Acknowledgments

The authors thank K. Skare for assistance with surgical procedures and M. Micke for his instrumental expertise.

References

Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911-917.
DeLuca, H. F., & Schnoes, H. K. (1976) *Annu. Rev. Biochem. 45*, 631-666.
DeLuca, H. F., Paaren, H. E., & Schnoes, H. K. (1979) *Top. Curr. Chem. 83*, 1-65.
Esvelt, R. P., Schnoes, H. K., & DeLuca, H. F. (1979) *Biochemistry 18*, 3977-3983.
Frolik, C. A., & DeLuca, H. F. (1971) *Arch. Biochem. Biophys. 147*, 143-147.
Frolik, C. A., & DeLuca, H. F. (1972) *J. Clin. Invest. 51*, 2900-2906.
Harnden, D., Kumar, R., Holick, M. F., & DeLuca, H. F. (1972) *Science 193*, 493-494.
Jones, G., & DeLuca, H. F. (1975) *J. Lipid Res. 16*, 448-453.
Kumar, R., & DeLuca, H. F. (1976) *Biochem. Biophys. Res. Commun. 69*, 197-200.
Kumar, R., & DeLuca, H. F. (1977) *Biochem. Biophys. Res. Commun. 76*, 253-258.
Kumar, R., Harnden, D., & DeLuca, H. F. (1976) *Biochemistry 15*, 2420-2423.
Kumar, R., Schnoes, H. F., & DeLuca, H. F. (1978) *J. Biol. Chem. 253*, 3804-3809.
Napoli, J. L., Fivizzani, M. A., Hamstra, A. H., Schnoes, H. K., & DeLuca, H. F. (1978) *Steroids 32*, 453-466.
Suda, T., DeLuca, H. F., & Tanaka, Y. (1970) *J. Nutr. 100*, 1049-1052.
Tanaka, Y., Castillo, L., DeLuca, H. F., & Ikekawa, N. (1977) *J. Biol. Chem. 252*, 1421-1424.
Webling, D. D., & Holdsworth, E. S. (1965) *Biochem. J. 97*, 408-421.
Webling, D. D., & Holdsworth, E. S. (1966) *Biochem. J. 100*, 652-660.

Factors Affecting the Binding of Chick Oviduct Progesterone Receptor to Deoxyribonucleic Acid: Evidence That Deoxyribonucleic Acid Alone Is Not the Nuclear Acceptor Site†

Cary L. Thrall‡ and Thomas C. Spelsberg*

ABSTRACT: Studies on the interaction of the chick oviduct progesterone-receptor complex (P-R) with various nuclear components revealed a variable, nonsaturable binding of P-R to pure deoxyribonucleic acid (DNA). In contrast, a receptor-dependent, saturable, high level of binding of P-R was observed with a nonhistone protein–DNA complex called nucleoacidic protein (NAP). Three categories of factors were identified which affected the binding of P-R to the DNA. These were (1) the conditions of the binding assay, (2) the properties of the receptor, and (3) the state of the DNA. The conditions in the binding assay which affect DNA binding are the choice of the blanks, the salt concentration, and the pH of the assay. The receptor preparations display their own characteristic levels of binding to native DNA. The basis of this DNA binding capacity by each preparation is unknown. Lastly, the purity and the integrity of the DNA itself determine the level of binding of the P-R. Protein impurities, moderate degradation of the DNA by enzymatic or physical fragmentation, and ultraviolet (UV) light treatment greatly enhance the receptor binding to the DNA. The extent of binding to DNA depends on the degree of damage. Interestingly, totally denatured (single-stranded) DNA displays little or no binding of the P-R. Seasonal differences which are observed for the binding of P-R to chromatin in vivo and in vitro and to NAP in vitro do not occur with DNA whether it is undamaged or damaged. It is concluded from these studies that under controlled conditions and by using DNA preparations as native as possible, minimal binding of P-R to pure DNA occurs. The numerous reports in the literature describing marked binding of the steroid-receptor complex to DNA may well be due to conditions described in this paper. Further, it is concluded that native or partially degraded DNA alone does not appear to represent the native nuclear acceptor sites for the chick oviduct P-R. In contrast, the DNA–nonhistone protein (acceptor protein) complexes do show characteristics of the native-like acceptor sites.

A primary action of steroid hormones is the regulation of specific gene expression in target cells. The alteration in gene expression has been attributed to the direct interaction of steroid receptors with the chromosomal material of several tissue systems (Mueller et al., 1958; Means & Hamilton, 1966; Hamilton et al., 1968; O'Malley et al., 1969, 1972; Glasser et al., 1972; Baxter & Tomkins, 1971; Knowler & Smellie, 1971; Palmiter, 1972; Kurtz et al., 1976; Tata, 1976; Spelsberg, 1976; Spelsberg et al., 1971, 1972; Martial et al., 1977; Thrall et al., 1978). Since the discovery of steroid receptors, their translocation to the nucleus, and their binding to chromatin, a growing interest has arisen as to the nature of their nuclear binding sites. One of the ultimate goals in such studies has been to determine if the steroid receptor binds directly to deoxyribonucleic acid (DNA) or whether other nuclear components are involved in the high-affinity interaction.

† From the Department of Cell Biology, Mayo Clinic, Rochester, Minnesota 55901. *Received November 27, 1979.* Supported by HD 9140-B from the National Institutes of Health and the Mayo Foundation.

‡ Recipient of National Institutes of Health Postdoctoral Fellowship HD 05480. Present address: Department of Neurology, Neurochemical Research, Mayo Clinic, Rochester, MN 55901.

0006-2960/80/0419-4130$01.00/0

It has been reported by many laboratories that a variety of steroid-receptor complexes bind to DNA (Baxter et al., 1972; Clemens & Kleinsmith, 1972; King & Gordon, 1972; Musliner & Chader, 1972; Spelsberg et al., 1971, 1976a,b; Toft, 1972; Yamamoto & Alberts, 1974, 1975; Sluyser et al., 1974; Andre & Rochefort, 1975; Rousseau et al., 1975; Webster et al., 1976; Thrower et al., 1976; Alberga et al., 1976; Simons et al., 1976; Bugany & Beato, 1977; Simons, 1977; Cidlowski & Munck, 1978; Thanki et al., 1978; Kallos & Hollander, 1978; Kallos et al., 1978). There have been reports of specific interactions in terms of preferences of steroid receptors for substituted over unsubstituted DNAs (Kallos et al., 1978), for native over denatured DNAs (Andre et al., 1976; Kallos & Hollander, 1978), for poly(dA-T) (Sluyser et al., 1974; Kallos & Hollander, 1978), oligo(dT), or oligo(dC) (Thrower et al., 1976; Thanki et al., 1978) over other synthetic poly(deoxyribonucleotides), for eukaryotic over prokaryotic DNA (Clemens & Kleinsmith, 1972), and finally for native DNA over RNA (Toft, 1972; Yamamoto & Alberts, 1974; Rousseau et al., 1975). However, many laboratories have reported no differences in the binding of steroid-receptor complexes to the native DNAs of a variety of sources (Yamamoto & Alberts, 1974; Alberga et al., 1976; Kallos & Hollander, 1978; Rousseau et al., 1975; Simons, 1977). Further, in most instances the binding to pure DNA is not saturable, suggesting a nonspecific interaction (Sluyser et al., 1974; Yamamoto & Alberts, 1974; Andre & Rochefort, 1975; Alberga et al., 1976; Thanki et al., 1978; Rousseau et al., 1975; Simons et al., 1976; Spelsberg et al., 1976b; Webster et al., 1976).

It has been postulated that this lack of DNA specificity could be ude to the inability to detect a few specific sites among an overwhelming number of slightly lower affinity nonspecific sites (Yamamoto & Alberts, 1975, 1976). In any event, the role of DNA in binding steroid-receptor complexes has not been resolved.

This laboratory has been investigating the binding of P-R[1] to various nuclear components from chick and hen tissues. Conditions were established in a cell-free assay whereby a saturable, tissue-specific binding to oviduct nuclei and chromatin occurred which mimicked that measured in vivo (Pikler et al., 1976; Spelsberg et al., 1977). The binding to certain residual DNA–nonhistone protein complexes called nucleoacidic protein (NAP) markedly exceeded that to pure DNA and was saturable. This binding required the steroid to be bound to an activated receptor (Spelsberg et al., 1976a; Webster et al., 1976). Further seasonal variations in the binding of P-R to chromatin were shown to occur both in vivo and in vitro and to NAP in vitro. However, no such seasonal differences were observed with pure DNA (Boyd & Spelsberg, 1979). This has been interpreted to mean that nonhistone proteins may play a role in the specific binding of P-R to the avian genome. During the course of these investigations, the binding of P-R to pure DNA was conducted routinely as a background control. By use of a variety of DNA and receptor preparations over the years, a pronounced variation in the extent of binding to DNA with no apparent rhythm was observed. Since a relatively constant level of DNA binding was expected, a systematic study of defined parameters that affect DNA binding was pursued and is presented in this report. The identification of many of the factors which affect binding of the P-R to DNA should allow more accurate chemical analysis of the acceptor sites in the nuclei of target cells as well as help to explain many of the previously published results on DNA binding by steroid receptors in a variety of systems. These results, together with the evidence in this and other laboratories that binding of steroid-receptor complexes to pure DNA is unsaturable, support that DNA alone does not appear to represent the native acceptor site but rather a nonspecific binding site. The possibility that a specific binding of P-R occurs with a very limited number of sites on the DNA has not been ruled out.

Materials and Methods

Preparation of [^2H]Progesterone Receptor Complex. The detailed procedure for the isolation and labeling of the P-R from estrogen-stimulated chick oviduct has been described previously (Pikler et al., 1976). Briefly, immature chicks were treated with diethylstilbestrol for 4 to 5 weeks and the developed oviducts excised. These oviducts contain cytosolic receptor protein specific for progesterone with little endogenous progesterone present. The oviducts were homogenized in 3 volumes (v/w) of 10 mM Tris, 1 mM EDTA, and 12 mM thioglycerol, pH 7.4 (TESH buffer), and a 100000*g* supernatant was prepared. The resulting cytosol was incubated for 2 h at 4 °C with 2×10^{-8} M [^{3}H]progesterone at 1 μCi/mL. The [^{3}H]P-R was then precipitated with 35% $(NH_4)_2SO_4$ (Schrader & O'Malley, 1972), and the pellets were stored at −80 °C until needed.

Isolation of DNA. A modification of the Marmur procedure (Marmur, 1963) for DNA isolation was used, starting with hen spleen nuclei as described previously (Spelsberg et al., 1971). All procedures were performed at room temperature unless otherwise specified. Briefly, the nuclei were suspended in 0.15 M NaCl and 0.1 M EDTA, brought to 1 M in $NaClO_4$, and deproteinized repeatedly with chloroform–isoamyl alcohol. Aliquot 1 was taken at this step for P-R binding analysis. The DNA was precipitated with 2 volumes of cold ethanol, spooled on a glass rod, dried, and resuspended in 15 mM NaCl and 1.5 mM sodium citrate, pH 7.0 (0.1 × SSC), for 12 h. The solution was then made 0.33 M in sodium acetate and 0.1 mM in EDTA and the DNA precipitated with 0.6 volume of isopropyl alcohol. The precipitate was resuspended in SSC, and aliquot 2 was taken for P-R binding analysis. The remaining material was treated with RNase and Pronase. After enzyme treatment, the solution was made 1 M in $NaClO_4$ and 1% (w/w) in sodium dodecyl sulfate. The solution was then deproteinized repeatedly with chloroform–isoamyl alcohol. The DNA was precipitated with ethanol, spooled, resuspended in 0.1 × SSC overnight, adjusted to 0.33 M sodium acetate, and precipitated again with isopropyl alcohol. This pellet of DNA was resuspended in 1 × SSC buffer and represented the purified "native" DNA used for the studies described in this paper. Aliquot 3 was taken for P-R binding analysis. In certain instances, the above steps of this DNA purification, beginning with the enzyme treatments, were repeated. Aliquot 4 was taken from this repurified DNA.

Care was taken not to homogenize or stir the DNA solution too vigorously since this causes extensive fragmentation which would reduce the yield and cause altered receptor binding (described in this paper). The final product was analyzed for purity by measuring DNA with the diphenylamine reaction (Burton, 1956), RNA by the orcinol reaction (Ceriotti, 1955), and protein by the Lowry method (Lowry et al., 1951). An acceptable DNA preparation contained about 0.5% (w/w)

[1] Abbreviations used: P-R, progesterone-receptor complex; TESH buffer, 10 mM Tris-HCl–1 mM EDTA–12 mM thioglycerol, pH 7.4; SSC, 150 mM NaCl–15 mM sodium citrate, pH 7.0; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, disodium ethylenediaminetetraacetate; Tris, tris(hydroxymethyl)aminomethane; PMSF, phenylmethanesulfonyl fluoride; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-methyl(5-phenoxazolyl)]benzene; Gdn·HCl, guanidine hydrochloride.

protein to DNA or less. If >1% protein was present, further deproteinization steps were carried out by repeating treatments of Pronase, chloroform-isoamyl alcohol, and $NaDodSO_4$.

Preparation of Chromatin and Nucleoacidic Protein. Shortly after the chicks or hens were sacrificed, the oviducts were excised, cleaned, and frozen in dry ice. When needed, the tissue was homogenized while still frozen in a Waring Blendor in buffered sucrose solutions, and the nuclei and chromatin were purified as described previously (Pikler et al., 1976; Spelsberg et al., 1972, 1977). All steps were performed at 0–4 °C. Briefly, the nuclei were sedimented in a buffered 0.5 M sucrose solution (0.05 M Tris, 0.025 M KCl, and 0.002 M $MgCl_2$, pH 7.5) at 40000*g* for 10 min, resuspended in a buffered 2.0 M sucrose solution (0.05 M Tris, 0.025 M KCl, and 0.002 M $MgCl_2$, pH 7.5) by using a Teflon pestle–glass homogenizer, and resedimented at 60000*g* for 1 h. The nuclei were resuspended in 0.5 M sucrose solution containing 0.2% Triton X-100, passed through 100-mesh organza cloth, and resedimented at 15000*g* for 20 min. These nuclei were used to isolate chromatin via suspension in solutions containing (1) 0.08 M NaCl and 0.02 M EDTA, pH 6.3, (2) 0.3 M NaCl, and (3) 2 mM Tris-HCl and 0.1 mM EDTA, pH 7.5, as described elsewhere (Spelsberg & Hnilica, 1971; Spelsberg et al., 1971). The chromatin was then used to prepare chromatin–cellulose (described below). For preparation of NAP, the chromatin was extracted with 4 M Gdn·HCl solution containing 0.01 M 2-mercaptoethanol, 0.005 M PMSF, 0.001 M EDTA, and 0.01 M phosphate buffer, pH 6.0. The solution was centrifuged for 24 h at 4 °C at $100000g_{max}$. The pellet was resuspended in 2 MM Tris-HCl and 0.1 mM EDTA, pH 7.5, and dialyzed vs. the same solution for 4 h, and the insoluble protein was removed by centrifugation for 10 min at $5000g_{av}$. The supernatant represents the NAP which has been the subject of studies with P-R binding as described elsewhere (Webster et al., 1976; Spelsberg et al., 1976a,b, 1977).

Preparation of DNA–Cellulose. The procedure for preparation of the cellulose and UV attachment of chromatin or DNA to cellulose have been described in detail elsewhere (Webster et al., 1976; Spelsberg et al., 1978). Briefly, a high-intensity UV light treatment in absolute alcohol was used to couple DNA covalently to cellulose (Litman, 1968). This yielded a resin with a high content of DNA which was stable to treatments of high salt and urea concentrations or high concentrations of Gdn·HCl (Spelsberg et al., 1978). Resins prepared without UV light treatment were unstable to these reagents. Two approaches in preparing DNA–cellulose have been used. One involves coupling of purified native DNA directly to the cellulose (Spelsberg et al., 1978), and the other involves coupling of whole chromatin to the cellulose with the subsequent removal of the protein, as described elsewhere (Webster et al., 1976). In the latter case, the chromatin–cellulose was treated with 0.2% $NaDodSO_4$ or 6 M Gdn·HCl at 1 mL/mg of cellulose for 2 h or overnight at room temperature to remove protein. The resin was then washed again with these reagents and then washed extensively with 2 mM Tris-HCl and 0.1 mM EDTA, pH 7.5.

Fragmentation of DNA. (1) Shearing. Native DNA at 25 μg/mL in dilute buffer was physically sheared in a Virtis 60 homogenizer at 40000 rpm for 30 min at 4 °C with five blades attached to the rotating shaft.

(2) Mild Nuclease Treatments. Micrococcal nuclease (Worthington NFCP) was used at a concentration of 1.5 μg/mL (11 000 units/mg) with native DNA at a concentration of 500 μg/mL in 10 mM sodium borate and 1 mM $CaCl_2$, pH 8.8. The mixture was incubated for 10 min at room temperature and the reaction stopped by the addition of EDTA to a concentration of 5 mM.

Pancreatic deoxyribonuclease (Worthington DPFF) was also used at a concentration of 1 μg/mL (2200 units/mg) with native DNA at a concentration of 500 μg/mL in 10 mM Tris, 10 mM NaCl, and 3 mM $MgCl_2$, pH 7.5. The mixture was incubated for 30 min at room temperature. The reaction was stopped with 10 mM EDTA. These samples were also dialyzed against 2 mM Tris and 0.1 mM EDTA, pH 7.5, to remove salt and bring the pH to neutrality in preparation for receptor binding analysis, since unbuffered 10 mM EDTA lowers the pH significantly. The extent of shearing or nuclease digestion was monitored by acid precipitation and by gel electrophoresis in 2.0% acrylamide and 0.5% agarose (Peacock & Dingman, 1968). More than 90% of the DNA was rendered acid insoluble under the conditions used. The DNA fragments produced a mean size of 750 base pairs as determined by polyacrylamide gel electrophoresis with *Hin*cII restriction fragments of phage λ for a reference (Landy et al., 1974).

Binding of [^{3}H]Progesterone-Receptor Complex to DNA or to Nucleoacidic Protein. The two methods used in this study for measuring the interaction of P-R and native and treated DNA have been previously described (Webster et al., 1976; Spelsberg et al., 1977). These methods involve the rendering of the DNA insoluble, to facilitate its separation from unbound [^{3}H]P-R. One procedure utilizes the antibiotic, streptomycin sulfate, at the end of incubation to precipitate the DNA together with any DNA-bound P-R. The other method involves DNA coupled to cellulose (prepared from chromatin–cellulose as described above) in the binding assays which renders the DNA insoluble. Both binding techniques allow rapid processing of multiple samples. In the streptomycin method, 60 μg of DNA (as pure DNA or as NAP) is incubated in buffered 0.1 M KCl with a given amount of receptor in a 1-mL total volume for 90 min at 4 °C (Webster et al., 1976; Spelsberg et al., 1977). In some instances, a 2-mL reaction containing 120 μg of DNA and double the amount of receptor is used. Higher concentrations of KCl result in the dissociation of the streptomycin–DNA complex. The antibiotic is then added to a final concentration of 1.0 mg/mL in the assay which renders the DNA insoluble within a few minutes. This level of the antibiotic has minimal effects on the receptor while precipitating maximal amounts of the DNA (T. C. Spelsberg, unpublished experiments). In the cellulose method, 25 μg/mL DNA is similarly incubated in buffered 0.18 M KCl at 4 °C. The DNA is sedimented by centrifugation at 1000*g* for 10 min, and the pellets are washed twice in dilute buffer. The pellet of DNA is collected on nitrocellulose membrane filters (24 mm, 0.45-μm pore size), dried, and counted for radioactivity in a toluene–PPO–POPOP scintillation solution. After counting, the filters are removed and dried, and the DNA is quantitated by using the diphenylamine reaction of Burton (1956) as described elsewhere (Spelsberg et al., 1971; Webster et al., 1976). The blanks represent assays containing no DNA or NAP. In the cellulose method, pure cellulose is added in the same amount to the blank assays as was added to the experimental assays. After the incubation, the blank assay tubes are washed in a manner similar to that of the experimental assay tubes with the last wash transferred to the filters. The radioactivity per blank filter is subtracted from the respective experimental filter values (i.e., those with the same ratio of receptor to DNA in their assays). The remaining radioactivity is then adjusted per milligram of DNA. The results are expressed as cpm/mg of DNA on the filter. When fragmented DNA is used,

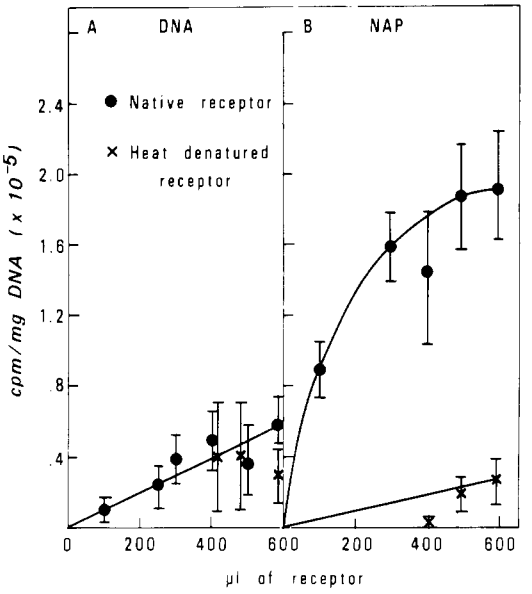

FIGURE 1: Effect of using a denatured receptor as a blank on [^{3}H]P-R binding to DNA and NAP. The receptor was denatured by mild heat treatment at 50 °C for 30 min. Bindings were performed as described under Materials and Methods with increasing levels of P-R by using a 2-mL streptomycin assay with (A) 120 μg of DNA or (B) 120 μg of NAP. The values represent the difference between total binding of the complete assay and that of a blank consisting of the native receptor without DNA or NAP. The blank assays were handled in a manner similar to that of the experimental assays as described in detail under Materials and Methods. About 50% of the original DNA added to the assays was recoverable on the filters. The radioactivity on the filters was counted by liquid scintillation spectrometry with 50% efficiency. The mean and range of five replicate analyses at each level of receptor are presented.

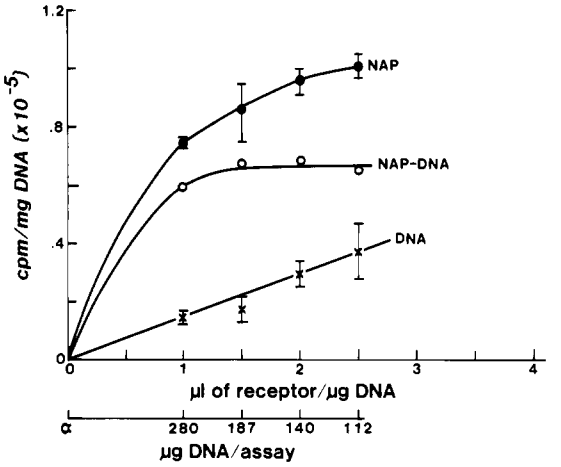

FIGURE 2: Effects of varying DNA levels with constant receptor levels on [^{3}H]P-R binding. The binding assays were performed as described under Materials and Methods and in the legend of Figure 1 except that 1-mL streptomycin assays were performed by using varying amounts of DNA as (●) NAP and (×) pure DNA with 280 μL of receptor in each assay. After the incubation, the streptomycin was added to 2 mg/mL in the binding assay instead of the usual 1 mg/mL to assure the precipitation of the greater amounts of the DNA in some of the assays. (○) represents the difference in NAP and DNA binding or the binding to protein–DNA complexes, which is termed "acceptor activity", used in subsequent figures. The mean and standard deviation of four replicate analyses at each level of the NAP or DNA are presented.

modifications of the streptomycin method are necessary to improve the efficiency of the DNA recovery. In this instance, a 50% increase in DNA and streptomycin concentrations was used to enhance the efficiency of precipitation. The assay mixture was also centrifuged at 5000*g* for 10 min to adequately pellet the fragmented DNA–streptomycin complexes.

Results

Studies in this laboratory on the binding of P-R to a variety of nuclear preparations revealed that the P-R binds to the greatest extent to the partially deproteinized chromatin preparation, NAP, followed by the binding to chromatin and pure hen DNA (Spelsberg et al., 1976a,b, 1977; Webster et al., 1976). Although the level of binding to DNA generally was found to be minimal, occasionally an enhanced binding occurred which attained levels equivalent to that of NAP. Consequently, it has been difficult to assign either a low-capacity or high-capacity binding of P-R to DNA.

During several years of study, several parameters were discovered which affect the degree of DNA binding by P-R. These were (1) the conditions of the binding assay, (2) the receptor preparation itself, and (3) the state of the DNA. The effects of each of these parameters on the DNA binding by P-R are presented below.

First, two characteristics of the P-R binding to pure DNA were observed which suggest that DNA alone does not represent the native binding site. The binding to DNA was dependent on the selected blank (or background radioactivity) of the binding assays. Figure 1 shows the binding levels of intact P-R to DNA (A) and NAP (B), obtained by using the streptomycin method and 2-mL assays and, as background, values obtained from assays containing no DNA or NAP as described under Materials and Methods. Similar patterns were observed when blank assays consisting of free [^{3}H]P (no receptor) with either the DNA or NAP were used. However, when the blanks contained the same respective DNA or NAP but a denatured receptor preparation (i.e., denatured by mild heat treatment of 50 °C for 30 min), the background radioactivity was sufficiently high to eliminate the apparent binding of P-R to DNA (Figure 1). In contrast, the binding to NAP remains significant and saturable. Therefore, by use of the complete assay containing denatured receptor as a blank, there is no apparent binding of P-R to DNA but significant binding to NAP. Thus, there may not be any real binding of P-R to pure DNA. Unfortunately, the use of the heat-denatured receptor was not always practical. Even though the denaturation of the receptor was carefully monitored by the charcoal assay of Rochefort & Baulieu (1971), the aggregation of protein in the receptor preparation which resulted in excessive levels of absorbed or trapped [^{3}H]P sometimes occurred. The question remains as to which is the more accurate blank. The denatured receptor could be considered the ideal background assay, and, if used, no measurable P-R binding to DNA is found.

Since there are several reports of artifacts when whole cytosol which can either create or prevent saturable binding to nuclear sites in cell-free assays was used (Chamness et al., 1974; Milgrom & Atger, 1975), we performed the cell-free assays by using a constant receptor level and varying the DNA (or NAP) levels. Figure 2 shows that by using the streptomycin method a nonsaturable binding to the pure DNA and a saturable binding to NAP is again observed. Thus, the lack of saturable binding of [^{3}H]P-R to DNA does not appear to be due to cytosol factors in the receptor preparations.

Another line of evidence suggesting that DNA alone may not represent the native acceptor site for P-R in the avian oviduct is the lack of correlation between DNA binding in vitro and the nuclear binding of P-R in vivo. It has recently been reported that the nuclear binding of P-R to chromatin, both in vivo and in vitro, and to NAP in vitro displays an annual rhythm with periods in the winter which show no nuclear

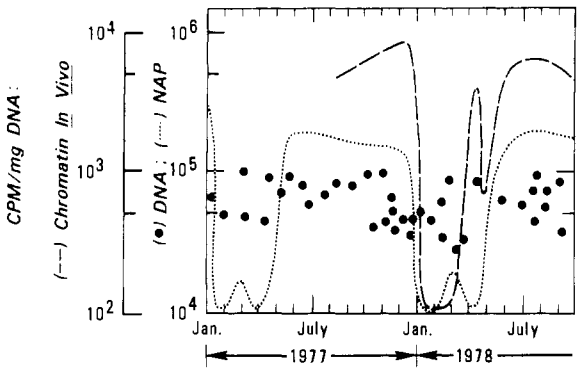

FIGURE 3: Relative binding of various [^{3}H]P-R preparations to the same isolated DNA sample and comparison with NAP binding in vitro and chromatin in vivo. Bindings were performed by a 1-mL streptomycin method in 0.1 M KCl by using 300 μL of stored receptor at 3×10^5 cpm/mL, equivalent amounts of DNA and NAP, and a titration of the receptor as described under Materials and Methods. Results are plotted by the date on which each particular receptor was prepared. Bound receptor is expressed on a logarithmic scale. (●) represents the means of four replicate analyses for DNA binding with each receptor preparation. (···) represents binding to protein–DNA complexes on the NAP in vitro. It is obtained by subtracting the binding to pure DNA from the binding to NAP as described in the legend of Figure 2. The latter binding was performed similarly to the DNA binding. (---) represents chromatin binding in vivo. The latter two plots are data taken from another report (Boyd & Spelsberg, 1979) and are used here for comparison.

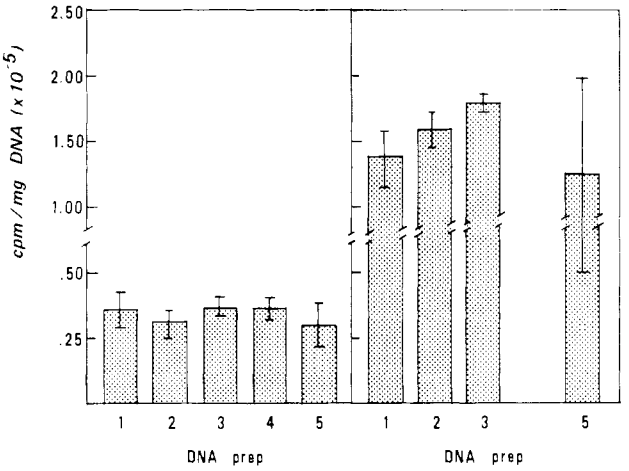

FIGURE 4: Characteristic DNA binding capacities of two different receptor preparations measured with several native DNA preparations. Bindings were performed as described in Figure 3. The abscissa represents five different DNA preparations. The two panels represent two different receptor preparations, one which characteristically displays high levels of DNA binding and one which displays low levels of DNA binding. The mean and standard deviations of four replicate analyses are given for each DNA preparation.

binding [Figure 3 and Boyd & Spelsberg (1979)]. The rhythms were found to be due to changes in the receptor itself and not to the nuclear preparations. In contrast, as shown in Figure 3, the binding of P-R to pure DNA displays no annual rhythms. These results support the conclusion that the P-R binding to chromatin and NAP in vitro represents binding to the native acceptor sites, while the binding to pure DNA alone represents some other form of interaction.

Interestingly, each of the receptor preparations displays its own characteristic level of binding to pure DNA (Figure 3). That the different capacities for binding of the various P-R preparations to DNA are inherent in the receptor preparations and not in the different preparations of native DNA is shown in Figure 4. In this case, five different preparations of native DNA were each bound with two separate receptor preparations, one displaying a low capacity and one a high capacity for DNA binding. These two receptor preparations contained equivalent concentrations of [^{3}H]P-R. It can be seen that the different preparations of native DNA display similar levels of binding by a particular receptor preparation. However, marked differences are observed between the two receptor preparations. Thus, each receptor preparation maintains its own characteristic level of binding, the basis of which is obscure.

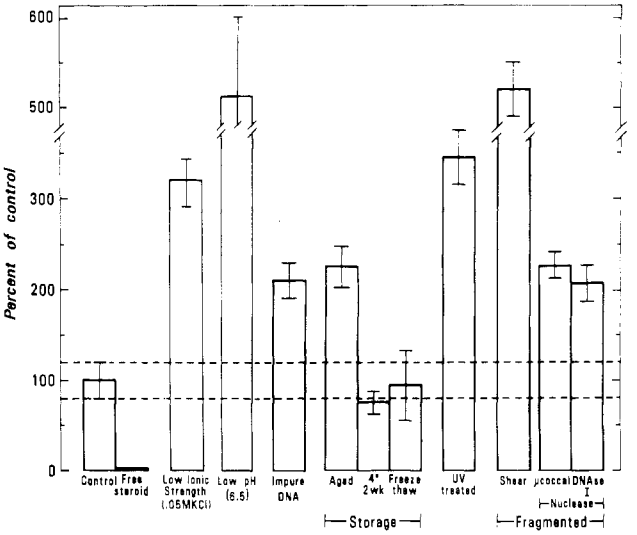

FIGURE 5: Composite showing the relative [^{3}H]P-R binding to DNA preparations under different conditions or DNA treatments. Binding analyses were performed as described in the legend of Figure 3, under Materials and Methods, and below by using a 1-mL streptomycin assay method with 60 μg of DNA and 300 μL of receptor. Results are presented as a percent of the control (binding to native DNA) (i.e., control conditions as described under Materials and Methods with untreated, purified DNA). The mean and standard deviations encompassing all controls are indicated by the broken lines. The same is shown for each bar. The low ionic strength and low pH represent binding assays with untreated DNA conducted under low-salt or low-pH conditions, respectively. The *impure DNA* represents isolated DNA with >1% protein contamination. *Aged DNA* represents a stock sample (2 mg/mL) which was stored frozen but repeatedly thawed, aliquots being taken, and refrozen over a 3-month period. The binding level shown was measured at the end of that time period. The *4 °C, 2-week* sample represents a stock sample stored undisturbed in a refrigerator for 2 weeks and then assayed. The *freeze–thawed* sample represents a stock sample that was frozen and thawed 10 times in a 24-h period. The *UV-treated DNA* represents DNA coupled directly to cellulose with 5×10^6 erg/cm^2 by using a 254-nm UV lamp as described under Materials and Methods. The *fragmented DNAs* were prepared and assayed as described under Materials and Methods.

The level of binding to the DNA not only varies with the particular receptor preparation but is dependent on the conditions of the assay and the state of the DNA. Figure 5 represents a composite of factors which were found to affect the DNA binding by P-R. The ionic strength and pH of the assays and the impurities in the DNA, as well as the state of the DNA itself, alter the binding of P-R to the DNA. The low-pH and low-salt conditions in the binding assay result in an enhanced, nonsaturable binding of P-R to the DNA.

During extended periods of analysis of P-R binding to DNA and NAP, marked differences in the binding to DNA were also found to correlate with the extent of handling of the DNA samples. When a DNA preparation was stored frozen with intermittent thawing over a long period, the extent of P-R binding often increased over that of unthawed DNA samples. Figure 5 displays a typical binding level of such stored samples. Attempts to generate similar increases in DNA binding by storage of the DNA at 4 °C over 2 weeks or by multiple

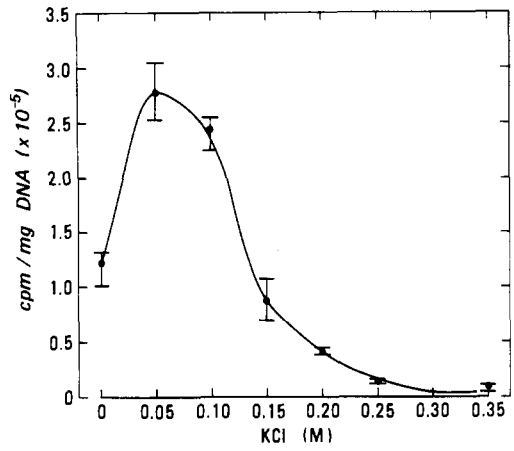

FIGURE 6: [^{3}H]P-R binding to DNA–cellulose at varying salt levels. Binding was carried out by using the DNA–cellulose method with 300 μL (4 × 10^5 cpm) of [^{3}H]P-R per assay and "native" DNA bound to cellulose prepared from chromatin–cellulose as described under Materials and Methods. The range and mean of three replicate analyses at each salt concentration are shown. The abscissa represents the salt content (ionic strength) of the binding assay.

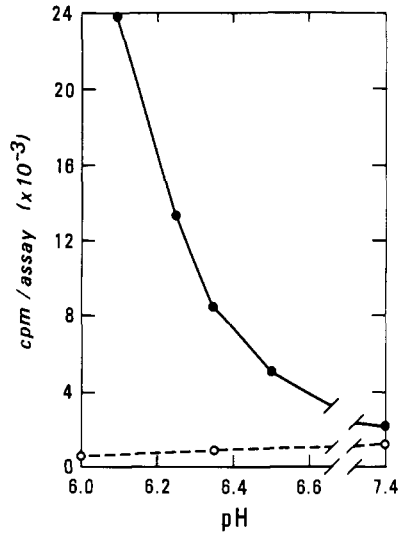

FIGURE 7: Effect of different pH conditions on the level of [^{3}H]P-R binding to DNA. The binding of [^{3}H]P-R to DNA was performed by using the 1-mL streptomycin assay method with 60 μg of DNA and 300 μL of receptor. (●) represents the radioactivity in the complete assay containing native receptor and untreated DNA; (○) represents the radioactivity in the assays containing no DNA (the blanks). A pH of 7.4 is the normal working pH of typical control assays. pH values of 6.0 and 6.1 were achieved by using dilute acetic acid. Other pH values in the pH 6.0 range as measured in the final binding assay were achieved by using weakly buffered 5–10 mM EDTA in the sample buffer. Results are expressed as total bound cpm per assay. The means of four replicate analyses at each pH level are shown.

freezing and thawing of the DNA preparation over a 24-h period failed (Figure 5). This phenomenon may be due to nuclease activity, as shown in Figure 5; however, the exact cause of the enhanced binding of "aged" DNA remains obscure. During studies to evaluate the cause of the high level of binding by aged DNA, it was found that other factors affected the degree of P-R binding to pure DNA. As shown in Figure 5, DNA damaged by UV light, mechanical shear, and nuclease treatment also markedly enhanced the binding of P-R to DNA.

The degree of change in P-R binding to DNA was found to be "dose" dependent for most of the treatments of the DNA and the conditions of the assay. The results of these studies are described below. Figure 6 illustrates the level of binding to DNA as a function of salt concentration. At high salt concentrations (>0.2 M KCl), a dissociation of the receptor–DNA complex occurs, and little or no binding is observed. At salt concentrations below 0.15 M KCl, an increase in binding to DNA is observed which peaks at 0.05 M KCl. At very low ionic conditions (e.g., 0.01 M KCl), a relatively lower level of P-R binding was measured. The latter observation appears to be due to extensive adsorption of the receptor to the cellulose at low ionic strength and may be due to a partial denaturation of the DNA (i.e., a generation of single-stranded DNA) which occurs at very low ionic conditions (Dove & Davidson, 1962) and which lowers the P-R binding (discussed later). By use of experiments involving a titration of P-R levels, the extensive binding which occurs under low ionic strength was shown not to be saturable and, therefore, may be nonspecific.

Figure 7 illustrates the effect of pH on the apparent level of receptor bound to DNA. A marked increase in binding of P-R to DNA is observed when the reaction is carried out in the pH 6.0 range. This high binding only occurs when the receptor is incubated in the presence of DNA. Receptor incubated alone at pH 6.0 does not cause any enhanced bound radioactivity upon subsequent binding to DNA at pH 7.4. Similarly, when the DNA is pretreated alone at pH 6.0 and then subjected to P-R binding at pH 7.4, no enhanced binding is observed. Thus, when the DNA and receptor are incubated together under moderate ionic strength and at a pH below 6.5, the extent of DNA binding is significantly increased. When the binding assays are performed at pH 7.4 under moderate ionic strength, the binding to native DNA is usually minimal. By use of experiments involving the titration of P-R levels with a constant amount of DNA, the extensive "binding" which occurs at low pH was shown to be nonsaturable and, therefore, probably nonspecific.

The concentrations of the receptor in the binding assay were also found to affect the level of DNA binding. When small amounts of P-R are added to the binding assay or when the assays are diluted (which lowers the concentration of P-R), much of the steroid dissociates from the receptor, resulting in unexpectedly low levels of binding to the DNA. In contrast, when large excesses of the P-R are used in the assays, unexplainable increases in apparent binding to DNA are observed. We believe the latter phenomenon is due either to the removal of much of the salt by the excess protein, resulting in a lower effective KCl concentration which enhances the level of binding, or to simple aggregation of the receptor. The DNA concentration in the assays was not found to be a factor in altering the DNA binding.

Further studies revealed that DNA preparations contaminated with protein greater than 1 to 2% (w/w) resulted in elevated binding by the P-R. Figure 8 shows that as the protein is removed from the DNA by the various solvents, the P-R binding to the residual DNA–protein complex increases to a protein to DNA ratio (w/w) of 0.01. As the remaining protein is removed, the P-R binding then begins to markedly decrease. This "unmasking" of binding sites as well as the extensive P-R binding by the residual DNA–protein complex using different methods to remove the proteins has been reported elsewhere (Webster et al., 1976; Spelsberg et al., 1976a,b, 1977).

Marked effects of UV light treatment on the DNA binding of P-R were discovered during studies involving the direct covalent attachment of isolated DNA to cellulose with the use of high-intensity, short-wavelength UV light (Figure 5). The

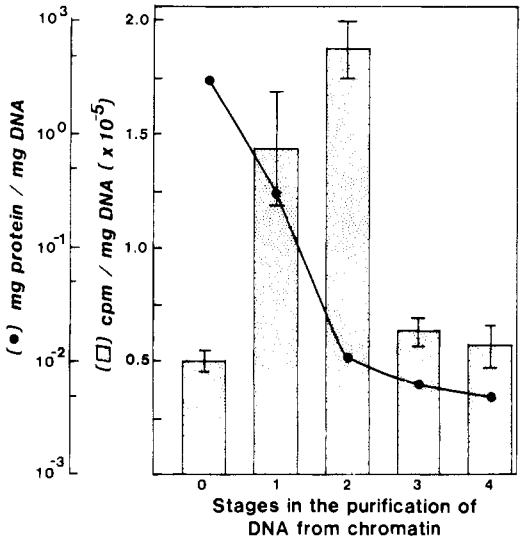

FIGURE 8: Binding of [^{3}H]P-R to DNA at various stages of purification. The binding of [^{3}H]P-R was performed by the 1-mL streptomycin assay method as described under Materials and Methods. The abscissa represents the DNA taken at various stages of purification as identified under DNA Purification. 0 represents whole chromatin, 3 the final purified native DNA, and 4 a repeat of the enzymatic treatments. The bars represent the P-R binding with the mean and standard deviation of four replicate analyses given. (●) represents the mg of protein/mg of DNA for each sample of preparation.

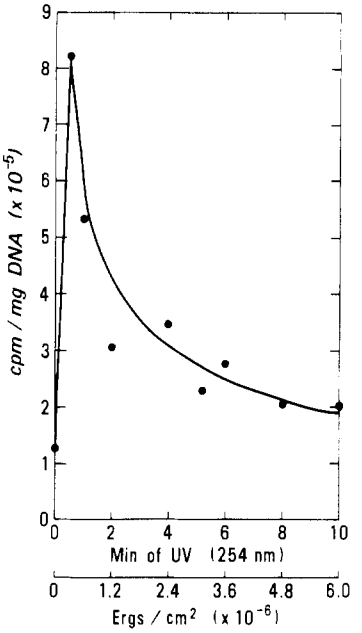

FIGURE 9: Binding of [^{3}H]P-R to DNA coupled directly to cellulose as a function of UV dose. Pure DNA was coupled directly to cellulose at the times designated on the abscissa as described under Materials and Methods and elsehere (Spelsberg et al., 1978). For the 0-min UV treatment, the DNA was mixed with cellulose and lyophilized. All resins were washed to eliminate unbound DNA as described under Materials and Methods. Amounts of cellulose equivalent to the DNA–cellulose were used as a blank for the individual cellulose binding assays. Bindings were carried out at 0.15 M KCl with 300 μL (4 × 10^5 cpm) of [^{3}H]P-R per assay. The means of three replicate analyses of [^{3}H]P-R binding to DNA–cellulose samples prepared at each dose of UV light are shown.

utilization of UV light for coupling DNA or chromatin results in a more stable complex than that prepared by simpler adsorption methods (Spelsberg et al., 1978). The direct irradiation of DNA in preparing DNA–cellulose, however, resulted in a significantly higher binding than the DNA–cellulose prepared from the chromatin–cellulose as described under Materials and Methods. Apparently the protein on chromatin protects it from UV damage. The effect of the dose of UV light on the cellulose was then studied. The doses of UV light selected were shown to damage the DNA (Spelsberg et al., 1978). Figure 9 shows that the milder damage of the DNA with the lower doses of UV light results in marked receptor binding to DNA. Higher doses of UV light cause greater damage to the DNA and a corresponding decrease in P-R binding but to a level still above that of the control. Experiments involving a titration of P-R levels with a constant level of the UV-treated DNA–cellulose in the assay show nonsaturable binding. The exact mechanism by which the UV light treatment produces enhanced but unsaturable P-R binding to the DNA–cellulose is unknown. In contrast, the UV light treatment of cellulose alone (used as blanks in the binding assays) has no effect on the P-R binding to the cellulose.

Figure 5 showed that mechanical shearing and nuclease treatments cause an increase in P-R binding to DNA. In these studies, the extent of shearing or nuclease fragmentation was analyzed by size distribution on polyacrylamide gel electrophoresis. Treatments with the nucleases were purposely mild, minimizing exonucleolytic activity and resulting in a fairly uniform population of double-stranded DNA fragments (mean size of 750 base pairs). The shearing of the DNA resulted in a more diverse population of DNA fragments, many of which were smaller than those generated by the particular nuclease treatment utilized. In general, the greater the fragmentation of the double-stranded DNA, the greater the binding of P-R to that DNA. Binding assays involving receptor titration and a constant level of DNA indicate that the binding to each of the fragmented DNAs is also unsaturable. Since the degree of P-R binding increased with increasing fragmentation of the DNA, it was of interest to determine how well P-R bound to completely denatured DNA (i.e., single-stranded DNA). The methods of binding described previously do not allow direct binding analysis with totally single-stranded DNA because (1) streptomycin does not precipitate single-stranded DNA in the presence of the added receptor protein preparation and (2) the direct attachment of single-stranded DNA to cellulose could be subject to UV-induced binding as described above. Therefore, an indirect competitive binding assay was performed between free, single-stranded DNA and native DNA by using the DNA–cellulose method. As shown in Figure 10, single-stranded DNA does not effectively compete with DNA–cellulose, while double-stranded DNA does compete. Thus, it appears that totally denatured single-stranded DNA does not bind P-R, while fragmented double-stranded DNA does bind. These results may explain the initial increase followed by a decrease in DNA binding with increasing doses of UV light, shown in Figure 9. The larger doses of UV light result in severely damaged DNA involving production of single strands which do not bind the P-R. The smaller doses of UV light cause DNA fragments and some base unpairing which apparently bind P-R significantly.

Since the UV light treatment did create an enhanced binding to the DNA to a level equal to or above that observed with NAP, it was of interest to compare its binding to that of chromatin in vivo and in vitro. As stated above, it was found that P-R binding to the partially damaged DNA was unsaturable, reflecting a nonspecific interaction. Further, the nuclear binding of P-R in vivo and in vitro to chromatin and to NAP in vitro has been shown to display a seasonal variation, based on the period of the year the receptor was isolated from the oviduct (Boyd & Spelsberg, 1979). The oviducts in the winter contain receptor which fails to translocate and bind to

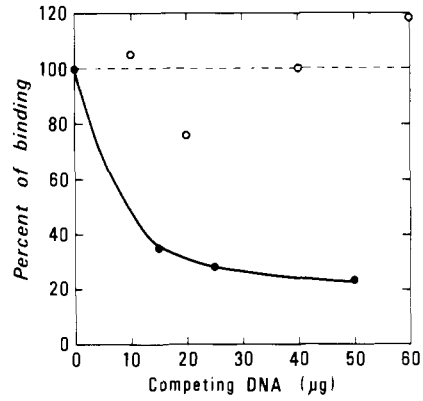

FIGURE 10: Competition between native or denatured DNA for [^{3}H]P-R binding to native DNA–cellulose. DNA–cellulose prepared by NaDodSO$_4$ extraction of chromatin–cellulose (described under Materials and Methods) was assayed by the routine binding procedure at 0.1 M KCl and 300 μL (4×10^5 cpm) of [^{3}H]P-R. Included in the assay mixture during the incubation period were increasing amounts of native DNA (●) or heat denatured (single-stranded) DNA (○). The denaturation was shown to be complete by hyperchromicity during and after melting of the DNA. Results are presented as the percent of the control of P-R binding to native DNA–cellulose prepared from chromatin–cellulose with no free DNA added. The data represent binding only to the DNA–cellulose since free DNA is soluble and is removed during the washing. The mean of four replicate analyses at each level of competitor is shown.

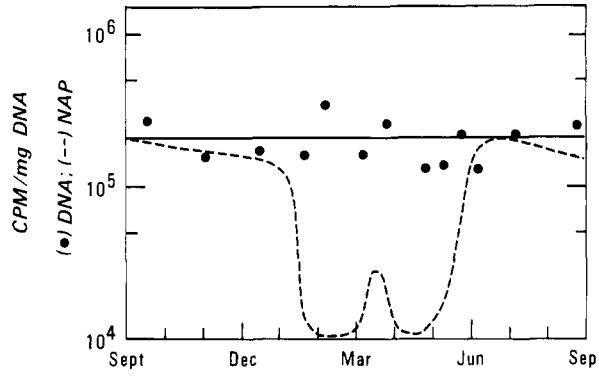

FIGURE 11: Seasonal binding of [^{3}H]P-R to a common preparation of denatured DNA and intact NAP. The experiment was performed essentially as described in the legend of Figure 9 and under Materials and Methods by using the cellulose method except that damaged DNA [i.e., DNA treated wtih 6×10^6 erg/cm^2 UV light (2600 Å)] and receptors isolated at different times of the year are used. (●) represents binding to the UV-treated DNA. (---) represents binding to protein–DNA complexes represented by the difference between total NAP binding and DNA binding as described elsewhere (Boyd & Spelsberg, 1979) and shown here for the sake of comparison. The mean of four replicate analyses is presented for the DNA binding.

nuclear acceptor sites, whereas the receptor from the summer displays marked binding. Therefore, it was of interest to see if such a variation occurred with the UV-treated DNA. Figure 11 demonstrates that no such seasonal differences occur with the damaged DNA as with NAP. Therefore, these generated binding sites on the damaged DNA appear to differ from those on NAP or chromatin.

Discussion

These studies demonstrate that much of the binding of a P-R or any steroid-receptor complex to DNA may be artificially induced. It is important to establish appropriate assay conditions for measuring receptor affinity. First, tests for saturability should include titration of both the receptor and the DNA or nuclear component since it has been shown that the former could create artificial saturation or prevent saturation (Chamness et al., 1974; Milgrom et al., 1975). Both approaches are used in this report to demonstrate a saturable binding with the NAP and a lack of saturation with pure DNA in the cell-free binding assays. Problems with the receptor titration were not anticipated since most reports concerning artifiacts in this approach utilized crude cytosol, whereas a more purified receptor preparation was used in the studies in this paper. It has been reported that the partially purified (ammonium sulfate precipitated) estrogen receptor from calf uterus seemed to lack the nuclear binding inhibitor apparent in the crude cytosol receptor preparation (Sala-Trepat & Vallet-Strouve, 1974). Since the binding of [^{3}H]progesterone to NAP requires intact receptor, a variety of assay conditions used for blanks (e.g., assays with no DNA or NAP and assays with no receptor or denatured receptor) resulted in only a minor contribution to the total radioactivity in each of the assays.

As was shown here for DNA and has been described previously for chromatin and NAP (Spelsberg et al., 1976a–c, 1977; Webster et al., 1976), the ionic strength markedly affects the level of binding. The pH of the binding assay also affects the degree of binding. Often dilute buffers cannot maintain the buffering capacity in the crude cytosol of whole tissue homogenates. In our hands, the use of such cytosols in DNA binding assays lowers the pH of the binding assay and increases the level of binding. Further, care must be taken to maintain the nativeness of the components of the binding assays. This has been dramatically shown for the methods of isolation and handling of DNA used in these binding assays. Impurities, severe fragmentation, and certain chemical modifications can all lead to substantial increases in P-R binding. The size of eukaryotic DNA precludes the isolation of totally native DNA, but we have shown that the careful handling of the DNA to avoid extreme damage can yield a preparation that displays a minimal level of P-R binding. The particular receptor complex preparation also appears to be variable with regard to DNA binding. Significant variations among different receptor preparations for DNA binding were found, the exact level of which was characteristic for each receptor preparation. The exact cause of this preparation-specific binding is unknown. The variation in DNA binding for individual receptor preparations requires the characterization of each receptor preparation for this capacity.

When all parameters are carefully controlled, the extent of the binding of the P-R to DNA is minimal compared to the binding to NAP. Further, DNA binding is eliminated under certain conditions such as when complete reactions containing denatured receptor are used as blanks. It is interesting that a seasonal rhythm in P-R binding to nuclear acceptor sites occurs in vivo and in vitro with chromatin and in vitro with NAP but not with native (untreated) DNA (Boyd & Spelsberg, 1979; Spelsberg et al., 1979). The above results, together with the fact that under physiologic ionic strength the chromatin and NAP but not the DNA display a saturable binding and that the chromatin binding in vitro saturates at levels similar to that measured in vivo (Webster et al., 1976; Spelsberg et al., 1976a, 1977), support the view that DNA alone is not the native acceptor site. The capability of the receptor to interact to a greater extent with damaged DNA than with intact DNA indicates the introduction of additional "binding sites" of unknown relative affinity or biological relevance. However, these additional sites on damaged DNA do not appear to be similar to those on NAP or chromatin since the latter two, but not the damaged DNA, display a saturable binding as well as a seasonal rhythm in binding.

In conclusion, many factors affect the binding of this progesterone-receptor complex to pure DNA. Some of these factors may have played a role in the observations of other laboratories on the marked levels of DNA binding by steroid-receptor complexes. These studies, however, do not rule out a role of DNA as part of a complex in the native nuclear acceptor sites for steroid-receptor complexes, nor do they rule out the existence of a group of a very limited number of DNA sites with specific sequences which serve as these acceptor sites as postulated by Yamamoto & Alberts (1975, 1976).

Acknowledgments

We thank Brad Syverson, Barb Gosse, and Kay Rasmussen for their excellent technical assistance and Evelyn Gardner for her clerical support.

References

Alberga, A., Ferrez, M., & Baulieu, E. E. (1976) *FEBS Lett. 61*, 223–226.

Andre, J., & Rochefort, H. (1975) *FEBS Lett. 50*, 319–323.

Andre, J., Pfeiffer, A., & Rochefort, H. (1976) *Biochemistry 15*, 2964–2969.

Baxter, J. D., & Tomkins, G. M. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 932–937.

Baxter, J. D., Rousseau, G. G., Bensen, M. C., Garcea, R. L., Ito, J., & Tomkins, G. M. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1892–1896.

Boyd, P. A., & Spelsberg, T. C. (1979) *Biochemistry 18*, 3685–3690.

Bugany, H., & Beato, M. (1977) *Mol. Cell. Endocrinol. 7*, 49–66.

Burton, K. (1956) *Biochem. J. 62*, 315–323.

Ceriotti, G. (1955) *J. Biol. Chem. 214*, 59–70.

Chamness, G., Jennings, A. W., & McGuire, W. L. (1974) *Biochemistry 13*, 327–331.

Cidlowski, J. A., & Munck, A. (1978) *Biochim. Biophys. Acta 543*, 545–555.

Clemens, L. E., & Kleinsmith, L. J. (1972) *Nature (London) New Biol. 237*, 204–206.

Dove, W., & Davidson, N. (1962) *J. Mol. Biol. 5*, 467–478.

Glasser, S. R., Chytil, F., & Spelsberg, T. C. (1972) *Biochem. J. 130*, 947–957.

Hamilton, T. H., Teng, C. S., & Means, A. R. (1968) *Proc. Natl. Acad. Sci. U.S.A. 59*, 1262–1272.

Kallos, J., & Hollander, V. (1978) *Nature (London) 272*, 177–179.

Kallos, J., Fasy, T., Hollander, V., & Bick, M. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4896–4900.

King, R. J. B., & Gordon, J. (1972) *Nature (London), New Biol. 240*, 185–187.

Knowler, J. T., & Smellie, R. M. S. (1971) *Biochem. J. 125*, 605–614.

Kurtz, D. T., Sippel, A. E., & Feigelson, P. (1976) *Biochemistry 15*, 1031–1036.

Landy, A., Ruedisueli, E., Robinson, L., Foeller, C., & Ross, W. (1974) *Biochemistry 13*, 2134–2142.

Litman, R. M. (1968) *J. Biol. Chem. 243*, 6222–6233.

Lowry, O. H., Rosebrough, N. J., Farr, A. C., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Marmur, J. (1963) *Methods Enzymol. 6*, 726–738.

Martial, J. A., Baxter, J. D., Goodman, H. M., & Seeburg, P. H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1816–1820.

Means, A. R., & Hamilton, T. H. (1966) *Proc. Natl. Acad. Sci. U.S.A. 56*, 686–693.

Milgrom, E., & Atger, M. (1975) *J. Steroid Biochem. 6*, 487–492.

Mueller, G. C., Herrnanen, A. M., & Jervell, K. J. (1958) *Recent Prog. Horm. Res. 14*, 95–139.

Musliner, T. A., & Chader, G. J. (1972) *Biochim. Biophys. Acta 262*, 256–263.

O'Malley, B. W., McGuire, W. L., Kohler, P., & Korenman, S. (1969) *Recent Prog. Horm. Res. 25*, 105–160.

O'Malley, B. W., Rosenfeld, G. C., Comstock, J. P., & Means, A. R. (1972) *Gene Transcription Reprod. Tissue, Trans. Karolinska Symp. Res. Methods Reprod. Endocrinol., 5th*, 388–395.

Palmiter, R. D. (1972) *J. Biol. Chem. 247*, 6450–6461.

Peacock, A. C., & Dingman, C. W. (1968) *Biochemistry 7*, 668–674.

Pikler, G. M., Webster, R. A., & Spelsberg, T. C. (1976) *Biochem. J. 156*, 399–408.

Rochefort, H., & Baulieu, E.-E. (1971) *Biochimie 53*, 893–907.

Rousseau, G. G., Higgins, S. J., Baxter, J. D., Gelfand, D., & Tomkins, G. M. (1975) *J. Biol. Chem. 250*, 6015–6021.

Sala-Trepat, J. M., & Vallet-Strouve, C. (1974) *Biochim. Biophys. Acta 371*, 186–202.

Schrader, W. T., & O'Malley, B. W. (1972) *J. Biol. Chem. 247*, 51–59.

Simons, S. S. (1977) *Biochim. Biophys. Acta 496*, 349–358.

Simons, S. S., Martinez, H. M., Garcea, R. L., Baxter, J. D., & Tomkins, G. M. (1976) *J. Biol. Chem. 251*, 334–343.

Sluyser, M., Evers, S. G., & Nijssen, T. (1974) *Biochem. Biophys. Res. Commun. 61*, 380–387.

Spelsberg, T. C. (1976) *Biochem. J. 156*, 391–398.

Spelsberg, T. C., & Hnilica, L. S. (1971) *Biochim. Biophys. Acta 228*, 202–211.

Spelsberg, T. C., Steggles, A. W., & O'Malley, B. W. (1971) *J. Biol. Chem. 246*, 4186–4197.

Spelsberg, T. C., Steggles, A. W., Chytil, F., & O'Malley, B. W. (1972) *J. Biol. Chem. 247*, 1368–1374.

Spelsberg, T. C., Pikler, G. M., & Webster, R. A. (1976a) *Science (Washington, D.C.) 194*, 197–199.

Spelsberg, T. C., Webster, R. A., & Pikler, G. M. (1976b) *Nature (London) 262*, 65–67.

Spelsberg, T. C., Webster, R. A., Pikler, G. M., Thrall, C. L., & Wells, D. W. (1977) *Ann. N.Y. Acad. Sci. 286*, 43–63.

Spelsberg, T. C., Stake, E., & Witzke, D. (1978) *Methods Cell Biol. 17* 303–324.

Spelsberg, T. C., Knowler, J. T., Boyd, P. A., Thrall, C. L., & Martin-Dani, G. (1979) *J. Steroid Biochem. 11*, 373–379.

Tata, J. R. (1976) *Cell (Cambridge, Mass.) 9*, 1–14.

Thanki, K., Beach, T., & Dickerman, H. (1978) *J. Biol. Chem. 253*, 7744–7750.

Thrall, C. L., Webster, R. A., & Spelsberg, T. C. (1978) *Cell Nucl. 6*, 461–529.

Thrower, S., Hall, C., Lim, L., & Davison, A. (1976) *Biochem. J. 160*, 271–280.

Toft, D. O. (1972) *J. Steroid Biochem. 3*, 515–522.

Webster, R. A., Pikler, G. M., & Spelsberg, T. C. (1976) *Biochem. J. 156*, 409–419.

Yamamoto, K. R., & Alberts, B. (1974) *J. Biol. Chem. 249*, 7076–7086.

Yamamoto, K. R., & Alberts, B. (1975) *Cell (Cambridge, Mass.) 4*, 301–310.

Yamamoto, K. R., & Alberts, B. (1976) *Annu. Rev. Biochem. 45*, 722–746.