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pBR322 CONTAINS GLUCOCORTICOID REGULATORY ELEMENT DNA CONSENSUS SEQUENCES

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SUMMARY: A computer search of the pBR322 DNA sequence identified five sites matching reported glucocorticoid regulatory element (GRE) DNA consensus sequences and three related sites. A pBR322 DNA fragment containing one GRE site was shown to bind immobilized HeLa S_3 cell glucocorticoid receptor and to compete for receptor binding in a competitive binding assay. Conversely, a pBR322 DNA fragment devoid of GRE sites showed barely detectable interaction with glucocorticoid receptor in either of these assays. These results demonstrate the importance of GRE consensus sequences in glucocorticoid receptor interactions with DNA, and further identify a cause for high background binding observed when pBR322 DNA is used as a negative control in studies of glucocorticoid receptor-DNA interactions.

Glucocorticoids regulate transcription of a variety of specific genes through a direct interaction of the hormone receptor complex with specific high-affinity DNA binding sites in or near the promoter regions of regulated genes (1,2). These DNA binding sites, termed glucocorticoid regulatory elements (GREs), have been most extensively characterized where they occur in the Long Terminal Repeat of Mouse Mammary Tumor Virus (MMTV-LTR) (3-5). Gene transfer experiments demonstrate that constructs prepared from large DNA fragments containing these GREs can confer glucocorticoid-regulated expression onto heterologous genes (3,6). DNA footprinting and methylation protection studies with partially

Abbreviations : bp, base pair(s); GRE, glucocorticoid regulatory element; kDa, kiloDalton; MMTV-LTR, mouse mammary tumor virus long terminal repeat; PB, phosphorylase b.

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purified glucocorticoid receptor preparations have led to the identification of a small set of closely related 6-8 bp GRE core consensus sequences. In the MMTV-LTR, Payvar et al. (4) reported an octanucleotide GRE consensus sequence, 5'-AGA($^{A}/_{T}$)CAG($^{A}/_{T}$)-3', in which the indicated nucleotides occurred with a frequency of 11, 13, 9, 12, 12, 10, 8 and 12 of 13, respectively, while Scheidereit et al. (5) reported a hexanucleotide GRE, 5'-TGTTCT-3'. [If the complementary strand of the octanucleotide is written, i.e. 5'-($^{T}/_{A}$)CTG($^{T}/_{A}$)TCT-3', then the hexanucleotide, 5'-TGTTCT-3' occurs within it as one form of the ($^{T}/_{A}$) ambiguity.] More recently, DNA footprinting analyses of glucocorticoid receptor binding to the human growth hormone gene (7) and to the human metallothionein gene (8) have indicated a slight variant of the hexanucleotide GRE core sequence, i.e. 5'-TGTCCT-3'.

We had previously studied the ability of HeLa S_3 cell glucocorticoid receptors electroblotted onto nitrocellulose to interact with DNA (9). In the course of these studies, we observed that, while a DNA fragment derived from the MMTV-LTR consistently bound strongly to the nitrocellulose-immobilized glucocorticoid receptor, fragments from sonicated pBR322 DNA also bound at low, but detectable levels. This low level of binding of radiolabeled pBR322 DNA fragments persisted, even when experiments were conducted in the presence of large excesses of sonicated E. coli DNA.

We now report that pBR322 contains multiple copies of GRE core consensus sequences, and that human glucocorticoid receptors interact specifically with a DNA fragment containing one of these sequences under two different sets of experimental conditions. This selective binding of the human glucocorticoid receptor to a DNA fragment containing a single GRE core consensus sequence suggests that glucocorticoid receptor interactions with DNA may be even more dependent on these small DNA sequence patterns than previously recognized.

MATERIALS AND METHODS

Plasmid DNA and Radiolabeling. Plasmid pLTR-190, containing a 326 bp Hae III-Hpa II DNA fragment derived from the 5' LTR of MMTV was the generous gift of J. Majors (3). The insert in this plasmid corresponds to nucleotides -222 to +104 relative to the primary transcription start site in the 5' LTR of MMTV, and was cloned into the Bam H1 site of pBR322 by the addition of Bam H1 linkers. pLTR-190 and pBR322 were grown in E. coli K12, HB101 and extracted using standard methods (10). Plasmids pLTR-190 or pBR322 were digested with the appropriate restriction enzymes (Bethesda Research Laboratories) and electrophoresed on 5% polyacrylamide gels containing 90mM Tris-borate, 1mM EDTA, pH 8.3. The desired DNA fragments were excised from the gel and extracted as described (11). DNA fragments were labeled with alpha [^{32}P] dCTP (ICN) to a specific activity of 10^8 cpm μg^{-1} by nick translation (12).

Blotting Procedure. Crude cytosolic extracts containing glucocorticoid receptor labeled with [^{3}H] dexamethasone mesylate (New England Nuclear) were prepared from HeLa S_3 cells as previously described (9,13). Cytosolic proteins were electrophoresed on 7.5% polyacrylamide gels which were subsequently incubated in a buffer containing 50mM NaCl, 10mM Tris-Cl, pH 7.0, 20mM EDTA, 0.1mM dithiothreitol and 4M urea to remove sodium dodecylsulfate and enable partial renaturation of the proteins in the gel (9,14). The partially renatured proteins were then electrophoretically transferred onto nitrocellulose filters (Schleicher & Schuell, BA-85). Filter pieces containing the immobilized glucocorticoid receptor were incubated with ^{32}P labeled DNA fragments in a buffer containing 5% nonfat dry milk (9,15), washed, dried and autoradiographed.

DNA-cellulose Competitive Binding Assays. HeLA S_3 cells were incubated with 2×10^{-8} M triamcinolone acetonide (New England Nuclear) and cytosols were prepared as previously described (9,13). DNA-cellulose competitive binding assays were performed essentially as described (16,17). Briefly, aliquots of calf thymus DNA-cellulose (Sigma Chemical Co.) containing 2µg of adsorbed calf thymus DNA were incubated with increasing amounts of glucocorticoid receptor cytosol. A titration curve was plotted as shown in Fig. 3A and an aliquot of cytosol was chosen from the linear region of this curve for use in subsequent competitive binding assays. For these, a 2µg aliquot of calf thymus DNA-cellulose and the chosen aliquot of cytosol were incubated with increasing concentrations of free, competing DNA. For each sample, the DNA-cellulose was washed, centrifuged and the amount of [^{3}H] triamcinolone acetonide labeled glucocorticoid receptor bound determined by liquid scintillation counting.

RESULTS

The DNA sequence of pBR322 (18,19) was searched for GRE sites using the Cornell sequence analysis package (20) on an Apple IIe microcomputer. Results of this search showed a single site which exactly

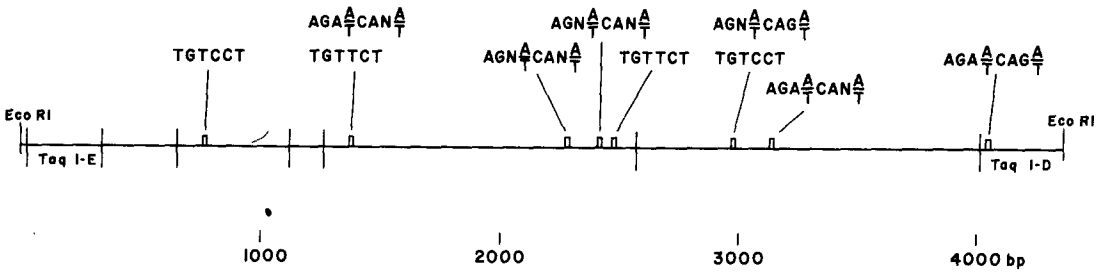

Figure 1. Locations of glucocorticoid regulatory element core DNA consensus sequences in the DNA sequence of pBR322. A computer search of the pBR322 DNA sequence identified the following sites matching or resembling the GRE consensus sequence: a single site (4044-4051) exactly satisfied the constraints of the octanucleotide GRE consensus sequence described in the MMTV-LTR (4). If base pair mismatches were allowed at either or both of the two least frequently occurring nucleotide positions of the GRE, additional sites were found. Two sites (1376-1383 and 3133-3140) contained a single base pair mismatch at position 7, a third site (2975-2982) contained a mismatch at position 3, and two additional sites (2279-2286 and 2416-2423) contained mismatches at both positions 3 and 7. Additionally, two sites (1376-1381 and 2472-2477) exactly matched the hexanucleotide 5'-TGTTCT-3' observed in MMTV-LTR (5) and two sites (760-765 and 2975-2980) matched the hexanucleotide 5'-TGTCCT-3' described in the human growth hormone gene (7) and the human metallothionein gene (8).

satisfies the constraints of the octanucleotide GRE, and four additional sites which exactly match one of the two forms of the hexanucleotide GRE core consensus sequence. When the stringency of the computer search was relaxed to allow base pair mismatching at either or both of the two least frequently occurring nucleotides of the octamer, three additional sites were found (Fig. 1).

To test the selectivity of DNA binding by the nitrocellulose filter immobilized glucocorticoid receptor, three distinct DNA fragments were purified. The fragment corresponding to nucleotides -222 to +104 of the MMTV-LTR was isolated from pLTR190. This 326 bp DNA fragment shows a complex pattern of three regions protected in nuclease footprinting studies (4,5). Plasmid pBR322 was digested with Taq I and fragments similar in size to the MMTV-LTR fragment were purified. The Taq I-D fragment (4020 to 24) is a 368 bp fragment which contains the single exact match to the octanucleotide GRE, while the Taq I-E fragment (25 to

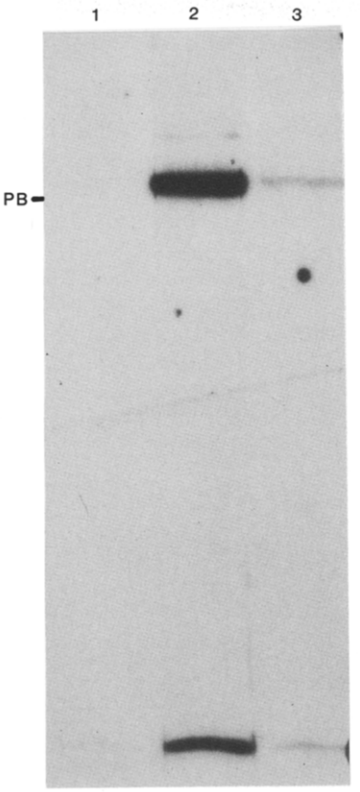

Figure 2. The binding of ^{32}P labeled DNAs to nitrocellulose filter immobilized glucocorticoid receptor. Lane 1 the Taq I-E fragment of pBR322; Lane 2, the MMTV-LTR DNA fragment; and Lane 3, the Taq I-D fragment of pBR322 containing the single occurrence of the octanucleotide GRE consensus sequence. The position of the 97kDa phosphorylase b marker is indicated by PB.

339) is a 315 bp fragment which contains no sequence matching the search criteria described above. These DNA fragments were labeled with alpha [^{32}P] dCTP by nick translation and incubated with HeLa S_3 cell glucocorticoid receptor immobilized on nitrocellulose filters as indicated in Materials and Methods. Autoradiographs obtained from one such experiment are shown in Fig. 2. The filter strip incubated with the MMTV-LTR DNA fragment (lane 2) showed a strong band of DNA binding activity at a position consistent with the migration of the glucocorticoid receptor at a molecular weight slightly greater than phosphorylase b. Filter lanes 1 and 3 were incubated with pBR322 DNA fragments Taq I-E or Taq I-D,

respectively. The nitrocellulose filter immobilized glucocorticoid receptor showed appreciable binding of the Taq I-D fragment containing the single GRE consensus sequence (lane 3), while binding of the Taq I-E fragment was barely detectable (lane 1). DNA binding by an approximately 18kDa protein is seen in all three lanes. The identity of this DNA binding protein is unknown. Since its apparent pattern of specificity in binding these three DNA fragments is similar to that of the glucocorticoid receptor, the possibility exists that this low molecular weight protein could be a proteolytic fragment of the receptor. This possibility has not been experimentally investigated.

As a further test of the selectivity of glucocorticoid receptor interaction with these DNA fragments, DNA-cellulose competitive binding assays were performed. To insure that binding competition occurred under conditions of DNA-cellulose excess, a titration experiment was performed on each preparation of receptor cytosol, and a subsaturating aliquot of cytosol was chosen from the linear region of the titration curve. Results of a typical titration experiment are shown in Fig. 3, Panel A. As indicated, the amount of heat-activated [^{3}H] triamcinolone acetonide labeled glucocorticoid receptor binding to DNA-cellulose increases in proportion to the volume of cytosol used. In contrast, much less binding is seen with the non-activated cytosol. For the competitive binding assays, the chosen aliquot of cytosol was incubated with 2 μg aliquots of calf thymus DNA-cellulose in the presence of increasing concentrations of free, competing DNA. Results from four experiments conducted with the pBR322 Taq I-E fragment, the MMTV-LTR DNA fragment and with the pBR322 Taq I-D fragment are summarized in Fig. 3, Panels B, C and D, respectively. Strongest competition was seen with the MMTV-LTR fragment (Panel C) and moderate competition with the pBR322 Taq I-D fragment containing the single GRE consensus sequence (Panel D). By contrast, very little competition was seen with the pBR322 Taq I-E fragment (Panel B). These results indicate that HeLa S_3 glucocorticoid receptor can recognize and

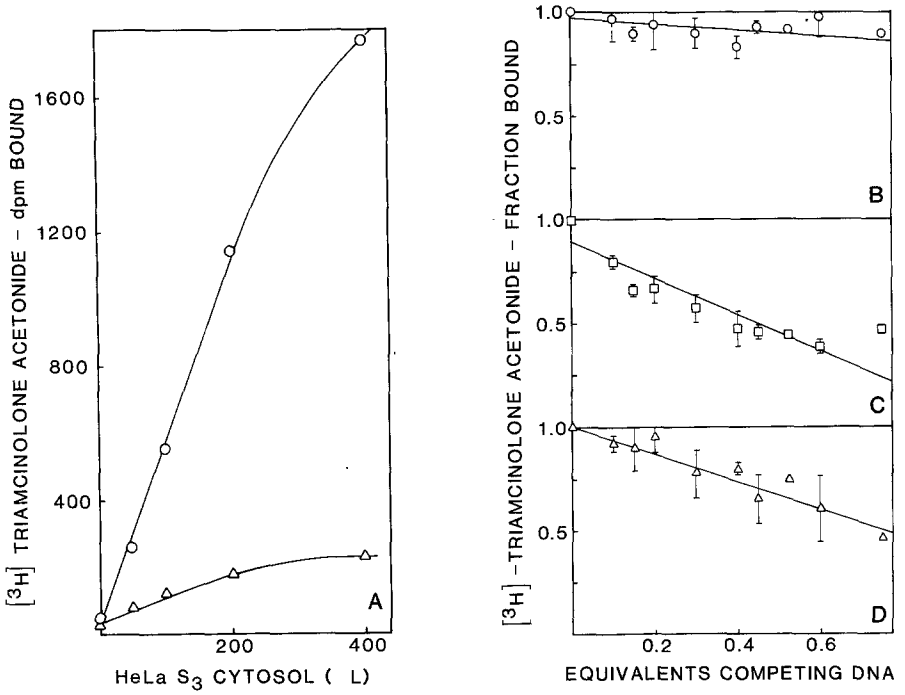

Figure 3. Glucocorticoid receptor interaction with calf thymus DNA-cellulose or free competing DNAs. Panel A, Interaction of calf thymus DNA-cellulose with heat-activated (○—○) or non-activated (△—△) HeLa S_3 cell glucocorticoid receptor. Panels B-D, Competition of isolated DNA fragments with calf thymus DNA-cellulose for binding of heat-activated HeLa S_3 cell glucocorticoid receptor. Panel C, MMTV-LTR (□—□); Panel B, pBR322 Taq I-E (○—○); and Panel D, pBR322, Taq I-D (△—△).

preferentially bind a pBR322 DNA fragment containing only a single copy of the GRE consensus sequence.

DISCUSSION

Although from the perspective of biological function, it is surprising to find GRE sites in pBR322, simple probability calculations predict that the octanucleotide 5'-AGA($^{A}/_{T}$)CAG($^{A}/_{T}$)-3' may occur by chance with a frequency of 1/8192 bp and any given hexanucleotide at a frequency of 1/2048 bp. Thus the frequencies of occurrence that we observe for GRE consensus sequences in pBR322 are reasonably consistent with a simple probability model. This relatively frequent chance occurrence of GREs

suggests that obtaining appropriate hormonal regulation of gene expression is likely to require more than the simple presence of a GRE consensus sequence. What we believe to be equally important, however, is the evidence that, whether they occur by chance or by design, these sequences seem to show preferential interaction with the glucocorticoid receptor. The ability of HeLa S_3 cell glucocorticoid receptor to show preferential binding of the pBR322 Taq I-D fragment (containing a single copy of the octanucleotide GRE) compared to the pBR322 Taq I-E fragment in both the nitrocellulose filter binding and DNA-cellulose competitive binding experiments demonstrates the importance of this core consensus sequence to glucocorticoid receptor interaction with DNA. On the other hand, there is a large difference in binding of the pBR322 Taq I-D fragment relative to the MMTV-LTR fragment by the nitrocellulose filter immobilized glucocorticoid receptor, as seen in Fig. 2. Since the MMTV-LTR fragment contains at least four GRE sites, much of this difference in binding might be due to a simple difference in the number of binding sites. However, there appears to be an even greater than four-fold difference in binding of these two DNA fragments by the nitrocellulose filter immobilized glucocorticoid receptor. This again suggests that the MMTV-LTR fragment may contain features in addition to the simple presence of the GRE core consensus sequence that lead to higher affinity binding by glucocorticoid receptor. Such features might relate to the close juxtaposition of multiple GRE sites seen in the MMTV-LTR. Alternately, for many of the GRE sites in the MMTV-LTR, as well as for GRE sites in the human growth hormone gene and the human metallothionein gene, plausible DNA hairpin structures involving the GRE core consensus sequences can be drawn. Since similar hairpin structures are not apparent for the pBR322 Taq I-D fragment, it is tempting to speculate that DNA secondary structure may be involved in the differential binding of these DNA fragments by the glucocorticoid receptor. Results of the current study cannot distinguish among these possibilities.

Nonetheless, the observation that glucocorticoid receptor shows preferential binding of a DNA fragment containing a single copy of the GRE core sequence suggests that glucocorticoid receptor interactions with DNA may be even more sequence dependent than previously recognized. That these sequence patterns may occur by chance at moderate frequency suggests that much of what in the past has commonly been attributed to a nonspecific interaction of glucocorticoid receptor with random DNA, may instead be a somewhat more specific interaction of glucocorticoid receptor with DNA sequence patterns occurring at unexpected locations.

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