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Immunological detection of left-handed Z DNA in isolated polytene chromosomes. Effects of ionic strength, pH, temperature and topological stress

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We have searched for the presence of left-handed Z DNA in unfixed polytene chromosomes isolated from the salivary glands of *Chironomus thummi* larvae. Physiological as well as fixation conditions were explored to assess the effects of a variety of factors known to influence the B-Z equilibrium. At neutral pH and physiological ionic strength, a weak immunofluorescence staining confined to the periphery of chromosomal bands is elicited but only by using high concentrations of anti-Z DNA immunoglobulin (IgG). The accessibility of internal highly condensed structures, as monitored with antibodies against core histones, is very limited under these conditions. Increasing the ionic strength exposes core histone determinants but results in a decondensation of the bands. The staining for Z DNA is still weak and primarily restricted to regions resisting decondensation or undergoing collapse. Dramatic changes in anti-Z DNA immunofluorescence intensities occur upon short exposure to low pH. Adjustment of the pH between 2.5 and 2.0 leads to an abrupt large increase in antibody binding, at first confined to a few specific bands and then generalized to bands throughout the chromosomes in a pattern very similar to that elicited in classical acid-fixed squash preparations. The acid-mediated effects are influenced by ionic strength, temperature and prior removal of histones; they can be mimicked by exposure to high temperature at neutral pH. The 'transition pH' assessed with a monoclonal IgG specific for left-handed $d(G\text{-}C)_n$ sequences is slightly lower than in the case of polyclonal antibodies which also recognize $d(A\text{-}C)_n \cdot d(G\text{-}T)_n$. Topoisomerase or S1 nuclease digestion obliterates the antibody binding potentiated by low pH. In addition, u.v. and c.d. spectral studies show that the Z conformation of poly[d(G-C)] is preferentially induced and stabilized in acid. These results imply that generation of topological stress, base protonation and/or local strand separation provide driving forces for the B-Z transition. Candidates for factors which could promote and stabilize the left-handed conformation *in vivo* are discussed.

Key words: Z DNA/antibodies/polytene chromosomes/*Chironomus*/topoisomerases

Introduction

One of the most dramatic changes in the conformational state of DNA is the interconversion of right-handed (R,B) and left-handed (L,Z) helices which can be induced in alternating purine-pyrimidine sequences. This transition is elicited in solution by varying the ionic conditions and/or the water activity as demonstrated for poly[d(G-C)] (Pohl and Jovin, 1972; Pohl, 1976; van de Sande and Jovin, 1982; van de Sande *et al.*, 1982; Zacharias *et al.*, 1982) and, more recently, for derivatives of poly[d(A-C)·d(G-T)] (McIntosh *et al.*, 1983; Jovin *et al.*, 1983a; McIntosh and Jovin, in preparation). The transition can occur at physiological ionic strength in linear molecules methylated in the C5 position of cytosine (Behe and Felsenfeld, 1981) or in negatively supercoiled circular DNAs with insertions of certain alternating purine-pyrimidine sequences (Klysik *et al.*, 1983; Peck *et al.*, 1982; Nordheim and Rich, 1983b; Haniford and Pulleybank, 1983).

Left-handed polynucleotides differ from B polymers in a number of physicochemical and biochemical properties: (i) the formation of left-handed structures relaxes negatively supercoiled DNA (Wang *et al.*, 1983); (ii) Z DNA sequences show a pronounced tendency to self-associate and form large aggregates (Z* formation; van de Sande and Jovin, 1982; Jovin *et al.*, 1983c); (iii) linear polymers in the Z conformation are transcribed with reduced efficiency by RNA polymerase compared with the corresponding B forms (van de Sande and Jovin, 1982; Durand *et al.*, 1983); and (iv) Z DNA shows reduced sensitivity to endonucleases (Behe *et al.*, 1981; Nickol *et al.*, 1982; Jovin *et al.*, 1983c). Thus, B→Z transitions could be involved in the control of the structural state of the chromosomes, in the regulation of the functional state of particular chromosomal loci and in recombination events. Intracellular factors likely to stabilize the left-handed form are: divalent cations (van de Sande and Jovin, 1982; van de Sande *et al.*, 1982); polyamines (Behe and Felsenfeld, 1981; Klevan and Schumaker, 1982; Ivanov and Minyat, 1981); and chromosomal proteins (Miller *et al.*, 1983; Nordheim *et al.*, 1982b; Russell *et al.*, 1983). Models for the possible involvement of Z sequences as structural and regulatory elements in chromosomes and chromosomal subunits have been presented by Rich (1983) and Jovin *et al.* (1983a).

Specific antibodies raised against Z-polynucleotides have facilitated the search for left-handed helices in natural DNA. More than 20 publications have documented the presence of Z DNA in closed circular DNAs and cytological preparations of chromatin from a variety of sources using such antibodies (see Discussion, Table I). However, in all these studies, the DNA was subjected to extraction or fixation conditions which may have a profound influence on the conformational state (see e.g., Courey and Wang, 1983). An understanding of the effects of the extraction and fixation procedures on the B-Z transition and on chromatin structure has become a prerequisite for assessing the presence of Z DNA sequences *in vivo*.

To approach the *in vivo* situation of natural chromatin as closely as possible, we have isolated unfixed polytene chromosomes from *Chironomus* salivary glands according to a technique developed by Robert (1975). This system provides

Results

Antibody binding to isolated polytene chromosomes at physiological ionic strength and pH

Isolated unfixed polytene chromosomes from insect larvae provide a unique system for studying the distribution of Z DNA sequences under conditions as close as possible to the physiological. However, in carrying out immunological experiments on these chromosomes, we found that the physical exposure of DNA determinants for antibody binding may change, depending on a variety of environmental factors. We have used polyclonal antibodies to histone H3 and a monoclonal antibody to histone H1 to assess the general degree of chromatin accessibility under given experimental conditions.

The immunofluorescence pattern obtained with anti-histone H3 antibodies in isolated polytene chromosomes at physiological pH and ionic strength is shown in Figure 1A. By serial focusing through the chromosome structure, we observe the fluorescence to be distributed at the periphery in a sheath-like fashion. Similar results are obtained with a monoclonal anti-histone H1 antibody.

A weak peripheral binding of anti-Z DNA antibodies is also seen after indirect staining of isolated polytene chromosomes in the same medium (Figure 1B,C). Use of direct fluorescein-labeled anti-Z DNA antibodies shows the same fluorescence distribution suggesting that a limited penetrability of the first antibody may be the primary cause of the observed outside binding. It follows that this pattern is unlikely to reflect the actual distribution of Z DNA tracts in the chromosomes. Therefore, we tested a variety of factors which might enhance the accessibility of determinants in chromosomal substructures.

the following advantages: (i) the signals obtained by immunofluorescence are amplified due to the high degree of polytenization; (ii) there exists detailed knowledge about the effects of ionic strength and pH on the structural state of the chromosomes (Robert, 1971; Lezzi and Robert, 1972); (iii) conditions are known for optimal transcriptional activity (Hameister, 1977; Mähr *et al.*, 1979; Sass, 1980); (iv) the ionic concentrations prevalent *in vivo* in the salivary gland nuclei have been measured (Kroeger *et al.*, 1973; Palmer and Civan, 1975); (v) the conditions necessary to deplete specific types of chromosomal proteins have been determined (Bastian, 1983); and (vi) numerous studies on the binding of anti-Z DNA antibodies using fixed preparations of the same chromosomes have been carried out (Jovin *et al.*, 1983a, 1983c; Robert-Nicoud *et al.*, 1983; Zarling *et al.*, 1984a, 1984b) including quantitative immunofluorescence microscopy (Arndt-Jovin *et al.*, 1983; Jovin *et al.*, 1983b). The latter studies have demonstrated that at saturating antibody concentrations, one antibody binds per 3000 – 15 000 bp in the bands and that the immunofluorescence pattern reflects the sequence specificity of the anti-Z DNA antibody. We have exploited the unfixed polytene chromosomes to investigate the influence on anti-Z DNA immunofluorescence of conditions known to effect the B → Z transition, such as ionic strength and temperature, protein extraction and DNA supercoiling. In parallel, we have studied the effect of acid pH on the B → Z transition of polynucleotides in solution and documented the consequences of acid treatment both on protein extraction from, and anti-Z DNA immunofluorescence in, the chromosomes so as to determine the role fixation plays in the frequency and distribution of Z DNA in natural sequences.

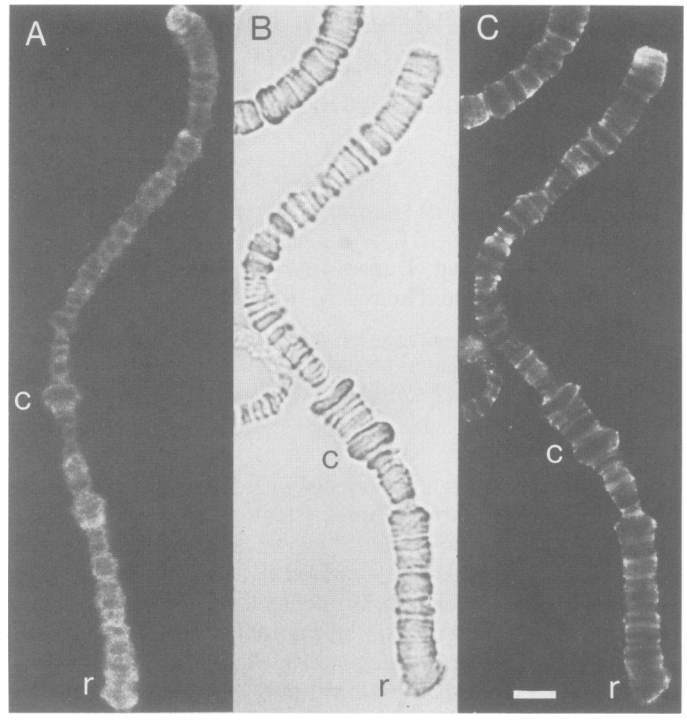

Fig. 1. Binding of anti-Z DNA and anti-histone H3 antibodies to isolated salivary gland chromosomes of *C. thummi* at physiological ionic strength and pH. Indirect immunofluorescence patterns obtained after staining in $CR_{7.3}$-medium (see Materials and methods). **(A)** polyclonal anti-H3 antiserum, 1/100 dilution, **(C)** polyclonal anti-Z DNA IgG, T4, 200 μg/ml, followed by fluoresceinated IgG fraction of goat anti-rabbit IgG (F1-GARIG); **(B)** bright field image of chromosome in **(C)**. Chromosome III; c, centromere; r, right-hand end. Bar denotes 10 μm.

Influence of ionic strength

Raising the salt concentration to 0.35 M NaCl (in the presence of 10 mM $MgCl_2$ and 10 mM Tris-HCl, pH 7.5) is known to elicit a partial and differential decondensation of the bands (Robert, 1971; Lezzi and Robert, 1972). After indirect staining with anti-histone H3 antibodies, the entire chromosomes including the decondensed regions display a bright fluorescence (Figure 2A,B) showing that H3 determinants are accessible under these environmental conditions.

At the same salt concentrations only a weak fluorescence staining is obtained with concentrations of anti-Z DNA antibodies 20 times higher than those used to stain fixed chromosomes preparations. This staining appears to be confined to chromosomal regions which have either persisted or collapsed into a condensed state (Figure 2C). Treatment at higher ionic strength (0.6 M, 0.9 M and 1.2 M NaCl) leads to a greater decondensation of the chromosomes and abolishes all perceptible anti-Z DNA antibody binding both in high salt and after return to physiological ion concentrations. This decondensation and concomitant change in chromosome structure is presumably due to the dissociation of certain types of chromosomal proteins known to occur at these ionic strengths (see Disucssion).

Influence of low pH

Removal of chromosomal proteins without concomitant decondensation can be achieved by lowering the pH. Acid fixatives have been extensively employed for the preparation of cytological samples for the purpose of increasing the permeability and accessibility of structures without undue loss

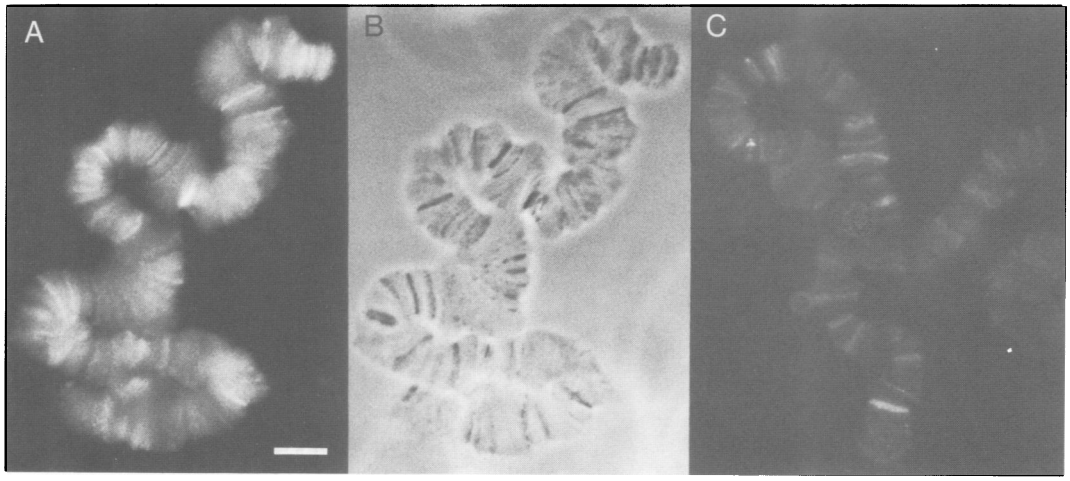

Fig. 2. Binding of anti-Z DNA and anti-histone H3 antibodies to isolated *Chironomus* polytene chromosomes at elevated ionic strength and neutral pH. Indirect immunofluorescence of chromosomes stained in 0.35 M NaCl, 10 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.3. **(A)** anti-histone H3; **(B)** phase contrast image of chromosome in **(A)**; **(C)** anti-Z DNA IgG, T4. Antibody concentrations as in Figure 1. Bar denotes 20 μm.

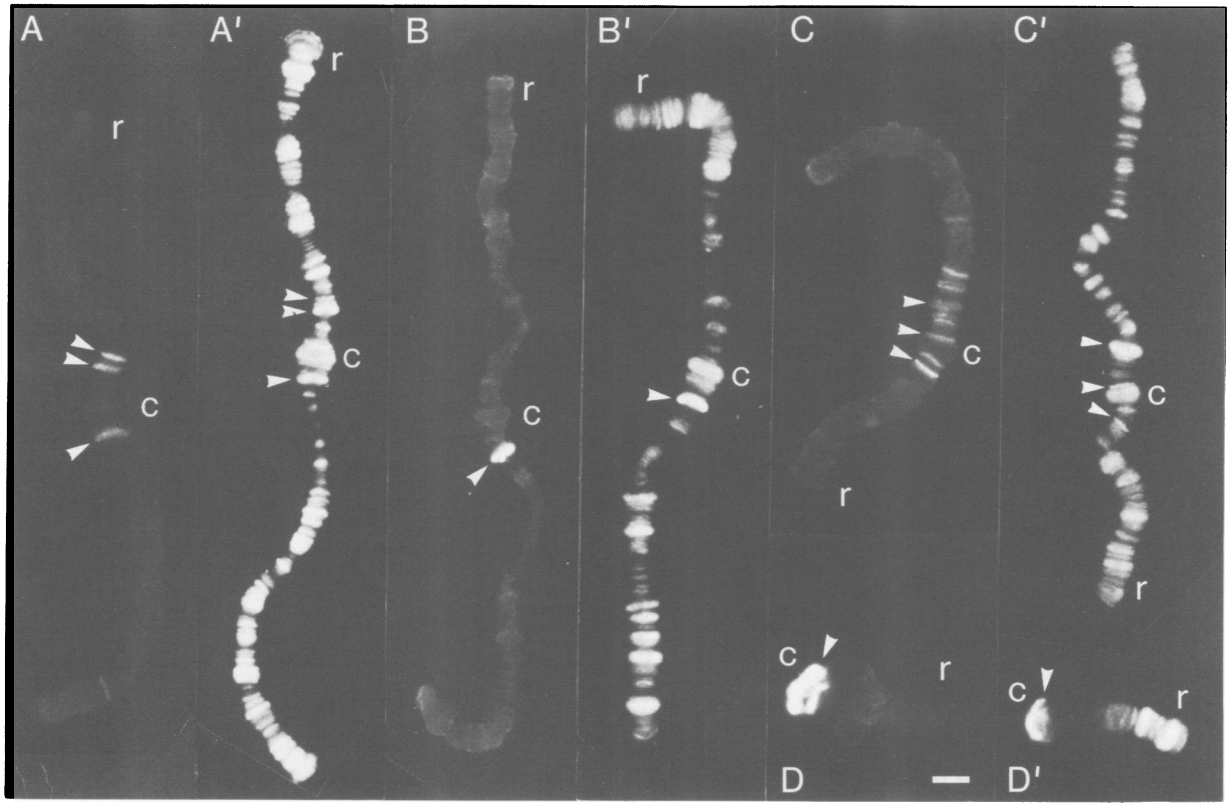

Fig. 3. Sequential changes in the Z DNA-specific immunofluorescence staining of bands in isolated polytene chromosomes of *C. thummi* mediated by exposure to decreasing pH. Chromosomes were exposed to pH 2.45 **(A–D)** or pH 2.2 **(A′–D′)**, returned to neutral pH and stained with anti-Z DNA IgG, T4, at 10 μg/ml followed by F1-GARIG. **AA′, BB′, CC′, DD′**: chromosomes I, II, III, IV, respectively. Arrows in **A′–D′** denote corresponding bright bands in **A–D**. Notation as in Figure 1. Bar denotes 10 μm.

of morphological relationships.

A dramatic change in the staining of isolated polytene chromosomes with anti-Z DNA antibodies occurs upon exposure to solutions buffered between pH 3 and 2. After treatment at pH 3, only a weak indirect immunofluorescence associated with the periphery of the chromosomes is detected, similar to that seen at pH 7. Gradually lowering the pH to values between 2.5 and 2.0 elicits an abrupt increase in the staining of the bands (Figure 3). Brief exposure to pH 2.45 results in a specific intense immunofluorescence of a limited set of bands in the telomeres and in the vicinity of the centromeres (Figure 3A–D). The latter are located in regions Id3-e1, IIc4, IIIb2-4, and IVe2 (designation according to Keyl, 1957). In contrast, treatment at pH 2.2 leads to strong antibody binding to bands distributed throughout the chromosomes (Figure 3A′–D′).

The immunofluorescence patterns obtained with anti-Z DNA antibodies in isolated chromosomes treated in glycine

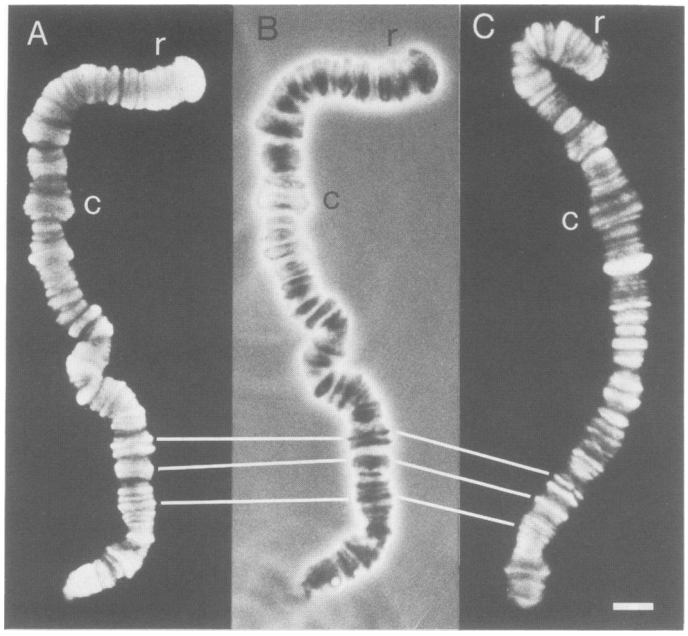

Fig. 4. Comparison of Z DNA-specific immunofluorescence in acid-treated isolated polytene chromosomes and chromosomes of fixed salivary gland squashes. **(A)** Indirect immunofluorescence pattern obtained after exposure of isolated chromosomes to 20 mM glycine-HCl, pH 2.0, 80 mM NaCl; **(B)** phase contrast image of chromosome shown in **(A)**; **(C)** indirect immunofluorescence in a squash preparation after fixation of salivary glands in EtOH:acetic acid (3:1) followed by 45% acetic acid. Staining performed at neutral pH with anti-Z DNA IgG, T4, 10 μg/ml followed by Fl-GARIG. Chromosome III. Notation as in Figure 1. Lines connect corresponding bands in region IIId. Bar denotes 10 μm.

or citrate buffers (at pH 2.2–2.0), in 0.01 N HCl-0.1 M NaCl (pH 2.0), or in 45% acetic acid (~pH 1.6) are similar in terms of distribution and intensity to those seen on squash preparations of salivary glands (see Figure 4 and cf. Figure 1 in Arndt-Jovin *et al.*, 1983). Comparison of the phase contrast and immunofluorescence staining of a chromosome exposed to pH 2.0 in Figure 4A,B clearly demonstrates that the staining is in the bands.

The effect of low pH treatment on the subsequent binding of anti-Z DNA antibodies is not restricted to isolated polytene chromosomes or even to polytene systems in general. We have observed that squashing salivary glands directly in 80 mM NaCl, 20 mM glycine-HCl, pH 2.2 (but not pH 2.5) and in the presence of 0.1% Triton X-100 to permeabilize the cells, results in preparations which stain strongly with anti-Z DNA antibodies. Cells with diploid nuclei from a variety of organisms show the same effects of low pH. A complete description of these results will be presented elsewhere.

Factors influencing the effects of low pH on the stainability of isolated chromosomes

The immunofluorescence pattern obtained with anti-Z DNA antibodies varies depending upon the time of the exposure to the low pH buffer. The pattern shown in Figure 3A′–D′ is elicited by treatment of the chromosomes at pH 2.2 for at least 5 min. Chromosomes exposed to this pH for shorter periods of time (10 s to 1 min) display the strong fluorescence of the bands elicited at pH 2.45 but the rest of the chromosome is stained weakly.

The critical pH at which the changes in staining occur is shifted to higher values (by 0.2–0.3 units) at higher ionic strength (2 M NaCl or 1 M glycine). Treatment of the chromosomes under ionic conditions known to cause the release of the majority of the chromosomal proteins including the core histones (e.g., at 0.6 M NaCl, pH 4.3; Zueger, 1971) prior to the exposure to low pH has the same effect.

At low temperature the pH-dependent process is retarded. For example, exposure of isolated chromosomes to pH 2.2 at 4°C for 5 min does not elicit the subsequent strong binding of antibody, although the same treatment applied to protein-depleted chromosomes leads to the complete and intense immunofluorescence. We conclude that the temperature-dependent step is likely to be the acid-mediated release of chromosomal proteins.

The extraction at acidic pH of chromosomal proteins was monitored by immunofluorescence using antibodies to histone H1 and H3. Histone H1 specifically dissociates from most of the chromosomal bands at pH 2.3 and is completely lost by pH 2.0. On the other hand, we find that the chromosomes stain strongly and uniformly with anti-histone H3 antibodies after treatment at pH 2.5–2.0. Fixation of the isolated chromosomes with 3% formaldehyde, pH 7, for 5 min at room temperature diminishes but does not abolish the anti-Z DNA immunofluorescence potentiated by low pH treatment.

Anti-Z DNA antibodies with sequence specificity

The pH-dependent changes in the stainability of bands in isolated polytene chromosomes shown in Figure 3 are observed using a polyclonal antibody (T4) raised against the chemically brominated alternating copolymer br-poly[d(G-C)]. This IgG recognizes the left-handed (Z) conformations of both the $d(G\text{-}C)_n$ and the $d(A\text{-}C)_n \cdot (G\text{-}T)_n$ sequence

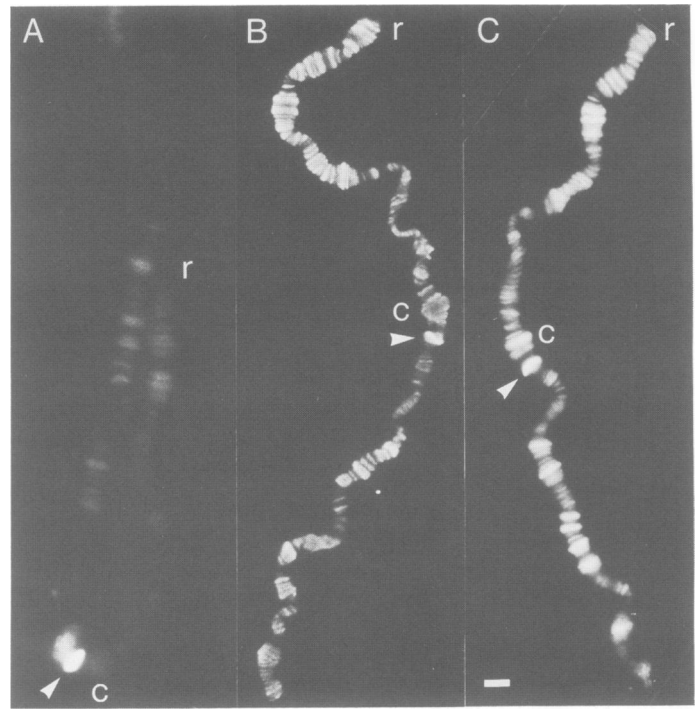

Fig. 5. Binding of anti-Z DNA antibodies with different sequence specificities to isolated polytene chromosomes exposed to low pH. **(A,B)** Immunofluorescence pattern obtained with a monoclonal antibody, D11, which recognizes only $d(G\text{-}C)_n$ sequences; staining at 5 μg/ml IgG; **(C)** sequence-independent anti-Z DNA polyclonal IgG, T4; staining at 10 μg/ml. Isolated chromosomes were treated in 80 mM NaCl, 20 mM glycine-HCl buffer **(A,C)** at pH 2.2, and **(B)** at pH 1.8, respectively. Chromosome II. Notation as in Figure 1. Arrows denote strongly stained region in IIc4. Bar denotes 10 μm.

families (Zarling *et al.*, 1984a). Similar changes can be visualized by using antibodies with a more restricted pattern of recognition. D11, for example, is a monoclonal antibody generated by R.Thomae (Thomae *et al.*, 1983) which displays strong sequence specificity, recognizing only members of the $d(G\text{-}C)_n$ but not of the $d(A\text{-}C)_n \cdot (G\text{-}T)_n$ families (Zarling *et al.*, 1984a). As seen in Figure 5A, D11 stains only bands in the vicinity of the centromere after treatment at pH 2.2, a pH which elicits staining of the entire chromosome with the sequence-independent antibody, T4, (Figure 5C). Treatment of chromosomes at pH 1.8 is required in order to obtain an immunofluorescence staining pattern with D11 (Figure 5B) similar to that observed in acetic acid-fixed squash preparations of salivary glands. As in the case of the sequence-independent antibody, a prior removal of chromosomal proteins (in 0.6 M NaCl, pH 4.3) raises the critical pH value by 0.2–0.3 units. The patterns of immunofluorescence seen with T4 and D11 IgGs in isolated acid-treated polytene chromosomes show the same differences we reported for squash preparations (Jovin *et al.*, 1983b, 1983c). As an example, T4 antibodies bind strongly to telomeres of chromosomes I, II and III, whereas the monoclonal D11 does not bind to these regions.

B-Z transition of poly[d(G-C)] at low pH

The acidic conditions potentiating the immunospecific staining of the polytene chromosomes were applied to the prototype Z-forming polynucleotide, poly[d(G-C)]. The conformational states of the DNA were assessed by u.v. absorption and c.d. spectroscopy as a function of ionic strength and pH. The results of an experiment performed at pH 3 and using NaCl concentrations of 0, 1 and 2 M are shown in Figure 6. In 0.05 M glycine buffer, spectral features previously reported as indicative of protonation of guanine residues (Narasimhan and Bryan, 1975) are observed: hyperchromism at 255 nm, hyperchromism around 275 nm, and a positive c.d. peak near 260 nm. Upon addition of NaCl to 1 M, the absorption and c.d. spectra reverted to those characteristic of the B conformation at neutral pH (Pohl and Jovin, 1972; van de Sande and Jovin, 1982). However, at 2 M (and 3 M, data not shown) NaCl, the spectra indicate that the predominant form of the DNA is the left-handed Z conformation. Thus, the A_{295}/A_{260} ratio (0.44) and the c.d. minimum at 297 nm are close to the corresponding values observed with the high-salt form of poly[d(G-C)] at neutral pH. Furthermore, at 2 M NaCl, a perceptible kinetic process follows the addition of the DNA to the salt solution. The reaction is rapid at 22.4°C, proceeding with half-lives of 0.5–1.5 min; in contrast, the half-life at neutral pH and in 3 M NaCl is ~9 min. In other experiments, the pH was varied at constant salt concentration. For example, in 3 M NaCl and pH 2.1, denaturation of the DNA occurs at temperatures above 10°C, as characterized by a λ_{max} of 281 nm and development of turbidity. At pH 2.4 and at the same salt concentration, the spectrum of the partially protonated B form is observed. In 2 M NaCl, the Z conformation appears to dominate between pH 2.9 and 3.5 but reverts to the B form above pH 4. We conclude that the Z form of poly[d(G-C)] is preferentially stabilized in the acidic range (~pH 2.9–3.5) relative to the situation at neutral pH.

Influence of enzymes affecting the degree of DNA supercoiling

The loss of proteins resulting from exposure to low pH is expected to place the DNA in topologically constrained domains of the chromosomes under torsional stress, thereby facilitating a B–Z transition in sequences with high potential for such a conformational change (see Discussion). In order to test if the Z conformation detected by immunofluorescence in acid-treated isolated chromosomes is dependent upon torsional stress, we have used enzymes known to relax DNA either by introducing permanent breaks (S1 nuclease), or by the transient strand cutting and religation activities of topoisomerases I and II (Gellert, 1981). We have shown elsewhere (Zarling *et al.*, 1984b) that anti-Z DNA antibody binding to SV40 Form I DNA is abolished by relaxation of supercoiling by topoisomerase activity. Treatment with calf thymus topoisomerase I or *Drosophila* topoisomerase II in the presence of ATP of histone-depleted polytene chromosomes after exposure to low pH leads to a strong reduction of the immunofluorescence intensity in most chromosomal regions (Figure

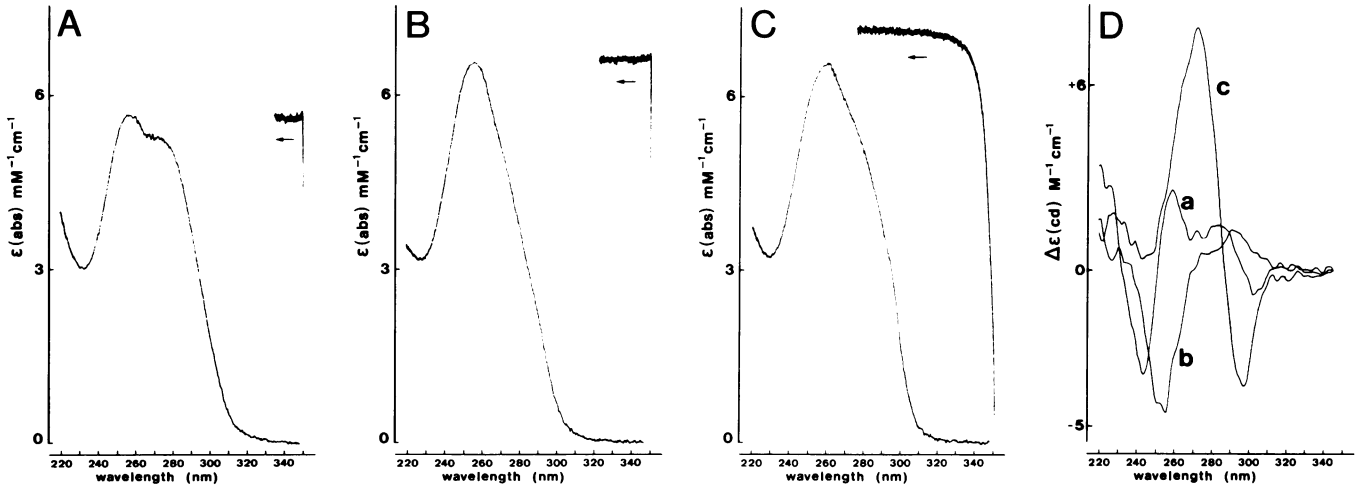

Fig. 6. Salt-induced conformation transitions in poly[d(G-C)] at pH 3. **(A–C)** u.v. absorption spectra of 50 μm poly[d(G-C)] at 22.4°C in 48 mM glycine-HCl containing **(A)** no added salt, pH 3.03; **(B)** 1 M NaCl, pH 3.15; **(C)** 2 M NaCl, pH 3.14. The kinetics at 295 nm upon addition of the DNA is shown with a 4-fold expanded scale and shifted origin. The arrows point in the direction of increasing time; their length equals 2 min. The curve in **panel C** shows two components with $t_{1/2}$ of ~0.5 min (80%) and 1.5 min (20%). **(D)** c.d. spectra (a–c) corresponding to solutions in **panels A–C**, respectively. A_{295}/A_{260} ratios and λ_{max} values: **(A)** 0.44, 261 nm; **(B)** 0.44, 261 nm; **(C)** 0.22, 256 nm. λ_{min} (nm), λ_{max} (nm) values in **(D)**: (a) 244, 260; (b) 253, 291; (c) 297, 273. Tentative assignment of predominant DNA conformations (see text): (A,a) protonated B form; (B,b) B form ; (C,c) Z form.

7). Telomeric and centromeric regions as well as small localized regions strongly attached to the glass surface remain fluorescent but the rest of the chromosome no longer binds antibody indicating that it has undergone relaxation. S1 nuclease, an enzyme known to cut at single-stranded sites in general, and at junctions between B and Z DNA tracts in particular (Singleton *et al.*, 1983), eliminates the staining in chromosomes previously treated at low pH, whereas exposure to S1 nuclease before the acid treatment has no effect.

Influence of high temperature and denaturing agents

Exposure of isolated chromosomes to pH values between 2.5 and 2.0 is likely to result in a partial and reversible denaturation of certain DNA sequences (see Discussion). We explored other conditions leading to reversible denaturation of the chromosomal DNA. We find that exposure of isolated polytene chromosomes to elevated temperatures (70–90°C) elicits similar changes in the specific anti-Z DNA immunofluorescence staining as those observed with low pH. The effects are seen only if the treatment is carried out at low ionic strength in the absence of divalent cations (10 mM Tris-HCl, pH 7.5, 1 mM EGTA) and are maximal if the chromosomes have been depleted of proteins.

By gradually increasing the temperature to 75–90°C, sequential changes in the stainability of specific chromosomal regions are observed. After treatment at 75°C only bands in telomeric and centromeric regions display bright immunofluorescence. After exposure to 80–90°C all the bands show strong binding of anti-Z DNA antibodies. Treatment of the chromosomes at high temperature without prior removal of proteins results in similar changes but, presumably due to reduced accessibility, the antibody binds primarily at the periphery of the bands. The denaturation occurring in this temperature range appears to be fully reversible since there is no increase in the metachromatic staining of the chromosomes by acridine orange (indicative of single-stranded DNA) after the treatment. On the other hand, conditions leading to an irreversible denaturation, e.g. exposure to 50–60% dimethylsulfoxide (DMSO) at 37°C for 10 min, do not result in an increased staining with anti-Z DNA antibodies. Incubation in 60, 65, or 70% ethanol in the presence of 10 mM $MgCl_2$ and 0.1 M NaCl also does not lead to increased antibody binding.

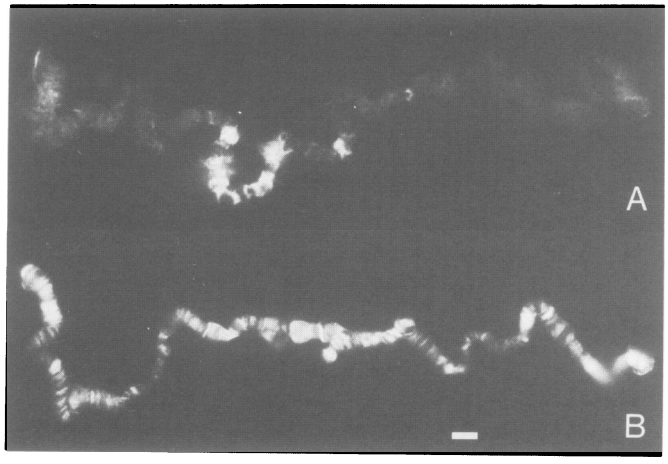

Fig. 7. Effect of DNA relaxation by *Drosophila* topoisomerase II on the anti-Z DNA immunofluorescence of isolated *Chironomus* polytene chromosomes. Chromosomes were depleted of proteins in 0.6 M NaCl, 50 mM Na citrate, pH 4.3, treated at pH 2.2 in 20 mM glycine-HCl, 80 mM NaCl and incubated **(A)** with and **(B)** without enzyme in digestion buffer at 30°C for 4 h (see Materials and methods). Staining performed at neutral pH with anti-Z DNA IgG, T4, 10 μg/ml followed by Fl-GARIG. Bar denotes 10 μm.

Discussion

Experiments using antibodies to search for left-handed helices in natural DNA have been carried out almost exclusively with extracted DNA or fixed cytological preparations. The studies reported to date are summarized in Table I. In both cases, the procedures used are likely to affect the conformational state of the DNA. Thus, positive results do not necessarily imply the existence of Z DNA *in vivo*. In this and earlier studies (Robert-Nicoud *et al.*, 1982; Jovin *et al.*, 1983c) we have used isolated unfixed polytene chromosomes from *C. thummi* and Z DNA-specific antibodies to search for the presence of left-handed DNA tracts under conditions approaching the physiological as closely as possible, and to study the effects of ionic strength, pH, temperature and enzymes known to affect the topological state of the DNA. We have specifically investigated the problems derived from the use of acids in extraction and fixation procedures, i.e., their effects on the B-Z equilibrium of polynucleotides in solution and their consequences on the state of chromatin and on the subsequent binding of specific antibodies to chromosomes.

At physiological ionic strength and pH and using high concentrations of IgG, only a low level of anti-Z DNA antibody binding can be visualized by immunofluorescence at the periphery of the chromosomes. The similar distribution of anti-histone H3 and anti-histone H1 antibodies indicates that the accessibility of determinants can be very limited under these conditions. This may be attributed to the fact that immunoglobins are unable to penetrate the chromosome substructures, which are highly condensed, and/or specific ligands physically mask determinants.

At 0.35–0.6 M NaCl, dissociation of high mobility group non-histone proteins (HMG proteins; Nicolas and Goodwin, 1982; Bosshard, 1979) and of histone H1 (Bolund and Johns, 1973; Kühne, 1977) occurs causing unfolding of the nucleosome filament. Under these ionic conditions, a partial and differential decondensation of the chromosomal bands is observed by light microscopy (Robert, 1971; Lezzi and Robert, 1972). Core histone determinants are made available for antibody binding as shown by the large increase in the amount of anti-histone H3 antibodies bound and the overall distribution of the fluorescence. In contrast, no perceptible increase in the fluorescence is observed with anti-Z DNA antibodies; in this case, the staining appears to be restricted to regions which have not undergone decondensation. These observations may be interpreted as follows: (i) Z DNA is absent from most chromosomal regions or present at low density only in very restricted sites; (ii) the decondensation of the chromosomes results in a dilution of the Z sequences which can no longer be detected by immunofluorescence; (iii) a conformational change of the DNA accompanies the decondensation process; and (iv) Z DNA helices are present but determinants are still masked by specific ligands.

At higher ionic strength (0.6–1.2 M) anti-Z DNA antibody binding can no longer be detected by immunofluorescence. This is not due to a direct effect of the ionic strength on the binding itself since the same observation was made for chromosomes treated at high ionic strength and returned to physiological ionic conditions. (The return to lower salt con-

Table I. Anti-Z DNA antibody binding to natural DNAs

Extracted closed circular DNAs		Fixed cytological preparations			Unfixed chromosomes
Phage and Virus	Plasmid[1]	Haploid and Diploid		Polyploid and Polytene[2]	
		Interphase	Metaphase		
M13(RF)[27]	pBR322[6,27]	rat[15]	*Gerbillus*[25]	*Stylonichia*[14]	*Chironomus* SG[9,21,22]
ϕX174(RF)[27,28]	pColE1[28]	human[5]	human[5,26]	*Drosophila* SG[4,16,18]	*Drosophila* SG[8]
PM2[23,27]	*E. coli*/pUC-8[24]	simian[5]	*Cebus*[26]	*Glyptotendipes* SG[11]	SV40 minichromosome[28]
SV40[17,27,28]	Form V[3,12,19]	bovine[5]	bovine[5]	*Chironomus* SG[4,9–11,13,21,22,27]	
		plant[20]	hamster[5]		
		Chironomus[5]	*Chironomus*[5]		

[1]Excluding plasmids with $d(G\text{-}C)_n$ or $d(A\text{-}C)_n$ inserts. [2]SG = salivary gland polytene chromosomes. [3]Form V DNA constructs of pBRβG2.17 or pBR322. [4]Arndt-Jovin *et al.*, 1983; [5]Arndt-Jovin *et al.*, in preparation; [6]Azorin *et al.*, 1983; [7]DiCapua *et al.*, 1983; [8]Hill and Stollar, 1983; [9]Jovin *et al.*, 1983c; [10]Jovin *et al.*, 1983b; [11]Jovin *et al.*, 1983a; [12]Lang *et al.*, 1982; [13]Lemeunier *et al.*, 1982; [14]Lipps *et al.*, 1983; [15]Morgenegg *et al.*, 1983; [16]Nordheim *et al.*, 1981; [17]Nordheim and Rich, 1983a; [18]Pardue *et al.*, 1983; [19]Pohl *et al.*, 1982; [20]Rich, 1983; [21]Robert-Nicoud *et al.*, 1983; [22]This paper; [23]Stockton *et al.*, 1983; [24]Thomae *et al.*, 1983; [25]Viegas-Péquignot *et al.*, 1982; [26]Viegas-Péquignot *et al.*, 1983; [27]Zarling *et al.*, 1984a; [28]Zarling *et al.*, 1984b.

centrations, however, does not result in a complete recondensation of the chromosomes.) If Z DNA tracts exist *in vivo*, one possible explanation for the absence of immunofluorescence would be that Z DNA binding proteins dissociate at these salt concentrations, thereby causing a destabilization of the left-handed conformation. In fact, Z DNA binding proteins isolated from *Drosophila* cells are eluted from left-handed br-poly[d(G-C)] affinity columns above 0.6 M NaCl (Nordheim *et al.*, 1982b).

In addition to salt-mediated changes in DNA-protein interactions, direct effects of ions on the conformation and topological state of the chromosomal DNA should also be taken into consideration. Indeed, both promoting and inhibiting effects of mono- and divalent cations on the B-Z interconversion in free polynucleotides and in closed circular DNA with inserted blocks of alternating purine-pyrimidine sequences have been shown to occur at physiological as well as at high ionic strength (Singleton *et al.*, 1982; Stirdivant *et al.*, 1982; Klysik *et al.*, 1983; Azorin *et al.*, 1983; Pohl and Jovin, 1972; Zacharias *et al.*, 1982; Zarling *et al.*, 1984b). The ionic requirements for the conformational transition depend in a complex fashion on the degree of negative supercoiling, base modifications, the nature of the cations and the presence or absence of flanking B regions. The mechanism of action of ions is likely to involve the formation of specific coordination complexes, ionic strength and dehydration effects, as well as changes in the free energy of supercoiling. Clearly, understanding these effects in chromatin will require the investigation of model systems comprised of closed circular DNAs with specific Z tracts reconstituted *in vitro* with chromosomal proteins.

The most dramatic changes in the immunofluorescence staining of isolated chromosome with anti-Z DNA antibodies are observed after exposure to low pH. The effects, which occur in a narrow pH range (2.5 – 2.2) and are dependent upon time of incubation, temperature and salt concentration, are likely to involve both changes in the accessibility of determinants in condensed chromosomal substructures, as well as a facilitation of the B→Z transition.

Changes in accessibility are likely to be due to depletion of proteins. At pH 3.0 – 2.3, non-histone proteins and histone H1 are released from isolated chromatin and nuclei while dissociation of core histones occurs below pH 2.0 (Murray, 1966; Lawson and Cole, 1979). Our observations with isolated polytene chromosomes show that histone H1 is lost from most of the bands between pH 2.5 and 2.3. In contrast to the situation at elevated ionic strength, the acid treatment does not result in a decondensation of the chromosomes (see Robert, 1971), but clearly increases the accessibility of core histone determinants which are detectable by immunofluorescence down to pH 2.0. Below this value, the fluorescence is lost due to extraction of the proteins.

It is not known whether Z DNA binding proteins dissociate at low pH; however, this would seem likely. Assuming this is the case, we would postulate that, in the absence of masking proteins, pre-existing Z sequences which are stabilized at low pH (see below) will be unmasked and will show an increased tendency to interact with neighboring regions on the same or on adjoining chromatids so as to form cross-links and networks (Z*-associated form; see van der Sande and Jovin, 1982; Jovin *et al.*, 1983c).

Two processes could serve to trigger the formation of new Z DNA sites at low pH: (i) protonation of bases followed by local and transient strand separation is likely to facilitate rotation of the base pairs, a prerequisite for the B→Z transition (Olson *et al.*, 1983; Harvey, 1983); (ii) dissociation of chromosomal non-histone and histone proteins is expected to leave the DNA in constrained domains of the chromosomes under topological stress (Benyajati and Worcel, 1976; Igo-Kemenes and Zachau, 1978; Germond *et al.*, 1975; Javaherian *et al.*, 1978), thus facilitating the formation of the left-handed conformation. The results of our enzymatic digestion with topoisomerases confirm that in acid-treated chromosomes antibody binding occurs primarily to topologically stressed DNA, as has been demonstrated for various circular DNAs *in vitro*. The fact that S1 nuclease reduces the staining only if applied after, but not before, the pH treatment implies that new B-Z junctions have been formed.

In parallel solution studies using the linear alternating copolymer poly[d(G-C)], we find that acidic conditions favour the formation of the left-handed conformation. Both the salt-mediated equilibrium state and the transition kinetics are affected. Thus, at pH 3 and in 2 M NaCl, the Z state is formed in a few minutes at 22°C whereas at neutral pH, this salt condition is insufficient for forming an appreciable fraction of the left-handed form (the transition midpoint is 2.3 M NaCl). Furthermore, even in 3 M NaCl, the transition half-life at neutral pH is ~10 min compared with <1 min at pH 3. It appears likely that the acid-mediated potentiation of the Z form proceeds via protonation of the N7 position of guanine. The spectral, equilibrium and kinetic properties of the putative acidic Z form of poly[d(G-C)] described here (Figure

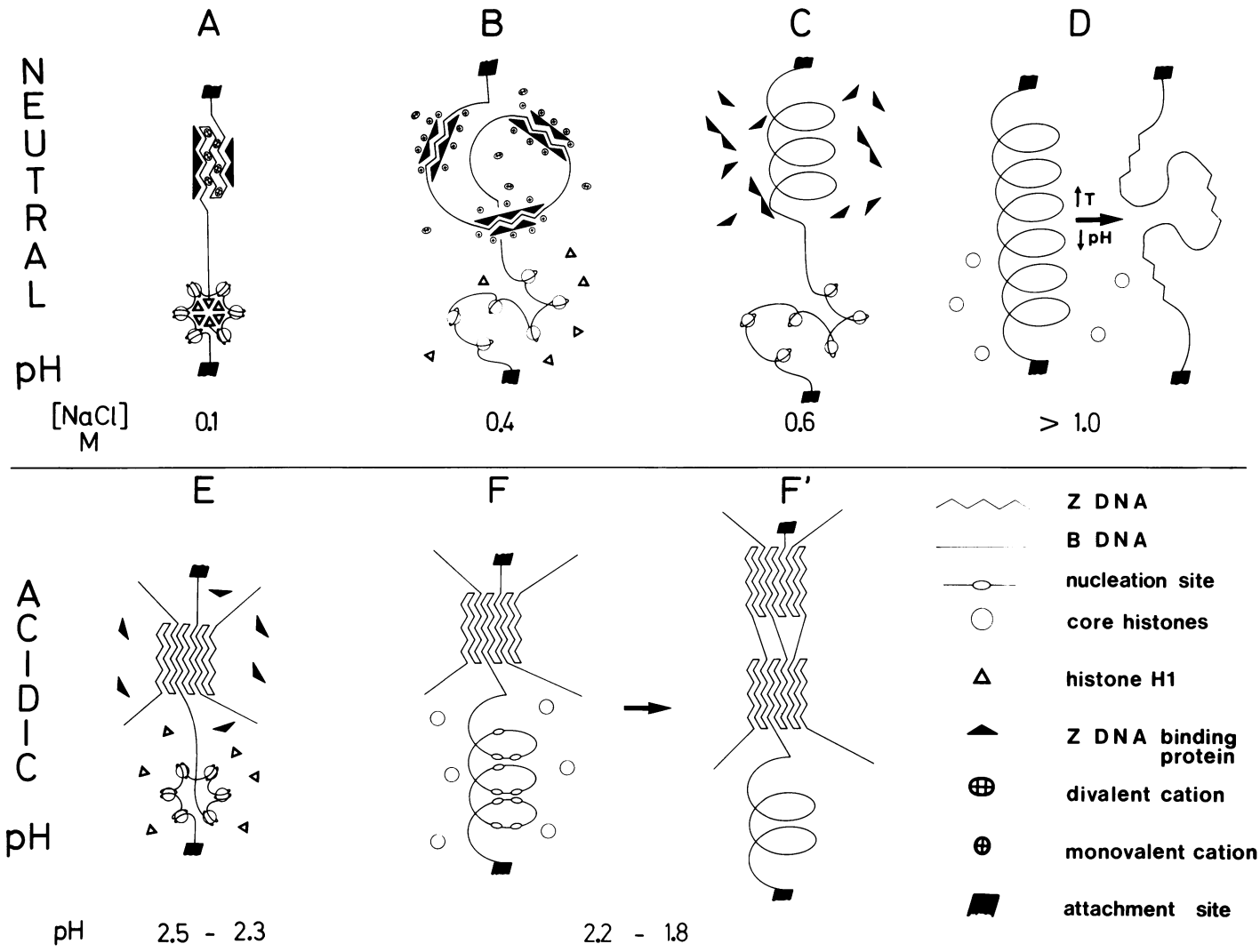

Fig. 8. Schematic representation of changes induced in the structural state and interactions of chromosomal components by variations in ionic strength and pH. In **(A)**, two regions of a topologically constrained domain of chromosomal DNA are represented: one with DNA in the B conformation organized into nucleosomes and condensed by histone H1, the other with DNA in the Z conformation stabilized by Z DNA binding proteins and forming Z*-associated structures. **(B–F')** show possible changes occurring in the DNA-protein interactions, in the topological state and conformation of the DNA, as a consequence of variations of ionic strength **(B–D)** and pH **(E–F')**, and the resulting structural modifications. In **(B)**, the dissociation of histone H1 causes a decondensation of the B region; displacement of divalent cations by monovalent cations destabilizes the Z* aggregates; Z DNA binding proteins remain bound and could mask antibody binding sites. In **(C)**, the release of Z DNA binding proteins destabilizes the left-handed conformation; due to the Z–B transition, the DNA is left in a topologically stressed state. In **(D)**, the release of core histones increases the degree of negative supercoiling; the B–Z transition may occur but in the absence of specific ligands the transient Z conformation will not be stabilized; Z formation can be induced by exposure to high temperatures or low pH. In **(E)**, histone H1 and Z DNA binding proteins are released; the Z conformation is stable at this low pH in the absence of stabilizing proteins; the formation of Z* networks is promoted. In **(F,F')**, core histones are released; supercoiling and local strand separation due to protonation of bases trigger the formation of new Z DNA sites. This schematic representation does not attempt to show the actual localization nor the relative size of the single chromosomal components.

6) closely resemble those of the chemically methylated polymer (Möller *et al.*, 1981), although we do not observe the large reduction in c.d. intensities reported to occur upon increasing degrees of methylation. The right-handed spectra are in agreement with those previously reported during the acid titration of poly[d(G-C)] (Narasimhan and Bryan, 1975). Protonation at N7 of guanine favors the assumption of the *syn* conformation about the glycosidic bond (Son *et al.*, 1972), and lowers the interstand repulsive interactions between phosphate groups. Both effects would be expected to potentiate the B–Z transition. Our spectral data indicate that the conformational states in the acid range are mixed, presumably reflecting the statistical distribution and/or clustering of protonation sites.

Protonation of A-T base pairs and consequent loss of hydrogen bonding appears to precede the disruption of G-C base pairs during the acid titration and ultimate denaturation of DNA (Hermann and Fredericq, 1977). Therefore, alternating $d(C\text{-}A)_n$ sequences are likely to undergo strand separation at slightly higher pH than $d(G\text{-}C)_n$ sequences. This is consistent with our observation that the changes in stainability seen with anti-Z DNA antibodies recognizing only sequences in the $d(G\text{-}C)_n$ family occurs at lower pH than with antibodies which also recognize $d(A\text{-}C)_n \cdot d(G\text{-}T)_n$ sequences.

Since we were unable to generate Z DNA staining regions in the chromosomes by stripping of proteins including core histones, we surmise that base protonation followed by local denaturation causes nucleation foci for a facilitated transition to the Z state. Further support for the importance of local denaturation in the triggering of the conformational transi-

tion is provided by the results of our studies at high temperature and low ionic strength which generate immunofluorescence staining similar to that induced by low pH treatment. It is possible that the 'protonation pathway' described above is exploited at neutral pH *in vivo* by proteins acting catalytically, i.e., in the form of 'DNA conformases' (Jovin *et al.*, 1983a).

It follows from the above that exposure of chromosomes to pH 1.8 should result in the expression of the majority of sequences with Z-forming potential. The frequency of Z DNA sequences in acid-fixed polytene chromosome preparations of *Chironomus* has been estimated to be 0.02–0.1% by anti-Z DNA antibody titration and quantitative laser microphotometry (Arndt-Jovin *et al.*, 1983). Some chromosomal bands appear to be particularly rich in sequences with Z-forming potential. Telomeres of *Chironomus* polytene chromosomes stain strongly with Z DNA-specific and sequence-independent antibodies but not with the monoclonal D11 which does not recognize left-handed $d(A\text{-}C)_n$ sequences (Jovin *et al.*, 1983c; Zarling *et al.*, 1984b). Since these regions can be detected even after treatment at pH 2.45 (Figure 3) they may constitute unique structural elements. In fact, eukaryotic telomeres contain repeating tracts of alternating $d(A\text{-}C)_n$ (Walmsley *et al.*, 1983) the function of which is not understood. Other bands which stain preferentially after pH 2.45 treatment are located in the vicinity of the centromeres and correspond to those designated as constitutive heterochromatin by Hägele (1977) on the basis of their selective staining with two Giemsa banding methods (C and RB bandings). The high degree of compaction of heterochromatin and its particular higher order structure stabilized by divalent cations (Weith, 1983) may be the consequence of an increased number of Z potential sequences.

During the preparation of this manuscript, a report on the effects of acetic acid on Z DNA antibody binding to isolated *Drosophila* polytene chromosomes was published by Hill and Stollar (1983). Their conclusions are in general agreement with ours; however, we would like to stress several points. Their Figure 1C does not provide convincing evidence for a localization of immunofluorescence in interbands and puffs. We find that short treatments at acid pH result in a selective staining of a particular set of bands (see above), at least in the case of *Chironomus*. Furthermore, their claim that DNA is freely accessible to antibodies in isolated chromosomes at physiological pH and ionic strength based on studies with an anti-B DNA antibody is also not supported by the published pictures (Figure 2, Hill and Stollar, 1983). This antibody appears to bind to puffs and interband regions more intensely, not to the condensed, DNA-richer chromosomal regions. Moreover, the immunofluorescence was drastically reduced after treatment of the chromosomes in 45% acetic acid for 30 s which indicates that the binding may have been to complexes of DNA with chromosomal components which are extracted at low pH.

The low frequency of potential Z DNA tracts in natural DNA (<1%, see above) has precluded direct detection by physicochemical means. Immunological detection offers the advantage of high specificity and amplification, but does not permit a quantitative assessment of the frequency and occurrence of Z DNA under physiological conditions due to the very nature of chromosomes themselves. However, with the combination of specific antibodies and unfixed chromosomes, we have been able to determine the nature of the factors which could trigger and/or stabilize Z formation *in vivo*.

In Figure 8, we present a model describing molecular changes which may occur in hypothetical B and Z regions of a chromosome domain as a consequence of the variation of the ionic and pH conditions and which are consistent with our experimental results on immunological detection of Z DNA. The model emphasizes the possible structural role of Z DNA tracts in chromosomes. The formation of Z* associates and networks may mediate the interaction between homologous chromomeres and chromatids in polytene chromosomes, the association between non-homologous chromosomes, the compaction of heterochromatic regions, and the condensation of metaphase chromosomes (see also Jovin *et al.*, 1983a). In addition, however, conformational changes occurring at specific sites may provide a way to regulate the activity of specific genes and the B-Z transition would be expected to be exploited *in vivo* by proteins acting catalytically.

The extent to which potential Z DNA tracts express the Z conformation under physiological conditions in native chromatin will be directly related to the interplay between Z stabilizing and destabilizing factors. These appear to involve on the one hand: (i) torsional stress induced by local dissociation of chromosomal proteins and, possibly, by gyrase-type enzymes: (ii) specific Z DNA binding proteins which would trap transient Z structures; (iii) higher order Z DNA:Z DNA (Z*) interactions; and (iv) enzymes (conformases, helicases) and/or single-strand binding proteins capable of local strand separation which would potentiate Z DNA formation. Destabilizing factors, on the other hand, would include the action of cellular topoisomerases, relaxation of negative supercoiling by nucleosome formation, and proteins or ligands specific for B DNA. Reconstitution of partially stripped chromosomes with cellular components stabilizing Z DNA should permit the experimental assessment of some of these conclusions.

Materials and methods

Isolation of polytene chromosomes by micromanipulation

Unfixed polytene chromosomes were isolated from explanted salivary glands of 4th instar larvae of *C. thummi* according to the technique of Robert (1975). This procedure consists of the following steps: (i) short incubation of explanted salivary glands in physiological saline ($CR_{6.3}$-medium: 87 mM NaCl, 3.2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Tris-maleate pH 6.3) containing non-ionic detergents (in this study we have used a mixture of 0.1% Nonidet P-40 and 0.05% Triton X-100 instead of digitonin); (ii) homogenization of the salivary glands with micropipettes and collection of the nuclei; (iii) washing the nuclei in the same solution buffered at pH 7.3 with 10 mM Tris-HCl ($CR_{7.3}$-medium) without detergents; and (iv) breaking the nuclear membrane through homogenization with micropipettes and collection of free chromosomes. For further treatment, the isolated chromosomes were positioned onto the surface of a cover glass to which they readily adhere. The cover glass was then inverted onto a slide with two parallel strips of double-sided tape forming a channel through which the solution can be exchanged.

Treatment of isolated chromosomes

Changes in ionic strength were accomplished by washing the chromosomes with the appropriate buffer for 20 min at room temperature and monitored by observation of the chromosomes with phase microscopy. pH treatments were carried out by washing the preparations for 10 min at room temperature in 20 mM glycine-HCl, 80 mM NaCl buffers of the appropriate pH unless otherwise noted. Chromosomes were then returned to neutral pH buffer and washed for 20 min before other manipulations. The restoration of neutral pH is immediate, as monitored by the effluent pH, except after treatment with 45% acetic acid in which case 1–2 min of washing with saline is required to restore neutrality. The removal of chromosomal proteins including core histones was achieved by washing the chromosomes in 0.6 M NaCl, 50 mM Na citrate, pH 4.3 for 30–60 min at room temperature. The dramatic decondensation of the chromosomal bands can be monitored by light microscopy. Restoration of neutral pH in 100 mM NaCl does not result in a recondensation of these regions. Temperature treatment was accomplished by pre-equilibration of the preparations in 10 mM NaCl, 10 mM Tris-HCl,

pH 8, 1 mM EGTA for 30 min at room temperature followed by immersion in the same buffer at the elevated temperature for 5 min and return to physiological salt solutions at room temperature.

Enzymatic treatment

The chromosomes were digested with nuclease S1 from *Aspergillus cryzae* (PL Biochemicals) in 75 mM NaCl, 30 mM Na acetate, pH 4.7 and 0.5 mM $ZnCl_2$ for 1 h at 37°C. Control preparations were taken through all of the same manipulations without addition of enzyme. Reaction volumes were 70 μl and as little as 58 U/ml was sufficient to abolish almost completely the immunofluorescence from anti-Z DNA antibody staining of chromosomes which had been stripped of core histones and exposed to pH 2.2. Most reactions were carried out at 580 U/ml. Chromosomes remained intact by visual inspection after digestion with as much as 5800 U/ml S1 nuclease. DNA relaxing enzyme topoisomerase I from calf thymus was obtained from BRL. *D. melanogaster* DNA topoisomerase II, an enzyme which relaxes both positive and negative supercoils in the presence of ATP (Hsieh, 1983; Sander and Hsieh, 1983) was the kind gift of T.-S.Hsieh. Chromosome preparations with and without prior removal of histones (see above) were subjected to pH 2.2 for 10 min, reneutralized and then treated with the enzymes. Reactions with topoisomerase I were carried out at 37°C for 90 min – 3 h in 50 mM NaCl, 50 mM Tris-HCl, pH 8. 0.5 mM dithiothreitol (DTT) and 30 μg/ml bovine serum albumin (BSA) with 70 μl reaction volumes and an enzyme concentration of 1.5 U/μl. Assay conditions with topoisomerase II were: 10 mM Tris-HCl, pH 7.9, 50 mM KCl, 100 mM NaCl, 10 mM $MgCl_2$, 1.25 mM ATP and 50 μg/ml BSA at 30°C for 5 h, 2000 U/ml, reaction volum 50 μl. Control preparations were carried through all manipulations without addition of enzymes.

Antibodies and immunofluorescence staining

All staining of the isolated chromosomes unless otherwise stated was carried out in $CR_{7.3}$-medium containing 1 mg/ml BSA at 37°C for 30 – 60 min. A sequence-independent Z DNA-specific polyclonal antibody (T4) was used at 10 μg/ml with indirect immunofluorescence visualization by an IgG fraction of fluoresceinated goat anti-rabbit IgG (GARIG) obtained from TAGO. For most of the conditions tested, directly fluoresceinated T4 at 20 μg/ml was used in parallel experiments to test if penetration of the second antibody was a limiting factor. A monoclonal antibody (D11; the kind gift of R.Thomae) which recognizes only $d(G\text{-}C)_n$ sequences in their Z conformation was used at 4 μg/ml followed by Texas Red-conjugated sheep anti-mouse $F(ab')_2$ (New England Nuclear). A polyclonal anti-histone H3 antibody (the kind gift of M.Bustin) and a monoclonal antibody raised against *Chironomus* histone H1 (the kind gift of E.Mohr) were used to assess the accessibility of determinants under the various experimental conditions in this study, and to test the effects of low pH on the release of chromosomal proteins. Second antibodies were the same as above.

Under all the conditions tested in this study, the immunofluorescence was abolished by competition of the anti-Z DNA antibody with polynucleotides in the Z conformation or by omission of first antibody, thus demonstrating the specificity of the staining.

Spectroscopic measurements of poly[d(G-C)] at acid pH

Poly[d(G-C)] was synthesized with *Escherichia coli* DNA polymerase I. U.v. absorption and c.d. measurements were carried out in 10 mm pathlength cells using a Kontron Uvikon 820 spectrophotometer and a Jobin-Yvon Mark IV Dichrograph. Conditions are given in Figure 6.

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