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A Supercoil-Dependent Structural Alteration within the Regulatory Region of the Human Transferrin Receptor Gene

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Summary. The transferrin receptor gene is transcribed at low levels in quiescent cells and at much higher levels in growing or transformed cells. This regulation involves elements located within the first 114 base pairs upstream of the major transcriptional start site. This region is specifically recognized by several transacting factors and contains an element that is composed of alternating purines and pyrimidines. In vitro this element can adopt a non-B DNA conformation in a supercoil-dependent manner. Similar elements, with nearly identical spacing relative to a protein recognition sequence, can be observed in several other proliferation dependent gene promoters.

DNA is a structurally dynamic macromolecule able to adopt a number of unusual conformations in addition to the familiar B-form. These alternative conformations have been extensively studied and characterized in vitro and it is widely believed that they have important regulatory roles in processes such as replication, recombination, and transcription. However, evidence for in vivo functions of such structures is very limited. Recently, critical advancement was made when it was shown that both Z-form DNA and cruciforms can exist in living E. coli cells (1,2).

In vitro, these structures are stabilized by negative supercoiling of the DNA molecule, and this is probably the driving force allowing them to exist within the cell. In prokaryotes it is well known that the level of supercoiling can play a regulatory role in promoter usage (for a review see ref. 3). In eukaryotic systems most DNA supercoils are thought to be constrained within nucleosomes. However, it is very likely that localized regions of supercoiling do exist in the chromatin of a eukaryotic cell. Liu and Wang (4) have proposed that a major factor influencing the level of supercoiling is the transcriptional process itself. They suggest that the resistance to rotation of the

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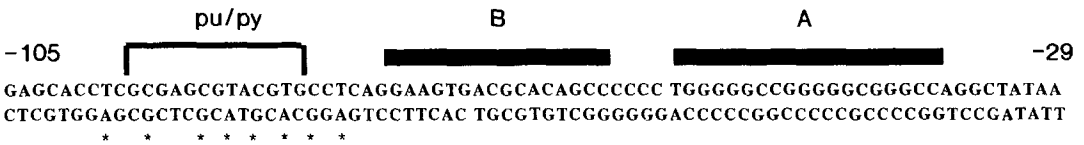
transcriptional apparatus around the template DNA results in negative supercoiling upstream and positive supercoiling downstream of the region being transcribed. Direct evidence supporting this hypothesis has been obtained in both prokaryotic and eukaryotic systems (5-9). In this model the highest level of localized negative supercoiling would be found just upstream of the transcriptional start site within the promoter region and would reach levels sufficient to stabilize non-B structures (4). In support of this Rahmouni and Wells (10) have shown that, in vivo, a (CG) tract as short as 12 base pairs adopts the Z form when placed upstream of the tet gene but that a tract as long as 74 base pairs will not undergo a transition to the Z form when placed at the 3' end of the same gene.

We have observed a region of alternating purines and pyrimidines (pu/py, a potential Z-forming motif) within the promoter of the human transferrin receptor (TR) gene. This gene is expressed at low levels in quiescent cells and activated upon mitogen stimulation (11-13). This is a late response in the mitogenic pathway reaching a maximum near the beginning of S phase (14,15). By deletion mutation analysis and microinjection assays, we have found that promoter sequences between -24 and -78 are critical for the proliferation dependent response of the gene (16 and unpublished data). We have extensively characterized this region by DNase I footprinting and found that two elements within this region are bound by nuclear factors (14 and unpublished data). These are shown by solid bars in Fig. 1.

The possibility that the region of alternating pu/py (bracket Fig. 1) in the TR promoter can undergo a structural transition is of



TR5-7
GAGCACCTCGCGcGCGcgCGcGCCTCAGGAAGTGACG
CTCGTGGAGCGCgCGCgcGCgCGGAGTCCTTC ACTGC

TR6-8
GAGCACCTCGCGAGgGTAgGTGCCTCAGGAAGTGACG
CTCGTGGAGCGCTC cCATcCACGGACTCCTTCACT GC

Figure 1. Diagram of the TR promoter sequence. The top shows the sequence of the wildtype TR promoter region from -105 to -29. The bars labelled A and B indicate protein binding sites determined by DNase I footprinting experiments (ref. 14) and correspond to the region of the promoter that is responsive to mitogen stimulation. The bracket indicates a region composed largely of alternating purines and pyrimidines. The asterisks indicate the bases chemically modified by DEPC in supercoiled DNA (see Fig. 2). The sequence changes in two site specific mutants, TR5-7 and TR6-8, are shown at the bottom. The mutated bases are indicated by lower case letters.

considerable interest because of its position just upstream of the transcriptional start site and immediately adjacent to the mitogen responsive region of the promoter.

METHODS

Diethylpyrocarbonate (DEPC) Modification. The plasmids pTRSaf, pTR5-7, and pTR6-8 contain either the wildtype (pTRSaf) or mutated (pTR5-7 and pTR6-8) TR promoter region extending from -114 to +251. For DEPC modification, these plasmids were used either in supercoiled form as isolated from the bacterial host or in linear form after digestion with Sal I which cleaves at a single site 19 base pairs upstream of the TR promoter insert. The plasmids (~20 μg) were dissolved in 200 μl of 50 mM sodium cacodylate (pH 8.0), 1 mM EDTA. DEPC (3 μl) was added and the tubes vortexed. After a 15 minute incubation at room temperature the samples were ethanol precipitated. End labelled fragments were isolated by digestion with Sal I, end filling with Klenow fragment in the presence of ^{32}P-dCTP, and then digestion with Bam HI. The labelled fragment was purified by agarose gel electrophoresis and then treated with 1 M piperidine at 90° C for 30 minutes. The cleavage products were analyzed by electrophoresis on a 7 % acrylamide sequencing gel.

RESULTS

We have used a chemical modification procedure to determine if a structural transition can occur within the putative Z-forming region of the TR promoter. Plasmid DNA containing the TR promoter region to -114, either linearized by a single cut restriction enzyme or in native supercoiled form as isolated from the host bacteria, was treated with diethylpyrocarbonate (DEPC). This reagent has previously been utilized to discriminate between B-form and Z-form DNA (16,17). It modifies the N7 position of purine bases which are exposed in Z-DNA but inaccessible in the B conformation. After DEPC treatment, end-labelled fragments were isolated, subjected to piperidine cleavage, and the fragments were analyzed on sequencing gels. Fig. 2 shows that in the linearized sample there is a faint ladder of bands that are slightly enhanced at purine residues. In contrast, the supercoiled lane shows a prominent series of intense bands that map precisely in the region of alternating purines and pyrimidines. Within this region every purine base shows enhancement, with A residues being more prominent than G residues. This is exactly the pattern expected if the region has undergone a B-Z transition since the purines should be in the syn configuration exposing the N7 position on the outside of the molecule. Obviously the structural perturbation extends beyond the strictly alternating pu/py region in both directions. This is consistent with the findings of Johnston and Rich (17) who have shown that bases out of alternation can form Z-DNA and that the B-Z junctions can move depending on the supercoil density. Thus the TR promoter can undergo a supercoil-dependent structural transition that is most probably a Z conformation.

To further address this question we have constructed two site specific mutants in which the putative Z-forming region described above

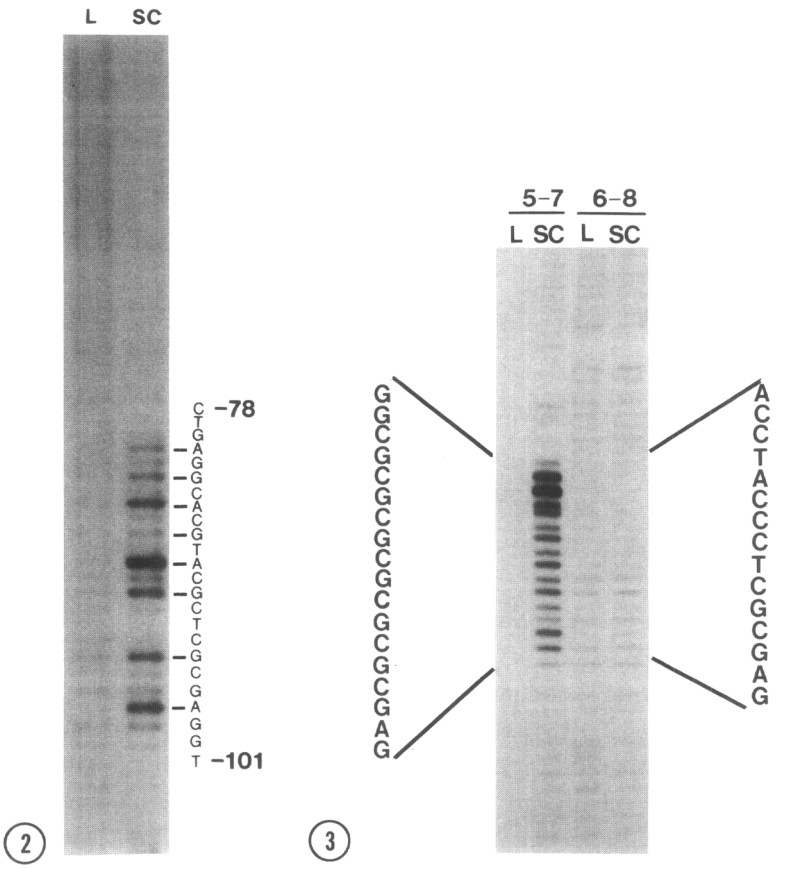


Figure 2. Chemical modification of either linear (L) or supercoiled (SC) plasmids carrying the TR promoter region. The sequence of the region that undergoes modification in the supercoiled sample is shown at the right and can be compared to the sequence in Fig. 1.

Figure 3. DEPC modification of TR promoter mutants. Analyses were carried out as described for the wildtype promoter. Two site specific mutants, TR5-7 (left) and TR6-8 (right), carrying alterations within the alternating pu/py region were tested in either linear or supercoiled form. The sequence within the mutated region is shown for each construct. The bands within the CpG region of TR5-7 migrate anomalously and appear compressed on the sequencing gel.

has been modified. For mutant TR5-7, four base pairs were mutated resulting in a 15 bp region consisting entirely of alternating G/C from position -97 to -83. This is the most favorable sequence for adopting the Z conformation and should readily undergo a B-Z transition. When this mutant is subjected to DEPC modification analysis (Fig. 3), TR5-7 is modified over exactly the same region as seen for the wildtype TR promoter. This modification occurs only in the supercoiled sample.

For mutant TR6-8, the C's at positions -91 and -87 were converted to G's. This disrupts the wildtype pattern of alternating pu/py. Thus if the observed pattern of DEPC modification in the wildtype promoter is due to a transition to the Z-form, this mutations should not undergo such a transition. As predicted, mutant TR6-8 is not modified by DEPC in either the supercoiled or linear form (Fig. 3).

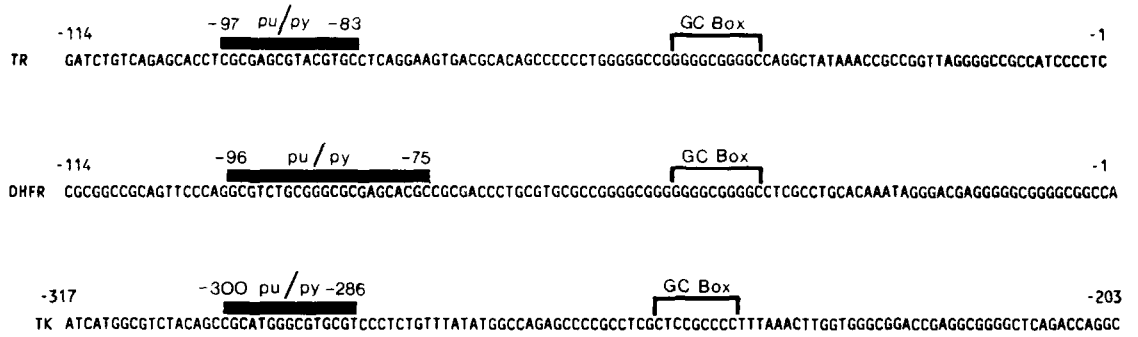


Figure 4. Comparison of the TR promoter region with promoter regions of the human DHFR and human TK genes. The solid bars indicate sequences composed largely of alternating purines and pyrimidines. The brackets indicate GC-boxes encoding high affinity Sp1 binding motifs that are embedded within more extensive GC-rich regions. The sequence for the TK gene is from ref. 18 and that for DHFR is from ref. 21.

Since the region of the TR promoter that undergoes a supercoil-dependent structural transition is adjacent to cis-acting elements that are required for growth dependent expression of the gene, we have examined analogous regions of other genes that are expressed in a similar manner (i.e late genes). Sequences for only a few of these are available. However two, the human dihydrofolate reductase (DHFR) and human thymidine kinase (TK) genes, appear to have elements strikingly similar to that seen in the TR promoter (Fig. 4). Both of these genes are transcriptionally activated by mitogen stimulation and, as with the TR gene, activation is a late event. The DHFR gene contains a region that is composed substantially of alternating pu/py, and the position of this region relative to the major transcriptional start site is nearly identical to that of the Z-forming region of the TR promoter. There are several out of alternation bases within this 21 base pair region, but it contains a high level of CpG dinucleotides. We have tested the ability of this region to undergo a structural transition by chemical modification with DEPC (Fig. 5). Precisely within the region of alternating pu/py a series of modified bases are observed. This modification requires supercoiling and is not observed in the linearized sample. Due to the distance of the motif from the labelled end, the resolution of this experiment is not sufficient to determine which bases within the region of alternating pu/py are modified. Interestingly, the DEPC modified region in the human DHFR promoter region is found nearly the same distance upstream from a GC-box as the TR Z-forming region. This consensus high affinity Sp1 binding sequence is embedded within a more extensive GC-rich motif that is found in both genes.

Kim et al. (18) have localized a cell cycle regulatory element within the promoter region of the human TK gene. This element resides within a 378 base pair fragment which extends from -63 to -441 and is able to confer proliferation dependent expression to a heterologous gene. We have examined this sequence and found that it contains a single

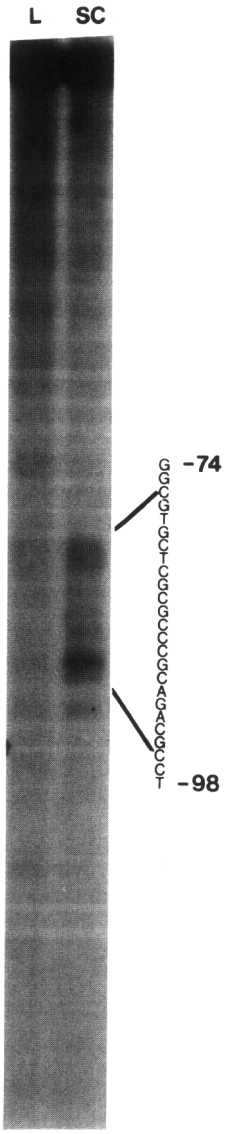


Figure 5. DEPC modification of the human DHFR promoter region. The plasmid insert contained 544 base pairs of DHFR sequences extending from -328 to +216. The fragment was labelled on the lower strand near the 5' end of the insert. The sequence of the modified region is shown at the right.

alternating pu/py motif (Fig. 4). This element, like that of the DHFR gene, is the same distance upstream from a GC-box as the Z-forming region of the TR promoter. At present we do not know if the TK motif can undergo a supercoil-dependent structural transition.

DISCUSSION

The finding that a motif within the TR promoter is able to adopt a supercoil-dependent non-B DNA conformation is of interest for two reasons. First, it is located just upstream of the major transcriptional

start site between positions -83 and -97 (Fig. 1) and therefore in a region that would be predicted by the model of Liu and Wang (4) to be highly negatively supercoiled when the TR gene is being transcribed. Supercoil-dependent structures within this region are therefore very likely to exist transiently.

Second, it is located within a region that is required for proliferation dependent expression of the TR gene, being immediately adjacent to cis-acting elements that are required for mitogen responsiveness.

At present we can only speculate on the role that such a transition might play in determining the level of transcription. One possibility is that it could modulate transcription by influencing the binding of specific transacting factors to adjacent cis-acting elements or by changing protein-protein interactions by altering the spacing or helical twist within the region. Such changes could lead to either positive or negative effects on promoter usage.

The position of the putative Z-forming region within the TR promoter suggests that a transition would be responsive to the level of TR transcription itself. It is therefore possible that the enhanced level of transcription associated with mitogen activation of cell proliferation leads to a structural transition. This in turn could further modulate the transcriptional process, perhaps to limit the maximal level of TR mRNA synthesis. Alternatively, it is possible that DNA synthesis induces a transition within the TR element as a consequence of the translocation of the replication machinery along the DNA. It has been shown that Z-DNA is not packaged into nucleosomes (19). Therefore a B-Z transition in response to the replication process could prohibit chromatin assembly within the region and maintain the promoter in an accessible state for the next cell cycle.

It is of interest that putative Z-forming regions are found in similar contexts in each of 3 proliferation dependent genes. None of these motifs are optimal Z-forming regions in that they are relatively short and have out of alternation bases. However, it is this type of sequence, rather than more stable Z-forming sequences, that would be most responsive to changes in supercoil density and therefore be capable of playing a role in gene regulation.

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