Binding Specificity of the Two Major DNA-binding Proteins in Human Serum*

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The two major DNA-binding proteins of human serum (DNA-binding protein 1 and DNA-binding protein 2) were shown to bind preferentially to single-stranded polynucleotides rich in guanine residues. Equilibrium competition experiments using a nitrocellulose filter assay system containing labeled human lymphocyte DNA and various competing natural and synthetic polynucleotides indicated that both proteins recognized sequences of bases containing a keto group in either position 6 (purines) or 4 (pyrimidines) and that these keto groups must be readily accessible for effective binding to occur. Guanine was shown to be the preferred nucleotide through inhibition experiments using a series of synthetic homopolymers and a series of bacterial DNAs of differing G + C content. The relationship between protein affinity and G + C content was shown to be directly proportional. The equilibrium constants for the binding of the human lymphocyte DNA by both proteins were on the order of $10^{-6}$ M, and the length of the nucleotide sequence necessary for effective binding was found to be 12 to 18 bases using a series of oligomers of poly(dG).

When human serum is fractionated by affinity chromatography on DNA-cellulose, 2 major protein species are identified with more than 20 minor species. The two major DNA-binding proteins, designated DBP-1' and DBP-2, were first isolated and characterized in this laboratory (1, 2). DBP-1 is a glycoprotein rich in glycine and acidic at neutral pH. It has a molecular weight of 128,000 determined both by sedimentation equilibrium and SDS-polyacrylamide slab gel electrophoresis. DBP-2 has been identified as Factor B of the alternate pathway of complement activation.3

Using these proteins in purified form, we have undertaken the present study to characterize the binding of both DBP-1 and DBP-2 with respect to the type of nucleic acid preferred, conformational restrictions, the size of the polynucleotide bound, and nucleotide sequence specificity. A nitrocellulose filter assay system (3, 4) was developed; by making use of equilibrium competition experiments, we were able to determine the affinity with which DBP-1 or DBP-2 bound a number of polynucleotides, both natural and synthetic. Double-stranded DNA is not retained by the nitrocellulose filter unless it is bound to protein. By adding an unlabeled polynucleotide to the labeled DNA, we were able to rank possible substrates as to their ability to compete with the labeled double-stranded DNA for the protein and thus were able to establish their relative affinities.

These studies establish that DBP-1 and DBP-2 are capable of binding DNA with specificity based at least in part on conformation of the substrate and its base sequence. This in itself supports the possibility that the binding of polynucleotides may indeed be one of the in vivo functions of these proteins. Such evidence is important because the relationship between DNA-binding ability and physiological function has yet to be elucidated for either of these two proteins.

MATERIALS AND METHODS

Purification of DNA-binding Proteins

All operations were carried out at 4°C and all buffers contained 1 mM 2-mercaptoethanol, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride, except as noted. The original purification protocol for DBP-1 (1) was modified as follows.

Step 1: QAE-Sephadex Chromatography—The original protocol was unchanged.

Step 2: DNA-cellulose Chromatography (Step Elution at pH 6.8)—The major DNA-binding protein fraction was not eluted in bulk in 10 mM potassium phosphate, pH 6.8, containing 500 mM NaCl. Rather, a series of salt elution steps was employed, using NaCl concentrations of 50, 100, 200, and 500 mM. DBP-1 was eluted in 10 mM potassium phosphate, pH 6.8, containing 200 mM NaCl.

Step 3: Ammonium Sulfate Fractionation—DBP-1 was concentrated from the 200 mM NaCl eluate in the 0 to 40% and the 40 to 55% ammonium sulfate saturation fractions. The precipitates were resuspended in 10 mM potassium phosphate, pH 6.8, containing 50 mM NaCl and 20% glycerol (v/v).

The experiments described in this report were carried out with DBP-1 from the 40 to 55% ammonium sulfate fractionation. Normally the protein would next be passed over a Sephadex G-25 column in 10 mM Tris-HCl, pH 7.5, containing 100 mM NaCl, 1 mM EDTA, 1 mM sodium azide, and 15% glycerol (v/v) to remove the 2-mercaptoethanol. This allows the DBP-1 to form high molecular weight aggregates which are then chromatographed on a Sepharose 6B column in the same buffer. DBP-1 elutes in the void volume. Addition of 2-mercaptoethanol to this protein restores DNA-binding activity. These final steps proved to be unnecessary in the purification of the DBP-1 used in these studies since it was greater than 95% homogeneous after Step 3 as judged by SDS-polyacrylamide slab gel electrophoresis.

The purification protocol (1) for DBP-2 was modified as follows.

Step 1: QAE-Sephadex Chromatography—The original protocol was unchanged.

Step 2: DNA-cellulose Chromatography—DBP-2 was eluted in 10 mM potassium phosphate, pH 6.8, containing 100 mM NaCl.

Step 3: Ammonium Sulfate Fractionation—DBP-2 was concentrated from the 100 mM NaCl eluate in the 60% to saturation ammon-

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§ Recipient of Research Career Development Award CA00004 from the National Cancer Institute.

1 The abbreviations used are: DBP-1, DNA-binding protein 1; DBP-2, DNA-binding protein 2; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetate; sodium salt; QAE, diethyl-(2-hydroxypropyl)aminoethyl.
nium sulfate fraction. The precipitate was resuspended in 10 mM potassium phosphate, pH 6.5, containing 20% glycerol (v/v), and dialyzed overnight against 50 volumes of this buffer.

**Step 4: DEAE Chromatography**—The second DNA-cellulose chromatography step is no longer necessary. The dialysate is applied directly to the Whatman DE52 column as described in the original protocol, with the modification that all buffers used contain 20% glycerol (v/v). The incorporation of glycerol in the buffer maximizes the recovery of purified DBP-2 from the 30 mM NaCl eluate of this column.

**Preparation of DNA**

Human lymphocyte DNA was prepared from WIL2 cells grown for us by Dr. William Meinke, Scripps Clinic and Research Foundation, according to his procedure (5). At a cell density of 2.5 × 10⁶ cells/ml, 0.1 M of [2-14C]thymidine (>10 mCi/mmol, New England Nuclear) or 2.5 M of [methyll-H]thymidine (90 Ci/mmol, New England Nuclear) were added, and the cells were allowed to continue growing for another 24 h. The cells were then harvested and lysed by the urea/SDS/isoamyl alcohol method (6). The lysate was applied to an hydroxypatite (Bio-Rad, HTP DNA Grade) column at room temperature in EDTA. All DNA preparations were stored over chloroform at 4°C.

Lysozyme (4 mg) was added and the suspension incubated at 37°C for 15 to 25 min. During this incubation period, the filters were placed in a Millipore sampling manifold and the suction started. The DNA thus isolated was dialyzed overnight against 0.1 M sodium phosphate, pH 6.8. The DNA fraction was made 1 M in EDTA, treated 30 min with RNase (20 pg/ml) at 37°C, 30 min with 0.48 M sodium phosphate, pH 6.8. The DNA fraction was made 1 M in EDTA, treated 30 min with RNase (20 pg/ml) at 37°C, 30 min with pronase (20 μg/ml), and extracted with an equal volume of phenol; chloroform (3:1). The phenol was redistilled and saturated with 0.2 M Tris-HCl, pH 7.4, containing 10 mM EDTA. The DNA-containing aqueous phase was added to a 50:50 mixture of chloroform and isoamyl alcohol and extracted with 0.5 M NaCl and phenol/chloroform (3:1). The DNA was dialyzed overnight against 1 M sodium chloride. The dialysate was applied to a hydroxypatite column at room temperature in buffer containing 0.1 M sodium phosphate, 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0. The DNA was dialyzed overnight against 0.1 M SSC (0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0) made 1 M in EDTA. All DNA preparations were stored over chloroform at 4°C.

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The specific activities of the "C- and "H-labeled DNAs were 5 × 10⁶ cpm/μg and 1 × 10⁶ cpm/μg, respectively.

**Clostridium perfringens DNA** (Warthington) and Micrococcus lysodeikticus DNA (Sigma) were dissolved in SSC buffer, diluted 1:15 with 0.24 M sodium phosphate, pH 6.8, containing 8 μM urea, and purified on two hydroxypatite columns as described above. DNA from Bacillus subtilis BSt3 (int, trpK) was prepared by suspending 2.0 g of frozen cells in 40 ml of 0.2 M EDTA, 0.2 M NaCl, pH 8.1. Lysosyme (4 mg) was added and the suspension incubated at 37°C for 30 min. At that time, a second aliquot of lysosyme was added and the incubation continued for 1 h. SDS (2 ml of a 10% solution) was added and the mixture shaken until clear. Immediately, 80 ml of distilled water was added and the mixture centrifuged in a clinical centrifuge for 10 min at room temperature. The top layer was ex- tracted with an equal volume of phenol-chloroform (3:1) as described above. The DNA was precipitated by adding 2 volumes of 95% ethanol to the top layer, pooled on a stirring rod, redissolved in SSC, and subjected to hydroxypatite chromatography as described above.

Human placental DNA was prepared by the Sevag method (8).

**RESULTS**

**Determination of the Equilibrium Constants for the Binding of DBP-1 and DBP-2 to WIL2 DNA**—The nitrocellulose filter assay system was used to determine the equilibrium constants for the binding of double-stranded WIL2 DNA to both DNA-binding proteins. The conditions of the filter assay were developed to maximize DNA-protein binding. The assay system is quite sensitive to ion strength and pH changes. Sodium chloride concentrations above 0.05 M lead to increased background levels of binding as well as decreased levels of specific protein binding. Likewise, the specific binding decreased as the pH of the reaction mixture was raised; maximal binding occurred in a range of 0.5 unit centering on pH 6.0. At pH 6.0 and at sodium chloride concentrations below 0.05 M, the background levels were less than 2% of the total counts. These results appear to be a consequence of the nature of the interaction of the DNA-protein complex with the nitrocellulose filter since, when adsorbed to DNA-cellulose affinity columns during purification, DBP-1 and DBP-2 require a salt concentration of 0.2 and 0.1 M, respectively, for complete elution in a buffer at pH 6.8. Using the described assay, the equilibrium constant for DBP-1 was determined to be 2.84 × 10⁻⁶ M, and that of DBP-2 to be 4.88 × 10⁻⁶ M (Fig. 1).

A consequence of this sensitivity of the filter assay system to changes in ionic strength or pH (or both) is the apparent inhibition observed of the binding of the DNA by DBP-2 at the highest DNA concentration (Fig. 1b). DBP-1 elutes from DNA-cellulose in buffer containing 200 mM NaCl while DBP-2 is eluted in buffer containing 100 mM NaCl. The 10 mM potassium phosphate buffer used in the assay has a limited

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buffering capacity at pH 6.0. Thus, the system could well be sensitive to relatively small changes in ionic strength or pH. Because of this, the various inhibiting polynucleotides were dissolved in assay buffer whenever possible and were kept at sufficiently high concentrations to make unnecessary the addition of large amounts of solutions of differing ionic strengths or pH (or both). This was not possible for the curve depicted in Fig. 1b. The apparent lack of linearity is the result, and in fact, can be magnified at higher salt concentrations.

Specificity Based on Nucleotide Structure—The ability of DBP-1 and DBP-2 to differentiate among the various nucleotides commonly found in DNA and RNA was examined using synthetic homopolymers. Table I summarizes the relative affinities of both proteins for a series of polyribonucleotides. The inhibition curves for the interaction of DBP-1 and DBP-2 with the series of ribose-containing homopolymers are shown in Fig. 2. DBP-1 showed a 140-fold difference in relative affinity from most effective to least effective inhibitor, and DBP-2 showed a 60-fold difference. Both proteins show less affinity for poly(rA) and poly(rC) than for double-stranded WIL1 DNA. Thus, these two nucleotides do not appear to be significantly involved in the protein-substrate interactions. When the structures of the nucleotides tested were examined, it was seen that all substrates which were bound effectively by the proteins have a keto group either in position 6 for the purines or position 4 for the pyrimidines. Neither poly(rA) nor poly(rC) have such a keto group.

Looking only at the effective competitors, some general trends are evident. Both proteins show higher affinities for purines than pyrimidines and for ribose-containing substrates than deoxyribose-containing ones. That these are only general trends is illustrated by the fact that DBP-1 has a higher affinity for poly(rU) than poly(dG) while DBP-2 shows the opposite order of affinities. The differences in the relative affinities of the effective competitors, while not nearly as large as the differences between effective and noneffective competitors, are still significant. DBP-1 shows a 6-fold higher affinity for poly(rG) than poly(dT), and DBP-2 shows an 11-fold difference. The lower absolute values of the relative affinities of DBP-2 may indicate that the binding sites of the two proteins are different or that different environments around the same site are causing variations in the binding strength.

Specificity Based on Mole Per Cent G + C—By examining three bacterial DNAs with widely differing mole per cent G + C contents, it was possible to assess the specificity of DBP-1 and DBP-2 for sequences rich in guanine residues. Fig. 3 displays the relationship between relative affinity and G + C content. The bacterial DNAs used were those mentioned above—Cl. perfringens, B. subtilis, and M. lysodeikticus—and contained, respectively, 26%, 43%, and 72% G + C. The use of bacterial DNAs, which are highly unlikely candidates to be natural substrates for human serum proteins, tends to rule out the presence of unique specific sequences. These DNAs should give information based only on the statistical probability of a recognized sequence being present which, in turn, should be merely a function of a number of guanine residues found in the given substrate. For these three DNAs in the single-stranded form, there is a relationship between mole per cent G + C and the ability to be bound by both DBP-1 and DBP-2. Relative affinity increases in direct proportion to the amount of G + C present. The similarity in the slopes of the two lines could indicate that the two proteins are binding at the same site(s) on the DNA but that they are not binding with the same strength as shown by the difference in absolute values. It could also indicate that each protein sees approximately the same number of compatible sequences, although those sequences are different. With respect to the

### Table I

<table>
<thead>
<tr>
<th>Competing polynucleotide</th>
<th>Relative affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>WIL2 DNA (double-stranded)</td>
<td>1.00</td>
</tr>
<tr>
<td>Poly(rG)</td>
<td>56.0</td>
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<tr>
<td>Poly(rT)</td>
<td>47.7</td>
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<tr>
<td>Poly(dG)</td>
<td>12.6</td>
</tr>
<tr>
<td>Poly(dT)</td>
<td>9.77</td>
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<tr>
<td>Poly(rU)</td>
<td>18.3</td>
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<tr>
<td>Poly(rA)</td>
<td>0.87</td>
</tr>
<tr>
<td>Poly(rC)</td>
<td>0.40</td>
</tr>
<tr>
<td>Poly(dG-dC)</td>
<td>0.71</td>
</tr>
<tr>
<td>Poly(dA-dT)</td>
<td>0.51</td>
</tr>
</tbody>
</table>

a Since double-stranded WIL2 DNA was the standard from which all relative affinities were calculated, it is defined as having relative affinity of 1.00.

b The [50] was found by linear extrapolation.

c ND, not determined.
TABLE II
Specificity of Major Serum DNA-binding Proteins

<table>
<thead>
<tr>
<th>Competing polynucleotide</th>
<th>Relative affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBP-1</td>
<td>DBP-2</td>
</tr>
<tr>
<td>WIL2 DNA (double-stranded)*</td>
<td>1.00</td>
</tr>
<tr>
<td>Human placental DNA</td>
<td></td>
</tr>
<tr>
<td>Double strand</td>
<td>1.00</td>
</tr>
<tr>
<td>Single strand</td>
<td>2.65</td>
</tr>
<tr>
<td>Cl. perfringens DNA</td>
<td></td>
</tr>
<tr>
<td>Double strand</td>
<td>2.00</td>
</tr>
<tr>
<td>Single strand</td>
<td>4.61</td>
</tr>
<tr>
<td>B. subtilis DNA</td>
<td></td>
</tr>
<tr>
<td>Double strand</td>
<td>1.11</td>
</tr>
<tr>
<td>Single strand</td>
<td>5.37</td>
</tr>
<tr>
<td>M. lysodeikticus DNA</td>
<td></td>
</tr>
<tr>
<td>Double strand</td>
<td>0.77</td>
</tr>
<tr>
<td>Single strand</td>
<td>7.92</td>
</tr>
<tr>
<td>B. subtilis ribosomal RNA</td>
<td>6.81</td>
</tr>
</tbody>
</table>

* Since double-stranded WIL2 DNA was the standard from which all relative affinities were calculated, it is defined as having relative affinity of 1.00.

Discussion

Both DBP-1 and DBP-2 exhibited the highest affinity for the synthetic homopolymer poly(dG), but the results also indicated that substantial binding occurred with all homopolymers containing a critical keto group found at position 6 for purines and position 4 for pyrimidines. Under the assay conditions used, it is unlikely that the differences in homopolymer binding were related to the formation of multistrand complexes (10, 11). Thus, it appears that the binding of protein to polynucleotide is mediated by these carbonyl oxygens. Although the binding ability of both proteins is related to guanine content, experiments with DBP-1 using the double-stranded synthetic polynucleotides poly(dG) and poly[(dA-dT)-(dA-dT)] indicate that the number of guanine residues alone does not account for the effective binding of high G + C substrates. Both polynucleotides inhibit in the binding assay at approximately the same level and both are poor inhibitors as indicated in Table I. Two explanations could account for these observations. First, the effective binding of a substrate might require a sequence of continuous bases each possessing a keto group in the correct position as previously discussed. Were that the case, both synthetic substrates would be poorly bound because the keto group appears only on alternating bases. Second, DBP-1 is single-strand-specific and might require the guanine to be more accessible with respect to secondary structure. True denatured single-stranded substrates are not attainable under these assay conditions because of the tendency for each strand to self-annal giving a double-strand-like molecule.

A comparison of the binding properties of DBP-1 and DBP-2 with those of other characterized DNA-binding proteins reveals a number of similarities. First, the equilibrium constants of these proteins are similar to those of the lac repressor for nonoperator DNA (12). Reported values for the lac repressor of 10^{-8} to 10^{-9} m depending on the base composition compare favorably with values of 10^{-8} m for double-stranded WIL2 DNA with both DBP-1 and DBP-2, and it is apparent that for polynucleotides with a high percentage of guanine residues the equilibrium constants are even lower. Second, the implied preference of both proteins for single-stranded substrates corresponds to data reported for the DNA-unwinding proteins (13-17). However, the number of nucleotides bound per protein molecule for DBP-1 and DBP-2 discourages the idea that these are unwinding proteins. Assuming a molecular weight for DBP-1 of 126,000 (1), a protein molecule binds approximately once every 500 nucleotides using double-stranded WIL2 DNA as the substrate. A similar calculation for DBP-2 with a molecular weight of 86,000 yields a value of 1 protein molecule bound every 320 nucleotides. Third, there is the question of binding length. Turning again to the lac repressor, we find reported values of 11 to 16 nucleotides (18) and this is comparable to the value of 12 to 18 observed for
both DBP-1 and DBP-2. In contrast to these similarities, the preference of both proteins for polynucleotides rich in guanine residues sets them apart from the lac repressor and most of the unwinding proteins which have much higher affinities for (A + T)-rich substrates.

The specificity of nucleic acid binding shown by both DBP-1 and DBP-2 lends support to the possibility of an actual in vivo function which utilizes this ability. The existence of a definite hierarchy of affinities apparently based on nucleotide sequence and secondary structure rather than purely electrostatic interactions argues in favor of such a function and against coincidence as the reason for this binding ability. The presence of these proteins in the serum in the concentrations measured, coupled with the fact that they have the ability to bind such an important macromolecule as DNA with some specificity, indicates that the possibility of such binding having in vivo significance should not be ignored.

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