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Oligodeoxynucleotide Base Recognition by Steroid Hormone Receptors

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Oligodeoxynucleotides covalently linked to cellulose were used as probes of the DNA-binding domains of mouse steroid holoreceptors. With uterine cytosol estrogen receptor (E_2R) the relative binding order, in prior studies, was oligo(dG) > oligo(dT) $\geqq$ oligo(dC) >> oligo(dA) > oligo(dI). The binding reactions were salt-sensitive with an optimal KCl concentration of 0.1–0.2 M. There was no enhancement of binding by activation, either temperature- or salt-induced. In the present study, using the oligomer ligands at a lower concentration, oligo(dT) binding was greater than that to oligo(dC). Quantitative differences in oligodeoxynucleotide binding were elicited by a number of inhibitors. These differences are again seen by exposure of E_2R to chaotropic salts such as SCN^-, ClO_4^- and NO_3^- as well as to putative modifiers of receptor amino acids, ie, iodoacetamide, 1,2 cyclohexanedione, and Rose Bengal. These results, and the quantitative differences following heat and purification, led to a designation of two types of subsites within the DNA-binding domain of uterine E_2R. These are stable G sites, which interact with oligo(dG); and labile N sites, which bind to oligo(dT), oligo(dC) and oligo(dA). Stimulation of binding to N sites and stabilization of the holoreceptor was effected by histones H2A and H2B. However, the differential response to incubation at 37°C was not altered by addition of H2B. Treatment of uterine E_2R by limited proteolysis also eliminated the stimulatory response to H2B. The above data, as well as prior studies, indicate that steroid holoreceptors can discriminate between the structural features of deoxynucleotide bases and this recognition process can be modulated by accessory proteins.

Key words: oligodeoxynucleotides, cellulose, DNA-binding, holoreceptors, estrogen

With detection of high-affinity binding sites confined to limited sequences of cloned DNA of steroid-regulated genes [1–3], an increased appreciation of cytosol steroid holoreceptors as DNA-binding proteins is under way. In the cases of the chick oviduct progesterone receptor A subunit [4] and rat liver glucocorticoid receptor [5], purification to homogeneity or near-homogeneity yielded products which retained the

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property of DNA binding. With impure steroid receptors, including those for estrogen, evidence has accrued that they contain at least two separate domains, the binding sites for steroid ligands and polynucleotides. Limited proteolysis eliminated DNA binding without significant loss of the receptor-bound steroid [6–8]. Functional distinctiveness of these sites was observed after interaction of pyridoxal 5-phosphate [9], cibacron blue F3GA [10], aurintricarboxylic acid [11], 1,2 cyclohexanedione [12], or diethylpyrocarbonate [12] with a variety of steroid receptors. In all cases these compounds inhibited binding to DNA or oligodeoxynucleotides without disruption of steroid binding.

In order to probe the functional properties of the DNA-binding domains of mouse steroid holoreceptors, we have used oligodeoxynucleotides covalently linked to cellulose as binding templates. In addition to stability of the ligand attachment, these matrixes have the advantages of relatively similar ligand length and uniformity of ligand polarity, as they are attached to cellulose through the 5′ phosphate. Using the immobilized oligodeoxynucleotides we found that the binding of mouse uterine cytosol estrogen receptor (E_2R) was salt-stimulated; the optimal KCl concentration was achieved at 0.1–0.2 mM [13]. Furthermore, uterine E_2R discriminated between the base structures of the oligodeoxynucleotides such that binding to oligo(dG) > oligo(dT) $\geqslant$ oligo(dC) >> oligo(dA) > oligo(dI) [14]. The ligand interaction was selective for intracellular receptors, as neither a low-affinity, high-capacity binder, serum albumin, nor the estrophilic mouse α-fetoprotein was bound [13]. In a comparative study, mouse liver dexamethasone receptor required thermal or salt-induced activation for optimal binding, a requirement not evident with the kidney testosterone receptor, kidney E_2R or uterine E_2R of the same animal [15]. These results indicated that estrogen holoreceptor binding to oligodeoxynucleotides is a salt-dependent reaction involving nucleotide base discrimination, a prerequisite for specific DNA sequence recognition.

Differential oligodeoxynucleotide binding offers a method of examining the consequences of perturbation of the receptor's microenvironment on its capacity to interact with DNA. Not all the oligodeoxynucleotide binding activities were affected to the same degree by the inhibitors pyridoxal 5-phosphate [16], cibacron blue F3GA [10] and diethylpyrocarbonate [17]. With all these reagents oligo(dG)-cellulose binding was most resistant. Qualitative differences in residual binding were seen after heating or partial purification of mouse uterine E_2R; oligo(dG) cellulose binding was retained, while those for the other oligodeoxynucleotides were lost [14].

An interpretation of these data is that there are at least two types of subsites within the DNA-binding domain of uterine E_2R. These are stable G sites, which interact with oligo(dG), and labile N sites, which bind to oligo(dT), oligo(dC) and oligo(dA). Stimulation of E_2R binding to N sites and stabilization of the holoreceptor were effected by histones H_2A and H_2B [14,18].

In the present report we expand upon the differential recognition of mouse uterine E_2R, the sensitivity of the binding activities to chaotropic salts and a series of group inhibitors and the influence of histones on the temperature sensitivity of oligodeoxynucleotide binding of uterine E_2R.

MATERIALS

Female Nya:NYLAR mice 21 days old and weighing approximately 12 g were obtained from Griffin Laboratories of this Center. [2,4,6,7,16,17-^{3}H]Estradiol,

specific activity 145 Ci/mmol, was purchased from Amersham. Nonradioactive steroids were products of Steraloids, Wilton, New Hampshire. Oligodeoxynucleotide celluloses were purchased from Collaborative Research Inc, Waltham, Massachussetts. The oligonucleotide chain lengths were in the range of 2–18 (mean length, 10), and the total nucleotide content varied from 10 to 20 mg/g of cellulose. Cellulose used to determine nonoligonucleotide binding was obtained from the same source. Rose Bengal and iodoacetamide were procured from Sigma Chemicals. 1,2-Cyclohexanedione (98%) was a product of Aldrich Chemical Co. Histone 2B was from Worthington. Trasylol was from Mobay Chemical Corp.

METHODS

Mouse Uterine Cytosol

Mouse uterine horns were dissected free of fat, minced, weighed and homogenized with a Polytron homogenizer (Brinkman) in 2.5 vol of TEDG-sucrose-chloroquine buffer (0.01 M Tris HCl, pH 7.6; 0.001 M EDTA; 0.001 M dithiothreitol; 10% glycerol; 0.25 M sucrose; 30 mg/L chloroquin) containing 5,000 units of Trasylol/ml. Five bursts of 15 sec each at a setting of 8 accomplished complete homogenization. The homogenate was centrifuged twice, at 14,000g for 10 min in a Sorvall RC-5B and then at 105,000g for 60 min in a Beckman LS-65 centrifuge. The supernatant was used to prepare [^{3}H]estradiol receptor complexes.

Preparation of [^{3}H]estradiol receptor complexes (E_2R). Uterine cytosol was incubated at 4°C for 90 min with [^{3}H]estradiol (100-125 cpm/fmol) at a final concentration of 9 nM. Testosterone was included at 1.2 μM to saturate nonestradiol binding sites. Unbound estradiol was separated from macromolecule-bound steroid with charcoal dextran as previously described [10,13,14]. In a parallel incubation a 200-fold excess of unlabeled estradiol was included. Specific hormone binding was calculated as the difference between protein-bound estradiol in the presence and absence of unlabeled hormone. The specific binding was generally between 90% and 95%. The estradiol receptor complexes thus prepared were stored under liquid nitrogen.

Assay of E_2R binding to oligodeoxyribonucleotide celluloses. Binding of E_2R to oligodeoxynucleotide celluloses was performed batch-wise in polypropylene tubes (1.5-ml capacity). The reaction mixture (0.6 ml total volume) consisted of 0.15 M KCl, 30–35 fmol of estradiol receptor and oligodeoxynucleotide cellulose representing 180–190 nmol of organic phosphorus or an equal volume of unmodified cellulose (as control). After an incubation at 4°C for 60 min on a multipurpose rotator, the cellulose suspensions were centrifuged and washed, and the radioactivity was measured in a scintillation counter. Specific oligonucleotide-bound radioactivity was estimated as the difference in counts bound to oligonucleotide celluloses and blank celluloses.

Other experimental details are described in figure legends and the table.

RESULTS

In our initial studies on the capacity of mouse uterine E_2R to bind the oligomers of DNA constituent bases, 200 nmol of each of the various ligands was used to assess the receptor's nucleotide base preferences. Under those conditions the relative order

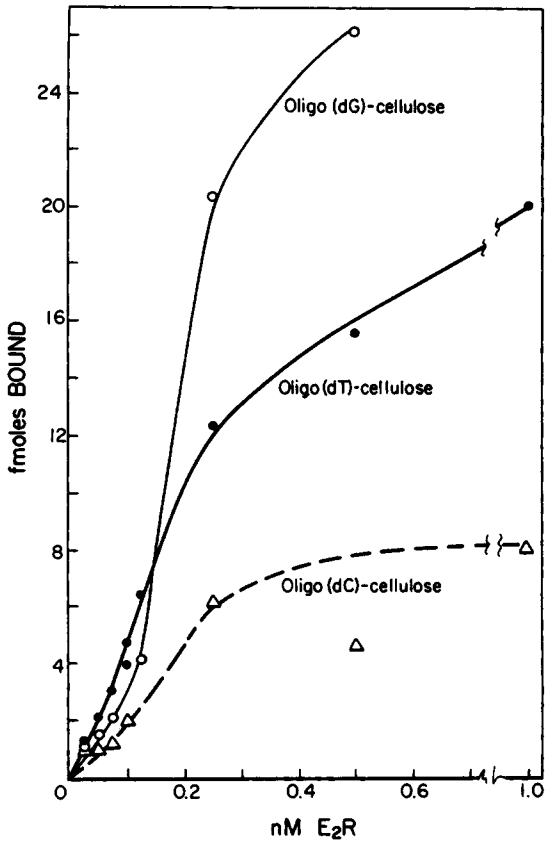


Fig. 1. Binding of mouse uterine cytosol estradiol receptor (E_2R) to oligodeoxynucleotide celluloses. Reaction mixtures (0.6 ml, total volume) contained the indicated concentrations of E_2R with a suspension of oligodeoxynucleotide cellulose (representing 20 nmol of organic phosphorus) or an equal volume of blank cellulose in TED (10 mM Tris HCl, pH 7.6; 1.5 mM EDTA; and 1.0 mM DTT). The contents were mixed on a rotator at 4°C for 60 min. The mixture was then filtered through GF/C filter discs and washed three times with 10 ml of TED-0.15 M KCl. E_2R bound to the cellulose matrix was determined by measuring the radioactivity retained on the filter discs. Binding was calculated as the difference between radioactivity bound to the oligonucleotide celluloses and to blank cellulose. ○—○, Oligo(dG); ●—●, oligo(dT); △---△, oligo(dC).

of binding was oligo(dG) > oligo(dT) ≧ oligo(dC) >> oligo(dA) [14]. Those studies were compromised because of our inability to measure binding efficiencies at low concentrations of immobilized ligand. However, with adoption of a filtration technique to recover minute amounts of the affinity matrixes, the relative binding of E_2R to ligand inputs of 20 nmol could be measured (Fig. 1). Again the relative order of binding was oligo(dG) > oligo(dT) > oligo(C), but a clear distinction between the oligodeoxypyrimidines appeared at the lower ligand concentrations. Also a distinct cooperative effect was observed with oligo(dG)-cellulose as the ligand. These data confirm the functional separateness of the nucleotide-binding activities at the DNA-binding domain of uterine E_2R.

Another method of demonstrating the heterogeneous character of DNA binding of steroid holoreceptors was to observe the results of perturbations on the receptors' interactions with individual oligodeoxynucleotides. The binding of mouse uterine E_2R is a salt-stimulated reaction with an optimal KCl concentration of ~ 0.15 M. The identity of the cation is of little consequence (data not shown), but the choice of anion has significant consequences (Table I). Generally the lyotropic nature of the anion and the binding affinity were correlated. Anions which promote ordered water structure (ie, CH_3COO^-, F^-, Cl^-, Br^-) stimulated E_2R:oligodeoxynucleotide interaction, while those which disrupt water structure (ie, SO_4^{2-}, NO_3^-, ClO_4^-, SCN^-) were inhibitory.

TABLE I. Effect of Chaotropic Salts on Binding of Mouse Uterine Estradiol Receptor to Oligo-(dT)- and Oligo(dG)-Cellulose*

Salt (0.15 M)	Oligodeoxynucleotide-bound estrogen receptor (fmol)		Ratio (2)/(1)
	Oligo(dT)-cellulose (1)	Oligo(dG)-cellulose (2)	
NaSCN	0.99	3.92	3.9
$NaClO_4$	0.54	4.38	8.1
$NaNO_3$	9.48	16.69	1.8
KI	0.64	8.05	12.6
KBr	14.14	21.26	1.5
KCl	26.41	34.07	1.3
K_2SO_4	5.57	7.98	1.4
$KOOCCH_3$	27.81	21.35	0.8
KF	22.87	25.45	1.1

*Oligodeoxynucleotide cellulose containing 195 nmol of nucleotide phosphorus and 88 fmol of mouse uterine cytosol [^{3}H]estradiol receptor were used in each assay. Radioactivity bound specifically to the oligodeoxynucleotide cellulose was measured. Nonnucleotide binding was measured by substituting the corresponding quantity of blank cellulose. In each case oligonucleotide binding refers to the difference between the amount bound to derivatized cellulose and blank cellulose.

Significant differences were also apparent between the interactions of E_2R with oligo(dG)- and oligo(dT)-celluloses as the extent of binding decreased. The extent of binding to oligo(dT) followed the order: $CH_3COO^- > Cl^- > F^- > Br^- > NO_3^- > SO_4^{2-} > SCN^- > I^- > ClO_4^-$. The order for oligo(dG) binding was $Cl^- > F^- > CH_3COO^- = Br^- > NO_3^- > I^- > SO_4^{2-} > ClO_4 > SCN^-$. With the chaotropic anions SCN^-, ClO_4^- and I^- there was a greater impairment in the binding of E_2R to oligo(dT)- than to oligo(dG)-cellulose. The observed differences between the formation of complexes of E_2R with the two ligands are consistent with the view that functionally different subsites exist in the DNA-binding domain of mouse uterine E_2R.

In prior experiments the mouse uterine cytosol E_2R binding affinity for oligo(dG)-cellulose was the most stable after exposure to inhibitors of DNA binding [10,16,17]. Also binding to oligo(dT)-cellulose was usually more stable than oligo(dC)-cellulose interactions. We have extended these studies to other inhibitors of DNA-binding: iodoacetamide, 1,2,-cyclohexanedione, and Rose Bengal. These are putative modifiers of protein sulfhydryl groups, arginine, and histidine residues, respectively. The results are shown in Figure 2. In the case of iodoacetamide a new pattern of stability was observed. Oligo(dT)-cellulose binding by E_2R was most stable, while oligo(dG)- and, to a greater extent, oligo(dC)-cellulose binding were lost more readily. With 1,2-cyclohexanedione the G sites were again more stable; little distinction was seen in the decay of binding involving the oligodeoxypyrimidines. At lower concentrations of the reagent, activation of all the binding activities was observed. This pattern of inhibition, excluding a range of activation, was also seen after reaction with Rose Bengal. These data support the concept of functional heterogeneity of the sites involved in E_2R binding to DNA.

The most extensive difference between the binding activities of mouse uterine E_2R is the response to incubation at 37°C for 30 min. While binding to the oligodeoxypyrimidine ligands was rapidly lost, the interaction with oligo(dG)-cellulose was essentially retained [14]. In a similar loss of the labile N binding sites after partial

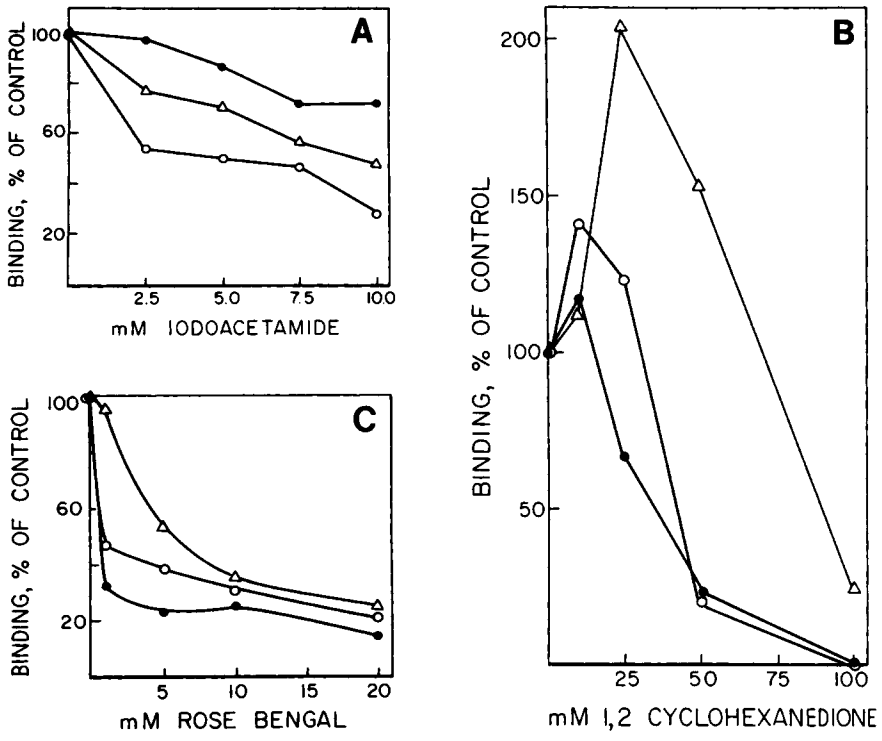
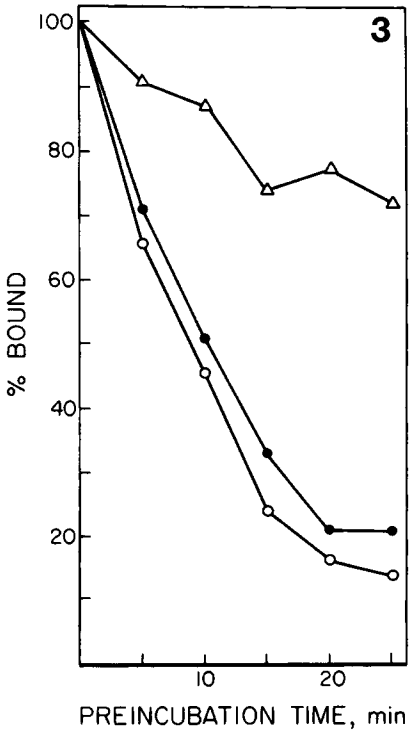


Fig. 2. Effect of iodoacetamide (A), 1,2-cyclohexanedione (B), and Rose Bengal (C) on oligodeoxynucleotide-cellulose binding of mouse uterine cytosol estradiol receptor (E_2R) complexes. Aliquots containing 32 fmol of E_2R were incubated with the indicated concentrations of inhibitor for 30 min at 4°C in 100 mM Tris HCl, pH 7.6 (total volume, 0.6 ml). A pelleted aliquot of a suspension of oligodeoxynucleotide cellulose representing 190 nmol of organic phosphorus was added, and the reaction mixture was brought to 0.15 M KCl (final concentration). Oligodeoxynucleotide cellulose-bound E_2R was assayed by the standard procedure. In the absence of the reagent (control) E_2R bound to oligodeoxynucleotide celluloses was as follows: 12 fmol to oligo(dG), 8 fmol to oligo(dT), and 6.5 fmol to oligo(dC). These values are each scored as 100% in the figure. △—△, Oligo(dG); ○——○, oligo(dC); ●——●, oligo(dT).

purification of E_2R, the activities were restored by addition of histone 2A or 2B, cationic proteins which also stabilize steroid retention by the receptor [18]. Could histone 2B prevent the loss of the N sites after exposure of cytosol E_2R to heat? Essentially no protection of binding to oligo(dT)- or oligo(dC)-cellulose was seen by adding histone 2B at a concentration which was effective in restoring these activities of the partially purified receptor (Fig. 3).

Finally there was a question as to the relationship of the sites on uterine E_2R for interaction with DNA and histones. Kallos et al [19] have shown preferential binding of partially purified rabbit uterine cytosol E_2R to histones 2A and 2B. Limited proteolysis caused a loss of DNA-binding, but E_2R binding to histone 2B-Sepharose was retained, indicating that these sites are independent. These data are consistent with a "bridge" mechanism of E_2R-histone-DNA interaction in which the binding of the steroid receptor and the polydeoxynucleotide is through the histone. We examined



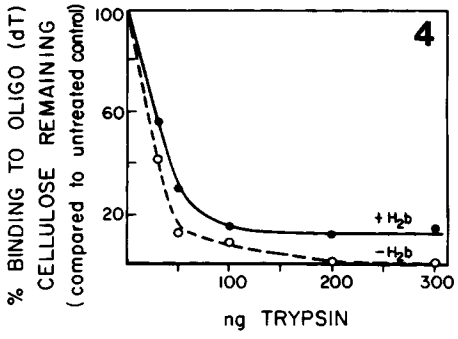


Fig. 3. Effect of preincubation of E_2R at 37°C in the presence of histone H_2B. E_2R was incubated for the times indicated in the presence of histone H_2B (50 μg/0.6 ml of reaction mixture). Free estradiol was removed by treatment with charcoal/dextran, and the binding assays were carried out with aliquots representing equal quantities of E_2R ($\simeq$ 20 fmol). In each case the amount of nonpreincubated E_2R which bound to the oligodeoxynucleotide cellulose was taken to represent 100% (0 min control). △—△, Oligo(dG); ○—○, oligo(dC); ●—●, oligo(dT).

Fig. 4. Effect of trypsin treatment of E_2R on oligo(dT)-cellulose binding in the presence or absence of histone H_2B. E_2R was incubated with the indicated concentrations of trypsin for 30 min at 25°C. Free estradiol was removed by charcoal/dextran treatment. Equal quantities of E_2R (15–20 fmol) were used for each oligo(dT)-cellulose binding assay. Histone $_2B$ (50 μg/0.6 ml of binding assay mixture) was used where indicated. 100% represents the amount of untreated E_2R bound to oligo(dT)-cellulose (5–6 fmol).

this effect of histone 2B by exposing mouse uterine cytosol E_2R to very low concentrations of trypsin and observing the effects on E_2R binding to oligo(dT)-cellulose (Fig. 4). Near-obliteration of the binding was seen after incubation with 50 μg of trypsin at 4°C. Addition of 50 μg of histone 2B had little or no effect upon the loss of oligo(dT)-cellulose binding, suggesting that the histone 2B effect is mediated through the DNA-binding domain of E_2R and not as an intermediate in a ternary complex.

DISCUSSION

Recent studies indicate that steroid receptors, at least some for glucocorticoids and progesterone, recognize specific nucleotide sequences of double-stranded DNA. While most attention is focused on the composition of these sequences and their

position relative to steroid-regulated genes, the role of receptors as DNA-binding proteins should not be overlooked. No longer is the binding merely a matter of nonspecific electrostatic interactions between cationic amino acid residues of the receptor and the polyphosphate backbone of DNA, although such forces are of great importance. Certain properties of the DNA-binding domains underlie specific base recognition. In the absence of knowledge of the detailed amino acid sequences comprising these domains, indirect approaches must suffice to gain information about their functional characteristics.

Immobilized oligodeoxynucleotides are binding matrixes composed of two structural elements of DNA, the polyphosphate backbone and a nucleotide array capable of self-interaction. To a putative binding ligand, such as a steroid holoreceptor, they present some of the problems in association and recognition that DNA does. In the present and previous studies using the mouse uterine cytosol E_2R, certain features of the nucleotide recognition reactions were delineated. The reaction itself, not as a means of activation, has a strong dependence on the appropriate salt concentration [18]. A definite hierarchy of oligodeoxynucleotide binding preferences was found: oligo(dG) > oligo(dT) $\geqslant$ oligo(dC) >> oligo(dA). In all cases except oligo(dG) there was no discernible indication of cooperative binding of E_2R. At very low concentrations of E_2R and oligo(dG)-cellulose there was a suggestion of cooperativity. So within the limited structures of these oligomers, E_2R can easily distinguish between the oligodeoxypurines and between them individually and the oligodeoxypyrimidines.

Functional heterogeneity of the DNA-binding domain or regions of the receptor which influence the DNA-binding domain has been demonstrated by the use of a series of putative modifiers of constituent amino acids, chaotropic salts, elevation of temperature or the less-defined processes of purification. As a rule the G sites are most stable, but this is not always true. Preincubation with the sulfhydryl-blocking reagent iodoacetamide leads to more extensive inhibition of oligo(dG)-cellulose binding at lower concentrations than in the interaction with oligo(dT)-cellulose. With other perturbants the N sites, which bind to oligo(dT), oligo(dC), and oligo(dA), are more labile and, in the case of heat treatment, irretrievably lost, while the G sites remain. Excluding the participation of nonreceptor molecules, these findings indicate that the DNA-binding domain contains discrete binding subsites of differing stability.

The restoration of the N sites by histones 2A or 2B [14] indicates that the DNA-binding domain is malleable. The present study indicates that the histone effect does not extend to protection against thermal inactivation of the N sites. Furthermore the failure of histone 2B to restore E_2R binding to oligo(dT)-cellulose after limited proteolysis suggests that an intact DNA-binding domain is a requirement for histone 2B modulation. The sum of the results of these indirect experiments suggests that the DNA-binding domain of E_2R exists in dynamic equilibrium, with N sites easily capable of distortion but equally capable of reactivation by appropriate interaction with neighboring cationic proteins.

Furthermore a practical consequence of the oligodeoxynucleotide-binding studies was recognition that the chaotropic anions SCN^- and ClO_4^- are denaturants even at concentrations lower than those used in published purifications of estrogen receptors [20,21].

Finally, our studies have developed evidence that steroid receptors contain distinct polynucleotide binding domains composed of subsites with different affinities

for the nucleotide base components of DNA. While the general order of preference—oligo(dG) > oligo(dT) > oligo(dC) > oligo(dA)—is a property shared by all the steroid holoreceptors that we have examined [14,15,17], there were significant differences between these receptors. In an earlier paper [15], we reported that mouse kidney testosterone receptor bound to oligo(dA) relatively better than did the liver dexamethasone receptor or kidney E_2R. At higher oligodeoxynucleotide inputs, E_2R and testosterone receptors bound to oligodeoxypyrimidines equally well, but the dexamethasone receptor bound to oligo(dT)-cellulose preferentially. The oligodeoxynucleotides are, of course, limited probes for exploring the polynucleotide domains of steroid receptors, as they do not mimic the complete three-dimensional array of a DNA helix binding site. In fact, the avidity for oligo(dG)-cellulose may reflect a feature of holoreceptor binding to abortive rather than effective nuclear sites [22]. But they do afford a means of assessing nucleotide recognition by steroid holoreceptors and those factors, ie, inhibitors, accessory proteins, etc, which modulate this recognition.

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