Accessing binding sites in DNA wrapped around histones in condensed chromatin is an obstacle that transcription factors must overcome to regulate gene expression. Here we demonstrate cooperativity between two transcription factors, the glucocorticoid receptor (GR) and nuclear factor 1 (NF1) to bind the mouse mammary tumor virus promoter organized as regular chromatin in vivo. This cooperativity is not observed when the promoter is introduced transiently into cells. Using RNA interference to deplete NF1 protein levels in the cells, we confirmed that NF1 promotes binding of GR to the promoter. Furthermore, we observed a similar synergism between GR and NF1 binding on the endogenous 11β-hydroxysteroid dehydrogenase promoter, also regulated by GR and NF1. Our results suggest that the chromatin architecture of the promoters does not permit strong association of GR in the absence of NF1. Therefore we propose that cooperativity among DNA binding factors in binding to their cognate recognition sites in chromatin may be an important feature in the regulation of gene expression.

Eukaryotic DNA is condensed and highly compacted into chromatin, which allows multiple levels of transcriptional regulation (1). Chromatin architecture can inhibit transcription by preventing the access of transacting factors to their binding sites on the DNA (2). The mechanism by which transcription factors access their cognate binding sites buried in the nucleosome is not clear. Chromatin modifying enzymes such as the ATP-dependent chromatin remodeling factors are known to aid unwrapping or translocation of DNA on the nucleosomes such that binding sites for transcription factors are exposed (3). Recent evidence suggests that remodeling complexes may be recruited to the target promoters by site-specific DNA binding proteins, which initially engage their target sites (4). One such class of transcription factors is the steroid hormone receptors that can interact with the repressive chromatin structure and remodel the chromatin by recruiting chromatin-remodeling complexes to eventually allow other transcription factors to bind (5).

The hormone-inducible mouse mammary tumor virus (MMTV) promoter has been used extensively as a model to study the relationship between chromatin structure and transcriptional activation by the steroid hormone receptors (6). When stably integrated into mammalian cells, MMTV promoter assumes a phased array of six nucleosomes (A to F). The minimal promoter is made up of nucleosomes A and B, which encompasses binding sites for glucocorticoid receptor (GR) hormone response elements (HREs), nuclear factor 1 (NF1), octamer transcription factor (OTF), and the transcription factor IID complex. In the absence of hormone, the chromatin architecture of the promoter excludes the binding of NF1 and OTFs. Upon hormone administration, GR recruits ATP-dependent BRG1 remodeling complex and multiple coactivator proteins to the promoter to remodel the chromatin (7). This remodeling or “opening” of the promoter permits binding of NF1 and OTFs, which eventually leads to the assembly of a transcription initiation complex and the subsequent induction of mRNA (6).

Previously we established that NF1 protein is required for both transcription and chromatin remodeling of the MMTV promoter (8). Furthermore, we demonstrated that NF1 promotes the binding of GR with the stably integrated promoter. This synergism in binding by transcription factors may be a common occurrence on regulatory regions in the genome. Indeed, most eukaryotic promoter regions contain binding sites for several DNA-binding proteins, which probably allows multiple levels and combinatorial control of gene expression (9). Therefore we wanted to investigate the role of cooperative binding by transcription factors to their cognate binding sites within an in vivo chromatin context. In this study, we report that cooperative binding by GR and NF1 is observed only in the context of chromatin because the synergism in binding is absent on a transiently transfected promoter. We confirmed these results by using RNA interference to deplete NF1 protein levels in the cell followed by assessment of GR binding to the promoter. Furthermore we show that GR and NF1 cooperativity is not unique to the MMTV promoter but is also observed on the GR-regulated 11β-hydroxysteroid dehydrogenase (11βHSD2) promoter.

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2 The abbreviations used are: MMTV, mouse mammary tumor virus; GR, glucocorticoid receptor; ChIP, chromatin immunoprecipitation; HRE, hormone response element; NF1, nuclear factor 1; BRG1, brahma-related gene 1; 11βHSD2, 11β-hydroxysteroid dehydrogenase 2; sgk, serum/glucocorticoid inducible kinase; OTF, octamer transcription factor; dex, dexamethasone; WT, wild-type; mNF1, mutation in NF1 binding site; siRNA, small interfering RNA; PEPCK, phosphoenolpyruvate carboxykinase.
**RESULTS**

**GR Requires an Intact NF1 Binding Site to Bind to an Integrated MMTV Template but Not a Transiently Transfected Template**—Previous studies with the stably integrated MMTV promoter have shown that binding of NF1 is hormone-dependent (6). We demonstrated previously that binding of GR to the stably integrated promoter is dependent on NF1 binding (8). To
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Figure 1. GR requires NF1 to bind to MMTV only on stably integrated template. A, schematic of the MMTV promoter depicting the nucleosomes A, B, and C. Hatched square represents the NF1 binding site, closed circles denote HREs, and the arrows represent the real-time PCR primers. B, GR binding requires binding of NF1 on a stably integrated MMTV promoter. WT and mNF1 cell lines (C127) were treated with dex for 1 h, and ChIP assays were performed. The lysates were immunoprecipitated by the normal rabbit IgG (NS) and GR (M-20) (Santa Cruz Biotechnology) antibodies. Real-time PCR using nucleosome B primers were performed. C, GR binding does not require NF1 on a transient promoter. MCF7M cells were transiently transfected with WT and mNF1 MMTV constructs for 24 h, treated with dex for 1 h and isolated nuclei. ChIP assays were performed as shown in B.

We begin to investigate this interdependency of GR and NF1 for binding their recognition sites, we first examined the potential contribution of promoter chromatin architecture. We tested the binding of GR to the transiently transfected MMTV promoter in the absence of NF1 binding. It has been shown earlier that in contrast to the stably integrated template, when MMTV promoter DNA is introduced transiently into cells, the DNA fails to acquire the same phased array of nucleosomes. The transient MMTV promoter permits constitutive binding of NF1 but despite the apparent open conformation of the transient template, the promoter remains hormone-inducible.

We transiently transfected the wild-type MMTV promoter constructs and a construct with mutation in NF1 binding site (mNF1) into MCF7M cells. As illustrated in the schematic, the MMTV promoter contains four HREs; all located within nucleosome B of the phased array. The NF1 binding site also resides within nucleosome B extending to the linker region with nucleosome A (Fig. 1A). We then performed a series of ChIP assays using an anti-GR antibody and specific primers amplifying the nucleosome B region of the MMTV promoter (Fig. 1B). For stable template, we performed ChIP assay to detect GR binding on integrated copies of MMTV promoter in WT and mNF1 cell lines. As shown previously on the stable template the binding of GR is dependent on NF1 binding (Fig. 1B) (8). In contrast to what we observe on the stable template, the binding of GR to the transiently transfected promoter is not affected by the mutation in NF1 binding site (Fig. 1C). Therefore the requirement for a functional NF1 bind site to allow GR binding is observed only on the stably integrated promoter. Consistent with this idea, in the absence of organized chromatin, the GR is able to bind the promoter independent of NF1. These data suggest that NF1 binding is required for GR to overcome chromatin architecture and to bind its cognate site within the promoter.

Depletion of NF1 Levels by siRNA Transfection—The exclusion of GR from the promoter in the absence of NF1 binding is intriguing, given the fact that under in vitro conditions in the absence of NF1, GR is able to bind to its site on the promoter and remodel the chromatin with SWI/SNF complex (16, 17). Although we have previously shown that the deletion in the mNF1 construct does not affect its ability to position nucleosome accurately (8), the above results could be explained by any of the following reasons. 1) There could be topological constraints in the mutant construct that may prevent the binding of GR efficiently in the absence of NF1 when stably integrated. 2) Alternatively, by mutating the NF1 site, binding site for another unknown transcription factor may be impaired in this construct or 3) positional effects on the stably integrated promoter constructs based on the integration locus. To address these concerns, we employed RNA interference to deplete NF1 protein levels in the cells followed by chromatin immunoprecipitation assays. By this approach, the synergy in binding of these two transcription factors can be assessed without any possible structural alteration to the promoter. This experimental design also allows us to test the universality of this phenomenon on other GR-regulated genes.

The NF1 gene family contains four different, ubiquitously expressed, highly related genes: NF1A, NF1B, NF1C, and NF1X (18). Because of the tissue-specific expression of these isoforms and their splice variants it is not clear which isoform is required for cooperation with GR (19). Because NF1-C isoform has been shown to be most abundant in the mouse mammary gland (20) (Fig. 2A), we tested the role of NF1-C isoform in GR-mediated gene regulation in MCF7M cells. Using ChIP assay and NF1-C-specific antibody, we showed that NF1-C isoform binds to the MMTV promoter in a hormone-dependent manner (Fig. 2, A and B). Therefore, we used siRNA targeted to NF1-C transcript to deplete NF1 protein levels required for activation of the promoter. MCF7M cells were transfected with siRNA targeting lamin A/C (control) and NF1-C mRNA. Transfection with NF1-C siRNA resulted in significant reduction in NF1-C mRNA levels (Fig. 2C), although there is some knockdown of other NF1 isoform-specific transcripts as well. In agreement with the mRNA levels, we also saw significant reduction in NF1 protein levels when Western blots were probed with antibody that recognized all isoforms (Fig. 2D). This suggests that by targeting siRNA to NF1-C mRNA, we depleted significant levels of total NF1 proteins in these cells.
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Effect of NF1 Depletion on MMTV Transcription and GR Binding to the Promoter—Using this in vivo model for NF1 depletion, we examined the effect of NF1 diminution on GR-mediated MMTV transcription. As expected, reduced NF1 protein levels resulted in a decline in the level of MMTV transcription after hormone treatment (Fig. 3A) compared with control siRNA. This decrease in transcriptional activation is not associated with changes in global GR levels in the cell (Fig. 3B). To assess the effect of NF1 reduction on chromatin remodeling of the MMTV promoter, restriction enzyme hypersensitivity assay was performed. Consistent with our previous result (8), we observed reduced hormone-dependent increase in accessibility of SstI enzyme in NF1-depleted samples compared with the control samples (Fig. 3D, compare lanes 1 and 2 with lanes 3 and 4). This implies that NF1 is required for chromatin remodeling of the MMTV promoter.

Next, we performed ChIP assays with GR and NF1 antibodies after NF1 depletion (Fig. 3E). In control siRNA transfections, GR and NF1 bound to the promoter in a hormone-dependent manner. As expected, NF1 binding to the MMTV promoter was diminished upon NF1 knockdown in both control and hormone-treated samples. Thus it appears that there is some basal NF1 binding in the absence of GR as measured by ChIP assay. Consistent with our hypothesis, we observed reduction in GR binding to the promoter upon depletion of NF1 proteins suggesting that GR required NF1 to stabilize its association with its binding site on the MMTV promoter organized as chromatin.

GR and NF1 Co-occupy the Promoter—For GR and NF1 to bind cooperatively to the MMTV promoter, we would expect these proteins to co-occupy the promoter. To demonstrate concurrent binding of GR and NF1 on the same promoter, we performed a ChIP-reChIP experiment (Fig. 4). We performed the first ChIP with GR antibody and as expected there was increased amount of GR binding to the promoter in the presence of hormone. Using the hormone-treated sample immunoprecipitated with GR antibody, we reChIPed with IgG or NF1 antibody. NF1 antibody immunoprecipitated the complexes immunoprecipitated with GR antibody in the first ChIP. This suggests that GR and NF1 co-exist on the same MMTV promoter.

Effect of NF1 Depletion on Expression of Other GR-regulated Genes—To further understand the dependence of NF1 and GR in binding the MMTV promoter, we wanted to understand the universality of this phenomenon. NF1 is known to function as an accessory transcription factor for genes regulated by a number of signal transduction pathways including those controlled by cAMP, insulin, TGFβ, other steroid hormones, vitamin D, and others. Also GR and NF1 have been shown to co-regulate a number of promoters (18). Interestingly, both GR and NF1-B knock-out mice have very similar phenotype with defects in lung maturation, and there are a number of genes affected by these two proteins (21, 22). Therefore to understand the cooperation between GR and NF1 on other GR-regulated genes, we analyzed two GR-regulated promoters, sgk and 11βHSD2. We scanned the sgk and 11βHSD2 promoters with the Transfac software for putative GR and NF1 binding sites (Fig. 6, A and C). As reported previously, sgk promoter is a primary glucocorticoid responsive gene (23) and has four putative NF1 binding sites but their role in GR-mediated activation has not been demonstrated. When we monitored transcription from sgk promoter under conditions of NF1 depletion, we failed to see any effect on GR-dependent increase in sgk mRNA levels (Fig. 5A). This suggests that despite the presence of putative binding sites, NF1 may not play a role in GR-mediated transactivation of the sgk promoter. This result also implies that depletion of NF1 does not universally impair ability of the GR to stimulate gene expression.
In contrast to the sgk promoter, NF1 was identified as a strong stimulatory factor for 11βHSD2 promoter activity, and its binding site at −498 was shown to be important for this activation (24) (Fig. 6A). There are four additional putative NF1 binding sites, and one HRE within the proximal promoter. Glucocorticoids has been shown to stimulate expression of 11βHSD2 in a number of cell lines (7, 25, 26). Indeed when we analyze 11βHSD2 mRNA levels under NF1 depletion conditions, there is a decline in hormone-mediated response (Fig. 5B) suggesting that NF1 is required for GR-mediated activation of this promoter.

GR and NF1 Cooperativity on Endogenous GR-regulated Promoters—Using our in vivo model to understand cooperativity between GR and NF1 proteins on other endogenous promoters, we analyzed the binding of GR to NF1-dependent 11βHSD2 promoter and to NF1-independent sgk promoter. We performed ChIP assays with GR antibody under NF1-depleted conditions and probed for these two promoters. The sgk promoter region has at least four putative HREs between −1000 and −1500 (Fig. 6A), and we used real-time PCR primers to this region. Consistent with the sgk mRNA levels (Fig. 5A), GR binding to the sgk promoter in the presence of hormone was not reduced upon NF1 knockdown (Fig. 6B). Therefore NF1 depletion seems to have no effect on sgk mRNA levels or on GR binding to the promoter.

Consistent with the hormone-dependent increase in 11βHSD2 mRNA levels, we observed a significant recruitment of the GR at the bona fide HRE present in the promoter. In sharp contrast to what was seen with the sgk promoter, when we amplified the region on 11βHSD2 promoter with a putative HRE, the binding of GR in the presence of hormone was reduced upon NF1 knockdown (Fig. 6, C and D). This implies that binding of GR to 11βHSD2 promoter is also NF1-dependent. This is similar to what we found on the MMTV promoter, which suggests that the binding of GR to promoters that is regulated by both GR and NF1 may be dependent on NF1 binding. These mechanistic similarities between the 11βHSD2 and MMTV promoters may define a subset of GR-regulated genes with a critical role for NF1 with respect to GR binding its cognate site in vivo.

DISCUSSION

Gene transcription requires multiple molecular machines the activities of which are often directed to promoters by target-specific transcription factors. We have used the GR and NF1 transcription factors within the context of the MMTV promoter to explore the contributions of chromatin to combinatorial protein–protein interactions in vivo. We previously demonstrated that NF1 plays a dual role, one as a classical transcription factor and the other as an architectural protein for chromatin remodeling of the promoter (8). Furthermore we...
showed that NF1 binding is required for stable interaction of GR with the promoter (8). In this current study, we found that binding of GR on transiently transfected MMTV promoter was not dependent on NF1 binding. However, the GR did require NF1 to bind to HREs only in the context of organized chromatin architecture. We also demonstrate that both these transcription factors co-occupy the promoter, which is a prerequisite for cooperativity. However, in vitro studies demonstrated that GR but not NF1 can bind nucleosomes (27). Therefore, we propose that not only is GR necessary to permit NF1 binding but also that bound NF1 in turn facilitates further occupancy or increases stronger GR association with the promoter in vivo. Thus there is a reciprocal and sequential synergism between GR and NF1 on MMTV promoter, which we propose may be necessary for the dynamic interaction of GR with the promoter (28). Interestingly, this cooperation does not appear to require direct protein-protein interaction between GR and NF1 as we fail to co-immunoprecipitate these proteins together (data not shown).

This interdependence of GR and NF1 binding to the promoter they both regulate provides interesting insights into how transcription factors cooperate to bind to their recognition sites on DNA organized as chromatin. The presence of combinatorial binding sites for multiple site-specific transcription factors on many promoters may be an indication of the generality of cooperation between them. Consistent with our results, a similar cooperativity has been suggested for phosphoenolpyruvate carboxykinase (PEPCK) promoter where GR binding has been shown to be facilitated by accessory factors (29). Further, in breast cancer cells, the Forkhead protein, FoxA1, interacts with cis-regulatory regions in combination with adjacent estrogen response elements to facilitate the interaction of estrogen receptor with chromatin (30). Our observations with GR and NF1 on the MMTV promoter are particularly exciting because this is the first study in which two transcription factors have been shown to cooperate to specifically overcome the chromatin architecture. We believe a similar cooperation may occur on the 11βHSD2 promoter. Indeed, as seen with the MMTV promoter, 11βHSD2 promoter is shown to require BRG1 remodeling complex for GR-mediated activation (7). Despite the differences in the layout of these two promoters with respect to binding sites for GR and NF1, the chromatin architecture may be similar such that cooperation between GR and NF1 may be essential to permit stable association of the activation complex with the promoter.

Unlike the MMTV and 11βHSD2 promoters, the absence of GR and NF1 cooperativity on the sgk promoter is striking. One possibility for such a differential requirement may be that the chromatin architecture of the sgk promoter permits...
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FIGURE 6. NF1 depletion has differential effect on GR binding to sgk and 11βHSD2 promoters. A, schematic of the sgk promoter. Closed circle, putative HREs; open squares, putative NF1 binding sites. Arrows denote real-time PCR primers. B, CF7M cells were transfected with lamin A/C (control) or NF1 siRNA for 48 h and treated with dexamethasone (Dex) for 1 h. ChIP assays were performed as described in the legend to Fig. 1B using normal rabbit IgG and GR (N499) antibodies. ChIP DNA was amplified with primers shown in A. C, schematic of the 11βHSD2 promoter. Closed circle, putative HRE; open squares, putative NF1 binding sites; hatched square, known NF1 binding site. Arrows denote real-time PCR primers. D, ChIP DNA samples from B were amplified with primers shown in C.

FIGURE 7. Proposed models for GR and NF1 cooperative binding to the MMTV promoter (1). In the initial factor binding model, hormone-bound GR and NF1 initially bind cooperatively to their sites on the promoter. The binding of these two transcription factors targets the BRG1 complex to the promoter, which leads to remodeling and subsequent transcriptional activation of the promoter (2). In the remodeling model, we propose that in the presence of hormone, GR binds to its site, which results in the recruitment of BRG1 complex to the promoter. Binding of NF1 to its site may be an essential step in the BRG1-mediated chromatin remodeling process itself or in maintaining the remodeled state of the promoter after the remodeling process.

stable association of GR even in the absence of cooperative binding. Conversely, GR may utilize other transcription factor(s) to stabilize its interaction with the chromatin. Although the chromatin architecture and other transcription factor requirement for GR-mediated activation of sgk promoter is still largely undefined, there are putative binding sites for Sp1, AP1 transcriptional complex, CCAAT enhancer-binding protein, GATA, and OTF transcription factors on the sgk promoter (based on transfactor analysis). It will be interesting to evaluate whether any of these factors have similar cooperative binding properties as NF1.

The differential requirement of cooperativity between NF1 and GR on the sgk and the 11βHSD2 promoters may represent another contributing factor to the cell and promoter-specific effect of the hormone. The sequence of the HRE, recruitment of coactivators/corepressors, and the composition of other associated regulatory components are known to mediate a promoter-specific response to hormone (31). Recent data suggest that differential recruitment of mediator complex, MED14 and MED1, by GR also facilitates gene-specific transcription (32). Furthermore, recent studies show that different ligands can selectively affect the ability of GR to bind HRE and result in differences in the subunit composition of regulatory complexes recruited to the promoter (33). Accordingly, a number of factors appear to promote cell and promoter-specific control by GR, and we propose that the cooperative binding of different site-specific DNA binding proteins can be another contributing factor to achieve this regulation.

We present two models to explain the cooperativity between GR and NF1 on promoters they both regulate (Fig. 7). In the first model, cooperativity between transcription factors can be envisioned to be the first step in initial target site recognition in which both GR and NF1 bind cooperatively to their sites on the promoter. Based on this hypothesis, one would expect cooperativity even in the absence of chromatin remodeling. Therefore in the absence of NF1 binding, we observe loss of GR binding and attenuated chromatin remodeling. In this context, it will be important to explore whether the DNA binding properties of NF1 are sufficient to stabilize GR binding to the MMTV promoter.
In the second model, we speculate that NF1 plays a role in a step subsequent to initial factor binding. In this model, GR initially binds to the promoter and then recruits BRG1 remodeling complex, which remodels the promoter. Consistent with this idea, GR is shown to bind to the promoter even in the absence of BRG1 remodeling complex (7). We propose that NF1 may play an active role in the remodeling process itself or may be necessary to maintain the remodelled state of the promoter because in the absence of NF1 binding, chromatin remodeling is reduced. Therefore based on this model, cooperation between GR and NF1 occurs at the remodeling step. The dynamic nature of the association of the GR with the promoter in the continued presence of ligand (28) might necessitate the requirement of a factor such as NF1, which can maintain the remodelled state. Hence it is conceivable that in the absence of NF1 binding to the promoter, the rapid reassociation of GR with the promoter is impaired. This loss in reassociation would then result in the observed reduction of both chromatin remodeling and GR binding on the promoter as measured in the ChiP assay. Future studies addressing the role of BRG1 remodeling complex in cooperative binding of GR and NF1 can aid in elucidating a mechanism for this cooperativity.

Consistent with the idea that NF1 has a role in stabilizing GR binding, there is evidence that NF1 interacts with histone H3 and is suggested to restructure chromatin of target promoters and replication origins (34). Furthermore, interaction of NF1 with histone H3 is shown to prevent the propagation of silent chromatin structure (35). This function of NF1 is perhaps analogous to the barrier protein function of CCCTC-binding factor DNA-binding protein on the chicken β-globin locus (36). Taken together, all these findings suggest that NF1 has an architectural role in transcriptional activation, and such a function may facilitate cooperativity between GR and NF1 in overcoming chromatin organization. These results suggest a significant advancement in our understanding of the roles of these two well studied transcription factors (5). Previously the GR was seen as the initiating agent that recruited the remodeling complex to open the chromatin structure (6). The binding of NF1 was then thought to be subsequent to this chromatin remodeling step. Our new data suggest that NF1 has a much earlier role such that the initial functional interaction of the GR and remodeling complex requires both the NF1 protein and a functional NF1 binding site. This represents a fundamental shift in previously proposed functions of the GR, NF1, and the BRG1 remodeling complex on this well studied promoter (6). Our new data suggest a molecular mechanism inherent to cooperative binding function of very diverse groups of transcription factors that is central to understanding gene regulation.

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