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ROLE OF NUCLEAR PROTEINS AS HIGH AFFINITY SITES ("ACCEPTORS") FOR PROGESTERONE IN THE AVIAN OVIDUCT

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SUMMARY

Administering multiple doses of progesterone to immature chicks results in the detection of several classes of oviduct nuclear binding sites. Comparisons of serum progesterone levels and responses to RNA polymerase I and II activities with the nuclear binding, indicate that the highest affinity classes of sites are the biologically important ones. *In vitro* binding studies using isolated progesterone receptor complex also reveal the presence of these multiple classes of sites. The highest affinity class of nuclear sites in the oviduct, representing 6000 to 10,000 sites per cell with a $K_D \sim 10^{-9}$ M, appear to be tissue specific. These sites are present but completely masked in the chromatin of non-target organs (spleen and erythrocyte), while 70% are masked in the target tissue (oviduct). Further fractionation studies involving DNA affinity chromatography using chromatin-cellulose resins and molecular sieve-chromatography using Agarose-GuHCl resins reveal that the "acceptor" activity is associated with two molecular weight-proteins between 12,000 and 17,000. These proteins are bound with very high affinity to DNA. The "acceptor-proteins" must be reannealed to DNA to achieve binding activity. These results support that acidic proteins determine the high affinity nuclear binding sites for steroids.

INTRODUCTION

At present, the mechanism by which steroids alter gene expression is obscure. Obviously, the events occurring between administration of the steroid and the appearance of specific mRNA are the ones involved in gene regulation. We have been studying the earliest known event which occurs in the avian oviduct nucleus before the progesterone induced alteration of gene activity occurs, i.e., the binding of the progesterone–receptor complex to isolated nuclei *in vitro*. The nuclear (or chromatin) component which binds the progesterone–receptor complex (P–R) is being explored since there is the possibility that the macromolecules representing this binding site may be involved in the steroid induced modifications of gene expression.

Practically every component in the nucleus has been suggested (with supporting evidence) as the nuclear "acceptor" which binds steroid–receptor complexes *in vitro*. Examples are the nuclear envelope [1, 2], the ribonucleoproteins [3], basic (histone) proteins [4–6], the acidic (non-histone) proteins [7–15] and DNA [16–20]. There have also been reports of a specific binding of free steroids (not complexed with receptor) to nuclear material [1, 14, 21]. In short, the chemical identity of the nuclear acceptors which bind steroids *in vitro* has yet to be demonstrated. One problem is that the conditions of these *in vitro* assays have varied markedly resulting in a saturable tissue specific binding in some systems [8, 22–25] but not in others [4, 26, 27]. The majority of studies on DNA binding demonstrate a nonspecific, nonsaturable binding by steroid–receptor complexes *in vitro* [16, 17, 19, 20, 22]. It is likely that any nonsaturable binding measured *in vitro* probably represents nonspecific adsorption as opposed to specific binding. Before investigating the identity of the nuclear binding sites (or "acceptor") we attempted to clarify the discrepancies in specific vs non-specific binding *in vitro*.

We have found that the low ionic conditions (0.01–0.05 M KCl) in the binding assays performed by the majority of laboratories results in a pattern of nuclear binding which is neither saturable nor tissue specific. These results are obtained whether one uses the nuclear material from the oviducts of immature chicks [7–9] or adult hens [10–12], and a crude or purified progesterone–receptor complex [P–R]. Under these low ionic conditions, the P–R binds to a multitude of components including, all nuclear material in a nonsaturable fashion. However, when the ionic strength of the binding assays is increased to 0.15 M or 0.2 M KCl, we observe a saturable, tissue specific binding which selectively measures only the highest affinity class of binding sites [10–12]. Some of the results concerning the selection of proper conditions for assaying high affinity, specific nuclear binding are presented in this report. This section is followed by our results of the purification and characterization of the high affinity binding sites in oviduct nuclei which we term "acceptors".

EXPERIMENTAL

The majority of materials and methods used in this report have been previously described [10–12, 28, 29]. For clarity, the more important methods will be outlined in the figure legends.

RESULTS

Chronology of events of steroid hormone action on gene expression

Studies were performed in the chick oviduct to assess the chronology of events which occur after steroids enter target cells and bind to their receptor proteins [30]. These studies demonstrated that within minutes after steroid administration, the hormone binds to the oviduct nucleus and modifies the activity of nucleolar and nucleoplasmic RNA polymerases. Either accompanying or shortly following these events are changes in the degree of DNA restriction (chromatin template capacity). Subsequent to these occurrences (2 h after administration), we detect the synthesis of ovalbumin mRNA. This chronology of events is summarized in Fig. 1. We find no correlation between the expression of a specific gene and the gross changes reflected in total polymerase activities or chromatin template capacity. This is not surprising since the latter parameters undoubtedly represent the sum total of the response of thousands of genes to thousands of molecules of a single effector molecule (steroid).

Detection of multiple classes of nuclear binding sites in vivo: *identification of the biologically important sites*

We have recently demonstrated the presence of more than once class of binding sites in chick oviduct nuclei *in vivo* and in hen oviduct nuclei and chromatin *in vitro* [10–12, 28]. Selecting a 2 h incubation period after injecting 100 μCi (0.718 μg) of labelled progesterone either alone or together with increasing concentrations of unlabelled progesterone, the number of molecules of hormone bound to oviduct nuclei was assessed. As shown in Fig. 2A, use of multiple levels of unlabelled progesterone together with the fixed amount of [^{3}H]-progesterone results in the detection of more than one type or class of binding sites in the oviduct nucleus. The saturation of the first class of binding sites appears to occur when 10–15 μg of the hormone are injected and involves approximately 10^3 molecules of progesterone per cell nucleus. The saturation of the second class of binding sites occurs by injecting between 15 and 100 μg of the hormone and involves about 10^4 molecules of

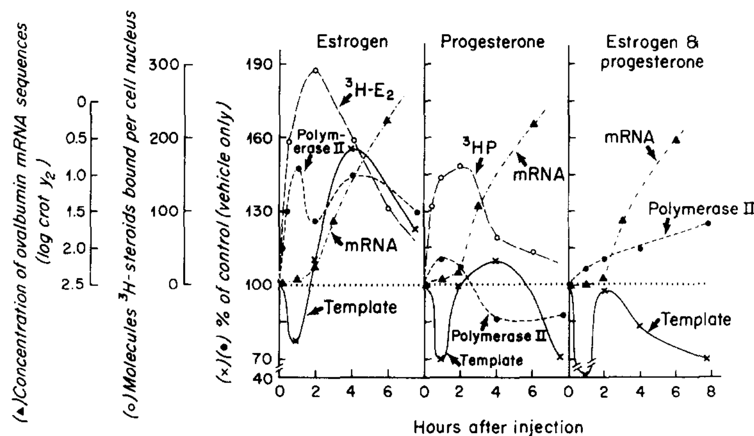

Fig. 1. Effects of estrogen and progesterone on transcriptional events in the oviducts of withdrawn chicks. The effects on several parameters of transcription, as well as uptake of E and P by oviduct nuclei are plotted for each individual hormone treatment on a chronological basis. Control chicks were treated with vehicle (sesame oil) only and sacrificed at the same time intervals as the treated chicks. At various times after injection, groups of chicks were sacrificed and the RNA polymerase activities in isolated nuclei, and template capacities of isolated chromatin were analyzed. The data is plotted with the mean of the control values representing 100%. After various times of treatment with E, P, or E + P, total cellular RNA was isolated from excised oviducts. To estimate the ovalbumin mRNA concentration in each sample, RNA (20–50 μg) and cDNA$_{ov}$ (0.6 ng; 500 c.p.m.) were then hybridized at 67°C for up to 48 h in sealed glass capillaries and the percentage of cDNA$_{ov}$ resistant to digestion by S1 nuclease was determined. The small fraction of cDNA$_{ov}$ which was resistant to S1 nuclease in the absence of added RNA (5–10% of the total radioactivity) was subtracted for each hybridization time. The percentage of cDNA$_{ov}$ hybridized as a function of the product of initial RNA concentration (C_{ro}) and time (t) was plotted as a double-reciprocal plot [40] to estimate $C_{ro}t_{1/2}$ values (when 50% of the input cDNA$_{ov}$ become hybridized) for each sample. The concentration of ovalbumin mRNA sequences in total RNA samples is plotted for convenience as the log of the $C_{ro}t_{1/2}$ value for each sample. [Reproduced with permission: T. C. Spelsberg and R. F. Cox, *Biochim. Biophys. Acta* **435** (1976) 376–390.]

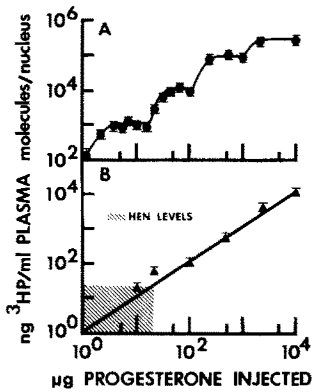

Fig. 2. Nuclear binding vs the plasma levels of [^{3}H]-progesterone *in vivo*. Figure 2A represents the molecules of progesterone bound to oviduct nuclei while Figure 2B represents serum levels of progesterone in response to the dose of the hormone given to immature chicks, which were previously treated with diethylstilbestrol (DES) for 20–25 days [8, 9, 28]. Groups of four chicks were given injections subcutaneously of 100 μCi (or 0.718 μg) of [^{3}H]-progesterone alone or in combination with specified concentrations of unlabelled hormone. Stock [^{3}H]-progesterone (47.8 Ci/mmol) in benzene was mixed with a specified amount of unlabelled progesterone in benzene as described elsewhere [28]. The solutions were frozen, freeze-dried and resuspended in sesame oil for injection. Each chick received a 100 μl injection for doses under 100 μg and a 200 μl injection for doses over 100 μg. In A at 2 h after injection, the oviducts of each group were excised, combined, and the nuclei isolated as described elsewhere [11, 28]. Their radioactivity was measured and the results plotted as molecules [^{3}H]-progesterone/cell nucleus calculated as described elsewhere [28]. The average and range of three replicates of analysis of values obtained for each group are presented. In B at 2 h after injection of [^{3}H]-progesterone, blood was taken from the wing vein into heparinized capillary tubes, the tubes were centrifuged for 2 min at 200 *g* and 10–50 μl portions of the plasma removed for measurement of radioactivity and protein analysis [28]. The amount (ng) of progesterone/ml of plasma was calculated from the radioactivity (d.p.m./ml plasma). The average and range of two replicate analyses for each group are presented. [Reproduced with permission, Spelsberg T. C. *Biochem. J.* **156** (1976) 391–98.]

progesterone per cell nucleus. Further increases in the dose of progesterone reveal two more classes of "weaker" binding sites, representing approximately 10^5 and 3×10^5 molecules per cell nucleus.

The physiological concentrations of progesterone in the plasma of laying hens range between 0.88 and 17.21 ng/ml [31, 32]. Figure 2B shows the concentrations (ng/ml) of progesterone in the plasma of immature chicks 2 h after injecting the hormone. The injection of 20 μg of progesterone into immature chicks results in concentrations of the hormone in the plasma which approximate the maximal level measured in the plasma of laying hens. This dose results in the nuclear binding of progesterone which involve the two highest affinity class of sites, representing 1000 to 10,000 molecules of progesterone per cell nucleus.

Comparisons of the amount of nuclear binding with the RNA polymerase activities using the numerous increasing doses of progesterone were also performed. As shown in Fig. 3, an initial enhancement of RNA polymerase I activity over that of controls is observed, beginning at a 10 μg dose of progesterone. At doses greater than 100 μg, a decrease in RNA polymerase I activity compared with that of controls is observed. Doses greater than 200 μg of progesterone have no further effect on the RNA polymerase I activity. The RNA polymerase II activity decreases, beginning at a 6 μg dose of progesterone. This decrease continues up to the 100 μg dose. Doses of the hormone greater than 100 μg fail to alter further the RNA polymerase II activity. Comparing the amounts of nuclear-bound progesterone with the RNA polymerase activities, it is seen that only the binding of progesterone to the two highest-affinity classes of sites results in some response in these enzyme activities. Binding to the weaker classes of sites results in no further alteration in the RNA polymerase activities.

The results support that the two highest affinity class of nuclear binding sites, consisting of about 10,000 molecules of progesterone per cell nucleus, represent the required sites for direct transcriptional response. The "weaker" binding could be involved in the steroid induced alterations in transcription but also may be artifacts obtained after tissue homogenization.

Detection of multiple classes of nuclear binding sites in vitro: *Specific binding to the highest affinity class*

A search for different classes of binding sites in oviduct nuclei *in vitro* as found *in vivo* was then initiated.

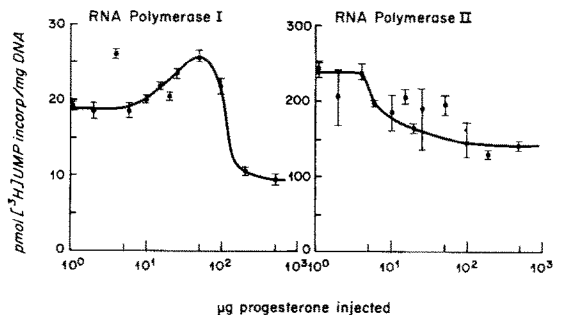

Fig. 3. Effects of the dose of progesterone on RNA polymerase activity. The preparation of the progesterone for injection into chicks is described in the legend to Fig. 2 and elsewhere [28]. The doses of 200 μg and 1000 μg were administered in 200 μl of sesame oil, and the lower doses in 100 μl. These experiments were carried out as described in the legend to Fig. 2. Groups of six to ten chicks were each injected (subcutaneously) with a specified amount of the hormone. Two groups were injected with vehicle only and used for determining the non-injected controls. At 2 h after injection, the oviducts from each group were excised, combined, and the nuclei isolated as described elsewhere [43]. The activities are expressed as pmol of [^{3}H]-UMP incorporated/mg of DNA. The average and range of three replicates of analysis are presented for all values. [Reproduced with permission, Spelsberg T. C.: *Biochem. J.* **156** (1976) 391–98.]

In the following experiments, isolated [^{3}H]-progesterone–receptor complex (P–R) was incubated with various nuclear components at 4°C for 90 min as described elsewhere [10–12]. The nuclear material, bound with P–R, was then separated from the free P–R, and the amount of nuclear bound P–R measured. The application of many different levels of the P–R in the binding assays was employed in hopes to detect the presence of more than one class of nuclear binding sites. As shown in Fig. 4, the presence of several classes of binding sites with differences in binding affinities is seen. Evidence of even more classes of sites has been obtained by using even greater amounts of the hormone–receptor complex. The presence of these different classes of binding sites may be responsible for the non-saturability of the nuclear binding sites under the low-ionic-strength conditions. Since increasing ionic concentrations cause a decrease in total binding of the [^{3}H]-progesterone–receptor complex to nuclei, it is possible that different classes of binding sites are involved. Data in Fig. 4 support this hypothesis in that an increase in the ionic strength from 0.05 M to 0.15 M KCl causes the elimination of the weaker classes of binding sites, leaving only a highest-affinity class. The equilibrium disssociation constants (K_D) for each class of sites are also given in Fig. 4. These constants were calculated by using Scatchard-plot analysis [33]. An equilibrium dissociation constant, $K_D = 1.2 \times 10^{-9}$ M, representing approximately 5000 sites/cell nucleus, was calculated for the first (highest-affinity) class of binding sites [10, 11]. The second (next-highest-affinity) class of binding sites has a tenfold less affinity with an equilibrium dissociation constant, $K_D = 1.3 \times 10^{-8}$ M, representing approximately 20,000 sites/cell nucleus. Scatchard-plot analysis of the third class of binding sites was too variable, so an estimate of the K_D ($\sim 4.0 \times 10^{-8}$ M), and the number of sites per cell ($\sim$ 35,000) were roughly calculated from a plot of bound versus free receptor, by using estimated half-saturation and saturation values, respectively. In any case, more than one class of binding sites is detectable *in vitro* as well as *in vivo*. The interaction between P–R and oviduct nuclei in each class of sites is primarily electrostatic in nature, with each selectively dissociated at specific ionic strengths [10]. Under physiological ionic conditions (and higher), only the highest affinity class of binding sites exists.

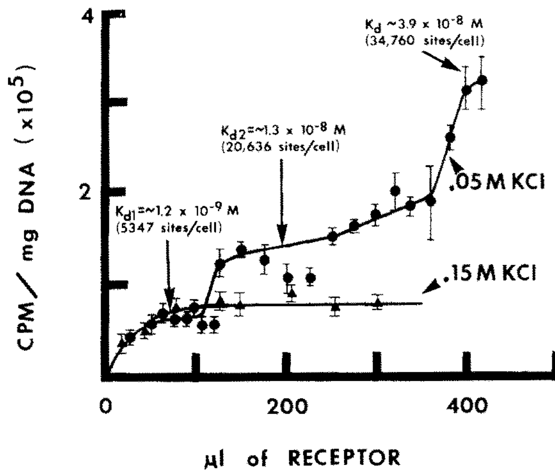

Fig. 4. Detection of multiple classes of nuclear binding sites *in vitro* in the hen oviduct: effects of ionic strength. These experiments were performed with the oviducts from mature hens as described elsewhere [10, 11]. The assay mixtures contained either (●) 0.05 M KCl or (▲) 0.15 M KCl (final concentration). The assays were performed using 25 µg of DNA per assay and the standard assay method. The equilibrium dissociation constants and the number of binding sites per cell for the first two plateaux were calculated from Scatchard plot analysis [11, 33]. The values for the third plateau were obtained from the estimated saturation (sites/cell) and half-saturation (K_D) concentrations in a plot of bound versus free [^{3}H]-progesterone-receptor complex. The DNA content per hen organ cell was taken as 2.5 pg/cell [39]. The range and average of three replicates of analysis for each receptor concentration are shown. [Reproduced with permission, Pikler G. M., Webster R. A. and Spelsberg T. C.: *Biochem. J.* **156** (1976) 399–408.]

For better comparisons between nuclear binding *in vivo* and *in vitro*, the data in Fig. 2 and 4 were replotted as molecules of P bound/cell nucleus (see Fig. 5). The levels of progesterone bound to the higher affinity classes do not differ greatly between the *in vivo* and *in vitro* conditions (Fig. 5A and 5B). In addition, the higher affinity classes of binding sites appear to be localized with chromatin isolated from oviduct nuclei (Fig. 5C). In summary, studies *in vivo* and *in vitro* demonstrate the presence of more than one class of binding sites of which the highest affinity classes involve the binding of 10,000 molecules or less of progesterone per cell nucleus. These highest affinity sites

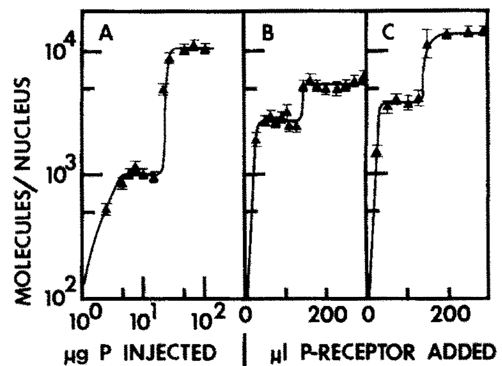

Fig. 5. Comparison of the binding of [^{3}H]-progesterone to the high affinity sites in oviduct nuclei *in vivo* and *in vitro*. The data in Fig. A and B are taken from Fig. 2 and 4 respectively and represent binding to nuclei *in vivo* (A) and *in vitro* (B). Figure C represents binding to isolated chromatin as described in the legend of Fig. 4 for nuclei. The molecules/cell nucleus were calculated as described elsewhere [11, 12, 28]. In A, B, and C, the range and average of three replicates of analysis for each receptor concentration are shown. [Reproduced with permission, Spelsberg T. C. *et al.*, New York Academy of Sciences, in press.]

are suspect as being the biologically important sites, because: (1) they are the only ones which bind P–R *in vitro* under physiological ionic conditions (0.15 to 0.20 M KCl); (2) they are the only ones bound *in vivo* when the steroid is at physiological levels in the serum, and (3) when the majority of these sites are bound *in vivo* with the P–R, the total response of the RNA polymerase I and II activities is observed.

Tissue specificity of the high affinity classes of nuclear binding sites

Studies to assess the tissue specificity of the various classes of binding sites revealed a marked tissue specificity with respect to the high affinity sites. However, the lower affinity binding sites appear to be common to the chromatin of all tissues [10, 12, 34]. As shown in Fig. 6A under low ionic conditions, the higher affinity classes of binding sites are not detected in the spleen chromatin as they are in the oviduct chromatin. Using the higher ionic conditions (which selectively measure the higher affinity classes of binding sites), the differences in binding between the chromatins from non-target and the target tissue are evident (Fig. 6B). When the ionic conditions in the binding assays reach physiological levels (~0.18 M KCl as in Fig. 6C), the binding of P–R is absolutely tissue specific, with binding occurring only with the target tissue (oviduct) chromatin. Interestingly, while the level of binding of P–R to DNA is greater than that to oviduct chromatin under low ionic conditions (Fig. 6A), the situation is reversed in the intermediate ionic conditions with the binding to DNA almost eliminated (Fig. 6B). In short, the high affinity binding sites appear to be tissue specific. They are not found in erythrocyte, liver, spleen or lung chromatin (see ref. 12 and Fig. 7). The DNA alone does not appear to represent the high affinity sites. Also, the ionic conditions of the nuclear binding assays appears to be very important in term of levels of binding, the class of sites bound, and tissue specificity of binding.

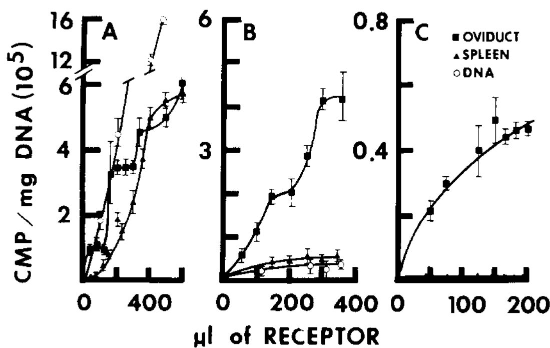

Fig. 6. The binding of the [^{3}H]-progesterone receptor complex under varying ionic conditions. Varying amounts of isolated P–R were incubated with (■) hen oviduct chromatin, (▲) hen spleen chromatin, and (○) hen organ DNA under (A) 0.05 M KCl, (B) 0.10 M KCl, or (C) 0.18 M KCl conditions. The chromatin binding assays in (A) and (C) were performed by the standard method while chromatin binding assay in (B) and DNA binding assays were performed by the streptomycin method [10–12]. The average and range of three replicates of analysis for each receptor concentration are given. [Reproduced with permission, Pikler G. M., Webster R. A. and Spelsberg T. C.: *Biochem. J.* **156** (1976) 399–408 and 409–416.

Masking of high affinity binding sites (acceptors) in the chromatins of hen tissues: Role of acidic proteins

From this point on, all binding assays will involve the high ionic conditions to selectively analyze the highest affinity binding sites. This class (or classes) of sites was termed "acceptors" several years ago [7, 9, 10], a term which will be used in the remaining sections of this paper.

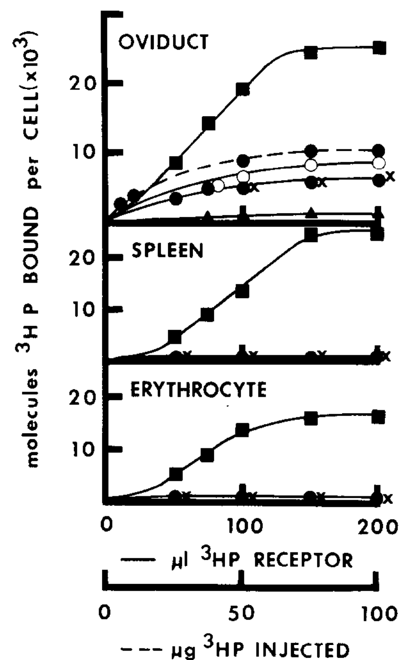

Fig. 7. Binding of [^{3}H]-progesterone to high affinity sites in the nuclear material of oviduct, spleen, and erythrocytes of the chicken. The (●--●) represents the binding to nuclei of the chick oviduct performed *in vivo* as described elsewhere [28]. The (——) represents the binding under *in vitro* conditions using isolated nuclear components and [^{3}H]-progesterone–receptor complex as described elsewhere [12]. The *in vitro* binding to the soluble DNA or DNA-protein complexes was performed using the streptomycin method or the cellulose method, as described elsewhere [10, 12]. The high affinity binding *in vitro* was assayed under 0.15 M KCl using 0 to 200 µl of labelled receptor and 50 µg DNA (as chromatin or protein–DNA complexes). The removal of total histones as well as AP_1, AP_2 or total protein has been described elsewhere [7–9, 12]. The efficiency of removing these fractions from chromatin DNA and the integrity of the nuclear components were assayed by polyacrylamide gel electrophoresis and quantitation of the protein:DNA using the Lowry [41] and diphenylamine method [42]. The symbols represent binding to the following: (●——●) isolated nuclei; (×——×) whole chromatin, (○——○) chromatin deficient of histone, (■——■) chromatin deficient of histone, AP_1, AP_2, and (▲——▲) chromatin deficient of total protein (pure DNA). The molecules of [^{3}H]-progesterone bound per cell was calculated as described elsewhere [12]. [Reproduced with permission, Spelsberg T. C., Webster R. A. and Pikler G. M. (1976) *Nature* **262**, 65–67.]

In Fig. 7, the high affinity binding or "acceptor" activity (i.e., binding assayed in 0.15 to 0.2 M KCl) of P–R to hen oviduct nuclei and chromatin *in vitro* is shown. The "acceptor" activity of chick oviduct nuclei measured *in vivo* closely approximates that measured *in vitro*. When the histones are removed the level of acceptor sites is only slightly elevated over that of whole chromatin. However, when the acidic protein fractions AP_1 and AP_2 are also removed, the number of acceptor sites in the oviduct nuclear material is enhanced 4 to 5 fold. A similar increase in acceptor sites for DNA-AP_3 complexes was reported earlier using the chick oviduct chromosomal material [8]. Interestingly, the histones and the AP_1 and AP_2 fractions can be reconstituted back to the DNA-AP_3 fraction and the "masking" (70 to 80% of the total acceptor sites) reinstated in the reconstituted chromatin as found in the native chromatin [7, 8, 34].

Also shown in Fig. 7, the nuclei and chromatins of non-target tissues (hen spleen and erythrocyte) display no acceptor sites. However, the removal of histones and acidic protein fractions AP_1 and AP_2 results in a level of binding approximating that of the target tissue (hen oviduct). Thus, even though the nontarget tissues display none (or a few) nuclear acceptor sites, these sites in reality are present but completely masked. Table 1 shows the extent of masking of these sites in the chromatins of these tissues. Assuming that all the acceptor sites are unmasked in the chromatins devoid of histones, AP_1 and AP_2, the *in vivo* assays involving oviduct nuclei shows 58% masking. The *in vitro* assays involving hen oviduct nuclei/chromatin exhibits about a 60 to 80% masking while that to hen spleen or erythrocyte nuclei/chromatin, essentially shows 100% masking.

Biological implications of the regulation of nuclear binding

The biological need for an apparent whole animal distribution of the high affinity binding sites and the masking of these sites remains obscure. It is possible that the extent of masking in the target tissue varies under normal biological influences. As shown in Fig. 8, the high affinity binding to the chromatin of the developing oviduct of immature chicks does vary with the stage of development [35]. At 20 days of estrogen treatment, the oviduct of immature chicks is fully developed [36]. The number of acceptor sites in this fully developed oviduct of the chick closely approximates that measured in the hen oviduct. Again, chick and hen spleen display very little (or no) acceptor activity. Whether or not this variable level of high affinity binding occurs during a changing endocrine state remains to be determined.

High affinity binding ("acceptor" activity) in oviduct chromatin associated with acidic proteins

As shown in Fig. 7, the removal of the remaining acidic proteins (representing the AP_3 fraction) results in a loss of the majority of acceptor sites. Similar results are displayed in Fig. 6B. These results support those reported previously for chick oviduct chromatin wherein the "acceptor" activity (i.e., high affinity binding) is associated with the acidic chromatin protein fraction termed "AP_3" [9]. These results strongly support a role of acidic chromatin proteins both in con-

Table 1. Extent of masking of high affinity sites. These data were taken from values in Fig. 7. The number of binding sites measured in each of the chromatin minus histone, AP_1, AP_2 preparations was assumed to represent the total sites in the respective chromatin and thus each of these preparations was assigned a 0% masking. The values at saturation for each preparation were taken at 100 μg of injected hormone for the *in vivo* binding and at 200 μl of labelled receptor preparation for the *in vitro* binding. The preparations of the nuclear components as well as the hormone binding assays were performed as described in the legend of Fig. 7.

Source	Level of Binding at Saturation (molecules/cell)	Per cent of Total Sites Masked
1. Oviduct Nuclei *in vivo*	10,600	58%
2. Oviduct Nuclei	5951	76%
3. Oviduct Chromatin	7441	71%
4. Oviduct Chromatin -histone	9378	58%
5. Oviduct Chromatin -histone -AP_1, AP_2	25,290	0%
6. DNA	2055	—
1. Spleen Nuclei	60	100%
2. Spleen Chromatin	48	100%
3. Spleen Chromatin -histone -AP_1, AP_2	24,106	0%
1. Erythrocyte Nuclei	357	100%
2. Erythrocyte Chromatin	416	100%
3. Erythrocyte Chromatin -histone -AP_1, AP_2	15,018	0%

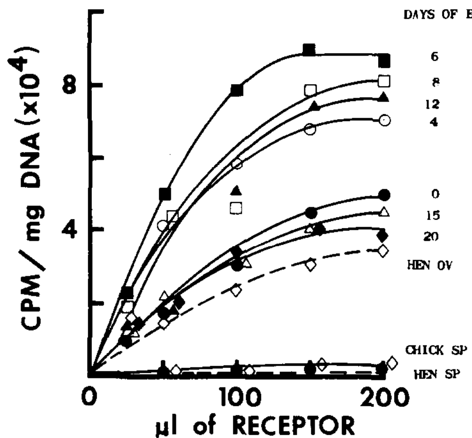

Fig. 8. High affinity binding of [^{3}H]-progesterone–receptor complex to chromatin from the developing chick oviducts. The binding assay used in this experiment was the standard method [9, 11], except that crude cytosol preparation of [^{3}H]-progesterone–receptor complex was used as described elsewhere [8]. The symbols represent binding to the chromatins of oviducts from chicks treated with diethylstilbestrol (DES) for (●) 0 days, (○) 4 days, (■) 6 days, (□) 8 days, (▲) 12 days, (△) 1 day and (◆) 20 days. At 0 day, the oviducts are undeveloped while at day 20 they are fully developed [36]. The (◇——◇) represents the chromatin from chick spleen treated 20 days with DES while the (◇– – –◇) represents chromatin from hen oviduct and (○– – –○) chromatin from hen spleen. [Reproduced with permission, Spelsberg T. C., Steggles A. W., O'Malley B. W. *Biochim. Biophys. Acta* **254** (1971) 129–134.]

stituting part of the "acceptor" sites and the masking of these sites in the oviduct (target tissue) nucleus. If the dissociated AP_3 proteins are reconstituted back to the DNA, the high affinity sites can be reinstated. Reconstituted whole hen oviduct chromatin displays the same relative levels of high affinity binding as the untreated hen oviduct chromatin, i.e., the high affinity sites as well as the masking are restored. This ability to restore the levels of binding to reconstituted chromatins similar to that found in untreated chromatins was previously demonstrated for the chick oviduct system [7–9]. Thus, evidence is presented which suggests that one fraction of the acidic chromatin proteins (called AP_3) contains the high affinity binding activity in the hen oviduct chromatin while other fractions (AP_1 and AP_2) are involved in masking of these sites. It also appears that the "acceptor" sites (high affinity binding) also exist in the chromatins of nontarget tissues of the hen but are completely masked.

Purification and characterization of nuclear "acceptor" which binds the progesterone–receptor complex

Further purification of the "acceptor" activity was desired. A chromatin-cellulose resin was prepared wherein the chromosomal proteins are eluted from the chromatographic column according to their affinity for either the DNA or other chromatin components still attached to the resin [10]. When this resin is subject to a gradient of guanidine hydrochloride in a solvent containing a high concentration of a reducing agent at pH 8.5, multiple peaks of protein are eluted from the column (Fig. 9). As shown in Fig. 10, the majority of the protein is eluted by 4 M guanidine hydrochloride. The absorption observed in Fig. 9 under conditions of a 4–7 M guanidine hydrochloride extract which yields only small amounts of protein is due primarily to the elution of ribonucleoprotein as well as small amounts of DNA. The protein eluted from the column was pooled according to the extraction as units of molarity of GuHCl (see Fig. 9) and reannealed to DNA. The resulting nucleo pro-

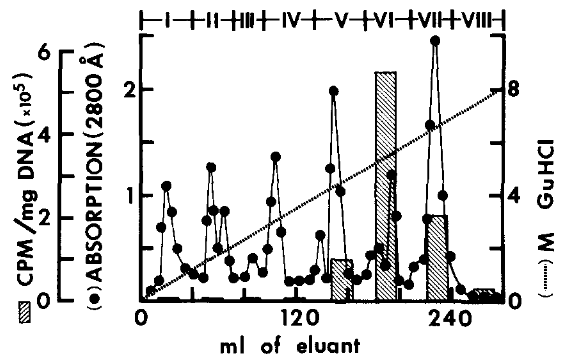

Fig. 9. Selective dissociation of proteins and "acceptor" from chromatin-cellulose resins. Hen oviduct chromatin-cellulose resin was prepared as described elsewhere [10, 12]. Twenty grams of this resin containing approximately 60 mg of DNA as chromatin was resuspended in 100 ml of cold solution containing 0.005 M sodium bisulfite + 0.01 M Tris–HCl (pH 8.5) and allowed to hydrate for 8 to 12 h with gentle stirring at 4°C. The resin was then collected on a column and a gradient of 0–8 M guanidine hydrochloride passed through this column within a 4 h period using constant levels of the buffer + 0.1 M HSETOH. The tubes were monitored by absorption at 280 nm. The fractions were also monitored for conductivity as well as refractive index, and the gradient level of guanidine hydrochloride plotted. The fractions were collected according to their elution with each unit of concentration of guanidine hydrochloride (e.g., 1 M, 2 M, 3 M, etc.). Pooled samples were then dialyzed thoroughly versus water and lyophilized. The lyophilized material was resuspended in a small vol. of water, homogenized in a Teflon pestle glass homogenizer, assayed for protein by Lowry[41], and reannealed to pure hen organ DNA. The protein:DNA to begin these reannealings was 1:10 (v/v). The reannealing consisted of a gradient dialysis from 6.0 M GuHCl in the above described buffer to 0.0 M GuHCl in the same buffer and finally dialysis versus 0.01 M Tris HCl, pH 7.5. The nucleoproteins were then analyzed for "acceptor" activity using the streptomycin method, subtracting the values obtained with pure DNA. This activity is presented as bar graphs. Total recoverable protein after dialysis and lyophilization was estimated to be 30 to 50% of the total protein placed on the column as chromatin-cellulose. [Reproduced with permission, Spelsberg T. C. *et al.* (1975). In *Chromosomal Proteins and Their Role in Gene Expression* (G. Stein & L. Kleinsmith Eds.) 153–186. Academic Press. New York.]

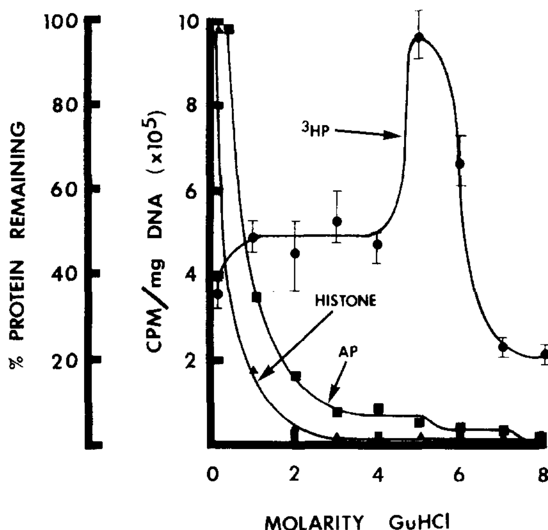

Fig. 10. The binding of [^{3}H]-progesterone–receptor complex to hen oviduct chromatin-cellulose treated with GuHCl. In this experiment, aliquots of chromatin-cellulose were washed twice in 20 vol. of solution which contains 0.1 M HSETOH + 0.005 sodium bisulfite + 0.01 M Tris–HCl (pH 8.5) and a specified molarity of guanidine hydrochloride. These resins were then washed in dilute Tris–EDTA buffer several times, frozen and lyophilized. Twenty to 50 mg amounts of the resin were then assayed for protein by resuspending the resin in the Tris–EDTA buffer, allowing it to hydrate several hours with gentle mixing, and assaying for (▲) histone using a 0.4 N H_2SO_4 extract with subsequent analysis of the (■) acidic proteins (AP) with a 0.1 N NaOH extract. These guanidine-treated resins were also tested for their (●) acceptor activity using 20–25 μg of DNA (bound to the cellulose) together with 300 μl of the hormone–receptor complex solution. The binding assay was performed essentially as described elsewhere [12] and the counts per mg DNA calculated. The average and range of 3 replicates of analysis for each assay of the hormone binding are shown. Spelsberg T. C. *et al.* (1975) In *Chromosomal Proteins and Their Role in Gene Expression.* (G. Stein and L. Kleinsmith Eds.) pp. 153–186. Academic Press. New York.

tein was analyzed for high affinity binding. As shown in Fig. 9 only the material which elutes from the column between 5 and 7 M GuHCl contains the high affinity binding activity.

After the extraction with increasing levels of guanidine hydrochloride, samples of the resin itself were removed, washed thoroughly, and subjected to the hormone binding assay. As shown in Fig. 10, the removal of all of the histone and most of the acidic proteins from the chromatin by 4 M guanidine hydrochloride fails to alter significantly the number of acceptor sites in the oviduct chromatin. These results support those shown previously in which the removal of all of the histones failed to unmask this class of binding sites. Further extraction of the chromatin-cellulose with 5 M and 6 M guanidine hydrochloride does unmask many of the acceptor sites. However, the 6 M guanidine hydrochloride extraction begins to reduce the number of acceptor sites on the DNA. So as the high affinity binding activity is lost from the resin (5–7 M GuHCl) (Fig. 10), the majority of the "acceptor" activity is detected in the eluant (Fig. 9). Table 2 demonstrates that almost 100,000 fold purification (from the starting whole tissue) is achieved at this stage.

Further purification has recently been achieved with molecular sieve chromatography using 100 cm. columns containing agarose – 1.5 m, with 6.0 M GuHCl according to the procedure of Mann *et al.*[37]. Figure 11 shows the pattern of a typical chromatography of the 5 to 7 M GuHCl extract of chromatin. The eluted material was pooled according to peaks of absorbing material and reannealed to DNA. The nucleoproteins were then analyzed for "acceptor" activity. The acceptor activity coelutes with standard molecular weight proteins (histone IV and ribonuclease) in a molecular weight range of 12,000 to 16,000 Daltons. Polyacrylamide-SDS gel electrophoresis of the fraction IV from this column is shown in Fig. 12. Gel I represents the protein from a 5–7 M GuHCl extract of whole chromatin while gel II represents the fraction IV from the Agarose column. Multiple bands are seen in the 5–7 M GuHCl extract. However only two major bands are observed in Gel II which migrate with molecular weights

Table 2. Summary of purification of "acceptor"

	Binding at saturation to highest affinity sites			
	c.p.m./mg DNA	Protein/DNA	pmols/mg Protein	Purification
Whole Tissue	75,00	24.21	0.004	1.0
Nuclei	75,00	3.05	0.972	243.0
Chromatin-Cellulose	125,000	2.14	2.3	575
NAP-Cellulose	425,000	0.36	46.7	11.675
Chromatin Cellulose Extraction				
1.0 M GuHCl	496,546	0.80	24	6,131
2.0 M GuHCl	452,960	0.31	58	14,434
3.0 M GuHCl	522,967	0.15	138	34,440
4.0 M GuHCl	446,927	0.15	115	28,668
5.0 M GuHCl	829,112	0.13	287	71,823
6.0 M GuHCl	652,609	0.07	390	97,677
7.0 M GuHCl	176,602	0.06	116	29,000

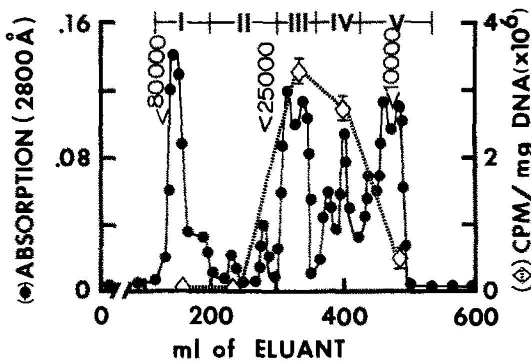

Fig. 11. Agarose – 1.5 m Molecular Sieve Chromatography of the "Acceptor" Protein Fraction in the 5–7 M GuHCl Chromatin Extract. The acceptor fraction representing the 5 to 7 M GuHCl extract of chromatin resin was dialyzed against deionized water and lyophilized. The material was resuspended in a 6.0 M GuHCl solution as described elsewhere [37] at 2 mg protein per ml. This protein solution was clarified by centrifugation at 2000 *g* for 10 min and then applied to a 2.6 × 94 cm. column of Agarose – 1.5 m. The eluted fractions were pooled according to the (●) absorbing peaks as shown in the figure. The pooled fractions were then reannealed to DNA and assayed for (◇) "acceptor" activity using the streptomycin assay as described elsewhere [12]. Values for DNA binding was subtracted from all values obtained with nucleoprotein. The average and range of three replicates of analysis are shown. A selectivity curve for this Agarose – 1.5 m column (2.6 × 94) was calculated using standard proteins, purchased from commercial sources. Their subunit molecular weights were obtained from I. M. Klotz and D. W. Darnall, P. L. Biochemicals Chart. The molecular weight distributions are given in the figure. [Reproduced with permission, Spelsberg T. C. *et al.*, New York Acad. Sci. In press.]

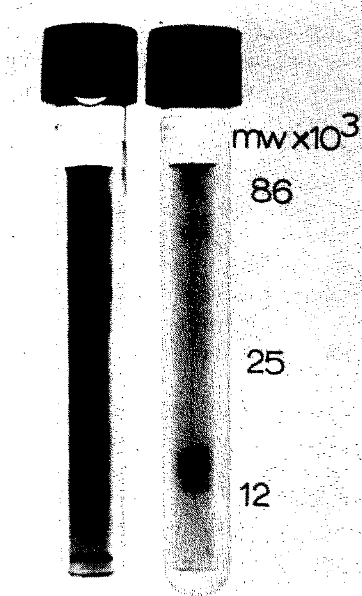

Fig. 12. Polyacrylamide-SDS-Gel electropherogram of the purified "acceptor" activity from Agarose 1.5 m column. The gels represent the protein patterns of whole chromatin from (I) 5–7 M GuHCl extract and (II) of fraction IV of the A-1.5 m chromatography (94 cm. long) described in Fig. 11. Approximately 150 μg of protein was applied to the gel I while only 30 μg of protein was applied to gel II. The gels represent 10% acrylamide, 9.5 cm. long and were run (top to bottom direction) until the bromophenol blue tracking dye migrated to within 0.5 cm. of the bottom. The gels were stained with Coomassie blue. The distribution of standard molecular weight protein in this SDS system is shown.

between 13,000 and 17,000 Daltons. This molecular weight range is in close agreement with that estimated from molecular sieve chromatography (Fig. 11).

Since the high affinity binding activity of these preparations is destroyed by pronase, and since the reannealing to DNA is required for high affinity binding, we conclude that the high affinity binding of the progesterone–receptor complex *in vitro*, and probably *in vivo*, is due to low molecular weight nonhistone proteins bound to DNA. These proteins themselves have a high affinity for DNA which yields a rapid effective approach to the purification of the "acceptors".

DISCUSSION

The identification of the nuclear acceptors which bind steroid–receptor complexes is important since this interaction represents the next step after steroid–receptor activation and binding, as well as the first step in steroid induced changes in genetic transcription. The small molecular weight proteins observed in Fig. 12 represent the only detectable protein species in these "acceptor" fractions. The presence of minute amounts of higher molecular weight proteins (as observed in Fig. 12) is somewhat of a mystery as to their source. More recent experiments involving further purification removes these proteins without significant loss in the "acceptor" activity. There remains the possibility that (1) the two protein species are breakdown products of larger "native" macromolecules and (2) the two protein species predominant in the gels are themselves not the "acceptors" but other molecules in the preparation actually induce the "acceptor" activity. The fact that the "acceptor" activity coelutes with these proteins from chromatographic resins and that the "acceptor" activity is destroyed by pronase treatment certainly supports their role as the nuclear "acceptors". Our results suggest that the "acceptor" protein must be reannealed to DNA to achieve high affinity binding, i.e., non reannealed protein does not significantly bind to P–R. In any case, we propose that the progesterone–receptor complex migrates to the nucleus and binds to a acidic proteins-DNA complex which is followed by alterations in RNA polymerase activities and gene expression [7, 8, 30].

The high affinity sites ("acceptors") consist of about 6000 to 10,000 sites in whole oviduct chromatin. Removal of the masking acidic proteins (fractions AP_1, AP_2) increases the number of binding sites 4

to 5 fold or to 24,000 to 50,000 sites per cell nucleus. Assuming one receptor–steroid complex binding to one acceptor protein, we would have 20 to 50 thousand acceptor molecules per oviduct cell. Since the "acceptor" protein has an average weight of about 15,000 Daltons (by our experiments), and there is 2.5 pg DNA per hen somatic cell [39], we have about 10^{-15} g acceptor protein–cell or 0.2 to 0.5 μg of acceptor protein per mg DNA. Since we obtain about 1 mg chromatin DNA/g hen oviduct, it would take 5 kg of oviduct to obtain 1 mg acceptor assuming 100% yield. We require about 5 times that amount or 1 kilogram of oviduct to obtain 40 μg acceptor protein.

It is interesting that a 70% to 80% "masking" of acceptor sites occurs in the target tissue chromatin. The varying number of acceptor sites measured in the chick oviduct chromatin during development (Fig. 8) suggests that a regulation of steroid action on target cells may occur at the level of nuclear binding. One must wonder whether or not transformed cells in culture or in mammary tumors which contain steroid receptors but which do not respond to the steroids, have "masked" nuclear "acceptor" sites. Thus, these cells may be nonresponsive since the genome cannot bind the steroid–receptor complexes.

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DISCUSSION

Clark. Have you done the McGuire–Chamness type experiment? That is, inject hormone *in vivo* in order to fill nuclear acceptor sites and then add receptor hormone complex to these nuclear preparations. One would expect to see reduced binding if indeed acceptor sites were filled by the *in vivo* injection.

Spelsberg. We've attempted to do this. We have performed this 4 times and 2 out of the 4 times, analyzing the high affinity sites, we observed a 30% competition. In one experiment, we observed nothing and in another we observed a 15% difference between controls and injected. We don't know why we are not getting better competition,

maybe the sites between *in vitro* and *in vivo* conditions are completely different. It will probably take years of work on our part to prove the relationship. If the reports on *in vitro* activation of RNA polymerases by steroids are correct, then these *in vitro* sites must be in part biological.

Munck. Is there any evidence that the hormone–receptor complex, once it gets to a nuclear site, remains attached at that point?

Spelsberg. No there is no evidence. We tried to do things like fixing the receptors to chromatin with biofunctional agents. Dr. Webster spent several months trying to fix the *in vivo* bound hormone-receptor to chromatin with bifunctional reagents but we encountered problems trying to hook the few thousand receptor molecules out of hundreds of millions in the nucleus. Theoretically and practically, it takes days of incubation to accomplish this, but this long period results in destruction of receptor–steroid complex. We appeared to be able to fix a few of the receptors to the chromatin but it was not enough to analyse. I will mention one thing we are doing just out of interest. We can take small amounts of protein and reanneal it to the DNA at ratios of protein to DNA of 0.05 where we observe maximum of binding. As we reanneal more of this purified preparation to the DNA, we actually observe an inhibition of binding. So we feel (based on these observations) that there may be specific sites on the DNA in which the acceptor anneals.

Clark. The fact that you can uncover sites must mean that you can grind up a whole chicken.

Spelsberg. We thought of this, Jim, but even though we see the same level of binding to high affinity sites in partially deproteinized chromatins of all tissues, we are a bit afraid that these sites may be different between the tissues, so right now we are working only with oviduct. We would like to homogenize the whole hen—the cost per mg DNA would be lower.