

# Site-specific Interactions of JBP with Base and Sugar Moieties in Duplex J-DNA

EVIDENCE FOR BOTH MAJOR AND MINOR GROOVE CONTACTS\*

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**$\beta$ -D-Glucosyl-hydroxymethyluracil, also called base J, is an unusually modified DNA base conserved among Kinetoplastida. Base J is found predominantly in repetitive DNA and correlates with epigenetic silencing of telomeric variant surface glycoprotein genes. We have previously identified a J-binding protein (JBP) in *Trypanosoma*, *Leishmania*, and *Crithidia*, and we have shown that it is a structure-specific binding protein. Here we examine the molecular interactions that contribute to recognition of the glycosylated base in synthetic DNA substrates using modification interference, modification protection, DNA footprinting, and photocross-linking techniques. We find that the two primary requirements for J-DNA recognition include contacts at base J and a base immediately 5' of J (J-1). Methylation interference analysis indicates that the requirement of the base at position J-1 is due to a major groove contact independent of the sequence. DNA footprinting of the JBP-J-DNA complex with 1,10-phenanthroline-copper demonstrates that JBP contacts the minor groove at base J. Substitution of the thymine moiety of J with cytosine reduces the affinity for JBP ~15-fold. These data indicate that the sole sequence dependence for JBP binding may lie in the thymine moiety of base J and that recognition requires only two specific base contacts, base J and J-1, within both the major and minor groove of the J-DNA duplex.**

In the DNA of kinetoplastid flagellates, a fraction of thymine is replaced by the modified base  $\beta$ -D-glucosyl-hydroxymethyluracil (called base J)<sup>1</sup> (1–3). In all kinetoplastids, J is abundantly present in telomeric repeats (1). In the parasite *Trypanosoma brucei*, J is also found in the telomeric variant surface glycoprotein (VSG) gene expression sites involved in antigenic variation (4, 5). The presence of J in inactive telomeric VSG gene expression sites but not in the active site suggests that J may be involved in the transcriptional repres-

sion of VSG gene expression sites and thus antigenic variation (1, 4–8).

Our discovery of a J-binding protein (JBP) in kinetoplastids that specifically bind J-containing DNA indicates that proteins mediate J function (9). These proteins may then directly or indirectly lead to gene silencing and/or suppression of DNA recombination (6, 10), both of which are involved in the mechanism of antigenic variation. Understanding how JBP specifically recognizes and binds J-DNA may represent a first step in elucidating the function of J and its mechanism of action.

We have recently characterized the binding properties of recombinant JBP from *Crithidia* using synthetic J-DNA substrates that contained the glycosylated base in various sequences and contexts (11). These studies indicated that the JBP/J-DNA interaction is not just simple glucose recognition but rather requires the presentation of the glucose moiety within the major groove of a double-stranded DNA helix. The JBP/J-DNA interaction is not competed by free glucose or free base J, and JBP fails to recognize single-stranded J-DNA or a J-DNA/RNA duplex. Furthermore, the minimal J-DNA substrate for JBP recognition represents base J in the center of one helical turn of double-stranded DNA. These findings, as well as the relative sequence independence of JBP/J-DNA interaction in contrast to a 4-fold higher affinity of JBP for telomeric repetitive DNA, suggested that JBP may represent a structure-dependent binding protein.

To examine how JBP specifically recognizes and binds the unique modified base in DNA, we analyzed the site-specific interactions of JBP with base and sugar moieties in duplex J-DNA using modification interference-protection, DNA footprinting, and photocross-linking techniques. We report that JBP makes only two critical base contacts with J-DNA as follows: with base J itself and with the base immediately 5' (position J-1) on the same strand. The requirement for the base at position J-1 is due to a sequence independent contact in the major groove. Footprint analysis indicates that JBP also contacts the minor groove at base J, and additional analysis suggests that this contact may be sequence-specific. That JBP recognition includes both major and minor groove interactions may allow some indication of a potential J-DNA binding motif and help explain the effect of DNA structure on the affinity for JBP.

## EXPERIMENTAL PROCEDURES

**Enzymes and Chemicals**—T4 DNA polynucleotide kinase and T4 DNA ligase were purchased from Invitrogen. [ $\gamma$ -<sup>32</sup>P]ATP was from PerkinElmer Life Sciences. All other chemicals were obtained from Sigma.

**Preparation of Oligonucleotide Substrates**—J-containing DNA oligos were synthesized as described previously (11). Synthesis of 5-( $\beta$ -D-glucopyranosyloxymethyl)-2'-deoxycytidine-containing DNA oligo was

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<sup>1</sup> The abbreviations used are: base J,  $\beta$ -D-glucosyl-hydroxymethyluracil; JBP, J-binding protein; VSG, variant surface glycoprotein; DMS, dimethyl sulfate; oligo, oligonucleotide; Br, bromo; HTH, helix-turn-helix.

performed using a similar procedure as described for J (12). Standard non-modified oligonucleotides were purchased from Invitrogen. Bromodeoxyuridine and bromodeoxycytidine-modified oligos were purchased from Oligos Etc. Oligos were 5'-end-labeled with [ $\gamma$ - $^{32}$ P]ATP and purified by exclusion chromatography. Labeled oligos used in the modification-protection assays were gel-purified on a 20% sequencing gel. For double-stranded DNA substrates, the labeled oligo was annealed to its non-labeled complementary strand by heating in a 3-fold excess of the complementary strand for 5 min at 95 °C in 10 mM Tris-HCl, pH 7.9, 50 mM KCl, followed by slow cooling to room temperature. The oligos were verified by native gel analysis to be double-stranded. The DNA strand corresponding to the base J-containing strand will be referred to as the J-strand and the complementary strand as the A-strand.

**Purification of Recombinant JBP—***Crithidia fasciculata* JBP was expressed in *Escherichia coli* and purified by metal affinity chromatography as described previously (11).

**Electrophoretic Mobility Shift Assays—**The standard binding reaction (20  $\mu$ l) contained 35 mM Hepes-NaOH, pH 7.9, 1 mM EDTA, 1 mM dithiothreitol, 50 mM KCl, 5 mM MgCl<sub>2</sub>, 10  $\mu$ g of bovine serum albumin, 2  $\mu$ g of poly(dI-dC)-poly(dI-dC), 4  $\mu$ g of  $\alpha$ -casein and the indicated amounts of protein and radiolabeled DNA substrates. The reactions were incubated for 15 min at room temperature and analyzed on a 4.5% nondenaturing polyacrylamide gel (19:1) using 0.5 $\times$  TBE at 150 V for 60 min at room temperature. After drying, the gels were exposed to film and to a PhosphorImager screen for quantitation. Distamycin A (100 mM) was prepared in distilled water and stored at -20 °C. Chromomycin A<sub>3</sub> (100 mM) was prepared in dimethyl sulfoxide and stored at -20 °C.

**Determination of the Apparent Equilibrium Dissociation Constants of the JBP-J-DNA Complex—**The  $K_d = [\text{protein}][\text{DNA}]/[\text{protein-DNA}]$  was measured as the concentration of JBP at which half of the maximal target J-DNA was bound as described previously (11).

**Missing-base Interference Assay—**This method was adapted from Ordling *et al.* (13). End-labeled DNA was resuspended in 15  $\mu$ l of H<sub>2</sub>O in a microcentrifuge tube. Partial depurination was obtained by adding 1.5  $\mu$ l of piperidine formate, pH 2, and incubating at 25 °C for 15 min. Partial depyrimidation was obtained by adding 20  $\mu$ l of hydrazine and incubating for 25 min at 25 °C. Both reactions were stopped by adding 200  $\mu$ l of stop solution (0.3 M NaAc, 0.5 mM EDTA). After two ethanol precipitations, the pellet was washed with 95% ethanol, dried, and resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8). The reaction times and reagent concentrations here are approximate and were adjusted depending on the length or base composition of the oligonucleotide.

The modified oligo was then annealed to the complementary strand (present at 2–3-fold molar excess) and used as a substrate in the JBP binding reaction, as described above. The reaction conditions were such that ~80% of the input DNA was bound by the enzyme. Binding was allowed to proceed for 5 min at 25 °C, after which time the different samples were loaded onto the 6% native gel. After autoradiography of the wet gel, bound and free DNA were excised, eluted in TE buffer, phenol/chloroform extracted, ethanol-precipitated, and dried. Further purification was performed using the QIAquick Nucleotide Removal Kit (Qiagen), as directed by the manufacturer. The isolated modified DNA was then subjected to piperidine cleavage at 95 °C for 30 min (14) followed by drying and resuspension in 30  $\mu$ l of H<sub>2</sub>O. After another cycle of drying and resuspension, the samples were dried and dissolved in 15  $\mu$ l of formamide loading buffer and loaded on a 20% sequencing gel (14). The cleavage patterns were visualized by autoradiography and quantitated by phosphorimager. G + A and C + T Maxam-Gilbert sequencing ladders of the labeled DNA were loaded on the same gel for comparison (but not shown).

To quantitate the degree of interference at each base following modification, percent bound was determined by phosphorimager and normalized to the position where modification had the least effect on JBP binding. This resulted in a degree of interference for each band or base. JBP does not bind DNA that lacks base J (11). Therefore, by representing base J removal as the maximal level of inhibition (100%), the data are expressed as relative percent inhibition.

**Methylation Interference Assay—**The labeled DNA oligo was pre-methylated by treatment with 0.5% dimethyl sulfate for 5 min at 25 °C. Modification was halted by the addition of  $\beta$ -mercaptoethanol (14), and the DNA was recovered by ethanol precipitation as described above. The modified oligo was hybridized to the complementary strand and incubated with purified JBP protein as described above. Protein-bound and free DNA fractions were recovered after native gel electrophoresis, cleaved with piperidine, and analyzed by denaturing gel electrophoresis as described above for the missing-base interference assay.

TABLE I  
Summary of dissociation constants ( $K_d$ ) of JBP bound to J-DNA of various sequences and context

Substrate	K <sub>d</sub> (nM)
Tel-1J ACCCTAACCCJAAACCCTAAC TGGGATTGGGATTGGGATTG	40 ± 2.6 <sup>a</sup>
VSG-1J CAGAAGGCAGCJGCAACAAG GTCTTCCGTCGACGTTGTTC	140 ± 8.3 <sup>a</sup>
VSG-G CAGAAGGCAGGJGCAACAAG GTCTTCCGTCACGTTGTTC	120 ± 2.1 <sup>b</sup>
VSG-C TTCTTGTJGCAGCTGCCTTCTGTTTCTGCT AAGAACAACGTCGACGGAAGACAAAGACGA	143 ± 15 <sup>b</sup>
BSK-1J GGAAACAGCJACGACCATG CCTTTGTCGATGCTGGTAC	110 ± 2 <sup>b</sup>
Glc-T CATTACTACJGGAACCTCAG GTAATGATGACCTTGAGTC	131 ± 5 <sup>b</sup>
Glc-C CATTACTAXTGGAACTCAG GTAATGATGACCTTGAGTC	2180 ± 100 <sup>b</sup>

<sup>a</sup> See Ref. 10.

<sup>b</sup> This study.

<sup>c</sup> X refers to the modified base  $\beta$ -D-glucosyl-hydroxymethylcytosine.

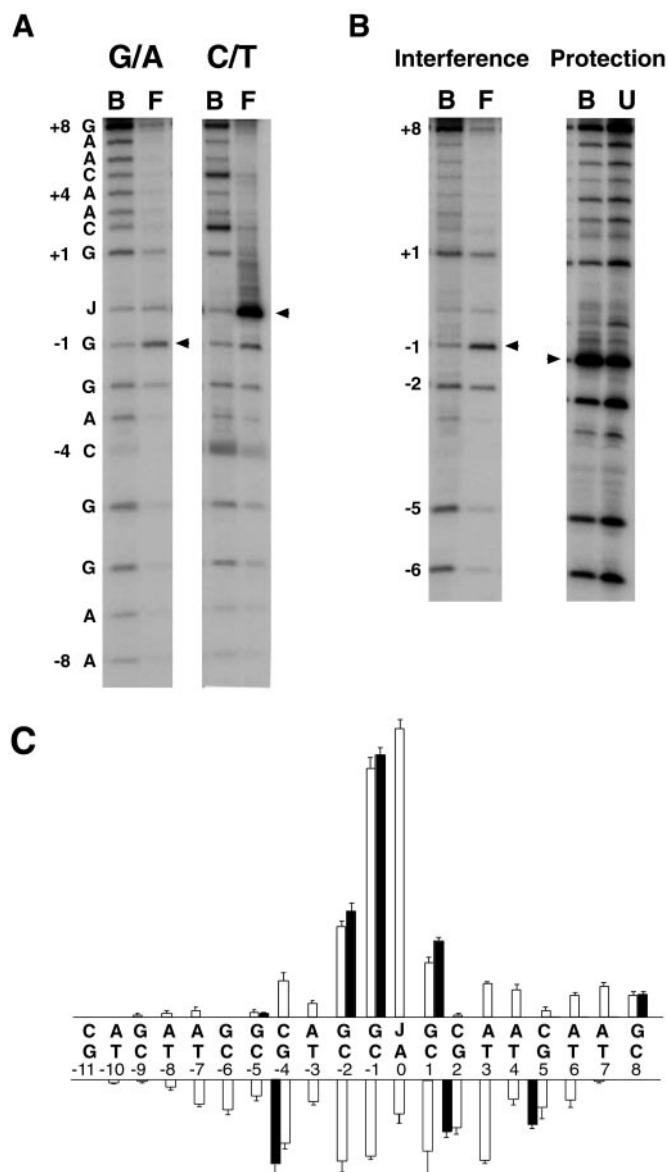
**Methylation Protection Assay—**Binding reactions were scaled up 5-fold and fractionated by native gel electrophoresis. The reactions were constituted such that about half of the input DNA was bound by the enzyme. A discrete JBP/[ $^{32}$ P]J-DNA complex was resolved from free labeled J-DNA and excised separately from the gel. The gel slices were then soaked in 300  $\mu$ l of TE containing 0.5% DMS for 10 min at 25 °C. The reaction was terminated by washing the gel slice 2 $\times$  with stop buffer (1.5 M sodium acetate, pH 7, 1 M  $\beta$ -mercaptoethanol) and eluting the DNA by soaking overnight at 37 °C in stop buffer. DNA was precipitated, cleaved with piperidine, and analyzed by denaturing gel electrophoresis as described above.

**1,10-Phenanthroline Protection Assay—**Binding reactions were scaled up and fractionated by polyacrylamide gel electrophoresis as described for the methylation protection assay. The free and bound DNAs were then digested *in situ* by the nuclease activity of the 2:1 1,10-phenanthroline-cuprous complex (15). Briefly, after electrophoresis, the entire gel was immersed into a clean Pyrex dish containing 200 ml of 10 mM Tris-HCl, pH 8.0. Next, 20 ml of a solution containing 2 mM 1,10-phenanthroline, 0.45 mM CuSO<sub>4</sub> (prepared just prior to use by mixing 1 ml of 40 mM 1,10-phenanthroline in 100% ethanol, 1 ml of 9 mM cupric sulfate in water, and 19 ml of H<sub>2</sub>O) was added to the solution. *In situ* digestion of the free and complexed J-DNAs was initiated by the addition of 20 ml of a freshly prepared 58 mM 3-mercaptopropionic acid solution (1:200 dilution of 3-mercaptopropionic acid in H<sub>2</sub>O) and was allowed to proceed for 20 min at 25 °C. The reaction was quenched by adding 20 ml of freshly prepared 28 mM 2,9-dimethyl-1,10-phenanthroline in 100% ethanol and allowing the resulting solution to stand for 2 min. The gel was then rinsed with distilled water, and free and bound J-DNAs were eluted from the gel as described above. The DNA was extracted with phenol/chloroform, precipitated with ethanol, and run on a 20% sequencing gel and analyzed as described above.

**UV Cross-linking Analysis—**The 20-mer bromo-substituted DNA substrate was prepared by 5'-end labeling of the Br-containing A-strand, followed by hybridization to the unlabeled J-strand. Cross-linking reactions (20  $\mu$ l) containing 0.1 pmol of DNA and 0.5 pmol of JBP in a standard reaction mixture were incubated for 5 min at 25 °C in a microtiter plate. The samples were then irradiated for the times indicated using a 312 nm transilluminator situated 2.2 cm above the samples (on ice). The samples were denatured in 1% SDS followed by 10% SDS-PAGE. The cross-linked adducts were visualized by autoradiography of the dried gel. The extent of cross-linking is expressed as percent of input DNA in the protein adduct representing an average of three independent experiments.

## RESULTS

**Missing-base Interference—**We have shown previously that JBP recognizes J only in duplex DNA and that the binding is relatively independent of the DNA sequence context (11) (Table I). To characterize further the sequence dependence of JBP binding, we tested the ability of JBP to recognize the thymine moiety of base J. A synthesized DNA substrate containing the



**FIG. 1. Chemical probing of the JBP-J-DNA complex.** **A**, missing-base interference. The J-strand of the VSG-G substrate was pre-modified and analyzed for effects on JBP binding as described under "Experimental Procedures." **B** and **F**, refer to the "bound" versus "free" DNA population, respectively. The sequence of the oligo and position of residues (*i.e.* +8 to -8) are indicated on the left. *Arrowheads* indicate residues that are over-represented in the free population and under-represented or absent in the bound population. Notice that base J is hypersensitive to hydrazine relative to T (34), explaining the relative increase in cleavage at this position. **B**, methylation interference-protection. Methylation-interference (*left panel*). The J-strand was pre-treated with DMS (see "Experimental Procedures") and analyzed for effects on JBP binding as in **A**. *Arrowhead* indicates the guanine at position J-1 where a methyl group in the major groove resulted in the greatest interference with JBP binding. Thus, the guanine at this position is over-represented in the free population as described in **A**. Methylation-protection (*right panel*); the effect of JBP binding on methylating the guanine residues of the J-strand in the major groove was determined as described in "Experimental Procedures." Notice that here **B** or **U** refers to methylation profiles of DNA that is either bound (**B**) or unbound (**U**) by JBP. The *arrowhead* indicates the increase in DMS modification at J-1 upon JBP binding. **C**, summary of modification-interference data for the VSG-G substrate. Interference data from **A** and **B**, and data not shown, were quantified by PhosphorImager analysis as described under "Experimental Procedures." The distance from the line indicates the degree of interference that modification of that particular residue has on the ability of the DNA substrate to bind JBP. The degree of interference is represented as percent inhibition relative to 100% inhibition due to the removal of base J. *Open boxes* and

glucose linked to cytosine binds JBP with ~17-fold lower affinity (Table I) than J-DNA in which the glucose was attached to the thymine base. Thus, JBP was able to distinguish between thymine and cytosine bases containing the glucose moiety. The difficulty in synthesizing modified DNA substrates containing glucosylated A or G has precluded further analysis of the thymine specificity of JBP binding. However, this result suggests JBP makes a specific contact with the thymine moiety of base J.

To identify precisely the bases in J-DNA that contribute to the binding of JBP, we used a missing-base footprint-interference analysis (13, 16). This method allows the importance of every base in the J-DNA substrate for JBP binding to be analyzed. In this approach the DNA is sparingly depurinated or deprimidinated and then reacted with JBP. The bound and unbound fractions of J-DNA were separated, cleaved with piperidine, and analyzed on a sequencing gel to determine the positions of base removal. Positions where removal of the base greatly reduced binding of the JBP protein were taken as bases directly involved in J-DNA recognition. When the VSG-G J-DNA substrate was used, the missing-base interference analysis revealed that two bases on the J-containing strand are important for JBP binding (Fig. 1A). The data for both the J- and A-strand (not shown) are summarized in Fig. 1C. As expected, the removal of base J (position 0) has the greatest effect on JBP binding, essentially shifting all the DNA to the unbound fraction (Fig. 1A). However, unexpectedly, the specific removal of the adjacent base at position -1 on the same J-strand (referred to as position J-1) resulted in a similar inhibition of JBP binding (Fig. 1, A and C). This effect is independent of sequence, because we see a similar result if the base at position J-1 is G, C, or T (Figs. 1 and 2 and data not shown). In other positions surrounding J and J-1 on the same J-strand as well as all positions of the A-strand, only a minor or less striking effect of base removal was seen. This minor effect extends to ~4–6 bp flanking base J, depending on the substrate analyzed (see below). This is similar to the minimal J-DNA substrate of 5 bp of duplex required flanking base J for JBP binding (11). Two potential exceptions to this minor effect are positions J -2 and J +1 (Fig. 1A). However, when these effects are quantitated (Fig. 1C), we see that removal of these bases result in ~30% inhibition or less (31 and 19% for J -2 and J +1, respectively) compared with the maximal interference due to J removal. Therefore, we include these as minor or less important effects (see "Discussion").

Disregarding the obvious effect at J-1, overall the substrates analyzed indicate a relatively symmetrical binding site surrounding base J (Figs. 1C and 2A). However, the interference pattern for the telomeric substrate may indicate an asymmetrically bound protein with minor interference effects extending preferentially along the 5'-side (minus positions) of base J (Fig. 2B). It may be significant that this apparent differential binding pattern reflects the differential binding affinity of JBP for this substrate (11) (Table I). However, further studies are needed to test this hypothesis.

**Methylation Interference and Protection**—Whereas the missing-base interference analysis allows the investigation of every base in J-DNA and its requirement for JBP binding, it does not distinguish between potential critical minor or major groove interactions. Treatment of DNA with dimethyl sulfate (DMS) methylates the N-7 of guanines in the major groove and the N-3 of adenosines in the minor groove (14, 17). We performed the

solid boxes represent results from base removal and methylation, respectively. *Error bars* representing the S.E. of the results in three independent experiments are shown.

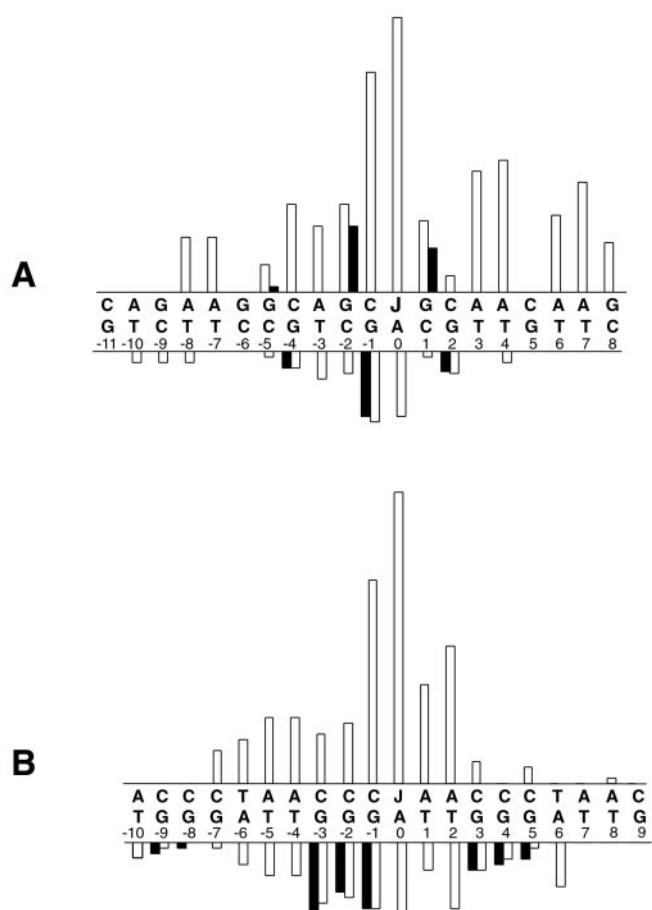


FIG. 2. **Modification-interference analysis of VSG-1J and Tel-1J.** Analysis of J-DNA substrates VSG-1J and Tel-1J was performed as in Fig. 1. A summary of the data is shown, using the same symbols as in Fig. 1C. The results presented are representative of three independent experiments.

reaction under conditions where only guanine methylation led to strand breakage. This procedure allowed us to address specific contacts in the major groove by testing whether pre-methylation of certain guanines affects JBP binding. By using the VSG-G substrate, we found that the presence of methylated guanine at position J-1 significantly interfered with JBP binding (Fig. 1, *B* and *C*). This suggests that the requirement for the base at position J-1 for JBP binding represents a specific contact in the major groove with JBP. However, once JBP is bound none of guanines on either strand, including the J-1 position, are protected from methylation (Fig. 1*B* and data not shown). On the contrary, we see hypermethylation at the J-1 position, indicative of a local conformational change upon JBP binding. This lack of methyl protection, especially at J-1, suggests that once JBP is bound, it does not tightly interact with DNA surrounding base J.

JBP binding requires the presence of the glucose moiety in the major groove of DNA (11, 19). Thus, it is not surprising that the methylation interference pattern indicates decreasing effects with increasing distance from base J along the DNA substrate (Figs. 1*B* and 2). This is consistent with the idea that JBP lies within the major groove of J-DNA. It is also apparent from the methylation interference pattern that this major groove occupancy is biased toward the minus positions in J-DNA (Figs. 1*B* and 2). This is also reflected in the missing-base interference data and is especially evident in the analysis of VSG-G and Tel-1J substrates (Figs. 1*B* and 2*B*). These results are consistent with the asymmetric orientation of JBP in

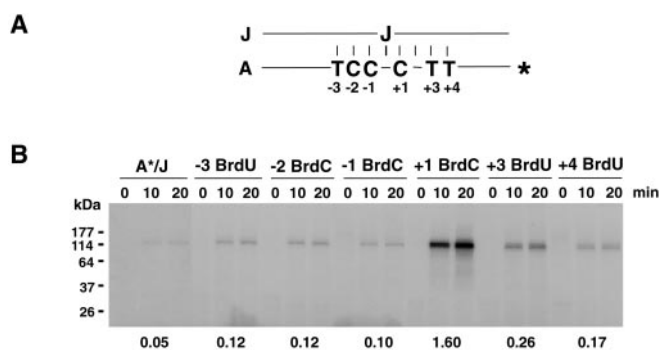


FIG. 3. **UV cross-linking of JBP to bromopyrimidine-substituted J-DNA.** The 20-mer bromo-substituted J-DNA substrate (VSG-G), singly substituted in the A-strand at positions indicated in A, was prepared by 5'-end-labeling the Br-containing strand, followed by hybridization to the unlabeled complementary J-strand oligo. Cross-linking reactions were performed for 0, 10, and 20 min as described under "Experimental Procedures," and samples were run on a 10% SDS-PAGE. An autoradiograph of the gel is shown in B. The singly bromo-substituted substrates are indicated above each of the set of reactions. A\*/J represents the non-brominated J-DNA control. The length of time for each cross-linking reaction is indicated above each lane. The extent of cross-linking for each DNA substrate after 20 min, as described under "Experimental Procedures," is indicated below each set of reactions. Protein size standards were run on the same gel and are indicated on the left.

the major groove, preferentially bound on the minus positions, or 5', of base J.

**Photocross-linking**—Specific contacts between JBP and purine bases in the major groove can be inferred from the DMS protection and interference studies described above. To probe pyrimidine-specific contacts and further elucidate the major groove occupancy by JBP, we have investigated the photocross-linking of JBP to duplex J-DNA molecules substituted at single positions with either 5-bromocytosine or 5-bromouracil. The bromine in the pyrimidine ring is situated within the major groove of the DNA helix. Upon excitation by UV light, a covalent adduct between the activated pyrimidine and protein can result if a substituent of the polypeptide is situated in close proximity. The substrates used for UV cross-linking were 20-mer J-DNA duplexes (VSG-G). Bromopyrimidines were introduced at six positions in the A-strand which was then 5'-end-labeled and hybridized to an unlabeled complementary J-oligo (Fig. 3A). Control experiments established that bromo substitution had no effect on the ability of JBP to bind J-DNA (data not shown).

Cross-linking of bromo-J-DNA to JBP was assayed by the formation of an SDS-stable complex of ~120 kDa detected by SDS-PAGE (Fig. 3B). For all substrates tested, the formation of a protein-DNA adduct was absolutely dependent on 312 nm UV irradiation, and the yield of photoproduct (especially in the case of the +1 BrdUrd substrate) increased with duration of UV exposure (Fig. 3B). Cross-linking of control J-DNA lacking the brominated nucleotide (A\*/J) was minimal (0.05% cross-linking). Obvious variability in the efficiency of cross-linking was observed according to the position of the bromo-substituted pyrimidine base. The highest level of cross-linking (1.6%) was obtained for the +1 bromodeoxycytidine J-DNA (Fig. 3B), suggesting that JBP was in intimate contact with the +1C base in the major groove of the J-DNA helix. Taken together with the missing-base interference data, the residue at positions +1 on the A-strand is in close proximity with JBP in the major groove but does not make an essential base contact. The remaining base substitutions on the minus positions of the J-DNA substrate resulted in little increase in cross-linking than the non-modified control. This lack of cross-linking on the minus posi-

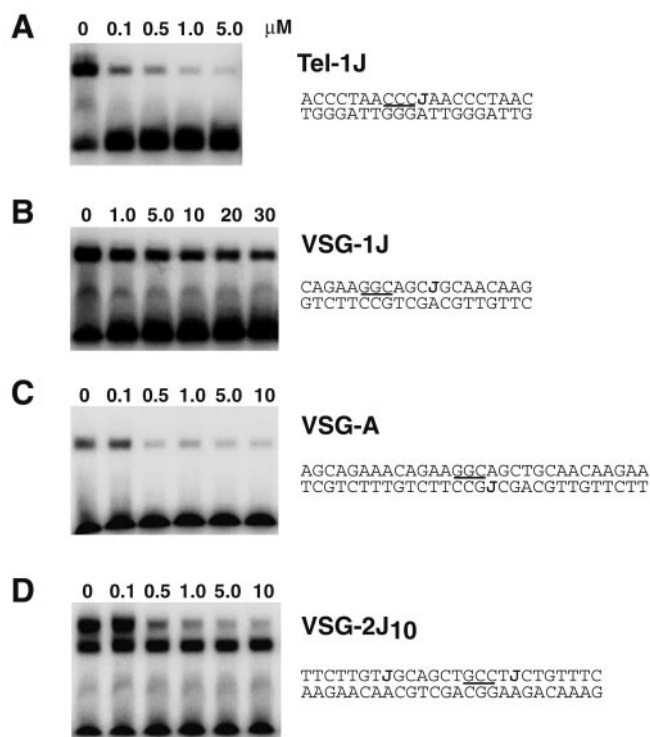


FIG. 4. **Chromomycin  $A_3$  inhibition of JBP binding.** Increasing amounts of chromomycin  $A_3$  were tested for their ability to inhibit JBP binding to various J-DNA substrates (A–D). JBP binding is illustrated by the gel shift assay as described previously (11). The concentration of chromomycin  $A_3$  ( $\mu\text{M}$ ) is indicated above each lane. The J-DNA substrate tested and its corresponding sequence are indicated on the right. Base J is in *bold*. The underlined GC-rich sequences represent putative chromomycin-binding sites.

tions includes the base in the  $-1$  position on the A-strand which is hydrogen-bonded to the essential J-1 base.

**Minor Groove Interactions**—Chromomycin  $A_3$  and distamycin A are reversible minor groove-binding drugs with sequence preference for GC- or AT-rich regions, respectively. This specificity in DNA interaction has proven useful for selective disruption of the DNA binding activity of minor groove-binding proteins (18–20). With the presence of several GC-rich regions present in our J-DNA substrates, we asked whether chromomycin  $A_3$  would specifically disrupt JBP binding.

As we demonstrated previously by using the gel shift assay, JBP binds with high affinity to short J-DNA substrates resulting in a shifted JBP·J-DNA complex (11). Here we used the gel shift assay to ask whether minor groove-binding drugs would inhibit JBP binding. Chromomycin  $A_3$  at 0.5–1.0  $\mu\text{M}$  nearly completely blocks JBP binding to the Tel-1J substrate (Fig. 4A). However, using another J-DNA substrate with a GC-rich element farther away from base J (VSG-1J), we see an inability of chromomycin  $A_3$  to inhibit effectively JBP binding even up to 30  $\mu\text{M}$  (Fig. 4B). When base J is moved to replace a thymine adjacent to this GC-rich element (*i.e.* VSG-A), we now see nearly 90% inhibition of JBP binding at 0.5  $\mu\text{M}$  chromomycin  $A_3$  (Fig. 4C). In contrast, distamycin A at concentrations up to 10  $\mu\text{M}$ , 5-fold greater than the concentration which completely blocks the interaction of TBP with the TATA element (19), had no effect on JBP binding to any of the J-DNA substrates tested (data not shown).

Inhibition of protein binding by minor groove binders did not directly indicate the importance of minor groove interactions. In fact, it has been demonstrated that minor groove binders at high concentrations can induce structural changes in DNA that are propagated over 100 bp and compete with binding of pro-

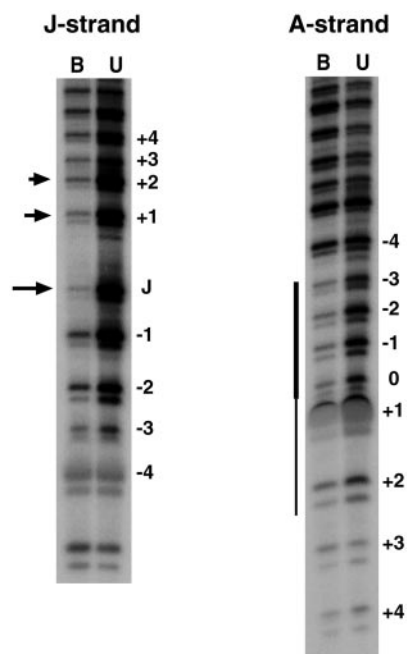
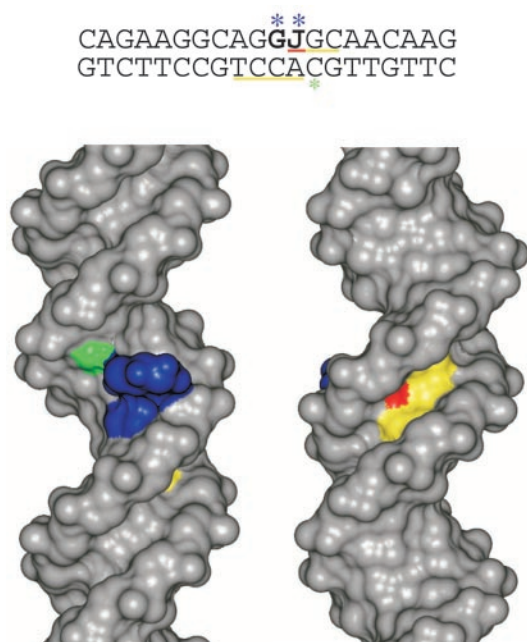


FIG. 5. **Minor groove DNA footprinting.** Pre-formed JBP·J-DNA complexes were treated with 1,10-phenanthroline-copper as described under “Experimental Procedures.” The resulting cleavage patterns were visualized by running the samples on a 20% sequencing gel. An autoradiograph of the gel corresponding to protection on the J-strand (*left panel*) and A-strand (*right panel*) of the J-DNA duplex is shown. B and U refer to fractions of J-DNA that were bound *versus* unbound, respectively, by JBP. Arrows indicate residues with reduced cleavage due to JBP binding in the minor groove at these positions on the J-strand. The length of the arrow indicates the relative degree of protection. The black line indicates that protection on the A-strand extends from +2 to  $-3$ . The thicker line indicates that the degree of protection is greatest at positions 0 to  $-3$ . Note that chemical cleavage by 1,10-phenanthroline-copper may yield two products, a 3'-phosphorylated and 3'-phosphoglycolated-terminated polynucleotide. As a result, the cleavage products may appear as a doublet in this analysis.

teins in the major groove (21–23). To test whether the chromomycin-induced inhibition of JBP binding can spread farther along DNA, we analyzed a J-DNA substrate containing two J molecules 10 bp apart (VSG-2J<sub>10</sub>). This substrate is able to bind to two molecules of JBP (11). By using this substrate we only see inhibition of the second molecule of JBP binding, presumably due to the single proximal GC-rich sequence, even at up to 30  $\mu\text{M}$  chromomycin (Fig. 4D and data not shown). Thus, this substrate is able to bind the drug but not propagate the inhibitory effects any further than  $\sim 7$ –9 bp along the DNA.

To address directly whether JBP makes specific contacts in the minor groove, we performed DNA footprinting analysis with 1,10-phenanthroline-copper.

1,10-Phenanthroline-copper is a chemical nuclease that selectively cleaves the phosphodiester bond in the floor of the minor groove through oxidative attack of the C-1' and to a lesser extent the C-4' hydrogen of deoxyribose in a sequence-independent manner (24, 25). Any protein that blocks access to the C-1' or C-4' hydrogen will prevent cleavage by this reagent. By using this footprint reagent, we find protection by JBP on the J-strand at J, J + 1, and to a lesser extent J + 2 and on the A-strand from +2 to  $-3$  positions (Fig. 5A). A summary of the potential contacts is presented in Fig. 6. Quantitation of the footprint indicates the protection to be significant for base J and positions 0 to  $-3$  on the opposing A-strand (Fig. 5A). We see no evidence of minor groove interaction at the base at J-1. Because only the removal of base J had a significant effect on JBP binding (Figs. 1 and 2), it follows that the only critical minor groove interaction is at base J. The ability of chromomy-



**FIG. 6. Model of JBP/J-DNA interactions.** A summary of the contacts identified in our studies as described in the text is indicated on the DNA sequence (*above*) and two different views of the full atom model of J-DNA substrate (*below*), based on the crystal structure of J-DNA (26). Linear VSG-G substrate sequence (*above*). In **bold** are the essential bases at position J-1 and the glucosylated base J. The **blue asterisks** indicate essential major groove contacts. The **green asterisk** indicates the residue in close proximity to JBP but not essential for JBP binding. Residues **underlined in yellow and red** indicate minor groove contacts. The potentially essential sequence-specific contact at base J is highlighted in **red**.

cin A<sub>3</sub> to inhibit JBP binding independent of its ability to interact on the minus or plus side of J (Fig. 4, *B* and *C*) further supports the single critical minor groove interaction at base J. Thus, the minor groove footprint of bases on the opposing A-strand may simply be the consequence of JBP accessing the minor groove for the essential base J contact. The apparent sensitivity of JBP binding to the nature of the glycosylated base (*i.e.* T *versus* C, Table I) also suggests that this critical minor groove interaction is base-specific.

#### DISCUSSION

The present findings show that JBP interacts with the major and minor grooves of the J-DNA helix at the modified base J and its immediate vicinity. Bases for which there is clear evidence for major groove contacts are highlighted in *blue* and *green* on the B-form J-DNA structure (26) in Fig. 6. These include the base in position J-1, which was shown to be essential for JBP binding by chemical modification with DMS and base removal, the +1C residue on the A-strand that was photocross-linked to JBP, and the glucose moiety of base J, which was shown to be essential for JBP binding (11). The essential contacts are shown in *blue*, and the non-essential contact in the major groove is indicated in *green*. Bases for which there is clear evidence for minor groove contact are highlighted in *yellow* and *red*. These include base J, J + 1, and J + 2 on the J-strand and positions 0 to -3 on the opposite A-strand. The potential essential sequence-specific contact at base J in the minor groove is indicated in *red*.

It must be noted that the model presented here may not include all potential base interactions with JBP. For example, according to the missing-base analysis, position J-2 may contact JBP to some degree upon complex formation. However, for the purposes of this analysis we conclude that missing-base

interference results of less than 40% inhibition as less striking and represent minor contacts that contribute little to overall binding (see Fig. 1C). Therefore, we have not included these potential contacts in our model. Furthermore, it should be noted that a negative cross-linking result does not indicate a lack of intimate contact between JBP and that base because the degree of cross-linking under the conditions used may be restricted by the amino acid residues of JBP contacting a base. Although the requirements for successful DNA-protein cross-linking (type of amino acid residues and their distances from DNA) are still not completely understood, it is known that halogenated pyrimidines cross-link preferentially to electron-rich amino acid residues (27, 28). The lack of the appropriate proximal residue may therefore limit the ability of a base to cross-link to JBP. Thus, we cannot interpret the lack of JBP cross-linking at other positions along the J-DNA substrate (including the base hydrogen bonded to the essential J-1 position; Fig. 5).

Previous results (11) indicated that JBP is a relatively sequence-independent and structure-specific binding protein. The results presented here are consistent with this conclusion. By using several independent DNA footprinting methods, we find that JBP does not make any sequence-specific contacts with the bases surrounding the modified base J. We find that the only critical contacts in J-DNA required for JBP binding are present on the J-containing strand and include major and minor groove interactions at base J and a major groove contact at the base at position J-1 (highlighted in *blue* and *red*, respectively, in Fig. 6). The contact at J-1 appears to be sequence-independent, whereas substitution of glucosylated hydroxymethyluracil with glucosylated hydroxymethylcytosine resulted in a 17-fold reduction in affinity for JBP, suggesting that the contact at base J is thymine base-specific. These results indicate that the sole sequence dependence of the JBP/J-DNA recognition may lie in the thymine moiety of base J itself. DMS interference studies suggest that the requirement for the J-1 base is due to contact with JBP in the major groove. However, once JBP is bound to J-DNA none of the guanines in the J-DNA duplex, including the one at J-1, are protected from methylation. Rather, there is an increase in methylation at the J-1 position, indicative of a local conformational change upon JBP binding. This hypermethylation corresponds to approximately a 2-fold increase in the degree of methylation at the J-1 position, relative to the adjacent guanine residues, following JBP binding. Although this increase is not large, it is significant if we take into account the essential nature of the base at this position and the effect of pre-methylation on JBP binding (see Fig. 1C). This lack of methyl protection, especially at J-1, suggests that JBP, once bound, does not interact tightly with DNA surrounding base J. Taken together, the data suggest that it is the base as well as the glucose moiety of base J that are the key identity elements of the JBP/J-DNA interaction.

Whereas JBP may not make many essential contacts or any sequence-specific contacts with the surrounding bases, the sequence context of J does affect the ability of JBP to recognize the J-DNA substrate. We have reported previously (11) that JBP has an ~4-fold higher affinity for base J in a telomeric repetitive sequence context. In addition, we found that JBP binding requires base J within at least one helical turn of the B-form double-stranded helix. Thus, these differences in binding may be due to structural differences in DNA that enhance the JBP/J-DNA interaction. It is possible that certain sequences (*i.e.* repetitive DNA elements) may allow an increased accessibility to both major and minor grooves as well as have an inherent increase in overall DNA flexibility, thus enhancing the conformational change in the DNA helix upon binding JBP.

Although this idea is consistent with the data, it is highly speculative at the present time.

JBP is a novel protein, and the JBPs of *T. brucei*, *C. fasciculata*, and *Leishmania tarentolae* show similarity throughout their sequence (9). Included is a region present in all three JBPs that is homologous to the Myb DNA-binding motif (9). This binding motif is a derivative of the helix-turn-helix (HTH)-type DNA-binding domains which, in its simplest form, is composed of a two to three turn helix (H2), a four residue turn (T), and a four turn "recognition" helix (H3). Upon binding DNA, the recognition helix is localized within the DNA major groove of its target site and responsible for most of the base-specific DNA contacts (29). While at the present time there is no direct evidence for an essential role of the Myb domain in binding of JBP to DNA, the requirement for the glucose moiety in the major groove of DNA and the chemical probing of the JBP-J-DNA complex presented here are consistent with a single HTH-like motif being involved in JBP/J-DNA interaction.

To ensure stable and proper recognition of the target DNA, several HTH-type proteins must make additional, and sometimes extensive, specific and nonspecific DNA contacts. In many cases residues in the primary sequence distal to the HTH are involved in additional contacts with DNA (29). For example, the HTH motifs of the eukaryotic homeodomains (30, 31) and the telomeric binding proteins (32, 33) make base contacts in both grooves of DNA with the recognition helix of the HTH in the major groove and the N-terminal arm in the adjacent minor groove. The predicted contacts between JBP and both grooves of J-DNA that we have reported here are remarkably similar. Therefore, sequence comparison and biochemical analysis of the JBP/J-DNA interaction suggests that JBPs contain a DNA-binding fold very similar to that of the Myb/homeodomain/telomere-binding type. To examine this in further detail, efforts are underway to crystallize the JBP-J-DNA complex.

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**Site-specific Interactions of JBP with Base and Sugar Moieties in Duplex J-DNA:  
EVIDENCE FOR BOTH MAJOR AND MINOR GROOVE CONTACTS**

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