Histone H1 Binding at the 5′ End of the Rat Albumin Gene†

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ABSTRACT: Cloned DNA containing the first nine exons of the rat albumin gene was digested with *Eco*RI and *Hin*dIII, and the resulting fragments were used to screen for regions with relatively high affinity for protein. Of three restriction fragments preferentially bound, the fragment containing the first two exons of the albumin gene was consistently bound over others by heat-stable protein extracted from liver nuclei with 0.35–1.0 M NaCl. Proteins extracted with lower and higher ionic strength buffers bound the DNA fragments, but with little specificity. The DNA fragment that was preferentially bound consistently by the 1.0 M nuclear extract was subcloned into pBR325 and was used to isolate the specific DNA-binding activity. After purification, histone H1 was the polypeptide with preferential DNA-binding activity. Histone H1 has a high-affinity binding site in the 5′ end of the rat albumin gene within 440 5′-flanking base pairs and the first two exons of the gene.

Selective eukaryotic gene transcription resides in the upstream 5′ sequences for genes transcribed by RNA polymerase II (Breathnach & Chambon, 1981). DNA sequences that increase transcriptional efficiency are relatively independent of their position and orientation with respect to the nearby gene (Benoist & Chambon, 1980; Gillies et al., 1983). These transcriptional control regions appear to be associated with DNase I hypersensitive sites in the chromatin of cells expressing the gene (Wu et al., 1979; Wu, 1980; Chung et al., 1983). An attractive view of DNase I hypersensitivity is that short stretches of chromosomal DNA are open to the environment, which become available for interaction with regulatory macromolecules (McGhee et al., 1981; Keene & Elgin, 1981). At least two factors interact preferentially with gene promoter regions, which are required for stable preinitiation complexes (Davison et al., 1983). It appears possible that the nucleosomal organization mimics DNA sequence organization of the gene (Mengeritsky & Trifonov, 1983).

If sequence reflects the organization of chromosomal proteins along the DNA, the presence of nucleosomal DNA binding proteins at precise positions along the control DNA sequence may occur. By applying known characteristics of specific DNA binding proteins (Lin & Riggs, 1975; Yamamoto & Alberts, 1975), we have shown in a salt extract of rat liver nuclei the presence of preferential DNA-binding activity within the 5′ end of the rat albumin gene (Sargent et al., 1979). By subcloning one of the preferred regions of the 5′ end of the albumin gene, the cloned sequence was then used to purify the preferential DNA-binding protein. The albumin gene is suited to this approach since the rat liver albumin gene is constitutively expressed in all hepatocytes of the adult liver (Guillouzo et al., 1982), making the presence of the binding protein reasonably high. Electrophoretic analysis of the DNA-binding protein shows that histone H1 can interact with specific regions within the 5′ end of the rat liver albumin gene.

Materials and Methods

Animals, Cell Fractions, and Protein Extracts. The livers from 150-g male Sprague-Dawley rats (ARS Sprague-Dawley) were processed for nuclei (Castro & Sevall, 1980). The washed nuclei were suspended at a DNA concentration of 2 mg/mL in buffer containing 10 mM potassium phosphate (pH 7.0), 10 mM sodium chloride (NaCl), 1.5 mM magnesium chloride ($MgCl_2$), 0.5 mM dithiothreitol (DTT), and 1 mM phenylmethanesulfonyl fluoride (PMSF). The nuclei were homogenized for 45 s with a Tekmar tissumizer at a setting of 75%. The solution was then centrifuged for 15 min at 12000*g*. The supernatant, designated the 0.01 M extract, was saved. The pellet was resuspended in the same volume of the above buffer containing 0.35 M NaCl with 10 strokes of a Teflon pestle in a glass homogenizer. The suspension was centrifuged for 15 min at 12000*g*. The supernatant, designated the 0.35 M extract, was saved. The pellet was resuspended in half the volume of the initial step in the above buffer containing 1.0 M NaCl with a glass–glass homogenizer. The suspension was stirred for 1 h before centrifugation for 2 h at 245000*g*. The supernatant, labeled the 1.0 M extract, and the two preceding supernatants were dialyzed against 20 mM tris(hydroxymethyl)aminomethane (Tris) (pH 7.4) containing 0.15 M NaCl, 0.1 M DTT, and 1 mM PMSF. The 1.0 M extract pellet was resuspended in one-fourth the volume of the initial step in the above buffer containing 4.0 M NaCl with a glass–glass homogenizer. The suspension was stirred for 1 h before centrifugation overnight at 245000*g*. The supernatant, designated the 4.0 M extract, was dialyzed against the same buffer as for the previous extracts. Any precipitated material was removed by brief centrifugation before all the extracts were stored in small aliquots at −70 °C. All steps were done at 4 °C. Proteins were measured by the method of Lowry et al. (1951) as modified by Peterson (1977) with bovine serum albumin (BSA) as a standard. For basic proteins, the fluorescamine detection of primary amines (Udenfried et al., 1972) with chymotrypsinogen as the standard was used to quantitate protein levels.

Substrate DNAs. The DNA from the clone λRSA30 (Sargent et al., 1979) was a generous gift from Dr. Tom Sargent. The individual subclones of λRSA30 of restriction fragments JB and JC subcloned in pBR325 and pBR322, respectively, were gifts from Dr. Linda Jagodzinski.

DNA from λRSA30 was digested with *Eco*RI and *Hin*dIII as recommended by the manufacturer (Bethesda Research Laboratory). The restriction fragments were dephosphorylated with calf intestinal alkaline phosphatase (Boehringer Mannheim) and 5′ labeled with polynucleotide kinase (P-L Biochemicals, Inc.).

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To obtain labeled fragments JB and JC, plasmids pJB and pJC were first digested with restriction enzymes *Eco*RI and *Hin*dIII or with *Hin*dIII, respectively. The restriction fragments were terminally labeled with DNA polymerase I Klenow fragment (Rigby et al., 1977). Approximately 10 μg of the restricted fragments from each of the plasmids was incubated in a total volume of 0.1 mL for 10 min at 37 °C with 4 units of the Klenow fragment in buffer containing 40 mM potassium phosphate (pH 7.5), 3 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 25 μM each of the unlabeled deoxynucleoside triphosphates, and the appropriate labeled deoxynucleoside triphosphate. Fragments were ^{3}H labeled by incubation with 20 μM each of [8-^{3}H]dATP (17 Ci/mmol) and [methyl-^{3}H]TTP (60 Ci/mmol). Incubation with 2.5 μM each of [α-^{32}P]dCTP (200 Ci/mmol) and [α-^{32}P]TTP (200 Ci/mmol) was performed when obtaining ^{32}P-labeled fragments. After incubation, the labeled fragments were electrophoresed on 1% low melting point agarose gels (Sea Plaque Agarose Marine, Colloids), and labeled JB and JC were extracted from the gels as directed by Bethesda Research Laboratories. The specific activities of [^{3}H]JB and [^{3}H]JC typically were 280 and 200 cpm/ng, respectively. [^{32}P]JB and [^{32}P]JC typically were labeled to 4900 and 2700 cpm/ng. These specific activities were adjusted to values closer to the activities of the ^{3}H fragments by addition of unlabeled JB or JC. The ^{3}H- and ^{32}P-labeled JB and JC could be visualized after electrophoresis on 1% agarose gels either by staining with ethidium bromide or by autoradiography after treatment of the gel with EN^{3}HANCE (New England Nuclear). To demonstrate the affinity of DNA fragments for protein was not altered by the labeling procedure, the binding at one level of protein as a function of specific activity of the labeled fragment was measured. The total amount of DNA remained constant while the specific activity was altered by changing the ratio of labeled to unlabeled DNA fragment. A constant percentage of DNA bound by the protein at all specific activities was interpreted as indicating the unlabeled and labeled DNA competed equally well for the protein. The preferential binding was independent of isotope used to label the DNA, demonstrating that observed binding was not an artifact of the labeling procedure. DNA concentration was measured by absorbance at 260 nm using the molar extinction coefficient E_{260} = 6600 M^{-1} cm^{-1} or by a fluorescence assay using diaminobenzoic acid (Vytasek, 1982).

Filter Binding Assay. The procedure used to detect DNA–protein complexes was based on the technique developed by Riggs et al. (1970), which takes advantage of the fact that DNA is retained by nitrocellulose filters only if it is associated with protein. The nitrocellulose filters (Schleicher & Schuell, BA 85, 0.45-μm pore size) were prewashed with 0.5 M potassium hydroxide for 20 min before equilibration with the binding buffer without BSA. The binding buffer consisted of 25 mM Tris-HCl (pH 8.3), 50 mM KCl, 1 mM $MgCl_2$, 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 100 μg/mL BSA. The labeled DNA fragments in 20 μL of the binding buffer in siliconized polypropylene microtubes were mixed with 2 μL of an appropriate dilution of the DNA-binding protein extract. Protein dilutions were made with 20 mM Tris-HCl buffer (pH 7.4), containing 0.15 M NaCl, 0.1 mM DTT, and 10% glycerol, immediately before use. After 15 min at room temperature, 1 mL of binding buffer without BSA was added, and the solution was passed through the nitrocellulose filter at a vacuum of 4–5 cmHg. Filters were washed with an additional milliliter of buffer.

When the specificity of binding to the *Eco*RI–*Hin*dIII digestion fragments from the albumin gene region was tested, the protein was mixed with 30–70 ng of the fragments from the λRSA30 DNA. After filtration of the mixture, the filters were blotted, counted, and further processed as follows. The bound DNA was eluted from the filters by washing with 2 mL of 0.1% sodium dodecyl sulfate (SDS) for 1 h at room temperature, after which time 100 μg of tRNA and 0.1 mL of buffer containing 10 mM Tris-HCl (pH 8.3), 5 M NaCl, and 1 mM EDTA were added. The DNA was precipitated by the addition of 6 mL of ethanol. The precipitate was dissolved in a total of 0.7 mL of 0.2 M sodium acetate, and the solution was transferred to a polypropylene microtube and extracted with phenol–chloroform. One volume of 2-propanol was added to the aqueous layer, and the precipitated DNA was washed with ethanol and lyophilized. The DNA pellets were counted and dissolved in an appropriate volume of 10 mM Tris-HCl (pH 7.4). An equal number of cpm from each sample was applied to each lane of a 1% agarose gel except when the total cpm from the protein-free control was too low. After electrophoresis, the DNA bands were visualized by autoradiography of the dried gel. The relative intensity of the bands was occasionally measured by densitometry with a Beckman DU-8 spectrophotometer and the gel scan compuset.

The specificity of binding to DNA fragments JB and JC was assayed by the addition of protein to tubes containing 5 ng each of JB and JC, either ^{3}H or ^{32}P labeled. After filtration of the mixture, the filters were blotted, placed into liquid scintillation vials, and dissolved by the addition of 0.5 mL of 0.5 N HCl and 1 mL of ethyl acetate. After approximately 15 min with occasional mixing, 4 mL of Aquasol-2 was added and the vials were mixed well. The cpm due to [^{3}H]DNA and [^{32}P]DNA were determined by liquid scintillation counting with a Beckman LS 7500 counter.

Histone H1 Purification and Polyacrylamide Gel Electrophoresis. A crude rat liver H1 component was isolated from the 0.35–1.0 M NaCl extract of rat liver nuclei by adjusting the buffer to 0.4 M NaCl, 20 mM Tris, pH 7.0, and 0.1 mM PMSE and fractionating over the cation exchanger Bio-Rex 70 (van den Broek et al., 1973). To purify the H1 component, the basic fraction of the 0.35–1.0 M extract was chromatographed over a Bio-Rex 70 column (1.8 × 50 cm) and eluted with a linear 2-L gradient of guanidine hydrochloride (7–14%) in 0.1 M phosphate (pH 6.8) (Kinkaid & Cole, 1966). Absorbance at 218 nm detected individual fractions that were dialyzed to 0.01 N HCl and concentrated by lyophilization. The lyophilized fraction was dissolved in a small volume (0.5–1.0 mL) of 0.01 N HCl and chromatographed over a G-100 Sephadex (1.5 × 100 cm) molecular sieve column. The fractions were read at 204 nm, collected, and concentrated by lyophilization. Individual fractions were stored at −20 °C in distilled water.

Isolated fractions were characterized on two polyacrylamide slab gel electrophoretic systems. Acid–urea gel electrophoresis in 15% polyacrylamide slabs (40 × 15 × 0.15 cm) was carried out as described by Balhorn & Chalkley (1975). The slabs were preelectrophoresed (12–24 h at 15 mA) and electrophoresed (48 h at 6 mA) at room temperature. The gels were stained with amido black overnight and destained by diffusion. SDS gel electrophoresis was carried out by the method of Laemmli (1970) in discontinuous slab gels. The gels were stained with Coomassie blue and destained by diffusion.

Results

Identification of Preferential Binding at the 5′ End of the Rat Albumin Gene. The DNA-binding specificity of ionic strength released chromosomal proteins was determined with respect to the 5′ end of the rat albumin gene. The albumin

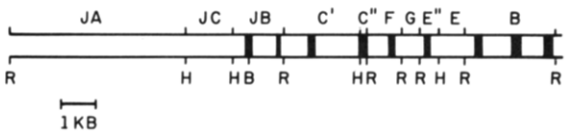

FIGURE 1: Restriction map of the rat albumin gene insert in λRSA30. Black bars are exons. The letters R, H, and B below the map are the restriction sites for the enzymes *Eco*RI, *Hin*dIII, and *Bst*EII, respectively. Letters above the map are the designations given to the fragments generated after digestion with *Eco*RI and *Hin*dIII (Sargent et al., 1981).

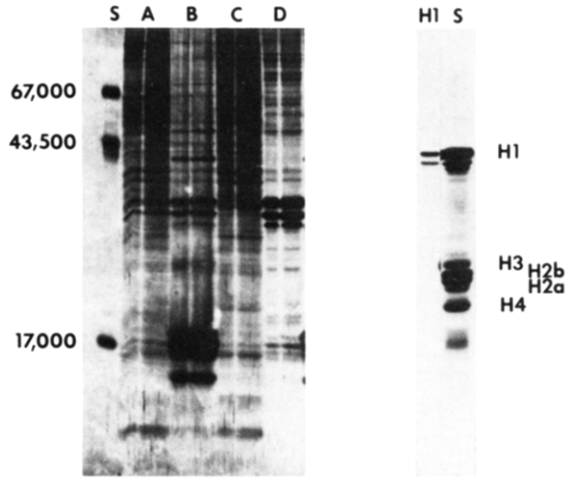

FIGURE 2: SDS–polyacrylamide gel analysis of the nuclear protein extracts. In part A, lanes A, B, C, and D, the samples were the 0.35–1.0 M NaCl nuclear extract, the 1.0–4.0 M NaCl nuclear extract, the acidic fraction of the 0.35–1.0 M NaCl extract, and the basic proteins of the 0.35–1.0 M NaCl nuclear extract, respectively. Lane S was standard polypeptides of M_r 67 000 (bovine serum albumin), M_r 43 000 (ovalbumin), and M_r 17 000 (cytochrome *c*). Polyacrylamide gel concentration was 11.5%. Part B shows the electrophoresis of the H1 purified fraction from Bio-Rex 70 chromatography and standards (S) of histones isolated by acid extraction of rat liver nuclei. The gel was 15% polyacrylamide.

genomic clone studied covers about 17 kilobases of rat DNA, including 7.8 kilobases of the 5′-flanking sequences. Figure 1 shows a map of the exons and restriction sites for *Eco*RI and *Hin*dIII present in the λRSA30 clone (Sargent et al., 1979, 1981). The protein extracts were prepared from freshly prepared nuclei. Fifty milligrams of rat DNA was selectively extracted with successive extractions of 0.01, 0.35, 1.0, and 4.0 M NaCl. The protein contents of the nuclear extracts were 7.7, 21, 5, and 21 mg, respectively. The DNA-binding preference and capability were not altered by freeze–thawing nor by storage for up to 6 months at −70 °C. One-dimensional denaturing gel electrophoresis (Figure 2, lanes A and B) shows the 0.35–1.0 M and 1.0–4.0 M salt extracts were composed of a heterogeneous group of chromosomal proteins. The 1.0–4.0 M extract was dominated by the presence of the core histone polypeptides. The high mobility group non-histones have been purified (data not shown) from the 0.01–0.35 M salt extract (Goodwin et al., 1980).

The interaction of labeled albumin gene fragments and the protein extracts from liver nuclei was measured by the nitrocellulose filter binding assay (Riggs et al., 1970). Less than 1% of the DNA was retained on the nitrocellulose filter when the DNA was filtered in the absence of protein. Greater than 80% of the DNA retained on the filter in the presence of added protein was eluted by the SDS solution, and 60–80% of the eluted DNA was recovered after processing of the DNA for subsequent agarose gel electrophoresis. Figure 3A shows at high protein levels (low dilution) the retention of DNA was high and the pattern of the protein-bound DNA fragments was the same as that of the input DNA. As the amount of protein from the 1.0 M NaCl extract was decreased (higher dilution) and the total retention dropped to 40% or less, a selective loss or enrichment of specific bands was observed. Initially, the smaller restriction fragments are lost. Because the number of nonspecific DNA binding sites is directly related to the size of the DNA fragment, the initial lost of the smaller DNA fragments is consistent with nonspecific DNA binding (McGhee & von Hipple, 1974). At higher protein dilution JA, JB, and B are retained over similarly sized restriction fragments, one arm of the charon 4A phage, C′, and JC, respectively. The band consistently seen to be enriched was the DNA fragment JB for several protein preparations. This fragment contains the first two exons of the gene and about 440 5′-flanking base pairs that contain the CAAT and the TATATTA box (Sargent et al., 1980). Quantitation of the bands from the autoradiograms by densitometry showed that JB could be enriched 5–8-fold compared with JC, a fragment similar in size to JB. Multiple exposures of each autoradiograph indicated the representative autoradiographs shown in Figure 3 are not an artifact of exposure. Fragment B, a 2700 base pair region from the middle of the albumin gene, and fragment JA, a 5200 base pair region 1700 base pairs from the initiation site of the albumin gene, were occasionally enriched. The nuclear protein extracts from previous 0.01 and 0.35 M NaCl washes were also tested for binding specificity. Figure 3B shows that extracts could retain DNA on membrane filters but no albumin restriction fragment was consistently enriched.

Characterization of the Protein–DNA Interaction. The laborious nature of the filter binding dictated the use of a more rapid assay for characterization of the protein–DNA interaction. The assay depends on the retention of protein–DNA complexes on nitrocellulose filters with the DNA labeled by nick translation (Rigby et al., 1977) with either [^{32}P]dNTP's or [^{3}H]dNTP's. Since JB contained the start site of transcription, equal amounts of two albumin restriction DNA fragments, JB and JC, were used in the assay. The preference of the 1.0 M NaCl extract for ^{32}P-labeled JB or ^{3}H-labeled JC was tested by mixing various amounts of protein with an equal amount of [^{32}P]JB and [^{3}H]JC. The assay is reported as the retention of both DNAs as a function of protein (Figure 4A) and the preference for JB with respect to JC as a function of total DNA bound (Figure 4B). As in the electrophoresis assay with the labeled albumin gene fragments, a greater binding of JB than JC was seen when less than 40% of the total DNA was bound. The proteins in the 0.35 M NaCl extract and 4.0 M extract showed a ratio close to 1 for protein levels that retained 5–75% of the input DNA, indicating little preference between JB and JC (Figure 5). The proteins in the 0.01 M NaCl extract had very little DNA-binding activity with a maximum of 6.5% of the DNA retained and no DNA fragment preference.

The effect of pH on the total binding of DNA and preference of the protein for JB or JC was tested by mixing the 1.0 M NaCl extract with DNA in Tris-HCl buffer ranging in pH from 7.2 to 9.0. Within this pH range no effect on bound DNA or on the ratio of bound JB to bound JC was observed. The effect of ionic strength seen in Figure 6 was apparent in both the total amount of bound DNA and the specificity of binding. Little binding of either JB or JC was seen at 0 mM KCl. At physiological KCl concentrations (25–100 mM) more JB was retained, reflected by the increase in the ratio of bound JB to bound JC. At higher KCl con-

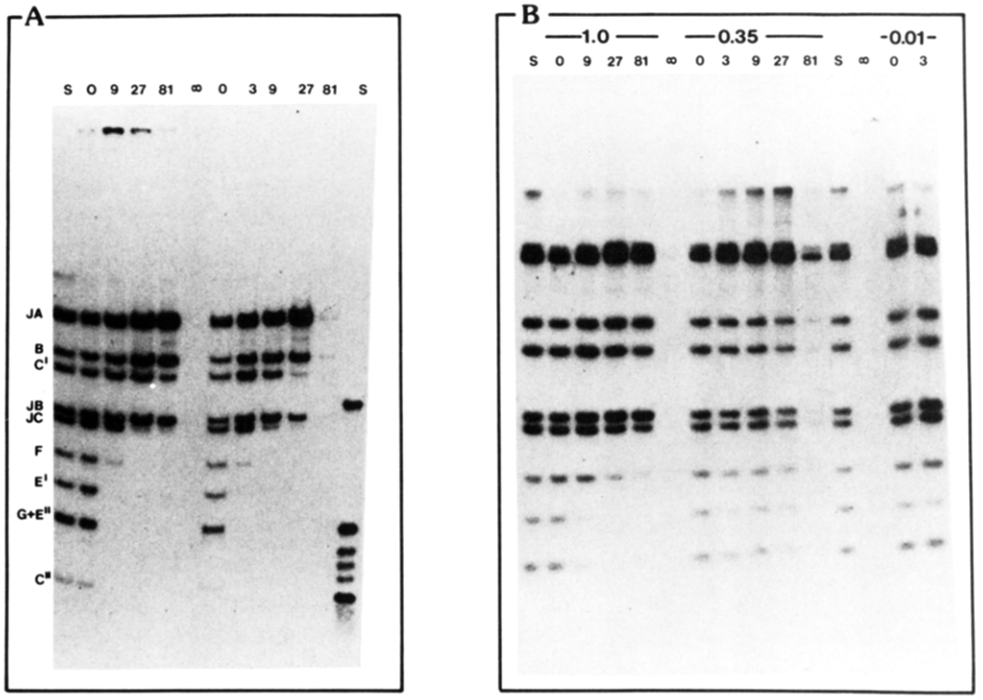

FIGURE 3: Filter binding assay with the digestion fragments from λRSA30. The selective binding of λRSA30 digestion fragments by nuclear extracts was tested by mixing the labeled fragments with serial dilutions of the protein samples, electrophoresis of the filter retained cpm, and autoradiography. (A) Preferential retention of bands by two 1.0 M NaCl nuclear extracts. The numbers at the top of the lanes refer to the dilution of protein extract that was mixed with 67 ng (15 000 cpm) of DNA. An equal number of cpm was applied to each protein sample lane, except for the infinite dilution lane and the 81-fold dilution lane, because of the very low level of cpm present in these samples. The left-hand lane marked S is the pattern of the input DNA, and the right-hand lane marked S is pBR322 digested with *Hin*fI. The percentage of the total cpm bound by the filter for each protein sample was, from left to right, 60.4, 40.4, 20.9, 6.9, 0.8, 81.7, 37.2, 14.9, 5.6, and 1.5. (B) Binding of λRSA30 digestion fragments by 0.01, 0.35, and 1.0 M NaCl nuclear extracts. The numbers at the top of the lanes refer to the dilution of protein extract that was mixed with 30 ng (47 000 cpm) of DNA fragments. An equal number of cpm was applied to each protein sample lane except for the two infinite dilution lanes and the 81-fold dilution of the 0.35 M NaCl extract because of the low level of cpm in these samples. The lanes marked S are two different levels of the input DNA as standards. The percentage of the total cpm bound by the filter for each protein sample was, from left to right, 59.2, 45.0, 12.8, 2.4, 0.5, 25.7, 20, 7.3, 3.2, 1.5, 0.4, 18.1, and 3.3.

centrations (250 mM), the amount of bound DNA decreased but the ratio of JB to JC remained high.

Identification of the Polypeptide with Preferential DNA-Binding Activity. Treatment of the 1.0 M extract with proteinase K (100 μg/mL) at 37 °C for 1 h destroyed the DNA-binding activity. The protein retained its DNA-binding activity and preference toward JB over JC when boiled for 10 min. However, boiling the extract did not appreciably purify the DNA-binding protein as determined by gel electrophoresis (data not shown). The 1.0 M extract was fractionated into basic or acidic polypeptides by cation-exchange chromatography (Bio-Rex 70) in 0.4 M NaCl–20 mM Tris, pH 8.0 (van den Broek et al., 1973). The acidic fraction and basic fraction were characterized by one-dimensional, sodium dodecyl sulfate gel electrophoresis. Figure 2C,D indicated the acidic fraction (lane C) was highly heterogeneous yet retained little of the DNA-binding activity or JB preference (Figure 7). The basic fraction (Figure 2D) was also heterogeneous but was characterized by the presence of an M_r 30 000 polypeptide and retained the DNA-binding activity as well as the preference of JB over JC (Figure 7).

Since histone H1 is characterized on one-dimensional gels as a group of polypeptides at around M_r 30 000, it was purified (Kinkade & Cole, 1966) and characterized by polyacrylamide gel electrophoresis (Figure 2, H1) on sodium dodecyl sulfate gels and acid–urea gels. The purified H1 polypeptides were composed of two to three H1 variants on SDS gels; however, isolated H1 electrophoresed on acid–urea gels showed a single component (data not shown). The ratio retention assay as shown in Figure 8 showed DNA-binding activity was associated with histone H1, with a 38-fold preference of JB over JC at 4–5% bound DNA. This ratio was a 4-fold increase over the preferential binding activity associated with the basic fraction of the 1.0 M NaCl extract. Purification of the H1 class of polypeptides retained the preferential affinity toward the 5′ end of the albumin gene. Electrophoretic assays with 5′-labeled *Eco*RI–*Hin*dIII fragments of λRSA30 indicated the preference resides within the JB fragment.

Discussion

The experiments described in this paper demonstrate the presence of the histone H1 site of interaction within the 5′ region of the rat albumin gene. The physiological significance is not yet determined, yet the involvement of H1 with DNA regions at the 5′ end of a structural gene may affect the structural organization of the gene (Thoma et al., 1983). DNA-binding proteins are characterized by a specific (K_s) and a nonspecific (K_{ns}) binding constant whose ratio (K_s/K_{ns}) remains independent with moderate variation in pH and physiological ionic strength (Lin & Riggs, 1975). These characteristics were found with the DNA-binding activity of the 0.35–1.0 M NaCl extract from rat liver nuclei. The preference for JB over JC at limiting protein was independent of pH (7.2–9.0). Ionic strength between 50 and 250 mM KCl decreased the total DNA binding, but JB preference was generally observed. At very low ionic strength (0 mM KCl) little DNA binding or preference for JB was observed, probably due to protein–protein interaction. To detect the DNA-binding activity in eukaryotic systems, the presence of genomic DNA, which adds such a large concentration of nonspecific binding sites that the specific interaction is effectively outcompeted by the nonspecific interactions, had to be reduced.

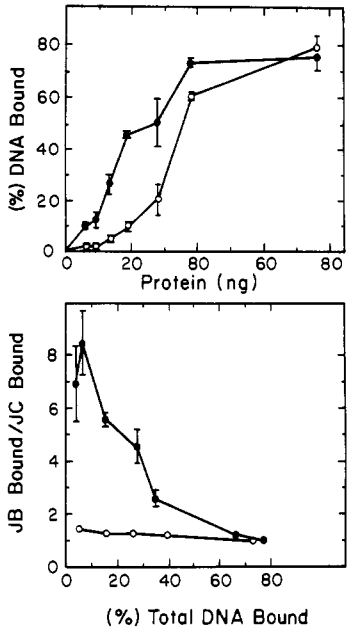

FIGURE 4: Effect of protein level on the binding of JB and JC. Various amounts of protein were mixed with a solution containing 5 ng each of [^{32}P]JB and [^{3}H]JC, and the amount of each fragment bound to protein under the standard conditions was determined by the double-label filter binding assay. (A) Binding of [^{32}P]JB (●) and [^{3}H]JC (○) by various amounts of protein from the 1.0 M NaCl extract is shown. (B) The ratio of [^{3}H]JB bound to [^{32}P]JC bound by various amounts of protein in the 1.0 M NaCl extract (●) and the 0.35 M NaCl extract (○) is shown as a function of the percentage of total DNA bound.

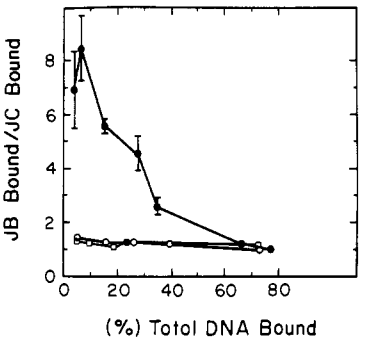

FIGURE 5: Effect of protein level on the binding of JB and JC. Various amounts of protein were mixed with a solution containing 5 ng each of [^{32}P]JB and [^{3}H]JC, and the amount of each fragment bound to the protein under the standard conditions was determined by the double-label filter assay. The ratio of [^{32}P]JB bound to [^{3}H]JC bound by various amounts of protein in the 0.01–0.35 M NaCl extract (○), the 0.35–1.0 M NaCl extract (●), and the 1.0–4.0 M NaCl extract (□) is shown as a function of the percentage of total DNA bound.

Cloned eukaryotic genes reduce the concentration of nonspecific binding sites such that the specific site of interaction can be detected within the rat ribosomal gene (Olson et al., 1983) or the major heat-shock protein gene in *Drosophila melanogaster* (Jack et al., 1981). Using this rationale, we demonstrated the presence of DNA-binding activities in rat liver nuclear extracts with preference toward several restriction fragments of the 5′ end of the rat albumin gene. One fragment was the *Eco*RI–*Hin*dIII restriction fragment containing the first two exons of the rat albumin gene flanked by 440 base pairs of the 5′-end "control sequences" (Sargent et al., 1980).

Since restriction fragment JB contains the RNA initiation site and the CAAT and the TATATTA sequences, characterization of the Pronase-sensitive DNA-binding activity in the 1.0 M salt extract was initiated. The DNA fragment, which was preferentially bound, was subcloned and used to

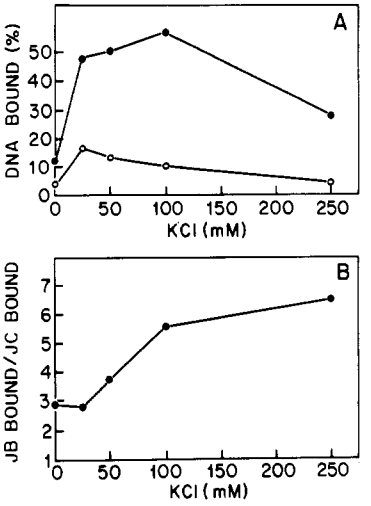

FIGURE 6: Effect of ionic strength on the binding of JB and JC. Protein (19 ng) from the 1.0 M NaCl extract was mixed with a solution containing 5 ng each of [^{32}P]JB and [^{3}H]JC, and the amount of each fragment bound to protein at different ionic strengths was determined by the double-label filter binding assay. (A) Binding of [^{32}P]JB (●) and [^{3}H]JC (○) was determined at different ionic strengths. (B) The ratio of [^{3}H]JC bound to [^{32}P]JB bound is shown as a function of ionic strength.

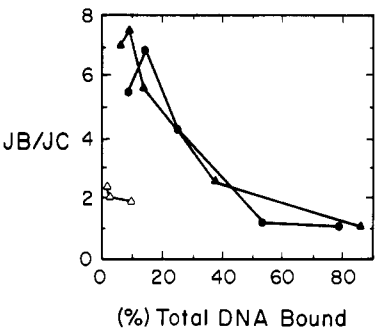

FIGURE 7: Effects of protein level on the binding of JB and JC. Various amounts of protein were mixed with a solution containing 5 ng each of [^{32}P]JB and [^{3}H]JC, and the amount of each fragment bound to the protein was determined by the double-label filter assay. The ratio of [^{32}P]JB bound to [^{3}H]JC bound by various amounts of proteins in the 0.35–1.0 M NaCl extract (●), the acidic proteins of this extract (△), and the basic proteins of the nuclear extract (▲) is shown as a function of the percentage of total DNA bound.

assay for the specific DNA-binding protein (Hsieh & Brutlag, 1979). The basis for this assay was the direct competition for the protein by the fragment of interest (JB) and another similarly sized DNA fragment (JC). The competitor DNA was the same size since the amount of nonspecific binding that might occur is directly proportional to the length of the DNA fragment (McGhee & von Hipple, 1974). Linear DNA defined the secondary and tertiary structure of the DNA, simplifying the interpretation of the retention assay. The protein component that can interact with high affinity toward the 5′ end of the albumin gene was identified as histone H1. Confirmation was made by isolating H1 to high homogeneity and showing that the preference for JB over JC was increased with respect to the protein extract.

Histone H1 interacts with chromatin at all levels of DNA packaging; H1 stabilizes the two turns of nucleosomal DNA (Simpson, 1978; Thoma et al., 1979) and it is required for the formation of intermediate higher order structures (Thoma & Kollar, 1977) as well as for the compact chromatin fibers (Finch & Klug, 1976; Brasch, 1976; Renz et al., 1977; Thoma et al., 1979; Thoma & Kollar, 1981). Little is known about how H1 molecules interact in chromatin to form higher order

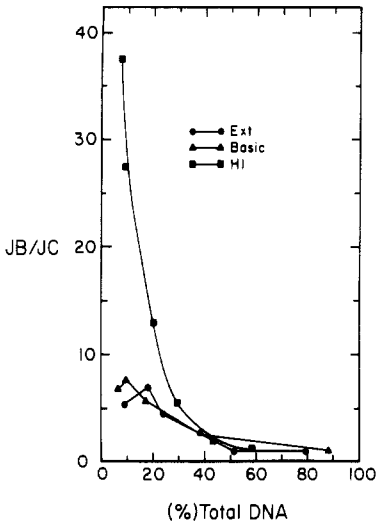

FIGURE 8: Effect of protein level on the binding of JB and JC. Various amounts of protein were mixed with a solution containing 5 ng each of [^{32}P]JB and [^{3}H]JC, and the amount of each fragment bound to the protein was determined by the double-label filter assay. The ratio of [^{32}P]JB bound to [^{3}H]JC bound by the protein in the 0.35–1.0 M NaCl nuclear extract (●), the basic proteins of the 0.35–1.0 M NaCl nuclear extract (▲), and the purified rat liver H1 histones (■) is shown as a function of the percentage of the total DNA bound.

structures and aggregates of chromatin fibers (Thoma et al., 1983). However, the interaction between calf thymus histone H1 and DNA can selectively precipitate homologous DNA at a limited number of binding sites. This suggests histone H1 can maintain specific interactions with certain DNA sequences that could act as nucleation points for high-order levels of chromatin organization (Diez-Caballero et al., 1981). The potential of H5 or H1 to induce a more "native" spacing between reconstituted or randomly aligned nucleosomes in an in vitro system also suggests a major structural role (Stein & Künzler, 1983; Künzler & Stein, 1983).

Histone H1 is composed of a number of closely related subcomponents that vary in amino acid sequence, molecular size, and postsynthetic modifications (Langan, 1982). These H1 variants can interact differently with DNA and nucleosomal structures (Welch & Cole, 1979; Liao & Cole, 1981; Ball et al., 1983). Hence, localization of the site of interaction of the subcomponent rat liver H1 and the subsequent identification of the protein domain involved in nucleic acid binding may yield precise information on the physiological role histone H1 has on the structure and function of chromatin and its relation to gene activity.

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Electronic Effects in the Acylation of α-Chymotrypsin by Substituted *N*-Benzoylimidazoles†

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ABSTRACT: Rate constants for the acylation of α-chymotrypsin by a series of acyl-substituted *N*-benzoylimidazoles have been determined by proflavin displacement from the active site. The second-order acylation rate constants k_2/K_m are large [e.g., that for *N*-(*m*-nitrobenzoyl)imidazole is 1.7×10^4 M^{-1} s^{-1} at pH 7.5], even though K_m must be quite large (plots of k vs. $k/[S]_0$ have infinite slopes). The values of k_2/K_m are nearly independent of pH in the range 5.0–9.0 when the substituent group is electron donating. Electron-withdrawing substituents produce an increase in k_2/K_m with increasing pH until a maximum is reached near pH 7. This is also the case in acylation by the *N*-[*p*-(dimethylamino)benzoyl]-*N*′-methylimidazolium ion (pK_{app} = 6.5). While the reaction of the N′-methylated derivative is via a positively charged species at all pH values, the unmethylated compounds react through both the neutral species and the conjugate acids, with the observed pH dependence depending on the relative values of the rate constants. The limiting value of k_2/K_m for the *N*-[*p*-(dimethylamino)benzoyl]-*N*′-methylimidazolium ion is 2.1 times less in D_2O than in H_2O. Thus, His-57 must be participating in the acylation reaction as a general base. The limiting values of k_2/K_m for the corresponding N′-methylated and unmethylated derivatives differ by a factor of only 150, which is similar to the difference in the second-order rate constants for nonenzymatic OH^--catalyzed hydrolysis. A plot of log k_2/K_m vs. the Hammett substituent constant σ is linear with a slope ρ of 0.9 for rate constants obtained at pH 7.5 and nearly zero for rate constants determined at pH <5.0. These ρ values are considerably smaller than those in nonenzymatic hydrolysis reactions of *N*-benzoylimidazoles. As a consequence, in the enzymatic acylation reactions, there must be less bond making with the nucleophile (Ser-195) in the transition state than in the hydrolysis reactions. The transition state for acylation must approximate the reactants. This is especially the case in acylation reactions of the *N*-benzoylimidazole conjugate acids.

Reactions catalyzed by α-chymotrypsin follow the scheme of eq 1, where ES′ is an acyl-enzyme intermediate. It has been

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_2 \qquad (1)$$
$$\qquad\qquad\qquad\qquad\quad + \qquad\qquad\qquad\qquad\quad P_1$$

well established that an acyl-enzyme intermediate is formed during the hydrolysis of both specific and nonspecific ester and amide substrates (Bender & Kezdy, 1964; Gutfreund & Sturtevant, 1956; Bender & Zerner, 1962; Zerner & Bender, 1964; Zerner et al., 1964; Kezdy et al., 1964). The acyl-enzyme is undoubtedly an ester of serine-195 (Bruice & Benkovic, 1966; Bender & Kezdy, 1964). Histidine-57 is also located at the active site and participates in both acylation and deacylation. Electronic substituent effects have been determined in deacylation of substituted benzoylchymotrypsins (Caplow & Jencks, 1962) and in acylation by substituted phenolic esters (Bender & Nakamura, 1962; Hubbard & Kirsch, 1972) and anilides (Inagami et al., 1965). Acylation of the enzyme by ester substrates is characterized by Hammett ρ values (Hammett, 1940) that are large and positive (Bender & Nakamura, 1962; Hubbard & Kirsch, 1972). In contrast, substituted anilides give a highly negative ρ value (−2.0) in acylation (Inagami et al., 1965), although nonproductive binding of the anilide substrates may be important (Fastrez & Fersht, 1973). A nitrogen isotope effect in the α-chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tryptophanamide requires that the C–N bond of the amide is broken in the rate-determining step (O'Leary & Kleutz, 1972). Breakdown of a tetrahedral intermediate formed in acylation reactions of amide substrates might therefore involve general acid catalysis by the His-57 conjugate acid since protonation of the leaving group is a requirement in hydrolysis reactions of amides to avoid expulsion of an amine anion. If the rate-determining step is breakdown of a tetrahedral intermediate, then a mechanistic role for His-57 in assisting nucleophilic attack by Ser-195 cannot be specified since formation of the tetrahedral intermediate will be an equilibrium step.

N-Acylimidazoles are kinetically favorable amide substrates for α-chymotrypsin (Schonbaum et al., 1961; Bender et al., 1962; Kogan et al., 1982). The rate constants k_2/K_m for acylation can be very large (Kogan et al., 1982) [e.g., the value of k_2/K_m for acylation by *N*-(β-phenylpropionyl)imidazole at pH 7.5 is 1.2×10^6 M^{-1} s^{-1} (30 °C)], even though binding to the enzyme must be weak (an ES complex cannot be experimentally detected). In these reactions, mechanisms involving the His-57 conjugate acid acting as a general acid can be ruled out (Kogan et al., 1982). Furthermore, it is unlikely that a kinetically significant tetrahedral intermediate is formed in nonenzymatic hydrolysis (Fife, 1965; Fee & Fife, 1966a,b; Fee, 1967) and alcoholysis (Oakenfull & Jencks, 1971) reactions of these compounds, and it is reasonable that this would

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