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Interaction of the Chick Oviduct Progesterone Receptor with Deoxyribonucleic Acid†

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ABSTRACT: The purified DNA binding component (receptor A) of the chick oviduct progesterone receptor has been analyzed for its ability to bind to the cloned ovalbumin gene and to plasmid DNA of various structural compositions. The rapid equilibrium filter adsorption assay of Riggs et al. [Riggs, A. D., Suzuki, H., & Bourgeois, S. (1970) *J. Mol. Biol. 48*, 67] has been used to demonstrate high affinity binding of the protein to DNA (K_{diss} = 10^{-10} M at 50 mM KCl, pH 7.2). Studies of association rates are consistent with equilibrium measurements ($t_{1/2}$ = 40–80 min). Association of purified receptor with DNA and the kinetics of the interaction have been verified independently by velocity sedimentation techniques. Direct binding assays were performed with the ovalbumin structural gene (cDNA), the entire natural ovalbumin gene containing seven intervening sequences, and various ovalbumin gene fragments coding for the 5′ end of the nuclear precursor RNA, intron–exon junctions, and the 3′-noncoding region of the gene. No DNA-sequence specificity was identified for the binding of the receptor protein to any region of ovalbumin gene DNA. In contrast, the structural integrity of the DNA template greatly affected receptor binding. The poorest affinity was to supercoiled DNA and to blunt end, linear duplex gene fragments. The receptor bound saturably to DNA containing limited nicks but became nonsaturable as nicks were increased. Binding of the protein to double-stranded DNA increased susceptibility of the DNA to digestion by the enzyme S_1, a single strand specific nuclease. On the basis of preferential receptor binding to single-stranded DNA, a possible mechanism involving DNA helix destabilization is discussed.

Our laboratory has been studying the progesterone receptor of chick oviduct [for a review, see Vedeckis et al. (1978)]. This protein is a potential gene regulatory factor in oviduct cells (O'Malley et al., 1972; Buller et al., 1975) on the basis of its appearance in oviduct nuclei in vivo and in vitro following administration of the hormone (O'Malley et al., 1970). In experiments performed in vitro, receptor–hormone complexes bind with high affinity to oviduct chromatin (Spelsberg et al., 1971). Progesterone induces the specific egg-white protein avidin (O'Malley, 1967), and, when administered to estrogenized immature chicks, progesterone will also induce ovalbumin (Palmiter, 1972). These hormonal events involve rapid increases in the rate of synthesis of ovalbumin mRNA and its accumulation (Swaneck et al., 1979a,b; Harris et al., 1975). The close temporal coupling between receptor occupancy in the nucleus and the induction of mRNA (Tsai et al., 1975) has led to the hypothesis that the receptors may act directly as inducers of specific gene transcription (O'Malley et al., 1972; Palmiter, 1972). One test of this idea would be to examine the interaction of the progesterone receptor protein with the DNA it potentially regulates. We have previously reported the purification of this protein and measurements of its interaction with DNA–cellulose and with labeled chick embryo fibroblast DNA in solution (Coty et al., 1979). Due to the lack of DNA fragments of discrete composition, it was not possible to determine kinetic parameters of this interaction. These characteristics are of particular interest for steroid receptor studies in view of reports in the literature using crude receptor preparations which suggested that receptor–DNA binding was weak and transitory (Yamamoto & Alberts, 1976) and preferential for double-stranded DNA (Kallos & Hollander, 1978). In the present communication, we have investigated the interaction of the DNA-binding component (receptor A)[1] of the progesterone receptor with cloned fragments of the chicken ovalbumin gene (Gannon et al., 1978; Dugaiczyk et al., 1978, 1979; Roop et al., 1978, and 1980)

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[1] Abbreviations used: receptor A and A protein, purified DNA binding component of the chick oviduct progesterone receptor; pOV4.5, pOV2.4, pOV1.8, and pOV9.2, cloned fragments of the chick ovalbumin gene in the bacterial plasmid pBR322 (refer to Figure 5); dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; HDP, helix destabilizing protein; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; bp, base pairs.

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and with other DNAs such as the plasmid pBR322.

The receptor A protein (Schrader & O'Malley, 1972) is one of two high-affinity progesterone binding molecules in oviduct cells. This protein has been purified to apparent homogeneity from both the immature chick and the adult laying hen (Coty et al., 1979; Schrader & O'Malley, 1978). The protein is a single polypeptide chain of molecular weight 79 000. Based upon structural (Birnbaumer et al., 1979; Schrader et al., 1975) and functional tests (O'Malley et al., 1973; Buller et al., 1975), we have hypothesized that the A component of the progesterone receptor may function as a gene regulatory protein in the chick oviduct. One possible mechanism for the control of gene expression in this tissue follows directly from the extensive work performed in prokaryotic systems and involves the binding of receptor molecules directly to DNA at a defined nucleotide sequence. Such specific interactions are exemplified by the binding of repressors and activators to gene operator regions, RNA polymerase to promoter sites, and restriction enzymes and methylases to exact nucleotide sequences (Maniatis et al., 1975; Gilbert et al., 1976; Chamberlin, 1974). Consequently, our initial experiments, reported in this paper, were designed to test for the possible binding of the progesterone receptor to a defined primary sequence(s) of the cloned ovalbumin gene.

Another potential mechanism for the control of gene expression involves the nonspecific association of neutral or slightly acidic proteins with DNA in a manner which destabilizes the DNA helix (Champoux, 1978). DNA helix destabilizing proteins are widely distributed in nature and have been associated with replication, recombination, repair, and transcription, all processes involving the formation and manipulation of transient single-stranded nucleic acid conformations (Alberts & Sternglaz, 1977). During the course of this investigation, it was found that the receptor A protein exhibits some of the common features of DNA-destabilizing proteins. This paper analyzes these characteristics and provides preliminary evidence which may help in understanding the mechanism by which this specialized receptor molecule transfers regulatory information from the steroid hormone to the target gene.

Experimental Procedures

Materials

[^{3}H]Progesterone (55 Ci/mmol) and [^{32}P]deoxynucleotide triphosphates (2000–3000 Ci/mmol) were obtained from Amersham/Searle. T_4-DNA ligase and DNase-free bovine serum albumin were from Bethesda Research Labs. All DNA restriction endonucleases were from Bethesda Research Labs except enzyme *Ava*II which was obtained from New England Bio Labs. T_4-polynucleotide kinase, *Escherichia coli* DNA polymerase I, and the "large fragment" of DNA polymerase I were from Boehringer Mannheim. All gel filtration media were from Pharmacia except resin AcA54 which was from LKB. All ion-exchange resins were from Reeve-Angel. DNA nuclease S_1 (*Aspergillus orzyae*) was purchased from Miles Laboratories and bacterial alkaline phosphatase from Worthington. Nitrocellulose filter sheets (type HAWG, 0.45 μm) was from Millipore, Aquasol II scintillation fluid from Beckman, and diethylstilbestrol from Sigma.

Methods

Receptor Purification. White leghorn chickens were implanted weekly with a 20-mg pellet of diethylstilbestrol (DES) until the stimulated oviduct tissue reached about 2 g per 5-week-old bird. This tissue was processed exactly as described previously (Coty et al., 1979). The resulting receptor A protein was monitored for purity by sodium dodecyl sulfate gel electrophoresis (7% acrylamide). When only the peak fractions from the ion-exchange columns were pooled, the resultant preparation exhibited a single major band of protein at 79 000. Overall yield of labeled receptor A was 1–2%. The receptor A concentration used in the filter binding assays was determined from the amount of bound [^{3}H]progesterone. Counting efficiency for ^{3}H was 35%. Hormone–receptor complexes were measured in the final column (phosphocellulose) eluate, and the receptor A was used immediately. DNA-binding activity remained stable for at least 16 h at 4 °C.

Restriction Endonuclease Cleavage of Double-Stranded DNA. Digestion of the plasmid and ovalbumin gene with restriction endonucleases followed the protocol provided by the supplier. Enzymes were added at 1 unit/μg of DNA substrate and incubated at 37 °C for 2–6 h. Reactions were stopped by adding EDTA to 50 mM followed by precipitation or by heating at 68 °C for 10 min.

Preparation of Ovalbumin Gene Fragments. The 583 and 750 base-pair ovalbumin gene fragments were prepared from pOV4.5, a recombinant DNA containing the left half of the natural ovalbumin gene contained in plasmid pBR322 (Roop et al., 1978). Chimeric plasmids were digested with *Ava*II, labeled at the 5′ termini by the polynucleotide kinase procedure, digested with *Pvu*II (583-bp fragment) or *Pst*I (750-bp fragment), and separated by electrophoresis through a 4% polyacrylamide gel. Polyacrylamide vertical slab gels (40 × 20 × 0.1 cm) were run in 50 mM Tris–borate, pH 8.3, and 1 mM EDTA. Gels were run 16 h at 200 V, and DNA fragments were eluted by the method of Maxam & Gilbert (1977).

Labeling of DNA by Nick Translation. DNA was labeled by using a modification of the procedure of Mackey et al. (1977). The reaction was performed in a final volume of 25 μL containing 50 mM Tris (pH 7.8), 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.5 μg of bovine serum albumin (BSA), 5 μg of DNA, 0.03 mM dATP and dGTP, and 0.005 mM [^{32}P]dCTP and [^{32}P]dTTP (2000–3000 Ci/mmol each, evaporated to dryness). These components were combined at 4 °C and preincubated at 14 °C for 10 min. No exogenous DNase was added in order to minimize nicking of the DNA template; each lot of DNA polymerase I was independently analyzed for endogenous DNase contamination, and the incubation time and temperature were adjusted accordingly. *E. coli* DNA polymerase I (5 μL, 20 units) was then added, and the reaction was incubated an additional 20 min at 14 °C. The reaction was stopped by addition of 25 μL of 0.2 M EDTA and 50 μL of redistilled phenol saturated with 10 mM Tris (pH 7.8) and 1 mM EDTA. After three phenol extractions to ensure removal of DNA polymerase I (necessary for low background binding in the filter assay), unincorporated dNTPs were separated from labeled DNA by gel filtration on a column (5-mL disposable pipet fitted with glass wool plug) of AcA54 by using 10 mM Tris (pH 7.8) and 1 mM EDTA. Radioactive fractions (0.25 mL) eluting in the void volume were pooled. The DNA was evaluated for extent of nicking by sucrose gradient centrifugation under neutral and alkaline conditions. In some experiments, the extent of nicks was reduced by incubation with bacteriophage T_4-ligase. The reaction (0.02 mL) contained 66 mM Tris (pH 7.6), 6.6 mM $MgCl_2$, 0.4 mM ATP, 10 mM dithiothreitol, 1–5 μg of nick-translated DNA, and 0.1 unit of T_4-DNA ligase. Incubation was for 16 h at 4 °C. This method dramatically reduced the amount of slow-sedimenting DNA from the alkaline sucrose gradient. The specific activity of the nicked-translated DNA

ranged from 1×10^6 to 8×10^6 cpm/μg.

Labeling of DNA 3′ Ends by Proteolyzed DNA Polymerase I. Supercoiled pBR322 was digested with *Eco*RI (cuts the plasmid once) to produce linear nick-free molecules containing four single-stranded nucleotides on each 5′ end of the duplex. The labeling reaction (0.025 mL) containing 50 mM Tris (pH 7.8), 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.5 μg of BSA, and 5 μg DNA was heated to 60 °C for 10 min to denature any reannealed ends and then cooled on ice. The reactants were added to [^{32}P]dATP and [^{32}P]TTP (labeled in the α position, evaporated to dryness; 0.005 mM in final incubation), and the reaction was initiated by the addition of 25 units of DNA polymerase I—"large fragment". This enzyme lacks 5′ to 3′ exonuclease activity but contains 3′ to 5′ single strand specific nuclease activity (Klenow et al., 1971) and was used to fill in the *Eco*RI-digested DNA by using the 3′ ends as primer. Incubation was for 3 h at 14 °C after which the reaction was stopped and treated as described for nick translation. The specific activity of the DNA ranged from 9×10^6 to 11×10^6 dpm/μg. The theoretical maximum specific activity, if all eight nucleotides were incorporated, was 13.5×10^6 dpm/μg; hence, the DNA was considered to be largely blunt ended.

DNA 5′ End Labeling. Restriction fragments of plasmid DNA (20 μg) were digested with bacterial alkaline phosphatase (20 μg) in a reaction volume of 200 μL containing 0.01 M Tris (pH 9.3) and 0.01 M $MgCl_2$. Incubation was at 68 °C for 30 min to inactivate any DNase contamination in the phosphatase. The mixture was then extracted twice with 200 μL of phenol and precipitated with 3 volumes of 95% ethanol. The pellet was redissolved in 50 μL of 50 mM glycine–NaOH (pH 9.5), 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM spermidine, and 25% glycerol. The DNA solution was added to 400 μCi of dried [γ-^{32}P]dATP (2000–3000 Ci/mmol), and 5 μL of T_4-polynucleotide kinase, 4000 units/mL, was added. The reaction was incubated at 37 °C for 30 min, stopped by the addition of 0.1 mL of 0.2 M EDTA, and immediately chromatographed on AcA54 to remove mononucleotides.

Sequence Determinations. After ethanol precipitation, the DNA was resolubilized in the appropriate buffer (200 μL) and redigested with suitable restriction enzyme to remove label from one of the 5′ termini. Fragments were separated by electrophoresis through a 4% polyacrylamide gel and eluted by the method of Maxam & Gilbert (1977). Sequences were determined (Maxam & Gilbert, 1977) to confirm the identity of the isolated ovalbumin gene fragments prior to binding assays.

Nitrocellulose Filter Assays. Direct binding assays of receptor with DNA were performed by modifications of the original procedure (Jones & Berg, 1966; Riggs et al., 1970a). Interassay and interfilter variation could be kept to a minimum if the nitrocellulose paper (type HAWG) was purchased in 10-ft continuous sheets, ensuring that all filters would originate from the same source and lot. When filter material containing low background (<2% input DNA binding adsorbed in the absence of added protein) was identified, 2-cm squares were cut from the roll by using a paper cutter. Filters were washed with distilled H_2O, soaked in 0.5 M KOH (0.75 mL/filter) for 15 min, and then washed exhaustively with distilled H_2O. Filters were soaked in buffer containing the appropriate ionic strength prior to use.

Binding reactions (0.8 mL) contained 10 mM Tris (pH 7.4), 10 mM $MgCl_2$, 0.125 mM EDTA, 1.5 mM thioglycerol, 100 μg/mL bovine serum albumin, 5% dimethylsulfoxide (Me_2SO), 6.25–200 mM KCl (binding buffer), and 25 ng/mL [^{32}P]DNA. The mixtures were cooled to 4 °C, and the reaction was initiated by the addition of purified receptor A. After incubation, each reaction was divided into three 0.2-mL aliquots and separately filtered on nitrocellulose membranes at 0.4 mL/min. Each filter was then washed twice with successive 0.2-mL aliquots of wash buffer containing 10 mM Tris (pH 7.4), 10 mM $MgCl_2$, 0.125 mM EDTA, and the appropriate molarity of KCl (wash buffer). Care was taken not to allow the filters to dry excessively between sample and wash applications. Filters were placed into 22-mL scintillation vials to which 0.5 mL of 0.5 HCl and 1 mL of ethyl acetate was added to dissolve the membrane. Samples were counted in 10 mL of Aquasol II scintillation fluid by using a Beckman LS 8000 counter. Data are expressed as the average of the triplicate-filtered reaction aliquots. Interfilter variation was less than 5%; interassay variation between replicate reaction tubes was less than 8%.

Sedimentation Velocity Analysis. Binding reaction mixtures containing various combinations of DNA and freshly prepared receptor A protein having bound [^{3}H]progesterone were incubated for 60 min at 4 °C. The binding buffer consisted of 1 mM Na_xPO_4 at pH 7 and included 0.1 mM EDTA and 100 mM NaCl. A small quantity (about 5000 cpm) of identical DNA, end labeled with ^{32}P to a specific activity of 10^6 cpm/μg, was included as a tracer. Samples (140 μL) were layered onto 5-mL, 5–20% sucrose density gradients made with binding buffer, sedimentated at 65 000 rpm for 35 min in a Beckman VTi 65 vertical tube rotor, fractionated, and counted for tritium and ^{32}P in a Beckman Model LS8000 liquid scintillation counter. In addition, the radioactivity representing material that sedimented to the bottom of the gradients was determined by counting the empty tube.

Nuclease S_1 Digestion of Protein–DNA Complexes. Nick-translated pBR322 (10^6 cpm/μg; 20 ng/reaction; 4×10^{-11} M double-stranded molecules) was incubated with increasing concentrations of receptor A protein in standard binding buffer supplemented with 20 μg/mL bovine serum albumin (DNase free), 1 mM $ZnCl_2$, and 20 mM sodium acetate (pH 5.8) to allow for subsequent digestion. Receptor ranged from 27 to 270 ng per reaction, resulting in concentrations of 1.7–17 nM. After incubation at 37 °C for 30 min to produce protein–DNA complexes, DNA nuclease S_1 (*Aspergillus orzyae*, 30 units) was added, and incubation was continued for an additional 30 min at 37 °C. Parallel tubes were included to determine (1) background digestion in the absence of added receptor A (16%), (2) the extent of digestion of heat-denatured, quick-cooled DNA (97%), and (3) digestion by receptor A alone in the absence of added S_1 enzyme (6.1%). The background digestion value of 16% has been subtracted from the data depicted in Figure 11. Preliminary time–course experiments indicated that digestion was complete with 20–30 min with this large excess of enzyme at this slightly elevated pH (pH 5.8 vs. the recommended pH 4.5). It was desirable to complete the digestion as rapidly as possible to avoid any significant protein exchange on the DNA. After digestion, two 100-μL aliquots were removed directly into 3 mL of cold 10% Cl_3AcOH containing 100 μg of BSA. After 45 min, the contents were filtered through nitrocellulose disks. Filters were washed with 5 mL of 5% cold Cl_3AcOH and prepared for scintillation counting as described above.

Results

Purification of the Progesterone Receptor DNA-Binding Protein. The chick oviduct progesterone receptor A protein was purified over 4500-fold by a procedure previously de-

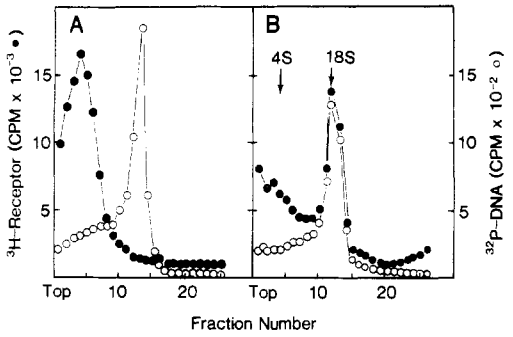

FIGURE 1: Sedimentation velocity analysis of receptor binding to DNA. The individual components and a mixture of DNA and purified receptor protein were analyzed on sucrose density gradients as described under Experimental Procedures. (A) A composite of the patterns obtained with 2 nM purified receptor A protein alone (●) or 4 nM (10 μg/mL) *Eco*RI-digested pBR322 DNA alone (○). (B) The pattern found after sedimentation of a mixture of DNA and receptor at these concentrations. The radioactivity which sedimented to the bottom of these gradients (and the percent of the total) was (A) 1300 cpm ^{3}H (10%) and 200 cpm ^{32}P (4%) and (B) 1100 cpm ^{3}H (10%) and 400 cpm ^{32}P (8%).

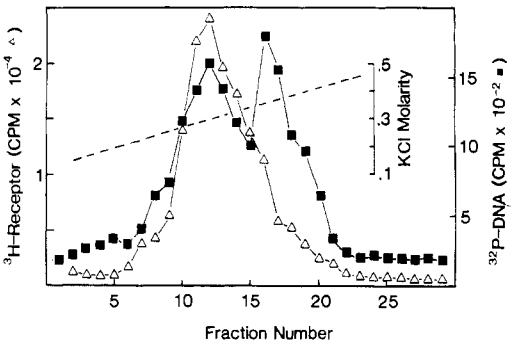

FIGURE 2: Elution of receptor A and DNA binding activity on the final phosphocellulose purification column. Aliquots (50 μL) of the eluate (1-mL fractions) from the final receptor A purification phosphocellulose column (0.5-mL bed volume) were removed and counted for [^{3}H]progesterone receptor complexes (△). Similar aliquots were removed, adjusted to 280 mM KCl, and analyzed for DNA-binding activity in the filter adsorption assay (■). Assay conditions were at 4 °C and 50 mM KCl. Data represent the average of triplicate filtrations.

veloped in this laboratory (Coty et al., 1979). The method capitalizes on the observation that in the native form the progesterone receptor is aggregated and does not itself bind to DNA (Schrader et al., 1977). When dissociated from the aggregate, receptor A binds strongly to DNA at physiological ionic strength (Schrader et al., 1972). Consequently, the procedure termed differential chromatography can be employed; in this procedure the receptor A protein is applied twice to phospho- and DNA–cellulose, first as an aggregated form and then, after dissociation, as a 4S monomeric species. The resulting material, when assessed for purity by polyacrylamide and acid–urea gel electrophoresis, exhibits a single major band of protein with a molecular weight of 79 000 (Coty et al., 1979).

We have used the nitrocellulose filter adsorption procedure (Jones & Berg, 1966; Riggs et al., 1970) to analyze the interaction between the receptor and DNA. In this assay [^{32}P]DNA is bound to nitrocellulose filters only when it is bound to protein. Since the receptor A purification scheme utilizes DNA–cellulose adsorption as a step in the isolation, small amounts of contaminants which copurify with the receptor would also be DNA-binding proteins. Thus a minor contaminating protein possessing very high affinity for DNA could mask the DNA-binding activity of the receptor. Several approaches were used to ascertain if the [^{3}H]progesterone-labeled receptor was responsible for the DNA binding observed with the nitrocellulose filter assay. The DNA-binding property of highly purified receptor A protein was demonstrated directly in sedimentation velocity experiments (Draper & von Hippel, 1979). In this experiment, rapid separation between free receptor protein and receptor–DNA complexes was obtained by sedimentation with a vertical tube rotor at 65 000 rpm for 35 min. As shown in Figure 1A, in the absence of DNA, the tritiated receptor sedimentated only a short distance into the gradient whereas the DNA alone, consisting of ^{32}P-end-labeled pBR322 linearized with the restriction endonuclease *Eco*RI, moved half way to the bottom (18 S). The gradient in Figure 1B was obtained with a mixture of receptor A protein and plasmid DNA and shows ^{3}H-labeled receptor sedimentating as a bound complex with the DNA molecules.

Another approach was to analyze the DNA-binding activity of the eluate from the final phosphocellulose column in the receptor A purification. Each fraction was assayed for DNA binding activity as shown in Figure 2. Analysis of the fractions shows a peak of labeled progesterone receptor eluting at 280 mM KCl; coincident with this is a peak of DNA-binding activity. The profile also reveals a second DNA-binding moiety eluting at 330 mM KCl. This contaminating protein, which appears as a faint band on overloaded polyacrylamide gels of the final receptor A preparation, was excluded from the present study by pooling only the peak-labeled receptor fractions. Thus, analyses of the purified receptor preparation show (1) predominantly one polypeptide chain by electrophoresis, (2) migration of the receptor with DNA in sedimentation experiments, and (3) coincidence between DNA binding and the [^{3}H]progesterone-labeled receptor elution profile from the final phosphocellulose column. We conclude from these experiments that receptor A protein is responsible for the observed DNA adsorption to nitrocellulose filters reported in this paper.

Filter Retention Efficiency and the Kinetics of Receptor–DNA Complex Formation. The interaction between the receptor protein and plasmid DNA was examined in detail by using the nitrocellulose filter assay. Since receptor A was labeled with bound [^{3}H]progesterone, the use of DNA labeled with [^{32}P]dNTPs permitted dual monitoring of both components in solution and on the filter. ^{32}P-Labeled DNAs (for example, pBR322 labeled by nick translation to specific activity of (1–8) × 10^6 cpm/μg) were incubated for 1 h at 4 °C in buffer containing 50 mM KCl with increasing concentrations of receptor A protein. Hyperbolic binding curves were obtained, typical examples of which can be found throughout this paper (see Figures 6, 8, and 11). In such experiments, receptor binding to the DNA appeared to be saturable. This was not due to reaction of all the DNA since the figures show that less than 100% of the input DNA was retained. This is a typical observation with this assay (Riggs et al., 1970b). In this case, it is probably due to the efficiency of the nitrocellulose filter to adsorb the complexes (wide fluctuations have been observed between filter lots) and the dissociation of the receptor–DNA complexes during the washing procedure. As long as these factors are constant throughout the binding curve, they can be corrected for, and the absolute concentration of receptor–DNA complexes can be calculated.

Another experiment was designed to analyze the effect of filter washing on the shape of the binding curve since the loss of protein–DNA complexes in this manner would affect the validity of the measurement. The results (not shown) demonstrate that, although the plateau of the saturation curve decreased with additional washes, the receptor concentration

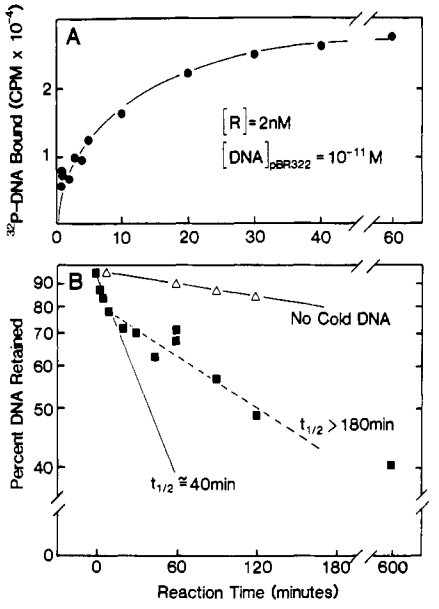

FIGURE 3: Association and dissociation rates of receptor–DNA interaction. (A) Purified receptor A (2 nM) was incubated for the designated length of time at 4 °C with nick-translated pBR322 DNA (25 ng; 10^{-11} M molecules). Reactions were stopped by filtration on nitrocellulose membranes. (B) Identical incubations were performed for 60 min after which a 100-fold mass excess of highly nicked, unlabeled DNA was added to provide a large surplus of protein binding sites (■). Control incubations contained no added nonradioactive DNA (△). At specified time intervals, aliquots were removed and filtered to quantitate remaining [^{32}P]DNA–receptor complexes.

necessary to achieve one-half saturation on the binding curve (10^{-10} M at 50 mM KCl; pH 7.2) was independent of this parameter. Thus, the retention efficiency does not vary across the binding curve.

Since receptor–DNA complexes dissociate slowly (discussed in the next paragraph), ^{32}P-labeled complexes were allowed to form, and then various size aliquots were removed, diluted to a constant volume, and filtered immediately. The percentage of labeled complexes retained by the filter was constant throughout the binding curve and over a wide range of receptor–DNA concentration. Hence, these experiments indicated that the filter assay can be reliably used to analyze receptor–DNA interaction and to determine the concentration of these complexes in solution.

The time course of the receptor–DNA binding reaction is shown in Figure 3A. Receptor protein (2 nM) was incubated with [^{32}P]pBR322 DNA, and aliquots were removed at various time points and filtered. Figure 3A shows that the protein binding reaction was essentially complete in 45 min. Incubation times of 60 min were used in all subsequent experiments.

The dissociation of the receptor–DNA complexes was analyzed as shown in Figure 3B. The receptor was allowed to bind to nick-translated [^{32}P]DNA for 60 min. Filtration over a period of several hours showed the complexes to be quite stable at 4 °C. In companion tubes, a 100-fold excess of unlabeled nicked DNA was added. The reaction was filtered at various times after this addition to determine the amount of receptor–[^{32}P]DNA complexes remaining. The results illustrated in Figure 3B show that receptor–DNA complexes dissociate slowly ($t_{1/2}$ = 40–180 min) under these pseudo-first-order conditions compared to the time required for filtration (20 s). The displacement curve apparently contained at least two components. This was the first indication that the DNA template contained more than one type of receptor binding configuration (discussed later in this paper). From

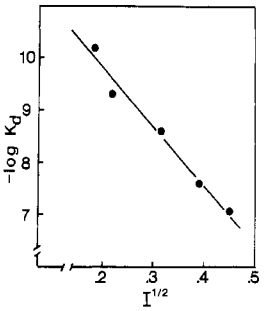

FIGURE 4: Effect of ionic strength on the dissociation constant. Complete binding curves were performed with receptor A (2 nM) and labeled pBR322 (10^{-11} M DNA chains) at various concentrations of KCl. The dissociation constant was calculated by Scatchard analysis.

these two experiments of Figure 3, we conclude that the receptor–DNA complexes are not transitory but rather are stable, and thus the nitrocellulose filter assay is, in fact, an equilibrium determination.

Effect of Ionic Strength on Receptor–DNA Interaction. The binding of receptor A to DNA is extremely sensitive to the ionic strength of the solution. Figure 4 illustrates the dramatic effect of the ionic strength on complex formation. A 5-fold increase in salt concentration results in a weakening of the dissociation constant by more than 2 orders of magnitude. Previous reports (Coty et al., 1979) have shown that receptor A binds strongly (at neutral pH) to DNA–cellulose and phosphocellulose and that elution from these resins requires a KCl concentration greater than 200 mM. Taken together, these findings indicate that strong electrostatic interactions are involved in the binding of receptor to DNA.

Receptor Binding to Ovalbumin Gene DNA. Having established the conditions necessary to use the nitrocellulose filter assay to study receptor A–DNA binding, we tested for possible nucleotide sequence specificity of the interaction. As a source of DNA, we used specific fragments of the chicken ovalbumin gene cloned in the bacterial plasmid pBR322. A diagram of the natural ovalbumin gene with relevant restriction endonuclease cleavage sites is illustrated in Figure 5 (Gannon et al., 1978; Lai et al., 1978; Breathnach et al., 1978). These fragments have been individually cloned (Woo et al., 1978; Dugaiczyk et al., 1978) and sequenced (McReynolds et al., 1978; Catterall et al., 1978).

Studies performed in our laboratory and others support the hypothesis that this gene is under the hormonal control of estrogen and progesterone; these hormones induce the in vitro and in vivo syntheses of new mRNA transcripts for ovalbumin (Cox et al., 1974; Harris et al., 1975; McKnight et al., 1975; Spelsberg & Cox, 1976; Swaneck et al., 1979a,b) prior to the hormone-induced appearance of ovalbumin (Means et al., 1972). If steroid receptor protein regulates the gene via direct DNA binding, the most likely gene regulatory regions where the protein might potentially interact would be the 5′ end of the gene, the intron–exon junction points, or the 3′ terminus noncoding region.

Plasmids containing the appropriate ovalbumin gene fragment (pOV2.4, pOV1.8, pOV4.5, pOV9.2) were labeled by nick translation and used as probes in the filter binding assay. Two progesterone receptor preparations were tested: receptor A, possessing evident DNA-binding activity (Coty et al., 1979), and receptor B, a similar protein thought to be a companion subunit of receptor A in the cytosolic receptor complex but lacking DNA-binding activity at ionic strength above 0.03 M KCl (Vedeckis et al., 1980). Figure 6 illustrates the titration of the [^{32}P]DNAs with purified receptor A and receptor B.

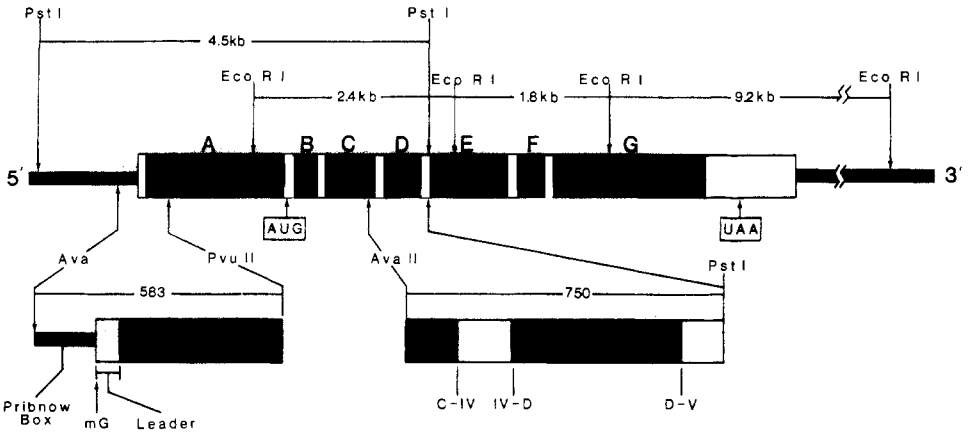

FIGURE 5: Physical map of the natural ovalbumin gene. The map shows the restriction fragments used in the receptor binding assays. Eight structural exons (unshaded) are interspersed by seven intervening sequences labeled A–G (shaded). Splice junction points in the 750-base-pair fragment are labeled according to Robertson and co-workers (1979).

Under standard incubation (50 mM KCl, 4 °C for 1 h) and binding assay conditions, the B protein fails to bind to the 2.4-kb fragment (not shown), to the parent plasmid, or to any other DNA tested. In sharp contrast, receptor A demonstrates high-affinity binding to these nicked DNA templates. The number of receptor binding sites on the DNA is unknown, and, therefore, it is not possible to determine a precise receptor–DNA dissociation constant. An approximate value of $K_{diss} = 2 \times 10^{-10}$ M receptor can be calculated if it is assumed that one receptor molecule bound to the DNA is sufficient to cause retention of the protein–DNA complex on the filter; the validity of this assumption is discussed below. This value of the dissociation constant is in agreement with our previous estimate (O'Malley et al., 1973) using gel filtration. Figure 6A also shows that there is no detectable difference in receptor A binding characteristics to any of the gene regions examined.

A small restriction fragment was desirable which would contain the DNA sequence coding for the 5′ end of the ovalbumin gene (Roop et al., 1980). Plasmid pBR322 containing a 4.5-kb fragment (Figure 5) of the ovalbumin gene (Dugaiczyk et al., 1978) was used to prepare this probe. The chimeric plasmid was cleaved with restriction endonuclease *Ava*II, digested with bacterial alkaline phosphatase, and labeled at the 5′ termini with bacteriophage T_4-polynucleotide kinase (Maxam & Gilbert, 1977). The fragments were then cleaved with *Pvu*II or *Pst*I (leaving only one labeled 5′ end of the dsDNA) and run on a 4% polyacrylamide slab gel. Two fragments of particular interest and similar size were eluted from the gel and their identity was confirmed by sequence analysis; one was a 583-bp piece containing the "Hogness Box" region (Goldberg, 1979; Pribnow, 1975), the $mRNA_{ov}$ cap site, and the AUG start codon for the message (Catterall et al., 1978); the other fragment (750 bp) contained three exon–intron junctions near the middle of the ovalbumin gene. Both fragments (and others not described here) were then tested for receptor binding with the filter assay. The results shown in Figure 6B demonstrate high-affinity binding to both DNA fragments, but no detectable differences between the putative regulatory region and the internal gene piece. Similar results were found when comparing other sections of the gene in this manner.

In addition, we have used several other approaches to investigate potential sequence-specific receptor A binding sites in the ovalbumin natural gene. One technique utilizes the observation that the rate of nucleotide base methylation by dimethyl sulfate can be altered when a protein is bound to the DNA (Gilbert et al., 1976). Such Me_2SO_4 protection experiments have been used to demonstrate that a proteolytic

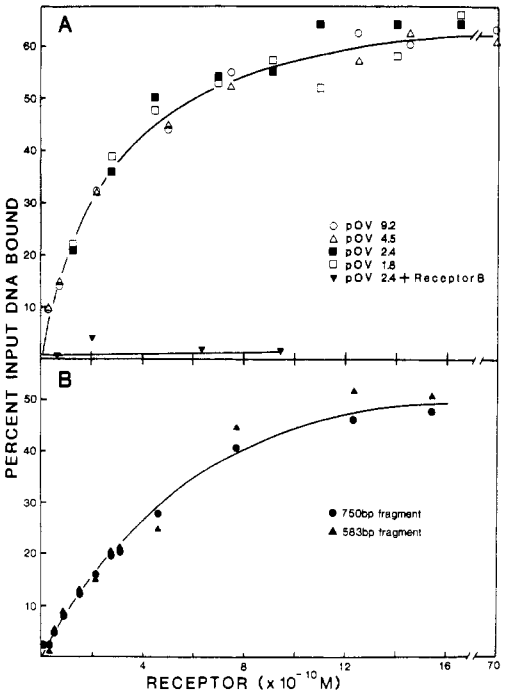

FIGURE 6: Direct equilibrium binding curves of the progesterone receptor interaction with the chick ovalbumin gene. (A) Increasing amounts of receptor A were added to binding buffer containing 50 mM KCl and 25 ng of [^{32}P]DNA. Symbols represent plasmids containing the appropriate ovalbumin gene fragment depicted in Figure 5. The DNA binding of a companion progesterone-binding protein (receptor B) is shown for comparison. (B) Two representative binding curves of receptor A complexes with isolated fragments of the ovalbumin gene. Binding buffer contained 50 mM KCl and 10 ng of 5′-end-labeled [^{32}P]DNA. Symbols represent fragments described in Figure 5. Triplicate determinations (SEM < 4%) are plotted as the percentage of input radioactivity per sample after subtracting background binding (<12%).

fragment of the *lac* repressor specifically binds to *lac* operator, even though this specific binding could not be detected with the filter assay (Ogata & Gilbert, 1978; Geisler & Weber, 1977). More recently, similar studies were reported (Sauer et al., 1979) for the specific binding of an amino-terminal portion of the λ repressor to λ operator DNA. When this technique is utilized, labeled restriction fragments of pOV4.5 were methylated in the presence or absence of receptor protein in an attempt to detect preferentially protected nucleotide sequences. The receptor appeared to bind nonspecifically throughout all the gene fragments tested (data not shown).

The technique termed DNA footprinting has also been employed to search for specific receptor A binding sites on the

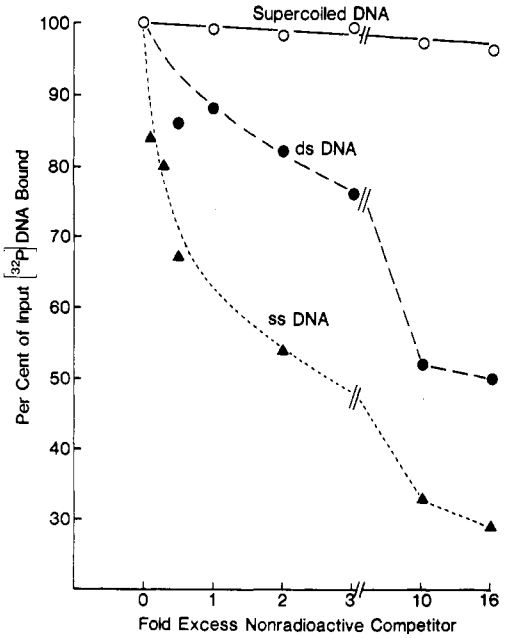

FIGURE 7: Equilibrium competition curves. Increasing concentrations (mass, ng/ng) of supercoiled pBR322 (○), linear, double-stranded pBR322 (●), or linear, heat-denatured pBR322 (▲) were included in binding reactions containing 25 ng of ^{32}P-labeled pBR322 and 5 × 10^{-10} M receptor A. Results are expressed as a fraction of the DNA adsorbed in the presence of cold competitor to the DNA adsorbed in its absence (38% at this receptor concentration).

ovalbumin gene (Schmitz & Galas, 1979). The footprinting method analyzes the susceptibility of DNA to DNase digestion in the presence or absence of the binding protein of interest; subsequent electrophoretic analysis reveals the sequence and location of the protected DNA (Maxam & Gilbert, 1977). Restriction fragments of the ovalbumin gene were ^{32}P-labeled at the 5′ ends with polynucleotide kinase, incubated with receptor A, digested with DNase I, and sequenced to produce footprint patterns (data not shown). As seen with the filter assay and Me_2SO_4 protection experiments, this method shows that, although the receptor binds with high affinity to DNA, there is no evidence for preferential sequence-specific interaction with the ovalbumin gene. We therefore turned our attention to examining the nature of receptor binding to plasmid DNA.

Preferential Receptor Binding to Single-Strand DNA. The interaction of receptor A with structural features of the DNA was investigated next. Our initial experiments (Figures 1–3) utilized DNA containing nicks and single-stranded gaps; we reasoned that receptor A might be associating with these structural features in a manner analagous to the DNA binding of catabolite activator protein (Mitra et al., 1975; Riggs et al., 1971) and RNA polymerase (Freeman & Jones, 1967).

Further analysis of the binding of receptor to labeled nicked DNA was obtained by using nonradioactive DNA in competition experiments. Three plasmid pBR322 DNA configurations were tested: (1) heat-denatured, linear DNA, (2) linear, double-stranded DNA, and (3) covalently closed, supercoiled DNA. The latter was of interest because it contains no cohesive 5′ ends and no nicks. A receptor concentration which produced about 40% maximum DNA binding was added to tubes containing a constant amount of randomly labeled, moderately nicked pBR322 (specific activity = 5 × 10^5 cpm/μg) plus increasing amounts of nonradioactive DNA competitor. As seen in Figure 7, both double-stranded and heat-denatured linear DNA competed effectively for receptor A binding whereas supercoiled DNA did not. This result

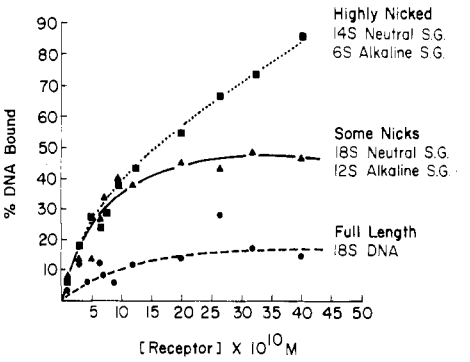

FIGURE 8: Receptor A binding curves to DNA containing nicks. Plasmid DNA was subjected to mild DNase digestion to produce single-strand scissions. Filter assays were performed as described in Figure 6. Aliquots of each DNA preparation were subjected to sucrose gradient ultracentrifugation under both neutral and alkaline (0.05 M NaOH) conditions as an index of nuclease digestion.

demonstrates that receptor A binds preferentially to polynucleotide ends or single-stranded gaps in the DNA. A further preference for single-stranded DNA is indicated since 50% competition required less heat-denatured DNA than intact double-stranded DNA.

This observation was substantiated by the following experiment. Supercoiled pBR322 was linearized with *Eco*RI, producing a four-nucleotide single-stranded region on each 5′ end. This DNA was labeled with [^{32}P]dATP and [^{32}P]TTP by using a large proteolytic fragment of DNA polymerase I (Klenow et al., 1971) to fill in the restriction cuts and produce blunt-ended DNA. This DNA was largely devoid of internal nicks as indicated by its 18S sedimentation coefficient on alkaline and neutral sucrose gradients (data not shown). Receptor titration showed a low level of binding to this DNA (Figure 8). The labeled DNA was then progressively nicked with pancreatic DNase I and tested for binding. Figure 8 illustrates that binding becomes more extensive even with only a few nicks introduced and finally becomes nonsaturable when nicking is extensive. Sedimentation coefficients of the nicked DNAs are indicated on the figure. The curves are representative of results obtained with a wide range of digestion conditions.

Nuclease Activity Is Absent from Receptor A. It is important for interpretation of our binding data that no enzymatic release of radioactivity occurred during the course of the filter assays. To test for endonuclease activity, receptor A was incubated with in vivo labeled supercoiled DNA (*φX174*, RFI) at several temperatures (0–42 °C), salt concentrations (6–150 mM KCl), and molar ratios (protein/DNA 1–200) under conditions similar to those used throughout this paper. The DNA was subsequently analyzed by the filter method of Kuhnlein et al. (1976) which is capable of detecting as little as one single-strand scission in supercoiled DNA. In control reactions, DNase I (0.02 μg/mL; Wang et al., 1977) introduced nicks into 95% of the DNA molecules after 15 min. In contrast, less than 1% of the DNA became nicked in the presence of receptor A, even after 60 min. Furthermore, endonuclease activity was not evident in velocity density gradients either, where no increase in slowly sedimenting ^{32}P was observed after incubation of receptor A with 5′- or 3′-end-labeled, full-length linear pBR322 (see Figure 1, for example). This observation also indicates the absence of exonuclease and phosphatase activity in the protein solutions. Finally, results from the filter assay show no decrease in labeled DNA bound to nitrocellulose filters even at high input ratios of receptor to DNA and incubation for 4 °C for 1 h (see Figure 6, for example), or overnight, or after 1 h at 25 °C (data not

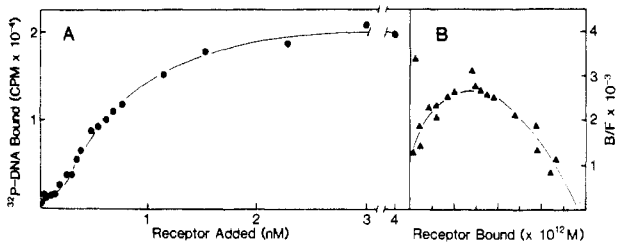

FIGURE 9: Binding to nick-free, blunt-ended DNA. (A) Supercoiled pBR322 was linearized by *Eco*RI digestion and labeled at the 3′ ends by using the large fragment of DNA polymerase I. Binding reactions were performed by using 5×10^{-12} M DNA chains. Background binding (8%) in the absence of added protein has been subtracted from each assay. (B) Scatchard plot of the data of panel A.

shown). We conclude that insignificant nuclease activity was present in the receptor A preparations.

Sigmoidal Binding of Receptor to Nick-Free DNA. Since receptor binding decreased when the single-stranded ends of the DNA were removed, it seemed possible that the protein was binding to these cohesive ends. This idea was tested by direct receptor binding assays performed with blunt-ended, nick-free [^{32}P]dsDNA. Incubations were performed at 4 °C and 100 mM KCl in order to reduce the incidence of transient base unpairing, particularly at the DNA ends. As expected, titration of this DNA template with receptor resulted in very poor binding, as shown in Figure 9 by the low percentage of input DNA bound at the plateau. The most striking feature of the binding curve is its sigmoidal shape, prominent at these low receptor concentrations. This effect is most probably due to one of two phenomena: (i) the retention efficiency of the filter assay requires that several protein molecules be bound to the DNA strand in order for the complex to be retained on the membrane, as has been shown to be the case for RNA polymerase binding to DNA (Freeman & Jones, 1967), or (ii) the positive cooperativity is observed because the binding of the first receptor molecule alters the DNA (by melting, bending, relaxing, etc.) in a manner which facilitates the binding of subsequent receptor proteins. Similar sigmoidal binding curves were recently reported by Tjian (1978) for the binding of T-antigen to SV40 DNA and shown by other methods to be a result of multiple interacting binding sites on the DNA. The filter assay is not capable of distinguishing between these two possibilities. The velocity sedimentation technique was used to analyze this more closely.

Receptor Binding Affects the Hydrodynamic Properties of DNA. Experiments were performed to measure the effect of increasing concentrations of receptor A protein on its interaction with DNA by using velocity sedimentation analysis on sucrose density gradients. In Figure 10 are shown the distributions of [^{3}H]progesterone and ^{32}P-labeled, *Eco*RI-cut pBR322 which resulted after sedimentation of mixtures containing increasing concentrations of receptor A and a constant concentration of DNA molecules (4 nM). It is apparent that the amount of receptor bound to the sedimenting DNA increases as expected when the receptor concentration is tripled from 2 to 6 nM. In addition, a small but significant change in sedimentation rate of the labeled DNA also occurs. Moreover, when the receptor A concentration is increased to 10 nM, the bulk of the DNA, together with most of the bound receptor protein, sediments to the bottom of the tube. The few percent of nucleic acid still found in the gradient is associated with a small peak of bound receptor A and has an *s* value about 25% greater than that of free DNA. Further increases in protein concentration lead to the absence of a bound receptor peak in the gradient and the appearance of

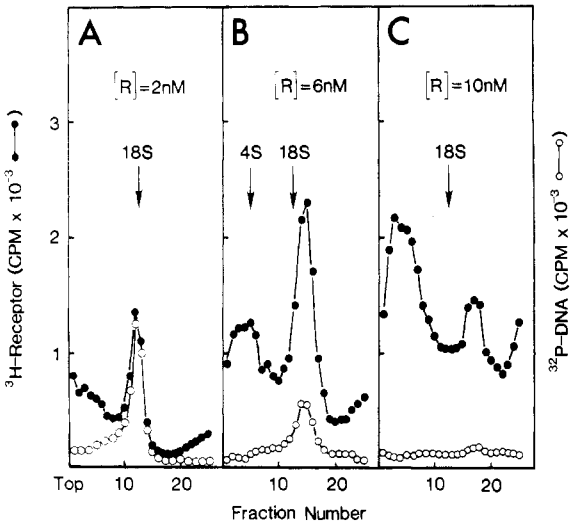

FIGURE 10: Receptor A interaction with linear pBR322 DNA studied by sedimentation velocity. Binding reaction mixtures containing the indicated concentrations of receptor A protein and 4 nM ^{32}P-labeled pBR322 digested with *Eco*RI were analyzed on sucrose density gradients. Receptor A is represented by the amount of ^{3}H present (●) and the DNA by ^{32}P (○). The radioactivity which sedimented to the bottom of each gradient, expressed also as a percentage of the total counts in each gradient, was the following: (A) 1100 cpm of ^{3}H (10%) and 400 cpm of ^{32}P (8%); (B) 3200 cpm of ^{3}H (15%) and 1350 cpm ^{32}P (26%); (C) 12000 cpm of ^{3}H (25%) and 3100 cpm of ^{32}P (65%).

all the DNA on the bottom of the gradient (data not shown).

In Figure 3B we showed that, once formed, receptor–DNA complexes remain stable for a time period which exceeds that of sedimentation. Consequently, the data in Figure 10 can be used to calculate the concentration of free vs. DNA-bound receptor A in the initial reaction mixtures. When the amount of inactive receptor is accounted for (by achieving 100% binding with a large DNA excess), the K_D obtained for receptor A binding to this DNA is about 5×10^{-9} M. This value is in good agreement with that obtained by the nitrocellulose filter binding assay at a similar pH (7.2) and ionic strength (100 mM; see Figure 4).

The change in sedimentation rate of the bound receptor–DNA complexes is apparently correlated with the average molar ratio of receptor bound to the DNA. This ratio is about 0.2, 1.2, and 3.0 mol/mol for the receptor–DNA peaks in Figure 10, A–C, respectively. In experiments at greater input ratios of receptor to DNA, more rapidly sedimenting complexes have been observed where the bound ratio of receptor to DNA molecules was greater than 3. This large change in sedimentation rate which occurs when only a few receptor molecules (two to ten) are bound on average per DNA molecule cannot be accounted for solely by the increment in molecular weight from the protein. Rather, the hydrodynamic shape of the complexes change either because (1) the DNA molecules are more compact in the presence of receptor, (2) several DNA molecules become linked together by the receptor protein, or (3) a combination of these interactions occurs.

Digestion of the Receptor–DNA Complex by S_1 Nuclease. The observations above suggested that receptor A binds preferentially to nicked DNA templates and also has a high affinity for single-stranded DNA. We have tested this latter finding by a method independent of the nitrocellulose filter binding assay, namely by digesting with the enzyme S_1, a single-strand specific nuclease, to detect single-strand regions of DNA in the presence or absence of receptor. Plasmid pBR322 DNA was labeled by nick translation under mild conditions (specific activity = 10^6 cpm/μg) to minimize the

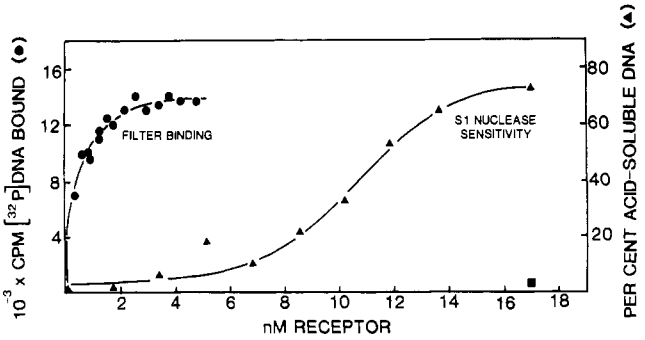

FIGURE 11: Nuclease S_1 digestion of DNA in the presence of receptor A. Nick-translated pBR322 (20 ng; 4×10^{11} M double-strand molecules) was incubated with increasing concentrations of receptor A protein as described in the text and under Experimental Procedures. Aliquots were then assayed for DNA binding activity (●). A parallel set of reactions was subjected to digestion by enzyme S_1 followed by precipitation with 10% trichloroacetic acid (▲). Values for control incubations are listed under Experimental Procedures. pBR322 DNA incubated with receptor A alone (no S_1 enzyme) under the same conditions (■).

single-stranded regions of the template, yet provide adequate receptor binding. Ten percent of the labeled DNA was soluble in trichloracetic acid (10% Cl_3AcOH), and after S_1 digestion this level had risen to 16%. Hence, approximately 84% of the DNA is S_1 insensitive (Cl_3AcOH precipitable) and considered to be double stranded in nature.

Receptor A was added to reaction tubes containing 20 ng of this DNA and incubated at 37 °C for 60 min followed by filter binding analysis. The results, shown in Figure 11, yield the expected dissociation constant of about 10^{-10} M. Parallel tubes were incubated at 37 °C for 30 min to form protein–DNA complexes after which S_1 nuclease (or counter buffer) was added followed by an additional incubation at 37 °C for 30 min. The data demonstrate that receptor A alone (no S_1 enzyme) does not digest the DNA, a finding which supports the previous conclusion that the receptor preparation contains little, if any, DNase activity. However, when receptor–DNA complexes are digested with S_1 nuclease, a 4-fold increase in Cl_3AcOH-soluble ^{32}P is observed. The molar input ratio of protein to pBR322 DNA chains ranges from 20 to 210 in this experiment and results in a 5-fold increase in Cl_3AcOH-soluble nucleotides.

The most dramatic increase in S_1 susceptability is seen at protein concentrations between 9 and 15 nM, representing input protein–DNA ratios of about 100 and 180. This may simply be a function of assay sensitivity where less added protein does not associate with enough ^{32}P-labeled polynucleotide to be measured by acid precipitation. Increasing the specific activity of the DNA leads to additional nicks and single-stranded gaps which, in turn, raises the digestion background and complicates the filter assay profiles (see Figure 8). Another explanation for this dramatic effect of receptor A at concentrations 50-fold greater than the observed binding constant may be related to cooperative localized interactions of individual receptor molecules on the DNA. The shape of the digestion curve is consistent with the idea that nonuniform receptor binding densities may exist along the DNA strand. Receptor A binding to double-stranded DNA may either destabilize the helix, producing localized single-strand regions, or bind to transient, preexisting single-stranded loops in otherwise duplex DNA. In either case, the binding renders the DNA more sensitive to digestion by a single-strand specific nuclease. Taken together with the results obtained by the membrane filtration assay and the velocity sedimentation technique, these data support the hypothesis that receptor A protein binds tightly and preferentially to single-stranded DNA.

Discussion

Although steroid hormone receptors are thought to regulate gene activity [reviews include Jensen & De Sombre (1973), O'Malley et al. (1973), Gorski & Gannon (1976), and Yamamoto & Alberts (1976)], the exact mechanism by which the receptor protein might mediate expression is virtually unknown. Previous attempts to investigate the interactions of receptors with DNA have relied on nucleic acid that is poorly defined (DNA cellulose), enormously complex (total DNA), or completely nonspecific (bacterial or phage DNA). Another major problem has been the impurity of the receptor protein being tested. Most reports have used crude cytosol or preparations in which the receptor represents less than 0.1% of the total protein. The present study eliminates these two major criticisms by using cloned fragments of the hormone-regulated ovalbumin gene together with the progesterone receptor purified to near homogeneity.

We have utilized the nitrocellulose filter adsorption technique of Jones & Berg (1966). This method has been used to study a myriad of DNA-binding proteins, including *lac* repressor (Riggs et al., 1970), λ repressor (Sauer et al., 1979; Sussman et al., 1978), *E. coli* catabolite activator protein (Riggs et al., 1971; Nissley et al., 1972), and SV-40 T-antigen (Tjian, 1979), to name only a few. A major advantage of this technique is its extreme sensitivity; nanogram quantities of protein can be used to generate a considerable amount of binding information. This is important in the chick oviduct system where the progesterone receptor represents only about 0.02% of the cellular protein and must be freshly prepared due to poor storage characteristics. Filter binding analyses were performed by using DNA fragments from throughout the entire natural ovalbumin gene including (i) the "Hogness (Pribnow) Box" sequence (Goldberg, 1979; Pribnow, 1975) and coding regions up to 1200 bp to the left of the 5′ end of the $mRNA_{oval}$, (ii) sequences coding for the 3′ end of the ovalbumin messenger, and 7000 bp beyond the 3′ end of the gene, and (iii) numerous internal ovalbumin DNA sequences containing from one to three splice–junction points joining gene introns and structural exons. No demonstrable binding specificity was detectable to any of these DNA probes.

Zubay and co-workers [see Riggs et al. (1971)] experienced similar difficulties using this assay to identify the site of *E. coli* CAP protein binding near the *lac* promoter. The specific CAP binding sequence was not detectable until a small (200 nucleotide), nick-free, blunt-ended *Hae*III fragment was used (Majors, 1975). A similar requirement was reported (Lin & Riggs) for *lac* repressor binding to total *E. coli* DNA; enrichment of operator sequences was a prerequisite to specific repressor binding in vitro. In the present experiments, precautions were taken to ensure that the ovalbumin gene probes were unique, nick free, and relatively small. In addition, the more recent techniques of Me_2SO_4 protection (Ogata & Gilbert, 1978) and DNA footprinting (Schmitz & Galas, 1979), which avoid potential filter assay artifacts, resulted in the same conclusions even when the DNA sequence being investigated was the only template present in the reaction. This would seem to rule out the masking of a specific nucleotide sequence by an enormous number of nonspecific sites (Yamamoto & Alberts, 1975). Other possibilities such as improper DNA conformation, the absence of obligatory chromosomal proteins, the denaturation of the receptor during purification, or the loss of a required cofactor or subunit cannot be excluded by these results.

Although specific binding was not observed, the foregoing results demonstrate that receptor A does bind with high affinity to general DNA and with marked preference for nicks and single-stranded regions. It is not possible to calculate an exact dissociation constant since we are not analyzing a unique DNA binding site and since the number of bound receptors required to retain the DNA protein complex on the filter is unknown. Freeman & Jones (1967), for example, determined that at least five RNA polymerase molecules were necessary to retain a single molecule of T_7 DNA on a filter and that numerous independent DNA sites were involved in polymerase binding. This may be the case with receptor binding to DNA nicks since Figure 8 displays binding curves indicating that each nick acts as an independent binding site. In contrast, receptor association with blunt-end, nick-free DNA produces sigmoidal curves (Figure 9) indicative of multiple binding events interacting on the same DNA molecule. These binding data yield a Hill coefficient of 1.9 ± 0.3 (Hill, 1910), suggesting that perhaps two receptors are sufficient to retain this DNA on the filter.

The velocity sedimentation experiments presented here served two purposes. First, by demonstrating comigration of [^{3}H]progesterone receptor with [^{32}P]DNA, the method provided further evidence that the purified receptor preparation retains its functional DNA-binding activity. Estimates of the equilibrium constant by this method and by filter binding are in agreement, indicating that the receptor is not contaminated with other, even more active, DNA binding proteins. Second, the perturbation of the sedimentation coefficient of the plasmid DNA by receptor allowed estimation of the stoichiometry of this reaction. As the sedimentation coefficient increases above 18 S, calculation of receptor:DNA ratios reveals a value of ≥ 1. At large receptor excess, the DNA sediments at >50 S. This behavior is consistent with the cooperative-binding concept developed from the nitrocellulose filter and nuclease digestion experiments.

These findings of weak protein binding to native DNA but strong binding to single-strand DNA lead us to speculate that the receptor can act as a helix-destabilizing protein (HDP). By definition (Alberts & Sternglanz, 1977; Champoux, 1978), a HDP displays preferential binding to ssDNA. Although the receptor A protein meets this qualification, there is no evidence at the present time implicating helix destabilization as a functional mechanism of action of the protein. Nevertheless, there are other similarities between receptor A and the more thoroughly characterized HDPs which are worth noting. Both the progesterone receptor and bacteriphage T_4 HDP (gene 32 protein) have been shown to form protein–protein complexes in which the nucleotide binding site is apparently occluded (Vedeckis et al., 1978; Coty et al., 1979; Carroll et al., 1975; Kelly & von Hippel, 1976). Progesterone receptor undergoes self-aggregation in solution; high molecular weight receptor complexes readily appear during receptor manipulations, especially as the species is purified (Coty et al., 1979). This observation is also evident with T_4 HDP where the protein displays a concentration-dependent indefinite self-aggregation in solution in the absence of DNA. This aggregation may be involved in the cooperative binding of the protein to single-stranded polynucleotides (Carroll et al., 1972, 1975).

Perhaps the most informative data regarding receptor–DNA binding come from the S_1 nuclease digestion experiment shown in Figure 11. Under the conditions of the experiment, very little of the plasmid dsDNA would be expected to open spontaneously to single strands; the control incubation of S_1 enzyme supports this notion. When receptor A is added to the incubation and allowed to form protein–DNA complexes prior to nuclease addition, the conformation of the DNA template is altered significantly, as evidenced by marked S_1 susceptibility. It is yet to be determined whether both DNA strands are exposed to digestion. Clearly, however, these findings are unlike results obtained for *E. coli* HDP (Molineux & Gefter, 1975) and T_4 HDP (Curtis & Alberts, 1976) where these proteins rendered the DNA resistant to digestion by several nucleases including S_1 enzyme. Other steroid receptor proteins such as the estrogen and glucocorticoid receptors have been shown to contain DNA-binding moieties (Andre et al., 1976; Sakaue & Thompson, 1977; Yamamoto, 1974), considerable asymmetry (Puca, 1971), and subunit structure (Notides & Nielsen, 1974; Erdos & Fries, 1974). These common features may indicate a common mechanism by which steroid receptors interact with DNA to alter gene expression. When the techniques recently developed for analyzing other protein–DNA interaction such as direct visualization in the electron microscope and DNA melting profiles are used, the hypothesis that steroid receptors act via helix destabilization can be more rigorously tested.

Acknowledgments

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