Antibody·Nucleic Acid Complexes. Identification of the Antigenic Determinant of a Murine Monoclonal Antibody Specific for Single-Stranded Nucleic Acids†

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ABSTRACT: Cloned hybrid cells, selected for their ability to secrete an IgG 2a immunoglobulin specific for single-stranded (ss) nucleic acids, were obtained by fusion of spleen cells from an unimmunized autoimmune MRL/1pr male mouse with nonsecreting myeloma cells (MOPC-21, line Sp2/0-Ag14). Designated MRss-1, this monoclonal antibody was (i) propagated by intraperitoneal injection of hybrid cells into pristane-treated, Balb/c mice, (ii) purified from the bulk of other proteins in ascites extracts by chromatography with DEAE-Sephacel adsorbent, and (iii) radiochemically labeled via reductive methylation using NaB^3H_4 and formaldehyde. The binding of ^{3}H-labeled antibody to immobilized (ssDNA–agarose, calf thymus) or soluble (fd DNA) ssDNA was rapid and dependent upon ssDNA and ionic strength, but not hydrogen ion concentration. Optimal binding occurred in both low and intermediate salt concentrations (0.01–0.25 M NaCl), yet was completely abolished above 0.30 M NaCl. The presence of guanine (Gua)-containing mono-, oligo-, and polynucleotides also abolished and/or decreased ^{3}H-labeled antibody binding to ssDNA–agarose. In these competition assays, the amount of Gua-containing mono- and oligonucleotides required to inhibit antibody binding by 50% (0.2–1.0 mg/mL) exceeded those of poly(G), rRNA, and fd DNA (i.e., 0.03–0.1 μg/mL) by 4 orders of magnitude. In contrast, (deoxy)ribose 5′-phosphate as well as other nucleic acid derivatives devoid of Gua failed to inhibit antibody binding. The above findings were substantiated by the observation that ^{3}H-labeled antibody bound to guanosine (G)- and guanidylate (pG)-conjugated Sepharose, yet not to other nucleoside (A, C, and U)- or nucleotide (pA, pC, and pU)-conjugated adsorbents. Last, the introduction of a methyl group at the N-2, O-6, and N-7 positions in the Gua ring system completely abolished antibody binding. Collectively, these results demonstrate that the MRss-1 antibody recognized single-stranded nucleic acid substrates by virtue of their content of guanidylate residues and, more specifically, by the presence of the Gua base moiety.

The presence of anti-nucleic acid antibodies in the serum of humans and various laboratory animals possessing various autoimmune traits is of considerable interest, both clinically and experimentally (Stollar, 1973, 1975; Notman et al., 1975; Provost, 1979; Lerner et al., 1981). Generally such sera contain a multitude of antibody populations with varying antigenic specificity (Stollar, 1975; Gilliam et al., 1980). Attempts to define individual specificities are often difficult or indeed impossible in view of this vast heterogeneity. Further, assessments of specificity have generally relied upon poorly defined, complex substrates such as calf thymus DNA. So that many of these inherent problems could be surmounted, various laboratories have focused attention upon the use of hybridoma technology (Kohler & Milstein, 1976; Galfre et al., 1977) to generate monoclonal antibodies specific for either double-stranded (ds) or single-stranded (ss) nucleic acids (Eilat et al., 1980; Andrzejewski et al., 1980; Hahn et al., 1980; Lerner et al., 1981). Subsequent selection of hybrid clones has resulted in the production of monoclonal antibodies that have distinctive binding specificities toward ds- and ssDNA[1] (Andrzejewski et al., 1980; Hahn et al., 1980), rRNA (Eilat et al., 1980; Lerner et al., 1981), and oligonucleotides (Andrzejewski et al., 1980). While the above investigations have suggested that monoclonal anti-DNA antibodies possess unique specificities for polynucleotides and/or for the ribose phosphate "backbone", the identity of the antigenic determinant within a nucleic acid remains to be ascertained.

We have concentrated our efforts, therefore, on defining the antigenic specificity of a single monoclonal antibody that appears equally specific for ssDNA and ssRNA substrates (Hahn et al., 1980). Previously designated D_2D_4 [see Hahn et al. (1980)], this particular antibody has been reclassified as MRss-1 to reflect its source (MRL/1pr mouse) and specificity (ssDNA). Specificity was assigned on the basis of competition assays in which unlabeled single-stranded nucleic acids (both RNA and DNA) inhibited the binding of MRss-1 to ^{125}I-labeled DNA to a greater extent than unlabeled dsDNA. While this particular DNA binding assay [commonly referred to as the "Farr assay"; see Wold et al. (1968) and Riley et al. (1979)] is useful for detecting anti-DNA antibodies, it provides only limited and/or no information as to the (i) quantity of antibody present, (ii) stoichiometry of the reaction(s) involved, (iii) nature of the antibody·nucleic acid complex, and (iv) identity of the antigenic determinant. Further, results obtained from these assays must be interpreted with caution since the ^{125}I-labeled DNA employed as substrate is usually undefined in terms of its content of single-stranded sequences.

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[1] Abbreviations: SLE, systemic lupus erthematosus; $NaDodSO_4$, sodium dodecyl sulfate; PO_4/NaCl, phosphate-buffered saline; Tris, tris(hydroxymethyl)aminomethane; Tris/NaCl, Tris-buffered saline; G–BSA, guanosine conjugated to bovine serum albumin; G–BSA/Sepharose, G–BSA conjugated to Sepharose; ssDNA and dsDNA, single- and double-stranded DNA, respectively; ssDNA–agarose, single-stranded DNA conjugated to agarose; fd DNA and φX174 DNA, circular, ssDNA genomes of bacteriophages fd and φX174; Ad II DNA, dsDNA genome of adenovirus type II; m_2^2G, N^2,N^2-dimethylguanosine; m^6G, O^6-methylguanosine; m^7G, 7-methylguanosine. Abbreviations for bases, nucleosides, nucleotides, oligonucleotides, and polynucleotides appear under Materials and Methods and are symbols recommended by the IUPAC–IUB Commission on Biochemical Nomenclature.

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To circumvent the latter obstacle and to obtain quantitative information regarding antibody binding to nucleic acids, we examined the ability of a [^{3}H]IgG fraction containing the MRss-1 antibody to bind to well-defined DNA substrates (ssDNA–agarose, Ad II DNA, fd DNA, etc.). As described herein, this approach has permitted us to (i) quantitate the binding of MRss-1 to numerous DNA substrates, (ii) purify MRss-1 to apparent homogeneity, and (iii), most important, identify the determinant within nucleic acids responsible for MRss-1 binding, namely, the entire heterocyclic ring system of guanine.

Materials and Methods

Materials. A variety of nucleosides, nucleotides, oligonucleotides, and polynucleotides were employed in this investigation and unless otherwise stated were purchased from Sigma Chemical Co. (St. Louis, MO). Nucleosides included guanosine (G), 7-methylguanosine (m^{7}G), N^2,N^2-dimethylguanosine (m$_2^2$G, which was obtained from Vega Fox), and O^6-methylguanosine (m^{6}G), the last nucleoside (m^{6}G) being synthesized in our laboratory by the method of Briscoe et al. (1978). Nucleotides included the 5′-PO$_4$ of G (GMP), deoxy-G (dGMP), adenosine (AMP), deoxy-A (dAMP), cytidine (CMP), deoxy-C (dCMP), uridine (UMP), and thymidine (TMP). Dinucleotides include thymidyl(3′–5′)deoxyadenosine (dTA), thymidyl(3′–5′)deoxycytidine (dTC), thymidyl(3′–5′)deoxyguanosine (dTG), thymidyl(3′–5′)thymidine (dTT), and guanidyl(3′–5′)guanosine (GG). Trinucleotides were adenylyl(3′–5′)adenylyl(3′–5′)uridine (AUU) and adenylyl-(3′–5′)uridylyl(3′–5′)guanosine (AUG). Homopolynucleotides included polyadenylate [poly(A)], polycytidylate [poly(C)], polyguanidylate [poly(G)], and polythymidylate [poly(T)]. Naturally occurring DNAs included the genomes of bacteriophage fd and ϕX174 (both the + strand and the replicate form or RF) and adenovirus type II (Ad II DNA) and were obtained from Miles and Bethesda Research Laboratories. The 5′-PO$_4$'s of both ribose and deoxyribose were purchased from Sigma. Naturally occurring RNAs (both rRNA and tRNA) were obtained from KB cells and prepared as previously described (Munns et al., 1974).

Various nucleosides (G, m$_2^2$G, m^{6}G, and m^{7}G) and 5′-nucleotides (AMP, CMP, GMP, and UMP) were coupled to bovine serum albumin (BSA) via periodate oxidation techniques (Erlanger & Beiser, 1964) and these conjugates, e.g., G–BSA and GMP–BSA, in turn coupled to Sepharose (G–BSA/Sepharose and GMP–BSA/Sepharose). The above coupling reactions have been described in detail [see Munns et al. (1977) and Munns & Liszewski (1980)]. Other adsorbents include protein A coupled Sepharose (protein A/Sepharose), single-stranded DNA coupled agarose (ssDNA–agarose), and DEAE-Sephacel and were purchased from Pharmacia and/or Bethesda Research Laboratories.

Production of MRss-1 Monoclonal Antibody. Procedures resulting in the production of various cloned hybrid cell lines secreting IgG monoclonal antibodies specific for either ssDNA or dsDNA substrates have been described (Hahn et al., 1980). Briefly, 10^8 spleen cells from male MRL/1pr mice (Bar Harbor, ME), 12–16 weeks old and possessing high levels of circulating anti-DNA antibodies, were fused with 10^7 myeloma cells (nonsecreting MOPC-21, line Sp2/0-Ag14). Hybrid cells were propagated in 24-well culture dishes in hypoxanthine/aminopterin/thymine selection medium. Supernatants from confluent monolayers were screened for the presence of anti-ssDNA and -dsDNA antibodies by a modified Farr assay (described below) and positive cultures cloned in soft agar over feeder layers (Coffino et al., 1972). One such clone, secreting monoclonal antibodies (designated MRss-1), is the subject of this paper.

To obtain preparative quantities of MRss-1 (2–10 mg), we propagated cloned hybrid cells in monolayer culture and subsequently injected them into the intraperitoneal cavity of pristane-treated Balb/cBy mice (10^8 cells/mouse). Approximately 10 days after inoculation, the abdominal swelling in mice was sufficient to collect about 10 mL of ascites fluid. After removal of cellular debris by centrifugation (1000g, 20 min), ascites fluid was adjusted to contain 5 mM $MgSO_4$ and 50 μg/mL DNase I (Worthington) to remove endogenous DNA (24 h, 4 °C). Digests were dialyzed against phosphate-buffered saline (PO_4/NaCl-15; 10 mM PO_4 and 15 mM NaCl, pH 7.4), and the IgG fraction was obtained by DEAE-Sephacel chromatography (Fahey & Horbett, 1959).

Characterization of MRss-1 Monoclonal Antibody. Conformational specificity (single-stranded nucleic acids), isotype (IgG 2a), and isoelectric focusing points (pI values of between 8.0 and 8.3 for MRss-1) were determined previously by Hahn et al. (1980). Specificity was assigned on the basis that unlabeled single-stranded nucleic acid substrates (vs. dsDNA) preferentially inhibited the binding of MRss-1 to ^{125}I-labeled DNA as evaluated by a modified Farr assay. As described in detail by Riley et al. (1979), this assay is based upon the ability of poly(ethylene glycol) to selectively precipitate antibody-bound nucleic acid substrates. For our purpose, endonuclease-treated ^{125}I-labeled calf thymus DNA (New England Nuclear) was incubated with various antibody-containing preparations and the percent of ^{125}I-labeled DNA precipitated by poly(ethylene glycol) used as semiquantitative measure of antibody concentration and specificity. Additionally ^{125}I-labeled DNA was characterized via benzoylated–naphthylated, DEAE-cellulose chromatography to evaluate its ssDNA content (Locker et al., 1977). As previously reported by Ebling & Hahn (1980), 60% of this material eluted as dsDNA and 40% as dsDNA that contained an undetermined amount of internal single-stranded regions.

MRss-1 isotype was determined via pH-dependent elution of its IgG fraction which had previously been bound to protein A/Sepharose (Ey et al., 1978). As evaluated by the Farr assay (see above), only those proteins eluting as IgG 2a bound ^{125}I-labeled DNA. The pI values for MRss-1 (between 8.0 and 8.3) were determined by electrophoresis of IgG fractions in isoelectric focusing gels (Nicolotti et al., 1980). The migration of MRss-1 to specific pH regions in the gel was determined by an ^{125}I-labeled DNA overlay technique previously described by Hahn et al. (1980). Additional details regarding this technique appear in the legend of Figure 3.

Purification and Radiochemical Labeling of MRss-1 Antibody. IgG fractions obtained by DEAE-Sephacel chromatography of ascites fluid and possessing moderate to high levels of anti-DNA antibody (via Farr assay) were labeled via reductive methylation using NaB^3H_4 and formaldehyde (Tack et al., 1980). Upon removal of unincorporated radioactivity (Sephadex G-25 and exhaustive dialysis), the specific activity of various preparations of [^{3}H]IgG ranged from 1×10^5 to 6×10^5 cpm/μg of protein. A control IgG fraction obtained from Balb/cBy mice and lacking anti-DNA antibodies was labeled in an identical manner (specific activity 4×10^5 cpm/μg). These preparations (1–2 mg/mL) were stored at −20 °C in 40% glycerol, aliquots of the former being further processed for purification of MRss-1 antibody.

MRss-1 purification consisted of (i) incubating the [^{3}H]IgG fraction with ssDNA–agarose for 30 min at 24 °C in Tris-buffered saline (Tris/NaCl-150; 10 mM Tris and 150 mM

NaCl, pH 7.4), (ii) removal of unbound protein by extensive washings of the adsorbent (via centrifugation and resuspension) with Tris/NaCl-150, and (iii) removal of immunospecifically adsorbed antibody by reincubating the ssDNA–agarose in Tris/NaCl-500 (10 mM Tris and 500 mM NaCl, pH 7.4) containing 1 mg/mL BSA. After centrifugation (2000*g*, 2 min), the ^{3}H-labeled antibody in the supernatant was dialyzed against two successive changes of Tris/NaCl-15 (for electrophoresis) or Tris/NaCl-150 containing 40% glycerol. The specific activity of this purified preparation (lacking carrier protein) ranged from 1×10^5 to 4×10^5 cpm/μg. IgG fractions containing the MRss-1 antibody as well as other affinity-purified preparations were characterized by electrophoresis in isoelectric focusing (IEF) and $NaDodSO_4$–acrylamide gels according to procedures described by Nicolotti et al. (1980) and by Gordon et al. (1977). Specific details regarding these electrophoretic systems appear in the legend of Figure 3.

Antibody Binding Assay. Quantitative binding assays consisted of incubating either [^{3}H]IgG or purified ^{3}H-labeled MRss-1 with an immobilized DNA substrate or with various well-defined soluble nucleic acids, i.e., substrate not immobilized. In the latter assay, ^{3}H-labeled antibody·nucleic acid complexes were separated from unbound ^{3}H-labeled protein by gel filtration chromatography using a Sepharose 4B matrix. While the unbound ^{3}H-labeled protein is retarded by the gel matrix, the ^{3}H-labeled antibody·nucleic acid complex is eluted in the void volume. Additional details regarding this procedure appear in the legend of Figure 1.

A second binding assay consisted of measuring the amount of ^{3}H-labeled MRss-1 bound to an immobilized DNA substrate (ssDNA–agarose). Incubations were conducted in 1.5-mL polypropylene tubes in a volume of 0.3 mL. Upon completion of the desired incubation period (usually 30 min at 24 °C), unbound ^{3}H-labeled protein was removed by repeated centrifugation (2000*g*, 2 min) and resuspension of the adsorbent with incubation buffer (usually 1.0 mL of Tris/NaCl-150). The quantity of ^{3}H-labeled antibody remaining bound to ssDNA–agarose was determined by digesting the adsorbent with 0.5 mL of NCS/H_2O (9:1) (NCS from Amersham/Searle) prior to the addition of organic scintillant. Alternatively, bound antibody could be quantitatively recovered by reincubating the adsorbent in Tris/NaCl-500. Other details regarding this batchwise adsorption and elution process appear in the legend of Figure 1.

Binding studies also were performed with nucleoside–BSA adsorbents (G–BSA/Sepharose and GMP–BSA/Sepharose). Assay conditions and subsequent measurements of ^{3}H-labeled antibody binding to these adsorbents were identical with those employing ssDNA–agarose. Nonspecific binding was routinely monitored by using an [^{3}H]IgG preparation obtained from Balb/cBy mice serum, i.e., from mice used to propagate the MRss-1 antibody which lack endogenous anti-DNA antibodies. Nonspecific binding in Tris/NaCl-150 was not detected in the gel filtration procedure and never exceeded 0.3% of the total input counts per minute when incubated with the various agarose or Sepharose adsorbents.

Results

Binding of [^{3}H]IgG to Soluble and Immobilized DNA Substrates. So that sufficient quantities of MRss-1 antibody could be obtained for these studies, the corresponding hybrid cell line was injected into mice and the resulting ascites fluid processed for the isolation of an IgG fraction via DEAE-Sephacel chromatography. While two major and two minor absorbance peaks were discernible (data not shown), only those proteins coeluting in the first or IgG-containing peak were capable of binding ^{125}I-labeled DNA as evaluated by the Farr assay, the IgG representing approximately 25% of the total protein in ascites fluid. Aliquots of this IgG preparation were subsequently labeled via reductive methylation, and the resulting [^{3}H]IgG was assessed in terms of its ability to bind to fd DNA and ssDNA–agarose. The results of these studies and the manner in which they were obtained are depicted in Figure 1. Whereas Sepharose 4B was employed to separate ^{3}H-labeled antibody·fd DNA complexes from unbound protein (Figure 1A), ^{3}H-labeled antibodies retained by ssDNA–agarose were quantitated after repeated washings of the adsorbent to remove unbound protein (Figure 1B).

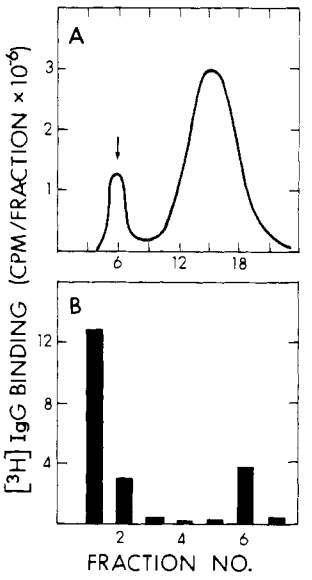

FIGURE 1: Isolation of ^{3}H-labeled antibody·nucleic acid complexes via gel filtration (A) and affinity (B) chromatographic techniques. For gel filtration, 25 μg of fd DNA was incubated in the presence of [^{3}H]IgG (21×10^6 cpm) in Tris/NaCl-150 (0.25 mL) containing 1–2 mg/mL carrier BSA. At the conclusion of the incubation (30 min, 24 °C), ^{3}H-labeled antibody·DNA complexes were separated from unbound ^{3}H-labeled protein via gel filtration (column, 25 × 0.6 cm) by using a Sepharose 4B matrix. Quantitation of ^{3}H-labeled antibody·nucleic acid complexes was determined by absorbance (254 nm) and radioactivity measurements of eluted material (0.2 mL/fraction), the arrow denoting the elution of fd DNA. In panel B, incubation conditions were the same as those in (A) except 30 μL of ssDNA–agarose (bed volume) was substituted for fd DNA. ^{3}H-Labeled protein not bound to the adsorbent was removed by repeatedly washing the ssDNA–agarose via centrifugation and resuspension with 1.0 mL of Tris/NaCl-150 (fractions 1–5). ^{3}H-Labeled antibody retained by ssDNA–agarose was recovered by reincubation the adsorbent in Tris/NaCl-500 and repeating the above washing procedure in Tris/NaCl-500 (fractions 6 and 7).

Effects of pH and Ionic Strength on [^{3}H]IgG Binding to ssDNA. Upon establishing that approximately 20% of the [^{3}H]IgG preparation could bind to the above substrates (Figure 1A,B), we reevaluated antibody binding as a function of pH and salt concentration, i.e., parameters that might provide some insight into antibody purification techniques. As the data in Figure 2 illustrate, binding was not significantly influenced by pH. Increased binding at pH 5.0 (25%) was attributed to nonspecific binding and confirmed with a control [^{3}H]IgG fraction devoid of anti-DNA antibodies. In contrast, optimal binding of the MRss-1 IgG preparation to ssDNA substrates occurred in low to intermediate salt concentration (0.01–0.25 M NaCl), yet was completely abolished when the salt concentration was increased to 0.3 M NaCl or above. This observation suggested that the MRss-1 antibody present in the [^{3}H]IgG fraction could be purified simply by altering the salt concentration during the adsorption, washing, and elution

Table I: Purification of MRss-1 Antibody by Affinity Chromatography with ssDNA–Agarose[a]

[^{3}H]IgG preparation	input radioactivity (cpm × 10^{-6})	input protein A_{280}	retained radioactivity (cpm × 10^{-6})	retained protein A_{280} (% of input)	sp act. of retained protein (cpm × 10^{-6}/ A_{280} unit)[b]
UNFX[^{3}H]IgG	450	1.19	85.4 (19.0)[c]	0.28	305
NR[^{3}H]IgG	250	0.62	<0.01 (<0.1)	<0.01	
IR[^{3}H]IgG	40	0.13	38.4 (96.0)	0.12	320

[a] Unfractionated [^{3}H]IgG (UNFX[^{3}H]IgG) was incubated with 250 μL of ssDNA–agarose in Tris/NaCl-150 for 30 min at 24 °C in a final volume of 0.5 mL. At the conclusion of the incubation period, the adsorbent was processed to obtain nonretained (NR[^{3}H]IgG) and immunospecifically retained (IR[^{3}H]IgG) fractions as described in the legend of Figure 1 and under Materials and Methods. Aliquots of these fractions were reincubated with ssDNA–agarose and the amounts of ^{3}H-labeled protein retained by the adsorbent determined. [b] A specific activity of 320 × 10^6 cpm/A_{280} equates to 230 000 cpm/μg of IgG assuming $E^{1\%}_{280}$ for IgG is 14.0. [c] Percent of input.

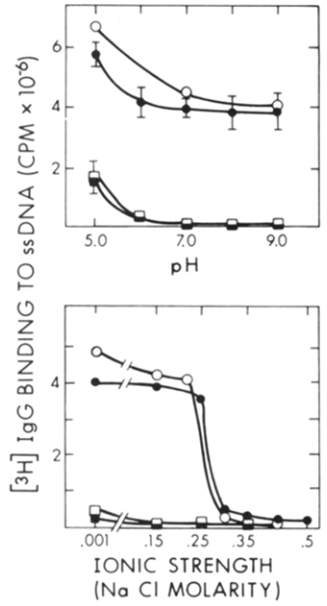

FIGURE 2: MRss-1 and [^{3}H]IgG binding to soluble (fd DNA) and immobilized (ssDNA–agarose) substrates: effects of pH and NaCl concentration. Incubation conditions and determination of [^{3}H]IgG binding were identical with those described in the legend of Figure 1. All assays for assessing pH were conducted in 0.15 M NaCl and buffered with either 0.01 M sodium acetate (pH 5.0–6.0), 0.01 M Tris (pH 7.0–8.0), or 0.01 M sodium borate (pH 9.0). Opened and closed circles represent binding of [^{3}H]IgG to fd DNA and ssDNA–agarose, respectively. Opened and closed squares reflect the binding of a control [^{3}H]IgG preparation to fd DNA and ssDNA–agarose, respectively. Control [^{3}H]IgG was obtained from Balb/cBy mouse serum.

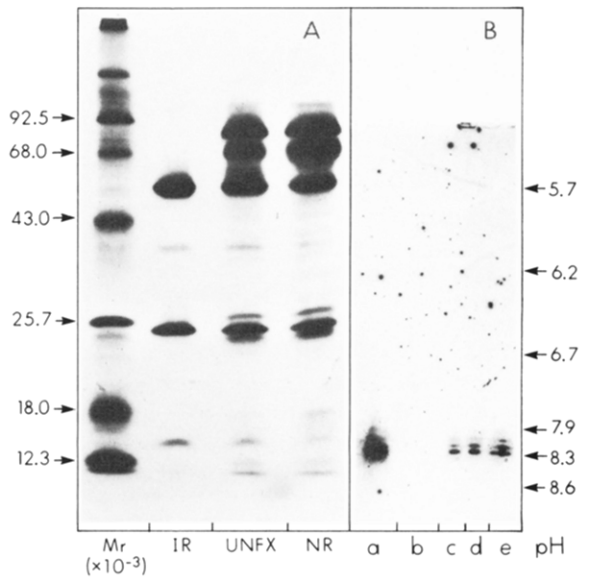

FIGURE 3: $NaDodSO_4$ (A) and IEF (B) electrophoretic characterization of MRss-1 IgG preparations representative of unfractionated (UNFX), nonretained (NR), and immunospecifically retained (IR) fractions. Prior to electrophoresis in $NaDodSO_4$ gels [15% polyacrylamide/0.3% bis(acrylamide)], ^{3}H-labeled preparations were heat denatured (100 °C for 3 min) in the presence of 2% $NaDodSO_4$ containing 0.1 M dithiothreitol. ^{14}C-Labeled proteins used as molecular weight standards were obtained from Bethesda Research Laboratories. Kodak film (XAR-5) was used for autoradiographs (5-day exposure). The quantity of radioactivity electrophoresed was 1.2 × 10^5, 2.0 × 10^5, and 3.0 × 10^5 cmp for IR, NR, and UNFX, respectively. Electrophoresis of unlabeled IgG preparations in IEF gels (5% polyacrylamide) was conducted without modification according to the procedure of Ebling & Hahn (1980). Upon completion of electrophoresis, immunoglobulins were precipitated with 21.5% Na_2SO_4 and immobilized within the gel matrix with dimethyl suberimidate, and the gel was incubated in the presence of [^{3}H]DNA (6 × 10^6 dpm). After extensive washings, the gel was autoradiographed (48-h exposure) to detect DNA-binding immunoglobulins. Tracts identified by lower case letters represent various IgG preparations: tract a, UNFX (120 μg); tract b, NR (100 μg); tracts c–e, IR (approximately 1, 2, and 5 μg, respectively). Other IEF gels not shown in Figure 3B revealed that IgG preparations (0.1–1.0 mg) obtained from Balb/cBy mice did not contain DNA-binding immunoglobulins.

phases of this affinity chromatographic procedure. The data presented in Table I verified this assumption by demonstrating that (i) a maximum of 20% of the unfractionated [^{3}H]IgG preparation was retained by ssDNA–agarose, (ii) nonretained [^{3}H]IgG reincubated with the adsorbent did not bind, and (iii) immunospecifically retained [^{3}H]IgG eluted with Tris/NaCl-500 and reincubated with ssDNA–agarose (in Tris/NaCl-150) bound to an extent of 95% or greater.

Electrophoretic Characterization of MRss-1 [^{3}H]IgG. For examination of the nature of the ^{3}H-labeled protein(s) retained by ssDNA–agarose as well as the effectiveness of this antibody purification scheme, aliquots representative of unfractionated (UNFX), nonretained (NR), and immunospecifically retained (IR) [^{3}H]IgG protein were denatured and subsequently characterized by $NaDodSO_4$ gel electrophoresis. Autoradiographs of these gels (Figure 3A) were purposely overexposed (150 h) to emphasize the extent of antibody purification. Whereas the UNFX preparation contained four major polypeptides (approximate molecular weights 25 000, 50 000, 70 000, and 85 000), only the lower molecular weight peptides representative of the heavy (50 000) and light (25 000) chains of IgG were present in the IR fraction. The appearance of two additional peptides in this preparation was of minor concern since they represented <0.5% of the total radioactivity electrophoresed. On the basis of their molecular weights (14 000 and 36 000) and reappearance in other IgG preparations radiochemically labeled with NaB^3H_4, we suspect they reflect minor breakdown products of the heavy chain of immunoglobulins.

Unlabeled preparations representative of UNFX, NR, and IR fractions were also characterized by electrophoresis in IEF gels. At the conclusion of electrophoresis, gels were incubated with ^{125}I-labeled DNA to detect for the presence of the MRss-1

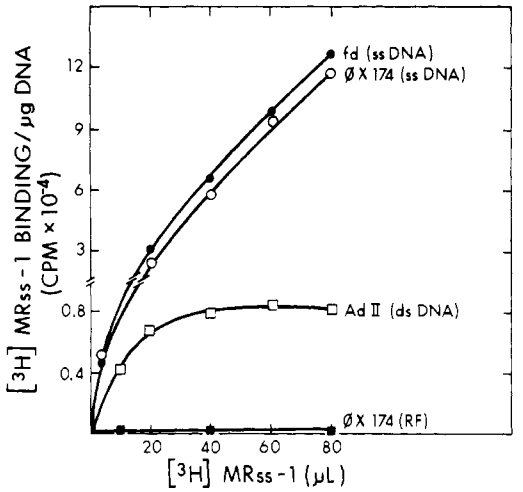

FIGURE 4: Binding of ^{3}H-labeled MRss-1 to soluble nucleic acid substrates representative of ssDNA (fd and ϕX174), dsDNA (Ad II DNA), and supercoiled DNA (ϕX174 RF DNA). Experimental conditions (i.e., incubation, chromatography, etc.) were identical with those described in the legend to Figure 1A. The quantity of nucleic acid used for each assay was 20 μg. The amount of ^{3}H-labeled MRss-1 used was variable and expressed in microliters (each microliter contained 0.14 μg of MRss-1 with a specific activity of 230000 cpm/μg; see Table I).

antibody. An autoradiograph of a representative IEF gel is presented in Figure 3B. These results indicated that the UNFX preparation (gel tract a) contained three prominent immunoglobulins (MRss-1 antibody) which bound ^{125}I-labeled DNA and possessed p*I* values of and between 8.0 and 8.3. Similar characterization of the NR (tract b) and IR (tracts c–e) proteins revealed that only the latter contained the MRss-1 antibody. Collectively, these electrophoretic systems emphasize the extent of purification of the MRss-1 antibody simply by employing various salt concentrations in conjunction with the ssDNA–agarose adsorbent. The presence of multiple proteins possessing closely related p*I* values was anticipated and appears to be a common feature of murine monoclonal immunoglobulins (Hahn et al., 1980; Nicolotti et al., 1980).

Conformational Specificity of MRss-1 Antibody. On the basis of ^{125}I-labeled DNA binding assays (Hahn et al., 1980), it was tentatively assumed that the MRss-1 antibody was specific for single-stranded nucleic acids. To confirm this assumption, we evaluated the binding of purified ^{3}H-labeled MRss-1 to well-defined DNA substrates representative of ssDNA (fd and ϕX174 DNA), dsDNA (Ad II DNA), and supercoiled DNA (ϕX174 RF DNA). This was accomplished by (i) incubating ^{3}H-labeled MRss-1 with each DNA substrate, (ii) separating the ^{3}H-labeled antibody·DNA complexes from unreacted antibody via Sepharose 4B chromatography, and (iii) determining the amount of antibody bound per microgram of DNA as a function of antibody concentration. These results (illustrated in Figure 4) revealed that the ssDNAs were the preferred substrates, with the amount of antibody bound being directly proportional to its concentration. In marked contrast, no binding was apparent with supercoiled DNA, and only minimal binding was observed with dsDNA (Ad II DNA). Of considerable interest, however, was the finding that MRss-1 binding to this particular preparation of Ad II DNA was saturable. Based upon the specific activity of ^{3}H-labeled MRss-1 and the molecular weight of Ad II DNA (24×10^6), we have determined that an average of five antibody molecules were bound to each Ad II DNA molecule. Whether this binding is random or restricted to specific locations within the Ad II genome remains to be determined. Not shown in Figure 4 was the additonal finding that MRss-1 binding to various heat-denatured Ad II DNA preparations was increased 10–20-fold and comparable to that obtained with identical quantities of fd DNA.

Identification of the Antigenic Determinant. While the above data provide convincing evidence that MRss-1 is specific for single-stranded nucleic acids, the identity of the determinant(s) responsible for antibody binding remained to be resolved. To obtain information in this regard, we investigated the ability of various well-defined, mono-, oligo-, and polynucleotides to inhibit the binding of ^{3}H-labeled MRss-1 to ssDNA–agarose. The results of these competition studies are displayed in Figure 5 and reveal two important findings. *First*, only those nucleic acid derivatives possessing a guanine (Gua) base effectively competed with ssDNA–agarose for MRss-1 antibody. *Second*, the quantity of Gua-containing constituents required to inhibit antibody binding by 50% spanned a con-

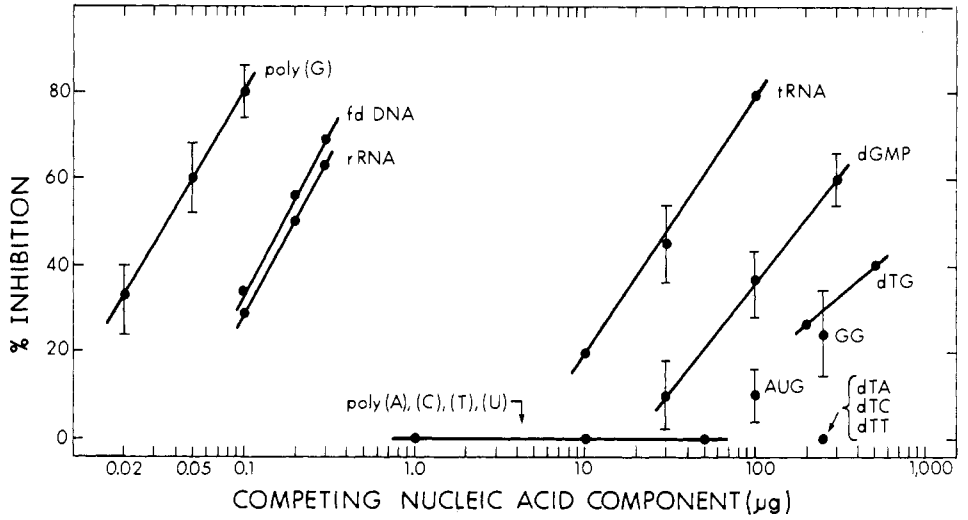

FIGURE 5: Inhibition of ^{3}H-labeled MRss-1 binding to ssDNA–agarose by various unlabeled nucleic acid components. Details regarding incubation conditions and measurement of bound ^{3}H-labeled antibody appear in the legend of Figure 1B and under Materials and Methods. Incubations were conducted in a reaction volume of 0.3 mL (Tris/NaCl-150) and contained 10 μL (bed volume) of ssDNA–agarose, 480000 cpm of [^{3}H]IgG, 1 mg/mL carrier BSA, and various quantities of competitors as listed (the latter were preincubated with [^{3}H]IgG for 10 min prior to adding ssDNA–agarose adsorbent). ^{3}H-Labeled antibody binding in control assays, i.e., without competing nucleic acid components, was 20% of the input radioactivity. Standard deviations reflect the results from three independent experiments. Not shown in the above figure was the finding that deoxyribose 5′-phosphate at 10 mg/mL did not inhibit the binding of [^{3}H]IgG to ssDNA–agarose.

Table II: Binding of ^{3}H-Labeled MRss-1 Antibody to Nucleoside(-tide)-Conjugated Sepharose Adsorbents[a]

adsorbent	retained ^{3}H-labeled protein (% input)	
	UNFX[^{3}H]IgG	IR[^{3}H]IgG
ssDNA-agarose	19.0	96.7
AMP-BSA/Sepharose	NB[b]	NB
CMP-BSA/Sepharose	NB[b]	NB
GMP-BSA/Sepharose	18.4[c]	94.2
UMP-BSA/Sepharose	NB	NB
A-BSA/Sepharose	NB	NB
G-BSA/Sepharose	18.9[c]	96.2
C-BSA/Sepharose	NB	NB
m_2^2G-BSA/Sepharose	NB	NB
m^6G-BSA/Sepharose	NB	NB
m^7G-BSA/Sepharose	NB	NB

[a] Aliquots of UNFX[^{3}H]IgG and IR[^{3}H]IgG were incubated with 25 μL (bed volume) of the above adsorbents in Tris/NaCl-150 for 30 min at 24 °C in a volume of 0.5 mL. Adsorbents were processed as described under Materials and Methods (also see legends to Figure 1 and Table I) for determination of immunospecifically retained ^{3}H-labeled protein and expressed as percent of input radioactivity. [b] NB is no binding or binding <0.3% of input radioactivity. [c] Electrophoretic characterization of the ^{3}H-labeled protein retained by Gua-containing adsorbents yielded results identical with those illustrated in Figure 3 (data not shown).

centration range (micrograms per milliliter) of approximately 4 orders of magnitude. For example, when identical incubation conditions were used, 0.035 and 1000 μg of poly(G) and dTG, respectively, were required to inhibit the binding of ^{3}H-labeled MRss-1 to ssDNA–agarose by 50%, i.e., a 1.4 × 10^4-fold difference. Noteworthy also were the findings that the quantities of fd DNA and rRNA (comprised of both 18S and 28S species), tRNA, and dGMP required to achieve the same degree of inhibition as poly(G) exceeded those of poly(G) by 4–5-fold (fd DNA and rRNA), by 850-fold (tRNA), and by 8500-fold (dGMP). The differences noted above are attributed to (i) base composition, (ii) size, and (iii) degree of secondary structure associated with each nucleic acid derivative and will be discussed in the next section.

The ability of poly(G) to be approximately 4 times more inhibitory than either fd DNA or rRNA suggests that the antigenic determinant is restricted to the size of a mononucleotide or less. For example, if the determinant was GG, then fd DNA and rRNA would be considerably less inhibitory relative to poly(G) since the presence of this dinucleotide occurs less frequently in fd DNA and rRNA; i.e., the theoretical occurrence of G is 25% (one-fourth) that of GG is 6.7% (one-sixteenth). This assumption is further supported by the findings in Figure 5 which reveal that GG is a very poor competitor relative to dGMP.

The above results, together with those which indicate a lack of significant inhibition associated with competitors lacking a Gua residue (e.g., deoxyribose 5′-phosphate, dTA, dTC, dTT, AUU, etc.), imply that the MRss-1 antibody recognized single-stranded nucleic acids by virtue of their content of Gua bases. To further substantiate this assumption, we conducted a series of binding studies in which ssDNA–agarose was replaced with various nucleoside(-tide)-coupled adsorbents. These results are presented in Table II and indicate that only those adsorbents containing immobilized Gua residues (dGMP/Sepharose and G–BSA/Sepharose) were capable of retaining the MRss-1 antibody. Elution and electrophoretic characterization of the ^{3}H-labeled protein retained by dGMP/Sepharose yielded autoradiographs identical with those obtained with the ssDNA–agarose adsorbent (see Figure 3).

In an attempt to further localize the major determinant(s) within the purine ring system of Gua, we included adsorbents containing guanosine (G), yet modified by the presence of a methyl group at N-7 (m^7G), O-6 (m^6G), and N-2 (m_2^2G), in these binding assays. The inability of any of these adsorbents to retain ^{3}H-labeled MRss-1 antibody further suggests that the determinant is not restricted to a specific portion of the heterocyclic ring system but rather encompasses the entire base structure.

Discussion and Conclusions

The reactions involved in the binding of an antibody to a nucleic acid are complex. For further complication of these types of reactions, many investigations have employed relatively undefined nucleic acid substrates and/or preparations of protein that contain a heterogeneous population of anti-nucleic acid antibodies. In an attempt to resolve many of these ambiguities, we focused our attention on a single monoclonal antibody (MRss-1) that appeared to be specific for single-stranded nucleic acids on the basis of various ^{125}I-labeled DNA binding assays (Hahn et al., 1980). To provide a more quantitative insight into antibody–nucleic acid interactions, we radiochemically labeled an IgG fraction containing MRss-1 antibody, and this ^{3}H-labeled preparation was assessed in terms of its ability to bind to ssDNA–agarose. Such evaluations (Figure 1) permitted us to purify MRss-1 to apparent homogeneity (Table I and Figure 3) and to identify both its conformational specificity (Figure 4) and the antigenic determinant responsible for antibody binding (Figure 5 and Table II).

Simplicity, speed, and adaptability to simultaneously process a large number of individual assays were the reasons for using ssDNA–agarose in most of our binding studies. However, since the ssDNA (calf thymus) immobilized to an agarose matrix is itself quite complex and relatively undefined, additional assays were performed with well-characterized soluble DNA substrates. While each method is distinctly different, both yielded comparable results when assessing ^{3}H-labeled MRss-1 binding as a function of pH, ionic strength (Figure 2), and nucleic acid concentration. In the latter instance, excess amounts of fd DNA, ssDNA–agarose, and GMP–BSA/Sepharose all bound a maximum of 20% of the [^{3}H]IgG preparation.

Similar assays using purified ^{3}H-labeled MRss-1 and Ad II DNA suggested that this substrate possesses a saturable number of antibody binding sites (Figure 4). While this limited binding (five antibodies per Ad II DNA molecule) can be attributed to variable quantities of single-strand nicks in the Ad II DNA, it is also plausible that the antibody is detecting specific nucleotide sequences within the genome that represent unpaired base regions within cruciform structures (Gierer, 1966; Wells et al., 1980). In the former instance, MRss-1 binding would be random; in the latter, it may be restricted to specific areas of the Ad II DNA genome.

Paramount in determining the specificity of MRss-1 antibody was the use of size- and sequenced-defined mono-, oligo-, and polynucleotides in competition assays. In this manner, it was easily demonstrated that only Gua-containing constituents were effective competitors. Once established, a simple and direct binding assay with nucleoside(-tide)-coupled adsorbents was utilized to verify these findings (Table II) as well as predict that the determinant encompassed the entire heterocyclic ring system of guanine. Quite possibly these and/or similar techniques can be applied to a host of other proteins associated with nucleic acids (polymerases, helix-destablizing proteins, ribosomal proteins, etc.) in an attempt to identify

those nucleotides and/or polynucleotide sequences responsible for protein-nucleic acid interactions.

Antibody specificity, however, is not the only useful information that can be obtained from competition assays. For example, by noting the difference in the amounts of various competitors [poly(G), fd DNA, etc.] required to inhibit antibody binding to the same extent (usually 50%), it is possible to make certain conclusions regarding the base composition, size, and degree of secondary structure associated with these molecules. First, nucleic acids large enough to span the intramolecular distance between antibody combining sites are more potent inhibitors than similar molecules lacking this size requirement [e.g., compare poly(G), fd DNA, and rRNA vs. tRNA in Figure 5]. Second, Gua-enriched molecules of sufficient size to span antibody combining sites are more potent inhibitors than similarly sized, Gua-poor molecules [compare poly(G) vs. fd DNA and rRNA vs. poly(A) etc.]. While the latter conclusion is based upon the concentration of the Gua determinant within each nucleic acid, the former is illustrative of the concepts of avidity and affinity [see Karush (1970)]. Simply stated, if an antigen has multiple antigenic determinants which are sterically oriented to be available to both antibody combining sites, then the strength of binding (or avidity) will be significantly stronger than that predicted from the affinity of a single binding site.

It is generally accepted that the intramolecular distance between antibody combining sites ranges from 60 to 100 Å (Nisonoff et al., 1975), while the distance between adjacent bases in nucleic acids is approximately 3 Å (Fisher & Williams, 1979). Conceivably, therefore, a tRNA molecule containing 80 nucleotides could possess a maximum length of 240 Å, i.e., a size more than sufficient to span intramolecular antibody combining sites. As the data in Figure 5 illustrate, tRNA is a very poor competitor in our binding assays. This, of course, is attributed to the secondary structure associated with tRNA which constricts the maximum length of these molecules to 80 Å (Ladner et al., 1975; Clark, 1978) and minimizes the actual number of unpaired Gua residues available for antibody binding.

On the basis of the example cited above, it appears probable that the MRss-1 antibody could be a useful reagent for fractionating small nucleic acids on the basis of their size, base composition, and secondary structure. Other potential applications include the (i) detection and location of potential transient and/or stable single-stranded sequences within ds-DNA and -RNA molecules and (ii) delineation of those nucleotide sequences present in ribonucleoproteins and exposed at the surface of these particles.

Whether most if not all anti-ssDNA and -ssRNA antibodies recognize nucleic acids by virtue of an individual base determinant remains to be elucidated. However, if this is true, then the employment of nucleoside-conjugated/Sepharose adsorbents such as those used in the present investigation (Table II) should prove quite valuable. Thus, if a unique subpopulation of antibodies specific for only one of the five bases present in nucleic acids is more prevalent in SLE (e.g., Gua) or in certain clinical subsets of SLE such as patients with nephritis, its identification and concentration could be readily determined with the appropriate nucleoside-conjugated adsorbent (e.g., G-BSA/Sepharose).

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Hydrodynamic Characterization of the Triton X-100 Solubilized Lactogenic Hormone Receptor of Rat Liver†

Randal C. Jaffe

ABSTRACT: Rat liver plasma membranes, prelabeled with radioactive human growth hormone, were extracted with Triton X-100. The solubilized lactogenic hormone receptor had a Stokes radius of 54.2 Å on Sepharose 6B chromatography. Numerical integration of the results of sedimentation experiments on the solubilized hormone–receptor–Triton X-100 complex in sucrose/H_2O and sucrose/^2H_2O density gradients produced an $s_{20,w} = 5.05 \times 10^{-13}$ S and a partial specific volume of 0.791 $cm^3\ g^{-1}$. From these data a molecular weight of 148 000 and frictional ratio of 1.40 for the hormone–receptor–Triton X-100 complex were calculated. Triton X-100 was calculated to comprise 32.4% of the complex, and thus, the hormone–receptor complex has a molecular weight of 99 800. These results indicate that the lactogenic hormone receptor, as would be expected of an integral membrane protein, has the capacity to bind a large amount of detergent.

The action of the lactogenic hormone prolactin is generally believed to be initiated by the binding of the hormone to a specific binding protein, known as the receptor, in the cell membrane. It is unknown, however, how the response of the cell is elicited by the binding of the hormone to its receptor on the cell surface. Specific lactogenic hormone binding sites have been described in a number of tissues in a wide variety of species (Posner et al., 1974a). The lactogenic hormone receptor in the female rat liver membrane has been characterized (Posner et al., 1974b; Herington et al., 1976), and its binding of hormone has been shown to be only slightly altered on solubilization with Triton X-100 (Bonifacino et al., 1981). The kinetics of binding of lactogenic hormones to rat liver membranes are similar to those found in another target tissue for the hormone, the rabbit mammary gland (Shiu & Friesen, 1974a).

We report here the hydrodynamic characterization of the lactogenic hormone receptor–detergent complex from rat liver membranes. Gel filtration and sedimentation velocity determination in sucrose/H_2O and sucrose/^2H_2O gradients were used to determine the size and weight of the receptor–detergent complex and to estimate the amount of detergent bound.

Experimental Procedures

Materials. ^{125}I for iodination of human growth hormone and ^3H_2O were obtained from Amersham Corp. Human growth hormone (hGH; HS2243E)[1] and ovine prolactin (oPRL; PS-14) were gifts from the NIAMDD, NIH. Triton X-100, toluene, and PPO–POPOP were purchased from RPI International. Sepharose 6B, Sephadex G-50, Blue Dextran 2000, and protein standards for the calibration of columns were obtained from Pharmacia Fine Chemicals. The proteins used as standards for the density gradient sedimentation experiments were purchased from Sigma, and ^2H_2O was obtained from Aldrich. Sprague-Dawley rats were obtained from King Animal Labs.

Preparation of Plasma Membranes. Rat liver membranes were prepared according to the method of Costlow & Gallagher (1977). In brief, tissue was excised and then homogenized in 10 volumes of 1 mM sodium bicarbonate and 0.5 mM calcium chloride, pH 7.0 (buffer A) at 4 °C. The homogenate was then diluted to 1 g of tissue/100 volumes of buffer A and filtered through cheesecloth. The homogenate was centrifuged at 900*g* for 20 min at 4 °C, and the resulting supernatant was then centrifuged at 20000*g* for 20 min at 4 °C. The pellet was resuspended in 1 volume of 25 mM sodium phosphate, 10 mM magnesium chloride, and 0.1% BSA, pH 7.0, and frozen until used.

Preparation of ^{125}I-hGH. Iodinated hGH was prepared by the lactoperoxidase method of Thorell & Johannson (1971) and purified by chromatography on Sephadex G-50. The specific activity, determined as described by Shiu & Friesen (1974a), ranged from 90 to 120 Ci g^{-1}.

Binding Reaction. Membranes were resuspended by homogenization, and 180–300 μg of membrane protein was incubated with ^{125}I-hGH (~1.2×10^6 cpm) in 10 mM Tris, 10 mM $MgCl_2$, and 0.1% BSA, pH 7.4, for 20 h at 19 °C. The total volume was 1.5 mL. The extent of nonspecific binding was determined by adding an excess of unlabeled oPRL (6 μg) to an identical set of tubes. After incubation, the tubes were cooled on ice and 2 mL of 10 mM sodium phosphate, pH 7.0, was added. The tubes were then centrifuged at 20000*g* for 15 min at 0–5 °C, the supernatant was removed, and the pellets were counted. Total binding was generally between 25% and 35% of the counts added of which approximately 50% represented specifically bound hormone.

Solubilization. Solubilization was performed by adding 1 mL of 1% Triton X-100 in 10 mM Tris, pH 7.4 (made up in ^2H_2O when the material was to be applied to sucrose/^2H_2O

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[1] Abbreviations: hGH, human growth hormone; ^{125}I-hGH, ^{125}I-labeled hGH; oPRL, ovine prolactin; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin; PPO–POPOP, 2,5-diphenyloxazole–1,4-bis(5-phenyloxazol-2-yl)benzene; cpm, counts per minute.

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