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Multiple Specific Binding Sites for Purified Glucocorticoid Receptors on Mammary Tumor Virus DNA

Farhang Payvar, Gary L. Firestone, Susan R. Ross, Vicki L. Chandler, Örjan Wrange, Jan Carlstedt-Duke, Jan-Åke Gustafsson, and Keith R. Yamamoto

Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143 (F.P., G.L.F., S.R.R., V.L.C., K.R.Y.), and Department of Medical Nutrition, Karolinska Institutet, S-10401 Stockholm 60, Sweden (Ö.W., J.C.-D.,J.-Å.G.)

Glucocorticoid hormones selectively stimulate the rate of transcription of integrated mammary tumor virus (MTV) sequences in infected rat hepatoma cells. Using two independent assays, we find that purified rat liver glucocorticoid receptor protein binds specifically to at least four widely separated regions on pure MTV proviral DNA. One of these specific binding domains, which itself contains at least two distinct receptor binding sites, resides within a fragment of viral DNA that maps 110–449 bp upstream of the promoter for MTV RNA synthesis. Three other binding domains lie downstream of the promoter and within the MTV primary transcription unit. Restriction fragments bearing separate binding domains have been introduced into cultured cells; transformants have been recovered in which the introduced fragments are expressed under glucocorticoid control. Thus, it appears that this assay will be useful for assessing the biological significance of the receptor binding sites detected in vitro.

Key words: glucocorticoid receptors, protein–DNA interactions, transcriptional regulation, steroid hormone action, mouse mammary tumor virus

The biological effects of glucocorticoids and other steroid hormones are mediated by intracellular receptor proteins that are hormone specific; the receptor–ligand interaction increases the affinity of the receptor for nuclear binding sites in vivo and for purified DNA in vitro (see [1] for review). In several experimental systems, the mechanisms of receptor action are beginning to emerge. For example, in cultured hepatoma tissue culture (HTC) cells bearing stably integrated murine mammary tumor virus (MTV) genes, the glucocorticoid–receptor protein complex selectively increases the rate of MTV RNA synthesis [2,3]. In these cells, MTV RNA is transcribed from a single promoter, Pm, which initiates synthesis 1196 bp from the end of the 9 kb proviral element [3,4]. In the simplest view, it appears that all of the information required for a receptor-dependent response is encoded within MTV DNA sequences, and that the receptor exerts its transcriptional effect solely by increasing the efficiency of utilization of Pm [4].

Glucocorticoids appear to affect the expression of only a few specific genes; what determines the great selectivity of receptor action? We have presented evidence

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that purified glucocorticoid receptors bind selectively in vitro to cloned fragments of MTV DNA [5]. Thus it seems possible that receptor action in vivo may be mediated, at least in part, by DNA sequence recognition. In this report, two independent methods–nitrocellulose filter binding and electron microscopy—are employed to map in greater detail the regions within the MTV element to which the receptor binds with high affinity. In addition, preliminary DNA transformation experiments that begin to assess the biological significance of the receptor binding sites are described.

MATERIALS AND METHODS

Purification of the rat liver glucocorticoid receptor protein [6] and conditions for specific receptor–DNA binding and identification of the bound fragments by nitrocellulose filter binding [5] were as described previously. Receptor preparations were 20–85% pure; all contained saturating levels of bound hormone. Binding mixtures contained 20–50 pM DNA fragments and 10–100-fold molar excess of receptor monomers. Preliminary receptor and DNA titration data suggest that active receptor complexes are not in excess under these conditions, consistent with the finding that receptors appear to bind as multimer complexes (Firestone, unpublished observations) to multiple DNA target sites. Fixing, spreading, staining, and shadowing of DNA–protein complexes for electron microscopy was carried out according to the procedures of Williams [7]. Cloned fragments of MTV DNA in recombinant λ phages were provided by J. Majors and subsequently transferred to pBR322 and subcloned as indicated. DNA cotransformations into mouse Ltk⁻ cells were essentially as described by Wigler et al [8].

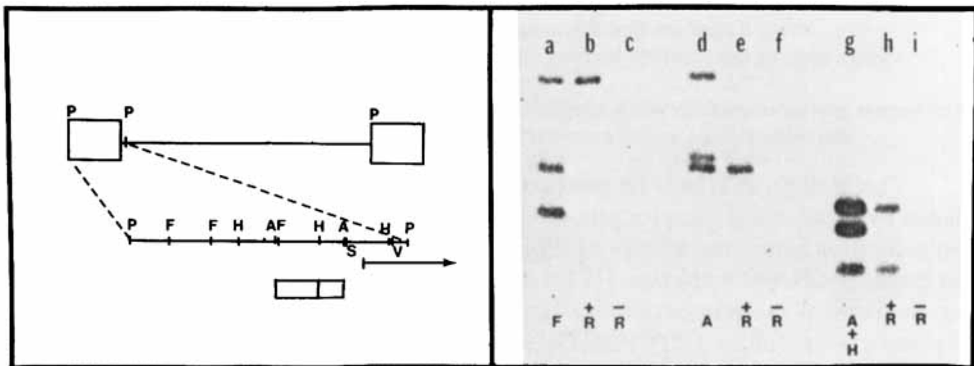

Fig. 1. Mapping the receptor binding domain within the MTV LTR by nitrocellulose filter binding. The diagram at left shows the 1.45 kb PstI fragment containing the MTV LTR (shown as boxes in the upper map), with restriction sites shown for PstI (P). Hinf (F), HaeIII (H), Sau3A (A), SacI (S), and PvuII (V). The arrow locates the MTV promoter and shows the direction of transcription. The boxes below the diagram show the two Sau3A-HaeIII fragments that contain specific receptor binding sites as shown by the data in the right panel. a–c, subfragments end-labeled after Hinf digestion; d–f, subfragments end-labeled after Sau3A digestion; g–i, subfragments end-labeled after Sau3A plus HaeIII digestion; a,d,g, fragments before receptor binding; b,e,h, fragments retained on nitrocellulose after receptor binding; c,f,i, fragments retained in nitrocellulose in the absence of added receptor. Elution from filters, electrophoresis in agarose gels and autoradiography were as described [5].

RESULTS

In a previous report [5], we monitored the receptor-DNA interaction using the nitrocellulose filter binding assay [9], in which DNA is retained on the filter only if it is associated with protein; after filter binding, the cloned fragments were eluted and analyzed on agarose gels. Those experiments revealed that purified glucocorticoid receptor protein binds selectively a cloned fragment of MTV DNA containing about half of the sequences present in the intact viral element, including the region encoding Pm; furthermore, a subfragment that maps several kilobases downstream from Pm appeared to contain a high affinity receptor binding site.

Figure 1 shows the results of a nitrocellulose filter binding experiment that tests receptor association with ^{32}P-labeled cloned DNA fragments from defined regions of the 1.3 kb MTV long terminal repeat (LTR) sequence. It was of particular interest to determine whether specific receptor binding sites reside proximal to Pm, which is encoded within the LTR [3,4]. These experiments identify two contiguous restriction fragments, together covering the region 110–449 bp upstream of the transcription start site, each of which contains specific receptor binding activity; the receptor fails to associate with other subfragments of the LTR.

In separate experiments, receptor binding to the MTV LTR sequences was also examined by electron microscopy. The binding conditions were identical to those used above [5]; complexes were then fixed, spread on polylysine-coated grids, negative stained with uranyl acetate, and rotary shadowed with tungsten [7]. The orientation of the LTR relative to the bound receptor was established by examining binding to the 1453 bp PstI fragment that contains the entire LTR, as well as to the 1079 bp subfragment produced by cleavage with SacI, which cuts near Pm (Fig. 2). The results, examples of which are presented in Figure 2a–c, are fully consistent with those from the filter binding assay: on 71 of 74 DNA molecules containing bound receptor, the complex was associated with one of two distinct regions of the LTR that are coincident with the two restriction fragments retained on nitrocellulose (Fig. 1).

No DNA fragments were detected that contain more than one bound receptor complex. This may reflect the fact that binding studies have not yet been carried out at high molar ratios of active receptor-DNA. In this initial study, it appeared that approximately equal numbers of fragments are found with receptor associated at each of the two binding sites (Firestone, unpublished observations), consistent with the view that the receptor has a similar intrinsic affinity for each site. In any case, the MTV LTR appears to contain at least two closely spaced receptor binding sites located in a discrete region upstream from the transcription start site.

Nitrocellulose filter binding assays revealed previously that at least one receptor binding site resides in a region of viral DNA that maps far downstream from Pm [5]. When restriction fragments from that region were exposed to purified receptor and examined in the electron microscope, receptor complexes were detected within a specific subfragment; an example is shown in Figure 3. Although our present results in this region of the MTV map are still preliminary, both nitrocellulose and electron microscopic experiments imply that as with the LTR, at least two closely spaced binding sites may reside in a region located 200–600 bp upstream from the beginning of the "righthand" LTR sequence (data not shown).

Additional nitrocellulose filter assays (Payvar, unpublished observations) have confirmed the existence of the predicted receptor binding locus in the righthand LTR,

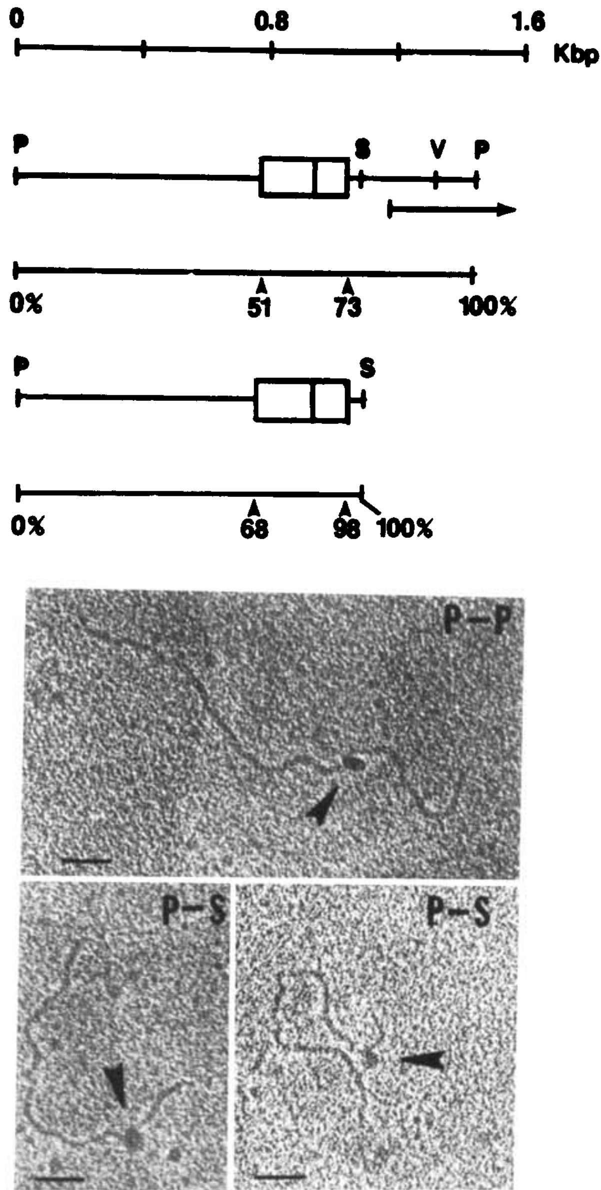

Fig. 2. Mapping the receptor binding domain within the MTV LTR by electron microscopy. The PstI fragment containing the MTV LTR (Fig. 1) was employed for receptor binding either as an intact fragment or after secondary cleavage with SacI. The boxes denote the two Sau3A-HaeIII subfragments that specifically bind receptor in the nitrocellulose assay (Fig. 1). After receptor binding, the complexes were prepared for electron microscopy [7]. The micrographs shown reveal receptor complexes associated with the intact (P–P) or truncated (P–S) fragments, and give examples of receptor complexes associated with each of the two binding sites identified by the nitrocellulose assay. The bar represents 0.5 μm.

and also revealed an additional region of receptor binding centered approximately 3 kb distal to the start site of transcription (Fig. 4). Thus, our current view is that the receptor selectively recognizes at least four widely separated binding domains on the MTV DNA element, and that the domains may themselves be composed of multiple, closely spaced binding sites.

To begin evaluating the biological significance of these in vitro results, cloned fragments of MTV DNA containing receptor binding sites were introduced into cultured cells and stable transformants were tested for hormone responsive transcription of the introduced sequences. In initial experiments, three such fragments, each containing a single binding domain and cloned at the PstI site of pBR322, were introduced into mouse L cells by cotransformation with cloned HSV *tk* DNA; tk^+ transformants were recovered in selective medium [8].Cotransformants were identified, and total cell RNA from hormone treated and control cultures was screened by RNA-dot hybridization [10] for transcripts homologous to the introduced MTV recombinant. The results, summmarized in Figure 4, reveal that consistent with previous reports [11–13], expression from a fragment containing the LTR receptor binding domain and Pm is hormonally regulated in the transformants. Surprisingly, two fragments containing receptor binding domains that map far downstream from Pm also appear to yield dexamethasone responsive transformants by this assay. Thus, hormone responsive transcription of these isolated regions of the MTV genome seems to occur in the absence of Pm, perhaps reflecting in vivo a functional activity of the receptor binding sites detected in vitro.

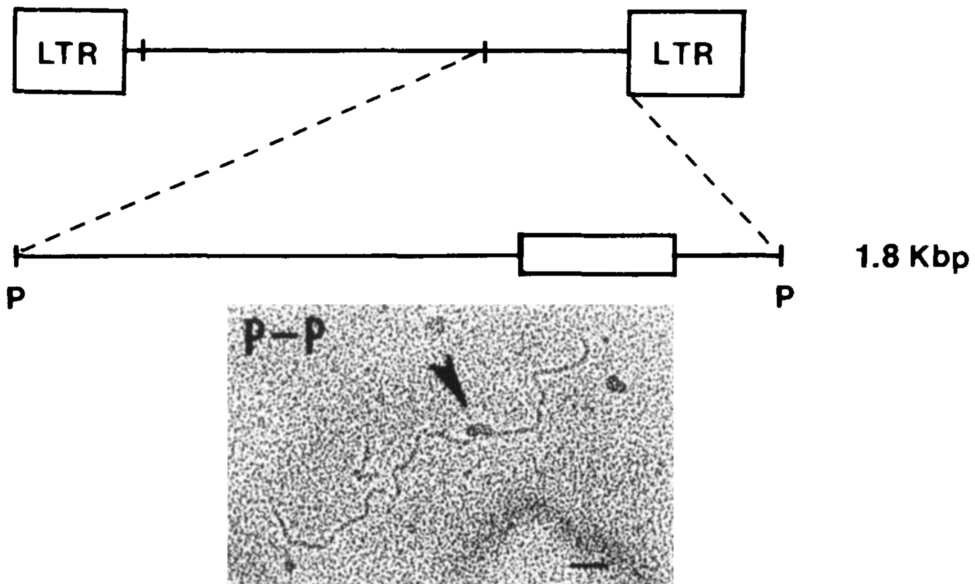

Fig. 3. Mapping a receptor binding domain within the transcribed region of MTV by electron microscopy. A 1.8-kb PstI restriction fragment that maps just upstream of the righthand MTV LTR was subjected to receptor binding and electron microscopy as described in Figure 2. The micrograph shown is a typical example of a specifically bound receptor complex; the box in the diagram depicts its relative location within the fragment. Nitrocellulose assay data (not shown) confirm this interpretation.

DISCUSSION

Our results demonstrate that highly purified glucocorticoid receptor protein binds selectively and with high affinity in vitro to a number of widely dispersed loci within and adjacent to sequences encoding a transcriptional unit that is strongly regulated by glucocorticoid receptors in vivo; at least one of these binding domains contains a minimum of two distinct binding sites. Recent results (Payvar, unpublished observations) indicate that the receptor associates with the different domains with approximately similar affinities, and that the binding is highly selective relative to DNA fragments from several cloned cellular and viral genes that are not glucocorticoid regulated; we are currently attempting to identify the exact nucleotide sequences that are recognized by the receptor protein. Preliminary DNA transformation experiments with cloned MTV subfragments indicate that this approach will be useful for determining the biological functions, if any, that can be ascribed to these binding sites.

It is intriguing that fragments bearing receptor binding domains but lacking known promoters appear to be transcribed in a hormone-responsive fashion in transformants; the significance of these observations is not yet understood. If these results are confirmed, the start sites of the hormone-responsive transcripts will be determined as a preliminary test of the possibility that the fragment, when associated with a receptor complex, is acting at a distance on a nearby cellular or vector-encoded promoter. One alternative view is that these regions may contain cryptic promoter sequences that are inactive in the intact proviral element.

It is also conceivable that only the promoter proximal receptor binding domain is associated with biological activity in vivo, and that the receptor may increase promoter efficiency by a mechanism analogous to those proposed for prokaryotic

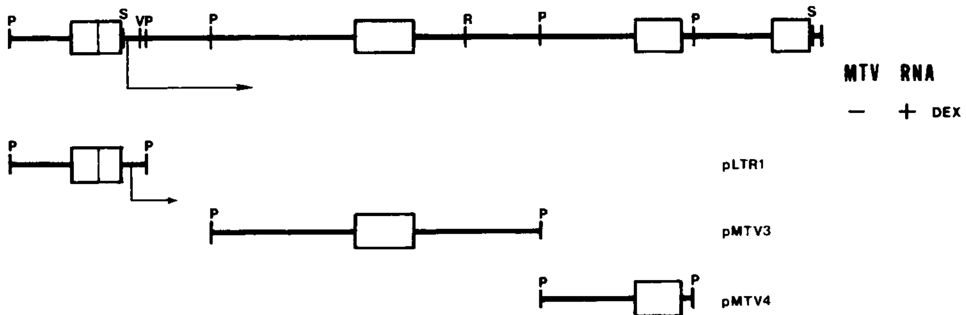

Fig. 4. Hormone responsive expression in vivo of MTV fragments containing specific receptor binding domains in vitro. The upper diagram summarizes the four receptor binding domains that have been identified on MTV DNA to date. Three PstI subfragments, each containing a single receptor binding domain and cloned at the PstI site of pBR322, were introduced into mouse L cells by cotransformation [8]. Stable transformants containing the MTV fragment were recovered, and RNA purified from cultures grown in the presence or absence of dexamethasone was assayed on RNA dot blots for transcripts homologous to the introduced sequences. The results from the transformants shown reveal that all three fragments are expressed in a hormone responsive fashion. The apparent difference in the magnitude of the hormone response in the different transformants is not readily interpretable, since each contains different numbers of inserts integrated at distinct chromosomal loci; Feinstein et al. [16] have described strong position effects on the expression and regulation of MTV sequences.

transcriptional regulatory proteins that bind near promoters. In fact, apparently fortuitous and nonfunctional binding sites for certain prokaryotic regulatory proteins have been detected within structural gene sequences that are regulated at promoter proximal sites [14]. However, in view of our results suggesting multiple dispersed binding domains on MTV DNA for glucocorticoid receptors, together with similar conclusions by Mulvihill et al. [15] for progesterone receptors on ovalbumin sequences, it is intriguing to speculate that the multiple sites might be functional. For example, a proposal put forth on theoretical grounds [1] suggested that multiple receptor binding events might somehow alter chromatin structure across the entire transcriptional unit, and thereby allow or induce an increase in its rate of transcription. Consistent with this view, Feinstein et al. [16] have shown that different chromatin configurations can have striking effects on the competence of the glucocorticoid receptor to stimulate MTV transcription, and preliminary data suggest that glucocorticoid stimulation of MTV transcription may in some cases be accompanied by detectable changes in chromatin structure (Feinstein, unpublished observations).

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