λ Phage *cro* Repressor Interaction with Its Operator DNA: 2′-Deoxy-5-fluorouracil O_R3 Analogues†

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ABSTRACT: The experiments here show that chemically synthesized DNA containing fluorine at selected sites can be used to test specific predictions of a model for *cro* repressor–operator interaction. This is done by observation of the perturbation to the fluorine-19 NMR spectra of analogues of O_R3 synthesized with 2′-deoxy-5-fluorouracil at specific positions in the DNA helix. Although the three-dimensional structure of the *cro* repressor from phage λ has been determined by Matthews and co-workers [Anderson, W., Ohlendorf, D., Takeda, Y., & Matthews, B. (1981) *Nature (London) 290*, 754–758], direct structural observations on the complex of the protein with its specific DNA recognition sequence, O_R3, are limited. From that structure of the protein, *alone*, a model of its complex to DNA was built by fitting B-form DNA, with some distortion [Ohlendorf, D., Anderson, W., Fisher, R., Takeda, Y., & Matthews, B. (1982) *Nature (London) 298*, 718–723]. That model proposes that the *cro* repressor contacts only one side of this DNA double helix and a number of specific protein–DNA contacts. To test the model, 2′-deoxy-5-fluorouracil was used to place the fluorine-19 nuclear spin-label on the side of the DNA contacting the *cro* repressor and on the opposite side facing away from the *cro* repressor. The results presented here are consistent with the prediction that λ phage *cro* repressor contacts only one side of the DNA double helix.

Temperate bacteriophages and their mechanism of lyosogeny play a central role in our understanding of the macromolecular interactions in gene regulation (Ptashne et al., 1982; Jacob & Monod, 1961) and recombination [R. Weisberg and A. Landy, personal communication in Hendrix et al. (1983, pp 211–250); G. Smith, personal communication in Hendrix et al. (1983, pp 175–210); Meselson & Weigle, 1961]. Among temperate or lysogenic phages, the *Escherichia coli* phage λ has certainly been the most intensively studied (Hendrix et al., 1983; Hershey, 1971). Of its gene products, the *cro* repressor (also referred to as *tof* protein in the literature) is the only one whose structure is known in almost complete atomic detail (Anderson et al., 1981; Pabo & Sauer, 1984). The structure of the DNA binding domain, or the amino terminal 40%, of the λ repressor (cI protein) has also been examined by crystal diffraction (Pabo & Lewis, 1982). The similarities of portions of the three-dimensional structure of the two proteins along with primary structure similarities with several other specific gene regulatory proteins (Anderson et al., 1982; Matthews et al., 1982; Steitz et al., 1982; Sauer et al., 1982; von Wilckem-Bergmann & Muller-Hill, 1982; Weber et al., 1982; Ohlendorf et al., 1983a–c; Laughon & Scott, 1984; Ljungquist et al., 1984; Pabo & Sauer, 1984; Postle et al., 1984) as well as its proposed, very widely published, and generalized model for interaction with DNA by Matthews and co-workers (Matthews et al., 1982, 1983a,b; Ohlendorf et al., 1982; Takeda et al., 1983; Ohlendorf & Matthews, 1983) invite further and detailed evaluation.

Although Matthews et al. (vide supra) determined the three-dimensional structure of the *cro* repressor in the absence of DNA, they took the bold step of suggesting the structure of its complex with DNA assuming minimal variation from B-DNA (Ohlendorf et al., 1982). The structure proposed for the complex of *cro* protein with DNA suggests an interaction where the protein is primarily on one side of the DNA helix with α-helical portions of the protein engaged in the wide groove for potential, specific intermolecular contact. This is illustrated in Figure 1 and schematically in Figure 2. In particular, Figure 2 indicates a number of potential amino acid side-chain contacts with the O_R3 operator DNA sequence, the primary site of *cro* repressor action (Johnson et al., 1978), including the methyl group of the thymine at position −5, according to the nomenclature of Matthews and co-workers (Ohlendorf et al., 1982). We describe here the synthesis of a double helical O_R3 DNA fragment where the thymine at position −5 is replaced with 2′-deoxy-5-fluorouracil (5FU),[1] resulting in the replacement of the thymine methyl group with a fluorine atom that serves as a nuclear spin-label. From the model of Matthews and co-workers in Figure 1, one sees that the protein DNA interaction involves only one side of the DNA. Therefore, as a control, we synthesized another DNA double helix that contains an identical substitution at a thymine five base pairs (bp) away at position −10. As illustrated in Figure 2, this places a fluorine at a position 180° away from the potential DNA protein interaction to be probed with the fluorine at position −5.

A crystal structure of the endonuclease *Eco*RI–DNA complex has been reported (Frederick et al., 1984). In that case the entire DNA recognition sequence is engulfed by the protein. Thus, a test of the one-sided contact of the DNA by *cro* protein becomes of even greater interest.

The use of ^{19}F NMR in biological systems has been reviewed (Sykes & Weiner, 1980) and offers advantages of large chemical shift dispersion compared with protons, 100% natural abundance, and extreme sensitivity to electronic environments. The ribose form, 5-fluorouracil, has been biosynthetically incorporated into RNA for ^{19}F NMR studies (Hills et al., 1983; Marshall & Smith, 1980), resulting in resonances from all of

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[1] Abbreviations: bp, base pair(s); 5FU, 2′-deoxy-5-fluorouracil; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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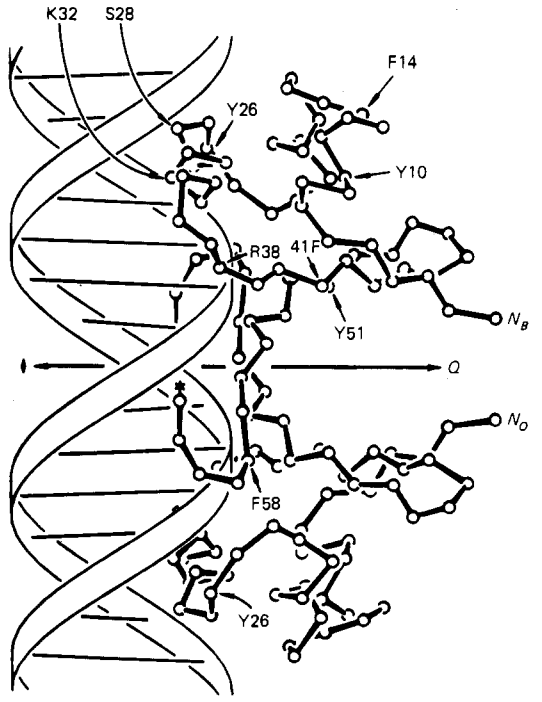

FIGURE 1: Positions of the α-carbons of the *cro* repressor (dimer of 66 amino acids per subunit) superimposed upon B-form DNA in the model proposed by Matthews and co-workers [figure adapted from Anderson et al. (1981)].

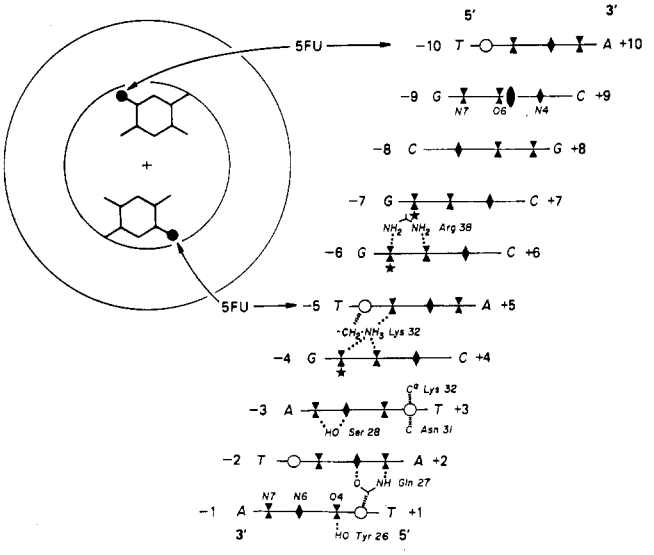

FIGURE 2: Schematic presentation, adapted from Ohlendorf et al. (1982), of the wide groove of the DNA from one of the almost symmetrical halves of the model of Figure 1 indicating the locations of the thymine methyl groups (open circles), hydrogen-bond donors (A6, C4 protons) (diamonds), and hydrogen-bond acceptors (N7 and O6 of G, O4 of T and A) (hourglasses). The right-hand side of the figure shows the relative positions of the two thymine methyls of interest here when viewed along a projection down the helix axis on the left. Note that they are 180° apart. The center of the 2-fold symmetry of Figure 1 is at base pair ±9 at the top right.

the uracil positions. In the application demonstrated here, 2′-deoxy-5-fluorouracil is chemically synthesized into the DNA recognition sequence one position at a time so that the spectra have only a single resonance, overcoming ambiguities of resolution and assignment. Even with the advent of two-dimensional proton NMR methods, the resolution and assignment of proton resonances from nucleic acid–protein complexes still are a formidable task that remains to be demonstrated as a generally soluble problem. For this reason most ^{1}H NMR observations of gene repressor–operator complexes have been

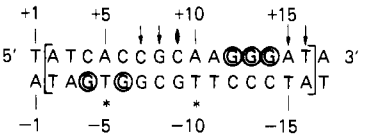

FIGURE 3: O_R3 DNA sequence of the specific binding site for *cro* repressor. The circles indicate G's protected against methylation in modification experiments and the asterisks represent where thymine methyl groups have been substituted via 2′-deoxy-5-fluorouracil, respectively. The arrows indicate known sites of mutations.

limited to aromatic side-chain protons of proteolytic repressor fragments and imino protons of the DNA (Nick et al., 1982; Buck et al., 1983; Scheek et al., 1983; Kirpichnikov et al., 1984). Since the natural line width of the NMR spectral lines is dependent on the molecular size, the general approach used here with single fluorine as a nuclear spin-label is also applicable to larger protein–DNA systems as well as to systems that evade crystallization.

Materials and Methods

Figure 3 illustrates the three fragments of DNA that were synthesized for these experiments. For the sake of economy we chose to synthesize 15-bp fragments that cover the sequence +2 through +16 of the upper strand of Figure 3 and two strands corresponding to the sequence −2 through −16 of the lower strand in that figure. The methyl groups that have been substituted with fluorine are indicated at the T's with the asterisk at positions −5 and −10.

Preparation of the Fully Protected Fluorouridine. (*1*) *5′-O-(Dimethoxytrityl)-5-fluorodeoxyuridine.* The synthesis of 5′-*O*-(dimethoxytrityl)-5-fluorodeoxyuridine was carried out essentially as described (Gait et al., 1982) except that the elution of the desired product during flash chromatography (Still et al., 1978) was achieved with 400 mL of chloroform–0.1% pyridine, 400 mL of 3% methanol–chloroform–0.1% pyridine, and 400 mL of 5% methanol–chloroform–0.1% pyridine. Pure fractions were pooled, evaporated to a foam, redissolved in chloroform, and precipitated with hexane.

(*2*) *Phosphorylation of 5′-O-(Dimethoxytrityl)-5-fluorodeoxyuridine.* The preparation of the fully protected mononucleoside was performed as described (Narang et al., 1980); however, flash chromatography was used in place of the short column of silica gel. Elution of the desired product was achieved with 160 mL of chloroform–0.1% pyridine, 160 mL of 3% methanol–chloroform–0.1% pyridine, and 700 mL of 5% methanol–chloroform–0.1% pyridine. Each fraction was checked by thin-layer chromatography (TLC), and the fractions containing the desired product (R_f = 0.36 in 5% methanol–chloroform on silica gel TLC) were pooled, evaporated to a foam, redissolved in chloroform, and precipitated with hexane.

DNA Synthesis. The 17-bp O_R3 and its 15-bp 5FU analogues were chemically synthesized by a modified phosphotriester method using a solid support (Ito et al., 1982). Monomers were purchased from Vega Biochemicals. Resins and dimer blocks were purchased from Bachem Inc.; 50 mg of resin (0.15 mmol/g) was used for each single-strand synthesis. After synthesis, the protected oligonucleotides were deblocked, purified by reversed-phase high-performance liquid chromatography, and desalted by Sephadex (G-25) column chromatography. The deblocking used 50 °C in the NH_4OH step rather than 60 °C to reduce loss of fluorine from 5FU. The percentage of DNA containing fluorine was checked by comparing the area of the single ^{19}F resonance in a solution of 5FU of known concentration with the area of the ^{19}F resonance from the DNA solution where the concentration was measured by

absorption of 260-nm light using a molar extinction coefficient of 6.6×10^3/mol of bases. Loss of fluorine through the protection, coupling, and deprotection process was thus measured to be about 10%. The ^{19}F NMR spectrum of the single strands showed one resonance slightly downfield from the single resonance in Figure 7I in the case of substitution at position −5 and three resonances for the substitution at position −10. The latter was probably the result of several possible intrastrand hydrogen-bonded forms with loops. Figure 7 shows the double-stranded forms both yield a single resonance.

Isolation of cro Repressor. The isolation procedure for *cro* repressor was essentially that of Takeda et al. (1977) as modified by Boschelli et al. (1982). Fractions from the Sephadex G-75 column containing *cro* repressor were concentrated by loading them in 0.1 M KCl onto a 3-mL phosphocellulose column and eluting with 0.8 M KCl. The *cro* repressor was then further purified by rechromatographing on a 30 × 3 cm Sephadex G-75 column.

Fluorescence Measurements. All measurements were performed with a Hitachi Perkin-Elmer MPF-4 spectrofluorometer as previously described (Boschelli et al., 1982) in 10 mM Tris-HCl (pH 7.4 at 4 °C), 0.1 mM EDTA, and 50 mM KCl, initially.

^{19}F NMR Measurements. Equimolar amounts of complementary strands were mixed, heated to 55 °C, and allowed to anneal for 4 h. The duplex DNA was then diluted in buffer (0.04 M Tris, 0.125 M KCl, 10 mM EDTA, 25% D_2O, pH 7.3) to yield a final DNA concentration of 0.124 mM (15 bp). The buffer conditions were chosen to keep the *cro* repressor soluble.

^{19}F NMR experiments were performed on either a Bruker CXP-200 spectrometer at 188 MHz equipped with a 10-mm probe or a Nicolet NT-150 spectrometer at 141 MHz equipped with a 20-mm probe. Spectra were obtained with a 10000-Hz sweep width represented by 8K data points. Forty thousand scans were accumulated with a delay of 0.5 s between each acquisition. Chemical shifts are reported with respect to the chemical shift of trifluoroacetic acid as an external standard.

cro repressor was added directly to the samples in small increments to give the desired ratios of *cro* dimer:DNA. The salt-induced dissociation of the *cro* repressor–DNA complex was performed by removing the sample from the NMR tube, dissolving the appropriate amount of KCl (to make the sample 2 M in KCl), and then returning the sample to the NMR tube. Final concentrations are listed in the figure legends.

Results

Since we anticipated extending the approach of using specific fluorine labels on nucleic acid analogues for exploring protein–DNA contacts, economy suggested the use of shorter fragments of DNA, if possible, to do these experiments. The fact that λ operators are normally referred to as 17 bp long is based on a consensus and symmetry observed in the three leftward and three rightward operators (Ptashne et al., 1976). If one allows some variations, as seen in the center of the consensus sequence, it is possible to argue that the consensus is 19 bp long. Although the model proposed by Matthews et al. (Ohlendorf et al., 1982) includes interactions of the protein with position 1, the operator mutations furthest away from the center of symmetry that affects *cro* repressor function occurs at positions 5 and 16 of O_R3 (Meyer et al., 1980) (see Figures 2 and 3). Thus, we chose to synthesize 15 bp long DNA double helices that leave out base pairs 1 and 17. There is chemical modification evidence that the λ cI repressor does indeed use 17 bp in its contact with the various operators [G. Gussin, A. Johnson, C. Pabo, and R. Sauer, personal communication in Hendrix et al. (1983)]. In order to show that this shorter DNA double helix and its analogues containing single fluorine substitutions still interact with *cro* repressor in a manner that is comparable to that of the 17-bp piece, we exploited the tyrosine fluorescence quenching assay developed for *cro* repressor by Boschelli et al. (1982).

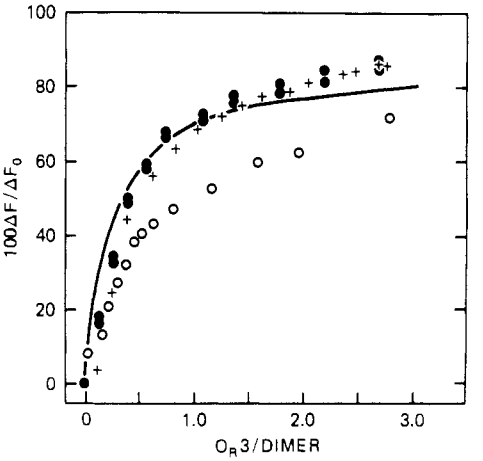

FIGURE 4: Addition of DNA to a solution of *cro* repressor protein results in a quenching of tyrosine fluorescence, which is plotted here in the standard manner: $\Delta F = F_0 - F_{(+DNA)}$, where F_0 is the initial fluorescence intensity and $F_{(+DNA)}$ the intensity with the added DNA. The solid line represents the quenching of fluorescence when a 17-bp DNA double helix corresponding to the O_R3 operator is added. The curve defined by the (+) symbols is the quenching caused by the addition of the 15-bp O_R3 fragment containing no fluorines. The (●) symbols represent 15-bp O_R3 with fluorine at position −5 thymine and the open circles (○) 15-bp O_R3 with fluorine at position −10 thymine. The initial *cro* repressor dimer concentration was 1.2 μM and the final concentration 1.5 μM. All of the titrations were done in 10 mM Tris-HCl (pH 7.3 at 4 °C) and 0.1 mM EDTA, starting with 50 mM KCl.

Figure 4 shows the quenching of the *cro* repressor tyrosine fluorescence excited at 280 nm and monitored at 310 nm. There are 3 tyrosines (at 10, 26, and 51) in each 66 amino acid monomer of the dimeric *cro* repressor (Roberts et al., 1977; Hsiang et al., 1977). The solid line represents the quenching caused by the addition of a 17-bp DNA fragment with the O_R3. All three 15-bp fragments of O_R3 quenched the tyrosine fluorescence almost identically when compared with the 17-bp fragment. We have found that the concentration of potassium chloride required to dissociate the complex is diagnostic of the specific interaction between DNA and *cro* repressor (Boschelli, 1982; Boschelli et al., 1982). Thus, KCl was added to the sample at the end of the fluorescence quenching titration from Figure 4, with 2.8 O_R3 DNA fragments per *cro* repressor dimer. The fluorescence returns to the original level. A comparison of this return for KCl added to the 17-bp and the 15-bp O_R3–*cro* repressor complexes is shown in Figure 5. We note that the shorter fragment dissociates at lower KCl concentrations. This is a length dependence that we have observed and will describe in greater detail in a separate publication. For the purposes of the experiments here, the important observation is shown in Figure 6 where the salt concentration required to dissociate the fluorine-substituted 15-bp fragments of DNA is compared with that required for the unsubstituted 15-bp O_R3 fragment. One notes that the KCl concentration required for 50% dissociation of the fluorinated analogues is similar to that for the unsubstituted DNA double helix of the same length.

Figure 7 shows the effect on the ^{19}F NMR spectra of the two DNA analogues by added *cro* repressor at a ratio of two dimers per O_R3 15-bp fragment. It is clear that the larger effect occurs at position −5 as one would anticipate from the

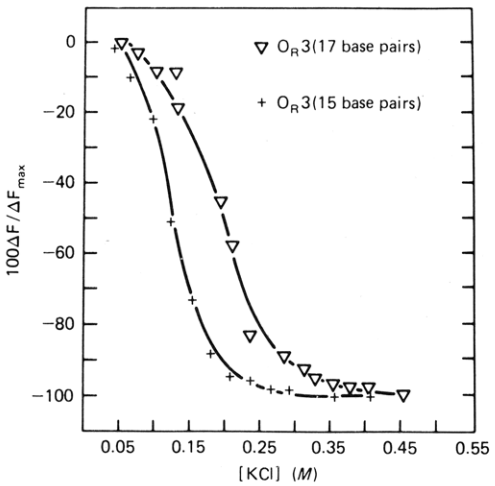

FIGURE 5: Quenching of fluorescence can be reversed by the addition of salt: $\Delta F = F_{(DNA)} - F_{(+KCl)}$, where $F_{(DNA)}$ is the final point from Figure 4 and $F_{(+KCl)}$ the fluorescence intensity with added KCl. This is indicated for both 17 (▽) and 15 (+) bp DNA's without fluorine to indicate that the use of slightly shorter operators gives qualitatively the same results. As anticipated, the shorter operator dissociates at slightly lower salt concentrations. The initial *cro* repressor dimer concentration was 1.15 μM and the final concentration 1.0 μM.

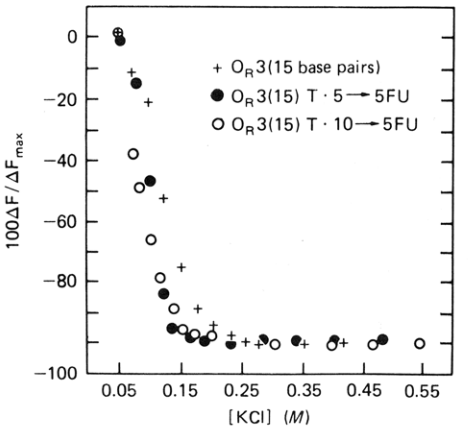

FIGURE 6: Fluorescence quenching by O_R3 operators with fluorouracil are also reversed by KCl addition. The initial *cro* repressor dimer concentration was 1.15 μM and the final concentration 1.0 μM.

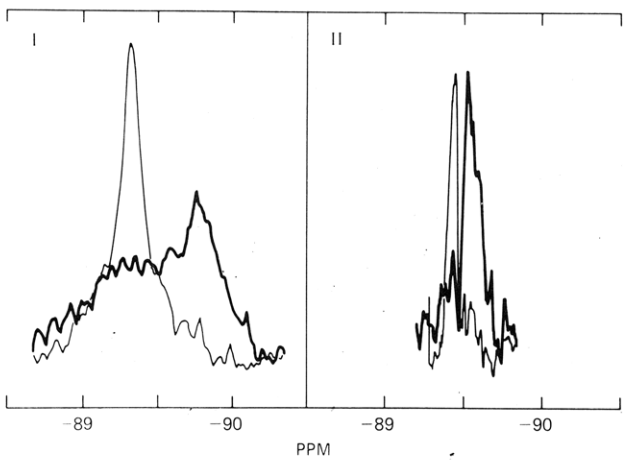

FIGURE 7: Changes in the NMR spectra of fluorine-substituted O_R3. Panel I (141 MHz) is the fluorine at position −5 and panel II (188 MHz) is fluorine at position −10. The thin spectra are in the absence of *cro* protein. The thick spectra are in the presence of two *cro* repressor dimers per DNA fragment. The initial O_R3 DNA concentration was 120 μM (thin spectra). Final concentrations: O_R3 DNA, 110 μM; *cro* repressor dimer, 220 μM.

model illustrated schematically in Figures 1 and 2. The broad shoulder at −89.25 ppm, the position of uncomplexed DNA,

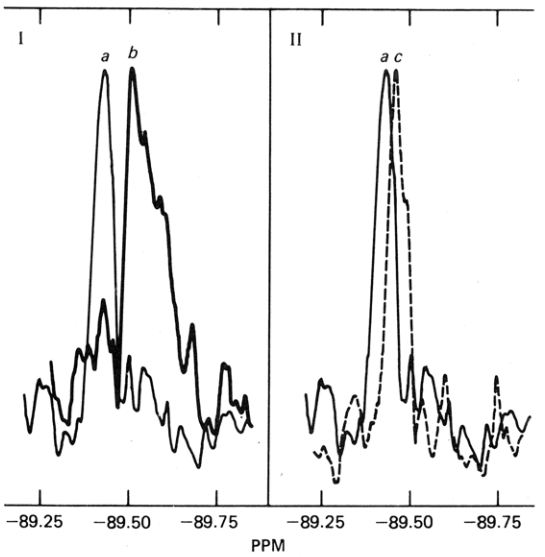

FIGURE 8: Expanded version of the spectra of Figure 7II is shown in panel I. (a) is the DNA with fluorine at position −10 alone (120 μM); (b) is the spectra with the added *cro* repressor dimer (110 μM), two dimers per DNA (220 μM). In panel II, spectrum a is the same as in panel I. Spectrum c is the sample from panel Ib with 2.0 M KCl.

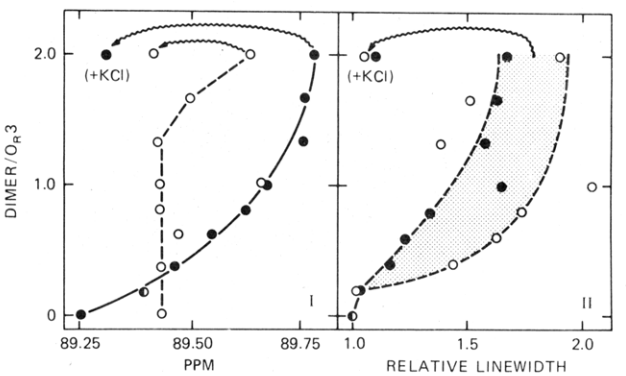

FIGURE 9: Titration of the DNA with *cro* repressor dimers. The left side shows the change in chemical shift whereas the right side shows the change in relative line width. The open circles (○) are position −10 fluorine, and the solid circles (●) are position −5. The wiggly line with the arrowhead indicates the change that occurs upon addition of KCl to 2.0 M.

suggests that the complex may have alternate forms at these high concentrations and protein to DNA ratios. One notes, however, that there is a slight chemical shift alteration at position −10, which is on the backside of the DNA helix in the Matthews model. In both cases, the addition of KCl to 2 M returns the chemical shift and the line width back to the uncomplexed position in the respective spectra. This is illustrated for the case of the fluorine substitution at position −10 in the expanded form in Figure 8. Figure 8I is an expansion of Figure 7II, and Figure 8II shows that the addition of salt returns the chemical shift almost back to the starting point. The residual small change in chemical shift is due to the effect of the added salt on free DNA with 5FU. One clearly sees that the line width that increases from spectrum a to spectrum b reduces again in spectrum c corresponding to *cro* repressor–O_R3 complex formation followed by dissociation.

An essential feature of the Matthews model is confirmed by these data. This is clearly visualized when one plots the chemical shift as a function of added *cro* repressor dimer as shown in Figure 9I. One notes that the actual chemical shift of the fluorines at the start of the titration, before addition

of protein, is slightly different for the two DNA molecules. This is very likely a sequence-dependent variation reflecting the sensitivity of fluorine to its environment. The striking feature of Figure 9I is that the addition of *cro* repressor changes the chemical shift only at position −5 and *not* at position −10 until an excess of one dimer is added per O_R3 DNA fragment. This suggests that the preferred binding mode perturbs the environment at position −5 for ratios of *cro* repressor dimer to DNA up to 1:1. However, as more *cro* repressor is added, complexes that contain more than one dimer per DNA may be possible (Boschelli, 1982). This may be the reason for the slight chemical shift that is illustrated in Figure 7II, which was taken from the final titration point in Figure 8.

One anticipates a line width increase if a protein–DNA complex is actually forming under these conditions as the molecular weight of the free O_R3 operator (M_r 9000) increases to that of the complex of *cro* repressor and the DNA (M_r 14 700 + 9000). Although the spectra are somewhat noisy, we estimated the relative line width by comparing the areas of the individual spectra for each titration point scaled to the same height relative to the corresponding spectrum with no protein. This is plotted in Figure 9II. The shaded area indicates the qualitative trend of the line width increase. Most important, in both panels of Figure 9, the addition of KCl to 2 M returns both the chemical shift and line width value to the starting point as indicated by the wavy lines. There appears to be a slight discontinuity in the trend of the line width points at one dimer per O_R3, but we cannot rule out experimental variation due to the noise. However, the fact that it occurs with both samples suggests that further experiments at higher concentrations need to be pursued. It could conceivably be due to the addition of the second protein molecule to the complex. The possibility of a second molecule being added is underscored by the fact that the line width seems to, in general, continue to increase after a 1 to 1 ratio of protein to O_R3 has been achieved.

Discussion

These data demonstrate the practicality of using fluorinated deoxyribonucleotide analogues to probe protein–DNA complexes. Since single lines are involved, it is clear that it will be possible to look at relatively large molecules, well beyond the 20 000-dalton size that can be approached by ^1H NMR in solution. NMR studies of 5-fluorocytidine and 2-fluoroadenosine have been reported (Alderfer et al., 1982). The enzymatic syntheses of poly(2′-deoxy-2′-fluoroadenylic and -cytidylic acids) have been reported as well (Gulshbauer et al., 1977; Ikehara et al., 1978). Thus this approach can be extended to positions in the narrow DNA groove and the backbone of DNA helix.

This also complements the use of fluoro amino acid substituted analogues of the repressor protein for ^{19}F NMR examination of the protein–DNA complex (Arndt et al., 1983; Nick et al., 1982). The model proposed by Matthews et al., suggesting the interaction of *cro* protein with O_R3 occurs due to the tangential contact of the protein of one side of DNA helix, is supported here. This assumes average B-DNA conformation, a reasonable one supported by solution NMR data with both O_R3 (Chou et al., 1983) and O_L1, one of the other five λ operator sites (Weiss et al., 1984). The range of helical twist angles for B-DNA is 28–42° and for A-DNA 16–44° with an average of 33°. The angle subtended by the fluorines five base steps apart can at most range from 80° to 180°, leaving a minimum projected gap of a quarter circle and most likely more. Thus, the interaction of O_R3 with *cro* repressor appears to be more like a rack and pinion than like the lead screw and half-nut arrangements seen in the *Eco*RI endonuclease complex (Frederick et al., 1984). Anthropomorphically, *cro* repressor contacts DNA more like a sober man leaning on a lamp post than a drunk one embracing it.

A possible concern might be that the length of DNA that we are using is 15 bp rather than 17 bp, as seen in the consensus sequence for λ phage operators. As we pointed out above, the operator mutation in O_R3 furthest away from the center of symmetry occurs at position 16. This suggests that a 15-bp DNA is sufficient to span the specific recognition region. Furthermore, analysis of cocrystals of *cro* repressor with DNA shows that complexes can be formed with a stoichiometry of 1:1 if pieces as short as 9 bp span the sequence from base pair 5 to base pair 13 (Anderson et al., 1983). If a fragment DNA were used that spanned base pairs 2–7, one gets cocrystals containing two DNA fragments per protein dimer. This suggests that the deletion of base pairs 1 and 17 as we have done here will not alter the stoichiometry. Since the 15-bp O_R3 fragment dissociates at lower KCl concentrations, there is the possibility of nonspecific ionic contacts beyond the central 15 bp used here. In a closely related system, the phage 434 and its repressor, good cocrystals were obtained with a 14-bp DNA fragment (Anderson et al., 1984).

These results also extend the genetic and biochemical data on specific groups at the O_R3 operator that are experimentally implicated for potential interaction with the *cro* repressor [Johnson et al., 1978; G. Gussin, A. Johnson, C. Pabo, and R. Sauer, personal communication in Hendrix et al. (1983)]. The genetic information shows that a mutation at base pair 5, which converts the AT to a GC, v3C, reduces the affinity of *cro* repressor for O_R3 10-fold. The methylation protection experiment shows the N-7 positions of the neighboring G's at positions −4 and −6 are protected from dimethyl sulfate by the *cro* repressor when bound to DNA. As Figure 2 schematically shows, the N-7 positions of both of these G's are the closest functional groups in the wide groove on neighboring base pairs to the fluorine being used to probe the thymine methyl environment.

The observation that the relative line width continues to increase somewhat beyond 1:1 stoichiometry requires additional comment. The concentrations used in the NMR experiments, greater than 100 μM, are well above the estimated dissociation constant for O_R3 of about 10^{-10} M, when contained on intact viral DNA (Takeda et al., 1977; Johnson et al., 1980), even when one allows a 3–4 order of magnitude increase due to the short operator DNA fragments. We have noted the possibility of multiple *cro* repressor dimers bound nonspecifically to a short fragment of DNA with two specific binding sites in our titrations that began with excess protein (Boschelli et al., 1982). A similar observation is seen in the chemical shift at the position 10 fluorine for methyl substitution, which occurs at the high *cro* repressor dimer of concentrations. It suggests that additional binding of the protein to the DNA fragment is possible on the backside of the helix. At this stage, it is not possible to compare our findings at position −10 with the tentative observation that the imino proton of the CG base pair at position ±9 shifts significantly downfield (Kirpichnikov et al., 1984). The *cro* repressor dimer to O_R3 DNA ratios used in those experiments were 1 or lower and do not overlap into the region where we observe the change at the fluorine on the T analogue of position 10. Although these experiments do not specifically identify the amino acid side chains causing the perturbations seen here, given the broad, intense interest in the λ *cro* repressor–O_R3 system

among NMR spectroscopists (Iwabashi et al., 1982; Kurochkin & Kirpichnikov, 1982; Kirpichnikov et al., 1982; Arndt et al., 1983; Bolotina et al., 1983; Chou et al., 1983; Ulrich et al., 1983; Lee et al., 1983; Meyer et al., 1983; Kirpichnikov et al., 1984) as well as cocrystal structure determination along with chemical modification experiments by the X-ray crystallographers (Anderson et al., 1983; Takeda et al., 1984), the answers will be forthcoming shortly.

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Distribution of 5-Methyldeoxycytidine in Products of Staphylococcal Nuclease Digestion of Nuclei and Purified DNA†

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ABSTRACT: We have compared the distribution of 5-methyldeoxycytidine (m^5dC) between staphylococcal nuclease (SN) sensitive and resistant regions of human diploid fibroblast chromatin to the corresponding distribution in purified DNA. After SN digestion of fibroblast nuclei or purified DNA, nuclease-resistant products were separated from sensitive products by perchloric acid or ethanol precipitation; the radioactively labeled nucleosides were then fractionated by high-performance liquid chromatography and quantitated. Our results indicate that m^5dC is preferentially associated with SN-resistant regions of both chromatin and purified DNA. The magnitudes of these preferences in fibroblast chromatin and DNA are similar; we find that the enrichment of m^5dC content in SN-resistant fractions of nuclei and DNA relative to the corresponding sensitive fractions is approximately 2–3-fold. Therefore, highly methylated regions of DNA have an intrinsic resistance to digestion by SN that is of sufficient magnitude to explain the high degree of nuclease resistance of chromatin containing highly methylated DNA.

One approach to elucidating the biological role of DNA methylation in eukaryotes is to investigate the interaction of methylated and unmethylated regions of the genome with chromosomal proteins. A probe used widely in the study of the interaction of DNA and nucleosomal proteins is the enzyme SN.[1] By preferentially cutting between nucleosome cores, SN facilitates the fractionation of chromatin into a nuclease-resistant, core-enriched fraction and a nuclease-sensitive, linker-enriched fraction (Felsenfeld, 1978). Previous investigations of the distribution of m^5dC between SN-sensitive and -resistant regions of chromatin have led to differing conclusions. Adams et al. (1977) found no significant difference between the m^5dC content of nuclease-resistant chromatin and total DNA of CHO cells and concluded that the distribution was random. Using a variety of cell types, Cedar and collaborators (Razin & Cedar, 1977; Solage & Cedar, 1978) demonstrated an enrichment of m^5dC in SN-resistant chromatin regions and suggested that m^5dC residues are preferentially localized within nucleosomal core particles.

We have reexamined this issue of the distribution of m^5dC between SN-resistant and -sensitive regions of chromatin in human diploid fibroblasts. In order to evaluate whether or not chromosomal proteins are responsible for the specific distribution of m^5dC between resistant and sensitive regions of human fibroblast chromatin, we have studied this distribution in purified human fibroblast DNA. Our findings indicate that m^5dC is preferentially associated with SN-resistant fractions in both chromatin and purified DNA, suggesting that chromosomal proteins are not responsible for the enrichment of m^5dC in SN-resistant regions of chromatin. These results further emphasize the complexity of the action of SN and the difficulty in using this enzyme as a probe in detailed studies of chromatin.

Materials and Methods

Cell Culture. Human diploid fibroblasts (AG1518; Institute for Medical Research) were grown as previously described (Dresler et al., 1982). Cells were labeled during exponential growth with 10 nCi/mL [^{14}C]dC (480 mCi/mmol; Amersham) or 250 nCi/mL [6-^{3}H]dC (5–6 Ci/mmol; Moravek Biochemicals). To reduce the conversion of labeled dC to dT, unlabeled dT (100–400 μM final concentration) was added to the medium. In all experiments, the radioactivity was chased with unlabeled medium after 1 week of labeling, and

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[1] Abbreviations: SN, staphylococcal nuclease; m^5dC, 5-methyldeoxycytidine; dC, deoxycytidine; dT, thymidine; PCA, perchloric acid; HPLC, high-performance liquid chromatography; dG, deoxyguanosine; dA, deoxyadenosine; SEM, standard error of the mean; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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