THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 2, Issue of January 11, pp. 958–966, 2002
Printed in U.S.A.

Recognition of Base J in Duplex DNA by J-binding Protein*

Received for publication, September 18, 2001, and in revised form, October 24, 2001
Published, JBC Papers in Press, November 7, 2001, DOI 10.1074/jbc.M109000200

Robert Sabatini‡§¶, Nico Meeuwenoord‖, Jacques H. van Boom‖, and Piet Borst‡

From the ‡Division of Molecular Biology and Centre for Biomedical Genetics, The Netherlands Cancer Institute, 1066 CX Amsterdam, The Netherlands, the ‖Leiden Institute of Chemistry, Gorlaeus Laboratories, 2300 RA Leiden, The Netherlands, and the §Division of Geographic Medicine, University of Alabama at Birmingham, Birmingham, Alabama 35294

β-D-Glucosylhydroxymethyluracil, also called base J, is an unusual 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 found a J-binding protein (JBP) in *Trypanosoma*, *Leishmania*, and *Crithidia*. We have now characterized the binding properties of recombinant JBP from *Crithidia* using synthetic J-DNA substrates that contain the glycosylated base in various DNA sequences. We find that JBP recognizes base J only when presented in double-stranded DNA but not in single-stranded DNA or in an RNA:DNA duplex. It also fails to interact with free glucose or free base J. JBP is unable to recognize nonmodified DNA or intermediates of J synthesis, suggesting that JBP is not directly involved in J biosynthesis. JBP binds J-DNA with high affinity (K_d = 40–140 nM) but requires at least 5 bp flanking the glycosylated base for optimal binding. The nature of the flanking sequence affects binding because J in a telomeric sequence binds JBP with higher affinity than J in another sequence known to contain J in trypanosome DNA. We conclude that JBP is a structure-specific DNA-binding protein. The significance of these results in relation to the biological role and mechanism of action of J modification in kinetoplastids is discussed.

In the DNA of kinetoplastid flagellates, a fraction of thymine is replaced by the modified base β-D-glucosylhydroxymethyluracil, called J (1–3). In all kinetoplastids, J is abundantly present in telomeric repeats (3). In the parasite *Trypanosoma brucei*, J is also found in the telomeric variant surface glycoprotein (VSG)[1] 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 repression of VSG gene expression sites and thus antigenic variation (3–9).

It has been suggested that J is involved in long term transcriptional repression and that J could also suppress unwanted recombination between repetitive sequences in the genome (3, 8, 9). Whether this is true and whether J is the cause or the consequence of this silencing remain to be determined. However, consistent with both ideas, the protrusion of the sugar group of the major groove of DNA, at specific locations, could allow the recognition and binding of proteins that would mediate J function. These proteins could lead to gene silencing and/or suppression of DNA recombination, both of which are involved in the mechanism of antigenic variation. Our recent discovery of J-binding proteins (JBPs) in kinetoplastids that specifically bind J-containing DNA is compatible with this idea (10). By understanding how JBP specifically recognizes and binds the unique modified base in DNA could represent a first step in elucidating the function and mechanism of J action.

In this report, we use recombinant JBP to study the interaction between JBP and J-DNA. We utilize DNA duplexes with base J at defined positions to determine the kinetics and specificity of the interaction between J-DNA and JBP. We show that JBP recognizes with very high specificity the glucosylated base only when present in double-stranded DNA. The inability for free base J to compete with JBP-binding J-DNA, the requirement for one helical turn of double-stranded DNA for optimal binding, and the higher affinity of JBP for repetitive DNA suggest that DNA structure is an essential component of the recognition of base J by JBP. Thus, there is more to the JBP/J-DNA interaction than just glucose recognition. Our findings suggest it is the glucosylated base in DNA, rather than DNA or base J itself, that is recognized by JBP. We conclude that JBP is a structure-specific DNA-binding protein.

EXPERIMENTAL PROCEDURES

Enzymes and Chemicals—T4 DNA polynucleotide kinase and T4 DNA ligase were purchased from Invitrogen. [γ-^{32}P]ATP was from PerkinElmer Life Sciences. All other chemicals were obtained from Sigma.

Preparation of Oligonucleotide Substrates—The DNA substrates used in this study are listed in Table I. J-containing DNA oligos were synthesized using standard oligonucleotide synthesis protocols using the fully protected J derivative 5-(2,3,4,6-tetra-*O*-benzyl-β-D-glucopyranosyl)oxymethyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyuridinyl-3′-*O*-(2-cyanoethyl-(*N*,*N*-diisopropyl)-phosphoramidite (11). The modified oligos were purified by strong anion exchange chromatography (Mono Q, Amersham Biosciences), followed by desalting with Sephadex G-25 (Amersham Biosciences). Standard nonmodified oligonucleotides were purchased from Invitrogen. Oligos were end-labeled with [γ-^{32}P]ATP and purified by exclusion chromatography. For double-stranded DNA substrates, the labeled oligo was annealed to its nonlabeled 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. Double-stranded oligo competitors were prepared in a similar manner except that both strands were nonlabeled.

Preparation of Multiple J Substrates—The VSG-2J$_{19}$ substrate (Table I) was generated by ligation of two molecules of the VSG-1J J-oligo using the corresponding 40-mer complementary "splint" oligo. For ligation 20 pM of the J-oligo kinased with [γ−^{32}P]ATP was hybridized to 10

* This work was supported in part by The Netherlands Foundation for Chemical Research and The Netherlands Organization for Scientific Research (to P. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed. Tel.: 205-934-1971; Fax: 205-975-2547; E-mail: sabatini@uab.edu.

[1] The abbreviations used are: VSG, variant surface glycoprotein; base J, β-D-glucosylhydroxylmethyluracil; JBP, J-binding protein; amino-T, aminomethylthymine; oligo, oligonucleotide; dsDNA, double-stranded DNA; ss, single-stranded; nt, nucleotide.

TABLE I
Duplex oligonucleotides used in this study

Sequence	Oligo[a]
5′-CAGAAGGCAGC**J**GCAACAAG-3′ GTCTTCCGTCGACGTTGTTC	VSG-1J
5′-ACCCTAACCC**J**AACCCTAAC-3′ TGGGATTGGGATTGGGATTG	Tel-1J
5′-ACCC**J**AACCC**J**AACCC**J**AACCC**J**A-3′ TGGGATTGGGATTGGGATTGGGAT	Tel-4J
5′-ACCCTAACCCTAACCCTAAC-3′ TGGGATTGGGATTGGGATTG	Tel-T
OH OH OH OH 5′-ACCCTAACCCTAACCCTAACCCTA-3′ TGGGATTGGGATTGGGATTGGGAT	Tel-T-OH
5′-ACCCTAACCC**J**AACCCTAAC-3′	ssTel-1J
5′-CAGAAGGCAGC**J**GCAACAAG-3′ GTCTTCCG**J**CGACGTTGTTC	VSG-isoJ
5′-TTCTTGTTGCAGC**J**GCCTTCTGTTTCTGCT-3′ AAGAACAACGTCGACGGAAGACAAAGACGA	VSG-A
5′-TTCTTGT**J**GCAGCTGCCTTCTGTTTCTGCT-3′ AAGAACAACGTCGACGGAAGACAAAGACGA	VSG-B
5′-TTCTTGT**J**GCAGC**J**GCCTTCTGTTTCTGCTGC-3′ AAGAACAACGTCGACGGAAGACAAAGACGACG	VSG-2J_5
5′-TTCTTGT**J**GCAGCTGCCT**J**CTGTTTCTGCTGC-3′ AAGAACAACGTCGACGGAAGACAAAGACGACG	VSG-2J_{10}
5′-CAGAAGGCAGC**J**GCAACAAGCAGAAGGCAGC**J**GCAACAAG-3′ GTCTTCCGTCGACGTTGTTCGTCTTCCGTCGACGTTGTTC	VSG-2J_{19}

[a] Substrate Tel-T-OH is also known as Tel-V; see Ref. 10.

pmol of unlabeled complementary oligo, and the resulting nick between the two molecules of VSG-1J was ligated with T4 DNA ligase. The ligation product was gel-purified on an 8% polyacrylamide, 8 M urea gel, DNA recovered by ethanol precipitation, and hybridized to unlabeled complementary oligo (40 pmol). VSG-2J_5 and VSG-2J_{15} were chemically synthesized as described above.

Purification of Recombinant JBP—Crithidia fasciculata JBP was expressed in *Escherichia coli* and purified by metal affinity chromatography as described previously (10). However, to increase the purity of the affinity-purified JBP fraction, the His_6 tag was increased to His_{10} by PCR. The affinity-purified JBP was concentrated to 2 ml in a Centricon-100 apparatus and loaded onto a Sephadex S-200 (Amersham Biosciences 16/60) column equilibrated with buffer A (50 mM Hepes, pH 7.0, 100 mM NaCl, 1 mM dithiothreitol). The fractions containing JBP were pooled and concentrated to 200 μl by Centricon-100 (10–20 mg/ml final concentration). JBP purified in this manner was more than 95% pure as judged by its appearance on the Coomassie-stained SDS-PAGE. In this procedure, we are able to obtain ~2 mg of JBP from 5 liters of bacterial culture.

Electrophoretic Mobility Shift Assays—The standard binding reaction mixture (20 μl) contained 35 mM Hepes-NaOH, pH 7.9, 1 mM EDTA, 1 mM dithiothreitol, 50 mM KCl, 5 mM MgCl, 10 μg of bovine serum albumin, 2 μg of poly(dI-dC)-poly(dI-dC), 4 μg of α-casein, and 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× TBE at 150 V for 60–90 min at room temperature. After drying, the gels were exposed to film and to a PhosphorImager screen for quantitation of the bands.

Determination of the Apparent Equilibrium Dissociation Constants of the Complex of JBP with J-DNA—Under conditions in which [DNA] < K_d, the concentration of the protein that results in 50% maximal formation of a protein-DNA complex is approximately equal to K_d. The K_d = [protein][DNA]/[protein-DNA] was measured as the concentration of JBP at which half of the maximal target J-DNA is bound. To keep the J-DNA concentration below K_d, 10 fmol of labeled oligonucleotide was used in a total reaction volume of 20 μl with varying amounts of JBP. The data from three titration gels were averaged to obtain the reported K_d values.

Determination of the On/Off Rates of JBP—JBP was incubated with the VSG-1J duplex (Table I) and immediately loaded onto a native gel to examine the on rate for complex formation. To determine the off rate, a complex was formed between the labeled VSG-1J substrate and JBP. Following the 10-min incubation, 300-fold molar excess of unlabeled VSG-1J substrate was added, and the reaction was immediately loaded onto the native gel.

Competition Assays—For competition assays 40 fmol of ^{32}P-labeled DNA substrates were used together with 10–500-fold excess of unlabeled substrate. The protein (25 nM) was added to the mixture last. Identical results were obtained if the labeled substrate was added last, after the preincubation of cold competitor and JBP.

RESULTS

JBP Recognition of Base J in dsDNA—We have developed previously a rapid isolation procedure for His-tagged recombinant JBP produced in *E. coli* (10). We have further improved this procedure by replacing the His_6 tag by a His_{10} tag. The recombinant JBP obtained was ~95% pure, as judged by its appearance in Coomassie-stained gels, and thus was suitable for investigating the specific interaction of JBP and J-modified DNA (J-DNA). Table I lists the modified substrates used in this study, which vary in the amount and sequence context of base J as well as in the overall size and sequence of the oligos. The specificity of the interaction of JBP and J-DNA was determined by competition and direct binding assays. Fig. 1 shows the inability of JBP to bind to unmodified DNA. We see no competition of the shifted complex using unlabeled unmodified substrate (identical sequence with thymine instead of J) at concentrations sufficient for J-DNA to result in 90% inhibition (Fig. 1*B*). There is also no competition with up to 500-fold excess of nonmodified DNA. This was further analyzed by a direct binding assay using labeled unmodified DNA. Unmodified DNA does not result in a shifted complex with JBP (Fig. 1*C*). Thus, our highly purified recombinant JBP has similar binding specificity as native JBP assayed in kinetoplastid nuclear extracts (10).

Indirect evidence indicates that J is made in two steps. A thymine in DNA is converted to 5-(hydroxymethyl)uracil, which in turn is glycosylated to form J (Fig. 1*A*; Ref. 8). To determine whether JBP has some affinity for the putative intermediate in J synthesis, we tested whether DNA containing 5-(hydroxymethyl)uracil could inhibit JBP binding. Substrate Tel-T-OH, which is the same as Tel-1J but has 4 of the

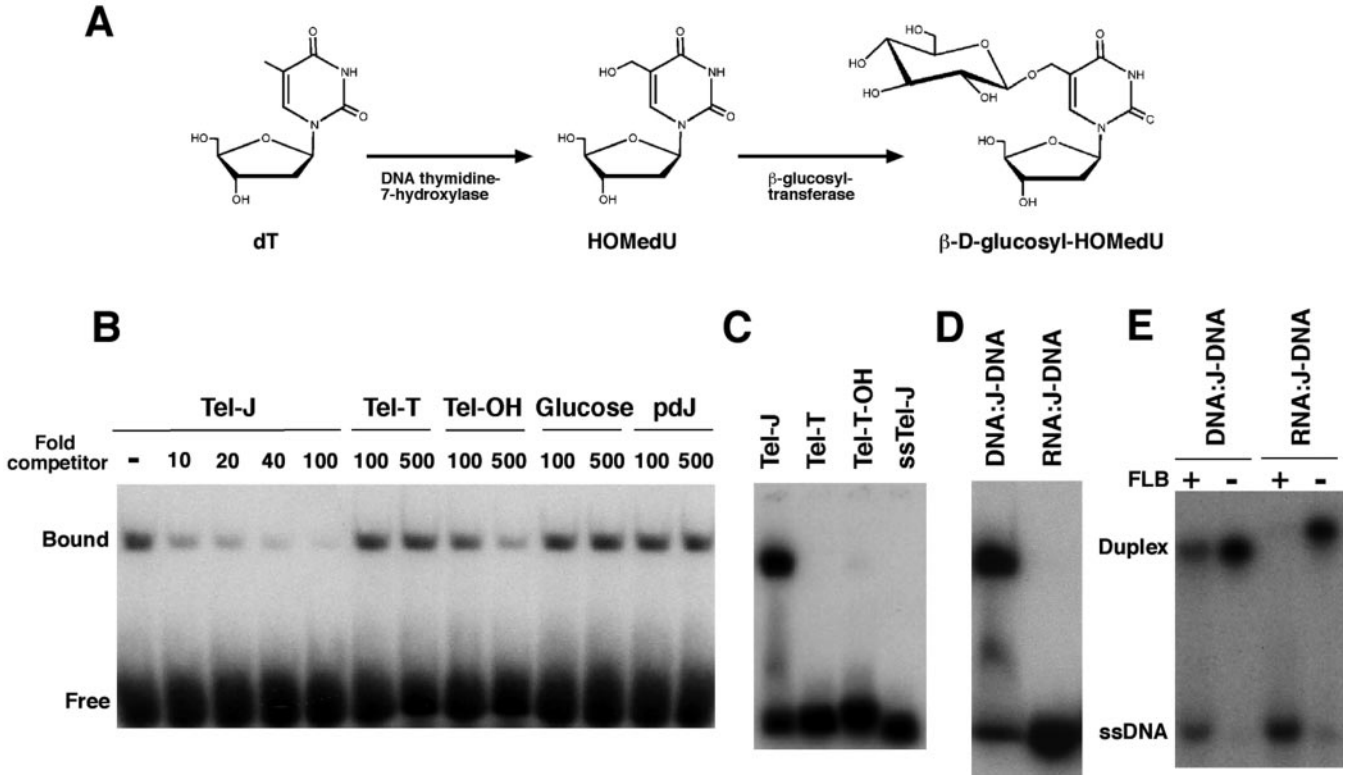

FIG. 1. **Specificity of JBP binding.** *A*, putative biosynthetic pathway for J. First, a thymine (*dT*) residue in a certain context in DNA is converted into 5-(hydroxymethyl)uracil by a DNA thymine-7-hydroxylase. Second, 5-(hydroxymethyl)uracil in DNA is converted into β-D-glucosyl-5-(hydroxymethyl)uracil (*dJ*) by a β-glucosyltransferase, which is not sequence-specific. *B*, competition assays. Gel shift assays consisted of radiolabeled J-DNA incubated with JBP in the absence or presence of the indicated fold excess of unlabeled competitor. The reactions were performed and analyzed as described under "Experimental Procedures." An autoradiograph of the gel is shown. The position of the JBP/J-DNA complex (*Bound*) and free J-DNA substrate (*Free*) is indicated on the *left*. *pdJ* represents free base J. *C* and *D*, direct binding assay. *C*, gel shift binding assays were performed as above using 10 pmol of JBP and 15 fmol of the radiolabeled substrate indicated *above* the lanes. *D*, binding assays were performed using radiolabeled J-DNA (VSG-1J sequence) annealed to a complementary DNA (*DNA:J-DNA*) or RNA (*RNA:J-DNA*) strand. *E*, the duplex nature of the substrates in *D* were analyzed by electrophoreses in a 17% native gel in the absence of JBP. Regions of the gel corresponding to the duplexed J-DNA (*Duplex*) *versus* the single-stranded J-DNA oligo (*ssDNA*) are indicated on the *left*. *+FLB* indicates the sample was boiled in the presence of formamide loading buffer prior to loading on the gel. See Table I for a description of all substrates used above.

thymines replaced with 5-(hydroxymethyl)uracil, was unable to compete significantly for JBP binding (Fig. 1*B*). 500-Fold excess of Tel-T-OH failed to complete to similar levels as 10-fold Tel-1J. Furthermore, if we test the Tel-T-OH substrate in the direct binding assay, we see what appears to be a very small amount of shifted complex (Fig. 1*C*). By PhosphorImager analysis, this apparent shift is ~100-fold less than the complex formed with J-containing DNA (Tel-1J). We also tested the free glycosylated nucleotide (pdJ), glucose, and activated nucleotide sugars potentially involved in J synthesis, UDP-Glc, and GDP-Glc. None of these were found to compete with J-oligos for JBP binding up to 500-fold molar excess (Fig. 1*B* and data not shown). Glucose and pdJ fail to compete even up to 1,000-fold molar excess (data not shown).

Next, we tested single-stranded J-oligonucleotides to examine the requirement of base J in duplex DNA for JBP binding. As shown in Fig. 1*C*, there was no detectable binding of the ssTel-J substrate. Fig. 1*D* shows that JBP does not bind to an RNA:J-DNA hybrid, whereas the same J-DNA strand annealed to a complementary DNA strand readily formed a complex with JBP. JBP binding to the RNA:J-DNA duplex could not be induced by inclusion of magnesium or mangenese over a concentration range of 0.5–10 mM (data not shown). Identical results were obtained with the VSG-1J (Fig. 1*D*) or Tel-1J substrates (not shown). The failure of the RNA:J-DNA substrate to bind JBP is not because of the failure of the radiolabeled J-DNA strand to hybridize to the RNA complement, as shown in Fig. 1*E*. The mobility of the hybridized substrates is lower than that of substrates subjected to boiling in formamide. Taken together, these results demonstrate that JBP specifically recognizes J only in the context of dsDNA. The failure of JBP to recognize the RNA:J-DNA duplex may reflect the sensitivity of JBP to the global conformation of the helix (*e.g.* A-form *versus* B-form).

Binding of JBP to Various DNA Sequences Containing J—To determine the relative binding affinity of JBP for J-DNA, we used the gel shift assay to investigate the binding of JBP to J-DNA duplexes (VSG-1J and Tel-1J) that have a single centrally located J modification. The dissociation constants (K_d) were determined by titrating under conditions of relatively low concentrations of J-DNA duplex ([J-DNA] $\ll K_d$). The DNA substrates were incubated with increasing amounts of JBP protein, and the complex was analyzed on native gels. The results of the gel shift assay in Fig. 2, *A* and *C*, show that the amount of free J-DNA decreases with increasing concentrations of JBP. The binding curve (Fig. 2, *B* and *D*) is hyperbolic, indicative of a true equilibrium process. We calculate the dissociation constants for JBP with the VSG and telomeric substrates of 160 and 43 nM, respectively (Table II). Thus, JBP has a 3.5-fold higher affinity for the telomeric than for the VSG substrate.

It is clear from these binding studies that JBP is not able to bind 100% of the DNA substrate. The inability to shift all input DNA into complex is due to the impurity of the J-oligo following chemical synthesis of J-DNA. During the de-blocking step of synthesis a small fraction of each oligo preparation undergoes

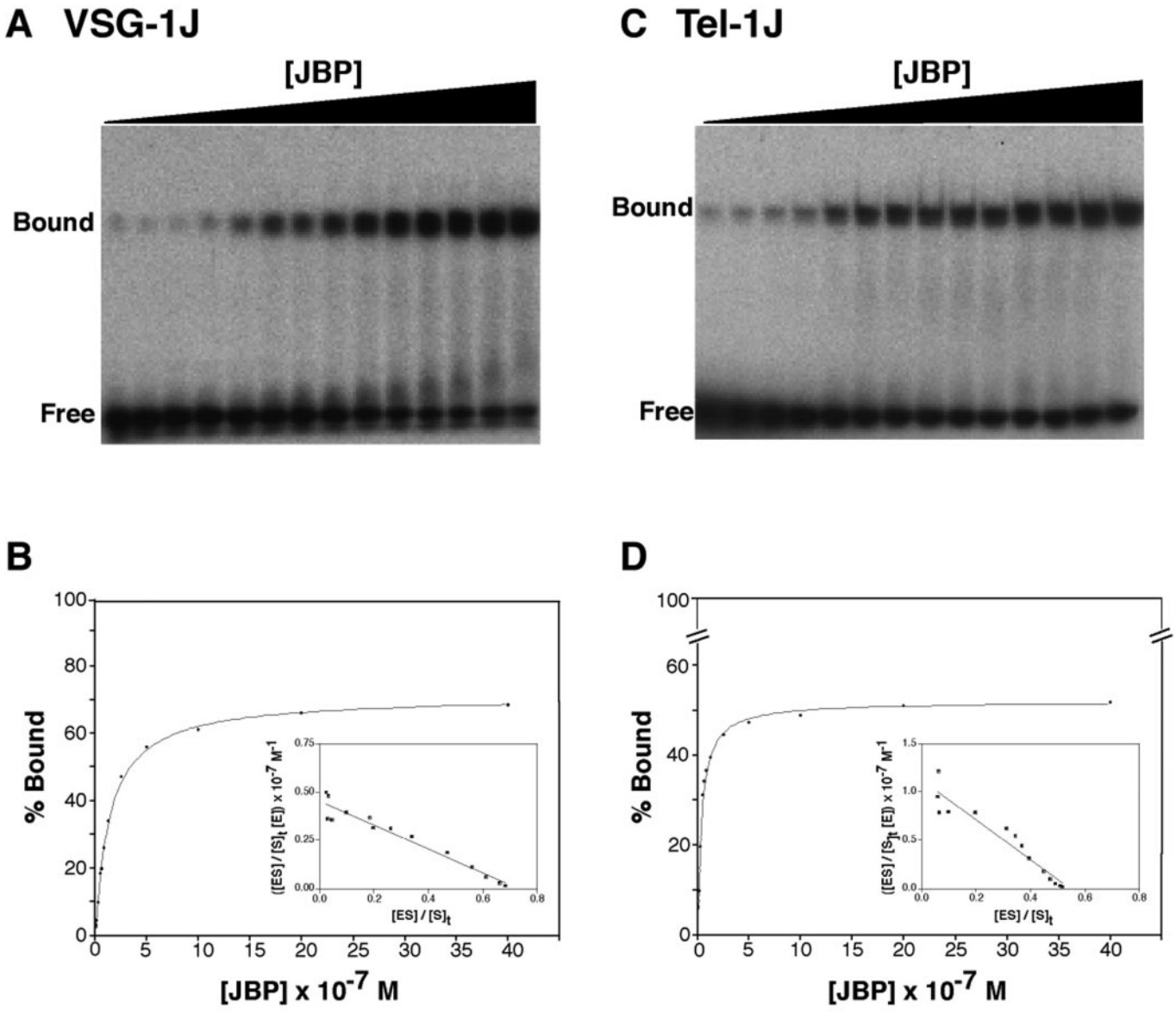

FIG. 2. **K_d analysis of JBP binding to single J-containing substrates.** *A* and *C*, gel shift assays for the VSG-1J (*A*) and Tel-1J (*C*) substrates interacting with JBP. 0.5 nM radiolabeled J-DNA was incubated with 0.005, 0.0065, 0.0083, 0.0125, 0.025, 0.05, 0.0625, 0.0835, 0.125, 0.25, 0.5, 1, 2, and 4 μM JBP. *B* and *D*, determination of K_d from titration data. Plot of JBP concentration *versus* percent complex formation from the titration data. The *inset* is a Scatchard plot.

TABLE II
Summary of K_d of JBP bound to J-DNA of various sequences and context

Oligo	Length	K_d
	nt	*nM*
Tel-1J	20	40 ± 2.6
Tel-4J	24	37 ± 1.5
VSG-1J	20	140 ± 8.3
VSG-A	30	176 ± 20
VSG-B	30	166 ± 26
VSG-isoJ	20	120 ± 4
VSG-$2J_5$	32	130 ± 6
VSG-$2J_{10}$	32	120 ± 2.5
VSG-$2J_{19}$	40	110 ± 8

a nucleophilic attack by ammonia resulting in the replacement of the glucose moiety in base J by an amino group. This fraction of oligo preparation is unable to bind JBP. Small variations in the fraction of aminomethylthymidine in the J-oligos can explain the variability in maximal percent binding that depends on the oligo tested. However, the ~4-fold difference in affinity reported here is not due to the quality of each of the J-DNA substrates because the lower affinity data are from the substrate that resulted in the higher maximal percent binding (~70% maximal binding by the VSG-1J substrate compared with ~50% for the Tel-1J). In addition, the Tel-4J substrate, containing 4 J molecules, has a higher percentage of substrate bound by JBP (over 80%) but still results in a similar K_d as the single J-modified telomeric sequence (Fig. 4*A* and Table III).

In addition to equilibrium binding, we attempted to use the gel shift assay to determine the on/off rates of JBP interaction with J-DNA. The dissociation rate constant was examined by adding excess unlabeled substrate to a pre-equilibrated JBP-labeled substrate complex and measuring the decay of the preexisting complex as a function of time. However, we see maximal competition at the shortest time point possible (*i.e.* loading a running gel immediately following competitor addition). Similarly, we see maximal complex formation following the shortest reaction incubation period possible by the gel shift assay (data not shown). Therefore, the short time scales required for the association and dissociation do not allow us to

TABLE III
J-DNA truncation substrates and percent relative binding to JBP

Substrate	Sequence	Duplex	Binding
			%
13-16	TTCTTGTTGCAGC**J**GCCTTCTGTTTCTGCT AAGAACAACGTCGACGGAAGACAAAGACGA	+	100
13-7	TTCTTGTTGCAGC**J**GCCTTCTGTTTCTGCT AAGAACAACGTCGACGGAAGA	+	126 ± 10
13-6	TTCTTGTTGCAGC**J**GCCTTCTGTTTCTGCT AAGAACAACGTCGACGGAAG	+	122 ± 10
13-5	TTCTTGTTGCAGC**J**GCCTTCTGTTTCTGCT AAGAACAACGTCGACGGAA	+	81 ± 11
13-4	TTCTTGTTGCAGC**J**GCCTTCTGTTTCTGCT AAGAACAACGTCGACGGA	+	39 ± 8
13-3	TTCTTGTTGCAGC**J**GCCTTCTGTTTCTGCT AAGAACAACGTCGACGG	+	9 ± 5
13-2	TTCTTGTTGCAGC**J**GCCTTCTGTTTCTGCT AAGAACAACGTCGACG	+	27 ± 9
13-1	TTCTTGTTGCAGC**J**GCCTTCTGTTTCTGCT AAGAACAACGTCGAC	+	7 ± 3
13-0	TTCTTGTTGCAGC**J**GCCTTCTGTTTCTGCT AAGAACAACGTCGA	+	5 ± 3
13	TTCTTGTTGCAGC**J**GCCTTCTGTTTCTGCT AAGAACAACGTCG	−	4 ± 3
7-16	TTCTTGTTGCAGC**J**GCCTTCTGTTTCTGCT AACGTCGACGGAAGACAAAGACGA	+	100 ± 3
6-16	TTCTTGTTGCAGC**J**GCCTTCTGTTTCTGCT ACGTCGACGGAAGACAAAGACGA	+	100 ± 6
5-16	TTCTTGTTGCAGC**J**GCCTTCTGTTTCTGCT CGTCGACGGAAGACAAAGACGA	+	79 ± 10
4-16	TTCTTGTTGCAGC**J**GCCTTCTGTTTCTGCT GTCGACGGAAGACAAAGACGA	+	36 ± 8
3-16	TTCTTGTTGCAGC**J**GCCTTCTGTTTCTGCT TCGACGGAAGACAAAGACGA	+	47 ± 8
2-16	TTCTTGTTGCAGC**J**GCCTTCTGTTTCTGCT CGACGGAAGACAAAGACGA	+	38 ± 12
1-16	TTCTTGTTGCAGC**J**GCCTTCTGTTTCTGCT GACGGAAGACAAAGACGA	+	20 ± 3
0-16	TTCTTGTTGCAGC**J**GCCTTCTGTTTCTGCT ACGGAAGACAAAGACGA	+	4 ± 1
16	TTCTTGTTGCAGC**J**GCCTTCTGTTTCTGCT CGGAAGACAAAGACGA	+	5 ± 2
77	TTCTTGTTGCAGC**J**GCCTTCTGTTTCTGCT AACGTCGACGGAAGA	+	126 ± 11
66	TTCTTGTTGCAGC**J**GCCTTCTGTTTCTGCT ACGTCGACGGAAG	+	117 ± 13
55	TTCTTGTTGCAGC**J**GCCTTCTGTTTCTGCT CGTCGACGGAA	−	67 ± 10

obtain reliable values for these reaction constants using this method. It is interesting that even with this high off rate we are able to detect complex formation by gel shift analysis. This is believed to be due to a caging effect of the polyacrylamide gel matrix that stabilizes complexes that are formed prior to gel entry (12).

To examine the effects of multiple Js on JBP binding, we tested the telomeric substrate containing four telomeric repeats with all four of the thymines converted to base J (Tel-4J, Table I). Our K_d estimation indicates that JBP has similar binding affinities for the telomeric substrate independent of the amount of J modification (Table II and Fig. 4*B*). These results suggest that JBP contains a single binding site for J and that a single modified base in DNA is sufficient for maximal high affinity binding. Furthermore, JBP seems to have a preference for J in the context of telomeric *versus* a relatively nonspecific sequence. However, we find no effect on the affinity of JBP for J within several different sequence contexts other than the telomeric sequence (Tables I and II). Thus, JBP recognizes J-DNA in a relative sequence-independent manner with higher affinity for the telomeric sequence potentially due to its altered secondary structure.

Determination of the Minimal Substrate for JBP Recognition—To determine the minimal substrate for JBP recognition, we used a 30-mer J-oligo hybridized to complementary oligos containing various truncations from each end (Table III). As J in ssDNA does not detectably bind JBP (Fig. 1*C*), the truncations allowed us to quickly scan the extent of duplex DNA required at regions flanking base J without synthesizing different sized J-oligos. This also avoided the technical problems we have encountered in synthesizing the J-oligos. The use of a single J-oligo allowed us to quantitate the changes in binding due to the extent of DNA duplex rather than those due to potential varying J levels in each J-oligo preparation. If we express the percent binding relative to the control 30-mer duplex containing the full complementary duplex regions of 13 and 16 nt flanking 5′ and 3′ of base J, respectively (*i.e.* substrate 13–16 or VSG-A; Tables I and III), the largest decrease in complex formation occurs with a truncation from 5 to 4 nt of duplex on each side of base J (Fig. 3*A*; Table III). A low level of

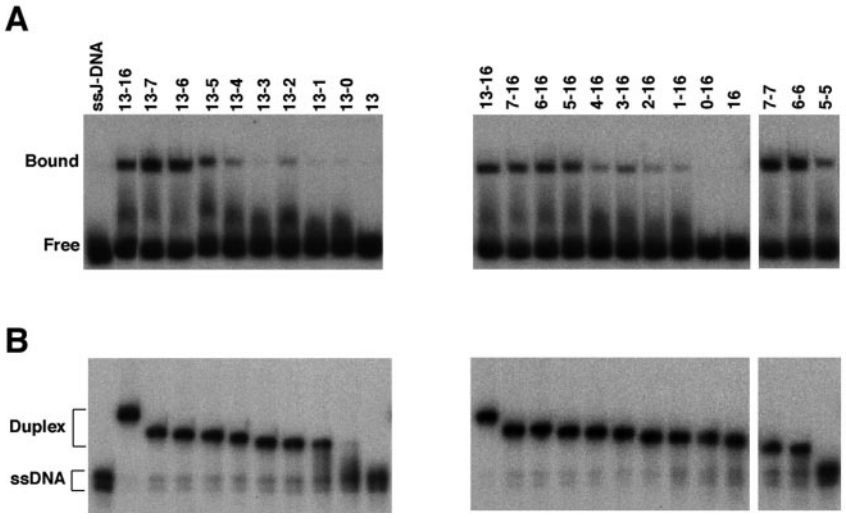

FIG. 3. **Determination of minimal substrate requirements for JBP binding.** *A*, gel shift assays contained 15 pM JBP and 15 fmol of the radiolabeled substrate indicated *above* the lanes. The J-DNA substrates were either in single-stranded (*ssJ-DNA*) or annealed to a complementary DNA strand of varying lengths (see Table II). The JBP·J-DNA complex (*Bound*) *versus* free DNA (*Free*) is indicated on the *left*. *B*, examination of the duplex nature of the DNA substrates used in *A*. The substrates, in the absence of JBP, were electrophoresed in a 17% native gel. An autoradiograph of the gel is shown. *Brackets* indicating regions of the gel corresponding to DNA duplex (*Duplex*) *versus* the single-stranded J-DNA oligo (*ssDNA*) are indicated on the *left*. Quantitation of the results are shown in Table II.

binding (20–30%) is still present with only 1–4 bp of duplex flanking base J. This is especially evident with truncations on the 5′ side of base J (substrates 4–16 to 1–16). This low level of binding requires at least 1 bp of duplex flanking J because we see no shifted complex formed with the 0–16 and 16 substrates. In contrast, the 3′ side of J requires at least 2 bp of duplex for this low level of binding.

We see an apparent stimulation in complex formation following the truncation from 16 to 7 nt of duplex on the 3′ side of J but not on the 5′ side of J from 13 to 5 nt of duplex. This stimulation is also found with the double truncated substrates 7-7 and 6-6 (Fig. 3*A*). No stimulation was observed, however, when we truncated oligo VSG-B, which is identical in sequence to VSG-A, but has base J at position 8 instead of 14 (see Table I). By using the VSG-B substrate, we have been able to confirm the minimal requirement for 5 bp of duplex on the 3′ side of J (data not shown). Because this substrate lacks the stimulatory effect described above, we therefore think that this unusual effect is only associated with oligo VSG-A and without significance for understanding the minimal duplex requirements for the binding of JBP to J-DNA.

These truncation effects on JBP binding could not be attributed to a failure of the radiolabeled J-strand to hybridize to its truncated complement. Analysis of the annealed strand by native polyacrylamide gel electrophoresis revealed that the mobility of the hybridized truncated substrates had been altered relative to that of J-DNA that had not been subjected to annealing (*ssJ-DNA*, Fig. 3*B*). We interpret this mobility shift as indicative of effective hybridization. The 13-0 and 13 substrate failed to form stable duplexes, which may explain their lack of JBP binding. However, 13-1 was duplexed but still relatively inactive for JBP binding (Fig. 3, *A* and *B*). It is interesting that the 5-5 substrate does not migrate as a duplex in the absence of JBP but nevertheless results in greater than 60% binding compared with the full-length duplex. Apparently the 5-5 substrate can form a duplex in solution in the presence of Mg^{2+} ions during the binding reaction, but this duplex dissociates during gel electrophoresis in low salt buffer. This suggests that JBP may stabilize J-DNA duplexes during the electrophoresis once the JBP·J-DNA complex is formed. Taken together, these results indicate that there must be at least 5 nucleotides of duplex flanking base J for maximal stable association of JBP. These 11 nucleotides represent approximately one helix turn of B-form DNA.

It is evident from Fig. 3*A* that at high concentrations of JBP (15 pmol) only 40% of this J-modified substrate (13–16 or VSG-A) is driven into a JBP·J-DNA complex that remains stable during gel electrophoresis. Again, this relatively low percent binding is due to the impurity of the J-oligo following chemical synthesis of J-DNA. In fact, the presence of two populations of J-modified DNA oligo can be visualized on the high percentage acrylamide gel in Fig. 3*B* (indicated as the *double bands* in the ssDNA region of the gel). We have demonstrated that only the upper band binds JBP (data not shown). Because this species only represents ~60% of the total J-DNA in the binding reaction, the maximal amount of complex formed would be 60%. Thus, 40% maximal binding in these reactions represents ~70% conversion of the "active" J-DNA into stable complex with JBP. The lower band of ssJ-DNA is thought to contain the chemically modified version of base J (as described above), thus making this substrate unreactive toward JBP.

The apparent smear of radioactivity in the gel shift represents dissociation of bound complex during electrophoresis indicating the relative instability of complex formation with this substrate. For purposes of this analysis, we only quantitate the stable complex indicated as *Bound* in Fig. 3*A*. It is interesting that the dissociation occurs earlier during electrophoresis for substrates containing minimal regions of duplex flanking base J, consistent with the quantitation results of product representing stable complex formation (Table III). This would explain the decrease in size of the smear as we assay from substrate 13-5 to substrate 13 and 5-16 to 16 (Fig. 3*A*). None of these problems with this particular J-modified substrate detracts from the qualitative nature of this assay, indicating optimal requirements of the duplex nature of J-DNA for JBP interaction.

Binding of JBP to DNA Substrates Containing Multiple Js—During the titration experiment, using the Tel-4J substrate, we see an additional slower migrating species in the native gel in reactions containing higher concentrations of JBP (Fig. 4*A*), presumably due to the binding of multiple JBPs to one oligo. Examination of the results in Fig. 4*A* and the corresponding binding curve in Fig. 4*B* gives no indication of cooperative binding. In addition, as mentioned above, there is no enhanced affinity of JBP for this substrate over the single J-containing DNA. Because the minimal substrate for JBP binding appears to require 5 nt flanking J (Fig. 3), we may not expect optimal binding of multiple JBPs to the Tel-4J substrate. This substrate contains 4 J molecules in an oligo of 24 nt in length. Thus, the separation between each modified base is maximally 5 nt. In addition, the Js located near the ends of the oligo have only 4 or 1 nt on the 5′ or 3′ side of the base, respectively (Table I). Thus, the optimal binding site for JBP would represent the two modified bases centrally located within the oligo. Whereas

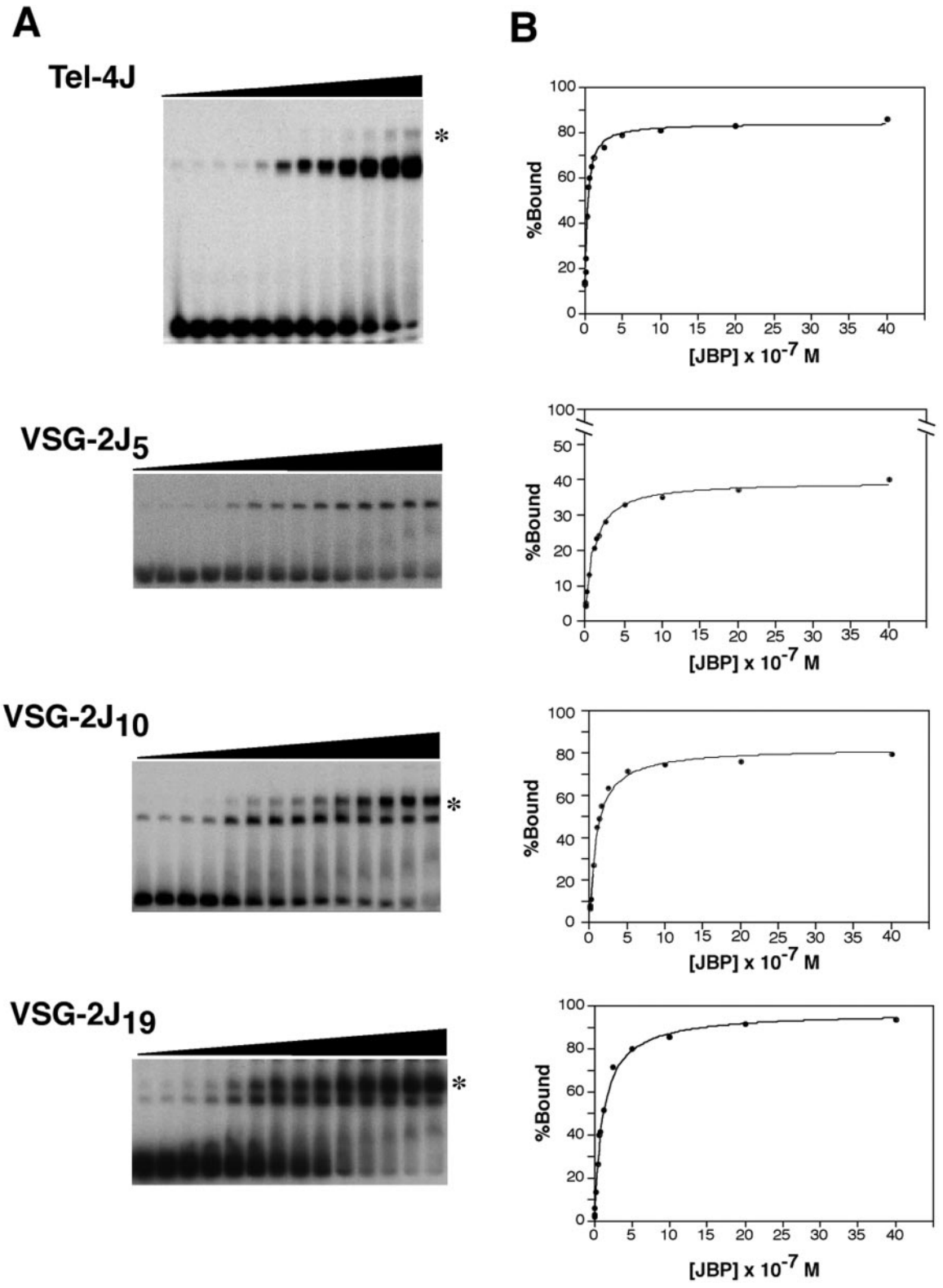

FIG. 4. **JBP binding analysis of DNA containing multiple J modifications.** *A*, gel shift titration analysis of JBP binding to the Tel-4J, VSG-$2J_5$, VSG-$2J_{10}$ and VSG-$2J_{19}$ substrates, as described in Fig. 3. The *asterisk* refers to the complex representing multiple JBP binding. *B*, plot of the titration data.

5 nt may be sufficient for JBP recognition, the physical size of JBP may not allow optimal binding of two molecules of JBP at these positions in the same substrate. This would explain the low amount of the large shifted complex in these reactions (Fig. 4*A*). In a similar experiment, a substrate with two J modifications separated by 3 nt on opposing strands (VSG-isoJ), thus placing J on opposite sides of the helix, is also unable to bind more than one molecule of JBP (data not shown) and has similar binding affinity as the 1 J substrate (Table III).

To examine more closely the effects of the distance of multiple Js in substrate oligos on JBP binding, we tested substrates containing two molecules of J separated by various distances.

By using a VSG substrate with two J molecules separated by 19 bp (VSG-2J_{19}), we see the formation of two shifted complexes thought to represent multiple JBP binding (Fig. 4*A*). The larger complex, with potentially two molecules of JBP bound, is indicated with an *asterisk*. Although it is clear that during the titration analysis greater than 90% the VSG-2J_{19} substrate is bound by JBP, we are unable to convert all the substrate to this larger double JBP-bound complex. It is interesting that the ratio of the smaller complex, containing a single molecule of JBP, and the larger double JBP complex, is similar to the ratio of bound and free DNA in the gel shift analysis using the single J-modified VSG-1J substrate (Fig. 2*A*). This is to be expected because the VSG-2J_{19} substrate was constructed by ligating two molecules of the VSG-1J substrate. In fact, because the VSG-1J results in a maximal binding of 70%, potentially due to the 30% that lacks the glucose moiety of base J, we would expect the ligated substrate, based on simple probability, to result in ~91% maximal binding. In Fig. 4*A* we see that over 90% of the substrate is bound by at least one molecule of JBP. Thus, the effects of the quality of the J-oligos on JBP binding is reflected in results using the substrate following ligation. However, analysis of the gel shift and the corresponding binding curve, quantitating both the single and double JBP binding events, gives no indication of any cooperative effects of multiple JBP binding (Fig. 4, *A* and *B*). Determination of the K_d with this multiple J-containing substrate indicates a similar JBP binding affinity as substrates containing a single J modification (Table III).

It is possible that the separation of 19 bp between the modified bases is too great a distance to allow interaction between the bound JBPs. However, separation of 10 bp (VSG-2J_{10}) also fails to allow interaction between two bound JBPs (Fig. 4, *A* and *B*), whereas a substrate containing the modified base 5 bp apart (VSG-2J_5) gave no apparent shifted complex representing multiple JBP binding events (Fig. 4*A*). Thus, a separation of 5–10 bp between adjacent J molecules is required for the binding of two molecules of JBP. With sufficient separation to allow multiple JBP binding, the modified bases represent independent binding sites with no interaction between the bound JBP molecules.

DISCUSSION

We have analyzed how JBP recognizes its target in a background of unmodified DNA. We find that the minimal size of J-DNA for strong complex formation with JBP is 11 bp in length with 5 nt flanking base J. This is consistent with our modification/interference analysis indicating that nucleotides up to 5 nt on each side of base J are important for JBP binding.[2] Comparison of the affinity of JBP for Tel-1J and Tel-4J indicates a lack of cooperative effects on JBP binding to J-DNA. This suggests that for protein-DNA interactions studies using other physical techniques, such as NMR and x-ray crystallography, a J-DNA substrate containing a single J modification within a length of at least 11 nts is necessary and sufficient to form stable JBP·J-DNA complexes.

The minimal substrate for JBP recognition was determined using a substrate, oligo VSG-A, contaminated with an oligo containing aminomethylthymidine (amino-T) instead of J. Because oligo VSG-A contains only a single J (or amino-T), we are confident that the amino-T-containing fraction of VSG-A, which does not bind JBP, had no effect on the binding assay or on our interpretation of the requirements of the duplex nature of J-DNA for optimal JBP interaction.

The quality of the J-oligos is also reflected in the failure to bind 100% of the input DNA substrate during the JBP titration analysis (Figs. 2 and 4). Although each oligo differs in the percentage maximally bound by JBP, this difference in quality does not affect our interpretation of binding affinities. For example, 65% of the VSG-1J and 50% of the Tel-1J substrate are maximally bound, but the Tel substrate nevertheless binds with a 3.5-fold higher affinity. In addition, the Tel-4J oligo (over 80% binding) and Tel-1J (50% binding) yield a similar K_d value (Table II).

The variable quality of J-oligos is due to a chemical modification of the glycosylated base following DNA synthesis.[3] The glucose moiety of base J is synthesized with benzoyl groups at each of the hydroxyl positions (12). To remove these groups, the resulting oligo is de-protected by ammonolysis (50 °C, 16 h), and this results in the formation of a side product to varying degrees in each synthesis reaction. This aminomethylthymidine is formed by the nucleophilic displacement of the glucose with ammonia, and it is inactive for JBP binding (data not shown). This explains why the percent maximal binding increases with increasing J content of the oligo (*i.e.* Tel-1J *versus* Tel-4J; Figs. 2 and 4). The formation of this side product to varying degrees in each synthesis reaction explains the varying percent maximal binding, but as discussed above, we think that the contaminant has not affected the interpretation of the results presented here.

The gel shift assay shows that JBP specifically recognizes J-modified DNA only when J is presented in the context of dsDNA (Fig. 1). JBP does not bind ssJ-DNA, an RNA:J-DNA duplex, or free base J. The inability to compete the specific binding of JBP and J-DNA with high concentrations of glucose underlines that the JBP/J-DNA interaction is not just simple glucose recognition. The inability of high concentrations of free base J to compete for JBP binding, the minimal requirement of one helical turn of dsDNA, and the apparent sensitivity of JBP binding to the nature of the DNA duplex all indicate that there is more to the interaction of JBP with J-DNA than just base J recognition. We conclude that the JBP recognizes the glycosylated base in DNA and that DNA structure is an essential component of the recognition.

The crystal structure of J-DNA has indicated that glucose is present in the major groove of B-form DNA (13). The inability of JBP to recognize effectively the RNA:J-DNA duplex may reflect its sensitivity to the global conformation of the helix or the presence of a 2′-OH group on the individual sugars interfering with specific protein-nucleic acid backbone interactions. However, we have yet to detect specific contacts of JBP with the DNA backbone or phosphate interactions along the length of the J-DNA duplex.[4] Thus, we think that the inability to bind the RNA:J-DNA duplex is due to its altered structure (not that of B-form DNA).

The increase in affinity for telomeric sequences may also reflect the sensitivity of JBP to the DNA structure (Fig. 2 and Table II). During the purification of native JBP from nuclear extract, we were unable to elute JBP from the J-DNA affinity column when the column contained the Tel-4J substrate (10). With a VSG-1J affinity column, we were able to elute the JBP with 300 mM KCl. The data presented here show that this difference in apparent affinity was not due to the presence of multiple J molecules but to the sequence of the telomeric substrate. This property of JBP binding may result from the repetitive nature of the telomeric sequence and the unique structures inherent in repetitive DNA, because the several different contexts of J tested here all have similar 3–4-fold lower affin-

[2] R. Sabatini, N. Meeuwenoord, J. H. van Boom, and P. Borst, manuscript in preparation.

[3] R. Sabatini, N. Meeuwenoord, J. H. van Boom, and P. Borst, unpublished results.

[4] R. Sabatini, unpublished results.

ities for JBP than the telomeric sequence context (Tables I and II). This remains to be tested with other simple repeats.

Structure-specific DNA repair proteins have dissociation constants in the nanomolar range (15–19), in contrast to sequence-specific enzymes, such as restriction endonucleases, which have dissociation constants in the picomolar range (20). The relatively lower affinity of the structure-specific binding proteins for DNA is thought to be due to the requirement of these proteins to recognize a single damaged base independent of its sequence context, to catalyze the repair reaction, and to dissociate (15–19, 21). In contrast, the sequence-specific binding proteins, such as transcription factors or restriction endonucleases, need to recognize a specific sequence, and they require more specific contacts and consequently have a higher binding affinity than structure-specific proteins. The binding affinity of JBP for J-modified DNA reported here (40–140 nM) puts JBP in the class of structure-specific binding proteins, in agreement with its ability to recognize a single modified base independent of sequence context and its apparent sensitivity to the DNA helix structure.

The structure-specific nature of JBP binding may provide some indication of a relationship between JBP and the presence of J in the genome. *In vivo*, ~50% of the total J is in telomeres with the remaining J found in imperfect simple sequence repeats as follows: the minichromosomal 177-bp repeats, the long array of 50-bp repeats upstream of the expression site promoter, and the 70-bp repeat arrays directly upstream of telomeric VSG genes (4, 5, 22). Here we show that JBP binding is sensitive to the structure of DNA and has 3.5-fold higher affinity for telomeric repetitive elements than for other J-containing sequences. Based on its selective binding, JBP may influence the presence of J at repetitive sequences in the genome. This may be a consequence of the direct involvement of JBP in J synthesis, stabilization of the J-modified DNA by JBP binding, propagation of J modification within repetitive DNA by JBP binding, or JBP binding may contribute to the stable inheritance of the modified base. The data presented here suggest that JBP is not an enzyme directly involved in J-synthesis, and there is no known "J-ase" that removes J (22).[5] Hence, our current hypothesis is that JBP is involved in the selective propagation and/or inheritance of J within repetitive DNA domains potentially by recruitment of the enzyme(s) involved in J -synthesis (*i.e.* DNA thymine hydroxylase). The involvement of JBP in the propagation or stable inheritance of base J along DNA would link the affinity of JBP for J-DNA and the level of J modification, thus potentially explaining the higher density of J at telomere repeats and other repetitive arrays in the *T. brucei* genome.

[5] M. Cross, R. Kieft, R. Sabatini, A. Dirks-Mulder, I. Chaves, and P. Borst, manuscript in preparation.

Acknowledgments—We thank Jayleen Grams, Rudo Kieft, Rainer Mussmann, and Sebastian Ulbert for critical reading of this manuscript. We also thank Steve Hajduk and members of the Borst and Hajduk laboratories for helpful advice and support.

REFERENCES

1. Gommers-Ampt, J. H., Van Leeuwen, F., de Beer, A. L., Vliegenthart, J. F., Dizdaroglu, M., Kowalak, J. A., Crain, P. F. & Borst, P. (1993) *Cell* **75,** 1129–1136
2. Borst, P. & van Leeuwen, F. (1997) *Mol. Biochem. Parasitol.* **90,** 1–8
3. van Leeuwen, F., Taylor, M. C., Mondragon, A., Moreau, H., Gibson, W., Kieft, R. & Borst, P. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 2366–2371
4. van Leeuwen, F., Wijsman, E. R., Kuyl-Yeheskiely, E., van der Marel, G. A., van Boom, J. H. & Borst, P. (1996) *Nucleic Acids Res.* **24,** 2476–2482
5. van Leeuwen, F., Wijsman, E. R., Kieft, R., van der Marel, G. A., van Boom, J. H. & Borst, P. (1997) *Genes Dev.* **11,** 3232–3241
6. Bernards, A., van Harten-Loosbroek, N. & Borst, P. (1984) *Nucleic Acids Res.* **12,** 4153–4170
7. Pays, E., Delauw, M. F., Laurent, M. & Steinert, M. (1984) *Nucleic Acids Res.* **12,** 5235–5247
8. van Leeuwen, F., Kieft, R., Cross, M. & Borst, P. (1998) *Mol. Cell. Biol.* **18,** 5643–5651
9. Gommers-Ampt, J., Lutgerink, J. & Borst, P. (1991) *Nucleic Acids Res.* **19,** 1745–1751
10. Cross, M., Kieft, R., Sabatini, R., Wilm, M., de Kort, M., van der Marel, G., van Boom, J., van Leeuwen, F. & Borst, P. (1999) *EMBO J.* **18,** 6573–6581
11. Wijsman, E. R., van den Berg, O., Kuyl-Yeheskiely, E., van der Marel, G. A. & van Boom, J. H. (1994) *Rec. Trav. Chim. Pays-Bas* **113,** 337–338
12. Cann, J. R. (1989) *J. Biol. Chem.* **264,** 17032–17042
13. Gao, Y.-G., Robinson, H., Wijsman, E. R., van der Marel, G. A., van Boom, J. H. & Wang, A. H.-J. (1997) *J. Am. Chem. Soc.* **119,** 1496–1497
14. van Leeuwen, F., Dirks-Mulder, A., Dirks, R. W., Borst, P. & Gibson, W. (1998) *Mol. Biochem. Parasitol.* **94,** 127–130
15. Castaing, B., Boiteux, S. & Zelwer, C. (1992) *Nucleic Acids Res.* **20,** 389–394
16. O'Handley, S., Scholes, C. P. & Cunningham, R. P. (1995) *Biochemistry* **34,** 2528–2536
17. Hitomi, K., Kim, S. T., Iwai, S., Harima, N., Otoshi, E., Ikenaga, M. & Todo, T. (1997) *J. Biol. Chem.* **272,** 32591–32598
18. Wilson, D. M., III, Takeshita, M. & Demple, B. (1997) *Nucleic Acids Res.* **25,** 933–939
19. Miao, F., Bouziane, M. & O'Connor, T. R. (1998) *Nucleic Acids Res.* **26,** 4034–4041
20. Terry, B. J., Jack, W. E., Rubin, R. A. & Modrich, P. (1983) *J. Biol. Chem.* **258,** 9820–9825
21. Husain, I. & Sancar, A. (1987) *Nucleic Acids Res.* **15,** 1109–1120
22. Blundell, P. A., van Leeuwen, F., Brun, R. & Borst, P. (1998) *Mol. Biochem. Parasitol.* **93,** 115–130

Recognition of Base J in Duplex DNA by J-binding Protein

Robert Sabatini, Nico Meeuwenoord, Jacques H. van Boom and Piet Borst

J. Biol. Chem. 2002, 277:958-966.
doi: 10.1074/jbc.M109000200 originally published online November 7, 2001
