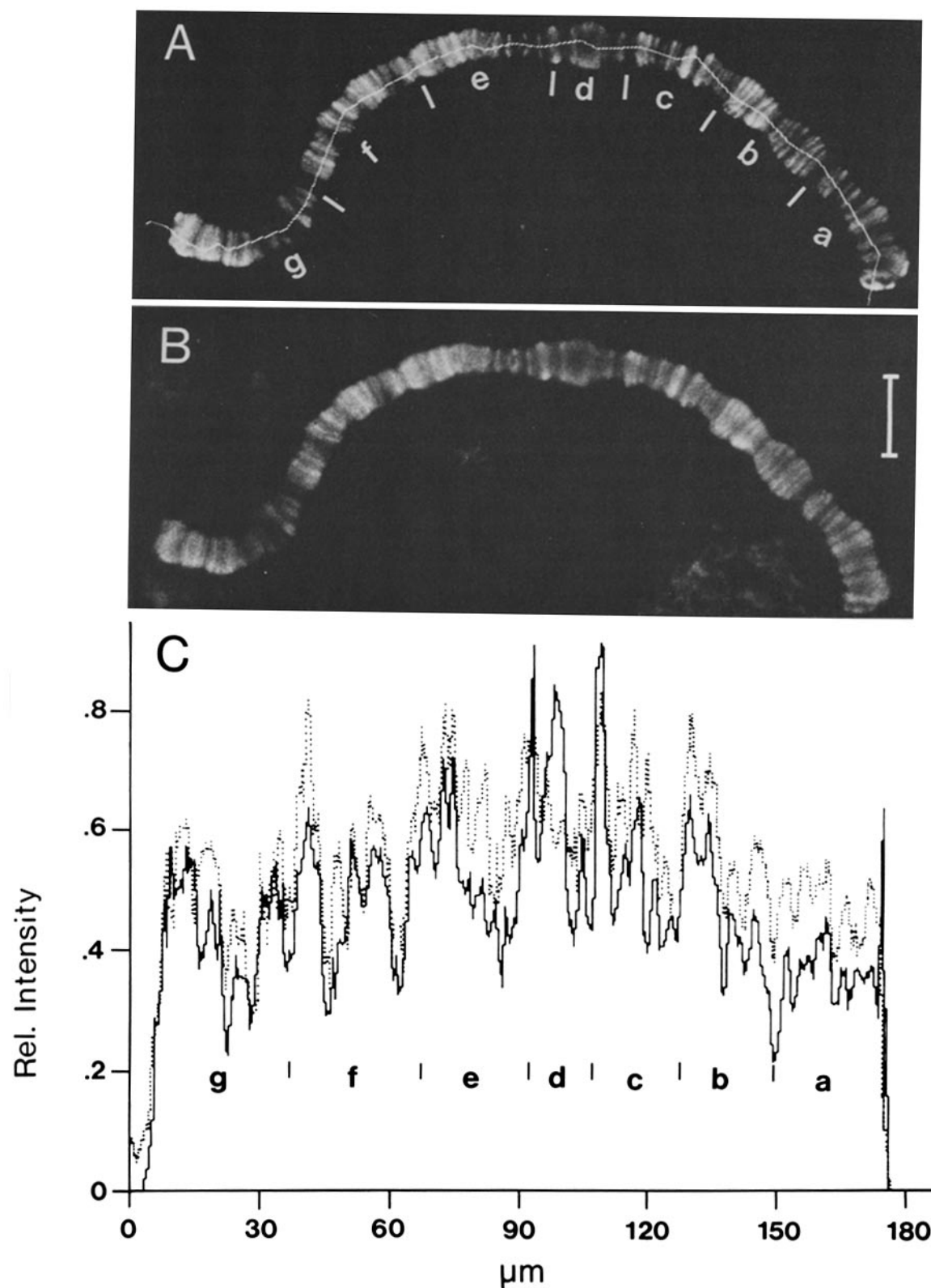


FIGURE 4 Scanning microphotometry of *C. thummi* polytene chromosome I stained with both class 1 and class 2 anti-Z-DNA IgG. (A) Class 1 antibody, T4, 30 $\mu\text{g}/\text{ml}$, indirect staining with fluorescein-labeled goat anti-rabbit IgG. The actual photometric scan line superimposed on the fluorescence image and the map designations of Keyl (32) are indicated below. (B) Class 2 antibody, D11, 30 $\mu\text{g}/\text{ml}$, indirect staining with Texas red-labeled goat anti-mouse IgG. Scan line (not shown) was the same as in A. (C) Intensity profiles obtained by microphotometric scanning (Zeiss UMSP 80). —, fluorescein emission; ····, Texas red emission. Ordinate: the curves were arbitrarily set to equivalence at regions Ide, If2 and are displayed on a linear scale of fluorescence intensity. *Rel.*, relative. Abscissa: distance along the scan line; map designation as in A. Bar, 20 μm . $\times 640$.

acridine orange fluorescence of the highly methylated GC-rich satellite DNA is seen.

Quantitative Assessment of Differential Binding of Class 1 and 2 Antibodies to Polytene Chromosomes

By titration to saturation of fixed polytene chromosomes with anti-Z-DNA antibodies and measurement of the fluorescence emission of chromosomal regions excited by a laser spot, we were previously able to estimate that about one antibody is bound per 3,000–15,000 bp of DNA (26). To compare the relative distribution of the antibody with the DNA content throughout the polytene genome rather than restrict ourselves to discrete spots we have performed scanning photometry of the DNA staining and anti-Z-DNA immunofluorescence on polytene chromosomes. The differential binding of sequence specific antibodies can be compared by using chromosomes doubly stained with a polyclonal rabbit antibody of one class and a monoclonal mouse antibody of another. The indirect fluorescein immunofluorescence pattern of the sequence independent class 1 polyclonal antibody for *C. thummi* chromosome 1 is seen in Fig. 4A with the superimposed scan line of the focused excitation beam. The chromosome map notation of Keyl (32) is shown as well. In

Fig. 4B, the indirect Texas red immunofluorescence of the mouse anti-Z-poly[d(G-C)] class 2 antibody for the same chromosome is seen. The measured fluorescence intensities for the two antibody scan lines are superimposed in Fig. 4C. The qualitative differences in the antibody binding seen in Fig. 2 are quantitatively borne out.

To compare the two immunofluorescence patterns with the total DNA distribution, we employed a procedure involving three-wavelength excitation and emission scanning photometry (Fig. 5). The right-hand end of *C. thummi* chromosome 1 is shown at higher magnification than in Fig. 4 using the class 1 (fluorescein) immunofluorescence (Fig. 5A). The three photometric scan lines are superimposed in Fig. 5B for class 1 and 2 immunofluorescence and DNA dye fluorescence. Normalizing to the total DNA content as reflected by the dye fluorescence we find that the sequence-independent class 1 anti-Z-DNA antibody has 6–10 times the relative fluorescence of the monoclonal class 2 antibody in the telomere. In the region just before the telomere the class 2 antibody binding is slightly enhanced (20%) as compared with the class 1 antibody binding. One of the striking features of the quantitative microscopy on *C. thummi* chromosomes is the finding that to a large extent the staining intensity parallels the total DNA content, the implication being that the potential Z sequences are ubiquitous in the genome. Few bands appear to be devoid

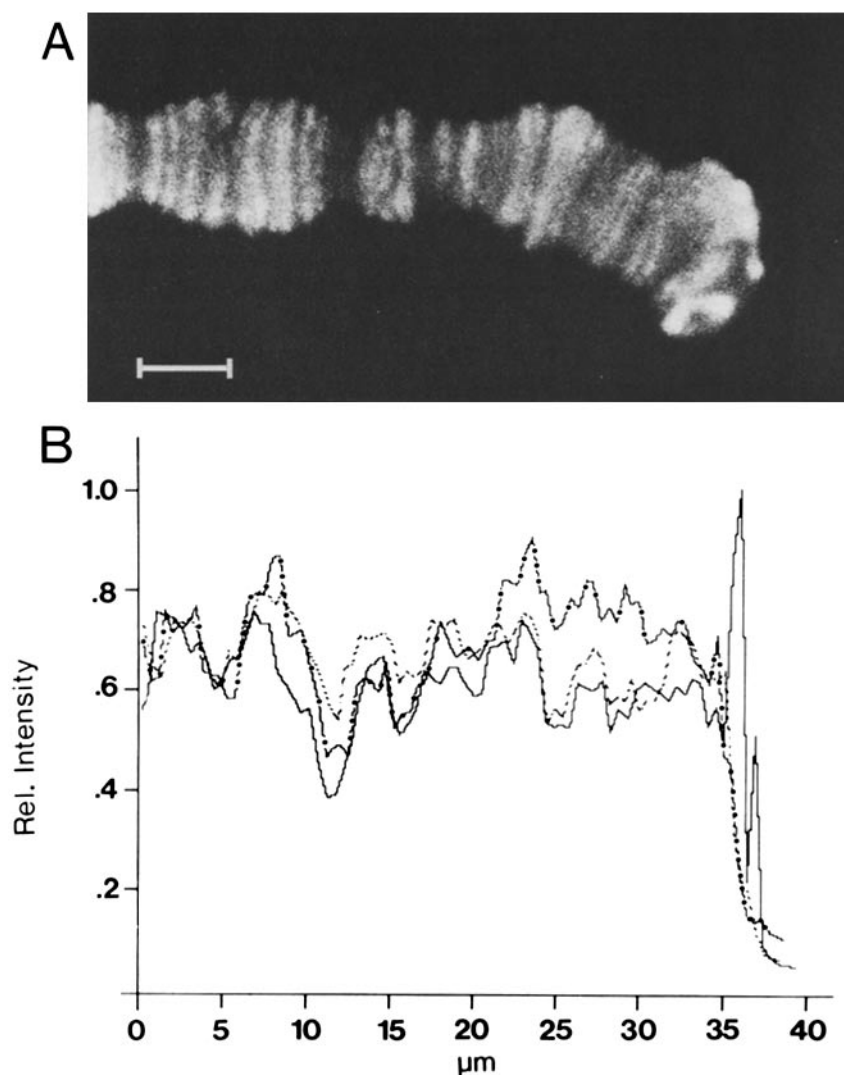


FIGURE 5 Scanning photometry of Z-DNA and total DNA distribution in the right-hand end of *C. thummi* polytene chromosome 1. (A) Indirect immunofluorescence pattern with class 1 antibody; class 2 antibody staining and DNA staining with H-33342 are not shown. (B) Superimposed relative (Rel.) fluorescence intensity scan profiles. —, class 1 fluorescein indirect immunofluorescence; ----, class 2 Texas red indirect immunofluorescence; —•—, 3 μM H-33342 DNA fluorescence. Conditions as in Fig. 4. Curves were set to equivalence at 1b2. Bar, 5 μm. × 1,000.

of binding by both antibody classes. In the telomeres of the three large chromosomes, alternating d(G-C)_n tracts cannot be detected, and alternating d(A-C)_n tracts are presumably overrepresented.

Scanning photometry is limited to structures that do not overlap or intersect. In addition, the relatively long exposure of the chromosome to the excitation light source during three-wavelength scanning can result in a deterioration of the image due to bleaching. Both of these problems can be overcome by whole image acquisition from an epi-illumination fluorescence microscope with a low light level TV camera and a dedicated computer image acquisition system. Integrated (256) fluorescence images can be acquired in <6 s at low light levels such that no bleaching occurs even for exposures of >1 min. The spectrally separated images and background images are stored in memory using the quantitative fluorescence image analysis system described in Materials and Methods. Subsequent image superposition, background subtraction, and normalization can be performed, as can arithmetic functions such as subtraction, division, filtering, and line scanning.

We have used this system to quantitate class 1 and 2

antibody staining of the *Chironomus* karyotype under conditions of staining with a single antibody or with varying ratios of class 1 and 2 antibodies present simultaneously. The differences in binding of either class 1 or class 2 antibody throughout each chromosome can be quantitated from ratios of images or from line scans through double-stained preparations. Binding of a large excess of the class 1 antibody eliminates the binding of the class 2 antibody as expected. It is interesting that high levels of the class 2 antibody potentiate the binding of the class 1 antibody at very low antibody concentrations. That is, class 1 antibody at 0.4 μg/ml in the presence of 100 μg/ml of class 2 antibody binds to most of the condensed DNA bands, whereas class 1 antibody binding alone at this concentration is restricted to the very heterochromatic regions of the chromosome. However, we find the maximum level of binding of the class 1 antibody in the presence of saturating class 2 antibody to be reduced by an average of 40% over most of the bands.

A few regions do not show reduced binding in the presence of class 2 antibody. These regions should represent the areas that have only A-C tracts in the Z conformation. Images were

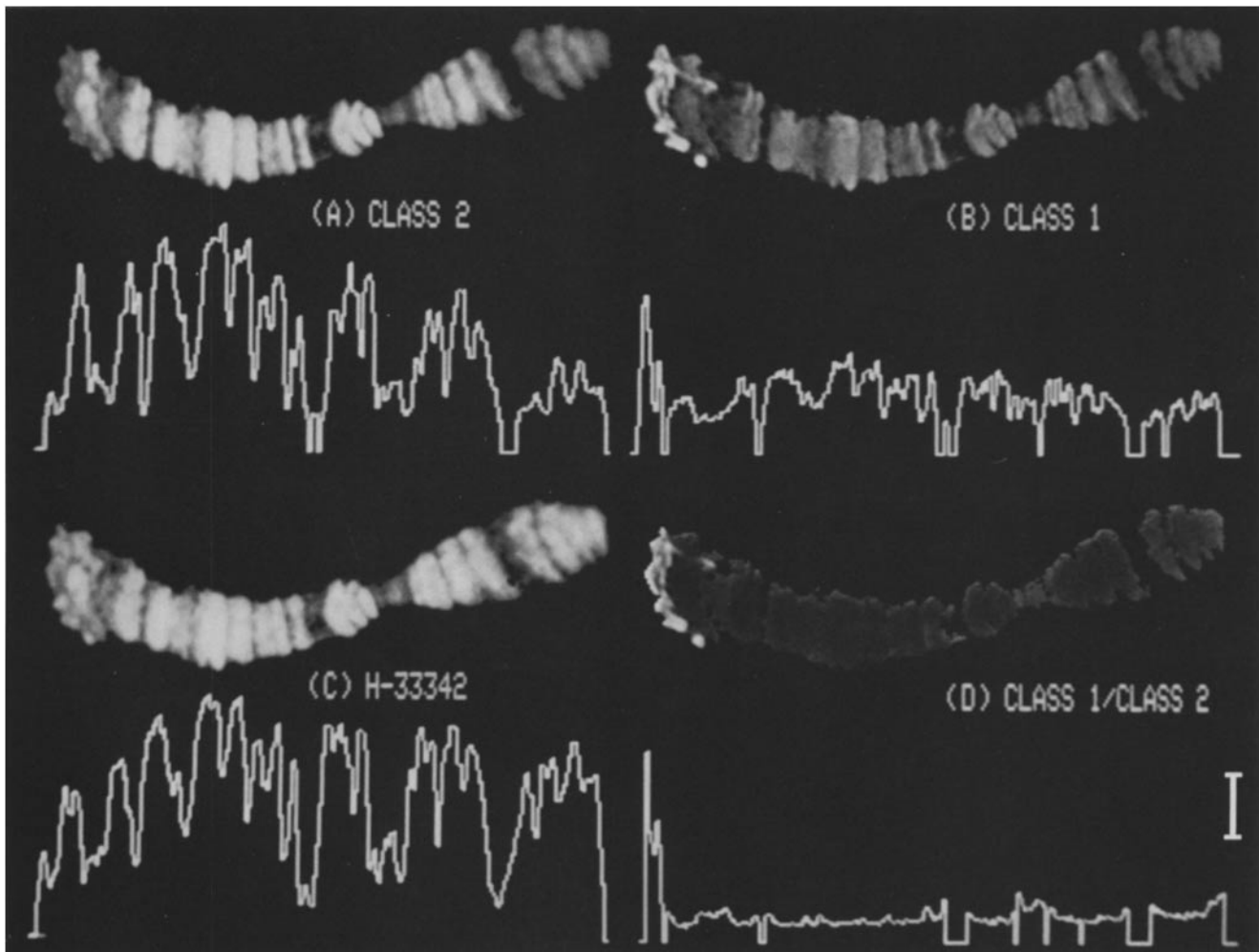


FIGURE 6 Localization of AC-rich Z-DNA sequences in polytene chromosomes of *C. thummi* by image analysis of triple-stained preparations. (A) Class 2 monoclonal antibody, D11, 75 μg/ml, indirect staining with Texas red-labeled goat anti-mouse IgG; (B) class 1 polyclonal antibody, Z6, 9.5 μg/ml, indirect staining with fluorescein-labeled goat anti-rabbit IgG; (C) H-33342 (3 μM) fluorescence. Note that the monoclonal antibody is here in great excess. Images were averaged over 256 successive frames (50 Hz frequency), background subtracted, and normalized (contrast stretched). In D, arithmetic division was performed and the ratio was displayed with a fivefold amplification. A line was drawn with the cursor along the central axis of the chromosome, and the corresponding grey level distribution along this line is displayed for all four images. Bar, 10 μm. × 1,000.

acquired from preparations stained with high levels of class 2 antibody and subsaturating levels of class 1 antibody as well as a DNA dye after background subtraction and normalization (Fig. 6). Each image (A, class 2; B, class 1; and C, DNA) has been scanned with a cursor line aligned along the longitudinal chromosome axis. The ratio of the class 1 to class 2 immunofluorescence images (Fig. 6, D) accentuates the very strong class 1 binding of the telomeric region. The enhancement of class 1 antibody binding in this region is ~ 15 -fold, thus obscuring more subtle differences that appear in the ratio along the rest of the chromosome.

High but subsaturating levels of class 1 antibody restrict class 2 binding to the strongly heterochromatic bands (33)

and a number of regions in the number IV chromosome (data not shown). These bands presumably have extensive tracts of alternating G-C basepairs.

Both Class 1 and 2 Antibodies Bind to Polytenic Chromosomes of Other Tissues and Other Species

In tissues of lower polyteny such as the Malpighian tubule cells of *C. thummi* the same strong correlation of the immunofluorescence with DNA content along the polytene chromosomes is observed (data not shown). Polytene chromosomes of this lower ploidy take on a more extended mor-

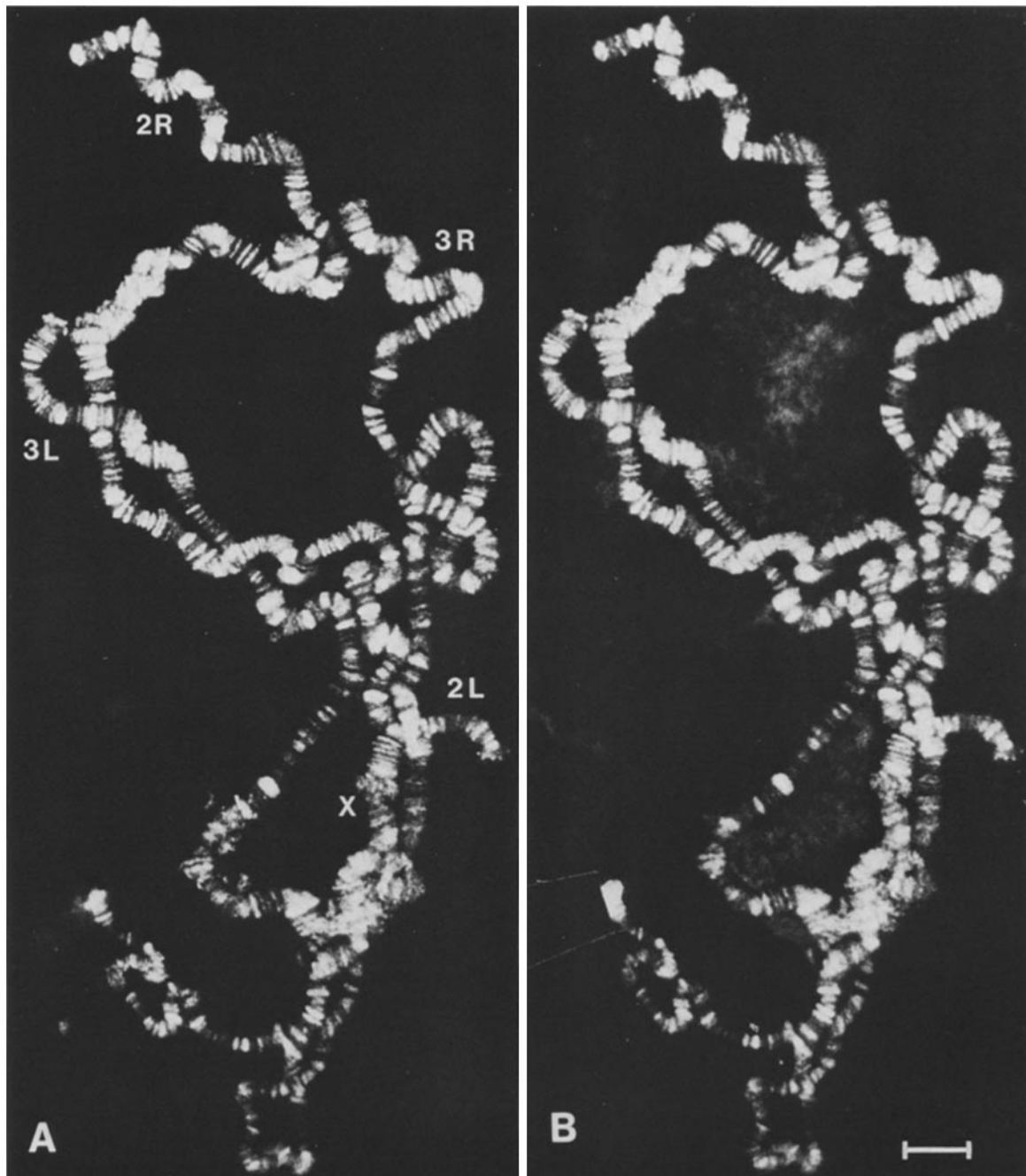


FIGURE 7 Double immunofluorescence staining of polytene chromosomes from *D. melanogaster* salivary glands with anti-Z-DNA antibodies (A) Class 1 antibody, T4, 30 $\mu\text{g/ml}$, indirect staining with fluorescein labeled goat anti-rabbit IgG; (B) class 2 antibody, D11, 7.5 $\mu\text{g/ml}$, indirect staining with Texas red-labeled goat anti-mouse IgG. Chromosome arms are designated in A. Bar, 10 μm . $\times 640$.

phology by stretching in the interband region and thus make possible higher resolution quantitative immunofluorescence localization, a feature of particular interest in the case of the species *D. melanogaster* for which a large body of sequence and genetic data is accumulating.

Staining of acetic acid squashed polytene chromosomes of *D. melanogaster* salivary glands with the sequence-independent, class 1 antibody (Fig. 7A) or the monoclonal, class 2 antibody (Fig. 7B) produces very similar immunofluorescence patterns. The class 2 antibody staining can be completely competed against by a large excess of class 1 antibody as was the case for *C. thummi*. There are no striking differences in the telomeric staining of the number 2R and 3L chromosomes, in contrast to the case of the three large chromosomes of *C. thummi*.

Drosophila polytene chromosomes are not as amenable to laser scanning photometric measurements due to their interconnection at the chromocenter and their highly irregular morphology in squashes. A quantitative comparison of the immunofluorescence images can be made using the computer-assisted image analysis system. After background sub-

traction and normalization of the antibody-stained images, we can compare the fluorescence patterns by division or subtraction. Such a procedure was followed for preparations stained with class 1 and 2 antibodies and yielded deviations of >20% in only a few bands. Regions in the images that are well delineated can be scanned in the image processing mode by describing a cursor line through the image. The intensity profiles can then be compared and differences quantitated to a resolution of 1–2 μm i.e., from one to a few bands. Fig. 8 is a composite of the three fluorescence images (A, DNA fluorescence; B, class 2 antibody immunofluorescence; C, class 1 antibody immunofluorescence) and their respective intensity profiles for the left end of chromosome 3. The antibody fluorescence patterns are strikingly similar. Increased binding of the class 1 antibody can be seen in the fourth major peak from the left (band 67A), whereas the class 2 antibody shows increased binding in a group of six peaks (region 61–62) adjacent to the telomere. More generally, the immunofluorescence staining does not reflect the local variations in DNA content as closely as in the case of the *C. thummi* polytene chromosomes.

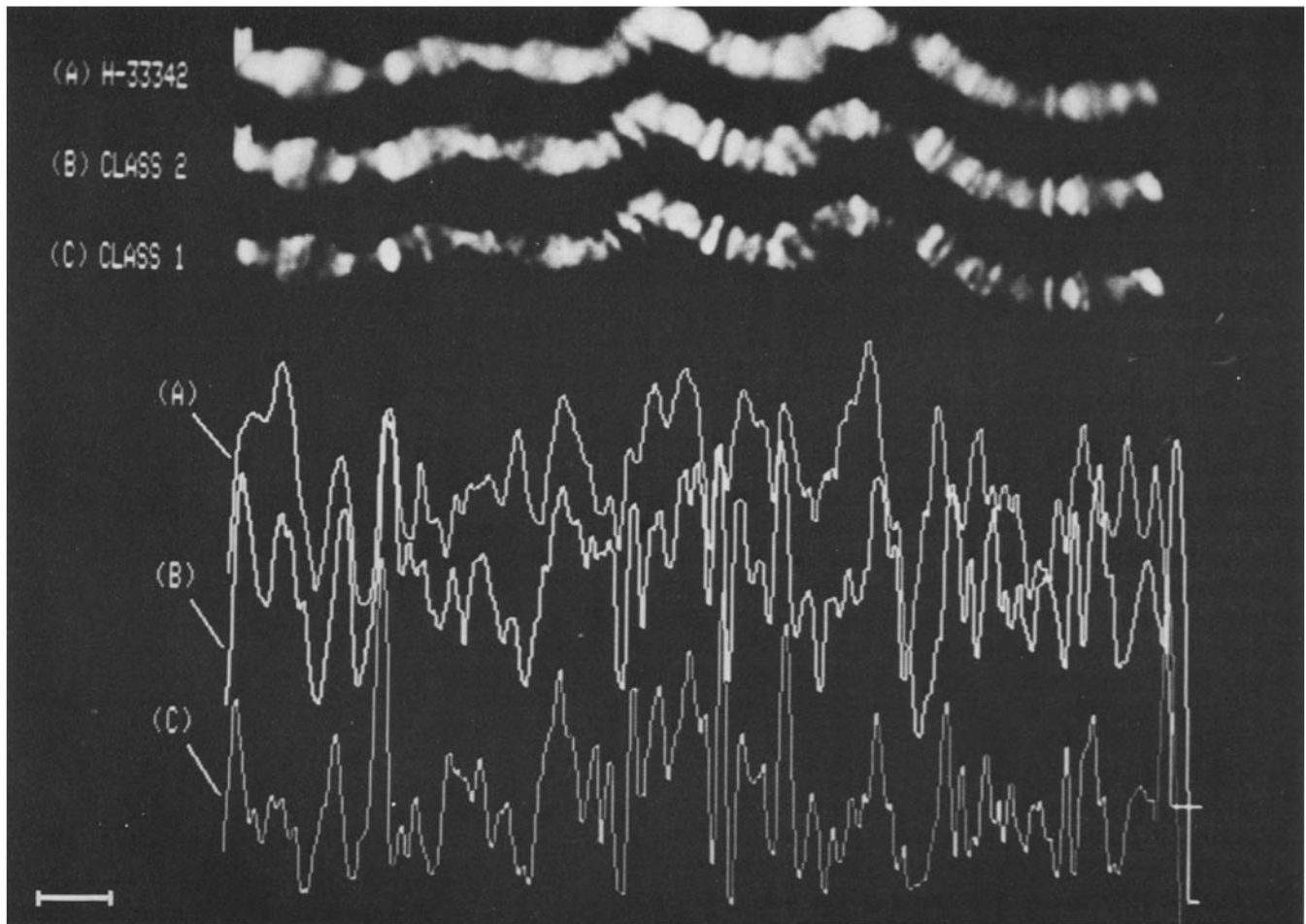


FIGURE 8 Quantitative image analysis of the immunofluorescence patterns obtained in *D. melanogaster* polytene chromosomes by triple staining with H-333342 and 2 classes of anti-Z-DNA antibodies. (A) DNA staining with H-333342 (3 μM); (B) class 2 antibody, D11, 6 $\mu\text{g}/\text{ml}$, Texas red-labeled goat anti-mouse IgG; (C) class 1 antibody, Z6, 9.5 $\mu\text{g}/\text{ml}$, fluorescein-labeled goat anti-rabbit IgG. Images were acquired and processed as in Fig. 6. The figure shows images from the end of the 3R chromosome. The intensity profiles along the chromosome axis were obtained as in Fig. 6 and are shown superimposed but with the respective base lines displaced vertically (see right-hand margin). Bar, 10 μm . $\times 1,000$.

Anti-Z DNA Antibody Staining Is Not Restricted to Polytene Chromosomes

In polytene chromosomes, the signals obtained by indirect immunofluorescence are amplified due to the high copy number of chromatids (2^{9-13}). However, potential Z-DNA sequences are detectable at all levels of ploidy. This can be demonstrated by binding of antibodies to acid-fixed $2n$ interphase nuclei and metaphase chromosomes. *Drosophila* tissue culture, D2, cells stain strongly with anti-Z-DNA antibodies. Various tissues (brain, testes, embryos) from a number of species (*Locusta*, *Chorthippus*, *Sarcophaga*, *Chironomus*, *Glyptotendipes*, and *Drosophila*) show the same strong antibody binding as *Drosophila* tissue culture cells (data not shown). The quantitative relationship of antibody binding to DNA content at various levels of DNA ploidy can be normalized by measuring the ratio of the antibody immunofluorescence to the DNA fluorescence in a double-stained preparation. Such data for *C. thummi* brain preparations were evaluated by arithmetic integration of the total fluorescence for a single nucleus and subsequent division of the immunofluorescence intensity by the DNA fluorescence. This ratio was found to be highest for $2n$ nuclei, decreasing slightly with increasing ploidy level over a range of $2-16n$. In cell nuclei with ploidy values $>8n$ the mean of the ratio of antibody fluorescence/DNA drops by 25% as compared with the mean determined for $2n$ nuclei.

DISCUSSION

Antibodies against Z-DNA that have sequence specificity (Fig. 1) provide a means to determine the relative distribution and frequency of potential Z-DNA sites in natural DNA. We have shown that these antibodies can be used to distinguish sites in closed circular DNA of extracted phage and viral genomes (12, 13) as well as in plasmids with cloned inserts (15). For example, closed circular Simian virus 40 DNA at extracted negative superhelical density binds class 1 antibodies better than class 3 antibodies and is not recognized by class 2 antibodies (13). Sequence analysis of the Simian virus 40 binding site (21, 22) shows that it belongs to the family of alternating sequences that contain A·T as well as G·C base-pairs, thus supporting the antibody binding data. On the other hand, Φ X174 DNA is recognized by all three antibody classes (12, 13). Recent anti-Z-DNA antibody binding and electron microscopic mapping of Φ X174 DNA (25) shows that 53% of a class 1 antibody binding is to a region containing a 14-bp sequence at position 763 and two other 8-bp sequences of mixed alternating purines and pyrimidines. There are also two pure G·C alternating sequences (at positions 5,345 and 2,363) which account for 22% and 6% of the class 1 antibody binding, respectively.

Polytene chromosomes in which the genome is arranged in an ordered array and where amplification increases the occurrence of all sequences 8,000–16,000-fold provide a system in which the localization of potential Z-DNA tracts and the affinity of sequence specific antibodies can be elucidated. In this paper we demonstrate how we have been able to determine the frequency and location of Z tracts in acid fixed polytene chromosomes of *C. thummi* and *D. melanogaster* using scanning microphotometry (Figs. 4 and 5) and quantitative fluorescence computer-assisted image analysis (Figs. 6 and 8). It is striking that most of the phase-contrast dark

bands stain with all classes of antibodies tested in this study, which indicates that potential Z sequences with different base composition are interspersed throughout most of the genome. The right-hand end of the number I chromosomes of *C. thummi*, regions *a* and *b* in Fig. 4, show no bands that are not present in both antibody scan profiles (Fig. 4C) except the telomere. Throughout these regions the ratio of the relative intensities of the two antibodies varies by only 20%. However, a band adjacent to the telomere has 30% more class 2 antibody binding. A dramatic reversal of the antibody frequency occurs in the telomere itself, where the class 1 antibody is bound much better than the class 2 antibody. In fact, scanning at higher spatial resolution reveals that the ratio of class 1 antibody/DNA is >20 times that of the ratio of class 2 antibody/DNA in the telomere (Fig. 5). These data are consistent with sequence data for eukaryotic (yeast) telomere clones, which contain stretches of alternating A·C basepair repeats (18).

Figs. 4–6 demonstrate other more subtle quantitative differences along the chromosomes. In studies on the effect of acid fixation on polytene chromosomes (27) we were able to demonstrate by progressive reduction of the fixation pH that the first bands to bind antibody are the heterochromatic bands described by Hägele (33). Both class 1 and class 2 antibodies are bound strongly to these bands. In fact, very high concentrations of class 1 antibody are required to saturate these regions. The D11, class 2 monoclonal antibody has a stronger affinity for G·C tracts in the Z conformation than does the T4, class 1 polyclonal antibody. Thus, at subsaturating levels of class 1 antibody the heterochromatic bands can be specifically highlighted by the class 2 antibody.

The predominance of Z DNA potential sequences in the telomeres and heterochromatin of *Chironomus* suggests that left-handed DNA could assist in the condensation of these regions of chromatin. Solution studies of polynucleotides in the Z and B conformations have shown that Z-DNA has a strong tendency to self-associate and form large aggregates (7, 30) which can be visualized in the electron microscope (34, 35). This property may facilitate the interaction between homologous or similar sequences and mediate the pairing of chromatids and end-to-end associations.

Under conditions of simultaneous binding of both class 1 and class 2 antibodies to *D. melanogaster* polytene chromosomes, the ratio of the normalized antibody immunofluorescence micrographs indicates that there are no large differences in the binding patterns of the two antibodies such as in the case of the *C. thummi* telomeres described above. However, differences in the frequency of G·C and A·C tracts in specific bands are demonstrated by the small variations in the two immunofluorescence images. These are easily distinguished with the aid of line scanning through the contour images (Fig. 8). The immunofluorescence patterns follow closely the DNA distribution in *C. thummi*. This is not the case for *D. melanogaster* (Fig. 8). Our effective resolution in the localization of the antibody binding is actually reduced for *Chironomus* salivary gland chromosomes as compared with *Drosophila* polytene chromosomes. The lower polyteny level of the latter allows them to assume a more extended morphology in the preparations, stretching especially in the interband regions.

Our image analysis system has a spatial resolution of one or a few bands, assuming an average band of $0.5 \mu\text{m}$ and an average interband distance of $1.5 \mu\text{m}$. Since, as determined by saturation titration with class 1 antibody, $<1\%$ of the DNA

is comprised of Z-potential sequences (26), and since a band of the polytene chromosomes has an average of 50,000 bp per chromomere, we expect <50 bp of Z-potential sequences per chromomere for an average band. Thus, differences at this resolution would result from over-representation of one sequence or the distribution of different sequences at opposite ends of a gene or in adjacent genes.

Many of the Z-potential tracts in these chromosomes may be mixed alternating sequences, as found in viral and phage genomes. The finding that binding of high levels of class 2 antibody actually potentiates class 1 antibody binding at low concentrations is compatible with this idea. We have shown previously (7, 12) that antibody binding to an equilibrium mixture of B and Z conformations will shift the equilibrium towards the Z. Not all potential Z-DNA sites in natural DNA that are found at low pH or high temperature (27) will remain in the Z conformation at neutral pH, at which antibody binding is carried out. However, high concentrations of class 2 antibody would hold G-C tracts in the Z conformation and could shift contiguous or adjacent A-C tracts to this conformation, thereby facilitating the binding of class 1 antibody. Immunoelectron microscopy of the class 1 antibody with Φ X174 RFI DNA (25) demonstrates that the antibody-mediated intramolecular cross-linking stabilizes regions of the DNA supercoiling even after restriction endonuclease digestion.

The potential Z-DNA sequences express themselves at low copy number. Diploid cells from a number of insect tissues (brain, testes, and embryos) and from tissue culture from five insect species (*Locusta*, *Chorthippus*, *Sarcophaga*, *Drosophila*, and *Chironomus*) have been investigated. All bind anti-Z-DNA antibodies very strongly after acid fixation. Relative measurements of the immunofluorescence intensity and of the DNA staining made on *Chironomus* cells with various degrees of ploidy were made. The ratio of antibody bound to DNA content plateaus above $8n$ to a value $\sim 25\%$ below the ratio for $2n$ nuclei. This observation may reflect a reduced accessibility of binding sites in the more tightly packed chromatin fibers of oligotene and polytene chromosomes.

In conclusion, we have found a distribution of Z-DNA potential sequences throughout the genome by quantitative scanning photometry and quantitative image analysis of insect polytene chromosomes. The heterochromatic bands and telomeres have higher frequencies of these sequences. Differences in the distribution of G-C and A-C tracts exist, as do deviations of the antibody distributions from the total DNA content profile along the chromosomes at the present resolution of $\sim 0.5 \mu\text{m}$.

Alternating G-C sequences in the Z conformation have been shown to support reduced transcriptional activity (36-38). Z-DNA potential sequences dispersed throughout the genome could serve functional roles in interphase (e.g., regulation of transcriptional activity of genes) as well as a structural role in metaphase or in inactive chromatin by exploitation of its inherent tendency to associate.

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