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Proteins That Mask the Nuclear Binding Sites of the Avian Oviduct Progesterone Receptor†

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ABSTRACT: The binding of a steroid receptor to specific nuclear sites (i.e., nuclear acceptor sites) represents the immediate event preceding the steroid regulation of gene transcription. How the same steroid receptor regulates different genes in different tissues is unknown. Since a major fraction of the nuclear acceptor sites for a variety of steroid receptors has been reported to be masked in the chromatins of a variety of tissues, the differential expression of the nuclear acceptor sites may explain this regulation of different genes. In the avian oviduct, the removal of a subfraction of chromosomal non-histone proteins, termed CP-2, results in the unmasking of the nuclear acceptor sites for the progesterone receptor (PR). Further, the extent of masking of these nuclear acceptor sites for PR has been reported to vary during cytodifferentiation of the avian oviduct. This paper describes a method for the reconstitution of the masking of PR nuclear acceptor sites in the avian oviduct chromatin using a partially purified chromosomal protein fraction (CP-2b). The reannealling of the CP-2b fraction to unmasked avian oviduct chromatin (termed nucleoacidic protein or NAP) results in the "remasking" of about the same number of nuclear acceptor sites for PR as found in intact chromatin. Because some of the PR acceptor sites on the NAP cannot be remasked, these sites either must be protected from masking or not be recognized by the masking proteins. The masking activity apparently involves only protein(s) because the unmasking of acceptor sites can be achieved with protease but not ribonuclease activities and because the dissociated masking activity is destroyed only by proteases. The masking appears to be reversible because the reconstituted masked sites can again be unmasked. Preliminary purification and characterization of the masking activity in fraction CP-2b by molecular sieve chromatography indicate a heterogeneity of size with the activity eluting in a molecular weight range of from 60 000 to >150 000. Whether the masking proteins prevent the binding of the progesterone receptor by directly binding the acceptor sites or by binding neighboring domains to condense the chromatin is unknown. It is speculated that the masking of acceptor sites may be responsible in part for determining the tissue-specific gene expression induced by steroids and/or may play a role in the unresponsiveness of certain human tumors containing steroid receptors.

Steroid hormones are best known for their effects on gene transcription and the resulting modification of the metabolism of many cellular components in their target cells (O'Malley & Means, 1974). Of the many events which must occur prior to hormone response, the nuclear event immediately preceding the alteration of gene activity is the binding of steroid–receptor complexes to the nuclear acceptor sites. In most instances, these acceptor sites have been localized on isolated chromatin (Thrall et al., 1978; Spelsberg, 1982).

Early studies in this laboratory demonstrated that the majority of the nuclear binding sites for the avian oviduct progesterone receptor are "masked". Thus, in intact avian oviduct

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chromatin, these sites are inaccessible for binding by the progesterone–receptor complex (Spelsberg et al., 1971b, 1972, 1976, 1983; Spelsberg, 1982; Webster et al., 1976). Using techniques described by Webster et al. (1976), a similar masking of nuclear acceptor sites for several different steroids in a variety of target tissue systems of mammals has been reported (Klyzsejko-Stefanowicz et al., 1976; Perry & Lopez, 1978; Ruh et al., 1981; Ruh & Spelsberg, 1983). Thus, the masking of chromatin acceptor sites for steroid receptors may be universal.

Studies in this laboratory have shown that when chromatin is partially deproteinized with chaotropic agents, the number of sites available for progesterone receptor (PR)[1] binding increases from 6000 to as many as 20 000 sites per cell (Spelsberg et al., 1976, 1983). The masked acceptor sites in intact chromatin appear to have properties identical with the "unmasked sites" with respect to receptor requirement for binding, the affinity of binding, and patterns of binding. The latter includes similarities in PR nuclear binding patterns which occur during the seasons of the year (Boyd & Spelsberg, 1979), and during different stages of oviduct development and estrogen withdrawal (Spelsberg et al., 1984; Thrall et al., 1978; Boyd-Leinen et al., 1984). Preliminary studies have suggested that the chromatins of nontarget tissues such as spleen and erythrocyte, which bind the PR at a very low level (e.g., less than 500 sites per cell), can also be unmasked by removal of protein. This unmasking of nuclear acceptor sites in the chromatin of nontarget tissues results in a level of PR binding comparable to that which occurs in unmasked oviduct chromatin (20 000 sites per cell) (Webster et al., 1976; Spelsberg et al., 1976, 1984). However, the properties and specificity of these masked binding sites require further study.

The biological function of masking of the nuclear binding sites for steroid receptors in target cell nuclei is unknown. Interestingly, the degree of masking of acceptor sites for the avian oviduct progesterone receptor has been shown to vary during cytodifferentiation of the oviduct (Spelsberg et al., 1983). Therefore, one possibility is that masking may explain the mechanism by which the same steroid and receptor within an animal affect different patterns of gene expression in different tissues. This paper investigates the conditions required for the reconstitution of the masking of the nuclear acceptor sites for the avian oviduct PR. Such a method is a prerequisite to the purification and chemical characterization of the masking activity. Preliminary characterization of the masking factors for the avian oviduct progesterone receptor is presented.

Materials and Methods

Preparation of [^{3}H]Progesterone–Receptor Complex (PR). [1,2-^{3}H]Progesterone (47.8 Ci/mmol) was obtained from New England Nuclear Corp. (Boston, MA) and examined for purity by high-pressure liquid chromatography using a reverse-phase system consisting of C_{18} μBondapak resin and a linear gradient of 40–80% (v/v) acetonitrile and H_2O. The receptor was isolated from the developed oviducts of estrogen-treated immature chicks as described elsewhere (Boyd & Spelsberg, 1979a; Boyd-Leinen et al., 1984). Ammonium sulfate precipitates of the receptor were stored in a Revco at −70 °C. When needed, they were resuspended in solution A at 4 °C for 1 h, dialyzed against solution A for 1 h, and then clarified by centrifugation at 10000*g* for 10 min. These preparations of PR were used directly in the nuclear binding assays (described below). The preparations contained about 10^6 dpm/mL with 60–70% of the [^{3}H]progesterone remaining specifically bound to receptor during the nuclear binding assay.

Isolation of Chromatin. Tissues from adult laying hens (Silver Lake Hatcheries, Silver Lake, MN) served as the primary source of nuclei. Shortly after the hens were sacrificed, oviducts were removed, cleaned, and frozen on solid CO_2. The tissues were stored at −80 °C until needed. The isolation of nuclei and chromatin is as described elsewhere (Spelsberg et al., 1971a,b, 1972; Boyd & Spelsberg, 1979b). Chromatin was stored in solution B at −90 °C until needed.

Isolation of the Chromosomal Protein Fractions. The isolation of chromosomal protein fractions and residual protein–DNA complexes was accomplished by three different methods.

(A) Sedimentation of Free Chromatin in NaCl–Gdn-HCl. Histones were selectively removed by resuspending oviduct chromatin in solutions containing varying concentrations (0–3.0 M) of NaCl in 0.05 M phosphate buffer, pH 6.0, as reported previously (Spelsberg & Hnilica, 1970). The total histone fraction (CP-1) was extracted by buffered 3.0 M NaCl (solution C). The solution was centrifuged for 24 h at 10^5g to separate the CP-1 (supernatant) from the dehistonized chromatin (pellet). The pellet of dehistonized chromatin (deficient in CP-1) was resuspended in 4 M Gdn-HCl (solution D) to dissociate the CP-2 fraction of non-histone proteins (NHP). This extract was centrifuged for 24 h at 10^5g, and the supernatant (containing protein fraction CP-2) was dialyzed thoroughly against H_2O, frozen, and lyophilized. The pellet containing the residual DNA and associated protein was resuspended in solution B and dialyzed for 5–6 h against the same buffer to remove the Gdn-HCl. Centrifugation at 1000*g* for 5 min separated the soluble DNA with bound protein [termed nucleoacidic protein (or NAP)] in the supernatant from the free (insoluble) protein in the pellet. The NAP, therefore, represents chromatin devoid of the histones (CP-1) and a fraction of non-histone protein (CP-2). This residual DNA–protein complex (NAP) still maintains the saturable PR binding sites measured in whole chromatin. The pellet of insoluble protein was further fractionated by resuspending the pellet in 6 M Gdn-HCl in solution E and centrifuging 24 h at 10^5g. The solubilized protein (supernatant) represents the CP-2b fraction (which contains the masking activity). The final pellet of insoluble protein was termed CP-2c and was discarded because it was insoluble in all solutions. All protein fractions were dialyzed, frozen, lyophilized, and stored after dialysis vs. water at 4 °C.

(B) Extraction of Immobilized Chromatin. The second method involved immobilizing chromatin by covalent linkage to insoluble cellulose resin using UV light treatment. The preparation and use of the chromatin–cellulose resin are described elsewhere (Webster et al., 1976; Spelsberg et al., 1977, 1978; Thrall et al., 1978). The wet resin was dried under vacuum and stored in air-tight containers until needed. The chromatin–cellulose was extracted with NaCl and Gdn-HCl

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; Gdn-HCl, guanidine hydrochloride; solution A, 50 mM Tris, 0.5 mM EDTA, 12 mM thioglycerol, and 1 mM PMSF, pH 7.4; solution B, 2 mM Tris and 0.1 mM EDTA, pH 7.5; solution C, 3 M NaCl and 0.05 M sodium phosphate, pH 6.0; solution D, 4 M Gdn-HCl and 0.05 M sodium phosphate, pH 6.0; solution E, 0.01 M EDTA, 0.5 mM PMSF, 0.10 M β-mercaptoethanol, and 0.05 M sodium acetate, pH 6.0; solution F, 0.01 M NaCl, 0.5 mM PMSF, and 0.01 M Tris-HCl, pH 7.5; solution G, 7.5 M urea and 5 mM Tris-HCl, pH 8.3; solution H, 4 M NaCl, 0.8 M sodium phosphate, and 4 mM PMSF, pH 6.0; solution I, 0.5 M NaCl, 0.1 M sodium phosphate, and 0.5 mM PMSF, pH 6.0; PR, progesterone receptor; NAP, nucleoacidic protein; NHP, non-histone proteins(s); SDS, sodium dodecyl sulfate; DHT, 5α-dihydrotestosterone.

as described for free chromatin except that the chromatin was collected on filters instead of sedimented by centrifugation.

(*C*) *Sedimentation of Chromatin in 7.5 M Urea.* The third method involves treatment of chromatin with a buffered solution containing 7.5 M urea (solution G) to remove most of the non-histone proteins but not the histones according to Zama et al. (1978). The suspension contained a DNA concentration of 0.2 mg/mL and after an hour extraction was centrifuged at 100000*g* for 24 h. The pellet was resuspended in a small volume of solution G at a concentration of 1 mg/mL and centrifuged at 100000*g* for 10 min. The supernatant containing the residual DNA and bound proteins (consisting primarily of histones) was subjected to a regressing gradient dialysis beginning at 7.5 M urea (solution G) and ending with 0 M urea in solution E over a 10-h period. This method reportedly allowed the re-forming of the nucleosome structures (Zama et al., 1978).

Treatment of Chromatin and NAP with Protease and Ribonuclease. The protease treatment involved limited digestion of oviduct chromatin with Pronase (a nuclease-free protease from *Streptomyces griseus*, Calbiochem-Behring Corp., 70000 PUK/g dry weight at 40 °C at pH 7.4). One PUK (i.e., one proteolytic unit) is the amount of enzyme that liberates a digestion product equivalent to 25 μg of tyrosine per minute at 40 °C, pH 7.5. The optimal treatment for unmasking of PR binding sites involved adding the protease at a ratio (w/w) of Pronase to DNA of 0.054, in solution B, followed by incubation at room temperature for 10 min. This ratio was determined empirically to achieve a workable time frame for sampling the effects on the subsequent steroid receptor binding. Reactions at various enzyme to DNA (as chromatin) ratios and at various time intervals were stopped by 20-fold dilution of the reaction assay with solution B at 4 °C and addition of excess albumin (at a protein to enzyme ratio of 20) as well as $^1/_8$th volume of solution H. This solution was quickly centrifuged at 10^5g for 12 h to sediment the residual protein–DNA complexes. The Pronase activity largely remained in the supernatant. The pellets were again resuspended in solution B, $^1/_8$th volume of cold solution H was added, and the suspension was recentrifuged. The pellets were rinsed with cold water and then resuspended at 0.2 mg of DNA/mL in solution I and assayed for PR binding (acceptor activity).

Oviduct NAP was also treated at a ratio of Pronase to DNA (w/w) of 0.0036. Again, the ratios were determined empirically to achieve a workable time frame. Reactions were terminated as described above for chromatin. The extent of proteolysis was monitored by treatment of a chromatin solution containing [^{14}C]ovalbumin with the enzyme followed by analysis of acid solubility and radioactivity profiles of the [^{14}C]ovalbumin upon SDS–polyacrylamide gel electrophoresis. In other experiments, oviduct chromatin was treated with ribonuclease (RNase) (Millipore Corp., Freehold, NJ, 3000 units/mg of protein) at a RNase to DNA ratio (w/w) of 0.14 at 4 °C. Reactions were stopped at various times by centrifugation at 10^4g for 5 min, resuspending pellets in solution E, and recentrifuging. Pellets were gently washed 3 times, resuspended in solution B, and assayed for PR binding (acceptor activity). RNase activity was assayed by addition of *Escherichia coli* RNA to the chromatin solutions and measurement of absorbance at 2600 Å in the acid-soluble supernatant.

Quantitation of the DNA and Proteins. For DNA analysis, 10–20-mg amounts of dry chromatin–cellulose resins or varying aliquots of free chromatin were incubated in 1.0 mL of 0.5 N $HClO_4$ at 90 °C for 30 min. When the resins were analyzed, equivalent amounts of pure cellulose were included in the blanks and in the DNA standards. After incubation, the samples were cooled on ice and centrifuged at 1000*g* for 5 min. Aliquots of 300 μL were removed from the supernatants for analysis of DNA by the diphenylamine method (Burton, 1956; Spelsberg et al., 1971a,b). For analysis of histone levels, 4–6 mg of dry resin or 10–50 μg of free chromatin (as DNA) was extracted with 1.0 mL of 0.4 N H_2SO_4 for 30 min at 4 °C. The solutions were centrifuged at 1000*g* for 5 min. The supernatants were neutralized and assayed for histone protein by the method of Lowry (1951). For analysis of non-histone protein or total protein, the resins of the dehistonized chromatin were resuspended in 1.0 mL of 0.1 N NaOH and the supernatant was quantitated as above by the method of Lowry (1951).

Reconstitution of Masking Activity. The method for reconstituting the masking activity utilized a modification of a method described previously for reconstituting acceptor sites for PR to pure DNA (Spelsberg et al., 1984). The method was modified for optimal reconstitution of the masking activity to the NAP. Reconstitution of the chromatin proteins to remask the PR binding sites on the NAP was performed by reannealling the various protein fractions at increasing ratios of protein to DNA (as NAP). This was accomplished by dialysis against a decreasing gradient of 6–0 M Gdn-HCl in solution E over a 24-h period followed by dialysis vs. solution E for several more hours. Reconstituted samples were diluted with 1 volume of solution E and centrifuged at 10000*g* for 10 min to remove insoluble (unbound) protein. The supernatants were then centrifuged at 10^5g for 24 h to sediment the reconstituted nucleoproteins. The pellets were resuspended in solution B at about 1 mg/mL of DNA. The concentrations of DNA and protein were determined as described above. About 70% of the DNA (as nucleoprotein) was recovered after this reconstitution procedure.

Methods Used for Measuring Binding of PR to Nuclear Acceptor Sites. Two methods were used to assay for PR binding to native chromatin and DNA.

(*A*) *Streptomycin Method.* The streptomycin assay has been described previously (Spelsberg, 1983). Varying amounts of partially purified [^{3}H]progesterone receptor, [^{3}H]PR, were added to initiate the reactions. Tubes were incubated on ice for 90 min with occasional mixing. The amounts of receptor and the incubation period were selected to achieve saturable binding with minimal degradation of the chromatin and steroid–receptor complexes. Assays involving chromatin were terminated by sedimentation of the chromatin followed by washes. The assays involving NAP or DNA were terminated by the addition of streptomycin sulfate (Upjohn Co., Kalamazoo, MI) which forms salt linkages with the DNA and thus causes DNA or NAP to precipitate. These complexes were sedimented by centrifugation and washed similar to chromatin. In both cases, the sedimentation allows rapid separation of the bound (to NAP or chromatin) [^{3}H]PR from unbound [^{3}H]PR.

(*B*) *Cellulose Method.* This method has been described previously (Webster et al., 1976; Thrall et al., 1978). Native, partially purified, dehistonized chromatins or pure DNA was attached to cellulose as described elsewhere (Spelsberg et al., 1978). After the 90-min incubation with [^{3}H]PR, the chromatin–cellulose material was treated in the same manner as chromatins in the streptomycin method, except no addition of streptomycin was necessary since the resins were insoluble. In these experiments, pure cellulose was added to separate binding assay tubes, and the resulting PR "binding" values were subtracted as background from the values obtained from

Table I: Unmasking of Nuclear Acceptor Sites for the Avian Oviduct PR Using Chaotropic Agents

treatment	residual protein bound (protein/DNA w/w) histones	non-histones	total	fraction removed	PR binding (cpm/mg of DNA × 10^{-5})
(A) Chromatin Attached to Cellulose[a]					
none (intact chromatin)	1.0	2.0	3.0		0.90
+1.0 M NaCl (pH 6)	0.45	1.98	2.53	part of CP-1	1.92
+2.0 M NaCl (pH 6)	0.10	1.95	2.05	all of CP-1	1.40
+3.0 M NaCl (pH 6)	0.00	1.93	1.93	all of CP-1	1.40
+4.0 M Gdn-HCl (NAP)	0.00	0.25	0.25	CP-2	3.80
+7.0 M Gdn-HCl (DNA)	0.00	0.05	0.05	CP-3	1.50
(B) Free Chromatin[b]					
one (intact chromatin)	1.06	2.23	3.29		0.95
+2.0 M KCl (pH 6)				CP-1	1.55
+4.0 M Gdn-HCl (NAP)				CP-1, CP-2	3.10
+7.0 M Gdn-HCl (DNA)				CP-1, CP-2, CP-3	0.35
7.5 M urea (pH 8.3)	1.05	0.84	1.94	"CP-2" and "CP-3"	3.70

[a] Unmasking of progesterone acceptor sites in selectively deproteinized chromatin–cellulose samples. Chromatin–cellulose resins were treated with increasing levels of NaCl followed by 4 or 7.0 M Gdn-HCl to dissociate different groups of chromosomal proteins. The resins were washed with dilute buffer to remove traces of the salts. PR binding assays were then performed on each of the extracted resins as described under Materials and Methods. CP-1 represents all histones and about 5% of the non-histone proteins. CP-2 and CP-3 represent about 8.0% and 15% of the total non-histone chromosomal proteins. The NaCl and Gdn-HCl solutions contained 0.05 M phosphate buffer, pH 6.0. The values represent the mean of five replicate binding assays using one saturating level of PR. The values are not corrected for DNA binding. The residual protein was determined by the method of Lowry (1951). DNA was quantitated by the method of Burton (1956). [b] Unmasking of progesterone acceptor sites in free ("unattached") hen oviduct chromatin. Hen oviduct chromatin was treated with NaCl or Gdn-HCl, in sequential order, to dissociate different groups of chromosomal proteins as described above. Alternatively, the chromatin was treated with 7.0 M urea. After the treatments, the residual DNA and bound proteins were separated from dissociated proteins by ultracentrifugation at 10^5 g, resuspended in buffer B, and bound with PR by using the streptomycin assay. The mean and standard deviations of five replicate assays using one saturating level of PR are presented for each sample. The values are not corrected for DNA binding. (The CP-2 fractions of non-histone proteins extracted by urea and 4 M Gdn-HCl are roughly equivalent.)

assays containing cellulose with chromatin, NAP, or DNA. Similar results were obtained by using either the streptomycin or the cellulose methods.

Results

Unmasking of Nuclear Acceptor Sites by Removal of a Specific Chromosomal Protein Fraction. Previous studies have shown that the masked PR binding sites in chromatin, once unmasked, have the same high affinity for PR binding and the same low level of nonspecific binding of nonfunctional receptors as the available sites in intact chromatin. Pure DNA, on the other hand, did not display saturable, high-affinity PR binding or differences in binding between nonfunctional and functional receptors (Boyd & Spelsberg, 1979b; Spelsberg & Halberg, 1980; Boyd-Leinen et al., 1984; Spelsberg et al., 1976, 1984). Table I shows the results of selective dissociation of the various chromosomal proteins on the amount of PR binding (acceptor sites) to the residual chromatin. Chromatin, attached to cellulose, was treated sequentially with increasing molarities of NaCl to selectively extract discrete groups of histones from the DNA (Table I, section A). Alternatively, aliquots of unattached chromatin were treated with 2.0 M KCl or different molarities of Gdn-HCl to extract the proteins followed by ultracentrifugation to sediment the residual chromatin (Table I, section B). Acid–urea–polyacrylamide gel electrophoresis of the protein extracted from the chromatin showed that different groups of histones were extracted by increasing amounts of NaCl with all histones extracted by 2.0 M NaCl (data not shown).

Cell-free binding assays were performed on each of the salt-treated chromatin preparations to determine whether removal of any or all of the histone species resulted in unmasking. Table I (section A) shows that selective removal of histones (CP-1) from chromatin–cellulose caused only a moderate increase in PR binding. This was speculated to be due to the PR binding to the exposed DNA. Studies using unattached chromatin gave similar results (Table I, section B). In contrast, treatment of both bound and free chromatin with 4.0 M Gdn-HCl, which removes the CP-2 fraction of non-histone proteins, resulted in a major (4-fold) increase (unmasking) in PR binding (Table I). The chromatin deficient in the CP-1 and CP-2 protein fractions has been termed nucleoacidic protein (NAP). These experiments indicate that non-histone proteins, but not the histones, are responsible for the major masking of the specific acceptor sites. As further proof that the masking activity involves the non-histone proteins but not histones, treatment of chromatin with high concentrations of urea was performed. Zama et al. (1978) reported that treatment of chromatin with high concentrations of urea extracts the majority of the non-histone proteins but not the histones. Hen oviduct chromatin was treated with 7.5 M urea in dilute buffer at pH 8.3, and the residual protein–DNA complex was dialyzed against dilute Tris buffer to regain the nucleosome structures (Zama et al., 1978). As shown in Table I (section B), the urea treatment resulted both in the removal of a large fraction of the non-histone proteins and in the unmasking of the acceptor sites. The histones were not extracted.

Table I also shows that the removal of the remaining proteins from the chromatin by extracting with 7 M Gdn-HCl caused a marked decrease in the PR binding to a level similar to that observed with pure DNA (Table I). This loss of PR binding has previously been shown to be due to removal of the tightly bound non-histone protein fraction CP-3 from the DNA (Webster et al., 1976; Spelsberg et al., 1976, 1977, 1979, 1984; Thrall et al., 1978).

Determination of the Chemical Nature of the Masking Activity. To determine whether proteins (or RNA) play a role in the masking of acceptor sites, the effects of protease and RNase treatments on the PR binding to chromatin were examined. Figure 1 shows the results of studies of PR binding to the residual deoxyribonucleoproteins after treatment of oviduct chromatin with Pronase or ribonuclease. As shown in Figure 1 (panel A), treatment with RNase, which decreased the chromatin RNA by 75% (data not shown), had no effect on the PR binding. In contrast, when chromatin was treated

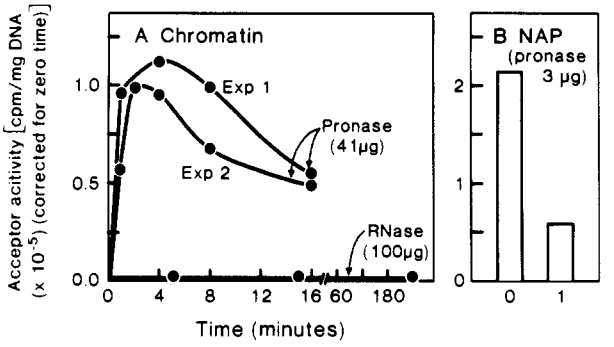

FIGURE 1: Susceptibility of masking activity and acceptor activity in oviduct chromatin to Pronase and ribonuclease. Oviduct chromatin and NAP were treated with Pronase at a Pronase to DNA ratio (w/w) of 0.054 at room temperature in solution C. Chromatin was treated with ribonuclease (RNase) at an RNase to DNA ratio (w/w) of 0.14 at 4 °C. Reactions were stopped at various times after treatment, and the residual material was washed by ultracentrifugation as described under Materials and Methods. PR binding to the residual material after Pronase and RNase treatment was performed by the streptomycin assay. The results of two experiments for Pronase treatment and one for the RNase treatment are presented in panel A. Each value was corrected for zero time of enzyme treatment; that is, the value of PR binding to chromatin in which the reaction was stopped immediately after addition of enzyme (zero time) was subtracted from each of the other values. The mean of five replicate binding assays performed within the same experiment is presented for each value. In panel B, the bar graph represents PR binding to untreated and Pronase-treated NAP. The values are not corrected for zero time.

with Pronase, an enhanced PR binding occurred with time of incubation. The amount of protein in chromatin decreased only 10% at the 4-min incubation period when the optimal unmasking of PR binding occurred (data not shown). As the Pronase treatment progressed, the PR binding eventually began to decrease. The increase in binding with mild protease treatment is interpreted as due to destruction of proteins which are responsible for masking the acceptor sites. The decrease in binding after extended protease treatment is speculated to be due to the destruction of acceptor protein required for PR binding (Spelsberg et al., 1983, 1984; Boyd & Spelsberg, 1979a; Boyd Leinen et al., 1984). This destruction of acceptor sites is supported by the fact that Pronase treatment of NAP, in which the acceptor sites are chemically unmasked, caused a marked loss in PR binding (see Figure 1, panel B). The maximum unmasking seen with Pronase treatment never reached that obtained by the salt extractions (panel A compared to panel B). This is not surprising since the protease action would simultaneously digest acceptor protein as well as masking proteins although at different rates. Control studies were performed to verify that Pronase-treated chromatins or NAPs did not degrade the receptor in the subsequent PR binding assays. The residual DNA material was washed extensively and did not affect the integrity of the receptor during subsequent incubations as determined by steroid binding to the receptor and by sucrose gradient analyses (data not shown).

To determine whether artifacts in the binding of PR were being generated by proteolysis, chromatin was subjected to partial Pronase treatment to achieve partial unmasking. This Pronase-treated chromatin was then subjected to 4 M Gdn-HCl treatment, the commonly used salt extraction to unmask the acceptor sites. The resulting "NAP" displayed a PR binding equivalent to that of 4 M Gdn-HCl treatment alone [i.e., the effects were additive, and no "new" binding sites are created by Pronase treatment (data not shown)]. These data support the conclusion that the macromolecule responsible for the masking is a protein(s).

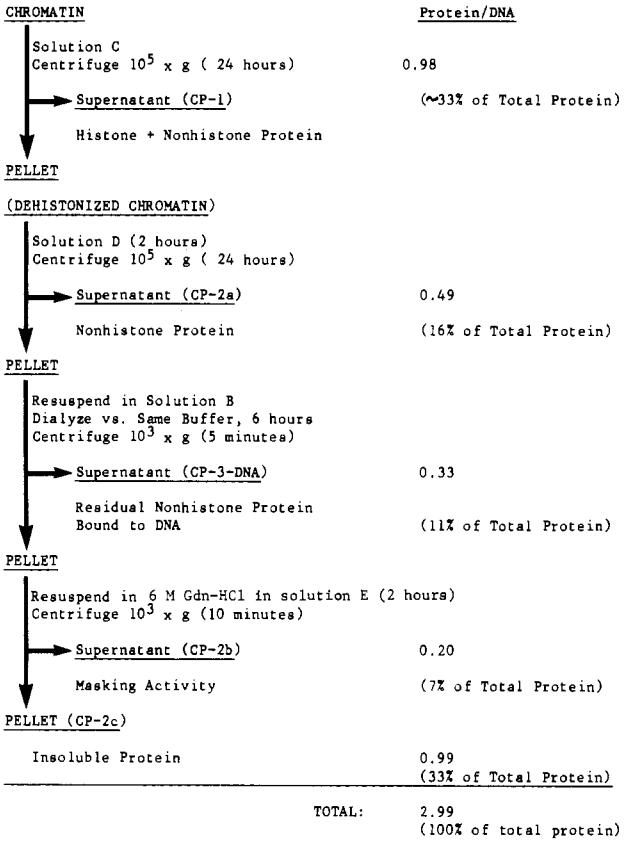

FIGURE 2: Subfractionation of the CP-2 fraction for masking activity. Chromatin was extracted with 3.0 M NaCl, pH 6.0 (solution C), for 2 h centrifuged 12 at 4 °C at $10^5 g$. The supernatant represents the CP-1 fraction. The pellet was again extracted with 4.0 M Gdn-HCl and centrifuged at $10^5 g$ for 24 h to solubilize the CP-2a fraction. The pellet was resuspended in dilute buffer (solution B), dialyzed against solution B for several hours, and centrifuged at $10^4 g$ for 5 min. The NAP (DNA + bound CP-3 protein fraction) remains in the supernatant. The pellet, which contains the CP-2b and CP-2c fractions, is then extracted with 6.0 M Gdn-HCl (pH 6.0) to solubilize the CP-2b fraction. The CP-2c fraction remains insoluble and is discarded. The amount of protein recovered in each fraction is listed on the right side as the ratio of protein to DNA and as the percent of the total recovered protein.

Masking of Nuclear Acceptor Sites in Nontarget Tissues. It should be mentioned that treatment of chromatin from a nontarget tissue (spleen) with Pronase resulted in a similar unmasking of PR binding sites (data not shown). The masking of PR binding sites in the chromatins of nontarget tissues has previously been shown by using salt extraction procedures (Spelsberg et al., 1976, 1977, 1979, 1984).

Partial Purification of the Masking Proteins. The CP-2 fraction was further fractionated to partially enrich the masking proteins. Most of the non-histone proteins are extremely insoluble in aqueous solutions after their removal from chromatin. A fractionation procedure based on differential solubility of the various CP-2 proteins in Gdn-HCl was found to be a rapid and easy method for the initial enrichment of the masking activity. Figure 2 outlines the separation of CP-2 into three subfractions as described under Materials and Methods and shows the distribution of protein in these fractions. Unfortunately, CP-2c, which represents about 33% of the total chromosomal protein, was insoluble even in 6 M Gdn-HCl and thus could not be analyzed for masking activity.

Reconstitution of Masking Activity (Proteins). To determine which CP-2 protein subfraction actually contains the masking activity, the two protein fractions were reconstituted to the unmasked (CP-2 deficient) chromatin, termed NAP. Thus, conditions for reconstituting the masking proteins to

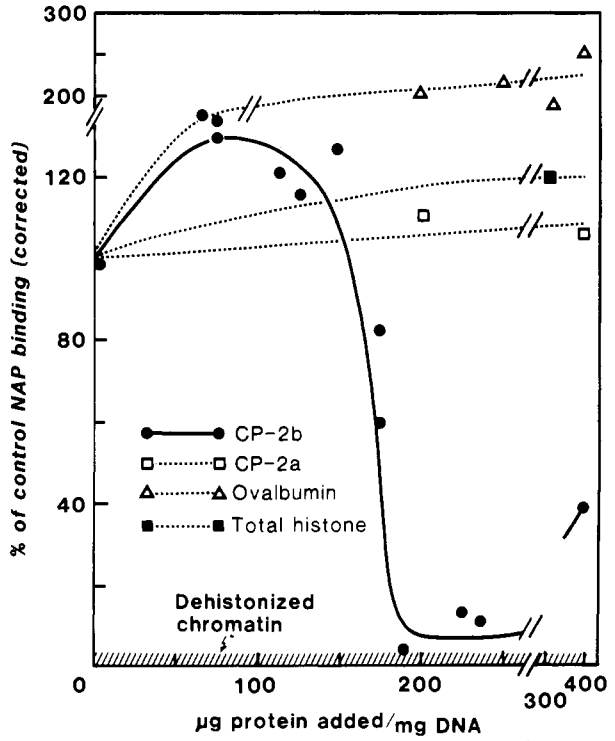

FIGURE 3: Reconstitution of various chromatin components to NAP: analysis of masking activity. Two fractions of chromosomal proteins, CP-2a and CP-2b, ovalbumin, and total histone were reconstituted separately to NAP at increasing ratios of protein to DNA. The reconstitutions and binding assays were performed as described under Materials and Methods. After reconstitution, the unbound protein was removed by centrifugation, and the DNA–protein complexes were assayed for PR binding. Each point represents the mean of five replicate binding assays. PR binding to NAP reconstituted with fractions (●) CP-2b, (□) CP-2a, (△) ovalbumin, and (■) histone is presented. All binding values were corrected for binding to dehistonized chromatin; i.e., the binding values for dehistonized chromatin were subtracted from binding values obtained for the reconstituted nucleoproteins. All values were normalized by using PR binding to native NAP as 100% of control.

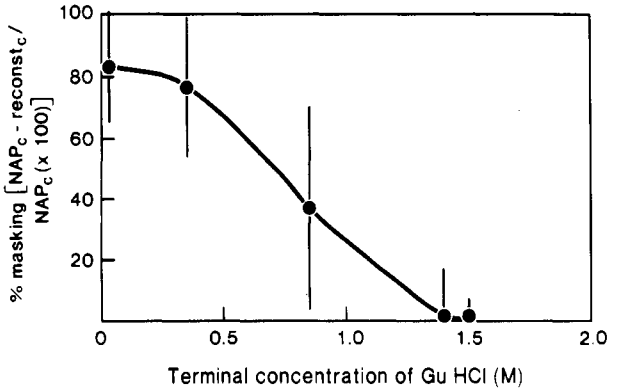

FIGURE 4: Appearance of masking activity as a function of the final Gdn-HCl concentrations during the reconstitution process. CP-2b was reconstituted to NAP at a protein to DNA ratio of 0.230 as described under Materials and Methods. Dialysis was terminated at various Gdn-HCl concentrations (shown on the abscissa as determined by conductivity measurements). The time required to reach these concentrations was 3 (4 M Gdn-HCl), 4 (3 M Gdn-HCl), 8 h (2 M Gdn-HCl), 12 (1 M Gdn-HCl), 16 (0.5 M Gdn-HCl), and 24 h (0.1 M Gdn-HCl). Each point represents the average and standard deviation of the means of PR binding to the three replicate nucleoproteins reconstituted in separate assays at each of the concentrations of Gdn-HCl. The values are expressed as the percent masking, calculated as the PR binding to control NAP (NAP$_c$) minus that to the reconstituted NAP (+CP-2b) (reconst) divided by the NAP$_c$. This value was multiplied by 100. Therefore, 100% masking is equivalent to PR binding to intact chromatin. The 0% masking is equivalent to PR binding to totally unmasked nuclear sites on partially deproteinized chromatin, termed NAP.

NAP to regenerate the masked PR binding sites were a modification of a method previously reported by this laboratory to reconstitute the specific binding sites (Thrall et al., 1978; Spelsberg & Halberg, 1980; Spelsberg et al., 1983, 1984). In the experiments presented here, many different conditions were examined. One of the most important conditions was the prevention of proteolysis during the reconstitution process. This protease activity was associated with the CP-2 protein fraction and thus probably represents chromatin-associated proteases. The addition of the protease inhibitor PMSF was found to inhibit much of the proteolysis and permitted complete and efficient reconstitution of the masking activity. Without this inhibitor, the extent of masking activity was reduced. This proteolytic activity was monitored by analyses of degraded [^{14}C]ovalbumin, added to the reconstitution assays, on SDS gel electrophoresis. Thus, PMSF was included in all reconstitution assays as well as in the cell-free nuclear binding assays with the PR.

For reconstitution of masking activity, 0.5–1-mg aliquots of NAP were mixed together with increasing amounts of each of the CP-2 protein subfractions (CP-2a, CP-2b). The NAP and CP-2 subfractions were dissolved in 6 M Gdn-HCl at varying ratios of protein to DNA and the samples dialyzed against a gradient of 6–0 M Gdn-HCl as described elsewhere (Spelsberg et al., 1984). While the free CP-2 proteins by sedimentation of the former were insoluble in aqueous solution, those bound to the DNA (NAP) were relatively soluble. After the reconstitution process, the unbound (insoluble) proteins were separated from the DNA-bound CP-2 proteins by low-speed centrifugation. Each sample was then analyzed for PR binding activity to assay for masking activity. Figure 3 shows a composite of many reconstitution experiments. As shown by the solid line, reconstitution of only CP-2b resulted in remasking of the PR binding sites. The protein specificity of this masking phenomenon was supported by the fact that CP-2a and the egg white protein, ovalbumin, as well as the histones displayed no masking activity (Figure 3). As the ratio of CP-2b to DNA in the reconstitution increased per mass of DNA, the binding activity first increased and then sharply declined. The basis of this early rise in binding activity was observed with all proteins and is speculated to be due to a protection of acceptor proteins needed for PR binding. The maximum decrease in binding was caused by a ratio of CP-2b to DNA in the range of 0.20–0.23, which is approximately the same ratio at which CP-2b is calculated to be present in native chromatin (Figure 3).

Interestingly, the maximal degree of masking occurs at a level of binding similar to that measured with dehistonized chromatin even when an excess of CP-2b is applied. Often a reversal of the masking effect is observed. The reason for this decrease in masking is not clear but may be due to some degree of aggregation of the CP-2b proteins at the higher ratios of protein to DNA. Figure 4 shows that the reconstitution of the masking activity of the NAP occurs during the reconstitution process at low concentrations of Gdn-HCl. Although the reconstitution process is begun at 6.0 M Gdn-HCl in order to solubilize the CP-2 proteins, the masking activity actually is only reconstituted below 1.0 M Gdn-HCl. Therefore, the masking activity appears to correlate with the presence or absence of the CP-2b proteins bound to the NAP. Also, the unmasking using chemical solvents such as 4.0 M Gdn-HCl must involve removal of these masking protein(s).

Correlation of Masking with Bound Protein during Reconstitution. Although the majority of the reconstitution

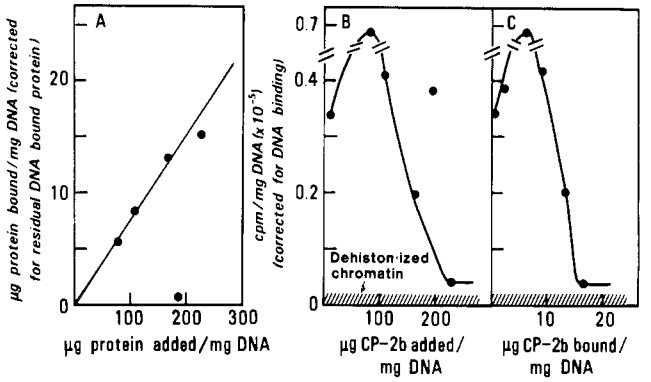

FIGURE 5: Relationship of masking activity to levels of "bound" CP-2b protein. Reconstitutions of CP-2b to NAP were performed as described under Materials and methods. For each level of CP-2b added, determinations of protein bound to NAP were performed. After the reconstitution process, the insoluble (unbound) CP-2b was separated from soluble NAP with bound CP-2b by low-speed centrifugation. The ratio of protein to DNA in native NAP was subtracted from the ratios in the reconstituted NAPs to obtain the amount of CP-2b protein bound to NAP as a result of the reconstitution process. Bound protein was quantitated by the method of Lowry (1951). DNA was quantitated by the method of Burton (1955). Panel A represents a plot of the amount of CP-2b added to the reconstituted assays vs. the amount of CP-2b bound to NAP during the reconstitution. In panel B, PR binding to the reconstituted samples is plotted against the amount of CP-2b *added* to the reconstitution assay as in panel A. In panel C, PR binding to reconstituted samples is plotted against the amount of CP-2b *bound* to NAP as a result of the reconstitution. The shaded areas represent PR binding to dehistonized chromatin. Each point represents the mean of five replicate binding assays. PR binding to DNA was subtracted from each value to give "corrected" cpm per milligrams of DNA.

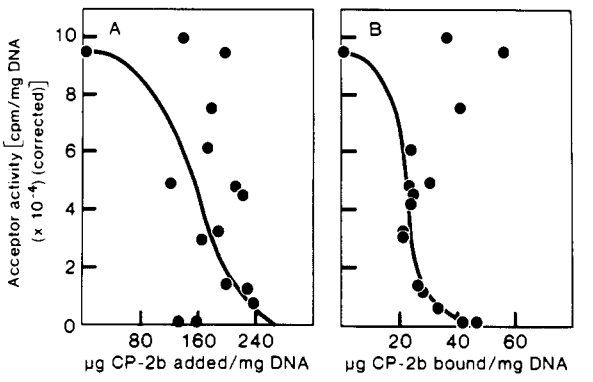

FIGURE 6: Efficiency of the reconstitution of masking activity of the acceptor sites for the progesterone receptor. This figure represents a composite of experiments using multiple ratios of CP-2b protein to DNA as described in the legend of Figure 6. In panel A, the PR binding is plotted against the ratio of CP-2b added to the DNA (as NAP) whereas in panel B, the PR binding is plotted against the ratio of CP-2b bound to the DNA (as NAP). The reconstitution assays contained 200 μg of ovalbumin to offset the early increase in PR binding shown in Figure 6.

samples displayed a masking of binding sites, about 20% of the samples failed to display any masking activity. This 20% failure in remasking could be due to failure of the CP-2b proteins to reanneal to NAP during reconstitution or to some residual proteolytic activity which destroys the masking proteins. Experiments were performed to determine the extent of CP-2b proteins bound to the NAP as a result of the reconstitution process. The free protein was separated from the NAP-bound protein after each reconstitution (described under Materials and Methods), and the amount of protein bound over that contained in the NAP itself was then determined. Figure 5 (panel A) represents a plot of the amount of CP-2b added to the reconstitution assay vs. the amount of protein which is actually bound to the NAP. In most reconstitution assays, there appears to be a linear increase in the amount of CP-2b bound to NAP vs. the amount of protein added to the reconstitution mixtures. Figure 5 (panel B) represents a plot of the extent of PR binding to these reconstituted samples vs. the amount of protein added to the reconstitution assay. In general, the amount of CP-2 protein added to the reconstitution assay correlates with the decrease in PR binding (i.e., masking). However, exceptions were found. In the reconstitution assay containing 190 μg of CP-2b protein in Figure 5, panel A, most of the added protein failed to bind to the DNA. This was accompanied by a high level of PR binding (i.e., no masking) for this same reconstituted sample (see panel B). However, when the PR binding activity is plotted against the amount of CP-2b actually bound to NAP, this stray point at the 190-μg level better fits the pattern (Figure 5, panel C). These results show that there is a better correlation between the extent of masking and the amount of CP-2b protein actually bound to NAP as opposed to the amount of CP-2b protein added to the reconstitution assay. Thus, the actual binding of the CP-2b proteins appears necessary for masking to occur. The increase in PR binding at the low ratios of CP-2b protein to DNA is inexplicable but might be due to protection of the DNA from damage during the reconstitution process as described previously (Thrall & Spelsberg, 1980; Spelsberg et al., 1984). It is interesting that the saturation of the masking of sites is still achieved even though less than 10% of the CP-2b protein added is actually bound to NAP during the reconstitution procedure (Figure 5). This could be explained by the fact that the masking proteins represent only a small fraction of the proteins in CP-2b.

Figure 6 shows a composite of several binding assays comparing the extent of masking vs. the quantity of CP-2b protein added to the assays (panel A) or the quantity actually bound to the NAP (panel B). Again, a better correlation is observed between the extent of masking and the CP-2b protein actually bound. However, these more extensive analyses also show that about 10–15% of the reconstitution assays with adequate bound CP-2b protein still fail to mask the acceptor sites (Figure 6, panel B). The reason for the failure to remask the PR binding sites in these reconstituted samples is not known but possibly could be due to the degradation or loss of the actual masking proteins in the CP-2b protein.

Reversibility of Masking. It was conceivable that the CP-2b proteins were not masking the PR binding sites but rather that some of the acceptor sites were being destroyed during the reconstitution process. The reversibility of the masking activity was then examined. The acceptor sites on the NAP were remasked by reconstitution with the CP-2b fraction. Both the NAP reconstituted alone and the NAP reconstituted with the CP-2b fraction were then assayed for PR binding. Table II shows that the reconstituted CP-2b fraction causes decreased PR binding. The "remasked" samples were then treated with 4 M Gdn-HCl to again unmask the acceptor sites. As shown in Table II, the 4 M Gdn-HCl treatment of the reconstituted CP-2b–NAP complex resulted in a high PR binding resembling the level achieved with the unmasked sites on NAP. These results indicate that the reconstituted masking of the acceptor sites is reversible and does not involve damage to the acceptor sites. Instead, a physical blocking of PR binding sites generated by the CP-2b proteins appears to be involved.

Molecular Sieve Chromatography of the Masking Activity. Preliminary characterization of the masking activity in the CP-2b fraction was performed by using molecular sieve chromatography on CL-Sepharose-6B in 6 M Gdn-HCl.

Table II: Reversibility of Masking by the CP-2b Fraction[a]

treatment	protein fraction removed or reconstituted	[^{3}H]PR binding (cpm/mg of DNA × 10^{-4})
none (native chromatin)	none	0.60 ± 0.02
4.0 M Gdn-HCl extraction of native chromatin to obtain NAP	CP-1 + CP-2 removed	1.43 ± 0.1
NAP reconstituted to remask sites	CP-2b reconstituted	0.70 ± 0.15
4.0 M Gdn-HCl extraction (NAP) of reconstituted NAP	CP-2b removed	1.35 ± 0.3

[a] Chromatin was treated with 4 M Gdn-HCl to unmask acceptor sites as determined by the PR binding assay (first unmasking). This NAP was reconstituted with CP-2b at optimal conditions to remask the acceptor sites. Subsequent treatment of the reconstituted NAP (bound with CP-2b proteins) with 4 M Gdn-HCl restored the original unmasked level of acceptor activity (second unmasking). All values represent the mean ± SE of five replicate binding assays in the same experiment, using one saturating level of PR and the streptomycin assay (Spelsberg, 1983). The values are not corrected for DNA binding.

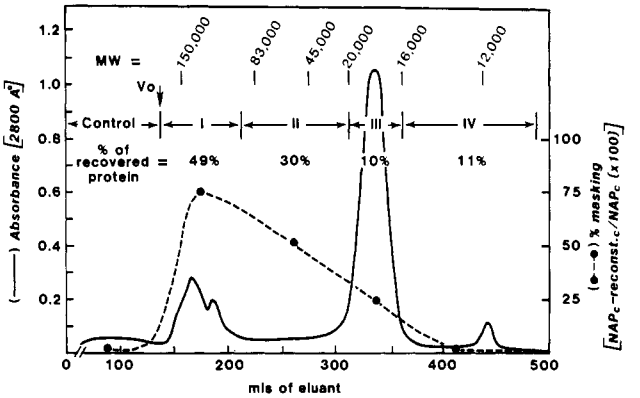

FIGURE 7: Molecular sieve chromatography (CL-Sepharose-6B) of the CP-2b fraction: elution of the masking activity of the acceptor sites of the progesterone receptor. The CP-2b fraction was applied to a CL-Sepharose-6B column in 6 M Gdn-HCl in 0.01 M phosphate buffer, pH 6.0. The fractions were reconstituted to NAP as described under Materials and Methods at several ratios (v/w) of the fraction volume to the amount of the DNA (as NAP). The lowest ratio in any of the column fractions which gave marked >60% masking of the NAP acceptor sites was selected as the ratio for all fractions to be plotted. The solid line (—) represents the A_{280} profile of the eluted fractions. The dashed line (●---●) represents the masking activity found in the eluted fractions. Molecular weights of standard proteins from a calibration curve for this column are presented at the top.

Figure 7 shows the patterns of protein and masking activity eluted from this column. The masking activity was detected over a broad range of molecular mass from 150 000 (or greater) to 50 000 daltons. This indicates either that a heterogeneous population of masking activity exists or that the masking activity is sticking to the resin and "bleeding" off the resin during the elution process. Higher concentrations of reducing agent (0.1 M β-mercaptoethanol) failed to alter this elution pattern. Further purification and characterization of these masking proteins are planned.

Discussion

The method presented here can be used to identify a macromolecule(s) which mask(s) the nuclear acceptor sites for the avian oviduct progesterone receptor. Methods for the unmasking and remasking of these PR acceptor sites are described. While there are occasional failures in the reconstitution of masking of the PR binding sites, the method is sufficiently successful to allow identification of such activity in various chromatin fractions. Determination of the amount of bound CP-2b protein presented a more accurate correlation with the masking activity than did the amount of protein added to the reconstitution assay. However, some samples still failed to show masking even though some protein was bound during reconstitution. Overall, about 10% of the reconstitution assays still failed to regenerate masking activity even when the amount of bound protein was considered. The reconstitution method represents a modification of that reported previously for the reconstitution of the acceptor proteins to DNA to generate nuclear binding sites (acceptor sites) for PR (Spelsberg et al., 1984). However, the acceptor activity (proteins) is reconstituted at 2.0 M Gdn-HCl while the masking activity is reconstituted below 1.0 M Gdn-HCl. The reconstitution of both the acceptor sites and the masking of these sites utilize the general approach for reconstituting the chromatin ultrastructure described earlier by Chae (1975) and Woodcock (1977). The fact that the addition of excess masking proteins to the reconstitution assays failed to totally block PR binding indicates that the acceptor sites, which are normally exposed in native chromatin, may be protected.

On the basis of data from previous studies, the properties of the masked sites for the avian oviduct PR appear to be identical with the unmasked sites in native chromatin (Webster et al., 1976; Boyd & Spelsberg, 1979b; Spelsberg & Halberg, 1980; Boyd-Leinen et al., 1984; Spelsberg et al., 1976, 1977, 1979, 1983, 1984). The studies presented in this paper show that the masking activity is proteinaceous. Therefore, one group of non-histone proteins (CP-3) is involved in the binding of PR to chromatin, and another group (CP-2) is responsible for masking of these binding sites. These results indicate that the non-histone proteins are involved in both positive and negative control of the steroid receptor binding to nuclear acceptor sites.

It is interesting that the loss of masking activity by proteolysis occurs on oviduct or spleen chromatin even before the degradation of histones occurs. Thus, the masking proteins are extremely susceptible to proteolytic digestion. This property emphasizes the importance of suitable conditions for the isolation and handling of intact chromatin to be used in the hormone binding assays to achieve an accurate estimate of the number of steroid receptor binding sites on native chromatin. Conditions must be established which prevent even slight proteolytic action on chromatin or nuclei during their isolation or during their subsequent incubation in the cell-free binding assay. Otherwise, unmasking of acceptor sites might occur. The possible unmasking of nuclear acceptor sites by proteolysis may explain why some laboratories have reported that steroid receptors bind to the chromatin of nontarget tissues (Chamness et al., 1974; Higgins et al., 1973; Milgrom & Atger, 1975), while other laboratories report no such binding (Spelsberg et al., 1976; Thrall et al., 1978). The former studies utilized long incubation times, higher temperatures, and crude cytosolic preparations, all of which encourage proteolysis which could unmask the acceptor sites.

It should be mentioned that evidence for the masking of nuclear acceptor sites has been found in several other steroid target-tissue systems in other animals. Klyzsejko-Stefanowicz et al. (1976) found that removal of a protein fraction, similar to the CP-2 proteins, from rat testicular and prostatic chro-

matin, using high-salt–urea solutions, resulted in an increase in the number of acceptor sites available for binding by 5α-dihydrotestosterone (DHT)–receptor complexes. They did not, however, observe such an increase in a nontarget tissue such as liver. Similarly, Perry & Lopez (1978) observed a 4–5-fold increase in binding of both estrogen– and progesterone–receptor complexes to sheep hypothalamic chromatin after removal of the CP-2 proteins from chromatin using 2 M NaCl + 5 M urea. Also, Ruh and co-workers have observed a masking of acceptor sites for estrogen receptors (Ruh et al., 1981; Singh et al., 1984) and for glucocorticoid receptors (Ruh et al., 1985) using 2–4 M Gdn-HCl to unmask target tissue chromatin. Similarly, Ruh & Spelsberg (1983) reported an unmasking of acceptor sites for the estrogen receptor in avian oviduct chromatin. Thus, it seems that masking of nuclear acceptor sites for steroid receptors may be a universal phenomenon.

The exact biological relevance of masking remains unclear. The fact that in nontarget tissue chromatins the acceptor sites may be totally masked and that in the developing oviduct chromatin the degree of masking changes (Spelsberg et al., 1983) suggests that masking may be tissue specific and thus possibly involved in a regulation of transcriptional responses to steroids. Quantitative changes in masking during cytodifferentiation are consistent with the idea that masking might play some regulatory function with respect to which genes will or will not respond to the constitutive steroid–receptor complex. This mechanism would explain how the same steroid and the same receptor are capable of affecting different sets of genes in different tissues. Thus, masking may be responsible for the differential response to progesterone and estrogen by the avian oviduct at different stages of development (O'Malley et al., 1969). In support of this theory, Sarkar & Lydigsen (1976) have reported that hydrocortisone elicits an age-dependent induction of glutamine synthetase in the embryonic chick retina. They found hydrocortisone receptors in retinas from both noninducible (7 days) and inducible (12 day) stages of embryonic development. However, the number of specific nuclear binding sites for the glucocorticoid receptors in the 12-day retina decreased as did the steroid induction of the enzyme. Similarly, Teng & Teng (1975) have reported that nuclear binding sites for the estrogen receptor are present in the chromatin of both the left and right Mullerian ducts of the chick during differentiation. However, in later stages of development, when the right duct becomes nonfunctional, the specific nuclear binding sites in the right duct markedly decrease. At the same time, the binding to the nuclear sites from chromatin from the left duct, which develops into the future functional oviduct, does not decrease. Thus, a model is proposed whereby during cytodifferentiation, the nuclear binding sites of steroid receptors are selectively masked and unmasked to generate tissue-specific responses involving tissue-specific gene expression in response to steroids. Irregularities in masking might serve as the basis of some diseases, such as the steroid resistance of certain tumors, which contain functional steroid receptors.

There are many aspects of the masking phenomenon which remain obscure: (1) Do the quantitative changes in nuclear binding in different tissues actually reflect qualitative changes in binding sites or to different degrees of masking? (2) By what mechanism are the masking proteins positioned, and what are the chemical properties of the masking protein? (3) What prevents certain acceptor sites (approximately the same numbers which are exposed in native oviduct chromatin) from being masked when ample masking proteins are reconstituted to oviduct NAP? (4) Do other proteins or the genome location play a role in this "protection"?

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Calcium-Dependent and Calcium-Independent Interactions of Prothrombin Fragment 1 with Phosphatidylglycerol/Phosphatidylcholine Unilamellar Vesicles†

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ABSTRACT: We have measured the phase behavior of mixed dipentadecanoylphosphatidylglycerol ($DC_{15}PG$)/dimyristoylphosphatidylcholine (DMPC) small unilamellar vesicles (SUV) in the presence of saturating (>98% occupancy of binding sites) concentrations of bovine prothrombin fragment 1 and 5 mM Ca^{2+}. Binding of fragment 1 in the presence of Ca^{2+} was verified by an increase in 90° light scattering. Only in the cases of $DC_{15}PG$/DMPC SUV below their phase transition and of pure DMPC SUV were such light scattering measurements not reversible upon addition of ethylenediaminetetraacetic acid to complex Ca^{2+}. Phase-behavior changes of $DC_{15}PG$/DMPC SUV as monitored by diphenylhexatriene fluorescence anisotropy occurred in concert with the binding of fragment 1. The major effects of peptide binding on SUV phase behavior were to raise the phase-transition temperature by 2–15 °C, depending on vesicle composition, and, in general, to make the phase diagram for these small vesicles closely resemble that of large vesicles. No evidence was obtained for the existence of lateral membrane domains with distinct compositions induced by the binding of prothrombin fragment 1 plus Ca^{2+}. Surprisingly, fragment 1 without Ca^{2+} also altered the phase behavior of $DC_{15}PG$/DMPC SUV. Most striking was the effect of fragment 1 (with or without Ca^{2+}) on DMPC SUV phase behavior. Freeze–fracture electron microscopy demonstrated that pure DMPC vesicles were induced to fuse in the presence of fragment 1, while vesicles containing $DC_{15}PG$ remained intact. The rate of DMPC SUV fusion (followed by 90° light scattering) increased with increasing fragment 1 concentration but was not saturable up to 40 μM fragment 1, suggesting a weak, nonspecific interaction between fragment 1 and the neutral phospholipid vesicle. We conclude that Ca^{2+}-dependent binding of prothrombin fragment 1 to $DC_{15}PG$/DMPC vesicles does not induce the formation of thermodynamically stable $DC_{15}PG$-rich domains but does involve Ca^{2+}-independent interactions in addition to the commonly accepted electrostatic one inherent in the Ca^{2+}-bridging model.

The activation of prothrombin to thrombin is a membrane-mediated process requiring the tight association of both the substrate (prothrombin) and enzyme (factor Xa) with a negatively charged membrane surface (Suttie & Jackson, 1977; Zwaal, 1978). While platelets probably provide the requisite membrane surface in vivo, synthetic phospholipid vesicles provide a more convenient model system for examining this association in vitro.

Ca^{2+} is required for tight binding of either prothrombin or its N-terminal proteolytic fragment (fragment 1) to a charged membrane surface (Jackson et al., 1975; Nelsestuen, 1976). The dissociation constants for binding of either prothrombin or prothrombin fragment 1 to PG-containing membranes have been reported to be identical (Dombrose et al., 1979). The ion presumably acts to "bridge" γ-carboxyglutamic acid residues of prothrombin fragment 1 to negatively charged lipids within a membrane (Nelsestuen, 1978; Dombrose et al., 1979). This view has spawned a detailed molecular picture in which prothrombin binding is seen to induce formation of membrane domains that are rich in charged lipids (Dombrose et al., 1979; Nelsestuen, 1978; Barton & Findley, 1976). While this view is currently the most popular, others feel that nonelectrostatic interactions may be involved (Madar et al., 1982). Figure 1 summarizes the several possible models for the interaction of prothrombin fragment 1 with mixed-phospholipid membranes that will be considered in this paper. These models all recognize the probable contribution of Ca^{2+} bridging but differ as to the size of possible membrane domains and as to the occurrence of nonelectrostatic interactions.

In this paper, we begin to distinguish between these possible models of prothrombin binding by determining the effect of prothrombin fragment 1 with and without Ca^{2+} on the phase behavior of small unilamellar vesicles (SUV)[1] composed of mixtures of dipentadecanoylphosphatidylglycerol ($DC_{15}PG$) and dimyristoylphosphatidylcholine (DMPC). The results

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[1] Abbreviations: DMPC, 1,2-dimyristoylphosphatidylcholine; $DC_{15}PG$, 1,2-dipentadecanoylphosphatidylglycerol; DPH, 1,6-diphenyl-1,3,5-hexatriene; TES, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; LUV, large unilamellar vesicles; LMV, large multilamellar vesicles; SUV, small unilamellar vesicles.

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