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INTERPLAY OF STEROID HORMONE RECEPTORS AND TRANSCRIPTION FACTORS ON THE MOUSE MAMMARY TUMOR VIRUS PROMOTER

M TRUSS, G CHALEPAKIS* and M BEATO†
Institut fur Molekularbiologie und Tumorforschung, Emil-Mannkopff-Str 2, 3550 Marburg, Germany

Summary—The mouse mammary tumor virus (MMTV) promoter, that responds to glucocorticoids and progestins, contains a complex hormone response element (HRE) in the long terminal repeat (LTR) region covered by a phased nucleosome Hormone treatment leads to alterations in chromatin structure that make the HRE region more accessible to digestion by DNase I and permit binding of transcription factors, including nuclear factor I (NFI), immediately downstream of the HRE NFI acts as a basal transcription factor on the MMTV promoter *in vitro* but competes with the hormone receptors in terms of binding to free DNA In uninduced chromatin, the precise positioning of the DNA double helix on the surface of the histone octamer precludes binding of NFI to its cognate sequence while still allowing recognition of the HRE by the hormone receptors We postulate that receptor binding to the nucleosomally organized MMTV promoter disrupts the chromatin structure enabling NFI binding and subsequent formation of a stable transcription complex Whether the receptor remains bound to DNA during induction or is displaced by NFI is not conclusively known, but our evidence supports a "hit and run" mechanism

NFI is not the only factor involved in hormonally induced transcription of the MMTV promoter Two degenerated octamer motifs located immediately upstream of the TATA box are recognized by the ubiquitous transcription factor OTF-1 (Oct-1, NFIII), and are also important *In vitro*, mutations in these motifs do not influence basal transcription, but completely abolish the stimulatory effect of purified progesterone receptor Progesterone receptor bound to the HRE facilitates binding of OTF-1 to the two octamer motifs Thus, OTF-1 is a natural mediator of progesterone induction of the MMTV promoter and acts through cooperation with the hormone receptor for binding to DNA

INTRODUCTION

The proviral genome of mouse mammary tumor virus (MMTV) is transcribed from a promoter region located in the long terminal repeat (LTR) region, that arises as a consequence of the mechanism of reverse transcription (Fig 1) The LTR region of MMTV is longer than that of most other retroviral proviruses and exhibits an open reading frame with the capacity to encode a 36 kDa polypeptide, as well as the elements responsible for tissue-specific expression and hormonal regulation (for a recent review see [1]) Induction of MMTV transcription by glucocorticoids has been a classical system to study the mechanism of action of steroid hormones [2] Treatment with glucocorticoids of tissue explants or cells in culture derived from primary mouse mammary tumors leads to an accumulation of MMTV particles and MMTV-RNA [3–6] After the demonstration that hormone administration enhances the rate of transcription of MMTV-DNA [7–9], attempts were directed to elucidate the molecular mechanism of this process Utilizing gene transfer methods, several groups succeeded in localizing the nucleotide sequences relevant for mediating transcriptional activation to the LTR region of the proviral genome [10–13] Deletion analysis allowed to delimit the responsible sequences of the LTR to the region between −200 and −50 upstream of the transcription start point [14–16]. The partially purified glucocorticoid receptor binds to a region overlapping the functionally relevant sequences [17–21] In DNase I protection experiments, four areas sharing the hexanucleotide motif TGTTCT were identified in the hormone responsive region of the MMTV-LTR in the GR mice strain [21] Later it was shown that mutation

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*Present address Max-Planck-Institut fur Biophysikalische Chemie, Nikolausberg, D-3400 Gottingen, Germany
†To whom correspondence should be addressed

of any of these hexanucleotide motifs has a dramatic influence on hormonal induction [22] Thus, these experiments demonstrated the existence of a complex array of binding sites for the hormone receptors on the MMTV-LTR that mediates hormonal induction of the provirus In addition, this element confers hormone inducibility to a heterologous promoter This was the first demonstration of the existence of hormone response elements (HRE) as a defined nucleotide sequence in the vicinity of regulated promoters

Though originally MMTV induction was used as a classical example of glucocorticoid regulation, the MMTV promoter has been shown to respond to progestins [23] as well as to mineralocorticoids [24, 25] and to androgens [26, 27] Induction by progesterone may be physiologically relevant [28], as expression of MMTV in the mammary gland correlates with high levels of progesterone, and mammary tumors originate only after pregnancy Binding experiments with purified progesterone receptor (PR) from rabbit uterus demonstrated that the region protected against DNase I is very similar, but not identical, to that covered by the glucocorticoid receptor (GR) [22, 29] Similar experiments have not been performed with the receptors for the other steroid hormones, but mutational analysis indicates a similar relevance of the individual TGTTCT motifs for response to the various other hormones [30] The fact that the different steroid hormone receptors bind to the same regulatory elements was unexpected, and raises the question of how hormone specificity is achieved *in vivo* While induction by different steroid hormones is observed in many systems, including MMTV, in other systems, only one hormone is able to induce a particular gene even if the cells are equipped with other hormone receptors able to interact with the regulatory elements Therefore, either quantitative differences in affinity of the different receptors for particular HREs are functionally important or other mechanisms (additional factors) mediate the specificity of the hormonal response

In more general terms, the regulation of gene activity by steroid hormones can be affected by signals and factors acting through different transducing pathways The induction of MMTV expression by glucocorticoids can be inhibited by activation of endogenous Fos or Jun or by co-transfection of the appropriate expression vectors [31–35] This effect could explain the observed negative influence of various oncogenes, including *v-mos* and *H-ras*, on the regulation of MMTV-LTR by glucocorticoids [36, 37] The reverse is also true, in that glucocorticoid treatment can inhibit induction of promoters carrying an AP1 binding site [31] Transrepression of the *c-fos* promoter by Fos is also inhibited by glucocorticoids bound to their receptor The DNA binding domain of the receptor and the N-terminal region of Fos are required for reciprocal transrepression [31] It is interesting to note, that the relevant region between amino acids 40 and 111 of Fos has not been assigned a function yet This region is not conserved in FosB, and FosB is not capable of transrepressing the GR mediated activation of transcription [31] Although no evidence for complex formation between Fos and the GR has been produced, a direct interaction between Jun and the GR has been detected in cells overexpressing both proteins [32, 33, 35], suggesting a mechanism for regulation independent of direct DNA binding of the AP1 complex These interactions do not necessarily lead to mutual inhibition of transcription Depending on the cell type and the promoter used one can observe functionally different interactions that also include synergistic effects [38, 39] These observations may represent an example for a rather general mechanism of cross-talk among different signal transduction pathways that suggest a new degree of complexity in combinatorial gene regulation

MATERIALS AND METHODS

Receptors and DNA fragments

Rabbit PR and rat GR were prepared as described previously [19, 29]

DNA-fragments used for band shift and interference experiments were obtained by restriction digestion of plasmids containing the linker scanning mutant LS-155 that generates a *Sal* I restriction site between the promoter proximal cluster and the promoter distal site of the MMTV promoter A double mutation containing this restriction site and an additional mutation of binding site 3 (LS-98), or two direct repeats of the promoter distal HRE of the MMTV promoter all cloned into pTKCAT 3 [22, 40] were also used

Interference experiments

$KMnO_4$ interference and methylation interference with PR and GR have been performed

as described [40, 41] The *Sal* I/*Hind* III fragment containing the three promoter proximal binding sites of the MMTV promoter was 5'end-labeled with T4 kinase or 3'end-labeled with klenow For methylation interference, these fragments were treated with dimethyl sulfate for 3 min as described by Maxam and Gilbert [42] For $KMnO_4$ interference experiments these fragments were first dissolved in 5 μl of Tris (30 mM, pH 8), denatured by heating to 95°C for 2 min, and modified by adding 20 μl of a $2\,5 \times 10^{-4}$ M $KMnO_4$ solution After 10 min at 20°C, the reactions were stopped with 225 μl of 0 22 M β-mercaptoethanol, 0 33 M sodium acetate (pH 7 0) and 750 μl of ethanol After one reprecipitation the DNA was dissolved in 10 μl of 10 mM Tris (pH 8), 1 mM EDTA, 30 mM NaCl, heated to 60°C for 5 min and hybridized by slowly cooling to room temperature The premodified DNA-fragments were added to preparative (50 μl) binding reactions and subsequently electrophoresed through a 4% polyacrylamide gel DNA was electroblotted onto Whatman DE 81 paper in 0 5 × Tris–borate–EDTA (TBE) buffer at 100 mA at 18 V overnight in a Bio-Rad Trans-Blot System Bands were localized by autoradiography of the wet DE 81 paper, excised and the DNA eluted in 300 to 500 μl in TE with 1 5 M NaCl for 2 h at 65°C After chloroform–isomalyalcohol extraction and precipitation the DNA was cleaved with piperidine and subsequently electrophoresed on 15% acrylamide sequence gels

DNA binding assays

DNA binding experiments have been performed as described [41] Purified PR or GR were incubated for 15 min at room temperature in a 10- to 50-μl assay containing 15 mM Tris (pH 7 5), 10% glycerol, 100 mM NaCl, 1 mM dithiothreitol (DTT), 3 mg/ml bovine serum albumin, 0 1 ng labeled DNA-fragment and 1 μg poly (dIdC) Free DNA and DNA–protein complexes were resolved on 4% polyacrylamide gels

RESULTS AND DISCUSSION

Structural and functional analysis of the HREs

The amino acid sequence analysis of members of the nuclear receptor superfamily suggests that they can be divided into two subfamilies according to the structure of their DNA binding domains [43] One subfamily includes the receptors for glucocorticoids, progestins, androgens and mineralocorticoids, whereas the other includes, among others, the receptors for estrogens, ecdysterone, thyroid hormones and retinoic acid A comparison of the HREs described in many genes reveals a consensus nucleotide sequence for the glucocorticoid/progesterone responsive element (GRE/PRE) [44] This sequence is composed of 15 bp organized as an imperfect palindrome with 2 unequally conserved half sides separated by 3 non-conserved nucleotides [45–47] Each half palindrome exhibits the general structure TGTYCT but only one half of the palindrome, usually the one closer to the promoter, is perfectly conserved In similar studies, the consensus sequence for estrogen induction (ERE) has been identified and found again to be a palindrome with 2 conserved halves separated by 3 non-conserved nucleotides The general structure of the ERE half side is TGACC [45–48] The main difference among various members of this subfamily resides in the optimal distance between the 2 half palindromes Whereas in the ERE the distance is 3 bp as in the GRE/PRE, in the TRE optimal response is observed with no spacing between the 2 half palindromes An exception seems to be the retinoic acid regulatory element Here optimal response is observed with a direct repeat rather than with a palindromic element [49–51]

Efficient receptor binding to the HRE does not only require a palindromic HRE, but is also dependent on the length of the flanking DNA sequences In experiments with oligonucleotides of different lengths containing the HRE either in a central position or at the borders, a clear dependence of the receptor binding affinity on the length of the sequences flanking the HRE is found [52] Optimal binding requires at least 8 bp on each side of the HRE, but the nature of the sequence appears to be irrelevant, suggesting that no specific interaction with the nucleotides takes place in the flanking region This result is in agreement with the lack of conservation of the region flanking the HRE [44] Even in the absence of base-specific binding, the interaction with the sequences flanking the HRE could have important functional consequences, in cases when these flanking sequences encompass binding sites for other transcription factors

Discrimination between various HREs

The nucleotide sequence recognized by the GR subfamily contains the half palindrome

TGTYCT, whereas the ER subfamily interacts with the half palindrome TGACCN We have recently found that the sixth nucleotide of the conserved half palindrome of the GRE/PRE, usually a T, can be substituted by a C without affecting binding or transactivation [40] Therefore, the two classes of HREs share several conserved positions including the T at position 1, the G at position 2, and the C at position 5 The main differences between the two types of HREs reside in positions 3 and 4 In position 3, a T is always found in GREs/PREs, and an A is the most common nucleotide in EREs, although a C or a G are also tolerated [53–55] Thus, the main difference in this position resides in the prohibitory nature of a T for a productive interaction with the estrogen receptor As for the fourth position in the half palindrome, it can be a T or a C in GRE/PREs, but is always a C in EREs Using a newly developed technique to analyze contacts between hormone receptors and methyl groups of thymines on DNA [40, 41], we have demonstrated that the T at the first position of the half palindrome is contacted through its methyl group in GRE/PRE as well as in ERE In GRE/PRE there is another contact of the hormone receptor with the T in the third position of the half palindrome, whereas the T opposite to the A in this position of the ERE is not contacted by the estrogen receptor These data suggest a model according to which the DNA binding domain of the hormone receptors will interact with the major groove of the half palindrome that can accommodate an α-helical structure providing hydrogen bonding to several of the base pairs and also hydrophobic interactions with the methyl group of these two conserved Ts

The subtle differences in the recognition mechanism for the two subclasses of steroid hormone receptors reflect differences in the amino acid sequence of the DNA binding domains of these two subfamilies of receptors [43] Three essential amino acids in the knuckle of the first zinc finger of the DNA binding domain [56–58] are important for discrimination between an ERE and a GRE/PRE In addition, amino acids in the knuckle of the second zinc finger also probably play an important role in generating the appropriate positioning of the DNA recognition helix [58], possibly by specifying dimerization

Our molecular understanding of the interactions between different amino acid side chains and the relevant base pairs of the HRE will improve when the low resolution structural data, based on 2D-NMR analysis and crystal structure of the DNA binding domain [59–61] are refined The available data clearly demonstrate that the DNA binding domains of the GR and the estrogen receptors bind to their respective HREs as homodimers in head to heat orientation, confirming previous biochemical results with the intact receptors [62–66] Apart from the dimerization function located in the DNA binding domain, there is another hormone-dependent dimerization interface in the C-terminal half of the steroid hormone receptors Recently this function has been delimited to a 23 amino acid sequence that is distinct from the steroid binding region [67]

Interaction of GR and PR with the MMTV-HRE

Receptor homodimers can also cooperate when binding to adjacent HREs [68–74] In the MMTV-HRE there is a functional interaction between the promoter distal strong palindromic site, and a downstream block of receptor binding sites composed of three incomplete palindromes (Fig 1), as demonstrated by deletion mutants and by the influence of inserting oligonucleotides of different length between the two blocks [22] These effects are dependent on the topology of the transfected DNA, suggesting that negatively supercoiled DNA favors the interaction between receptor molecules bound to each block [75] Though the exact stoichiometry of receptor binding to the promoter proximal block is not completely clear [76], there are indications for a strong functional cooperativity between the individual sites in this region [22, 76] Mutation of any of the three TGTTCT motifs has a strong inhibitory effect on receptor binding, though this effect is less dramatic with the most proximal site

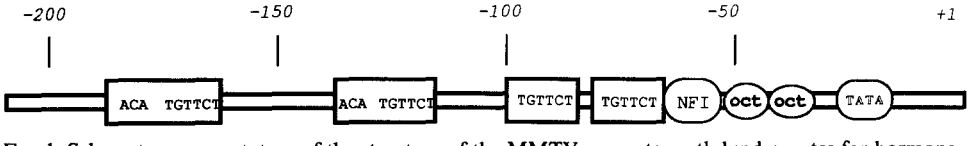

Fig 1 Schematic representation of the structure of the MMTV promoter with binding sites for hormone receptors, NFI, OTF-1 and TATA box

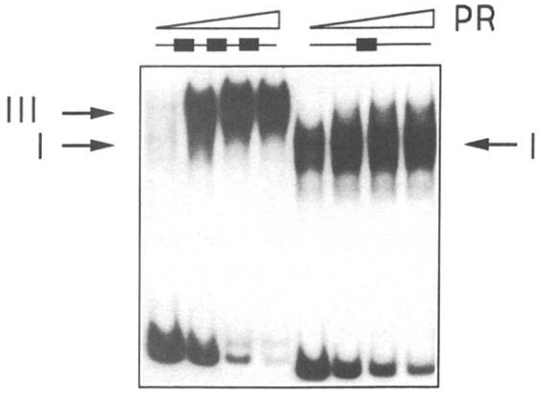

Fig 2 Binding of the PR to the promoter proximal cluster of binding sites compared to the strong promoter distal site Radioactive labeled DNA-fragments containing either the promoter distal HRE or the three promoter proximal HREs of the MMTV promoter were incubated with 1, 2, 4 or 8 ng PR Receptor binding was assayed by electrophoresis on a 4% native polyacrylamide gel Arrows indicate positions of receptor/DNA-complexes, the number of boxes in the schematic drawing above the lanes corresponds to the number of HREs of the DNA-fragments

To analyze in more detail the interaction of receptor molecules with the promoter proximal cluster of binding sites on the MMTV promoter we have performed band retardation studies and interference experiments A comparison of the retarded complexes formed with the PR and DNA fragments of similar length containing either the group of promoter proximal sites or the strong promoter distal site is shown in Fig 2 At the lowest receptor concentration, a single complex is detected with the promoter distal DNA fragment whereas two complexes, labeled I and III, are seen with the promoter proximal DNA fragment The relative intensity of these two complexes is comparable At twice the receptor concentration, the total amount of retarded complexes increases dramatically mostly due to an increment of complex III Complex I does not increase with receptor concentration and is even less abundant at the highest concentration used This behavior suggests a strongly cooperative binding of PR to the 3 sites on the promoter proximal cluster of the MMTV, with the faster complex representing single occupation of a site and the slower complex full occupation of the 3 sites.

The low resolution of the gel retardation experiments does not allow to estimate the number of receptor molecules bound to the promoter proximal group of receptor binding sites To address this question we have tried different binding interference techniques, using DNA-fragments modified with either dimethyl sulfoxide or potassium permanganate [40, 77, 78] One example of the results obtained with the upper strand is shown in Fig 3 Confirming previous results with other binding sites [62, 79], methylation of the G at the second position of the TGTTCT half palindrome interferes with binding of the PR [Fig 3(A), compares lanes 1 and 2] Similarly, modification of the T at the first or the third position of the half palindromes with $KMnO_4$ also interferes with receptor binding [Fig 3(A), compares lanes 3 and 4] This effect is more pronounced in the binding sites 2 and 3 than in the most promoter proximal binding site 4 A quantitation of the binding interference data with both DNA strands is shown in Fig 3(B)

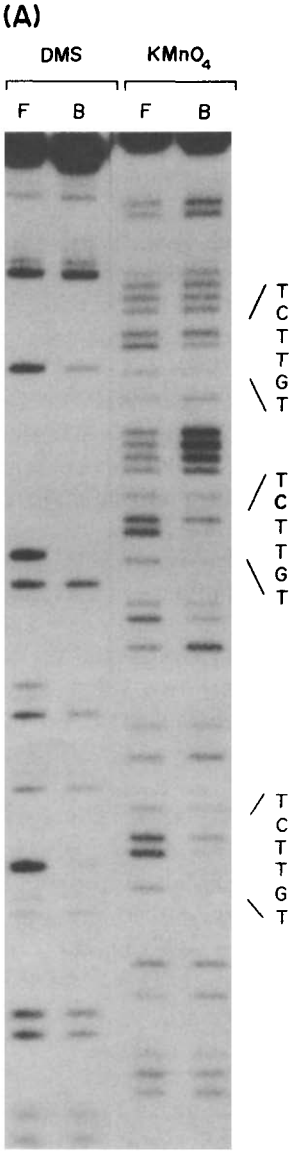

Fig 3(A) —*legend overleaf*

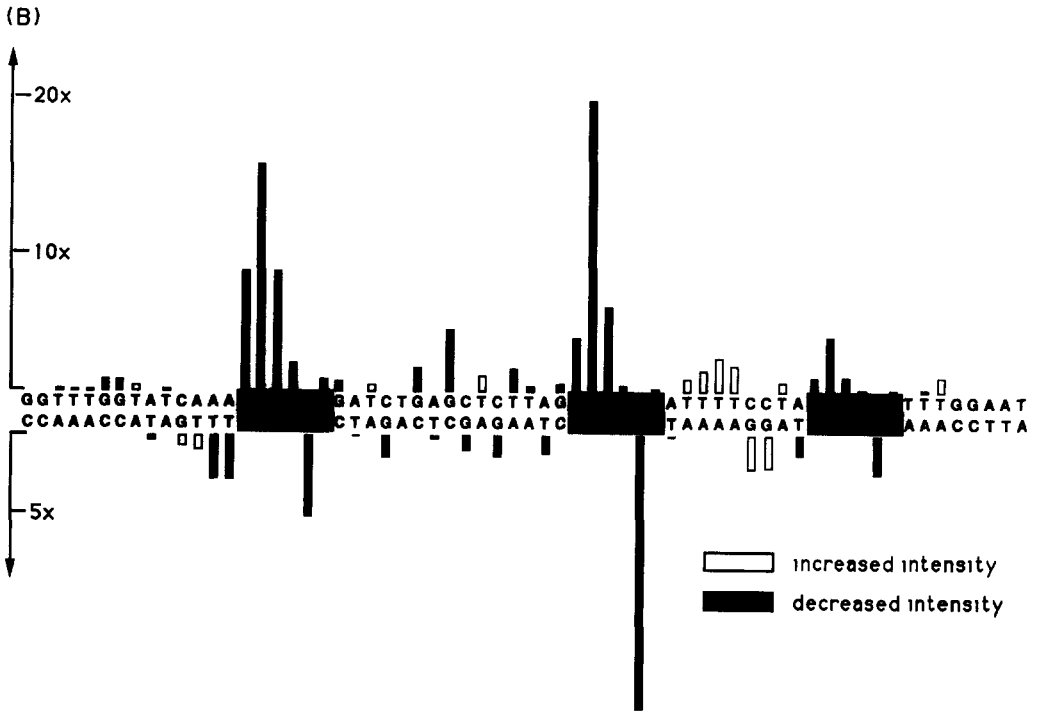

Fig 3(B)

Fig 3 Dimethylsulfate and $KMnO_4$ interference experiment with PR and the promoter proximal cluster of receptor binding sites (A) The 5'-end labeled 90 bp *Sal* I/*Bam* HI fragment of plasmid pLS-155 containing the three promoter proximal HREs of the MMTV promoter, was modified either with dimethylsulfate (DMS) or potassium permanganate ($KMnO_4$) This premodified DNA was then used for preparative gel retardation assays with PR Bound (B) and free (F) DNA were recovered, cleaved with piperidine and analyzed by electrophoresis in a 15% sequencing gel The positions of the three TGTTCT-hexanucleotides are indicated (B) Summary of data from these DMS and $KMnO_4$ interference experiments The magnitude of the effect is by the size of the bar above or below that position in the DNA fragment The position of the TGTTCT-hexanucleotides is indicated by arrows

From these results it is clear that the G opposite to the C at position five of the half palindrome is also important for receptor binding, specially in binding site 3, but also in binding site 2 Outside of the conserved half palindromes there is no dramatic effect of base modification on PR binding, except for the G at position −106 Similar results were obtained with the GR (data not shown) It seems that all important contacts of the receptor with the DNA take place within the conserved hexanucleotide motifs, and that all 3 sites are occupied

The results discussed above suggest that hormone receptors bind to the three sites cooperatively, although their affinity for binding sites 2 and 3 is higher than for binding site 4 To test this idea more directly we studied the receptor binding to DNA-fragments of similar size carrying either the strong binding site 1 or combinations of the 3 proximal binding sites A comparison of the band shift results obtained with the wild type-fragments shows that receptor binding to the cluster of 3 proximal sites exhibits a high degree of cooperativity (Fig 4) The initial affinity for the fragment containing the promoter distal site is higher than for the proximal cluster but the increment in retarded complexes observed with increasing concentrations of receptor is much more pronounced with the promoter proximal cluster (Fig 4, compare lanes 2 to 4 and 5 to 7) The cooperativity of receptor binding to the promoter proximal cluster of binding sites is further documented by the dramatic effect of mutating the central binding site 3 This mutation almost completely abolishes binding of the PR (Fig 4, lanes 8 to 10) We conclude that the functional cooperativity observed between the 3 promoter proximal sites may be explained in part by cooperative binding of receptor molecules

In an attempt to determine the stoichiometry of receptor molecules bound to the promoter proximal cluster of incomplete binding sites we compared the electrophoretic mobility of retarded complexes formed with this cluster to the mobility of complexes formed on fragments carrying either a single string palindrome or two properly spaced copies of the palindrome

(Fig 4, lanes 11 to 13 and 14 to 16, respectively) The observed mobility of the retarded complexes in fragments containing two GREs/PREs is very similar to that found with the promoter proximal cluster of MMTV, suggesting that two dimers of the PR may be associated with this region of the MMTV (Fig 4, compare lanes 7 and 16) However, the very slow mobility and the consequent short migration of these complexes in the gel precludes a precise determination of the number of receptor molecules bound per fragment

Binding sites for other transcription factors on the MMTV-LTR

How do the receptor molecules bound to the HRE mediate enhanced promoter utilization? The prevalent model of gene regulation implies that activation is achieved by direct or indirect interaction among regulatory proteins bound to DNA [80] Accordingly, one would expect that binding of the receptor to the HRE favors, either directly or with the help of adaptor molecules, the interaction of other transcription factors with the promoter and the subsequent formation of a transcription complex

Nuclear factor I A possible candidate for this function is the transcription factor nuclear factor I (NFI), which has been shown to be important for transcription of the MMTV promoter [81–85] The binding site for NFI is located immediately downstream of the HRE, and mutations of these NFI binding sites that inhibit NFI binding *in vitro* have been reported to strongly reduce glucocorticoid induced transcription [82, 83] We have confirmed these findings using T47D cells and either the endogenous PR or a cloned GR Mutation of the NFI binding site reduces response to either glucocorticoids or progestins by 80–90% [85]

These results suggested that NFI is actually operating as a transcription factor in the MMTV promoter In cells that contain low levels of functional NFI, transfection of a reporter plasmid containing the MMTV promoter linked to the CAT gene does not yield significant induction by either glucocorticoids or progestins If, however, the cells are co-transfected with an expression vector for NFI, marked induction by glucocorticoids and progestins can be detected [85] Moreover, hormonal induction is inhibited by mutation of the NFI binding site in the MMTV promoter [85] Thus, NFI acts as a transcription factor in the MMTV promoter and is needed to obtain optimal hormonal induction

It has been shown previously that NFI and steroid hormone receptors can cooperate in transactivation of artificial promoter carrying binding sites for the two proteins in the correct spacing [73, 86] It was therefore expected that the NFI site in the MMTV promoter would participate in similar interactions However, we found that purified hormone receptors do not cooperate but rather compete with NFI

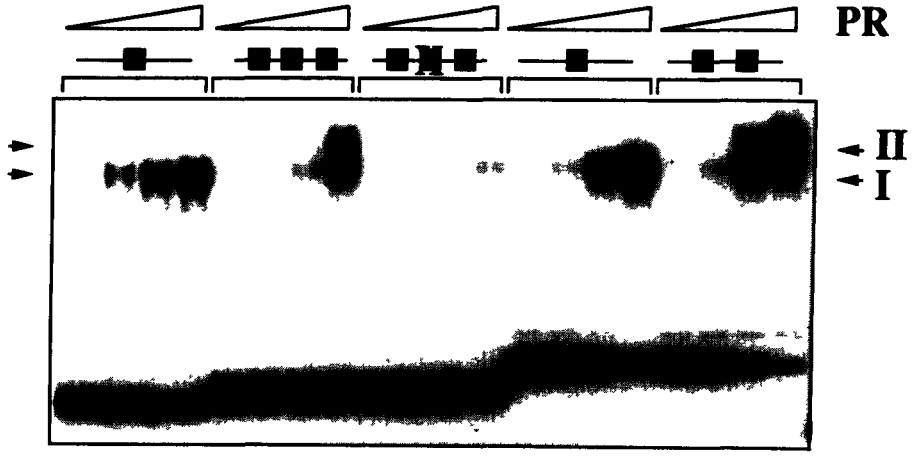

Fig 4 Binding of the PR to DNA-fragments containing various combinations of GREs Gel-retardation experiments with various DNA-fragments that have been incubated with 0 5, 1, 2 or 4 ng PR DNA binding was assayed by electrophoresis on a 4% native polyacrylamide gel From left to right 45 bp fragment containing the promoter distal HRE, 90 bp fragment containing the three promoter proximal HREs, the same 90 bp fragment in which the central of the three HREs is mutated (LS98), 130 bp fragment with one copy of a symmetric perfect GRE (TGTCCT) [41], 130 bp fragment with two copies of the same GRE Arrows indicate positions of receptor/DNA-complexes, the number of boxes in the schematic drawing above the lanes corresponds to the number of HREs of the DNA-fragments

for binding to the MMTV promoter and vice versa [85] Under no experimental conditions have we detected a synergism of the two types of DNA binding proteins in terms of interaction with the MMTV promoter In fact, the observed competition is not unexpected as the sequences protected against DNase I by the hormone receptors overlap by several bases with the footprint generated by NFI Given the observed requirement of sequences flanking the HRE for efficient binding of the receptor [52], a steric hindrance in the interaction of both proteins with the MMTV promoter would be expected [85] Thus, we were faced with the paradox, that although NFI acts as an essential transcription factor for the MMTV promoter and seems to be required for optimal hormone induction, there is no direct cooperation between steroid hormone receptors and NFI in terms of DNA binding Therefore, either the synergism between the two proteins is mediated by an adaptor molecule that is missing in our partially purified fractions, or mechanism other than DNA binding synergism mediate NFI-dependent transcription in response to steroid hormones

Octamer binding factors Even after complete elimination of the NFI binding site, the mutant MMTV promoters are still able to respond to hormone administration, albeit with only one tenth of the efficiency of the wild-type promoter [85] Thus, in addition to NFI there must be other factors that can mediate induction of the MMTV by steroid hormones A search of the MMTV promoter led to the identification of two octamer motifs between the NFI binding sites and the TATA box [87] Mutations at these sites result in a significant reduction of the hormonal induction of the MMTV promoter in gene transfer experiments [87, 88] The two octamere sites are functionally not equivalent The promoter distal site exhibits a single mismatch and binds octamer transcription factor-1 (OTF-1) with an affinity similar to that of the consensus octamer motif Mutation of this site has a significant effect on MMTV transcription in hormone treated cells 3- to 4-fold in HeLa cells and 5- to 6-fold in T47D cells The promoter proximal site exhibits two mismatches, mutations at this site are virtually silent in HeLa cells and show only a 50% reduction in activity in T47D cells [87] Nevertheless this site binds OTF-1 in the wild type MMTV promoter as demonstrated by the presence of a dimer of OTF-1 bound to the corresponding oligonucleotide containing both octamer motifs [87] Whether OTF-2, the lymphoid-specific octamer transcription factor, can bind to the octamer motifs in the MMTV promoter, remains to be established, but will not be unexpected given the observed expression of the MMTV promoter in lymphoid cells [89]

Contrary to the results with NFI, OTF-1 binds weakly to the MMTV promoter in the absence of receptor However, when either PR or GR is preincubated with the MMTV-DNA, binding of OTF-1 is strongly enhanced, as demonstrated in DNase I footprinting experiments [87] Since these experiments were performed with highly purified preparations of receptor and OTF-1, it is unlikely that the DNA binding cooperativity is mediated by additional factors Therefore, in respect to OTF-1, the steroid hormone receptors behave as expected in terms not only of their functional synergism but also in terms of their cooperative binding to their respective sites on the MMTV promoter [Fig 5(B)] As we will see below, this effect can be reproduced under cell-free conditions

Other factors In addition to the hormone receptors, NFI and OTF-1, other factors are involved in the regulated transcription of the MMTV promoter Mutations of the TATA box region diminish the activity of the MMTV promoter in response to glucocorticoids [88] After hormone administration on exonuclease stop is detected at position +1 of the promoter suggesting the presence of a protein bound to the region of the TATA box [90] In other systems it has been shown that a general transcription factor TFIID, in cooperation with several other factors, is responsible for both binding to the TATA box region and for its functional utilization in cell-free transcription [91] Minimal promoters containing only a TATA box and binding sites for the hormone receptors immediately upstream respond to hormone treatment in gene transfer experiments [92] These findings suggest that under certain conditions the receptors can interact, directly or indirectly, with TFIID or other components of the basal transcription machinery Whether this interaction plays a role in induction of the wild type MMTV promoter, or whether the effect in this case is exclusively mediated by other transcription factors (NFI and/or OTF-1) remains to be studied

The existence of negative regulatory sites within the MMTV-LTR has been repeatedly

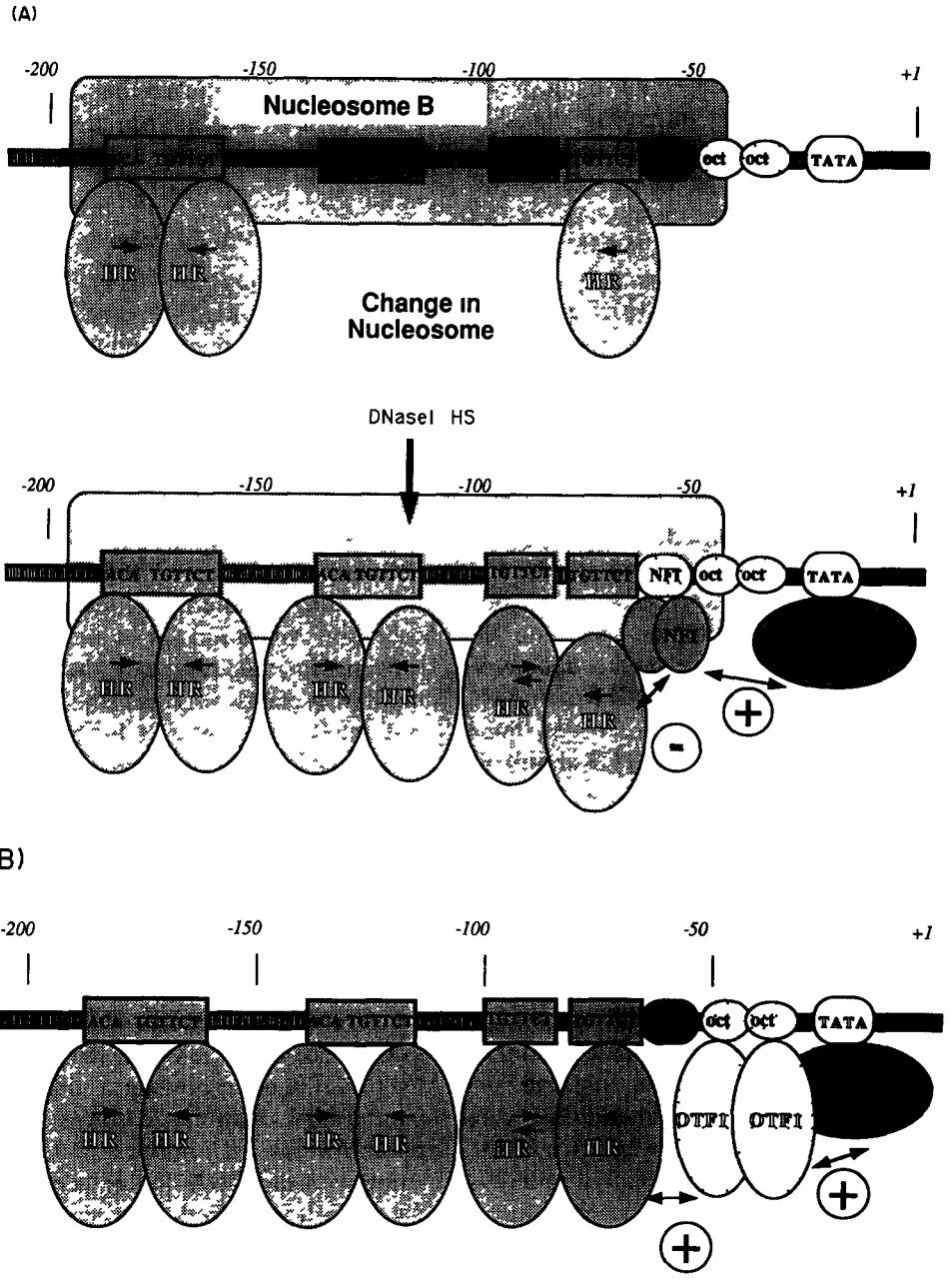

Fig 5 Two pathways for hormonal induction of the MMTV promoter (A) Removal of nucleosome-mediated repression and binding of NFI followed by eventual displacement of receptors (B) Synergistic DNA binding of receptors and OTF-1

reported In transfection experiments, it has been shown that the MMTV enhancer can block the action of the HaMuSV enhancer upon a reported gene [93] Most interestingly, in certain lymphatic leukemias MMTV provirus has been found that carry a deletion encompassing the sequences upstream of the HRE that leads to constitutive expression of the promoter [89] These findings suggest the existence of negative regulatory elements upstream of the HRE, although they could also be explained in terms of the particular chromatin structure of the LTR (see below)

Cell-free transcription experiments

Two possibilities have been considered for transcriptional activation either enhanced level of activity of individual promoters after hormonal treatment, or recruitment of inactive promoters into the active state Recent experiments with cells carrying stably integrated copies of a *lacZ* gene driven by the MMTV

promoter, support the second mechanism [94] It seems that the intrinsic activity of each individual promoter is not changed during hormone induction, but that with increasing doses of glucocorticoids an increasing proportion of promoters is found in the active state This means that the hormone receptors act to recruit other transcription factors to the MMTV promoter turning it into the active state, but they do not modulate the level of activity of each individual promoter This mechanistic distinction is important as it has clear consequences for the interpretation of transcription experiments

To reproduce hormonal induction mechanism in a soluble cell-free system, we have initiated transcription studies using free DNA as a template and nuclear extracts from HeLa cells as a source of transcription factors In this system, we obtain a very efficient transcription of the MMTV promoter, that can be stimulated about 10-fold by preincubation of the DNA template with purified PR from rabbit uterus [95] In agreement with the *in vivo* findings, mutation of a single TGTTCT motif on the HRE has a dramatic influence on the PR-mediated transcriptional stimulation [95] Surprisingly, however, mutation of the NFI binding site of the MMTV promoter, though it reduces transcriptional efficiency about 10-fold, has no influence on the effect of added PR These *in vitro* experiments confirm the role of NFI as transcription factor on the MMTV promoter, but do not allow to detect its participation in hormone-dependent transcription observed *in vivo* This could be due to the relatively high basal activity of the promoter when free DNA is used as template, which contrasts with the almost silent state of the promoter in uninduced cells *in vivo* The *in vitro* effect of PR can be completely abolished by mutations in the two octamer motifs [Fig 5(B) and Ref [87]] Therefore, under cell-free conditions, we reproduce only part of the *in vivo* induction process, namely the residual induction found in gene transfer experiments with templates carrying mutations on the NFI binding site [85] *In vivo*, a negative regulatory mechanism operates that prevents transcription of the MMTV promoter in the uninduced state We postulate that constitutive repression is due to the above mentioned chromatin organization of the LTR that precludes binding of the transcription factor NFI Under *in vitro* conditions, with free DNA as template, the promoter is accessible to NFI, and therefore active, in the absence of added receptor Thus, what we have not reproduced in our cell-free system is the repression due to chromatin structure To reach this goal we will have to use as template appropriately reconstituted minichromosomes rather than free DNA

Chromatin structure and transcriptional repression of the MMTV promoter

It has previously been shown that the binding of NFI to the MMTV promoter *in vivo* is dependent on hormone induction [90] In the absence of hormone, there is no binding of NFI to its cognate site in the MMTV promoter, and this is not due to lack of NFI binding activity [96] One possible explanation for this behavior will be that hormone administration and receptor binding to the HRE changes the chromatin structure and thus enables NFI to bind to the promoter This idea was originally postulated by Gordon Hager and his colleagues, following their finding that the MMTV-LTR in BPV minichromosomes is organized into positioned nucleosomes [97] One of the three nucleosomes on the LTR appears to cover the regulatory region including the HRE After hormone administration this region becomes hypersensitive to DNase I [98], suggesting that the hormone has induced a change in chromatin structure, possibly the removal of a nucleosome [97]

Based on these observations, we initiated *in vitro* chromatin studies aimed at analyzing the influence of nucleosome structure over the MMTV-LTR upon binding of hormone receptors and NFI Mononucleosomes reconstituted on the MMTV-HRE adopt a preferred conformation with the DNA double helix following a very precise path on the surface of the histone octamer [99, 100] We have also reconstituted dinucleosomes with longer linear or circular fragments of the MMTV-LTR including part of the transcribed region [101] These dinucleosomes exhibit the expected rotational positioning of the DNA double helix over the HRE and the NFI binding site [101] Thus, it seems that the nucleotide sequence of the corresponding region of the LTR is designed to bend around the histone octamer in a preferred way This idea is confirmed by the analysis of minicircles generated by ligation of naked DNA-fragments containing the regulatory region of the MMTV-LTR In these minicircles the DNA double helix adopts the same path as in reconstituted nucleosomes [101] Therefore the nucleotide sequence of the MMTV promoter is the main determi-

nant of its rotational setting around the nucleosome This prediction is further supported by the possibility to use simple algorithms, based on the dinucleotide matrix of Drew and Calladine [102], to precisely predict the preferred rotational phasing of the MMTV double helix around the histone octamer [103]

The precise path of the DNA double helix around the octamer of histones was also analyzed using hydroxyl radical footprinting, and was shown to be compatible with binding of the hormone receptors to two of the four TGTTCT motifs but incompatible with binding of NFI to its cognate sequence [100] These predictions were experimentally confirmed and support the idea that rotational positioning of the DNA helix around the histone octamer precludes transcription of the MMTV promoter by masking the NFI binding site When *in vitro* reconstituted nucleosomes are incubated with purified hormone receptors we observe an alteration in the 3′ limit of the nucleosome which becomes more accessible to digestion by exonuclease III [100] This could indicate that the DNA helix changes its path on the surface of the histone octamer upon binding of the hormone receptors *in vitro* does not result in removal of the histone octamer [100] This is not unexpected, as removal of a nucleosome *in vitro* may require the use of larger minichromosomes on circular DNA molecules and possibly other factors, such as nucleoplasmin, and/or enzymes, such as topoisomerases In addition, the receptors only bind to two of the four TGTTCT motifs on reconstituted nucleosomes The other two motifs, that have been shown to be essential for hormonal induction *in vivo*, are not accessible in the surface of the nucleosome In order to bind to these two masked sites the structure of the nucleosome must be altered, a process that may require other factors and ultimately lead to exposure of the NFI binding site [Fig 5(A)]

Although the functional significance of these studies remains to be conclusively established, the observations collected so far are compatible with a model according to which the MMTV promoter is silent in the absence of hormonal stimulation due to its particular array in nucleosomes that precludes binding of NFI Upon hormonal administration and binding of the hormone receptors to the MMTV-HRE, an alteration of the nucleosome structure would take place that exposes the NFI binding site and leads to the formation of stable transcription complexes [Fig 5(A)]. How the nucleosomal organization of the MMTV promoter influences the interaction of OTF-1 with its two cognate sites is an interesting question to be studied in the future According to our nucleosome reconstitution experiments, the two octamer motifs should be located in the linker region between nucleosomes B and A [101] If this nucleosome organization prevails *in vivo*, binding of OTF-1 to these sites may be influenced by the interaction of histone H1 with the linker DNA A similar situation applies for the interaction of the basal transcription factors, including TFIID, with the TATA box of the MMTV promoter in chromatin In general, the role of histone H1 in modulating the interaction of transcription factors with promoters has not been carefully studied, with the possible exception of the 5S-RNA genes [104] In the case of the MMTV, the location of many relevant sites in or near the nucleosome linker suggests that histone H1 could play a crucial role in promoter utilization

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