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A model for hormone receptor binding to the mouse mammary tumour virus regulatory element based on hydroxyl radical footprinting

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ABSTRACT

The mouse mammary tumour virus long terminal repeat region contains regulatory sequences able to mediate transcriptional induction by different steroid hormones. Two clusters of binding sites for the glucocorticoid and the progesterone receptors have been identified in the region between -70 and -190, the so called hormone responsive or regulatory element . To understand the molecular details of the interaction between the receptors and the DNA we have used the high resolution technique of hydroxyl radical footprinting (1). Both in the promoter distal site and in the promoter proximal cluster additional contacts between the proteins and the double helix are detected by this technique, outside of the region identified by methylation protection. The pattern of contacts in the promoter distal region is compatible with a model involving the interaction of a receptor dimer with the major grooves of four subsequent turns of the double helix, each turn being contacted by a separate zinc finger. This model is illustrated by computer graphical methods and discussed in terms of sequence homologies with other hormone regulatory elements.

INTRODUCTION

The mechanism by which steroid hormones induce transcription of hormone-responsive genes involves an interaction of the hormone receptors with a set of DNA sequence elements, the hormone responsive or regulatory elements (HRE), located at variable distance from the regulated promoter (for a review see Ref. 2). A comparison of the nucleotide sequence protected by the glucocorticoid receptor against DNaseI cleavage in 24 DNA binding sites studied so far yields the consensus 15-mer 5'-GGTACANNNTGTYCT-3, that has been shown to mediate both glucocorticoid and progesterone inducibility of an adjacent promoter (3, 4). This 15-mer exhibits a dyad symmetry element centered at position 8. On the basis of protection against methylation of purines by dimethyl sulfate it has been

postulated that the glucocorticoid receptor binds to the 15-mere as a dimer in head-to-head orientation (5). However, it has been later reported that a molecule of receptor binds per each HRE element in the MMTV promoter (6). Thus, the precise stoichiometry of the interaction between hormone receptor and DNA remains to be established.

In an attempt to further characterize this interaction at the molecular level we have used the technique of hydroxyl radical footprinting (1). Since the hydroxyl radicals attack the deoxyribose moiety of the DNA helix independently of the base sequence, this method yields information on contacts between proteins and DNA at each single nucleotide of the sequence. In addition, the small size of the reagent, similar to a water molecule, yields a high resolution footprint and delimits very precisely the regions contacted by regulatory proteins. Using this technique we detect protein contacts to the consensus HRE that confirm previous results (5). In addition we find contacts between hormone receptors and the major groove of the DNA double helix in the regions flanking the conserved 15-mer. It is difficult to interprete these findings in terms of a monomer of the receptor being the DNA binding entity. In conjunction with the proposed structure of the DNA binding domain of the receptor molecule, these results suggest a refined model for a dimer of the receptor complexed with each 15-mer consensus, that in addition to the central base specific binding to the 15-mer involves base independent interactions with the flanking DNA sequences. This model is illustrated by computer graphics analysis of the contact points, and its functional significance is discussed.

MATERIALS AND METHODS

Purification of hormone receptors. Glucocorticoid receptor was purified from liver cytosol of adrenalectomized rats and progesterone receptor from uterine cytosol of rabbits treated with estrogen for 1 week, according to previously published procedures (8,14).

Plasmids. The plasmid pLTR-wt has been previously described (8).The BamHI/HinfI fragment from p13-13 (15) was made blunt

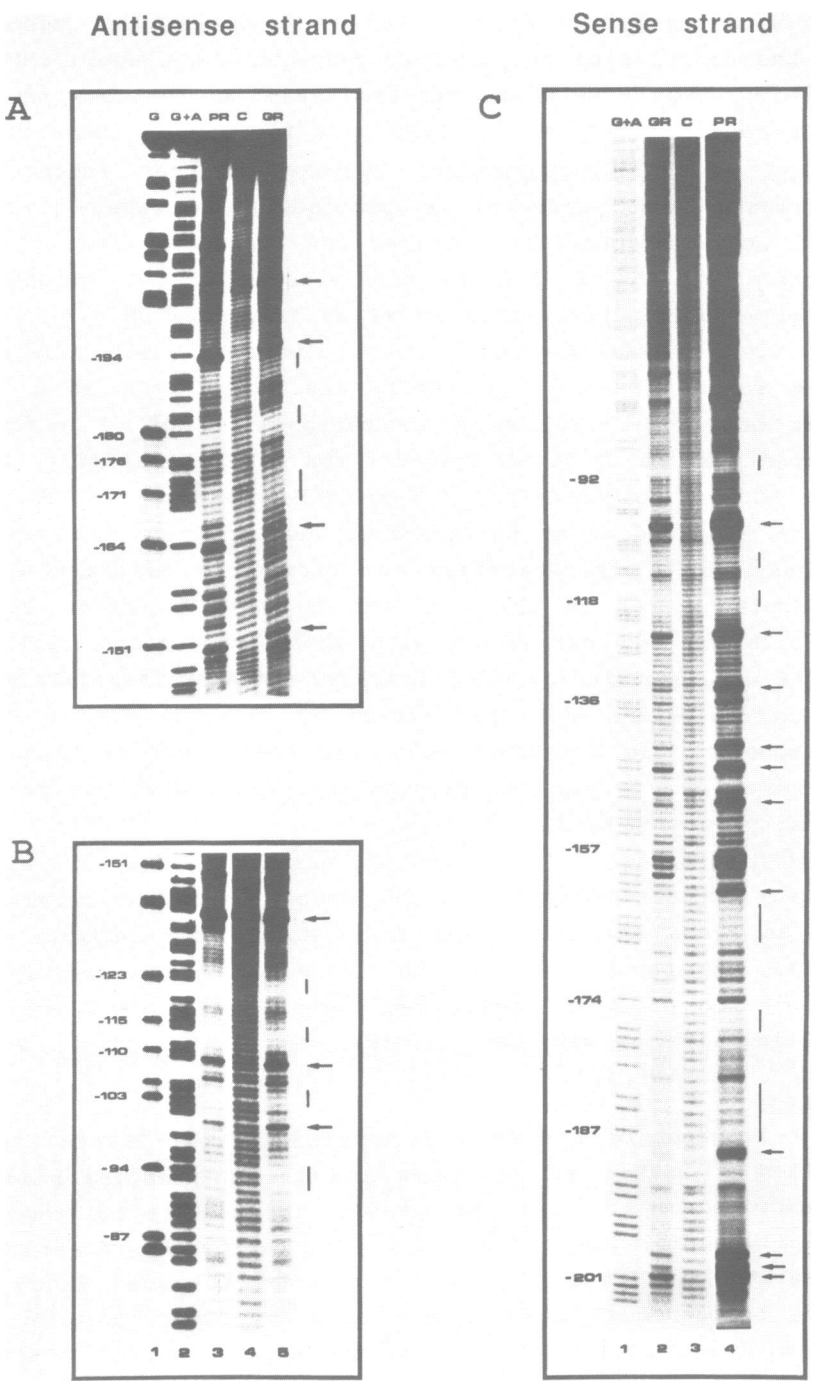
ended with the Klenow fragment of DNA polymerase and subcloned into the HincII site of pUC9 to generate the plasmid pMMTV-wt containing the MMTV-LTR region from -236 to -70. The promoter proximal HRE was near the of EcoRI site in the polylinker.

Hydroxyl radical footprinting. Hydroxyl radical footprinting experiments were performed as previously described (1) with slight modifications. End labelled DNA fragments (2-4 ng) were incubated at 25° C for 40 min with different amounts of partially purified receptors in 12 mM Tris-HCl, pH 7.5, 0.9 mM EDTA, 0.9 mM dithiothreitol, 1.9 % glycerol (v/v), 0.02 mg/ml bovine serum albumin, 1 mM $MgCl_2$ and 80 mM NaCl in a final volume of 160 ul. Following incubation , 40 ul of a freshly prepared mixture of 12 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 80 mM NaCl, 500 ng poly[d(A-T)], 4.5 mM $(NH_4)_2Fe(SO_4)_2$, 9 mM EDTA, 0.55 mM H_2O_2 and 45 mM Na-ascorbate was added and the reaction continued for 1 min. Reactions were stopped by adding 45 ul 1.2 M NaAcetate, 180 mM thiourea and 7 ug tRNA. After ethanol precipitation and extraction with phenol:chloroform (1:1) the samples were analyzed on 6.5 % polyacrylamide-8M urea gels (16). The autoradiograms were quantitated by scanning with a video-densitometer (Bio Rad Model 620), and the extent of protection determined with a computer program that calculates the relative intensity of each band.

Computer graphics. The computer graphics analysis of the receptor contact sites on the DNA double helix was carried out with an Evans and Sutherland Color Multipicture System and a Digital Equipment VAX 11/780 computer. For display and manipulation of B-DNA double helices the UCSF MIDAS molecular modelling software was used (17, 18).

RESULTS.

A fragment of the MMTV-LTR extending from -236 to -70 was labelled at either end and used for the footprinting reactions as described in Material and Methods. The results obtained with partially purified glucocorticoid and progesterone receptors are illustrated in Figure 1. In the absence of added receptor a nearly homogeneous ladder with only a few hypersensitive sites is seen (Fig. 1 lanes C). One of the hypersensitive sites maps


Antisense strand
Sense strand
A
G G+A PR C GR
-194
-180
-176
-171
-164
-151
B
-151
-123
-115
-110
-103
-94
-87
1 2 3 4 5
C
G+A GR C PR
-92
-118
-136
-157
-174
-187
-201
1 2 3 4

to -207 in the lower or antisense strand (Fig. 1A), and around -200 in the upper or sense strand (Fig. 1C). There is also a group of strong cleavage sites at around -150 and isolated strong bands at -134, -121/-122, -115, -111 and further downstream in the upper strand. This deviation from random cleavage may reflect some structural peculiarities of the corresponding DNA region (1).

In the presence of either glucocorticoid or progesterone receptor a complex pattern of protection and enhancement is observed over the whole HRE region from -200 to -76 (Fig. 1). Evaluation of the autoradiogram by high resolution densitometry was used to calculate the relative changes in intensity for each band in the presence of either receptor. A summary of these results is shown in Figure 2 and a computer graphics representation of the contact points is shown in Figure 3. For a description of these changes we will divide the HRE into two blocks that are separated by the a cluster of enhanced cleavage sites between -159 and -134. This seems appropriate since using DNaseI two different footprint regions are found upstream and downstream of these enhanced cleavage region (7, 8).

The promoter proximal region between -133 and -76, shows six sets of contacts point in the lower strand centered at -132/-133, -122/-123, -113, -101/-102, -92/-93 and -82, roughly separated by 10 bp intervals. In the upper strand five sets of contacts are detected showing a similar 10 bp spacing: -118/

Figure 1: Hydroxyl radical footprint analysis of the binding of progesterone and glucocorticoid receptors to MMTV-LTR.
A and B. The plasmid pMMTV-wt was digested with BamHI, 5'labelled to analyze the antisense strand and redigested with HindIII. A long (A) and a short (B) run were performed to have the required resolution. Lanes 1 and 2: guanine and purine specific sequencing reactions respectively; lane 3: 260 ng progesterone receptor; lane 4: control reaction incubated with buffer; lane 5: 400 ng glucocorticoid receptor.
C. The plasmid pLTR-wt was digested with HindIII, labelled in 5' to analyze the sense strand and redigested with BglII. Lane 1: purine specific sequencing reaction; lane 2: 400 ng glucocorticoid receptor; lane 4: control reaction; lane 5: 260 ng progesterone receptor.
The vertical lines indicate the main regions protected by receptor binding and the arrows point to positions hypersensitive in the presence of receptor. Numbers refer to distance from the transcription start point in the MMTV-LTR.

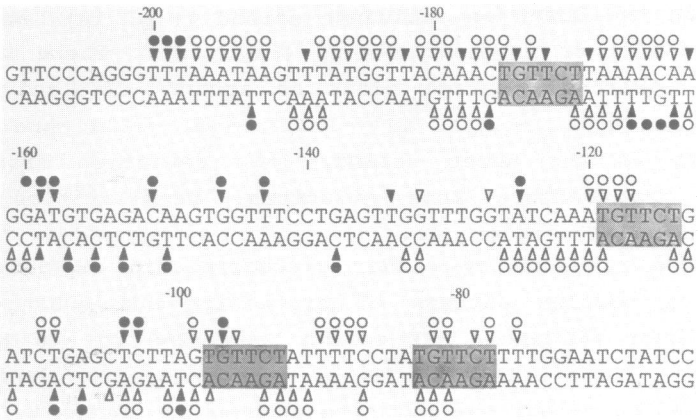


Figure 2: Nucleotide sequence of the promoter region of the MMTV-LTR
Numbers refer to the distance from the transcription start point. Bases whose deoxyriboses are protected by bound progesterone receptor from attack by hydroxyl radical are indicated by open triangles, those protected by glucocorticoid receptor are indicated by open circles. Black symbols indicate the hypersensitive positions respectively. The TGTTCT motives are indicated by shadowed boxes.

-119, -109/-110, -97/-98, -88/-89 and -79. In addition there are two isolated contacts at -127 and -132. These results are compatible with an interaction of the bound receptors with six subsequent turns of the DNA double helix (Fig. 3A). With the exception of the contacts at positions -132, -127, -105, -98, -96 and -79 that are only detected with the progesterone receptor all other forty contacts are also found with the glucocorticoid receptor. In addition there are a series of positions that are preferentially modified in the presence of either receptor (Fig. 2).

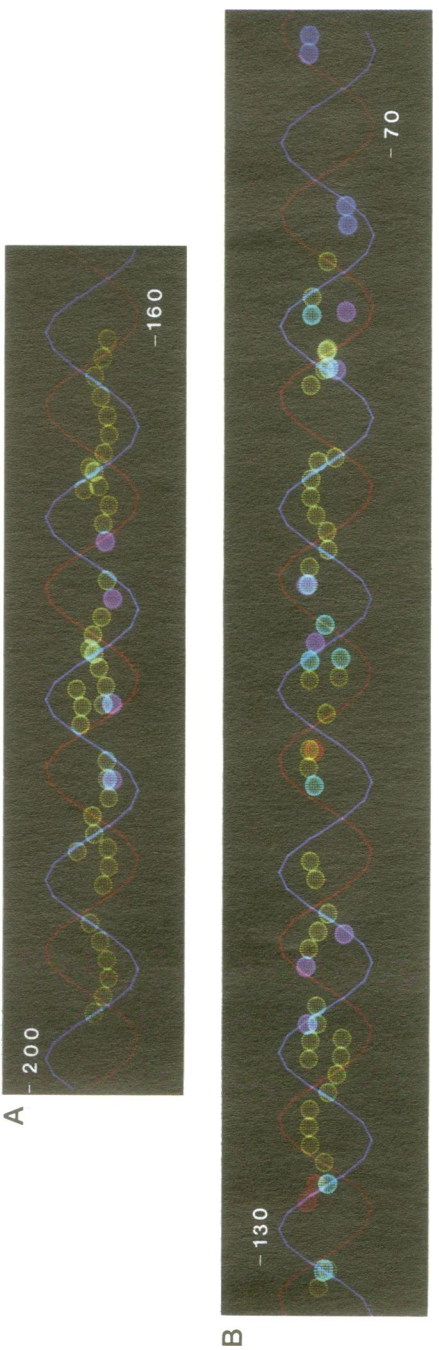
In the promoter distal region a simpler pattern of protection is found. The limits of the protected region are defined by the enhanced cleavage sites at around -200 and -160 (Fig. 2). In this region of about 40 bp there are 39 contact points by either receptor arranged in four blocks of contacts separated by approximatedly 10 bp (Fig. 2). In the upper strand the contacts are centered around: -195, -185, -175 and -165, and in the lower strand around: -189, -178/-179, -168/-169 and -161.

In addition there is a set of three contacts around -180 (upper strand) that does not fit the 10 bp pattern. Some of these enhanced sites in the flanking region are specific for either the glucocorticoid (-160, -164, -165) or the progesterone receptor (-159, -162). As illustrated in the computer graphics model (Fig. 3B) this array of contacts is compatible with four subsequent turns of the double helix being contacted by either receptor predominantly through the major groove.

DISCUSSION

The results obtained with hydroxyl radical footprinting confirm previous findings with DNase I and methylation protection experimets, and precisely define the limits of the HRE in the MMTV-LTR between -200 and -76 upstream of the transcription start point. In addition these data also confirmed that the HRE is composed of two sets of receptor binding sites located at -200 to -160 and -133 to -76. Although the total number of contacts between receptors and DNA is very similar for both regions their detailed array is different in the promoter proximal and distal regions (Fig. 3).

In the promoter distal binding region there are four sets of contacts between the receptors and the DNA each separated by roughly ten base pairs (Fig. 2). Together, these contacts cover four subsequent turns of a B-DNA double helix (Fig. 3B). The two central sets of contacts include the guanines that have been shown to be protected by the receptors in methylation protection experiments with dimethyl sulfate (7, 8), and cover the conserved 15-mer sequences found in other hormone regulated genes (2). The two outer sets of contacts lay in flanking AT-rich sequences, that are not conserved in other receptor binding sites mapped so far (2). However, we have preliminary evidence from experiments with synthetic oligonucleotides suggesting a need for flanking sequences to achieve efficient receptor binding (G. Chalepakis, unpublished). Since there is no specific sequence requirement in these outer DNA regions we postulated that these sequences are involved in base-independent interactions with the DNA double helix, probably of ionic nature.


A
-200
-160
B
-130
-70

To explain these results we propose the model shown in Figure 4. This model is based on the hypothetical formation of two so-called "zinc-fingers" in the DNA binding domain of the hormone receptor. This hypothesis is based on the comparison of the primary amino acid sequences of the hormone receptors that shows a striking conservation of a set of cysteines within the central DNA binding region. The array of some of these cysteines is compatible with the formation of two zinc-fingers similar to those found in other DNA binding proteins (9, 10). Instead of the characteristic Cys/His repeating pattern found in the transcription factor TFIIIA of Xenopus laevis (9) all the coordinates of the zinc atom will be occupied by cysteines in the case of the receptors (for a Review see Ref. 11 and 12). In fact, a recent detailed mutational analysis has made very probable that the cystein residues involved in metal binding by the glucocorticoid receptor are those at positions 440, 443, 457, 460, 476, 482, 492 and 495 (13). As shown in our model, this information can be used to generate two zinc-fingers of different size. Unfortunately attempts to model the receptor zinc-fingers according to the proposed structure of TFIIIA (11,12) have not been successful. Therefore, no details of the polypeptide chain structure are incorporated in our model but rather the zinc-fingers are shown as globular structures. We postulate that Finger 1, which is 13 amino acids large and has few basic residues, does interact specifically with one half of the conserved 15-mer, whereas Finger 2, which is only 9 amino acids long and very basic, would interact electrostatically with the DNA backbone of the helix turns flanking the 15-mer. We

Figure 3: Computer graphics representation of the contact sites between hormone receptors and the DNA double helix.
A. Binding region between -200 and -160.
B. Binding region between -130 and -70.
Only the linked phosphorus atoms of the helix backbone are shown. Blue: sense strand; red: antisense strand. The van der Waals spheres of the deoxyribose C-4 atoms contacted by both receptors are shown in yellow, and those contacted only by the progesterone receptor are shown in green. The guanine N-7 atoms protected against methylation by both receptors are shown in magenta, and those protected only by the progesterone receptor in red (8). Shown in blue are the van der Waals spheres of the guanine N-7 atoms protected by binding of nuclear factor I against methylation by dimethyl sulfate (19)

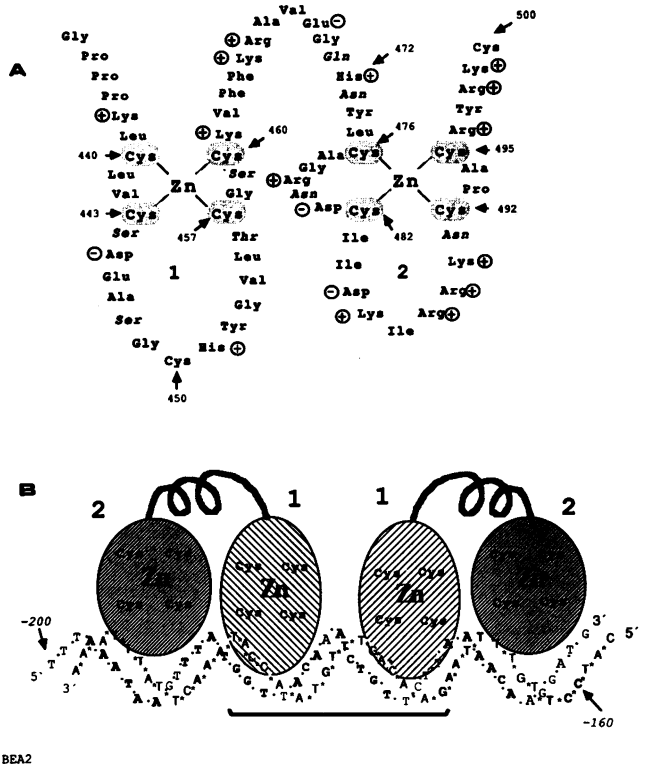


Figure 4: Model of the DNA binding domain of the glucocorticoid receptor.

A) Amino acid sequence of the DNA binding domain of the glucocoricoid receptor with two Zn-atoms coordinated as proposed by Severne et al. (13).

B) Hypothetical model illustrating the interaction of a receptor DNA dimer with the promoter distal binding site of the MMTV-LTR. The two zinc fingers are numbered as shown in A). The conserved 15-mer of the HRE is indicated by the horizontal bracket. Those nucleotide positions protected against hydroxyl radical attack are shown in bold letters.

believe that finger 2 probably does not penetrate the major groove, since binding of the receptor does not protect purines in this region against methylation by dimethyl sulfate and methylation at these purines does not interfere with receptor binding (5). Although, according to this model, sequence recognition would be accomplished exclusively by finger 1, the interaction of finger 2 with DNA will not be irrelevant, as it would contribute significantly to the binding energy. It is also

conceivable that this "division of labor" between the two fingers may facilitate the scanning of long DNA stretches in searching for HREs. For this function the small finger 2 may play a role in guiding the receptor along the DNA double helix.

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