Hormone-mediated repression: a negative glucocorticoid response element from the bovine prolactin gene

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We have defined and characterized a region upstream of the bovine prolactin gene that confers repression by glucocorticoids. This 'negative glucocorticoid response element' (nGRE) contains multiple footprinting sites for purified glucocorticoid receptor protein between -51 and -562 bp. A strong consensus sequence for receptor binding within the nGRE has not yet been defined, but it is apparent that nGRE sequences differ from the GRE consensus elements that confer positive glucocorticoid regulation. Unlike 'positive' GREs, the nGRE enhances promoter activity in the absence of glucocorticoids or receptor, presumably through the action of a protein that binds in the same region and activates transcription. The hormone-receptor complex appears to negate this enhancement by competing or inactivating the second factor. As with positive GREs, nGRE sequences confer hormonal regulation upon linked heterologous promoters within various cell types; a 34-bp subfragment containing a single receptor binding site is sufficient for nGRE activity. We speculate that nGRE sequences might alter the structure of bound receptor, thereby preventing it from functioning as a positive regulator when bound at those sites.

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The glucocorticoid receptor protein transduces the signaling information of hormonal ligands into specific changes in gene expression: upon hormone binding, the receptor-hormone complex undergoes nuclear localization (Picard and Yamamoto 1987), associates with genomic sites, and alters the efficiency of transcriptional initiation at specific promoters (for review, see Yamamoto 1985). As with other transcriptional regulatory factors (Borrelli et al. 1986; Bram et al. 1986; Johnston 1986; Ptashne 1986), the glucocorticoid receptor stimulates transcription from some promoters while down-regulating others. Its positive regulatory effects are conferred by binding to a specific class of transcriptional enhancers termed glucocorticoid response elements (GREs) (Chandler et al. 1983; Ponta et al. 1985; DeFranco and Yamamoto 1986; Miksicek et al. 1986). Mutational analyses of a mouse mammary tumor virus (MTV) GRE suggested that the receptor is the only specific binding factor required for GRE enhancer activity (DeFranco et al. 1985; Buetti and Kühnel 1986). The GRE-bound receptor presumably interacts with some component of the transcription initiation apparatus, thereby stimulating its activity (Yamamoto 1985). GREs that mediate positive regulatory effects have now been shown to reside within or near a variety of genes (e.g., Karin et al. 1984; Renkawitz et al. 1984; Moore et al.

1985; Danesche et al. 1987; Jantzen et al. 1987), and a common consensus sequence, similar to that identified within MTV GREs (Payvar et al. 1983; Scheidereit et al. 1983), has been identified.

Negative regulation of transcription by glucocorticoids has been clearly documented (Camper et al. 1985; Israel and Cohen 1985; Charron and Drouin 1986; Freneau et al. 1986; Frisch and Ruley 1987; Weiner et al. 1987), but the role of specific receptor-DNA interactions and the mechanistic relationships of positive and negative control have not been defined. In this report, we identify sequences upstream of the bovine prolactin gene that confer negative glucocorticoid regulation upon the prolactin promoter in anterior pituitary cells (Camper et al. 1985). In particular, we describe properties of a 'negative GRE' (nGRE) in this region, test glucocorticoid receptor binding to the nGRE, and examine nGRE activity in heterologous cells and on heterologous promoters.

Results

Glucocorticoid receptor binding sites upstream of the bovine prolactin gene

Camper et al. (1985) showed that the bovine prolactin promoter and 1 kb of 5' flanking DNA are sufficient to mediate glucocorticoid-repressible expression of a linked chloramphenicol acetyltransferase (CAT) gene in

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transfected GH3 rat pituitary cells. To determine whether the receptor binds selectively within that region, we carried out DNase I footprinting assays with glucocorticoid receptor purified from rat liver (Payvar et al. 1983; Wrange et al. 1986). Seven footprints were detected between -50 and -562 relative to the transcription start site. Receptor protected both strands of DNA and displayed different binding efficiencies at the different sites (Fig. 1); for example, footprint 7 is >10-fold stronger than the relatively weak footprint 3. In general, the range of apparent affinities was similar to that observed on MTV DNA with similar receptor preparations (data not shown; Payvar et al. 1983). The DNA sequences within the footprints lack extensive similarity, although each includes sequences that are weakly related to the octanucleotide consensus element (AGA^AT CAG^AT) implicated in receptor binding and enhancer activity at 'positive' GREs (Fig. 2). Whether these consensus-like sequences are in fact determinants of specific receptor binding within the prolactin upstream region is unknown; in any case, the putative nGRE se-

quences do not adhere as closely to the consensus as those associated with positive GRE function.

Although the receptor is encoded by a single-copy gene (Miesfeld et al. 1984), functionally distinct forms could arise in principle post-transcriptionally. Different receptor isoforms might then recognize different classes of DNA sequences and mediate either negative or positive transcriptional regulation. To test this possibility, we performed competition footprinting experiments in which the labeled DNA fragment contained high-affinity receptor binding sites derived from an MTV GRE (Payvar et al. 1983) cloned adjacent to the prolactin footprint 3 region (PRL3; -247 to -214) described above (Fig. 3; see also Fig. 6). As shown in Figure 3, footprinting on PRL3 required approximately 10-fold more receptor than that needed for a similar level of occupancy at the MTV-derived binding sites (MTV46). Nevertheless, both the prolactin and MTV46 footprints were competed by excess unlabeled DNA from either the weak PRL3 or the strong MTV46 binding sequences, whereas a control prolactin fragment (-230 to -188)

Figure 1. DNase I footprint analysis of glucocorticoid receptor binding to prolactin DNA. Prolactin DNA was (A) 3'-end-labeled or (B) 5'-end-labeled at a *Bam*HI site (+22) or (C) 5'-end-labeled at a *Bgl*II site (-248) and footprinted with 0–0.4 μ g purified glucocorticoid receptor as indicated. (Lane G) Probe DNA submitted to guanosine-specific cleavage. Sequences protected from DNase digestion are indicated by brackets; sites of enhanced cleavage are marked with arrows. Diagram shows positions of these features relative to the prolactin transcription initiation site.

Figure 2. DNA sequences of the bovine prolactin 5'-flanking region bound by glucocorticoid receptor and location of receptor binding sites. The nucleotide sequence of bovine prolactin DNA from plasmid pPRLcat-1 (Camper et al. 1984, 1985) is presented. Receptor footprints are demarcated by lines above and below the DNA sequence for the coding and noncoding strands, respectively. Sites hypersensitive to cleavage in the presence of receptor are indicated by arrows. Dashed lines indicate putative TATA box and CCAAT box sequences at -25 and -80, respectively, and a sequence at -120 that has been observed in all members of the growth hormone gene family (Rottman et al. 1986).

not recognized by receptor failed to compete. Thus, the relative affinities of receptor binding *in vitro* to these MTV-derived and prolactin sequences are within approximately one order of magnitude, and receptor binding near positively and negatively regulated genes apparently is accomplished by a common pool of receptor rather than by distinct, noncompeting forms.

Effects of the receptor binding region on prolactin promoter activity

To assess whether the DNA segment bound specifically by glucocorticoid receptor *in vitro* might modulate prolactin promoter activity *in vivo*, we constructed a series of nested 5' deletion mutants that progressively truncated the flanking DNA upstream of the prolactin promoter, which was fused at +16 to the CAT coding region. Plasmids carrying these constructs were transfected into GH3 cells, and CAT activities measured in transient expression assays (Gorman et al. 1982). In addition, accurately initiated prolactin-CAT mRNA was monitored by RNase mapping (Melton et al. 1984), and relative transcript levels were calculated by normalization to an internal control mRNA expressed from a co-transfected plasmid bearing a Rous sarcoma virus pro-

motor-CAT gene fusion, which is not glucocorticoid regulated (see Fig. 4).

Consistent with previous results (Camper et al. 1985), we found that dexamethasone treatment reduces by three- to four-fold the level of prolactin-CAT transcripts produced by constructs containing at least 310 bp of 5' flanking sequence (see Fig. 4). Further deletion, however, resulted in a progressive decline in both basal (i.e., 'unrepressed' or glucocorticoid independent) expression and in the extent of repression. Deletions to positions -290 to -236, which remove footprint 4 but not footprint 3, are repressed 2- to 2.5-fold by hormone treatment. Low levels of hormone regulation were observed with deletion mutants containing between 229 and 173 bp of flanking sequence, which retains only footprints 1 and 2; deletion to -45 abolished detectable transcription. Thus, we conclude that sequences encompassing footprints 1-4 yield hormonal regulation that is indistinguishable from that seen when all seven receptor footprints are present (Fig. 4). The decreased repression observed with deletion mutants containing less than 310 bp of 5' flanking DNA suggests that sequences within or near footprint sites 2, 3, and 4 contribute to optimal hormonal regulation; footprint 1 alone (deletions -152 to -78) fails to confer significant glucocorticoid regulation in this context.

Figure 3. GRE and nGRE competition footprinting analysis of glucocorticoid receptor binding to prolactin and MTV sequences. A 650-bp fragment from plasmid MTV-152 (see Fig. 6) was 3'-end-labeled at a *Hind*III site (+18) and footprinted with 100 ng of rat liver glucocorticoid receptor in the absence or presence of competitor DNAs. Brackets indicate positions of receptor footprints within the PRL3 and MTV46 segments of the fragment. Competitor DNAs: (lanes c–e), a 58-bp *Xba*I fragment containing two copies of MTV nucleotides –123 to –105 (MTV46); (lanes h–j) 49-bp *Xba*I fragment containing prolactin nucleotides –247 to –214 (PRL3); (lanes m–o) 57-bp *Xba*I fragment containing a nonfootprinted prolactin region, nucleotides –230 to –188 (CON). Reactions contained 2.3 fmoles of ³²P-labeled DNA together with 0 (lanes a, b, f, g, k, l, p), 0.23 (lanes c, h, m), 0.7 (lanes d, i, n), or 2.3 (lanes e, j, o) pmoles of competitor DNA; reactions in lanes a, f, k, and p lacked receptor.

The gradual loss of glucocorticoid-mediated repression observed with progressive 5' deletion reflects predominantly a decrease in promoter activity observed in the absence of hormone, whereas the absolute level of transcription in the presence of dexamethasone remains, in general, relatively constant (Fig. 4). This implies that the prolactin 5'-flanking sequences comprise a complex regulatory region containing contiguous or interdigitated elements responsible both for conferring glucocorticoid responsiveness and for establishing a high level of promoter activity in the absence of glucocorticoids.

To characterize in greater detail the roles of receptor binding sites, 1, 2, and 3 in glucocorticoid-mediated repression, the three sites were mutated individually in constructs lacking footprint regions 4–7. In this context, we found that alteration of any of the three remaining sites decreased both basal transcription from the prolactin promoter and eliminated glucocorticoid regulation. Thus, 5' deletion to –212, which eliminates receptor footprint site 3, reduced basal expression (by

about four-fold in this experiment) relative to constructs extending to –248, and greatly diminished hormonal regulation (Fig. 5a,b; see also Fig. 4). Interestingly, reinsertion of the PRL3 region (–214 to –247) in the inverted orientation restored both basal transcription and hormone-mediated repression (Fig. 5c). This suggests that, as with the GREs that confer positive hormonal regulation, putative nGRE elements can function in both orientations. Linker substitution (LS) mutations that selectively disrupted footprints 2 or 1 were phenotypically similar to the footprint site 3 deletion. Thus, LS –170/–152 and LS –70/–59 interfere, with receptor footprinting at sites 2 and 1, respectively, in vitro (data not shown), and reduce basal expression and hormonal regulation in vivo (Fig. 5d,e). In contrast, a linker substitution mutation (LS –214/–202) that had no effect on receptor binding altered neither expression nor glucocorticoid responsiveness (Fig. 6 and data not shown).

Taken together, our results indicate that prolactin promoter activity is governed by a relatively large and complex region which, at the minimum, must include footprint regions 1, 2, and 3.

Linkage to an MTV GRE renders the prolactin promoter inducible by glucocorticoids

It seemed conceivable that GRE and putative nGRE sequences, per se, might be functionally indistinguishable, but that the prolactin promoter, within the physiological context of anterior pituitary cells, might 'interpret' the bound receptor as a negative regulator. As an initial test, we inserted MTV46, a 46-bp synthetic oligonucleotide, whose sequence was derived from an MTV GRE, into various positions within the prolactin flanking sequence. Neither induction nor repression was observed when MTV46 was positioned upstream of receptor footprint site 3 (Fig. 6c); this may indicate that the GRE and nGRE elements function with equal (i.e., offsetting) activity in this configuration. Consistent with this view, MTV46 strongly enhances, in a hormone-dependent fashion, other promoters into which it is similarly positioned (data not shown). With the GRE positioned between footprint sites 2 and 3, a three-fold induction by glucocorticoids was observed (Fig. 6d), and the induction increased to greater than 20-fold as the GRE was moved closer to the prolactin start site with concomitant deletion of footprint sites 2 or 1 (Fig. 6e,f). These results demonstrate a strong position dependence of GRE activity in the context of the prolactin promoter.

We conclude that the prolactin promoter does not, per se, respond negatively to the glucocorticoid receptor within pituitary cells. Rather, both positive and negative glucocorticoid regulation can be sensed by this promoter. Therefore, transcription factors or other components necessary for both modes of regulation must coexist within this cell type. Most importantly, these results suggest strongly that GRE and nGRE sequences themselves must somehow dictate whether a single regulatory protein, the glucocorticoid receptor, is to act as a positive or negative regulator.

Figure 4. Expression and glucocorticoid regulation from prolactin gene 5' deletion mutants. Test plasmids (15 μ g) bearing 5'-terminal deletions in the prolactin flanking region were cotransfected with a reference plasmid pRSVcat (1 μ g) into GH3 cells with (+) or without (–) 0.1 μ M dexamethasone. Poly(A)⁺ RNA was isolated 48 hr later and the 5' ends were analyzed by RNase protection. Autoradiographs representative of RNase protection assays are shown. The positions of probe fragments protected by accurately initiated transcripts from the test (PRL) and reference (RSV) genes are indicated; control transfections with 15 μ g of pPRLcat-2 (5' Δ -248) or pRSVcat alone are shown in lanes RSV and PRL. The graph depicts densitometric quantitation of prolactin–CAT transcripts. All values are normalized to the reference signal and represent the mean expression level from two to six independent transfections; relative expression of ≤ 0.05 was indistinguishable from film background in these assays. Diagram shows positions of the seven receptor footprints relative to deletion end points; also indicated is the probe fragment and the species protected by accurately initiated prolactin–CAT transcripts (146 nucleotides) and RSV–CAT transcripts (130 nucleotides). Note the 5' Δ -236 deletes most of footprint 3 sequence but does not abolish footprinting at that position (see text); this may reflect substantial similarity between the fused sequences (see Materials and methods) and the wild-type sequences (Fig. 2).

nGRE effects on a heterologous promoter in nonpituitary cells

As a direct test of our interpretation that GRE and nGRE sequences are functionally distinct, we fused various subfragments of the prolactin flanking region to the herpes simplex virus thymidine kinase (tk) promoter, and examined basal promoter and hormone regulatory capacity of these constructs in transiently transfected nonpituitary cells. Similar results were obtained with several different recipient cell lines. Here we present data from the monkey kidney cell line CV-1, which

lacks endogenous glucocorticoid receptor activity (Miesfeld et al. 1986). With this line, we could readily test the role of glucocorticoid receptor protein in nGRE function by assessing the requirement for cotransfected plasmids that express receptor coding sequences (see Materials and methods).

We found that a 34-bp prolactin subfragment containing receptor footprint region 3 (PRL3; –247 to –214) rendered the tk promoter glucocorticoid repressible in a receptor-dependent fashion when fused in either orientation upstream of the promoter (Fig. 7e–h) or (weakly) when situated downstream of the transcription

Figure 5. Expression and glucocorticoid regulation from linker substitution and inversion mutations of the prolactin regulatory region. Receptor binding sites 1, 2, and 3 are required for glucocorticoid repression. Site 3 functions in an inverted orientation. Test (prolactin-CAT fusions) and reference (pRSVcat) plasmids were cotransfected into GH3 cells and total cytoplasmic RNA isolated 48 hr later; transcript mapping and densitometry were as in Fig. 4. Diagram shows the prolactin regulatory region from nucleotides -248 to +16 relative to the transcription start site; numbered boxes locate the three most promoter-proximal receptor binding sites; thick lines designate *Bam*HI linker sequences.

unit (Fig. 7j,k). Similar results were obtained with the prolactin DNA fragments containing isolated receptor footprints 1, 4, 5, or 6 (data not shown); footprints 2 and 7 have not been tested in this manner. Likewise, prolactin sequences were able to confer negative hormonal regulation upon the SV40 early, rabbit β -globin, and mouse metallothionein promoters (data not shown). In contrast, fusion of a GRE rendered the tk promoter strongly inducible by glucocorticoids (Fig. 7c,d,i), whereas the tk promoter alone specified a low level of transcription which is unaffected by hormone treatment (Fig. 7a,b).

Importantly, nGRE sequences, unlike the MTV46 sequence, dramatically increase expression of the tk promoter in the absence of glucocorticoids, and this stimulation is independent of the glucocorticoid receptor (Fig. 7). Thus, these results imply that the prolactin nGRE

provides binding sites for at least two distinct components — a factor other than the glucocorticoid receptor that strongly enhances basal transcription efficiency, and the glucocorticoid receptor itself, which in the presence of hormone counteracts this constitutive enhancer activity.

Discussion

Identification of negative glucocorticoid response elements

We have employed several experimental approaches to identify regulatory elements associated with the bovine prolactin gene that are determinants of glucocorticoid-mediated transcriptional repression. Glucocorticoid receptor footprinting, deletion, and linker substitution mutagenesis, as well as heterologous promoter fusions,

Figure 6. The prolactin promoter is glucocorticoid inducible when linked to an MTV GRE derivative. Test (prolactin-CAT fusions) and reference (pRSVcat) plasmids were cotransfected into GH3 cells and total cytoplasmic RNA isolated 48 hr later. Transcript mapping and densitometry were as in Fig. 4. Diagram shows the prolactin regulatory region from -248 to +16; hatched boxes represent the MTV46 synthetic GRE fragment (see Materials and methods); thick lines designate *Bam*HI linker sequences.

Figure 7. The prolactin nGRE modulates transcription from a linked tk promoter. nGRES function in an orientation-independent manner. Glucocorticoid receptor is not required for basal enhancement by nGRES. CV-1 cells were cotransfected with the tk-CAT fusion test plasmid indicated and either glucocorticoid receptor-expression plasmid pRSVGR (+GR) or a control plasmid lacking receptor sequences (–GR). The autoradiogram shows CAT assays from a representative transfection; similar results were obtained in 4 (3' inserts) to 26 (5' inserts) repetitions. (Lanes a,b) Plasmid pOTCO, an enhancerless tk-CAT fusion; insertion of MTV46 at (lanes c,d) *Xba*I or (lane i) *Bam*HI; insertion of PRL3 (–247 to –214) at *Xba*I in the (lanes e,f) forward or (lanes g,h) reverse orientation, or at *Bam*HI in the (lane j) forward or (lane k) reverse orientation.

have identified a series of receptor-binding DNA sequences as functional components in the repression response. Demonstration of positive regulation of the prolactin promoter by an MTV-derived GRE, and of negative regulation conferred by the prolactin nGRE upon heterologous promoters in nonpituitary cells, indicate that the receptor binding sites themselves determine the nature of the hormonal regulation.

Mechanism of receptor-mediated repression

Our experiments demonstrate that the prolactin nGRE is able to function upon heterologous promoters at a distance, reminiscent of the behavior of other long-range operator elements (Gilmour et al. 1984; Brand et al. 1985; Gorman et al. 1985; Johnson and Herskowitz 1985; Goodbourn et al. 1986; Laimins et al. 1986; Larsen et al. 1986; Muglia and Rothman-Denses 1986; Nir et al. 1986; Siliciano and Tatchell 1986; Simpson et al. 1986; Hammer et al. 1987). The prolactin nGRE, however, is distinct from the operator elements described previously in that it appears to be a compound element that includes a constitutive enhancer-like component that activates transcription in the absence of glucocorticoids, together with a novel class of receptor binding sequences that negate the enhancement effect upon receptor binding.

We suggest that receptor binding at the prolactin nGRE results solely in the reversal of constitutive enhancer action, presumably by removing or otherwise

neutralizing the actions of an enhancer-activating protein bound at an overlapping site; that is, it appears that the direct effect of receptor at the nGRE is to modulate enhancer activity rather than promoter activity. Consistent with this view, we have not observed repression of transcription to levels below that of the original basal promoter activity (Fig. 7 and data not shown). In addition, the constitutive enhancer activity associated with the prolactin nGRE is present both in pituitary and in some nonpituitary cells; however, in a cell line lacking that activity, the nGRE confers neither positive nor negative effects upon the linked promoter in the absence or presence of hormone (D. Sakai, unpubl.).

Adler et al. (1988) have reported that regions of the rat prolactin 5'-flanking region also confer glucocorticoid repressibility upon the tk promoter. In contrast to our results, however, the repression observed does not appear to correspond to a reversal of enhanced basal promoter activity. As the sequences of the rat and bovine genes differ considerably in the 5'-flanking region, and as Adler and colleagues did not characterize receptor binding sites, the explanation for the apparent distinction in regulatory mechanisms cannot readily be discerned.

Positive and negative regulation by the glucocorticoid receptor

The observation that a single transcriptional regulatory protein can effect both positive and negative control is

not a novel one. The mechanisms that determine these distinct activities have been investigated in some detail for several prokaryotic regulators, such as the *Escherichia coli* cAMP receptor (Aiba 1983; Buc et al. 1987) and araC proteins (Martin et al. 1986; Lee et al. 1987), and the bacteriophage λ CI protein (Ptashne 1986). In addition, results from a number of eukaryotic systems are consistent with the view that regulatory strategies may commonly employ combinations of positive and negative activities (e.g., see Goodbourn et al. 1986; Shore and Nasmyth 1987; Sudhof et al. 1987; Keleher et al. 1988). In the case of the glucocorticoid receptor, our data suggest that it effects its two modes of control as a single protein species that recognizes two distinct classes of DNA sequence elements.

Perhaps the simplest interpretation of our results is that the receptor assumes alternative conformations upon binding to GRE and nGRE sequences. One or both classes of DNA sequences recognized by the receptor might serve as an allosteric ligand, altering receptor structure such that binding to the nGRE, for example, would preclude positive regulatory activity. According to this scheme, receptor bound at the nGRE would compete or neutralize the constitutive enhancer factor bound nearby, but would itself have no direct effect on promoter function. Obviously, more complex models can also be envisioned; for example, different auxiliary factors might associate with receptors bound to GREs and nGREs, thereby conferring the distinct modes of hormonal regulation.

Miesfeld et al. (1987a,b) have shown that a small region of the glucocorticoid receptor that encompasses the DNA binding domain is necessary and sufficient for both positive and negative regulation. This implies that a single DNA-binding region within the receptor is able to recognize both GRE and nGRE sequences. A yeast regulatory protein, HAP1, also appears to bind specifically to two distinct DNA sequences, although it has not yet been determined whether it utilizes a common DNA binding domain (Pfeifer et al. 1987).

Further studies will be necessary to define in detail the specific nGRE nucleotides essential for receptor binding, for association of the putative constitutive enhancer factor, and perhaps for binding of additional factors. In any case, it seems likely from our experiments that distinct configurations of protein-DNA complexes will be involved in receptor-mediated positive and negative regulation. In particular, we suggest that the mere binding of receptor to sites near a promoter is insufficient to specify the nature of the regulatory response. Consistent with this view, distinct regions of the glucocorticoid receptor protein have recently been identified that are essential for negative and positive regulation (P. Godowski and K.R. Yamamoto, unpubl.).

Materials and methods

Plasmid constructions

Plasmids pRSVcat (Gorman et al. 1982), pPRLcat-1 and

pPRLcat-2 (Camper et al. 1985), pOTCO (DeFranco and Yamamoto 1986), and pRSVGR (Miesfeld et al. 1986) have been described previously.

Nested 5' deletions of the bovine prolactin promoter were generated from pPRLcat-1 (5' Δ -983) by *Bal31* or exonuclease III resection from the *NdeI* site at -1 kb or *BglII* site at -248. Following blunting of the DNA, plasmids were digested with *BglII* and the fragments bearing the deletions were isolated by electrophoresis in low-melt agarose. The fragments were ligated to the *SmaI*-*BglII* fragment of pUC13 (Messing 1983), which regenerates the ampicillin resistance gene and fuses a polylinker to each deletion end point (Fig. 8). Deletions entering promoter sequences from the 3' side were generated in a similar manner using pPRLcat-2 (5' Δ -248) linearized at the *HindIII* site (Fig. 8). Deletion end points were determined by direct sequencing (Chen and Seeburg 1985) utilizing the M13 universal and reverse sequencing primers (Messing 1983); deletions are designated by the last wild-type nucleotide that is retained. Linker substitution mutants were derived by insertion of the small *BamHI* fragment from 5' deletions into the *BamHI* site of 3' deletions (Fig. 8). The plasmid (-214/-247)5' Δ -212 was generated by the insertion of the small *BamHI* fragment from 5'(Δ)-212 into the *BglII* site of 3' Δ -214.

For construction of tk promoter derivatives, prolactin promoter fragments were excised using existing restriction sites and sites within the polylinker of deletion derivatives, and subcloned into the *XbaI* or *BamHI* site of pOTCO (see Fig. 7). In some cases, fragments were initially subcloned into pUC12B (a derivative of pUC12 with an inverted duplication of the polylinker region; D. Sakai, unpubl.) to customize flanking linker sequences prior to insertion into the reporter plasmid. MTV46, a self-complementary synthetic 46-bp GRE generously provided by P. Searle and R. Palmiter (University of Washington, Seattle), contains an inverted duplication of MTV nucleotides -123 to -105: (5'-GATCCAAATGTTCTGATCTGAGC-CATGGCTCAGATCAGAACATTG-3'); it was similarly introduced into pOTCO and also into the *BamHI* or *BglII* site of linker substitution mutants to generate the plasmids depicted in Figure 6.

All plasmids were propagated in *E. coli* DH5 (Hanahan 1983) and purified by alkaline lysis (Birnboim and Doly 1979) followed by two or three successive bandings in CsCl-ethidium bromide gradients. The concentrations and purity of all plasmids were verified by UV absorption and agarose gel electrophoresis.

Cell culture and transfections

GH3 rat pituitary tumor cells (Tashjian 1979) were propagated in a 1:1 mixture of Dulbecco's modified Eagle medium and Ham's F-12 medium supplemented with 10% fetal bovine serum. Approximately 10^7 cells were plated in 15-cm dishes 48 hr prior to transfection (Camper et al. 1985). Monolayers were rinsed gently with phosphate-buffered saline (PBS) three times and overlaid with 15 ml of Ham's F-12 containing 50 mM Tris-HCl (pH 7.5), 100 μ g/ml of DEAE-dextran (Sompayrac and Danna 1981), 1 μ g of pRSVcat, and 15 μ g of test plasmid and incubated 1 hr at 37°C. The cells were then rinsed once with PBS, replenished with growth medium containing 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0 or 0.1 μ M dexamethasone, and incubated for an additional 48 hr.

CV-1 monkey kidney cells were propagated in Dulbecco's modified Eagle medium containing 5% fetal bovine serum. Transient cotransfections were done essentially as previously described (Miesfeld et al. 1986). In brief, freshly fed cultures (10^6 cells per 10-cm dish) were overlaid with 1 ml of calcium phos-

Figure 8. Construction of the bovine prolactin terminal and linker substitution mutants. See Materials and methods for details.

phate-DNA precipitate containing 5 μ g of pRSVGR and 1 μ g of test plasmid. Chloroquine was added to 0.1 mM [Luthman and Magnusson 1983] and the cells incubated 5 hr at 37°C. The monolayers were rinsed once with PBS, replenished with growth medium with or without 0.1 μ M dexamethasone, and incubated an additional 24 hr. Extracts were prepared and CAT assays were performed as described previously [Gorman et al. 1982; Camper et al. 1985].

Isolation and analysis of RNA

Monolayer cultures were rinsed twice with Tris-buffered saline and then lysed in 4 ml of 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 1% NP-40 and 10 mM vanadyl ribonucleosides [Berger and Birkenmeier 1979]. After 10 min at room temperature, the lysate was transferred to centrifuge tubes and extracted twice with equal volumes of buffered phenol/chloroform/isoamyl alcohol (25:24:1), once with chloroform/isoamyl alcohol (24:1), and ethanol precipitated. Poly(A)⁺ RNA was prepared by oligo(dT)-cellulose chromatography [Aviv and Leder 1972].

Transcript mapping was performed essentially as described

by Melton et al. (1984) except that nuclease digestion was performed at room temperature for 1 hr with RNase A and T₁ at concentrations of 4–10 μ g/ml and 40 U/ml, respectively. In the hybridizations, probe was present at 100-fold excess over CAT transcripts as determined by titration. Antisense RNA probes (sp. act. = 5×10^8 dpm/ μ g) were transcribed from the 466-bp PstI–RsaI fragment of pPRLcat-1 (which spans the prolactin start site, see Fig. 4), cloned into pSP65 [Melton et al. 1984; Camper et al. 1985], and purified on sequencing gels.

DNase I footprinting

Purified rat liver glucocorticoid receptor was prepared according to Wrangé et al. (1986). Probes were prepared from plasmid pPRLbgh-1 (D. Sakai, unpubl.) and from plasmid with MTV-152 (Fig. 6). DNAs were 5'-end-labeled or 3'-end-labeled polynucleotide kinase or DNA polymerase I (Klenow fragment). They were subjected to secondary digestion with restriction enzymes that yielded 650- to 1000-bp probe fragments that were then purified from low-melt agarose gels by the glass bead method [Vogelstein and Gillespie 1979].

DNA binding reactions [Payvar et al. 1983] included 2–5 fmoles of ³²P end-labeled DNA fragment in 100–250 μ l of binding buffer (20 mM Tris-HCl, (pH 7.5), 1 mM EDTA, 2 mM MgCl₂, 50 mM NaCl, 20 mM dithiothreitol, 100 μ g/ml of insulin, 20% glycerol, 1 μ M dexamethasone) and 20–400 ng of receptor. Competitor DNA was mixed with the probe DNA prior to receptor addition. Binding was for 15 min at room temperature. DNase I (3–60 ng/ml depending upon receptor concentration) was then added along with 1 mM CaCl₂, 1 mM MgCl₂, and 0.1 μ g/ml pBR332 DNA; incubation was for 1 min at room temperature. The reaction mixture was adjusted to 10 mM of Na₂EDTA, 0.1% SDS, and 200 μ g/ml proteinase K and incubated for 30 min at 37°C. The nucleic acids were extracted with buffered phenol/chloroform (1:1), ethanol-precipitated, and electrophoresed on 6% sequencing gels along with sequencing ladders.

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