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Z-DNA in transcriptionally active chromosomes

(polytene chromosomes)

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ABSTRACT **Due to the striking correlation between the distribution of transcriptionally active subdivisions of the polytene chromosomes and Z-DNA, we have addressed the question of whether the Z-DNA configuration exists in native, transcriptionally active chromosomes of *Drosophila hydei* prepared without interference by procedures known to induce the B to Z conformation. Our experiments indicate that Z-DNA forms are present in a specific set of sites on the native chromosomes. They occur on interbands and other subdivisions of dispersed DNA, but there is no correlation between the amount of Z-DNA detected and DNA compaction. The results suggest, moreover, that Z-DNA forms are restricted to specific genes, because various subdivisions induced to transcription in puffs show different patterns of Z-DNA. We show, in addition, that removal of chromosomal proteins by proteinase K has a strong influence on the level of anti-Z-DNA reactivity.**

In recent years the concept that DNA is not a static molecule but is a dynamic structure with considerable conformational flexibility represents a significant development in the biochemistry of DNA. In fact, soon after the formulation of the B-DNA model there was some discussion about the possible existence of alternative conformational variants. This discussion, never reflected in systematic studies, was stimulated by the unexpected discovery of a left-handed structure in a crystal of an alternative hexanucleotide (dC-dG)$_3$ (1, 2). The discovery initiated an intensive search for the possible biological significance of this conformation, named Z-DNA. The observations (*i*) that the transition from B to Z may occur in conditions related to hydration of the molecule (3–5) or to the type and number of the associated cations, (*ii*) that it is not restricted to poly(dC-dG)$_3$ (6–11), and (*iii*) the possibility that *in vivo* Z-DNA can be stabilized by negative supercoiling (7, 12–15) and specific binding proteins (16), indicated that the Z-DNA forms might be present in chromosomal structures. The most stimulating discovery was that potential Z-DNA sites are present in transcriptional enhancers (8) and structural genes (17, 18) and also function as a negative regulatory transcription signal (19, 20). Rich *et al.* (16) postulated, therefore, that the information content of the Z-DNA forms may be associated with specific locations in the chromosome relative to the structural genes or with their sequence composition. What the role of these DNA forms is and whether they exist and function *in vivo* is still unclear. The existence of specific Z-DNA binding proteins in the nuclei of *Drosophila melanogaster* and in minichromosomes of virus SV40 as well as in wheat germ (21) seems to indicate, however, that this DNA conformation does have a function in biological systems.

The availability of antibodies that react specifically with Z-DNA has stimulated the search for Z-DNA in chromosomal structures. It was found in the polytene chromosomes of *Drosophila* (22–25) and *Chironomus* (26, 27), in the interphase nuclei of *Stylonichia mytilus* (28), and in metaphase chromosomes of primates (29). The fact, however, that the solvents used during the manual preparative procedures (ethanol and acetic acid) induce different and reproducible patterns of Z-DNA (25, 26, 30) has raised some doubts about its existence in the native state. Hill and Stollar (30) and Robert-Nicoud *et al.* (26) reported that they could not detect Z-DNA in unfixed polytene chromosomes isolated by micromanipulation, and Gross *et al.* (31) indicated that specific Z-DNA conformations formed by (dT-dG)$_n$(dC-dA)$_n$ sequences do not significantly exist in isolated nuclei from exponentially growing mouse mastocytoma cells.

In this paper we have addressed the question of whether the Z-DNA configuration exists in native transcriptionally active chromosomes of *Drosophila hydei* prepared without interference by procedures known to induce the B to Z transformation. Our experiments indicate that in native conditions Z-DNA forms are detected in a specific set of sites on the chromosomes, that they occur on interbands and other subdivisions of dispersed DNA in a pattern similar to that found in acetic acid-squashed chromosomes, but also indicate that there is no correlation between the amount of Z-DNA detected and DNA compaction. The results suggest, moreover, that the cytological correlation observed between the Z-DNA configuration and active transcription in regions of dispersed chromatin is restricted to specific genes, because various subdivisions of the chromosomes induced to high transcription rates in puffs show different patterns of Z-DNA reactivity.

MATERIALS AND METHODS

Isolation of Nuclei. Forty pairs of salivary glands from *D. hydei* larvae were homogenized in 40 μl of 20 mM Tris·HCl, pH 7/80 mM KCl/4 mM $MgCl_2$ buffer by agitating on a mixer for 30 sec in a 1.5-ml Eppendorf tube containing a loosely fitting glass bead. After the homogenization, 160 μl of fresh medium was added, and the nuclei were filtered through nylon cloth (80-μm pore size). The filtrate was centrifuged at 20°C for 3 min at 500 rpm (MSE table centrifuge) to separate the nuclei. In some experiments this homogenization period was extended to 90 sec to break nuclear membranes.

Immunofluorescence Reaction of Z-DNA. Isolated nuclei in 40 μl of homogenization buffer were incubated for 15 min with goat anti-Z-DNA IgG at a dilution of 1:20 in phosphate-buffered saline (PBS; 0.01 M phosphate, pH 7.2/0.15 M NaCl). The IgG stock solution had a protein concentration of 200 μg per ml. After the incubation, the nuclear suspension was centrifuged for 2 min at 500 rpm, the supernatant was removed, the nuclei were washed twice with PBS and pelleted again at 500 rpm. Then nuclei were resuspended in 40 μl of PBS. Nuclear aliquots (≈100 nuclei) were placed on glass slides, fixed with ethanol/formaldehyde [100% EtOH/37% formaldehyde (vol/vol) 9:1] for 30 sec and with 50%

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Abbreviations: NHP, nonhistone proteins; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate.

acetic acid for 1–2 min. The chromosomes were squashed in the acetic acid solution, and the cover slip was snapped off after freezing the slides in liquid nitrogen. The chromosomes were immersed in 100% EtOH for 30 min, rehydrated through a series (from higher to lower) of ethanol/PBS concentrations, and incubated for 1 hr with fluorescein isothiocyanate (FITC)-labeled rabbit anti-goat IgG (Miles) at a dilution of 1:40. The fluorescence was visualized in a Zeiss epi-illuminated microscope. The fluorescent chromosomes were first photographed with a Zeiss oil immersion lens (40×), and then, after having been washed with tap water and stained with lactoacetic-orcein (Bachem Fine Chemicals, Torrance, CA), they were again photographed using the same lens. A Zeiss (model 03) scanning cytophotometer was used to locate on the negatives the exact position of the fluorescence with respect to DNA on the chromosomes. Control experiments were done by mock incubation of the isolated nuclei with nonimmune serum.

Immunofluorescence Visualization of Nonhistone Proteins (NHP) and Endogenous Chromosomal DNA·RNA Hybrids That Have Incorporated ^{3}H-Labeled UTP. The molecular integrity of the chromosomes with respect to NHP was studied using anti-NHP antibodies. For this test isolated nuclei, taken after incubation with anti-Z-DNA were washed with PBS, fixed, and applied to a slide by precipitation with ethanol/formaldehyde for 30 sec. Afterwards, the chromosomes were squashed in 50% acetic acid, postfixed in ethanol/formaldehyde for 15 min and rehydrated in PBS. The chromosomes were then incubated for 30 min with rabbit anti-NHP and anti-rabbit FITC-labeled antibodies. Chromosomal function was tested by analyzing for the presence of nascent RNA molecules able to form hybrids with their templates (38). For this test isolated nuclei, taken at the end of the period of incubation with the anti-Z-DNA antibody were attached to a slide by precipitation with 100% ethanol, postfixed with 50% acetic acid for 1 min, and squashed in the same solution. After dehydration in 100% ethanol for 4 hr the chromosomes were incubated with anti-DNA·RNA antibodies as described by Alcover and coworkers (32, 33). RNA synthesis was measured by determining the amount of trichloroacetic acid-precipitable radioactivity incorporated by isolated nuclei that were incubated in 250 μl of the buffer 8 mM KCl/20 mM NaCl/4 mM $MgCl_2$/16 mM Pipes, pH 7.5, supplemented with 4 mM CTP, GTP, and ATP and 15 μCi (1 Ci = 37 GBq) of ^{3}H-labeled UTP (45 Ci/mmol; Amersham). At different intervals the RNA from 50 μl of the nuclear suspension (≈2500 nuclei) was precipitated with 10 μl of 50% trichloroacetic acid containing 5 μg of bovine serum albumin.

Z-DNA Immunofluorescence in Nondenatured Chromosomes: Effect of Proteinase K Digestion on Z-DNA Formation. The influence of DNA denaturation and protein extraction caused by the acid-fixing procedures on Z-DNA reactivity was studied by comparing the anti-Z-DNA binding on chromosomes from isolated nuclei that had been prefixed either in PBS/3% formaldehyde or ethanol/formaldehyde for 2 min before the acetic acid squash with that of chromosomes fixed and squashed in acetic acid. The effect of protein extraction on Z-DNA induction was studied by proteinase K treatment of acetic acid-squashed chromosomes and of chromosomes prefixed in PBS/formaldehyde. The concentration of the enzyme was 5 μg or 20 μg per ml in 2× SSC (1× SSC = 0.15 M NaCl/0.015 sodium citrate, pH 7). Incubation time was 5 min at 25°C. The enzyme solution was self-digested for 30 min at 37°C and had no DNase activity.

RESULTS

As we have previously reported, specific chromosomal subdivisions cytologically identified as interbands in which the DNA is dispersed, from nuclei frozen and dihydrated in 100% ethanol, show anti-Z-DNA reactivity (25). Even though the chromosomes in these experiments had not been submitted to denaturation or protein removal, it was difficult to ascertain whether the pattern observed could be attributed to the *native* conformation of the DNA or to *induced* Z-DNA. To determine whether Z-DNA forms exist in nondenatured squashed chromosomes, we applied the anti-Z-DNA antibody to chromosomes under conditions that prevented DNA denaturation and protein extraction. For this purpose we used acetic acid-squashed chromosomes from isolated nuclei that had been prefixed for 2 min in either PBS/formaldehyde or ethanol/formaldehyde, because formaldehyde fixation prevents chromosomal denaturation and protein extraction by acid (34, 35).

Fig. 1*C* shows that after prefixation in PBS/formaldehyde, anti-Z-DNA, fluorescence is detected preferentially over subdivisions of dispersed DNA—with a distribution similar to that observed without prefixation (Fig. 1*A*). After prefixation in ethanol/formaldehyde the same pattern was observed, but the intensity of the fluorescence was higher (Fig. 1*D*). When the isolated nuclei were squashed in acetic acid without formaldehyde prefixation, the fluorescence was very intense over all the chromosomal structure (Fig. 1*B*). Because (*i*) acetic acid does not denature the chromosomal DNA in chromosomes prefixed in PBS/formaldehyde, (*ii*) there was not removal of chromosomal proteins in chromosomes prefixed in ethanol/formaldehyde, and (*iii*) the cytological distribution of anti-Z-DNA reactivity observed in all cases (Fig. 1*A*, *C*, and *D*) is similar, we think that the pattern of Z-DNA detected in acid-squashed chromosomes or in formaldehyde-fixed chromosomes, if induced, is not the result of the release of DNA torsional strain due to DNA denaturation.

To avoid the interference of solvents that may influence the B to Z transition we have incubated isolated nuclei with goat

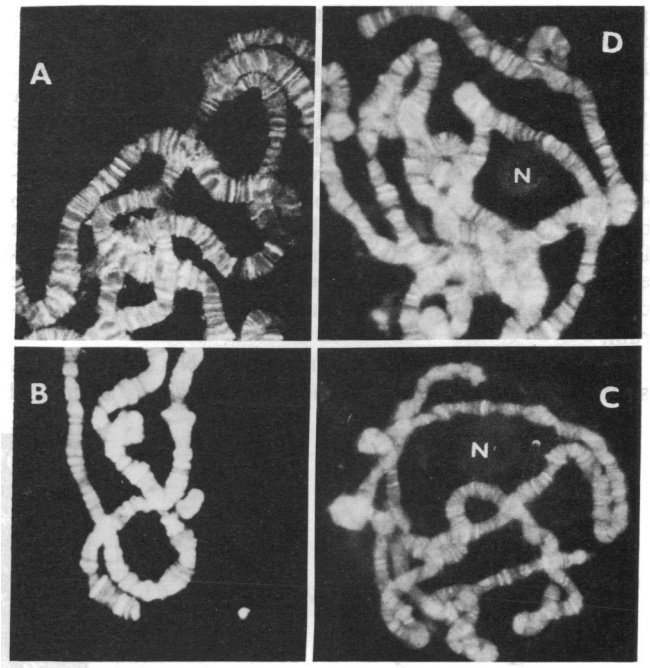

FIG. 1. Z-DNA reactivity of polytene chromosomes of *D. hydei*. (*A*) Chromosomes in glands were fixed in 50% acetic acid and squashed in the same solution; (*B*) chromosomes in isolated nuclei were fixed and squashed in 50% acetic acid; (*C*) chromosomes in isolated nuclei were fixed in 0.01 M PBS formaldehyde and squashed in 50% acetic acid; (*D*) chromosomes in isolated nuclei were fixed in ethanol/formaldehyde and squashed in 50% acetic acid. N, nucleolus. In all cases rehydrated squashed chromosomes were incubated for 1 hr with goat anti-Z-DNA followed by incubation with FITC-labeled rabbit anti-goat IgG.

anti-Z-DNA antibody before any prefixation procedure. We assumed that once the antibody binding took place the nuclei could be fixed in ethanol/formaldehyde before squashing in 50% acetic acid. The advantage of this procedure is two-fold: on the one hand, intact chromosomes could be in contact with the antibody during the period in which they were considered to be in native condition, and, on the other hand, the formaldehyde would help stabilize binding of the anti-Z-DNA antibody to the Z-DNA segments during the second period of incubation with the second antibody. Any Z-DNA segments induced by the ethanol during the fixation step of the nuclei would not be visualized because binding with the anti-Z-DNA antibody was already completed. As a control we used goat nonimmune serum.

To ensure that chromosomes were in native state during the period of incubation with the antibody we tested their ability to incorporate ^3H, and we also checked for the presence of NHP and nascent RNA molecules in the chromosomes after the incubation period. Although the isolated nuclei incorporated radioactivity for 90 min (Fig. 2*a*) DNA·RNA hybrids and NHP could only be detected in the chromosomes for the first 30 min after isolation (Fig. 2 *b* and *c*). When the chromosomes were incubated for a longer period of time (40 min) the chromosomes lost the ability to bind anti-NHP and, moreover, nascent RNA molecules could not be detected when the DNA was denatured and formation of DNA·RNA hybrids was encouraged. Thus the chromosomes could not be considered native 30 min after isolation. The pattern of distribution of Z-DNA in these chromosomes may be seen in Fig. 3. Although the intensity of fluorescence was in general lower than that seen in acetic acid-squashed chromosomes or in chromosomes that had been prefixed in ethanol/formaldehyde previous to incubation with the anti-Z-DNA antibody, the patterns are very similar. In native chromosomes, the anti-Z-DNA sites locate in interbands or subdivisions of dispersed DNA, but there is no correlation between DNA dispersion and intensity of anti-Z-DNA fluorescence (Fig. 4). For example, subdivision 4-93A has very dispersed DNA but a very low level of Z-DNA fluorescence, whereas in subdivision 4-92A with also a very dispersed DNA the intensity of fluorescence is higher. Similar relationships are observed in chromosomes fixed and squashed in 50% acetic acid. Z-DNA reactivity was never detected over any chromosomal subdivisions in chromosomes that had lost capacity to bind anti-NHP antibodies or in which we could not demonstrate DNA·RNA molecules. Moreover, anti-Z-DNA was not detected over the nucleolar DNA, nor was it observed on the chromosomes in the controls incubated with nonimmune serum.

The influence of protein removal on Z-DNA induction was studied on acetic acid-squashed chromosomes incubated

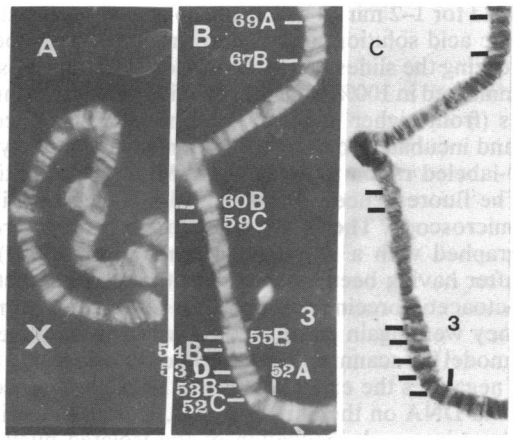

FIG. 3. Z-DNA reactivity on native polytene chromosomes of *D. hydei*. Isolated nuclei were incubated for 15 min with goat IgG directed against Z-DNA. Afterwards the nuclei were washed in PBS, fixed with ethanol/formaldehyde for 30 sec and squashed in 50% acetic acid. Then the chromosomes were incubated for 1 hr with FITC-labeled rabbit anti-goat IgG. After being photographed in a Zeiss epi-illuminated microscope the chromosomes were stained with aceto-orcein. (*A*) Pattern of anti-Z-DNA reactivity of the X chromosome; (*B*) pattern of Z-DNA reactivity of chromosome 3; (*C*) aceto-orcein staining of the same chromosome 3 as in *B*. Bars indicate subdivisions of disperse DNA showing intense Z-DNA reactivity.

with proteinase K. Fig. 5*B* shows the distribution of anti-Z-DNA seen after digestion with 5 μg of proteinase K per ml for 5 min. The pattern of Z-DNA reactivity is similar to that observed before treatment with the enzyme in that it located over regions of dispersed DNA (Fig. 5*A*), but the intensity of the fluorescence is significantly higher. When the chromosomes were treated with a higher concentration of the enzyme (20 μg/ml) for the same period of time, anti-Z-DNA binding extended over the whole chromosomal structure, even to DNA-rich bands (Fig. 5*C*). In contrast, after longer periods of incubation with 20 μg/ml (10–15 min), the anti-Z-DNA reactivity significantly decreased everywhere. However, increase in Z-DNA reactivity was not seen after 15 min of proteinase K digestion (20 μg/ml) in chromosomes fixed in formaldehyde in spite of protein removal (data not shown).

The high levels of Z-DNA induced in chromosomes treated with proteinase K allowed us to observe different levels of Z-DNA in active loci. In subdivision 4-78B, induced to high transcription rates during late-third instar, there are high levels of anti-Z-DNA binding, whereas in subdivision 4-81B, induced to puff by a heat-shock treatment, the Z-DNA

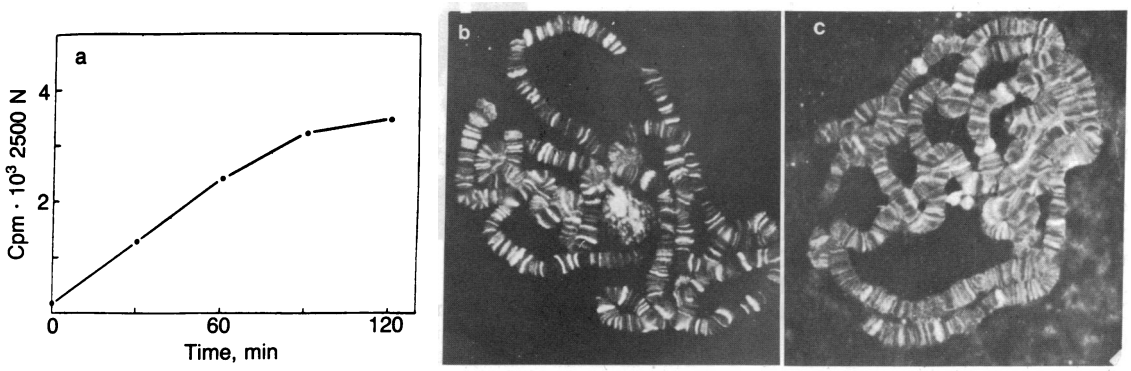

FIG. 2. Kinetics of [^3H]UTP incorporation by isolated nuclei and immunofluorescence of DNA·RNA hybrids and NHP in polytene chromosomes of *D. hydei*. (*a*) Trichloroacetic acid-precipitable radioactivity; (*b*) immunofluorescence of DNA·RNA hybrids; (*c*) immunofluorescence of NHP. In (*b*) and (*c*) isolated nuclei taken at the end of the incubation period with the goat anti-Z-DNA antibody were fixed and squashed as described elsewhere. They were incubated with goat anti-DNA·RNA hybrids and rabbit anti-NHP. Then they were labeled with FITC-labeled rabbit anti-goat and goat anti-rabbit IgG, respectively.

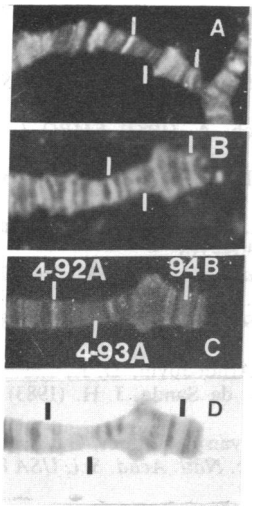

FIG. 4. Z-DNA reactivity in a fraction of chromosome 4 (regions 91–94). (*A*) Chromosomes were fixed in ethanol/formaldehyde before squashing in 50% acetic acid; (*B*) chromosomes were fixed and squashed in 50% acetic acid; (*C*) chromosomes were incubated with the antibody and then fixed as described for Fig. 3. In all cases the chromosomes were incubated with goat anti-Z-DNA followed by FITC-labeled rabbit anti-goat IgG. In (*D*) the chromosome of *C* was stained with aceto-orcein.

reactivity is at background level. On the other hand, in puff 2-48B (also heat-inducible) there is higher Z-DNA reactivity. This is clearly seen in Fig. 6 *d* and *c* where densitometer profiles of the Z-DNA fluorescence (*b*) and DNA bands (*a*) are shown. Therefore, the Z-DNA configuration is not always associated with high transcription, and it is not induced by DNA decondensation *per se*, as Pardue also reported (23).

DISCUSSION

In a recent paper Lancillotti *et al.* (25) indicated that in the polytene chromosomes of both *D. hydei* and *D. melanogaster* anti-Z-DNA binding could be detected in dense bands or interbands depending on the method used for fixation. Although these experiments suggested that Z-DNA conformations could exist in native chromosomes where DNA is dispersed, that ethanol could drive the B–Z equilibrium towards the Z form (36) as reported for deproteinized DNA (37) was not excluded. The data presented in this paper demonstrate that, indeed, Z-DNA configurations can be induced in the chromosome structure, because removal of chromosomal proteins by mild proteinase K treatment increases significantly the level of anti-Z-DNA reactivity. Because short incubation times with proteinase K cause the Z-DNA reactivity to increase only over subdivisions of disperse DNA, whereas longer incubation times extend the effect to compact regions (bands), we think that the release of torsional strain in nucleosomes or higher-order chromatin structures due to protein removal, may allow DNA mobility, introduce changes in superhelical densities, and thus induce the Z-DNA conformations. These data indicate, moreover, that potential Z-DNA-forming sites are distributed throughout the chromosome—in interbands as well as in bands. Removal of small amounts of protein may lead to changes in superhelical densities only in interbands, whereas to produce

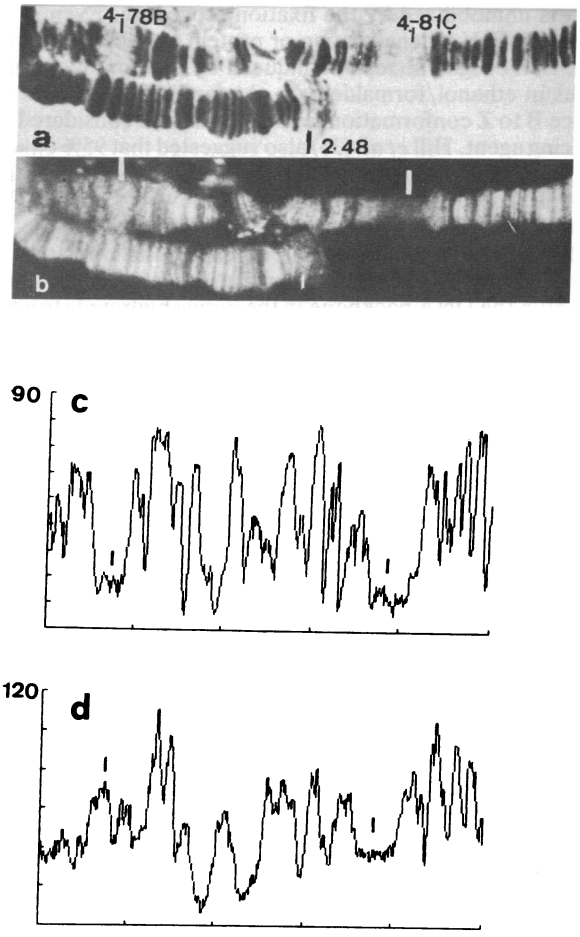

FIG. 6. Z-DNA in polytene chromosomes of *D. hydei* (subregions 76C–84A). The larvae were heat-shocked at 35°C for 20 min. Chromosomes were fixed in 50% acetic acid and squashed in the same solution. Afterwards the rehydrated chromosomes were treated with 5 μg of proteinase K per ml for 5 min. After extensive washing in 0.01 M PBS the chromosomes were incubated with goat anti-Z-DNA followed by FITC-labeled rabbit anti-goat IgG. (*a*) Aceto-orcein staining; (*b*) Z-DNA reactivity; (*c*) densitometer profile of the staining of the chromosome of *a*; (*d*) densitometer profile of the fluorescence of the chromosome of *b*. The intensity of staining and fluorescence is given in arbitrary units on the ordinates.

the same effect in very compact bands larger amounts of protein must be removed.

Because, after a long incubation period with proteinase K, the level of Z-DNA reactivity decreases significantly throughout the chromosome, we think that extensive relaxation of the DNA supercoiling favors the equilibrium toward the B conformation. Probably Z-DNA forms cannot be induced in formaldehyde-treated chromosomes because the

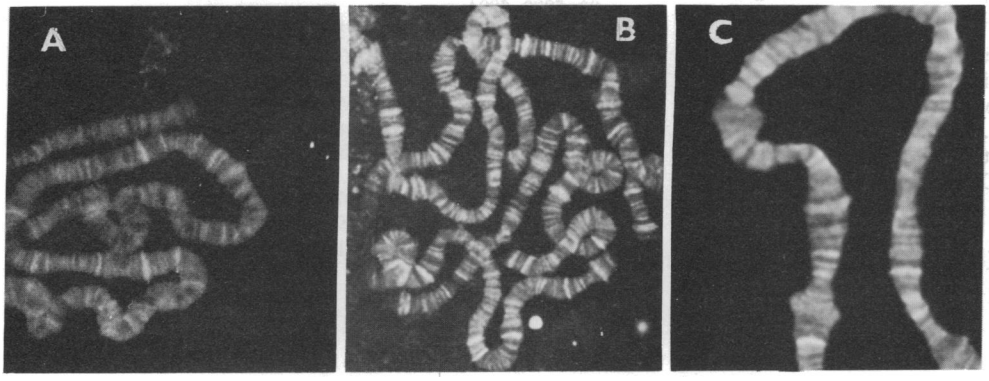

FIG. 5. Z-DNA reactivity of the polytene chromosomes of *D. hydei*. All chromosomes were fixed and squashed in 50% acetic acid. Chromosomes were treated with 5 μg (*B*) and 20 μg (*C*) of proteinase K per ml. All chromosomes were incubated with goat anti-Z-DNA and FITC-labeled rabbit anti-goat IgG.

DNA is immobilized by the fixation procedure. Even in the absence of DNA denaturation or protein extraction, however, Z-DNA segments can be induced by fixation of chromosomes in ethanol/formaldehyde. As formaldehyde does not induce B to Z conformation, ethanol has to be considered the inducing agent. Hill *et al.* (36) also suggested that 95% ethanol may enhance Z-DNA reactivity in chromosomes. Since ethanol solutions do not increase the level of anti-Z-DNA fluorescence of chromosomes prefixed in formaldehyde, we think that the inducing effect of ethanol is operative by neutralization of the negative repulsion of the phosphate groups in the DNA backbone in the *native* hydrated structure or by protein denaturation.

There is no doubt that the crucial question is whether or not Z-DNA segments exist in functionally active chromosomes. Because our data demonstrate that Z-DNA segments can be visualized in unfixed chromosomes, but only while they maintain a normal pattern of NHP and nascent RNA molecules, it may be, as indicated by Rich *et al.* (16) that removal of specific subsets of chromosomal factors may have been the reason why Z-DNA conformations were not visualized in chromosomes isolated by micromanipulation (26, 30). The nature of these factors is difficult to determine because a nonspecific removal of chromosomal proteins increases the Z-DNA signal. We think that the absence of Z-DNA forms in chromosomes obtained by micromanipulation must be correlated with the loss of their functional *native* state.

Because the distribution of Z-DNA conformations in native chromosomes is similar to that detected in acetic acid-squashed chromosomes, we think that the original observations of Nordheim *et al.* (22) may, at least partially, reveal the native condition. Although the reason why Z-DNA segments *in vivo* are mainly localized in specific subdivisions of dispersed DNA involved in transcription is not obvious, it probably should be correlated with specific changes in the electrostatic environment of potential Z-DNA segments during the transcriptional process. The finding of strong anti-Z-DNA reactivity in transcriptionally active subregions induced by ecdysone (25) supports this suggestion.

On the other hand, it is clear that neither the accumulation of NHP in some chromosomal loci nor the transcription process *per se* induces Z-DNA conformations, because in some potential transcriptionally active subdivisions, which can be induced to puff, there is no Z-DNA. Moreover, in these loci Z-DNA cannot be experimentally induced. Probably in these regions there are no potential Z-DNA-forming DNA segments. The role of the Z-DNA forms *in vivo* and of the potential Z-DNA-forming segments localized at specific transcription sites is still to be discovered. Because removal of chromosomal proteins may induce Z-DNA conformations, it is possible that specific chromatin-bound factors, interacting with the DNA or proteins of specific loci, may introduce changes in the conformation of the DNA to regulate the level of transcription, that these conformational changes may also be a consequence of changes in nucleosome structure due to histone modification, and that specific Z-DNA-forming sequences are required as recognition sites for those factors.

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