# Methylation of Cytosine in the 5-Position Alters the Structural and Energetic Properties of the Supercoil-Induced Z-Helix and of B–Z Junctions†

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ABSTRACT: The structural and energetic consequences of cytosine methylation in the 5-position on the supercoil-dependent B–Z equilibrium in alternating dC-dG sequences cloned into recombinant plasmids were investigated. The helical parameters determined with the band shift method for right-handed [10.7 base pairs (bp)/turn] and left-handed (12.8 bp/turn) 5MedC-dG inserts were different from the helical repeat values for unmethylated dC-dG inserts (10.5 bp/turn in the right-handed and 11.5 bp/turn in the left-handed form). We analyzed the thermodynamic parameters $\Delta G_{BZ}$ (free energy difference per base pair between right-handed and left-handed helix structure), $\Delta G_{jx}$ (free energy for formation of one B–Z junction), and $b$ (helix unwinding at a junction region) for varying lengths of dC-dG inserts by two-dimensional gel electrophoresis and application of a statistical mechanics model. A comparison of plasmids fully methylated in vitro with *Hha*I methylase and their unmethylated counterparts revealed that $\Delta G_{jx}$ is not significantly changed by cytosine methylation. However, this base modification results in an approximate 3-fold decrease of $\Delta G_{BZ}$ and an approximate 2-fold decrease of the unwinding $b$ at B–Z junction regions. Analysis of a pair of related plasmids, each containing two dC-dG blocks, revealed qualitatively different transition behaviors. When the two dC-dG blocks were separated by 95 bp of a mixed sequence, they underwent independent B to Z transitions with separate nucleation events and junction formations. When the two blocks were separated by only a 4 bp GATC sequence, only one nucleation event was necessary, and the Z-helix spread across the nonalternating GATC region. These structural and energetic alterations demonstrate that methylation of cytosine in the 5-position may be an important switch mechanism for influencing the B–Z equilibrium and DNA topology in general, thus potentially affecting DNA–protein interactions and gene regulation at physiological levels of DNA supercoiling.

The supercoil-induced B to Z transition in recombinant plasmids has been the subject of several investigations in the recent past. The structural inversion of a DNA segment from a right-handed to a left-handed helix form was observed in segments of alternating dC-dG (Stirdivant et al., 1982; Singleton et al., 1983; Peck & Wang, 1983; Nordheim et al., 1982; O'Connor et al., 1986; Klysik et al., 1981), 5MedC-dG (Klysik et al., 1983), (dT-dG)·(dA-dC) (O'Connor et al., 1986; Haniford & Pulleyblank, 1983; Singleton et al., 1984), and several synthetic or naturally occurring DNA regions containing interruptions of the purine–pyrimidine alternation (McLean et al., 1986; Ellison et al., 1985; Kilpatrick et al., 1984). The B to Z transition in a supercoiled plasmid, although usually limited to only a few percent of the sequence, has profound effects on the overall properties of the plasmid. Generation of a new type of helix structure necessitates the formation of B–Z junctions with unique structural features (Singleton et al., 1983, 1982, 1984; Stirdivant et al., 1982). Both events provide new potential DNA–protein interaction sites and also influence the topology of the closed circular plasmid domain. The cause–effect relationship between DNA supercoiling and the B–Z equilibrium has also been analyzed by a variety of theoretical approaches (Frank-Kamenetskii & Vologodskii, 1984; Vologodskii & Frank-Kamenetskii, 1984; Sarai, 1984; Sarai & Jernigan, 1985; Benham, 1980).

Cytosine 5-methylation is believed to play a fundamental signaling role in eucaryotic gene expression (Doerfler, 1983) and in the protection and self-defense mechanisms of procaryotes against invasion by foreign DNA (Adams & Burdon, 1985). Cytosine 5-methylation also facilitates the B to Z transition in vitro in synthetic linear DNA polymers (Behe et al., 1985; Chen et al., 1984, 1982; Nickol et al., 1982; Behe & Felsenfeld, 1981; Pack et al., 1986; Roy & Miles, 1983) and in supercoiled recombinant plasmids (Klysik et al., 1983). These observations support the hypothesis that the two phenomena, i.e., the B–Z equilibrium and site-specific cytosine methylation, may constitute a concerted mechanism for the modulation or fine tuning of DNA topology and DNA–protein interactions, and thus for the regulation of gene expression in vivo.

To further evaluate the feasibility of this hypothesis, it is necessary to determine whether the thermodynamics of this system would allow its function under in vivo conditions. We therefore performed a systematic investigation on the effects of cytosine 5-methylation on the thermodynamic parameters of the supercoil-dependent B–Z equilibrium in alternating dC-dG sequences and on the structural alterations of the Z-helix induced by this base modification.

Our results show that cytosine 5-methylation mainly affects the free energy difference between the right-handed and left-handed helix form of dC-dG segments without significant alteration of the free energy for B–Z junction formation. We also show that the helical repeat parameters of both the right-handed and left-handed forms of 5MedC-dG are not

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identical with those of the unmethylated dC-dG helices.

## Materials and Methods

*Plasmids.* The constructions and features of the following recombinant plasmids were described previously: pRW751 (Klysik et al., 1981), pRW755, pRW756, and pRW757 (Klysik et al., 1982), and pRW471 (Hsieh & Wells, 1987). pRW53 was constructed by digesting 2 μg of the parent plasmid, pRW759 (Singleton et al., 1983), with *Cla*I and *Sal*I restriction endonucleases and creating blunt ends by filling in the sticky ends by using 5 units of Klenow fragment DNA polymerase with deoxynucleoside triphosphates. The ends were then joined by using 4 units of T4 DNA ligase in a ligation buffer containing spermidine to facilitate the blunt-ended ligation. The DNA was transformed into *Escherichia coli* HB101 after 15-h ligation at 20 °C (Maniatis et al., 1982). The transformation mix was spread onto agar plates containing ampicillin for selection of colonies. Approximately 50 colonies were observed, and 12 of those colonies were grown in 10-mL miniprep cultures in LB medium under ampicillin selection. Ten of the miniprep cultures contained DNA of the anticipated size.

The relevant inserts of these plasmids are shown in Figure 2. The plasmids were grown in *E. coli* HB101 and purified as described (Maniatis et al., 1982). Plasmids pLP316, pLP324, pLP332, and pLP342 that were used for the helical repeat experiments were kindly provided by Dr. L. Peck (MIT) and were described previously (Peck & Wang, 1983).

*Enzymes. Hha*I, *Bss*HII, *Pst*I, and *Hha*I methylase were obtained from New England Biolabs. Calf thymus topoisomerase I was isolated and purified according to a published procedure (Ferro & Olivera, 1984).

*Chemicals.* Chloroquine phosphate, *S*-adenosylmethionine, and agarose were from Sigma Chemical Co.

*Preparation of Methylated Topoisomer Populations.* Purified plasmid DNA was relaxed with topoisomerase I at room temperature for 2 h, phenol and ether extracted, and precipitated by ethanol (O'Connor et al., 1986). Methylation with *Hha*I methylase of these relaxed populations was performed as described (Zacharias et al., 1984). These populations were completely (>95%) resistant to cleavage by *Hha*I and *Bss*HII restriction enzymes, as judged by agarose gel electrophoresis of digestion products. In addition, we previously determined in a direct way the extent of methyl group incorporation by *Hha*I methylase into dC-dG blocks in plasmids. Determination of the dMeCMP/dCMP ratio in the reaction products after enzymatic degradation to mononucleotides showed that more than 90% of all cytosine bases were methylated (Klysik et al., 1983). Topoisomer populations with defined average superhelical densities were prepared with the topoisomerase/ethidium bromide technique (Singleton & Wells, 1982).

*Two-Dimensional (2D) Gel Electrophoresis.* 2D gels were run in 1% agarose and 1 × TBE buffer [89 mM tris(hydroxymethyl)aminomethane (Tris)–borate, 2 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0] at room temperature (first dimension, 85 V for 40 h; second dimension, 100 V for 20–24 h in the presence of 5 or 10 μM chloroquine). Other details of this procedure were published previously (O'Connor et al., 1986; Zacharias et al., 1984).

*Statistical Mechanics Calculations.* The topoisomer distributions from the 2D gels were analyzed by using the statistical mechanics model described by Peck and Wang [1983; also O'Connor et al. (1986)].

*Helical Repeat Experiment.* For the determination of the helical repeat *h* of dC-dG and 5MedC-dG sequences, the band shift method (Wang, 1979; Peck & Wang, 1981; Strauss et al., 1981) was applied to one- and two-dimensional gel separations. Four plasmids were used that were identical in the vector component but contained dC-dG inserts of varying lengths cloned into the single *Bam*HI site of the parent vector pTR161 (Peck & Wang, 1983). The plasmids (and the lengths of their dC-dG inserts) were pLP316 [16 base pairs (bp)], pLP324 (24 bp), pLP332 (32 bp), and pLP342 (42 bp). The preparation of relaxed or negatively supercoiled topoisomer populations and the preparation of fully methylated plasmids were as described above. Positively supercoiled topoisomers were generated by relaxation of plasmid DNA with topoisomerase I in 10 mM Tris, 0.1 mM EDTA, and 0.2 M NaCl, pH 8.0 at 15 or 4 °C, overnight.

Two-dimensional gels were run in 1% agarose, 89 mM Tris–borate, and 2 mM EDTA, pH 8.0, at 80 V for 50 h in the first dimension, and at 80 V for 25 h in the second dimension with 5 μM chloroquine included in the agarose and buffer. In some cases, 0.7% or 1.5% agarose gels were run to obtain better resolution of certain topoisomer populations. On each gel a mixture of two of the above plasmids (0.8–1.0 μg of total DNA) was electrophoresed in both dimensions; the distances in the vertical (first dimension migration) direction between corresponding topoisomer band pairs were determined and analyzed as described (Wang, 1979; Peck & Wang, 1981).

Analysis of the helical repeat on one-dimensional (1D) gels was essentially as described (Wang, 1979; Peck & Wang, 1981) after densitometric tracing of Polaroid type 55 negative films with a Bio-Rad Model 620 video densitometer.

## Results

*Helical Repeat h of dC-dG and 5MedC-dG.* The interpretation of our supercoil relaxation data and statistical mechanics calculations (see below) requires an exact knowledge of the helical repeat *h* (bp/turn) of the inserts under consideration. We determined *h* of 5MedC-dG inserts and also, as a control for our method and as a reevaluation of published data (Peck & Wang, 1983), of dC-dG inserts in supercoiled plasmids when these inserts are either in a right-handed or a left-handed helix conformation.

The band shift method (Peck & Wang, 1983, 1981; Wang, 1979) has so far been used only in conjunction with one-dimensional gel electrophoresis. However, for sequences that cause relaxation of supercoils, the one-dimensional band pattern is complicated by overlapping regions of topoisomers which are difficult to identify. We therefore applied the 2D gel technique (O'Connor et al., 1986; McLean et al., 1986; Peck & Wang, 1983; Lee & Bauer, 1985) since in this way positive topoisomers can be separated from negative topoisomers unambiguously. More importantly, topoisomers after completion of a structural transition (which migrate slower due to the relaxation accompanying the transition) can be distinguished from topoisomers before this transition (which also migrate slower simply due to the lower number of supercoils that they possess, Figure 1).

In some cases, we also determined *h* by 1D gel analyses for positive and negative topoisomers with the inserts in a right-handed structure in order to test the quality and reliability of the data from 2D gels (data not shown).

The helical repeat values obtained in this way are summarized in Table I. For each individual gel, two of the four plasmids (pLP316, pLP324, pLP332, and pLP342) were mixed together. This resulted in six combinations of plasmids, the difference in the lengths of the dC-dG inserts in each mixture ranging from 8 (pLP316 plus pLP324) to 26 bp (pLP316 plus pLP342). It is clear that the values for right-handed dC-dG (10.5 ± 0.1) and left-handed dC-dG (11.6 ± 0.3) (Peck &

Table I: Helical Repeat Values for Cloned dC-dG and 5MedC-dG Sequences in a Right- or Left-Handed Helix Form[a]

| insert | dC-dG | | | 5MedC-dG | | |
|---|---|---|---|---|---|---|
| topoisomers | positive | negative | negative | positive | negative | negative |
| helix sense of insert | right handed | right handed | left handed | right handed | right handed | left handed |
| 1D gels | 10.6 ± 0.1 | 10.4 ± 0.4 | ND[b] | 10.7 ± 0.1 | ND | ND |
| 2D gels | 10.7 ± 0.4 | 10.6 ± 0.5 | 11.5 ± 0.7 | 10.7 ± 0.2 | ND | 12.8 ± 1.0 |
| overall averages | 10.5 ± 0.4 | | 11.5 ± 0.7 | 10.7 ± 0.2 | | 12.8 ± 1.0 |

[a] As determined by application of the band shift method (Wang, 1979) to analyze plasmid topoisomer distributions on one-dimensional (1D) or two dimensional (2D) agarose gels. All values are in bp/turn. [b] ND, not determined.

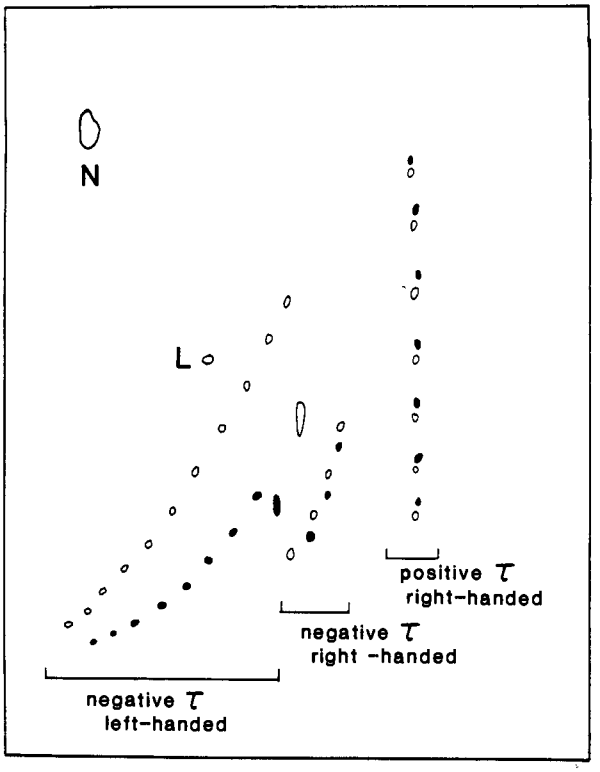


FIGURE 1: Tracing of a representative 2D gel for determination of the helix repeat of 5MedC-dG. A mixture of methylated pLP324 (closed spots) and methylated pLP342 (open spots), both positive topoisomer families and negative topoisomer families around the B to Z transition region for each plasmid, was run in one lane in the first dimension (top to bottom). The second dimension (left to right) was run in the presence of chloroquine, thus resolving different populations that are composed of positive topoisomers or negative topoisomers before and after the B to Z transition, as indicated by the brackets. The helix handedness of the 5MedC-dG inserts in each of these distributions is also indicated with the brackets. N = nicked circular form; L = linear form; $\tau$ = supercoils.

Wang, 1983) were closely reproduced with our method. The small discrepancy of 11.6 bp/turn (Peck & Wang, 1983) versus 11.5 bp/turn (our value) may have been caused by the presence of $Co(NH_3)_6Cl_3$ present in their buffer in order to stabilize the inserts in a left-handed helix structure. We also want to emphasize that our experimentally determined supercoil relaxation data (see below) gave a better fit with the amounts of expected relaxation calculated on the basis of the values in Table I instead of using 10.5 and 12.0 bp/turn for right-handed and left-handed 5MedC-dG, respectively (Table II).

It is clear that cytosine 5-methylation does have an effect on both the right- and left-handed form of the insert (10.5 versus 10.7 bp/turn and 11.5 versus 12.8 bp/turn, respectively). Therefore, the left-handed 5MedC-dG sequence has a helix structure different from the left-handed unmethylated dC-dG. This is true at least in the supercoil-induced structure without the stabilizing effect of high ionic strength or transition metal ions.

| Plasmid: | Total Size (bp): | Insert: |
|---|---|---|
| pRW755 | 4108 | $(CG)_{13}$ |
| pRW756 | 4114 | $(CG)_{16}$ |
| pRW757 | 4092 | $(CG)_5$ |
| pRW471 | 4128 | $(CG)_{23}$ |
| pRW751 | 4515 | $(CG)_{13}$lac 95$(CG)_{16}$ |
| pRW53 | 3967 | $(CG)_{13}$GATC$(CG)_{16}$ |

FIGURE 2: Relevant features of plasmids and inserts used for thermodynamic analysis of the supercoil-dependent B–Z equilibrium.

*Effects of Cytosine 5-Methylation on Supercoil Relaxation.* We investigated the supercoil relaxation behavior during the B to Z transition of plasmids containing 5MedC-dG inserts of varying lengths and, for comparison, that of their unmethylated counterparts. Figure 2 presents the total sizes of the plasmids (all derived from pBR322 as vector DNA) as well as the lengths and sequence features of the inserts to be analyzed.

The procedure to obtain relaxation data from two-dimensional agarose gel electrophoresis is illustrated in Figure 3, showing a representative 2D gel (unmethylated pRW471, Figure 3A) and a tracing of all the detectable topoisomers (Figure 3B). From this tracing, the change in writhe, i.e., the change in the amount of supercoiling, during the B to Z transition can be determined for each topoisomer, and a transition curve can be constructed (Figure 3C). In some cases (see below) the derivative of this transition curve was plotted from its change of slope in order to better illustrate the width (cooperativity) and midpoint of a transition (Figure 3C).

pRW755, pRW756, pRW757, and pRW471 in the unmethylated and fully methylated state were analyzed as described above in terms of transition midpoint and total amount of relaxation caused by the B to Z transition (Figure 4).

A summary of the quantitative analyses of these results is presented in Table II (plasmids pRW751 and pRW53 will be discussed below). As can be seen, the superhelix density ($-\sigma$) at the transition midpoints is substantially lower for the methylated inserts compared to their unmethylated forms, demonstrating the pronounced stabilization of the left-handed form by cytosine 5-methylation (Klysik et al., 1983; Behe & Felsenfeld, 1981). Also, it is obvious that an increasing length of the insert with Z-form potential results in a lower superhelix density required for the transition to occur. Both these factors together explain why we were not able to resolve in our gel system the B to Z transition for the shortest insert $(dC\text{-}dG)_5$ when unmethylated; however, it was detected for the same segment containing 5MedC.

The total amounts of supercoil relaxation observed in these experiments are in good agreement with the expected relaxation, calculated with the standard parameters for the B- and Z-helix (10.4 and 12.0 bp/turn, respectively), which do not distinguish between cytosine- and 5-methylcytosine-containing inserts. However, it must be pointed out that the experimental

Table II: Summary of the Quantitative Analysis of the B–Z Equilibrium in Structurally Related Plasmids As Determined by Two-Dimensional Gel Electrophoresis

| plasmid[a] | dC-dG insert (bp) | $-\sigma$ at transition midpoint | no. of supercoils relaxed | | |
|---|---|---|---|---|---|
| | | | experimental | predicted[b] | predicted[c] |
| pRW755U | 26 | 0.033 | 4.8 | 4.67 | 4.74 |
| pRW755M | 26 | 0.028 | 4.5 | 4.67 | 4.46 |
| pRW756U | 32 | 0.032 | 5.9 | 5.74 | 5.83 |
| pRW756M | 32 | 0.025 | 5.5 | 5.74 | 5.49 |
| pRW757U | 10 | ND[g] | ND | 1.79 | 1.82 |
| pRW757M | 10 | 0.039 | 1.3–1.8 | 1.79 | 1.72 |
| pRW471U | 46 | 0.030 | 8.3 | 8.26 | 8.38 |
| pRW471M | 46 | 0.021 | 8.1 | 8.26 | 7.89 |
| pRW53U | 26 + 32 | 0.032/0.042[d] | 5.3/10.9[d] | 5.74/10.41[e]<br>11.13[f] | 5.83/10.57[e]<br>11.30[f] |
| pRW53M | 26 + 32 | 0.028/0.038[d] | 4.9/10.0[d] | 5.74/10.41[e]<br>11.13[f] | 5.49/9.95[e]<br>10.64[f] |
| pRW751U | 26 + 32 | 0.032/0.045[d] | 5.4/10.5[d] | 5.74/10.41[e] | 5.83/10.57[e] |
| pRW751M | 26 + 32 | 0.024/0.037[d] | 4.8/9.6[d] | 5.74/10.41[e] | 5.49/9.95[e] |

[a] U = unmethylated, M = methylated. [b] With 10.4 and 12.0 bp/turn for the B and Z forms of the inserts, respectively. [c] With 10.5 and 11.5 bp/turn for the unmethylated B and unmethylated Z forms and 10.7 and 12.8 bp/turn for the methylated B and methylated Z forms, respectively. [d] The two numbers refer to the two subtransitions in the biphasic transition profiles. [e] The two numbers refer to the extent of relaxation after the first and second subtransition in the biphasic transition profiles. [f] Assuming that the central GATC sequence is part of the left-handed helix and contributes to the relaxation in the same way as the alternating dC-dG sequence. [g] ND, not determined.

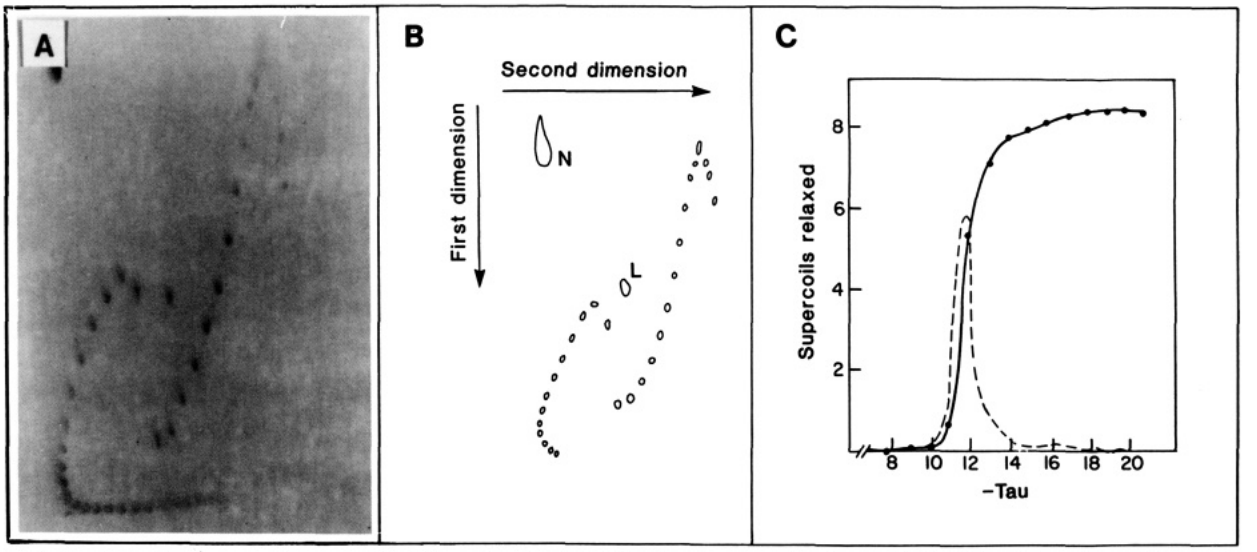


FIGURE 3: Analysis of the supercoil-induced B to Z transition by 2D gel electrophoresis. (A) 2D gel of pRW471. (B) Tracing of the topoisomer distribution on the gel in (A). (C) Transition curve (amount of supercoil relaxation as a function of topoisomer number; solid line) and derivative of the transition curve (dashed line; arbitrary units on the $y$ axis). Only data points around the transition region are shown in panel C. N = nicked circular form; L = linear form; $\tau$ = topoisomer number.

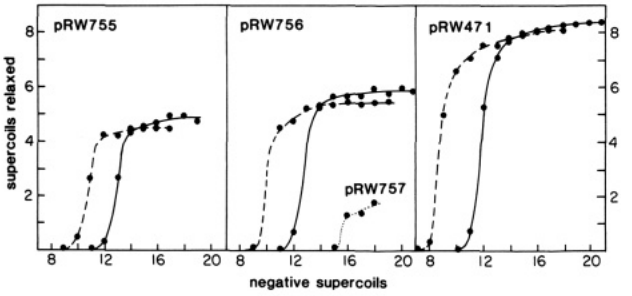


FIGURE 4: B to Z transition curves of pRW755, pRW756, and pRW471 which were used to analyze the thermodynamics of the supercoil-induced B–Z equilibrium (solid lines, unmethylated plasmids; dashed lines, methylated plasmids). Experiments were performed and analyzed as described in Figure 3. Only data points around the transition regions were used for these plots.

relaxation data for the methylated plasmids in general fit even closer to the expected amounts of relaxation, provided that our newly determined helix repeat values for right-handed and left-handed 5MedC-dG are used for the calculation (Table II). In addition, Figure 4 shows that the transition curves for each of the methylated plasmids reflect a slightly lower amount of total relaxation than their respective unmethylated counterparts. We consider both observations as a confirmation of our helix repeat parameters determined in the previous section.

*B–Z Equilibrium in Plasmids Containing Two dC-dG Blocks.* pRW751 and pRW53 are structurally related plasmids since they both are pBR322 derivatives containing the same two dC-dG blocks, 26 and 32 bp in lengths (Figure 2). The difference between them is that in pRW751 the two Z-form blocks are separated by 95 bp of a DNA segment from the *E. coli lac* control region, whereas in pRW53 they have only a 4 bp GATC sequence separating them. We investigated potential cooperative interactions between these two blocks in both plasmids in the unmethylated or methylated state.

Figure 5 shows the 2D gels of these plasmids obtained with methylated topoisomer populations, as well as the transition curves and derivative curves for both methylated and unmethylated plasmids. In all four cases, a biphasic transition is observed, as expected for a plasmid containing two separate segments with Z-form potential (Stirdivant et al., 1982; O'Connor et al., 1986; Zacharias et al., 1984; Kelleher et al.,

Table III: Thermodynamic Parameters for the B to Z Transition in Supercoiled Plasmids Containing dC-dG or 5MedC-dG Inserts[a]

| | pRW755U | pRW755M | pRW756U | pRW756M | pRW471U | pRW471M | average over all plasmids U | average over all plasmids M |
|---|---|---|---|---|---|---|---|---|
| $\Delta G_{BZ}$[b] | 0.32 | 0.13 | 0.34 | 0.06 | 0.29 | 0.15 | 0.32 | 0.11 |
| $\Delta G_{jx}$[c] | 4.6 | 4.8 | 5.1 | 5.1 | 5.0 | 4.3 | 4.9 | 4.7 |
| $b$[d] | −0.20 | −0.13 | −0.65 | −0.02 | −0.56 | −0.62 | −0.47 | −0.26 |

[a] The values were obtained by analyzing the experimental data from two-dimensional gel electrophoresis with the statistical mechanics model (Peck & Wang, 1983; O'Conner et al., 1986). U = unmethylated plasmids; M = methylated plasmids; the lengths of the inserts are indicated in Figure 2. [b] In kcal/(mol·bp). [c] In kcal/(mol·junction). [d] $b$ is the unwinding at B–Z junction regions, measured in helix turns per junction.

1986). The amounts of relaxation observed in the two subtransitions show that, as expected (Stirdivant et al., 1982; Singleton et al., 1983), the shorter dC-dG block (26 bp) inverts to a Z form only after the longer block (32 bp) has completed this transition. When one compares the methylated with the unmethylated state of each plasmid, the same features reflecting the effects of cytosine 5-methylation can be observed as with the plasmids described above (i.e., lower superhelix density at the transition midpoint, slightly lower amount of total relaxation).

However, a significant difference is observed when comparing pRW751 with pRW53. This different behavior is independent of the state of methylation and therefore must be caused by the spacer sequence in the arrangement of the two dC-dG blocks. The transition curves for pRW751 (Figure 5, middle) show that the two transitions, corresponding to the $(dC\text{-}dG)_{16}$ and $(dC\text{-}dG)_{13}$ blocks, are separated by a short, nearly horizontal region (i.e., we observe two independent transitions). On the other hand, this intermediate region for pRW53 never approaches a horizontal (i.e., the two transitions are overlapping). The derivative curves (Figure 5, bottom) are even more revealing. The peaks for the two transitions in pRW751 are separated by approximately six superhelical turns, corresponding to the amount of relaxation caused by the first transition of the longer $(dC\text{-}dG)_{16}$ segment into a left-handed helix. For pRW53, however, the two peaks are separated by only approximately four superhelical turns, i.e., significantly less than the relaxation that had occurred during the first transition. It is also important to note that, although the lengths of the pairs of dC-dG blocks in pRW751 and pRW53 are identical, the total amount of relaxation is slightly larger for pRW53 (11.0 turns) than for pRW751 (10.4 turns) (Table II and Figure 5, middle).

This type of behavior is observed in both the methylated and unmethylated plasmids (dashed versus solid lines in Figure 5). We believe that these results reflect a qualitatively different transition behavior of the two related plasmids, as discussed below.

*Effects of Methylation on the Energetics of the B–Z Equilibrium.* We used the topoisomer distributions from the 2D gel analyses described above to determine the thermodynamic parameters $\Delta G_{BZ}$, $\Delta G_{jx}$, and $b$ for the supercoil-dependent B–Z equilibrium in plasmids containing either dC-dG or 5MedC-dG inserts. $\Delta G_{BZ}$ is the free energy change of a base pair accompanying its inversion from a right-handed to a left-handed helix structure; $\Delta G_{jx}$ is the free energy for the formation of one B–Z junction; and $b$ is the unwinding in helix turns at each junction. A statistical mechanics treatment was developed (Peck & Wang, 1983; O'Connor et al., 1986) that allows the determination of these values from a set of topoisomer bands around or after the B to Z transition region observed on a 2D gel.

This method was applied to the 2D results obtained with three different lengths of inserts (26, 32, and 46 bp of dC-dG or 5MedC-dG contained in pRW755, pRW756, and pRW471,

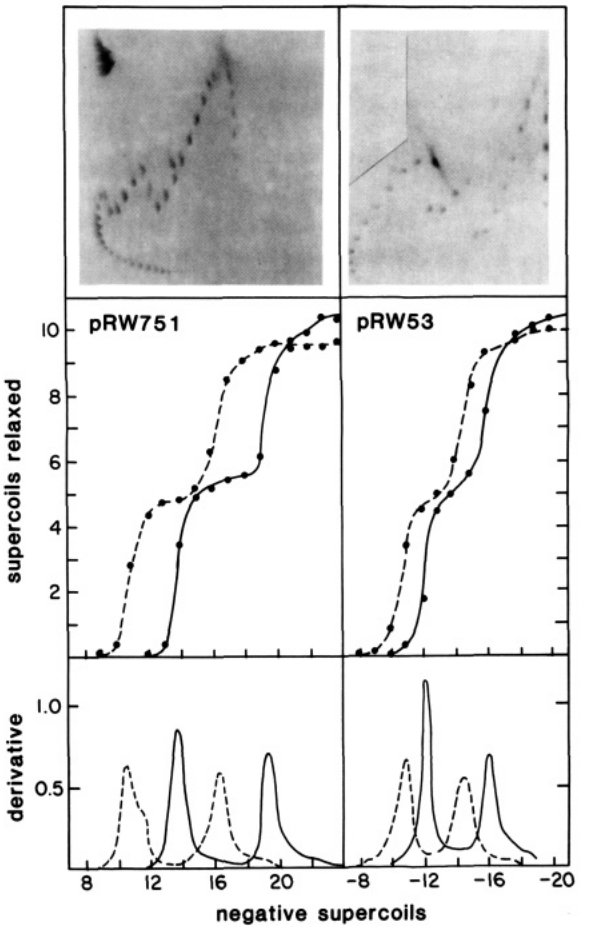


FIGURE 5: 2D gel analysis of the B–Z equilibrium in plasmids containing two blocks of alternating dC-dG (first dimension, top to bottom; second dimension, left to right). pRW751 (left panel) contains $(dC\text{-}dG)_{16}$ and $(dC\text{-}dG)_{13}$ separated by 95 bp of *E. coli lac* sequence. pRW53 (right panel) contains the same two dC-dG blocks separated by only a 4 bp GATC sequence as part of a *Bam*HI recognition site. Top row: 2D gels of methylated plasmids. Middle row: Transition curves for unmethylated (solid lines) and methylated (dashed lines) plasmids. Bottom row: Derivatives of the corresponding transition curves; $y$ axis is in arbitrary units. Only the data points covering the B to Z transition regions were used to construct the plots.

respectively). The energetic parameters for both methylated and unmethylated forms of these plasmids are presented in Table III, together with the average values from all three plasmids.

The values for $\Delta G_{BZ}$, $\Delta G_{jx}$, and $b$ obtained for unmethylated dC-dG sequences are in good agreement with previously published data from both our laboratory (Stirdivant et al., 1982; O'Connor et al., 1986; McLean et al., 1986) and another laboratory (Peck & Wang, 1983). The energetic parameters for 5MedC-dG containing Z-form segments have not been determined previously with this statistical mechanics treatment. For 5MedC-dG inserts, the free energy of junction formation [4.7 kcal/(mol·junction)] is not significantly different from

that of the unmethylated state [4.9 kcal/(mol·junction)]. However, the free energy required to invert 1 bp of 5MedC·dG from a B-type to a Z-type structure is approximately 3 times less [0.11 kcal/(mol·bp)] than required for 1 bp of unmethylated dC·dG [0.32 kcal/(mol·bp)]. In a recent investigation on $Ca^{2+}$ binding to poly(5MedC-dG)·poly(5MedC-dG) in the B and Z forms, a value of $\Delta G_{BZ} = 0.12 \pm 0.03$ kcal/(mol·bp) was found for this methylated DNA polymer (Ruggiero et al., 1987).

Our finding is a quantitation of the stabilizing effect of 5-methylcytosine on Z-DNA which has already been described qualitatively for synthetic DNA polymers (Behe & Felsenfeld, 1981; Nickol et al., 1982) and recombinant plasmid inserts (Klysik et al., 1983). In addition, the amount of unwinding at B–Z junction regions neighboring 5MedC-dG segments was approximately half of that found for dC-dG segments. The significance of these structural and energetic alterations caused by 5-methylcytosine is discussed below.

## Discussion

*Helical Repeat Measurements.* Our helical repeat value of 12.8 bp/turn for left-handed 5MedC-dG is different from the one for left-handed dC-dG determined by us (11.5 bp/turn) or published previously (11.6 bp/turn) (Peck & Wang, 1983). Also, with the hydroxylapatite/micrococcal nuclease method applied to synthetic DNA polymers in the Z form, it was found that the helical repeat of poly(5MedC-dG)·poly(5MedC-dG) (13.6–14.0 bp/turn) is higher than that of poly(dC-dG)·poly(dC-dG) (13 bp/turn) (Behe et al., 1981). However, from the crystal structure of $(5MedC\text{-}dG)_3$ it can be deduced that, at least in the crystallized state, $h$ is not much different from the value found in the $(dC\text{-}dG)_3$ crystal (12.2 versus 12.0 bp/turn) (Fujii et al., 1982).

Our results show that in methylated alternating dC-dG sequences the solution structure is different from that of the unmethylated state. A difference of 0.2 bp/turn between the right-handed helices of dC-dG and 5MedC-dG is equivalent to a change in winding angle between two consecutive base pairs from 34.3°/bp to 33.6°/bp; i.e., the methylated helix is slightly underwound compared to the unmethylated sequence. This difference corresponds to an unwinding of 0.7° per methyl group, in close agreement with the value of 0.6° per methyl group determined by others by quantitating the circularization probabilities of small DNA fragments (Goulet et al., 1987). For the left-handed forms, the winding angle changes from −31.3°/bp for dC-dG to −28.1°/bp for 5MedC-dG. This corresponds to an unwinding of 3.2° per methyl group, showing that the underwinding effect of 5MedC is more pronounced than in the right-handed helix, probably due to the substantially different stacking arrangements in the left-handed helix structure. This underwinding tendency of methylated helices may be caused by the increase in hydrophobic character of the cytidine ring after 5-methylation, thus promoting a stronger base stacking arrangement among neighboring bases (Gill et al., 1974; Chaires & Sturtevant, 1986).

The helical repeat values that we determined for dC-dG from 2D gel separations are in good agreement with published values that were obtained by 1D gel analysis. Therefore, we believe that our 2D method is a valuable extension of the band shift method with one-dimensional gels. Both positive and negative topoisomers as well as topoisomers before and after a structural transition with accompanying relaxation can be analyzed under identical conditions, i.e., in one sample and in one gel. The presence of the intercalator chloroquine in the second dimension clearly separates these different types of topoisomers and allows their unambiguous identification.

The standard deviations in Table I show that the scattering of data points is broader for the measurements on left-handed inserts than for their right-handed forms. The helix repeat for left-handed inserts must be measured on topoisomers with relatively high superhelical densities, where the gel separation of topoisomers is less pronounced than for more relaxed topoisomers. In addition, the number of interpretable bands is usually limited due to the presence of bands in which the B to Z transition has not been fully completed (see also transition curves in Figure 4). Finally, the band shift method assumes that a change in the linking difference by 1 always results in a twist of the primary helix that is constant and independent of the actual linking difference itself, and this assumption is only fulfilled for topoisomers close to the relaxed state. All these factors probably contribute to the large standard deviation for the $h$ values of left-handed helices as opposed to the right-handed forms.

The methylation-induced variation of helix parameters could be one of the reasons for the lack of cleavage of methylated recognition sites by their corresponding restriction endonucleases (Adams & Burdon, 1985), or for the methylation-dependent repression of gene expression in eucaryotes (Doerfler, 1983). It also further documents the existence of a family of left-handed structures with varying helix parameters, as suggested previously (Zacharias et al., 1982). The effects of cytosine methylation on the supercoil-dependent B–Z equilibrium and its energetic parameters will be discussed below.

Finally, we want to point out that other helix structures with drastically altered helix repeat have recently been characterized: poly(dA-ds$^4$T)·poly(dA-ds$^4$T) with 14 bp/turn (Boekemeier & Lezius, 1986), oligo(dA-dT)·oligo(dA-dT) inserts in supercoiled plasmids with 11.5–11.7 bp/turn (McClellan et al., 1986), and DNA wrapped around the histone-like HU protein from *E. coli* with 8.5 bp/turn (Broyles & Pettijohn, 1986).

*Effects of Cytosine 5-Methylation on Supercoil Relaxation.* During the B to Z transition each turn of right-handed primary helix is converted into a left-handed form, thereby decreasing the total twist of the molecule by approximately two helical turns. A more precise quantitative analysis requires exact knowledge of the helical repeat parameters $h$ of the insert under consideration in both its right- and left-handed structure, according to the formula $n/h_B + n/h_Z = r$ ($r$ = total amount of relaxation; $n$ = length in base pairs of the insert; $h_B$ and $h_Z$ = helical repeat of the insert in its right- and left-handed form, respectively).

We have used the $h$ values determined in the previous section to compare our experimentally observed relaxations with the expected amounts calculated on the basis of two different sets of helical repeat values. On one hand, using $h_B$ = 10.4 bp/turn and $h_Z$ = 12.0 bp/turn for both methylated and unmethylated dC-dG inserts for the calculations, we find good agreement between predicted and observed relaxation for all plasmids (Table II). As shown previously, the amount of relaxation is directly proportional to the length of the dC-dG insert (Stirdivant et al., 1982; Klysik et al., 1983; McLean et al., 1986). On the other hand, using the $h$ values summarized in Table I results in a still better agreement between observed and predicted relaxation for most of the plasmids, and especially for 5MedC-dG inserts. This supports our suggestion in the previous section that both the right-handed and left-handed forms of dC-dG sequences have different structures

depending on whether the 5-position of cytosine is methylated or not.

This methylation-induced structural variation could be one of the principles by which enzymes from bacterial restriction-modification systems (Adams & Burdon, 1985) or eucaryotic proteins that bind specifically to 5MedC-containing DNA regions (Wang et al., 1986; Zhang et al., 1986) discriminate between methylated and unmethylated target sites.

The difference in the total amount of relaxation for methylated versus unmethylated dC-dG inserts can also have a secondary effect by determining the overall superhelical density within a topological domain. Since 5MedC-dG inserts cause less relaxation than unmethylated inserts, the final negative superhelix density after completion of a B to Z transition must be slightly higher in a plasmid containing a methylated Z region compared to the same molecule containing this region in its unmethylated state. Although not very pronounced, this effect could be sensed by proteins whose binding to or action on DNA is supercoil-dependent (repressors, RNA polymerase, recombination factors) and could thereby be used for methylation-mediated DNA–protein interactions (Hamada & Bustin, 1985).

It should be pointed out here that, according to theoretical considerations, pRW471 with 46 bp of alternating dC-dG may be a borderline case in which the transition behavior of a long dC-dG insert qualitatively approaches the multistep behavior of long (dT-dG)·(dA-dC) inserts (Haniford & Pulleyblank, 1983; Frank-Kamenetskii & Vologodskii, 1984). It was predicted from energy potential calculations (Sarai & Jernigan, 1985; Vologodskii & Frank-Kamenetskii, 1984; Sarai, 1984) that at approximately 42–46 bp of total length of a dC-dG stretch the transition behavior should switch from an all-or-none type (monophasic transition) to a different type of behavior: after a limited number of base pairs, less than the whole length of the insert, has converted to a Z form (accompanied by a certain amount of relaxation), the further elongation of the Z region over the total length of the insert will require additional superhelical energy (thus proceeding without any net supercoil relaxation). This type of behavior was observed in a 64 bp (dT-dG)·(dA-dC) tract (Haniford & Pulleyblank, 1983; Singleton et al., 1984) but could not be detected for dC-dG inserts due to the in vivo instability of long dC-dG segments (Klysik et al., 1982). However, this theoretically predicted phenomenon, and the borderline length of 46 bp of dC-dG, could be the reason why our experimental and theoretical amounts of relaxation for pRW471 are not as close as for the other (shorter) plasmid inserts.

*B–Z Equilibrium in Plasmids Containing Two dC-dG Inserts.* We investigated the B to Z transition behavior of the two related plasmids pRW751 and pRW53, each containing a pair of dC-dG inserts of 26 and 32 bp in length (Figure 2). The important difference between the two molecules is that in pRW751 these two blocks are separated by 95 bp of the *E. coli lac* promoter/operator region, whereas in pRW53 they are separated by only 4 bp of the sequence GATC which forms the internal tetranucleotide of a *Bam*HI recognition site. The GATC region also interrupts the purine–pyrimidine alteration in the neighboring dC-dG blocks.

As described under Results and shown in Figure 5, both plasmids undergo biphasic B to Z transitions. The longer dC-dG block (32 bp) converts to a Z form first, followed by the shorter block (26 bp), as judged by the amounts of relaxation in the two transitions (Figure 5; also Table II). For pRW751 it is obvious that the relaxation caused by Z formation in the 32 bp block (approximately six turns) must be compensated for prior to initiation of the second transition; i.e., approximately the same superhelix density is required for both transitions. We conclude that in pRW751 each block requires a separate nucleation event including separate junction formation for its B to Z conversion and that the two transitions in this plasmid occur independently from each other (Stirdivant et al., 1982; Kelleher et al., 1986; O'Connor et al., 1986).

For pRW53, however, the second transition occurs at a lower negative superhelix density than in pRW751, after compensation of only approximately four out of the six turns relaxed in the first transition. We conclude from this that in pRW53 no new nucleation event is required for the shorter dC-dG block but that with increasing supercoiling the Z-helix simply spreads from the 32 bp block across the nonalternating GATC region into the shorter 26 bp dC-dG block, thus requiring less supercoil energy than would be needed for new nucleation and formation of two additional junctions. In other words, in pRW751 we observe the formation of two independent Z-helices, whereas in pRW53 the two dC-dG blocks are able to communicate across a short distance of 4 bp in order to find the energetically more favorable pathway to obtain maximum relaxation (i.e., Z-helix formation in a nonalternating GATC sequence as opposed to two separate nucleations and additional junction formations). The behavior of pRW53 also supports the previous observations that a DNA segment that is not an alternating purine–pyrimidine sequence can adopt a left-handed helix structure under the stress of negative supercoiling (Singleton et al., 1983; Kilpatrick et al., 1984; McLean et al., 1986; Ellison et al., 1985).

*Energetics of the B–Z Equilibrium in 5MedC-dG and dC-dG Sequences.* The two energy components governing the Z-DNA formation in supercoiled plasmids are the free energy difference of a base pair in the B and Z form ($\Delta G_{BZ}$) and the free energy cost for the establishment of B–Z junctions ($\Delta G_{jx}$). Two-dimensional gel electrophoresis provdes a detailed picture of the equilibrium distribution of topoisomers around the B to Z region. In combination with the statistical mechanics analysis, it allows us to quantitate these thermodynamic parameters, together with the amount of unwinding at B–Z junctions ($b$), from the experimentally measured twist changes in individual topoisomers as a function of increasing negative linking difference (Table III).

The values for $\Delta G_{BZ}$, $\Delta G_{jx}$, and $b$ obtained here for unmethylated dC-dG inserts are in good agreement with previously published values from our laboratory (Stirdivant et al., 1982; Klysik et al., 1983; O'Connor et al., 1986; McLean et al., 1986) as well as other groups (Peck & Wang, 1983; Nordheim et al., 1982). The energetic and junction unwinding parameters for 5MedC-dG inserts have not been determined previously under these conditions and with this analytical method. It is obvious from Table III that cytosine 5-methylation has two major consequences for the 5MedC-dG Z-helix in supercoiled plasmids.

First, the free energy $\Delta G_{BZ}$ required to convert 1 bp of 5MedC-dG from a right-handed to a left-handed structure is approximately 3 times lower than that required for 1 dC-dG bp. This pronounced stabilization of the supercoil-induced left-handed helix after cytosine methylation parallels the previously observed facilitation of the salt-induced B to Z transition in 5MedC-containing linear DNA polymers like poly(5MedC-dG)·poly(5MedC-dG) (Behe & Felsenfeld, 1981; Behe et al., 1985; Chen et al., 1984; Chaires & Sturtevant, 1986) or poly(5MedC-dA)·poly(dG-dT) (McIntosh et al., 1983), although the stabilizing factors for a supercoil-induced and salt-induced Z-helix must not necessarily be identical

(Frank-Kamenetskii et al., 1985; Sarai & Jernigan, 1985).

Second, the calculated amount of unwinding (*b*) at B–Z junctions neighboring a 5MedC-dG Z-form helix is roughly half of that calculated for a dC-dG insert (0.26 and 0.47 helix turns, respectively). This could be a reflection of the increased thermal stability of 5MedC-containing DNA polymers compared to their counterparts containing unmethylated dC (Gill et al., 1974; Chaires & Sturtevant, 1986), which would not allow the junction regions to partially spread into the 5MedC-dG segment. It should be interesting to determine whether the differences in junction unwinding between 5MedC-dG and dC-dG Z-form helices can be detected by junction-specific enzymatic or chemical probes like S1 nuclease (Singleton et al., 1982; McLean et al., 1986), bromoacetaldehyde (Kang & Wells, 1985; McLean et al., 1987), or osmium tetroxide (Galazka et al., 1986).

It must be pointed out, however, that the free energy for junction formation, $\Delta G_{jx}$, is not significantly altered by cytosine methylation (Table III). This is consistent with our assumption that most of the junctional helix deformation is located outside the Z-helix, since otherwise we would expect a more pronounced effect of cytosine 5-methylation on $\Delta G_{jx}$.

Both parameters, the 3-fold lower $\Delta G_{BZ}$ and the approximately equal $\Delta G_{jx}$ for 5MedC-dG segments relative to dC-dG inserts, result in a substantially lower amount of negative supercoiling required for a methylated segment to invert to a Z form compared to the same insert in its unmethylated state (Table II). This alteration of the energetic factors of the Z-helix caused by cytosine 5-methylation can have profound biological consequences. First, it can be used to establish a structural switch mechanism for a dC-dG segment exposed to a certain amount of superhelical strain within a topological domain. The superhelicity in the domain may not be enough to invert the unmethylated dC-dG sequence to a left-handed form but after methylation may indeed be sufficient to enable the B to Z transition in the same DNA segment. Second, methylation can be an indirect effector of the formation of other DNA secondary structures. The B to Z inversion of a DNA segment after methylation decreases the actual superhelix density within a domain, thus preventing or reversing the formation of a supercoil-dependent secondary structure at a different location within the same topological domain. Third, the methylation of dC-dG regions with subsequent relaxation by Z-DNA formation may be considered as a general mechanism for the transient modulation of supercoiling in vivo, thus either enhancing or diminishing supercoil-dependent DNA–protein interactions, promoter activities, or recombination events.

All these postulated in vivo effects outline the broad potential of cytosine 5-methylation, in conjunction with the B–Z equilibrium, to influence biological processes by a variety of different mechanisms.

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**Registry No.** Cytosine, 71-30-7; poly(dC-dG), 36786-90-0; poly(5MedC-dG), 51853-63-5.

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# DNA Binding Properties of Dioxin Receptors in Wild-Type and Mutant Mouse Hepatoma Cells†

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ABSTRACT: The current model of action of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin) entails stimulation of target gene transcription via the formation of dioxin–receptor complexes and subsequent accumulation of the complexes within the cell nucleus. Here, we have analyzed the DNA binding properties of the dioxin receptor in wild-type mouse hepatoma (Hepa 1c1c7) cells and a class of nonresponsive mutant cells which fail to accumulate dioxin–receptor complexes within the nucleus in vivo. In vitro, both the wild-type and mutant [$^3$H]dioxin–receptor complexes exhibited low affinity for DNA–cellulose (5–8% and around 4% retention, respectively) in the absence of prior biochemical manipulations. However, following chromatography on heparin–Sepharose, the wild-type but not the mutant dioxin receptor was transformed to a species with an increased affinity for DNA (40–50% retention on DNA–cellulose). The gross molecular structure of the mutant, non DNA binding dioxin receptor did not appear to be altered as compared to that of the wild-type receptor. These results imply that the primary deficiency in the mutant dioxin receptor form may reside at the DNA binding level and that, in analogy to steroid hormone receptors, DNA binding of the receptor may be an essential step in the regulation of target gene transcription by dioxin.

The polychlorinated aromatic hydrocarbon TCDD[1] is a potent inducer of aryl hydrocarbon hydroxylase, a cytochrome P-450 dependent enzyme activity involved in the oxidative metabolism of polycyclic aromatic hydrocarbons such as benzo[*a*]pyrene. Evidence suggests that the induction of specific isozymes of cytochrome P-450 by TCDD (i.e., cytochromes P-450c and P-450d in the rat) is mediated by an intracellular, soluble receptor protein [for review, see Whitlock (1986) and Poland and Knutson (1982)]. Binding of TCDD causes an increase in the affinity of the receptor for nuclear target elements and a rapid increase in the rate of cytochrome P-450c transcription (Whitlock, 1986). The model of action

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[1] Abbreviations: TCDD and dioxin, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDF, 2,3,7,8-tetrachlorodibenzofuran; r$^-$, receptor-less phenotype; nt$^-$, nuclear transfer deficient receptor phenotype; nt$^i$, nuclear transfer increased receptor phenotype; $R_s$, Stokes radius; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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