Glucocorticoid Receptor Transcriptional Activity Determined by Spacing of Receptor and Nonreceptor DNA Sites*

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The glucocorticoid receptor (GR) displays distinct modes of regulation when bound at glucocorticoid response elements (GREs) bearing different binding sequences and arrangements of binding sites. For example, it has been shown to activate transcription synergistically with itself or with other regulatory factors, such as AP1, when bound to a consensus palindromic element or "simple GRE" that is multimerized or linked tightly with an AP1 site. In contrast, at certain "composite GREs" GR and AP1 bind to nonconsensus sequences, and GR either activates or represses depending on the subunit composition of AP1. To uncouple the contributions to regulatory behavior of binding sequences and binding element arrangements, we examined GR action at "paired elements," combinations of a simple GRE and a consensus AP1 site, separated by different distances. We found that GR synergized with either c-Jun or c-Jun-c-Fos at paired elements with GRE-AP1 site separations of ≥26 base pairs. In contrast, paired elements with separations of 14–18 base pairs mimicked the composite GRE, i.e. GR synergized with c-Jun and repressed c-Jun-c-Fos. In DNA binding studies, GR and AP1 cooccupied the paired elements. We conclude that the arrangement of binding sites within a compound response element can be a major determinant of regulatory factor action.

Gene regulatory regions frequently encompass several kilobase pairs of DNA and contain numerous binding sites for transcriptional regulatory factors (1). The rate of transcription initiation for any particular gene is determined by a combination of synergistic and inhibitory interactions among the regulatory proteins that are brought, directly or indirectly, to the DNA (2–5). In some cases, binding sites for interacting factors may be separated by hundreds if not thousands of base pairs (bp), whereas in others two or more regulatory factors may bind in close proximity to each other. In yet other cases, only one of the factors may bind, whereas interacting factors are tethered to the DNA through protein-protein interactions. The glucocorticoid receptor (GR) is a hormone-dependent transcription regulator that uses all of these types of DNA sites to achieve a complex array of context-dependent regulatory effects (6, 7): 1) Synergistic activity results when binding sites for a variety of factors are paired with a consensus GR-binding site (also called a simple hormone response element (HRE)) (3); 2) GR represses the activity of certain activators such as AP1 and NF-κB at their respective consensus sites (8, 9). This repression does not require binding of GR to the sites that bind only the target factor and are thus termed "tethering" elements. GR activity at this type of element appears to require direct protein-protein interaction between GR and the repressed factor; 3) GR can either repress or stimulate transcription at composite GREs (cGREs). For example, the proliferin cGRE has juxtaposed binding sites for GR and AP1 family members, and the composition of the AP1 dimer bound determines whether the receptor represses or activates transcription (4, 10–12). When AP1 is comprised of c-Jun-c-Jun homodimers, the receptor activates transcription from a linked gene, and when AP1 is comprised of c-Jun-c-Fos heterodimers, the receptor represses transcriptional activity.

Thus, the regulatory response of each of these sequences differs qualitatively from the others. The first two types are typically induced (simple and paired elements) or repressed (tethering elements) by the receptor, whereas the third, the cGRE, can activate or repress in a context-dependent fashion. Consistent with its distinct regulatory behavior, the sequence of the prototypic cGRE, plfG, is also quite different from that of the simple HRE. Only short stretches (up to 3 bp) of the consensus HRE can be discerned, whereas a near-consensus AP1 site (5 of 7 match) can be recognized (4). Both AP1 and GR bind to the element in vitro. More recently, other cGREs have been identified, also characterized by juxtaposed or overlapping GR- and AP1-binding sites and the same biphasic response to GR, depending on the composition of AP1 (10, 11).

In principle, there are several possible mechanisms that could underlie the distinct behavior of the cGRE. We consider here four classes: 1) The DNA could have an allosteric effect on the receptor, AP1, or both that influences their activities. This type of effect could be either sequence-driven or DNA topology-driven (see below); 2) The relative position of the factors on the DNA could introduce constraints that permit only certain interactions; 3) The DNA site could bind a third factor that specifies activation or repression; 4) The composition of AP1 could specify activation versus repression whenever DNA-binding sites for both GR and AP1 are present, regardless of spacing or sequence. According to this mechanism, GR would synergize with c-Jun and repress c-Jun-c-Fos at any element that has binding sites for both.

The above considerations led us to ask whether the sequence of the binding sites, the composition of the AP1, or the relative positions of the factor-binding sites was the critical determin-
nant of composite element behavior. We therefore constructed a series of paired response elements consisting of a simple HRE at varying distances from a consensus AP1 site (see Fig. 1) and tested their transcriptional activity in the presence of GR, c-Jun, and c-Fos, as well as their ability to bind these factors simultaneously in vitro. We found that paired elements, appropriately spaced, can demonstrate cGRE behavior and thus that the relationship of DNA sites is an important determinant of transcription factor interactions. Moreover, paired elements that demonstrate composite behavior are able to bind both GR and AP1, simultaneously suggesting that the switch from activation to repression does not involve disruption of DNA binding but rather that the factors mutually influence each other's activities.

**EXPERIMENTAL PROCEDURES**

**Construction of Paired Element Reporters**—Paired element oligonucleotides, Gl(n)A, where n is the center-to-center distance, in nucleotides, between the two sites, were constructed by annealing single-stranded oligonucleotides containing the sequences shown in Fig. 1 (and their reverse complements). The top strand sequences contained SalI restriction sites at the 5’ ends, and the bottom strands contained XhoI sites at their 5’ ends. The double-stranded oligonucleotides were then ligated into the vector ΔODCO, which had been digested with SalI restriction enzyme and treated with calf intestinal phosphatase (0.5 units for 15 min) followed by heat treatment to kill the phosphatase (70 °C for 10 min). ΔODCO contains the Drosophila Adh minimal promoter driving expression of chloramphenicol acetyltransferase (4). Insertions were digested by digestion with appropriate restriction enzymes, and then positives were confirmed by sequencing (Biomedical Molecular Resource Center, UCSF). The same oligonucleotides were end labeled using T4 DNA kinase for gel shift experiments. Two forms of G16A were synthesized, both of which were tested in transfection assays. One was also used to make probe for gel shift experiments (see below) whereas the other was made with a unique BglII restriction site between the HRE and AP1 sites. It has the sequence: TCGAGGTGTTAGGATGTCAG (HRE and AP1 sites are underlined). Larger insertions (n = 39 nucleotides or more) were made by digesting this plasmid with BglII and ligating in size fractionated fragments. The fragments for insertion were generated by digesting pBluescript DNA with SnaAI (four-base recognition sequence) followed by removal of the melt gel isolation. Minipreps were performed, and plasmids that appeared to have desired size were sequenced.

**Cell Culture and Transfection Experiments**—60–80% confluent culture plates of murine F9 cells (Cell Culture Facility, UCSF) were grown at 5% CO2, Rh-90%, 37 °C in Dulbecco’s modified Eagle’s medium H-16 (Cell Culture Facility, UCSF) supplemented with 10% fetal calf serum (Life Technologies, Inc.). Cells were transfected in 6-cm plates in 1 ml of calcium phosphate precipitation, as described previously (13) with the mammalian expression plasmid, pmc1 (Life Technologies, Inc.). Cells were harvested, and extracts were prepared and assayed for chloramphenicol acetyltransferase and β-galactosidase activities as described previously (13). Representative experiments are shown in the figure legends. As shown in Fig. 2A, we found that the TAM HRE separated by 39 bp from a consensus AP1 site served as a weak GR reporter in the absence of cotransfected c-Jun or c-Jun-c-Fos. Both c-Jun and c-Jun-c-Fos stimulated reporter activity, as described previously for consensus AP1 sites (17) and, importantly, GR stimulated both c-Jun-c-Jun homodimers and c-Jun-c-Fos heterodimers in a hormone-dependent manner. The augmentation

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**Fig. 1. Sequence and relative positions of HRE and AP1 site in paired elements.** HRE sequence is that of the TAT GRE, whereas AP1 site sequence is that of the collagenase TPA-responsive element (16, 30). N, any nucleotide; N’, complement to N; n, center-to-center distance in nucleotides between HRE and AP1 site. The sequences of intervening nucleotides are shown under “Experimental Procedures.”

**RESULTS**

GR acts synergistically with various unrelated transcription factors such as Sp1, NF1, and estrogen receptor when their binding sites are near each other (3). However, synergy does not always result when binding sites for GR and another factor are near each other. Indeed, GR represses c-Jun-c-Fos activity at both consensus AP1 sites, to which GR does not bind, and at cGREs, to which both GR and AP1 bind. The GR- and AP1-binding sites in the proliferin (4), α-fetoprotein (10), and papilloma virus (11) cGREs are closely juxtaposed with center-to-center distances of less than 20 nucleotides. These observations raise the question of whether GR represses c-Jun-c-Fos in all contexts, regardless of the arrangement of their respective binding sites or if either the sequence or binding site spacing in the composite element is important for repression.

To address this question, we first asked if repression or synergy would occur when a simple HRE and an AP1 site were paired at a greater distance than in the composite elements. We chose, as a “typical” simple HRE, the sequence found 2.5 kilobase pairs upstream of the transcription start site of the TAT gene. This element has been found to mediate transcriptional activation, never repression, by GR in all contexts examined, whether in the setting of the endogenous gene or fused to various heterologous promoters (16). We juxtaposed this HRE with a consensus AP1 site (similarly mediating activation by AP1) at varying separations and placed the resulting “paired elements” upstream of a minimal promoter driving chloramphenicol acetyltransferase expression (Fig. 1). These reporters were co-transfected with GR and c-Jun or c-Jun and c-Fos together into AP1-deficient F9 cells (4).
of c-Jun-c-Jun activity exceeded that of c-Jun-c-Fos largely because the c-Jun-c-Fos basal was higher, consistent with the greater activity of the heterodimer (4, 8, 18). GR action in conjunction with c-Jun-c-Fos was more than the sum of the activity of either alone and was therefore synergistic. However, it was substantially less than multiplicative, whereas GR action in conjunction with c-Jun-c-Jun was substantially more than multiplicative. Various other spacings were also tried with similar results (Fig. 2B). Notably, both G(26)A and G(31)A, which place the two sites in opposite phases, mediated hormone-dependent augmentation of both c-Jun-c-Jun and c-Jun-c-Fos. It is notable, however, that the hormone-induced activity in the presence of c-Jun-c-Fos was greater for G(26)A than for G(31)A.

This type of synergistic behavior is similar to that identified previously for GR and other activators (3) but is quite distinct from the behavior that GR had demonstrated previously at consensus AP1 sites (repression of either c-Jun-c-Jun or c-Jun-c-Fos) or cGREs (activation with c-Jun and repression of c-Jun-c-Fos) (4, 8, 10, 19). These results indicate that GR does not repress c-Jun-c-Fos activity in all contexts but rather that either the spacing or sequence of the factor-binding sites (or surrounding sequence) must confer the repression phenotype.

The prototypic cGRE, plfG, was identified by the DNaseI footprint produced by either purified bacterially expressed GR DNA-binding domain (DBD) or full-length GR expressed in HeLa cells (4). Within plfG is a near-consensus AP1 site, which is protected by purified c-Jun or c-Jun-c-Fos in DNase I footprinting experiments. However, the plfG sequence bears little resemblance to the consensus sequence for a simple HRE (Fig. 1), and thus the relative spacing of the GRE and the AP1 site cannot be precisely determined. It is clear, however, that they are closely juxtaposed. With these observations in mind, we wanted to determine whether at smaller separations the paired elements would function as a cGRE. We first tested paired elements with a TAT HRE separated from a consensus AP1 site by 16 nucleotides. As shown in Fig. 3A, a TAT HRE separated by 16 nucleotides from a consensus AP1 site produces a response element, G(16)A, whose response to AP1 and GR is qualitatively identical to that of a cGRE: It is activated by GR in the presence of c-Jun, repressed by GR in the presence of c-Jun and c-Fos, and minimally affected in the absence of AP1 factors. Spacings of 14, 17, and 18 nucleotides also demonstrated this type of regulatory switch (Fig. 3B). Smaller sepa-
Transcriptional Response of Paired Elements

As a first step toward understanding the mechanism of the response of G(16)A, we examined the influence of GR and AP1 factors on each other's DNA binding. We examined first the ability of GR to co-occupy this element with c-Jun-c-Fos heterodimers and c-Jun homodimers in gel shift assays. We performed gel shift experiments using purified bacterially expressed GR DBD and full-length c-Jun and c-Fos proteins. GR DBD, rather than full-length, was used because the different retarded species could not be discerned using full-length receptor, consistent with previous reports (20, 21). As shown in Fig. 4 (lanes 1 and 6), c-Jun-c-Fos and GR DBD, when incubated separately, each shifted G(16)A to distinct positions. When the three proteins were added together, a third species appeared (lane 2). The uppermost band and the receptor band were competed by cold TAT HRE (lane 3), whereas the uppermost band and the AP1 band were competed by cold AP1 competitor DNA (lane 4). Finally, all three bands were competed by cold G(16)A (lane 5). We conclude that the upper band represents DNA co-occupied by GR and c-Jun-c-Fos heterodimers. This conclusion was further supported by antibody supershift experiments using anti-c-Jun, anti-c-Fos, and anti-GR antibodies (data not shown). Similar results were obtained when c-Jun homodimers were incubated with GR-DBD (lane 7), indicating that GR-DBD and the AP1 factors are capable of co-occupying the paired element under activating as well as repressing conditions. Not surprisingly, GR-DBD and AP1 (c-Jun or c-Jun-c-Fos) were able to co-occupy G(16)A as well (data not shown).

DISCUSSION

Spacing between DNA-binding Sites as a Determinant of Factor Activities—Our results show that the relative positions of a paired HRE and AP1 site profoundly influences hormone-induced activity. When a site for only one of the factors is present, then the other has an inhibitory effect (mutual inhibition) regardless of whether AP1 is composed of c-Jun-c-Jun or c-Jun-c-Fos (8, 18, 22). In contrast, when the two sites are both present but not closely juxtaposed (from 26 to at least 210 bp), they act synergistically regardless of the composition of AP1. However, when binding sites for both GR and AP1 are closely juxtaposed (separated by 14–18 bp), they behave as cGREs. With respect to cGRE function, several points are of interest: First, the paired elements are composed of a simple HRE and a consensus AP1 site that mediate transcriptional activation when present alone or separated from each other by 26 bp or more. Second, the surrounding sequences can be altered without affecting the response. Third, multiple different paired element spacings support a composite response. Thus, our data suggest that the relative position of the factor-binding sites is a critical determinant of composite behavior.

API-induced Changes in DNA Topology: a Putative Regulatory Switch—When AP1 and GR DNA sites are paired but not closely juxtaposed, synergy may result from several possible mechanisms. These include cooperative recruitment of a coactivator, action at distinct rate-limiting steps in transcription initiation, or cooperative DNA binding (23). Although the mechanistic basis of synergy between GR and AP1 has not been determined, it is interesting to note that both interact physically with CREB-binding protein, and this interaction has been suggested to explain mutual inhibition at their respective sites by titration of this important coactivator (24). When sites for both are present they can, in principle, cooperate in the recruitment of CREB-binding protein, providing a plausible unifying mechanism for synergy at paired sites and mutual inhibition at single sites. However, this type of mechanism does not account for composite behavior because it does not account for the opposite responses to GR of c-Jun-c-Jun and c-Jun-c-Fos.

The simplest explanation for the composite response is that due to the proximity of the sites GR disrupts c-Jun-c-Fos (but not c-Jun-c-Jun binding), as has been suggested previously to explain GR repression of cAMP-response element-binding protein-driven transcription at an overlapping HRE and cAMP response element (25). Our gel shift experiments suggest, however, that GR and c-Jun-c-Fos heterodimers can bind simultaneously to the paired element. Because these experiments were done with partial length GR protein, we cannot rule out a role of the N or C terminus in disrupting DNA binding by c-Jun-c-Fos. However, two observations suggest that this is not the case: 1) Full-length GR and AP1 co-occupy plfG (4); 2) mineralocorticoid receptor has a similar ligand-binding domain and a substantially larger N terminus and binds with equal affinity to the same site yet does not repress AP1 activity at either plfG or G(16)A (13).2

These arguments suggest that GR and c-Jun-c-Fos are bound simultaneously to G(16)A but that their activities are altered by virtue of their proximity to each other. With this idea in mind, we consider four possible mechanisms that could account for such a proximity-dependent effect: 1) c-Jun-c-Jun and c-
Jun-c-Fos could cause distinct local changes in DNA topology that are transmitted to GR bound nearby. Interestingly, there is evidence to support the idea that c-Jun-c-Jun homodimers and c-Jun-c-Fos heterodimers bend DNA in opposite directions, in vitro (26, 27). Although the question of DNA bending by AP1 remains unresolved (28), it is appealing to speculate that the distinct local topologies have differential allosteric effects on GR conformation and activity; 2) GR, which has also been found to bend DNA (29), could exert local effects on DNA topology that influence c-Jun-c-Fos activity differently from that of c-Jun-c-Jun; 3) GR bound to the paired element might make specific base contacts with the AP1 site, resulting in a sequence-driven (as opposed to a local topology-driven) conformational change in GR as proposed for Oct1-VP16 activity at TAATGARAT sequences; 4) Constraints imposed by their proximity when bound to adjacent sites might force the GR-c-Jun-c-Fos complex (but not the GR-c-Jun-c-Jun complex) into an inactive conformation that, for example, interacts poorly with coactivators or the general transcription machinery.

In view of the multiple paired element spacings that are tolerated, it seems unlikely that GR is contacting the AP1 site. Rather, mechanisms based on either a factor-driven change in local DNA topology or constrained protein-protein interaction seem more likely. These latter two possibilities cannot be distinguished by our data. In principle, experiments using reporter driven by paired elements separated by intrinsically bent DNA (26) should be able to distinguish these mechanisms. Such studies have been initiated, but preliminary results have been equivocal. However, a local topology-driven DNA allosteric effect is still quite plausible and presents an intriguing extension of the concept of DNA-induced allosteric effects on transcription factor activity (6). Perhaps, by exerting distinct effects on local DNA topology, c-Jun-c-Fos heterodimers and c-Jun-c-Jun homodimers differentially influence the allosteric effect of DNA on GR activity. In any case, whether we attribute the findings to changes in protein-protein interaction or to alterations in local DNA topology, we conclude in either case that local DNA architecture plays an essential role in controlling transcription factor activities.

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