Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 31–35, January 1991
Cell Biology

A potential Z-DNA-forming sequence is located between two transcription units alternatively expressed during development of *Drosophila hydei*

(DNA conformation/torsional stress/polytene chromosomes)

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Communicated by M. M. Green, September 10, 1990 (received for review June 15, 1990)

ABSTRACT **Recent studies have demonstrated that Z-DNA exists *in vivo* in *Escherichia coli* as well as in *Drosophila* and mammalian cells. In the present paper, we show the existence *in vivo* of Z-DNA epitopes in the developmentally regulated subregion 4-75C of polytene chromosomes in *Drosophila hydei*. The Z-DNA epitopes were detected in subdivision C_2 only during late third instar when the transcriptional activity of the locus was high. Accumulation of nonhistone chromosomal proteins in that locus was also detected during late third instar only at the time of the Z-DNA formation. Northern blot data and nucleotide sequence analysis indicated that the Z-DNA-forming sequence is located between two transcription units whose expression is regulated during the third instar. Our results suggest that in subdivision 4-75C_2 a B- to Z-DNA flux occurs at a specific time during late third instar and that this flux may play a negative as well as a positive role in gene expression.**

In vitro, specific DNA sequences may adopt the left-handed conformation and other non-B-DNA structures may also be generated in response to local cellular environments (1, 2). Among the unusual non-B forms, the best characterized and understood DNA structure is the Z-conformation. Most of the studies concerning this structure have been focused on the analysis of sequence requirements, conditions for Z-DNA stabilization, thermodynamics, kinetics, B–Z junctions, specificities of Z-DNA binding proteins, and the interaction of Z-DNA with small ligands (1–6). It is probable also that binding to left-handed DNA may be a common property of recombination–initiation proteins (7, 8).

However, recently direct proof was obtained for the existence *in vivo* of Z-DNA. Z-DNA is, at least transiently, present *in vivo* in *Escherichia coli* (9) and is detected in metabolically active mammalian cells (10) as well as in native polytene chromosomes of *Drosophila* (11). On the other hand, there is no evidence of the *in vivo* function of the Z-DNA form although it has been suggested that Z-DNA is involved with genetic recombination (12) and other important biological processes such as transcription (13–15). For example, $(CG)_{32}$ in left-handed Z-DNA introduces strong transcriptional blocks in plasmids (16) and $d(TG)_n \cdot d(CA)_n$ of the rat prolactin gene forms Z-DNA and inhibits gene transcription (17). On the other hand, in mouse cells, enhanced transcription of the bacterial chloramphenicol acetyltransferase gene is observed when it is cotransfected with poly(dG-m^5dC)·poly(dG-m^5dC) or poly(dG-dC)·poly(dG-dC) (where m^5dC is 5-methyldeoxy cytidine), which forms Z-DNA (18).

Thus, it is likely that specific Z-DNA-forming sequences may play a negative as well as a positive control of gene transcription depending on their local genomic environment since the negative or positive twin supercoiling waves generated during transcription may be sufficient to drive structural transitions (19). Recent studies have demonstrated that indeed domains of negative supercoiling exist *in vivo* in *E. coli* and that short $(G\text{-}C)_n$ tracts adopt the Z-DNA conformation when inserted upstream from a transcribed gene, whereas no Z-DNA was detected when cloned downstream of the gene (20).

In the present paper, we show that, in subregion 4-75C of the polytene chromosomes of *Drosophila hydei*, a strong Z-DNA epitope is generated *in vivo* when high transcription is induced during late third instar and after β-hydroxyecdysterone (β-OH-ecdysterone) administration and that nonhistone chromosomal proteins (NHCPs) accumulate in that subregion when Z-DNA forms. We show, moreover, that the Z-DNA-forming sequence† is located between two transcription units whose transcription is regulated during third instar. We suggest that Z-DNA may play negative as well as positive roles in the expression of these genes.

MATERIALS AND METHODS

A laboratory stock of *D. hydei* was used. The larvae were raised at 25°C and 70% humidity.

Autoradiography and DNA·RNA Autoimmunograms of Polytene Chromosomes. Middle and third instar larvae were injected with 0.2 μl of 0.1× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) containing 1 μCi of [^{3}H]uridine (specific activity, 45 Ci/mmol; 1 Ci = 37 GBq). After 20 min of incubation with the precursor, the salivary glands were fixed and squashed in 45% (vol/vol) acetic acid and postfixed in ethanol/37% (vol/vol) formaldehyde (9:1) for 4 hr. The chromosomes were covered with AR10 autoradiographic film (Kodak). The exposure time was 4 days. The relative transcriptional activity of each loci was estimated by normalization to the labeling of the nucleolus. Autoimmunograms of DNA·RNA hybrids were prepared from salivary glands as described (21). Chromosomal DNA denaturation was carried out in 0.1× SSC for 20 s at 95°C and annealing of DNA·RNA molecules was done in 50% (vol/vol) formamide/2.5× SSC for 10 min. The preparations were washed in isotonic phosphate-buffered saline and incubated for 1 hr with goat anti-hybrid IgG and with fluorescein isothiocyanate-labeled anti-goat IgG. Autoimmunograms were also prepared from salivary glands incubated for 20 min in 100 μl of Grace's medium containing 1 μM β-OH-ecdysterone.

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Abbreviations: NHCP, nonhistone chromosomal protein; ORF, open reading frame; β-OH-ecdysterone, β-hydroxyecdysterone.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M55173).

Isolation of NHCPs and Preparation of Specific Antisera. NHCPs were prepared from chromatin isolated from nuclei of *D. hydei* embryos (100 g) as described by Silver and Elgin (22). Chromatin was dissociated in 5 M urea/2 M NaCl/1 mM sodium phosphate, pH 7.00. Histones, NHCPs, and DNA were eluted sequentially from a hydroxylapatite column with 0.001, 0.1, and 0.5 M sodium phosphate, respectively (23). The fraction eluted at 0.1 M phosphate was loaded into a new hydroxylapatite column and eluted as before. This fraction was dialyzed against 5% acetic acid and lyophilized. Rabbit anti-NHCP IgGs were obtained. IgGs were precipitated with 40% (wt/vol) ammonium sulfate and purified by affinity chromatography.

Immunofluorescent Staining of NHCPs. Salivary glands were incubated for 30 s in 5% (vol/vol) Tween 20 and fixed for 2 min in 3.7% formaldehyde. Then, the glands were transferred to 45% acetic acid and squashed. After removal of the coverslip the chromosomes were immersed in absolute ethanol for 1 hr and rehydrated in PBS. The chromosomes were incubated with the NHCPs and with fluorescein-labeled anti-rabbit IgG for 1 hr.

Immunofluorescent Staining of Z-DNA. The immunofluorescence reaction of Z-DNA was carried out in whole isolated nuclei prior to fixation as described (11). About 100 nuclei were incubated for 15 min with 20 μl of anti-Z-DNA IgG and for 40 min with the fluorescein-labeled anti-goat IgG (5 μg/ml). Densitometry was carried out on photographic negatives as described (24).

Construction and Screening of a *D. hydei* Genomic Library. DNA from third instar larvae was extracted and partially digested with *Sau*3A restriction enzyme. The fragments obtained [≈12 kilobases (kb) long] were inserted into the *Bam*HI cloning site of λ EMBL3. The library was screened using as probe the insert from clone pF17 isolated from libraries of *D. hydei* nuclear DNA using anti-Z-DNA antibodies (25) according to standard procedures (26). The largest positive isolated clone was further subcloned in pUC18.

RNA Hybridization. The RNA was extracted from embryos and larvae according to Crowley (27). Poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose chromatography. The larval stages were determined by the form of the jaws and the distinction between middle and third instar was done by the puffing pattern (28). Six micrograms of an RNA sample was electrophoresed, transferred to Hybond-N membranes (Amersham), hybridized, and washed as described (32). Single-stranded probes were obtained by primer extension from the direct primer 1211 (New England Biolabs).

Sequence Analysis. The nucleotide sequence was determined by the dideoxynucleotide chain-termination method (30). Both strands were sequenced.

RESULTS

Z-DNA Epitopes. We have reported (24, 25) that in acetic acid-squashed chromosomes Z-DNA epitopes could be detected in subregion 4-75C during late third instar and after β-OH-ecdysterone induction and that a DNA sequence that hybridizes *in situ* with these subdivisions adopts the Z-DNA conformation when included in supercoiled plasmids. To ensure that the Z-DNA epitopes were present in native transcriptionally active chromosomes, metabolically active nuclei from middle and late third instar larvae were incubated with anti-Z-DNA IgG (11) at a concentration that does not induce the Z-DNA form in nuclei (10). Fig. 1 *A* and *B* shows that a strong Z-DNA epitope is present on subregion 4-75C in the chromosomes of late third instar larvae, and the anti-Z-DNA reactivity of that locus in the chromosomes of younger larvae is very low, respectively. In chromosomes from late third instar larvae, the fluorescence in 4-75C increased ≈10-fold relative to that observed during middle third instar. The Z-DNA epitope is located in the interband of the telomeric section of band C_2 [according to the map of Berendes (31)]. The analysis of the *in situ* hybridization experiments using as probe the Z-DNA-forming sequence reported (25) showed that the silver grains accumulated also in the same subdivision. We observed, moreover, a Z-DNA fluorescent band in the ecdysone-inducible subregion 4-78B (28). However, the intensity of fluorescence in this subregion in late third instar was similar to that observed in chromosomes from middle third instar when the puff is not induced.

Since RNA transcription is likely to be one of the major factors that contributes to the level of local DNA torsional stress affecting non-B-DNA structural transitions, we have analyzed the transcriptional activity of subregion 4-75C. Fig. 2 shows the pattern of [^{3}H]uridine incorporation from larvae injected with the tritiated precursor and of chromosomal DNA·RNA hybrids. We observed that although in middle third instar larvae the amount of silver grains accumulated over subregion 4-75C is low (Fig. 2*B*), the labeling was very intense when the larvae were in late third instar (≈165 hr old; Fig. 2*A*). The ratio of silver grains accumulated was about 2:11. Also the DNA·RNA reactivity of subregion 4-75C in chromosomes from late third instar larvae (Fig. 2*C*) is significantly higher than that detected in chromosomes from younger larvae (Fig. 2*D*). The fluorescent band is located in the interband of the telomeric section of band 4-75C_2. High transcriptional activity in subregion 4-75C was also detected by DNA·RNA autoimmunograms of chromosomes from middle third instar larvae after a 30-min treatment with 1 μM β-OH-ecdysterone (Fig. 2*E*), relative to untreated larvae (Fig. 2*F*). The increase in newly synthesized RNA molecules occurred 5–10 min after β-OH-ecdysterone incubation since an intense DNA·RNA fluorescent band could be detected at that moment. Increase in DNA·RNA reactivity was also observed in the ecdysone-inducible subregion 4-78B.

The presence of Z-DNA epitopes and the increase in transcription of locus 4-75C was accompanied by the binding of NHCPs. An intense fluorescent band was present in 4-75C in chromosomes from late third instar (Fig. 3*A*) and the band was absent in chromosomes from younger larvae (Fig. 3*B*). The fluorescence was located also in the interband of the telomeric section of band C_2. Staining for NHCPs was, moreover, observed in the ecdysone-inducible subregion 4-78B in late third instar larvae (Fig. 3*B*). However, in 4-78B a band of NHCPs was also observed in noninduced chromosomes during the middle third instar (Fig. 3*A*). Moreover, accumulation of NHCPs was also detected in these subregions after β-OH-ecdysterone administration (data not shown). The cytological data indicate, therefore, that in 4-75C_2 there is strong correlation among induction to higher transcription, accumulation of NHCPs, and B- to Z-DNA transition and that, although specific sequences from this

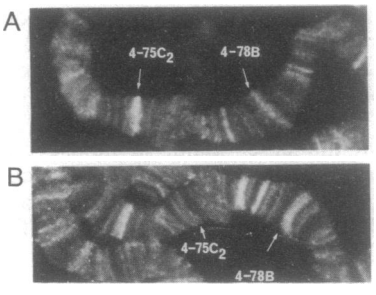

FIG. 1. Z-DNA reactivity on the telomeric end of polytene chromosome 4 of *D. hydei* in native conditions. The isolated nuclei were incubated with 20 μl of a solution containing polyclonal anti-Z-DNA IgGs (5 μg/ml). (*A*) Salivary glands from late third instar larvae. (*B*) Salivary glands from middle third instar. Regions 4-75C and 4-78B are indicated.

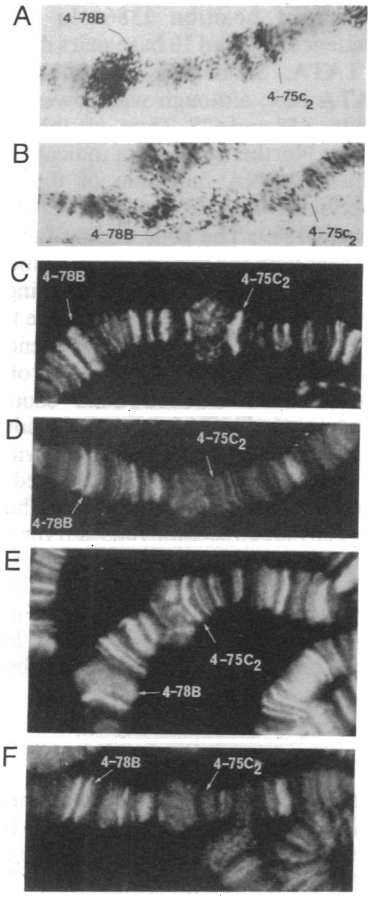

FIG. 2. Transcriptional activity of the telomeric end of polytene chromosome 4 of *D. hydei*. Autoradiographs after 20 min of [^{3}H]-uridine *in vivo* (1 μCi per larva) are as follows. (*A*) Salivary glands from late third instar. (*B*) Salivary glands from middle third instar. Autoimmunograms of DNA·RNA hybrids are as follows. (*C*) Salivary glands from late third instar larvae. (*D*) Salivary glands from middle third instar. (*E*) Salivary glands from middle third instar after 30 min of 1 μM β-OH-ecdysterone treatment *in vitro*. (*F*) Salivary glands from middle third instar without β-OH-ecdysterone treatment. The hybrids were visualized with anti-DNA·RNA IgGs. Regions 4-75C and 4-78B are indicated.

subregion are in transient B–Z flux at a specific time in development between middle and third instar, the potential Z-DNA sequences in subregion 4-78B are in a rather more static Z-form. It should be noticed, however, that the level of transcription activity detected in 4-78B in nonpuffing form is higher than in 4-75C in the same condition.

Transcription Units in 4-75C$_2$. To determine whether the Z-DNA-forming sequence from 4-75C$_2$ is located in the neighborhood of any of the transcripts synthesized in this subdivision, large genomic DNA fragments hybridizing with the Z-DNA-forming sequences (25) were isolated from a library of *D. hydei*. Fig. 4 shows the restriction map of one of the clones containing a 12-kb fragment (named B11) and the position of the subclone containing the Z-DNA-forming sequence (named pF17).

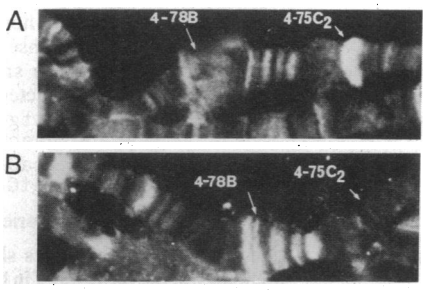

FIG. 3. Visualization of NHCPs in the telomeric end of polytene chromosome 4 of *D. hydei*. (*A*) Salivary glands from late third instar larvae. (*B*) Salivary glands from middle third instar. The NHCPs were visualized by anti-NHCPs IgGs.

The existence of transcription units adjacent to the Z-DNA-forming sequence was revealed by hybridization of clone pH71 containing the 2.7-kb *Pst* I–*Hin*dIII DNA stretch to poly(A)$^+$ RNA. Three transcripts of ≈2.7, ≈1.6, and ≈0.60 kb were revealed (Fig. 5*A*). The 2.7- and 1.6-kb transcripts were detected in 4- to 24-hr embryos and in all larval stages up until late third instar. The 0.60-kb transcript was only present in larvae from late third instar. Thus, it is likely that the high level of expression of the 0.60-kb transcript may correlate with the inducibility of subdivision 4-75C$_2$. The direction of transcription goes from left to right in the restriction map shown in Fig. 4, as indicated by hybridization with a single-stranded probe labeled by primer extension from *Sph* I to *Pst* I.

Fig. 5*B* shows that the Z-DNA-forming sequence is an integral part of the 2.7- and 1.6-kb transcripts since both RNA bands were labeled by pF17. The 0.60-kb transcript has to be transcribed from a DNA sequence located downstream of the Z-DNA-forming sequence in the direction of transcription, since pF17 does not hybridize with this RNA band and a DNA fragment located to the right of pF17 (pH7s2) between *Taq* I and *Sph* I hybridizes only with the 0.60-kb RNA band (Fig. 5*C*). Since, moreover, neither pH7s1 nor any other clone located to its right hybridizes with the 2.7-, 1.6-, and 0.6-kb transcripts, the 3′ end of the 2.7- and 1.6-kb and of the 0.6-kb transcripts should be located upstream from *Taq* I and *Sph* I, respectively. At present we do not know the 5′ end of the transcription unit coding for the 2.7- and 1.6-kb transcripts since all the subclones from B11 extending upstream from pF17 hybridize with these RNA bands. It is likely that the 2.7- and 1.6-kb RNA bands represent two transcripts synthesized from a single structural unit having different transcription origins or alternative splicing and that the transcript of 0.60 kb is synthesized from an independent unit of transcription. Thus, the molecular data indicate that the Z-DNA-forming sequence is located in between two units of transcription, one of which, containing the potential Z-DNA

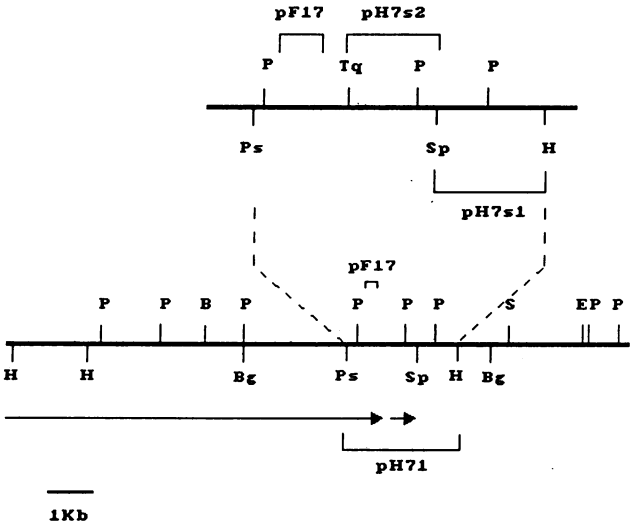

FIG. 4. Restriction map of the 12-kb *Drosophila* insert. The position of pF17 and subclones pH71, pH7s1, and pH7s2 are indicated. Arrows indicate the direction of transcription of both transcription units. S, *Sal* I; P, *Pvu* II; H, *Hin*dIII; B, *Bam*HI; E, *Eco*RI; Bg, *Bgl* II; Ps, *Pst* I; Tq, *Taq* I.

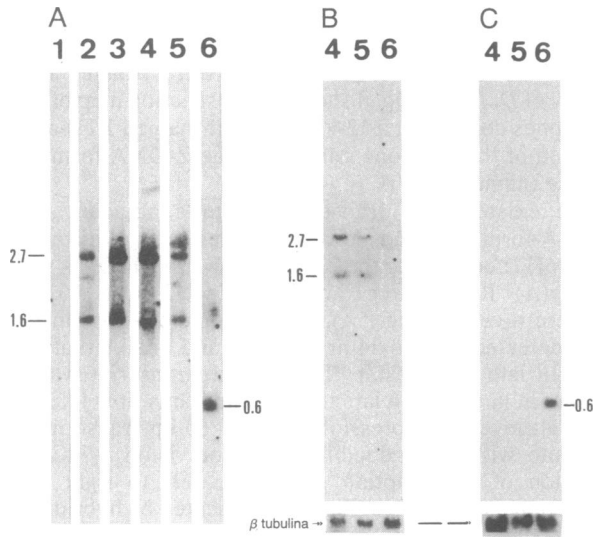

FIG. 5. Northern blot analysis of the transcripts in 4-75C_2. Poly(A)$^+$ RNA from different stages of development was hybridized to clone pH71 (*A*), to clone pF17 (*B*), and to clone pH7s2 (*C*). Lanes: 1, 0- to 4-hr embryos; 2, 4- to 24-hr embryos; 3, first instar; 4, second instar; 5, middle third instar; 6, late third instar. Under each lane in *B* and *C* hybridization to β-tubulin RNA is shown as an internal control.

sequence, is expressed in embryonic and early larval development but specifically repressed during late third instar while the other is active at that moment.

Sequence Analysis of the Z-DNA Flanking Regions. The analysis of the coding strand between *Pst* I and *Sph* I (Fig. 6) indicates the presence of three putative AATAAA polyadenylylation signals within an A-rich environment at positions 668, 1389, and 1452. According to the Northern blot data, we think that the signal at position 668 located downstream of the Z-DNA-forming sequence probably marks the 3′ end of the 2.7- and 1.6-kb transcripts and those at positions 1386 and 1452 indicate the 3′ end of the 0.6-kb RNA. We found a probable Hogness box at position 884 with a 5′-TATATA AT-3′ sequence 550 base pairs upstream of the polyadenylylation sequence at position 1386. In addition, a 5′-CCAAT-3′ sequence is found 10 base pairs downstream from the putative TATA. Sequences that could also match a consensus TATA box, although with lower homology, are found in positions 658 and 678. Thus, we think that the DNA sequence and the Northern blot data indicate that the corresponding inducible RNA molecule of 0.60 kb could be entirely transcribed from the sequence between nucleotide positions 668 and 1389. The gene coding for this transcript should be collinear with the mature mRNA since we have not identified any consensus sequence for splicing.

Since subregion 4-75C is induced during late third instar and by β-OH-ecdysterone, we searched for sequences with significant similarity to the DNA binding domain of the ecdysone receptors. We found a 5′-TGCACTTG-3′ sequence 550 base pairs upstream from the TATA box at nucleotide 318, included within the 60-nucleotide fragment of pF17 shown to be sufficient for induction of B–Z transitions in supercoiled plasmids (25). That sequence is identical to the right half of the DNA-binding domain of the ecdysone receptor reported for the *hsp27* gene (33). Motifs similar to the TGCACTTG sequence but with an inverse orientation were also found at nucleotide positions 853 and 789 flanking a (dC-dA)-rich DNA stretch (Fig. 5). This DNA stretch adopts the Z-conformation in supercoiled plasmids, as indicated by its binding to anti-Z-DNA antibodies (data not shown).

DISCUSSION

The results presented in this paper not only show that Z-DNA domains exist *in vivo* in subdivision 4-75C_2 in the polytene chromosomes of *D. hydei* but also that specific sequences from this subdivision are in transient B–Z flux during late third instar. Since no other sequence from the 12-kb DNA fragment from 4-75C_2, except F-17 and the (dC-dA)-rich DNA stretch, shows specific affinity for anti-Z-DNA antibodies when included in plasmids, it is likely that the Z-DNA epitopes detected in 4-75C_2 derive from the Z-conformation adopted by any one or both of these sequences. However, our data cannot rule out the possibility that the Z-DNA reactivity observed in 4-75C_2 may originate from another Z-DNA-forming sequence located in the interband of C_2 since

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CTGCAGCCATCGGATGTGGAACTATGGCTAGGCAGGGCATGGGCATGTGCATGGGCCTGCGACTCCTCCCTAATAAGCTATTTTGTCCGTTTTTAGAAAC 100

AATTCCTATGCCTCAAGGCGGCTGCGGGGTGGCAGCGGCACGCAGCGAAACTAGTGTGTGTGGGTGGGAGATGATGTATACAGCTGCTGGCTGGGGGCTA 200

AGGGCTGGGGGCTGGGGGCTGGGGGCTGGGGGCGTGTGTGCCATACTGCTGTGAGACCATGGGCGGCCAGCTGCTGAGCATCGAACATGAATTGCCGTGC 300

     F17[-----------------------------------------------------------------------------------------
TCGTCGTGGATCGTTCGtgcacttgAGTTCCGGGCCACTCGCTCCACCAGCGCGCTCGCTCGCTCGCCTGTCCGTTTGGGCCTCTCGCTCGCACCGCTCC 400
-----------------------------------------------------------------------------------------
CAAAAATTGACTATCCTTCTCTTACAGTTCGTTTCGTTTCGTGTCGGCATTTCAATATTTTTTTGCTTCTGCTTCTTCTTTCGTTTTGTGAATAGAATTT 500
-----------------------------------------------------------------------------------------]
TTCAACAATTTGGCTGTCTTAGAGTTTTCGAAATTTTGGAGCGTAGTTGCAGTGTGTGCCGACGCGTCAGCGTATACGGATCGGTTCGCTATAGTTTTTC 600

AATCAACCCTACAAAATAGACCACATCAAGGAGTATTCCAAAAACTAAAAAAAAAACTATAAATGTTAATAAAAAGTTATAAACTATTTGTGGTTGTTTC 700

                                                                                          [---
ACAAATCGAAGTGCTTTGATATACATCAGGATATATGGGTAGGAAGTGTTTCAGTCAACTGCGGAAGTGGGCGTTGTGCCTGTTGCCAAGTTCcaaatgc 800
-----------------------------------------------------------------]
aCATATATATAGACACACATAGACACACACACACACTCACACACACAcatatgcaCACATATATGTATATAATTTTCGGCTTTCCAATATTTCACTC 900

                                                  *
AACTCTGCGCTGCCTTTTGGCTAAAAATATTTTCCGTTAAAAACGTCGAAAAGTCCAAAAAGAAATATGAAAAAATCCACTCGTCTCAGCGCCTGCCTGC 1000
                                  *                                                *
CGCCTGTTTTTCATAATTTTTCATCTTTATTTATTTATCGTATAGATGAAAATTTTGCTCACGAACTTGCGTTCTCATTTGATTTCGCATGTCGTCATTT 1100

TTGTTTATCCCCCACCACAGCCCAACAATCTGCACAGCTTGCCGCAGCCTTTGTCTCGTGGGAGGAGCAAAAGGAATACACTGTCTTCGTATTGCCAGCC 1200

TAATGAAACAAATCACAATTTAATAGGCACAGCGCGCAGTTTACAACTTCCTTGATATGACTATATATATAGAGAGCGAGATATATATATATATTTATAT 1300

ATGTTACATATATCTGTGTGGCATGGGAAGACAGCTGTGTGCTCTTTGGCAGATGCCATGCTGCGTATGTGGGGGAAGTCCATAAAAAAATAAAAGAAAA 1400

AATGCAAAGAAACAGAACTAAAACAAAATCAAACCAGCACACAGTTATAAAAATAAAACTATTTTTGTATTTTTGATATTTGTATTTATGTATTTTTGTA 1500

CTTCTGTTTGGCATGC  1516
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FIG. 6. Nucleotide sequence of the DNA fragment between *Pst* I and *Sph* I. The Z-DNA-forming sequences are overlined between brackets. The putative TATA boxes, polyadenylylation signals, and CCAAT box are underlined. The sequences with homology to the ecdysone response element are indicated in small letters. The homology is detected in the direct ($^{5'-Gtgcacttg-3'}_{3'-Cacgtgaac-5'}$) and inverse ($^{5'-caaatgcaC-3'}_{3'-gtttacgtG-5'}$) and ($^{5'-catatgcaC-3'}_{3'-gtatacgtG-5'}$) orientation. These three sequences share homology in the tgcac motif in both orientations. The asterisks indicate the first AUG of each of the longest ORFs. The first sequence between brackets (F-17) corresponds to the insert in pF17 (25).

the DNA content of this subdivision is somewhat larger (15–17 kb; unpublished data) than the cloned 12-kb fragment.

The cytological and molecular data suggest that the B–Z transition in 4-75C_2 might have been generated by affecting the local level of DNA supercoiling during induction of transcription of the gene specific for late third instar. This hypothesis can be endorsed by the sequence data that show that the potential Z-DNA-forming sequences are located upstream of the transcription unit coding for the 0.6-kb RNA molecule induced during late third instar and by the expectations deduced from the twin-supercoiled-domain model of transcription (19). According to this model short purine-pyrimidine alternations would only be induced to adopt the Z-form when located upstream of a transcribed gene in response to the negative supercoiling "waves" generated behind the moving RNA polymerase (34). Thus, it is likely that F-17 and the (dC-dA)-rich DNA fragment could adopt the Z-DNA form when negative supercoiling "waves" are generated during synthesis of the 0.6-kb transcript and that the local torsional stress in the neighborhood of the potential Z-DNA-forming sequences should be similar to or higher than that experienced by these sequences in plasmids. The presence, moreover, of NHCPs in the transcriptionally induced state of 4-75C_2 may be an additional factor influencing either Z-DNA formation or stabilization.

At present we cannot make any firm statement on whether the Z-transition in 4-75C_2 is only a consequence of increased transcription or whether it also plays a specific physiological function. We think that, since the transcriptional unit located upstream from the induced gene is repressed at the developmental period in which the Z-DNA forms, it may well be that the Z-DNA form in F-17 may function as a biological silencer of the gene located upstream from it by inhibiting the transcriptional movement of the RNA polymerase toward the polyadenylylation site. If this hypothesis were true, the conformational flexibility of the DNA in 4-75C_2 could be envisioned as an evolutionary developed molecular mechanism that could function as a transcriptional block when the Z-DNA generates as a consequence of increased transcription of the gene located 3′ downstream. We cannot exclude, however, that repression of the large transcripts at the time of the Z-DNA formation may be due to a control located at the 5′ end of the gene.

We think that the Z-DNA form in 4-75C_2 may also enhance the induction of the gene by affecting DNA topology or its affinity with regulatory factors or proteins, since sequences homologous to the right half of the ecdysone-responsive element of the *hsp27* gene (33) are found in F-17 and at both sides of the (dC-dA)-rich DNA fragment. In fact, it was suggested that a 22-fold increase in the binding of the estrogen receptor to poly(dA-dC)·poly(dG-dT), in the presence of polyamines, is the result of induction of the Z-DNA form (35) and that poly(dC-dA)·poly(dG-dT) sequences may be involved in the control of the rate of transcription (36, 37). Also, DNA stretches of poly(dC-dA)·poly(dG-dT) have been detected in the regulatory regions of particular genes (13, 15, 38, 39) including those induced by steroids (17). The existence of some correlation between Z-form and ecdysone induction may be reinforced, however, by the fact that all of the early ecdysone-inducible loci of the polytene chromosomes of *D. hydei* (27) have Z-DNA epitopes (ref. 24; unpublished observations). Thus, our results suggest that under physiological conditions short Z-DNA-forming sequences from particular chromosomal domains may adopt the Z-form through generation of negative supercoiling and that this conformation may negatively or positively affect DNA template function (19, 38, 40) depending on their position within structural genes.

We are grateful to Dr. B. D. Stollar for providing the antibodies against Z-DNA and DNA·RNA hybrids. We are also indebted to M. A. Fuertes for technical assistance. This work was supported by Grant PB88-0011. The institutional financial support of Fundacion Ramòn Areces is also acknowledged.

1. Wells, R. D. (1988) *J. Biol. Chem.* **263**, 1095–1098.
2. Rich, A., Nordheim, A. & Wang, A. H. (1984) *Annu. Rev. Biochem.* **53**, 791–846.
3. Lafer, E. M., Sousa, R. J. & Rich, A. (1988) *J. Mol. Biol.* **203**, 511–516.
4. Azorin, F. & Rich, A. (1985) *Cell* **41**, 365–374.
5. Nordheim, A. & Rich, A. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 1821–1825.
6. Zacharias, W., Jaworski, A., Larson, J. E. & Wells, R. D. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 7069–7073.
7. Blaho, J. A. & Wells, R. D. (1987) *J. Biol. Chem.* **262**, 6082–6088.
8. Fishel, R. A., Detmer, K. & Rich, A. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 36–40.
9. Jaworski, A., Hsiel, W.-T., Blaho, J. A., Larson, J. E. & Wells, R. D. (1987) *Science* **238**, 773–777.
10. Witting, B., Dorbic, T. & Rich, A. (1989) *J. Cell. Biol.* **108**, 755–764.
11. Lancillotti, F., López, M. C., Arias, P. & Alonso, C. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 1560–1564.
12. Kmiec, E. B. & Hollorman, W. K. (1986) *Cell* **44**, 445–554.
13. Hamada, H. & Kakunaga, T. (1982) *Nature (London)* **298**, 396–398.
14. Vasicek, T. J., McDevitt, D. E., Freeman, M. W., Fennick, B. J., Hendy, G. N., Potts, J. T., Rich, A. & Kronenberg, H. M. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 2127–2131.
15. Kilpatric, M. W., Klysic, J., Singleton, C. K., Zarling, D. A., Jovin, T. M., Hanau, L. H., Erlanger, B. F. & Wells, R. D. (1984) *J. Biol. Chem.* **259**, 7268–7274.
16. Peck, L. J. & Wang, J. C. (1985) *Cell* **40**, 129–137.
17. Naylor, L. H. & Clark, E. M. (1990) *Nucleic Acids Res.* **18**, 1595–1602.
18. Banerjee, R. & Grunberger, D. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 4988–4992.
19. Tsao, Y.-P., Wu, H.-Y. & Liu, L. F. (1989) *Cell* **56**, 111–118.
20. Rahmouni, A. R. & Wells, R. D. (1989) *Science* **246**, 358–363.
21. Alcover, A., Izquierdo, M., Stollar, B. D., Kitagawa, Y., Miranda, M. & Alonso, C. (1982) *Chromosoma* **87**, 262–277.
22. Silver, L. M. & Elgin, S. C. R. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 423–427.
23. MacGillivray, A. J., Cameron, A., Krauze, R. J., Rickwood, D. & Paul, J. (1972) *Biochim. Biophys. Acta* **227**, 384–402.
24. Lancillotti, F., López, M. C., Alonso, C. & Stollar, B. D. (1985) *J. Cell. Biol.* **100**, 1759–1766.
25. Jiménez-Ruíz, A., Requena, J. M., Lancillotti, F., Morales, G., López, M. C. & Alonso, C. (1989) *Nucleic Acids Res.* **17**, 4579–4588.
26. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
27. Crowley, T. E., Mathers, P. H. & Meyerowitz, E. M. (1984) *Cell* **39**, 149–156.
28. Berendes, H. D. (1965) *Chromosoma* **17**, 35–77.
29. Lehrach, H., Wozney, D. J. M. & Boedtker, H. (1977) *Biochemistry* **16**, 4743–4751.
30. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
31. Berendes, H. D. (1963) *Chromosoma* **14**, 195–206.
32. Morales, G., Requena, J. M., Jiménez-Ruiz, A., López, M. C., Ugarte, M. & Alonso, C. (1990) *Gene* **93**, 213–219.
33. Riddihough, G. & Pelham, H. R. B. (1987) *EMBO J.* **6**, 3729–3734.
34. Liu, L. F. & Wang, J. C. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 7024–7027.
35. Thomas, T. & Kiang, D. T. (1988) *Nucleic Acids Res.* **16**, 4705–4720.
36. Shen, L. P. & Rutter, W. J. (1984) *Science* **224**, 168–171.
37. Hayes, T. E. & Dixon, J. E. (1985) *J. Biol. Chem.* **260**, 8145–8156.
38. Huijser, P., Hennig, W. & Dijkhof, R. (1987) *Chromosoma* **95**, 209–215.
39. Pardue, M. L., Lowenhaupt, K., Rich, A. & Nordheim, A. (1987) *EMBO J.* **6**, 1781–1789.
40. Peck, L. J., Nordheim, A., Rich, A. & Wang, J. C. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 4560–4564.