Covalent and Noncovalent Receptor–Glucocorticoid Complexes Preferentially Bind to the Same Regions of the Long Terminal Repeat of Murine Mammary Tumor Virus Proviral DNA†

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ABSTRACT: Dexamethasone 21-mesylate, an irreversible antiglucocorticoid in HTC cells, forms a covalent receptor–steroid complex which can be activated in cell-free systems. The molecular basis of its antiglucocorticoid activity is unknown; it might result from altered DNA sequence preferences and/or affinities of the covalent receptor–steroid complex. To test this hypothesis, the affinities of both covalent receptor–antagonist and noncovalent receptor–agonist complexes for defined DNA sequences were measured in a DNA binding competition assay. This assay requires neither purified complexes nor large quantities of DNA, yet it provides quantitative comparisons of the affinities of different double-stranded DNAs for binding receptor–steroid complexes. In this assay, activated covalent receptor–dexamethasone 21-mesylate complexes in crude cytosol bound to calf thymus DNA and cloned subregions of the long terminal repeat (LTR) of murine mammary tumor virus (MMTV) proviral DNA with approximately the same relative affinities as did noncovalent receptor–dexamethasone complexes. Both types of complex exhibited similar orders of preferential binding to DNA sequences. LTR subregions, as well as the entire LTR, were 2–20 times more potent competitors than calf thymus DNA. Cloned sequences from the 3′ terminus of the LTR were more effective competitors than either the entire LTR or comparably sized DNAs from the 5′ terminus. The DNA sequences with the greatest affinities for both covalent and noncovalent complexes are located within the region of −221 to −67. These studies support the theory that recognition by regulatory elements of specific DNA sequences upstream of responsive genes is an integral step of hormone action. However, the potent antiglucocorticoid activity of dexamethasone 21-mesylate cannot be explained by differences between the covalent and noncovalent receptor–steroid complexes in either the affinities for defined DNA sequences or the orders of DNA sequence preference.

Current models of glucocorticoid hormone action propose that the steroid diffuses freely into cells and binds specific cytoplasmic receptors with high affinity. After activation to a DNA binding form, receptor–glucocorticoid complexes are thought to translocate to the nucleus, bind site specifically to chromatin, and regulate its transcriptional activity (Baxter & Rousseau, 1979). In contrast, antiglucocorticoids bind specifically to the same receptor, yet fail to elicit a biological response (Simons et al., 1980; Simons & Thompson, 1981; Lamontagne et al., 1984). Several models have been proposed to explain the molecular basis of action of steroid hormone antagonists: the antagonist binds to a conformation of the receptor that cannot be activated (Rousseau et al., 1972); the rate of dissociation of the receptor–antagonist complex is too rapid to permit activation and/or translocation of the complex (Raynaud et al., 1980); the nuclear interactions of the receptor–antagonist complex differ from those of the receptor–agonist complex (Turnell et al., 1974; Golaz & Beck, 1983; Simons et al., 1983).

To further define the mechanism of antiglucocorticoid action, we have examined several new antiglucocorticoids (Simons et al., 1980, 1983; Simons & Thompson, 1981; Eisen et al., 1981; Chrousos et al., 1982; Lamontagne et al., 1984; L. Mercier and S. S. Simons, Jr, unpublished results). Dexamethasone 21-mesylate,[1] a potent irreversible antiglucorticoid in rat HTC (hepatoma tissue culture) cells, forms a covalent receptor–steroid complex (Simons & Thompson, 1981; Eisen et al., 1981). Thus, a rapid rate of dissociation of the receptor–dexamethasone 21-mesylate complex cannot explain its antagonist activity. The antiglucocorticoid activity of dexamethasone 21-mesylate is also not the result of an inability of the covalent receptor–steroid complex to undergo activation to a DNA binding form. Receptor–dexamethasone 21-mesylate complexes can be activated with the same efficiency as noncovalent receptor–agonist complexes (Simons et al., 1983), but the covalent complexes bind DNA with a 2-fold lower affinity (Simons & Miller, 1984). The last result suggests that the antiglucocorticoid activity of covalent dexamethasone 21-mesylate labeled receptors might result from altered DNA sequence preferences and/or affinities. To test this hypothesis, we have examined receptor–steroid complex binding to murine mammary tumor virus (MMTV) long terminal repeat (LTR) DNA.

MMTV has been used extensively as a model of glucocorticoid regulation of transcription. Dexamethasone, a synthetic glucocorticoid, selectively increases the rate of transcription of integrated DNA in both mouse mammary tumor and heterologously infected cells (Ringold et al., 1975; Young et al., 1975; Ringold et al., 1977). Through a variety of experimental approaches including deletion studies and transfections of subgenomic fragments of chimeric gene constructions, the LTR has been identified as the region required for hormonal regulation of adjacent genes (Lee et al., 1981; Huang et al., 1981; Ucker et al., 1981; Hynes et al., 1983;

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[1] Abbreviations: HTC, hepatoma tissue culture; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; TAPS, 3-[[tris(hydroxymethyl)methyl]-amino]propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid. The following are the trivial and systematic names of the steroids used in this study: dexamethasone, 9-fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione; dexamethasone 21-mesylate, 9-fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione 21-methanesulfonate.

Majors & Varmus, 1983; Buetti & Diggelmann, 1983). In addition, specific binding of both crude and purified preparations of receptor–glucocorticoid complexes to purified LTR DNA has been observed in vitro by electron microsopy, nuclease protection studies, filter hybridization, and DNA–cellulose competition assays (Payvar et al., 1981, 1983; Geisse et al., 1982; Pfahl, 1982; Govindan et al., 1982; Scheidereit et al., 1983; Pfahl et al., 1983). MMTV–LTR thus provides a convenient system to test our hypothesis of the molecular basis of antiglucocorticoid action.

In this study, we have used a DNA binding competition assay (Simons, 1977) to measure the relative affinities of both the covalent receptor–dexamethasone 21-mesylate and the noncovalent receptor–dexamethasone complexes in crude cytosol for short cloned subregions (100–300 base pairs) of MMTV–LTR. We report that both covalent receptor–dexamethasone 21-mesylate complexes and noncovalent receptor–dexamethasone complexes bind calf thymus DNA, LTR DNA, and subregions of the LTR with approximately the same relative affinities and preferentially bind sequences immediately 5′ to the site of transcription initiation with the greatest affinity.

Experimental Procedures

Chemicals, buffers, cell culture, preparation and labeling of cytosol, and the DNA–cellulose binding assay are described in the preceding paper (Simons & Miller, 1984). Additional procedures specific to this study are presented below.

Preparation of Cloned DNA Fragments for Binding Assays. DNA fragments were produced by appropriate restriction digestion of C3H MMTV–LTR cloned in recombinant plasmids (see Figure 4). Fragments were separated by electrophoresis in 5–8% polyacrylamide gels [20:1 acrylamide–bis(acrylamide)] using buffer consisting of 50 mM Tris, 10 mM boric acid, and 1 mM EDTA (1× TBE, final pH 8.3 at ambient temperature). After electrophoresis, DNA was located by ultraviolet shadowing, bands were excised from the gel, and the DNA was recovered by electroelution in 1× TBE. DNA recovered in this manner was further purified and concentrated by anion-exchange chromatography (Elutip-d; Schleicher & Schuell). Following ethanol precipitation, fragments were treated with S1 nuclease (Boehringer Mannheim; 5 units/μg of DNA) in order to remove single-strand regions (Mulvihill et al., 1982; Geisse et al., 1982), extracted once with phenol–chloroform–isoamyl alcohol (24:24:1) and once with chloroform–isoamyl alcohol (24:1), and ethanol precipitated. DNA was finally resuspended in 10 mM Tris-HCl–1 mM EDTA (pH 7.4) (TE) and the concentration determined by absorbance at 260 nm.

DNA Binding Competition Assay. The relative binding affinities of different double-stranded DNAs for receptor–steroid complexes can be assessed *qualitatively* in a "constant DNA" binding competition assay, in which the total amount of DNA present is constant, but the amounts of DNA in solution and DNA adsorbed to cellulose are varied inversely. This assay, which does not require purified complexes or large amounts of DNA, can be modified to permit a *quantitative* assessment of the relative binding affinities of different DNAs and to further reduce the amount of soluble DNA required. In the modified assay, the amount of DNA–cellulose is constant, but the amount of soluble DNA and thus the amount of total DNA vary. Both the constant DNA assay and its modification have been described previously (Simons, 1977). Briefly, in the constant DNA assay, if the binding capacities (affinity times number of sites) of the soluble DNA and the DNA adsorbed to cellulose were equal, the amount of receptor–steroid complexes bound to DNA–cellulose would be directly proportional to the amount of DNA–cellulose present (theory line, Figure 1). However, soluble DNAs whose binding capacities differ from that of the DNA–cellulose will yield binding curves that deviate from this line. A DNA with no affinity for receptor–steroid complexes has been mimicked experimentally by the measurement of binding of receptor–[^{3}H]dexamethasone complexes to different amounts of DNA–cellulose in the absence of soluble DNA. DNAs with binding capacities greater than that of the DNA adsorbed to cellulose would yield curves that deviated negatively from the theoretical line [see Simons (1977) for further detail]. Thus, in the modified assay, if a single amount (e.g., 0.625 unit) of DNA–cellulose were incubated with zero to infinite amounts of DNA in solution, the amount of binding to DNA–cellulose, expressed as a percentage of control binding (i.e., amount of binding to 1 unit of DNA–cellulose), would decrease as illustrated in Figure 1. The upper limit of binding has been established by the previous measurement of binding to DNA–cellulose in the absence of DNA in solution. When the data are replotted as percent of control binding vs. logarithm [DNA in solution (μg)] (Figure 2), it is possible to determine the amount of DNA in solution that is required to decrease the binding to 62.5% of control. This represents the amount of DNA in solution that has the same binding capacity as 0.375 (=1.0 − 0.625) unit of DNA–cellulose. Such determinations permit quantitative comparisons among different DNAs in solution. DNA in solution has a higher binding capacity than DNA–cellulose, presumably due to the physical blockage of sites on the DNA adsorbed to cellulose (Simons, 1977).

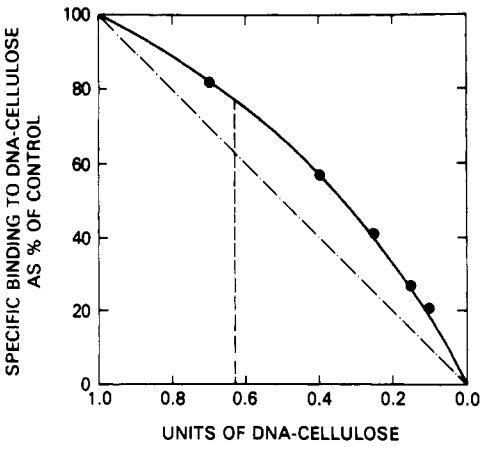

FIGURE 1: DNA binding competition assay. (●) Observed "competition" of receptor–^{3}H steroid complex binding to DNA–cellulose in the presence of no added DNA in solution, which mimics addition of soluble DNA with no capacity to bind complexes. (–·–) Theoretical curve for binding to DNA–cellulose in the presence of soluble DNA with in an identical binding capacity for ^{3}H-labeled complexes. (– – –) Theoretical curve for binding of ^{3}H-labeled receptor–steroid complexes to 0.625 unit of DNA–cellulose in the presence of various amounts (0–∞) of soluble DNA. Control binding is the amount of receptor–^{3}H steroid binding to 1 unit of DNA–cellulose, which was 100 μL of DNA–cellulose slurry prepared from a DNA–cellulose whose DNA concentration was 1.2 mg of DNA/mL of cellulose.

The assay was performed essentially as described for the DNA–cellulose binding assay (Simons & Miller, 1984). Activated, radiolabeled cytosol (250 μL) was incubated with 1 unit of DNA–cellulose (in 100 μL of $TAPS_0$ buffer, pH 8.8, and 50 μL of TE buffer for control binding) or with 0.625 unit of DNA–cellulose (in 100 μL of $TAPS_0$ buffer, pH 8.8) and soluble DNA (in 50 μl of TE buffer) for 0.75–2.5 h at 4 °C. Samples were processed as described in Simons & Miller

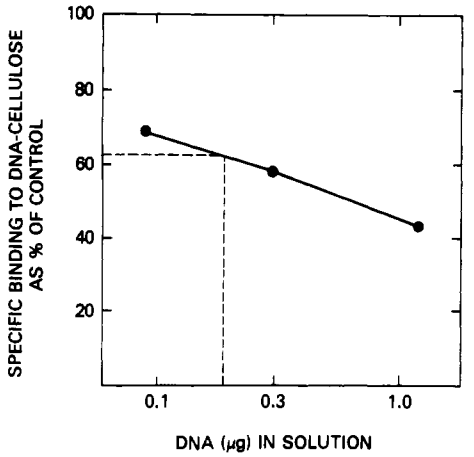

FIGURE 2: DNA binding competition by MMTV-LTR DNA. Activated, [^{3}H]dexamethasone-labeled cytosol was incubated with 1 unit of DNA cellulose (control binding) or 0.625 unit of DNA–cellulose and 0.09–1.2 μg of LTR DNA. Binding of receptor–[^{3}H]dexamethasone complexes to DNA–cellulose was measured as described under Experimental Procedures and expressed as a percentage of control binding. The amount of LTR DNA with the same binding capacity as 0.375 unit of DNA–cellulose is that amount which reduces the binding of receptor–[^{3}H]dexamethasone complexes to DNA–cellulose to 62.5% of control binding. Interpolation of this value is indicated by the broken lines.

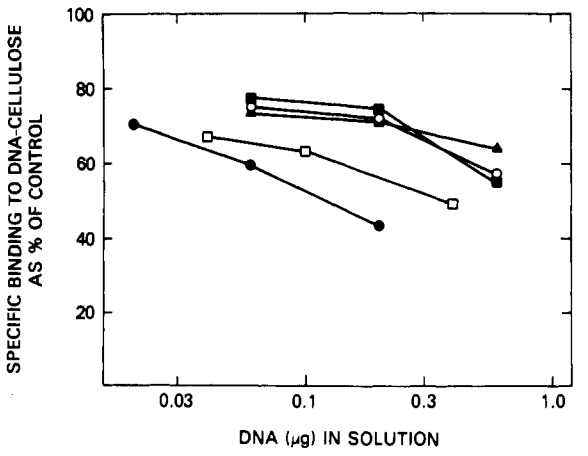

FIGURE 3: DNA binding competition by subregions of MMTV-LTR DNA. The amount of P1 DNA (●), P2 DNA (□), P2* DNA (▲), P3A DNA (○), or P4B DNA (■) with the same binding capacity for receptor–[^{3}H]dexamethasone complexes as 0.375 unit of DNA–cellulose was determined by interpolation of data from DNA binding competition assays performed as described under Experimental Procedures.

(1984). Nonspecific binding for each condition was determined by parallel incubations of the appropriate amounts of DNA–cellulose and soluble DNA with cytosol labeled in the presence of excess [^{1}H]dexamethasone. Specific binding was calculated as the difference in the amounts of binding to DNA–cellulose by cytosols labeled in the absence (total binding) or in the presence (nonspecific binding) of excess [^{1}H]dexamethasone. Specific binding in the presence of soluble DNA was then expressed as a percentage of specific binding to control (i.e., 1 unit of DNA–cellulose).

Results

DNA Binding Competition Assay. The affinities of different soluble DNAs, relative to that of calf thymus DNA immobilized on cellulose, for covalent and noncovalent receptor–steroid complexes were compared by determining the amount of each DNA that was required to reduce the binding of the complexes to 0.625 unit of DNA–cellulose to 62.5% of control binding (see Experimental Procedures for detailed explanation of assay). This particular amount of DNA–cellulose was chosen because it did not require large amounts of soluble DNA, yet provided considerable discrimination among different DNAs. The concentrations of soluble DNA employed in the assay were varied for each DNA such that the binding would closely approximate 62.5% of control binding (Figure 3).

Several possible artifacts of the assay were excluded. Yamamoto & Alberts (1974) have reported that purified DNA can cause aggregation of receptor–glucocorticoid complexes. DNA–cellulose does not cause precipitation of receptor–steroid complexes since, in the absence of soluble DNA, the amount of binding of receptor–steroid complexes to 0.625 unit of DNA–cellulose was 70–80% of control binding (Figure 1; data not shown) and represented only ~20% of the available receptors. Precipitation of the receptor–steroid complexes by soluble DNA would be detected as an apparent increase in the binding to DNA–cellulose. For each DNA examined, binding to DNA–cellulose in the presence of soluble DNA was less than or equal to the amount of binding observed in its absence (data not shown). Soluble DNA did not associate with the cellulose-bound DNA. After 4 h of incubation of soluble, ^{3}H nick-translated MMTV-LTR DNA with DNA–cellulose and nonradioactive receptor–steroid complexes under conditions comparable to those of the binding assay, the amount of radioactivity associated with the DNA–cellulose pellet was less than 2% of the added [^{3}H]DNA. Finally, we ascertained that soluble DNA does not affect the time course of binding for either receptor–dexamethasone or receptor–dexamethasone 21-mesylate complexes binding to DNA–cellulose (data not shown).

Preferential Binding of LTR DNA Sequences. A restriction endonuclease map of the LTR DNA from the C3H-S strain of proviral MMTV is presented in Figure 4. The entire nucleotide sequence of the LTR has been reported previously (Donehower et al., 1981). We analyzed the effectiveness of relatively small (100–300 base pairs) regions of MMTV–LTR DNA to competitively bind receptor–glucocorticoid complexes. Evaluation of such short, comparably sized regions is advantageous for two reasons: (1) the regions of high-affinity binding can be delineated more precisely, and (2) the receptor–steroid complex binding affinity for different DNAs can be quantitated without correction for disparities in size. Such corrections involve presently untested assumptions about receptor–steroid complex binding to a single high-affinity site in the presence of numerous low-affinity sites.

To determine whether covalent receptor–dexamethasone 21-mesylate complexes would demonstrate the same order of sequence preference and bind defined DNA sequences with the same relative affinities as noncovalent receptor–dexamethasone complexes, two protocols were used: (1) the binding of covalent and noncovalent complexes to two different DNAs was directly compared in a single experiment or (2) the relative binding abilities of several DNAs for a single type of complex were measured within a single experiment and then compared to the normalized values obtained for the other type of complex in a parallel experiment. Identical results were obtained with either experimental design. A summary of the data from both types of experiments is presented in Table I. Receptor–dexamethasone 21-mesylate complexes demonstrated a DNA sequence preference that is very similar to that of the receptor–dexamethasone complexes. In addition, both the covalent and the noncovalent complexes bind the various regions of the LTR with approximately the same relative affinities. When

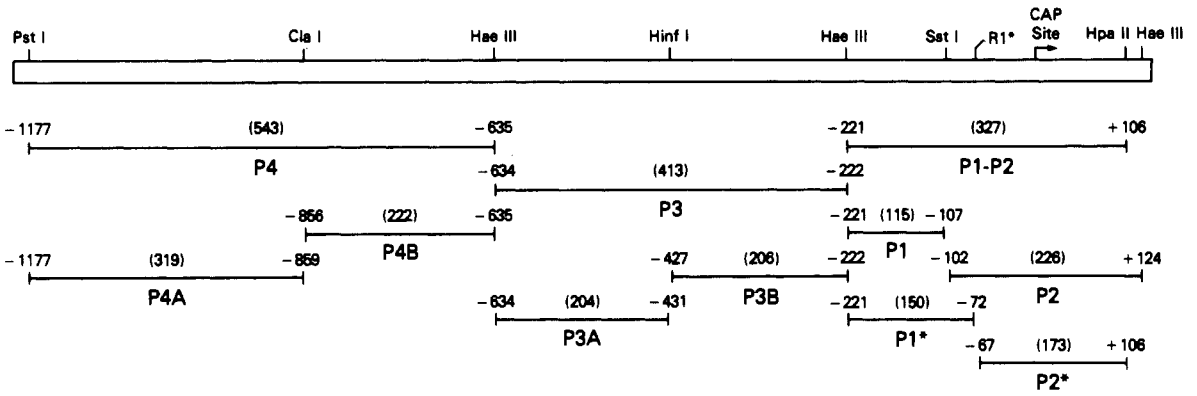

FIGURE 4: Restriction endonuclease map of MMTV-LTR DNA. Sites of the restriction endonucleases used to generate LTR fragments are presented in the upper portion of the figure. The position of each fragment is expressed relative to the cap site, and the size of the fragment, in base pairs, is indicated in parentheses. The 1283 bp LTR that was used is the segment generated by *Pst*I (−1177) and *Hpa*II (+106).

Table I: Comparison of Binding of Noncovalent and Covalent Complexes to Binding of Noncovalent and Covalent Complexes to MMTV DNAs and Calf Thymus DNA[a]

DNA	size[b]	amount of DNA required for competition[a]			DNA_x/DNA_{P1}
		dexamethasone	dexamethasone 21-mesylate	average	
P1	115	2.2 ± 0.5 (n = 7)	1.3 ± 0.4 (n = 7)	1.8 ± 0.6 (n = 14)	1
P2	226	4.6 ± 2.2 (n = 6)	3.3 ± 1.8 (n = 5)	4.0 ± 2.0 (n = 11)	2.2
P3B	206	5.4 ± 1.0 (n = 4)	5.6 ± 1.0 (n = 4)	5.5 ± 0.9 (n = 8)	3.1
LTR	1283	7.7 ± 2.6 (n = 5)	5.1 ± 2.5 (n = 4)	6.5 ± 2.8 (n = 9)	3.6
P4B	222	7.7 ± 2.5 (n = 4)	5.1 ± 3.3 (n = 3)	6.6 ± 3.0 (n = 7)	3.7
P3A	204	9.9 ± 2.3 (n = 5)	5.8 ± 0.7 (n = 4)	8.1 ± 2.7 (n = 9)	4.5
P4A	319	11.1 ± 4.2 (n = 3)	10.7 ± 4.7 (n = 3)	10.9 ± 4.0 (n = 6)	6.1
calf thymus	c	35.5 ± 8.0 (n = 4)	36.5 ± 16 (n = 4)	36.0 ± 12 (n = 8)	20.0

[a] Cloned MMTV DNAs and calf thymus DNA were compared in the DNA binding competition assay for the ability to bind receptor–glucocorticoid complexes. The amount of each DNA required to reduce the binding of complexes to 0.625 unit of DNA–cellulose to 62.5% of control binding was determined as described under Experimental Procedures. One unit of DNA cellulose was either 100 μL of DNA–cellulose slurry prepared from a DNA–cellulose whose DNA concentration was 1.2 mg of DNA per mL of cellulose or 80 μL of slurry from a 2.0 mg of DNA per mL of cellulose stock. The data have been normalized to reflect these differences in unit size and DNA concentration and are expressed as nanograms of DNA in solution per microgram of DNA adsorbed to cellulose (mean ± standard deviation, n = number of experimental determinations). [b] The lengths of LTR DNAs are presented in base pairs. [c] The molecular weight of calf thymus DNA was 9×10^6.

Table II: Ability To Bind Receptor–Dexamethasone Complexes[a]

DNA	size	amount of DNA required for competition[a]	DNA_x/DNA_{P1}
P1	115	2.4 ± 0.6	1
P1*	150	2.9 ± 0.4	1.2
P1–P2	327	4.2 ± 1.9	1.75
P2	226	7.0 ± 2.2	2.5
P2*	173	23.8 ± 1.7	9.9

[a] P1, P1*, P2, P2*, and P1–P2 were tested in the DNA binding competition assay for the ability to bind receptor–dexamethasone complexes as described under Experimental Procedures. The amount of each DNA required for competition, expressed as nanograms of DNA in solution per microgram of DNA adsorbed to cellulose (mean ± standard deviation), was determined in three independent experiments for all DNAs except P2*, which was measured in two experiments and is shown as mean ± range. The P1 and P2 data presented above were not included in Table I data.

the binding of covalent and noncovalent complexes to different DNAs was directly compared in the same experiments, there were no significant differences.

There were, however, significant differences in binding capacities among the LTR subregions. The best competitors, P1, P2, and P3B, are located at the 3′ end of the LTR, the region reported to be required for glucocorticoid responsiveness in gene transfection studies (Lee et al., 1981; Huang et al., 1981; Hynes et al., 1983). P1, P2, and perhaps P3B are all better competitors than the whole LTR; yet, the LTR is more efficient at binding the receptor–steroid complexes than P3A or P4A. The average value obtained experimentally for the amount of LTR DNA required for competition is in reasonable agreement with the amount predicted from the weighted average of its component DNAs (6.5 vs. 5.3 ng of DNA in solution/μg of DNA adsorbed to cellulose).[2] In the last

[2] The calculation of weighted averages is based on the following equations:

$$RS + DNA_{cell} + DNA_{sol} \rightleftarrows RS\text{-}DNA_{cell} + RS\text{-}DNA_{sol} \quad (1)$$

$$K_a^{DNA_{sol}} = [RS\text{-}DNA_{sol}]/([RS][DNA_{sol}]) \quad (2)$$

The amount of activated receptor–steroid complexes (RS; covalent or noncovalent) that was added to each competition assay solution and the amount of complexes that bound to DNA–cellulose (DNA_{cell}) were approximately the same in each experiment. When enough of each DNA in solution (DNA_{sol}) was added to reduce complex binding (at equilibrium) to DNA–cellulose to 62.5% of the control binding, the same amount of RS-DNA_{sol} and RS would be present in solution. Thus, in each case, [RS-DNA_{sol}]/[RS] would be a constant, and eq 2 would reduce to $K_a^{DNA_{sol}} = \alpha/[DNA_{sol}]$ where the added DNA_{sol} was in large excess so that $[DNA_{sol}] \simeq$ concentration of added DNA_{sol}. The theoretical amount of soluble LTR DNA required for 62.5% of control binding (X_{LTR}) was calculated from the data of Table I by using the weighted average equation:

$$\alpha/X_{LTR} = K_a^{LTR} = \sum_{i=1}^{6}\left[\left(\frac{\text{length}_i}{\text{length}_{LTR}}\right)\left(\frac{\alpha}{X_i}\right)\right]$$

where i = P4A, P4B, P3A, P3B, P2, or P1, X = amount of DNA required to reduce the binding of receptor–steroid complexes to 0.625 unit of DNA–cellulose to 62.5% of control binding, expressed as nanograms of DNA in solution per microgram of DNA adsorbed to cellulose, and α was arbitrarily assigned any constant value. Similarly, $X_{P1\text{-}P2}$ was calculated from this equation:

$$\alpha/X_{P1\text{-}P2} = K_a^{P1\text{-}P2} = \sum_{i=1}^{2}\left[\left(\frac{\text{length}_i}{\text{length}_{P1} + \text{length}_{P2}}\right)\left(\frac{\alpha}{X_i}\right)\right]$$

where i = P1 or P2 and X and α are as defined above.

Table III: Comparison of MMTV–LTR DNA Binding Sites for Glucocorticoid Receptor Complexes

authors	type of assay	receptor source	receptor purity	binding sites identified within LTR
Payvar et al. (1981, 1982)	nitrocellulose filter binding; electron microscopy	rat liver	40–60% (1981), 20–85% (1982)	110–449 base pairs upstream of promoter (two distinct sites within this region)
Govindan et al. (1982)	nitrocellulose filter binding; electron microscopy	rat liver	homogeneous	−150 to −100
Pfahl (1982)	DNA–cellulose competition	rat liver; murine lymphoid cell lines	crude cytosol; purified 1000-fold	−400 to −50
Geisse et al. (1982)	nitrocellulose filter binding	rat liver	partially purified (up to 90%)	556 base pairs at 3′ terminus of LTR
Scheidereit et al. (1983)	nitrocellulose filter binding; nuclease protection	rat liver	50–90% pure	−202 to −50; possibly −137 to −50 (filter assay) −124 to −72 −192 to −163 (nuclease protection)
Pfahl et al. (1983)	DNA–cellulose competition	rat liver	crude cytosol	−202 to −137 −137 to −50
Payvar et al. (1983)	nitrocellulose filter binding; nuclease protection; electron microscopy	rat liver	≥80% pure	−84 to −127 −135 to −159 weak −166 to −189 −269 to −283 −289 to −305
Miller et al. (1984)	DNA–cellulose competition	HTC	crude cytosol	−102 to −67 −102 to ≃ −67

column of Table I, the average abilities of different subfragments to competitively bind both types of receptor–steroid complex are expressed relative to the ability of P1, the most effective competitor. P4A is the least effective competitor: it requires more than 6 times the amount that P1 does to inhibit binding to DNA–cellulose to the same extent.

Preferential Binding within the P1–P2 Region of the LTR. A P1–P2 fragment lacking the 3′ 18 base pairs of P2 was constructed to determine whether receptor–steroid complex binding could be enhanced either through preparation of a combined P1-P2 region, which might permit cooperative binding of the complexes, or through a different cleavage of the combined P1–P2 region, which would preserve a potential binding site at the P1–P2 interface. In other experiments, the missing 3′ 18 base pairs of P2 were found not to alter the binding properties of this region of DNA (data not shown). The binding capacity of P1–P2 is consistent with that predicted from weighted averages of P1 and the entire P2 (4.2 vs. 4.2 ng of DNA/μg of DNA adsorbed to cellulose)[2] (Table II). These results suggest a lack of cooperativity between or among binding sites located in the P1 and P2 DNAs.

Digestion of the P1–P2 region with *Eco*RI* yields two fragments of lengths 150 and 173 base pairs, which were designated P1* and P2*, respectively. P1*, which contains the 115 base pairs of P1 and the additional 5′ 35 base pairs of P2 at the 3′ terminus, is slightly less effective as a competitor than P1, yet more effective than P2. P2*, which lacks the 35 base pairs at the 5′ terminus of P2, is a relatively ineffective competitor for receptor–glucocorticoid complexes. It requires 3.4 times more DNA to inhibit binding to DNA–cellulose to the same extent as P2 and 9.9 times more DNA than P1. Thus, the 35 base pairs at the 5′ end of P2 must be important for high capacity binding of receptor–steroid complexes. In preliminary experiments, we have observed similar competition behavior of these DNAs for receptor–dexamethasone 21-mesylate complexes (data not shown).

Discussion

We have used a modification of our constant DNA binding competition assay (Simons, 1977) to examine the binding of both covalent and noncovalent receptor–steroid complexes to sequences in the long terminal repeat of murine mammary tumor virus proviral DNA. In contrast to the receptor–DNA binding assays that immobilize DNA on nitrocellulose filters, the present assay provides quantitative as well as qualitative comparisons of the binding affinities of receptor–steroid complexes for defined DNA sequences. In addition, this assay may be used with either crude cytosol or purified receptor–steroid complexes. Crude cytosol was used in this study to circumvent the problems attendant with a purification protocol which might remove a factor(s) necessary for proper DNA sequence recognition by the receptor–glucocorticoid complexes. The main differences between our DNA competition assay and those used by others (Kallos & Hollander, 1978; Pfahl, 1982; Pfahl et al., 1983; Romanov et al., 1983) are that our assay requires somewhat less soluble DNA and that the data are analyzed differently.

The covalent receptor–dexamethasone 21-mesylate complexes bound calf thymus DNA, intact MMTV–LTR DNA, and six subregions of the LTR with approximately the same relative affinity and order of preference as did the noncovalent receptor–dexamethasone complexes. The average amounts of DNA required for competition of the covalent complexes were generally lower than those required for the noncovalent complexes. However, since the error limits of the data overlap and no differences were observed in simultaneous comparisons, the two sets of data have been combined to give average values of competition (Table I).

Two subregions near the 3′ terminus of the LTR were identified as the most effective competitors, and thus the most likely site(s) of receptor–glucocorticoid binding of DNA. P1, located from −221 to −107 base pairs upstream of the site of transcription initiation, has the greatest binding capacity for both receptor–dexamethasone and receptor–dexamethasone 21-mesylate complexes. P2, located from −102 to +124, is less potent than P1 but more effective than the whole LTR or any other subregion. Further analysis of this region provided no evidence for cooperativity between these sites. Additionally, the majority of the high-affinity binding region within P2 was localized to the 5′ 35 base pairs of P2, as shown by analysis of the shorter P2* fragment. It is unlikely that the decreased size of P2* could account for the drastic change in affinity, since the most potent competitor (P1) is even shorter, and comparably sized DNAs such as P3A and P3B

are also more effective competitors (Table I). A more plausible explanation is that P2* has lost most or all of the binding site(s) at the 5′ terminus of P2.

The DNA binding sites identified in this paper are in good agreement with those reported by other investigators (Table III). When all of the data are combined, it appears that there are at least two preferential binding sites located within a 200 base pair sequence proximal to the 3′ terminus of the LTR (i.e., −250 to −50).

Binding of receptor–steroid complexes to LTR sequences, or envelope sequences, with a higher affinity than to calf thymus DNA or pBR322 DNA (as control) has been interpreted as indicating the presence of other specific binding sites for the receptor–steroid complexes (Pfahl, 1982; Pfahl et al., 1983). By this criterion, we find many binding sites on the LTR since all of the fragments are 2–20-fold better binders of the complexes than is calf thyumus DNA.[3] We prefer to interpret this as representing a continuum of binding site affinities. A similar situation occurs in prokaryotic systems, where mutations in the sites for repressor binding cause considerable variation in the affinities of the sites for repressors and also alter repressor effectiveness (Ptashne et al., 1980; Sadler et al., 1983; Youderian et al., 1983; Kolb et al., 1983). The higher affinity of the whole LTR, relative to calf thymus DNA, may help to "funnel" the complexes into the highest affinity (and biologically active) sites.

Despite the ability to recognize and bind defined DNA sequences with approximately the same affinity as receptor–agonist complexes, receptor–dexamethasone 21-mesylate complexes behave as antiglucocorticoids in whole cells (Simons & Thompson, 1981). As previously noted, these complexes can be activated to a DNA-binding form under cell-free conditions (Simons et al., 1983). However, little nuclear binding by receptor–dexamethasone 21-mesylate is detectable in intact cells (Simons et al., 1983). These observations suggest that the difference(s) between agonist and antagonist behavior which accounts for the difference in biological responses occurs at a step or steps distal to the activation of the receptor–steroid complex. The present data indicate that the antagonist activity of dexamethasone 21-mesylate is not due to an alteration in the sequence-specific DNA binding of the receptor–steroid complexes. Thus, other factors must be involved in the expression of antiglucocorticoid activity by dexamethasone 21-mesylate.

The significance of specific DNA sequence binding by receptor–steroid complexes is not entirely clear. Through gene transfection studies, the region of LTR DNA required for hormonal regulation of adjacent sequences has been identified and specific binding of receptor–glucocorticoid complexes to this sequence has been observed (Table III). However, the small differences in complex affinity for the various DNAs do not appear to be sufficient to account for recognition of the few glucocorticoid responsive genes (Ivarie & O'Farrell, 1978) scattered throughout the genome. Additionally, appropriate developmental and tissue-specific regulation by glucocorticoids despite an almost ubiquitous distribution of glucocorticoid receptors suggests that additional determinants affect glucocorticoid responsiveness. In fact, chromosomal proteins have been shown to enhance the affinity of receptor–steroid complex binding and to decrease the number of acceptor sites (Simons et al., 1976; Spelsberg et al., 1983). The relative importance of DNA sequence, chromosomal proteins, and conformation of the chromatin (e.g., phasing of nucleosomes, chromatin folding) for recognition by receptor–glucocorticoid complexes and subsequent transcriptional regulation requires further investigation.

[3] Yeast tRNA was a far less potent competitor than calf thumus DNA; 7–10-fold more tRNA was required to reduce binding to DNA–cellulose to the same extent (data not shown).

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Regulatory Properties of Acetylcholine Receptor: Evidence for Two Different Inhibitory Sites, One for Acetylcholine and the Other for a Noncompetitive Inhibitor of Receptor Function (Procaine)†

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ABSTRACT: Does the acetylcholine receptor have a specific regulatory (inhibitory) site for the natural receptor ligand acetylcholine? This paper deals with this question. The inhibition of acetylcholine–receptor function by diverse organic cations including local anesthetics such as procaine has been well documented. Evidence indicates that these compounds are noncompetitive inhibitors, enter the open-channel form of the receptor, and block it and that the extent of this blockage depends on the transmembrane voltage of the cell. Recently we reported that in the electroplax of *Electrophorus electricus* the receptor-controlled transmembrane ion flux is inhibited by acetylcholine in a voltage-dependent, noncompetitive manner. We report here that the *Torpedo californica* receptor also has an inhibitory site for acetylcholine. The question of whether acetylcholine, which is an organic cation, binds to the same site as other organic cations such as the noncompetitive inhibitor procaine is important and is addressed. The results reported here of chemical kinetic investigations, with receptor-rich *E. electricus* and *T. californica* membrane vesicles, indicate that the inhibition of receptor function by acetylcholine and by a local anesthetic, procaine, involves two different receptor sites. The existence of a specific inhibitory site for the natural receptor–ligand acetylcholine suggests that this site can play an important role in the modulation of receptor function and in the regulation of transmission of signals between cells.

Noncompetitive inhibitors of the acetylcholine receptor, including positively charged local anesthetics such as procaine, appear to enter the open-channel form of the receptor and block it in a voltage-dependent manner (Neher & Steinbach, 1978; Oswald et al., 1983; Cox et al., 1984). High concentrations of acetylcholine and its analogues also appear to block the receptor channel (Takeyasu et al., 1983; Sine & Steinbach, 1984).

The voltage-dependent inhibition of the receptor-mediated ion flux in *Electrophorus electricus* by acetylcholine and suberyldicholine (Pasquale et al., 1983) has recently been reported (Takeyasu et al., 1983). The inhibitory site was found to be distinct from the sites that are involved in the opening of transmembrane receptor channels and in the inactivation (densensitization) of the receptor. We now report that a voltage-dependent inhibitory site for acetylcholine also exists in *Torpedo californica*. The question of whether positively charged acetylcholine merely acts like the positively charged local anesthetics such as procaine or whether there exists a

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