Articles

Characterization of Human Glucocorticoid Receptor Complexes Formed with DNA Fragments Containing or Lacking Glucocorticoid Response Elements†

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ABSTRACT: Sucrose density gradient shift assays were used to study the interactions of human glucocorticoid receptors (GR) with small DNA fragments either containing or lacking glucocorticoid response element (GRE) DNA consensus sequences. When crude cytoplasmic extracts containing [^{3}H]triamcinolone acetonide ([^{3}H]TA) labeled GR were incubated with unlabeled DNA under conditions of DNA excess, a GRE-containing DNA fragment obtained from the 5′ long terminal repeat of mouse mammary tumor virus (MMTV LTR) formed a stable 12–16S complex with activated, but not nonactivated, [^{3}H]TA receptor. By contrast, if the cytosols were treated with calf thymus DNA–cellulose to deplete non-GR-DNA-binding proteins prior to heat activation, a smaller 7–10S complex was formed with the MMTV LTR DNA fragment. When similar experiments were conducted under conditions of large receptor excess, using 3′ [^{32}P]-MMTV LTR DNA, the trace quantity of DNA formed a stable 10–14S complex with DNA–cellulose pretreated cytosols or with untreated cytosols in the presence of excess *Escherichia coli* competitor DNA. If trace quantities of the 3′ [^{32}P]-MMTV LTR DNA were incubated with untreated crude cytosols, much larger complexes were formed, indicating the association of other cytosolic proteins with the MMTV LTR DNA fragment. Activated [^{3}H]TA receptor from DNA–cellulose pretreated cytosols also interacted with two similarly sized fragments from pBR322 DNA, but with lower apparent affinities in the order MMTV LTR DNA fragment $\gg$ pBR322 fragment containing a single GRE DNA consensus sequence $>$ non-GRE-containing pBR322 fragment. Stability of the complexes formed between GR and these three DNA fragments was strongly affected by even moderate alterations in either the salt concentration or the pH of the gradient buffer. Under all conditions tested, the complex formed with the MMTV LTR DNA fragment was more stable than the complexes formed with either of the pBR322 DNA fragments. Together these observations indicate that the formation of stable complexes between activated GR and isolated DNA fragments requires the presence of GRE consensus sequences in the DNA.

Regulation of gene expression by glucocorticoids and other steroid hormones is believed to occur by means of a direct interaction of the steroid hormone receptor complex with specific sites in the DNA of target cells [for review, see Yamamoto (1985), Beato (1987), Gustafsson et al. (1987), or Burnstein and Cidlowski (1989)]. Upon entering a target cell, glucocorticoids are specifically bound by a soluble receptor protein that subsequently undergoes a poorly understood process termed "activation" or "transformation". The activated hormone receptor complex is then translocated into the cell nucleus where it interacts with specific regulatory sites in DNA to modulate expression of glucocorticoid regulated genes. The 5′ long terminal repeat of mouse mammary tumor virus (MMTV LTR)[1] contains a cluster of such regulatory sites, called glucocorticoid response elements (GREs), and has been widely used as a model system for studies of glucocorticoid regulated gene expression in transfected cells (Chandler et al., 1983), as well as for biochemical characterization of glucocorticoid receptor (GR) interactions with DNA. Nuclease protection experiments with MMTV LTR (Payvar et al., 1983; Scheidereit & Beato, 1984) and with other glucocorticoid regulated genes (Karin et al., 1984; Cato et al., 1984; Moore et al., 1985; Danesch et al., 1987; Klock et al., 1987) have led to the identification of a family of GRE DNA consensus sequences. Nuclease protection experiments are normally performed by using purified GR that is incubated with end-labeled DNA under conditions in which the molar concentration of receptor is in large excess with respect to the concentration of DNA. While these conditions are inherent to the design of nuclease protection experiments, they are far removed from the conditions likely to prevail in intact cells. We therefore wished to determine if [^{3}H]TA-labeled GR from crude cytosolic extracts could discriminate between DNA fragments either containing or lacking GRE sites under conditions in which the ratio of receptor to DNA is in a range that more closely approximates physiologic conditions. We have previously shown that a 368-bp Taq I fragment of pBR322 DNA, designated the Taq I-D fragment, contains a

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[1] Abbreviations: ALD, aldolase; [^{32}P]dCTP, deoxycytidine 5′-[α-^{32}P]triphosphate tetrakis(triethylammonium) salt; EDTA, ethylenediaminetetraacetic acid; GR, glucocorticoid receptor; GRE, glucocorticoid response element; JMEM, Joklik's minimal essential medium; LTR, long terminal repeat; MMTV, mouse mammary tumor virus; MYO, myosin; [^{3}H]TA, [6,7-^{3}H]triamcinolone acetonide; Tris, tris(hydroxymethyl)-aminomethane.

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single octanucleotide homologous to the GRE DNA consensus sequence described by Payvar et al. (1983). Although its interaction with GR is not as strong as that shown by the MMTV LTR DNA fragment, the pBR322 Taq I-D DNA fragment does show preferential interaction with activated GR in DNA–cellulose competitive binding assays (Tully & Cidlowski, 1987), as well as with GR immobilized on nitrocellulose filters by Southwestern blotting (Silva et al., 1987). Conversely, a comparably sized fragment of pBR322 DNA, designated the Taq I-E fragment, which contains no GRE sites, shows only weak or undetectable interaction with GR in both of these assays. We wished to determine if GR from crude cytosolic extracts could interact specifically with these small DNA fragments to form complexes that would remain stable on sucrose density gradients. We report here that activated, but not nonactivated, GR forms stable complexes only with GRE-containing DNAs and that crude cell extracts contain other DNA-binding proteins of unknown nature that participate in the complex formed with the MMTV LTR DNA fragment.

Materials and Methods

Chemicals. [6,7-^{3}H]Triamcinolone acetonide ([^{3}H]TA), specific activity 43.6 Ci/mmol, was obtained from New England Nuclear (Boston, MA). [α-^{32}P]dCTP, specific activity 3000 Ci/mmol, was from ICN Radiochemicals (Irvine, CA). Culture media and supplements were from GIBCO Laboratories (Grand Island, NY). Restriction enzymes and the Klenow fragment of DNA polymerase I were from GIBCO-BRL (Gaithersburg, MD). Calf thymus DNA–cellulose and reagent grade chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Culture and Cytosol Preparation. Cytosols were prepared from HeLa S_3 cells as previously described (Cidlowski & Richon, 1984), except for minor modifications. Briefly, HeLa S_3 cells 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 were harvested at 2500*g* in a Sorvall GSA rotor at 4 °C, washed in cold, unsupplemented JMEM, and resuspended in 4 mL of JMEM. Aliquots of the cell suspensions were counted by using a Coulter Model ZM cell counter (Coulter Electronics, Inc., Hialeah, FL), and the volumes of the cell suspensions were adjusted, if necessary, to yield a final cell density of 2–4 × 10^7 cells/mL. Approximately 2–4 × 10^8 cells were incubated with 40 nM [^{3}H]-triamcinolone acetonide ([^{3}H]TA) for 2 h at 0 °C 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 [20 mM sodium phosphate, pH 7.0, 50 mM NaCl, 10% (v/v) glycerol, 2 mM 2-mercaptoethanol, 1 mM EDTA], and homogenized by 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 either incubated with 0.05 g of calf thymus DNA–cellulose (4 mg of DNA/g of DNA–cellulose) for 5–10 min at 4 °C, followed by incubation 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 $MgCl_2$], or incubated directly with the dextran-coated charcoal. The cytosol–charcoal suspension was centrifuged at 12000*g* for 10 min at 0 °C, and the supernatant was collected. Charcoal-stripped cytosol was either maintained on ice (nonactivated) or heat activated by incubation for 30 min at 27 °C. Estimates for the concentration of GR in the crude cytosols were calculated from cell counts, based on earlier work from this laboratory indicating that HeLa S_3 cells contain approximately 20000 receptors/cell (Cidlowski & Cidlowski, 1981).

Plasmid DNA and Radiolabeling. The three DNA fragments used in these studies have been previously described (Tully & Cidlowski, 1987). Briefly, the MMTV LTR DNA fragment is a 326-bp fragment that includes 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). This DNA fragment contains three regions of sequence protected to varying degrees in nuclease protection experiments with purified GR (Payvar et al., 1983; Scheidereit & Beato, 1984). Two additional fragments were obtained from a Taq I digest of pBR322. The 368-bp Taq I-D DNA fragment extends from nucleotides 4019 to 24 of pBR322 and contains a single GRE site identified by a computer search of the pBR322 DNA sequence (Tully & Cidlowski, 1987; Sutcliffe, 1978; Peden, 1983). The 315-bp Taq I-E DNA fragment extends from nucleotides 24 to 339 and contains no sites matching the GRE DNA consensus sequence. These DNA fragments were excised by using the appropriate restriction enzymes and gel purified on 5% polyacrylamide gels as previously described (Maxam & Gilbert, 1980). The purified DNA fragments were 3′ end labeled by using [α-^{32}P]dCTP and the Klenow fragment of DNA polymerase I by a standard protocol (Maniatis et al., 1982). *Escherichia coli* DNA was sonicated and run on a preparative agarose gel. DNA fragments in the size range of 300–400 bp were extracted from the gel for use as competitor DNA.

DNA Binding and Sucrose Gradient Centrifugation. Aliquots (0.4–0.6-mL) of cytosol containing 0.4–0.9 pmol of GR were incubated with various quantities of purified DNA fragments for 1–2 h at 0 °C with gentle agitation and layered onto linear sucrose density gradients prepared in buffer A or modifications of buffer A as described in the figure legends. Gradients were centrifuged for 16 h at 189000*g* in a Beckman SW 50.1 rotor at 3 °C, fractionated into 10-drop fractions, and counted in a Beckman LS 3801 liquid scintillation counter. The tube bottoms were cut off and counted as the "zero" fraction.

Results

Sucrose density gradient shift assays were used to characterize the interactions between human glucocorticoid receptor cytosols and purified fragments of DNA either containing or lacking GRE sites. Figure 1 shows the sedimentation profiles of heat-activated [^{3}H]TA cytosol alone, after incubation with 500 ng of unlabeled MMTV LTR DNA, or after incubation with 5 ng of 3′ [^{32}P]-MMTV LTR DNA. The profile for activated cytosol alone showed a major peak of [^{3}H]TA sedimenting at approximately 4S with a smaller proportion of the [^{3}H]TA sedimenting at approximately 8S. The sedimentation profile for nonactivated cytosol run on a parallel gradient (data not shown) produced a well-defined symmetrical peak at 8S. Thus, the small proportion of [^{3}H]TA sedimenting at 8S represents nonactivated steroid–receptor complex, while the 4S peak is the activated DNA-binding form of glucocorticoid receptor (Holbrook et al., 1983; Vedeckis, 1983; Currie & Cidlowski, 1982). Incubation of a 0.5-mL aliquot of the activated cytosol containing 0.5 pmol of GR with 500 ng (2.35 pmol) of unlabeled MMTV LTR DNA, under conditions of DNA excess, resulted in a decrease in the amount of [^{3}H]TA sedimenting at 4S and the simultaneous formation of a new peak of [^{3}H]TA sedimenting in the region of 12–16S, while

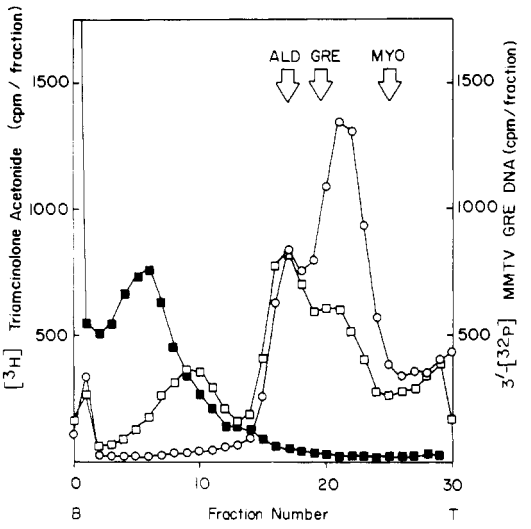

FIGURE 1: Sedimentation rate analysis of HeLa S_3 cell cytosol complexes with MMTV LTR DNA under conditions of DNA excess (□) or large [^{3}H]TA receptor excess (■). Aliquots of heat-activated, [^{3}H]triamcinolone acetonide labeled HeLa S_3 cell cytosol containing approximately 0.5 pmol of GR were incubated with buffer A (○), with 500 ng (2.35 pmol) of MMTV LTR DNA (□), or with 5 ng (0.024 pmol) of 3′ [^{32}P]-MMTV LTR DNA (■) for 1–2 h at 4 °C and layered onto 10–30% sucrose density gradients. 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 sliced and counted as the "zero" fraction. The positions of protein standards, aldolase (ALD, 7.8S) and myoglobin (MYO, 1.2S), and of the 3′ [^{32}P]-MMTV LTR DNA fragment (GRE, ~6S) run in parallel gradients are indicated by arrows.

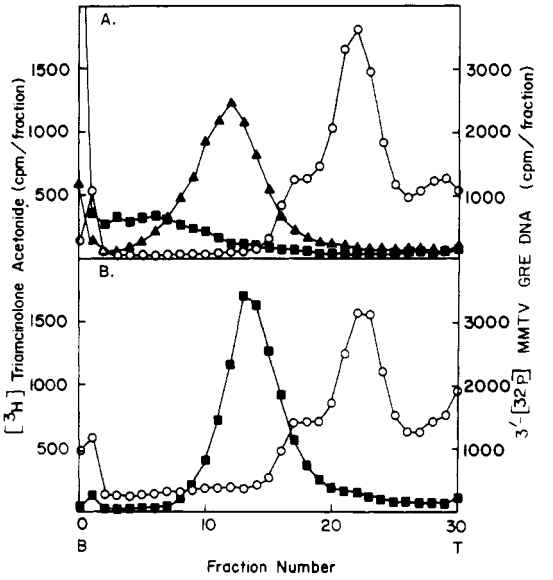

FIGURE 2: Effect of added competitor DNA or pretreatment of cytosol with calf thymus DNA–cellulose on [^{3}H]TA receptor complex formation with trace amounts of 3′ [^{32}P]-MMTV LTR DNA. Cytosol extracts were either prepared normally (A) or were treated with calf thymus DNA–cellulose prior to heat activation (B). Aliquots of each activated cytosol containing approximately 0.9 pmol of GR were incubated with 5 ng (0.024 pmol) of 3′ [^{32}P]-MMTV LTR DNA and centrifuged through 10–30% sucrose gradients. (A) Aliquots of the untreated cytosol were incubated with buffer A (○), with buffer A containing 5 ng of 3′ [^{32}P]-MMTV LTR DNA (■), or with buffer A containing 5 ng of 3′ [^{32}P]-MMTV LTR DNA plus 500 ng of sonicated *E. coli* competitor DNA (▲). (B) Aliquots of DNA–cellulose pretreated cytosol were incubated with buffer A (○) or with buffer A containing 5 ng 3′ [^{32}P]-MMTV LTR DNA (■).

the proportion of [^{3}H]TA sedimenting at 8S remained essentially unchanged. By contrast, incubation of the activated cytosol with 5 ng (0.024 pmol) of 3′ [^{32}P]-MMTV LTR DNA under conditions of large receptor excess produced no detectable change in the sedimentation profile of [^{3}H]TA-labeled receptor (data not shown). As shown in Figure 1, however, the ^{32}P-labeled DNA formed a new peak sedimenting at a somewhat greater *S* value in the region of 16–18S, and a large proportion of the labeled DNA was driven into the pellet. When run on a parallel gradient in the absence of cytosolic extract, the 3′ [^{32}P]-MMTV LTR DNA sedimented as a well-defined peak at approximately 6S. These results led us to consider the possibility that the crude cytosols might contain other DNA-binding proteins that could interact with the trace quantity of 3′ [^{32}P]-MMTV LTR DNA in addition to or instead of glucocorticoid receptor to produce the very large complexes seen under conditions of large receptor excess. We reasoned that if other DNA-binding proteins were present in the cytosols, binding by these proteins might be saturated by adding excess competitor DNA. Alternatively, since the nonactivated form of glucocorticoid receptor fails to bind DNA, then if the cytosols were incubated with DNA–cellulose prior to heat activation, other DNA-binding proteins should be removed while leaving the nonactivated GR free in solution.

Figure 2 shows the sedimentation profiles of a series of gradients run to address these questions. In panel A, as had been seen in Figure 1, the activated [^{3}H]TA receptor complex sedimented at 4S with a shoulder of [^{3}H]TA sedimenting at approximately 8S, coincident with the expected sedimentation position of nonactivated GR. Again, incubation of an equal aliquot of cytosol with 5 ng of 3′ [^{32}P]-MMTV LTR DNA shifted the trace quantity of labeled DNA into the pellet or to sedimentation positions greater than 16S. However, when the cytosol was incubated with 5 ng of 3′ [^{32}P]-MMTV LTR DNA in the presence of 500 ng of sonicated *E. coli* competitor DNA, the labeled MMTV LTR DNA now formed a smaller complex sedimenting in the range of 10–14S. The fact that a smaller complex was formed when the MMTV LTR DNA was incubated with activated [^{3}H]TA cytosol in the presence of excess competitor DNA than had been seen in the absence of the competitor DNA is consistent with the hypothesis that other DNA-binding proteins are present in the crude cytosol. Panel B shows that a similarly sized complex was formed when a trace quantity of 3′ [^{32}P]-MMTV LTR DNA was incubated with an aliquot of the cytosolic extract that had been treated with DNA–cellulose prior to heat activation. The formation of smaller complexes between the MMTV LTR DNA and [^{3}H]TA receptor from DNA–cellulose pretreated cytosols further supports the hypothesis that other DNA-binding proteins may be present in the crude cytosols.

Figure 3 shows the effect of pretreatment of cytosolic extracts with calf thymus DNA–cellulose on complex formation between the MMTV LTR DNA fragment and heat-activated or nonactivated [^{3}H]TA receptor under conditions of DNA excess. As seen in both panels of Figure 3, the nonactivated [^{3}H]TA receptor, whether from untreated cytosols or from DNA–cellulose pretreated cytosols, showed no detectable interaction with 750 ng of the MMTV LTR DNA fragment. This failure to bind DNA is consistent with the expected behavior for nonactivated GR. However, activated [^{3}H]TA receptor from untreated cytosols (panel A) interacted with the MMTV LTR DNA to form a large complex sedimenting in the region of 12–16S, while activated [^{3}H]TA receptor from DNA–cellulose pretreated cytosols (panel B) formed a somewhat smaller complex sedimenting in the range 7–10S. These results further suggest that other DNA-binding proteins present in the crude cytosols may participate in assembly of the larger complex formed between activated [^{3}H]TA receptor

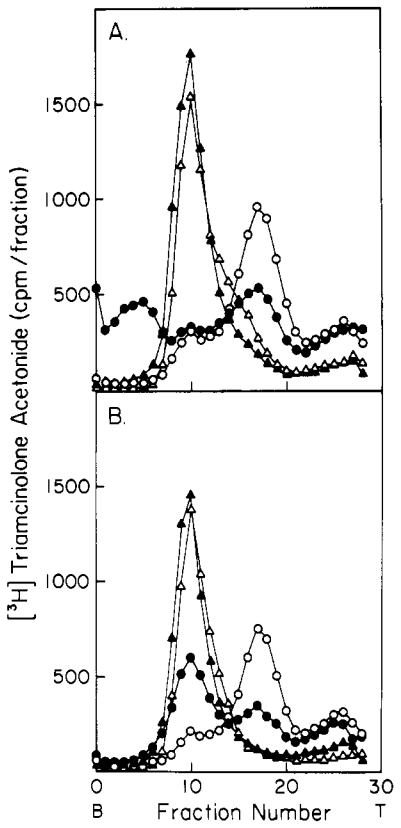

FIGURE 3: Effect of pretreatment of cytosolic extracts with calf thymus DNA–cellulose on complex formation between the MMTV LTR DNA fragment and heat-activated or nonactivated [^{3}H]TA receptor under conditions of DNA excess. Cytosol extracts were either used as prepared (A) or were treated with calf thymus DNA–cellulose prior to heat activation (B). Aliquots of both the untreated cytosol and of the DNA–cellulose-treated cytosol were maintained on ice (nonactivated) or were heat activated at 27 °C. Aliquots of each cytosol fraction containing approximately 0.7 pmol of GR were then incubated with buffer A alone or with buffer A containing 750 ng (3.5 pmol) of the MMTV LTR DNA fragment and subsequently centrifuged on 5–20% sucrose density gradients. (A) Untreated, activated [^{3}H]TA receptor alone (○) or incubated with 750 ng of MMTV LTR DNA (●). Untreated, nonactivated [^{3}H]TA receptor alone (△) or incubated with 750 ng of MMTV LTR DNA (▲). (B) DNA–cellulose-treated, activated [^{3}H]TA receptor alone (○) or incubated with 750 ng of MMTV LTR DNA (●). DNA–cellulose-treated, nonactivated [^{3}H]TA receptor alone (△) or incubated with 750 ng of MMTV LTR DNA (▲).

and the MMTV LTR DNA fragment, as seen in panel A. Since this 7–10S complex formed under conditions of DNA excess is even smaller than the 10–14S complex seen with DNA–cellulose pretreated cytosols under conditions of GR excess, these results raise the additional possibility that multiple receptors may bind a single molecule of the MMTV LTR DNA fragment under conditions of receptor excess. This result is not surprising, since the MMTV LTR DNA fragment used in these studies contains three distinct GREs identified by nuclease protection experiments (Payvar et al., 1983; Scheidereit & Beato, 1984). Additionally, Tsai et al. (1988) have presented evidence that two molecules of a truncated form of GR interact with a 27-bp oligonucleotide containing the GRE from the tyrosine aminotransferase gene in a cooperative manner. Having thus determined that pretreatment of crude cytosolic extracts with DNA–cellulose prior to heat activation could be employed to minimize binding of the MMTV LTR DNA fragment by non-GR-DNA-binding proteins, we next wished to ascertain whether the activated [^{3}H]TA receptor from DNA–cellulose pretreated cytosols could discriminate among DNA fragments containing either multiple or single GRE sites or lacking any GRE sites to form similar stable complexes.

The three panels of Figure 4 show the sedimentation profiles of DNA–cellulose-treated, heat-activated [^{3}H]TA cytosol incubated with 750 ng of one of three DNA fragments and subsequently run on 5–20% sucrose density gradients prepared in buffer A with or without added KCl. The DNA fragments used included two Taq I fragments of pBR322 DNA in addition to the MMTV LTR DNA fragment used in the preceding experiments. The pBR322 Taq I-D DNA fragment contains a single octanucleotide matching the GRE DNA consensus sequence, while the pBR322 Taq I-E DNA fragment lacks any recognizable GRE sites. The sedimentation profiles for samples run on gradients prepared in buffer A are shown in panel A. As seen before, the activated [^{3}H]TA receptor alone sedimented with a major peak of radioactivity at approximately 4S, with a smaller proportion of the radioactivity sedimenting at approximately 8S. Incubation of an equal aliquot of the cytosol with 750 ng of MMTV LTR DNA shifted most of the 4S activated receptor to a peak in the 8–12S region of the gradient. When equal aliquots of the activated cytosol were incubated with 750 ng of each of the pBR322 DNA fragments, the Taq I-D fragment containing a single GRE site shifted a slightly smaller proportion of the 4S activated receptor while the Taq I-E fragment containing no GRE sites shifted even less of the 4S activated receptor to the 5–9S region of the gradient. The apparent lower relative mobility of the complex formed between the activated [^{3}H]TA receptor and the Taq I-E fragment of pBR322 DNA may relate to a reduced stability of this complex during the course of the 16-h centrifugation. Since the pBR322 Taq I-E DNA fragment contains no recognizable GRE DNA consensus sequences, we suspected that any interaction of this DNA fragment with the activated [^{3}H]TA receptor must represent a non-sequence-specific interaction of GR with DNA. While sequence-specific interactions between proteins and DNA appear to require complementary hydrogen bonding between hydrogen-bond donors and acceptors on the protein and on the individual base pairs of the DNA, non-sequence-specific binding often involves a large electrostatic component (von Hippel & Berg, 1986). Since these kinds of electrostatic interactions are dependent on salt concentration and pH, we decided to test the relative stabilities of the complexes formed between activated GR and each of these three DNA fragments by running aliquots of the samples on sucrose gradients prepared in modifications of buffer A in which either the salt concentration or the pH of the buffer had been altered. Replicate samples were thus run on gradients prepared in buffer A supplemented with 50 mM KCl (panel B) or 100 mM KCl (panel C). As seen in panel B, addition of 50 mM KCl to the gradient buffer had no apparent effect on the stability of the complex formed with the MMTV LTR DNA fragment, but completely disrupted the complex formed with the pBR322 Taq I-E DNA fragment containing no GRE sites. The complex formed with the pBR322 Taq I-D DNA fragment containing a single GRE site was also almost completely disrupted, with only a slight shoulder remaining to suggest that any interaction had occurred. With 100 mM KCl added to the gradient buffer (panel C), some interaction with the MMTV LTR DNA fragment remained, but even this complex appeared to be somewhat less stable, while complexes formed with both of the pBR322 DNA fragments were completely disrupted. These results show that addition of as little as 50 mM KCl to the gradient buffer is sufficient to disrupt non-sequence-specific interactions of GR with DNA and further

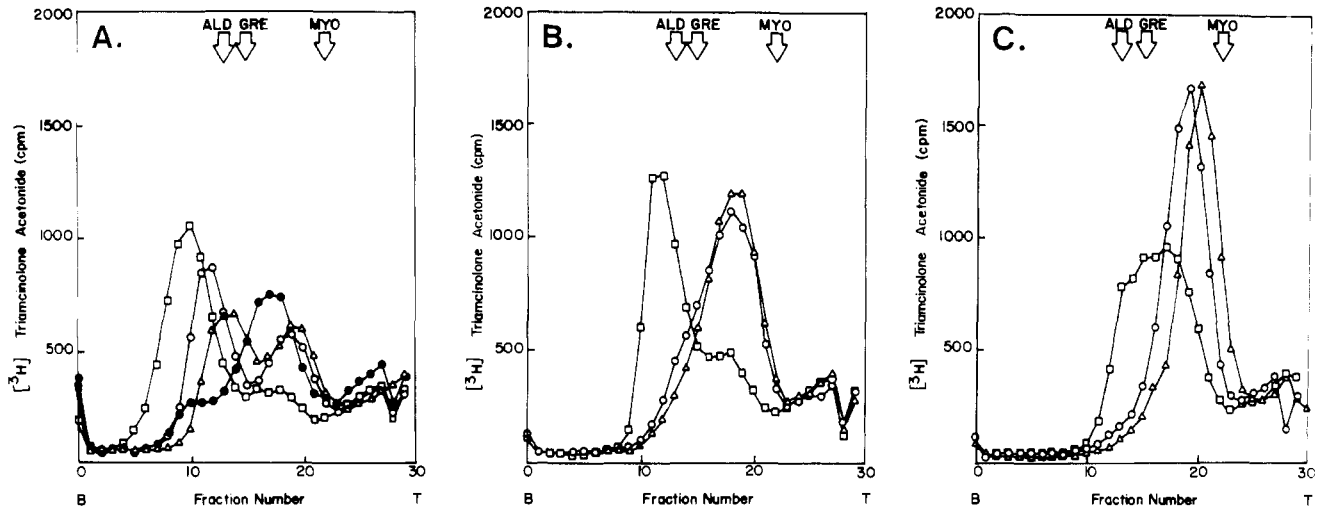

FIGURE 4: Effect of KCl concentration on the sedimentation coefficients of [^{3}H]TA receptor complexes with the MMTV LTR DNA fragment (□), with the pBR322 Taq I-D fragment containing a single site homologous to the GRE DNA consensus sequence (○), or with the pBR322 Taq I-E DNA fragment containing no GRE sites (△). Aliquots of DNA–cellulose-treated, heat-activated [^{3}H]TA cytosol containing approximately 0.7 pmol of GR were incubated with buffer A (●) or with buffer A containing 750 ng (3.1–3.7 pmol) of one of the three DNA fragments described above for 1–2 h at 4 °C. Samples were then centrifuged through 5–20% sucrose gradients prepared in buffer A containing no additional KCl (A), 50 mM added KCl (B), or 100 mM added KCl (C).

suggest that even the presence of the single octanucleotide GRE DNA consensus sequence in the pBR322 Taq I-D DNA fragment may not be sufficient to maintain a stable GR–DNA complex under these conditions of slightly elevated salt concentration.

To further test the relative stabilities of complexes formed between activated GR and each of these three DNA fragments, samples were run on sucrose gradients in which the pH of the gradient buffer had been adjusted. Figure 5 shows that the interaction of activated [^{3}H]TA receptor with these purified DNA fragments is also affected by alterations in pH. Aliquots of [^{3}H]TA cytosol incubated with buffer A or with buffer A containing 750 ng of MMTV LTR DNA, pBR322 Taq I-D DNA, or pBR322 Taq I-E DNA were run on 5–20% sucrose density gradients prepared in buffer A (panel A) or in buffer A adjusted to pH 8.0 (panel B). In panel A, the activated cytosol alone showed a major approximately 4S peak of radioactivity with a smaller amount of a second approximately 8S peak. Incubation of an aliquot of the cytosol with 750 ng of MMTV LTR DNA resulted in the formation of a 7–10S complex that shifted most of the 4S activated receptor. The pBR322 Taq I-D fragment containing a single GRE site shifted about half as much of the 4S activated receptor to a similar position in the gradient, while the pBR322 Taq I-E fragment shifted a smaller proportion of the 4S receptor to the 5–9S region of the gradient. When aliquots of the same samples were run on gradients buffered at pH 8.0, as shown in panel B, only the complex formed with the MMTV LTR DNA fragment remained stable, while complexes formed with both of the pBR322 DNA fragments were completely disrupted. These results show that elevation of the pH of the gradient buffer from 7.0 to 8.0 can disrupt non-sequence-specific interactions of GR with DNA in a manner similar to that seen when the salt concentrations were increased. These results also suggested that the presence of a single octanucleotide GRE site was not sufficient to maintain a stable GR–DNA interaction in the presence of the pH 8.0 buffer. To ascertain whether the interaction of activated [^{3}H]TA receptor with the pBR322 Taq I-D DNA fragment containing the single GRE DNA consensus sequence would remain stable to more moderate increases in the pH of the gradient buffer, six replicate aliquots of cytosol were incubated with 750 ng of the pBR322 Taq I-D DNA fragment containing a single GRE DNA consensus sequence and were then run on 5–20% sucrose gradients prepared in buffer A adjusted to pH 7.0, 7.2, 7.4, 7.6, 7.8, and 8.0 (data not shown). At pH 7.0, as was seen in panel A of Figure 5, the pBR322 Taq I-D DNA fragment formed a stable complex that shifted more than half of the 4S activated receptor to the 7–10S region of the gradient. This complex remained stable at pH 7.2, but clearly began to dissociate when run on gradients buffered at pH 7.4. If run on gradients buffered at pH 7.6 or greater, the complexes formed between GR and the pBR322 Taq I-D DNA fragment were completely disrupted during centrifugation.

Discussion

Sedimentation profiles for activated [^{3}H]TA receptor alone, whether from crude cytosols or from cytosols treated with DNA–cellulose prior to heat activation, usually showed a biphasic peak with a majority of the [^{3}H]TA-labeled material sedimenting at 4S and a smaller peak or shoulder sedimenting at 8S. These results are consistent with previously reported values for the sedimentation coefficients of activated and nonactivated GR (Currie & Cidlowski, 1982; Holbrook et al., 1983; Vedeckis, 1983). When crude cytosols were incubated with MMTV LTR DNA under conditions of DNA excess, the MMTV LTR DNA fragment formed stable 12–16S complexes with heat-activated but not with nonactivated HeLa S_3 cell [^{3}H]TA receptor. Under conditions of receptor excess, however, incubation of trace quantities of 3′ [^{32}P]-MMTV LTR DNA with crude cytosol extracts led to the formation of larger complexes either pelleting or sedimenting at 16–18S. The fact that these large complexes did not comigrate with the [^{3}H]TA receptor containing peak in the 12–16S region of the gradient and did not contain detectable quantities of [^{3}H]TA suggested that this dramatic shifting of the trace quantity of labeled DNA might be due more to interaction of the DNA with other DNA-binding proteins contaminating the cytosol extracts than to any interaction directly attributable to activated GR. If excess *E. coli* competitor DNA were included in the incubation along with the trace quantity of 3′ [^{32}P]-MMTV LTR DNA, or if cytosolic extracts had been treated with calf thymus DNA–cellulose prior to heat activation and incubation with the trace quantity of 3′ [^{32}P]-

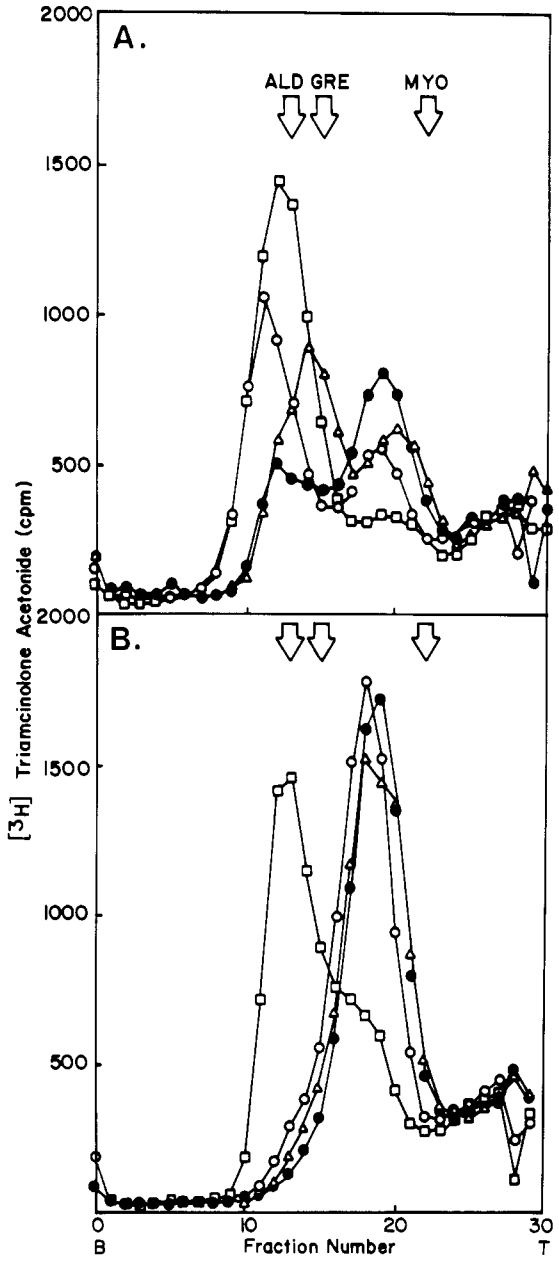

FIGURE 5: Effect of pH on the interaction of [^{3}H]TA receptor complexes with the MMTV LTR DNA fragment (□), with the pBR322 Taq I-D fragment containing a single site homologous to the GRE DNA consensus sequence (○), or with the pBR322 Taq I-E DNA fragment containing no GRE sites (△). Aliquots of DNA–cellulose-treated, heat-activated [^{3}H]TA cytosol containing 0.43 pmol of GR were incubated with buffer A (●) or with buffer A containing 750 ng (3.1–3.7 pmol) of one of the above DNA fragments for 1–2 h at 4 °C and centrifuged through 5–20% sucrose gradients prepared in buffer A at pH 7.0 (A) or buffer A adjusted to pH 8.0 (B).

MMTV LTR, then smaller 10–14S complexes formed. These observations provided additional experimental evidence that other DNA-binding proteins associated with the 3′ [^{32}P]-MMTV LTR DNA after incubation with crude cytosols under conditions of large receptor excess. The fact that virtually none of the 3′ [^{32}P]-MMTV LTR DNA was pelleted after incubation with the DNA–cellulose pretreated cytosol, while a large proportion of the labeled DNA had pelleted after incubation with the untreated crude cytosol, is a further indication that other DNA-binding proteins participate in formation of the very large complexes seen with the MMTV LTR DNA under conditions of receptor excess.

Interestingly, incubation of MMTV LTR DNA with DNA–cellulose pretreated cytosols under conditions of DNA excess produced even smaller complexes sedimenting in the range of 7–10S. This 7–10S complex observed with the DNA–cellulose pretreated HeLa S_3 cytosols may be similar to a 7.3S complex formed between purified rat GR and a smaller (185 bp) fragment of MMTV LTR DNA run on glycerol density gradients (Wrange et al., 1986). Since the label being followed in the DNA-excess experiments is the [^{3}H]TA associated with the glucocorticoid receptor, it is clear that receptor must be present in the 12–16S complex formed with crude cytosols, but some other DNA-binding protein(s) must also be bound to the MMTV LTR DNA fragment to account for the larger size of this complex. It is also noteworthy that the 12–16S complex has a well-defined, albeit large, size. In this regard, Cordingley and Hager (1988) have shown that two different transcription factors isolated from the MMTV-induced murine carcinoma cell line, 34I cl.101, also interact specifically with the MMTV LTR DNA fragment. One of these is a CAT-box binding transcription factor thought to be the mouse cell homologue of HeLa cell NF-1 (Cordingley et al., 1987). The second is a TATA-box binding factor thought to be equivalent to HeLa cell transcription factor TF II D as described by Sawadogo and Roeder (1986). While the present data cannot address the identity of other proteins besides GR that might be present in the 12–16S complex, it is possible that nuclear factors, such as these transcription factors, could be released to some degree during homogenization. Factors such as these, which could interact with specific sites on the MMTV LTR DNA fragment, would serve to account for the formation of a large, but distinct complex. Furthermore, one might expect that such factors would be removed by treating the cytosolic extracts with calf thymus DNA–cellulose. Thus, these results supported the hypothesis that other DNA-binding proteins may have been present in the crude cytosol extracts and suggested that binding by these proteins could be eliminated either by including excess *E. coli* competitor DNA or by treating the cytosol extracts with DNA–cellulose prior to heat activation.

We next sought conditions that would permit better discrimination between low-affinity interactions of activated GR with nonspecific DNAs and higher affinity interactions with DNA fragments containing GREs. We had previously used three DNA fragments in other studies of GR–DNA interaction (Tully & Cidlowski, 1987): a 326-bp fragment derived from the 5′ MMTV LTR described above, which contains multiple high-affinity binding sites for activated GR, and two similarly sized fragments from pBR322 DNA, one of which contains a single octanucleotide having homology with the GRE DNA consensus sequence and the second of which contains no GRE sites. Von Hippel and Berg (1986) have argued that sequence-specific interactions between proteins and DNA require complementary hydrogen-bonding between hydrogen-bond donors and acceptors on the protein and on the individual base pairs of the DNA, while non-sequence-specific binding more often involves a large electrostatic component. Since these kinds of electrostatic interactions are dependent on salt concentration and pH, we decided to test the relative stabilites of the complexes formed between activated GR and each of these three DNA fragments by running aliquots of the samples on sucrose gradients prepared in modifications of buffer A in which either the salt concentration or the pH of the buffer had been altered.

Results of these experiments showed that while the complex formed with MMTV LTR DNA remained stable on a gradient with 50 mM KCl added and retained some interaction with

the addition of 100 mM KCl, addition of even 50 mM KCl completely disrupted the interaction of activated GR with both the pBR322 Taq I-D DNA fragment containing a single GRE sequence and the pBR322 Taq I-E DNA fragment that contains no GREs. Increasing the pH of the gradient buffer from pH 7.0 to pH 8.0 produced similar effects, resulting in virtually complete disruption of GR interactions with both of the pBR322 DNA fragments, while retaining interaction with the MMTV LTR DNA fragment. More moderate increases in pH of the gradient buffer allowed some retention of activated GR interaction with the pBR322 Taq I-D DNA fragment containing a single GRE site up to pH 7.4. These results demonstrate that even moderate alterations in either the salt concentration or the pH of the gradient buffer can have pronounced effects on the stability of GR interactions with these small DNA fragments.

These results showing greater stability for the complex formed with the MMTV LTR DNA fragment, containing high-affinity binding sites for activated GR, and conversely showing essentially complete disruption of the complex formed with the pBR322 Taq I-E DNA fragment, containing no recognizable GRE sites, in the presence of increased pH or salt concentration were as expected from the data of von Hippel and Berg (1986). The disruption of virtually all interaction between activated GR and the pBR322 Taq I-D DNA fragment containing the single GRE DNA consensus sequence by these moderate increases in salt concentration or pH, however, was somewhat surprising. Recent results from several laboratories have indicated that glucocorticoid, progesterone, and estrogen response elements are closely related and may all require palindromic DNA sequence patterns containing at least partial dyad symmetry for high-affinity interaction with the respective steroid hormone receptor (Klock et al., 1987; Strahle et al., 1987; Martinez et al., 1987). At least one of the GRE sites in the MMTV LTR DNA fragment contains this dyad symmetry element, while the same dyad symmetry is not apparent in the vicinity of the GRE DNA consensus sequence in the pBR322 Taq I-D DNA fragment. Thus, the absence of this dyad symmetry element may account for the reduced affinity of activated [^{3}H]TA receptor for this DNA fragment under conditions of increased salt or pH. Alternatively, although the octanucleotide sequence (ACTGATCT) in the pBR322 Taq I-D DNA fragment exactly matches the GRE DNA consensus sequence described by Payvar et al. (1983) [($^T/_A$)CTG($^T/_A$)TCT], it contains A at both positions of $^T/_A$ ambiguity reported in the original consensus sequence. Recent results suggest that the presence of A, especially at the second position of $^T/_A$ ambiguity in this sequence, may convert the GRE to an estrogen response element (Klock et al., 1987). Thus, a small difference in DNA sequence between the pBR322 Taq I-D DNA fragment and the MMTV LTR DNA fragment may account for the difference in affinity of the activated [^{3}H]TA receptor for these two DNA fragments.

In summation, under conditions of receptor–DNA interaction analysis that reflect those most likely to occur in intact cells, our results demonstrate that even moderate alterations in either the salt concentration or the pH of the gradient buffer can have pronounced effects on the stability of GR interactions with these small DNA fragments. Together, these observations suggest that the physiologic conditions prevailing in intact cells may allow few if any interactions between GR and DNA lacking GRE sequences. The implications of these observations are that in vivo differences in the apparent affinities of activated steroid receptors for GRE-containing DNAs may be sufficient to provide selective regulation of gene expression.

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DNA Substrate Structural Requirements for the Exonuclease and Polymerase Activities of Procaryotic and Phage DNA Polymerases†

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ABSTRACT: A DNA duplex covalently cross-linked between specific bases has been prepared. This and similar duplexes are substrates for the polymerase and exonuclease activities of the Klenow fragment of *Escherichia coli* DNA polymerase I and T4 and T7 DNA polymerases. The action of Klenow fragment on these duplexes indicates that the polymerase site does not require that the DNA duplex undergo strand separation for activity, whereas the exonuclease site requires that at least four base pairs of the primer strand must melt out for the exonucleolytic removal of nucleotides from the primer terminus. The exonucleolytic action of T4 and T7 DNA polymerases requires that only two and three bases respectively melt out for excision of nucleotides from the primer terminus. Klenow fragment and T4 DNA polymerase are able to polymerize onto duplexes incapable of strand separation, whereas T7 DNA polymerase seems to require that the primer terminus be at least three bases from the cross-linked base pair. A DNA duplex with a biotin covalently linked to a specific base has been prepared. In the presence of the biotin binding protein avidin, the exonucleolytic activity of Klenow fragment requires that the primer terminus be at least 15 base pairs downstream from the base with the biotin–avidin complex. On the other hand, the polymerase activity of Klenow fragment required that the primer terminus be at least six base pairs downstream from the base with the biotin–avidin complex. These results suggest that the polymerase and exonuclease sites of Klenow are physically separate in solution and exhibit different substrate structural requirements for activity.

Escherichia coli DNA polymerase I (Pol I)[1] is a 109-kDa protein required for repair and replication in vivo (Kornberg, 1980). In addition to a 3′→5′ polymerase activity, requiring a template to be copied and a primer strand to which nucleotides are added, the enzyme possesses a 3′→5′ exonuclease activity capable of removing nucleotides from the primer strand and a 5′→3′ exonuclease activity which removes nucleotides in front of the growing primer strand (Jovin et al., 1969). Limited proteolysis of Pol I yields a 68-kDa fragment (the Klenow fragment) which retains the polymerase and 3′→5′ exonuclease activity (Brutlag et al., 1969; Klenow & Henningsen, 1970). This fragment has been studied extensively by a variety of kinetic (McClure & Jovin, 1975; Bambara et al., 1976; Bryant et al., 1983; Mizrahi et al., 1985, 1986; Kuchta et al., 1987, 1988), stereochemical (Burgers & Eckstein, 1979; Brody & Frey, 1981; Gupta & Benkovic, 1984), genetic (Freemont et al., 1986), and structural (Joyce & Steitz, 1987; Ollis et al., 1985a) methods and is the chief object of this study.

X-ray crystallography has provided structural data of the Klenow fragment with substrates and inhibitors at 3.3-Å resolution (Steitz & Joyce, 1987; Ollis et al., 1985a). The location of the 3′→5′ exonuclease site was determined in crystals containing a nucleoside monophosphate (dTMP). Nucleoside monophosphates are known to be competitive inhibitors of 3′→5′ exonuclease activity. In the absence of metals, a single-stranded tetranucleotide DNA also was observed to bind to this site. A helical cleft proposed as a second binding site for double-stranded DNA was located in the enzyme, which from footprinting experiments would cover about eight base pairs of double-stranded DNA. By use of the X-ray structural data and guided by data from enzymes constructed with point mutations at the putative active sites that had been found to have altered polymerase or exonuclease activity (Freemont et al., 1986), B-DNA was model-built into the enzyme to approximate the location of the polymerase and exonuclease sites. One particularly intriguing observation gleaned from the model was that the putative polymerase and exonuclease sites were about 30 Å apart, prompting questions of how the two sites work together during DNA synthesis.

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[1] Abbreviations: Pol I, *Escherichia coli* DNA polymerase I; kDa, kilodalton(s); TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography; EDTA, ethylenediaminetetraacetic acid; DMT, 4,4′-dimethoxytrityl; CNE, 2-cyanoethyl; TEAB, triethylammonium bicarbonate; TEAA, triethylammonium acetate; DTT, dithiothreitol, Tris, tris(hydroxymethyl)aminomethane; dNMP, 2′-deoxynucleoside monophosphate; dNTP, 2′-deoxynucleoside triphosphate.

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