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Long-Range Allosteric Effects on the B to Z Equilibrium by Daunomycin†

Jonathan B. Chaires
Department of Biochemistry, The University of Mississippi Medical Center, Jackson, Mississippi 39216-4505
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ABSTRACT: Spectroscopic and fluorometric methods were used to study the binding of the anticancer drug daunomycin to poly[d(G-C)] and poly[d(G-m^{5}C)] under a variety of solution conditions. Under high-salt conditions that favor the left-handed Z conformation, binding isotherms for the interaction of the drug with poly[d(G-C)] are sigmoidal, indicative of a cooperative binding process. Both the onset and extent of the cooperative binding are strongly dependent upon the ionic strength. The binding data may be explained by a model in which the drug preferentially binds to B-form DNA and acts as an allosteric effector on the B to Z equilibrium. At 2.4 M NaCl, binding of as little as one drug molecule per 20 base pairs (bp) results in the conversion of poly[d(G-C)] from the Z form entirely to the B form, as inferred from binding data and demonstrated directly by circular dichroism measurements. Similar results are obtained for poly[d-(G-m^{5}C)] in 50 mM NaCl and 1.25 mM $MgCl_2$. Under these solution conditions, it is possible to demonstrate the Z to B structural transition in poly[d(G-m^{5}C)] as a function of bound drug by the additional methods of sedimentation velocity and susceptibility to DNase I digestion. The transmission of allosteric effects over 20 bp is well beyond the range of the drug's binding site of 3 bp. Since daunomycin preferentially binds to alternating purine–pyrimidine sequences, which are the only sequences capable of the B to Z transition, the allosteric effects described here may be of importance toward understanding the mechanism by which the drug inhibits DNA replicative events. The results described are of more general interest as an illustration of long-range allosteric effects on DNA conformation, of perhaps general importance in the regulation of gene expression.

The transition of DNA from the right-handed B form to the left-handed Z form is a topic of intense current interest. Since the early observations of the Z structure in solution (Pohl & Jovin, 1972) and in the crystalline state (Wang et al., 1979), Z DNA has been shown to exist (or to be inducible) in polytene chromosomes (Nordheim et al., 1981; Hill & Stollar, 1983; Robert-Nicoud et al., 1984) and to exist in vivo (Lipps et al., 1983). Proteins that will bind specifically to Z DNA have been isolated (Nordheim et al., 1982). Models invoking Z DNA as a potential mediator of gene expression have followed these observations (Rich, 1983), although direct experimental verification of the involvement of Z DNA in the regulation of gene expression is still lacking. The chemistry and biology of Z DNA have recently been reviewed in detail (Rich et al., 1984).

Various aspects of the involvement of small molecules in the B to Z transition are under active investigation in many laboratories. Metal ions are, in general, promoters of the B to Z transition, but metals differ, in often striking ways, in the concentration required for the formation of Z DNA (Pohl & Jovin, 1972; Behe & Felsenfeld, 1981; van de Sande et al., 1982; Chen et al., 1984). In contrast, a number of compounds are inhibitors of the B to Z transition. Many of these are of medical or clinical interest and include intercalators (Pohl et al., 1972; Mirau & Kearns, 1983), carcinogens (Nordheim et al., 1983; Rio & Leng, 1983), and other compounds that bind to DNA (Zimmer et al., 1983). Studies of the effects

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of such compounds on the B to Z transition are of interest not only for understanding the possible molecular basis of the drug–carcinogen action but also for the information they provide about the structure of Z DNA and the dynamics of the B to Z equilibrium. The precise mechanism by which these ligands inhibit the B to Z transition remains incompletely described.

Previous results from this laboratory showed that the important anticancer drug daunomycin inhibits the rate of the B to Z transition in poly[d(G-C)] (Chaires, 1983b). Further, the drug acts as an allosteric effector of the B to Z equilibrium and converts Z DNA to the B form under solution conditions that would otherwise favor the Z form in the absence of drug. These studies were motivated, in part, by recognition that daunomycin will bind preferentially to DNA of alternating purine–pyrimidine sequence (Chaires, 1983a), which is the sequence required for the B to Z transition [reviewed in Rich et al. (1984)]. Preferential interaction with such sequences, with concomitant structural alterations in the DNA, could conceivably be an important part of the mechanism by which daunomycin inhibits DNA replication and transcription (Ward et al., 1965; Hartmann et al., 1964; Di Marco et al., 1971). Additional studies of the influence of adriamycin, a compound closely related to daunomycin, on the B to Z transition in poly[d(G-C)] (van Helden, 1983) and poly[d(G-m^{5}C)] (Chen et al., 1983) have appeared.

The results reported here extend these previous studies and show that the effect of daunomycin on the B to Z transition is strongly dependent upon ionic strength. Under ionic conditions that favor Z DNA, but which approach those found in vivo, daunomycin shows long-range allosteric effects on the B to Z equilibrium. Under some conditions, as little as one bound drug molecule per 20 bp is sufficient to convert either poly[d(G-C)] or poly[d(G-m^{5}C)], initially in the Z conformation, entirely to the B form. The structural transition was inferred from cooperative drug binding isotherms and demonstrated directly by circular dichroism, sedimentation velocity, and DNase I digestion studies. The range of these allosteric effects is of comparable magnitude to previous reports of long-range interactions in DNA. These results are of general interest as an illustration of the transmission of allosteric effects in DNA, which may be of general importance in the regulation of gene expression, and of more specific interest toward understanding the molecular basis by which daunomycin acts to inhibit replicative events.

Materials and Methods

Polynucleotides. Poly[d(G-C)] (lot 31910) and poly[d(G-m^{5}C)] (lot 782-26) were purchased from P-L Biochemicals (Milwaukee, WI). Polynucleotides were dissolved in buffer by gentle rotational mixing at room temperature for 24 h and fractionated by using Sepharose 4B as previously described (Chaires, 1983a). The following buffers were used for the fractionation and subsequent experiments: *BPES*, containing 6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 0.185 M NaCl, and 1 mM disodium ethylenediaminetetraacetic acid (Na_2EDTA), pH 7.0; *BP*, containing 6 mM Na_2HPO_4, 2 mM NaH_2PO_4, and 50 mM NaCl, pH 7.0.

Fractionated polynucleotides were characterized as previously described (Chaires, 1983a).

Polynucleotide concentrations were determined spectrophotometrically, assuming an extinction coefficient of 16 800 M^{-1} [base pairs (bp)] cm^{-1} at 255 nm for both the methylated and the unmethylated polymer.

Binding Studies. Daunomycin binding was measured by visible spectroscopy and fluorometric methods as previously described (Chaires et al., 1982; Chaires, 1983a, 1985). Stock daunomycin solution was added to polynucleotide solution in small increments. Following each addition, the absorbance at the isosbestic point, 540 nm, and at 480 nm was recorded after equilibration. The concentration of total drug (C_T) was calculated from

$$C_T = A_{540}/\epsilon_{540}$$

where ϵ_{540} = 5100 M^{-1} cm^{-1}. The concentration of bound drug (C_B) was determined from

$$C_B = \Delta A_{480}/\Delta\epsilon_{480} = (A_{480}^{\circ} - A_{480})/\Delta\epsilon_{480}$$

where $A_{480}^{\circ} = 11500C_T$, A_{480} is the observed absorbance at 480 nm, and $\Delta\epsilon_{480}$ = 4500 M^{-1} cm^{-1}. The free drug concentration was then determined by the difference. The values for the extinction coefficients were previously determined and found to be invariant with ionic strength (Chaires et al., 1982b; Chaires, 1985). Fluorometric titrations utilized a Perkin-Elmer 654-40 spectrofluorometer. The fluorescence intensity in the presence of DNA (I) and in the absence (I_0) was determined for λ_{ex} = 480 nm and λ_{em} = 555 nm and used to calculate the concentration of free drug (C_f) according to

$$C_f = C_T(I/I_0 - P)/(1 - P)$$

where C_T is the known total concentration of added drug and P is the ratio of the observed quantum yield of bound drug to that of free drug. P was previously found to be 0.05 and was invariant with temperature and ionic strength.

This procedure assumes negligible interaction of daunomycin with Z DNA and that changes in the extinction and fluorescence of the drug arise from its interaction with B-form DNA. These assumptions are supported by the following observations. A 2 μM solution of daunomycin in the presence of 40 μM (bp) poly[d(G-C)] in 3.5 M NaCl (conditions that favor the Z conformation) shows an apparent extinction at 480 nm within 5% of that seen for the drug alone. In contrast, the same concentration of drug in the presence of 40 μM calf thymus DNA in 3.5 M NaCl (conditions in which the DNA retains the B conformation) shows an apparent extinction of 7000 ± 200 M^{-1} cm^{-1}, a value identical with that previously determined (Chaires et al., 1982b) for the bound form of the drug. Fluorescence titration experiments, in which the intensity of fluorescence emission at 555 nm (with λ_{ex} = 480 nm) was monitored with increasing amounts of added daunomycin in the presence of either 68.8 μM (bp) poly[d(G-C)] or calf thymus DNA in 3.5 M NaCl, showed the following: The fluorescence of added drug increases linearly over the range 0–5 μM in buffer alone, and in an identical fashion in the presence of poly[d(G-C)]. In contrast, over the same drug concentration range in the presence of calf thymus DNA, the fluorescence intensity is nearly completely quenched, consistent with the previous characterization of the fluorescence properties of the bound, intercalated drug (Chaires, 1982b). Further, in the presence of excess poly[d(G-C)], the value for the fluorescence polarization was near the value seen for the free drug, and the Stern–Volmer quenching constant, K_{sv}, was found to be 12.6 M^{-1}, a value near that seen for free daunomycin (Chaires, 1983). These observations suggest that if daunomycin does interact with Z-form DNA, the absorbance and fluorescence properties of the bound form are nearly identical with those of the free drug. The relative affinity of daunomycin for B- and Z-form DNA was examined by competition dialysis. Identical amounts of daunomycin were added to poly[d(G-C)] and calf thymus DNA solutions in 3.5 M NaCl at an identical polymer concentration [80 μM (bp)]. Drug concentration were such that at least a 30-fold molar

excess of base pairs was present. The drug–poly[d(G-C)] solutions were then dialyzed directly against the drug–calf thymus DNA solutions in a Spectraum Medical Industries five-cell dialyzer. Following 48 h of dialysis, the total drug concentration was assayed by fluorescence following dissociation of bound drug by the addition of dimethyl sulfoxide (Graves & Krugh, 1984). In three experiments, over a range of drug concentrations, daunomycin partitioned preferentially into the calf thymus, B-form DNA side of the dialysis membrane to yield a 10–20-fold molar excess over the drug concentration found on the poly[d(G-C)], Z-form side of the membrane. Daunomycin thus strongly prefers B-form DNA over the Z form as a potential binding site. These experimental results are in contrast to a recent report that claims that ethidium bromide will intercalate into Z DNA with approximately the same affinity as its binding to B-form DNA (Shafer et al., 1984), a claim that is in conflict with other studies on the same system (Pohl et al., 1972; Walker et al., 1985). A recent NMR study supports the contention made here that the interaction of daunomycin with Z DNA is negligible (Neumann et al., 1985).

When appropriate, binding data were fit to the neighbor-exclusion model

$$r/C = K(1 - nr)\{(1 - nr)/[1 - (n - 1)r]\}^{n-1} \quad (1)$$

where K is the binding constant to an isolated DNA site and n is the exclusion parameter (Crothers, 1968; McGhee & von Hippel, 1974).

Self-association of daunomycin was neglected in these binding studies since the value of the drug self-association constant is unknown for the ionic strengths used. We previously determined that self-association is negligible up to free drug concentrations of 10 μM in modest ionic strengths (Chaires et al., 1982). Ten micromolar is the maximum free drug concentration encountered in these studies. Menozzi et al. (1984) report a slight salt dependence in the dimerization constant of daunomycin, with $K_{\text{dimerization}}$ doubling for an approximate 100-fold increase in ionic strength. The ionic strengths used in this study are 10-fold greater than those used in our previous determinations of the daunomycin self-association constant, so neglect of self-association probably does not introduce gross errors into our analysis. The self-association constant would need to increase by a factor of 5 relative to the previously determined value (Chaires et al., 1982a) to produce more than 10% aggregated drug at a total drug concentration of 10 μM. The predominate error resultant from neglect of drug aggregation is in the exclusion parameter, n; K is largely insensitive to neglect of aggregation (Chaires et al., 1982a). Errors from neglect of daunomycin self-association are, then, expected to be slight and certainly would not affect the overall conclusions of these studies.

Circular Dichroism Measurements. Circular dichroism (CD) measurements were made by using a Cary 60 spectropolarimeter equipped with a CD attachment. CD spectra of polymer samples (50–70 μM bp) were recorded by using a 0.2-cm path-length cell at ambient temperature (26 ± 2 °C). Typically, 0–0.04° full-scale sensitivity was used, with a time constant of 3 s. Duplicate or triplicate scans were recorded for all samples, as was a scan of buffer alone to provide a base line.

CD spectra were digitized by using facilities of the NIH PROPHET Computer Resource, and the molar ellipticity was calculated from

$$[\theta] = 100\theta/cl$$

where l is the path length, c is the concentration of polymer (expressed here in terms of base pairs), and θ is the measured ellipticity.

Sedimentation Studies. Sedimentation velocity experiments were performed by using a Model E analytical ultracentrifuge equipped with an electronic speed control, an RTIC unit, and a photoelectric scanner system. Typical experiments used an AN-F rotor, 12-mm double-sector centerpieces, and a rotor speed of 30 000 rpm. Sedimentation coefficients were calculated by using the radial positions at the half-height of the scanner trace and were corrected to standard conditions.

DNase Digestion Studies. DNase I digestion studies were performed in solutions containing BP buffer, pH 7.0, with magnesium added as indicated, following the procedures of Kunitz (1950a). The rate of digestion was monitored by absorbance measurements at 260 nm, using a Cary 219 spectrophotometer thermostated to maintain a temperature of 25 °C. Typical assay mixtures contained 30 μM (bp) polynucleotide and were initiated by the addition of DNase I (Sigma Chemicals, St. Louis, MO; lot 41F9515, 1900 Kunitz units/mg) to a final concentration of 4.0 μg/mL. The initial rate was determined by linear least-squares regression of the linear portion of the time course.

RESULTS

The poly[d(G-C)] sample used for the studies described here showed the spectroscopic properties expected for the B and Z conformation under appropriate ionic conditions. In 0.2 M NaCl, the CD spectrum was characteristic of the B conformation, and the absorbance ratio A_{260}/A_{295} was near 9.0. At NaCl concentrations greater than 2.35 M, the CD spectrum was nearly inverted relative to the low-salt spectrum, and the A_{260}/A_{295} ratio decreased to 3.3. The equilibrium transition curve, as measured by the A_{260}/A_{295} ratio vs. NaCl concentration, was sharp, with a midpoint at 2.3 M NaCl, in agreement with previous results (Pohl & Jovin, 1972; Pohl, 1983).

The binding of daunomycin to poly[d(G-C)] as a function of NaCl concentration, and consequently as a function of position along the B–Z transition curve, is shown in Figure 1. At 1.5 M NaCl, conditions that favor the B form, drug binding is rapid, and the equilibrium binding data may be fully accounted for by the neighbor-exclusion model (Figure 1A). In contrast, at 3.5 M NaCl, conditions initially favorable to the Z form, drug binding is slow, with each addition of drug requiring 45 min to equilibrate. The resultant binding isotherm is sigmoidal, indicative of a cooperative binding process (Figure 1D). Such behavior has been previously described for the binding of ethidium (Pohl et al., 1972) and daunomycin (Chaires, 1983b) to poly[d(G-C)]. Striking differences from these extremes are seen at the intermediate salt concentrations of 2.8 and 2.4 M NaCl. At 2.8 M NaCl (Figure 1C), the binding isotherm is partially sigmoidal, but the onset of drug binding occurs near 1.5 μM free drug, instead of near 5 μM as seen in 3.5 M NaCl. Further, after a binding ratio of 0.15 is reached, drug binding becomes rapid, and the subsequent binding data may be accounted for by the neighbor-exclusion model. At 2.4 M NaCl, drug binding is again slow for the initial additions, but at a binding ratio of 0.05, binding becomes rapid, and the subsequent binding data follow the behavior predicted by the neighbor-exclusion model (Figure 1B). For this salt concentration, the onset of drug binding occurs at a free drug concentration near 0.1 μM, and the cooperative transition is complete at a binding ratio of 0.05.

The structure of poly[d(G-C)] under the conditions of the binding experiments was probed by using circular dichroism, with the results shown in Figure 2. In 2.4 M NaCl (Figure

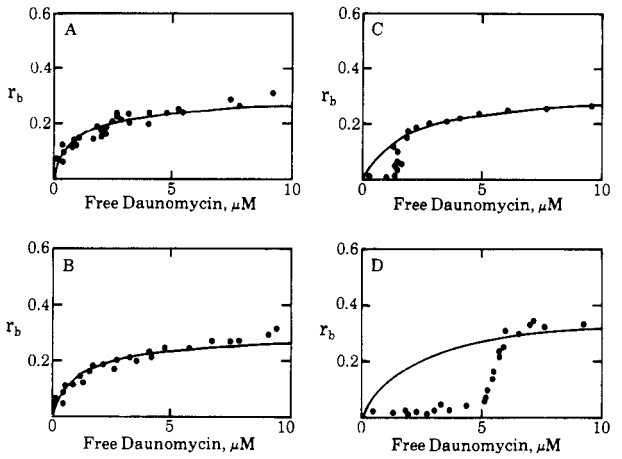

FIGURE 1: Binding of daunomycin to poly[d(G-C)]–poly[d(G-C)] as a function of NaCl concentration. Poly[d(G-C)] concentration was 35 μM (bp) for binding experiments, and the distribution of free and bound drug was determined by spectrophotometric or fluorometric methods described in the text. (A) 1.5 M NaCl. The solid line indicates the least-squares fit of the data to the neighbor-exclusion model (eq 1) with $K = 4.0 \times 10^5$ M^{-1} and $n = 2.8$ bp. (B) 2.4 M NaCl. The line is the fit to the neighbor-exclusion model with $K = 3.2 \times 10^5$ M^{-1} and $n = 2.8$ bp. (C) 2.8 M NaCl. The line is the fit of the data for free drug greater than 1.75 μM to the neighbor-exclusion model, with $K = 2.0 \times 10^5$ M^{-1} and $n = 2.5$ bp. (D) 3.5 M NaCl. The solid line is calculated for the neighbor-exclusion model with $K = 1.85 \times 10^5$ M^{-1} and $n = 2.0$ bp.

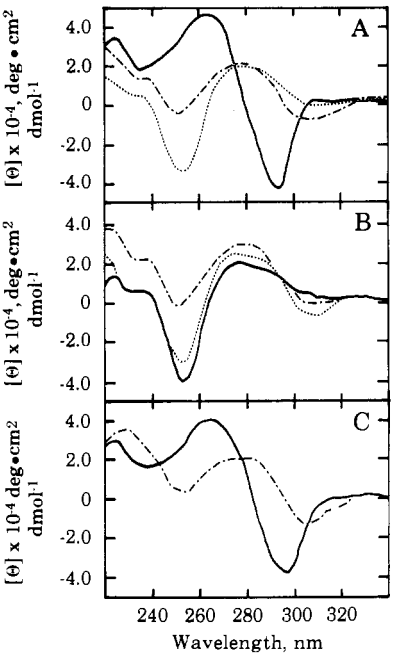

FIGURE 2: Circular dichroic spectra as a function of NaCl concentration and added daunomycin. (A) 2.4 M NaCl: (—) spectrum of poly[d(G-C)] with no added drug and characteristic of the spectrum associated with Z DNA; (···) spectrum that results from the addition of daunomycin to a binding ratio of 0.055 mol of drug/mol of bp; (–·–) saturation of the polymer with drug, $r = 0.33$. (B) 1.5 M NaCl with $r = 0$ (—), $r = 0.077$ (···), or $r = 0.33$ (–·–). (C) 3.5 M NaCl with $r = 0$ (—) and $r = 0.33$ (–·–). The spectrum for $r = 0.05$ superimposes exactly on that with no drug.

2A), the polymer shows a CD spectrum characteristic of Z-form DNA. Addition of daunomycin to a binding ratio of 0.055 results in an inversion of the CD spectra to one characteristic of B-form DNA. Thus, as little as one drug molecule per 20 bp is sufficient to convert the polymer from the Z to the B form. Saturating levels of drug further alter the CD spectrum, presumably reflecting the structure of the interca-

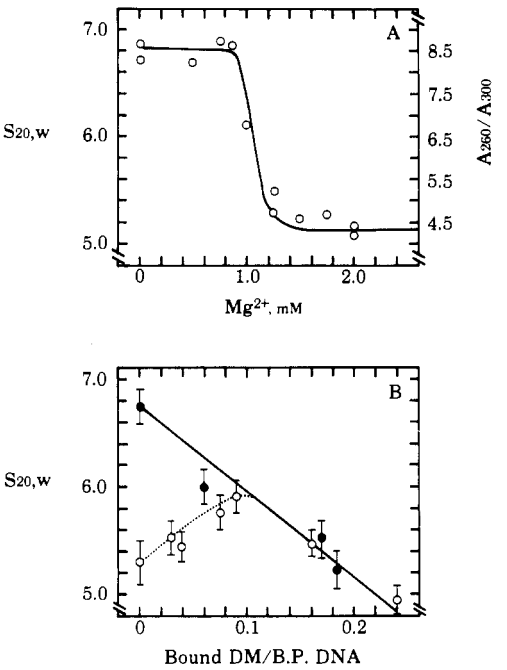

FIGURE 3: Sedimentation of poly[d(G-m^{5}C)] as a function of Mg^{2+} concentration and added daunomycin. (A) The sedimentation coefficient of the polymer is indicated by the symbols, while the line indicates the trend observed in the ratio A_{260}/A_{295}. (B) The closed symbols represent the sedimentation coefficient of the polymer at 0 mM $MgCl_2$ as a function of bound drug, with the trend indicated by the solid line. The open symbols show the sedimentation coefficient of the polymer in 1.25 mM $MgCl_2$, with the trend indicated by the dotted line. Experiments were performed at 22 °C, in a phosphate buffer (pH 7.0) containing 50 mM NaCl.

lated B form. Addition of low levels of drug to the polymer in 1.5 (Figure 2B) or 3.5 M (Figure 2C) NaCl results in little or no change in the original spectra. Saturating amounts of drug, however, yield CD spectra that are similar for all three salt concentrations.

Both the binding data of Figure 1 and the CD data of Figure 2 suggest that at a salt concentration of 2.4 M, low levels of daunomycin, near 1 drug molecular per 20 bp, can alter the structure of the entire polymer. In some cases, however, the CD method has produced misleading results. Spectra of poly[d(G-C)] in the presence of mitomycin were characteristic of the Z form, but other criteria showed that the polymer was in fact in the B conformation and that the observed CD spectra were apparently due to induced CD effects on the bound drug (Tomasz et al., 1983). Two additional methods, sedimentation velocity and susceptibility to DNase I digestion, were therefore developed and used to confirm the long-range allosteric effects suggested by the data of Figure 1 and 2. These studies required lower salt concentrations and were performed with poly[d(G-m^{5}C)], which will undergo the B to Z transition under less extreme ionic conditions.

Poly[d(G-m^{5}C)] undergoes the B to Z transition in phosphate buffer and 50 mM NaCl, pH 7.0, with a midpoint near 1.0 mM Mg^{2+} (Figure 3A). The binding of daunomycin to the methylated polymer was studied as a function of magnesium concentration, with the following results. In the absence of magnesium, drug binding is rapid and very tight, with a binding constant $K = 2.2 \times 10^6$ M^{-1} and a neighbor-exclusion parameter $n = 3.5$ bp. At magnesium concentrations of 1.25 and 2.5 mM, conditions that favor the Z conformation, initial additions of drug are bound slowly, until a binding ratio of $r = 0.05$–0.07 is reached, at which time binding becomes rapid and the binding isotherm nearly superimposes on that deter-

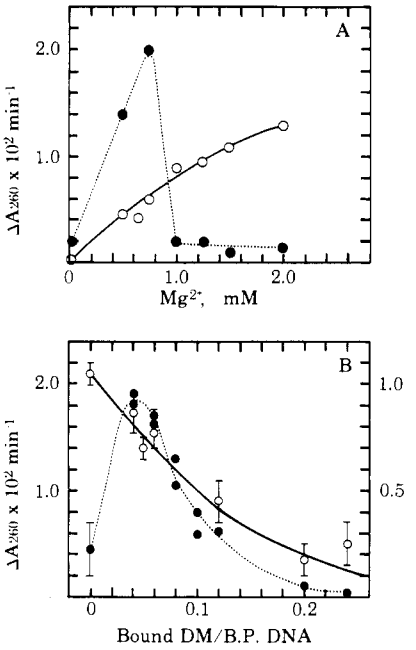

FIGURE 4: Initial rate of digestion of poly[d(G-m^{5}C)] and poly[d-(G-C)] by DNase I as a function of magnesium and added drug. Solution conditions were phosphate buffer (pH 7.0), 50 mM NaCl, 25 °C. DNaseé I was added to a final concentration of 4 μg/mL, and the total polymer concentration was 30 μM (bp). (A) Effect of magnesium on the digestion of poly[d(G-C)] (open circles) or poly-[d(G-m^{5}C)] (closed circles). (B) Effect of added drug on the initial rate of digestion at 1.25 mM $MgCl_2$, with the symbols as defined in panel A. The left y-axis scale refers to poly[d(G-m^{5}C)] data (closed circles), while the right y-axis scale is for poly[d(G-C)] (open circles).

mined in the absence of magnesium. The UV spectrum and derivative spectrum of poly[d(G-m^{5}C)] in 1.25 mM Mg^{2+} are characteristic of the Z conformation. Upon addition of daunomycin to a binding ratio of 0.05, the spectra revert to those characteristic of the B conformation. The behavior of the methylated polymer in 50 mM NaCl and 1.25 mM Mg^{2+} is thus analogous to that seen for the unmethylated polymer in 2.4 M NaCl.

Figure 3 illustrates the sedimentation properties of poly-[d(G-m^{5}C)] as a function of magnesium and added drug. The transition of the polymer from the B to the Z conformation results in an approximate 25% decrease in the sedimentation coefficient (Figure 3A). The change in the sedimentation coefficient parallels the decrease in the ratio A_{260}/A_{300}. Addition of daunomycin to poly[d(G-m^{5}C)] solutions not containing magnesium results in a linear decrease in the observed sedimentation coefficient (Figure 3B, closed symbols), a finding characteristic of the formation of an intercalation complex. Addition of drug to the methylated polymer in 1.25 mM Mg^{2+}, conditions that favor the Z conformation, results in an initial increase in the sedimentation coefficient until a binding ratio of 0.07–0.09 is reached, at which point $s_{20,w}$ decreases linearly and superimposes on the results obtained in the absence of magnesium. These results provide an alternate demonstration of a cooperative structural alteration resultant from the binding of daunomycin to Z DNA.

The susceptibility of poly[d(G-C)] and poly[d(G-m^{5}C)] to DNase I digestion as a function of magnesium and added drug is shown in Figure 4. Figure 4A shows the magnesium concentration dependence of the DNase digestion of the two polymers. The rate of digestion of poly[d(G-C)] by DNase increases steadily with added magnesium, consistent with the known magnesium requirement of the enzyme (Kunitz, 1950b). Poly[d(G-m^{5}C)] is initially more susceptible to DNase digestion than is the unmethylated polymer, with the initial rate of digestion increasing more sharply with added magnesium up to approximately 1.0 mM, at which point the activity sharply drops to nearly zero. This corresponds to the magnesium concentration at which the polymer shifts from the B to the Z form and confirms previous observations on the reduced susceptibility of Z DNA to nuclease digestion [reviewed in Rich et al. (1984)]. Addition of daunomycin to poly[d(G-C)] in 1.25 mM magnesium, in which the polymer is in the B conformation, decreases its susceptibility to DNase digestion (Figure 4B, closed symbols), consistent with previous observations on the effect of the drug on the digestion of natural DNA (Tsou & Yip, 1976). Addition of low levels of the drug to the methylated polymer under these conditions, however, increases the initial rate of digestion. After a binding ratio of about 0.04 is reached, the susceptibility to DNase digestion decreases, slightly more sharply than that seen for the unmethylated polymer.

These results may be summarized as follows: Daunomycin binding to poly[d(G-C)] under solution conditions favorable to the Z conformation is cooperative, but both the onset and the extent of cooperative drug binding are strongly dependent on the ionic strength. Under certain conditions, as little as 1 bound drug molecule per 20 bp is sufficient to alter the polymer to a form with drug binding characteristics of B-form DNA. Circular dichroism spectra are consistent with an alteration of the polymer from the Z form to the B form at these low binding ratios. Analogous behavior is seen for poly[d(G-m^{5}C)] at lower salt concentrations. Sedimentation velocity and DNase I digestion studies further support the observation that the binding of one drug molecule per 20 bp results in structural alteration of the polymer from the Z to the B form.

Discussion

The results reported here show that the effect of the anticancer drug daunomycin on the B to Z equilibrium is strongly dependent upon ionic strength. The drug will, under all conditions examined that would otherwise favor the Z conformation, convert Z DNA back to an intercalated B form. The amount of drug required for this conversion sharply decreases with decreasing ionic strength. While nearly 1 bound drug molecule per 3 bp is required for conversion of poly[d-(G-C)] from the Z to the B form in 3.5 M NaCl, only 1 bound drug molecule per 20 bp is required in 2.4 M NaCl. Poly-[d(G-m^{5}C)] is converted from the Z to the B form in 50 mM NaCl and 1.25 mM $MgCl_2$ by binding of 1 daunomycin molecule per 20 bp. These results are of interest both for understanding the possible molecular mechanism by which daunomycin inhibits DNA replication and transcription and as an illustration of long-range allosteric conformational transitions in DNA of potential importance in the regulation of gene expression.

Crothers and co-workers (Dattagupta et al., 1980) have proposed an allosteric model to account for cooperative drug binding to nucleic acids that is useful for understanding the results presented here. The model postulates that DNA is in equilibrium between two conformational states and that ligands may bind to either state with characteristic affinity, neighbor exclusion, and cooperativity. Preferential drug binding to one of the conformations may result in binding isotherms showing positive cooperativity, such as seen in Figure 1. A measure of such preferential binding is the ratio of the drug binding constants for each form. Long-range structural alterations may arise upon ligand binding, driven primarily by the tendency to avoid a thermodynamically unfavorable boundary

between incompatible structures (Crothers & Fried, 1983). The ionic strength dependence of the cooperative binding of daunomycin binding to poly[d(G-C)] described here may be understood in terms of that theory as arising from at least two contributions. First, daunomycin binding to B-form DNA is salt dependent (Chaires et al., 1982; Chaires, 1985). Increased ionic strength results in lessened affinity of the drug for B DNA and consequently may lessen the main driving force for the Z to B conversion. Second, the B to Z equilibrium is, of course, itself strongly dependent upon ionic strength (Pohl & Jovin, 1972; Pohl, 1983), with high salt favoring the Z form. Either the nucleation parameter or the equilibrium constant for the B to Z transition of a base pair, or both, may be dependent upon ionic strength. Preliminary analysis of the data of Figure 1 provides the following estimates for the ratio K_b/K_z (the ratio of drug binding constants for the B and Z forms) as a function of ionic strength: 3.5 M NaCl, $K_b/K_z = 47$; 2.4 M NaCl, $K_b/K_z = 24$. The decrease in K_b/K_z was unanticipated and suggests that the salt dependence of the allosteric effects seen in Figure 1 reflects primarily the salt dependence of the nucleation and/or propagation equilibrium constants for the B to Z transition. A more quantitative analysis of the data of Figure 1 awaits implementation of a computer program for the analysis of binding data in terms of the allosteric model of Dattagupta et al. (1980).

The data presented here indicate that under certain conditions, both poly[d(G-C)] and poly[d(G-m^{5}C)] will be converted entirely from the Z form to the B form by the binding of a drug molecule for every 20 bp. This corresponds to over two turns of the B-DNA helix, a distance considerably greater than the 3 bp binding site of the drug inferred from solution studies (Schutz et al., 1979; Chaires et al., 1982; Graves & Krugh, 1983) and visualized to atomic resolution by X-ray crystallography (Quigley et al., 1980). Notably, the cooperative length of the B to Z transition was found to be near 25 bp in low-salt conditions (Ivanov & Minyat, 1981). This number reflects the average number of base pairs present in a segment having a B or Z conformation at the half-transition point. The data presented here thus suggest that one daunomycin molecule, under the most favorable ionic conditions studied, will convert one cooperative unit from the Z to the B conformation.

The transmission of allosteric effects has been previously reported for other drug–DNA interactions, although the conformational transition resultant from drug binding in these cases is probably unrelated to the B to Z transition studied here. Daunomycin binding to calf thymus DNA in solutions containing 0.2 M Na^+ was reported to be cooperative (Graves & Krugh, 1983) and may be fit by the allosteric model described above with the result that the allosteric conversion of the DNA is complete upon binding of 1 drug molecule per 20–25 bp. Distamycin binds cooperatively to calf thymus DNA, with conformational changes in the DNA directly demonstrated by hydrodynamic methods (Dattagupta et al., 1980). Of more direct relevance are recent results from Krugh and co-workers reported after the work described here was completed. Walker et al. (1985) studied the interaction of ethidium, actinomycin, and actinomone with Z DNA and found long-range allosteric effects analogous to those reported here. For example, ethidium was found to convert 2–4 bp of poly[d(G-C)] per bound drug molecule from the Z to a right-handed form in 4.4 M NaCl. In 40 μM hexaminecobalt, however, each bound ethidium molecule converted approximately 20 bp of Z DNA to a right-handed form. For poly[d(G-m^{5}C)] in 2.0 mM $MgCl_2$, conditions near those reported here in Figures 3 and 4, ethidium converts approximately 7 bp of Z DNA to a right-handed form for each bound ethidium molecule. The results reported here are consistent with these general observations. Comparison of the daunomycin results with the ethidium results suggests that while intercalators may exert similar allosteric effects on Z DNA, pronounced quantitative differences may still be found, the origin of which remains undefined.

Two studies of the effect of adriamycin, a compound closely related to daunomycin, on the B to Z transition may be compared to the results presented here. Chen et al. (1983) studied the effect of adriamycin on poly[d(G-m^{5}C)] in the Z form in solutions containing 50 mM NaCl and 2.0 mM Mg^{2+}. They found that the drug cooperatively converted the polymer back to the B form, as judged by ^{31}P NMR and circular dichroism spectroscopy, with complete conversion reached with the binding of 1 drug molecule per 20 bp, in good agreement with the results presented here. Van Helden (1983) studied the effects of adriamycin on the high-salt transition of poly[d(G-C)] from the B to the Z form. The initial rate of the transition was totally inhibited by addition of 1 drug molecule per 9 bp, a slightly lower binding ratio than previously found for daunomycin (Chaires, 1983b).

The data of Figure 1 are unusual in that the value of the exclusion parameter, n, required to fit the binding data decreases with increasing ionic strength and approaches, at 3.5 M NaCl, the value of 2 bp normally found for simple intercalators. The exclusion parameter was previously found to be invariant (within experimental error) over the range 0.05–1.0 M NaCl (Chaires, 1985), but the value of 2 bp seen here in 3.5 M NaCl is in agreement with data reported previously at 4.0 M NaCl (Chaires, 1983b). The value of 2 bp is smaller than the estimates of 3–4 bp obtained at more modest ionic strength (Chaires et al., 1982; Chaires, 1985; Graves & Krugh, 1983; Schutz et al., 1979). It is possible that under these high-salt conditions an unusual intercalated B-form DNA may result in which drug molecules are more closely spaced than usual. The circular dichroic spectra of the intercalation complex in 1.5, 2.4, and 3.5 M NaCl do not show major differences, however, suggsting that the structure of the high-salt complex is not radically different from those at lower salt. The lower exclusion parameter at 3.5–4.0 M NaCl could conceivably arise from the minimization of polyelectrolyte effects by the enormous concentration of monovalent counterion. In this connection, Friedman & Manning (1984) have recently reported that actinomycin, another intercalator reported to have an exclusion parameter of several base pairs, may exclude as few as 2 bp once polyelectrolyte effects have been taken into account. Further, they cite unpublished model-building studies which indicate that an intercalation complex in which bound actinomycin molecules are separated by a single unoccupied base pair is sterically feasible. The decrease in the exclusion parameter inferred from the data in Figure 1 is reproducible, and the origin of the effect requires further study.

The sedimentation results shown in Figure 3A are consistent with previous observations that Z DNA is longer (Wang et al., 1979) and less flexible (Thomas & Bloomfield, 1983) than B DNA, both of which would contribute to a decrease in the sedimentation coefficient. The 25% decrease in s_{20} seen in Figure 3A is larger than the 8–11% decrease reported by Behe & Felsenfeld (1982), who used hexamminecolbalt to induce the B to Z transition in poly[d(G-C)] and poly[d(G-m^{5}C)]. It is possible that aggregation biased their estimate. The coincidence of the sedimentation data and the optical data in

Figure 3A lends confidence to the estimates of the sedimentation coefficients. The sedimentation method introduced in Figure 3 should provide a useful nonoptical method for accessing the influence of ligands on Z-DNA structure in future studies.

Z DNA has previously been reported to be less susceptible to digestion by nucleases (Rich et al., 1984). The results of Figure 4A are consistent with these previous reports. The enhanced susceptibility of poly[d(G-m^{5}C)] to DNase digestion relative to the unmethylated polymer is, as far as can be determined, a novel finding and may be of interest in connection with DNase hypersensitivity (Reeves, 1984). The results of Figure 4B are in full agreement with spectroscopic measurements and confirm that binding of 1 drug molecule per 20–25 bp will convert the polymer to the B form. It is possible that at low binding ratios sites particularly susceptible to nuclease attack, such as the B–Z junction, could form and may contribute to the very sharp increase seen in the initial portion of the dotted curve in Figure 4B.

The role of the B to Z transition in gene expression is at present unknown. Z DNA is transcribed with lower efficiency than B DNA (van de Sande et al., 1982; Durant et al., 1983; Butzow et al., 1984), and the insertion of sequences capable of forming Z DNA into enhancer regions of specific genes inhibits transcription (Hipskind & Clarkson, 1983; Santoro et al., 1984). The hypothesis has been put forth that the Z structure is important in the function of enhancer sequences, as a conformational signal to promote (or inhibit) the binding of promoter proteins (Rich, 1983). Previous results from this laboratory demonstrated that daunomycin will bind preferentially to alternating purine–pyrimidine sequences (Chaires, 1983b), which are common in promoter sequences (Breathnach & Chambon, 1983) and which are the only sequences capable of undergoing the B to Z transition [reviewed in Rich et al. (1984)]. The results reported here suggest that the binding of the drug to such sequences could profoundly alter the conformation of several turns of the DNA helix and could thus influence the binding of proteins important for transcription. While this is clearly speculative at present, such ideas warrant consideration as possible contributors to the mechanism by which the drug inhibits replicative events.

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Nucleoprotein Hybridization: A Method for Isolating Specific Genes as High Molecular Weight Chromatin†

Jerry L. Workman and John P. Langmore*
Biophysics Research Division and Division of Biological Sciences, The University of Michigan, Ann Arbor, Michigan 48109
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ABSTRACT: We describe a new technique designed to isolate specific eukaryotic genes as native oligonucleosome fragments. The isolation method consists of hybridization of single-stranded termini of chromatin restriction fragments to complementary mercurated DNA probes, followed by isolation of the hybrids by sulfhydryl-Sepharose chromatography. SV40 minichromosomes were used to test the effectiveness of the technique. About 80% of *Kpn*I- or *Bam*HI-restricted and λ exonuclease treated SV40 minichromosomes hybridized to an appropriate DNA probe after a 12-h hybridization reaction under mild conditions (0.1 M aqueous salt, 37 °C, pH 8). When the restricted minichromosomes were mixed with a 15-fold excess of "background" chromatin from sea urchin embryos, nucleoprotein hybridization was able to reisolate the SV40 chromatin to 88% purity with a 63% yield. This represented a 115-fold enrichment of specific genes as chromatin. Results of electron microscopy and polyacrylamide gel electrophoresis indicate that the hybridized SV40 chromatin has not lost the major chromosomal proteins characteristic of SV40 nor acquired significant amounts of protein due to exchange with background chromatin. Our experimental results show that it is currently possible to isolate repeated genes from higher eukaryotes for structural and biochemical study of the proteins involved with gene regulation.

While very significant progress has been made in the understanding of gene structure at the level of the nucleic acid sequence, little is known about the ways in which histone and non-histone chromosomal proteins interact with DNA sequences to control transcription. Largely, our ignorance about the control of transcription is the result of the inability to isolate and study specific sequences of DNA still bound to the protein and RNA molecules that are responsible for the structure and function of those sequences in vivo.

Currently, the only way to identify the regulatory proteins and to elucidate their molecular functions is to study the transcription of reconstituted genes in vitro using purified components or in vivo using the amphibian oocyte system [reviewed by Manley (1983), Wickens & Laskey (1981), and Gurdon & Melton (1982)]. To isolate putative regulatory proteins from a complex mixture of chromosomal proteins, DNA affinity techniques have been used to collect proteins that bind in vitro to regulatory DNA sequences [e.g., see Weideli et al. (1980) and Emerson & Felsenfeld (1984)]. This approach is undoubtedly very useful yet is susceptible to several potential artifacts due to the complexity of eukaryotic chromosomal proteins and to the likelihood that regulation in vivo involves the simultaneous or sequential binding of several proteins on a gene [reviewed by Brown (1984)].

There is a clearly expressed need [e.g., see Wickens & Laskey (1981) and Manley (1983)] to isolate individual genes as intact nucleoprotein molecules in the amounts necessary for biochemical and in vitro transcription studies, in order to complement and validate the gene reconstitution studies mentioned previously. Thus far, the attempts to isolate specific genes as chromatin have been limited to three types of chromatin, namely, satellite, ribosomal RNA, and 5S RNA genes (Zhang & Horz, 1982; Prior et al., 1983; Reynolds et al., 1983). These partial purifications were possible due to the special physical characteristics of the chromatin involved. Unfortunately, the strategies used for isolation of satellite, ribosomal, and 5S chromatin cannot be applied easily to other

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* Address correspondence to this author at the Biophysics Research Division, The University of Michigan.

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