Affinity of Interactions between Human Glucocorticoid Receptors and DNA: At Physiologic Ionic Strength, Stable Binding Occurs Only with DNAs Containing Partially Symmetric Glucocorticoid Response Elements†

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ABSTRACT: Sucrose density gradient shift assays were adapted to permit determination of the affinity of interaction between human glucocorticoid receptors (GR) and DNA under conditions of DNA excess. Saturation analyses were performed to ascertain dissociation constants for the interaction of activated human GR with each of five DNA fragments. Centrifugation of GR-DNA complexes on sucrose gradients under nearly isotonic salt conditions revealed similar affinities with dissociation constants in the range of 2-16 nM for GR interaction with DNA fragments containing glucocorticoid response elements (GREs) exhibiting partial dyad symmetry. By contrast, GR exhibited virtually no affinity for non-GRE-containing DNA or for DNA containing only GRE half-sites. Additionally, GR showed evidence of multiple-site interaction with a DNA fragment containing two partially symmetric GREs, but interacted at only one site of an MMTV LTR DNA fragment containing a single partially symmetric GRE along with a cluster of three half-GREs. Together these data indicate that under physiologically relevant conditions, glucocorticoid receptors have high selectivity and affinity only for DNA containing specific partially symmetric GREs and further suggest that this high affinity for such DNA sites may be sufficient to account for the selective regulation of gene expression observed in glucocorticoid-responsive cells.

Glucocorticoids, like other steroid hormones, modulate expression of specific gene products in hormone-responsive cells through a direct interaction of the hormone–receptor complex with specific regulatory sites, called glucocorticoid response elements (GREs),[1] in or near the promoter regions of regulated genes [for a review, see Beato (1989), Burnstein and Cidlowski (1989), Gustafsson et al. (1987), and Yamamoto (1985)]. Apparently, the number of glucocorticoid-regulated genes in hormone-responsive cells is rather low. For example, Ivarie and O'Farrell (1978) showed by two-dimensional gel electrophoresis that in 2 rat hepatoma cell lines glucocorticoids directly induced or repressed the expression of only 10–20 proteins. Similarly employing giant two-dimensional gels, Colbert et al. (1985) detected only 16 glucocorticoid-induced changes in the profiles of proteins expressed in normal hepatocytes. Recently, Harrison et al. reported finding a maximum of 300 GRE-containing genes in the entire mouse genome (Harrison et al., 1990). Thus, it seems unlikely that any particular cell is apt to contain more than 30, or so, transcriptionally active glucocorticoid-responsive genes. Since GRE DNA sequences are generally less than 30 base pairs (bp) in length (Beato et al., 1989), and mammalian genomes contain approximately 3×10^9 base pairs of DNA per haploid genome (Britten & Davidson, 1969), a typical cell contains enough DNA to have on the order of 2×10^8 nonoverlapping sites at which glucocorticoid–receptor complexes could potentially interact. This large number of potential binding sites far exceeds the number of glucocorticoid receptors typically present in steroid-responsive cells [e.g., approximately 20 000 GR/cell in HeLa S_3 cells (Cidlowski & Cidlowski, 1981), or approximately 5000 GR/cell in rat liver cells (Kalimi et al., 1973)]. Thus, a major task of receptor essential for rapid regulation of gene expression must be the identification and stable binding of its proper binding sites in the midst of the large excess of DNA residing in the cell nucleus.

Interestingly, in contrast to such in vivo conditions, most current techniques for analysis of GR-DNA interaction, particularly nuclease protection experiments, are performed under conditions in which purified receptor is present in large excess with respect to the amount of DNA. Such protocols are also often performed under conditions of relatively low ionic strength (Payvar et al., 1983; Chalepakis et al., 1988; Drouin et al., 1989).

We have previously shown that the stability of GR-DNA complexes on sucrose density gradients is markedly affected by even moderate alterations in either salt concentration or pH and that at physiologic ionic strength only complexes with GRE-containing DNA remain stable (Tully & Cidlowski, 1989). In an effort to gain further insight into how glucocorticoid receptors might interact with DNA in the environment of a cell, we have now employed sucrose density gradient shift assays to study the affinity of GR-DNA interactions under conditions in which defined fragments of DNA are present in excess with respect to GR. We have used this assay

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[1] Abbreviations: ALD, aldolase; B_{max}, maximum bound; CAT, catalase; EDTA, ethylenediaminetetraacetic acid; GR, glucocorticoid receptor(s); GRE, glucocorticoid response element; JMEM, Joklik's minimal essential medium; K_d, dissociation constant; LTR, long terminal repeat; MMTV, mouse mammary tumor virus; MYO, myosin; THY, thyroglobulin; [^{3}H]TA,[6,7-^{3}H]triamcinolone acetonide; tris, tris(hydroxymethyl)aminomethane.

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to determine dissociation constants (K_d) for the interaction of human GR with defined DNA fragments containing zero, one, two, or multiple GREs under nearly isotonic salt conditions. We find that, under physiologically relevant conditions, GR has high selectivity and affinity only for DNAs containing at least partially symmetric GREs.

Materials and Methods

Buffers. The buffers used were buffer A [20 mM sodium phosphate (pH 7.0), 50 mM NaCl, 2 mM 2-mercaptoethanol, 1 mM EDTA, and 10% (w/v) glycerol] or a modification of buffer A containing an additional 50 mM KCl. Additional components are indicated where appropriate.

Cell Culture and Preparation of Cytosols. Cytosols were prepared from HeLa S_3 cells as previously described (Cidlowski & Richon, 1984; Tully & Cidlowski, 1989), except for minor modifications. Briefly, HeLa S_3 cells were grown in suspension culture at 37 °C in Joklik's minimal essential medium (JMEM) containing 2 mM glutamine, 75 units/mL penicillin G, 50 units/mL streptomycin sulfate, and 3.0% of a 1:1 (v/v) mixture of fetal calf serum and calf serum. Cells were harvested at 2500*g* in a Sorvall GSA rotor at 4 °C, washed in 50 mL of cold, unsupplemented JMEM, pelleted again, and finally resuspended in cold, unsupplemented JMEM to a final cell density of (2–4) × 10^7 cells/mL. Aliquots of the final cell suspension were counted by using a Coulter Model ZM cell counter (Coulter Electronics, Hialeah, FL) to obtain accurate cell counts for each experiment, and (2–4) × 10^8 cells were incubated for 2 h at 0 °C with 40 nM [6,7-^{3}H]triamcinolone acetonide ([^{3}H]TA) (specific activity 43.6 Ci/mmol; New England Nuclear, Boston MA) with gentle agitation. The steroid-treated cells were pelleted at 2000*g* in a refrigerated centrifuge, resuspended in an equal volume of ice-cold buffer A, and homogenized using a prechilled Tekmar Ultra Turrax homogenizer (Tekmar Co., Cincinnati, OH) for three 10-s bursts with 5–10-s rests on ice. The homogenate was immediately centrifuged at 165000*g* in a Beckman 50 Ti rotor for 1 h at 0 °C. The high-speed supernatant (cytosol) was incubated for 5 min with the pellet from an equal volume of a suspension of dextran-coated charcoal [1.0% (w/v) activated charcoal in 0.1% (w/v) dextran/1.5 mM $MgCl_2$]. The cytosol–charcoal suspension was centrifuged at 12000*g* for 10 min at 0 °C, and the supernatant was collected. The charcoal-stripped cytosol was heat-activated by incubation for 30 min at 27 °C.

DNA Constructs and Preparation of DNA Fragments. The dimer GRE construct was prepared as follows: Complementary 47-mer oligonucleotides containing the DNA sequences needed to create a *Hin*dIII-cohesive end, two GRE sites separated by *Sph*I and *Xba*I restriction linkers, and terminating in a *Bam*HI-cohesive end (see Figure 1) were synthesized and cloned into the *Hin*dIII and *Bam*HI sites of the pGEM 3Zf(−) cloning/sequencing vector (Promega Corp., Madison, WI). The DNA sequence of the synthetic oligonucleotide inserted into this plasmid was verified by using DNA sequencing protocols provided by Promega Corp. To obtain the monomer GRE construct, the dimer GRE plasmid was digested with *Hin*dIII and *Sph*I to remove one of the GR binding sites and then treated with the Klenow fragment of DNA Pol I and religated, using standard techniques (Maniatis et al., 1982). *Pvu*II digests of the dimer GRE and monomer GRE plasmids produce 396 and 376 bp fragments, respectively, containing the oligonucleotide inserts. The remaining DNA fragments used in these studies have been previously described (Tully & Cidlowski, 1987, 1989). Briefly, the MMTV LTR DNA fragment is a 326 bp fragment including nucleotides −222 to +104 relative to the primary transcription start site in the 5′ long terminal repeat of the mouse mammary tumor virus (Majors & Varmus, 1983), which contains three or two regions of sequence protected to varying degrees in nuclease protection experiments with purified GR (Payvar et al., 1983; Scheidereit & Beato, 1984). The pBR322, *Taq*I-E DNA fragment is a 315 bp fragment (nucleotides 24–339 of pBR322) which contains no detectable GR binding sites (Tully & Cidlowski, 1987; Sutcliffe, 1978; Peden, 1983). The pBR322, *Taq*I-D DNA fragment is a 368 bp fragment (nucleotides 4020 to 24 of pBR322) which contains a single site (TGATCT) homologous to the GRE DNA consensus sequence initially identified by Payvar et al. (1983), but which differs by a single nucleotide from the more widely recognized TGTTCT GRE DNA consensus sequence (Beato, 1989). These DNA fragments were excised by using the appropriate restriction enzymes and purified on 5% polyacrylamide gels as previously described (Maxam & Gilbert, 1980).

DNA Binding and Sucrose Gradient Centrifugation. Aliquots of cytosol (0.3–0.5 mL) containing 0.4–0.9 pmol of [^{3}H]TA-labeled GR were incubated with various quantities of purified DNA fragments in the presence of 5 μg of poly(dI-dC) competitor DNA (Sigma Chemical Co., St. Louis, MO) for 2 h at 0 °C with gentle agitation. Following DNA binding, samples were loaded onto linear sucrose density gradients made up in buffer A supplemented with an additional 50 mM KCl, and centrifuged for 16 h at 189000*g* in a Beckman SW50.1 rotor at 3 °C. Following centrifugation, gradients were fractionated from the bottom by 10-drop fractions into scintillation vials and counted in 7 mL of Scinti-Verse E (Fisher Scientific Co., Raleigh, NC) using a Beckman LS 3801 liquid scintillation counter (^{3}H counting efficiency approximately 55%, Figures 2–4) or a Beckman LS 7000 scintillation counter (^{3}H counting efficiency approximately 30%, Figures 5 and 6). The tube bottoms, containing any pelleted material, were cut off and counted as the "zero" fraction. DNA fragments not treated with cytosol extracts and protein molecular mass markers were centrifuged on parallel gradients. Sedimentation coefficients of GR, DNA, and GR–DNA complexes were determined relative to the molecular mass markers (Martin & Ames, 1961).

Data Analysis. The fraction (Frac *X*) corresponding to the minimum point between 4S activated GR and residual 8S nonactivated GR was chosen as the demarcation between free GR and GR–DNA complex in each experiment. ^{3}H cpm for the fractions corresponding to regions of the gradient that would contain GR–DNA complex (Frac 1 to Frac *X*) were summed for each gradient. The sum of cpm over this region (including residual 8S nonactivated GR) for the "zero DNA" control gradient in each experiment was taken as background, and this amount was subtracted from the sum of cpm over the corresponding region of each of the remaining gradients. The difference obtained was used as a measure of the fraction of GR complexed with DNA. The total concentration of GR contained in the cytosol extract and in each assay sample was calculated from cell counts, based on earlier work in this laboratory indicating that HeLa S_3 cells contain approximately 20000 GR/cell (Cidlowski & Cidlowski, 1981). The number of picomoles of GR bound to DNA was calculated from the fraction of GR complexed with DNA multiplied by the total picomoles of GR in each sample. The number of picomoles of DNA bound to GR was calculated, assuming that glucocorticoid receptors occupied each GRE site on the DNA as a homodimer (Wrange et al., 1989). The number of picomoles of DNA free (i.e., not bound to GR) was calculated by sub-

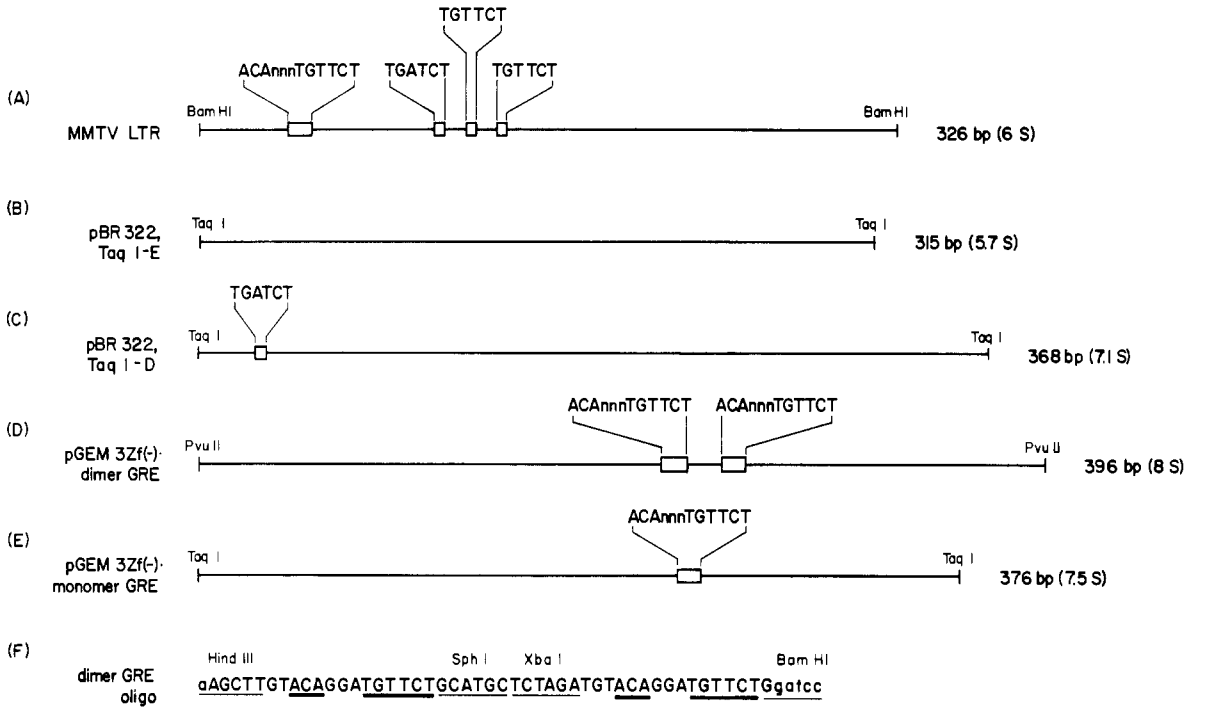

FIGURE 1: DNA fragments used in GR binding experiments. (A) The MMTV LTR DNA fragment is a 326 bp approximately 6S fragment derived from nucleotides −222 to +104 of the 5′ long terminal repeat of the mouse mammary tumor virus genome, and containing three GR footprints and three occurrences of the TGTTCT GRE DNA consensus sequence (Payvar et al., 1983). (B) The pBR322, *Taq*I-E DNA fragment is a 315 bp approximately 5.7S fragment which contains no detectable GREs (Tully & Cidlowski, 1987, 1989). (C) The pBR322, *Taq*I-D DNA fragment is a 368 bp approximately 7.1S fragment which contains a single occurrence of a hexanucleotide sequence (TGATCT) matching one of the half-GRE sites in the MMTV LTR DNA fragment. (D) The dimer GRE DNA fragment is a 396 bp approximately 8S fragment containing two linked copies of the GRE from the tyrosine aminotransferase gene promoter (Jantzen et al., 1987; Klock et al., 1987; Strahle et al., 1987). (E) The monomer GRE DNA fragment is a 376 bp approximately 7.5S fragment containing a single copy of the GRE from the tyrosine aminotransferase gene promoter. (F) DNA sequence of one strand of the 47-mer oligodeoxynucleotide synthesized for preparation of the dimer GRE and monomer GRE DNA constructs.

tracting the picomoles of DNA bound to GR for each gradient sample from the total picomoles of DNA added to that sample. The ratio of DNA bound to DNA free was then calculated for each sample, and the data were plotted by the method of Scatchard (1949). Linear regression calculations were used to determine the best-fit line through each Scatchard plot, and dissociation constants (K_d's) were calculated from the slope of the regression line. Estimates for the maximum concentration of stable GR–DNA complexes formed with each DNA fragment (B_{max}) were obtained by extrapolation of the linear regression line to its point of intersection with the horizontal axis of the Scatchard plot. The results of these calculations are summarized in Table I, and a representative example of the plots used in these experiments is given in Figure 7.

Table I: Affinities of Human Glucocorticoid Receptor Interaction with Selected DNA Fragments under Isotonic Salt Conditions[a]

DNA fragment used	K_d (nM)	B_{max} (nM)
MMTV GRE	11.1 ± 4.6	0.5 ± 0.25
pBR322, *Taq*I-E (no GRE)	indeterminate	<0.003
pBR322, *Taq*I-D (half-GRE)	indeterminate	<0.003
dimer GRE	2.5 ± 1.0	0.4 ± 0.1
monomer GRE	4.0 ± 1.0	0.5 ± 0.2

[a] The dissociation constants (K_d) for the interaction of human glucocorticoid receptors with each DNA fragment were calculated from Scatchard plots, as described under Materials and Methods. The theoretical maximum concentration of GR–DNA complexes formed with each DNA fragment (B_{max}) was estimated by extrapolating the linear regression line to its intersection with the horizontal axis of the Scatchard plot. Values reported in Table I were averaged from two independent experiments with each DNA fragment.

RESULTS

In an effort to gain further understanding of the means by which glucocorticoid receptors might select and bind specific GRE sites in the context of cellular DNA, we sought to measure the affinity of interactions between GR and various specific DNA fragments under conditions of DNA excess with respect to GR. Sucrose density gradient shift assays were employed to determine the affinity of interaction between GR and each of five purified DNA fragments. The DNA fragments used in these studies are shown schematically in Figure 1. The MMTV LTR DNA fragment (Figure 1A), derived from the 5′ long terminal repeat of the mouse mammary tumor virus, contains three regions of sequence protected to varying degrees by rat GR in nuclease protection experiments (Payvar et al., 1983). The most prominent protected region (−189 to −166) contains a GRE (ACAnnnTGTTCT) exhibiting partial dyad symmetry. Another protected region (−127 to −77) contains the sequence TGATCT, as well as two additional closely spaced sites containing the TGTTCT GRE DNA consensus sequence, but none of these sites show any apparent dyad symmetry. A third region of sequence in this DNA fragment (−159 to −135) was weakly protected by partially purified rat GR in experiments performed under slightly hypotonic conditions but shows no recognizable sequence homology with the GRE DNA consensus sequence. This 326 bp DNA fragment sediments at approximately 6 S when centrifuged on sucrose gradients in the absence of cytosolic extracts. The pBR322, *Taq*I-E DNA fragment (Figure 1B) is a 315 bp (approximately 5.7 S) fragment which contains no recognizable GRE sequences and which has shown no indication of selective interaction with activated GR (Tully & Cidlowski, 1987, 1989). The pBR322, *Taq*I-D DNA fragment (Figure 1C, 368 bp, approximatley 7.1 S) contains a single site (TGATCT) homologous to the GRE DNA consensus

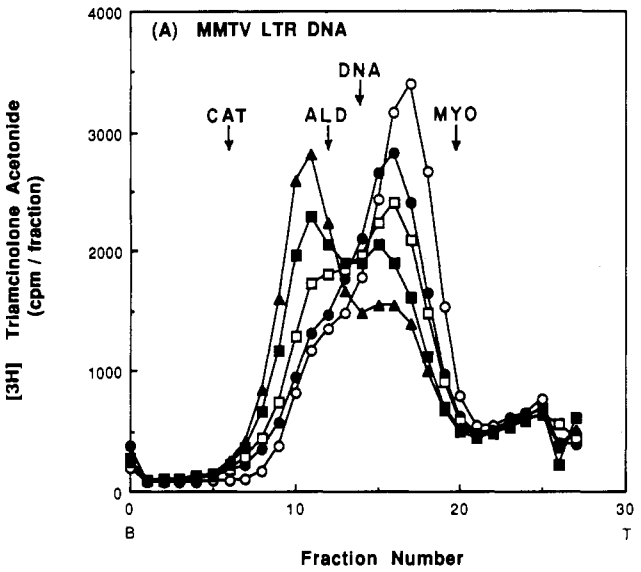

FIGURE 2: Sedimentation rate analysis of HeLa S_3 cell cytosol complexes with MMTV LTR (+GRE) DNA. Aliquots of heat-activated [^{3}H]triamcinolone acetonide labeled HeLa S_3 cell cytosol containing approximately 0.47 pmol of GR were incubated with buffer A containing 0.0 (○), 0.5 (●), 1.0 (□), 1.5 (■), or 3.0 (▲) pmol of MMTV LTR DNA fragment for 2 h at 4 °C and layered onto linear 5–20% sucrose density gradients prepared in buffer A containing 50 mM KCl. After centrifugation for 16 h at 3 °C in a Beckman SW 50.1 rotor at 189000*g*, gradients were fractionated into 10-drop fractions, and the tube bottoms were cut off and counted as the "zero" fraction. Positions of protein standards [myoglobin (MYO, 2 S), aldolase (ALD, 7.8 S), and catalase (CAT, 11.3 S)] and of the MMTV LTR DNA fragment (DNA, 6 S) run in parallel gradients are indicated by arrows. The gradient fractions were counted in a Beckman LS 3801 scintillation counter (^{3}H counting efficiency approximately 55%), and the data were analyzed on Scatchard plots, as described under Materials and Methods.

sequence initially identified by Payvar et al. (1983) but differs by a single nucleotide from the more widely recognized TGTTCT GRE DNA consensus sequence (Beato, 1989). The GRE in the pBR322, *Taq*I-D DNA fragment shows no apparent dyad symmetry and is referred to in these studies as a "half GRE" DNA fragment. The dimer GRE DNA fragment (Figure 1D, 396 bp, approximately 8 S) contains a 47-mer synthetic oligonucleotide insert containing two linked copies of a sequence matching the high-affinity GR binding site from the promoter region of the tyrosine aminotransferase gene (Jantzen et al., 1987; Strahle et al., 1987; Klock et al., 1987). This GRE exhibits partial dyad symmetry, similar to that seen in the −189 to −166 site of the MMTV LTR DNA. One copy of the GRE from the tyrosine aminotransferase gene has been deleted from the dimer GRE construct to produce the monomer GRE construct containing a single copy of this partially symmetric GRE in a 376 bp, approximately 7.5S, *Pvu*II fragment (Figure 1E). The DNA sequence of one strand of the oligodeoxynucleotide used in preparation of the dimer GRE and monomer GRE constructs is shown in Figure 1F.

Figure 2 shows the sedimentation profiles of heat-activated [^{3}H]TA cytosol alone, or after incubation with 0.5, 1.0, 1.5, or 3.0 pmol of MMTV LTR DNA, centrifuged on linear 5–20% sucrose gradients prepared in buffer A containing 50 mM KCl. Under these nearly isotonic conditions, the sedimentation profile for activated cytosol alone showed a predominant peak of [^{3}H]TA sedimenting at 4 S, with a small shoulder of [^{3}H]TA sedimenting in the 8S region of the gradient. We have previously shown that, when centrifuged under hypotonic conditions on sucrose gradients prepared in buffer A alone, heat-activated [^{3}H]TA-labeled GR from cytosols prepared by our standard protocol sedimented as two peaks with sedimentation positions of 4 S and 8 S, corresponding to 4S activated and 8S nonactivated forms of GR, respectively (Tully & Cidlowski, 1989; Holbrook et al., 1983; Vedeckis, 1983; Currie & Cidlowski, 1982). It has also been shown that a 5-fold excess of MMTV LTR DNA fragment fails to shift detectable quantities of the 8S nonactivated GR (Tully & Cidlowski, 1989). Since GR would be expected to undergo further activation to the 4S form as a consequence of exposure to increased salt, it is likely that the 4S peak seen here represents the 4S activated, DNA binding form of GR, while the shoulder of material sedimenting near 8 S corresponds to a small amount of residual 8S nonactivated GR which does not bind DNA. As shown here in Figure 2, incubation of the activated cytosol with increasing aliquots of MMTV LTR DNA prior to centrifugation on gradients prepared in isotonic salt resulted in progressive decreases in the amount of [^{3}H]TA sedimenting at 4 S and concomitant increases in the amount of [^{3}H]TA sedimenting as newly formed peaks at larger *s* values in the 9–10S region of the gradients. Scatchard plot analysis of data from two independent experiments with the MMTV LTR DNA fragment indicated dissociation constants in the range of 6.5–15.6 nM for interaction of GR with this DNA fragment under isotonic conditions (Table I). These results demonstrated that activated human GR could interact with the MMTV LTR DNA to form high-affinity complexes which remained stable during centrifugation on sucrose gradients under isotonic salt conditions. We wished to determine whether the stable complexes observed between GR and the MMTV LTR DNA could be attributed solely to interaction of GR at the specific GRE sites in this DNA fragment. We had previously found that, under hypotonic salt conditions, activated GR interacted to a lesser degree in an apparently non-sequence-specific manner with a DNA fragment from pBR322 which contains no recognizable GRE sites. We therefore wished to ascertain whether GR would have measurable affinity for this fragment under the isotonic salt conditions employed above.

Figure 3 shows the sedimentation profiles of heat-activated [^{3}H]TA cytosol alone, or after incubation with 0.5, 1.0, 1.5, 2.0, or 3.0 pmol of the pBR322, *Taq*I-E (−GRE) DNA, on linear 5–20% sucrose gradients prepared in buffer A containing 50 mM KCl. As seen before in Figure 2, the sedimentation profile for activated cytosol alone again showed a major peak of [^{3}H]TA sedimenting at approximately 4 S with a small shoulder of [^{3}H]TA sedimenting at approximately 8 S. In sharp contrast to the results obtained using the MMTV LTR DNA, however, incubation of activated GR with increasing amounts of pBR322, *Taq*I-E (−GRE) DNA and subsequent centrifugation on sucrose gradients prepared in buffer A containing 50 mM KCl showed no indication of the formation of stable complexes between GR and this DNA, even at the highest concentration of DNA, when the pBR322, *Taq*I-E (−GRE) DNA fragment was present at greater than 5-fold excess over GR. Analysis of data from experiments with this DNA fragment on Scatchard plots showed no measurable affinity for interaction of GR with this DNA (see Figure 7 for a plot of the data from one of the experiments with this fragment). These results indicated that, under isotonic salt conditions, non-sequence-specific interactions between GR and DNA were virtually completely disrupted and therefore suggested that the stable complexes observed between GR and MMTV LTR DNA must be due to binding at specific GRE sites.

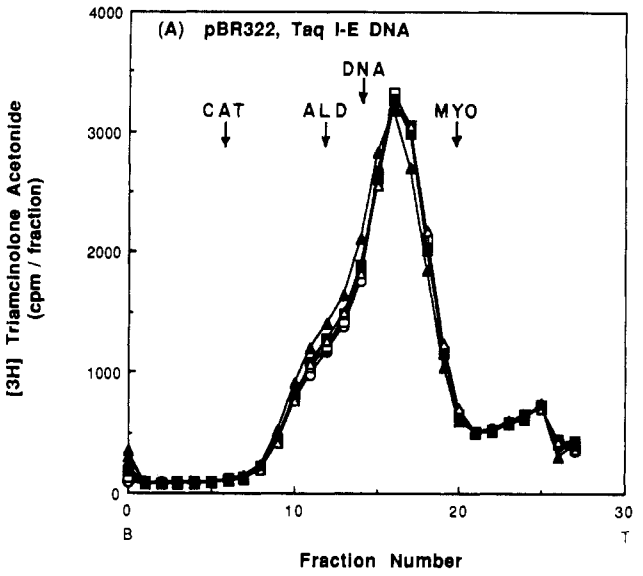

FIGURE 3: Sedimentation rate analysis of HeLa S_3 cell cytosol complexes with pBR322, *Taq*I-E (–GRE) DNA fragment. Aliquots of heat-activated, [^{3}H]triamcinolone acetonide labeled HeLa S_3 cell cytosol containing approxiamtely 0.47 pmol of GR were incubated with buffer A containing 0.0 (○), 0.5 (●), 1.0 (□), 1.5 (■), 2.0 (△), or 3.0 (▲) pmol of pBR322, *Taq*I-E DNA fragment for 2 h at 4 °C and subsequently centrifuged on linear 5–20% sucrose density gradients prepared in buffer A containing 50 mM KCl, as described in Figure 2. The positions of the protein standards [MYO (2 S), ALD (7.8 S), and CAT (11.3 S)] and of the pBR322, *Taq*I-E DNA fragment (DNA 5.7 S) are marked by arrows. Gradient fractions were counted in a Beckman LS 3801 scintillation counter (^{3}H counting efficiency approximately 55%).

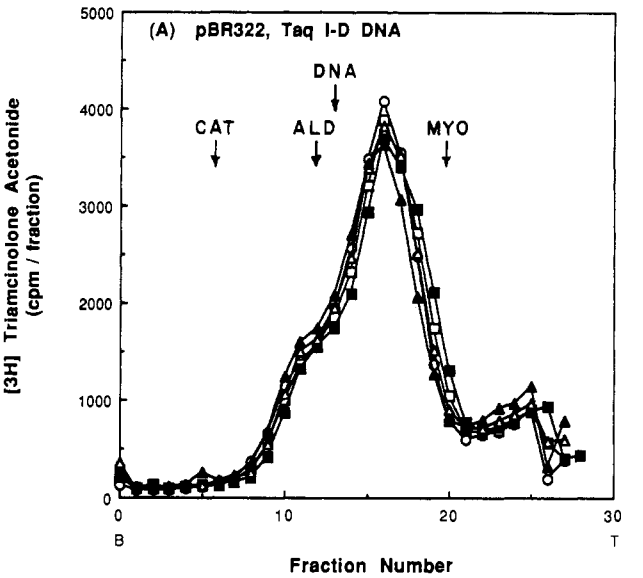

FIGURE 4: Sedimentation rate analysis of HeLa S_3 cell cytosol complexes with pBR322, *Taq*I-D (half-GRE) DNA fragment. Aliquots of heat-activated, [^{3}H]triamcinolone acetonide labeled HeLa S_3 cell cytosol containing approximately 0.81 pmol of GR were incubated with buffer A containing 0.0 (○), 0.5 (●), 1.0 (□), 1.5 (■), 2.0 (△), or 3.0 (▲) pmol of pBR322, *Taq*I-D DNA fragment for 2 h at 4 °C and subsequently centrifuged on 5–20% sucrose density gradients prepared in buffer A containing 50 mM KCl, as described in Figure 2. Again, arrows indicate the positions of the protein standards [MYO (2 S), ALD (7.8 S), and CAT (11.3 S)] and of the pBR322, *Taq*I-D DNA fragment (DNA, 7.1 S). Gradient fractions were counted in a Beckman LS 3801 scintillation counter (^{3}H counting efficiency approximately 55%).

Since the MMTV LTR DNA fragment contains a complex pattern of half-GRE sites that show no apparent dyad symmetry, in addition to a strongly protected site that exhibits partial dyad symmetry (−189 to −166), we next determined whether GR interacion at half-GRE sites could account for some of the stable complex formation we had seen with this DNA. To estimate the affinity of interaction of GR with a half-GRE, we performed similar experiments using the pBR322, *Taq*I-D DNA fragment (Tully & Cidlowski, 1987), which contains a single presumably fortuitous occurrence of the sequence TGATCT matching one of the half-GRE sites in the broadly protected region (−127 to −77) of MMTV LTR DNA (Payvar et al., 1983). Figure 4 shows the sedimentation profiles obtained when aliquots of activated GR cytosols were incubated with increasing quantities of the pBR322, *Taq*I-D DNA fragment and subsequently centrifuged on linear 5–20% sucrose gradients prepared in buffer A containing an additional 50 mM KCl. As evident from the figure, under these nearly isotonic salt conditions, virtually no stable complexes between GR and this DNA were observed. Furthermore, as might be expected from the overall appearance of the sedimentation profiles, Scatchard plot analysis of these data indicated no measurable affinity for interaction of GR with this DNA under isotonic conditions. Thus, even though this half-GRE DNA fragment shows some degree of selective interaction with activated GR under hypotonic conditions (Tully & Cidlowski, 1989), it is clear that GR complexes with this DNA fragment were not stable when centrifuged on sucrose gradients under isotonic conditions. These results suggested that GR binding at the GRE half-sites in the MMTV LTR DNA fragment was unlikely to account for the stable complexes observed with this fragment, unless there had been some effect due to the closely spaced pattern of multiple GREs that occur in this fragment.

To further assess the nature of GR–DNA interactions, we next wished to ascertain the affinity of GR binding to DNA fragments that contained one or two strong GR binding sites in a DNA sequence environment that did not contain other GREs of differing affinity or specific binding sites for other DNA binding proteins. To accomplish this, a DNA oligonucleotide containing two identical copies of the high-affinity GRE from the tyrosine aminotransferase gene promoter was synthesized and inserted into the pGEM3Zf(−) vector, as described under Materials and Methods. Figure 5 shows the sedimentation profiles of heat-activated [^{3}H]TA cytosol alone, or after incubation with 0.5, 1.0, 1.5, 2.0, or 3.0 pmol of the dimer GRE-containing *Pvu*II fragment from this plasmid, centrifuged on linear 10–30% sucrose gradients prepared in buffer A containing 50 mM KCl. In a corollary experiment, incubation of activated GR with 3.0 pmol of the corresponding *Pvu*II fragment from the pGEM3Zf(−) vector, used for preparation of the dimer GRE construct, showed no evidence of specific interaction between activated GR and the vector DNA fragment (data not shown). Sucrose gradients of 10–30% were used in experiments with these somewhat larger (approximately 8S) DNA fragments to give better resolution in the high molecular weight (i.e., 10–20S) range of the gradients. In contrast to the sedimentation profiles observed with the MMTV LTR DNA fragment, the sedimentation profiles for the dimer GRE DNA fragment, shown in Figure 5, indicate that GR interacted with this dimer GRE fragment to form complexes of two different sizes. Interestingly, at the lowest concentration of DNA used, where GR was present in nearly 2-fold excess over DNA, most of the new complexes formed sedimented at a larger *s* value in the 12–16S region of the gradient. As more DNA was added in the incubation, an increasing proportion of the new complexes formed sedimented in the 10–12S region of the gradient. Thus, the distribution of GR among complexes of at least two different sizes varied at least partly as a function of the relative proportions of DNA and GR present in the incubation mixture. These

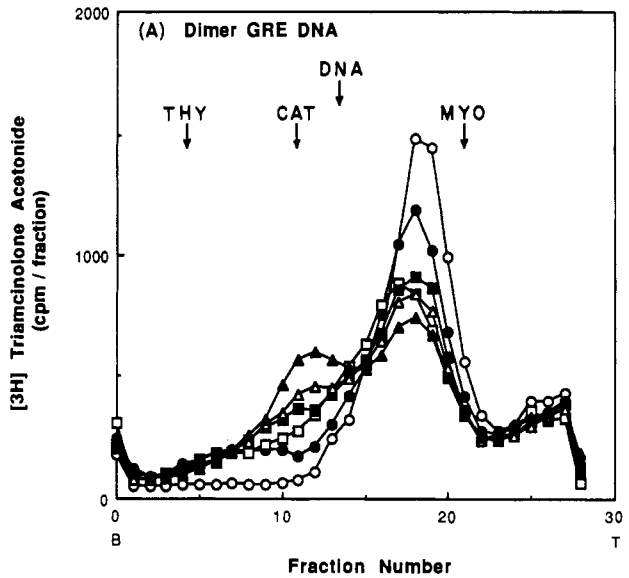

FIGURE 5: Sedimentation rate analysis of HeLa S_3 cell cytosol complexes with pGEM3Zf(−)·dimer GRE, *Pvu*II-B DNA fragment. Aliquots of heat-activated, [^{3}H]triamcinolone acetonide labeled HeLa S_3 cell cytosol containing approximately 0.73 pmol of GR were incubated with buffer A containing 0.0 (○), 0.5 (●), 1.0 (□), 1.5 (■), 2.0 (△), or 3.0 (▲) pmol of pGEM3Zf(−)·dimer GRE, *Pvu*II-B DNA fragment for 2 h at 4 °C and subsequently centrifuged on linear 10–30% sucrose density gradients prepared in buffer A containing 50 mM KCl, as described in Figure 2. Arrows indicate the positions of the protein standards [MYO (2 S), CAT (11.3 S), and thyroglobulin (THY, 19.4 S)] and of the dimer GRE DNA fragment (DNA, 8 S). Gradient fractions were counted in a Beckman LS 7000 scintillation counter (^{3}H counting efficiency approximately 30%).

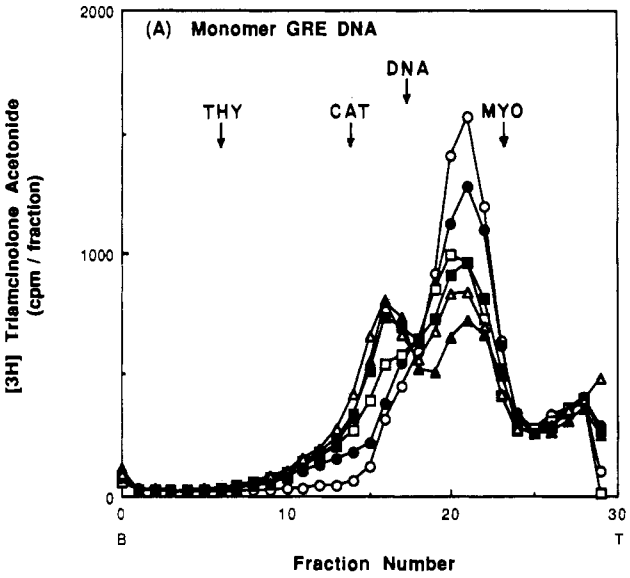

FIGURE 6: Sedimentation rate analysis of HeLa S_3 cell cytosol complexes with pGEM3Zf(−)·monomer GRE, *Pvu*II-B DNA fragment. Aliquots of heat-activated, [^{3}H]triamcinolone acetonide labeled HeLa S_3 cell cytosol containing approximately 0.92 pmol of GR were incubated with buffer A containing 0.0 (○), 0.5 (●), 1.0 (□), 1.5 (■), 2.0 (△), or 3.0 (▲) pmol of pGEM3Zf(−)·monomer GRE, *Pvu*II-B DNA fragment for 2 h at 4 °C and subsequently centrifuged on linear 10–30% sucrose density gradients prepared in buffer A containing 50 mM KCl, as described in Figure 2. The positions of protein standards [MYO (2 S), CAT (11.3 S), and THY (19.4 S)] and of the dimer GRE DNA fragment (DNA, 8 S) are shown by arrows. Gradient fractions were counted in a Beckman LS 7000 scintillation counter (^{3}H counting efficiency approximately 30%).

results suggested that when receptor was present in excess over DNA, large 12–16S complexes formed in which dimeric receptors may have occupied both of the high-affinity binding sites present in the dimer GRE fragment, and that as the ratio of DNA to receptor increased, conditions began to favor the formation of smaller complexes in which only one of the two GRE sites was occupied by receptor. If this interpretation were correct, one might expect that a DNA fragment containing only a single GRE would form the smaller 10–12S complexes but would fail to form the larger 12–16S complexes. To test this possibility, one of the GREs was excised from the dimer GRE plasmid construct, as described under Materials and Methods, and analogous experiments were performed with the *Pvu*II DNA fragment obtained from this plasmid.

Figure 6 shows the sedimentation profiles of heat-activated [^{3}H]TA cytosol alone, or after incubation with 0.5, 1.0, 1.5, 2.0, or 3.0 pmol of the monomer GRE DNA fragment run on linear 10–30% sucrose gradients prepared in buffer A containing 50 mM KCl. As predicted above, addition of increasingly greater amounts of of the monomer GRE DNA fragment in the incubation resulted in progressive decreases in the amount of 4S GR and the concurrent formation of new peaks of [^{3}H]TA sedimenting in the 9–11S region of the gradient. In contrast to the sedimentation profiles seen with the dimer GRE DNA fragment, however, the monomer GRE DNA fragment showed no indication of the formation of the larger 12–16S complexes seen with the dimer GRE fragment. The affinity of GR interaction with the monomer GRE DNA was estimated by Scatchard plots, as described under Materials and Methods, and suggested K_d's in the range of 3.0–5.0 nM (Table I).

Discussion

Table I summarizes the dissociation constants and B_{max} values obtained when aliquots of activated GR cytosol extracts

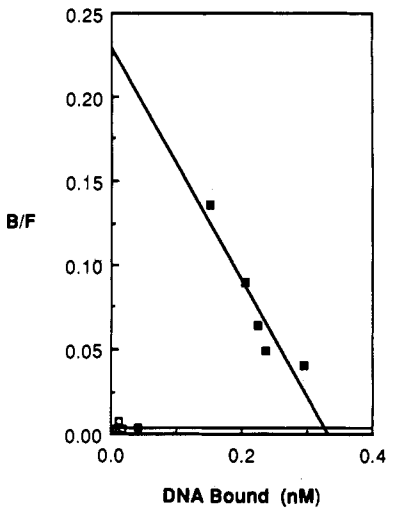

FIGURE 7: Scatchard plot presentation of data derived from saturation binding experiments with the dimer GRE, *Pvu*II-B DNA fragment (■) or the pBR322, *Taq*I-E (−GRE) DNA fragment (□). Data were analyzed as described under Materials and Methods and plotted according to the method of Scatchard (1949). Dissociation constants were calculated as the negative reciprocal of the slope of the linear Scatchard plot, and the B_{max} was determined from the intercept with the horizontal axis.

preincubated with various quantities of each of five DNA fragments were centrifuged on sucrose gradients prepared in isotonic buffer. These values have been calculated, as described under Materials and Methods, by plotting the data on Scatchard plots in which the DNA fragments have been treated as ligands. A representative example of these plots presenting data from binding experiments with the pGEM3Zf(−) dimer GRE, *Pvu*II-B DNA fragment (see Figure 5) and with the pBR322, *Taq*I-E (−GRE) DNA fragment (see Figure 3) is shown in Figure 7. As seen from

Table I, when centrifuged under nearly isotonic salt conditions, GR interacted with comparable affinities in the range of K_d = 2–16 nM with DNAs containing partially symmetric GREs, but showed no measurable affinity for interaction with either the non-GRE-containing pBR322, *Taq*I-E DNA fragment or the half-GRE pBR322, *Taq*I-D DNA fragment. The affinities that we report here for interaction of human GR with DNAs containing partially symmetric GREs are in reasonably close agreement with, although slightly lower than, the affinity (K_d = 1.37 nM) reported for interaction of partially purified rat GR with another DNA fragment containing 17 contiguous copies of an oligonucleotide matching the Strong GR binding site derived from nucleotides −191 to −163 of MMTV LTR (Wrange et al., 1989). Although details of the buffer composition employed in the quantitative nuclease protection experiments from which this K_d was calculated were not provided (Wrange et al., 1989), the reference cited suggests that their experiments may have been conducted under hypotonic conditions as described by Payvar et al. (1983). Under such hypotonic conditions, our unpublished results similarly indicate higher affinities for GR interaction with GRE-containing DNAs, but also suggest more limited DNA sequence specificity. Alternatively, the slightly lower affinities we have measured may derive from the fact that in order to be detected as bound to a given DNA fragment in the sucrose gradient shift assay, a GR–DNA complex would have to be stable throughout the course of gradient centrifugation. If there is some dissociation of GR from DNA during the course of centrifugation, as may well be the case, then the amount of GR–DNA complex observed would be reduced, leading to an underestimate of the actual affinity. Finally, since our experiments have used crude cytosol extracts, it is possible that degradation of the DNA fragments by contaminating nucleases could lead to an overestimate of the concentration of DNA actually present in the assays and result in the calculation of a lower apparent affinity. We consider this possibility unlikely, because cytoplasmic extracts should not be particularly enriched in nucleases; the buffers we have used all contained 1 mM EDTA, which should inhibit nuclease activity; the GR–DNA peaks observed are very homogeneous in size. Nonetheless, the sucrose gradient shift assay offers important advantages which offset its limitations: (1) This assay is conducted with crude cytosol extracts and does not require purification of GR which would be difficult to accomplish from cultured cells. (2) The sedimentation profiles for [^{3}H]TA-labeled GR permit accurate physical characterization of the GR present in the cytosolic extracts. (3) The assay can be conducted under DNA-excess conditions which more closely approximate the internal environment of cells. (4) Specific conditions of the assay, such as details of buffer composition, can easily be modified. (5) Stable GR–DNA complexes can readily be recovered from the gradient fractions for further study.

It is clear from the failure to detect any interaction at all between GR and the pBR322, *Taq*I-E (no GRE) DNA fragment, under isotonic salt conditions, that non-sequence-specific interactions of GR with DNA were virtually completely disrupted under physiological salt conditions. Sequence-specific interactions between proteins and DNA are generally believed to require complementary hydrogen bonding between hydrogen bond donors and acceptors on the protein and on the relevant base pairs of the DNA, while non-sequence-specific binding likely involves electrostatic interactions that would be disrupted by elevated salt conditions (Von Hippel & Berg, 1986). The present results showing no measurable affinity of GR for interaction with the pBR322, *Taq*I-E fragment are consistent with these general expectations and with our earlier results indicating that moderate elevations in salt concentration or pH could disrupt GR interactions with this DNA fragment (Tully & Cidlowski, 1989). These findings thus emphasize the importance of close attention to buffer conditions in studies of GR–DNA interaction.

Since the MMTV LTR DNA fragment also contains specific binding sites for nuclear factor I and the TATA box binding protein, transcription factor II D (Cordingley & Hager, 1988; Cordingley et al., 1987), in addition to its complex pattern of GRE sites (Payvar et al., 1983), there is a small possibility that these proteins could interact with the MMTV LTR DNA in addition to GR. If this should occur, it could alter the sedimentation position of the GR–DNA complexes. However, three points argue against the involvement of these other DNA binding proteins. First, it is unlikely that these nuclear proteins would consistently occur in cytosolic extracts in large enough quantities to affect the outcome of experiments conducted under conditions of DNA excess. Additionally, these experiments have all been conducted in the presence of excess poly(dI–dC) competitor DNA. Finally, the peak containing GR complexes with the MMTV LTR DNA under these nearly isotonic conditions is very homogeneous in size, and is probably not large enough to account for binding of these other proteins in addition to GR.

Several reports have indicated that steroid hormone receptors, including glucocorticoid receptor, bind DNA as homodimers of the hormone–receptor complex and have suggested that dimerization of the receptor protein leads to increased affinity for DNA (Wrange et al., 1989; Kumar & Chambon, 1988; Tsai et al., 1988; Notides et al., 1975; Yamaoto & Alberts, 1972). Wrange et al. (1989) further report that glutaraldehyde-cross-linked homodimers of purified rat liver GR sediment at 6 S on glycerol gradients, either in the absence of DNA or when complexed with a 35 bp DNA fragment containing a single copy of the partially palindromic GRE from −191 to −163 of the MMTV LTR DNA. While our data do not directly demonstrate the presence of GR homodimers, the sedimentation positions of the complexes we see between GR and the larger DNA fragments we have used are large enough to be consistent with GR dimerization, and we have therefore calculated the dissociation constants reported here based on the expectation that GR binds each GRE site as a homodimer. Nonetheless, the dissociation constants for GR–DNA interaction that we and others (Wrange et al., 1989) have measured are much higher than those obtained for interaction of some other DNA binding proteins with their cognate recognition sites [e.g., Rawlins et al. (1984) report an affinity of $K_d = 2 \times 10^{-11}$ M for interaction of nuclear factor I with DNA]. Part of the explanation for this difference may be that while GREs typically contain only partial dyad symmetry, nuclear factor I and many other DNA binding proteins interact with DNA recognition sites that have more extensive regions of dyad symmetry. This increased DNA contact area would be expected to lead to increased affinity for the DNA–protein interaction.

There have also been reports demonstrating cooperative binding and/or synergistic effects on transcriptional enhancement by steroid hormone receptors acting at closely spaced hormone response elements (Martinez & Wahli, 1989; Jantzen et al., 1987). Our results with the dimer GRE construct we have prepared offer strong evidence of GR interaction at both of the GRE sites in this DNA fragment, since we observe GR–DNA complexes of two different sizes (12–16

S and 10–12 S) in the experiments with this fragment (see Figure 5), but do not detect the larger 12–16S complexes in experiments with the monomer GRE fragment (Figure 6). These results do not, however, show clear evidence of the strong positive cooperativity phenomenon that other investigators have observed. If binding of GR to the dimer GRE fragment were highly cooperative, one might expect that the larger 12–16S GR–DNA complexes in which both of the GRE sites in this fragment appear to be occupied by GR would be the predominant species. While this is arguably the case at the lowest concentration of DNA used where GR was present in approximately 2-fold molar excess over the DNA fragment, it clearly does not remain true at higher DNA concentrations. The dissociation constant calculated for GR interaction with the dimer GRE DNA fragment (K_d = 2.5 ± 1.0 nM) is slightly lower than that obtained for the monomer GRE fragment (K_d = 4.0 ± 1.0 nM), which could be suggestive of cooperative binding, but the experimental variability observed in the determination of these values is great enough that these numbers may not actually be different. If the binding data for the dimer GRE DNA fragment are recalculated presuming that the ^{3}H-labeled GR appearing in the 12–16S region of the gradient indeed represents GR occupancy of both sites of the dimer GRE fragment, the recalculated K_d for the dimer GRE fragment (K_d = 3.5 ± 1.2) approaches the K_d for the monomer even more closely. The absence of evidence for cooperative interaction between GR and the dimer GRE DNA fragment in our experiments may stem from the specific distance separating the GREs in this fragment. The *Xenopus laevis* vitellogenin gene for which Martinez and Wahli observed cooperative binding by estrogen receptor contains two imperfect palindromic estrogen response elements in which the nucleotides that form the center of dyad symmetry for each binding site are separated by exactly 20 bp. This means that corresponding nucleotides of the two estrogen response elements in the vitellogenin gene lie on the same face of the DNA double helix and are separated by two turns of the helix. Similarly, the two GREs of the tyrosine aminotransferase gene shown to act synergistically in transcriptional enhancement are separated by 69 bp and thus lie very nearly on the same face of the DNA helix. By contrast, the nucleotides at the center of dyad symmetry of the two GREs in the dimer GRE DNA fragment we have used are separated by 27 bp which means that the binding sites are axially rotated with respect to one another by approximately 108°. The failure to observe cooperative binding effects between GR and the dimer GRE DNA fragment may therefore suggest that protein–protein contacts between closely positioned steroid hormone receptors are required to produce cooperativity effects. More work examining the effect of small alterations in the spacing between closely positioned GREs on GR binding to DNA would have to be done to further evaluate possible cooperative binding effects.

In summary, activated GR showed slightly higher affinity, but less stringent selectivity for DNA binding sites under hypotonic conditions. Under isotonic conditions more closely approximating the internal cellular milieu, however, activated GR retained high affinity in the range of K_d = 2–16 nM for specific partially palindromic GRE sites, but exhibited no measurable affinity for other regions of DNA, even those containing TGTTCT GRE half-sites. This indicates a difference on the order of 10^8–10^9-fold between the affinity of GR for specific partially palindromic DNA binding sites compared with its affinity for nonspecific DNA regions. Assuming that a typical cell likely contains less than 300 000 genes, of which approximately 300 may be glucocorticoid regulated, GR would have to be able to select on the order of 1 in 1000 genes. At least a 1000-fold difference in the affinity of GR for regulated genes with respect to nonspecific genes would be needed to accomplish this selection. The approximately 10^8–10^9-fold difference observed in our experiments between the affinity for GR for specific partially palindromic DNA binding sites, compared with its affinity for nonspecific DNA regions, may thus account for the rapid and highly selective transcriptional regulatory activity that GR exhibits in vivo.

Acknowledgments

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Proteolytic Activities of Human Fibroblast Collagenase: Hydrolysis of a Broad Range of Substrates at a Single Active Site†

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ABSTRACT: The action of human fibroblast collagenase (HFC) on six substrates of markedly different size, sequence, and conformation, including rat type I collagen, rat α1(I) gelatin, β-casein, and the three synthetic oligopeptides Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln, Asp-Val-Ala-Gln-Phe-Val-Leu-Thr-Pro-Gly, and Pro-Val-Gln-Pro-Ile-Gly-Pro-Gln, has been examined. The first peptide is a model for the collagenase cleavage site in the α1(I) chain of type I collagen, while the latter two peptides are models for the autolytic activation and degradation sites in pro-HFC, respectively. The goal of these studies was to assess whether HFC hydrolyzes all of these disparate substrates at the same active site. Individual kinetic parameters for the hydrolysis of all six substrates have been determined. Gel zymography experiments using collagen, gelatin, and casein as substrates show that all three activities are associated solely with HFC rather than impurities. Recombinant HFC expressed in *Escherichia coli* also exhibits caseinase activity, reinforcing the view that this activity is not due to a contaminating protease from fibroblasts. The ratios of these activities agree within experimental error for several independent HFC preparations and do not change when two additional affinity purification steps are employed. The inhibition of the hydrolysis of these substrates by both 1,10-phenanthroline and Boc-Pro-Leu-Gly-NHOH is identical within experimental error. A series of assays carried out in the presence of pairs of these substrates clearly shows that they compete for the same active site. On the basis of these kinetic experiments, it is concluded that HFC has a single active site that is capable of hydrolyzing a much wider variety of natural and synthetic substrates than previously believed.

The first vertebrate collagenase (EC 3.4.24.7) was discovered in tadpole tissue explants by Gross and Lapiere in 1962 (Gross & Lapiere, 1962). The enzyme was subsequently shown to hydrolyze all three α chains of native, triple-helical type I calf skin collagen at Gly-[Leu or Ile] bonds at a single locus located approximately three-fourths from the NH_2 terminus to give two collagen fragments referred to as TC^A, TC^B fragments (Gross & Nagai, 1965; Gross et al., 1974). At temperatures at which these fragments remain triple-helical, subsequent proteolysis was minimal. This characteristic pattern of hydrolysis is now accepted as the key distinguishing feature of vertebrate collagenases (Harris & Vater, 1982; Cawston & Murphy, 1981; Harris et al., 1984; Woolley, 1984; Birked-

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