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Artificial steroid hormone response element generated by dam-methylation

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ABSTRACT

Using the interaction of steroid hormone receptors with their palindromic response elements as an example, we show here that cloning in *dam*+ bacterial strains can lead to artifactual results due to methylation of adenine residues at the N-6 position. Substitution of the T by an A in the third position of the half palindromes of the hormone responsive element TGTTCT(1) yields a functional element only when amplification is made in *dam*+ bacteria. Mutant palindromes methylated at the N-6 position of this adenine exhibit the same affinity for progesterone and glucocorticoid receptors as the consensus response element, whereas their unmethylated counterpart binds with negligible affinity. These observations underline the significance of hydrophobic interactions between receptors and the major groove of the DNA for discrimination among various responsive elements, and point to the importance of using *dam*- bacterial strains for the correct identification of the nucleotide sequence of *cis*-acting elements.

INTRODUCTION

Gene expression is regulated primarily through the interaction of proteins with *cis*-acting elements within enhancer or promoter regions. Definition of the precise nucleotide sequence of such elements is usually achieved in transfection experiments and DNA binding studies. For functional studies, short pieces of the relevant DNA are cloned in bacterial plasmids and inserted upstream of standard reporter genes. These constructions are then transiently transfected into appropriate cell lines and the influence of the sequences in question on the expression of the reporter gene is determined. As a control, mutated elements are also tested in the same assay and help to define the relevant nucleotides. In parallel experiments, DNA fragments containing the regulatory sequences or mutated versions thereof are used for DNA binding studies. These findings are then correlated with the results of gene transfer experiments. In both types of experiments the eukaryotic DNA is usually amplified in laboratory strains of *E. coli*, most of which contain the two site-specific DNA methylases **Dam** and **Dcm** that respectively methylate the N^6 position of adenine residues in GATC and the C^5 position of the internal cytosine residues in CCAGG and CCTGG (2, 3).

This standard strategy has been used to define he nucleotide sequences responsible for induction of gene activity by steroid hormones, that have been called hormone responsive elements, HRE. The consensus element for glucocorticoid or progesterone induction (GRE/PRE) has been shown to be an imperfect palindromic structure with the half side sequence TGTYCT (1). In single base mutation experiments we observed that a palindrome with the half side sequence TGATCT was able to bind the hormone receptors and to confer responsiveness to glucocorticoids and progesterone in gene transfer experiments (4). These findings were unexpected as the T at position 3 of the half palindrome has been shown to be conserved among various GRE/PREs, and its methyl group is known to be directly contacted by the receptors (5). One possible explanation for our observation could have been an alternative contact of the receptor with the 5'-methyl group of the thymine on the opposite strand that is base paired with the adenine at position 3 of the mutant palindrome. However, no indication for this type of contact could be obtained in interference studies with $KMnO_4$ (5).

Another possible explanation for our findings could be a methylation of the A at position 3 of the mutant GRE/PRE by a ***dam***-methylase during the cloning of the corresponding sequences in ***dam***+ bacterial strains. This is possible since the central four bases of the mutant half palindrome form the consensus of bacterial ***dam***-methylase, namely GATC. To test this hypothesis we performed gene transfer and DNA binding experiments with DNA sequences cloned in ***dam***- bacterial strains. The results summarized in this paper clearly show that for receptor binding and functional activity as a GRE/PRE the mutant palindrome needs to be methylated at the N-6 position of the A. These observations underline the significance of hydrophobic interactions among receptors and the major groove of the HRE, and point to the importance of using ***dam***- bacterial strains for the correct identification of the nucleotide sequence of *cis*-acting elements.

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MATERIALS AND METHODS

Receptors

Rabbit-PR was isolated from uterine cytosol of rabbits treated with estrogen for 1 week and purified as described (6). Estrogen receptor and estrogen receptor mutants were expressed by transient transfection of COS-7 cells as previously described (4).

Plasmids and DNA fragments

Plasmids used in these experiments contain two copies of synthetic oligonucleotides (5'AGCTTAGTTTATTGGGTCACAGTGACCTTACCACAAGGATGG-3') named TGACCT, (5'AGCTTAGTTTATTGGGACACAGTGTCCTTACCACAAGGATGG-3') named TGTCCT and (5'AGCTTAGTTTATTGGATCACAGTGATCTTACCACAAGGATGG-3') named TGATCT, all cloned in pTK/CAT.3 (4). The plasmids were prepared from ***dam***$^+$ (HB101) or ***dam***$^-$ bacteria (GM 48) (7,8).

DNA-fragments used for bandshift and methylation analysis were obtained from these plasmids by digestion with *HindIII* that excised a 45 bp fragment including one of these two copies. After 3'endlabelling this fragments have been used for DNA binding experiments and restriction digests.

Methylation analysis

Radioactive labelled DNA-fragments were incubated with 10 U of the restriction endonuclease *MboI* (Boehringer Inc.) for 2 h. This enzyme exclusively recognizes the unmethylated sequence GATC (9). The reaction products were analyzed in a 10% polyacrylamide gel.

DNA binding assays

Bandshift experiments have been performed as described (4). Purified PR and whole cell extract (WCE) from COS-7 cells was incubated 15 min at room temperature in a 10- to 20- ml reaction mixture containing 15 mM Tris (pH 7.5), 10% glycerol, 100 mM NaCl, 1 mM dithiothreitol (DTT), 3 μg/ml bovine serum albumin, 0.1 ng labelled DNA and 1 μg poly (dIdC). Free DNA and DNA-protein complexes were resolved on 4% polyacrylamide gels.

Gene transfer experiments

T47D cells were grown in RPMI medium (GIBCO) supplemented with 5% fetal calf serum. Reporter plasmids and a β-galactosidase expression vector pRSV-βGal (10) were cotransfected by the DEAE-dextran method (11). When indicated, cells were treated with 10 nM R5020 for 48 h. CAT-assays were performed essentially as described (12).

RESULTS AND DISCUSSION

Expression of a reporter gene carrying the consensus response element for glucocorticoids or progestins (GRE/PRE) with the half palindromic sequence TGTTCT is induced by synthetic progestins after transfection in the human mammary carcinoma cell line T47D (4, 5) (Figure 1 B). Substitution of the **T** at the third position by an **A** does not alter the hormonal inducibility when the fourth position is a T and the plasmid used for transfection is derived from the widely used ***dam***$^+$ *E. coli* strain HB101 (4) (Figure 1B). However, when the ***dam***$^-$ bacterial strain GM48 (8) is used instead, this mutation of the GRE/PRE completely abolishes hormonal response (Figure 1B). This effect is not limited to progestin induction, but is also observed in cotransfection experiments with the glucocorticoid or the mineralocorticoid receptors (not shown).

In DNA binding experiments we detect efficient binding of the purified progesterone receptor to the mutated GRE/PRE when the labelled oligonucleotide is derived from plasmids amplified in HB101, but binding is drastically reduced when the plasmids were grown in GM48 (Figure 1C lanes 1 to 6). Binding is highly selective, as a single additional exchange (**T** to **C**) at the fourth position, that is tolerated in the context of the consensus TGTYC-

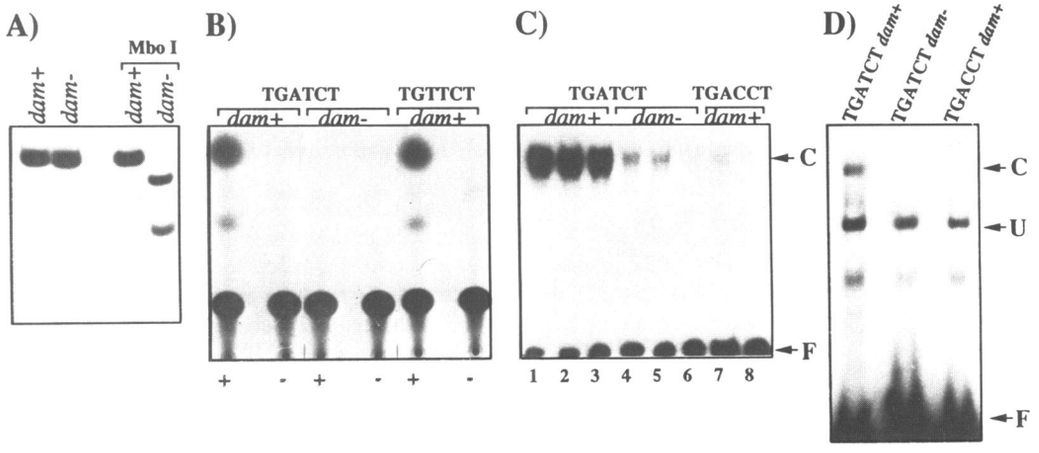

Figure 1. Effect of dam-methylation on steroid hormone induction and receptor binding. **A)** Analysis of the methylation state of DNA prepared from the *E. coli* strains HB101 (***dam***$^+$) or GM48 (***dam***$^-$). The radioactive fragments (1 ng) were incubated with 10 U of the the restriction enzyme *MboI* that exclusively recognizes the unmethylated sequence GATC and the products analyzed on a 10% polyacrylamide gel. **B)** Comparison of the ability of plasmids prepared from HB101 (***dam***$^+$) or GM48 (***dam***$^-$) to mediate induction by synthetic progestins in T47D-cells. Cells were transfected with 2μg of the indicated plasmids and incubated with (+) or without (−) R5020 for 48h. **C)** Comparison of progesterone receptor binding to dam-methylated or unmethylated DNA fragments. Purified receptor (lanes 1,4 and 7: 16 ng; lanes 2, 5 and 8: 8 ng and lanes 3 and 6: 4 ng) was incubated with TGATCT fragments methylated (lane 1−3), nonmethylated (lanes 4−6) and with TGACCT (lanes 7,8). C indicates the position of specific complexes between PR and DNA, F marks the position of the free DNA. **D)** Binding of the estrogen receptor double mutant HE91 to dam-methylated or unmethylated DNA-fragments. C indicates the complex formed by ER mutant HE91, U indicates an complex caused by an unspecific DNA binding protein and F the position of the unbound DNA.

T (4) but destroys the recognition site of the dam-methylase, completely abolishes binding (Figure 1C, lanes 7 and 8).

We interpret these findings as a consequence of methylation at the N-6 position of adenines by the dam-methylase in ***dam***$^+$ bacteria. That this type of methylation is actually taking place is demonstrated by the results of a restriction digestion with the methylation sensitive nuclease *MboI* (Figure 1 A). Only the plasmid obtained from the ***dam***$^-$ *E. coli* strain GM48 is digested at the relevant **TGATCT** site, whereas DNA obtained from HB101 is not. Therefore, the presence of an additional methyl group in the A at position three of the half palindrome seems sufficient to enable a strong specific interaction of the progesterone or glucocorticoid receptor with the major groove. The significance of hydrophobic interactions between glucocorticoid and progesterone receptors and position three of the HRE has been documented in binding studies with oligonucleotides containing either deoxyuridine or bromodeoxyuridine in place of thymine (5). The results of X-ray structural analysis of rat glucocorticoid receptor/DNA complexes demonstrate that Val-482, adjacent to the fourth cysteine of the first zinc finger is involved in this interaction (13). Our present results suggest that the 5′-methyl group of thymine can be partly replaced by a methyl at position N-6 of adenine. Methylated adenine can have two different configurations in which the amino group is coplanar with the purine ring and the methyl substituent is either directed toward or away from the imidazol ring of the base (14). In both configurations the position of this methyl group differs significantly from that of the thymine methyl group in the major groove. In the energetically unfavorable *trans* configuration (Fig 2), in which the adenines can base pair with thymines (9) this difference is smaller than in the cis conformation. The Valine-482 has two methyl groups, one of them interacting with the methyl group of thymine. In the crystal structure (13), the other methyl group is oriented in a way compatible with its hydrophobic interaction with the methyl group of N6-Me-adenine even in the energetically favorable cis configuration.

Dam-methylation seems to decrease the melting temperature of DNA, indicating a destabilization of the DNA-double helix. However, this destabilization does not result in significant changes of the conformation of the helix (15). Therefore, structural effects of Dam methylation on receptor binding are unlikely but can not be fully excluded.

Three amino acids at the knuckle of the first zinc finger in the DNA binding domain of the hormone receptors, in the case of rat glucocorticoid receptor including Val-482, are essential for the specificity of base sequence recognition and for discrimination among different hormone response elements (13, 16, 17, 18). To investigate the role of these amino acid residues in the interaction with the mutant GRE/PRE, we made use of estrogen receptor mutants carrying single and double amino acid exchanges in this region. In each case the mutant receptors have at the indicated positions the amino acid found in the glucocorticoid receptor (16). As previously reported, the wild type estrogen receptor does not recognize the mutant GRE/PRE, regardless whether the element is derived from ***dam***$^+$ (4) or ***dam***$^-$ bacterial strains (data not shown). A single amino acid exchange (**E** to **G**) at position 203 (corresponding to position 478 of the rat glucocorticoid receptor) generates a mutated receptor, HE84, that recognizes both the methylated (4) and the unmethylated TGATCT element, as well as the classical estrogen response element (ERE) with the half palindromic sequence TGACCT (not shown). An additional exchange (**A** to **V**) at position 207 (corresponding to position 482 of rat GR) yields a mutant receptor, HE91, that behaves qualitatively like the wild type glucocorticoid or progesterone receptors, interacting only with the methylated TGATCT element but not with the unmethylated variant nor with the consensus ERE (Figure 1 D).

Our observations may be relevant in conjunction with previous reports on the differences in nucleotide sequence among the regulatory elements for different steroid hormones (4, 13, 19). Indeed the main difference in the nucleotide sequence between GRE/PRE and ERE resides at the third position of the half palindrome. In the canonical GRE/PRE there is always a T, whereas an ERE can accommodate any base but a T at this position (4), suggesting that the 5′-methyl group of thymine

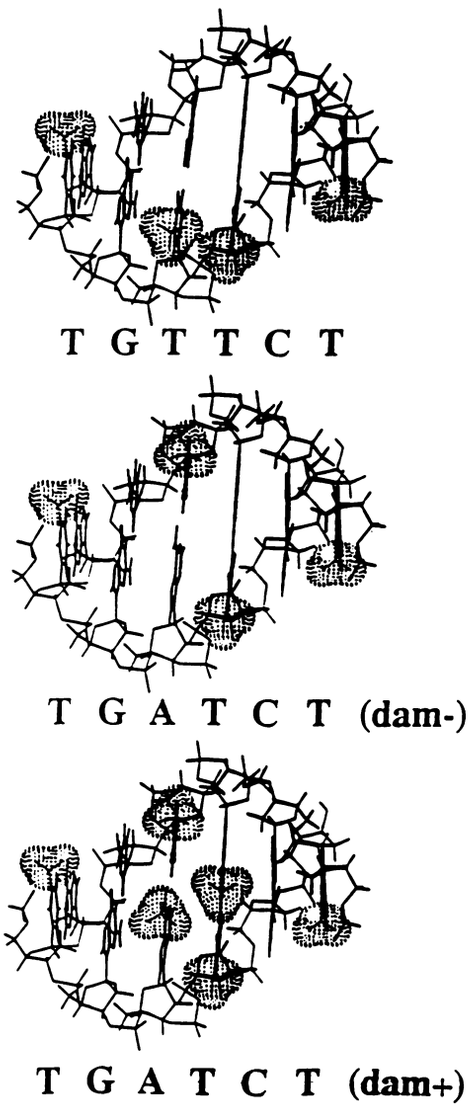

Figure 2. Representation of the distribution of methyl groups in the the major groove of the DNA hexanucleotides TGTTCT, TGATCT and the dam-methylated TGATCT hexanucleotide. The van der Waals spheres of the 5′methyl groups of thymines and the N7 methyl groups of dam-methylated adenosines are indicated as dotted surfaces. The methylated adenines are shown in the energetically unfavorable *trans* configuration, in which they can base pair with thymines (9).

generates a steric hindrance that prevents binding of the estrogen receptor. This idea is supported by the observation that elimination of the bulky side chain of glutamate at position 203 of the estrogen receptor, between the third and fourth cysteines of the first zinc finger (corresponding to position 478 of GR), is sufficient to allow binding to the dam-methylated TGATCT as well as to the canonical GRE/PRE (4 and data not shown).

It has been claimed that the regulatory elements mediating repression by glucocorticoid hormones differ from the positive GRE, in that the third position of the half palindrome is not a T but an A (1, 20). Since ***dam***$^+$ bacteria have often been used to clone and study these negative elements it is likely that the affinity of the receptors for these sites has been over-estimated due to dam-methylation. More generally, our results imply that the characterization of *cis*-elements that contain the recognition sequence of the dam-methylase, GATC (2) and possibly the dcm-methylase (3), GGWCC should be critically reevaluated taking into account the bacterial strains used for plasmid amplification. Because of the known disadvantages of ***dam***$^-$ strains (high recombination frequency, increased levels of spontaneous mutations) (2) they have not been widely used. However in view of our findings one should reconsider their use for the characterization of *cis*-elements.

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REFERENCES

1. Beato, M (1989)*Cell* , **56**, 335–344.
2. Geier, G. E. and Modrich, P. (1979) *J. Biol. Chem.*, **254**, 1408–1413.
3. Marinus, M. G. and Morris, N. R. (1973) J. Bacteriol., **114**, 1143–1150.
4. Truss, M., Chalepakis, G., Slater, E. S., Mader, S. and Beato, M.(1991) *Mol. Cell. Biol.*, **11**, 3247–3258.
5. Truss, M., Chalepakis, G. and Beato, M.(1990) *Proc. Natl. Acad. Sci. USA*, **87**, 7180–7184
6. von der Ahe, D., Janich, S., Scheidereit, C., Renkawitz, R., Schütz, G., und Beato, M. (1985) *Nature*, **313**, 706–709.
7. Boyer, H. W. and Roulland-Dussoix, D. (1969) *J. Mol. Biol.*, **41**, 459–472.
8. Yanish-Perron, C., Viera, J. & Messing, J. (1985) *Gene*, **33**, 103–109.
9. Barras, F. and Marinus M. G. (1989) *TIG*, **5**, 139–142.
10. Hall, C.V., Jacob, P.E., Ringold, G.M., and Lee, F. (1983) *J. Mol. Appl. Genet.*, **2**, 101–110.
11. Cato, A.C.B., Miksicek, R., Schütz, G., Arnemann, J., and Beato, M. (1986) *EMBO J.*, **5**, 2237–2240.
12. Gorman, C.M., Moffat, L.F., and Howard, B. H. (1982) *Mol. Cell. Biol.*, **2**, 1044–1051.
13. Luisi, B.F., Xu, W. X., Otwinowski, Z., Freedman, L. P., Yamamoto, K. R. and Sigler, P. B. (1991) *Nature*, **352**, 497–505.
14. Sternglanz, H. und Bugg, C. E. (1973) *Science*, **182**, 833–834.
15. Fazakerley, G. V., Teoule, R., Guy, A., Fritzsche und Guschlbauer, W. (1985) *Biochemistry*, **24**, 4540–4548.
16. Mader, S., Kumar, V., Verneuil, H. and Chambon, P. (1989) *Nature*, **338**, 271–274.
17. Umesono, K. and Evans, R.M. (1989) *Cell*, **57**, 1139–1146.
18. Danielsen, M., Hinck, L. & Ringold, G.M.. *Cell* (1989), **57**, 1131–1138.
19. Klock, G., Strähle, U. and Schütz, G. (1987) *Nature*, **329**, 734–736.
20. Akerblom, I.E. & Mellon, P. (1991) in: 'Nuclear Hormone Receptors' (ed, M.G. Parker) 175–196, Academic Press.