Iron(II) EDTA Used to Measure the Helical Twist Along Any DNA Molecule

Abstract. *A new method has been devised to measure the number of base pairs per helical turn along any DNA molecule in solution. A DNA restriction fragment is adsorbed onto crystalline calcium phosphate, fragmented by reaction with iron(II) EDTA, and subjected to electrophoresis on a denaturing polyacrylamide gel. A modulated cutting pattern results, which gives directly the helical periodicity of the DNA molecule. A 150–base pair sequence directly upstream of the thymidine kinase gene of the type 1 herpes simplex virus was found to have an overall helical twist of 10.5 base pairs per turn, which is characteristic of the* B *conformation of DNA. In addition, purines 3' to pyrimidines showed lower than expected reactivity toward the iron cutting reagent, which is evidence for sequence-dependent variability in DNA conformation.*

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High-resolution views of the DNA helix, through application to oligonucleotides of physical methods such as single-crystal x-ray diffraction (*1*, *2*) and nuclear magnetic resonance (*3*), have led to a deeper appreciation of the conformational plasticity of DNA. The helical twist, groove shape, base pair slide and roll, and handedness of DNA all have been found to depend on base sequence (*1*, *4*–*6*). It is difficult, though, to determine directly these same conformational details for larger DNA molecules. Digestion experiments, in which DNA molecules a few hundred base pairs long are cut with a chemical or enzymatic reagent, give information on the conformational variability of larger fragments of DNA (*7*). The structural features of DNA to which these reagents are sensitive are not always clear, however. We report a way to determine a quantitative measure of conformation, namely the number of base pairs per helical turn, along a DNA molecule of particular sequence in solution. An end-labeled restriction fragment of DNA, bound to an inorganic precipitate (*8*), is fragmented by reaction with the EDTA complex of iron(II). Analysis of the digestion pattern by gel electrophoresis, autoradiography, and densitometry gives the helical periodicity along the DNA molecule. As an example of the method, we have mapped the helical twist along a 207–base pair (bp) restriction fragment encompassing the DNA sequence upstream of the thymidine kinase (tk) gene of the type 1 herpes simplex virus (HSV-1) (*9*). We find that this DNA molecule has an overall helical periodicity of 10.5 bp per turn, characteristic of the *B*-DNA conformation. We find also that variations in the details of local DNA conformation are detectable by iron(II) EDTA [$Fe(EDTA)^{2-}$] fragmentation.

Rhodes and Klug determined the helical periodicities of random sequence DNA (*8*) and homo- and copolymers of DNA (*10*) by binding DNA to a crystalline inorganic surface, digesting the DNA with a nuclease, and separating the digestion products on a high-resolution, denaturing polyacrylamide electrophoresis gel. Since only the DNA backbone bonds most exposed to solution could be cut by the enzyme, a modulated cutting pattern was observed, with the periodicity of strong cutting sites directly translating into the helical periodicity of the DNA molecule. Random sequence DNA (*8*) and poly(dA-dT) (*10*) were found by this method to have helical twists of 10.6 ± 0.1 and 10.5 ± 0.1 bp per turn, respectively, while the helical repeat of poly(dA) · poly(dT) (*10*) was shown to be 10.0 ± 0.1 bp. Behe, Zimmerman, and Felsenfeld (*11*) used this technique to observe a change in helical periodicity from 10.5 to 13.6 bp when poly(dG-dC) underwent a transition from the *B*- to the *Z*-DNA conformation.

If we contemplate applying this experiment directly to DNA of particular sequence (for example, DNA restriction fragments containing sequences impor-

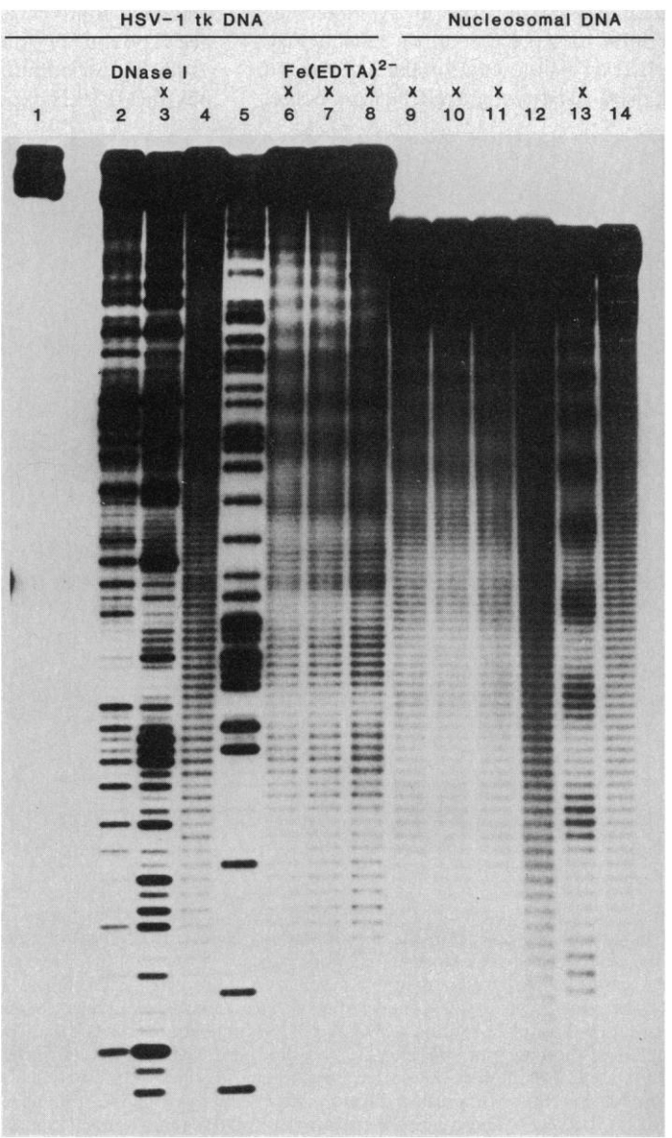

Fig. 1. Modulation of iron(II) EDTA–induced fragmentation of DNA by adsorption to calcium phosphate precipitate. (Lane 1) untreated 207-bp HSV-1 tk DNA; (lanes 2 and 3) HSV-1 tk DNA treated with DNase I; (lanes 4 and 6 to 8) HSV-1 tk DNA treated with iron(II) EDTA; (lane 5) products from a Maxam-Gilbert G-specific reaction (*22*) for HSV-1 tk DNA; (lanes 9 to 11 and 14) 146-bp nucleosomal DNA treated with iron(II) EDTA; (lanes 12 and 13) nucleosomal DNA treated with DNase I. Reaction times with iron(II) EDTA were lanes 4, 7, 10, and 14, 30 minutes; lanes 6 and 9, 5 minutes; and lanes 8 and 11, 60 minutes. The lanes marked with an "x" represent DNA samples bound to calcium phosphate during treatment; the unmarked lanes represent DNA samples free in solution during treatment.

tant for biological control, such as promoters or enhancers), a problem immediately arises. Enzymes that cut the backbone of DNA invariably have at least moderate sequence preferences (*7*), so a smooth cutting pattern will not be realized for such DNA free in solution. A modulation of enzyme digestion, caused by binding such DNA to a crystalline surface, is impossible to interpret accurately in terms of helical twist. A reagent is needed that will break the backbone of any DNA molecule equally at every position with no regard to sequence. Such a reagent is iron(II) EDTA (*12*). When allowed to react with dioxygen or hydrogen peroxide, iron(II) EDTA generates reduced oxygen species of high reactivity, including hydroxyl radical (*13*). It is thought that hydroxyl radical, by abstracting a hydrogen from a backbone deoxyribose, is the key species involved in iron-promoted DNA degradation (*14*). We have found that iron(II) EDTA–mediated breakage of the DNA helix is modulated when the DNA molecule is bound to a precipitate of calcium phosphate. This modulation pattern gives the helical twist along the DNA molecule.

We first compared iron(II) EDTA with deoxyribonuclease I (DNase I) as a cutting reagent for determination of the helical twist of random sequence 146-bp nucleosomal DNA (*15, 16*). Both DNase I and iron(II) EDTA give unmodulated cutting of nucleosomal DNA free in solution (Fig. 1, lanes 12 and 14), and both show clear modulation of cutting when the DNA is bound to a calcium phosphate precipitate (lanes 13 and 9 to 11). Comparison of densitometer tracings of lanes 11 and 13 of the autoradiograph of this gel (Fig. 2, inset) shows that both reagents give the same helical periodicity for random sequence DNA (*8*).

We then applied the helical determination method to a 207-bp restriction fragment containing the transcriptional control sequences of the HSV-1 tk gene (*17*). Figure 1 shows the results of cutting this DNA molecule with DNase I, both free in solution (lane 2) and bound to calcium phosphate (lane 3). Clear differences in the two cutting patterns are apparent, evidence that the DNA molecule is indeed bound to the precipitate in one azimuthal orientation (*18*), but it is not possible by this experiment to determine accurately the helical periodicity of the HSV-1 tk DNA. Iron(II) EDTA, by contrast, gives a smooth cutting pattern for unbound HSV-1 tk DNA (Fig. 1, lane 4). When this DNA molecule is bound to a calcium phosphate precipitate, we find clear modulation of the cutting induced by iron(II) EDTA (Fig. 1, lanes 6 to 8). In the densitometer tracing, the number of sharp bands between the prominent cutting sites found for DNA bound to calcium phosphate (Fig. 2) gives the number of base pairs per helical turn. For HSV-1 tk DNA bound to calcium phosphate, prominent DNase I cutting sites (Fig. 1, lane 3) occur at the same places in the sequence that prominent iron(II) EDTA cutting sites are found (Fig. 1, lanes 6 to 8).

We have mapped the helical twist along 150 bp of this 207-bp fragment. The overall helical periodicity is close to 10.5 bp per turn (147 bases per 14 turns, Fig. 2), consistent with this DNA molecule existing on average in the *B* conformation (*8, 10, 11, 19*). The broad sets of peaks in the densitometer tracings of Fig. 2 are not symmetrical, however, in striking contrast to the peaks for nucleo-

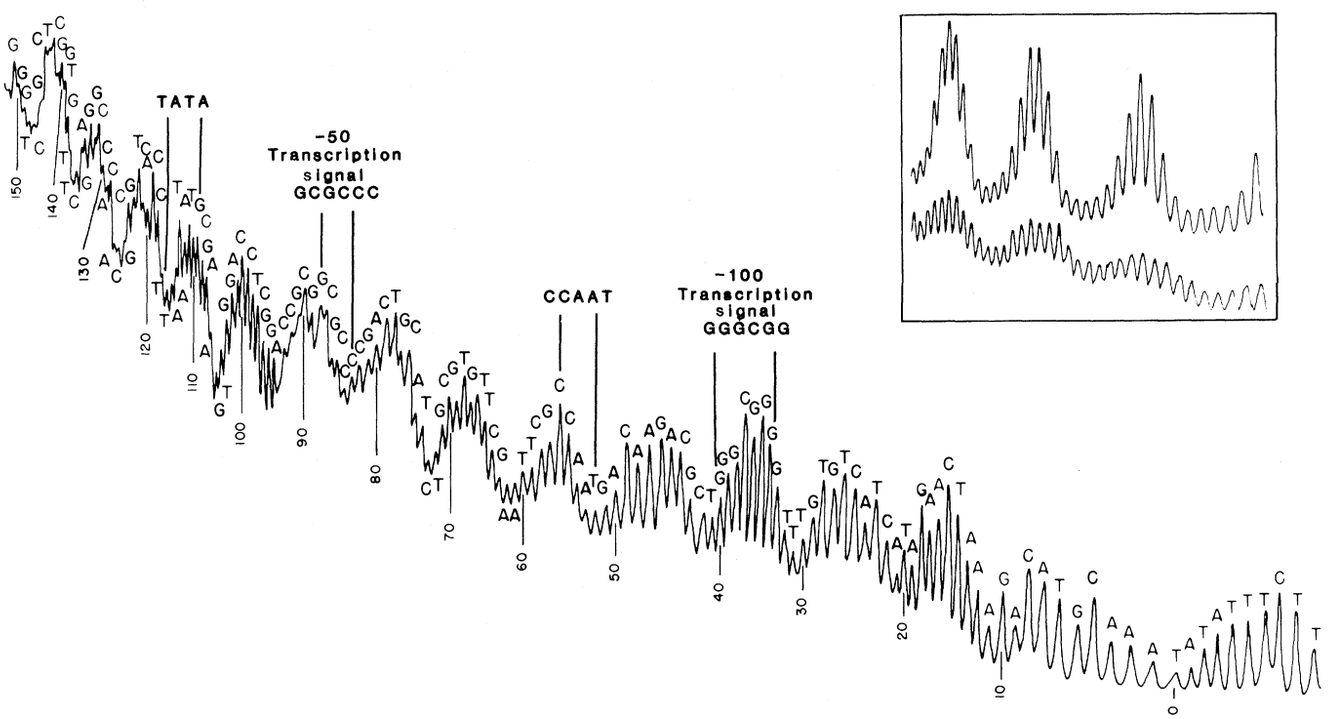

Fig. 2. Helical twist map of the HSV-1 tk DNA fragment. This map was produced by scanning lanes of autoradiographs of electrophoresis gels (such as the one shown in Fig. 1) with a densitometer (0.25-mm slit height, Joyce-Loebl Chromoscan 3 model). The numbers below the map are the number of bases from a position arbitrarily labeled "0." The letter above each sharp peak identifies the corresponding base whose deoxyribose was fragmented by reaction with iron(II) EDTA. The HSV-1 tk map is the result of combining scans from seven separate experiments, which is why the spacing between sharp peaks varies in a nonuniform manner. Scans were spliced together between base positions −1 and 0, 21 and 22, 41 and 42, 73 and 74, 93 and 94, and 115 and 116. Because the splices were made at minima in the pattern, each broad set of peaks is the result of one particular experiment, and the relative intensities of individual sharp peaks within one broad set of peaks can be compared. The intensities of broad sets of peaks cannot be compared with one another, since there was no absolute intensity standard for relating one experiment with another. (Inset) The helical twist of 146-bp nucleosomal DNA as determined by reaction with DNase I (top) and iron(II) EDTA (bottom). These maps were produced by scanning lanes 11 and 13 of Fig. 1.

somal DNA [Fig. 2, inset (*8*)]. The shape of each broad set of peaks is reproducible and amounts to a "fingerprint" of that particular turn of the DNA helix.

In particular, we observe that certain individual sharp peaks in the tracings for the HSV-1 tk DNA are less intense than would be expected from the intensities of nearby peaks. We find nearly all of the peaks of low intensity (representing backbone deoxyriboses of lower than expected reactivity toward hydroxyl radical) in sequences in which a pyrimidine nucleotide occurs to the 5′ side of a purine [pyrimidine(3′-5′)purine], with the purine giving rise to the peak of low intensity. An excellent example is the peak representing the guanine at position 36 in Fig. 2. Almost all pyrimidine(3′-5′)purine sequences have this characteristic. This observation recalls the analysis by Calladine (*4*) of the sequence-dependence of conformation of the dodecanucleotide d(CGCGAATTCGCG). Calladine explained variations in base pair twist angle, roll, propeller twist, and slide by showing that the steric clash of purines on opposite strands of the DNA helix could lead to perturbations in DNA structure. Pyrimidine(3′-5′)purine sequences are particularly susceptible to such steric clash and exhibit the largest deviation from the regular *B*-DNA conformation, according to Calladine's analysis. The sequence-dependence of the reactivity of hydroxyl radical toward deoxyribose residues along the helix is evidence for similar systematic variations (*4, 5*) of the shape of the surface of DNA in solution.

One reason for studying the helical periodicities of DNA molecules that contain regulatory sequences is to determine the relative orientations of proteins that bind to such sequences. The densitometer scan in Fig. 2 shows where on the helix the transcriptional control sequences (*9*) of the HSV-1 tk gene lie. After the "footprints" (sequence preferences) (*20*) of transcription factors bound to these regulatory regions (*21*) are determined, we should be able to deduce from our data the relative orientations of these proteins as they are bound along the helix.

The method we report here offers a simple way to determine the helical twist and other structural details of any DNA restriction fragment. We hope that its application to a wide variety of DNA molecules will provide a connection between high-resolution structural studies of oligonucleotides (*1–5*) and the conformations of regions of DNA in their natural contexts.

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15. Nucleosomal DNA was labeled at the 5′ ends by reaction with polynucleotide kinase and [γ-^{32}P]ATP. Solutions of 50 m*M* $CaCl_2$ and 80 m*M* K_2HPO_4 were prepared by dissolving $CaCl_2$ hydrate (Aldrich, Gold Label) and $K_2HPO_4 \cdot 3H_2O$ (Sigma) in 50 m*M* tris-HCl (*p*H 8.0, Sigma). Equal volumes of these solutions were mixed to generate a suspension of calcium phosphate microcrystals. Experimental samples consisted of calcium phosphate suspension, 1 μg of nonradioactive carrier nucleosomal DNA, and radioactive DNA in a total volume of 70 μl. [In samples without calcium phosphate precipitate, 50 m*M* tris-HCl (*p*H 8.0) was substituted for the calcium phosphate suspension.] DNA was allowed to bind to the precipitate for at least 1 hour before digestion. A solution of iron(II) EDTA was prepared immediately before use by mixing equal volumes of 0.2 m*M* $(NH_4)_2FeSO_4 \cdot 6\ H_2O$ (Aldrich) and 0.4 m*M* EDTA. The cutting reaction was initiated by placing iron(II) EDTA solution (10 μl), 0.3 percent H_2O_2 (10 μl, Baker), and 10 m*M* L-ascorbic acid (sodium salt) (10 μl, Sigma) on the inner wall of the 1.5-ml Eppendorf tube containing the DNA sample, allowing the reagents to mix, and then adding the reagent to the DNA-containing suspension. After the desired time the reaction was quenched by adding 0.1*M* thiourea (10 μl), 0.2*M* EDTA (32 μl), transfer RNA (5 μg), 0.3*M* sodium acetate (200 μl), and absolute ethanol (750 μl). The DNA was precipitated twice and then dissolved in a formamide-containing dye mixture. DNase I digestions (*8*) were performed for 5 minutes. Electrophoresis was performed at 1500 V on a high-bisacrylamide denaturing polyacrylamide gel (8.0 percent acrylamide, 1.2 percent *N,N′*-methylenebisacrylamide, 50 percent urea) prepared by the method of Lutter (*16*).
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17. The 207-bp HSV-1 tk DNA restriction fragment was isolated from a clone of the gene and its upstream and downstream sequences in plasmid pBR322 (*9*). The particular clone we studied has a single mutation (G changed to C) at position −50 relative to the cap site of the tk gene (*9*). Supercoiled plasmid DNA was linearized by digestion with Bam HI, radioactively labeled at the 3′ ends by reaction with the Klenow fragment of DNA polymerase I and [α-^{32}P]dGTP, and digested with Bgl II to give a 207-bp fragment labeled on the noncoding strand. The helical twist determination experiment was performed exactly as for nucleosomal DNA (*15*).
18. For DNA to adopt a particular azimuthal orientation on the inorganic surface (*8*) we find it important to use restriction enzymes that leave unpaired ends a few bases in length, in agreement with Rhodes and Klug (*8*), who reported that nucleosomal DNA, trimmed of its overhanging ends by S1 nuclease, did not orient in a particular way on the inorganic surface. Both Bgl II and Bam HI leave four-base 5′ overhangs.
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23. We thank S. McKnight for providing the clone of HSV-1 tk DNA and R. Morse for the nucleosomal DNA. This work was supported by the Searle Scholars Program of the Chicago Community Trust, by the Research Corporation, and by NIH grant S07 RR07041.

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Hypoglycemia-Induced Neuronal Damage Prevented by an *N*-Methyl-D-Aspartate Antagonist

Abstract. *The possibility that neuronal damage due to hypoglycemia is induced by agonists acting on the* N-*methyl-*D-*aspartate (NMDA) receptor was investigated in the rat caudate nucleus. Local injections of an NMDA receptor antagonist, 2-amino-7-phosphonoheptanoic acid, were performed before induction of 30 minutes of reversible, insulin-induced, hypoglycemic coma. Neuronal necrosis in these animals after 1 week of recovery was reduced 90 percent compared to that in saline-injected animals. The results suggest that hypoglycemic neuronal damage is induced by NMDA receptor agonists, such as the excitatory amino acids or related compounds.*

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Excitotoxins, in particular the excitatory amino acids glutamate and aspartate, have been implicated in the pathogenesis of brain damage in various neurological diseases, including temporal lobe epilepsy, Huntington's disease, olivopontocerebellar atrophy, and cerebral ischemia (*1*). Hypoglycemia, especially if severe enough to cause coma (*2*), is yet another pathological condition leading to extensive neuronal loss in selected brain areas (*3*). The mechanisms underlying the sensitivity of neurons to glucose deprivation are not understood. However, with the advent of a long-term recovery model of insulin-induced hypoglycemia in rats (*4*), mimicking the clinically most relevant conditions of hypoglycemia—those created by an intravenous overdose of insulin—it has become possible to study these mechanisms.

The brain needs a continuous supply of oxygen and glucose for functional integrity. A progressive decrease in blood glucose concentration leads to metabolic