

# Locations of Z-DNA in Polytene Chromosomes

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**ABSTRACT** In polytene chromosomes of *Drosophila hydei* and *D. melanogaster*, Z-DNA was identified in varying distribution after different conditions for fixation were used. When salivary glands were fixed and squashed in 50% acetic acid alone, Z-DNA was found in the less dense DNA regions, such as interbands, some puffs, and a few of the less dense bands. Prefixation that combined ethanol and acetic acid exposure led to prominent immunofluorescent staining of the bands, generally but not strictly correlating with the total DNA content. Separate exposure to ethanol and acetic acid did not cause this band to stain, but if residual ethanol was present after ethanol fixation, subsequent exposure to acid did cause it. Under the more selective acid fixation conditions, Z-DNA reactivity was seen in portions of certain ecdysone-inducible puffs in the induced but not in the resting state; in other inducible regions, the Z-DNA immunoreactivity was not changed on induction. Z-DNA was also identified in polytene chromosomes within isolated nuclei that had been frozen and fixed in ethanol without exposure to acid; this Z-DNA was present in regions of low DNA density.

The structure of left-handed Z-DNA was described by Wang et al. in 1979 on the basis of x-ray crystallography, at atomic resolution, of the hexamer (dCdGdCdGdCdG) (1). It was quickly recognized that this crystal structure corresponded to one of the conformations of the polymer poly(dG-dC), namely the form that existed in solutions of high ionic strength (2, 3). It has since been shown that other alternating (pyrimidine-purine) structures can exist in the Z-helical form (4-6) and that forces other than high ionic strength can drive the transition from the right-handed B-DNA helix to the left-handed Z-structure when the appropriate sequences are present (5, 7-9). It is notable that the torsional strain of supercoiling of closed circular DNA can lead to the formation of Z-DNA segments even under physiological conditions (8-10).

Soon after the discovery of the Z-DNA structure, the question arose as to whether it can exist in DNA in nature and whether it may play an important functional role, in combination with proteins that can recognize it specifically, in biological regulation. To test this, it was necessary to develop a probe that could detect Z-DNA as a small fraction of the total nucleic acid and when it was within complex biological materials such as chromatin. For this purpose specific antibodies were prepared, first by immunization with a stabilized Z-DNA, brominated-poly(dG-dC) (11), and later by other forms of Z-DNA polymers (12-15). With the resulting polyclonal or monoclonal antibodies, it was possible to detect the

presence of Z-DNA in acid- or ethanol-fixed chromosomes, most strikingly in the polytene chromosomes of *Drosophila* and *Chironomus* salivary glands (16-18). The presence of Z-DNA in these preparations was confirmed in several laboratories, but there were distinct differences in the reported patterns of localization of the Z-DNA within the chromosome. One group observed the staining primarily in interbands, some puffs, and a few bands (16), whereas another group saw the staining almost entirely in the dense bands, corresponding to the concentration of total DNA (17). A third laboratory observed primarily an interband distribution in *Drosophila* and a prominent staining of a small number of bands in *Chironomus* chromosomes (18). The differences between the findings of the first two groups were not resolved by an exchange of antibody reagents. This suggested that variations in the tissue fixation and preparation may be the basis for these differences. The importance of fixation was emphasized in studies in which chromosomes were dissected without exposure to acid or ethanol fixation; in such preparations the Z-DNA was not detected, or only weakly so, unless the chromosomes were then exposed briefly to these fixatives before immunofluorescent testing (19-21).

As part of the effort to evaluate the extent to which Z-DNA occurs in vivo, it is important to determine the conditions that unmask it or favor its formation during tissue preparation. To this end, it is also important to determine whether

the different distributions can be revealed by different fixation procedures in one laboratory. This article described such experiments, in which the predominantly interband pattern of staining was obtained after fixation with ethanol alone or acetic acid alone, and the predominantly band pattern of staining was obtained after prefixation with a combination of ethanol and acetic acid. Although Z-DNA forming sequences may occur throughout the genome, the left-handed structure is more readily unmasked or formed in the interband regions than in the bands.

## MATERIALS AND METHODS

**Polytene Chromosome Fixation:** Polytene chromosomes were isolated from salivary glands of *Drosophila hydei* and *D. melanogaster* during the third instar larval period. The glands were explanted in saline Poel's medium and fixed under different conditions: (a) fixed in ethanol/acetic acid (3:1) for 1 to 10 min and squashed after 1 to 10 min of treatment in 50% acetic acid; (b) fixed in 50% acetic acid for 1 to 10 min and squashed in the same solution; (c) fixed in 70% ethanol for 1 min and squashed after 5 min of treatment in 50% acetic acid; (d) fixed for 1 min in 50% acetic acid, postfixed in 70% ethanol, and squashed in 50% acetic acid after 5 min of treatment in that solution; or (e) fixed for 1 min in 70% ethanol, postfixed for 5 min in 50% acetic acid, postfixed again in ethanol/acetic acid (3:1), and squashed in 50% acetic acid after 5 min in that solution.

After fixation and squashing, the chromosomes were frozen in liquid nitrogen and transferred to 100% ethanol (Merck Chemical Div., Merck & Co., Inc. Isotopes, Rahway, NJ); after 0.5 h in ethanol the chromosomes were rehydrated through a series of decreasing concentrations of ethanol in phosphate-buffered saline (PBS).

**Isolation of Nuclei:** 25 pairs of salivary glands from *D. hydei* larvae in 200  $\mu$ l in saline Poel's medium were homogenized for 15 s in a 1-ml syringe with the aid of a loosely fitting glass bead which could freely rotate inside the syringe. Then the solution was filtered through a piece of nylon cloth with a pore of 80  $\mu$ m and maintained at 4°C for 5 min until the nuclei dropped to the bottom of an Eppendorf tube. 10- $\mu$ l aliquots were placed on glass slides, covered with coverslips, frozen in liquid nitrogen, and immersed in absolute ethanol. After 0.5 h the nuclei were rehydrated through a series of decreasing concentrations of ethanol in PBS.

**Immunofluorescence Reaction:** The squashed chromosomes or the isolated nuclei were incubated for 1 h with a rabbit IgG directed against Z-DNA, at a dilution of 1:40 in PBS (0.01 M phosphate, 0.15 M NaCl). After they were washed extensively in PBS, they were incubated for 1 h more with fluoresceinated goat anti-rabbit IgG (Miles Laboratories Inc., Elkhart, IN) and rinsed in PBS for 1 h. The Z-DNA reactivity was visualized under glycerol/1 M Tris (9:1) pH 8 in a Zeiss epiilluminated microscope. The chromosomes were photographed with a 40 $\times$  Zeiss oil immersion lens, then washed with tap water and stained with acetoorcein for 5 min. The stock solution for this stain

contained 2 g orcein in 50 ml acetic acid and 50 ml lactic acid. It was diluted 1:3 in acetic acid-lactic acid and chromosomes were stained for 5 min. The stain was washed out with three changes of 45% acetic acid (5 min each). The chromosomes were then photographed again at the same magnification. The photometer in the microscope and the developing times were set so as to maintain the image on the negative in the linear range of sensitivity of the film. Although acetoorcein can stain both DNA and protein, the density of staining under our conditions was found to be proportional to the DNA content measured by a more specific stain for DNA, gallocyanine-chromalum. The acetoorcein staining was much more rapid.

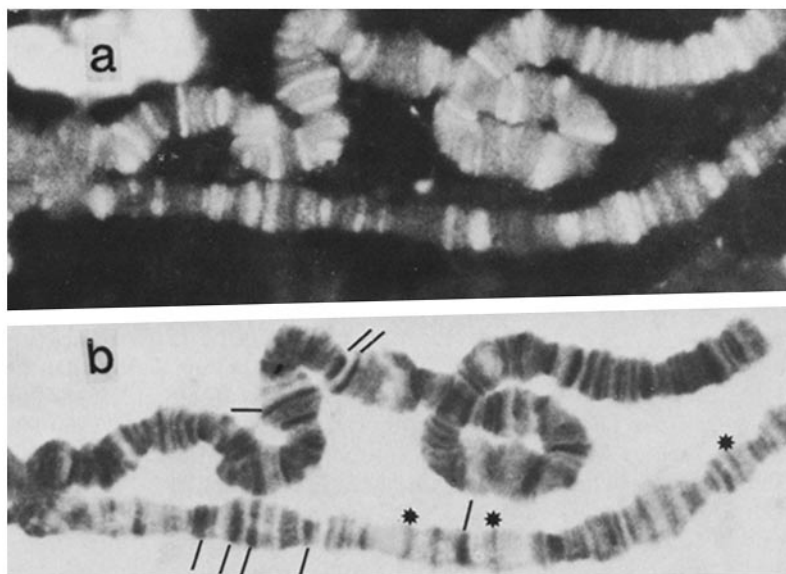
**Densitometry of Chromosomes:** Densitometry was carried out with the photographic negatives. The scanning was performed with a Zeiss scanning cytophotometer 03 connected to an HP 9816 computer. A Zeiss apamos computer program was used. The precise location of the Z-DNA staining relative to the bands or interbands was achieved by adjustment of the starting point of the scan at a specific coordinate within the photographic negatives of a given chromosome stained for Z-DNA and total DNA. A cytoplasmic marker or a particular feature of the chromosome was used for the determination of the starting point. Measurements were performed at 25  $\mu$ m intervals with a planar 2.5 $\times$  objective lens. Only one line was scanned. The negatives were placed on a slide and covered with a coverslip. The chromosomes to be measured were then relocated on the slide to the segment of interest and parallel to the direction of movement of the microscope stage. The aperture in the photometer corresponded to 1  $\mu$ m of the native chromosome diameter and to a length section of 0.1  $\mu$ m along the axis. The density values are given as quantities relative to 100% for the maximum value of a particular band within the area measured.

## RESULTS

### *Influence of the Ethanol/Acetic Acid Prefixation*

The Z-DNA immunofluorescence pattern obtained in *D. hydei* polytene chromosomes prefixed in ethanol/acetic acid (3:1) and squashed in 50% acetic acid is shown in Fig. 1. A comparison of the fluorescent staining for Z-DNA was superimposed over the DNA bands. Regions of low DNA density also showed reactivity, but the staining intensity was significantly lower. Analysis of the quantitative distribution of antibody binding showed that the intensity of fluorescence over bands correlated in general with the intensity of orcein staining. This correlation, however, was not always direct; some bands (shown by stars in Fig. 1) appeared to contain a low level of Z-DNA. We observed the same type of immunofluorescence pattern over bands with polytene chromosomes of *D. melanogaster* when they were squashed in 50% acetic acid after prefixation in ethanol/acetic acid 3:1, as was also ob-

FIGURE 1 Z-DNA in polytene chromosomes prefixed in ethanol-acetic acid. *D. hydei* chromosomes were prefixed in ethanol-acetic acid (3:1) and then squashed in 50% acetic acid, frozen in liquid nitrogen, and transferred to 100% ethanol for 30 min. They were rehydrated in steps with decreasing concentrations of ethanol in PBS, incubated with goat anti-Z-DNA (250  $\mu$ g/ml of IgG containing  $\sim$ 20  $\mu$ g/ml of antibody), washed, and incubated with fluorescein-labeled rabbit anti-goat IgG (a) and then stained with acetoorcein (b). Bars in b indicate examples of bands that are brightly stained in immunofluorescence in a. Stars in b indicate bands with low immunofluorescent staining in a.  $\times$  320.



served by Arndt-Jovin et al. (17). The Z-DNA fluorescence patterns were identical in chromosomes prefixed for either 1 or 10 min in the ethanol-acetic acid.

#### Z-DNA in Acetic Acid-fixed Chromosomes

On the other hand, when the chromosomes were fixed in 50% acetic acid without prefixation in ethanol-acetic acid, the immunofluorescence pattern was superimposed on the regions of low DNA density, mainly in the form of interbands (Fig. 2, *a* and *b*). This pattern was observed also with the polytene chromosomes of *D. melanogaster* (Fig. 2, *c-f*), as Nordheim et al. (16) and Pardue et al. (22) reported. Low levels of anti-Z-DNA antibody binding, however, were also located over band structures.

Fig. 3 shows a fraction of chromosome 2 between subregions 2-48c and 2-43a stained for total DNA (Fig. 3*a*) and Z-DNA (Fig. 3*b*). It is clear that the pattern of Z-DNA fluorescence coincided with the pattern of interbands or loosely packed DNA. In a densitometer tracing of the fluorescence and orcein staining from these subregions (Fig. 3, *c* and *d*),

the peaks of higher fluorescence intensity corresponded to regions of low DNA density (subregions 2-47c and 2-46c, b, a). There was not invariably, however, a direct correlation between subregions of low density of DNA and high intensity of fluorescence. In subregions 2-47c and 2-44c, the intensity of fluorescence clearly differed, whereas the density of DNA staining was similar (10% of the highest density seen). Also, the level of density of DNA staining in 2-48b was similar to that in subregion 2-47c, but the intensity of fluorescence was higher, by a factor of 2.5, in 2-47c. Both of these subregions are transcribed at the larval developmental stage at which the glands were explanted (23, 24) and both are also inducible. Subregion 2-48b forms a large puff when larvae are incubated at 37°C (heat shock) (25), and subregion 2-47c is induced to higher transcriptional activity by ecdysone (26) but does not form a large puff. In the chromosome shown in Fig. 3, the brightly fluorescent subregion 2-47c is in the induced form whereas 2-48b is not.

The background level of Z-DNA fluorescence over bands was ~25 to 30% of the level of fluorescence over the highest

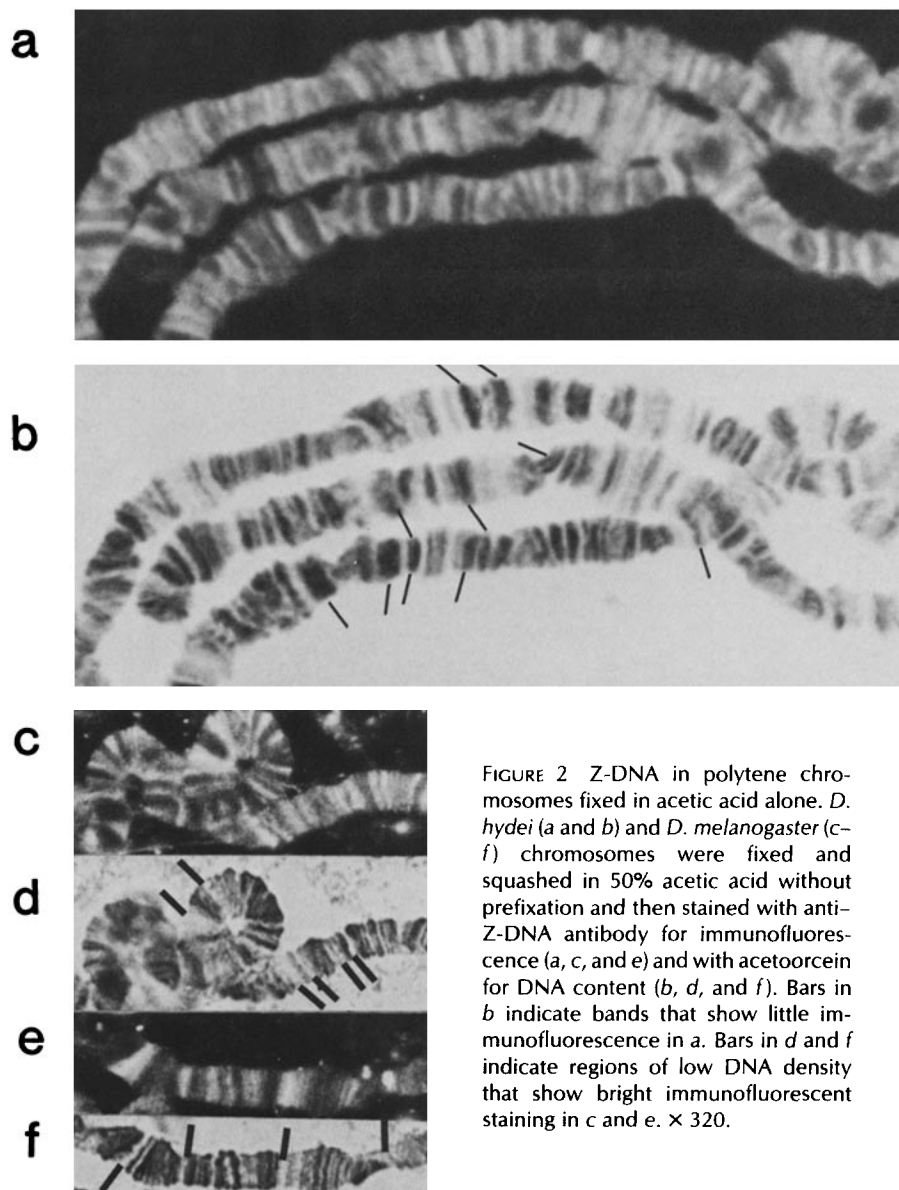


FIGURE 2 Z-DNA in polytene chromosomes fixed in acetic acid alone. *D. hydei* (*a* and *b*) and *D. melanogaster* (*c-f*) chromosomes were fixed and squashed in 50% acetic acid without prefixation and then stained with anti-Z-DNA antibody for immunofluorescence (*a*, *c*, and *e*) and with acetoorcein for DNA content (*b*, *d*, and *f*). Bars in *b* indicate bands that show little immunofluorescence in *a*. Bars in *d* and *f* indicate regions of low DNA density that show bright immunofluorescent staining in *c* and *e*.  $\times 320$ .

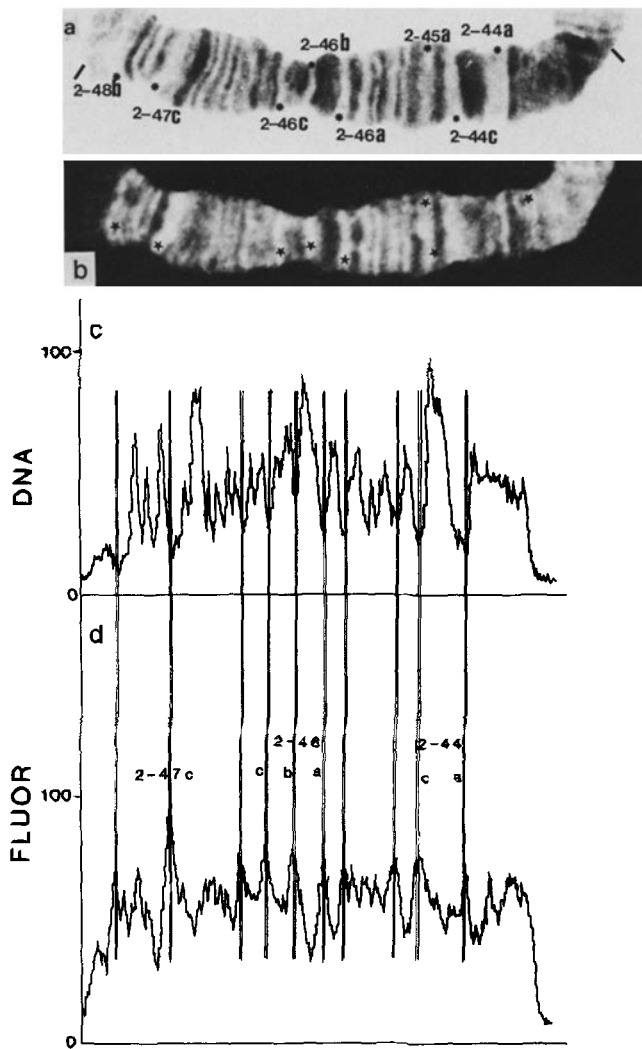


FIGURE 3 DNA content (a and c) anti-Z-DNA immunofluorescence (b and d) of *D. hydei* chromosome 2 between subregions 43a and 48c after fixation and squashing in 50% acetic acid only, without prefixation. The chromosomes were photographed (a and b;  $\times 320$ ) and negatives were scanned with a densitometer to allow precise alignment of DNA staining (c) in relation to peaks of immunofluorescence (d). Stars in a and b indicate corresponding regions in the two photographs.

fluorescent band. We have not found any difference in Z-DNA fluorescence patterns in chromosomes fixed in 50% acetic acid for as long as 1 or 10 min.

#### *Influence of Acetic Acid Treatment before or after Ethanol Prefixation*

In view of the differential pattern of Z-DNA reactivity obtained when the chromosomes were either fixed in ethanol-acetic acid (3:1) (stained in bands) or fixed in 50% acetic acid (stained in interbands), we wanted to investigate whether the ethanol or the acetic was the inducer of either of the patterns. To test that, the salivary glands were prefixed in 70% ethanol before they were squashed in 50% acetic acid or fixed in 50% acetic acid and then postfixed in 70% ethanol and squashed in 50% acetic acid. For the ethanol fixation, we chose the concentration of 70% because this is its concentration in the ethanol-acetic acid mixture. In addition, chromosomes prefixed in 100% ethanol are difficult to spread.

Under either of these sequences of fixation, when the exposure to fixatives was brief, the pattern of Z-DNA fluorescence was similar to that obtained from fixation and squashing in 50% acetic acid alone, but the fluorescence was less intense generally. The fluorescence was superimposed over regions of low DNA density in the form of interbands and less dense bands (Fig. 4). A similar pattern of Z-DNA reactivity was observed in the polytene chromosome of *D. melanogaster* (Fig. 5). As in the case of chromosomes fixed and squashed in 50% acetic acid, however, there was not a strictly inverse correlation between Z-DNA fluorescence and density of DNA staining in all subregions. In Fig. 4, the density of DNA staining in a subdivision within subregion 2-69d (indicated by a star) is higher than in subregions 2-70a (indicated by a bar), and the immunofluorescence is also higher. As the background level of Z-DNA fluorescence in bands was one-quarter to one-third of the highest fluorescent peak in interbands (Fig. 4), it appears that the band DNA is also a target for the antibodies.

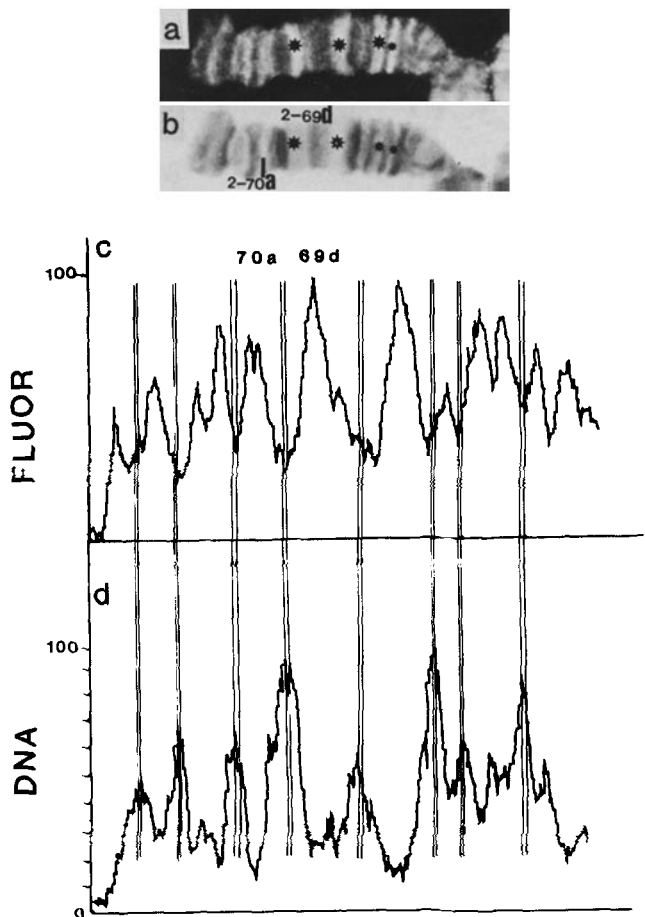


FIGURE 4 Z-DNA in chromosomes prefixed with ethanol. *D. hydei* chromosomes were fixed in 70% ethanol without acetic acid. This was followed by complete removal of ethanol, fixation for 5 min in 50% acetic acid, and squashing in 50% acetic acid. Chromosome 2 is shown stained with anti-Z-DNA antibody for immunofluorescence (a and c) and with acetoorcein for DNA content (b and d). The chromosomes were photographed (a and b;  $\times 320$ ) and negatives were scanned with a densitometer for precise alignment of fluorescent regions (a) in relation to peaks of DNA density (d). Stars in a and b indicate corresponding regions in the photographs. Fluor, fluorescence.

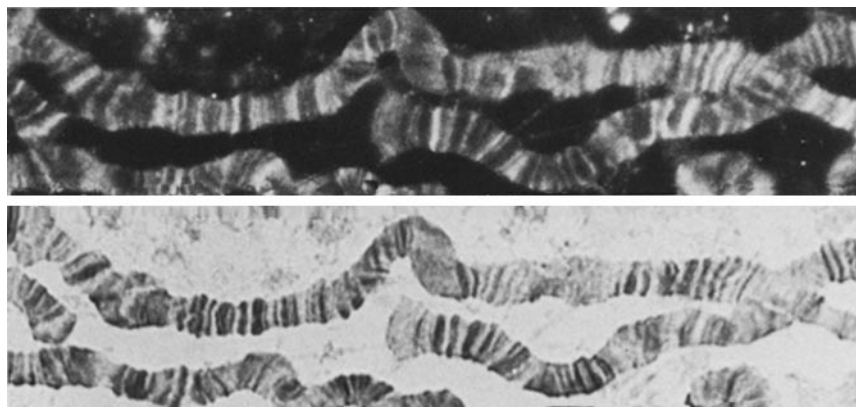


FIGURE 5 Z-DNA in *D. melanogaster* polytene chromosomes prefixed in 70% ethanol. Conditions were as for *D. hydei* chromosomes shown in Fig. 4.  $\times 320$ .

Prefixation periods  $>5$  min in 70% ethanol were able to generate a pattern of fluorescence similar to that observed after the simultaneous ethanol-acetic acid prefixation, but this pattern was restricted to  $<25\%$  of the nuclei from a given gland. All of the nuclei that were in a physiological stage in which the ecdysone-inducible puffs were developed showed the pattern characteristic of fixation with acetic acid alone.

These results indicated that neither the ethanol after acetic acid fixation nor the acetic acid after ethanol prefixation produced the same pattern of Z-DNA reactivity as the simultaneous ethanol-acetic acid prefixation. The importance of the combination of fixatives was shown in an experiment in which chromosomes were prefixed in 70% ethanol and after 2 min were further fixed and squashed in 50% acetic acid without complete removal of the ethanol (Fig. 6). This immunofluorescence pattern was identical to that caused by prefixation with the 3:1 ethano-acetic acid solution. We have also observed that a 1:1 or 0.5:1 ratio of ethanol to acetic acid will cause this pattern. Even though the band staining was most prominent, the level of fluorescence over interbands was higher than in chromosomes fixed and squashed in acetic acid alone. There was a correspondence between the levels of fluorescence and DNA staining in all subregions except in a group of bands on 1-17c (marked by a star and bar in Fig. 6). Moreover, the intensity of fluorescence over band 1-19a and 1-17c (Fig. 6, star) differed significantly, although the intensity of their DNA staining was similar. With this fixation, some sets of glands showed an intermediate pattern in which the fluorescence over bands was not significantly different from that over interbands. In a further test of the effect of varying the sequence of fixation steps, we found that once the chromosomes were fixed in acetic acid, subsequent exposure to combined acetic acid-ethanol did not cause the band staining pattern to appear.

### Z-DNA Reactivity and Transcription

Because Z-DNA immunoreactivity was associated with loosely bound DNA and it is known that the transcriptional activity in polytene chromosomes is associated preferentially with these regions, we investigated whether Z-DNA was also associated with loci induced to higher transcriptional levels in puffs. To visualize these loci, salivary glands from later third instar larval periods were explanted, fixed in ethanol (70%), postfixed in 50% acetic acid after complete removal of the ethanol and squashed in acetic acid 5 min later. As expected, in these chromosomes the low DNA density regions were fluorescent and the brightest spots were localized over devel-

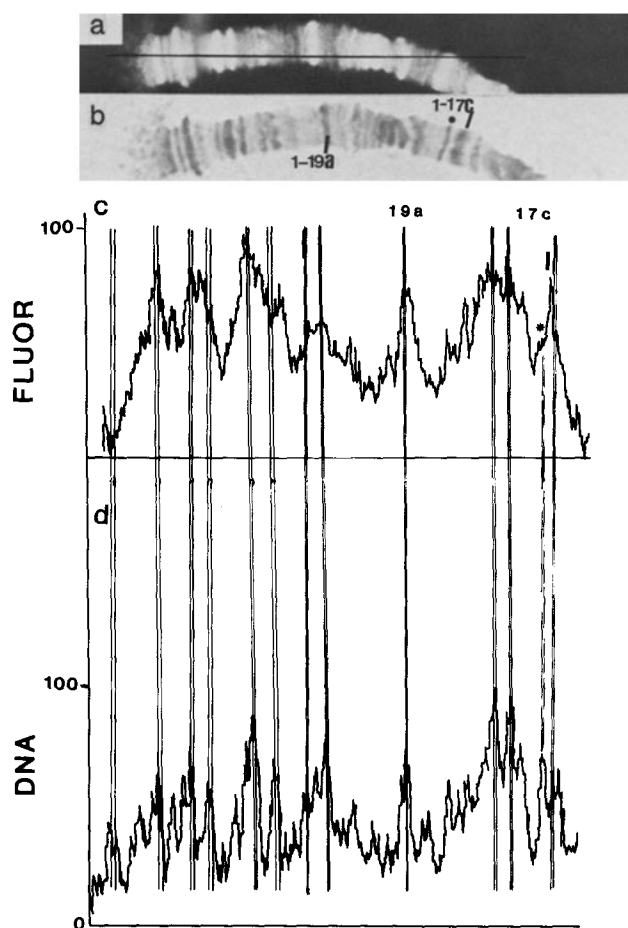


FIGURE 6 Effect of a 2-min prefixation in 70% ethanol and subsequent exposure to 50% acetic acid without complete removal of ethanol. *D. hydei* chromosome 1 stained for anti-Z-DNA immunofluorescence (a and c) and for DNA content (b and d). Regions of fluorescence (c) were aligned in relation to peaks of DNA staining (d) in densitometer scans. Markers show corresponding regions in the orcein-stained chromosome in photograph b and the fluorescence tracing (c). Fluor, fluorescence. (a and b)  $\times 320$ .

opmentally induced puffs. Fig. 7a shows the entire fourth chromosome of *D. hydei* in the induced state, with specific immunofluorescence at 75d; 78a, b, c; 87c; 90a, b; and 91a. The activity of all these regions is under the control of ecdysone. Fig. 7c shows a portion of the fourth chromosome in which these ecdysone-inducible regions are in the resting stage. We focused our attention on three subregions, two of

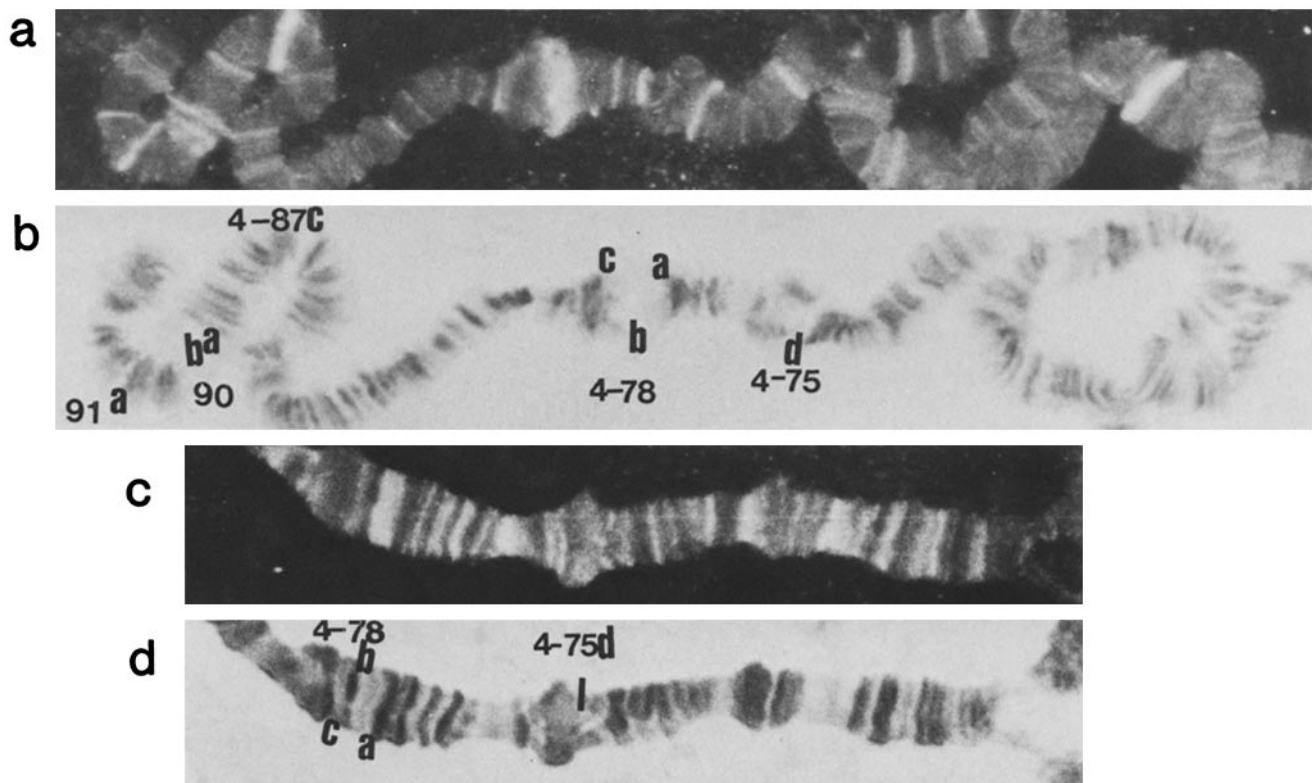


FIGURE 7 Z-DNA in chromosome 4 of *D. hydei* after induction by ecdysone (a and b) and in the resting state without induction (c and d). Chromosomes were fixed for 1 min in 70% ethanol alone and postfixed and squashed in 50% acetic acid after complete removal of the ethanol. They were then stained with anti-Z-DNA antibody for immunofluorescence (a and c) and by aceto-orcein for DNA content (b and d).  $\times 320$ .

which are among the first to be induced in vitro by the hormone (26). Subregion 4-78b forms a large puff 5 min after incubation whereas the DNA in subregion 4-75d decondenses without forming a visible puff. Both regions are very active in RNA transcription. The third subregion of interest is 4-78c. This subregion is not induced by ecdysone but transcribes RNA at a level similar to that in subregion 4-78b in the resting stage (24).

A comparison of both photographs reveals that (a) subregions 4-78a and b showed intense Z-DNA reactivity before puff induction; (b) the reactivity over region 4-78c was low even though its transcriptional activity and density of DNA staining was similar to that of 4-78b; and (c) the level of Z-DNA reactivity was also low in noninduced 4-75d (Fig. 7c). It is clear, however, that upon hormone induction, the level of Z-DNA reactivity became more intense in subregions 4-78a and b and 4-75d but remained low in 4-78c (Fig. 7a). Densitometry showed that the level of fluorescence over the induced 4-75d subregion increased three- to four-fold relative to the noninduced form, and became very similar to that over 4-78ab. It is interesting that the fluorescence over 4-78ab did not cover the entire surface of the puff, which initiates on subregion 4-78b and extends on both sides towards 4-78a and 4-78c. Pardue et al. have made a similar observation on a heat shock-induced puff (22). The peak of fluorescence was located over subregion 4-78b within the band from which the puff originates, and on which most of the transcriptional products accumulate (26). In the densitometry corresponding to Fig. 7c (not shown), we noted that the higher peak of

fluorescence was located over subregion b in noninduced form also.

These results indicate that in subregion 4-75d the increased reactivity is generated upon induction of transcription. On the other hand, the association of Z-DNA reactivity and puffing induction is not obligatory, as the level of Z-DNA activity is very low in the large puffs (2-48b and 4-81c) generated upon a heat shock treatment.

#### Z-DNA Reactivity in Non-Acid-fixed Chromosomes

The above results support the suggestion of Robert-Nicoud et al. (21) that protonation followed by local denaturation facilitates transition to the Z form. On the other hand, Z-DNA reactivity has been observed in chromosomes fixed with ethanol, without acid (20). In a further test of the requirement for denaturation, we tested isolated nuclei frozen in liquid nitrogen under a coverslip, dehydrated in 100% ethanol, and rehydrated through a series of decreasing concentrations of ethanol in PBS. Immunofluorescence with anti-Z-DNA antibody was observed in a large majority of the nuclei but was restricted to subregions of low density of DNA staining (Fig. 8). In nuclei, as in chromosomes, therefore, acid denaturation is not an absolute requirement for Z-DNA reactivity. Control experiments carried out with frozen and dehydrated *Drosophila* chromatin showed that the DNA was not denatured by these treatments. The ethanol may act by different means, inducing torsional strain, or by the dehydration that occurs during this treatment.

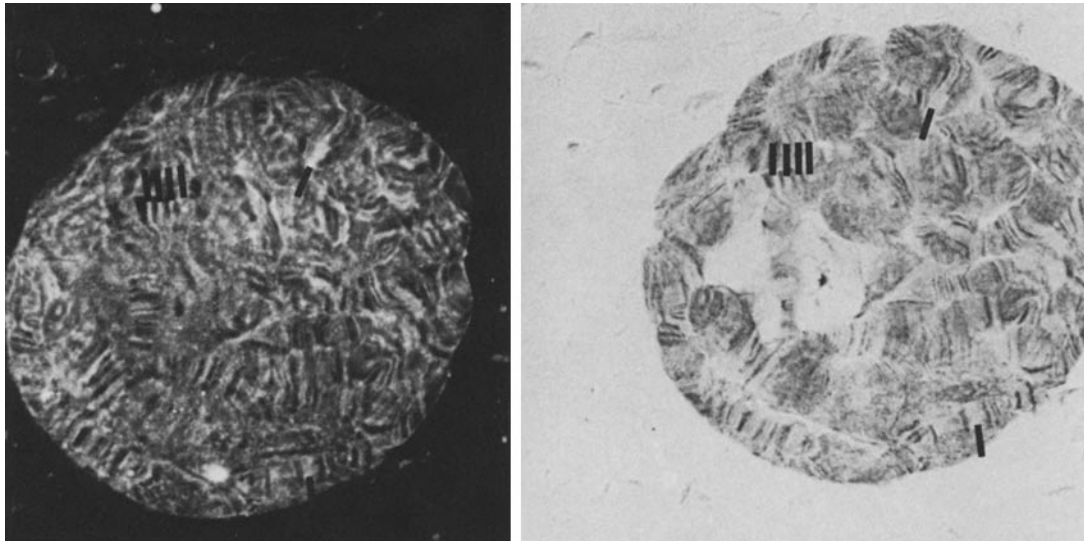


FIGURE 8 Z-DNA in ethanol-fixed nuclei of *D. hydei* larva salivary glands. Nuclei were isolated, frozen in liquid nitrogen, placed in 100% ethanol, and then rehydrated with decreasing concentrations of ethanol in PBS. They were stained with anti-Z-DNA antibody for immunofluorescence (left) and with acetoorcein for DNA (right).  $\times 320$ .

## DISCUSSION

In a rapidly developing series of experiments exploring the possibility that Z-DNA exists *in vivo*, several laboratories detected it in polytene chromosomes by immunofluorescent analysis (16–22). The meaningfulness of these findings has been questioned, however, because each laboratory reported a different localization of the Z-DNA (16–18) and because it was necessary to fix the chromosomes in order to demonstrate the Z-DNA (19, 21). The fixation usually involved exposure of the chromosomes to acid, although treatment with ethanol without acid could reveal the structure. The experiments reported in this article help to clarify the question of localization and confirm the detection of Z-DNA in ethanol-treated chromosomes, notably within isolated nuclei not exposed to acid.

In the first description of Z-DNA detection in polytene chromosomes, Nordheim et al. reported its presence primarily in interbands, some puffs, and a few bands (16). In further experiments, its occurrence in some puffs seemed to correlate with transcriptional activity, as only half of the heat shock-induced puff at location 87c was stained, and that corresponded to the half that was active in transcription (22). Throughout most of the chromosome the Z-DNA content did not correlate with the total DNA content. In contrast to these findings, Arndt-Jovin et al. reported that the Z-DNA content did in fact follow the total DNA distribution and was most abundant in bands (17, 21). Lemeunier et al. (18) found a distribution in *D. melanogaster* similar to that described by Nordheim et al. but found that predominantly a few bands stained in *Chironomus* chromosomes.

In the current work it became clear that the distribution observed with anti-Z-DNA antibody immunofluorescence depended on the fixation procedure. Prefixation with acetic acid–ethanol yielded the band-staining pattern reported by Arndt-Jovin et al. (17), whereas fixation with acetic acid without ethanol yielded the patterns reported by Nordheim et al. (16). The acetic acid–ethanol prefixation revealed Z-DNA in both bands and interbands, but the greater amount in the bands was by far the dominant pattern. It appears that this procedure, either by unmasking existing Z-DNA or by

favoring its formation during the fixation, reveals a large part of the Z-DNA-forming potential throughout the genome. It is possible that more proteins are extracted by the combination of acetic acid and ethanol than by either alone. A combined exposure was required to show the band-staining pattern. Prefixation with ethanol alone followed by complete removal of the ethanol and then fixation in acid did not do so; nor did fixation in acid, removal of acid, and exposure to ethanol separately. In fact, once the chromosomes were fixed with acetic acid, even subsequent exposure to the acetic acid–ethanol combination did not yield a typical band-staining pattern.

The fixation with acid alone also removes some protein, and Z-DNA formation is greatly favored at low pH (with a transition near the pH that also increases staining of the chromosomes by anti-Z-DNA antibody [21]). The low pH is not an absolute requirement, however, as ethanol alone allowed immunofluorescent detection of Z-DNA. This was striking in nuclei that were frozen and treated with ethanol and then rehydrated. Immunofluorescent staining within the nucleus was clearly present in the regions that did not contain high DNA density.

With a basis for understanding the variations in localization, the next question concerns the requirement for and the role of any fixation procedure. Chromosomes that were simply dissected into physiological solution with spermidine and a very low concentration of formaldehyde did not stain (19, 20) or at most gave only a weak stain with a high concentration of antibody (21). It is not possible in these experiments to determine whether the fixation is simply unmasking existing Z-DNA that may be inaccessible to antibody because of association with protein, or whether the fixation is actually driving the Z-DNA formation. The Z-DNA measured depends on torsional stress for its production or maintenance, as it is destroyed by topoisomerase I or by nicking concentrations of DNase I (20, 21).

Whatever the role of fixation, it appears that there is a subset of Z-DNA-forming sequences that is in a conformation or a chromatin location that makes it particularly likely to undergo the transition to the left-handed structure. It is re-

vealed in this conformation by procedures that remove less protein and are probably less denaturing. These sequences are located in interesting regions in relation to gene transcription activity, in interbands and puffs. Specific structural features seem to be involved, as this is not a simple correlation between low DNA density and Z-DNA staining; particular regions are selectively reactive. In this regard, it was of special interest that some such sites were ecdysone-inducible regions, which were stained much more strongly after induction than in the resting state. The experimentally induced and the spontaneously developing active states of these regions were similar morphologically and in their reactions with anti-Z-DNA antibody. Other sites did not undergo this increase in immunoreactivity. We hoped that focusing on specific sites of the chromosome in relation to physiological changes in activity will help reveal whether Z-DNA plays a role in regulation of these changes.

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