Nucleic Acids Research, 1992, Vol. 20, No. 24 6583–6587

A conserved DNA structural control element modulates transcription of a mammalian gene

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Received September 14, 1992; Revised and Accepted November 18, 1992

ABSTRACT

The mammalian dihydrofolate reductase (DHFR) gene promoters contain several conserved sequence elements which bind protein, and yet there are other conserved DNA sequences that do not footprint. We report here that mutation of one of these conserved non-footprinting regions increases transcription from this promoter both *in vitro* and *in vivo*. We show that this conserved region is flanked by sites hypersensitive to cleavage by methidiumpropyl-EDTA-Fe(II). Furthermore, multimers of a double-stranded oligonucleotide comprised of this region display faster migration through polyacrylamide than control DNA. The difference in mobility is not the result of bending, nor does the primary sequence contain features that would predict altered mobility. We propose that this 'Structural Control Element' is rigid and down-regulates transcription by inhibiting interactions between proteins binding adjacent to this region.

INTRODUCTION

Transcription of eukaryotic genes has been shown to be controlled by the interaction of specific proteins with DNA sequence elements in the promoter region. A large group of class II eukaryotic promoters is characterized by the presence of a TATAA element 25 to 35 bp upstream of the transcription start. However, a potentially larger group of genes lacks the TATAA element and is instead usually characterized by a GC-rich promoter and multiple binding sites for the transcription factor Sp1. A well characterized gene within the TATAA-less class is that encoding dihydrofolate reductase (DHFR). The DHFR promoters of the hamster (1,2), mouse (3) and human (4) have been studied; all three species have two perfectly conserved overlapping binding sites for the transcription factor E2F adjacent to the major transcription initiation site. E2F binding is required for efficient transcription *in vitro* and *in vivo* from the hamster DHFR promoter (5). The hamster and mouse DHFR promoters also have four similarly positioned Sp1 binding sites (Fig. 1), while the human DHFR gene has two Sp1 binding sites. We have found that Sp1 is absolutely required for DHFR transcription (6) and the interaction of Sp1 with the two most proximal GC boxes appears to regulate the efficiency of transcription initiation at the downstream start site (7). Thus, the sequence elements in the DHFR promoter that bind protein are required for maximal promoter activity and are conserved among species.

There is an additional sequence element, which is also highly conserved in these mammalian DHFR promoters, located immediately 3′ to the Sp1 binding GC boxes. There is one copy in human (4), three copies in mouse (3) and two copies in hamster (1,2). When these six sequences are aligned the consensus is revealed as 5′ CTNNGCTGCACAAATAGGANGCNGGGC 3′, where a base is specified if it occurs in at least four of the six sequences. This sequence, which we have designated 'SCE', lies between the Sp1 binding site closest to the major initiation site (designated GC box I, Fig.1) and the start of transcription which overlies the E2F binding site; it is also present 3′ to GC box II in the mouse and hamster promoters, and adjacent to GC box III in the mouse promoter, GC boxes being numbered in order of increasing 5′ distance from the start of translation. It is part of the '48 bp repeats' of the mouse promoter (3) and spans the conserved region that was previously designated 'Element 3' (1).

The SCE sequence in the hamster DHFR promoter does not show a DNAse I footprint with either HeLa or CHO nuclear extract (6,7, and Fig. 3A), implying that protein does not bind to this region; however, experiments in which this sequence has been mutated, including site-specific mutations *in vitro* (7,8) and linker-scanning mutations *in vivo* (9), suggest a potential, albeit subtle, repressive role in transcription. To further investigate the potential role of this region, we constructed a mutant (ETC) in which the majority of the SCE sequence closest to the major transcriptional initiation site (3′ to GC box I) was altered while leaving the GC box intact.

MATERIALS AND METHODS

Constructs

The plasmids used in these experiments are depicted in Fig. 1. The wild-type DHFR expression plasmid contains hamster DHFR promoter sequence from position −210 to −23 (+1 = A of ATG) joined to sequence coding for the bacterial gene

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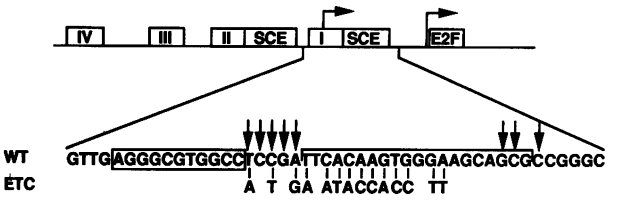

Figure 1. Diagrammatic representation of the hamster DHFR promoter. Boxes on the upper line designate regions of sequence conservation among the mouse, hamster and human DHFR promoters. The boxes designated by Roman numerals correspond to GC boxes, the binding sites for the transcription factor Sp1. The binding site for the transcription factor E2F is indicated. The conserved element 3′ to GC boxes I and II has been designated SCE. Arrows indicate the transcription start sites. The sequence below the diagram is that from position −116 to −71 in the hamster DHFR promoter (+1 is the start of translation). The sequence protected from MPE cleavage in the presence of nuclear extract is boxed, and is largely comprised of an Sp1 binding site. Vertical arrows indicate the nucleotides that are hypersensitive to MPE cleavage in the absence of nuclear extract (Fig. 3B). The bracket indicates the sequence synthesized to test the electrophoretic mobility in the experiments shown in Fig. 4. WT indicates the wild type sequence; ETC indicates the mutations present in the ETC mutant analyzed in Figs. 2 and 3 and corresponds to bacterial chloramphenicol acetyl transferase coding sequence between nucleotides +16 and +33 (A of ATG = +1).

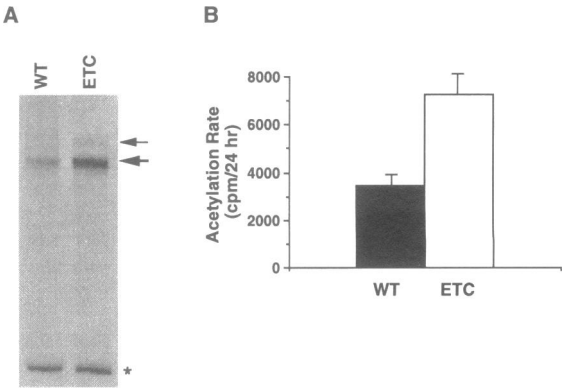

Figure 2. A. *In vitro* transcription. The products of transcription initiated *de novo* from linearized wild-type (WT) and mutant (ETC) plasmids are shown. The upper two bands shown by arrows (780 and 736 nucleotides respectively) are the transcription products which correspond to transcription initiated at the start sites shown in Fig. 1. The lowest band (shown by an asterisk) is an internal control for sample recovery. The gel shown is representative of 4 separate experiments. **B.** *In vivo* transient transfection. The acetylation counts resulting from transfection of plasmids containing the bacterial CAT gene driven by either wild type or mutant ETC promoters. Samples were normalized for transfection efficiency on the basis of cotransfected placental alkaline phosphatase activity. The bar represents the standard error of the mean (n=5).

chloramphenicol acetyl transferase (pDHF-210/CAT) as described previously (6). The plasmid ETC was constructed by site-directed mutagenesis (10). This mutation consisted of replacing part of the SCE with coding sequence from the bacterial chloramphenicol acetyl transferase gene as shown in Fig. 1.

In vitro transcription and *in vivo* transient expression

DHFR promoter wild-type and ETC mutant constructs digested with Sca I were used as templates for *in vitro* run-off transcription reactions (7). These templates were incubated with CHO nuclear extract in the presence of ribonucleotides (including α^{32}P-CTP), and the reaction mixtures were processed and electrophoresed on 4% polyacrylamide/urea gels. A pre-made 494-base Sp6 transcript was added to the reaction mixtures before processing as a control for sample recovery. CHO cells in monolayer were transfected by calcium phosphate co-precipitation (11) with 10 μg of either the wild-type construct ('WT') or mutated ('ETC') construct and 5 μg of an internal control RSV promoter/placental alkaline phosphatase reporter construct (12) (kindly provided by Tom Kadesch). CAT activity was determined in a transient transfection assay from 5 replicate plates 48 hours after transfection. The CAT activity was measured by the utilization of ^{3}H acetyl Co-A in a liquid scintillation assay (13) for 24 hours at room temperature, which is within the linear range of the assay.

DNAse I footprinting

DNA from wild type (WT) and ETC mutant (MU) constructs was digested with Hind III and Sac I to release 220 bp fragments. Fragments were asymmetrically end-labelled by filling in the Hind III site with α^{32}P-dATP and *E.coli* DNA polymerase I (Klenow fragment). A portion of the end-labelled fragments (100,000 cpm) was chemically modified with DMS and piperidine to generate a co-linear G-specific cleavage ladder (14). Footprinting reactions were carried out by incubating 0.1 to 1.0 ng of end-labelled DNA in the absence or presence of nuclear extract from CHO cells (60 μg) in binding buffer (7) containing 42 μg/ml poly(dI/dC). After incubation for 10 min. at room temperature, the reaction mixture was adjusted to 2 mM $CaCl_2$. DNAse I was added to final concentrations of 0.01 μg/ml for naked DNA and 1.0 μg/ml when nuclear extract was present. Digestion was allowed to proceed at room temperature for 4 minutes prior to stopping the reaction with addition of EDTA to a final concentration of 20 mM, followed by multiple phenol/chloroform extractions. Footprints were resolved by electrophoresis of digested DNA under denaturing conditions through 5% polyacrylamide/7 M urea gels and visualized by autoradiography after fixing and drying of the gel.

Methidiumpropyl-EDTA-Fe(II) footprinting

A wild type DNA fragment was end labelled and incubated with 10 μM MPE(Fe II) (kindly supplied by Peter Dervan) and 1 mM DTT for 7 minutes at room temperature, ethanol precipitated, resuspended in deionized formamide and electrophoresed on an 8% polyacrylamide/urea gel next to a co-linear G ladder as described above. The gel was then fixed, dried and exposed to X-ray film. The exposed film was then scanned with an LKB Ultroscan laser densitometer.

Electrophoretic mobility assay of mulitmerized double stranded oligonucleotides

20 μg of each oligonucleotide to be annealed as shown in Fig 4 were mixed and incubated 15′ at room temperature in ligation buffer. Annealed oligonucleotides were ligated into multimers in 50 mM Tris pH 7.5, 10 mM $MgCl_2$, 2 mM DTT, 5% PEG 8000, 800 μM ATP, 10 U T4 DNA ligase (kindly supplied by Jack Griffith) in a total volume of 110μl for 10 hours at 4°C. Half of each ligation reaction was then chloroform extracted and ethanol precipitated. The precipitates were resuspended in 20 mM Tris pH 8.0, 10 mM $MgCl_2$ and treated with 20 U shrimp alkaline phosphatase at 37°C in a 20 μl reaction volume for one hour and then incubated at 65°C for one hour to inactivate the phosphatase. The dephosphorylated multimers were kinased by the addition of 20 μCi γ^{32}P ATP, DTT to 5 mM, and 10 U T4

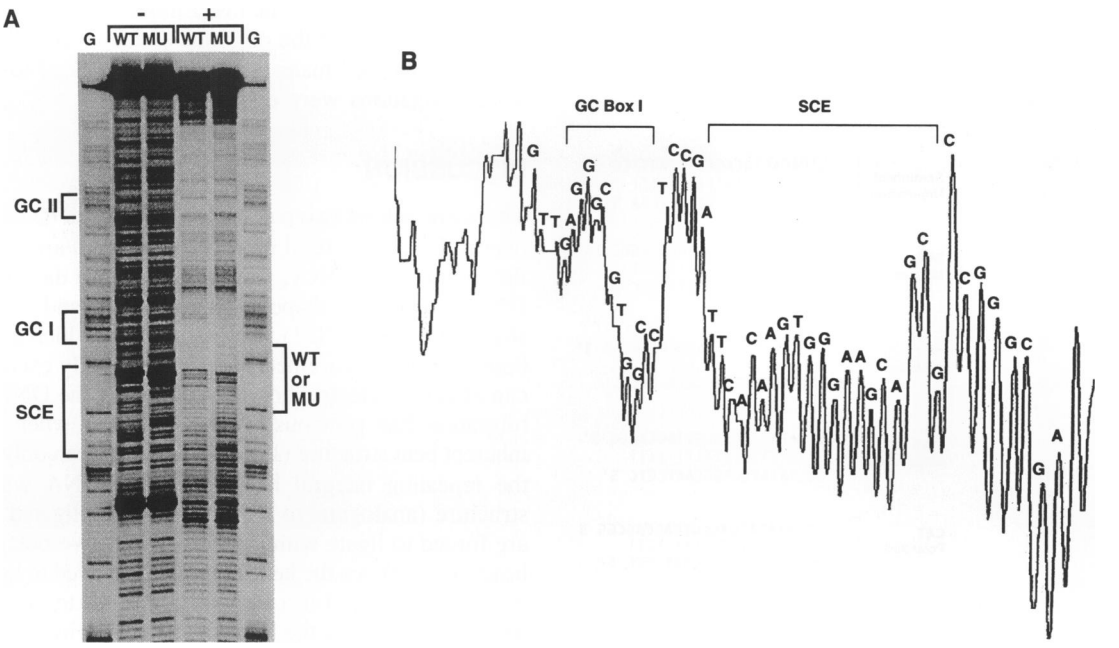

Figure. 3. A. DNAse I footprint. Wild-type (WT) and ETC mutant (MU) promoter footprints in the absence (−) and presence (+) of CHO nuclear extract. The co-linear G reaction ladder ('G') was included to localize the protein/DNA binding regions. Brackets on the left side demarcate the location of the various DNA sequence elements. 'SCE' indicates the region between the MPE hypersensitive sites shown in panel B; 'GC I' indicates the margins of GC box I; 'GC II' indicates the margins of GC box II. The bracket on the right side demarcates the region that was mutated in ETC. **B.** MPE cleavage of the DHFR promoter. The densitometer scan of an MPE footprinting gel of the wild-type promoter in the absence of nuclear extract is shown. The locations of GC box I and the SCE are indicated by brackets.

polynucleotide kinase at 37°C for one hour. Kinase reactions were stopped with 2 μl 500 mM EDTA. Glycogen (20 μg) was then added to the kinased multimers which were then ethanol precipitated, phenol/chloroform extracted, re-precipitated and resuspended in TE. Aliquots were run on a 5% native polyacrylamide (19:1 crosslink) gel that was subsequently fixed, dried and exposed to X-ray film.

RESULTS

The SCE represses transcription

Functional assays showed that transcription was significantly increased from the SCE-mutant ETC promoter compared to that from the wild-type promoter with an intact SCE. In an *in vitro* transcription assay, transcription from ETC was increased over that from a wild type promoter approximately two-fold (Fig. 2A). This increased activity was also observed in an *in vivo* transient transfection assay using Chinese hamster ovary (CHO) cells (Fig. 2B). These assays of the effect of this mutation therefore demonstrated a transcriptionally repressive function for the SCE sequence.

Mutation of the SCE does not alter protein footprints

Having shown that this sequence served to down-regulate DHFR transcription, we sought to address the nature of the repressive mechanism. To determine whether protein/DNA complexes forming on either side of the SCE were affected by the ETC mutation or whether this mutation resulted in new protein/DNA interactions, DNAse I and methidiumpropyl-EDTA-Fe(II) footprinting of the region between GC box I and the E2F binding site were performed in the wild type and mutated construct in the presence of nuclear extract. The ETC mutation did not affect the DNAse I footprint over the Sp1 binding site, nor was there any obvious change in the cleavage pattern within the mutated sequence (Fig. 3A). Using the more sensitive assay of *in vitro* MPE footprinting, no changes were seen in the boundaries of the region protected from MPE cleavage between wild type and ETC mutant sequence in the presence of nuclear extract, consistent with the DNAse I results (data not shown).

The SCE is bounded by hypersensitive MPE cleavage sites

Increased sensitivity to hydroxy radical attack has been shown to occur in kinetoplast DNA adjacent to sequences containing phased adenine tracts. It appears that this hypersensitivity is due to a localized 'relaxation' as the DNA progressively changes from a B form to a bent conformation (15). The localized transition in DNA topology has been modelled using synthetic oligonucleotides and appears to correlate well with a widening of the minor groove resulting in greater accessibility to chemical attack (16). In the absence of nuclear extract, clearly defined regions of MPE cleavage hypersensitivity flanked the SCE (Fig. 3B). These hypersensitive regions were located at the 3′ end of GC box I and at the 3′ end of the entire conserved region (Fig. 1). We inferred from these data that the increased MPE cleavage on either side of the SCE could indicate a structural transition in DNA conformation. In contrast to other DNA sequences that have been shown to possess altered conformation, the primary sequence of this region does not have obvious features that would predict altered structure. There is no alternating purine/pyrimidine motif such as that found in Z DNA (17 and references therein). The sequence does not possess inverted repeats that would be predictive of hairpin structures. There are no phased runs of adenines that would predict bent DNA (18 and references therein).

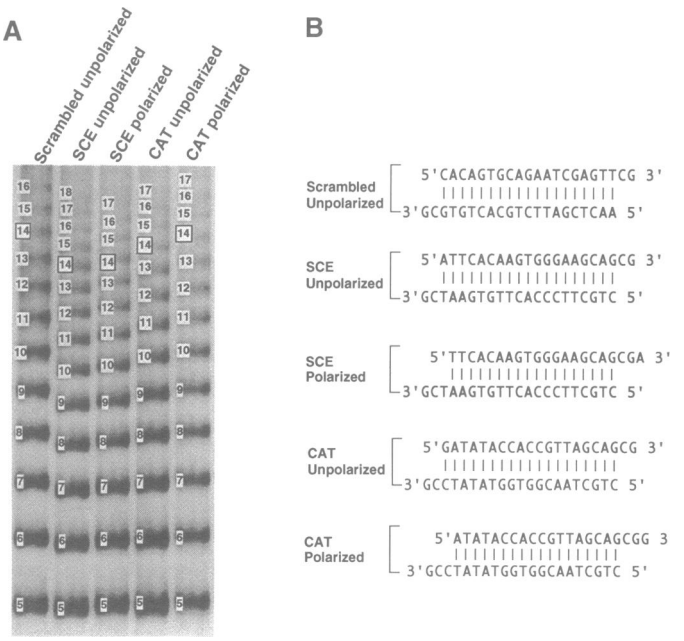

Figure 4. Electrophoretic mobility of multimerized oligonucleotides. Multimers of the various combinations of annealed oligonucleotides shown in panel **B** were electrophoresed through a polyacrylamide gel which was then dried and exposed to X-ray film. The bands are numbered according to the number of 21 base pair units that were ligated together to form the band. The gel shown is representative of 12 separate experiments.

The SCE shows anomalously fast electrophoretic migration

Measurement of electrophoretic mobility is a method used to detect altered DNA structure. Bent DNA, for example, displays retarded mobility through polyacrylamide gels (19). This change in mobility is revealed by use of a multimerization assay which involves measurement of ligation products that vary in length by multiples of the initial oligomer. To examine the DNA structure of the SCE, we synthesized several different 21 bp oligonucleotides (two helical turns) that were designed to test the effect of the primary sequence, the sequence composition, and the orientation of ligation products on electrophoretic mobility. The oligonucleotides used in this analysis are shown in Figure 4B. To test the effect of the primary sequence and the nucleotide composition, we synthesized two different control oligonucleotides to compare to the SCE; these included an oligonucleotide, identical in GC content to the SCE, consisting of the CAT coding sequence used in the ETC mutant, and an oligonucleotide of identical nucleotide composition as the SCE but in randomly scrambled order. To test the effect of the orientation of the ligation products, the SCE and CAT oligonucleotides were designed to concatenate in a polarized (head-to-tail) or an unpolarized (random) manner as shown in Fig. 4B.

Initially, we compared the mobility of the ligation products of the SCE and CAT oligonucleotides that were by design only able to ligate in a polarized (head-to-tail) manner. The faster migration of the SCE than that of the CAT control was detectable in the concatamers containing 8 oligomer units (168 bp), and became more apparent as the number of multimers increased to the point that a 14mer SCE comigrates with a 13mer CAT (Fig. 4A, lane 3 *vs.* lane 5). The faster migration of the SCE multimers also occurred when the oligonucleotides were allowed to ligate in an unpolarized manner in which both head-to-tail and head-to-head ligations were equally possible.

DISCUSSION

There are at least two parameters which can be used to describe overall DNA structural morphology. These are (i) the degree of flexibility of the DNA, which is related to the ease with which DNA can alter its shape in solution (20), and (ii) the preferred shape of the DNA (21), the extremes of which are straight and bent. These two parameters are not mutually exclusive and both can affect the electrophoretic mobility of the DNA. Anomalous migration has previously been observed when DNA has an inherent bent structure (19); however, this can only be seen when the repeating integral helical units of DNA with the altered structure (analogous to the 21 base units ligated in this study) are forced to ligate with a specific repetitive polarity, e.g. only head-to-tail. When the helical units are allowed to ligate in random orientation, a bend in one direction tends to be cancelled by a bend introduced in the opposite direction by ligation of a unit with reverse polarity. As can be seen in Fig. 4A, there is a difference between forms ligated with directed *vs* random polarity (lanes 2 and 3); however, a considerably larger discrepancy in mobility is seen between the randomly oriented ligation products of the wild type SCE sequence as compared to the randomly oriented ligation products of an oligonucleotide comprised of identical base composition but with the bases in scrambled order (Fig. 4A, lane 1 *vs.* lane 2). Since there is no overall polarity to either of these ligations, this larger mobility difference cannot be due to bent DNA structures. Further, it has been observed that bent DNA runs more slowly than DNA with normal structural characteristics (19), and our data show that the wild type sequence is in fact running *faster* than the control DNA with the same sequence composition but in scrambled order. Thus we conclude that the structure responsible for the anomalously fast migration is due to neither bending of the DNA nor to differences in G+C content of the constructs, but is instead the result of the primary sequence of the SCE.

We propose two models to explain why the conserved sequence migrates faster relative to random DNA and serves to repress transcription. The first model is that this sequence is more rigid than normal DNA. Theoretical models of DNA migration through polyacrylamide suggest that migration of species of identical length and molecular weight is proportional to the root mean square distance between the ends of each type of molecule (22). Rigid DNA molecules would have a longer persistence length which would serve to increase the root mean square end-to-end distance. Hence the DNA would migrate more rapidly (22). A region of rigid DNA in the DHFR promoter could repress transcription by inhibiting the DNA bending required for cross-talk between protein transcription factors binding to either side of this region (23). In the DHFR promoter, this region lies between the binding sites for Sp1 and E2F and between adjacent Sp1 binding sites (1, Fig. 1). It has also been proposed that producing rigid DNA triple helical regions could repress transcription in a similar manner (24).

The second model which would explain our results is that this conserved region is more compact than normal DNA. In this case, the ligation multimers migrate faster because they are physically

shorter than the scrambled sequence multimers. If this were the case, transcription could be repressed by forcing protein factors which bind adjacent to this region out of favorable helical phase with each other.

Although both models are attractive hypotheses to explain our data, we favor the rigid DNA model for several reasons. The DHFR promoter appears to be nucleosome free *in vivo*, displaying a lack of periodicity when cleaved with micrococcal nuclease (Azizkhan, unpublished). Regions of rigid DNA could help to maintain the promoter in a nucleosome free state by making the wrapping of DNA associated with nucleosomal binding more energetically unfavorable (25). Such a region has not been described in a promoter *per se*; however, it could fulfill a dual role in gene expression: 1) its repressive function would keep the rate of transcriptional initiation from this promoter low, while 2) the inhibition of nucleosomal binding could prevent transcription from this region from being silenced altogether. For a housekeeping gene such as DHFR, which requires low levels of transcription in all cell types, these two functions could account for the high degree of sequence conservation within this region of DNA. Transcription initiation in the hamster DHFR promoter appears to be regulated by the interaction of factors binding to the E2F site and to the Sp1 sites, and in fact the DHFR promoter is activated by a number of different signals such as growth stimulation (26,27), methotrexate treatment (28), and expression of viral immediate early gene products (29,30). These interactions require the appropriate helical relationship between the factor binding sites in order to maintain the correct levels of transcription initiation (Jambou and Azizkhan, unpublished). The SCE, as a result of its position between the binding sites for E2F and Sp1, may repress transcription by inhibiting the interaction between factors bound at these sites, without necessarily inhibiting the independent binding of these factors to their respective sites.

We have shown that a conserved sequence within the DHFR promoter which does not directly bind protein serves as a repressor of transcription both *in vitro* and *in vivo*. We have also shown that this region has an altered DNA structure not predicted by its primary sequence but as demonstrated by its anomalously fast migration through polyacrylamide. We have referred to this sequence as a 'Structural Control Element' (SCE) as it appears capable of influencing a cellular process at the molecular level through its inherent structure, and not through the interaction of a *trans*-acting factor binding to this region. Experiments are underway to characterize the nature of this anomalously migrating DNA structure.

ACKNOWLEDGEMENTS

This work was supported by grants from the NIH (RO1CA56890), the American Cancer Society (C.D. 317) and the March of Dimes (1-1158). AJP is the recipient of a Graduate Fellowship from the National Science Foundation. DEJ is supported by an NRSA NCI Training grant. The authors are indebted to Jeanne Kahn for technical assistance, Dr. Peter Dervan for generously providing MPE, to Michael Wade for review of the manuscript, and to Drs. Aziz Sancar and Jack Griffith for helpful suggestions and careful reading of the manuscript. The authors also thank Dr. Lee Pedersen for providing a computer model of the SCE. The first two authors contributed equally to this manuscript.

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