
H-DNA and Z-DNA in the mouse c-Ki-ras promoter

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Received August 6, 1991; Revised and Accepted November 5, 1991

GenBank accession no. M16708

ABSTRACT

The mouse c-Ki-ras protooncogene promoter contains a homopurine-homopyrimidine domain that exhibits S1 nuclease sensitivity *in vitro*. We have studied the structure of this DNA region in a supercoiled state using a number of chemical probes for non-B DNA conformations including diethyl pyrocarbonate, osmium tetroxide, chloroacetaldehyde, and dimethyl sulfate. The results demonstrate that two types of unusual DNA structures formed under different environmental conditions. A 27-bp homopurine-homopyrimidine mirror repeat adopts a triple-helical H-DNA conformation under mildly acidic conditions. This H-DNA seems to account for the S1 hypersensitivity of the promoter *in vitro*, since the observed pattern of S1 hypersensitivity at a single base level fits well with the H-DNA formation. Under conditions of neutral pH we have detected Z-DNA created by a (CG)₅-stretch, located adjacent to the homopurine-homopyrimidine mirror repeat. The ability of the promoter DNA segment to form non-B structures has implications for models of gene regulation.

INTRODUCTION

The transcriptional regulatory regions of many eukaryotic genes contain sites hypersensitive to the single-strand-specific nuclease S1 when present either in active chromatin or supercoiled DNA (1,2). Fine mapping has demonstrated that S1 hypersensitive sites are commonly located in homopurine-homopyrimidine domains, regions composed of one strand containing primarily purine residues and complementary pyrimidine-rich strand (for review see ref. 3,4). It was suggested, therefore, that homopurine-homopyrimidine stretches can adopt unusual DNA conformation(s) that determine S1 hypersensitivity (2). Recent studies concerned with the transcriptional regulation of eukaryotic genes have prompted speculation on the functional significance of S1 hypersensitive sites. Homopurine-homopyrimidine stretches have been found to be essential for optimal transcription of several genes including human *c-myc* (5,6), EGF-R (7), *ets-2* (8), chicken $\alpha 2(1)$ collagen (9), mouse *c-Ki-ras* (10), and *Drosophila hsp26* (11).

We and others have previously shown that homopurine-homopyrimidine sequences in supercoiled DNA may exist in a novel DNA conformation, termed H form DNA (3,12–20). The main element of the H form is an intramolecular triple helix formed by the entire pyrimidine strand and half of the purine strand, while the other half of the purine strand remains single-stranded. The existence of a large stretch of single-stranded DNA could explain the observed nuclease sensitivity. We have further proposed that H-DNA, rather than other possible structures, is responsible for the S1 hypersensitivity characteristic for eukaryotic genes (3). In a few cases this hypothesis has been confirmed for promoter DNA sequences. *In vitro*, H-DNA formation has been found for the *Drosophila hsp26* promoter (21), and the chicken β -globin promoter (22). Recently, it was also suggested that H-DNA with several mismatches could be formed in the human *c-myc* promoter (23). However, for most eukaryotic promoters the structural basis underlying the observed *in vitro* S1 hypersensitivity has not been elucidated.

In the present study, we sought to define the structural basis for the nuclease sensitivity exhibited *in vitro* by a region of the mouse *c-Ki-ras* promoter. The *c-Ki-ras* protooncogene is a member of the highly conserved *ras* gene family, whose products play a significant role in signal transduction and regulation of cellular proliferation (24). The promoter region of this gene is GC-rich, and does not contain TATA and CCAAT box elements. However, one functionally important element that has been identified is a homopurine-homopyrimidine region, that *in vitro* exhibits S1 nuclease hypersensitivity (10). We have extended the results of that earlier study to examine the structure of this homopurine-homopyrimidine domain in a supercoiled state using chemical probes specific toward non-B DNA conformations. These reagents include DEPC, OsO₄, CAA, and DMS (for review see ref.4). Identification of the modified sites at a sequence level allowed a determination of certain structural peculiarities associated with supercoiled promoter DNA. We found that two different non-B DNA structures could appear. Under mildly acidic conditions H-DNA is formed in the homopurine-homopyrimidine stretch, while Z-DNA is formed by the downstream d(C-G)₅-stretch under neutral pH. These data suggest that H-DNA and Z-DNA may play a functional role in the regulation of *c-Ki-ras* gene expression.

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MATERIALS AND METHODS

Plasmid DNA

Plasmid pKRS-413 containing an AhaII-MstII fragment of the mouse *c-Ki-ras* promoter in the pSVAOCat vector has been described in (10). The plasmid DNA was isolated by the alkali lysis technique followed by equilibrium centrifugation in cesium chloride-ethidium bromide gradients (25).

Chemical modifications of DNA

Supercoiled pKRS-413 DNA was modified under neutral (25 mM Na Cacodylate, pH 7.1; 1 mM EDTA; 100 mM NaCl) or mild acidic (20 mM Na Acetate, pH 4.5; 1 mM EDTA; 100 mM NaCl) conditions. Each reaction mixture contained 10 μ g of DNA in a 100 μ l volume. CAA was added up to 2%, and the samples were incubated 1 hr at 37°C. OsO₄ was added up to 2 mM in the presence of 2 mM of bipyridine, and the samples were incubated 15 min at 37°C. DEPC was added up to 3% followed by 30 min incubation at 20°C. At the end of modification 5 μ l of 1M NaCl was added to all samples, which was followed by two subsequent ethanol precipitation. The samples were then washed with 70% EtOH and dried. DMS was added up to a 0.5% for 1 min at 15°C. The reaction was stopped with 50 ml of standard DMS-stop solution (26) followed by ethanol precipitation. The samples were then washed with 70% EtOH and dried.

S1 nuclease treatment

5 μ g of supercoiled pKRS-413 DNA was treated with 1 U of the S1 nuclease (Sigma) at 10°C for 4 min in 20 mM Na Acetate, 100 mM NaCl, 1 mM ZnSO₄. The sample was then twice deproteinized with phenol, 2-times ethanol precipitated, washed with 70% ethanol and dried.

Mapping of the modified sites at a sequence level

Modified and control DNA samples were hydrolyzed by restriction enzyme AvaI (see Fig. 1). Top and bottom strands were ³²P-labelled using T4 polynucleotide kinase or Klenow fragment of DNA polymerase I, respectively. A second digestion with BglI was followed by isolation of 170 bp end-labelled fragments from a 6% native polyacrylamide gel. For a sequencing ladder we have made standard Maxam-Gilbert reactions (26). Samples modified with OsO₄, DEPC and DMS were treated with 1M piperidine for 30 min at 90°C. CAA-modified samples were treated with either formic acid, or hydrazine in high salt, followed by piperidine treatment. After piperidine treatment samples were dried, dissolved in 80% formamide, 1 mM EDTA and loaded on 8% polyacrylamide gel with 7M urea.

RESULTS

The DNA sequence of the mouse *c-Ki-ras* gene is presented in Fig. 1. This region has been cloned into a plasmid, referred to as pKRS-413 (10). Earlier it was shown (10) that this region contains a 27-bp-long homopurine-homopyrimidine mirror repeat (shown by arrows) that *in vitro* exhibits S1 hypersensitivity (underlined). Based on its sequence, this mirror repeat has potential to adopt the H-conformation, a structure that would be sensitive to single-strand-specific nucleases (3). Just downstream of the homopurine-homopyrimidine domain is a d(C-G)₅-stretch. This sequence could form Z-DNA, which may also contribute to nuclease hypersensitivity (27). These sequence elements

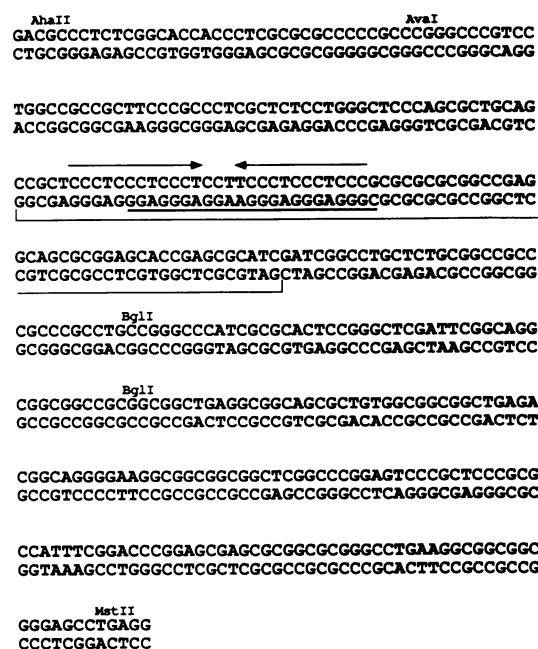


Fig. 1. Sequence of the mouse *c-Ki-ras* promoter cloned into the pSVAOCat vector. Arrows show homopurine-homopyrimidine mirror repeat. The region hypersensitive to S1 nuclease according to (10) is underlined. Brackets mark the area that was found essential for promoter activity in a transient transcription assay (10).

overlap by 1 bp. Deletion analysis shown that these elements are located in the part of the promoter that is essential for its activity (10). Thus, both sequence domains that have the potential to adopt unusual DNA conformations are located in the functionally important area of the promoter of this protooncogene.

Chemical hyperreactivity of the *c-Ki-ras* promoter in supercoiled DNA

Experiments were carried out to determine if unusual structures could indeed form in that region of the *c-Ki-ras* promoter involved in transcription control. To do this, supercoiled DNA of plasmid pKRS-413 was treated with the chemical probes C-AA, OsO₄, DEPC, and DMS. These reagents are reactive toward different bases and sensitive to particular DNA conformations, as previously detailed (for review see ref.4). Briefly, CAA preferentially interacts with single-stranded adenines and cytosines forming their ethenoderivatives. OsO₄ forms nonplanar esters with single-stranded thymines. DEPC carboxyethylates the N⁷ position of purines, when they are single-stranded, or in a *syn* conformation. DMS methylates the N⁷ position of guanines in all cases with the exception of Gs involved in Hoogsteen base pairing. Two types of environmental conditions were used: neutral (20 mM Na Cacodylate pH 7.0, 100 mM NaCl) and mildly acidic (20 mM Na Acetate pH 4.5, 100 mM NaCl). The last conditions are close to optimum for the S1 nuclease treatment. Samples were radioactively end-labelled and analyzed on sequencing gels. The primary data on chemical modification are presented in Fig. 2, and summarized graphically in Fig. 3.

At the neutral pH one can see a clear-cut chemical hypersensitivity only at the d(C-G)₅-stretch. All guanines in this stretch are hyperreactive toward DEPC. Two guanines located

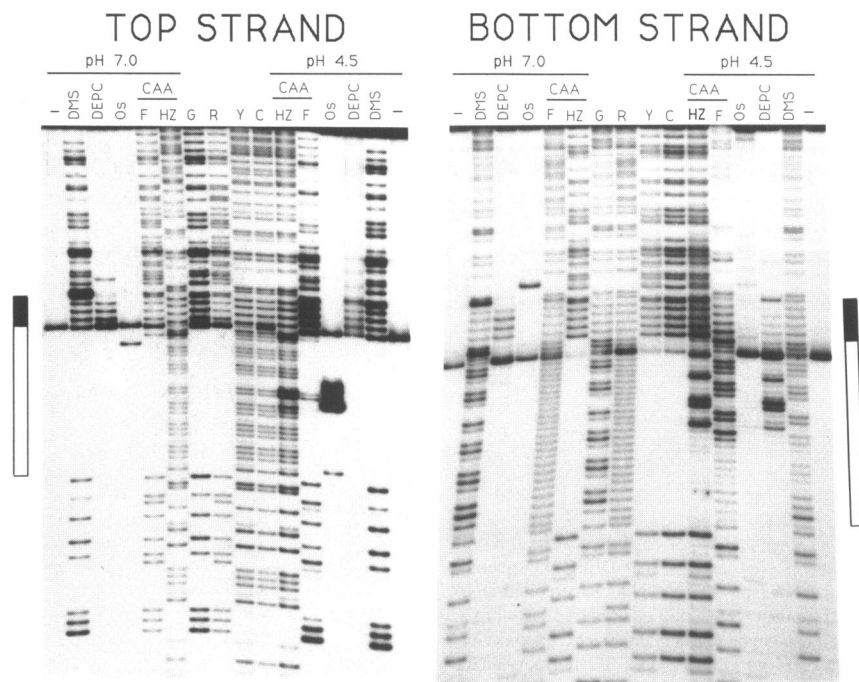


Fig. 2. Patterns of chemical modification of the mouse *c-Ki-ras* promoter in supercoiled DNA. Samples were treated with: —no chemicals, DMS, DEPC, Os—OsO₄, CAA (F—formic acid treatment prior to piperidine cleavage, HZ—hydrazine treatment prior to piperidine cleavage). G, R, Y, and C—standard Maxam—Gilbert sequencing ladders for guanines, purines, pyrimidines, and cytosines, respectively. Top and bottom strands according to Fig.1. Top strand was labelled at the 5'-end with the T4 polynucleotide kinase. Bottom strand was labelled at the 3'-end with the Klenow fragment of DNA polymerase I. Black box shows the position of the d(C-G)₅-stretch; white box shows the position of the homopurine-homopyrimidine stretch.

3' from the stretch are hypermethylated by DMS. A cytosine located 5' from the stretch at the top-strand is hyperreactive toward CAA. Finally, OsO₄ preferentially modifies adjacent thymines in both strands. One also can see modification by DEPC of guanines and adenine 3' from the stretch at the top-strand. Purines in the Z-conformation are hyperreactive toward DEPC (28,29), and DNA bases at B-to-Z junctions are preferentially attacked by single-stranded DNA-specific chemicals, including CAA and OsO₄ (29—31). Thus, our data provide evidence for formation of Z-DNA in the d(C-G)₅-stretch (see also Discussion).

At an acidic pH, the patterns of chemical modifications change dramatically (see Fig.2). The modification signals at the d(C-G)₅-stretch either disappear or become less prominent. Instead, very strong modification sites appear inside the homopurine-homopyrimidine tract. One can see that DEPC and CAA preferentially attack the 5'-part of the purine strand. Meanwhile, guanines in the 3'-part of the purine strand are partially protected against methylation with DMS. In the pyrimidine strand we detect a very prominent reactivity toward CAA and OsO₄ of three thymines and two cytosines at the middle part of the mirror repeat.

Previously we and others have observed (14—19) preferential modifications of the 5'-part of the purine strand, methylation protection in the 3'-part of the purine strand, and hyperreactivity of the central portion of the pyrimidine strand in H-form DNA. We have now demonstrated the same features in the *c-Ki-ras* promoter region. We conclude, therefore, that the homopurine-homopyrimidine domain in the *c-Ki-ras* gene adopts the H-conformation under acidic pH (see also Discussion).

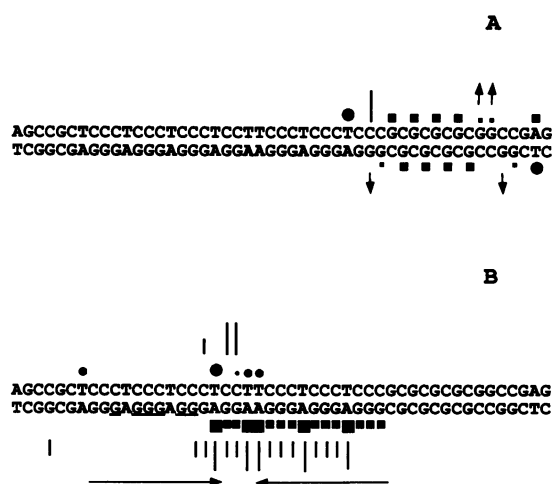


Fig. 3. Chemically hyperreactive sequences at the mouse *c-Ki-ras* promoter. A—pH-independent modification sites; B—pH-dependent modification sites. Modifications: squares—DEPC; circles—OsO₄; lines—CAA; vertical arrows—hypermethylation by DMS. Guanines protected against methylation are underlined. Horizontal arrows show the homopurine-homopyrimidine mirror repeat.

The structural transitions observed under both neutral and acidic pH were supercoil-dependent, because we didn't see any chemical reactivity in linear DNA (Fig.4A). This agrees well with previous observations that DNA supercoiling facilitates Z- and H-DNA formation (12,27).

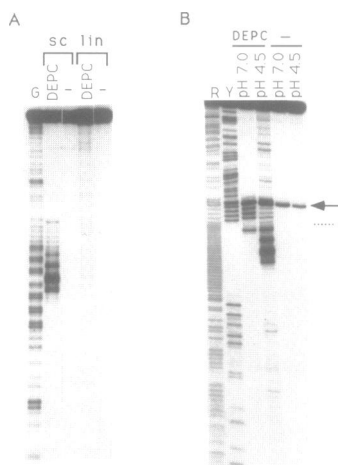


Fig. 4. A. Supercoil-dependence of chemical hyperreactivity of the *c-Ki-ras* promoter. Samples were treated with: —no chemicals or DEPC. G—guanine sequencing ladder; sc—supercoiled DNA; lin—linear DNA. Supercoiled DNA and end-labelled *Ava*I—*Bgl*II linear fragment were modified at pH 4.5 as in Fig.2. B. Dependence of mobility artifactual band upon polyacrylamide gel concentration. The same samples as in Fig.2 (8% gel) were loaded on a 5% polyacrylamide gel. R—purine ladder; Y—pyrimidine ladder; arrow indicates a novel position of the artifactual band, dotted line shows its relative position in an 8% gel.

A careful look at the data in Fig.2 shows a band that appears near the potential Z-H boundary in almost every lane, including the control. To understand whether this band reflects specific cleavage of DNA at this position or is an artifact of our experiments, we eluted the band from the gel and sequenced by Maxam—Gilbert protocol. We found that the sequence of this band corresponds exactly to the sequence of the whole 170-bp *Ava*I—*Bgl*II fragment we used in our chemical analyses (data not shown). We concluded that this band represents a portion of the *Ava*I—*Bgl*II fragment, which was not denatured under our electrophoretic conditions and, therefore, migrates faster than the corresponding single-stranded DNA fragment. This is not surprising because of the 81% GC content of this fragment. The co-migration of this band with the 'Z-H' boundary is an artifact of the 8% denaturing polyacrylamide gels we used in Fig.2. In a lower percentage polyacrylamide gel it migrated to a different position (Fig.4B), which allowed us to additionally clarify the patterns of chemical reactivity at the 'Z-H' junctions.

S1 hypersensitivity in the *c-Ki-ras* promoter

We have also extended the previous finding (10) that an S1 hypersensitive region of the *c-Ki-ras* promoter coincides with the position of the homopurine-homopyrimidine domain by examining the pattern of S1 cleavage at the nucleotide level. Supercoiled DNA was treated with the S1 nuclease to convert approximately half of the molecules into an open circular form. The amount of linear DNA was very low; thus, we believe that under our experimental conditions the enzyme makes predominantly single-stranded cuts in supercoiled DNA. The samples were then end-labelled and analyzed on a sequencing gel.

The data are presented in Fig.5 and demonstrate that the major cleavage sites are located within the homopurine-homopyrimidine stretch. We also detect minor cleavage sites at the boundaries of the d(C-G)₅-stretch. A comparison of the intensity of the major and minor signals suggests that at least 90% of the S1 hypersensitivity observed is associated with the homopurine-

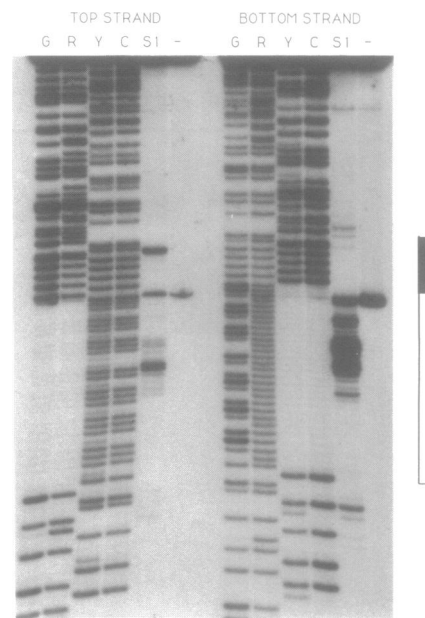


Fig. 5. S1 hypersensitivity of the mouse *c-Ki-ras* promoter in supercoiled DNA. Samples were treated with: —no enzyme; S1—nuclease S1. G, R, Y, and C—standard Maxam—Gilbert sequencing ladders for guanines, purines, pyrimidines, and cytosines, respectively. Top and bottom strands according to Fig.1. Black box shows the position of the d(C-G)₅-stretch; white box shows the position of the homopurine-homopyrimidine stretch.

homopyrimidine tract. Further, fine mapping of the cleavage sites shows that they are located at the 5'-part of the purine strand and middle portion of the pyrimidine strand. The cleavages in the purine strand are the most prominent. Thus, the pattern of the S1 hypersensitivity correlates well with the chemical reactivity described above. Taken together, these results provide evidence that the S1 hypersensitive structure in the *c-Ki-ras* promoter is the H-form DNA involving homopurine-homopyrimidine mirror repeat.

DISCUSSION

Our data show that two unusual DNA conformations, Z-DNA and H-DNA, can be formed under certain environmental conditions in the mouse *c-Ki-ras* promoter. At a neutral pH we detect chemical hyperreactivity in the d(C-G)₅-stretch that fits well with the Z-DNA formation. However, the exact length of the DNA segment adopting the Z-form is not clear. Indeed, the modification by DEPC (characteristic for Z DNA) is observed not only for guanines in the alternating GC-sequence, but for the adjacent guanines as well. Those guanines are also hypermethylated by DMS. We also observe chemical hyperreactivity of the AT base pairs located 4-bp upstream and 5-bp downstream from the d(C-G)₅-stretch. These data could be explained in two ways. The first explanation is that only the d(C-G)₅ stretch adopts the Z-form, while chemical reactivities of the adjacent DNA bases are due to the distortions at the B-to-Z junctions that are at least 5-bp long (Fig.6A). However, this cannot explain the hypermethylation of some guanines by DMS, and it is inconsistent with earlier observations that the B-to-Z junctions are less than 2 bp-long (32). The second possibility is that a larger DNA piece actually adopts the Z-conformation

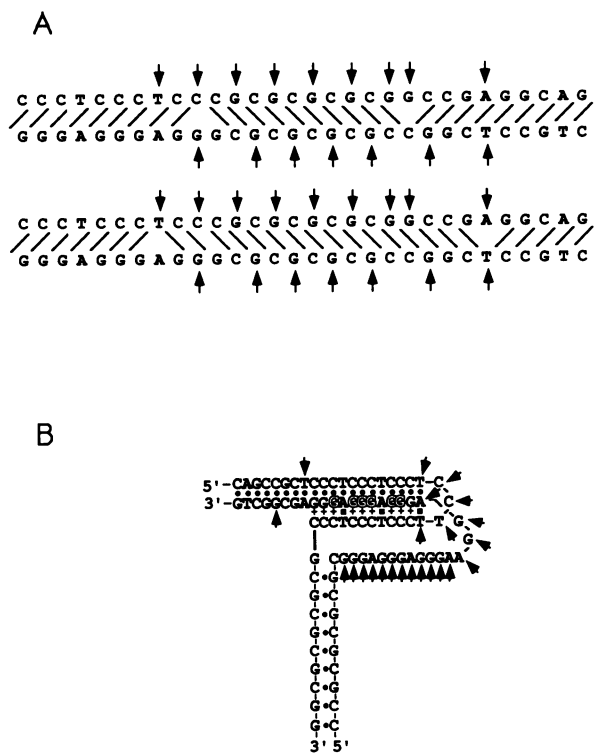


Fig. 6. Accordance between chemical hyperactivities and the formation of non-B DNA structures at the mouse *c-Ki-ras* promoter. **A**—Z-DNA; inclined lines show double helix handedness. Top and bottom figures differ in the length of the DNA segment involved in the Z-form formation. Arrows show hyperreactivity to one of the chemicals used. **B**—H-DNA, an isoform with the single-stranded 5'-part of the purine strand. Arrows—hyperreactivity to one of the chemicals used. Guanines protected against DMS are outlined.

(Fig. 6A). If this is the case, one CG base pair upstream of the d(C-G)₅ stretch and two GC base pairs downstream of this stretch would exist in an energetically unfavorable conformation while in Z-form (purine in *anti* and pyrimidine in *syn*). The energy difference between the B- and Z-form for the GC base pair in the unfavorable conformation is 2.6 kcal/mole, compared to 0.33 kcal/mole in favorable conformation (purine in *syn* and pyrimidine in *anti*) (33). This could be compensated for, however, because a 16-bp-long segment instead of 10-bp-long segment is now involved in the formation of the Z-form DNA, releasing 2.9 supercoils instead of 1.8 supercoils under structural transition. This possibility likely explains the observed methylation data, because guanines that are out of phase in the Z-conformation are preferentially methylated with DMS (29). The statistical mechanical consideration of Z-DNA formation in the *c-Ki-ras* promoter, provided by an algorithm described in (34), led to the following conclusions (A.V.Vologodskii, personal communication). The mid-point of the B-Z transition is at a superhelical density -0.045 . For $\sigma = -0.06$ (native superhelical density of isolated DNA samples) the probabilities of particular bases to be in the Z-form are:

T	C	C	C	G	C	G	C	G	C	G	C	G	C	C	G	A
0	0.2	0.2	0.8	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.8	0.1	0.1	0.1	0

This calculation fits well with our experimental data. We believe that our data agree with the second possibility, although the alternative explanation cannot be completely ruled out.

At an acidic pH we observe a hyperreactivity of the homopurine-homopyrimidine domain which suggests the formation of the H-DNA. Three kinds of evidence strongly support this conclusion. First, the hyperreactivity of the homopurine-homopyrimidine stretch is clearly pH-dependent. Second, one can see an asymmetry in chemical modification between the purine and pyrimidine strands. Only the central part of the pyrimidine strand is modified, while in the purine strand the modification is prominent throughout its 5'-half. H-DNA is the only structure that predicts such a strand asymmetry. Two isoforms of the H-form could in principle exist (3,13), and were detected for long homopurine-homopyrimidine sequences (20). Usually, however, the isoform in which the 5'-part of the purine strand is single-stranded would be dominant (14–18). This is also true for the homopurine-homopyrimidine sequence in the *c-Ki-ras* promoter. Third, the H-DNA model predicts the protection against methylation of guanines in the triple helix, because their N⁷-positions are involved in the Hoogsteen hydrogen bonding (14). For the *c-Ki-ras* sequence, we actually detect DMS protection of guanines in the 3'-part of the purine strand. This should be the case for the preferential isoform of the H-DNA. In Fig.6B we marked modification sites on the H-DNA model for the *c-Ki-ras* promoter. One can see that the results obtained with the chemical probes are in excellent agreement with the model.

The modification patterns under acidic pH show that chemical reactivities corresponding to the Z-DNA formation also exist, though less prominently than at neutral pH. We cannot say now whether this reflects the existence of Z-DNA and H-DNA in the same DNA molecules or different structures in different DNA molecules. The second possibility seems more attractive because of two reasons. First, Z- and H-forming DNA sequences are overlapped, so the formation of one structure could sterically prevent the formation of the other. Second, the formation of one structure would release torsional tension required for the formation of the other. Additional experiments are necessary to positively discriminate between the two possibilities.

It is intriguing to speculate on the way non-B DNA structures might affect gene expression if they were to occur *in vivo*. One attractive idea is that the formation of H-DNA may promote unwinding of GC-rich promoters like that of *c-Ki-ras*, thus facilitating the process of transcription. Alternatively, the formation of unusual DNA structures in the promoters could result from releasing of transcription-driven torsional tension (35). Finally non-B DNA structures could mediate or alter the binding of specific regulatory proteins. Indeed, there are data on protein binding to potential H-forming sequences in eukaryotic promoters, including *c-Ki-ras* (6,10,11,36).

An unusual feature of the mouse *c-Ki-ras* promoter is that it contains a Z-forming sequence that overlaps with the H-forming sequence. Z-triplex motifs have also been found in the nontranscribed spacer of the rat rDNA (37), the origin of replication of the Chinese hamster *dhfr* amplicon (38), and a site of unequal chromatid exchange in the mouse myeloma MPC-11 (39). The formation of H-DNA and Z-DNA under different environmental conditions was also detected in the human U1 gene, though in this case the corresponding DNA sequences were located at a distance (17). The biological role of Z-triplex motifs is not clear. One possibility might be that a competition between Z- and H-DNA could provide an effective regulatory switch. Careful deletion and mutational analysis combined with functional assays are needed to address this point.

It is interesting to note that one point substitution from T to C at the middle of the *c-Ki-ras* homopurine-homopyrimidine sequence makes it a simple repeat (CCCT)_n. A search of GeneBank DNA sequences shows that this motif is widespread among the non-coding regions of eukaryotic genes, including some promoters, many introns, and satellite DNA (data not shown). In most cases, however, there is no juxtaposition with the Z-like sequences. Homopurine-homopyrimidine motifs frequently occur in DNA regions involved in transcription control, recombination and replication. The abundance of these sequence elements and their likely role in mediating biologically important processes reinforces the need to better understand their ability to adopt unusual DNA conformations and the functional significance of these structures. Such studies may require the development of novel techniques or reagents to directly probe DNA conformations *in vivo*.

ACKNOWLEDGEMENTS

We thank Brian Johnston, Alex Vologodskii, Oleg Voloshin, and Charles Cantor for valuable discussions, and Angela Tyner for great help with the manuscript. This research was aided by grants from the American Cancer Society, Illinois Division, Inc., and the University of Illinois at Chicago to S. M. M.

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