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Purified glucocorticoid receptor–hormone complex from rat liver cytosol binds specifically to cloned mouse mammary tumor virus long terminal repeats *in vitro*

(steroid hormone receptor/DNA-protein interaction/transcription regulation)

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ABSTRACT Purified glucocorticoid hormone–receptor complex (HRC) from rat liver binds to specific DNA sequences contained in cloned mouse mammary tumor virus (MMTV) DNA. The binding site of the hormone–receptor complex is located in the long terminal repeat (LTR) of MMTV DNA as shown by filter binding studies with labeled restriction fragments and by visualization of DNA–receptor complexes with the electron microscope. The DNAs from cloned MMTV lacking the LTR sequences were neither retained on nitrocellulose filters nor bound specifically to HRCs examined in the electron microscope. The HRC also failed to bind to restriction fragments from pBR322 and phage λ. Specific binding of the HRC to LTR sequences is dependent upon occupancy of the receptor by a glucocorticoid. Previous work has demonstrated that the MMTV transcription is initiated within the LTR; additionally, MMTV transcription is known to be regulated by glucocorticoids. Our present results therefore support the hypothesis that HRC regulates hormone-induced transcription by binding to specific DNA sequences near the MMTV transcription start site.

Numerous studies have shown that glucocorticoid hormones bind to specific proteins [hormone–receptor complexes (HRCs)] present in their target cells (1, 2) and this binding apparently increases the rate of synthesis of specific mRNAs (3–5). DNA binding experiments using crude HRC preparations from cytosol as well as purified glucocorticoid HRC have demonstrated the association of HRCs with DNA (6–10). The major portion of HRC ($\approx 10^5$ per cell) appears to bind nonspecifically and with relatively low affinity to target cell nuclei *in vivo* (11) and *in vitro* (12).

Glucocorticoid hormones have been shown to regulate mouse mammary tumor virus (MMTV) production in mammary tumor cell lines (13–16). Induction of MMTV RNA synthesis and accumulation has been associated with a change in the rate of transcription of the proviral genes and may be a primary biochemical response to glucocorticoids (17, 18). DNA-mediated gene transfer of cloned proviral MMTV genes into cultured cells and analysis of their hormone-responsive transcription have provided evidence for the physical linkage of the regulated MMTV gene and the primary DNA sequences that mediate the hormonal response (19–21).

The availability of cloned viral sequences (22) and purified HRC preparations (10, 23, 24) should allow characterization of the specificity of the association between HRCs and MMTV DNA. Payvar *et al.* (25) recently used a nitrocellulose filter binding assay to show that HRC binds to MMTV DNA fragments that include either the *env* gene or the *env* gene and the long terminal repeat (LTR).

In this report, we describe use of both the filter binding assay and visualization of HRC–DNA complexes with the electron microscope to demonstrate specific binding of glucocorticoid HRCs to the MMTV LTR.

MATERIAL AND METHODS

Purification of Rat Liver Glucocorticoid HRC. HRC was purified from rat liver cytosol as described (10) except for the following modifications. The affinity eluate was extensively dialysed against 5 mM phosphate, pH 7.4/5 mM 2-mercaptoethanol/10% (vol/vol) glycerol, incubated with 0.5 μM [^{3}H]dexamethasone (5 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) at 25°C for 30 min, and applied to a column of DEAE-Sepharose-Cl 2B (20 ml) equilibrated in the same buffer (26). After thorough washing, the receptor was eluted from the column in 3-ml fractions with a linear gradient of 0–500 mM NaCl in the same buffer. A radioactive peak was eluted between 50 and 60 mM NaCl (Fig. 1). $NaDodSO_4$/polyacrylamide gel electrophoresis revealed the presence of a single polypeptide of M_r 90,000 (total, 880 μg) eluting in three fractions. This 9 ml of receptor solution was diluted to 20 ml with glycerol and, after the addition of 1 μM [^{3}H]dexamethasone (25 Ci/mmol), was stored at −20°C; it was stable for several weeks. The purified receptor could be stripped of dexamethasone by incubation for 5 min at 37°C followed by treatment with dextran-coated charcoal containing 1 mg of bovine serum albumin per ml (alb/dextran/C).

Cloned MMTV DNA. The MMTV DNA used in this study was derived from rat XC cells infected with the C3H strain of MMTV. 8H3TK3 contains a *Hin*dIII fragment with a partial MMTV provirus, probably resulting from reverse transcription of the *env* mRNA (22). Both LTRs are present within the fragment. 8H3TK3 also contains a 3.6-kilobase-pair (kb) *Bam*HI fragment harboring the thymidine kinase gene from herpes simplex virus. Plasmid clones 8-29 and 8-21 are *Pst* I subclones of the proviral sequences which contain a 1.45-kb LTR fragment (from the left end of the provirus) and a 1.75-kb *env* fragment, respectively. Plasmid clone 2-5 contains the 4-kb *Pst* I *gag–pol* fragment cloned directly into pBR322 by *Pst* I cleavage of circular DNA from XC cells (22).

DNA-Binding Experiments. HRC–DNA binding experiments were carried out at receptor concentrations of 0.01–10

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Abbreviations: MMTV, mouse mammary tumor virus; HRC, hormone–receptor complex; LTR, long terminal repeat; alb/dextran/C, bovine serum albumin-saturated dextran-coated charcoal; kb, kilobase pair(s).

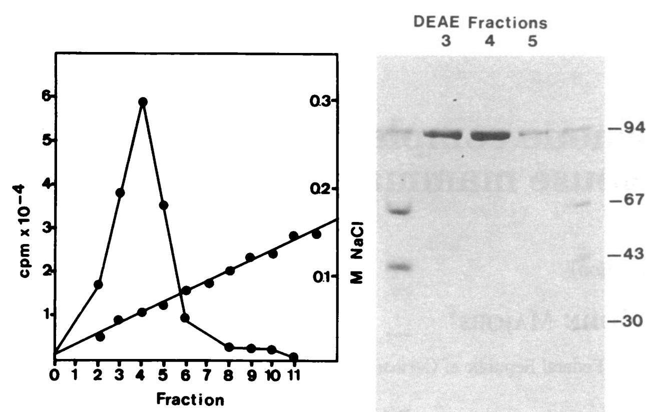

FIG. 1. (*Left*) DEAE-Sepharose Cl-2B elution profile of affinity-purified glucocorticoid receptor from rat liver. To a column filled with 20 ml of DEAE-Sepharose Cl-2B, 70 ml of affinity eluate was applied at a flow rate of 15 ml/hr. After thorough washing of the column, the receptor was eluted with a linear gradient of 5–500 mM NaCl in the equilibration buffer. Radioactivity was measured in 300-μl aliquots of 3-ml fractions. (*Right*) Samples (500 μl) from peak fractions 3, 4, and 5 were treated with 5 ml of 10% trichloroacetic acid to precipitate the proteins and applied to a 10% NaDodSO$_4$/polyacrylamide gel. The gel was stained with Coomassie blue. M_r markers are phosphorylase *b* (94,000), bovine serum albumin (67,000), ovalbumin (43,000), and carbonic anhydrase (30,000).

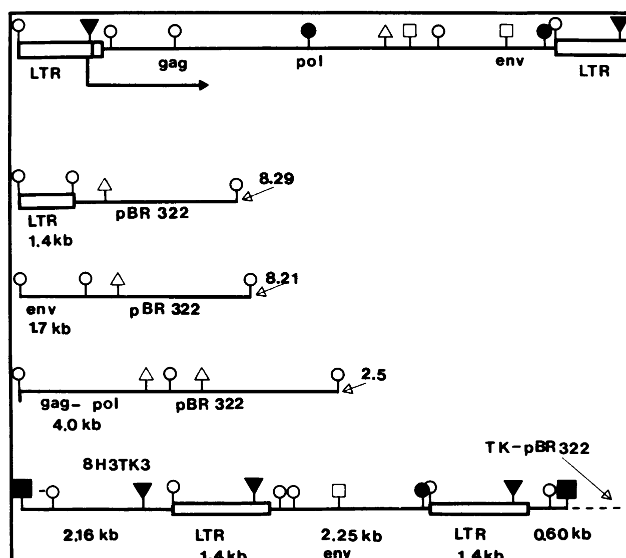

FIG. 2. Maps of restriction sites in MMTV C3H DNA (top line; arrow marks the apparent transcription start site) and of DNA inserted to pBR322 relevant to the present study (bottom four lines). Rectangles denote the 1.4-kb LTR sequences; ♀, *Pst* I; ●, *Bgl* II; ▼, *Sac* I; ⊓, *Bam*HI; △, *Eco*RI; ■, *Hin*dIII.

nM. Typically, the stock solution of receptor (0.5 μM) was diluted 1:10 with cold binding buffer (10 mM Tris·HCl, pH 8.0/50 mM KCl/1 mM MgCl$_2$/5 mM 2-mercaptoethanol/10% glycerol) and incubated for 5 min at 37°C. After cooling on ice for 10 min, unbound steroid was removed by alb/dextran/C treatment and diluted to the appropriate concentrations. After dilution, receptor devoid of hormone was prepared by treating the stock HRC solution with alb/dextran/C, incubating at 37°C for 5 min, cooling in ice, and treating a second time with alb/dextran/C. In 100 μl of 1 nM hormone-bound receptor there was 2,500 cpm (counting efficiency 50%); 100 μl of 1 nM hormone-free receptor contained no bound radioactivity. For some experiments, the receptor devoid of hormone was recharged with 0.5 μM [^{3}H]dexamethasone by incubating 90 min at 4°C, and unbound hormone was removed by alb/dextran/C treatment as above. The HRC prepared after recharging contained the same amount of bound radioactive hormone and bound to DNA with the same specificity as HRC prepared from stock receptor solution (data not shown).

The DNA concentration used in the binding experiments was 10 pM and the specific activity was adjusted to 2.5 mCi/nmol. Binding to nitrocellulose (SM 11306, size 30) was carried out as described (27), with the following modifications. The incubation volume was 100 μl, and the washing was done three times with 5 ml of binding buffer. Bound material was eluted with 0.2% NaDodSO$_4$ in binding buffer, precipitated with ethanol, and subjected to electrophoresis on a 1% agarose gel. The gel was dried and autoradiographed on Kodak XAR-5 x-ray film with an intensifying screen.

Electron Microscopy. Samples were incubated for 5 min at 37°C, cooled to 4°C, and diluted with binding buffer to 0.1–0.3 μg of DNA per ml. The Mg^{2+} concentration was adjusted to 10 mM and the DNA was adsorbed on mica (28). Subsequently, specimens were rinsed briefly in water, stained for 30 sec in 1% aqueous uranyl acetate, and rinsed again. After shadowing with platinum/carbon, the carbon-enforced replicas were mounted on sticky grids. Micrographs were taken with a Philips 400 electron microscope. The magnification was calibrated by a cross grating, and pBR322 DNA was used as length reference. Length measurements were made by tracing photographed molecules with a Tektronix graphics tablet.

RESULTS

Properties of Purified Glucocorticoid Receptor. Purified HRC had properties similar to those of the receptor preparations described before (10). We obtained a homogeneous receptor protein by DEAE-chromatography. The hormone binding site of the purified receptor could be occupied by [^{3}H]dexamethasone. From the specific activity of the [^{3}H]-dexamethasone (25 Ci/nmol), we calculate that 1 nmol of hor-

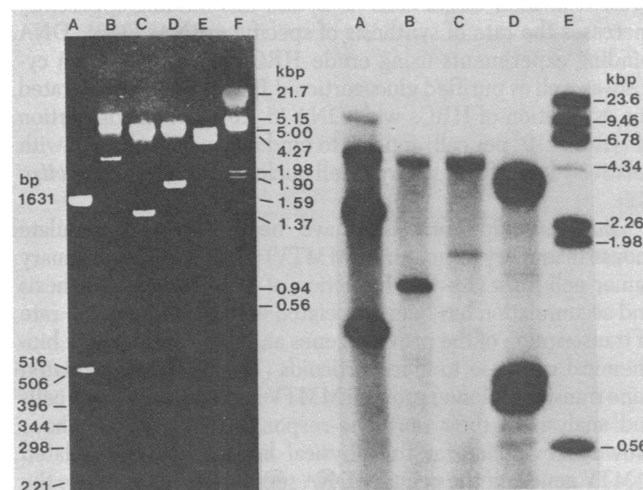

FIG. 3. Agarose gel electrophoresis. (*Left*) Of restriction fragments after ethidium bromide staining. Lanes: A, pBR322 *Hin*fI fragments; B, 8H3TK3 digested with *Hin*dIII followed by *Eco*RI; C, clone 8-29 cleaved with *Pst* I; D, clone 8-21 cleaved with *Pst* I; E, clone 2-5 cleaved with *Pst* I followed by *Eco*RI; F, *Hin*dIII/*Eco*RI fragments of phage λ. (*Right*) 5′-End-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (5,000 Ci/mmol). Note that labeling of the fragments was not even. Lanes: A, *Hin*dIII/*Eco*RI digest of 8H3TK3; B, clone 8-29 digested with *Pst* I; C, clone 8-21 digested with *Pst* I; D, clone 2-5 digested with *Pst* I/*Eco*RI; E, *Hin*dIII fragments of λ.

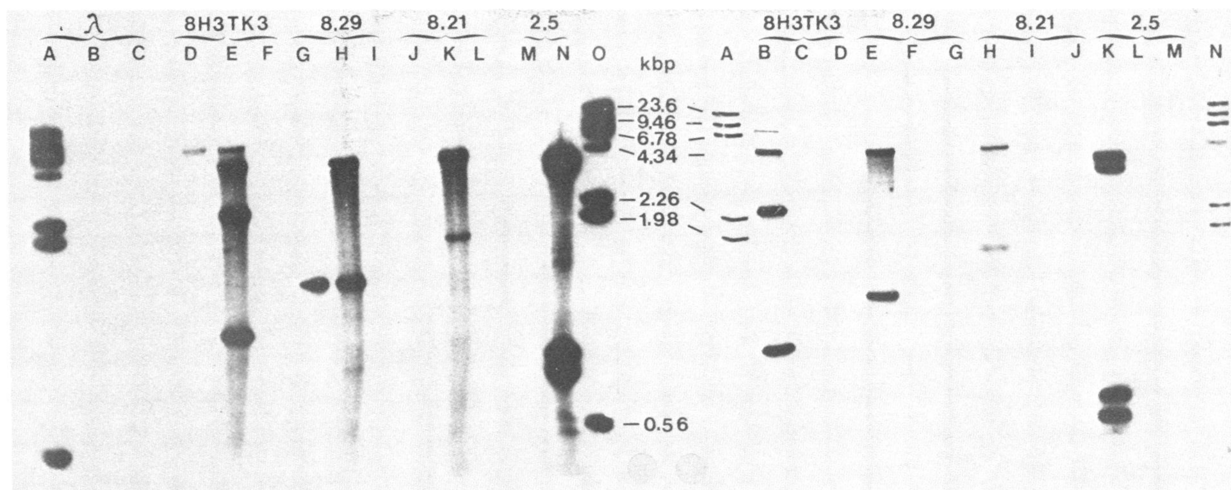

FIG. 4. (*Left*) Agarose gel electrophoresis of labeled *Hin*dIII fragments of λ (A, B, C, and O), *Hin*dIII/*Eco*RI fragments of 8H3TK3 (D–F), *Pst* I fragments of clone 8-29 (G–I) and clone 8-21 (J–L), and *Pst* I/*Eco*RI fragments of clone 2-5 (M and N). Lanes A, E, H, K, N, and O: DNA after prefiltration through nitrocellulose before incubation with the HRC. Lanes B, D, G, J, and M: 10 pM [^{32}P]DNA fragments (2.5 mCi/nmol) incubated with 1 nM HRC and bound to the filters. Lanes C, F, I, and L: DNA bound to filters in the absence of HRC. (*Right*) Agarose gel electrophoresis of labeled *Hin*dIII fragments of λ (A, N), *Hin*dIII/*Eco*RI fragments of 8H3TK3 (B, C, D), *Pst* I fragments of clone 8-29 (E, F, and G) and clone 8-21 (H, I, and J), and *Pst* I/*Eco*RI fragments of clone 2-5 (K, L, and M). Lanes C, F, I, and L: DNA bound to filter in the presence of receptor alone. Lanes D, G, J, and M: DNA bound to the filters in the presence of 1 nM hormone alone. Lanes A, B, E, H, K, and N: DNA prefiltered through nitrocellulose before binding to filter.

mone was bound per 90 μg of purified protein. As shown by the NaDodSO$_4$/polyacrylamide gel stained with Coomassie blue (Fig. 1 *Right*), the apparent M_r of the single polypeptide chain is 90,000. Thus, there can only be one hormone binding site per polypeptide chain. On sucrose gradient centrifugation in high-salt medium the HRC had a sedimentation coefficient of 4.5 S. After labeling of the purified receptor with [^{3}H]dexamethasone 21-mesylate (10, 29) the radioactivity comigrated with the receptor as a single band of M_r 90,000 on a NaDodSO$_4$/polyacrylamide gel (unpublished data). Receptor purified by DEAE-chromatography could be freed of bound hormone under conditions described above and then could be recharged with different dexamethasone derivatives (unpublished results). No differences were observed in binding studies with either HRC stored at −20°C in glycerol or fresh HRC preparations, but electron microscopic examination of HRC preparations stored frozen at −20°C or −70°C showed the formation of aggregates.

Binding of the Receptor–Dexamethasone Complex to Cloned MMTV DNA. The clone 8H3TK3 contains an LTR sequence and flanking cellular DNA on each side of the *env* gene (Fig. 2). *Hin*dIII/*Eco*RI digestion of 8H3TK3 released the expected 8.2-kb MMTV insert and the 4.5-, 2.5-, and 1-kb pBR-TK *Eco*RI fragments (Fig. 3). [The 1-kb fragment is too faint to be visualized on the photograph of the gel stained with ethidium bromide (Fig. 3 *Left*).] These fragments were end-labeled with ^{32}P, incubated with HRC, and filtered through nitrocellulose membranes. Only the labeled 8.2-kb fragment containing the viral coding sequences was retained on the nitrocellulose filter (Fig. 4 *Left*), even though the specific activity of this fragment was lower than that of the other three fragment (Fig. 3 *Right*). The viral DNA fragment was not retained when the DNA was filtered in the absence of HRC (Fig. 4 *Left*), with receptor alone, or with hormone alone (Fig. 4 *Right*). *Pst* I cleavage of clone 8-21 (Fig. 3) released DNA fragments of 4.4 and 1.7 kb; the first contained pBR322 sequences and the second contained *env* sequences. These fragments were not retained on nitrocellulose (Fig. 4 *Left*). Clone 8-29 (Fig. 2), which contained most of the LTR as a 1.4-kb *Pst* I fragment (Fig. 3), showed high specific HRC binding (Fig. 4 *Left*). This *Pst* I fragment therefore was used to study the effect of HRC and DNA concentrations on HRC–DNA binding.

After incubation with hormone alone or with receptor alone, this fragment was not retained on nitrocellulose (Fig. 4 *Right*). Titration of HRC with the labeled restriction mixture from clone 8-29 demonstrated that HRC at 1 nM gave maximal retention (Fig. 5 *Left*). At this concentration of HRC, optimal retention of DNA HRC was obtained at 10 pM DNA (Fig. 5 *Right*). Under such conditions, 35–40% of the DNA was retained on the nitrocellulose filter, suggesting that this percentage of DNA is complexed with HRC. However, only about

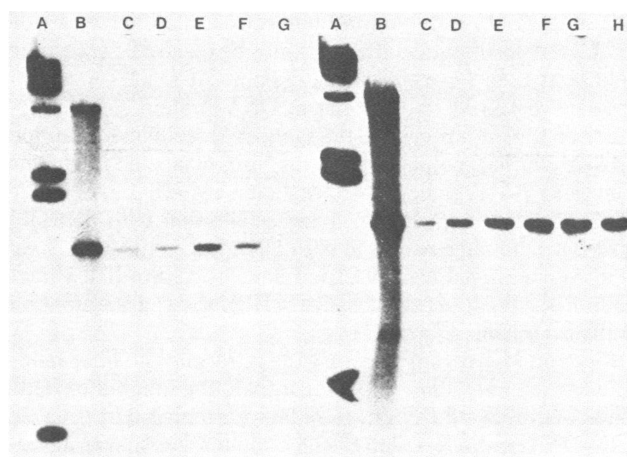

FIG. 5. (*Left*) Concentration dependence of HRC and DNA retention on nitrocellulose. Lanes: A, *Hin*dIII fragments of λ; B, *Pst* I fragments from clone 8-29 labeled at the 5′ end and prefiltered through nitrocellulose membrane; C–G, 10 pM DNA (2.5 mCi/nmol) incubated with 10 nM (C), 5 nM (D), 1 nM (E), 0.1 nM (F), or 0.01 nM (G) HRC and bound to nitrocellulose filters. (*Right*) Concentration optimum of DNA for retention on nitrocellulose membranes. Lanes: A, labeled *Hin*dIII λ fragments; B, labeled *Pst* I fragments from plasmid 8-29 prefiltered through nitrocellulose. Concentrations of DNA bound to the filters in the presence of 1 nM HRC were: C, 1 pM; D, 2.5 pM; E, 5 pM; F, 10 pM; G, 100 pM; and H, 1 nM.

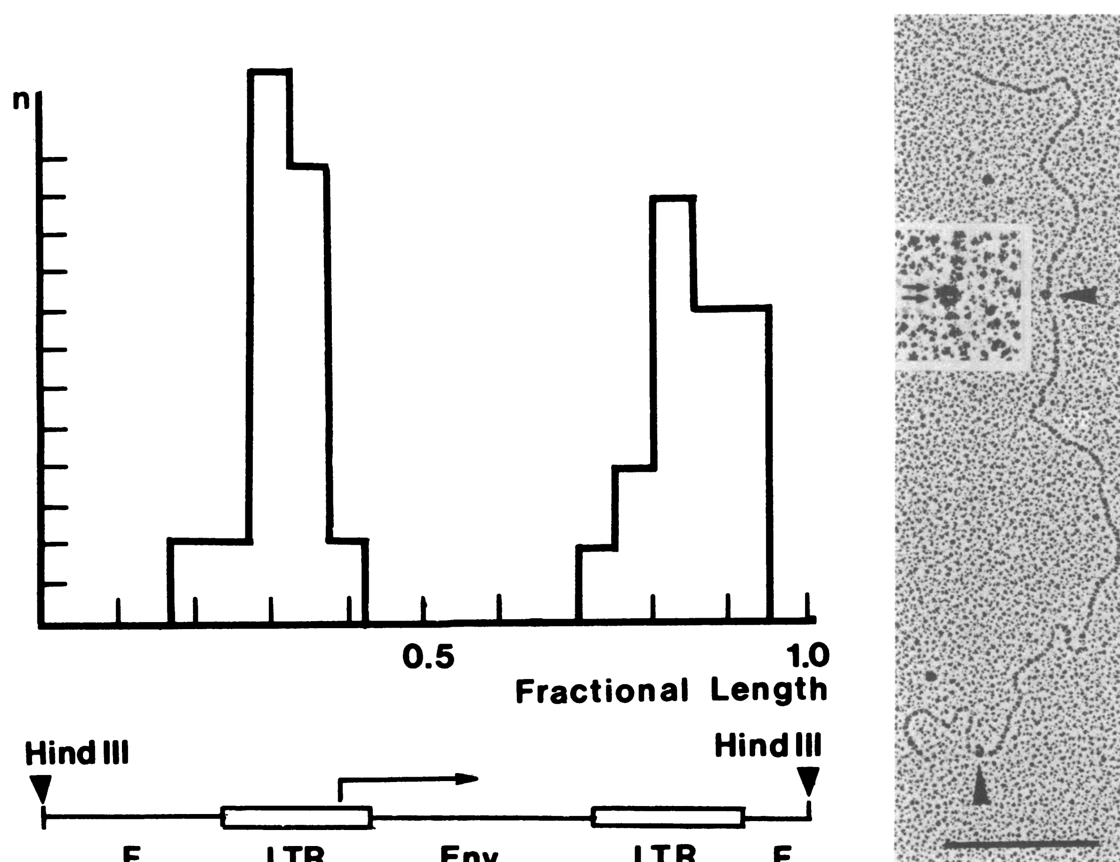

FIG. 6. Analysis of 8H3TK3 DNA-HRC by electron microscopy. Binding sites of HRC molecules were localized on *Hin*dIII/*Eco*RI-digested 8H3TK3 molecules and calculated in fractional length units. (*Left*) Histogram with data from 32 8H3TK3 DNA molecules exhibiting two HRCs. The map of the molecule (bottom) shows that the sites with maximal binding frequency correspond to the two LTR regions but F (flanking) and *env* sequences are free of HRC. The arrow in the map indicates the presumed transcriptional starting site. (*Right*) Electron micrograph from the shadowing of a representative 8H3TK3 molecule with two HRCs bound to it (arrowheads). (*Inset*) enlarged DNA-HRC site; the dimeric structure of the HRC is visible (arrows). Bar corresponds to 1 kb and 2 kb for the whole image and the *Inset*, respectively.

10% of the DNA observed in the electron microscope carried receptor molecules; the difference may be due to the spreading conditions used for electron microscopy. At DNA concentrations above 10 pM, the amount of DNA-protein complex retained on the filter remained the same. Nitrocellulose retention of DNA-protein complexes from mixtures of labeled fragments from clone 2-5 (Fig. 3) revealed that, at 1 nM HRC, no specific fragment was retained (Fig. 4 *Left*). Titration with 8H3TK3 DNA gave the same results as with DNA fragments from clone 8-29. An additional control experiment using *Hin*dIII λ fragments in the presence and absence of HRC showed no retention on the filter (Fig. 4 *Left*).

Electron Microscopy. Because the nitrocellulose filter binding assay does not allow one to quantitate the number of HRC bound to the cloned DNA fragments, we attempted to visualize HRC-DNA complexes with the electron microscope. In agreement with the filter binding experiments, HRC bound to a specific DNA fragment from 8H3TK3. Binding sites were found to be localized over the two LTRs of 8H3TK3 (Fig. 6). Clone 8-29, which contains a single LTR, showed a single distribution of binding (data not shown). The electron micrograph in Fig. 6 shows that individual DNA molecules appear to bind HRC at a single site within each LTR.

Examination of the isolated HRC by negative staining revealed a dimeric structure composed of equal rod-like 4.8 × 11 nm monomers which are connected by their long sides. In solution, dimers may aggregate to tetramers, in which case the dimers are arranged in two parallel layers (data not shown). The dimeric structure is also visible on HRC molecules bound to DNA (Fig. 6). From the size of the DNA-HRC complex it can be excluded that the HRC monomer builds up stable complexes with the DNA, but it cannot be excluded that the tetramers do so. Because of their morphology, dimers and tetramers would cover almost equal distances on the DNA. From the above data it is calculated that the HRC can cover a maximum of 35 ± 6 base pairs on the DNA.

DISCUSSION

A number of studies have demonstrated an association of HRC with chromatin *in vivo* (30). Binding studies with purified DNA (7, 8) and sequential purification of activated HRC (9, 10) on DNA cellulose (31) support the view that HRC may be analogous to some well-characterized prokaryotic regulatory proteins (32). This assumption is strengthened by studies of the accumulation of specific transcripts induced by the action of steroid hormones (3, 7, 33-42).

The availability of both purified glucocorticoid HRC and defined DNA sequences from MMTV has allowed us to localize the sequences within the LTR with which the HRC interacts. Our more recent DNA oligonucleotide pattern experiments suggest that a specific sequence of about 50 base pairs is protected by the HRC. This sequence maps to 100-150 base pairs 5′ to the MMTV transcription start site (43).

Our titration experiments using nitrocellulose filter binding show the concentration required for optimal binding. It should

be noted that receptor at 10 nM, a concentration 10 times the optimum, may be strongly aggregated and may not bind specifically. Incubation of 1 nM HRC with DNA at concentrations ranging from 1 pM to 1 nM demonstrated that optimal filter binding is reached at 10 pM DNA and remains constant at higher concentrations. The electron microscope mapping of the DNA binding site for HRC is in agreement with the data obtained in the nitrocellulose membrane assay. In addition, it suggests that LTR sequences in the 8H3TK3 bind the HRC independently and that there is only one strong binding region per LTR.

The glucocorticoid-bound HRC interacts with specific sequences in MMTV and may also interact with cellular DNA, functioning at these sites as a positive regulator of transcription. Analysis of other glucocorticoid-regulated genes will provide additional information regarding the specific nature of the HRC binding site(s) and will add to our understanding of hormone-regulated gene transcription.

In a similar study of HRC binding to MMTV DNA, Payvar *et al.* (25) found that HRC binds to DNA containing the *env* gene and to DNA containing the LTR and *env* gene. The source of the discrepancy between their results and the conclusions reported here remains unknown but may be the higher purity of the HRC used in our studies.

It will be important to establish whether these results are specific to MMTV or will apply to all glucocorticoid-regulated genes. Indeed, preliminary experiments indicate that HRC binds to specific sites near the 5′ end of cellular genes known to be transcriptionally regulated by glucocorticoids, such as the mouse metallothionein I gene.

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