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Use of Monoacetyl-4-Hydroxyaminoquinoline 1-Oxide To Probe Contacts between Guanines and Protein in the Minor and Major Grooves of DNA. Interaction of *Escherichia coli* Integration Host Factor with Its Recognition Site in the Early Promoter and Transposition Enhancer of Bacteriophage Mu†

G. B. Panigrahi and Ian G. Walker*
Department of Biochemistry, University of Western Ontario, London, Ontario, Canada N6A 5C1
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ABSTRACT: Monoacetyl-4-hydroxyaminoquinoline 1-oxide (Ac-HAQO) reacts with DNA to form adducts at the C8- and N^2-positions of guanine and with the N^6-position of adenine. Only the N^2-guanine adduct blocks the 3′-5′ exonuclease action of phage T4 DNA polymerase. Piperidine treatment cleaves the DNA at sites bearing C8-guanine adducts. The N^2-position of guanine lies in the minor groove of DNA, whereas the C8-position of guanine occupies the major groove. We have taken advantage of these characteristics to employ Ac-HAQO in conjunction with either T4 DNA polymerase or piperidine in a footprinting technique to probe the interaction of the *Escherichia coli* integration host factor (IHF) with its binding site. We show that when IHF binds to its recognition site both the N^2- and C8-positions of guanines are protected from modification by AcHAQO. In addition, the binding of IHF to DNA was prevented when either an N^2- or a C8-AQO adduct was present in the binding site. When dimethylsulfate was used as the footprinting reagent, IHF protected against methylation of the N3 position of adenine in the minor groove but not the N7 position of guanine in the major groove. The difference in results obtained with the two reagents is ascribed to their relative sizes. Both DMS and AcHAQO are excluded by IHF from the minor groove, but only the larger AcHAQO molecule is excluded from the major groove.

DNA footprinting studies with chemicals have provided valuable information about the contacts between proteins and DNA during specific interactions. Dimethylsulfate can be used to identify the guanines that interact with protein in the major groove and the adenines that interact with protein in the minor groove. Until this time there was no reagent which allowed one to determine contacts between guanines and protein via the minor groove. The present study describes the use of monoacetyl-4-hydroxyaminoquinoline 1-oxide (Ac-HAQO) to determine the contacts between guanines and protein in both the minor and major grooves of DNA. Ac-HAQO reacts with native, double-stranded DNA to form three purine adducts, two with guanine and one with adenine. The relative amounts of the N^2-guanine, the C8-guanine, and the N^6-adenine adducts formed are 50%, 30%, and 10%, respectively (Galiegue-Zouitina et al., 1985, 1986). However, preferential formation of the C8-guanine adduct is observed when Ac-4HAQO is reacted with denatured single-stranded DNA (70% of total modification; Galiegue-Zouitna et al., 1984). We exploited this difference in reactivity to study the inhibitory effect of the purine adducts on the 3′-5′ exonuclease activity of phage T4 DNA polymerase. It was found that only the N^2-guanine adduct halted the action of this enzyme (Panigrahi & Walker, 1990). When the digestion products of the T4 enzyme on the modified DNA are examined by denaturing polyacrylamide gel electrophoresis, a G-ladder is obtained,

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* To whom correspondence should be addressed.

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which is indicative of the N^2-guanine adducts formed. If the modified DNA is treated with hot 1 M piperidine and analyzed by electrophoresis, the G-ladder observed is indicative of the C8-guanine adducts formed. Since the N^2-position of guanine is located in the minor groove of DNA while the C8-position of guanine extends into the major groove of DNA, it seemed possible that Ac-HAQO could be used as a footprinting reagent to assess both minor-groove and major-groove interactions of DNA binding proteins with DNA. The protein whose interaction with DNA we chose to assess is the *Escherichia coli* integration host factor, IHF. This protein was first identified as one that was necessary for the integration of bacteriophage DNA at the *attP* site in the *E. coli* genome. Subsequently, it was found that IHF participated in a number of other DNA reactions [reviewed in Friedman (1988)]. There is evidence that IHF interacts with a specific sequence through the minor groove. Footprinting studies with dimethylsulfate showed that the N3 position of adenine, which lies in the minor groove, was protected against methylation by IHF, whereas the N7 position of guanine, which occupies the major groove, was not (Craig & Nash, 1984). This result shows that there is a close interaction between IHF and adenines in the minor goove but not between the protein and guanines in the major groove. Additional information on the interaction between IHF and guanines in the minor groove would be helpful in understanding the mechanism by which this protein bends DNA molecules and achieves its biological effects. Here we show the use of Ac-HAQO to probe the interaction of IHF with its recognition site in the early promoter–transposition enhancer of bacteriophage Mu.

Materials and Methods

Enzymes and Chemicals. [^{32}P]ATP (specific activity, 3000 Ci/mmol) was obtained from Amersham, Canada. *Bam*HI, *Hin*dIII, T4 DNA polymerase, alkaline phosphatase, and polynucleotide kinase were purchased from Pharmacia. 4-Nitroquinoline 1-oxide (4NQO) was obtained from Sigma. IHF protein was purified as described previously (Nash et al., 1987). Plasmid pMSIE1 containing the IHF binding site from the early promoter–transposition enhancer was constructed by taking the *Sau*3AI–*Bal*I fragment from 838 to 1242 of the left arm of phage Mu.

Preparation of Diacetyl-HAQO. 4-Hydroxyaminoquinoline 1-oxide (HAQO) was prepared essentially according to Kawazoe and Araki (1967) and was acetylated essentially according to Enomoto et al. (1968). 4NQO, 150 mg, was dissolved in 15 mL of ethanol followed by 750 mg of ascorbic acid. Then, 2.25 mL of a 1:1 mixture of concentrated NH_4OH and water was added with vigorous stirring. The yellow precipitate of HAQO was collected on a suction filter and washed with 12.5 mL of 25% (w/v) ascorbic acid and then 50 mL of water. The HAQO was dried in a vacuum and then dissolved in 6 mL of ice-chilled acetic anhydride with magnetic stirring under a stream of nitrogen. The solution was poured into 40 mL of ice-chilled water with vigorous stirring. The resulting precipitate was collected by filtration, washed with water, and dried under reduced pressure. The crude diacetyl-HAQO was dissolved in a minimum volume of acetone (approximately 1 mL), and 15 mL of anhydrous ethyl ether was added. Crystallization occurred at −20 °C. The crystals were collected, dried, and stored at −20 °C under nitrogen in the dark.

Preparation of AcHAQO. One milligram of diacetyl-HAQO was dissolved in 25 μL of DMSO and added to 0.8 mg of dithiothreitol dissolved in 25 μL of DMSO. After 15 min at room temperature, the preparation was ready to use

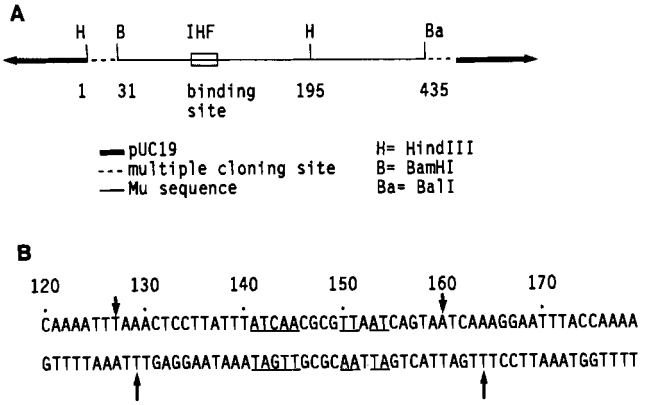

FIGURE 1: Map and sequence of IHF binding site used in this study. (A) Schematic representation of the Mu sequence containing the IHF binding site. The base pairs are numbered starting from the *Hin*dIII site of the multiple cloning site. The Mu sequence is taken from the left arm of phage Mu, base pairs 838–1242. (B) Nucleotide sequence of the IHF binding site and some restriction sites. The consensus sequence of the IHF binding site is underlined. Vertical arrows show the boundaries of the DNase I footprint.

(Galiegue-Zouitina et al., 1981; Panigrahi & Walker, 1990).

Substrates for AcHAQO and DMS Protection Study. (i) Top-strand-labeled DNA fragment (see Figure 1): Plasmid pMSIE1 was cut with *Bam*HI, the 5′-ends were labeled with ^{32}P by the phosphatase–polynucleotide kinase method, and the plasmid was cut again with *Hin*dIII. The *Bam*HI–*Hin*dIII 164-bp fragment was purified by electrophoresis in a 5% polyacrylamide gel, extracted, and precipitated with alcohol. (ii) Bottom-strand-labeled DNA fragment (see Figure 1): Plasmid pMSIE1 was cut with *Hin*dIII and a 194-bp fragment was isolated following polyacrylamide gel electrophoresis. The fragment was 5′-end-labeled and then cut with *Bam*HI. The fragment was extracted with phenol–chloroform and precipitated with alcohol.

Substrates for AcHAQO Interference Study. (i) Top-strand-labeled DNA fragment: Plasmid pMSIE1 was cut with *Bam*HI and the 5′-ends were labeled with ^{32}P. One microliter (20 μg) of AcHAQO solution was added to 50 μL of reaction mixture containing 21 pmol (25 μg) of ^{32}P-labeled DNA fragment in 2 mM citrate buffer, pH 7. After incubation at 37 °C for 15 min, the reaction was stopped by adding 0.1 volume of 3 M sodium acetate and 2 volumes of alcohol. The precipitated DNA was washed, dried, dissolved in appropriate buffer, and cut with *Hin*dIII. The DNA was phenol-extracted and precipitated. (ii) Bottom-strand-labeled fragment: Plasmid pMSIE1 was cut with *Hin*dIII and a 194-bp fragment was isolated following polyacrylamide gel electrophoresis. Fourteen picomoles of this fragment was 5′-end-labeled and modified with AcHAQO as described above except that 10 μg of AcHAQO was added to the reaction mixture. After modification the 194-bp fragment was cut with *Bam*HI and the DNA was phenol-extracted and precipitated.

Protection Study with AcHAQO. The reaction conditions were determined empirically. A higher salt concentration decreases the nonspecific reaction between IHF and DNA (Surette & Chaconas, 1989) but inhibits the reaction between AcHAQO and DNA (Galiegue et al., 1980). Nonspecific binding between IHF and DNA is minimized by providing a smaller than stoichiometric amount of IHF and by including salmon sperm DNA in the reaction mixture. Labeled top- and bottom-strand fragments (14–17 pmol) were dissolved in 40 μL of a buffer containing 5 mM HEPES–KOH, pH 7.6, 2.6 mM $MgCl_2$, 12.5 mM NaCl, and 25 μg/mL salmon sperm DNA. Twenty nanograms of IHF was added and the solution

was incubated for 10 min at room temperature. One microliter of AcHAQO preparation containing 40 μg of AcHAQO was added and the reaction mixture was incubated at 37 °C for 15 min. Four microliters of tracking dye was added, and the reaction mixture was loaded onto a 5% polyacrylamide gel and electrophoresed at 150 V for 2 h. The bound and unbound fractions of DNA were located by autoradiography. The bands were excised and the DNA was recovered by the crush and soak method. The DNA was precipitated and dissolved in water.

(a) Major-groove footprinting with AcHAQO and piperidine: Bound and unbound fractions (300K cpm) were dissolved in 90 μL of water plus 10 μL of 10 M piperidine and heated at 90 °C for 30 min. Piperidine was removed by lyophilization, and the residue was dissolved in tracking dye and loaded onto the gel.

(b) Minor-groove footprinting with AcHAQO and T4 DNA polymerase: Bound and unbound fractions (260K cpm) were dissolved in 30 μL of assay buffer (33 mM Tris–acetate, pH 7.9, 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM dithiothreitol, and 100 μg/mL BSA). T4 DNA polymerase, 10 units in 1 μL, was added and the mixture was incubated at 37 °C for 45 min. The reaction was terminated by adding 117 μL of 0.3 M sodium acetate and sonicated salmon sperm DNA to a concentration of 1 μg/μL. The DNA was precipitated and dissolved in tracking dye for gel electrophoresis.

Protection Study with DMS. The recipe followed is essentially that used previously (Craig & Nash, 1984). Labeled top- and bottom-strand DNA fragments (14–17 pmol) were dissolved in 40 μL of a buffer containing 5 mM Tris-HCl, pH 7.6, 12.5 mM NaCl, 2.5 mM $MgCl_2$, 1% glycerol, 100 mL of BSA, 50 mM sodium cacodylate, pH 8, 0.5 mM EDTA, and 25 mg/mL salmon sperm DNA. IHF, 40 ng, was added and the mixture was incubated for 10 min at room temperature. Then, 0.5 μL of DMS was added, and after a 1-min incubation at room temperature, 4 μL of tracking dye was added and the mixture was quickly loaded onto the gel. The gel was run as described above. The bound and unbound fractions were located and excised. DNA was depurinated at 90 °C and strand breaks were produced by heating with NaOH at 90 °C. The DNA was concentrated by alcohol precipitation and dissolved in 2 μL of dye for electrophoresis.

Interference Study with AcHAQO. Labeled top- and bottom-strand DNA fragments (14–21 pmol) that had been modified with AcHAQO were dissolved in 40 μL of a buffer containing 50 mM Tris-HCl, pH 8, 20 mM KCl, 10% glycerol, 100 mg/mL BSA, and 25 μg/mL sonicated salmon sperm DNA. IHF, 20 ng, was added and the mixture was incubated for 10 min at room temperature. Then, 4 μL of tracking dye was added and the mixture was loaded on a gel to separate the bound and unbound fractions by electrophoresis. The two fractions were localized by autoradiography and excised, and the DNA was extracted, precipitated, and dissolved in water. (a) Reaction with piperidine: Approximately 200K cpm of each DNA fractions was reacted with 1 M piperidine and the products were electrophoresed as described above. (b) Digestion with T4 DNA polymerase: Bound and unbound DNA fractions (200K cpm) were dissolved in 30 μL of buffer and treated with enzyme as described before. The DNA was precipitated, dissolved in buffer, and electrophoresed as described above.

Results

A schematic diagram of the plasmid construct carrying the early promoter and transposition enhancer of bacteriophage Mu is shown in Figure 1A. Panel B of Figure 1 shows the nucleotide sequence of the IHF recognition site region (Craig

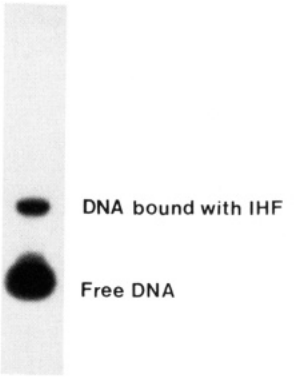

FIGURE 2: Electrophoretic separation of DNA bound to IHF from free DNA in order to determine the DNA bases protected by IHF. The top strand of the 164-bp fragment is labeled.

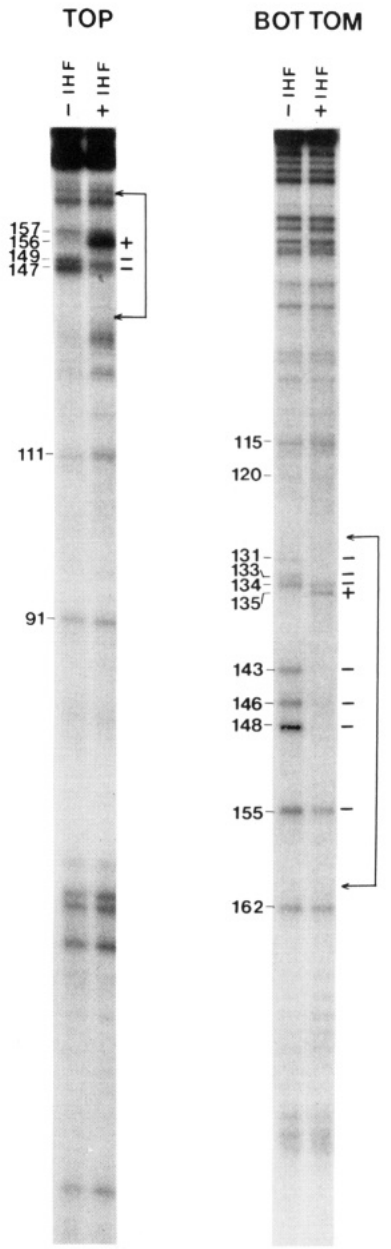

FIGURE 3: Protection by bound IHF against modification of guanines at the N^2 position by AcHAQO. The brackets indicate the DNase I footprint. The bases protected against chemical modification are shown by (–) signs; the bases that display enhanced modification are shown by (+) signs.

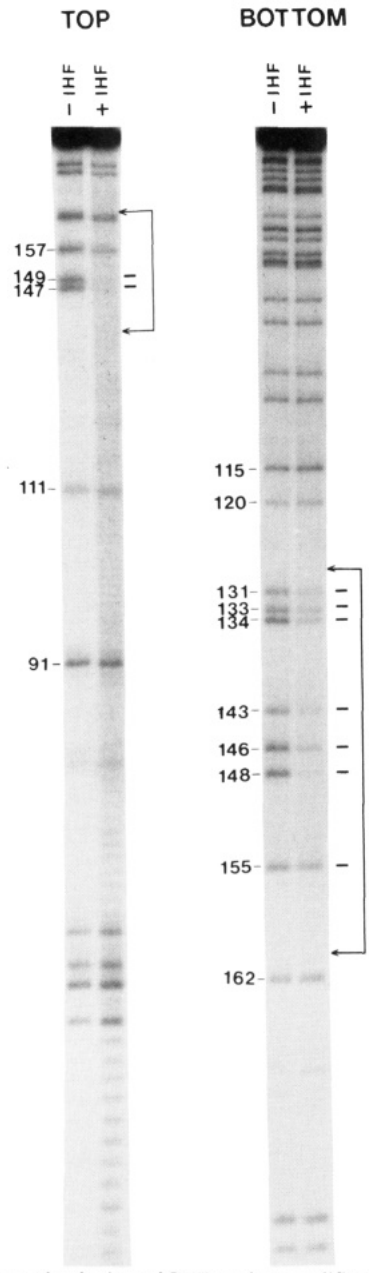

FIGURE 4: Protection by bound IHF against modification of guanines at the C8 position by AcHAQO. The brackets indicate the DNase I footprint. The bases protected against chemical modification are shown by (–) signs.

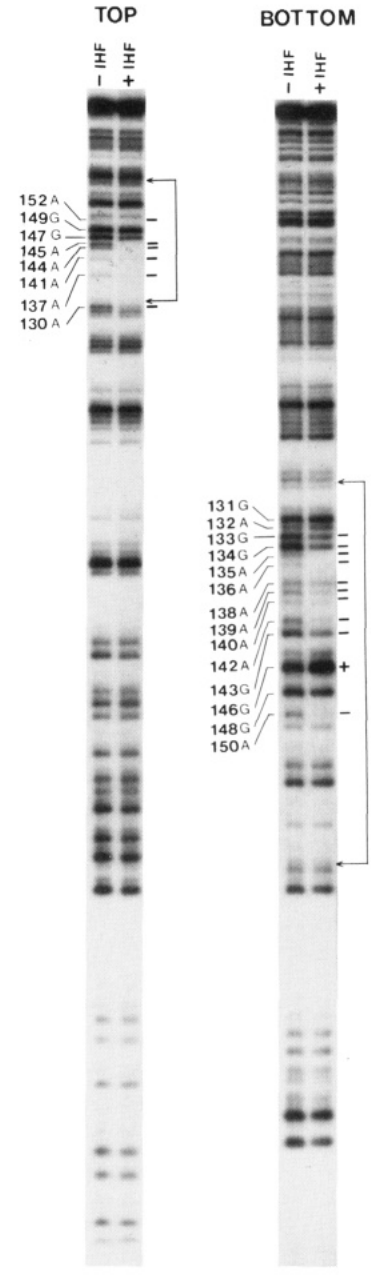

FIGURE 5: Protection by bound IHF against methylation of guanines at the N7 position and adenines at the N3 position. The brackets indicate the DNase I footprint. The bases protected against methylation are shown by (–) signs; the bases that display enhanced methylation are shown by (+) signs.

& Nash, 1984). The IHF binding site was defined by the sequence of nucleotides protected from digestion by DNase I when IHF is bound (experimental results not shown) is marked on the diagram by vertical arrows. The underlined nucleotides are identical to those in the consensus sequence defined previously (Craig & Nash, 1984).

T.PyAA ... PuTTGaT
A.PuTT ... PyAACtA

The protection afforded by bound IHF against attack by AcHAQO was determined with the 164-bp *Bam*HI–*Hin*dIII fragment labeled at the 5′-terminus of the *Bam*HI site for the top strand or the 5′-terminus of the *Hin*dIII site for the bottom strand. Each uniquely labeled fragment was added to a binding reaction mixture containing IHF in less than a stoichiometric amount. AcHAQO was added, and following the chemical reaction the free, modified DNA and the bound, modified DNA were separated by gel electrophoresis. Figure 2 is an example of the separation achieved when top-strand-labeled DNA was used. Each DNA fraction was isolated from the gel and divided into two parts. One part was treated with T4 DNA polymerase which is halted in its exonuclease action at N^2 guanine AQO sites. The other part was treated with piperidine which induces strand cleavage at C8 guanine AQO sites. The products of the T4 DNA polymerase reaction and the piperidine reaction were examined by gel electrophoresis as illustrated in Figures 3 and 4, respectively. Figure 3 shows that IHF protects the N^2 position of guanines lying within the DNase I footprint area against attack by AcHAQO. Figure 4 shows that IHF also protects the C8 position of the same guanines against attack by AcHAQO.

In an analogous manner the protection afforded by bound IHF against attack by DMS was analyzed. A Maxam and Gilbert A+G reaction was carried out on the two DNA fractions to reveal methylation of the N3 position of adenine and the N7 position of guanine (Figure 5). The banding

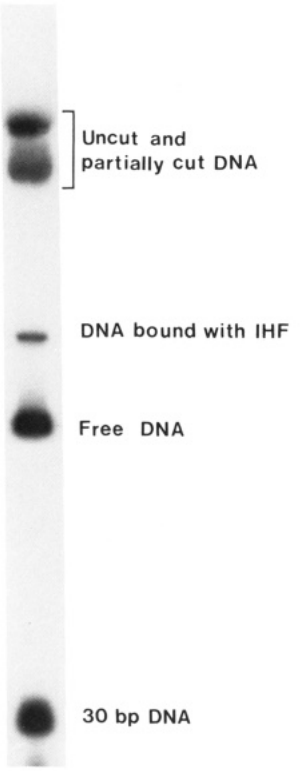

FIGURE 6: Electrophoretic separation of DNA bound to IHF from free DNA to determine which modified bases interfere with IHF binding. The top strand of the 164-bp fragment is labeled. Extra bands are seen because a preliminary isolation of the 164-bp fragment was not performed (see Materials and Methods).

patterns in this figure clearly show IHF protection of the N3 position of adenines within the DNase I footprint area.

Information about the nucleotides that play an important role in the DNA–protein binding reaction can also be obtained by determining which bases, when modified, interfere with the binding reaction. In a test of the interference technique, uniquely end-labeled DNA (the 164-bp *Bam*HI–*Hin*dIII fragment) was modified lightly with AcHAQO so as to yield less than one adduct per DNA strand. IHF was allowed to react with the DNA and the free and IHF-bound fractions of DNA were separated by gel electrophoresis. Figure 6 shows the result of one of these procedures. Each DNA fraction was isolated from the gel and divided into two parts. One part was treated with piperidine to induce strand cleavage at C8 guanine AQO sites. The other part was treated with T4 DNA polymerase to reveal N^2 guanine AQO sites. The products of the piperidine reaction and the T4 DNA polymerase reaction were examined by gel electrophoresis as illustrated in Figures 7 and 8, respectively. These patterns show that when guanines within the DNase I footprint area are modified by AcHAQO at either the C8 or N^2 positions, binding to IHF is inhibited.

The protection data obtained with both AcHAQO and DMS are displayed on a planar representation of the double helix (Yang & Nash, 1989) as shown in Figure 9. The sequence of the top strand is given and it is read in a 5′–3′ direction commencing at the bottom of the diagram. The planes of the base pairs are represented as horizontal lines; they extend across the lanes representing both the minor and the major grooves but for clarity only the former are shown. The protection symbols in the major and minor grooves are placed above the base-pair planes for the top-strand data and below the planes for the bottom-strand data. In order to describe the degree of protection, the X-ray films were scanned with a laser densitometer and the heights of the peaks were

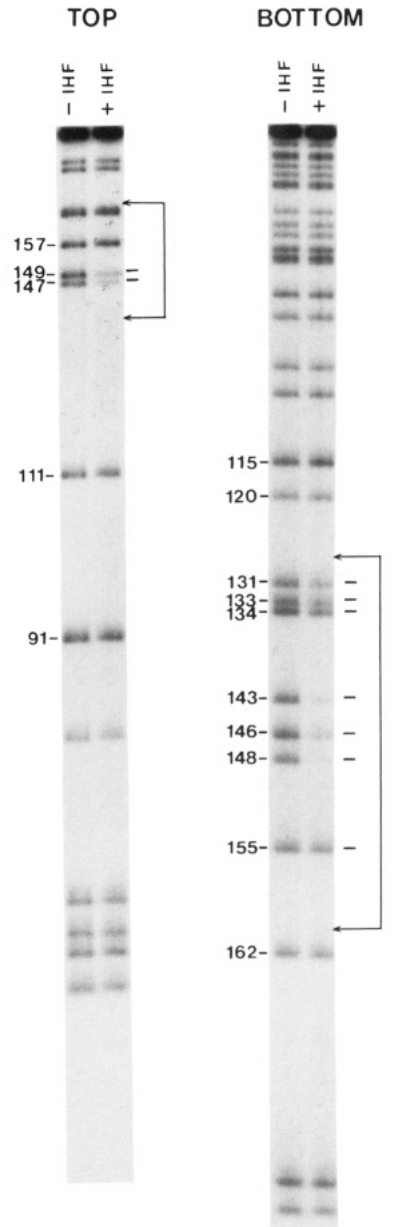

FIGURE 7: Interference with the binding of IHF to DNA which was modified by AcHAQO at the C8 position of guanines. The brackets indicate the DNase I footprint. The modified bases that interfere with IHF binding are shown by (–) signs.

measured. The peak heights obtained with bound DNA were expressed as a percentage of those obtained with free DNA and the following arbitrary rule was applied: >80%, no significant protection; 60–80%, weak protection; 40–59%, moderate protection; <40%, strong protection.

On a similar planar representation of the DNA molecule are plotted the AQO–guanine adducts that interfere with the binding of IHF (Figure 10). N2 guanine adducts are located in the minor groove and C8 guanine adducts are in the major groove.

Discussion

Considering first the protection results obtained with DMS, the summarized results of Figure 9 show that in the minor groove from base pairs 130 to 152 inclusive, 13 out of 15 of the adenines were well protected from methylation by DMS. The adenines not protected, 132 and 151, are both in the bottom strand and are at the extremes of the protected zone. In the major groove covering the same stretch of base pairs,

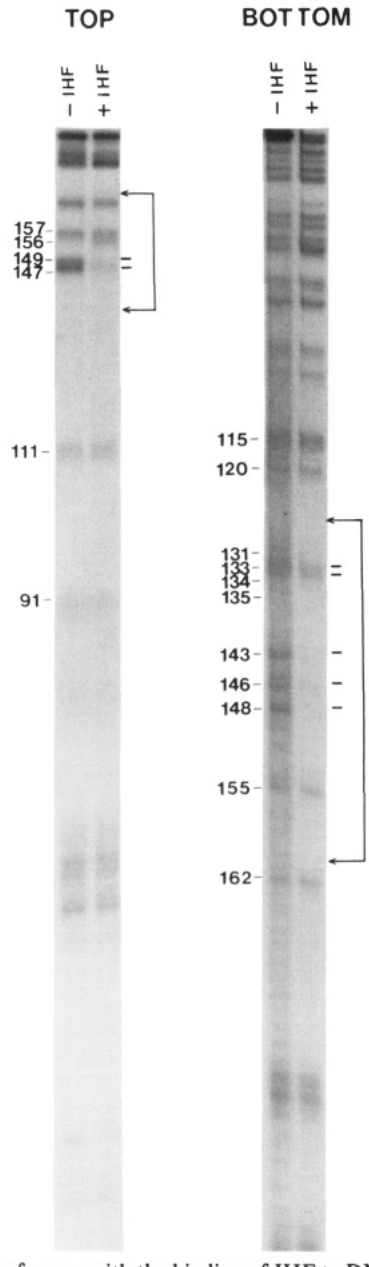

FIGURE 8: Interference with the binding of IHF to DNA which was modified by AcHAQO at the N^2 position of guanines. The brackets indicate the DNase I footprint. The modified bases that interfere with IHF binding are shown by (–) signs.

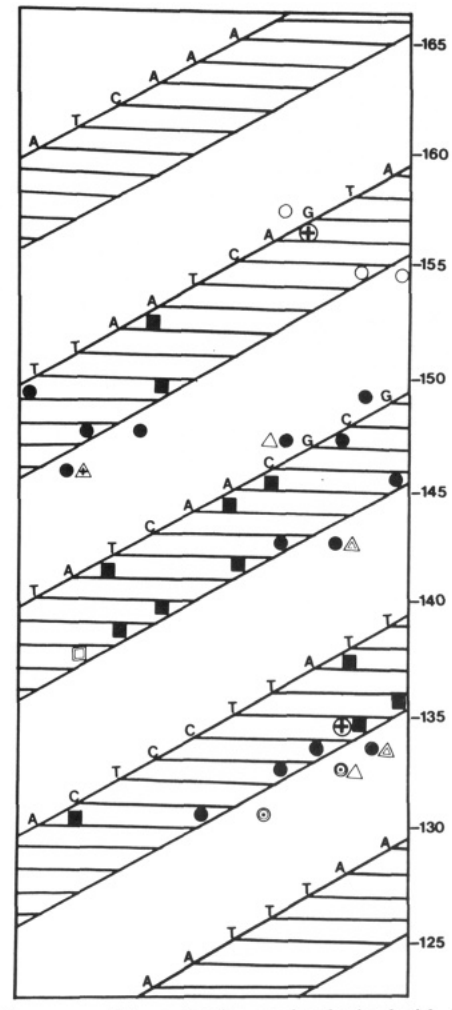

FIGURE 9: Summary of the protection results obtained with AcHAQO and DMS. The extent of protection is indicated as follows. Protection of guanines against AcHAQO: (solid circles) strong, (concentric circles) moderate, (open circles) weak. Protection of guanines against DMS: (concentric triangles) moderate, (open triangles) weak. Protection of adenines against DMS: (solid squares) strong, (concentric squares) moderate. Enhanced reaction of adenine with AcHAQO: circled plus. Enhanced reaction of guanine with DMS: plus in triangle.

4 out of 8 guanines are protected from methylation, but two of them are protected moderately and two of them lightly. The interpretation of this result is that when IHF binds to its recognition site it contacts the DNA intimately along its minor groove but only slightly along the major groove. This conclusion had previously been reached from studies with IHF and three binding sites within the λ phage attachment site, *attP* (Yang & Nash, 1989).

When AcHAQO was used as the footprinting reagent it was seen (Figure 9) and that all eight guanines within the sequence 130–152 were protected by IHF from attack on both the minor- and major-groove sides. The interference study with this reagent (Figure 10) also implicated both minor and major grooves as areas of close contact between IHF and its recognition site. That is, when any one of the guanines was modified at the C8 position or when seven out of eight of the guanines were modified at the N^2 position, binding of the protein was inhibited.

A likely explanation of the different footprinting results obtained with DMS and AcHAQO resides in the different sizes of the two probe molecules and in the inferred structure of the IHF molecule. IHF closely resembles HU protein, for which the structure has been solved by X-ray crystallographic analysis (Tanaka et al., 1984). Using the structural features of HU, models of the interaction between IHF and its recognition site have been proposed by Yang and Nash (1989) and independently by White et al. (1989), in which a three-stranded antiparallel β-pleated sheet covers the minor groove. Our results suggest that the proximity of the β-pleated sheet to the major groove is sufficient to prevent access of AcHAQO to the underlying nucleotides but insufficient to keep out the smaller DMS molecule. Thus, AcHAQO was not as discriminating as DMS in determining which of two regions of the DNA molecule was in closer contact with IHF. In the absence of the DMS data it would not have been possible to tell from the AcHAQO data which of the two DNA grooves was in closer proximity to IHF. Nonetheless, AcHAQO data has supplied structural information that was not apparent from the DMS data.

There are three nucleotides that display enhanced reactivity when the DNA is bound to IHF. G-146 in the lower strand

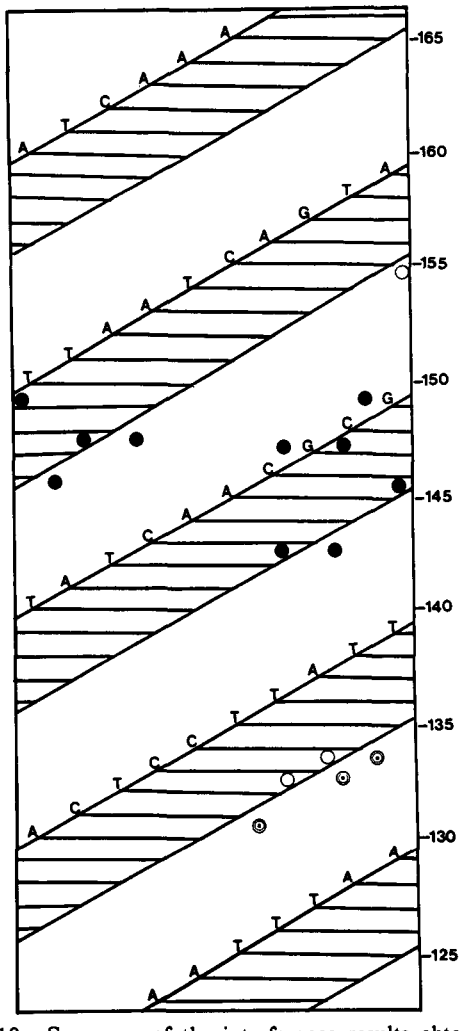

FIGURE 10: Summary of the interference results obtained with AcHAQO. The degree of interference is indicated as follows: (solid circles) strong, (concentric circles) moderate, (open circles) weak.

has an enhanced reactivity with DMS but is still protected against reaction with AcHAQO. This heightened reactivity could be the result of a protein-induced rotation that gives the guanine more of a syn conformation and hence a greater exposure of its N7 position. The adenines at positions 135 in the bottom strand and 156 in the top strand react strongly with AcHAQO as demonstrated by the T4 DNA polymerase–exonuclease reaction (Figure 8). In the latter case, the intensity of the band is so great that it obscures the precise location of the band and may actually reflect a reaction with three adenines in that region (adenines 152, 153, and 156). AcHAQO is thought to react predominantly with adenine at the N^6 position (Galiegue-Zouitina et al., 1985, 1986), which is in the major groove. We have never seen any evidence that the N^6 adenine–AQO adduct can block the 3′–5′ exonuclease action of T4 DNA polymerase (Panigrahi & Walker, 1990). By analogy with the enzyme-blocking action of the N^2 guanine–AQO adduct, which occupies the minor groove, it is suggested that the enzyme-blocking adenine adduct is the N3 derivative, which likewise is in the minor groove and whose reactivity has been enhanced by the DNA–protein interaction.

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