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A Putative Step in Steroid Hormone Action Involves Insertion of Steroid Ligands into DNA Facilitated by Receptor Proteins

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The hypothesis is advanced that hormonal ligands in the steroid/thyroid superfamily act through insertion between base pairs in partially unwound DNA. Using published X-ray coordinates of the complex of the glucocorticoid hormone response element (GRE) with the glucocorticoid receptor DNA binding domain, the interface between the protein and the gene was examined. The site 5′-TG-3′–5′-CA-3′ previously shown to accommodate cortisol was found in the first two bases of the GRE half sites, 5′-TGTTCT-3′. These base pairs were sufficiently exposed at the receptor–gene interface to permit access by the steroid. Docking of cortisol into the receptor/DNA complex resulted in a favorable van der Waals energy. Given the general lack of correlation of receptor binding with hormonal activity, we propose that hormone action involves an additional step in which the receptor protein in concert with other transcription factors inserts the hormone into the DNA. This notion provides an explanation for earlier paradoxical observations including structural analogies between base pairs and steroid hormones. The insertion hypothesis suggests that receptor bound ligand facilitates DNA unwinding, stereospecific control of donor/acceptor functional groups on the DNA followed by insertion and release of the ligand between base pairs at 5′-TG-3′–5′-CA-3′.

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INTRODUCTION

Historical background and unanswered questions regarding mechanism of action in the steroid/thyroid hormone superfamily including the role of the ligand

Not long after Watson and Crick's discovery of the structure of the B form of DNA [1], Allan Munck conducted pioneering studies to determine whether steroid hormones might interact with nucleic acids [2, 3]. The notion that interactions of steroids with double stranded DNA might be important in the genomic action of steroid hormones was further fueled by observations of Charles Huggins using physical space filling models [4]. Namely, the structures of steroid hormones possessed remarkably close relationships with those of base pairs. Despite a large number of published theoretical and experimental observations (e.g. [5, 6 and references in 7]), the discovery of protein receptors and their primacy in hormone action [8–10] obviated any serious consideration of the putative contact between the steroid and nucleic acid components of regulatory genes. In short, while many details of the mechanism remain to be elucidated, it has been clearly established and widely accepted that the genomic steroid hormone action involves binding of the ligand to its receptor coordinated with a highly specific interaction of the steroid/receptor complex with a given regulatory gene or genes.

In recent years, the advent of modern molecular biology techniques has led to the characterization of a superfamily of steroid/thyroid receptor proteins which have been extensively studied *in vitro and in vivo* [11–13, and references therein]. These receptors which range from approx. 400–900 amino acid residues in length act as transcription factors and have common structural features including unique zinc binding motifs located in the center of the protein that facilitate interaction of the receptor with DNA. Interestingly, the central highly conserved DNA binding domains (DBDs) of the receptors are relatively small spanning only about 70 amino acids. In contrast, the carboxy terminal end of the receptor which contains the ligand binding domain is variable and contains more than

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250 amino acids. The length as well as sequence of the amino terminal domain of the receptor is quite variable among the different receptors.

Conservation of structural features is also evident in the DNA sequences which bind to the DBDs. These sequences, which have been termed hormone responsive elements (HREs), are generally arranged in short repetitive sequences (e.g. palindromes, inverted repeats etc.) containing two half sites that can bind receptor homo and heterodimers. Various techniques including for example NMR and X-ray crystallography of the glucocorticoid and estrogen receptors have shown that only a few residues of the DBD actually contact the HREs [14–18, and references therein]. A particularly intriguing finding is that while all HREs are similar, they fall into two subclasses with identical sequences. Namely, the half sites HREs for the genes regulated by cortisol, aldosterone, testosterone and progesterone are the same. A second group with common HREs are regulated by estradiol, thyroid hormone, 1,25-dihydroxy-vitamin D_3 and retinoic acid. These groupings led Cooney and Tsai to raise the question "...how do the actions of receptors that bind different hormones and respond to different signaling pathways, but transactivate through common response elements, specify the correct differential genetic response dictated by their hormone?" [19].

Many additional factors have been shown to be involved in genomic steroid hormone action, e.g. receptor dimerization, phosphorylation, interactions with heat shock proteins and a steadily increasing role for other transcription factors. Despite the intense interest in unraveling the relative importance of these factors and understanding each of the steps involved in hormone action, the ultimate role as well as the fate of the ligand which appears to be essential for modulating transcriptional activity *in vivo* have not as yet been fully elucidated. The concentration and absolute stereochemistry of each hormone have been clearly shown to govern the magnitude and specificity of hormonal responses. It follows that the capacity to transfer information from the ligand to the gene should be manifest in the stereochemistry of the ligand binding site in any given receptor. Conformational changes associated with ligand–receptor–DNA binding should further elucidate this process. However, unlike the receptor DBDs and their complexes with HREs, detailed three dimensional structural information for ligand bound to intact receptor has been unavailable to date. Of additional concern has been the general lack of correlation between degree of receptor binding of various ligands and hormonal activity. For example, in the case of estrogens, certain super estrogenic ligands bind poorly to the estrogen receptor [20–24]. Conversely, certain strong binding ligands can have poor estrogenic activity [25–27]. Uptake of structurally related estrogens in hormone responsive tissues also does not always correlate with receptor binding [20]. Intracellular metabolism or the capacity to deliver a given ligand to target tissues cannot explain these results. Briefly, the character and extent of hormonal responses cannot be rationalized on the basis of receptor binding [28]. Given these observations it is even more difficult to understand how specific conformational changes in the large carboxy terminal domain of the receptor which reflect the precise concentration and stereochemistry of a relatively small ligand can be quantitatively transmitted to the distal DNA binding region of the receptor resulting in the highly fine tuned regulation of the gene. This mechanism is further puzzling when the apparent location and size of the various molecular components involved are taken into account. For example, the binding of the DBD of the receptor with the HRE may be several hundred angstroms apart from the ligand binding site. Recent findings with glucocorticoids have also shown that the level of transcription mediated by truncated receptors without the ligand binding domain can be enhanced 2-fold by application of ligand [29]. Taken as a whole, the weight of current evidence does not support the notion that hormonal responses can be fully explained by stereospecific interactions of ligands with receptors and the question must be raised as to whether an additional recognition step involving the ligand is taking place. That such a recognition step could involve a closer contact between the ligand and the gene than generally thought is supported by independent observations of covalent linkages formed between estrogens and DNA *in vivo* [30, 31], genotoxic damage caused by an estrogen metabolite [32] and radiotoxic damage caused by radioactively labeled iodinated estrogens in cells containing the estrogen receptor [33]. The latter findings are consistent with the radiolabel being within 10–15 Å of the DNA [34].

Chronological observations of stereochemical fit of steroid hormones into DNA resulting in the ligand insertion hypothesis

A new hypothesis for steroid hormone action is emerging based upon various experimental findings in our laboratories which suggest that an as yet unelucidated step involves direct, receptor-facilitated interaction of the ligand with DNA. This hypothesis has its roots in the discovery that steroid hormones fit remarkably well between base pairs in partially unwound double stranded DNA [35]. Namely, the size and shape of the steroid was accommodated well in the cavity formed between the base pairs and hydrophilic functional groups formed stereospecific hydrogen bonds to heteroatoms on the DNA. Evidence that such observations were not fortuitous included loss of fit when changes where made in either the chirality (asymmetry) of the natural steroid or the chirality of natural DNA. These findings which originated in the mid 1970s were derived from space filling models and were not initially based upon any rationale that they might be important in steroid hormone action. The primary

focus was on understanding why there were relatively narrow constraints on the structures of biologically active molecules in nature. Nevertheless, it did not escape notice that the observed recognition of steroid hormones and other small molecules by DNA modulated by chromosomal proteins might be involved in gene regulation [35].

In the early 1980s, it became possible to create physical models based upon X-ray coordinates of cavities in partially unwound DNA derived from different sequences. Hormonal steroids and thyroid hormones were found to fit best in the site 5′-dTdG-3′–5′-dCdA-3′ [7, 36–39]. Other sequences did not accommodate steroids well but fit other biologically active molecules. For example, plant hormones including gibberellic acid fit stereospecifically into the site 5′-dTdA-3′–5′-dTdA-3′. It was also discovered that the capacity of several classes of biologically active molecules including steroid and thyroid hormones to fit into DNA correlated with hormonal activity. Prospective *in vitro* and *in vivo* experiments as well as physicochemical studies were conducted to determine the relevance of the fit of steroids into DNA in hormone action and whether the modeling observations had predictive value. Using estrogen analogs as an example, hormonal activity was correctly predicted from fit into DNA [37]. However, only weak binding of steroids with DNA was found consistent with previously reported results of many other laboratories [40]. We concluded that it was unlikely that steroid hormones could act at the genetic level alone without the assistance of receptor proteins or other factors. That the binding of steroid hormone to its receptor could be solely responsible for the specificity and magnitude of hormonal activity was also unlikely given that the most active estrogen in these studies, i.e. 11β-acetoxyestradiol, bound poorly to the estrogen receptor [24, 37].

More recent studies have focused on the use of modern computer graphics and rigorous energy calculations to evaluate the stereochemical fit of hormonal ligands with DNA [41–47]. These studies have largely confirmed earlier observations made with physical models and indicate an even better complementary stereochemical fit of steroids into DNA than previously thought. It was also shown that changes in the nucleic acid structure to those which do not exist naturally, e.g., ent-DNA or homo-DNA which was recently synthesized by Eschenmoser [48], resulted in complete loss of fit of all hormones. A good correlation was observed between energetics of fit of ligands into DNA and hormonal activity. No such correlation was found between receptor binding and hormonal activity or receptor binding and fit into DNA. The correlations between DNA fit and activity led to the development of pharmacophores or three dimensional composites which can be used to predict activity of candidate hormone agonists as well as certain antagonists [49]. The discovery of pharmacophores and quantitative structure–activity relationships for ligands in the steroid/thyroid superfamily are providing a new and powerful tool for drug design. These current findings make it difficult to rationalize the close fit of compounds into DNA as simply the way that steroid hormones and other ligands evolved.

Our results based upon computer modeling and energy calculations, previous *in vitro* and *in vivo* findings in the context of physicochemical studies and historical evidence provided by numerous independent laboratories over the past 40 years have led to a more detailed investigation of whether hormonal ligands in the steroid/thyroid superfamily are inserted into DNA during their mode of action. To this end, structural data available on various hormone responsive elements and cognate DNA binding regions of the receptors were examined. We also studied the interface between the DNA binding domain of steroid receptors and the HREs of regulatory genes using as an example available data published for glucocorticoids. Here, we report: (1) the site 5′-dTdG-3′–5′-dCdA-3′ which was shown previously by modeling to best accommodate steroid/thyroid ligands is present, invariant and at the beginning of all HRE half sites; (2) the ligands in the steroid/thyroid superfamily that fall into two groups based upon closely related hormone responsive elements have structural features in common that fall into the same two groups based upon fit into DNA; (3) the site 5′-dTdG-3′–5′-dCdA-3′ is largely exposed in the complex formed between the DBD of the receptor and the HREs; (4) hormonal ligands can fit along the surface of the interface of the DBD–HRE complex at 5′-dTdG-3′–5′-dCdA-3′ and; (5) the van der Waals interaction of the glucocorticoid hormone cortisol with the interface of the glucocorticoid response element (GRE) and glucocorticoid receptor DNA binding domain (GR DBD) as measured by energy calculations is highly favorable. These findings are consistent with the possibility that the receptor protein upon binding both ligand and DNA folds in such a manner as to facilitate insertion and release of the ligand into the DNA. This putative addendum to the classical understanding of steroid hormone action has been termed the "Ligand Insertion Hypothesis" and does not to our knowledge contradict any existing experimental data. The hypothesis also provides an explanation for many puzzling observations including the historical reports of close structural relationships between steroids and DNA and the lack of good correlation between receptor binding and hormonal activity. Thus, ligand insertion is proposed to be a supplemental means by which the concentration and stereochemistry of the ligand can be transmitted to the gene thereby mediating transcriptional events.

MATERIALS AND METHODS

Molecular modeling was conducted on a Silicon Graphics Indigo Extreme using Sybyl molecular

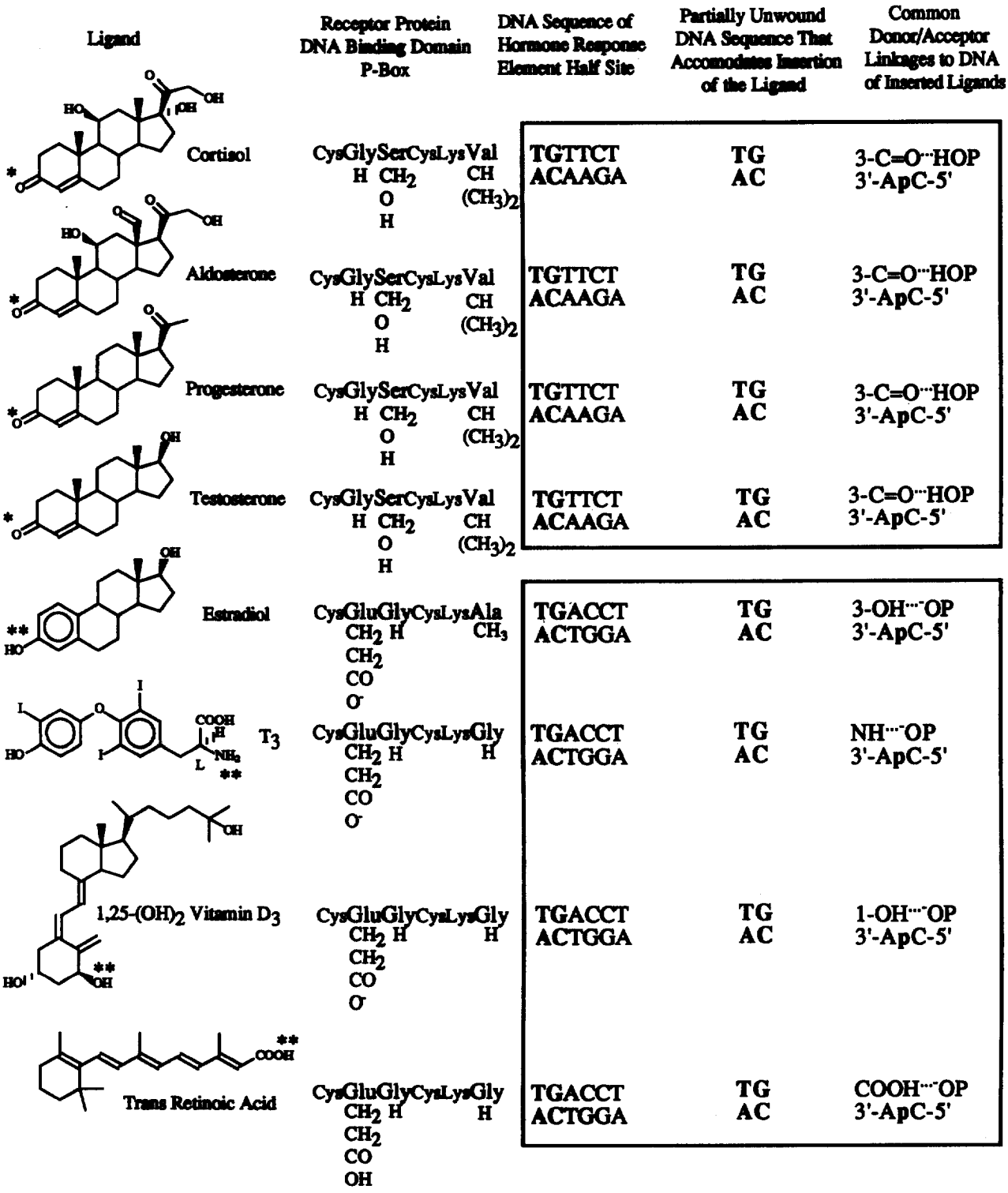

Fig. 1. Correlation of the stereospecific fit into partially unwound DNA of the ligands in the steroid/thyroid superfamily derived from molecular modeling [7, 35–47] with the sequences of the consensus HRE half sites and the amino acids in the DNA binding domain P-box of the corresponding receptor [11–13]. The site in DNA 5′-TG-3′–5′-CA-3′ which upon unwinding has been shown to accommodate all of the ligands has also been found to be invariant in the sequences of the HREs for all ligands. Functional groups on the ligands which form common hydrogen bond donor/acceptors to DNA fall into two classes designated by * and ** on the structures with the specific linkages listed in the right column. The boxes demonstrate that ligands within each of the two classes also fall into the same groupings based upon identical consensus HREs (see text for discussion).

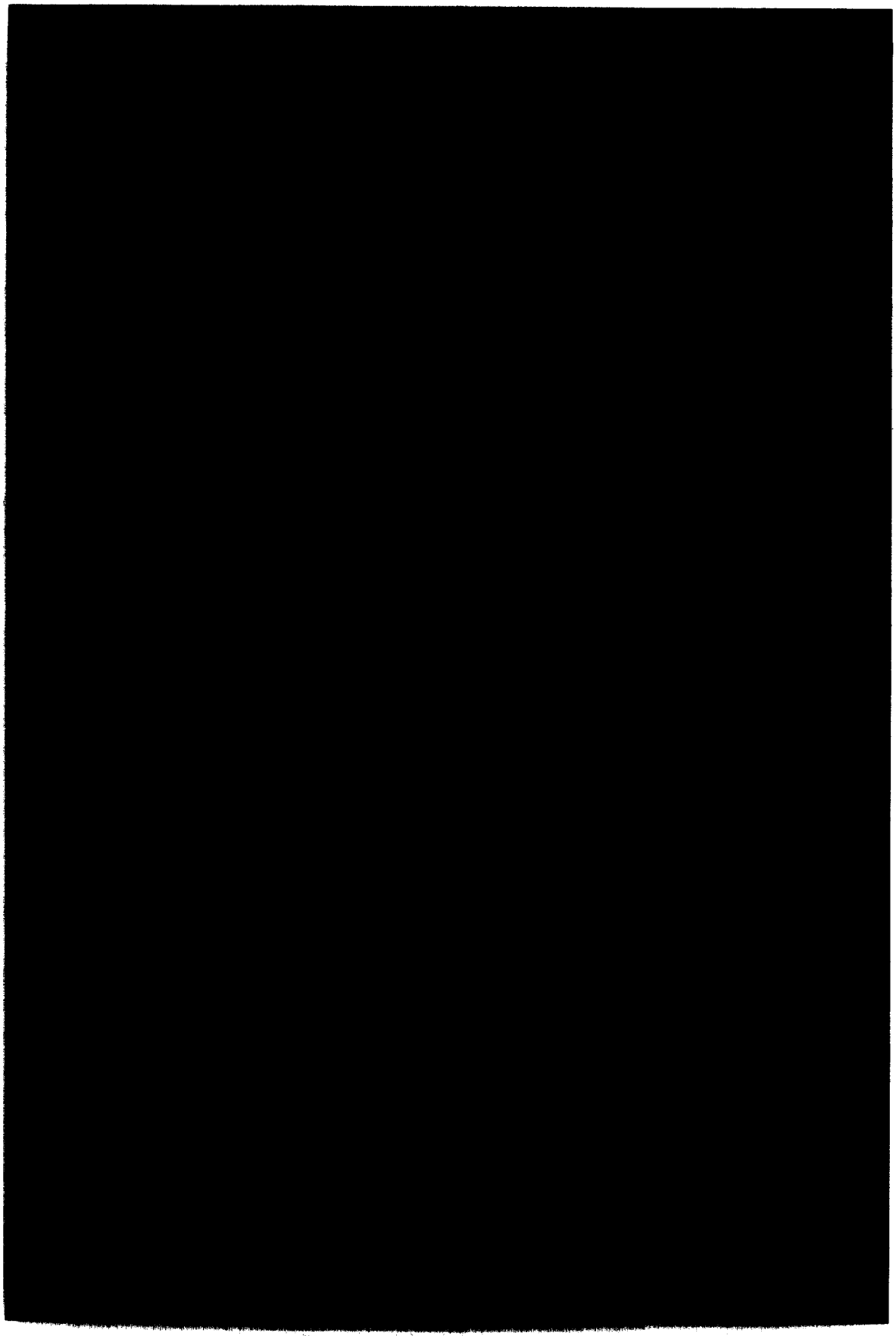

Fig. 2(A and B)—*legend on p. 179.*

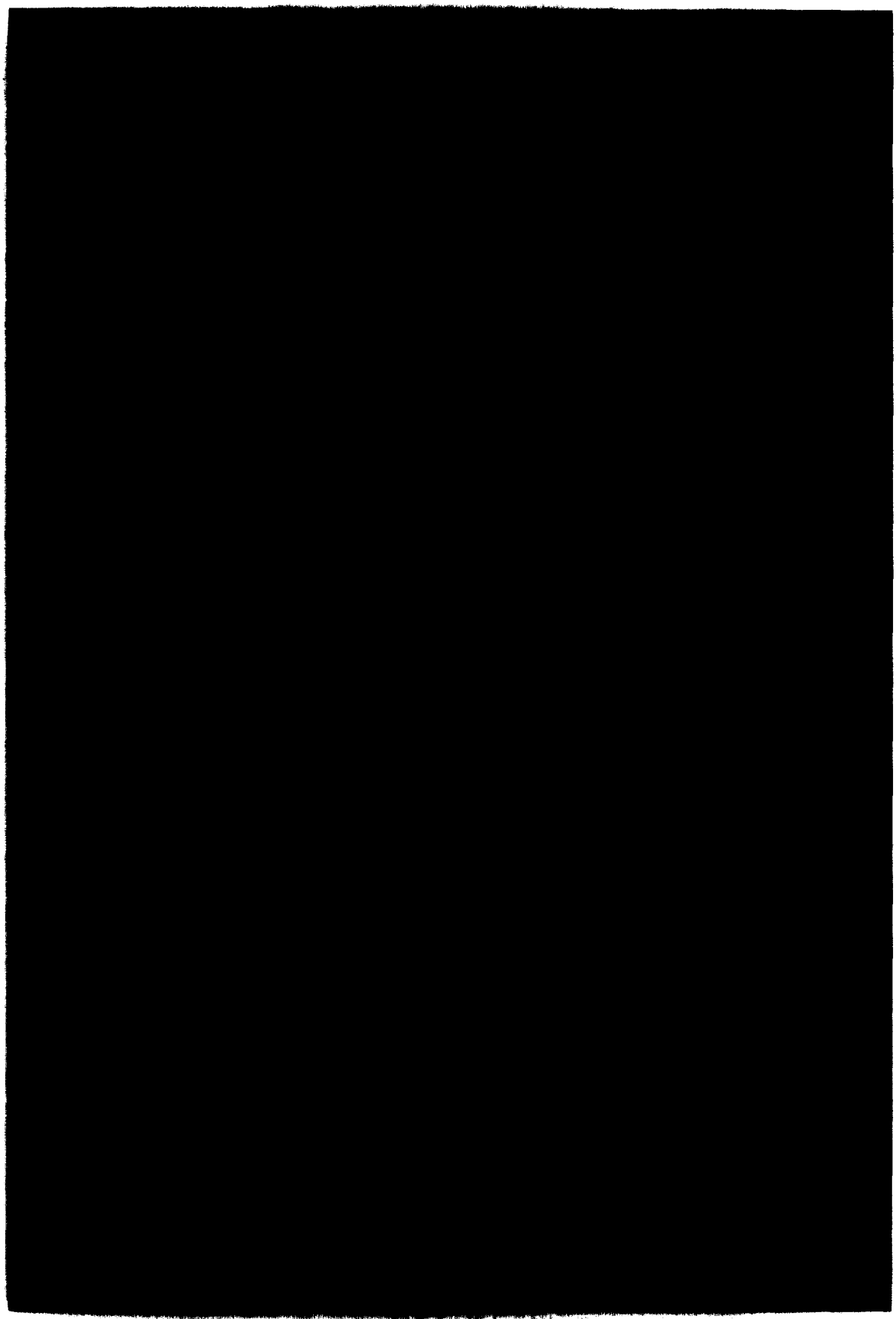

Fig. 2(C and D)—*legend opposite.*

modeling software version 6.1 from Tripos Associates (St Louis, MO). The structures for the GR DBD and the corresponding consensus GRE [15] were imported into Sybyl from coordinates [50] in the Brookhaven database [51, 52]. Appropriate atom and bond types were corrected and/or modified on these structures to correspond to those suitable for further analysis using the Sybyl program. Hydrogen atoms were added to satisfy appropriate valence requirements. Small molecular weight ligands were obtained from the Cambridge Database [53], construction from fragment libraries or modification of existing structures followed by energy minimization. The Sybyl force field was used in all energy calculations. Solvent accessible Connolly surfaces were generated at the receptor/DNA interface using a 1.4 Å probe atom.

Docking of ligands into the cleft observed in the receptor/DNA complex was accomplished using the autodocking program. The stereographics viewer and Connolly surfaces were employed to guide the docking procedure. Interactive measurements of surface interactions between ligands and the receptor/DNA complex were made using the force field and a 1.2 Å bond length for hydrogen. A negative change in van der Waals energies measured in kcal indicates a favorable interaction of molecular surfaces. A positive energy change indicates potential strain and poor interactions among candidate molecules.

RESULTS

Structures of ligands in the steroid/thyroid superfamily are shown in Fig. 1 along with the respective DBDs of the associated receptor proteins and consensus hormone responsive element (HRE) half sites. This data which were summarized from the literature demonstrate that the ligands are known to fall into two groups based upon common HREs [11–13]. In the first group, cortisol, aldosterone, progesterone and testosterone have the same sequence in the half sites, i.e. 5′-TGTTCT-3′. In the second group, estradiol, L-triiodothyronine (T_3), 1,25-dihydroxyvitamin D_3 and *trans*-retinoic acid have the consensus half site 5′-TGACCT-3′. The amino acid sequence of the DBDs are different among the groups but almost identical within each group. Figure 1 also summarizes the fit of hormones into partially unwound double stranded DNA based upon molecular modeling demonstrating that all of the ligands are capable of fitting between base pairs at the site 5′-TG-3′–5′-CA-3′ [7, 36–47]. Each of the ligands with the exception of retinoic acid had been previously shown to form unique stereospecific linkages to both strands of double stranded DNA. Structures which fit into the same site and formed the same linkages as a given hormone possessed the same hormonal activity. In searching for common features among the fit of the ligands, all of the structures were capable of linking to the phosphate oxygen attached to CpA which is bordering the cavity. The types of linkages fell into two distinct groups of donor/acceptor relationships. Cortisol, aldosterone, progesterone and testosterone required a protonated phosphate and were in each case linked via the common proton acceptor oxygen of the 3-carbonyl group. Estradiol, T_3, 1,25-dihydroxyvitamin D_3 and retinoic acid required a negatively charged phosphate oxygen and formed linkages involving the 3-hydroxyl, amino, 1-hydroxyl, and carboxylic acid, respectively.

Given the two classes of closely related HRE half sites, the question arises as to which bases are contacted by the receptor DBD. In the case of both the glucocorticoid and estrogen receptors, the three dimensional structures of the complexes of the GR DBD–GRE [15] and ER DBD–ERE [17] reveal multiple contact points at bases throughout the respective half sites 5′-TGTTCT-3′ and 5′-TGACCT-3′. With regard to the first two bases, TG, within the site which we have shown can accommodate hormones, an arginine residue in both receptors forms a hydrogen bond with G. No contact between the receptor and the first base T is observed in either receptor. The opposite strand 5′-CA-3′ also does not contact either receptor protein. The question arises as to whether the 5′-TG-3′–5′-CA-3′ site in the HRE is sufficiently exposed in the receptor/DNA complex to permit approach and subsequent insertion of the ligand. This question is answered by computer modeling of the GR DBD–GRE using the published X-ray coordinates [15]. Although difficult to view in two dimensions, stereo space filling models in Fig. 2(A) confirm that the GR DBD in green which contacts one side of the double helix shown in red does not block accessibility

Fig. 2 *(pp. 177–178).* Stereo computer generated models of the glucocorticoid response element half sites complexed with the glucocorticoid receptor DNA binding domain derived from published X-ray coordinates [15] demonstrating docking of the glucocorticoid hormone cortisol into the interface between the receptor and DNA. (A) Space filling model of the GR DBD (green)–GRE (red) complex. The GR DBD binds as a dimer with the specific recognition complex [15] shown at the top of the figure. The first two base pairs 5′-TG-3′–5′-CA-3′ of the consensus GREs are shown in blue. (B) Skeletal models as in A with the DNA sequence of the GRE labeled. The ribbon highlights the tertiary structure of the receptor. Note that the interface between the receptor protein and the DNA at the first two base pairs in the upper GRE half site 5′-TGTTCT-3′ as viewed from the major groove is largely exposed. (C) Space filling model showing that cortisol (magenta) can fit into the major groove at the first two bases pairs 5′-TG-3′–5′-CA-3′ of the upper half site in the GR DBD–GRE interface. (D) Skeletal models as in C above.

to the first two base pairs in the half site 5′-TG-3′–5′-CA-3′ shown in blue. The half site which has been shown to form specific contacts with the GR DBD [15] is at the top of the figure. That these base pairs are largely exposed in the GRE can also be seen from skeletal models in Fig. 2(B) which are provided in stereo with the base sequence labeled. Whether the hormone cortisol could come into contact with 5′-TG-3′–5′-CA-3′ with the receptor DBD still bound was assessed by docking the ligand into the GR DBD–GRE complex. As shown in Fig. 2(C) with stereo space filling models and in Fig. 2(D) with skeletal models, the cortisol colored magenta can come in close contact at 5′-TG-3′–5′-CA-3′ in the GR DBD–GRE complex. The docking of ligand does not interfere with binding of the GR DBD to the GRE and the ligand can approach the site easily from the major groove. If the base pairs were then unwound at 5′-TG-3′–5′-CA-3′, it is important to note that cortisol (as well as other hormones in the superfamily) would fit between the base pairs without necessitating disruption of the contact of G with the arginine residue of the receptor.

To further assess how well cortisol could fit into the protein–DNA complex, Connolly surfaces were created near the GR DBD–GRE interface at the exposed sequence 5′-TG-3′–5′-CA-3′. As shown in Fig. 3(A) the interface of the GR DBD and GRE in green forms a partial pocket. Cortisol shown in

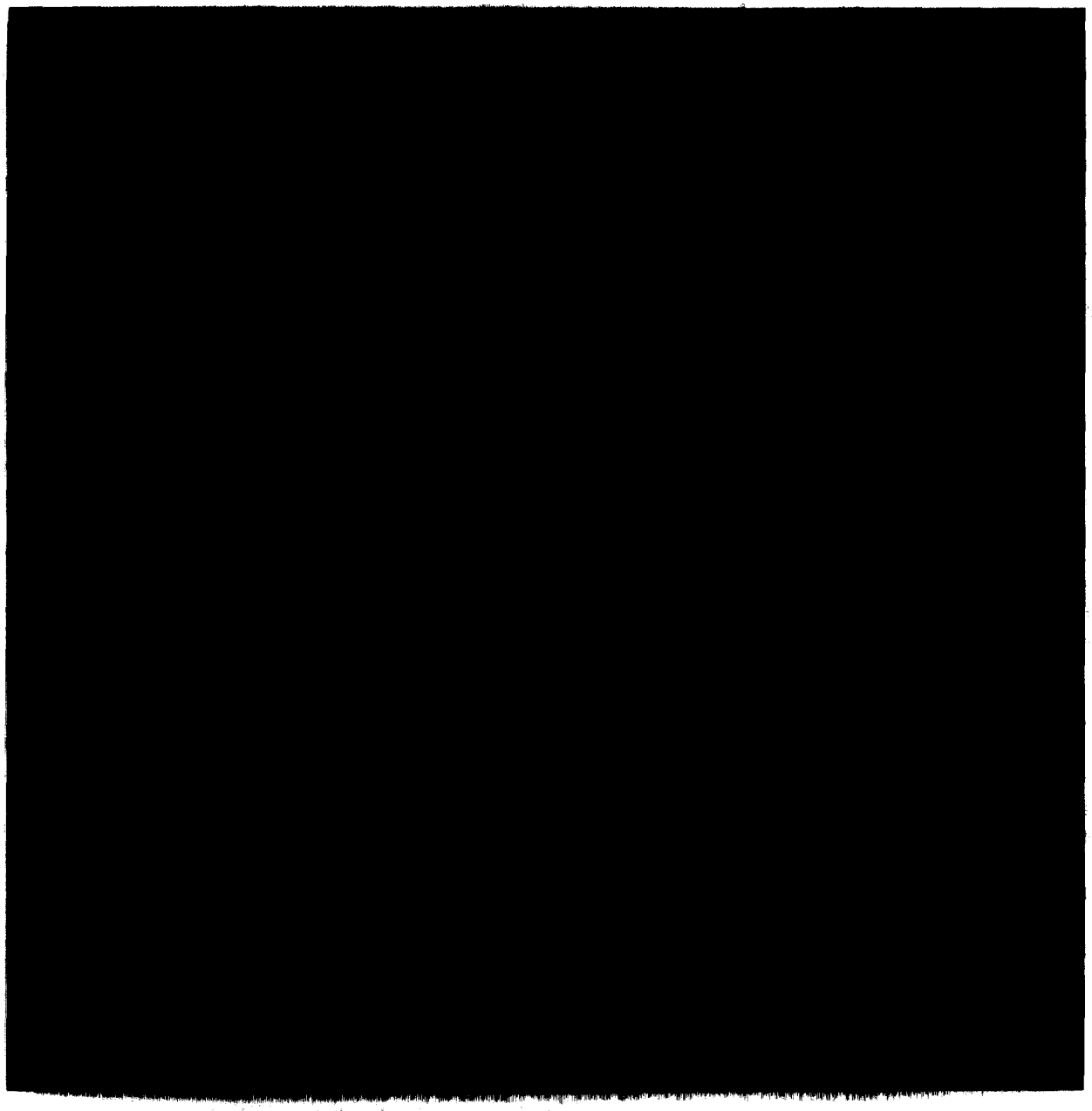

Fig. 3. Computer generated Connolly surfaces in stereo of: (A) the GR DBD–GRE delineating the interface (green) between the receptor and DNA at 5′-TG-3′–5′-CA-3′ of the upper half site 5′-TGTTCT-3′ magnified from Fig. 2; (B) fit of the glucocorticoid hormone cortisol (magenta) into the GR DBD–GRE interface as in A.

magenta in Fig. 3(B) fits particularly well into the interface. The van der Waals energy of docking of cortisol into the complex was approx. −12 to −18 kcal indicating a highly favorable interaction.

DISCUSSION

The goal of this study was to evaluate receptor–gene interactions in the steroid/thyroid superfamily in order to determine whether hormone action may involve insertion of ligands into DNA. Glucocorticoids were chosen as a specific example for detailed investigation given the extensive reports in the literature including available X-ray data on the interactions of GR DBD and the GRE [15]. The results clearly show that there is a cleft formed in the interface of the GR and GR DBD and that the natural glucocorticoid hormone cortisol is capable of coming in close contact with the DNA at the major groove of first two base pairs 5′-TG-3′–5′-CA-3′ which are uniquely exposed in the GRE half site, 5′-TGTTCT-3′ (Figs 2 and 3). The energetics of interaction of cortisol with the GR DBD–GRE complex at this locus is highly favorable. The orientation of the ligand and the GR DBD–GRE interface are such that if the DNA was unwound, insertion and release of the hormone between the base pairs at 5′-TG-3′–5′-CA-3′ could take place as previously shown by molecular modeling [7].

The X-ray data of the GR DBD–GRE complex [15] are consistent with NMR studies [14, 18] and are similar to those reported for the estrogen receptor DBD [17] and retinoic acid receptor DBD [54] which have three dimensional features in common. In each case, the first two base pairs 5′-TG-3′–5′-CA-3′ of the HRE half site appear to be largely exposed. Although not shown, other hormonal ligands in the steroid/thyroid superfamily are known to be about the same size as cortisol and can come in close contact with the HRE-DBD raising the possibility of insertion as a general mechanism. That such an interaction is fortuitous is unlikely since the insertion site which accommodates the ligands in the steroid/thyroid superfamily was reported prior to the publication of the sequences of many of the HREs [7, 36–39]. Moreover, many of the ligands are wedge shaped suggesting that the stereochemistry of the hormones may assist in unwinding and separating the base pairs. The findings summarized in Fig. 1 further demonstrate another remarkable correlation, namely, that the ligands in the superfamily can be divided into two groups based on previously reported common hydrogen bond donor/acceptor relationships upon insertion into 5′-TG-3′–5′-CA-3′ and that the ligands are grouped in the same manner based upon common sequences in their HRE half sites.

Taken as a whole, the above findings coupled with multiple lines of experimental and theoretical evidence in the literature are consistent with the hypothesis that ligands in the steroid/thyroid superfamily are inserted into DNA facilitated by the receptor protein and other transcription factors. One possible scenario is that the DBD of the receptor binds to the HRE and that the ligand upon binding to the receptor triggers a conformation change which folds the protein in a highly specific manner. Namely, such folding enables the distal ligand binding domain to bend back on itself and come in close proximity to the exposed site in the HRE, i.e. 5′-TG-3′–5′-CA-3′. It cannot be ruled out that the ligand bound receptor could conceivably interact at HREs which are not directly associated with its binding to its cognate consensus half site. In any case, the insertion hypothesis is consistent with the conclusions of Beekman *et al.* [55] that transcriptional activation requires conformational changes in the ligand binding domain of the estrogen receptor. The receptor ligand binding domain perhaps in concert with the amino terminal domain and other transcription factors may interact with the HRE stereospecifically governing certain donor/acceptor properties of the site and facilitating the insertion, release and full recognition of the ligand. If this scenario is correct, the receptor could function to select the proper ligand as well as to unwind the DNA. Recent experimental evidence suggests that hormone receptors may indeed bend DNA [56, 57]. Such bending of DNA followed by insertion and release of hormone is consistent with other independent experimental findings. Namely, chromosomal proteins have been shown to alter DNA conformation in a manner which creates preferred ligand binding sites in DNA for small molecular weight drugs [58]. While the interaction of the ligand with the receptor would be largely responsible for the specificity of the response, the magnitude of the response would be governed by degree of fit into DNA. This would explain why hormonal activity correlates well with fit into DNA measured by energy calculations but not with receptor binding. It would also explain why dexamethasone mesylate binds covalently to the GR in the steroid binding site, activates an apparently normal DNA binding complex yet acts as an irreversible glucocorticoid antagonist [59]. In our model, such ligands which bind covalently to the receptor would be incapable of release into the DNA and thus would have predicted antagonistic activity. It follows that molecules which interfere with the complementary stereochemical recognition of the natural hormone by DNA would also be antagonists as previously reported [7, 42, 44–47].

The ligand insertion hypothesis also provides an explanation for how transcription elicited by truncated receptors without the ligand binding domain can be enhanced by the presence of ligand as shown by Spanjaard and Chin [29]. It follows that ligand in the presence of full length receptor would, however, have a much greater effect on transcription which is also consistent with their findings. Ligand insertion can also

explain why various estrogens including radiolabeled analogs can have genotoxic effects [32, 33] as well as form covalent linkages with DNA [30, 31]. The hypothesis can also resolve the dilemma raised by Cooney and Tsai [19] (see Introduction) regarding how different ligands can transactivate through common response elements. An answer consistent with our model is that domains of the receptor other than the DBD are providing additional specificity by modulating the interaction with DNA and insertion of the ligand. With regard to glucocorticoids, it is particularly interesting that mutagenesis studies have shown that the first two bases of the hormone responsive element 5′-TGTTCT-3′ are required for transcriptional activity [60]. The first, T, has also been shown to be important in the binding of the glucocorticoid receptor [61, 62]. However, as reported previously [15–18] and shown here, the GR DBD does not contact this base. This finding necessitates that some other part of the ligand bound receptor recognizes T and can be explained by ligand insertion. Finally, it is clear that many factors will need to be considered in future studies including thermodynamics [63] of intact receptor–ligand–DNA complexes as well as dynamic modeling of the role of solvent. In this regard, recently published molecular dynamics simulations of the GR DBD–GRE complex using water droplets have led to the conclusion that the receptor protein unwinds the DNA [64] providing further evidence for ligand insertion.

NOTE ADDED IN PROOF

Shortly after submission of this manuscript, the first crystal structure of a LBD was reported (Bourguet W., Ruff M., Chambon P., Gronemeyer H. and Moras D.: Crystal structure of the ligand binding domain of the human nuclear receptor RXR-α. *Nature* 375 (1995) 377–382). As predicted by our model, considerable folding was observed in the LBD. This observation coupled with the finding that the putative ligand binding site is on the protein surface provide further support for the ligand insertion hypothesis.

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