Reconstitution of Nativelike Nuclear Acceptor Sites of the Avian Oviduct Progesterone Receptor: Evidence for Involvement of Specific Chromatin Proteins and Specific DNA Sequences†

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ABSTRACT: A specific fraction of avian oviduct chromosomal proteins can be reannealed to pure avian DNA to reconstitute nativelike specific nuclear binding sites (acceptor sites) for the oviduct progesterone receptor (PR). These specific nuclear binding sites represent the difference between the binding to the reconstituted NAP and that to pure DNA. The specific fraction of chromatin protein which contains the acceptor activity, fraction CP-3, is very tightly bound to hen DNA in a complex termed nucleoacidic protein (NAP). Removal of the CP-3 fraction from NAP results in a loss of specific PR binding sites. Resins containing chromatin adsorbed to hydroxylapatite are used as a rapid method to isolate the CP-3 fraction. Reconstitution of the CP-3 fraction to DNA by the described method involving a regressing gradient of 6–0 M guanidine hydrochloride (Gdn·HCl) results in a reconstituted NAP which displays specific PR binding sites identical with those in native (undissociated) NAP and whole chromatin. Optimal conditions and potential problems for reconstituting these nucleoproteins are described. Only partially purified receptor preparations were used in these cell-free binding analyses since they have been shown to bind with similar properties and patterns as the nuclear binding in vivo. Therefore, the binding of PR to the reconstituted NAPs was demonstrated to be receptor dependent, saturable, and of high affinity. Further, the pattern of binding to the reconstituted sites mimics those which are observed in vivo. Thus, nonfunctional receptors that cannot translocate and bind to the nuclear acceptor sites in vivo also failed to bind to the acceptor sites on the reconstituted NAPs generated by the acceptor proteins. In contrast, the binding to pure DNA does not reflect these receptor differences in receptor bindings. Specific binding of PR to reconstituted NAP can be reversed by again removing the protein fraction. Moreover, the specific binding can be destroyed by proteases and protected by protease inhibitors, indicating that acceptor activity is proteinaceous in nature. The reconstitution of the activity is both a concentration-dependent and time-dependent process. During the reconstitution, acceptor activity appears to reconstitute on the DNA when the Gdn·HCl concentration reaches 2.0 M. By use of the reconstitution method as an assay for acceptor activity, the activity in the CP-3 fraction was shown by molecular sieve chromatography to elute in a relatively broad molecular weight range between 13 000 and 25 000. The activity also focuses in isoelectric focusing resins with apparent p*I*'s of 5.2 and 6.4. Interestingly, only a limited number of acceptor sites can be generated on the hen DNA even at high ratios of CP-3 protein to DNA. The highest number of acceptor sites achievable in reconstituted NAP is in the range of that measured on native NAP. These results suggest the presence of a limited number of specific DNA sequences in the avian genome which can interact with the acceptor protein to generate a biologically active acceptor site for this receptor. This is supported by the fact that bacterial DNA does not generate PR acceptor activity when reconstituted with hen oviduct CP-3 protein. Purification of the acceptor "proteins" and the specific DNA sequences involved in the acceptor sites for the avian oviduct PR is under way.

Steroid hormones enter target cells from the vascular system and bind to receptors with high affinity and specificity (O'-Malley & Means, 1974; Gorski & Gannon, 1976; Spelsberg & Toft, 1976; Thrall et al., 1978). This steroid receptor complex then undergoes an "activation" step endowing the complex with the capacity to translocate and bind to nuclear binding sites on the genome termed "acceptor" sites (Spelsberg et al., 1971a). The result of this ternary complex (steroid–receptor–acceptor site) is an alteration of gene transcription. Thus, the nuclear acceptor sites for steroid receptors represent the focal point of steroid-induced alterations in gene expression. The receptors thus display properties of intracellular eucaryote gene regulatory proteins.

Nuclear acceptor sites for a variety of steroid receptors have been chemically characterized as (1) solely DNA sequences (Higgins et al., 1973a–c; Yamamoto et al., 1974; Simons et al., 1976; Payvar et al., 1978, 1981; Govidan et al., 1982; Mulvihill et al., 1982; Compton et al., 1982, 1983), (2) nonhistone basic proteins (Puca et al., 1974, 1975; Mainwaring et al., 1976), (3) ribonucleoproteins (Liao et al., 1973), (4) the nuclear envelope (Jackson & Chalkley, 1974a,b), (5) the nuclear matrix (Barrack et al., 1977; Barrack & Coffey, 1980, 1982), or (6) protein–DNA complexes (Alberga et al., 1971; Spelsberg et al., 1971a,b, 1972, 1976a–c, 1977, 1979a; Baxter et al., 1972; Defer et al., 1974; Klyzsejko-Stefanowicz et al., 1976; Perry & Lopez, 1978; Hamana & Iwai, 1978; Boyd & Spelsberg, 1979; Ruh et al., 1981; Spelsberg, 1982; Ruh & Spelsberg, 1983). For a review of these various interpretations of acceptor sites, the readers are referred to Thrall et al. (1978).

This laboratory has been studying the chemical composition of the nuclear acceptor sites of the avian oviduct progesterone receptor (PR) [for reviews, see Thrall et al. (1978) and Spelsberg (1982)]. During these studies, cell-free nuclear binding assays for [^{3}H]progesterone were found to require an intact activated PR (Pikler et al., 1976, Webster et al., 1976; Spelsberg et al., 1979a,b). Furthermore, overall patterns of binding were shown to mimic those observed in vivo, with regard to quantity of binding (Pikler et al., 1976; Spelsberg et al., 1976a,b, 1977), seasonal rhythms in binding (Boyd & Spelsberg, 1979; Spelsberg & Halberg, 1980), binding during

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estrogen withdrawal and aging (Boyd-Leinen et al., 1982), and binding during development and secondary estrogen stimulation (Spelsberg, 1982; Boyd-Leinen et al., 1984). Nuclear acceptor sites for PR were shown to consist of tightly bound protein–DNA complexes (CP-3 protein fraction bound to DNA)[1] which were given the general term of nucleoacidic proteins (NAP) (Webster et al., 1976; Thrall et al., 1978). NAP was shown to bind PR in a pattern similar to that of intact nuclei or chromatin. Removal of all protein (CP-3 fraction) from the DNA, however, resulted in a marked loss in PR binding. Residual binding to pure DNA was nonsaturable and displayed no in vivo-like patterns of binding (Webster et al., 1976; Boyd & Spelsberg, 1979; Spelsberg & Halberg, 1980; Thrall & Spelsberg, 1980; Spelsberg et al., 1979a,b; Boyd-Leinen et al., 1984). Thus, proteins in the CP-3 fraction appear to contain "acceptor activity" which endow DNA with the capacity for nativelike binding of PR. Similar fractions of tightly bound, non-histone proteins are associated with the nuclear matrix (Berezney & Coffey, 1977; Barrack & Coffey, 1980), with active-diffuse chromatin fractions (Wang et al., 1976) and with active structural genes (Bekhor & Mirell, 1979; Gates & Bekhor, 1980; Norman & Bekhor, 1981; Mirell & Bekhor, 1982). This same class of proteins has been reported to enhance transcription of DNA by increasing the binding of RNA polymerase to DNA (Bekhor & Samal, 1977). The DNA associated with similar tightly bound non-histone chromosomal proteins has also been reported to be enriched in middle repetitive sequences of DNA (Razin et al., 1979). The concept that tightly bound protein–DNA complexes contain nuclear acceptor sites for PR in hen oviduct has received support from other laboratories who found acceptor sites for various steroid receptors in similar complexes (Perry & Lopez, 1978; Klyzsejko-Stefanowicz et al., 1976; Hamana & Iwai, 1978; Ruh et al., 1981; Ruh & Spelsberg, 1983).

This paper describes the isolation and reannealing of the CP-3 fraction of chromatin proteins to pure hen DNA to reconstitute NAP with nativelike acceptor sites for PR. Properties of the reconstituted acceptor sites are described. We demonstrate that other protein fractions from oviduct chromatin do not contain acceptor activity. The number of acceptor sites achieved by reconstituting increasing amounts of the CP-3 proteins to a limited amount of DNA is saturable. This combined with the fact that bacterial DNA cannot generate the acceptor sites with hen oviduct CP-3 suggests that specific sequences of DNA exist which are also required for such acceptor activity. Previous studies have identified artifacts in the cell-free nuclear binding assay itself due to degradation or mishandling of DNA (Thrall & Spelsberg, 1980; Webster & Spelsberg, 1979) and variables in the binding assay due to the steroid receptor (Boyd & Spelsberg, 1979; Spelsberg et al., 1979a,b; Spelsberg & Halberg, 1980; Spelsberg, 1982; Boyd-Leinen et al., 1982, 1984). In this paper, the method involved and obstacles encountered in reannealing the chromosomal proteins to DNA to reconstitute the PR acceptor sites are described.

Materials and Methods

Preparation of [^{3}H]Progesterone Receptor Complex. Since only the partially purified progesterone receptor has been shown, in this laboratory, to bind in cell-free assays with similar patterns and properties as the nuclear binding in vivo, it was used in these studies. The procedures for isolation, labeling, and partial purification of PR from estrogen-stimulated chick oviduct used in this study were modifications of methods described previously (Schrader & O'Malley, 1972; Boyd & Spelsberg, 1979). Briefly, immature chicks were treated with diethylstilbestrol for 4–5 weeks, and the developed oviducts were excised 10 h after the last injection. These oviducts contain cytosolic receptor specific for progesterone with little endogenous progesterone present. Freshly excised oviducts were immediately homogenized in 3 volumes (v/w) of buffer A at 4 °C by using a Teflon pestle–glass homogenizer, and a $10^5 g_{av}$ supernatant or "cytosol" was prepared. The resulting cytosol was incubated for 2 h at 4 °C with 2×10^{-8} M [^{3}H]progesterone (60 Ci/mmol; New England Nuclear, Boston, MA) which gives approximately 1 μCi/mL. The [^{3}H]PR was then precipitated with 35% saturation of $(NH_4)_2SO_4$, and the pellets were stored at −80 °C until needed.

Preparation of Chromatin and Nucleoacidic Protein (NAP). Shortly after adult hens or estrogen-treated chicks were sacrificed, oviducts were excised, cleaned, and frozen in dry ice. Early studies utilized chromatin from mature oviducts of estrogen-treated chicks while the bulk of the studies presented in this paper utilized chromatin from oviducts of adult hens because they provided greater amounts of tissue. No differences were observed in the dissociation and properties of the acceptor activity from these two sources. When needed, tissues were prehomogenized while still partly frozen in a Waring blender in buffered sucrose solutions, and nuclei and chromatin were isolated and purified by using modifications of methods described previously (Pikler et al., 1976; Boyd & Spelsberg, 1979). All steps were performed at 0–4 °C. The isolation of the native nucleoacidic protein (NAP) has been described elsewhere (Webster et al., 1976; Spelsberg et al., 1977, 1979a,b). The chromatin and NAP were resuspended in solution B at 0.5–1.0 mg of DNA/mL.

Isolation of DNA. DNA was isolated from hen spleen nuclei as described previously (Spelsberg et al., 1972; Thrall & Spelsberg, 1980). Extreme care was taken during the DNA isolation to prevent damage to the DNA or NAP which affects PR binding (Thrall & Spelsberg, 1980). The final product in buffer C was analyzed for purity by measuring DNA with the diphenylamine reaction (Burton, 1956), RNA by the orcinol reaction (Cerriotti, 1955), and protein by the method of Lowry (Lowry et al., 1951) or Bradford (1976). An acceptable DNA preparation contained less than 1.0% (w/w) protein or RNA with respect to DNA. Larger contaminations of the DNA with residual chromatin protein (i.e., > 1.0% w/w) often resulted in high levels of saturable PR binding, represeing residual acceptor activity due to the tightly bound nature of the acceptor protein. If >1% protein was found, further deproteinization steps were carried out by repeating treatments of Pronase, chloroform–isoamyl alcohol, and NaDodSO$_4$.

Isolation of the CP-3 Protein Fraction Using Chromatin–Hydroxylapatite Resins. As outlined in previous studies

[1] Abbreviations: CP-1, chromosomal protein fraction 1 (total histones); CP-2, chromosomal protein fraction 2; CP-3, chromosomal protein fraction 3; NAP, nucleoacidic protein or complexes of DNA and CP-3 protein; [^{3}H]PR, [^{3}H]progesterone receptor complex from chick oviduct; NaDodSO$_4$, sodium dodecyl sulfate; EDTA, disodium ethylenediaminetetraacetate; HSETOH, 2-mercaptoethanol; Tris, tris(hydroxymethyl)aminomethane; PMSF, phenylmethanesulfonyl fluoride; PPO, 2,5-diphenyloxazole; Me$_2$POPOP, 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene; Gdn·HCl, guanidine hydrochloride; Bis, *N,N'*-methylenebis(acrylamide); Temed, *N,N,N',N'*-tetramethylethylenediamine; buffer A, 50 mM Tris-HCl, 1 mM EDTA, and 12 mM thioglycerol, pH 7.4; buffer B, 4 mM Tris-HCl and 0.2 mM EDTA, pH 7.5; Buffer C, 150 mM NaCl and 15 mM trisodium citrate, pH 7.0; buffer D, 7.0 M Gdn·HCl, 10 mM HSETOH, 1 mM EDTA, and 10 mM phosphate buffer, pH 6.0; buffer E, 10 mM EDTA, 1 mM HSETOH, 0.5 mM PMSF, and 50 mM sodium acetate, pH 6.0.

(Webster et al., 1976; Spelsberg et al., 1977, 1979a,b; Spelsberg 1982), the CP-1 fraction (total histones) is removed by treatment of chromatin with 3.0 M NaCl, pH 6.0, or 2.0 M Gdn·HCl (pH 6.0). CP-2 protein is extracted with 4.0 M Gdn·HCl in 0.01 M phosphate buffer (pH 6.0), leaving the CP-3 protein–DNA complex termed NAP. NAP (CP-3 fraction bound to DNA), isolated from oviduct chromatin as described above, is solubilized in buffer D at 4 °C. The solution is centrifuged at $(8 \times 10^4)g_{av}$ for 48 h to sediment the DNA or residual protein–DNA complexes. The supernatant contains the CP-3 fraction. In these studies the CP-3 protein fraction was isolated from hen oviduct chromatin with column chromatography using chromatin–hydroxylapatite resin. This method was derived from previous reports on the fractionation of chromatin proteins using hydroxylapatite and chaotropic agents (Bluthmann et al., 1975; Bloom & Anderson, 1978; Brown et al., 1978, Kilianska et al., 1980). The distinction of the method presented here was the mode of fractionating the proteins from the resin. The conditions described here allow fractionation of the proteins according to their affinity for the DNA and not the hydroxylapatite. In this method, whole chromatin was adsorbed to the resin via phosphate groups on the DNA and the proteins dissociated from DNA by using Gdn·HCl (Spelsberg et al., 1977, 1979a,b). The phosphate level (0.1 M) was empirically determined so as to maintain all the DNA, but only a fraction of the total protein bound to the resin hydroxylapatite at all concentrations of Gdn·HCl. Thus, at the phosphate concentration, the bulk of the proteins and the acceptor activity do not bind to the hydroxylapatite once they are dissociated from the DNA. The involvement of DNA affinity and the noninvolvement of the hydroxylapatite resins were tested by adsorbing free CP-3 protein to the hydroxylapatite followed by elution with a gradient of 0–6 M Gdn·HCl. In this instance, all detectable protein eluted from the resin at low levels of the Gdn·HCl. Hydroxylapatite (HA) (Bio-Rad Bio-Gel HTP) was prepared by gently mixing and hydrating 25 g of the resin in 100 mL of 0.05 M phosphate, pH 6.0, at 4 °C overnight. The resin was collected on a sintered glass funnel and resuspended in 125 mL of the same buffer at 200 mg of HA/mL. This resin was then mixed with chromatin by using 0.6 g of HA/mg of DNA (chromatin). A typical preparation, consisting of 300 g (dry weight) of hydrated HA and 500 mg of DNA as chromatin, was incubated in a beaker at 4 °C for 1 h with periodic gentle mixing. The resin was collected in a large column. This was treated with a linear gradient of increasing concentrations of Gdn·HCl (0–7.0 M) in 0.1 M phosphate buffer, pH 6.0 at 4 °C. The ratio (mL/g of resin) of solvent to resin was approximately 5 with a flow rate of 5.0 mL/min. Multiple fractions were collected, and the Gdn·HCl concentration was determined by refractive index. Fractions were pooled according to unit molarities of Gdn·HCl and the protein concentration and acceptor activity determined as described below.

Analysis of Protein and Acceptor Activity in the Chromatographic Fractions. The protein in all procedures was quantitated in the presence of Gdn·HCl by a modification of the method of Bramhall et al. (1969) substituting Coomassie Blue stain or that of Bradford (1976). In later studies, the method of Bradford (1976) was used for protein quantitation. In addition, protein concentrations and acceptor activity in the pooled fractions were performed after concentration of the seven fractions (i.e., 1–7 M Gdn·HCl) from the hydroxylapatite chromatography step described above. The fractions were dialyzed thoroughly over 36 h at 4 °C against deionized water by using prewashed dialysis tubing (M_r 12000 size cut off). These fractions were then lyophilized and resuspended in $^1/_{10}$ the original volume in 6 M Gdn·HCl for analysis of acceptor activity (see below).

Reannealing of Chromosomal Proteins to Pure DNA To Reconstitute Acceptor Sites for the Avian Oviduct Progesterone Receptor. Many approaches to the reconstitution of acceptor activity using the chromatin protein fractions were examined and are discussed later. The following represents the optimal conditions for reconstitution of the CP-3 protein fraction to pure DNA to obtain the optimal number of reconstituted acceptor sites for PR. Lyophilized CP-3 proteins, e.g., the 4–7 M Gdn·HCl extracted fraction from chromatin–hydroxylapatite chromatography, were resuspended in 6.0 M Gdn·HCl in buffer E. Protein concentration ranged from 1 to 2 mg of protein/mL. The solution was homogenized with a Teflon pestle–glass homogenizer and incubated for 2–3 h at 4 °C. Pure DNA in buffer C was mixed with CP-3 protein to maintain a DNA concentration of 0.5 mg/mL and protein to DNA ratios (w/w) usually ranging from 0.0 to 1.2.

The protein–DNA solutions were placed in dialysis bags (M_r (12000 exclusion limit; 4-mm diameter Spectropor-2 membranes; Fisher Scientific, Pittsburgh, PA) and an air pocket generated ($^1/_5$ the total volume of the dialysis chamber). Generally the bags contained 0.5–1.0 mg of DNA in a bag 0.5 cm wide by 2 cm long. These bags were placed in a specially designed reconstitution chamber (cylinders of 4 cm × 30 cm) with one end having a removable screw cap. Chambers with volumes ranging from 100 to 250 mL were usually used. These chambers have two open protruding apertures, one on each end and on the same side of the chamber. The cylinder was filled with 6 M Gdn·HCl in buffer E with an air pocket ($^1/_{10}$ the total volume of the cylinder). The whole cylinder was placed on a rocking platform at 4 °C. Rubber tubes were attached to each of the open plastic apertues, one leading to a peristaltic pump and the other to a vessel containing only buffer E. As the reconstitution chamber was rocked, the original solution containing 6 M Gdn·HCl in buffer E was continuously replaced with only buffer E (no Gdn·HCl) by using the peristaltic pump. The slow rocking allowed air pockets in the dialysis bags and the reconstitution chamber to continuously mix the solutions. The rate of outflow of the reconstitution buffer for optimal recovery of acceptor activity was found to be 1.0 mL/min. A more rapid rate (2.0 mL/min) or a slower rate (0.6 mL/min) of outflow resulted in a lower recovery of acceptor activity. The former is probably due to insufficient time to allow proper reannealing while the latter is speculated to be due to extended periods allowing residual proteolysis to occur. Over a 16-h period (1.0 mL/min) using the sized chambers, the Gdn·HCl concentration declined from 6.0 M to approximately 0.0 M when about 1000–2000 mL of replacement buffer was used.

After the removal of Gdn·HCl, the reconstitution mixture was removed from the dialysis bags and diluted with an equal volume of buffer E and the solution centrifuged at $(5 \times 10^3)g$ for 10 min to sediment unbound protein. These unbound chromatin proteins are insoluble in the absence of Gdn·HCl whereas reconstituted DNA–protein complexes are soluble. This was determined by analysis of the pellets and supernatants of the low-speed centrifugation for both DNA (Burton, 1956) and protein (Lowry, 1951) and the modified Bramhall method (1969) as described earlier. The supernatant was carefully removed by aspiration and recentrifuged at $(8 \times 10^4)g_{av}$ for 24–48 h to sediment DNA and any bound protein. This simple centrifugation approach to remove unbound protein yielded

reconstituted NAPs with protein/DNA ratios equivalent to those obtained by passage of the reconstituted NAPs through columns of agarose 15 or agarose 150 m (LKB Instruments, Sweden). The centrifugation method was utilized since it allowed simultaneous preparations of multiple samples. Reconstituted protein–DNA complexes (reconstituted NAP) were resuspended in a solution containing buffer B to give between 0.5 and 1.0 mg of DNA/mL. The preparations were dialyzed against the same buffer for several hours at 4 °C, analyzed for DNA concentration by the method of Burton (1956), and immediately analyzed for PR binding in the cell-free binding assay described below. The quantity of protein bound to DNA in reconstituted NAP was sometimes determined by the method of Lowry et al. (1951).

Binding of [^{3}H]Progesterone–Receptor Complex to DNA or to Nucleoacidic Protein (NAP) Using the Streptomycin Assay. Interaction of PR with DNA or NAP was measured by using the streptomycin assay. This method has recently been described in detail elsewhere (Spelsberg, 1983). In some studies, the nuclear material on the filters was first hydrolyzed in 0.5 N $HClO_4$ at 90 °C for 15 min. Aliquots for DNA analysis were taken and the remaining solution and filter dissolved in 2:1 (v/v) Phase combining System (Amersham)/xylene fluor. This solution was then counted for radioactivity in a scintillation spectrometer. This method was termed "direct hydrolysis" as opposed to the earlier method of "direct counting", whereby the filters were first counted and the DNA hydrolysis then performed. The direct hydrolysis approach eliminates quenching of radioactivity, but both procedures give the same corrected for DNA values and patterns of binding (B. A., Littlefield, R. Seelke, and T. C. Spelsberg, unpublished results). In all studies PR binding to pure DNA was performed, and the values were subtracted from those of NAP. This difference in binding is referred to as "corrected for DNA binding".

Nuclear Binding in Vivo. The procedure for quantitating the nuclear-bound [^{3}H]PR in vivo has been described elsewhere (Spelsberg, 1976; Boyd & Spelsberg, 1979b). Briefly 200 μCi of [^{3}H]progesterone (57 Ci/mmol) in sesame oil was injected (subcutaneously) into each estrogen-treated chick. After 1 h, oviducts were excised and purified nuclei isolated by a method designed to prevent loss of nuclear-bound receptor (Spelsberg, 1976). These nuclei were then collected on Millipore (type HA) filters, and the radioactivity was counted by scintillation spectrometry. Filters were then assayed for DNA content by using the diphenylamine reaction of Burton (1956). The amount of [^{3}H]PR bound per mg of DNA and molecules of [^{3}H]PR per cell were then calculated.

Molecular Sieve Chromatography of the Acceptor Activity. The lyophilized CP-3 protein fraction was resuspended in 6.0 M Gdn·HCl in buffer D at 22 °C at 2–4 mg of protein/mL. The sample was clarified by centrifugation at 10^5g for 1 h at 22 °C. The sample was then applied to a 2.6 × 100 cm column of agarose 1.5 m in the same solvent by using a flow rate of 2 mL/min and collecting 4.0-mL fractions. The fractions were pooled on the basis of the absorbance (2800 Å)/profile and on the basis of previous experiments, indicating the region where the activity elutes. The fractions were dialyzed and lyophilized, and the acceptor activity was determined by (1) reconstituting portions of each fraction to hen DNA at several ratios as described above by using total protein recovery as a basis for recovery of acceptor activity and (2) assaying for PR binding activity of each reconstituted NAP as described above by using the streptomycin method. The lowest ratio of eluted fraction volume to DNA which gave optimal acceptor activity was selected for plotting in the figures.

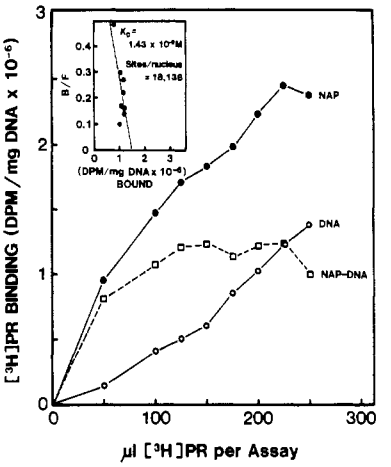

FIGURE 1: Cell-free nuclear binding of oviduct nuclear acceptor sites. NAP (●) and DNA (○) were bound as described under Materials and Methods with increasing amounts of [^{3}H]PR as indicated on the abscissa. PR binding is expressed as dpm bound per milligram of DNA recovered as measured by the direct hydrolysis method. Each point represents the mean of quadruplicate determinations. The difference between NAP and DNA binding was calculated and is expressed as (□). (Insert) The calculated difference between PR binding to NAP and DNA was transformed according to the method of Scatchard (1949). The dissociation constant for the reaction (K_D) and the estimated number of receptors bound per unit mass of DNA per nucleus were calculated from the following variable parameters: specific activity ([^{3}H]P) = 57 Ci/mmol; 2.5 × 10^{-12} g of DNA/cell nucleus; 0.1 mg of DNA/mL in the binding reaction; 2.5 × 10^6 dpm of [^{3}H]PR/mL of receptor preparation.

Isoelectric Focusing of the Acceptor Activity. The lyophilized CP-3 fraction was resuspended in 6.0 M Gdn·HCl in buffer D at 22 °C as described in the previous section. The sample was dialyzed at 4 °C against 50 volumes of 4.5 M urea in buffer B containing 0.5 mM PMSF over a 16-h period. The sample was then placed in a flat bed of Sephadex G-75 ultrafine containing 6.0 M urea and ampholines for isoelectric focusing in a pH 3–10 range (LKB, Sweden). The sample was mixed in the whole gel bed and the gel poured onto the multiphor flat bed unit (LKB, Sweden). The methods and conditions for isoelectric focusing and protein elution from the gel fractions are described elsewhere (Boyd & Spelsberg, 1979). The 15 fractions of the resin (across the pH gradient) were placed in columns and the proteins eluted. Each fraction was dialyzed, lyophilized, and analyzed for acceptor activity as described in the previous section.

Results

Cell-Free Nuclear Binding of [^{3}H]PR. The cell-free bindings were performed with only the partially purified PR since only this preparation has been thoroughly analyzed and shown to bind with in vivo-like properties (Boyd & Spelsberg, 1979; Boyd-Leinen et al., 1984). Figure 1 shows that the cell-free binding of [^{3}H]PR to NAP and DNA. Scatchard analyses of the binding show that the binding to NAP displays a biphasic pattern (curvilinear plot), indicating the presence of low-capacity high-affinity binding sites (data not shown). The binding to DNA, however, displayed a slight positive slope, indicating a lack of saturable sites and even a possible positive cooperativity of PR binding (data not shown). The insert in Figure 1 shows the Scatchard analysis of the more important values of the NAP binding (total binding) minus DNA bindings (we term nonspecific binding). The differences in these bindings are termed the "specific" acceptor sites which are generated by the acceptor proteins. These sites display

a high affinity (K_D = 10^{-9} M) and limited capacity (18 000 per cell nucleus) (Figure 1, insert). These results support those reported previously (Webster et al., 1976; Thrall et al., 1978). It should be mentioned that binding of [^{3}H]PR to NAP is markedly increased over that to chromatin. This increase in binding has been termed "unmasking" and is associated with fraction CP-2 (Webster et al., 1976; Spelsberg et al., 1979a,b; Spelsberg, 1982). Masking has also found in other steroid receptor-nuclear binding systems (Perry & Lopez, 1978; Klyzsejko-Stefanowicz et al., 1976; Hamana & Iwai, 1978; Ruh et al., 1981). Binding of [^{3}H]progesterone to NAP has been shown to be dependent on an intact, activated receptor, as reported previously (Webster et al., 1976; Spelsberg et al., 1977, 1979a,b; Spelsberg, 1982). Thus, the NAP, containing the CP-3 fraction bound to DNA, contains saturable, high-affinity binding sites for [^{3}H]PR. The removal of the CP-3 fraction from DNA results in a loss of this type of binding. It should be emphasized that the PR binding to pure DNA represents whole genomic DNA and that specific interactions of PR with a limited number of specific DNA sequences cannot be ruled out.

Isolation of Acceptor Activity from Chromatin. Previous studies had shown that the acceptor activity is dissociated from DNA at high Gdn·HCl concentrations (e.g., 4–6 M) (Spelsberg, 1983; Spelsberg et al., 1979a,b; Thrall et al., 1978). Figure 2 shows the fractionation of oviduct chromatin proteins using chromatin adsorbed to hydroxylapatite. This method involves the fractionation of proteins based on their affinity for DNA. As shown, the bulk of the acceptor activity elutes from the resin between 4 and 7 M Gdn·HCl. This is the same concentration range where the acceptor activity is dissociated from unattached chromatin by using centrifugation methods (Spelsberg et al., 1979a,b; Spelsberg, 1982). It should be mentioned here that many studies were performed to optimally measure the extracted acceptor activity, including binding of [^{3}H]PR to the free protein and to protein immobilized on resins and filter paper (Thrall et al., 1978; Spelsberg, 1982). Optimal acceptor activity was achieved only when the CP-3 fraction was reannealed to DNA by using methods as described in this paper.

Development of the Reconstitution Assay. The following conditions were established early in the studies: the chambers and the mixing rate during the reconstitution; the choice of chaotropic agent used in the reconstitution; the kinetics of the regressing gradient of the chaotropic agent; the buffers used in the reconstitution solutions; finally, the method devised to remove unbound protein from the reconstituted NAP. Several chaotropic agents such as NaCl–urea, formamide, sodium thiocyanate, and Gdn·HCl were also examined. The latter was selected on the basis of its reproducibility, lower toxicity, and the quantity of acceptor activity recovered. The screw-capped cylindrical reconstitution vessel, described under Materials and Methods, was created after examining a wide variety of chambers purchased or constructed. Solution mixing by air bubbles inside and outside the dialysis tubing by rocking gave better recovery of activity than using unmixed assays or stirring by magnetic stir bars. The kinetics of the regressing concentrations of Gdn·HCl are discussed later. The best activity was achieved when beginning with 6 M Gdn·HCl as opposed to 4 M or 2 M Gdn·HCl. It should be mentioned that the exposure of NAP or DNA to very low ionic strengths (<0.002 M NaCl equivalent) before or after reconstitution resulted in an irreversible rise in binding. This effect is probably due to damage or unwinding of the DNA due to the very low ionic conditions (Thrall & Spelsberg, 1980). On the basis of these

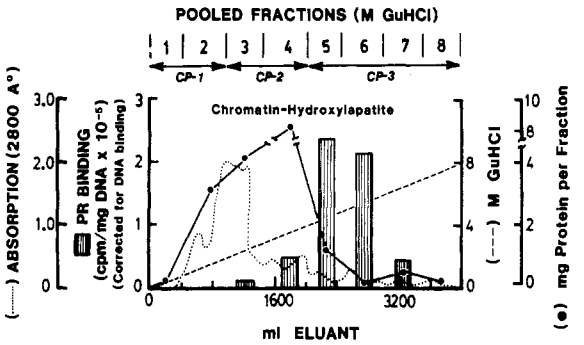

FIGURE 2: Fractionation of chromatin proteins and acceptor activity using immobilized chromatin resins. Chromatin–hydroxylapatite was prepared and used as described under Materials and Methods. The absorbance profile of the eluting buffer is shown by (···). (---) Represents the gradient of Gdn·HCl concentration. Multiple small fractions were pooled into eight larger fractions according to the unit molarities of Gnd·HCl as depicted at the top of the figure. The elution of protein fractions according to the previously reported terminology (CP-1, total histones; CP-2, bulk of the non-histone proteins containing masking activity; CP-3, fraction containing acceptor activity) is also listed under the larger fractions [for reference, see Spelsberg (1983) and Spelsberg et al. (1979a,b)]. The protein concentration (●) and acceptor activity (□) were then determined for each of these eight large fractions, as described under Materials and Methods. Acceptor activity (PR binding) is represented as the mean of four replicate binding analyses performed on one reconstituted NAP. The radioactivity was measured by using the direct counting method. The values are corrected for DNA binding as described under Materials and Methods. Since the reconstitution of acceptor activity was found to be titratable (see Figure 5), several reconstituted NAPs were prepared from each pooled fraction, each of the reconstituted NAPs representing a different ratio of aliquots (mL) of each fraction to DNA by using 1 mg of DNA. After the patterns of the cell-free [^{3}H]PR binding to all reconstituted NAPs were reviewed, the NAPs selected for illustration were those which, at the lowest ratios (i.e., fraction volume per mg of DNA) in the reconstitution assay, gave the highest PR binding. The binding to the reconstituted NAPs at this selected ratio (mL of fraction/mg of DNA) is plotted throughout the gradient. As expected, the exact protein to DNA ratio varied between each of the eight fractions since each fraction contained different quantities of protein.

results, the ionic strength in all solutions was kept equal to or greater than 4 mM NaCl equivalent. As mentioned earlier, reconstituted NAPs initially were separated from unbound proteins by using molecular sieve chromatography (agaroses 15 and 150 m; LKB Instruments, Sweden). It was subsequently found that the use of differential centrifugation resulted in NAPs with similar protein/DNA ratios as that obtained from the molecular sieve chromatographies but had the added advantage of application to multiple samples.

Effects of Endogenous Proteolytic Activity on the Recovery of the Acceptor Activity. It was discovered early in these studies that the CP-3 fraction contains significant proteolytic activity which negatively affected the recoverable acceptor activity during the reconstitution process. The presence of EDTA as well as PMSF and an acidic pH (pH 6) reduced nuclease and protease activities. The absence of PMSF in the reconstitution solutions resulted in a marked loss of recovery of acceptor activity while the addition of a protease to the assay totally removed the acceptor activity. Steps to reduce proteolysis such as the addition of "protecting proteins" (i.e., ovalbumin) helped the recovery of the acceptor activity. However, the inclusion of the serine protease inhibitor PMSF proved superior to all other approaches with the possible exception of 2.0 M Gdn·HCl. Interestingly, the acceptor activity appears to be regenerated when the Gdn·HCl reaches 2.0 M in the reconstitution assays (data not shown).

Effects of the Kinetics of the Regressing Gradient and Concentration of Constituents on Recovery of Acceptor Ac-

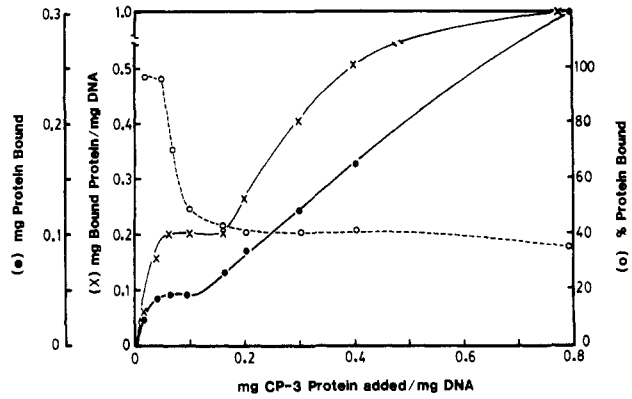

FIGURE 3: Protein bound to DNA in reconstituted NAP: effect of varying the CP-3 protein to DNA ratio. Reconstitution assays were performed as described under Materials and Methods by using varying amounts of CP-3 protein per milligram of DNA. DNA concentration was maintained at 0.5 mg/mL in all reconstitutions. Reconstituted NAPs were then analyzed for bound protein and DNA composition as described under Materials and Methods. The (●) represents total bound protein recovered after the reconstitution. The (×) represents the protein to DNA ratios in the reconstituted NAPs, and (○) represents the percent of the original input protein which is recovered as DNA bound protein. The mean of triplicate analyses of these values from one typical experiment is presented.

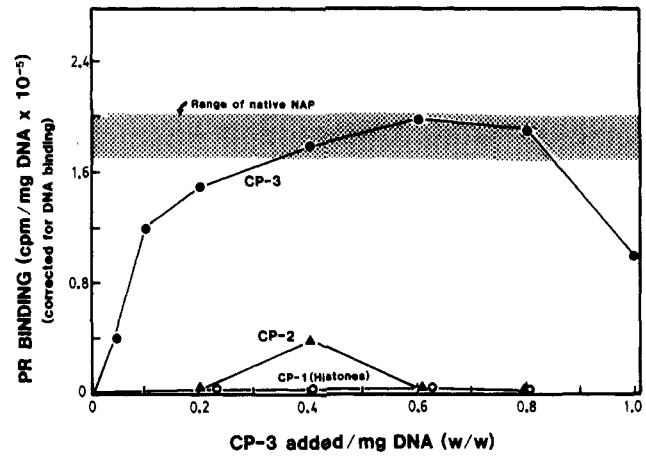

FIGURE 4: Recovery of acceptor activity as a function of the ratio of protein to DNA in the reconstitution assay and the chromatin protein fraction used. The CP-1, CP-2, and CP-3 protein fractions were reconstituted to hen DNA at varying concentrations as shown in the abscissa. Reconstituted NAPs with CP-1 (○), CP-2 (▲), and CP-3 (●) were analyzed for DNA recovery and acceptor activity. The mean of four replicate [^{3}H]PR binding assays in one typical experiment are shown. The radioactivity was measured by the direct counting method. The hatched area represents the range of [^{3}H]PR binding to native NAP (undissociated) obtained in these studies. Values are corrected for DNA binding as described under Materials and Methods.

tivity. Studies on the kinetics of the Gdn·HCl removal from the dialysis bags revealed that the rapid removal of Gdn·HCl (e.g., as 2.0 mL/min rate of outflow) from the reconstitution chambers resulted in reduced recovery of acceptor activity with marked increases in variability. The low recovery of acceptor activity using the rapid reconstitution gradients of Gdn·HCl might be due to insufficient time to allow appropriate reconstitution of functional acceptor sites at the selected concentrations of acceptor protein to DNA. This is supported by the fact that at low concentrations of DNA and/or CP-3 protein, the recovery of acceptor activity was markedly reduced. Thus, concentrations of DNA below 0.2 mg/mL and of CP-3 protein below 0.08 mg/mL protein gave less than optimal recoveries of acceptor activities in the 16-h reconstitution process (data not shown). Slower rates of Gdn·HCl removal such as 0.6 mL/min or slower also resulted in reduced recovery of acceptor sites. This is speculated to be caused by the extended periods allowing the action of residual proteases in the lower Gdn·HCl concentrations. A rate of 1 mL/min was found to give optimal recovery of acceptor sites with good reproducibility at the selected concentrations of DNA (0.5 mg/mL) and acceptor protein (0.1 mg/mL and higher). In addition, this rate is best suited for overnight reconstitutions.

Titration of the Acceptor Protein to DNA: Evidence for Roles of Specific Proteins and Specific DNA Sequences. The amount of total CP-3 protein needed to reconstitute an optimal number of acceptor sites on DNA was then addressed. Figure 3 shows the amount of protein that binds to DNA at the increasing ratios of CP-3 protein to DNA at the beginning of the reconstitution. At very low ratios, almost all the total CP-3 protein was reannealed to the DNA, whereas at higher ratios, only 40% of the total protein was bound to the DNA. There appears to be a saturation of this bound protein fraction (CP-3) at the low ratios followed by a linear nonsaturable increase in bound protein as the amount of added protein is increased. Reconstituted NAPs prepared with CP-3 protein to DNA ratios of 0.2–0.4 resulted in a ratio of bound protein to DNA (0.28–0.5) which resembled that found in native NAP (protein/DNA = 0.3–0.4). It was obviously of interest to also assess the recovery of PR acceptor activity when the CP-3 protein to DNA ratio was varied. Figure 4 shows typical results from one of many experiments on the pattern of PR binding to NAPs reconstituted at increasing ratios of CP-3 protein to DNA. First, it can be seen that a saturable number of acceptor sites on the DNA was achieved when the CP-3 to DNA ratios ranged between 0.2 and 0.4 (w/w). The saturation of PR binding also occurred at the same level as that of native NAP. Interestingly, only the CP-3 fraction and not the CP-1 or CP-2 fraction were able to restore PR acceptor sites. Two important points can be made from these findings: first, a specific chromosomal protein fraction is involved in generating PR acceptor sites, and second, there appears to be a limited number of sites (sequences) on DNA which can generate acceptor sites when bound with the appropriate protein fraction. To evaluate the latter, bacterial DNA was substituted for hen DNA in certain reconstitution assays using a hen CP-3 to DNA ratio of 0.6. Figure 5 shows that while the hen DNA did generate significant increases in PR binding, the bacterial DNA did not. Thus, specific DNA sequences appear to be involved in the nuclear acceptor sites as do the acceptor proteins. It should be mentioned that at the higher CP-3 protein to DNA ratios (approximately 1.0 or greater), an inhibition in the number of recovered acceptor sites on hen DNA was observed. The reason for this inhibition is unknown.

Characterization of Reconstituted NAP. It was of interest to quantitatively compare the binding of PR to reconstituted NAP with the binding to native NAP and chromatin. Table I shows that reconstituted NAP, prepared under optimal conditions, displays a receptor-dependent binding of [^{3}H]-progesterone characteristic of native NAP or intact chromatin (Webster et al., 1976; Spelsberg et al., 1979a,b). Heat-denatured receptor or free [^{3}H]PR in the presence of spleen cytosol resulted in a marked reduction in binding with native NAP, reconstituted NAP, and reconstituted DNA. The reconstituted DNA displayed a markedly lower level of binding of native [^{3}H]PR compared to that for NAP.

It had been shown previously that a seasonal pattern of binding of [^{3}H]PR to nuclear acceptor sites was revealed both in vivo and in the cell-free binding assays. The cause of this variation was shown to reside in the receptor rather than nuclear acceptor sites (Boyd & Spelsberg, 1979; Spelsberg &

Table I: Receptor Dependency of [^{3}H]Progesterone Binding to Reconstituted NAP and DNA[a]

sample bound	condition	bound [^{3}H]progesterone (cpm bound/mg of DNA × 10^3) (±SE)
reconstituted NAP	[^{3}H]PR	190 ± 30
	[^{3}H]P in spleen cytosol	18 ± 9
	heat-denatured [^{3}H]PR	28 ± 10
reconstituted DNA	[^{3}H]PR	60 ± 10
	[^{3}H]P alone or in spleen cytosol	18 ± 10
	heat-denatured [^{3}H]PR	30 ± 12

[a] Reconstituted NAP was prepared using by a CP-3 protein to DNA ratio of 0.6 (w/w) as described under Materials and Methods. Reconstituted DNA was prepared in the same way with ovalbumin to DNA ratio of 1.0 (w/w). [^{3}H]PR = binding of intact, native receptor (ammonium sulfate precipitated) to reconstituted NAP. [^{3}H]P in spleen cytosol = binding using spleen cytosol (i.e., $10^5 g$ supernatant of spleen homogenate) containing approximately the same protein concentration and [^{3}H]P equivalent to that in the [^{3}H]PR preparations. Heat-denatured [^{3}H]PR = native [^{3}H]PR heated at 37 °C for 1 h followed by centrifugation at $10^4 g$ for 10 min at 4 °C to clarify the solution. The reconstituted DNA was also bound by intact [^{3}H]PR, heat-denatured [^{3}H]PR, and [^{3}H]P in spleen cytosol as performed for NAP binding. The data shown represent the mean ± SE of four replicate PR binding assays for each receptor preparation, performed in the same experiment. The NAP binding values represent total binding (i.e., are not corrected for DNA binding).

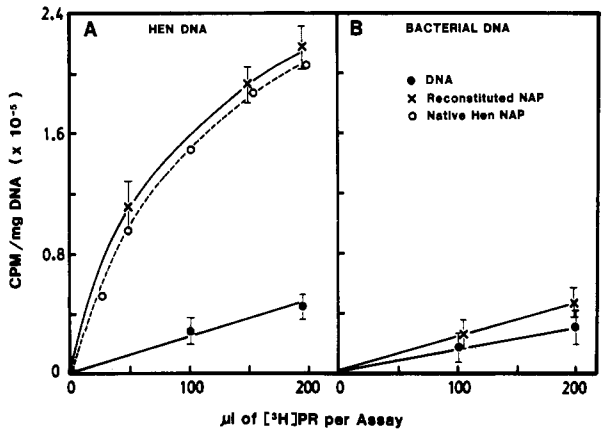

FIGURE 5: Inability of bacterial DNA to generate acceptor activity when reconstituted with hen oviduct CP-3 protein. Hen DNA (panel A) and bacterial DNA (*E. coli* DNA purchased from P-L Biochemicals, Milwaukee, WI) (panel B) were reconstituted with hen oviduct CP-3 protein fraction at a protein to DNA ratio of 0.6 (w/w) as described under Materials and Methods. These reconstituted NAPs were bound with various concentrations of [^{3}H]PR. the (●) represents binding to the reconstituted pure DNAs (no CP-3 protein), the (×) binding to reconstituted NAP, and (○) binding to native NAP. The mean ± SE of four replicate binding assays at each receptor level is presented.

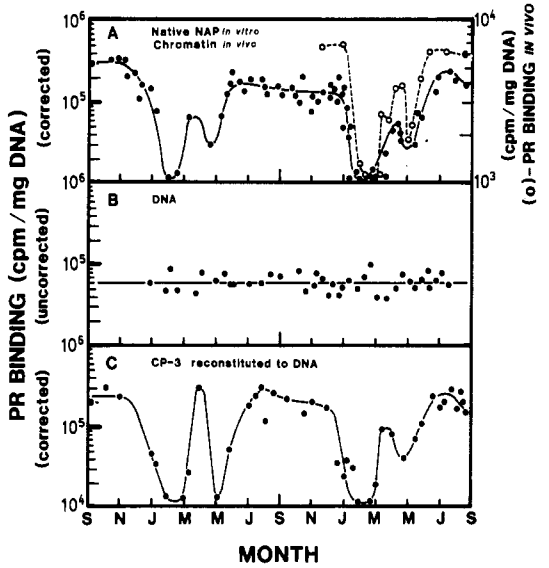

FIGURE 6: Seasonal patterns of binding of [^{3}H]PR to chromatin (in vivo) to native NAP, DNA, and reconstituted NAP. [^{3}H]PR, isolated at various periods of the year and stored as ammonium sulfate pellets at −70 °C, was used in the PR binding assays in six major studies as described under Materials and Methods. These assays utilized saturable levels of [^{3}H]PR. Studies were also performed throughout the year for analysis of chromatin binding of [^{3}H]P in vivo. In these studies, [^{3}H]P was injected for 1 h into the wing vein of DES-primed chicks as described under Materials and Methods. After 1 h, oviduct nuclei were isolated, and measurement of nuclear-bound [^{3}H]P was performed. This is shown in panel A as (○). The binding of the various [^{3}H]PR preparations to native NAP (●) in panel A, to pure DNA (●) in Panel B, and to reconstituted NAP (DNA + CP-3) (●) in panel C is shown. In all panels, the means of four replicate analyses of [^{3}H]PR binding, performed in a series of eight experiments, are shown. The values in each of the in vitro experiments were normalized to control NAP and DNA binding values by using one of the September receptor preparations used in each of the experiments. The bound radioactivity in the PR cell-free binding assay was measured by the direct counting method. Panels A and C are corrected for DNA binding as described under Materials and Methods, while panel B shows uncorrected data.

Halberg, 1980; Mulvihill et al., 1982). Figure 6 compares the seasonal patterns of binding of [^{3}H]PR to native NAP, pure DNA, and reconstituted NAP in vitro as well as to chromatin in vivo. The results show that PR, isolated in the winter, fails to recognize acceptor sites in native (panel C) or reconstituted NAP (panel A) in vitro or in whole chromatin in vivo (panel A). Panel B shows, however, that whole genomic DNA alone displays no such differences in the binding of winter receptor preparations vs. the summer preparations. Thus, reconstituted NAP displays the same patterns of seasonal binding as does native NAP or chromatin, but pure DNA preparations display no such patterns.

Nonfunctioning progesterone receptors have also been identified in the undeveloped oviducts of chicks under in vivo and in vitro conditions (Spelsberg et al., 1981; Boyd-Leinen et al., 1984). Figure 7 shows the results of a typical experiment comparing the binding of nonfunctional PR from the undeveloped oviduct with the functional receptor from the developed oviduct. The reconstituted NAP (panel C) displayed the same pattern of PR binding as the binding to nuclear acceptor sites in vivo (panel A) or to native NAP in vitro (panel B). In all instances, the PR from the undeveloped oviduct showed little or no binding to the NAP in vitro or to the nuclei in vivo whereas the PR from the developed oviduct did. In contrast, pure DNA, untreated or subjected to the reconstitution process, showed no difference in binding between the nonfunctional and functional receptor binding (panels B and C). Thus, reconstituted NAP displayed the same requirements of a functional receptor for nuclear binding as did the native NAP and nuclei while pure DNA displayed no such requirements. It should be stated that whole (genomic) DNA was used in these studies and that the binding to specific DNA sequences in enriched fragments of the genome may show a similar selectivity for receptors.

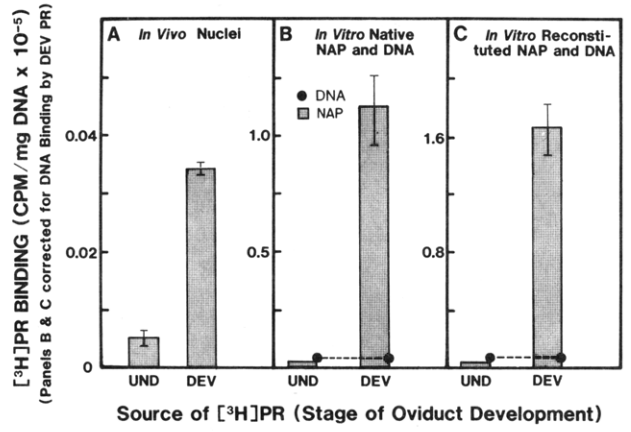

FIGURE 7: Binding of nonfunctional [^{3}H]PR from undeveloped oviducts and functional [^{3}H]PR from developed oviducts to the oviduct nuclei in vivo and to native NAP and reconstituted NAPs in vitro. [^{3}H]PR was isolated from undeveloped oviducts (UND) (from chicks receiving 4 days of estrogen treatment) and developed oviducts (DEV) (from chicks receiving the full 21 days of estrogen treatment) (Boyd-Leinen et al., 1983). These receptors were bound to native (undissociated) and reconstituted NAPs and to native and reconstituted DNAs in the cell-free assay as described under Materials and Methods. (Panel A) Binding of [^{3}H]P to nuclear acceptor sites in vivo in undeveloped oviducts (UND) and developed oviducts (DEV) as described under Materials and Methods and in the legend of Figure 9. (Panel B) Binding of UND and DEV ([^{3}H]PR to native hen oviduct NAP and hen DNA was measured by using (□) direct counting method. The direct hydrolysis method of measuring bound radioactivity as described under Materials and Methods gave similar results (data not shown). The binding to (●) native DNA by the two PR preparations was equivalent and is placed at zero values since the PR binding is plotted as corrected for DNA binding by DEV [^{3}H]PR. Panel C represents the same binding experiments as described in panel B except reconstituted NAP and reconstituted DNA were substituted for the native counterparts. Cell-free binding values in panels B and C are corrected for DNA binding by DEV [^{3}H]PR. The in vivo binding values in panel A were normalized to 10^5 cpm/mL of blood. In all panels, the mean ± SE of four replicate analyses of [^{3}H]PR binding, performed in one experiment, are presented.

We next wished to determine whether the reconstitution of acceptor activity is reversible, i.e., whether reconstituted acceptor sites can again be dissociated from DNA by removal of the reconstituted CP-3 proteins. Figure 8 shows that both native NAP and reconstituted NAP display a saturable, high-capacity binding of [^{3}H]PR. DNA isolated from reconstituted NAP displays the same reduced, nonsaturable binding of [^{3}H]PR that is characteristic of pure hen spleen DNA. Thus, reconstituted acceptor sites can be reconstituted and then redissociated in a manner which follows the presence or absence of the CP-3 protein fraction. It should be stated that DNA from reconstituted NAP always displays a slightly higher binding than pure hen spleen DNA. The enhanced [^{3}H]PR binding to DNA isolated from reconstituted NAP could be caused by (1) damage to DNA during handling and/or (2) exposure of the DNA several times to low ionic strength buffer (buffer B) (Thrall & Spelsberg, 1980). However, even partially damaged DNA was previously shown not to display saturable binding or any seasonal pattern of binding (Thrall & Spelsberg, 1980). Thus, nativelike acceptor sites were not being generated by simply damaging DNA.

Characterization of the Acceptor Activity in the CP-3 Fraction. The acceptor activity in the CP-3 protein fraction has been characterized in preliminary studies. It has already been stated that the activity is a protein based on proteolytic susceptibility. Figure 9 shows that the acceptor activity elutes from chromatographic column of agarose 1.5 m under denaturing conditions (i.e., 6.0 M Gdn·HCl) in the molecular mass range of 13 000–25 000 daltons. When the CP-3 fraction was

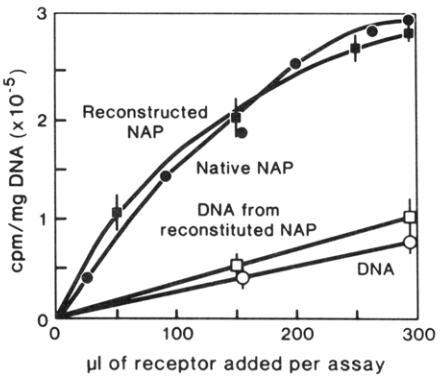

FIGURE 8: Dependence of the acceptor activity in reconstituted NAP on reannealed protein: reversibility of the reconstituted activity. Native and reconstituted NAPs and pure hen DNA were prepared and analyzed for [^{3}H]PR binding as described under Materials and Methods. DNA from reconstituted NAP was prepared by deproteinization using the chloroform–isoamyl alcohol–Pronase treatment as described for DNA isolation under Materials and Methods. Less than 0.5% (w/w) of the reannealed protein remained after these extractions. This DNA was also analyzed for [^{3}H]PR binding activity. All binding points except for the binding of native NAP (used as a reference) also contain the mean ● SE for the four replicate values at each receptor concentration. The radioactivity in the binding assays was measured by direct counting method and was not corrected for DNA binding. The following represent the [^{3}H]PR binding to native NAP (●), DNA isolated from whole hen spleen nuclei (○), reconstituted NAP (■), and DNA isolated from reconstituted NAP (□).

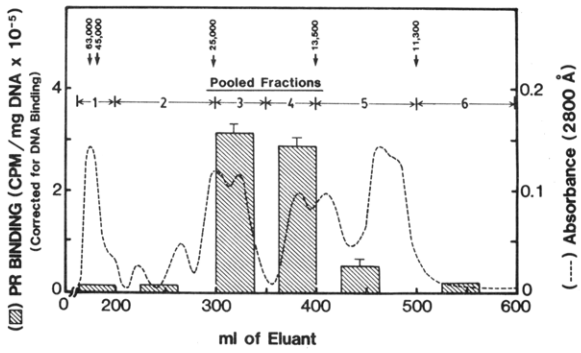

FIGURE 9: Molecular sieve chromatography of the acceptor activity on agarose 1.5 m in 6.0 M Gdn·HCl. The CP-3 protein fraction was applied to a 2.6 × 100 cm column of agarose 1.5 m with a 6.0 M Gdn·HCl in the eluting solvent as described under Materials and Methods. The fractions were pooled and analyzed for acceptor activity as described under Materials and Methods and in the legend of Figure 2. The radioactivity was measured by the direct counting method. The mean ± SE of four replicate analyses of the acceptor activity is presented. The recovery of protein after dialyses and lyophilization of each pooled fraction was 45%.

applied to isoelectric focusing, two peaks of activity were consistently identified, focusing at pHs of approximately 5.2 and 6.4 (Figure 10). Other studies have shown that the protein is only soluble in aqueous solutions containing chaotropic agents and detergents. Thus, the acceptor activity appears to be a small, acidic, hydrophobic protein.

Discussion

The [^{3}H]PR binding to NAP, but not to pure DNA, has been shown previously to mimic that found in vivo to whole chromatin (Boyd & Spelsberg, 1979; Spelsberg & Halberg, 1980; Boyd-Leinen et al., 1982, 1984; Spelsberg, 1982). This paper describes a method used to reconstitute the nativelike acceptor sites for avian oviduct PR by using the DNA and CP-3 protein fraction from chromatin. Procedures used to optimize recovery and activity are presented. The advantage of the approach taken is that correlations with biological ac-

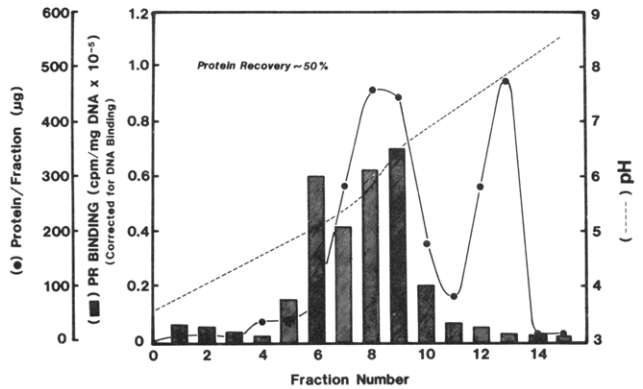

FIGURE 10: Isoelectric focusing of the acceptor activity in CP-3 protein fraction. The acceptor activity in the CP-3 protein fraction was analyzed by subjecting the CP-3 fraction of preparative isoelectric focusing in flat beds of Sephadex G-75 ultrafine using an LKB multiphor as described under Materials and Methods. The fractions were pooled according to 15 equal lengthwise sections of the bed resin across the pH gradient and analyzed for protein and PR acceptor activity as described under Materials and Methods and in the legend of Figure 2. The radioactivity was measured by the direct counting method. The protein per fraction was analyzed by the method of Bramhall et al. (1969) as described under Materials and Methods. The mean of triplicate analyses of the acceptor activity and protein concentrations was presented. The recovery of protein after dialysis and lyophilization of each pooled fraction was 50%.

tivity/function were utilized. The acceptor activity for PR was dissociated from avian oviduct chromatin at high Gdn·HCl concentrations (4.0–7.0 M) in the "CP-3" fraction. These concentrations of Gdn·HCl have been previously used to dissociate the most tightly bound chromosomal proteins from DNA in chromatin (Hill et al., 1971; Arnold & Young, 1972; Levy et al., 1972; Spelsberg et al., 1976a–c, 1977, 1979a,b). These tightly bound non-histone chromosomal proteins have been shown to be (1) bound to active genes (Gates & Bekhor, 1980; Norman & Bekhor, 1981), (2) bound to middle repetitive DNA sequences (Razin et al., 1979), (3) located in the active diffuse chromatin (Wang et al., 1976), (4) in the nuclear matrix (Berezney & Coffey, 1977; Barrack & Coffey, 1982), and (5) capable of enhancing the binding of RNA polymerase enzyme to DNA (Bekhor & Samal, 1977). These proteins have also been reported to serve as acceptor sites for other steroid receptors (Perry & Lopez, 1978; Hamana & Iwai, 1978; Klyzsejko-Stefanowicz et al., 1976; Ruh et al., 1981; Ruh & Spelsberg, 1983).

Although practically all proteins are denatured by 6 M Gdn·HCl, many can be renatured. The conditions used in this study to reconstruct the acceptor sites are similar to those used to renature proteins and nucleoproteins in general. Denatured enzymes and other nonenzymatic proteins have been renatured to their structurally native forms (Tanford, 1968; Teipel & Kochland, 1971; Weber & Kuter, 1971; Teipel 1972; Yazgan & Henkens, 1972; Carlsson et al., 1973; Ahmad & Salhuddin, 1976; Lykins et al., 1977). Proteins have also been recombined with DNA for reconstruction of nativelike deoxyribonucleoproteins (Spelsberg & Hnilica, 1970; Spelsberg et al., 1971a,b, 1972, 1976a,c, 1977, 1979a,b; Paul et al., 1973; Axel et al., 1974; Barrett et al., 1974; Stein et al., 1974, 1975; Chae, 1975; Gadski & Chae, 1976; Woodcock, 1977; Thrall et al., 1978; Spelsberg, 1982).

Optimal conditions are described in this paper for reconstituting the acceptor activity for the avian oviduct PR. The source of most of the variation in cell-free bindings performed in these laboratories was ultimately found to be due to differences in the receptor rather than to differences in the reconstituted NAP preparations (Boyd & Spelsberg, 1979; Spelsberg, 1982; Boyd-Leinen et al., 1982, 1984). However, some variations in the CP-3 preparations and replicate reconstitution assays were found. It is presently not possible to reconstitute acceptor sites with various preparations of CP-3 fraction and in different assays with different receptor preparations without a 10–20% variation in absolute PR binding values. However, the reconstituted NAP were shown in this study to contain acceptor sites which were quantitatively in the range and qualitatively very similar to those in native chromatin or NAP. The reconstituted acceptor sites (i.e., reconstituted NAP) displayed saturable receptor-dependent binding and patterns of binding (i.e., during the seasons and during oviduct development) similar to those of native acceptor sites (Boyd & Spelsberg, 1979b; Boyd-Leinen et al., 1984). The acceptor activity involves both protein and DNA. Conditions that prevented proteolytic damage enhanced the recovery of acceptor sites. Other factors such as the duration of the reconstitution process, the ratio of protein to DNA, and the concentrations of reactants also affected the reconstitution of acceptor sites. Conditions such as partial degradation of DNA (Thrall & Spelsberg, 1980), as well as the exposure of DNA to very low ionic strength buffers (described in this paper), were found to cause artifactual increases in PR binding. However, these high levels of binding "sites" on damaged pure DNA were previously shown to differ from the native acceptor sites by a lack of saturable PR binding, a lack of seasonal patterns of PR binding, and a lack of susceptibility to proteolytic activity (Thrall & Spelsberg, 1981; Spelsberg, 1982).

The studies presented here add further support to the concept that the acceptor sites for PR in the avian oviduct (i.e., the first sites of interaction of PR on chromatin) involve not only a specific class of tightly bound chromatin protein but also a specific sequence of DNA (Spelsberg et al., 1976, 1979a,b; Spelsberg, 1982). It is possible that some acceptor activity could be localized in the CP-1 and CP-2 fractions but remain undetectable. However, only when the CP-3 fraction was removed from the DNA was a loss in acceptor activity measured. Interestingly, a saturation of PR acceptor sites was generated when the reconstitutions included ratios of protein to DNA somewhere in the range between 0.2 and 0.4. Further, the amount of protein bound to DNA at this ratio was found to be in the range (e.g., 0.3–0.5) of that measured for native NAP (protein/DNA = 0.3–0.4). The apparent saturation of sites could be explained by a limited number of specific DNA sequences in the hen genome which are essential for acceptor activity. Alternatively, apparent saturation might be related to methodological problems such as inactivation of acceptor activity by proteolysis or aggregation of acceptor proteins. However, the substitution of the bacterial DNA for the hen DNA at these same ratios of protein to DNA resulted in no enhanced PR binding. These results support that specific DNA sequences play an essential role in the acceptor sites.

Modifications of these methods described in this paper were used to successfully reconstitute the acceptor sites for the avian oviduct estrogen receptor (Ruh & Spelsberg, 1983). Now that nativelike acceptor sites can be reconstituted by using isolated chromosomal proteins and DNA, the opportunity exists to purify and characterize both the DNA sequences and proteins involved in these binding sites.

Registry No. Progesterone, 57-83-0.

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Hydrogen Exchange and Macromolecular Motility†

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ABSTRACT: Only rarely have traditional studies of the rates of hydrogen exchange in macromolecules led to precise values of "motility". We show that if there are not too many participating conformers, if their concentrations are monitored, and if the rates of exchange and conformational interconversion are not too different, rate constants defining motility may be obtainable. Clearly, some substrates would not be appropriate for such studies. On the other hand, it is possible to manipulate the exchange rates widely (by choice of conditions) so that many macromolecules should qualify. The applicability of rate expressions in a variety of limiting situations is examined in detail.

The kinetics of peptide hydrogen exchange has often been used as a probe of the internal motions in complex molecules, e.g., proteins, nucleic acids, and polypeptides. The variety of descriptive terms given to these conformational changes, motility, (un)folding, breathing, fluctuation, flexibility, segmental mobility, opening/closing, and helix/coil transition, testifies to the breadth of interest in both the kinds of molecular motions and their types (Barksdale & Rosenberg, 1982; Woodward & Hilton, 1979; Englander et al., 1972; Woodward et al., 1982; Hvidt & Nielsen, 1966; Ghélis & Yon, 1982). Though the reviews just cited survey 3 decades of hydrogen exchange, the expectation that "standard data" (Altona, 1982) on conformational populations or relative stabilities ($\Delta H°$ and $\Delta S°$) *and* on barrier heights ($\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$) would be forthcoming has rarely been realized. Since observations on systems undergoing both hydrogen exchange and conformational interconversion may be difficult to relate to the individual processes, it seems useful to display both exact and approximate solutions of the kinetics that could be applied to the selection, design, and interpretation of experiments in the field.

A schematic representation of a flexible macromolecule containing an exchangeable proton is given in Figure 1. With respect to this proton, closed (C), partly closed (PC), and open (O) forms are indicated. In a real case, there may, of course, be several different exchangeable protons and many more conformations.

To simplify the treatment of the rate data, workers in the field normally catalyze the exchange, H for D or H for T, at constant pH. If the concentrations of the different conformations involving a single exchangeable hydrogen are written

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