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High-Resolution Analysis of a Histone H1 Binding Site in a Rat Albumin Gene†

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ABSTRACT: Interaction of rat liver histone H1 fraction with the 5′-end of the rat serum albumin gene was localized within a 346 base pair (bp) restriction fragment. Sequence analysis of the fragment showed the fragment was 72 mol % adenosine–thymidine, which is significantly greater than the mole percent adenosine–thymidine composition of the rat genome. Gel retardation assays of the histone H1–DNA interaction indicate the complex formed behaves as previously characterized H1–DNA and shows a high-affinity H1 binding site within the enriched albumin restriction site. Deoxyribonuclease I (DNase I) protection assays on the H1 binding site define three protected regions only on the template strand of the DNA fragment. The three sites lie 55 and 110 bp apart (165 bp between the first and third binding site) with a consensus binding sequence of 5′-GA-ATA-CTGGCTT-C-TT-CTA-G-3′. The sequences between the protected DNA regions are highly enriched in adenosine–thymidine bases (79.3 and 86 mol % adenosine–thymidine, respectively). The functional significance is not understood.

Histone H1 has been shown to interact with high preference toward several restriction fragments of the rat albumin gene (Berent & Sevall, 1984). Previous observations of lysine-rich histone–DNA interactions have shown that H1 histones differ in binding with adenosine–thymidine-rich or guanosine–cytosine-rich DNA (Sponar & Sormova, 1972; Hwan et al., 1975). Subfractions of histone H1 differ in their interaction with a given DNA (Welch & Cole, 1979; Corbett et al., 1980), and H1 binds more strongly to supercoiled than to linear DNA (Vogel & Singer, 1976).

The functional role of histone H1 is to maintain the higher order structure of chromatin (Allan et al., 1980; Thoma & Koller, 1981) in part by defining the repeat length between

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nucleosomes (Oshima et al., 1980; Stein & Kunzler, 1983; Stein & Bina, 1984). Histone H1 can also cooperatively interact, and as concentration of the protein is increased, large aggregates of protein are formed (Clark & Thomas, 1986; DeBernardin et al., 1986). This supports the reported function of histone H1 as a general repressor of transcriptional activity by stabilizing the higher order structure of chromatin in the condensed nonactive solenoidal conformation (Weintraub, 1984; Schlissel & Brown, 1984).

The interaction of histone H1 with a preferential binding region in the albumin gene is characterized in this report. It is confirmed that the region of high preference is adenosine–thymidine (A-T) rich but the region of deoxyribonuclease I (DNase I) cleavage protection is not appreciably A-T rich. The DNA between the characterized binding sites is extremely A-T rich, suggesting a possible role in stabilizing the curvature of the A-T-rich DNA regions. The protected DNA sequence appears to be on the template DNA strand, which implies the interaction may not block the transcription of RNA polymerase II in the albumin gene. The functional role of the interaction is yet to be demonstrated, but the location of the site of interaction near the initiation site of the albumin gene may have significance.

Materials and Methods

DNA and Protein Component. DNA fragment JB was isolated from pJB, a derivative of pBR325 containing the 1450 base pair (bp) *Eco*RI–*Hin*dIII restriction fragment, JB, cut from λ RSA30 genomic clone of the rat albumin gene (Sargent, 1981). The subfragment of JB, *Hpa*II–*Pvu*II, was inserted into pUC9 by blunt-end ligation into the *Sma*I site of pUC9 (Maniatis et al., 1982; Silhavy et al., 1984; Vieira & Messing, 1982). Restriction fragments were generated by three 1-h, 10-fold restriction endonuclease digestions (Bethesda Research Labs, Bethesda, MD; Borehinger Mannheim, Elkhart, IN) of the plasmid carrying the fragment insert in the appropriate digestion buffer. Insert was separated from the vector by agarose gel electrophoresis in 0.5 × TBE [1 × TBE = 90 mM Tris–borate pH 8.3, 1 mM EDTA] and isolated by electroelution with a Schleicher & Schuell "elutrap". The isolated fragments could be selectively end labeled at the 3′-end by Klenow DNA polymerase I or on the 5′-end with polynucleotide kinase. The restriction sites normally labeled at the 3′-end were *Hin*dIII (four unpaired TCGA) labeled with [α-^{32}P]dGTP (3000 Ci/mmol, ICN, La Costa, CA) and cold dCTP or *Eco*RI (TTAA) labeled with [α-^{32}P]TTP and cold dATP. For 5′-end labeling, the fragments were treated with calf intestine alkaline phosphatase and then with polynucleotide kinase and [α-^{32}P]ATP (>7000 Ci/mmol, ICN, La Costa, CA). Radiolabeled fragments were purified with Geneclean (Bio 101, La Jolla, CA), and the end-labeled fragment was made site specific by treatment with a restriction endonuclease that cleaves at a site close to one or the other labeled end.

The histone H1 fraction was prepared from ARS Sprague-Dawley male rat liver nuclei as described previously (Berent & Sevall, 1984). Fractionation of the H1 variants over reversed-phase, high-pressure liquid chromatography (HPLC) systems (Kurokawa & MacLeod, 1985) resulted in a loss of preferential DNA binding. Protein concentration was determined by the method of Bradford (1972) with the modification of Lea et al. (1984), using bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO) as the standard. Histone H1 was determined by a fluorescent assay with fluorescamine (Sigma Chemical Co., St. Louis, MO) using HPLC-grade acetonitrile or freshly distilled 1,4-dioxane. The protein standard was phosphorylase A (27 mol % lysine). Native DNA concentration was measured by absorbance at 260 nM with the molar extinction coefficient E^{260} = 6600 M^{-1} cm^{-1} or by fluorescence assay using diaminobenzoic acid (Aldrich, Milwaukee, WI) (Vytasek, 1982).

Filter Binding Assay. The high-affinity histone H1 binding site was localized within fragment JB by retention of end-labeled restriction fragments of JB on nitrocellulose filters with dilutions of histone H1 (Berent & Sevall, 1984). The rat genomic JB restriction fragment was electroeluted from agarose and digested witth the appropriate restirction enzymes. The subfragments were 5′- or 3′-end labeled. Histone H1 was mixed with 10–20 ng of the DNA fragment. The nitrocellulose filters (Schleicher & Schuell, BA 85, 0.45-μm pore size; or Whatman cellulose nitrate membrane filters, WCN, 0.45-μm pore size) were prewashed with 0.5 M potassium hydroxide (KOH) for 20 min before equilibration with the binding buffer without bovine serum albumin (BSA). The binding buffer was 25 mM Tris-HCl (pH 8.0), 50 mM KCl, 1 mM $MgCl_2$, 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 100 μg/mL BSA. In microfuge tubes, the labeled DNA fragments in 10 μL of the binding buffer were mixed with 1 μL of an appropriate dilution of the histone H1 fraction. H1 dilutions were made with 20 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 0.1 mM dithiothreitol (DTT), and 10% glycerol immediately before use. After 10 min at room temperature, 1 mL of binding buffer without BSA was added and the solution immediately passed through the nitrocellulose filter at a flow rate of 1 mL/30 s. Filters were washed with an additional 1 mL of buffer.

After filtration, the filters were blotted and counted, and the bound DNA was eluted. The bound DNA was eluted from the filters by washing with 0.5 mL of 0.1% sodium dodecyl sulfate (SDS) for about 1 h at room temperature with vigorous shaking in a 2-mL screw top microtube. After 1 h of shaking, the filters were removed, 50 μg of tRNA was added, and the total mix was adjusted to 2 M ammonium acetate. The mixture was extracted with phenol–chloroform, and the nucleic acids were precipitated with an equal volume of isopropyl alcohol. The DNA pellets were counted and dissolved in 10 mM Tris-HCl (pH 8.0). An equal number of cpm from each sample was applied to each lane of 1.5% agarose or 5% polyacrylamide gel except when the total counts from the protein-free control were too low. After electrophoresis, the DNA bands were visualized by autoradiography of the dried gel.

Centrifugation Assay. Fragment HP was 3′-end labeled at either or both ends of the DNA molecule. The binding mix was prepared as described in the filter binding assay except the total mix was increased threefold. After incubation at room temperature, the microtube was centrifuged for 2 min at 12000*g* on a Fisher Microcentrifuge. Two aliquots of 10 μL were removed and counted individually along with the last 10 μL of the assay mix. The data were reported as the percentage of cpm that remained in the supernatant (the average of the two supernatant aliquots) with respect to the label in the pellet and last 10 μL [(average cpm in supernate/pellet fraction) × 100 = percentage in the supernate]. If the label remained in the supernatant, the percentage was 100%. A low percentage indicated that most of the DNA was pelleted by the short centrifugation.

Retardation Assay. The ^{32}P-end-labeled DNA fragment was mixed with dilutions of the histone H1 fraction in 10 μL of the binding buffer. The mixture was incubated at room temperature for 15 min, and thereafter 1 μL of 50% glycerol and 0.2% bromophenol blue was added and the mixture immediately loaded onto a 4% polyacrylamide gel [acryl-

amide:bis(acrylamide) weight ratio of 30:1] containing 1 mM EDTA, 3.3 mM sodium acetate, and 6.7 mM Tris-HCl (pH 8.0) (Strass & Varshavsky, 1984). The gel (0.15 cm × 17 cm × 15 cm) was preelectrophoresed for 2 h at 12 V/cm. Electrophoresis was carried out at the same voltage gradient for 2–3 h at room temperature with tray buffer recirculation. The gel was dried and autoradiographed.

For competition experiments, the labeled DNA and unlabeled DNA were mixed with a limiting amount of histone H1. The competitor DNA was the unlabeled JB fragment, the 1600 bp *Hin*fI pBR322 fragment, a 390 bp *Hin*fI fragment isolated form *Hin*fI-digested pBR322, or the unlabeled *Hpa*II–*Pvu*II rat albumin DNA fragment.

Sequencing and Protection Assays. End-labeled DNA was chemically sequenced by the procedures of Maxam and Gilbert (1980). The DNase I protection assays were done under identical ionic conditions as the DNA filter assay. After incubation at room temperature, the histone H1 and DNA mixture was adjusted to 5 mM $MgCl_2$ and 1 mM $CaCl_2$ with the addition of $^1/_{10}$ volume of 50 mM $MgCl_2$ and 10 mM $CaCl_2$ for the DNase I protection assay. One nanogram of DNase I (electrophoretically purified) (Worthington Biochemicals, Elkhart, IN) was added at 20 °C for 30 s, and the digest was stopped with the addition of an equal volume of 5 M ammonium acetate, 50 mM EDTA, and 1 mg/mL tRNA and immediately extracted with phenol–chloroform. The digested DNA was precipitated with ethanol, washed with ethanol, dried, and resuspended in formamide, 0.02% bromophenol blue, and xylene cyanole. The samples were run on a 8% denaturing polyacrylamide gel. After electrophoresis, the gels were autoradiographed with intensifying screens for 24 h–1 week.

Results

Localization of the H1 Binding Site within Fragment JB. The high-affinity histone H1 binding site was localized within fragment JB of the rat albumin gene region of DNA. Fragment JB was investigated because of its preferential affinity by histone H1 over other *Hin*dIII–*Eco*RI restriction fragments from the rat albumin genomic clone λ RSA30 (Berent & Sevall, 1984; Sargent, 1981). Fragment JB contains the first two exons of the albumin gene, the Z–A intron, and 500 bp upstream from the initiation site of the albumin messenger RNA. A high-resolution restriction map of the JB fragment has been determined (Figure 1D). A set of four restriction endonuclease digestions subdivided the 1450 bp JB fragment into different groups of restriction fragments. The restriction fragments were individually end labeled and used in the filter binding assay with purified rat liver histone H1.

The experimental results are shown in Figure 1. Figure 1A–C shows the autoradiograph of three different assays resulting in enrichment of overlapping restriction fragments of JB. One set of DNA fragments generated by an *Hpa*II–*Dde*I digestion did not show any fragment enrichment by histone H1 retention on nitrocellulose (not shown). The filter retention assay demonstrates histone H1 high-affinity site(s) lies within the *Hpa*II–*Sau*96I restriction site. If the 350 bp fragment is cleaved in half by *Dde*I restriction, the preferential retention is apparently lost (data not shown).

The *Hpa*II–*Pvu*II DNA fragment was subcloned into the *Sma*I site in the multiple cloning region of the pUC9 vector (the actual fragment cloned was the 345 bp *Hpa*II–*Pvu*II fragment). The *Hpa*II–*Pvu*II fragment was confirmed by chemical sequencing on both strands of the DNA (Figure 2). Preparative amounts of the *Hpa*II–*Pvu*II fragment could be excised from the plasmid (pHP) by treatment with *Eco*RI and *Hin*dIII or *Bam*HI restrcition endonucleases and electrophoretically purified. The *Hpa*II–*Pvu*II fragment is characterized by a base composition of 28 mol % G-C, which differs significantly from a base composition of the rat genome of 41 and 37 mol % G-C of the "mother" restriction fragment JB.

Gel Electrophoresis Assay of the H1–DNA Complexes. The histone H1–DNA complex was characterized by electrophoresis of the protein–DNA complex in a low ionic strength 4% polyacrylamide gel (Chelm & Geiduschek, 1979; Fried & Crothers, 1981; Garner & Revzin, 1981; Strauss & Varshavsky, 1984). Figure 3 shows a protein titration of the *Hpa*II–*Pvu*II DNA. The fractional amount of the DNA that remained in the supernatant was determined by a 2-min centrifugation at 12000*g* in an Eppendorf tube and is listed in the legend. The data show, at high H1 levels, the DNA does not enter the gel on electrophoresis (lane B) and is almost completely pelleted by centrifugation. This is termed aggregation and represents a very large complex most likely formed by a large amount of nonspecific interaction. As the histone H1 concentration is lowered slightly, the complex can enter the electrophoretic gel as a diffuse complex but is still quickly pelleted by centrifugation (lanes B and C). This is termed a diffuse complex. At more dilute histone H1 levels, 33–60% of the DNA in the complex remains in the supernatant on centrifugation but has the electrophoretic mobility of two distinct entities (lanes E–I). The faster and major component (as histone H1 is diluted) has a mobility of free DNA standard (the samples showed a curvature in mobility due to the generation of heat during the electrophoretic run). The second and third component has a slightly slower mobility and is dependent on the level of histone H1. The second component can be seen in the lanes of the diffuse complex but represents a very minor portion of the total amount of DNA.

The radiolabeled *Hpa*II–*Pvu*II fragment was incubated with histone H1, under conditions that would form a diffuse complex, in the absence or presence of competitor DNAs. The resulting complexes were resolved from the free fragment by electrophoresis through a low ionic strength, nondenaturing polyacrylamide gel and visualized by autoradiography (Figure 4). In the absence of competitor, the *Hpa*II–*Pvu*II fragment runs as a diffuse complex as observed in the histone H1 titration (lane b, Figure 4). The presence of unlabeled competitor DNA disrupts the diffuse complex sufficiently to resolve three components. The fastest component coelectrophoreses with free DNA while the two slower components follow just behind the free DNA. The competitor DNAs are the 1450 bp JB albumin restriction fragment (A) from which the histone-bound DNA fragment (HP) is isolated and the 1630 bp *Hin*fI fragment (B) derived from pBR322. Both competitors can disrupt the diffuse H1–DNA complex into three electrophoretic components. The unlabeled JB fragment appears more efficient as a competitor since the diffuse protein–DNA complex is completely disrupted to free DNA at the highest level of JB competitor. The pBR322 *Hin*fI competitor at the same high level of competitor resolves the diffuse H1–DNA complex into two electrophoretic components. Faint bands running behind free DNA are present in both the free DNA (lane A) and in those lanes with high competitor DNA (lanes G–I), implying these faint bands are not H1–DNA complexes but are present in the DNA preparation. The data support the presence of unique H1–DNA complexes in the *Hpa*II–*Pvu*II DNA fragment, and the competitor fragment of pBR322 is slightly less efficient than the JB fragment in disrupting the complex(es).

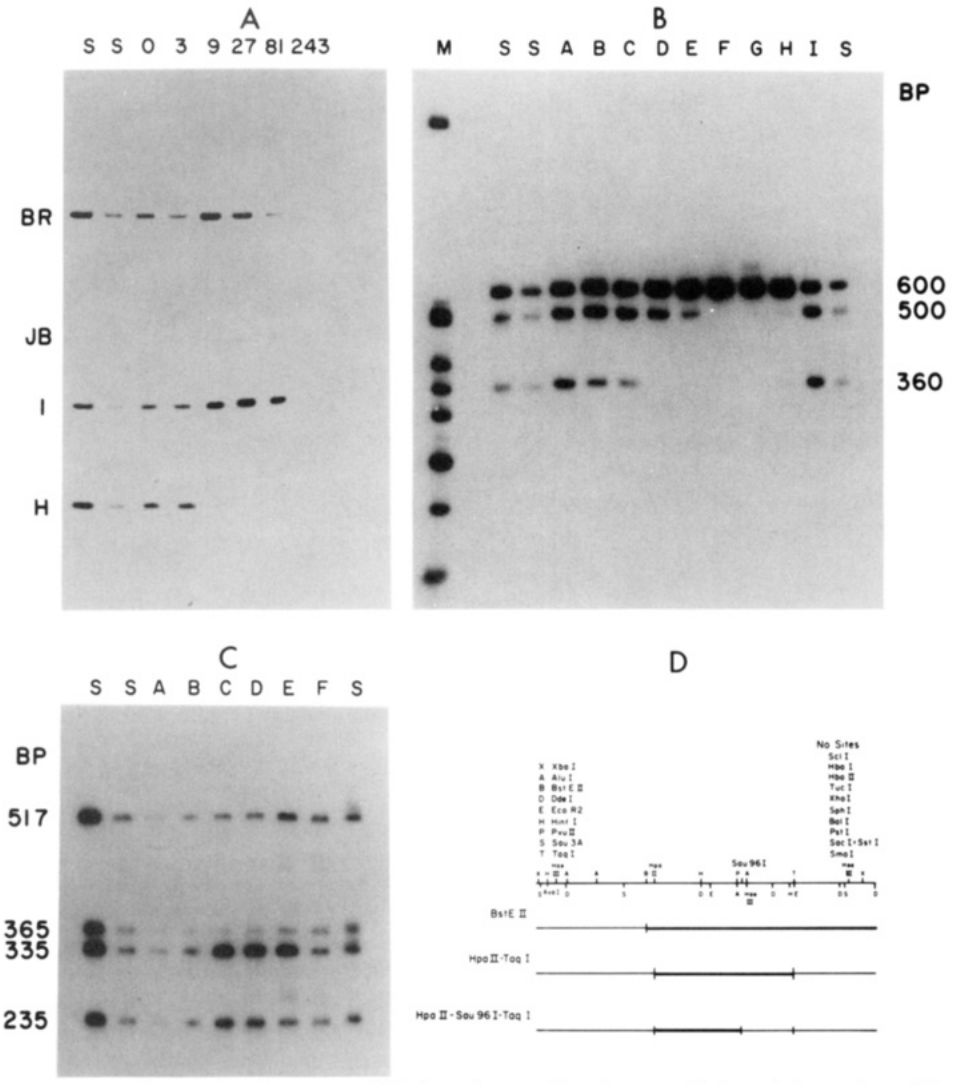

FIGURE 1: Preferential retention of restriction fragments of JB from the rat albumin gene. Preferential retention of three restriction digests of fragment JB of the albumin gene was mapped with purified histone H1 by the filter retention assay. JB or pJB (plasmid pBR325 harboring JB) was digested with the appropriate restriction enzyme, the fragments were 5′- or 3′-end labeled, and histone H1 preference was assayed by the electrophoresis assay. (A) *Bst*EII of pJB. Plasmid pJB was digested with *Eco*RI, *Hin*dIII, and *Bst*EII, and the fragments were end labeled and used in the assay for H1 preferential binding. Lane S is standards of the labeled restriction fragments, and lanes 0–243 are serial dilutions of a histone H1 fraction. The DNA retained on the filter purified for electrophoresis was 67.1%, 43%, 21%, 12.4%, 4.0%, and 0.01% of the input DNA. BR represents the DNA fragment of the vector pBR325, JB is uncut JB of 1450 bp, I is the 959 bp 3′-end pf JB cut by *Bst*EII, and H is the 490 bp 5′-end if the *Bst*EII cut JB. Fragment I represents 3′-end of JB that is preferentially retained by histone H1. The gel system was a 1.0% agarose gel. (B) *Hpa*II–*Taq*I digest of purified JB. Purified JB from a *Hin*dIII–*Eco*RI digest of pJB was digested with *Hpa*II and *Taq*I, and the digestion products were end labeled. Histone H1 preference toward the restriction fragments was assayed by the membrane filter retention assay. Lane M is a marker of *Hin*fI-digested pBR322 5′-end labeled. Lane S is a standard of the *Hpa*II–*Taq*I digest of JB. Lanes A–I represent dilutions of purified histone H1 of 100-fold (A), 200-fold (B), 400-fold (C), 500-fold (D), 800-fold (E), 1100-fold (F), 1500-fold (G), 1600-fold (H), and infinite dilution (I). The amount of DNA purified from the filter for electrophoresis on a 5% polyacrylamide gel is from 60.3%, 50.2%, 50.5%, 32.8%, 27%, 14.5%, 12.2%, 6.9%, and 4.4% of the input counts for lanes A, B, C, D, E, F, G, H, and I, respectively. (C) *Hpa*II–*Taq*I–*Sau*96I digest of JB. Purified JB was digested individually with *Hpa*II, *Taq*I, and *Sau*96I and end labeled, and H1 preference was assayed. Lane S is standard, and lanes A–F are 50-fold dilutions of H1 (A, B), 500-fold (C), 1000-fold (D), 2000-fold (E), and infinite dilution (no protein control; F). The DNA retained and recovered for electrophoresis from the input DNA was 27.8% (A, B), 12.0% (C), 4.0% (D), 3.37% (E), and 1.1% (F). The size of the DNA fragments was determined from marker fragments of a *Hin*fI digest of pBR322. (D) Restriction map of JB of the rat albumin gene. The 1450 bp sequence of JB was mapped for the restriction enzyme sites by computer analysis and by digestion of pure JB. The listed enzymes on the left were used to confirm those sites predicted by the computer analysis. Enzymes confirmed to have no sites are listed on the right. The summary of experiments A, B, and C is shown below on the restriction map. The fragment that was preferentially retained by histone H1 is shown as a solid bar in the restriction map. Fragment *Hpa*II–*Sau*96I is the common fragment retained by histone H1.

Support for a high-affinity interaction between histone H1 and HP is shown when a 396 bp *Hin*fI pBR322 fragment is used as a substrate for H1 binding (Figure 5). Under conditions where the histone H1 to DNA ratio is 4:1, the labeled 396 bp fragment is retained at the top of the gel. As unlabeled 396 bp is added to the protein–DNA mixture, the complex aggregates completely to yield the labeled DNA as free DNA on electrophoresis. The amount of DNA required to dissociate the complex is in the same range as required to resolve the electrophoretic components with labeled HP (8–10 ng with 396 bp DNA and 6–12 ng with HP DNA).

Delineation of the Histone H1 Binding Domain. The DNA fragment was labeled on the 5′- or the 3′-strand at either end of the molecule (5′-strand at the *Eco*RI site and 3′-strand at the *Hin*dIII site) with the Klenow DNA polymerase I. The specific site(s) of interaction was (were) determined by DNase I protection assays. Figure 6 demonstrates the protected regions in the labeled 3′-strand *Hpa*II–*Pvu*II fragment. At

```
1           11          21          31          41
CCCCGGTTCT  GCCTTTTCTA  GGGGTGTGTT  TCGCCGAGAA  GCACGTAAGC
GGGGCCAAGA  CGGAAAAGAT  CCCCACACAA  AGCGGCTCTT  GCTGCATTCG

51          61          71          81          91
ATTCTATGTT  TTCTCATCTC  TACTTTTATT  TTTCGTAGTA  ACGGAAGCCA
TAAGATACAA  AAGAGTAGAG  ATGAAAATAA  AAAGCATGAT  TGCCTTCGGT

101         111         121         131         141
GGTATTTCAA  AATTACTTAA  ATTTTCCTTT  GGTGATGATT  AATATCACCA
CCATAAAGTT  TTAATGAATT  TAAAAGGAAA  CCACTACTAA  TTATAGTGGT

151         161         171         181         191
TTATTATTAT  TATTATTATT  ATTATTATTA  TTATTATTAT  TACATTTGCA
AATAATAATA  ATAATAATAA  TAATAATAAT  AATAATAATA  ATGTAAACGT

201         211         221         231         241
TCTGAGAATC  CTTATGTGGT  TATATTATTG  TATTTTAGAT  AACTCGGATC
AGACTCTTAG  GAATACACCA  ATATAATAAC  ATAAAATCTA  TTGAGCCTAG

251         261         271         281         291
ACAATAATGG  GGGGACTTTG  AAAGATTAGT  TTTAAAATTT  CTTTTAATTA
TGTTATTACC  CCCCTGAAAC  TTTCTAATCAA  AAATTTTAAA  GAAAATTAAT

301         311         321         331         341
AAATAAAATG  CTAGGTAGAA  TGATTTGAAC  TATGTCAGAT  ACAGGGGAAT
TTTATTTTAC  GATCCATCTT  ACTAAACTTG  ATACAGTCTA  TGTCCCCTTA
```

FIGURE 2: Sequence of HP fragment of endonuclease fragment JB. The sequence of the *Hpa*II–*Pvu*II fragment from the JB restriction fragment of the rat albumin gene. The underlined sequence is the Z exon that codes for the 5′-end of the albumin messenger RNA. The double underlined sequences in the 3′-strand are the regions that are protected from DNase I cleavage by H1 interaction (Figure 6). 5′ to residue 1 is the *Hin*dIII and the *Bam*H1 site in the multiple cloning region of the vector pUC9. At the 3′-end is the *Eco*RI site of the vector.

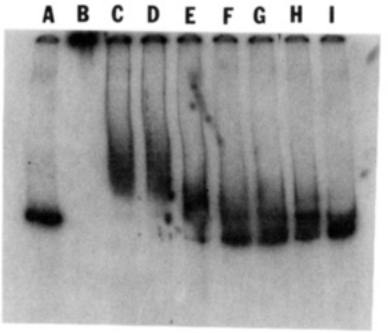

FIGURE 3: Histone H1 titration of the *Hpa*II–*Pvu*II albumin fragment. End-labeled HP fragment (10 ng) was mixed with increasing amounts of histone H1 in 10-μL total volume of binding buffer. Incubation was for 15 min at 23 °C followed by electrophoresis on a 4% gel [30:1 acrylamide:bis(acrylamide)] at 10 V/cm for 2 h after preelectrophoresis for 2 h. Lanes A, no H1; B, 476 ng; C, 95.3 ng; D, 47.6 ng; E, 23.8 ng; F, 15.8 ng; G, 11.9 ng; H, 5.9 ng; I, 3.2 ng of histone H1. The amount of labeled DNA remaining in the supernate after a 2-min spin at 12000*g* was as follows: A, 100%; B, 3.4%; C, 7.7%; D, 11.5%; E, 29.9%; F, 36.7%; G, 35.3%; H, 40.9%; I, 61.5%.

equimolar amounts of histone H1 and labeled DNA fragment, two sites are protected at residue 35–54 and 83–108 (double underlined in Figure 2) while two additional sites show clear alteration in their DNase I susceptibility at 135–145 and 210–215. When an unlabeled *Hpa*II–*Pvu*II fragment is included in the binding assay prior to the DNase I digestion, a fivefold excess of the DNA fragment (lane 3) does not abolish the protection. At 100-fold excess of the unlabeled DNA fragment (lane 4), the protected and altered sites are as accessible as the free DNA control (lane 1).

When the 5′-strand is labeled on its 3′-end at the *Eco*RI site, at H1 to DNA ratios of 4:1 (DNA concentration is 5–10 ng), the DNase I protection assays indicate that altered cutting occurs at position 50–100 with the rest of the DNase I pattern appearing similar to the control pattern (data not shown). The altered region covers the sequence on the 5′-strand which lies between the protected sites on the 3′-strand. As the histone

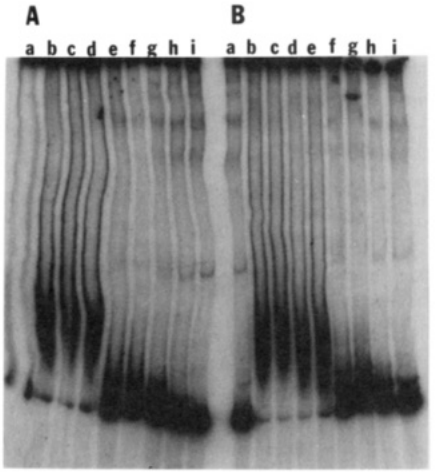

FIGURE 4: DNA competition of with the H1–*Hpa*II–*Pvu*II albumin fragment. End-labeled HP fragment (10 ng) was mixed with 47.6 ng of histone H1 and increasing amounts of competitor DNA in binding buffer. In panel A, fragment JB was the competitor. In panel B, the competitor is the 1500 bp *Hin*fI fragment of pBR322. Lane a is free HP DNA; lane b is HP DNA and histone H1; lanes c–i are dilutions 1:500, 1:250, 1:100; 1:50; 1:25, 1:10, and straight of the competitor (JB straight is 315 ng, and 1500 bp *Hin*fI pBR322 is 365 ng straight). The complexes were incubated at 23 °C for 15 min and electrophoresed as in Figure 1.

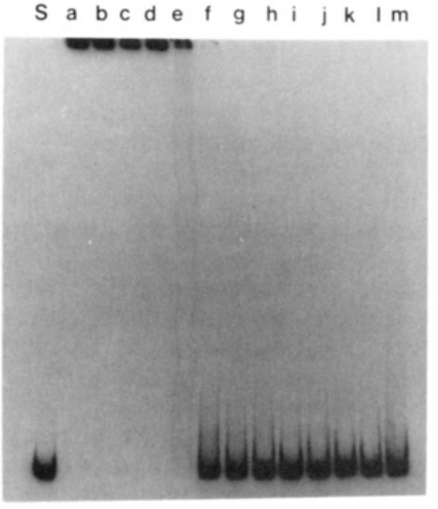

FIGURE 5: H1 interaction with a 396 bp *Hin*fI pBR322 fragment. A ^{32}P-end-labeled, 396 bp *Hin*fI restriction fragment of pBR322 at 5.7 ng was mixed with 37.6 ng of homogeneous histone H1 in 10 μL of binding buffer. Prior to the addition of histone H1, increasing amounts of unlabeled 396 bp *Hin*fI restriction fragment was added to the reaction mix. After 15 min at room temperature (23 °C), the sample was electrophoresed on a 4% low ionic strength polyacrylamide gel for 2 h at 200 V. Lane S is the free labeled DNA, and lanes a–m are 0, 1.25, 2.5, 5, 10, 25, 50, 100, 250, 500, 1000, 2000, and 3000 ng of the unlabeled 396 bp *Hin*fI pBR322 restriction fragment.

H1 to DNA ratio is lowered, the altered DNAse I region is lost and the DNase I pattern appears identical with the control pattern. At protein to DNA ratios where the DNA is not precipitable by centrifugation, the DNAse I pattern appears as the control. The apparent high-affinity binding is detectable when a large amount of the DNA is complexed in a precipitable or electrophoretically diffuse complex. A clear footprint is not observed and suggests that H1 interacts with preference on one strand of the DNA. Current work is addressing this observation.

Discussion

The results confirm the current concept of histone H1–DNA binding and initially characterize the observed preferential

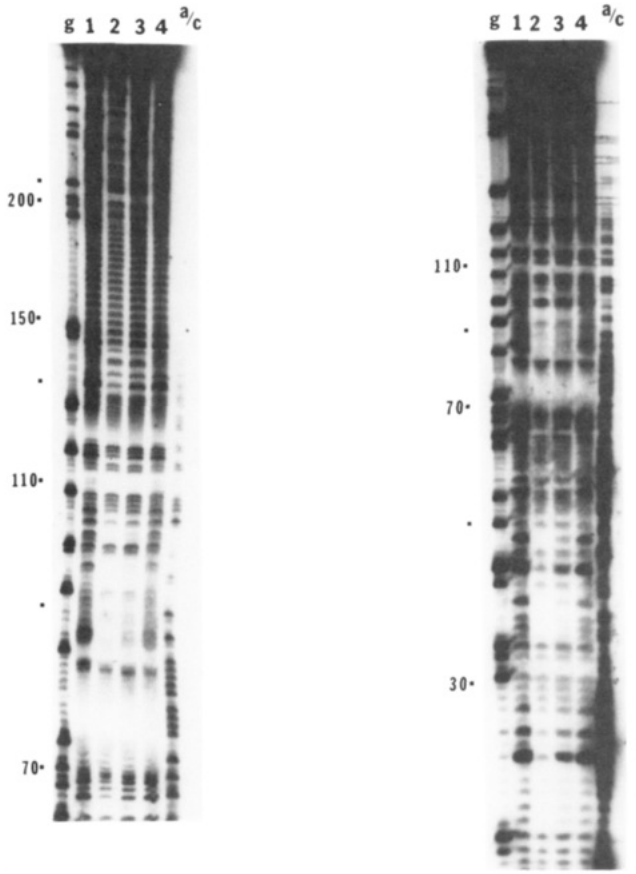

FIGURE 6: DNase I protection assay of the HP albumin restriction fragment. The 356 bp HP (Figure 2) fragment was labeled with ^{32}P on the 3′-strand at the *Hin*dIII site with Klenow DNA polymerase I. The DNA (8 ng) was mixed with 23.6 ng of histone H1 in 10 μL of binding buffer. After 15 min at 20 °C, 1 ng of DNase I was added with $MgCl_2$ (to 5 mM) and $CaCl_2$ (to 0.5 mM) for 30 s, when 25 mM EDTA stopped the reaction. Lane 1 had no histone H1 added, lane 2 had histone H1 added, and lanes 3 and 4 had unlabeled HP added at 80 ng (lane 3) and 800 ng (lane 4) prior to the addition of H1. The C and A/C are sequence reactions on the native HP as marked lanes.

binding of rat liver histone H1. The following conclusions can be made: (1) Histone H1 interacts preferentially with a restriction fragment that includes the rat albumin Z exon–intron border; (2) the histone affinity shows some sequence specificity since a specific site of protection can be defined by DNAse I digestion on the template strand of the albumin gene restriction fragment; and (3) the specificity does not apparently prefer A-T-rich DNA but more flexible DNA regions.

Gel electrophoresis of the H1–HP DNA complex shows a reduced mobility of the protein DNA complex (Figure 3). The retardation can be enhanced with the presence of competitor DNA probably by reducing the nonspecific interaction of histone H1 (Berent & Sevall, 1984) (Figure 4). The concept is that of the band competition assay (Sen & Baltimore, 1986) where excess unlabeled competitor DNA competes for nonspecific DNA interaction, allowing high-affinity interactions to be resolved. Competition assays show that either heterologous DNA (*Escherichia coli* DNA) or homologous DNA (JB fragment) at 1:1 to 30:1 competitor/HP DNA ratios effectively reduces the nonspecific histone H1–DNA interactions, allowing the resolution of the H1–HP DNA complex as the retarded DNA species. Support for the presence of a high-affinity H1 binding site in HP is the competition between ^{32}P-labeled 396 bp *Hin*fI pBR322 and the unlabeled 396 bp *Hin*fI pBR322 fragment. At comparable histone H1/labeled DNA ratios, namely, those required to form the diffuse complex, unlabeled 396 bp DNA completely disaggregated the H1–DNA complex and the DNA ran as free DNA. There is no indication that there is a high-affinity interaction in the *Hin*fI pBR322 fragment since there are no retarded DNA complexes at higher levels of competitor DNA.

Those regions protected under excess H1/HP DNA ratios at moderate competition with unlabeled HP DNA are three sites of interaction at residues 35–54, 83–108, and 201–215 (Figure 6) and are underlined in Figure 1. Histone H1 protected regions on the opposite DNA strand have not yet been identified. The bound sites are

```
54   GA AT gCTtaCgTgC TT CTc    36
     || ||  ||  | | | || ||
108  GAaATAcCTGGCTTcCgTTaCTAcG  84
       |||   || || |  | | | |
215    ATA  aGGaTT C  T C A G   202
     ga-ATa-ctggctT-C-tT-Cta-g
```

The H1-bound regions lack a high A-T base composition (the consensus sequence has a 57.9 mol % A-T). The sequences between the H1 binding sites are 79.3 mol % A-T and 86.0 mol % A-T, respectively. Apparently, the histone H1 preference for A-T-rich DNA is not direct and conforms more to the nonpreference core nucleosomes show for A-T-rich DNA (Chao et al., 1979). The H1 sequence preference is not for highly A-T rich DNA but rather for DNA with a lower A-T content. Adenine runs spaced with a 10 bp phase are characterized by a rigid bend conformation (Koo et al., 1986; Hagerman, 1986; Koo & Crothers, 1987). Thus, H1 appears not to bind preferentially to the very A-T rich DNA regions but to more flexible DNA regions. This contrasts with the earlier shown preference of poly(L-lysine) for A-T-rich DNA (Leng & Felsenfeld, 1966; Shapiro et al., 1969).

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Thiazolium C(2)-Proton Exchange: Structure–Reactivity Correlations and the pK_a of Thiamin C(2)–H Revisited†

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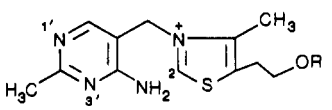
ABSTRACT: Rate constants for C(2)-proton exchange from thiamin, *N*(1′)-methylthiamin, and several 3-substituted-4-methylthiazolium ions catalyzed by D_2O and deuterioxide ion were determined by ^1H NMR at 30 °C and ionic strength 2.0 M. Values of pK_a for the thiazolium ions, including thiamin itself, were found to be in the range pK_a = 17–19; the pK_a values for N(1′)-protonated thiamin and free thiamin C(2)-H in H_2O are 17.7 and 18.0, respectively. The pK_a value for N(1′)-protonated thiamin was calculated from the observed rate constant for the pD-independent reaction with D_2O after correction for a secondary solvent deuterium isotope effect of k_{H_2O}/k_{D_2O} = 2.6. The pK_a value for free thiamin was calculated from the rate constant for catalysis by OD^- after correction by a factor of 3.3 = 8/2.4 for an 8-fold negative deviation of k_{OD} from the Brønsted plot of slope 1.0 for general base catalysis and a secondary solvent isotope effect of k_{OD}/k_{OH} = 2.4. Values of $k_{-a} = 2 \times 10^{10}$ and 3×10^9 M^{-1} s^{-1} were assumed for diffusion-controlled protonation of the C(2) ylide in the reverse direction by H_3O^+ and H_2O, respectively. The Hammett ρ_I value for the exchange reaction catalyzed by deuterioxide ion or D_2O is 8.4 ± 0.2. There is no positive deviation of the rate constants for free or N(1′)-substituted thiamin analogues in either Hammett correlation. This shows that the aminopyrimidinyl group does not provide significant intramolecular catalysis of nonenzymic C(2)-proton removal in the coenzyme.

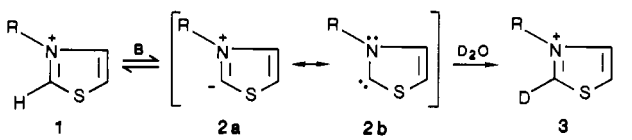
Thiamin pyrophosphate (TPP)[1] (**1a**) contains substituted pyrimidine and thiazolium heterocycles and is a coenzyme for the decarboxylation of α-keto acids, the formation of α-ketols, and transketolase reactions (Krampitz, 1969; Schowen & Schellenberger, 1987). The catalytic action of thiamin in both



1a, R = $P_2O_6H_3$
b, R = H

thiamin-dependent enzyme reactions and nonenzymic model reactions results from the base-catalyzed abstraction of the C(2) proton leading to the formation of the very reactive thiazolium ylide (**2**, Scheme I), which is not only a potent carbon nucleophile but also a reasonably stable leaving group (Breslow, 1962).

Scheme I



1 **2a** **2b** **3**

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[1] Abbreviations: TPP, thiamin pyrophosphate; Me_2SO, dimethyl sulfoxide.

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