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Unusual structure of a human middle repetitive DNA

Ratnasinghe, Duminda Devapriya, Ph.D.

East Tennessee State University, 1993

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Unusual Structure of a Human Middle Repetitive DNA

A Dissertation

Presented to

the Faculty of the Department of Biochemistry

James H. Quillen College of Medicine

East Tennessee State University

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy in Biomedical Science

by

Duminda D. Ratnasinghe

December, 1993

Approval

This is to certify that the Graduate Committee of

Duminda D. Ratnasinghe

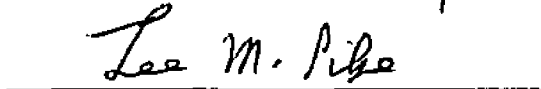
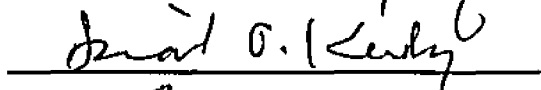
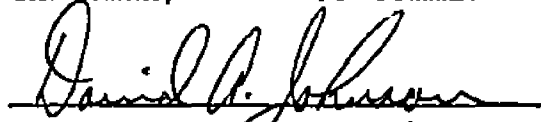
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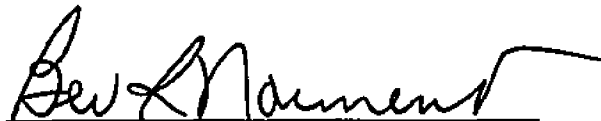
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Chairman, Graduate Committee



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for Research and Dean,
School of Graduate Studies

ABSTRACT

Unusual Structure of a Human Middle Repetitive DNA

by

Duminda D. Ratnasinghe

The L2Hs sequences are a polymorphic, interspersed, middle repetitive DNA family unique to human genomes. Genomic fingerprinting indicates that these DNAs vary from one individual to another and between tissues of the same individual. Sequence analysis reveals that they are AT-rich (76%) and contain many unusual sequence arrangements (palindromes, inverted and direct repeats). These sequence properties confer on the L2Hs elements the potential to fold into non-B-form structures, a characteristic of recombination hot spots. To test this hypothesis carbodiimide, osmium tetroxide and S_1 nuclease were used as single-strand specific probes to study a recombinant plasmid, pN6.4.39, containing a single L2Hs segment. Different forms of the plasmid substrate were analyzed, including linear molecules and circular forms of low, intermediate and high superhelical densities. In addition, plasmid DNA in growing *E. coli* cells were analyzed. Modified plasmid DNA was analyzed by primer extension in a sequencing-type reaction format. These studies demonstrate that the L2Hs sequences: 1. assume non-B-form structures both in vitro and in vivo, 2. map to predicted cruciform structures, 3. behave as C-type extrusion sequences and 4. that these unusual DNA structures are dependent on plasmid superhelicity.

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ABBREVIATIONS

β MCE	β -mercaptoethanol
bp(s)	base pair(s)
BSA	bovine serum albumin
CDI	1-cyclohexyl-3-(2-morpholinyl-1-ethyl)carbodiimide metho-p-toluenesulfonate or water soluble carbodiimide
CIAP	calf intestinal alkaline phosphatase
dH ₂ O	deionized water
DNase	deoxyribonuclease
DTT	dithiothreitol
<u>E. coli</u>	<u>Escherichia coli</u>
EBr	ethidium bromide
EDTA	ethylenediaminetetraacetic acid
ETOH	ethanol
kb	kilobase (1000 base pairs)
LB	Luria-Bertani broth
Lk	linking number
Lk ₀	linking number in a fully relaxed molecule
L2Hs	Line 2 Homo sapien sequence
OD	optical density
PCR	polymerase chain reaction
PEG	polyethylene glycol 8000
RNase A	ribonuclease A
SDS	sodium dodecyl sulfate
SOG	60% sucrose/0.1% orange G (3/1 : V/V)

SSC	0.15 M NaCl, 15 mM sodium citrate, pH 7.0
SSPE	0.18 M NaCl, 10 mM NaH ₂ PO ₄ , 1 mM EDTA, pH 7.5
T ₁₀ E ₁	10 mM Tris-HCl pH 8.4, 1 mM EDTA
T ₁ E _{0.1}	1 mM Tris-HCl pH 8.4, 0.1 mM EDTA
TAE	Tris-Acetate-EDTA
TBE	Tris-Boric acid-EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris-(hydroxymethyl) aminomethane
Tw	twist; winding of strands in double helix about axis
UV	ultraviolet
u	unit
Wr	writhe; crossing of the helix axis by DNA strand

CHAPTER 1

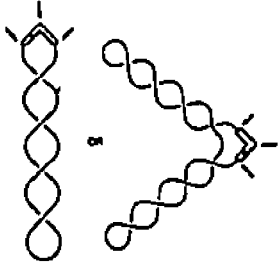
Introduction

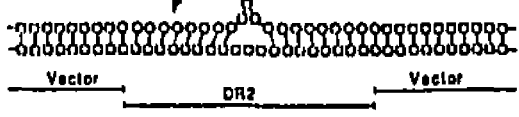
The structure of DNA and the regulation of gene expression remains an area of intense research. There has been an explosion in our understanding of promoter structures, enhancer effects, splicing and the specific interactions of regulatory proteins with these and other genetic elements. However, with this knowledge there has been an increasing appreciation for additional levels of regulatory interactions, some of which are quite subtle, but global in their impact.

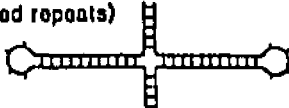
DNA forms one of the main determinants involved in these numerous interactions, defining the structure of a genome and, in large part, the regulation of its expression. In addition to specific DNA sequences (eg. promoters) which play obvious and crucial roles in gene expression, there are other motifs which are structure rather than sequence dependent. These structural features can be assumed by certain sequences under specific conditions and the conformation may form the main recognition motif rather than the primary sequence itself (Kennard and Hunter 1989; Wells 1988). Since these represent DNA conformations other than the standard B-form, as a group they are termed "non-B-form" structures. Such non-B form motifs include Z-DNA, purine-pyrimidine structures, anisomorphic DNA, bent DNA, slipped


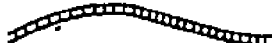
Left handed Z-DNA (therefore B-Z junctions)


Pur. Pyr. Structures


Anisomorphic DNA


Pyrimidine-rich Strand


Cruciforms (at inverted repeats)


Bent (curved) DNA
A-B junctions

Kinetoplast fragments



Slipped structures (at direct repeats)


FIGURE 1. Some unusual secondary structures of DNA (Adapted from Wells and Havey 1988).

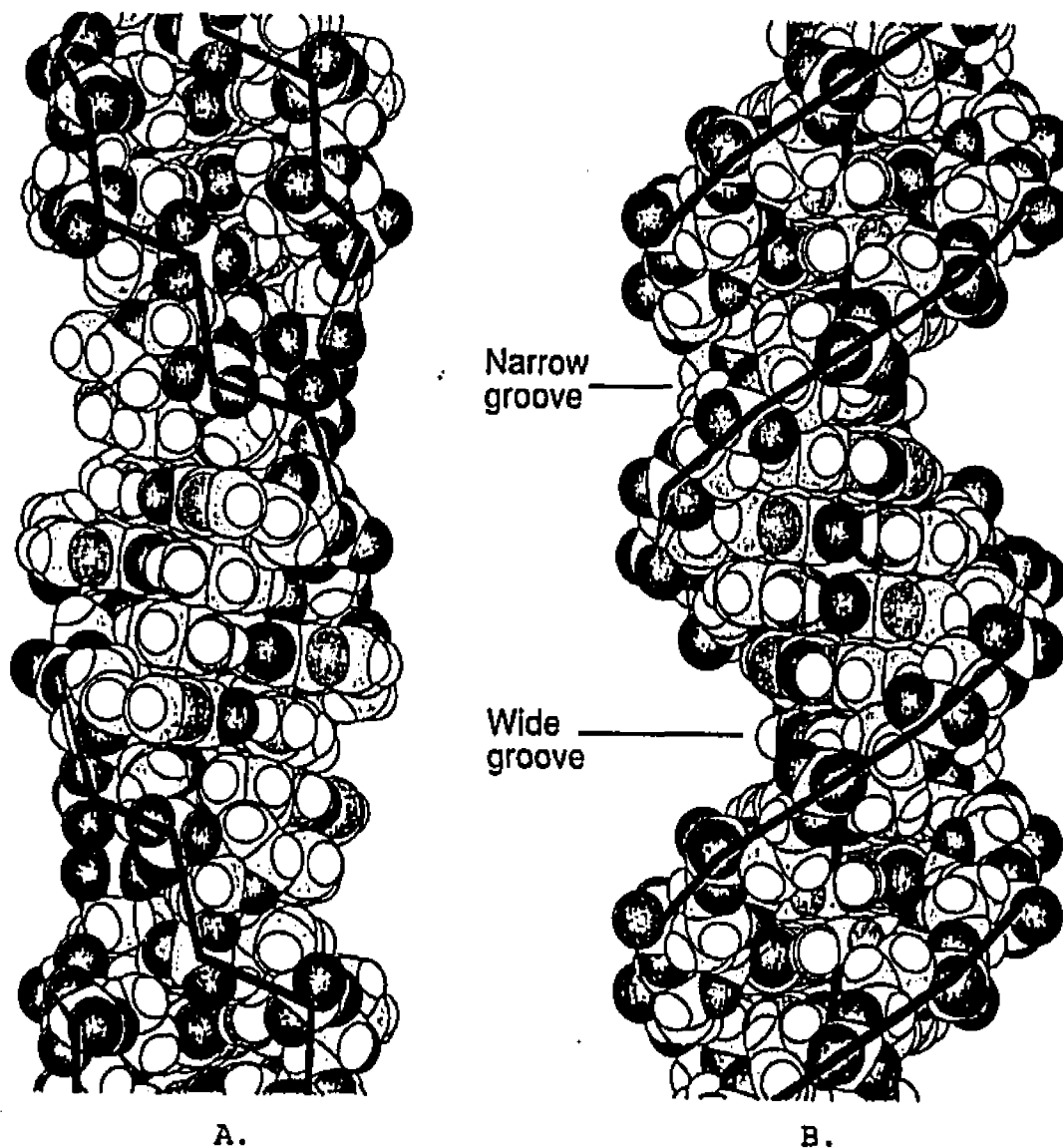


FIGURE 2. Space filling models of A. Z-DNA and B. B-DNA. The irregularity of the Z-DNA backbone is illustrated by heavy lines that go from phosphate to phosphate. In contrast the backbone of B-DNA is a smooth right-handed double helix (Adopted from Zubay 1993).

structures and cruciform DNA structures (Figs. 1 and 2). These non-B-form DNAs depend on certain sequence features plus the macromolecular effects of superhelical density (Cantor et al. 1988) and flanking sequences (Lilley et al. 1988) for their formation. Non-B-form DNAs appear to be critical elements for DNA replication (Benham 1993; Masse et al. 1992), recombination (Mizuuchi et al. 1982; Trinh and Sinden 1993) and transcription (Cantor et al. 1988; Gruskin and Rich 1993). They seem to provide unique binding sites for regulatory interactions and confer receptive DNA conformations on the adjacent sequence elements via their telestability effects (Sullivan et al. 1988). Consequently, elucidating the occurrence and behavior of these unusual DNA structures is necessary for understanding the structure, regulation and function of genomes (see Wells 1988 for review).

Furthermore, the tendency of some DNA sequences to assume unusual structures such as cruciforms may confer specific functional properties on these sequences. For example, topoisomerase II binds to cruciform structures and C-type cruciform-inducing sequences occur upstream from and in intron locations of genes and transposons (McClellan et al. 1986). The work presented in this dissertation concerns the potential for cruciform formation in a cloned human DNA segment. The next section defines the structural aspects of cruciforms and the mechanisms of their formation.

Cruciforms

The cruciform structure is a symmetrical double hairpin loop (Figs. 1 and 3). It was first proposed by Gierer (1966) and has attracted much attention as a potential genetic recognition site (see review in Lilley et al. 1988). The formation of a cruciform requires a dyad symmetrical arrangement of bases (i.e., inverted repeats or palindromes) to permit stem formation. The cruciform is a paranemic structure because two strands of B-form DNA must be physically separated before they can form the stem and loop structures. In closed circular and genomic DNA, cruciforms can be formed only by opening negative superhelical turns or by equivalent sources of linking deficit (Courey and Wang, 1988) (as described below in conditions necessary to stabilize unusual secondary structures). The bases along the cruciform stem are rewound to form an intrastrand B-form helix, so that the stem region becomes geometrically, but not topologically wound. The energy investment is limited to the unpairing of the bases in the loop and at the bottom of the stem. A transition into a cruciform is, therefore, less costly energetically for a supercoiled DNA molecule than is the formation of single-strands of proportional size. An alternating $d[(TG)_6(CA)_6]$ segment, which can form either a cruciform or left-handed Z-DNA, has been found to prefer the cruciform state over the Z state (McLean and Wells, 1988).

The formation of a single cruciform in a supercoiled molecule results in a gain in free energy. This energy is provided by relaxation of negative supercoiling and its magnitude depends on the length of the arms of the cruciform: a cruciform arm of 10.5 base pairs (bp) unwinds the supercoil by one turn. There is also a kinetic barrier to cruciform formation; and Furlong et al. (1989) have suggested two mechanisms for cruciform formation which have clearly distinct physical parameters and may be sequence dependent (see Fig. 3).

The faster process of cruciform formation, the S-pathway, has a ΔG of about 100 KJ/mol with a small positive entropy of activation. This more common pathway of cruciform formation requires a relatively small unpaired region. Following the formation of the single-stranded region a proto-cruciform intermediate is produced which then grows to equilibrium size by branch migration through the four-way junction. The slower mechanism, the C-pathway, involves the formation of a large bubble followed by its condensation to give the fully developed cruciform. This has a ΔG of about 180 KJ/mol with a large entropy of activation (Blackburn and Gait 1990).

The cruciform structure was the first unusual secondary structure demonstrated to exist in vivo (Panayotatos and Fontain, 1987). In fact, cruciform structures can be formed in eukaryotic genomes by the torsional stress generated when

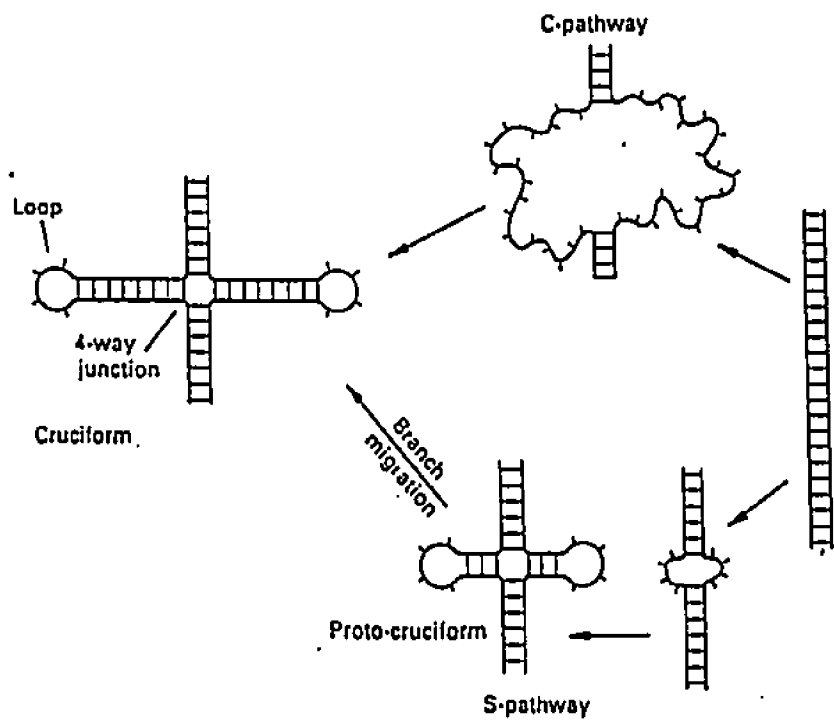


FIGURE 3. Structure of a cruciform and alternate pathways for its formation (Adopted from Blackburn and Gait 1990)

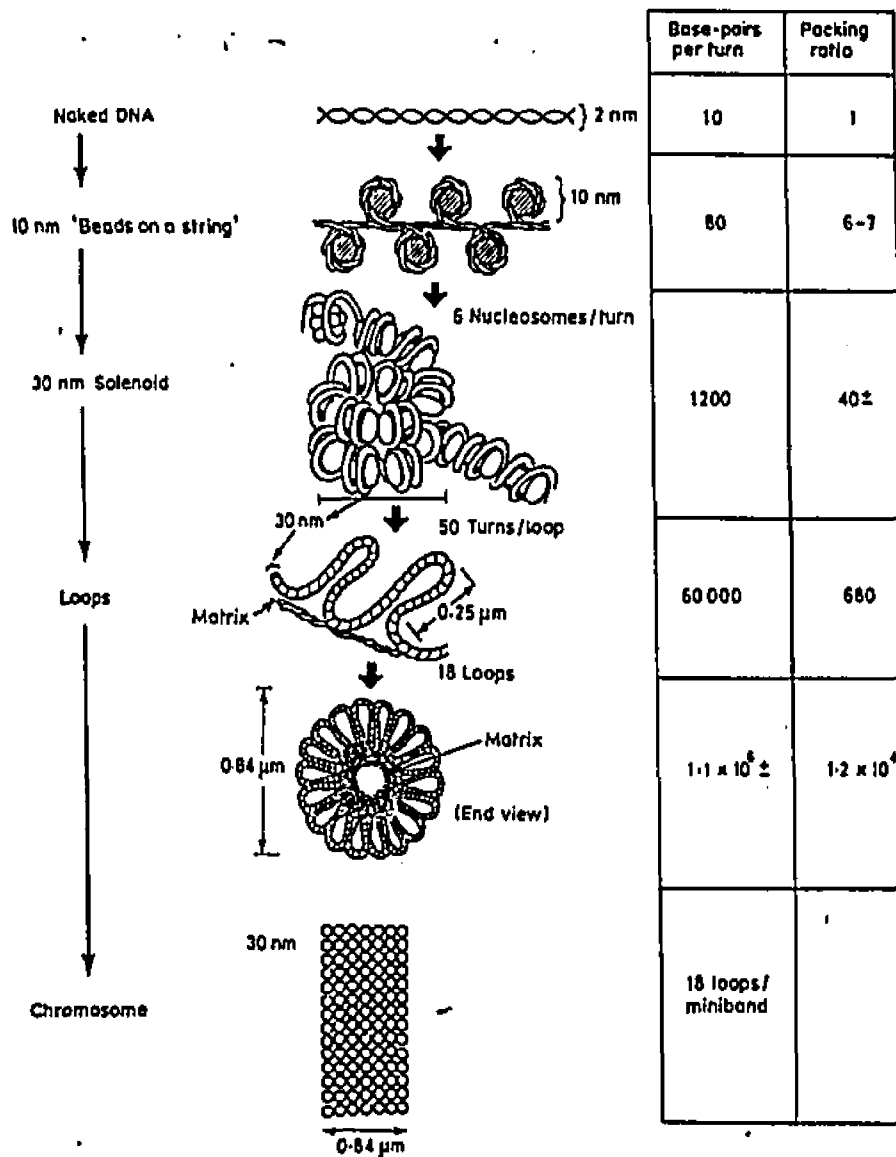


FIGURE 4. A schematic diagram of the higher-order organization of a chromatid. The packing ratio illustrates the degree of compaction attained with chromatid formation. Removal of histones from the 30 nm solenoid will result in a DNA structural form similar to that shown in Figure 5A (Adopted from Adams et al. 1992).

the DNA is wrapped around histones to form nucleosomes and higher order chromatin structures (Fig. 4). This packaging of DNA results in solenoidal (Fig. 5) type supercoiling (Richmond et al. 1984) which stabilizes cruciform structures. Hence, torsionally stressed DNA sequences can assume cruciform structures. These in turn can result in the formation of recombination hot spots. Cruciform structures and other non-B-form structures require specific conditions for their formation and stabilization. These conditions are discussed in the next section.

Conditions Necessary to Stabilize Unusual Structures

The conditions required to stabilize unusual secondary structures in DNA can be quite structure specific. The understanding of these conditions is particularly important since it provides further insight into the types of conformations formed. Negative supercoiling stabilizes several types of unusual structures including Z-DNA (McLean et al. 1986; Ellison et al. 1986) and cruciforms (Greaves et al. 1985). Although supercoiling is not the only stabilizing influence for unusual secondary structures, it is utilized to mediate DNA conformational transitions which may be similar to those induced in vivo by proteins, ionic gradients, temperature, etc... (see Wells and Harvey 1988).

To understand supercoiling, one must understand the properties of closed circular DNA. The linking number (LK)

is a property of DNAs that describes the intertwining of the Watson and Crick strands. Mathematically, it is usually more convenient to use the linking number of the Crick (or Watson) strand with respect to the helix axis (A) because writhe and twist are defined in terms of the axis. The LK of two closed curves (a circular DNA double helix) is a topological property, no matter how the curves are deformed (pulled, twisted etc.); as long as neither strand is broken, LK will remain the same. LK will be 0 for a pair of unlinked (not intertwined) closed curves, 1 for curves that loop together just once, 2 for curves that loop together twice, and so on. Each of these crossing points can be assigned an index number of +1 or -1 according to the direction in which the molecule rotates.

Therefore, for a molecule of relaxed closed circular DNA of 5,000 bp, with 10.5 bp per turn of the helix, the linking number will be $5,000/10.5 = +476.19$. By convention the number is positive because the DNA double helix is right handed.

The linking number can also be defined as a topological state that is the sum of two geometric properties, twist (Tw) and writhe (Wr). This is expressed in the well known equation of White (1969):

$$Lk = Tw + Wr$$

An intuitive feeling can be given for this relationship. Lk is the sum of the signed indices

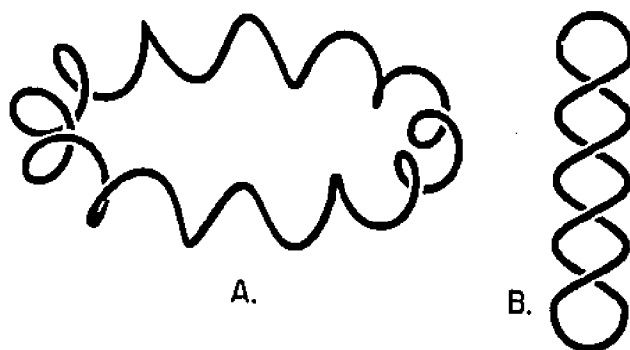


Figure 5. Two forms of supercoiled DNA. The double helix is depicted as a solid line and both DNAs are negatively supercoiled. A. Solenoidal or toroidal supercoiling in which the DNA wraps helically about a circular superhelical axis. B. In plectonemically supercoiled DNA, the DNA winds helically up and back down a superhelical axis. In the plectonemic form, regions of the DNA that are distant in primary sequence wind around each other (Adopted form Cozzarelli and Wang 1990).

associated with the crossing between the Crick strand and the DNA axis. These nodes can arise in either of two ways: the local winding of the strands of the double helix, which is related to Tw , and the crossing of the helix axis itself, which is measured by Wr .

Thus, for DNA, supercoiling refers to the coiling of the double helix, not the individual strands in space. There are two basic forms of DNA supercoiling, solenoidal (or toroidal) and plectonemic (interwound). Solenoidal supercoiling (Fig. 5A) is exemplified by DNA wrapped around histones in nucleosomes (Richmond et al. 1984); plectonemic supercoiling (Fig. 5B) is found in negatively supercoiled DNA in solution (Spengler et al. 1985).

Supercoiling can be defined in two different but related ways. If a DNA molecule free in solution is underwound, i.e., its Lk is less than Lk_0 (Lk_0 = number of base pairs divided by 10.5), the DNA generally assumes the plectonemic supercoiled form, and the number of supercoils is directly proportional to the linking difference, ΔLk , as defined by:

$$\Delta Lk = Lk - Lk_0$$

It is more convenient to describe the linking difference in terms of a length-independent descriptor, the specific linking difference or superhelical density (σ), which is equal to $\Delta Lk/Lk_0$. In addition to supercoiling

there are other stabilizing influences on unusual secondary structures such as salt concentration, pH, temperature, association of proteins with DNA, DNA packing in chromatin, etc. The conformational transitions induced by these conditions are similar to those induced by supercoiling (Wells and Harvey 1988).

Two critical elements in the formation of non-B structures are torsional stress and the presence of C-type extrusion inducing sequences (Murchie and Lilley 1992; Rajagopalan et al. 1990). C-type inducing sequences are generally of the alternating $d(A-T)_n$ form and $\geq 70\%$ AT-rich, permitting them to assume non-B-form conformations when torsionally stressed in superhelical plasmids or other constrained DNA domains (Schaeffer et al. 1989). Formation of this non-B-form motif can induce a nearby inverted repeat sequence to undergo C-type extrusion into paired stem loop structures (i.e. cruciforms); this second cruciform would not form without the telestability effect of the C-type inducing sequence of the first cruciform (Furlong et al. 1989; Sullivan et al. 1988).

The possible global influence of these C-type inducing sequences is suggested by their occurrence in numerous 5' upstream and intron locations in genes, in transposons and in some repetitive elements (McClellan et al. 1986). In addition to inducing cruciform extrusion, these sequences can induce alterations in Z-DNA structures (Rajagopalan et

al. 1990). Thus, these non-B-form AT-rich sequences may have numerous, subtle influences on genome structure.

We have described an interspersed, repetitive DNA in human genomes, termed the Line 2 Homo sapiens (L2Hs) sequence (Musich and Dykes 1986). Most of the work presented here is focussed on determining the presence and locations of unusual secondary structures in a cloned L2Hs element. Genomic fingerprinting reveals that these sequences exhibit qualitative and quantitative population polymorphisms suggesting that they are unstable and dynamically evolving elements of the human genome. Analysis of cloned L2Hs elements reveal that they are very AT-rich (76%) and contain numerous palindromic sequences, inverted and direct repeats. The inter- and intra-genomic polymorphisms, the AT-richness and the sequence structure of these DNAs suggest that they may assume unusual secondary structures. To test this hypothesis, we sought to map the non-B-form structures in an L2Hs segment cloned in a plasmid vector (pN6.4.39) and to measure the influence of superhelical density on such unusual structures.

CHAPTER 2

Materials and Methods

Materials

Sodium dodecylsulfate (SDS), dithiothreitol (DTT), N,N,N',N'-tetramethylethylenediamine (TEMED), Tris-(hydroxymethyl) aminomethane (Tris), β -mercaptoethanol (β MCE), ribonuclease A (RNase A), proteinase K, formamide, chloroquine, ferrous chloride, lysozyme, ampicillin and ethidium bromide (EBr) were purchased from Sigma Chemical Company.

Ethylenediaminetetraacetic acid (EDTA), ammonium sulfate, urea, acrylamide, polyethylene glycol 8000 (PEG), bovine serum albumin (BSA), agarose, CsCl, Triton X-100, ammonium acetate, bromophenol blue, formamide, potassium phosphate monobasic, potassium phosphate dibasic, sodium hydroxide, sodium chloride and boric acid were purchased from Fisher Scientific.

Water-soluble carbodiimide [1-cyclohexyl-3-(2-morpholinyl-1-ethyl) carbodiimide metho-p-toluenesulfonate] (CDI) and 2',2 bipyridyl were products of Fluka Chemical Company.

Bactotryptone and yeast extract were purchased from Difco Laboratories. Deoxyadenosine [α - 35 S] 5'-triphosphate (specific activity = 1355 Ci/mmol) was purchased from New England Nuclear. Hybridization reverse (5'-GTC ATA GCT GTT

TCC TGT GTG A-3') and hybridization forward (5'-GTC GTG ACT GGG AAA ACC CTG GCG-3') primers were purchased from National Biosciences. M13 reverse (5'-T CAC ACA GGA AAC AGC TAT GAC-3') and forward primers (5'-CGC CAG GGT TTT CCC AGT CAC GAC-3'), T_4 -polynucleotide kinase, calf intestinal alkaline phosphatase (CIAP), topoisomerase I, Magic Miniprep and Magic Maxiprep kits, and the fmol kit for thermocycle sequencing were obtained from Promega. The S_1 nuclease was purchased from Boehringer Mannheim. The Geneclean II kit was purchased from Bio 101, Inc. Osmium tetroxide (OsO_4) from Stevens Metallurgical Company was a generous gift from Dr. Ellen Rash.

The composition of solutions and buffers used in this study are listed in Appendix A.

Methods

1. Preparation of Plasmid DNA.

a. Magic DNA Miniprep: Plasmid DNA was isolated using the Magic Miniprep kit from Promega. Bacterial cultures (*E. coli* host cells, strain DH5 α IQ) were grown overnight in 10 ml of Luria-Bertani (LB) broth supplemented with 150 μ g/ml of ampicillin. Cells were collected by centrifugation for 10 minutes at 900-1000 x g and resuspended in 200 μ l of cell resuspension solution. The cells were lysed by adding 200

μ l of cell lysis solution and mixing by inversion. Then 200 μ l of neutralization solution was added and mixed. This mixture was spun in a microcentrifuge for 15 minutes and supernatants transferred to new microcentrifuge tubes. To this, 1 ml of magic miniprep DNA purification resin was added and the contents mixed by inverting the tube. For each sample, one magic miniprep mini-column was used to collect the resin. A 3 ml disposable syringe barrel was attached to the luer-lok extension of each minicolumn and the Magic Miniprep DNA purification resin containing the bound DNA was poured into the syringe barrel and the slurry pulled into the minicolumn by applying a vacuum. The resin was washed under vacuum by adding 2 ml of column wash to the syringe barrel. This was dried by continuing the vacuum for an additional 2 minutes. To elute the DNA, the column was transferred to a microcentrifuge tube and 50 μ l of $T_1E_{0.1}$, preheated to 65°C, was applied to the column. The minicolumn was spun in a microcentrifuge for 20 seconds to collect the eluted DNA. The DNA was stored at 4°C.

b. Magic DNA Maxiprep: Bacterial cultures were grown overnight in 500 ml of LB broth supplemented with 150 μ g/ml of ampicillin. The cells were pelleted by centrifugation at 900-1000 x g for 10 minutes and the cell pellet resuspended in 15 ml of cell resuspension solution. Cell lysis solution (15 ml) was added, mixed and 15 ml of neutralization

solution added. This mixture was centrifuged for 15 minutes at 10,000 rpm in a JA-20 rotor at 4°C. The clear supernatant was transferred to a fresh centrifuge tube, 0.6 volumes of isopropanol added and the DNA precipitate pelleted by centrifugation at 13,000 rpm for 25 minutes at room temperature. The pellet was resuspended in 2 ml of $T_1E_{0.1}$ and 10 ml of Magic Maxiprep DNA purification resin added. This was mixed well by inverting the tube several times. For each sample, one Magic Maxiprep column was used. The purification resin containing the bound DNA was poured into the column and a vacuum used to collect the slurry. The resin was washed under vacuum by adding 25 ml of column wash. The column was rinsed with 5 ml of 80% ethanol and the resin dried by continuing the vacuum for an additional 10 minutes. To elute DNA, the column was transferred to a 50 ml reservoir tube and 1.5 ml of $T_1E_{0.1}$ (preheated to 85°C) was added. The maxicolumn was allowed to stand for 1 minute before it was spun in a clinical centrifuge for 5 minutes at 2,500 rpm. The eluted DNA was stored at 4°C.

c. Triton X-100 Lysis/CsCl Banding Preparation of Plasmid DNA: This is a non-denaturing and gentle method for preparing DNA (Lilley 1992). Bacterial cultures were grown overnight in 500 ml of LB broth supplemented with 150 μ g/ml of ampicillin. Cells were collected by centrifugation at 900-1000 x g for 10 minutes and the cell pellet resuspended

in 5 ml of 25% sucrose, 50 mM Tris-HCl, pH 8.0, and 100 mM EDTA, pH 8.0. The suspension was transferred to a clean centrifuge tube (40 ml) and 1.5 ml of 25 mg/ml lysozyme solution, 2 ml of 0.5 M EDTA, and 25 μ l of 10 mg/ml DNase-free RNase was added and mixed by inversion. This mixture was incubated on ice for 15 min, overlaid with 2.5 ml Triton lytic mix (3% Triton X-100, 200 mM EDTA, 150 mM Tris-HCl, pH 8.0) and gently mixed by inversion. After standing for 20 min at 4°C, the cell debris was removed by centrifugation at 18,000 rpm in a Beckman JA-20 rotor. The supernatant was carefully transferred to a clean centrifuge tube; to this tube 0.97 g/ml CsCl (~10.67 g) and 250 μ g/ml of ethidium bromide were added. The refractive index of the mixture was adjusted to 1.3905 before the mixture was spun at 10,000 rpm for 10 min. The clear supernatant was separated from the dark pellicle and transferred into a screw cap ultracentrifuge tube. Centrifugation was continued in a Beckman Ti 70 rotor at 40,000 rpm for 16 hrs at 15°C. The EBr-stained DNA was visualized by illumination (in the dark) with 306 nm ultraviolet (UV) light; the lower plasmid band was collected and transferred to a fresh centrifuge tube. This tube was filled (~80%) with a $T_{10}E_1$ solution containing 0.97 g/ml CsCl and 250 μ g/ml ethidium bromide (refractive index adjusted to 1.3905). This was centrifuged at 40,000 rpm for an additional 10 hrs at 15°C. The plasmid DNA was recovered with minimum UV illumination

and placed in microcentrifuge tubes (in the dark). The EBr was removed by 1-butanol extraction seven times. The negatively supercoiled DNA was dialyzed against 10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA at 4°C for 36 hours, with five buffer changes to remove the CsCl. The DNA was transferred to microcentrifuge tubes, stored and maintained at 2-4°C at all times.

d. Boiling Miniprep: Bacterial cultures were grown in 80 ml of LB broth supplemented with 150 µg/ml of ampicillin to an optical density of 0.8 at 600 nm. Four 20 ml aliquots were spun separately and isolated cells treated with OsO₄ (see section on OsO₄ treatment of DNA in vivo). In order to isolate intact plasmid DNA without alkali treatment a quick boiling miniprep method (Ausubel et al. 1992) was used. The cells were washed and resuspended in 300 µl of 8% sucrose, 0.5% Triton x-100, 500 mM EDTA, 50 mM Tris-HCl (pH 8.4). To each sample, 20 µl of 25 mM Tris-HCl (8.4) containing 200 µg of lysozyme was added and the mixture placed on ice for 20 minutes. The samples were boiled for 2 minutes, spun in a microcentrifuge for 20 minutes and the supernatants transferred to new tubes. To each supernatant, 200 µl of 5 M ammonium acetate was added, the mixture spun for 15 minutes (to remove any residual cell debris) and supernatants transferred to new tubes. An equal volume of isopropanol was added and the samples placed at -20°C

overnight to precipitate DNA. The DNA was pelleted, washed with 70% ethanol and then resuspended in 21 μ l of $T_1E_{0.1}$.

2. Alcohol Precipitation of DNA.

Alcohol precipitation was used frequently for recovery and concentration of DNA or for removal of salt and/or unincorporated nucleotides from DNA samples. Either ammonium acetate (5.0 M) was added to a final concentration of 2-2.5 M or sodium acetate (3.0 M) was added to 0.3 M. Then, 2.5 volumes of ethanol or 1 volume of isopropanol was added. If ethanol was used, the solution was chilled for at least 10 min in an ethanol-dry ice bath or placed at -20°C overnight; for isopropanol precipitation the solution was placed in an ice-water bath for ≥ 10 min or placed at -20°C overnight. The DNA precipitated was collected by centrifugation in a microcentrifuge at 12,000 x g for 15 min at room temperature. The pellets were rinsed with 70% ethanol and dried in vacuo.

3. Gel Electrophoresis of DNA.

a. Standard Agarose Gel Electrophoresis: Standard agarose gels were prepared in 50 mM TBE buffer (50 mM Tris, 50 mM boric acid, pH 8.3, 1.25 mM EDTA) with or without EBr (0.5 $\mu\text{g}/\text{ml}$) at final agarose concentrations ranging between 0.7%-1.4%, depending upon the size of the DNA fragments to be separated. The agarose was melted in a microwave oven

and allowed to cool to about 65°C before being poured into an appropriate mold with a well-forming comb. DNA samples were mixed with 1/5 volume of SOG (60% sucrose, 0.1% orange G) loading buffer prior to loading. Gels were electrophoresed in 50 mM TBE buffer (with or without 0.5 µg/ml EBr) at constant voltage (25-100 Volts). The gels without EBr were post-stained with 50 mM TBE containing 0.5 µg/ml EBr. All gels were destained in deionized water (dH₂O). The DNA was visualized by UV transillumination and photographed with Polaroid type 55 positive/negative or type 667 high speed positive films.

b. Sequencing Gel Electrophoresis: An International Biotechnologies, Inc. model STS 45 apparatus with 32, 64, or 96 well combs was used to perform gel electrophoresis for DNA sequencing. The sequencing gels contained 5-8% polyacrylamide (acrylamide/bis : 19/1) and 42% urea in 50 mM TBE buffer or 5-6% Long Ranger Hydrolink and 42% urea in 106.8 mM TBE buffer.

The gel solutions were filtered and vacuum degassed. Just before pouring, ammonium persulfate and TEMED were added to 0.07% (w/v) and 0.03% (v/v), respectively. The gel solution was poured between two glass plates with 0.4-0.8 mm wedge spacers (for polyacrylamide) or 0.4 mm flat spacers (for Hydrolink) and allowed to polymerize for at least one hour, but usually overnight. The polyacrylamide gels were

preelectrophoresed at 1600 volts for a minimum of 20 min in 50 mM TBE buffer. The Hydrolink gels were preelectrophoresed for 10 min at 42 watts constant power in 89.4 mM TBE buffer.

An aliquot of 3-6 μ l of each sequencing reaction were loaded and electrophoresis continued until the tracking dye had reached the desired position or for an appropriate time. After electrophoresis, the gel was transferred onto filter paper and vacuum dried at 80°C before autoradiography.

4. DNA Cleavage by Restriction Enzyme Digestion.

The DNA subjected to SspI restriction enzyme digestion was diluted into the appropriate enzyme buffer (see appendix A). Nuclease-free BSA and β MCE were added to all reactions to final concentrations of ~100 μ g/ml and 6 mM, respectively. SspI restriction enzyme was added to ~1 unit/ μ g of DNA. The samples were incubated at 37°C for 2-12 hours.

5. Electroelution of DNA Fragments.

DNA restriction fragments or topoisomeric forms of DNA were separated by agarose gel electrophoresis. After staining with EBr the DNA band(s) of interest was located with 306 nm UV illumination. A gel slice containing the desired DNA fragment was excised with a razor blade and placed in the elution wells of the electroelutor

(International Biotechnologies, Inc.). After filling the chambers with TAE (50 mM Tris, 40 mM acetic acid, 1 mM EDTA) buffer, 150 μ l of 7.5 M ammonium acetate/0.01% xylene cyanol was placed in the V-channels of the electroeluter, and the DNA eluted from the gel at 50 volts for one hour. The eluted DNA in ammonium acetate was recovered by alcohol precipitation.

6. Isolation of DNA from Agarose Gel Slices using the Gene Clean II Kit.

DNA restriction fragments or topoisomeric forms of DNA were separated by electrophoresis through an appropriate agarose gel (generally, prepared and run in TAE buffer without EBr). After EBr staining, the DNA band(s) of interest was located with 306 nm UV illumination. A gel slice containing the desired DNA fragment was excised with a razor blade and treated with reagents from a Gene Clean II kit (Bio 101). Three volumes of NaI stock solution (6 M) was added to a final concentration 4 M and incubated at 50°C for about 10 minutes to dissolve the gel slice. An appropriate amount of glassmilk suspension (5 μ l/5 μ g of DNA) was added and the solution mixed intermitantly for 5 min. The glassmilk/DNA complex was pelleted by spinning for 20 seconds and washed with ice cold new wash buffer three times. The DNA was then eluted by adding an appropriate amount of $T_1E_{0.1}$, mixing and incubating the sample at 65°C for

5 min. The glassmilk was spun out and the supernatant containing the DNA transferred to a new tube.

7. S_1 Nuclease Treatment of DNA.

DNA samples prepared by alkaline lysis or the Triton X-100 lysis method were used for these studies. Digestion of DNA with S_1 nuclease was done as follows: to a 60 μ l reaction mixture containing 3.64 μ g of DNA, 50 mM sodium acetate (pH 5.0), 50 mM NaCl and 1 mM $ZnSO_4$, 14 units of S_1 nuclease were added. Incubations were performed at 16°C, 24°C or 37°C for appropriate time periods. The reaction was stopped by adding 6.6 μ l of 1 M Tris-HCl (pH 8.4) and 14 μ l of 0.1 M EDTA.

8. CDI Treatment of DNA.

A fresh solution of 1 M CDI in water (423.5 mg/ml) was prepared just before use. The reaction was carried out in a sialinated microcentrifuge tube by adding 13 μ l (1.69 μ g) of DNA (prepared by Magic Miniprep), 4 μ l of 1 M sodium borate (pH 8.0), and 10 μ l of 1 M CDI. The samples were incubated at 37°C for 3 hours. To remove the unreacted CDI, 20 μ l of 10 mM sodium phosphate buffer (pH 7.0) and 20 μ l of 7.5 M ammonium acetate were added, and the sample immediately extracted three times with 250 μ l of isoamyl alcohol freshly equilibrated with a solution of 2.5 M ammonium acetate in

$T_{10}E_1$ (7.4). The lower aqueous phase was transferred to a new sialinated microcentrifuge tube and the DNA precipitated by adding 180 μ l of ethanol and leaving it at -20°C overnight. The DNA precipitate was collected by spinning in a microcentrifuge for 15 minutes at room temperature, the supernatants discarded and the pellet rinsed with 70% ETOH. The pellet was dried in vacuo for only 5 min to avoid over-drying and taken up in 13 μ l of $T_1E_{0.1}$ (pH 7.4). The samples were heated in a boiling water bath for 5 minutes to destroy any residual CDI (Ganguly and Prockop 1990) and then cooled on ice for 5 minutes. This DNA was analyzed by primer extension.

Carbodiimide interacts non-covalently with DNA and alters its solubility; consequently CDI-treated DNA was difficult to precipitate with ethanol. In addition, CDI-treated DNA adheres to glass and plastic surfaces. Therefore, sialinated tubes were used and care was taken not to over-dry the samples. Because of the great losses during the processing of the CDI-treated samples, control samples not treated with CDI were usually diluted about four-fold before primer extension/sequence analysis.

9. OSO_4 Treatment of DNA In Vitro.

A 1.5 μ g aliquot of DNA prepared by the Magic Miniprep or Maxiprep method was ethanol precipitated, washed with 70% ethanol and dried in vacuo. The DNA samples for 10, 15, and

20 min time points were resuspended in 25 μ l of 0.2 M NaCl, 25 mM Tris-HCl buffer, pH 7.4, 2.5 mM EDTA, 2 mM 2',2'-bipyridyl and 2 mM OsO₄. The control DNA sample was resuspended in the same solution without the OsO₄. The samples tubes were quickly flicked about 10 times to resuspend the DNA and then incubated at 37°C for an appropriate time. The 20 min time point was resuspended (i.e. buffer added and flicked ten times) first, then the 15 min time point, etc. All the reactions were terminated at the same time by adding 75 μ l of water and extracting twice with ether to remove the OsO₄. The DNA was immediately precipitated by adding 100 μ l of 5 M ammonium acetate and 500 μ l of 95% ethanol. The DNA precipitate was collected by centrifugation, washed with 70% ethanol and then resuspended in 12 μ l of T₁E_{0.1}. This DNA was used for primer extension/sequence analyses.

10. OsO₄ Treatment of DNA In Vivo.

Bacteria were grown in 80 ml of LB broth supplemented with 150 μ g/ml ampicillin to an optical density of 0.8 at 600 nm. Four 20 ml aliquots were centrifuged separately for 10 min at 900-1000 x g and the cells resuspended in 2 ml of 0.15 M NaCl, 50 mM phosphate buffer. The suspension was centrifuged and the cells were resuspended in 2 ml of cold buffer. To the 5, 10 and 15 minute time point samples 1 ml

of 9 mM 2,2'-bipyridyl and 9 mM OsO_4 was added. To the 0 minute time point 1 ml of 9 mM 2,2'-bipyridyl only was added. After the appropriate time period, the cell suspensions were diluted with 25 ml of cold buffer. The cells were pelleted as above and washed with cold buffer twice to stop the OsO_4 reaction. The DNA was isolated using the non-alkaline boiling miniprep method described above. Alkaline lysis was not used to isolate OsO_4 -modified DNA because alkaline conditions induce DNA backbone cleavage at the OsO_4 -DNA adducts.

11. Topoisomerase I Treatment of DNA.

Topoisomerase I reactions were typically performed in a unit volume of 100 μl . It contained 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 20% glycerol, 50 mM NaCl, 10.4 μg of DNA and 2.5 units of topoisomerase I. The sample was incubated at 37°C for 45 minutes to generate lower topoisomeric forms of DNA and the reaction stopped by adding 100 μl of 2% SDS and 1 mg/ml proteinase K. This was incubated at 37°C for another 30 minutes for proteolytic digestion of the topoisomerase I. The reaction products were analyzed by 2-dimensional agarose gel electrophoresis to determine the superhelical density (σ) of the plasmid DNA.

12. Two-dimensional Gel Electrophoresis.

Two-dimensional gel electrophoresis was used to separate different topoisomeric forms of DNA and to measure σ values. In these analyses, the electrophoresis buffer was recirculated during the run. Approximately 2.5 μg of DNA in $T_{10}E_1/\text{SOG}$ (3/1) was loaded into a single slot of a 10 cm x 10 cm 1.4% agarose gel and run in 50 mM TBE buffer (no EBr). The sample was electrophoresed in the first dimension for 27 hrs at 28 volts (2.8 V/cm). Then, the gel was turned 90° and rerun at 28 volts for 17 hrs in the second dimension. The gel was soaked in 50 mM TBE with multiple buffer changes for 6 hrs to remove the chloroquine. The gel was stained with EBr for 1 hr, destained and photographed.

Alternatively, topoisomeric forms of DNA were separated by 2-dimension chloroquine agarose gel electrophoresis. Here the gel was run in the first dimension as before but electrophoresis in the second dimension was performed in buffer containing a mild intercalating agent, chloroquine. Before electrophoresis in the 2nd dimension, the gel was equilibrated in 50 mM TBE containing 10 $\mu\text{g}/\text{ml}$ chloroquine for 3.5 hrs. The gel was rotated 90° and electrophoresed in this buffer for 16.5 hrs at 25 volts (2.5 V/cm). The gel was stained and photographed as before.

13. Preparation of Different Topoisomeric Forms of pN6.4.39.

The topoisomerase I reaction was performed as described earlier with the addition of 0.8 mg of chloroquine per 100 μ l. The chloroquine reduced the amount of relaxed DNA generated and enhanced the amount of intermediate topoisomers formed. The reaction products were electrophoresed through a 1.4% agarose gel in TAE buffer without EBr. Edges of the gel were cut off and stained with EBr. The stained slices were used to locate bands containing different topoisomeric forms of the DNA. The appropriate bands were cut out and DNA was isolated either by electroelution or by the Gene Clean II procedure.

14. DNA Primary Sequence Analyses.

Gel autoradiograms containing DNA sequence patterns were scanned, compiled and analyzed using a Sun SparcStation IPX and Bioimage sequencing software (Millipore Corp. version 2.1). ΔG analyses were performed using a computer program described by Natale et al. (1992). Direct repeat and inverted repeat analyses were performed using the computer program DNASIS (Hitachi American)

15. DNA Dideoxynucleotide Thermocycle Sequencing.

All nucleotide sequencing was performed by the dideoxynucleotide sequencing method (Sanger et al. 1977)

using the fmol DNA sequencing kit according to the Promega Corp. protocols. The DNA was mixed with either M13 universal, reverse, hybridization forward or hybridization reverse primer in a total volume of 16 μ l containing 1X fmol sequencing buffer. This reaction mix contained 500 fmol of template DNA, 3.0 pmol of primer, 5 μ Ci of [α - 35 S]dATP and 5 u of sequencing-grade Taq DNA polymerase. Aliquots of four microliters of the reaction mix were placed into each of four tubes containing the appropriate deoxynucleotide-dideoxynucleotide mix (G,A,T,C). Each reaction mix was covered with 1 drop of mineral oil and the tubes placed in a thermal cycler (Precision Scientific, model GTC-1) preheated to 95°C. The reaction mixes were thermocycled according to the following profile:

95°C for 2 min, to ensure complete, initial denaturation, then cycled 30 times as follows:

95°C for 30 seconds (denaturation),

42°C for 30 seconds (annealing),

70°C for one minute (extension),

then cooled to 24°C.

After the thermocycling program was complete, 3 μ l of fmol sequencing stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol) was added. The samples were denatured by heating to 80°C for 2 min, then chilled on ice before loading onto a sequencing gel.

When the sequences analyzed were very AT-rich, primer

extension was found to terminate prematurely in the A and T lanes due to insufficient deoxynucleotides. This problem was corrected by adding one microliter of 20 mM dATP/dTTP mix to each tube along with the dideoxynucleotide mix in setting up the reactions.

16. Southern Blotting.

The appropriate DNA samples were electrophoresed through agarose gels in 50 mM TBE without EBr. The gels were stained in 50 mM TBE containing 0.5 $\mu\text{g/ml}$ of EBr for 15 minutes and photographed using transmitted UV light. The gel was soaked in 0.25 N HCl for 20 minutes (to partially depurinate the DNA), rinsed briefly with dH_2O , and soaked in 0.4 N NaOH, twice for 15 minutes each to denature and cleave the DNA backbone at the acid-depurinated sites. The gel was neutralized by soaking in 1 M Tris-HCl (pH 7.4) for 20 minutes. After a brief rinse with 10X SSPE, DNA in the gel was transferred onto an Immobilon-S membrane (Millipore) with 10X SSPE by standard capillary blotting (Southern 1975). The DNA was cross-linked to the membrane in a UV-crosslinker (Fisher scientific model FB-UVXL-1000) at the optimal setting. The filter with the crosslinked DNA was prehybridized with 6X SSPE, 2.5% Denhardt's reagent (Denhardt 1966), 0.5% SDS, 50% formamide at 42°C for 2.5 hrs and incubated at 65°C for 20 min. The DNA was hybridized in prehybridization solution containing biotinylated pN6.4.39

insert DNA as probe at 42°C overnight, then washed with 3X SSPE, 0.1% SDS at 65°C twice for 20 min. The insert of pN6.4.39 plasmid DNA was labelled with biotin-dUTP by the random primer method (Feinberg and Vogelstein 1983). The biotin in the hybridized DNA complex was detected by chemiluminescence using streptavidin, biotinylated alkaline phosphatase and lumigen-PPD following the manufacturer's protocols (Millipore Corp.).

17. Autoradiography.

Gels with ³⁵S-labelled DNA were dried onto Whatman 3 MM paper before exposure to Kodak (XAR or SB-5) or Fuji (RX) films at room temperature from 12 hours to 2 months. All films were developed for up to 7 minutes in Kodak GBX developer, fixed for 10 minutes in Kodak GBX fixer, and rinsed in running water for 15 minutes before air drying.

CHAPTER 3

Results

Construction of Plasmids

Recombinant plasmid pN6.4.39 was constructed by ligation of a 0.6 Kb L2Hs segment into the KpnI site of pTZ19U (US Biochemicals). The L2Hs segment was initially cloned in pUC19 plasmid vector (Musich and Dykes 1986). The plasmids pN6.4.39 and pTZ19U were used for all the work presented in this study. These plasmids were carried in *E. coli* host strain DH5 α IQ

Strategy Employed in These Studies

Unusual secondary structures, such as cruciforms and stem-loop structures, generally have single-strand regions which can be mapped by a variety of methods. These methods employ chemical or enzymatic probes which are specific for single-stranded DNA and particular bases. One enzymatic probe and two chemical probes were used to study the structure of the cloned L2Hs segment in pN6.4.39. The chemically-modified or enzymatically-cleaved DNA was sequenced to determine the positions of primer extension termination, indicative of single-stranded regions in unusual secondary structures. This approach is illustrated in Figure 6. CDI and OsO₄ are single-strand specific probes

which react with unpaired G and T or C and T bases, respectively. Due to the size of the base-CDI and base-OsO₄ adducts (Fig. 7), primer extension terminates at the modified site. This results in a hard stop across all four lanes (G,A,T,C) in the sequencing reactions of the modified, but not the untreated control DNA (Hard stop refers to a set of bands, one in each lane G,A,T,C at the same position). Positions of strand cleavage by S₁ nuclease also can be mapped by primer extension/sequencing, where extension terminates at the positions of template strand cleavage. Four different primers were used for primer extension/sequencing: forward (F) and reverse (R) primers were employed to analyze the insert sequence, and the hybridization-forward (HF) and -reverse (HR) primers were used to analyze the vector sequence (Fig. 6A).

Determination of Superhelical Density of pN6.4.39

The recombinant plasmid pN6.4.39 is 3469 bases long. Two dimensional agarose gel electrophoresis (Figs. 8 and 9) was used to determine the number of superhelical turns and superhelical density of the plasmid. In Figure 8 the band marked 1 is a combination of relaxed (0) and nicked (N) circular plasmid DNA along with pN6.4.39 containing one supercoil. These three forms of DNA can be resolved in two dimensional gels where the second dimension is performed in

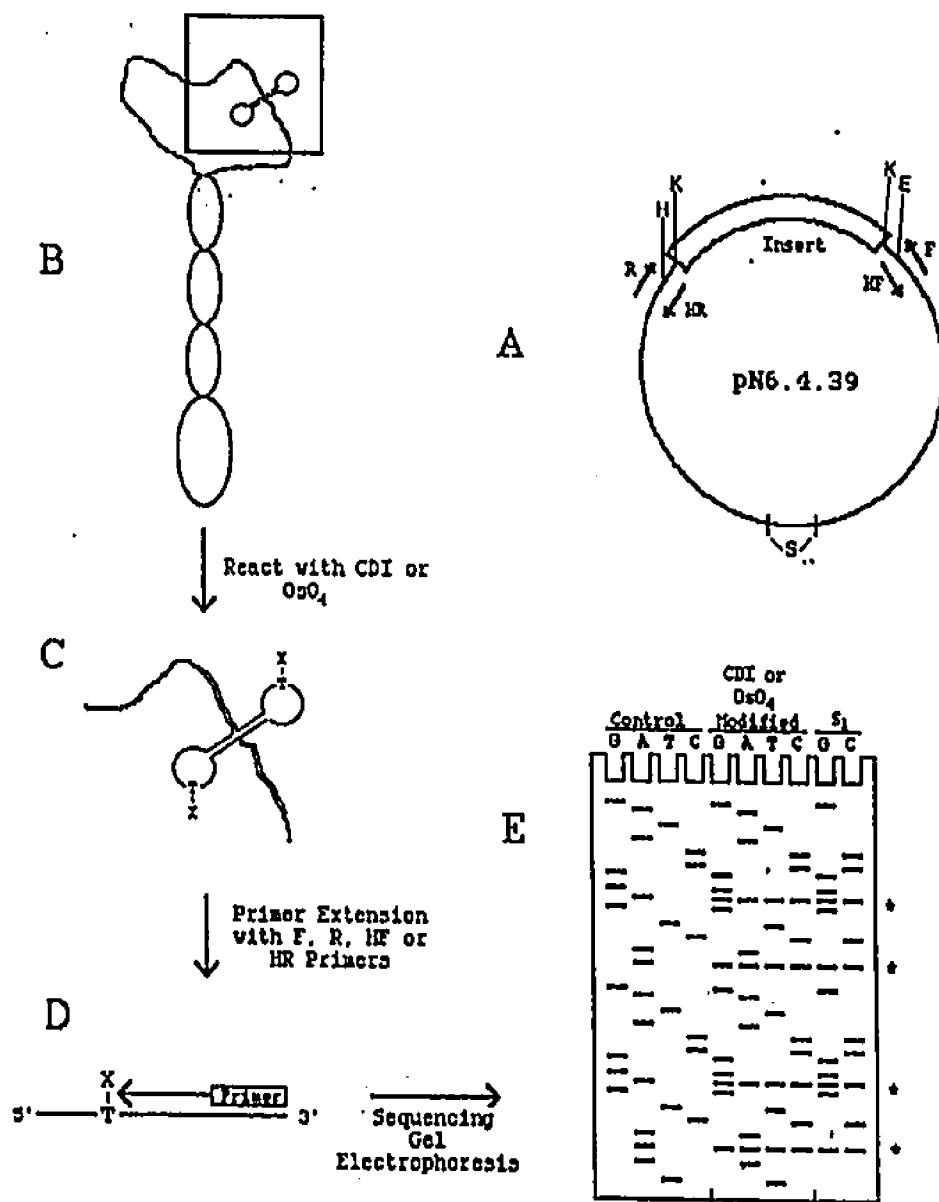
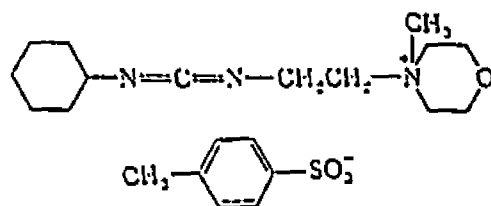
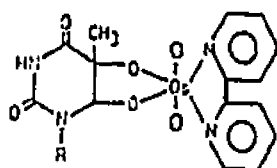


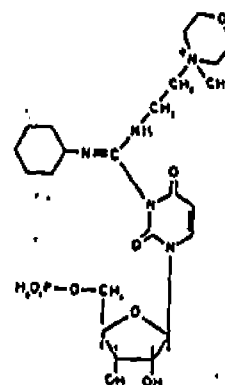
FIGURE 6. Scheme summarizing the mapping experiments. A. Primer binding sites and restriction enzyme cleavage sites in pN6.4.39 (not drawn to scale). B. Supercoiled DNA with cruciform structure. C. Single-stranded loop modified by chemical probes at unpaired T bases; an enlargement of the boxed structure shown in B. D. The position of adduct detected by primer extension/sequencing. Extension terminates just before the modified base. E. Sequencing gel with hard stops just before positions of modification. G, A, T, C lanes for untreated control, CDI- and OsO_4 -modified samples and G, C lanes for S_1 nuclease cleaved samples. Symbols used: * = position of hard stop, x = CDI- or OsO_4 -adduct, F = forward primer, R = reverse primer, HF = hybridization forward primer, HR = hybridization reverse primer, E = EcoRI site, H = HindIII site, K = KpnI site and S = SspI.



1-cyclohexyl-3-(2-morpholinyl-1-ethyl)carbodiimide
metho-p-toluenesulfonate
(CDI)



Osmium tetroxide and
2,2'-bipyridine complex
with thymine



Carbodiimide
and its adduct
with 5'-UMP

FIGURE 7. Structures of CDI and adducts formed between CDI, OsO_4 , and non-paired base.

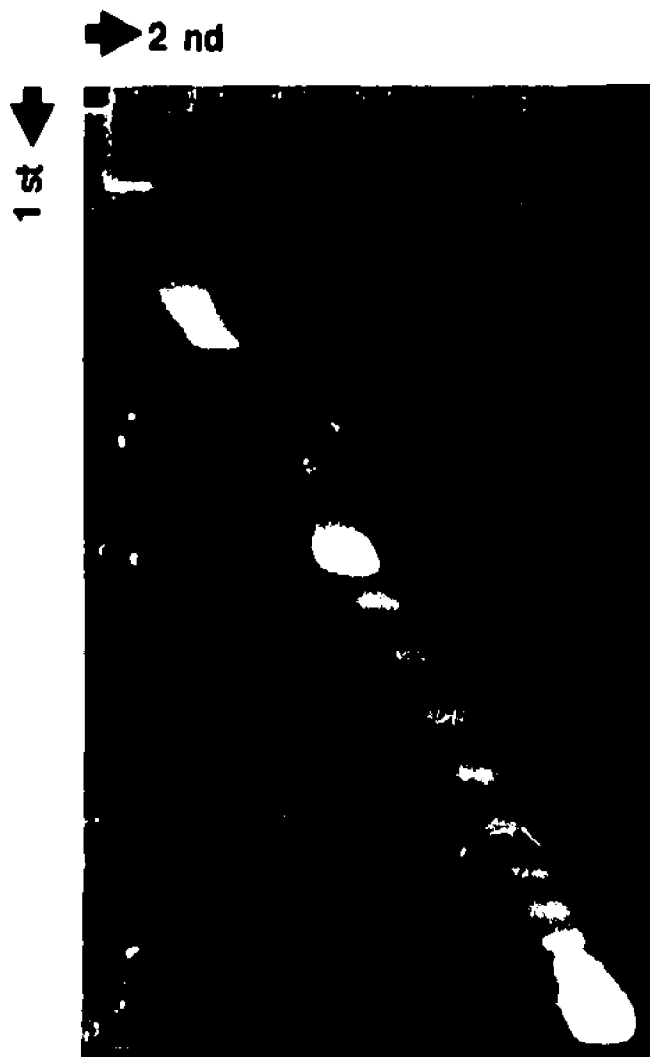


Figure 8. Two-dimensional agarose gel electrophoresis of pN6.4.39 partially relaxed by topoisomerase I. Electrophoresis in the first dimension (1st) was performed for 27 hrs at 28 volts. The gel was turned 90° and the gel run at 28 volts for 17 hrs in the second dimension (2nd). Both dimensions of the gel were run in 50 mM TBE buffer (no EBr); the gel was post-stained with 50 mM TBE containing 0.5 ug/ml EBr for 6 hours, destained and photographed. The numbers on the gel indicate the number of supercoils in the DNA contained in the marked bands.

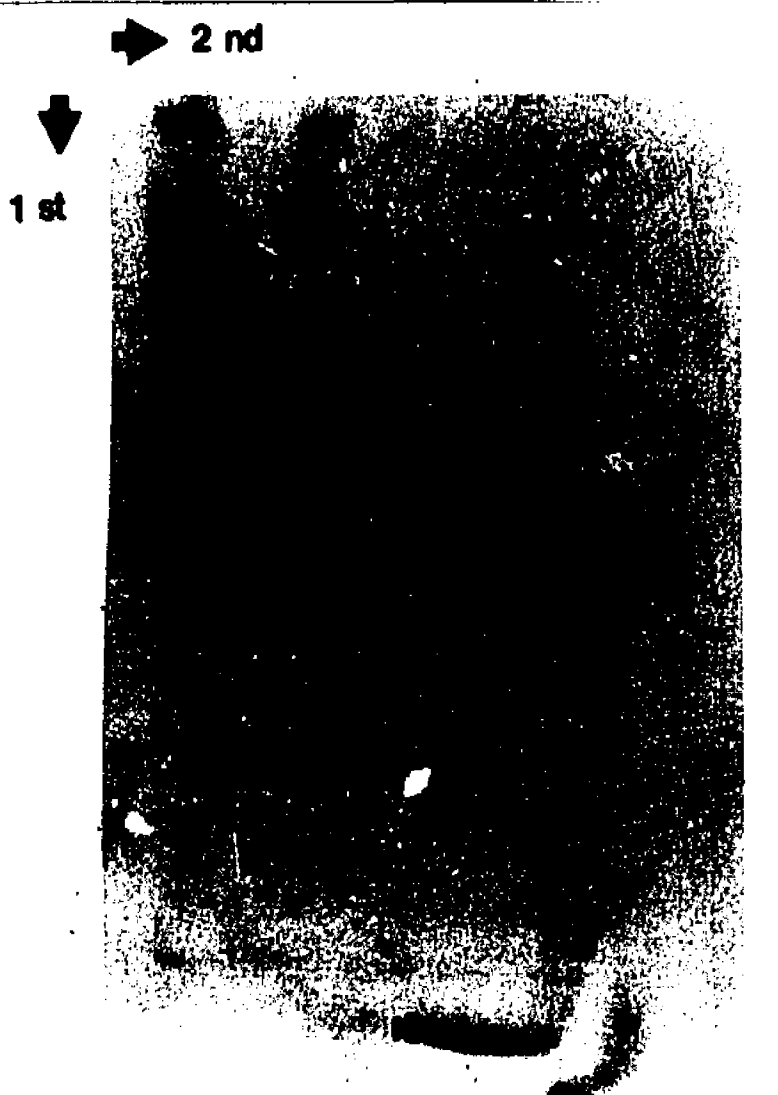


Figure 9. Southern blot of a two-dimensional agarose/chloroquine gel of topoisomerase I-treated pN6.4.39. Electrophoresis in the first dimension (1st) was performed for 27 hrs at 28 volts in 50 mM TBE, separation in the second dimension (2nd) was performed in 50 mM TBE containing 10 ug/ml chloroquine after soaking the gel in the same buffer for 3.5 hours. The DNA in the gel was blotted onto Immobilon-S membrane (Millipore), and hybridized to an L2Hs segment labelled with biotin. The image was then visualized by chemiluminescence and autoradiography. The numbers (0,1,13) indicate the number of supercoils in the DNA contained in the marked bands and N indicates the position of nicked circular form II DNA.

the presence of chloroquine. This cluster resolves into three separate bands, marked N, 0 and 1 in Figure 9.

The superhelical density (σ) of the recombinant plasmid was calculated:

$$\sigma = \text{number of superhelical turns/number of primary turns}$$

$$\text{Number of primary turns} = 3469/10.5 = 330.4$$

Number of superhelical turns = 13 (N.B. 3 bands as #13 in fig. 8)

$$\sigma = 13/330.4$$

$$= 0.039$$

The superhelical density of pN6.4.39 is -0.039 because the DNA is negatively supercoiled.

The Unusual Primary Sequence of the L2Hs Segment

Sequence analysis of the L2Hs segment shows that it is 76% AT-rich, and has many direct (Table 1) and inverted repeats (Table 2). The inverted repeats confer on the sequence a potential to generate an unusually large number of possible stem-loop structures (Table 2). The ΔG of strand separation (energy needed to separate the two strands) of the L2Hs sequence, in contrast to the flanking pTZ19U vector sequence, is illustrated in Figure 10.

Table 1: Direct repeats in the L2Hs insert of pN6.4.39

<u>Length^a:</u> <u>repeats</u>	<u>Start of long repeat and shorter overlapping</u> <u>[size]^b:</u>
24	414, 620
24	125, 339, 547, 383 [12]
23	215, 511
22	55, 520, 98 [15], 143 [15], 442 [15], 565 [13]
21	95, 517, 221 [17], 180 [12], 264 [11]
20	172, 510, 215 [18], 429 [10]
19	241, 458
18	528, 611, 64 [13]
16	337, 545
16	140, 562
15	321, 451, 412 [10]
15	117, 285, 583 [11]
14	93, 261
13	366, 575
12	284, 582
11	412, 456
11	353, 395
11	284, 582
11	239, 535, 618
10	354, 396
9	493, 534, 617

^aLength in bases of a repeat sequence: each repeat listed on a line is for the same sequence; different sequence repeats are listed on separate lines. Repeats of less than 9 bp were not scored.

^bPortions of a long repeat may be repeated as a shorter sequence at other positions in the L2Hs DNA; the positions of these shorter repeats are indicated with their length in brackets.

Table 2. Potential stem-loop, cruciform structures in the L2Hs insert of pN6.4.39 plus flanking vector sequences. The stems represent inverted repeats in the sequence.

#A..#B positions ^a	Stem match ^b	Stem size ^c	Loop size ^d	Stem energy ^e	#A..#B positions	Stem match	Stem size	Loop size	Stem energy
66..117	100	9	50	-----	71..151	100	6	79	-----
71..109	100	6	37	-----	83..125	100	6	41	-----
106..120	100	6	13	-0.61	109..117	100	9	7	-6.00
114..151	100	6	36	-----	114..193	100	6	78	-----
125..143	100	9	17	-5.19	125..185	100	7	59	-----
127..164	100	6	36	-----	129..206	100	7	76	-----
130..163	100	7	32	-----	130..205	100	8	74	-----
151..161	90	10	9	-5.40	152..158	90	10	5	-8.20
156..193	100	6	36	-----	161..167	100	6	5	-5.20
169..206	100	6	36	-----	169..248	100	6	78	-----
191..287	100	7	95	-----	198..277	100	6	78	-----
212..291	100	8	78	-----	213..241	100	7	27	-5.36
235..285	100	9	49	-----	239..340	100	6	46	-----
247..329	100	6	81	-----	252..339	100	6	86	-----
254..291	100	8	36	-----	254..329	100	6	74	-----
258..287	92	12	28	-6.56	274..288	100	6	13	-0.61
282..321	100	6	38	-----	292..313	100	7	20	-1.45
293..357	100	6	63	-----	294..339	100	7	44	-----
296..329	100	6	32	-----	298..371	100	6	72	-----
334..415	100	6	80	-----	344..371	100	6	26	-0.63
367..404	100	6	36	-----	367..449	100	6	81	-----
379..401	100	6	21	-0.21	402..498	100	6	95	-----
420..466	100	6	45	-----	420..459	100	6	38	-----
420..504	100	6	83	-----	456..556	100	7	99	-----
456..531	100	6	74	-----	464..538	100	6	73	-----
464..504	100	6	39	-----	469..497	91	11	27	-6.60
469..506	100	6	36	-----	469..547	100	6	77	-----
471..504	100	8	32	-----	471..538	100	6	66	-----
472..546	100	6	73	-----	503..563	100	6	59	-----
508..588	100	6	79	-----	509..538	100	6	28	-1.16
510..588	100	6	77	-----	512..586	100	8	73	-----
531..583	100	9	51	-----	536..556	100	7	19	-4.49
536..614	100	6	77	-----	543..621	100	6	77	-----
552..580	100	6	27	-0.60	556..583	90	10	26	-3.63
562..572	100	6	9	-3.00	562..613	100	7	50	-----
563..626	100	6	62	-----	570..586	100	6	15	-0.49
575..581	91	11	5	-5.70	* 642..702	100	7	59	-----

^a#A is the last paired base on the 5' side and #B is the first paired base on the 3' side of the duplex stem.

^bPercent of paired bases in the stem region.

^cSize of the duplex stem region in base pairs. Only stems \geq 6 bp are presented.

^dSize of the loop in bases, from positions #A to #B.

^eStem energy, in Kcal/mole in 1 M Na⁺ at pH 7.25, calculated by the Hitachi DNASIS program (Hitachi American).

*Stem formed between inverted repeats in the insert and the vector.

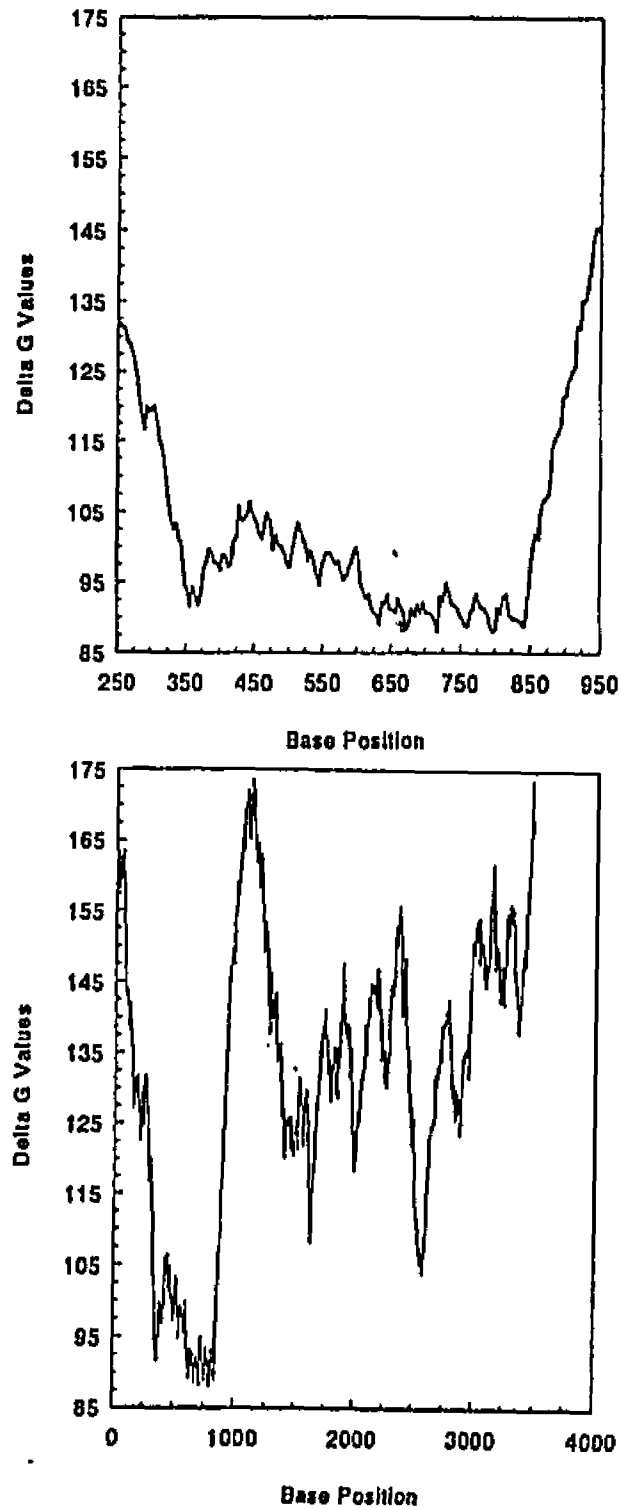


FIGURE 10. Plot of standard free energy change (ΔG) of strand separation in A. the L2Hs insert of recombinant plasmid pN6.4.39 and B. the entire recombinant plasmid pN6.4.39. The ΔG plot was performed using the computer program described by Natale et al. 1992.

CDI Analysis of pN6.4.39

Supercoiled pN6.4.39 prepared by alkaline lysis was treated with CDI and the positions of modification determined by primer extension/sequencing as described in Figure 6. The positions of hard stops occur 5' of CDI modification sites (Fig. 11) and reflect unpaired G and T bases in single-stranded regions in the L2Hs insert. The clustering of hard stops in Figure 11 may be due to imprecise termination of primer extension before the bulky CDI-modified base (Wilkins 1984) or to a cluster of modified bases in a mixed population of template molecules. The pattern indicates that there are numerous sites of CDI modification in the L2Hs segment of pN6.4.39.

The standard reverse primer consistently revealed base modification at non-paired bases in the multiple cloning site of the vector (HindIII to SmaI region) in supercoiled pN6.4.39, immediately adjacent to the KpnI site. This implies that the vector has single-stranded regions. Analysis of CDI-treated supercoiled pTZ19U vector (without the L2Hs insert) revealed no unpaired bases in this region (data not shown, see OsO_4 data in Fig. 18). Analysis of the same sample of CDI-treated DNA with hybridization primers reveals only one prominent hard stop (Fig. 12), showing that the single-stranded regions in this recombinant plasmid occur almost exclusively in the L2Hs sequence and not in the pTZ19U plasmid vector. The single-stranded regions detected

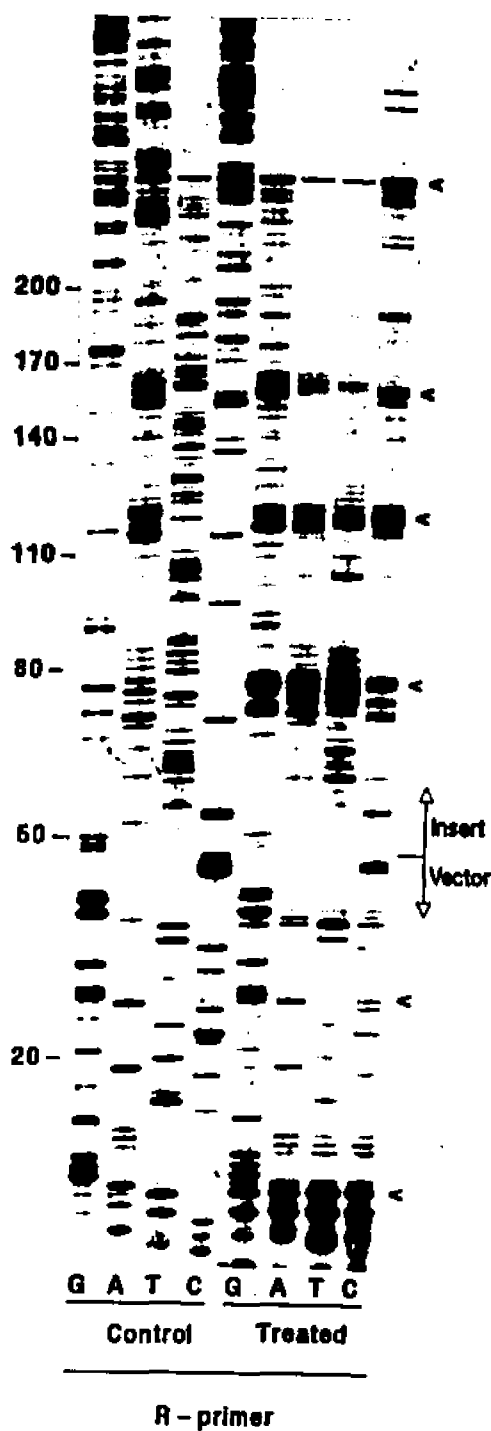


FIGURE 11. Primer extension analysis of untreated control and CDI-modified supercoiled pN6.4.39. The DNA ($\sigma = -0.039$) was modified for 3 hrs in 250 mM CDI. The pattern shown is for primer extension using the reverse primer. Hard stops (<) in treated samples are positions of modification. The numbers on the left represent positions in the sequence as depicted in Figure 25.

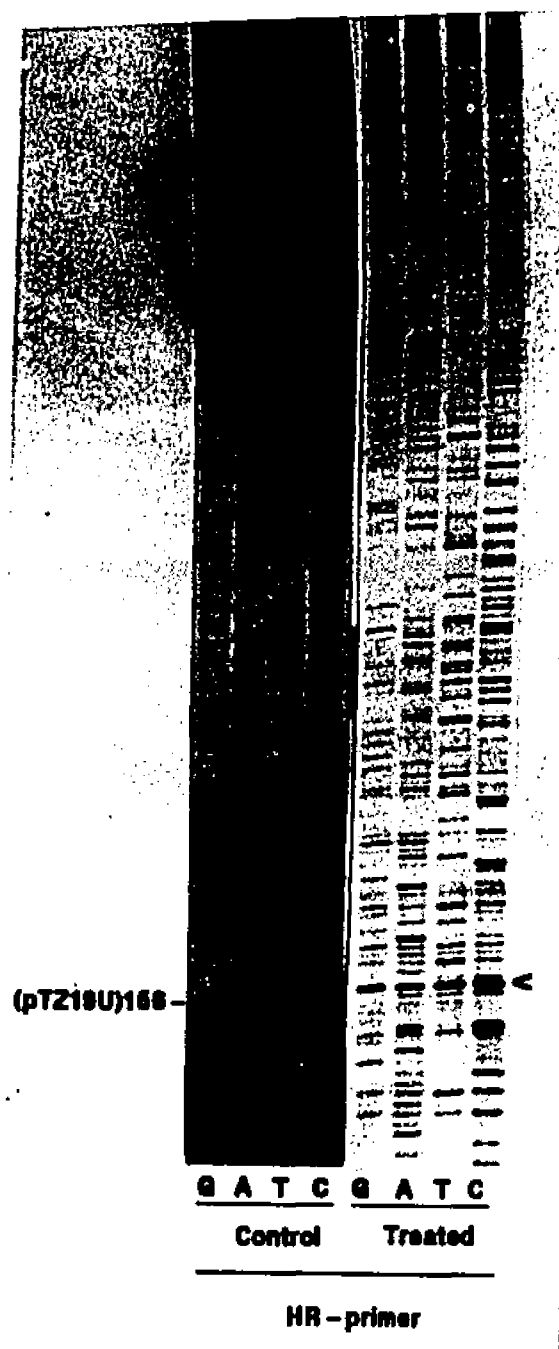


FIGURE 12. Analysis of vector sequence in CDI-treated pN6.4.39 and untreated control. The same template DNAs shown in Fig. 11 were used. The pattern shown is for primer extension using the hybridization reverse primer. The absence of hard stops in treated samples confirms that the unusual secondary structures are localized to the L2Hs insert. There is one pronounced hard stop in this gel (<), which is a position of an unusual secondary structure in the vector.

in the HindIII to SmaI region, therefore, seems to be induced by the non-B-form structure of the L2Hs element. This demonstrates that the L2Hs insert has a greater tendency to assume non-B-form DNA structures than does the vector sequence in supercoiled plasmid.

To determine whether the torsional stress in supercoiled pN6.4.39 was necessary for the formation of single-stranded regions, SspI linearized pN6.4.39 was analyzed. Hard stops were not observed in CDI-treated linear recombinant plasmid (Fig. 13), indicating the dependence of these non-B secondary structures on plasmid supercoiling.

These results, summarized in Fig. 25, reveal that the L2Hs sequence not only has the potential, but does assume unusual secondary structures in supercoiled pN6.4.39. However, it has been suggested (Murchie and Lilley 1992) that preparation of plasmid DNA by alkaline lysis induces the formation of non-B-form structures. Consequently, the structures detected in these CDI-experiments could be artifacts of DNA preparation. The OsO_4 and S_1 nuclease experiments, presented below, reveal that the non-B-form structures detected by CDI are not artifacts and that they indeed do exist in supercoiled L2Hs sequences.

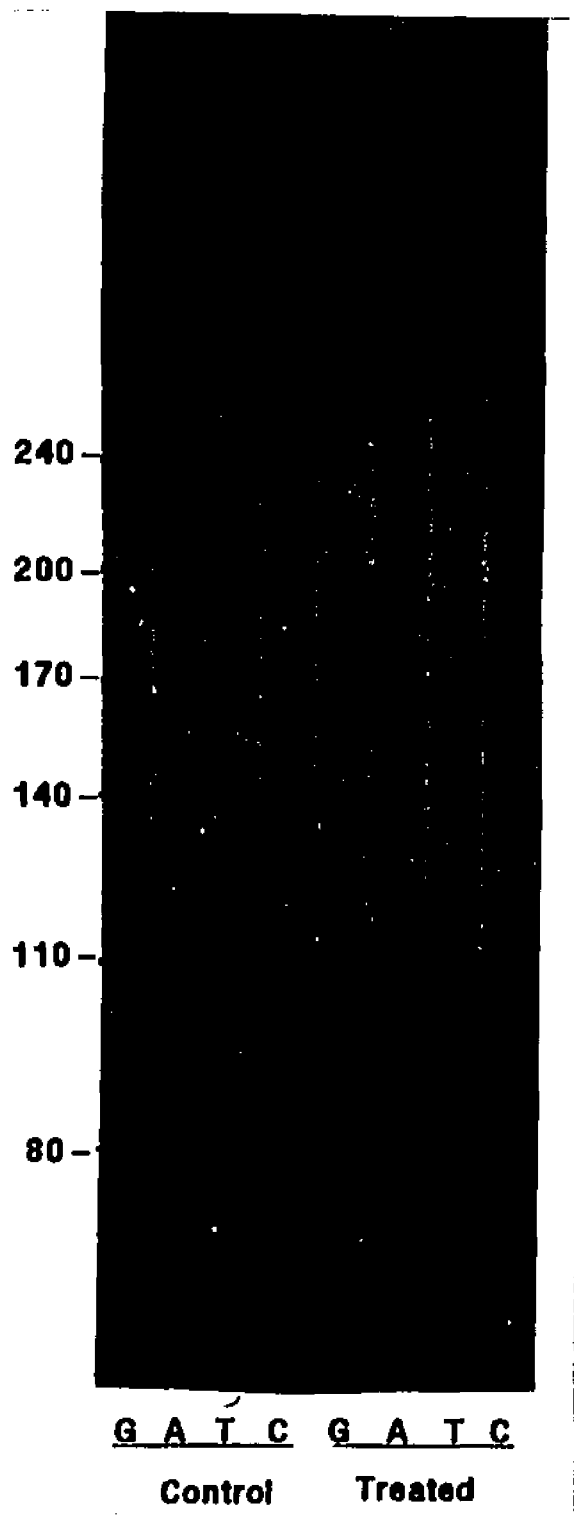


FIGURE 13. CDI-treated and untreated linear pN6.4.39 analyzed by primer extension. The CDI treatment and primer extension were done as described in Fig. 11. The absence of hard stops in treated samples reflects the requirement for supercoiling in the formation of unusual secondary structures.

OsO₄ Analysis of pN6.4.39

Supercoiled plasmid DNA was modified with OsO₄ in vivo and in vitro, and the positions of modification determined by primer extension/sequencing as in the CDI experiments (see Fig. 6). In vitro treatment with OsO₄ reveals that the L2Hs and immediate flanking vector sequences assume many non-B-form structures as seen in Fig. 14. In contrast, except for the reaction site at pTZ19U base 156 (Fig. 15), the more distant vector sequence appears not to assume any unusual secondary structures. None of these hard stops were seen in OsO₄-treated linear DNA (Fig. 16). This analysis of linear DNA confirms that supercoiling is necessary for the formation single-stranded regions in the L2Hs segment. These in vitro studies agree with the CDI experiments. They confirm that the L2Hs sequence has the potential to assume non-B-form DNA structures under the influence of torsional stress generated by DNA supercoiling.

To test whether these non-B-form structures reflect DNA conformations of functional cellular plasmid molecules, log phase cells were treated with OsO₄. This in vivo treatment also should confirm further that the in vitro results are not artifacts generated by the method of DNA preparation. Analysis of recombinant plasmid DNA modified intracellularly reveals that pN6.4.39 assumes unusual secondary structures in vivo (see hard stops in Fig. 17). The observed modification pattern agrees with the in vitro CDI and the

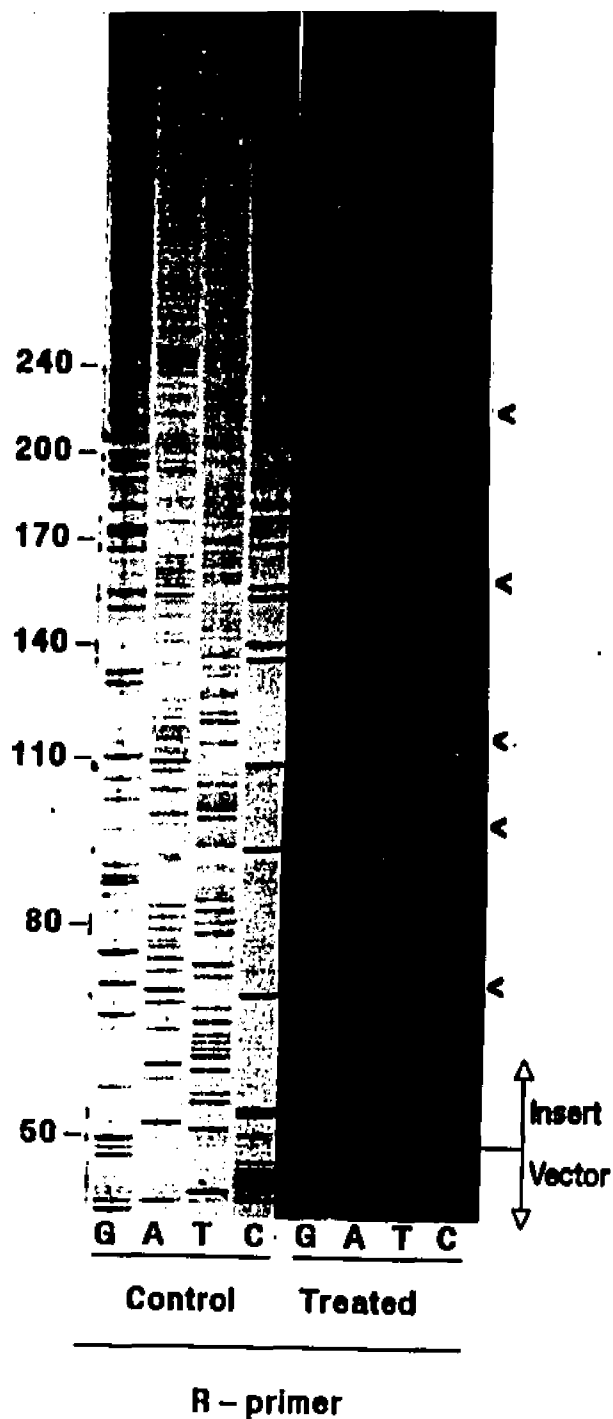


FIGURE 14. Primer extension analysis of in vitro OsO_4 -treated and control supercoiled pN6.4.39. The OsO_4 treatment was for 15 minutes as described in the methods. The pattern shown is for primer extension using the reverse primer. Hard stops (<) in the treated samples are positions of base modification; some but not all hard stops are indicated.

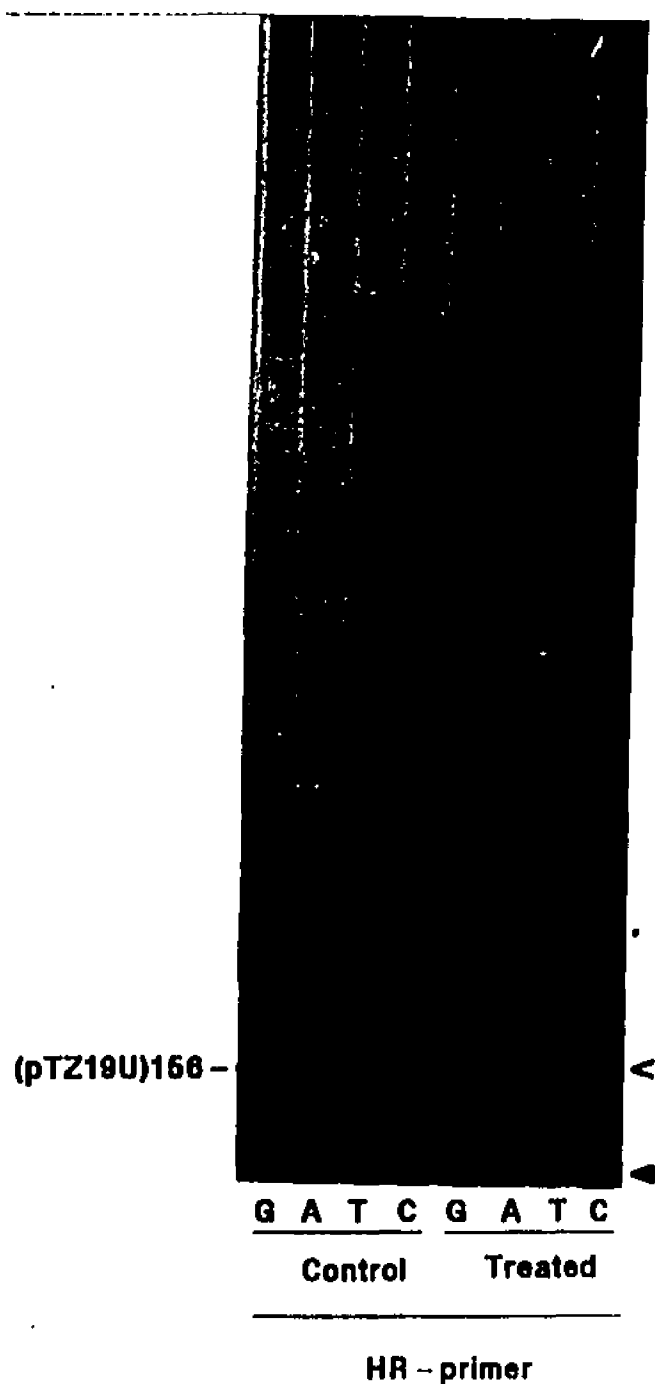


FIGURE 15. Vector sequence analysis of in vitro OsO_4 -treated pN6.4.39. The OsO_4 treatment was for 15 min as described in the methods. The pattern is for primer extension using the hybridization reverse primer. The absence of hard stops in the vector sequence confirms that the unusual secondary structures are localized to the L2Hs insert. There are two pronounced hard stops in this gel, one of which is a position of an unusual secondary structure in the vector (<); the second stop is seen in the control (<=) also.

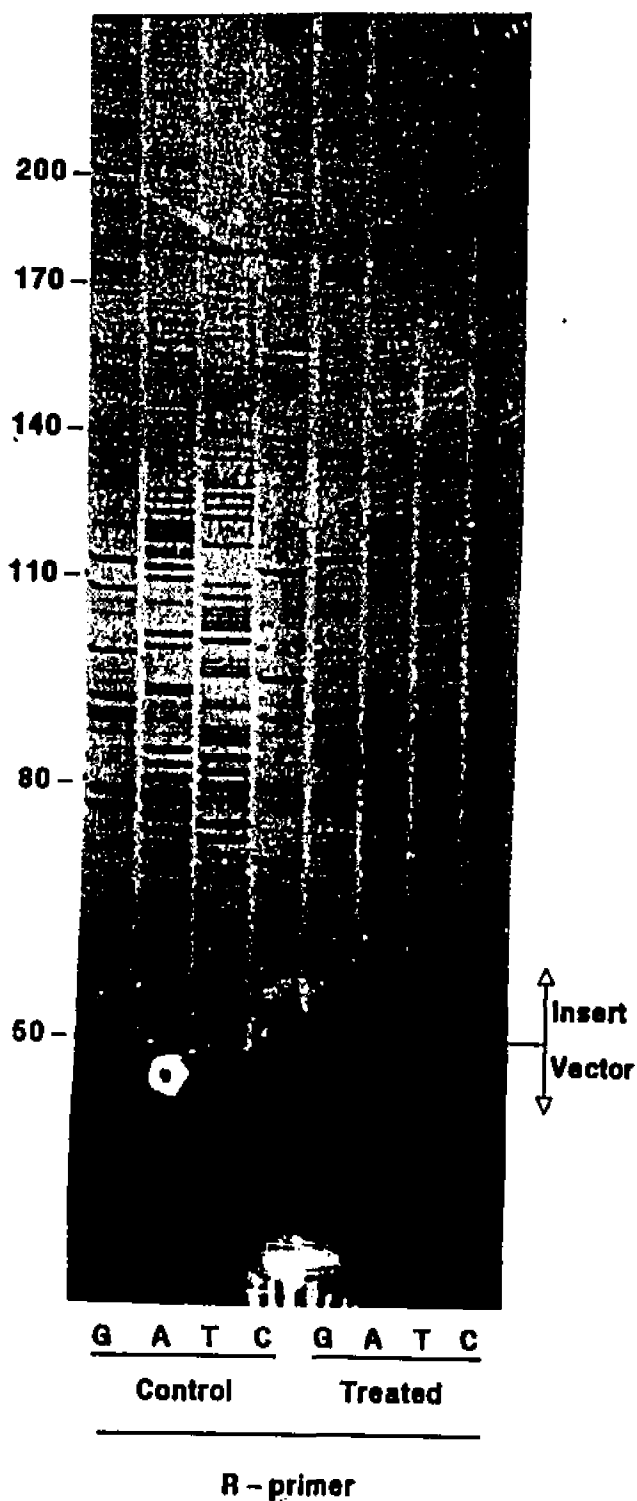


FIGURE 16. Gel pattern of in vitro OsO₄-treated linear pN6.4.39. The OsO₄ treatment was for 15 minutes as described in the methods. The shadow across the gel is an artifact generated by the fmol sequencing process using ³⁵S-dATP.

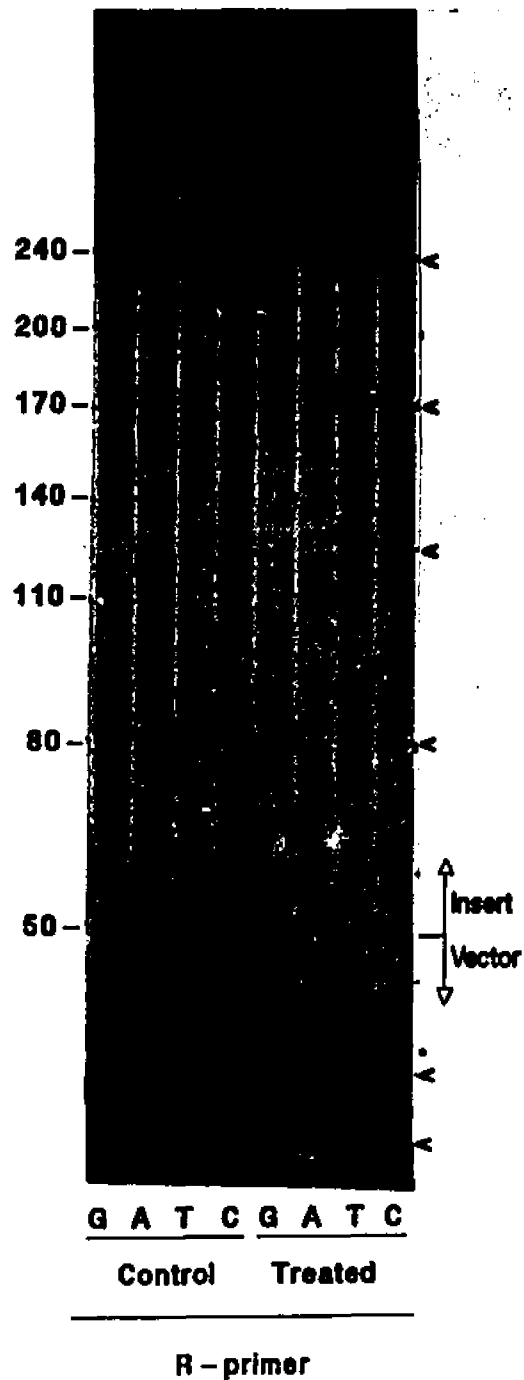


FIGURE 17. Primer extension analysis of in vivo OsO_4 -treated and control pN6.4.39. The treatments were done as described in the methods for 15 minutes. The pattern shown is for sequencing done with the reverse primer. Hard stops (<) in the treated samples are positions of modification. Not all hard stops are indicated.

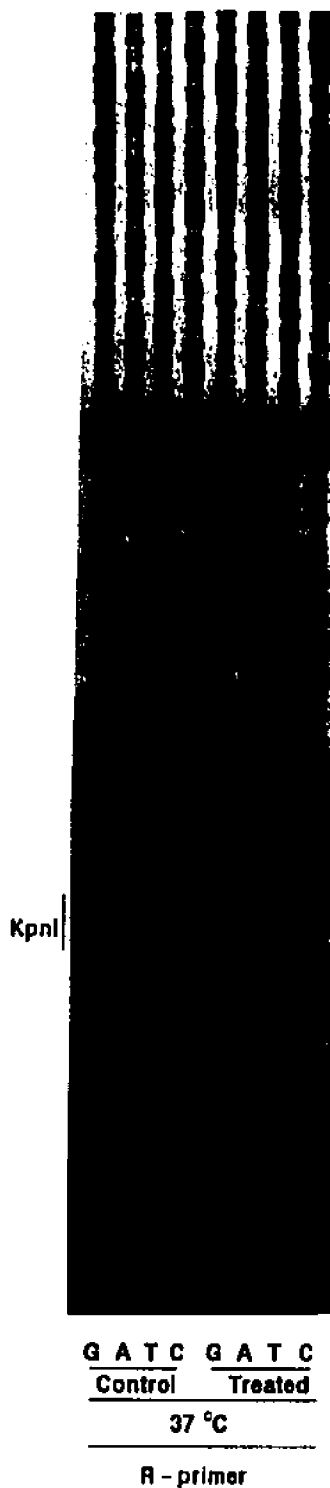


FIGURE 18. Analysis of in vitro OsO₄-treated pTZ19U. The treatment was for 15 min as described in the methods. The pattern is for primer extension using the reverse primer. The absence of hard stops in the treated sample confirms that unusual secondary structures are specific to the L2Hs insert.

OsO₄ data.

Furthermore, as in the CDI experiments, the standard reverse primer reveals single-stranded regions in the HindIII-to-SmaI region of the vector in supercoiled pN6.4.39. To test whether this is an inherent feature of this vector sequence, supercoiled plasmid vector pTZ19U, without an insert, was treated with OsO₄ in vitro. Primer extension analysis of OsO₄-treated pTZ19U vector revealed no unpaired bases in the HindIII to SmaI region (Fig. 18). This suggests that the non-B-form structure of the adjacent insert induces unusual secondary structures in the vector sequence.

These results, summarized in Figure 25, confirm the presence of unusual secondary structures in supercoiled pN6.4.39, both in vitro and in vivo. In addition, the presence of unusual secondary structures in vivo demonstrate that the in vitro results are not artifacts generated by the method of DNA preparation. This was documented further in the S₁ nuclease experiments described below.

S₁ Nuclease Analysis of pN6.4.39

S₁ nuclease sensitivity is widely used to test for single-stranded regions in DNA. This enzyme rapidly and specifically cleaves single-stranded regions without base preference in a variety of different non-B-form DNA secondary structures (Wells and Harvey 1988). Hence, it was

used to probe different topoisomeric forms of pN6.4.39. These include native superhelical DNA with high superhelicity ($\sigma = -0.039$), medium topoisomeric forms ($\sigma_{\text{AVE}} = -0.020$), low topoisomeric forms ($\sigma_{\text{AVE}} = -0.008$), completely relaxed plasmid ($\sigma = 0.0$) DNA, and linear DNA. These substrates were employed to map the positions of single-stranded regions and to determine the effect of superhelical density on the formation of unpaired bases. In addition, the effect of temperature on the formation of unusual secondary structures was analyzed.

S₁ Nuclease Cleavage Analysis at 37°C

Supercoiled plasmid DNA ($\sigma = -0.039$) prepared by the gentle, non-denaturing Triton X-100 lysis/CsCl method (Murchie and Lilley 1992) was treated with S₁ nuclease at 37°C. The recombinant plasmid is extremely susceptible to cleavage under these conditions. Initially (1 minute), ~50% of the DNA is converted from form I (supercoiled) to forms II (nicked circular) and III (linear). At the next time point (2 minutes), form I DNA is completely converted to forms II and III (Fig. 19).

These nicked DNAs were analyzed by primer extension to map the sites of S₁ nuclease cleavage (Fig. 20). Analysis with the forward and reverse primers revealed that unpaired bases were localized almost exclusively to the region of the

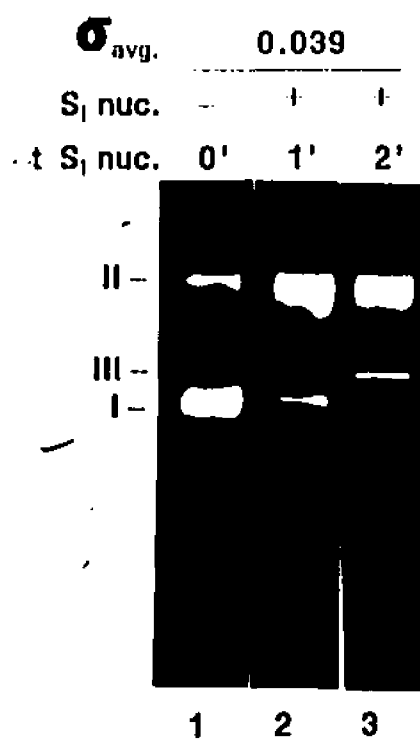


FIGURE 19. Gel pattern of supercoiled pN6.4.39 treated with S_1 nuclease at 37°C. Lane 1. control; Lane 2. 1 min time point; Lane 3. 2 min time point; supercoiled DNA (I), nicked circular DNA (II) or linear DNA (III).

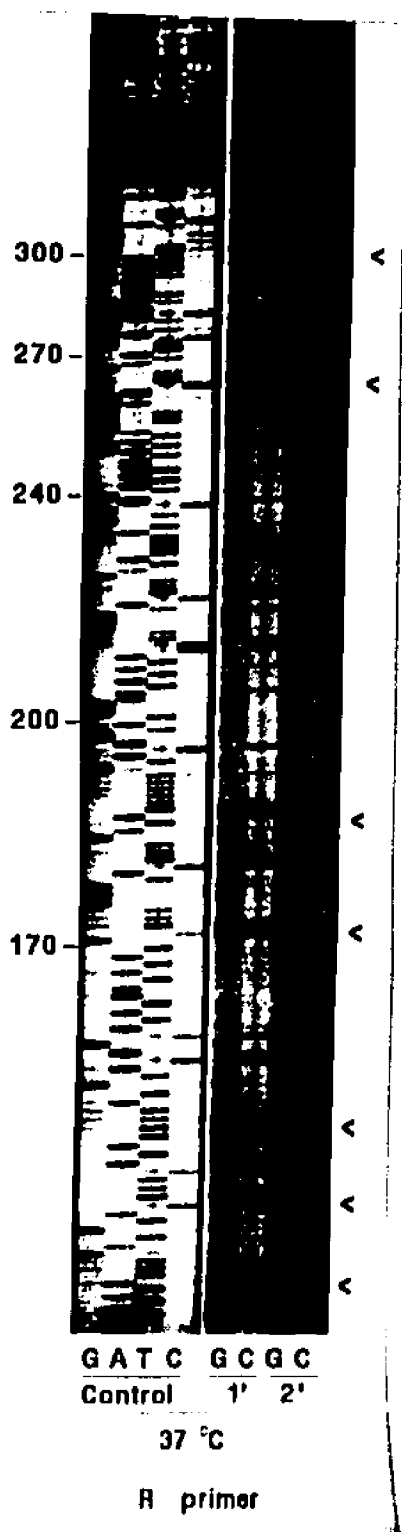


FIGURE 20. S_1 nuclease cleavage pattern of supercoiled pN6.4.39 at 37°C. The treatments were done as described in methods. The patterns shown are for primer extension using the reverse primer. Hard stops in the 1 min and 2 min time points are positions of S_1 nuclease cleavage (<). In the treated samples only G and C extension reactions need to be analyzed to visualize hard stops.

extended reverse primer. The number of overall hard stops observed were lower than those observed in the CDI and the OsO₄ experiments. This may be due to the collapse of the unusual secondary structures when S₁ nuclease introduces the first nick which permits the release of torsional stress. This data is summarized in Fig. 25.

As in the CDI and OsO₄ experiments, the S₁ nuclease experiments (at 37°C) show modification (cleavage) in the vector sequence adjacent to the L2Hs insert (see Fig. 25). However, S₁ nuclease analysis of pTZ19U vector without the L2Hs insert reveals no unpaired bases in this region (Fig. 21). The non-paired bases detected in the HindIII-SmaI region of pN6.4.39, therefore, must be induced by the non-B-form structure of the L2Hs element.

S₁ Nuclease Cleavage Analysis at 16°C and 24°C

Supercoiled DNA was treated with S₁ nuclease at 16°C and 24°C. pN6.4.39 is less susceptible to cleavage at these temperatures due to decreased enzyme activity at and to a reduction in the unusual secondary structures in the DNA substrate. At 16°C, about 75% of the DNA is converted to forms II and III within 8 hours. The form I DNA is almost completely converted to forms II and III by 16 hours (Fig. 22). At 24°C, the form I DNA is completely converted to forms II and III within 8 hours. At the 16 hour time point a significant proportion of the form II DNA was linearized

to form III (see Fig. 22). The degree of conversion in 16 hrs at 24°C is equivalent to the conversion by 2 min at 37°C (see Fig. 19).

The DNAs treated for 8 hr and 16 hr at 16 and 24°C were analyzed by primer extension. The patterns in Fig. 23 demonstrate an increased number of hard stops with a shift in temperature from 16°C to 24°C. The data, summarized in Fig. 25, demonstrate a decrease in single-stranded bases (i.e. unusual secondary structures) in supercoiled pN6.4.39 at reduced temperatures. Some single-stranded regions detected at 24°C, are not seen at 16 or 37°C. However, some sites are seen at all three temperatures. The number of single-stranded regions detected at 37°C is greater than the number detected at 16 or 24°C. It must be noted that an increase in temperature can aid in the melting of double-stranded regions to form single-stranded regions. More significantly, can result in an increase in superhelical density of the recombinant plasmid. An increase in both DNA melting and an superhelical density may account for the increased in non-B-form structures detected at 37°C. It must be noted that S_1 nuclease activity is reduced with a decrease in temperature.

S_1 Nuclease Analysis of Different Topoisomeric Forms of pN6.4.39

The goal of this experiment was to determine the effect



FIGURE 21. S₁ nuclease analysis of supercoiled pTZ19U plasmid vector at 37°C. The treatments were done as described in methods. The patterns shown are for primer extension using the reverse primer. There are no hard stops in the treated samples. The KpnI site is the location of the L2Hs segment in pN6.4.39.

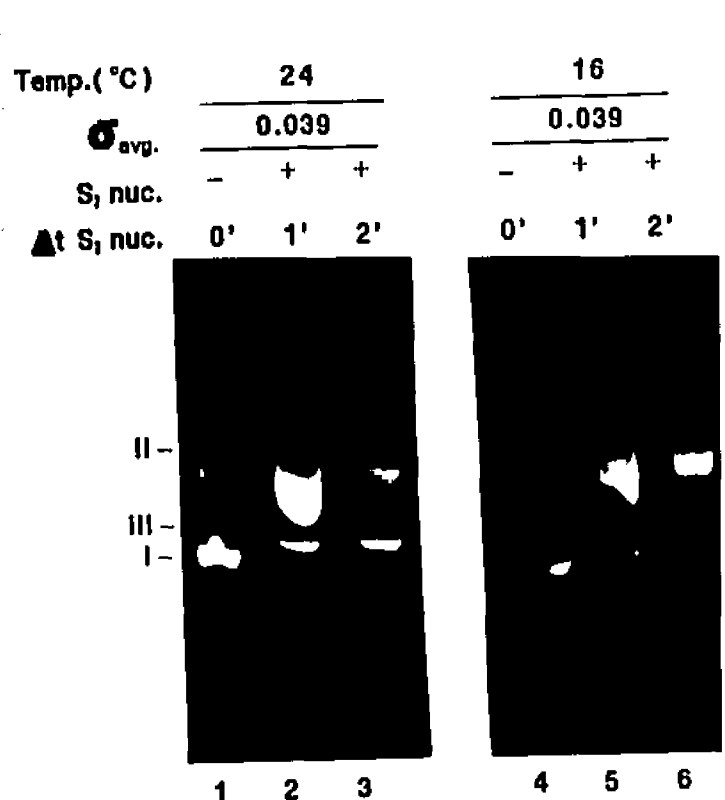


FIGURE 22. Gel pattern of supercoiled pN6.4.39 treated with S₁ nuclease at 16°C and 24°C. Lane 1. control (incubated at 16°C for 8 hrs in S₁ nuclease reaction buffer without S₁ nuclease); Lane 2. 8 hr-treated DNA at 16°C; Lane 3. 16 hr-treated DNA at 16°C; Lane 4. control (incubated at 24°C for 8 hrs in S₁ nuclease reaction buffer without S₁ nuclease); Lane 5. 8 hr-treated DNA at 24°C; Lane 6. 16 hr-treated DNA at 24°C. Supercoiled DNA (I), nicked circular DNA (II), linear DNA (III) are DNA forms indicated on the left.

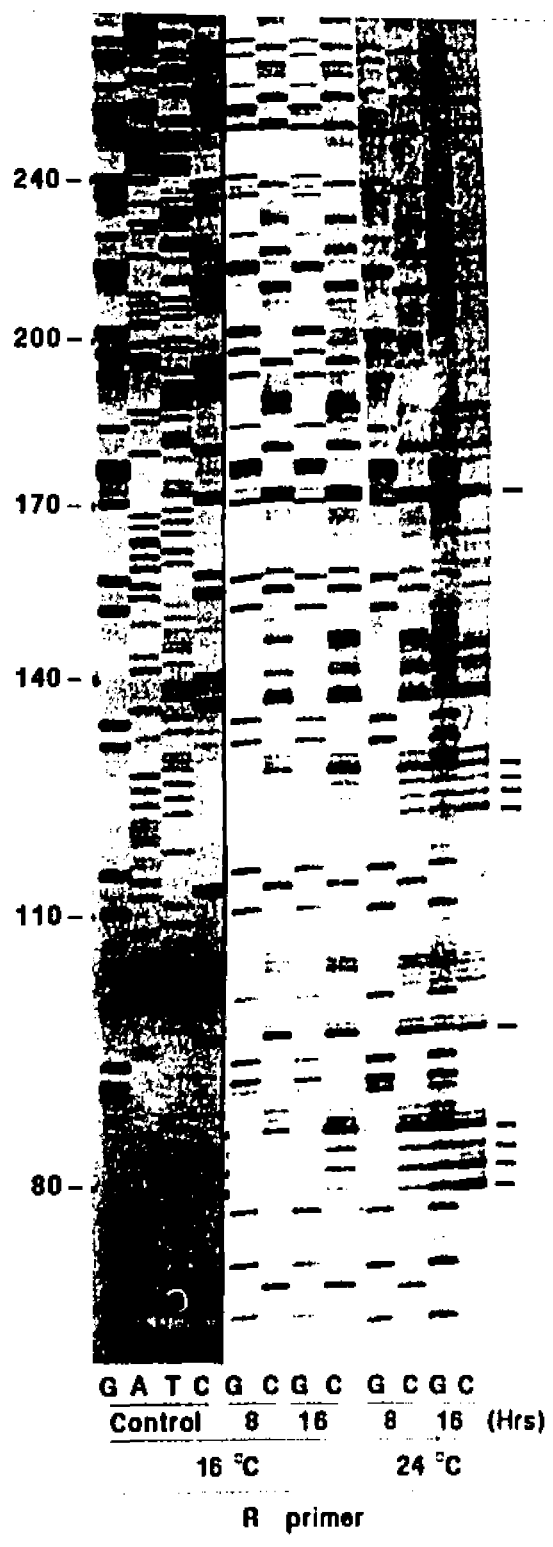


FIGURE 23. Primer extension analysis of supercoiled pN6.4.39 treated with S_1 nuclease at 16°C and 24°C. The treatments were done as described in the methods. - hard stops seen only at 24°C.

of superhelical density (σ) on the formation of single-stranded regions in the L2Hs element. Different topoisomeric forms of pN6.4.39 were treated with S_1 nuclease at 37°C. DNA with reduced superhelical density ($\sigma \leq -0.02$) was found to be significantly less susceptible to S_1 nuclease cleavage than was native, fully supercoiled ($\sigma = -0.039$) DNA. Agarose gel analysis reveals that DNA with superhelical densities less than -0.02 is relatively resistant to S_1 nuclease cleavage (compare Figs. 19 and 24). Primer extension analysis of the same DNA reveals no specific cleavage sites in this DNA (data not shown).

Data Summary

The CDI, OsO_4 , and S_1 nuclease experiments, summarized in Fig. 25, show a predominance of unusual secondary structures in the first hundred and fifty or so bases of the L2Hs insert in pN6.4.39. In addition, primer extension analysis with the reverse primer consistently revealed base modifications and S_1 nuclease cleavage at unpaired bases in the vector sequence immediately adjacent to the L2Hs insert. Such modifications were far fewer in the KpnI-to-EcoRI vector region of the recombinant plasmid. However, analysis of treated supercoiled pTZ19U vector DNA (without the insert) using the same primers revealed no unpaired regions. The non-paired vector bases detected in pN6.4.39 seem to be induced by the non-B-form structures in the adjacent AT-rich

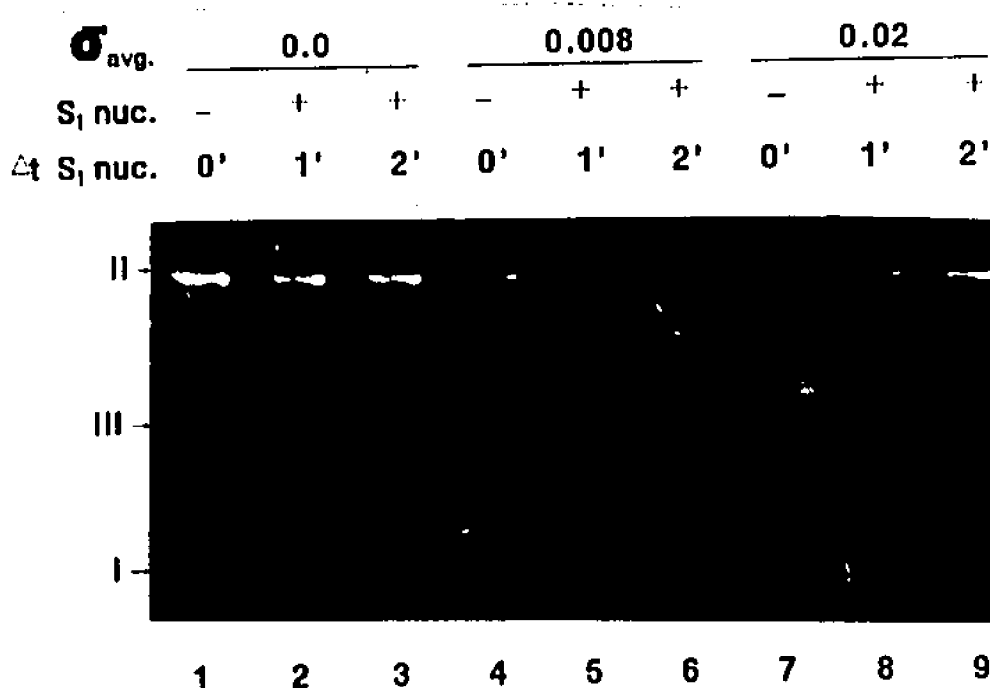


FIGURE 24. S_1 nuclease analysis of pN6.4.39 topoisomers at 37°C. The treatments were done as described in the methods. Lanes 1-3: Control, 1 min and 2 min S_1 nuclease-treated relaxed ($\sigma = 0$) plus linear DNA. Lanes 4-6: Control, 1 min and 2 min S_1 nuclease-treated low superhelical form ($\sigma_{AVE} = -0.008$) DNA. Lanes 7-9: Control, 1 min and 2 min S_1 nuclease-treated medium superhelical density form ($\sigma_{AVE} = -0.020$) DNA. Supercoiled DNA (I), nicked circular DNA (II) and linear DNA (III) are indicated.

L2Hs segment in supercoiled recombinant plasmid. By definition, then, the L2Hs element exhibits features of C-type extrusion-inducing sequences (Murchie and Lilley 1992).

Analyses of the vector sequence of treated DNA with hybridization primers revealed one possible unusual secondary structure at position 156 of vector DNA sequence. This non-B-form structure (at position 156) does not occur in supercoiled pTZ19U DNA. Consequently, this unusual secondary structure seems to be formed by the C-type extrusion-inducing activity of the L2Hs insert. Therefore most of the vector sequence does not appear to share the same structural characteristics as the L2Hs segment. Analysis of relaxed (or linear) pN6.4.39 DNA, and DNA with $\sigma \leq -0.002$ reveals that a certain degree of torsional stress is required to induce the formation of non-B-form DNA structures. Furthermore, the method of DNA preparation does not significantly effect the formation of these unusual secondary structures in the L2Hs element. The results presented here also show that the L2Hs sequences assume non-B-form structures in vivo as well as in vitro.

CHAPTER 4

Discussion

Genome structure and expression involve a multiplicity of macromolecular interactions. DNA is the critical specificity determinant in these interactions due to the H-bonding potentials present in the minor and major grooves of B-form DNA. In addition, certain sequence motifs assume non-B-form conformations, including Z-DNA, bent, single-stranded and cruciform DNA structures. Since these non-B-form structures present unique sites for regulatory interactions, elucidating their occurrence, function and behavior is necessary for understanding the regulation of genome structure and dynamics.

The L2Hs sequences, due to their unusual sequence properties, present a good model to study the role of unusual secondary structures in human genomes. For example, in *E. coli* the presence of inverted and direct repeats and palindromic sequences (like the L2Hs sequence) strongly influence recombination and replication events. These events lead to deletions and duplications of segments containing these sequences (Mizuuchi et al. 1982; Trinh and Sinden 1993). In addition, the transition into left helices of two alternating purine-pyrimidine tracts in the distal regulatory region of the rat prolactin gene appears to be mediated by a central AT-rich sequence (Kladde et al. 1993).

The L2Hs sequences, consequently, have the potential to participate in a myriad of cellular functions.

The L2Hs family consists of long, interspersed repetitive sequences. Although present in a few copies in other primates, the L2Hs elements exist only in human genomes as an intermediately repetitive sequence family (\geq 500 copies). In addition, these sequences exhibit extensive quantitative and qualitative polymorphisms among human genomes. This seemingly recent origin and ongoing evolution of the L2Hs family in the human lineage and the extensive population polymorphism observed indicate that these sequences remain dynamic elements in the evolving human genome (Musich and Dykes, 1986; Roudabush, 1989).

One 0.6 kb L2Hs segment cloned into the Kpn I site in pTZ19U, denoted pN6.4.39, was sequenced and used for most of the secondary structure analyses. This sequence is 76% AT-rich (Fig. 25) and internally very repetitious, with numerous palindromic, inverted and direct repeats (Table 1 and 2). Several of the sequence features of this L2Hs element may relate to the intra- and inter-genomic polymorphisms observed for this sequence family. In supercoiled plasmid, the insert may assume non-B-form DNA structures such as cruciforms with single-stranded loops. This hypothesis was confirmed by testing the sensitivity of pN6.4.39 to base modification by CDI, OsO₄ and S₁ nuclease, probes specific for unpaired bases. Furthermore,

computational analysis suggests that the sequence may form stem-loop structures (Table 2). Some of these are thermodynamically stable but most appear to be energetically unfavorable due to their large loop size. However, some large single-stranded loop regions could be involved in duplex formation with other single-strand regions of the insert, thus stabilizing stems with large loops. In fact, many of the hard stops shown in Figure 25 map to the loops of one or more predicted stem-loop structures (compare Table 2 to Fig. 25). Others have demonstrated that AT-rich sequences can form paranemic DNA structures (McCarthy et al. 1990; Price and Tullius 1993; Schaeffer et al. 1989) and that these may participate in replication and recombination events (Masse et al. 1992; Natale et al. 1992). In addition, somatic changes in repetitive DNAs are associated with mammalian development (Kelly et al. 1989) and tumor oncogenesis (Aaltonen et al. 1993; Peltomaki et al. 1993; Thibodeau et al. 1993). Thus, it is possible that the polymorphic features of the genomic L2Hs elements may stem directly from the dynamic behavior of the sequence itself and that these elements may participate in specific genomic and cellular events.

Analysis of pN6.4.39 With CDI

There are various chemical reagents that react with non-paired bases in single-stranded regions of unusual

secondary structures. These positions of modification can in turn be detected by primer extension (Fig. 6).

CDI is an electrophilic reagent, which reacts primarily with the imino N3 of guanine and with N1 of thymine in single-stranded regions of DNA (Mertz and Brown, 1969).

The bulky CDI-generated covalent adduct (Fig. 7) inhibits the progress of the Taq DNA polymerase in the primer extension reactions. Termination occurs at or over several nucleotides preceding an adduct (Wilkins, 1984). Both the pTZ19U vector and L2Hs insert regions were analyzed for the presence of single-stranded regions in these studies (Figs. 11, 12). This allows for comparison of the "unusual" L2Hs sequence and the "normal" vector sequence. We were able to show that single-stranded regions occur primarily in the L2Hs sequence and not in the vector. This technique allowed us to map the positions of possible unusual secondary structures and, hence, probable recombination hot spots and/or other regulatory structural motifs.

CDI generates a pattern of base modifications representing unpaired G or T bases in the single-stranded loop regions of cruciforms. Since the sequencing gels display bands of extended primer strands, we expect stops at or right before C or A bases (Figs. 6, 11). These single-strand specific modifications are dependent on plasmid superhelicity; linear (relaxed) pN6.4.39 is not sensitive to CDI modification (Fig. 13). The modified bases map to

single-stranded loop regions of the computer-generated stem-loop cruciform structures listed in Table 2. However, since the DNA for CDI analysis was prepared by alkaline lysis, which has been reported to induce unusual secondary structures (Murchie and Lilley 1992), it is possible that the structures detected are experimental artifacts. The *in vivo* OsO₄ and the *in vitro* S₁-nuclease experiments do not support this explanation. They reveal that unusual secondary structures are formed in the L2Hs sequence elements *in vivo* and *in vitro*, independent of the DNA preparation method.

Analysis of pN6.4.39 With OsO₄

Osmium tetroxide forms an addition compound with the C₅-C₆ bond of thymine (Fig. 7) and, to a minor extent, of cytosine in single-stranded regions of DNA (Yagil, 1991). The C₅-C₆ bond in B-form DNA resides in the major groove and is consequently unreactive toward OsO₄. In this set of experiments DNA was treated with OsO₄ both *in vivo* and *in vitro*. The treated DNA was analyzed by primer extension as in the CDI experiments. The vector sequence was also analyzed for formation of adducts and compared to the L2Hs insert. The results from these experiments were in full agreement with results from the CDI experiments.

OsO₄ generates a pattern of base modifications representing unpaired T or C bases. We expected and

observed stops at or just before A or G bases. As in the CDI experiments linear pN6.4.39 is not susceptible to OsO₄ modification (Fig. 16). Analysis of pN6.4.39 DNA treated with OsO₄ in vivo (Fig. 17) reveals that the L2Hs segment assumes non-B-form structures in cells, as well as in purified DNA treated in vitro (Fig. 14). This confirms the CDI results and shows that unusual secondary structures are present in vivo. Furthermore, they also confirm that the observed non-B-form structures are independent of the DNA isolation technique. This is confirmed further by the S₁ nuclease experiments using DNA prepared by the Triton X-100 lysis method, a gentle technique that does not induce unusual secondary structures (Murchie and Lilley 1992).

S₁ Nuclease Analysis of pN6.4.39

S₁ nuclease is a widely used probe to study unusual secondary structures. This enzyme has the ability to rapidly and sensitively detect a variety of different DNA conformations. Originally isolated from Aspergillus oryzae as a single-strand specific nuclease (Vogt 1980), S₁ nuclease also cleaves double-stranded DNA at specific, non-B-form sites, such as the junctions between right and left handed sequences (Kilpatrick et al. 1984; Hayes and Dixon 1985) and the center of inverted repeats in cruciform structures (Lilley and Kemper 1984; Muller and Wilson 1987). In addition, the enzyme seems to recognize polypyrimidine-

polypurine stretches (Selleck et al. 1984) and anisomorphic DNA (Wohlrab et al. 1987).

S_1 nuclease converted supercoiled pN6.4.39 DNA ($\sigma = -0.039$) prepared by the Triton X-100 lysis method, into nicked form II and linear form III DNA. Primer extension analysis of this DNA generated hard stops in the L2Hs segment at positions which overlap those of CDI and OsO₄ modification sites; however, the frequency of hard stops observed is reduced due to collapse of unusual secondary structures coincident with the first nick in the circular DNA and release of torsional stress. Reducing superhelical density ($\sigma \leq -0.020$) or linearizing the DNA abolished the S_1 nuclease sensitivity of pN6.4.39 (Fig. 24). Analysis of pN6.4.39 at lower temperatures (24°C and 16°C) revealed positions of possible unusual secondary structures (Fig. 23); however, the number of hard stops observed was lower than at 37°C (Fig. 25). This is probably due to a decrease in S_1 nuclease activity and a reduction in the melting of DNA at these lower temperatures. In addition, a reduction in superhelical density due to lower temperatures may also result in a net decrease in the number of non-B-form structures present.

Structure of pN6.4.39 Vector Sequence

Carbodiimide, osmium tetroxide and S_1 nuclease analysis of supercoiled pN6.4.39 vector sequences with hybridization

forward and reverse primers (Figs. 12, 15) reveals that the ability to form unusual secondary structures is dependent on specific sequence features such as high AT-content and the presence of inverted repeats. These features are present in the L2Hs insert and not in the vector. One non-B-form structure at position 156 in the vector sequence of pN6.4.39 was detected. This non-B-form structure is not detected in pTZ19U vector (without the L2Hs insert). Consequently the non-B-form structure detected at position 156 in the vector of pN6.4.39 is induced by the structure of the L2Hs insert.

In addition, the standard reverse primer consistently revealed base modifications and S_1 nuclease cleavages (Fig. 25) at non-paired bases in HindIII-to-SmaI region of the multiple cloning site of the vector in supercoiled pN6.4.39. This should be a relatively stable B-form region (69% G+C), even though it contains seven short palindromic restriction sites. Base modification and S_1 nuclease analysis of supercoiled pTZ19U vector without the L2Hs insert or linear/relaxed pN6.4.39 reveal no unpaired bases in this region (Figs. 18 and 16). The single-strand regions detected in the HindIII-SmaI region of supercoiled pN6.4.39, therefore, must be induced by the non-B structure in the adjacent AT-rich L2Hs segment. By definition, then, this L2Hs segment exhibits features of a C-type extrusion-inducing sequence (Furlong et al. 1989; Murchie and Lilley 1992; Sullivan et al. 1988). Consequently, the L2Hs

sequence elements, due to their unusual structure, can induce the formation cruciforms in adjacent DNA sequences.

Structure of pN6.4.39 In Vivo

Secondary structure analysis done with CDI and OsO_4 was performed on plasmid DNA prepared by alkaline lysis miniprep procedures. Non-B structures may have been induced during the alkaline denaturation step. However, plasmid DNA within cells can be treated with OsO_4 (Palecek, 1992). Such in vivo treatments confirm the in vitro studies (Fig. 17). In vivo, as in vitro, part of the multiple cloning site (HindIII-SmaI) of pN6.4.39 and the L2Hs insert, are modified, indicating an in vivo C-type extrusion inducing activity of the L2Hs sequence. Thus, the in vitro results, using the alkaline lysis DNA preparation, reflect the non-B-form structures of the L2Hs segment of pN6.4.39 as it occurs in rapidly growing cells.

The work presented here provides a molecular basis for the intra- and inter-genomic polymorphisms observed in the L2Hs sequence family because unusual secondary structures, as mentioned earlier, aid in recombination. Furthermore, the ability of these sequence elements to form unusual structures under torsional stress suggests that they could be involved in the release of torsional stress in actively transcribed and replicated chromosomes. In addition, L2Hs elements may serve to absorb torsional stress generated

during the packing of DNA in human chromosomes (Fig. 4). They also could be binding sites for enzymes such as topoisomerase I, reported to interact with sequences that are AT-rich and, in some instances, with sequences that assume non-B-form DNA secondary structures (Kirkgaard and Wang 1981). Consequently, it is possible that L2Hs elements temporarily relieve torsional stress in human nuclear DNA by assuming unusual secondary structures. These in turn could act as beacons for subsequent topoisomerase I binding and supercoil relaxation.

Prospects For The Future

The next step in this series of experiments will be to use a cruciform structure specific nuclease, a resolvase (such as T₄ endonuclease VII, T₄ endonuclease I or E. coli RuvC), to map cruciforms and not just single-stranded DNA regions in supercoiled pN6.4.39 (Lilley and Kemper, 1984). This would prove the existence of specifically cruciforms in the L2Hs elements.

Studies with the recombinant plasmid pN6.4.39-2, which has the same insert as pN6.4.39, but in the opposite orientation needs to be completed. This will provide some interesting data on the effects of orientation on non-B-form structure formation. It will also provide more information on the C-type extrusion-inducing ability of the L2Hs elements. For example, will a change in the orientation of

the insert effect its unusual structure and will this modify the C-type extrusion inducing activity of the L2Hs insert.

Furthermore, it will also be interesting to determine the presence of non-B-form structures in human genomic L2Hs DNA and nuclear chromatin. This would provide a very significant correlation to the *in vitro* and *in vivo* E. coli experiments. These experiments will provide more data on the structure of L2Hs elements as they occur naturally in the complex nuclear chromatin of human cells.

We have employed OsO_4 to successfully analyze non-B-form structural features of plasmid DNA within intact E. coli cells. Given the greater complexity of the genomic L2Hs elements in human cells this experiment will be less straight forward. Its basic design will be to treat human tissue culture cells with OsO_4 -bipyridine, washing out excess OsO_4 and embedding the cells in agarose plugs for DNA purification (Fritz and Musich 1990). As a control, purified intact genomic DNA embedded in agarose plugs will be OsO_4 -bipyridine treated in parallel to test for non-B-form structures. The DNAs then will be cleaved with appropriate restriction enzymes before strand cleavage at modified sites by incubation at 90°C in 0.1 M piperidine (Curiel et al. 1990). The denatured DNA fragments will be separated by alkaline agarose gel electrophoresis, Southern-blotted and hybridized with pN6.4.39 insert as probe. If the nuclear L2Hs sequences are in non-B-form DNA structures

with unpaired T and C bases, these sequences should be cleaved at the modified base by the hot piperidine treatment. These unpaired T and C modifications should be resolved in the DNA fingerprint as segments of reduced size relative to untreated nuclear DNA.

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APPENDIX A

Buffers and solutions

fmol sequencing reaction buffer

50 mM Tris-HCl, pH 9.0, 2 mM MgCl₂

fmol sequencing stop buffer

95% deionized formamide, 10 mM NaOH, 0.05
xylene cyanol, 0.1% bromophenol blue

LB broth

10 g bactotryptone, 5 g bacto-yeast extract, 5 g
NaCl, add dH₂O to 0.8 liters, adjust pH to 7.0
with NaOH and make up to 1 liter and autoclave to
sterilize

Prehybridization solution

6 X SSC (or SSPE), 2.5% Denhardt's reagent, 0.5%
SDS, 50% formamide.

RNase A

20,000 U/ml in 10 mM Tris-HCl pH 7.5, 15 mM NaCl,
boiled for 15 minutes

S₁ nuclease reaction buffer

50 mM NaOAc, 50 mM NaCl, 1 mM ZnSO₄, pH 4.9

SOG loading buffer

60% sucrose, 0.1% Orange G

SSC (1X)

0.15 M NaCl, 15 mM sodium citrate, pH 7.0

SSPE (1X)

0.18 M NaCl, 10 mM NaH₂PO₄, 1 mM EDTA, pH 7.4

SspI reaction buffer

20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 7mM MgCl₂,
0.1 mM DTT, 0.1 mg/ml BSA

T₁₀E₁

10 mM Tris-HCl pH 8.4, 1 mM EDTA

T₁E_{0.1}

1 mM Tris-HCl pH 8.4, 0.1 mM EDTA

50 mM TAE Buffer

50 mM Tris pH 8.4, 40 mM acetic acid, 1 mM EDTA

50 mM TBE Buffer

50 mM Tris pH 8.3, 50 mM boric acid, 1.25 mM EDTA

Topoisomerase I reaction buffer

50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 20%
glycerol, 0.05 M NaCl

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4. Unusual secondary structures in a human middle repetitive DNA. Duminda Ratnasinghe and Phillip R. Musich. (in progress)