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Purified glucocorticoid receptors bind selectively *in vitro* to a cloned DNA fragment whose transcription is regulated by glucocorticoids *in vivo*

(steroid hormone receptor/protein–DNA interaction/mammary tumor virus/transcriptional regulation)

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ABSTRACT Activated glucocorticoid receptor protein, purified to 40–60% homogeneity from rat liver extracts, binds selectively *in vitro* to a cloned fragment of murine mammary tumor virus (MTV) DNA. The DNA fragment tested contains about half of the sequences present in intact MTV DNA, and its rate of transcription, like that of the intact viral element, is strongly stimulated by glucocorticoids when it is introduced into the genome of a receptor-containing cell. In contrast, the receptor fails to bind selectively to DNA restriction fragments from *E. coli* plasmids pBR322 and RSF2124 or from bacteriophages λ and T4. Preliminary experiments to localize regions within MTV DNA responsible for selective binding have revealed thus far one subfragment that fails to bind the receptor and one selectively bound subfragment that maps far downstream from the 5′ terminus of the normal RNA transcript. These studies are consistent with the notion that steroid receptors may modulate rates of transcription by recognizing specific DNA sequences within or near the regulated genes.

In several experimental systems, it has been shown that steroid hormone–receptor complexes appear to increase rapidly and selectively the rate at which specific transcripts are synthesized. For example, in cultured rat hepatoma (HTC) cells bearing stably integrated murine mammary tumor virus (MTV) genes introduced by infection, glucocorticoid hormones specifically stimulate the rate of viral gene transcription (1) in a receptor-dependent manner (2).

Biochemical and genetic experiments have demonstrated that steroid receptors associate with DNA (3, 4) and that the hormone strongly increases the affinity of the interaction (5, 6); in addition, other chromosomal components have been proposed to participate in the nuclear binding event (for review, see ref. 7). Does receptor binding occur preferentially at specific high-affinity genomic sites? It appears that the bulk of the approximately 10^4 receptors per responsive cell associate nonspecifically and with relatively low affinity with nuclei *in vivo* (8) and *in vitro* (9), as well as with DNA *in vitro* (5). However, as elegantly demonstrated with prokaryotic transcriptional regulatory proteins, factors that bind with high affinity to specific DNA sequences also display a reduced but significant affinity for nonspecific sequences (10, 11). It was suggested from these observations and from theoretical considerations that the low-affinity nonspecific DNA binding of steroid receptors could preclude detection of a small number of high-affinity genome binding sites that might directly mediate the hormone response (7, 12).

According to this view, detection of the hypothetical specific binding sites would likely require either extensive enrichment relative to nonspecific sequences or special conditions for their direct visualization. Investigation of the process by which glucocorticoids stimulate MTV gene transcription in cultured cells has revealed that (*i*) sequences are present within the viral DNA itself that are required for hormonal regulation of MTV transcription (13), (*ii*) specific subregions of cloned viral DNA are competent to mediate hormonal regulation upon their reintroduction into cellular genomes by DNA transformation (ref. 14; unpublished data), and (*iii*) receptor action appears to stimulate the rate of initiation events from a single preexisting transcriptional start site within the viral DNA (13). Given this information, together with the availability of both pure viral sequences on recombinant vectors (14, 15) and purified preparations of glucocorticoid receptors (16), it was of interest to test the notion that the receptor might selectively recognize and associate with specific sequences within MTV DNA. As a first step, we have examined the selectivity of receptor binding to DNA by using an assay in which radioactively labeled DNA restriction fragments that bind to the receptor are selectively retained on a nitrocellulose filter.

MATERIALS AND METHODS

Binding Proteins. Glucocorticoid receptor from rat liver soluble extracts was purified in the presence of the synthetic glucocorticoid [^{3}H]triamcinolone acetonide (9 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) as described (16) by sequential chromatography on phosphocellulose and DNA-cellulose, followed by heat activation and chromatography on a second DNA-cellulose column; the eluate from this column (in 50 mM sodium borate, pH 8.1/3 mM $MgCl_2$/10% (wt/vol) glycerol/10 mM pyridoxal 5′-phosphate) was stored frozen (−45 to −80°C). Assuming a receptor of M_r 89,000 and a single hormone binding site per molecule (16, 17), purity was calculated to be 40–60%. In these preparations, 90% of the hormone dissociates after 20 hr at 0°C; highly purified human serum albumin (a gift from Kabi AB, Stockholm, Sweden) stabilizes the complex, so that only ≈ 10% dissociation occurs during 20 hr at 0°C. Therefore, human serum albumin (0.1–1.0 mg/ml) was added just before freezing.

Bacteriophage λ repressor protein, purified to homogeneity, was a gift of J. E. Anderson and M. Ptashne; purified calf thymus histones were a gift of L. Cousens.

DNA. The recombinant phage λ2.1a was recovered by J. Majors from *Eco*RI-digested unintegrated MTV DNA isolated from M1.54 (18), an MTV-infected HTC cell line; the 4.5-

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Abbreviations: MTV, murine mammary tumor virus; kbp, kilobase pairs.

kilobase-pair (kbp) MTV insert was transferred by V. Chandler to the *Eco*RI site of pBR322 and denoted pMTV2. ColE1-AP^r plasmid RSF2124 (19) was prepared as described (20). Bacteriophage T4 DNA containing cytosine rather than hydroxymethylcytosine, was a gift of J. Barry. *Eco*RI restriction fragments of various DNAs were terminally labeled by "filling in" the restriction site overhang with [^{32}P]dATP in the presence of dTTP, dGTP, dCTP, and T4 DNA polymerase (a gift of R. L. Burke) essentially as described (21); the presence of all four deoxynucleoside triphosphates prevents and competes with the 3' exonuclease activity of the polymerase.

Receptor–DNA Binding. Immediately before a binding reaction, an aliquot of purified receptor was rapidly thawed at 25°C, incubated at 4°C for 30 min in the presence of 100 mM dithiothreitol (22) and chromatographed on Sephadex G-75 gel filtration columns preequilibrated with 50 mM sodium phosphate buffer, pH 7.4/1 mM EDTA/2 mM 2-mercaptoethanol/10% glycerol/and 100 μg of human serum albumin per ml (G-75 buffer). The receptor, free of pyridoxal phosphate, was collected in the column void volume, which contained about 50% of the input [^{3}H]triamcinolone; removal of pyridoxal phosphate was required for subsequent DNA binding (unpublished results).

Typically, the binding reaction was carried out by adding DNA (final concentration, 200–400 ng/ml) to ^{3}H-labeled receptor (2–4 × 10^4 cpm; 250–500 ng) in 250–500 μl of G-75 buffer. Control reactions carried out in this same buffer established that the serum albumin carrier protein does not detectably participate in the nitrocellulose binding reaction. Moreover, DNA binding experiments in the absence of serum albumin showed that this protein is not required for the selectivity observed with the glucocorticoid receptor, although receptor stability was impaired without the carrier protein (data not shown). Binding to nitrocellulose filters (BA85, 27 mm; Schleicher & Schuell) was essentially as described (23). Bound material was eluted with NaDodSO$_4$-containing buffer (24), precipitated with ethanol, and dissolved in sample buffer for agarose gel electrophoresis. Nonbound material in filtrates also was examined on gels (data not shown) and confirmed that, under these conditions, 40–90% of the input DNA fragment bound by the glucocorticoid receptor was depleted by associating with the filter. Labeled DNA fragments recovered in the filtrates and filter eluants accounted for 85–95% of input radioactivity.

RESULTS

Receptor Purity and Integrity. Seven separate preparations of rat liver glucocorticoid receptors labeled with the synthetic glucocorticoid [^{3}H]triamcinolone acetonide and purified as described (16) were used. All preparations were estimated to be 40–60% pure, based on protein content and hormone specific activity. Several batches were reexamined after thawing the frozen stocks just prior to the DNA binding reactions; all gave results similar to those shown in Fig. 1. NaDodSO$_4$/polyacrylamide gel electrophoresis (25), followed by protein staining with ammoniacal silver nitrate (26), revealed a single major species of M_r 90,000 (Fig. 1A), consistent with the reported M_r of the hormone binding species (16). About five minor contaminants ranging in M_r from 30,000 to 110,000 could be visualized by extensively overstaining the gels; densitometric scans agreed with the 40–60% purity of the M_r 90,000 species calculated as noted above (data not shown). Sucrose gradient sedimentation (Fig. 1B) revealed a quantity of released hormone together with a single peak of receptor-associated [^{3}H]triamcinolone migrating at 3.5 S, as reported with purified material that had not been frozen and thawed (16).

Selectivity of Receptor Binding. Previous studies have indicated that the 4.5-kbp *Eco*RI fragment of MTV DNA that resides on recombinant plasmid pMTV2 is competent to mediate hormone-responsive transcription when introduced into a cellular genome by DNA transformation (ref. 14; unpublished data). Fig. 2 *Top* shows that pMTV2 contains the left-hand long terminal repeat sequence that encodes the apparent transcriptional start site (refs. 15 and 27; unpublished data) but lacks 4.4 kbp of DNA present elsewhere in intact MTV DNA. Because the transformation experiments suggested that the deleted sequences are not essential for hormonal regulation, we chose to carry out our initial binding studies with the smaller fragment. In all of the experiments described in this report, pMTV2 DNA was cleaved with *Eco*RI and end-labeled with [^{32}P]dATP. In the absence of further digestion, this yielded labeled fragments of 4.5 kbp (MTV2-EE) and 4.3 kbp (pBR-EE), only the larger of which contained viral sequences (Fig. 2 *Middle* and *Bottom*).

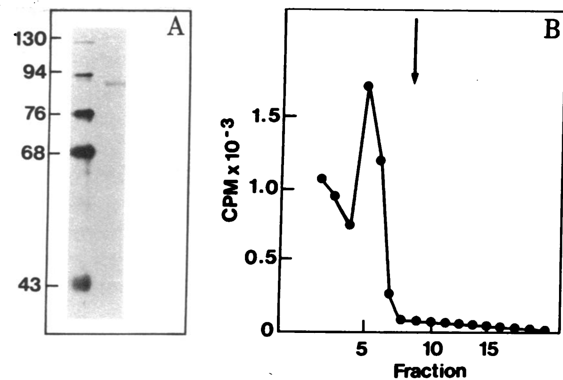

FIG. 1. Characterization of purified glucocorticoid receptor proteins. (*A*) Purified glucocorticoid receptor (25 ng) that had been stored frozen in the absence of added carrier protein was subjected to electrophoresis on a 10% NaDodSO$_4$/polyacrylamide gel (right lane). Molecular weight markers shown × 10^{-3} (left lane) are β-galactosidase (130,000), phosphorylase *a* (94,000), conalbumin (76,000), bovine serum albumin (68,000), and ovalbumin (43,000). After electrophoresis, gels were stained with ammoniacal silver nitrate. (*B*) Glucocorticoid receptor labeled with [^{3}H]triamcinolone and stored frozen in the presence of human serum albumin (300 μg/ml) was thawed and chromatographed on Sephadex G-75, and a 7000-cpm aliquot was sedimented through a 5–20% sucrose gradient in G-75 buffer (12 hr, 4°C, 165,000 × *g*). Arrow denotes migration of bacterial alkaline phosphatase activity, included in the gradient as an internal 6.2S marker.

To monitor the receptor–DNA interaction, we used the nitrocellulose filter binding assay (23), which takes advantage of the fact that DNA is retained on the filter only if it is associated with protein; after filter binding, the bound fragments were eluted and analyzed on agarose gels. Control experiments showed that under the conditions chosen for receptor binding, proteins that associated tightly and nonspecifically with DNA caused filter retention of any DNA fragment, whereas a protein known to bind site-specifically displayed appropriate selectivity. Thus, the two *Eco*RI fragments from pMTV2 and the six from phage λ were quantitatively bound to filters in the presence of calf thymus histones (Fig. 3, lanes C and H); in contrast, purified λ repressor protein bound with a high degree of selectivity to the 7.0-kbp phage λ operator-containing fragment (28) (Fig. 3, lanes D and I). In the absence of added protein (Fig. 3, lanes B and G), only a small amount of DNA (0.4–2% of input radioactivity) was retained by the filters.

When the purified glucocorticoid receptor was tested in the same experiment, the MTV2-EE fragment was preferentially bound relative either to pBR-EE or to the six phage λ DNA fragments (Fig. 3, lanes E and J). Thus, it appears from this result that the triamcinolone–receptor complex may recognize specific features within the MTV sequences present in the pMTV2 recombinant insert.

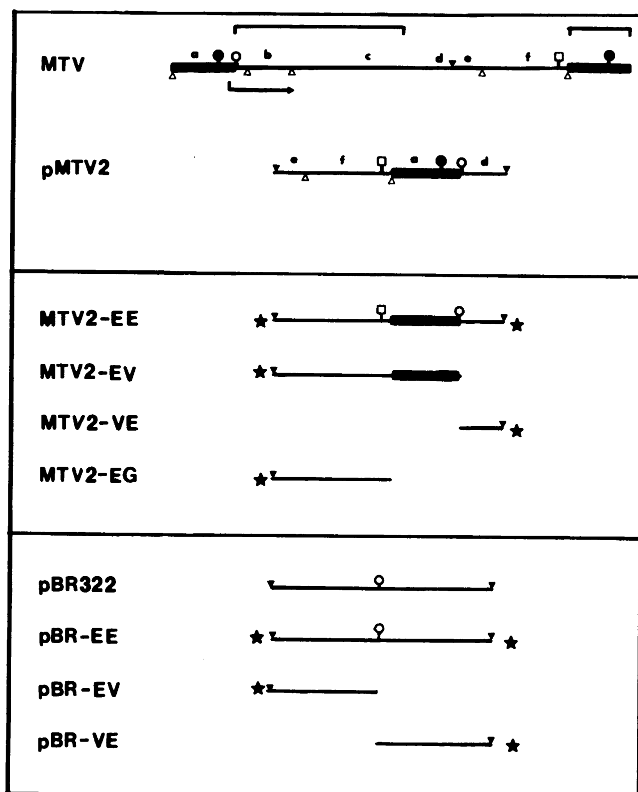

FIG. 2. Restriction endonuclease map of pMTV2 and end-labeled fragments used for receptor binding experiments. (*Top*, row 1) Map of intact MTV DNA arranged as found in the integrated proviral state. Heavy lines denote the 1.3-kbp long terminal repeat sequence; the arrow marks the apparent start site for MTV DNA transcription; brackets delineate the regions deleted in pMTV2; lower case letters are to facilitate orienting the permutation seen in pMTV2. (*Top*, row 2) Map of the MTV insert in pMTV2; note that it is permuted to the single *Eco*RI site in the MTV sequence. ▼, *Eco*RI; △, *Pst* I; ●, *Sac* I; ○, *Pvu* II; □, *Bgl* II. (*Middle* and *Bottom*) MTV and pBR322 fragments, respectively, ^{32}P end-labeled (★) at the *Eco*RI sites in the absence of further cleavage (MTV2-EE and pBR-EE) and after cleavage by *Pvu* II (MTV2-VE) and by *Pvu* II/*Bgl* II (MTV2-EG, pBR-EV, and pBR-VE).

Competition Experiments. To investigate further the selectivity of the receptor interaction with MTV sequences, three types of competition-binding experiments were carried out. In the first, the 38 end-labeled *Eco*RI fragments from phage T4 DNA (21) were mixed with end-labeled *Eco*RI fragments of pMTV2 that had then been digested with *Pvu* II (Fig. 4, lanes E and F). The most strongly bound fragment in this mixture was the MTV2-EV fragment (Fig. 4, lane H; see Fig. 2 *Middle* and Fig. 6); approximately four other fragments bound the receptor with apparently reduced affinity, whereas there was no detectable binding to the remaining fragments. As a control, when the phage T4 DNA fragments were mixed with end-labeled *Eco*RI fragments of phage λ DNA, the λ repressor protein selected in a highly specific manner the 7.0-kbp *Eco*RI fragment of λ DNA that carries a total of six high-affinity repressor binding sites (29) (Fig. 4, lanes A–D). Thus, although the overall selectivity of phage λ repressor appeared to exceed that of the glucocorticoid receptor under these conditions, the glucocorticoid receptor clearly selected MTV DNA sequences preferentially from a mixture of 40 different fragments.

In a second experiment, we assessed the ability of a 5-fold excess of either unlabeled pBR322 or pMTV2 DNA to compete for the selective binding of the receptor to the MTV2-EE [^{32}P]DNA fragment. Here, mixtures of labeled and unlabeled fragments were combined with receptor, after which binding of the labeled fragments to filters was monitored as before. Only pMTV2 DNA competed effectively for association of the receptor with MTV2–EE [^{32}P]DNA (Fig. 5, lanes A–G). Moreover, the reduction in binding of the labeled MTV2-EE DNA in the presence of a 5-fold excess of the same fragment (unlabeled) suggests that saturation of selective binding activity was approached at the concentrations of DNA and protein used in these experiments. This is consistent with other data showing that both DNA and receptor binding were in the linear range

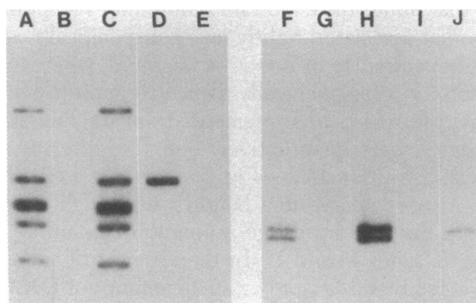

FIG. 3. Agarose gel electrophoresis of end-labeled *Eco*RI fragments of phage λ (*Left*) and pMTV2 (*Right*) DNA before and after nitrocellulose filter binding. Lanes: A–E, phage λ DNA; F–J, pMTV2 DNA; A and F, DNA prior to filter binding; B and G, DNA bound to filters in the absence of added proteins; C and H, DNA bound to filters in the presence of calf thymus histones; D and I, DNA bound to filters in the presence of phage λ repressor protein; E and J, DNA bound to filters in the presence of glucocorticoid receptor protein.

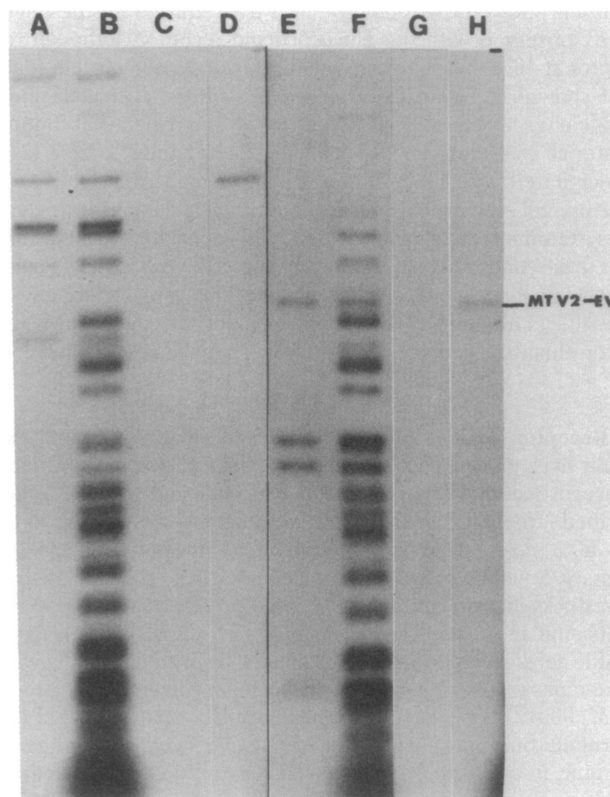

FIG. 4. Selectivity of DNA binding in the presence of ^{32}P end-labeled *Eco*RI fragments from bacteriophage T4 DNA. T4 DNA fragments (250 ng) were mixed with 100 ng of labeled *Eco*RI fragments of phage λ DNA (lanes A and B) or with labeled *Eco*RI fragments of pMTV2 DNA that had then been digested with *Pvu* II (lanes E and F). DNA fragments were then bound to nitrocellulose in the absence of added protein (lanes C and G), in the presence of phage λ repressor protein (lane D), or in the presence of glucocorticoid receptor protein (lane H).

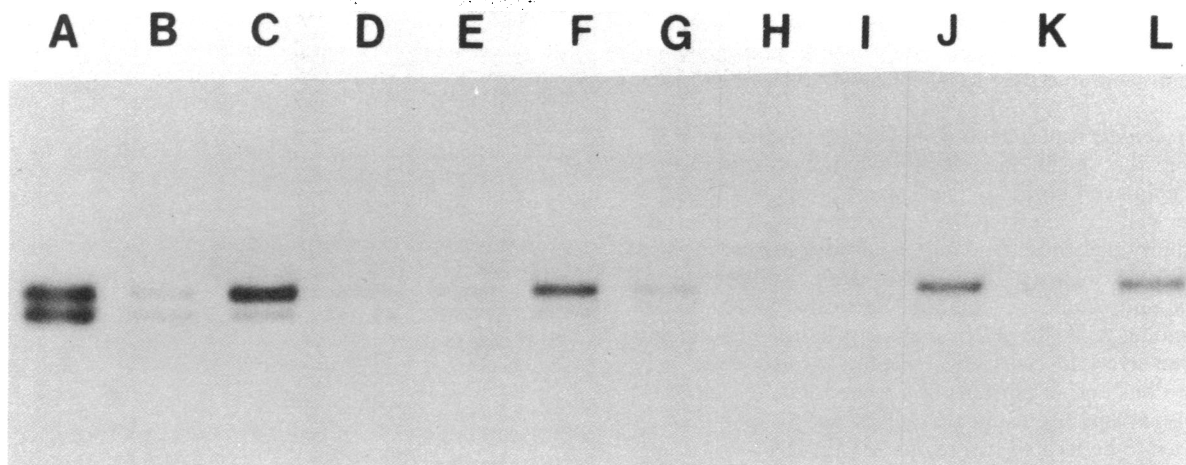

FIG. 5. Selectivity of receptor binding to MTV2-EE [^{32}P]DNA: competition by excess unlabeled DNA. In one experiment (lanes A–G), 100 ng of end-labeled *Eco*RI fragments from pMTV2 were incubated with or without 500 ng of unlabeled closed circular pBR322 or pMTV2 DNA either in the presence or absence of glucocorticoid receptor for 60 min at 4°C; DNA bound to nitrocellulose filters was eluted and analyzed on agarose gels. Lanes: A, DNA prior to filter binding; B, DNA bound in the absence of added protein; C, DNA bound in the presence of glucocorticoid receptor; D and E, DNA bound in the absence of glucocorticoid receptor but in the presence of a 5-fold excess of unlabeled pBR322 or pMTV2 DNA, respectively; F and G, DNA bound in the presence of glucocorticoid receptor together with a 5-fold excess of unlabeled pBR322 or pMTV2 DNA, respectively. In a second experiment (lanes H–L), 100 ng of the labeled pMTV2 fragments were first incubated in the presence or absence of glucocorticoid receptor for 30 min at 4°C; 500 ng of either RSF2124 or pMTV2 DNA was then added and incubation was continued for an additional 30 min at 4°C. Lanes: H and I, DNA bound in the absence of glucocorticoid receptor but in the presence of added pMTV2 or RSF2124 DNA, respectively; J, DNA bound in the presence of glucocorticoid receptor; K and L, DNA bound in the presence of glucocorticoid receptor and subsequently challenged with a 5-fold excess of unlabeled pMTV2 or RSF2124 DNA, respectively.

when present at these relative concentrations (unpublished results).

In a third competition experiment, receptor was allowed to react to equilibrium (30 min at 4°C; data not shown) with the labeled pMTV2 *Eco*RI fragments; the reactants were then challenged with a 5-fold excess of unlabeled calf thymus DNA, pBR322, pMTV2, or the ColE1 derivative plasmid RSF2124. After continued incubation for an additional 30 min, the remaining binding to the labeled fragments was assessed. pMTV2 DNA competed to a significant extent, whereas RSF2124 DNA

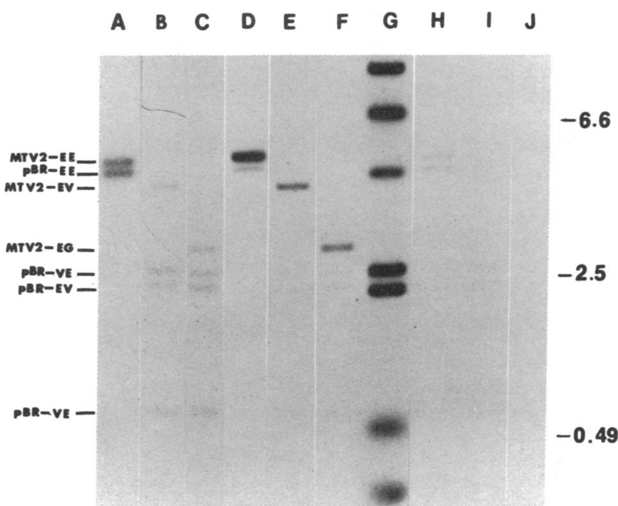

FIG. 6. Preliminary localization of subregions within MTV2-EE that selectively bind glucocorticoid receptors. Lanes: A–C, end-labeled pMTV2 *Eco*RI fragments were not further digested (lane A), cleaved with *Pvu* II (lane B), or cleaved with *Pvu* II/*Bgl* II (lane C) (the resulting labeled fragments originate as diagramed in Fig. 2 *Middle* and *Bottom*); D–F, DNA from samples A–C, respectively, bound to filters in the presence of glucocorticoid receptor; G, *Hin*dIII-digested phage λ DNA size markers (only relevant sizes shown); H–J, DNA from samples A–C, respectively, bound to filters in the absence of glucocorticoid receptor.

was not an effective competitor under these conditions (Fig. 5, lanes H–L); pBR322 and calf thymus DNA also failed to compete (data not shown). Thus, it appears that in the presence of excess MTV sequences, there is a substantial rate of dissociation of the receptor from MTV2-EE [^{32}P]DNA.

Binding to Specific Subfragments of MTV DNA. In an attempt to localize a smaller subregion of DNA that confers selective receptor binding, the end-labeled pMTV2 *Eco*RI fragments were further digested with *Pvu* II or with *Pvu* II/*Bgl* II; three labeled MTV-containing fragments (MTV2-EV, MTV2-VE, and MTV2-EG) and two labeled vector-containing fragments (pBR-EV and pBR-VE) were produced in these reactions (Fig. 2 *Middle* and *Bottom*). The receptor was found to bind selectively to the MTV2-EV and MTV2-EG fragments but not to the MTV2-VE or the vector fragments (Fig. 6). Thus, specific receptor binding sites appear to be located only within certain subregions of the MTV2-EE DNA fragment. The selective binding of the MTV2-EG fragment indicates that at least one binding site resides outside of the long terminal repeat sequence which encodes the transcriptional start site for MTV RNA (Fig. 2*A*); in fact, in intact hormone responsive proviruses, this fragment would map in the promoter-distal half of the MTV sequence.

DISCUSSION

The availability of highly radioactive steroids facilitated early observations that the hormone–receptor interaction promotes association of the complex with nuclear binding sites *in vivo* (reviewed in ref. 30) and with pure DNA *in vitro* (3, 4). Subsequently, it was demonstrated that these hormones affect the accumulation of specific transcripts by somehow altering the rates at which specific genes are transcribed (1, 31). Taken together, these data support the view that steroid hormone–receptor complexes may be analogous to well-characterized prokaryotic transcriptional regulatory proteins in that they specifically recognize and bind to DNA sequences within or near the regulated loci, and, thereby, effect a change in gene activity. Recently, the first direct evidence for specific chromosomal bind-

ing sites for a steroid (presumably complexed with its receptor) has been obtained in a cytogenetic study of *Drosophila* polytene chromosomes within ecdysone-responsive larval salivary gland cells (32).

The *Drosophila* experiments do not address the nature of the components that specify the receptor binding sites. In this report, we employed purified glucocorticoid receptors together with pure, well-characterized DNA fragments to begin to assess the possibility that these receptors might directly recognize a specific DNA sequence at their sites of action. The present results from a nitrocellulose membrane assay that monitored receptor binding to labeled DNA fragments indicate that highly purified rat liver glucocorticoid receptors interact selectively with some but not all portions of a cloned fragment of MTV DNA. It is perhaps interesting that at least one receptor binding site appears to be located in a region of viral DNA that is normally located several kbp from the apparent start site of MTV RNA synthesis.

Although our data suggest that glucocorticoid receptors purified nearly 10^4-fold bind in a sequence-specific fashion to DNA, it should be noted that our studies have been carried out with preparations that are not homogeneous. Moreover, because the receptor is purified only in its hormone-bound form and the hormone is not readily removed by the currently available techniques, we have not examined the predicted hormone dependence of the selectivity observed. Although it has not been ruled out that the selectivity resides in a minor contaminant in our receptor preparations, it is highly unlikely that a contaminating protein that adventitiously copurifies with the receptor would selectively associate with a hormone-regulated DNA sequence that itself has been purified more than 10^5-fold relative to the mammalian genome. Therefore, we believe that the receptor itself is responsible for selective binding of the MTV sequences.

Perhaps the most fully understood eukaryotic transcriptional regulatory protein to date is the T-antigen protein encoded by simian virus 40; it has been shown to bind to a specific sequence on simian virus 40 DNA and appears to be a negative regulator of early gene transcription (33, 34). It seems conceivable from our preliminary results that the cell-encoded glucocorticoid receptor protein might similarly interact with specific sequences in MTV and cellular DNA; functioning at those loci as a positive regulatory factor. The relationship of the present results to receptor action on MTV transcription in whole cells has not yet been addressed; clearly, it will be crucial to test rigorously the functional role of the putative receptor binding sites in gene regulation. In this study, we used a cloned fragment of MTV DNA that is known to be hormone-responsive when integrated at many sites into the genome of cultured cells (35); this approach might be extended by introducing various cloned DNA subfragments either bearing or lacking the putative receptor binding site into receptor-containing cells to assess their competence for hormone responsiveness. Thus, by combining additional biochemical and biological analyses, the details and possible function in gene regulation of the selective binding observed in these preliminary experiments may emerge.

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