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Independent Glucocorticoid Induction and Repression of Two Contiguous Responsive Genes

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Specific DNA sequence elements which contain binding sites for the glucocorticoid receptor mediate the action of glucocorticoid hormones on gene transcription. In glucocorticoid-inducible genes, these glucocorticoid-responsive elements behave as hormone-inducible enhancers of transcription. We have taken advantage of the bovine papillomavirus (BPV) system to test the stringency of glucocorticoid regulation of transcription. BPV episomes were constructed to contain two hormone-regulated transcription units in close proximity; one transcription unit is under control of a glucocorticoid-inducible promoter (mouse mammary tumor virus) while the other is under control of a glucocorticoid-inhibited promoter (pro-opiomelanocortin). Glucocorticoids independently regulated transcription of the two physically linked transcription units, irrespective of their relative orientation and of their proximity on the BPV episomes. This result contrasts with the so-called position-independent activity of enhancers and suggests that the multicomponent organization of eucaryotic promoters restricts the action of hormone-responsive regulatory elements to a specific transcription unit, thus accounting for the stringency of hormonal regulation observed in vivo.

The analysis of eucaryotic gene regulatory sequences has revealed the presence of unique regulatory elements, enhancers, which stimulate transcription independently of position and orientation (reviewed in references 21 and 36). Although enhancers are active over distances of several kilobases, their activity can be attenuated both by distance (14, 24, 39) and by the presence of another promoter between the enhancer and the promoter (18, 38). Since most eucaryotic genes appear to be separated from unrelated genes by at least a few tens of kilobases, distance may contribute to restrict the action of cellular enhancers. However, distance alone cannot account for the specificity of gene expression observed in vivo; indeed, distance is insufficient to prevent the action of the immunoglobulin heavy-chain gene enhancer over at least 17.5 kilobases (37). Alternatively, chromatin structure may serve to define domains for regulation of expression and in some way limit the effect of cellular enhancers to specific domains. Indeed, chromosomal DNA is organized into supercoiled looped domains of 50 to 100 kilobases of DNA (3, 27). The localization of enhancers close to chromosomal loop anchorage and to topoisomerase II sites is suggestive of a relationship between these elements and chromatin loop structure (6, 13). While a putative involvement of these various parameters has been proposed, the mechanism by which enhancer activity is restricted to one gene remains unclear.

Hormone-inducible genes which contain hormone-responsive enhancer elements provide a convenient system for assessing the promoter specificity of transcriptional enhancement. The mouse mammary tumor virus (MTV) long terminal repeat (LTR) was shown to confer glucocorticoid inducibility of transcription (15, 20), and, like other glucocorticoid-inducible genes (8, 17, 19, 22, 23, 31, 34), the MTV promoter contains conserved DNA sequences which bind the glucocorticoid receptor (2, 28, 33) and behave as hormone-inducible enhancers (4, 16, 29). We have recently shown that transcription of the pro-opiomelanocortin (POMC) gene is inhibited by glucocorticoids (12) and that a segment of the rat POMC gene promoter confers glucocorticoid repression (5, 10, 35). This segment contains a glucocorticoid receptor-binding site which overlaps the putative CCAAT box, thus suggesting that inhibition of transcription may involve a repressor mechanism.

Having previously shown that glucocorticoid-inducible MTV transcription and glucocorticoid-inhibited POMC transcription are appropriately regulated by glucocorticoids when present independently on bovine papillomavirus (BPV) episomes (5, 26), we have now used similar BPV episomes containing both POMC and MTV transcription units to assess the effect of transcriptional enhancement of one gene (MTV) on transcription and repression of another gene (POMC). Our results indicate that even when they are physically linked on the same episome, these two transcription units retain independent glucocorticoid regulation.

BPV-transformed cells. The chimeric genes used in the present work were previously shown to be glucocorticoid responsive in the BPV system (5, 26); they are composed of a glucocorticoid-inhibited rat POMC promoter fragment fused to early coding sequences of simian virus 40 which encode T antigen (POMC-Tag) and of glucocorticoid-inducible MTV LTR sequences fused to chloramphenicol acetyltransferase coding sequences (MTV-cat). Four POMC-Tag/MTV-cat/BPV plasmids were constructed so that the POMC-Tag and MTV-cat transcription units would be in all four possible orientations relative to each other. The structure of the episomes present in C127 cells transformed with these plasmids is illustrated in Fig. 1. The pBR322 plasmid sequences which were present in the plasmid constructs were deleted by digestion with the restriction enzyme *Sal*I before transfection. The four plasmids produced similar

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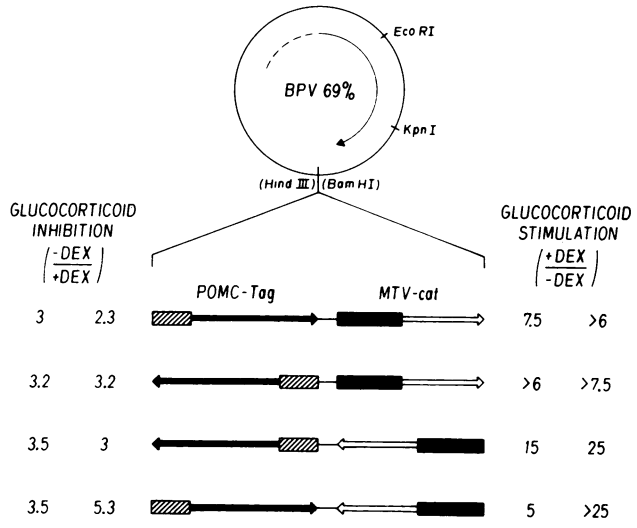

FIG. 1. Schematic representation of POMC-Tag/MTV-cat/BPV episomes and quantitation of glucocorticoid response. As illustrated, the four episomes contain the 69% transforming fragment of BPV and the POMC-Tag and MTV-cat transcription units in four different orientations relative to each other. They were derived from previously described plasmids (5, 25, 26). Promoter fragments are represented by boxes, while Tag and *cat* sequences are represented by arrows which indicate the orientation of transcription. The POMC promoter fragment extends from bp −706 to +63 of the rat POMC gene (9). The MTV fragments include the complete LTR from bp −1189 to +104. The magnitude of POMC-Tag mRNA inhibition and of MTV-cat mRNA stimulation by glucocorticoids is reported for the two cellular clones analyzed in Fig. 2. Glucocorticoid effects were measured by densitometric scanning of S1 nuclease mapping autoradiograms and were corrected for the levels of β- and γ-actin mRNA measured as an internal control. The data for the cell lines are presented in the same order as in Fig. 2; i.e., the data on the first line are from cell lines 2280 and 2319, respectively, etc.

numbers of foci (about 100 per μg of DNA per 3×10^5 cells) upon transformation of C127 cells, as did BPV control DNA. For each construct, 12 foci were isolated and propagated for analysis. As observed previously for similar episomes carrying either the MTV-cat (26) or the POMC-Tag (5) transcription units, most clonal lines contained only episomal DNA, which was present at 10 to 200 copies per cell as determined by Southern blot using MTV LTR or large T-antigen probes, independently; see the legend to Fig. 2 for the average episome copy number per cell for each cell line. The integrity of each transcription unit (MTV-cat and POMC-Tag) and of BPV sequences was assessed independently in all cell lines by using specific probes and at least two different restriction digests of total cell DNA (not shown). Of the cell lines for which data is presented in the figures, line 2280 is the only one which presented a slight rearrangement of the episomes, but this rearrangement appears to be a short deletion of about 100 base pairs (bp) within a 300-bp segment of pBR322 sequences; neither transcription units nor BPV sequences were altered by this slight rearrangement.

Glucocorticoid regulation of transcription. In order to assess the effects of glucocorticoids on episomal transcription, the levels of cytoplasmic POMC-Tag, MTV-cat, and cellular β- and γ-actin transcripts were quantitated by Northern (RNA) blot for each BPV episome in three to five clonal lines. All cell lines contained the expected POMC-Tag mRNAs of 2,322 and 2,602 nucleotides (nt) encoding large and small T antigens, respectively, and the MTV-cat transcripts of ≃1,610 nt (data not shown). While these were the major POMC-Tag and MTV-cat transcripts detected, the blots also revealed minor hybridizing bands which may represent aberrant transcripts. Treatment of cells overnight with 0.1 μM dexamethasone (DEX) decreased the levels of POMC-Tag transcripts about two- to fourfold while it increased MTV-cat transcripts from undetectable to detectable levels (not shown). Cellular actin mRNA levels measured in the same samples were not affected by DEX and served as an internal control (Fig. 2C). Transcript analyses for two representative clones of each POMC-Tag/MTV-cat/BPV episome are presented in Fig. 2. To quantitate more precisely the effects of DEX on correctly initiated cytoplasmic transcript levels, the 5′ ends of POMC-Tag and MTV-cat transcripts were analyzed by S1 nuclease mapping (Fig. 2A and B) and quantitated by densitometric scanning of the autoradiograms (not necessarily the exposures shown in Fig. 2B because, in most cases, longer exposures had to be used to quantitate −DEX samples). In all cell lines, the DNA probe used for mapping the 5′ ends of the POMC-Tag transcripts (5) protected predominantly the expected 87-nt fragment (Fig. 2A). For analysis of MTV-cat transcripts, a DNA probe of 359 bp was end labeled within *cat* sequences; in all cell lines, this probe protected the expected fragment of 252 nt (Fig. 2B). To provide a data base for comparison, the magnitude of glucocorticoid-dependent induction or repression was also quantitated for BPV episomes containing either MTV or POMC transcription units, respectively (Table 1).

At first approximation, both glucocorticoid inhibition of POMC-Tag transcripts (Fig. 2A) and glucocorticoid stimulation of MTV-cat transcripts (Fig. 2B) are similar for all clones containing any of the four episomes depicted in Fig. 1. For each construct, three to five cell lines were analyzed and gave similar results (only two are shown). As reported in Fig. 1 for each clone illustrated in Fig. 2, densitometric analysis of S1 nuclease mapping autoradiograms indicated that, in all cases, glucocorticoid inhibition of POMC-Tag transcripts was about threefold. After correction for the levels of β- and γ-actin mRNA, mean repression values of 3.0 ± 1 (n = 11, range 1.5 to 4.4), 2.6 ± 1.1 (n = 16, range 1.1 to 4.7), 2.6 ± 1.1 (n = 9, range 1.2 to 4.0) and 2.6 ± 1.1 (n = 16, range 1.4 to 5.3) were calculated for each construct depicted in Fig. 1 (top to bottom, respectively). Glucocorticoid induction of MTV-cat transcripts was more variable, ranging from about 5- to 25-fold. The magnitude of these glucocorticoid effects on POMC-Tag and MTV-cat transcripts is indistinguishable from those observed in cell lines which contain BPV episomes carrying only one glucocorticoid-sensitive transcription unit (Table 1) or in stable transformants of MTV transcription units (4, 15, 16, 20). Comparison of the magnitude of glucocorticoid stimulation of MTV-cat transcripts from cell lines containing the different episomes does not reveal any significant difference between the episomes even when the POMC and MTV promoter fragments are contiguous. Thus, transcription and regulation by glucocorticoids is essentially independent for each transcription unit despite the close juxtaposition of the two promoters in some episomes.

Discussion. We had previously shown that both the glucocorticoid-inducible MTV promoter (26) and the glucocorticoid-inhibited POMC promoter (5) are hormone responsive when present on BPV episomes. In this episomal system, the hormone-responsive transcription units are in a well-defined DNA sequence and chromatin environment (minichromo-

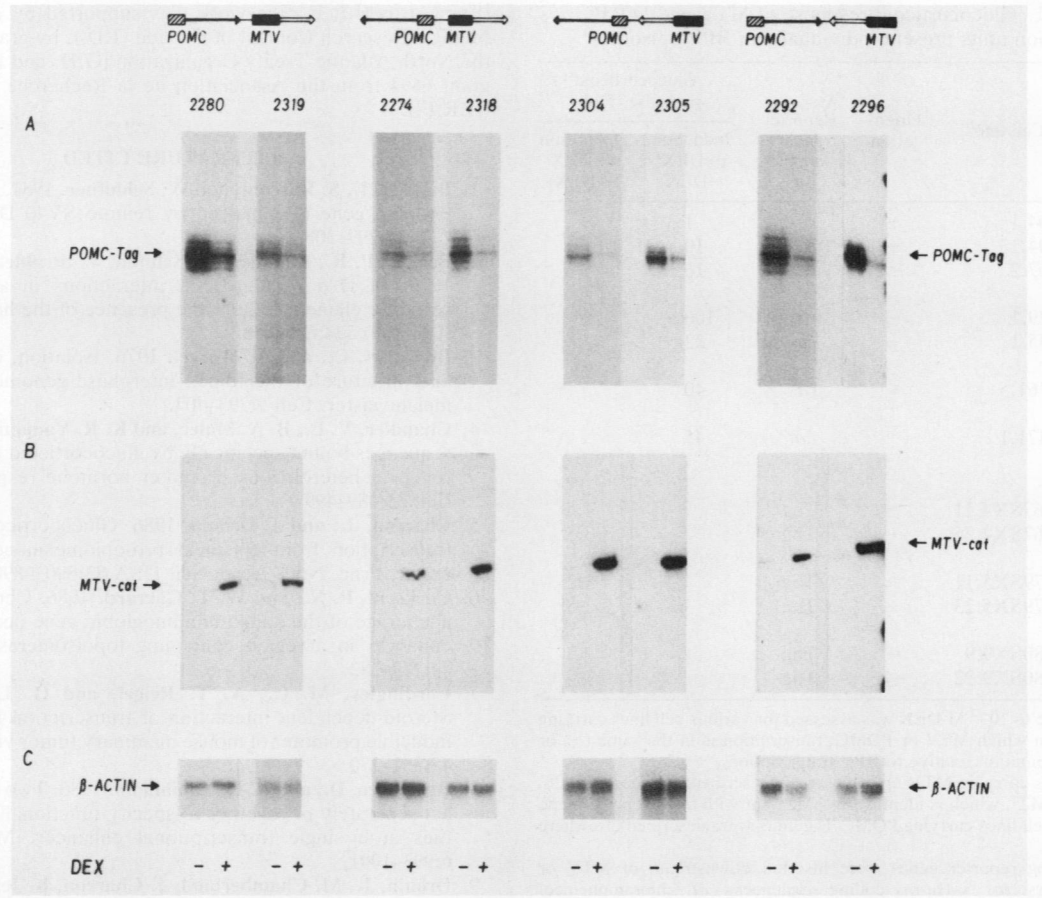

FIG. 2. Effect of glucocorticoid treatment on POMC-Tag and MTV-cat transcripts. For each of the four BPV episomes (illustrated at the top of the figure as in Fig. 1), data are presented for two (of three to five clones which were analyzed) BPV-transformed cellular clones analyzed for their responses to DEX; each cell line is identified at the top and was treated with DEX (+) or was untreated (−) overnight before extraction of cytoplasmic RNA. (A) S1 nuclease mapping autoradiograms showing the position of the 87-nt fragment protected by POMC-Tag transcripts (5). (B) S1 nuclease mapping autoradiograms showing the position of the 252-nt fragment protected by MTV-cat transcripts. The MTV-cat probe was a 359-bp fragment labeled at the *Pvu*II site within *cat* sequences and extending up to a *Sac*I site within the MTV LTR. (C) Northern blot analysis of cellular β- and γ-actin mRNA measured as an internal control, using a rat brain β-actin cDNA probe. The average number of episomes per cell for each cell line was 50 (line 2280), 25 (line 2319), 200 (line 2274), 80 (line 2318), 200 (line 2304), 175 (2305), and 25 (lines 2292 and 2296); the same copy number was measured by using either an MTV LTR probe or a large T-antigen probe. All BPV DNA sequences detected in these cell lines appear to be episomal.

somes). We have now used the same system to physically link these two transcription units on the same episome and to demonstrate that transcriptional regulation of each unit is completely independent of the other despite the enhancer properties (i.e., active at a distance) of the MTV glucocorticoid response element (4, 29). Indeed, both glucocorticoid induction of MTV-cat transcription and glucocorticoid repression of POMC-Tag are of the same magnitude whether these transcription units are present individually (Table 1) or together (Fig. 1 and 2) on BPV episomes. Furthermore, since episomal POMC-Tag transcripts are inhibited to the same extent (threefold) as the POMC transcription rate in primary cultures of anterior pituitary cells (12) and as POMC mRNA levels in AtT-20 cells (10), it is likely that most, if not all, BPV episomes which are transcribed are also subject to glucocorticoid inhibition. Independent hormone responsiveness is observed whether the MTV-cat and POMC-Tag transcription units are in the same (Fig. 1, first and third constructs from top) or opposite (Fig. 1, second and fourth constructs) orientations and even when the two promoter fragments are placed back-to-back (Fig. 1, second construct from top). It is also noteworthy that convergent transcription does not interfere with glucocorticoid regulation of both units (Fig. 1, fourth construct). Transcriptional interference between the two transcription units was not expected, since both units include transcription termination and polyadenylation sequences (30).

These observations contrast with the known properties of enhancer elements (1, 11, 24, 38) and, in particular, with observations made on the glucocorticoid-inducible MTV enhancer (4, 29) which indicated that these elements are active in an orientation- and position-independent fashion. In the experiments reported here, hormone-dependent changes in transcription were restricted to one gene and did not exhibit any long-distance effects. By extrapolation to our system, observations like those of Kadesh and Berg (18) on the effect of the simian virus 40 enhancer on multiple transcription units may have been interpreted to predict that glucocorticoid induction of MTV-cat transcription should have affected POMC-Tag transcription. This was not observed. Specific protein-protein interactions which are an integral part of the mechanism of hormone induction in the intact MTV promoter may contribute to restrict hormone inducibility to that same transcription unit. We demon-

TABLE 1. Glucocorticoid response of MTV and POMC transcription units present individually in BPV episomes[a]

Episome	Cell line[b]	Orientation	Reporter gene[c]	Glucocorticoid response (fold)[d]	
				Induction (+DEX/−DEX)	Inhibition (−DEX/+DEX)
pM18	904.1	+	*ras*	1.3	
	904.13	+	*ras*	10	
	907.2	+	*ras*	16	
pM19	929.1	−	*ras*	100	
	935.1	−	*ras*	22	
pM23	1361.5	−	*ras*	40	
pM25	1471.1	−	*cat*	45	
pJA67SX	C67SX4.11	+	Tag		1.5
	C67SX4.26	+	Tag		3.5
pJA79SX	C79SX5.11	+	Tag		3.2
	C79SX5.23	+	Tag		1.8
pJA80SX	C80SX9.9	−	Tag		3.7
	C80SX9.22	−	Tag		3.4

[a] The response to 10^{-7} M DEX was assessed for various cell lines carrying BPV episomes in which MTV or POMC transcription is in the same (+) or opposite (−) orientation relative to BPV transcription.

[b] The cell lines carrying MTV episomes were described previously (7, 26, 32) except for pM25, which is identical to pM23 but with a *cat* reporter gene instead of *ras*. Cell lines carrying POMC-Tag units were described elsewhere (5).

[c] The following reporter genes were inserted downstream of MTV or POMC promoters: *ras*, v-Ha-*ras* coding sequences; *cat*, chloramphenicol acetyltransferase-coding sequences; Tag, simian virus 40 T antigen-coding sequences.

[d] All fold inductions or repressions were calculated by densitometric quantitation of appropriate bands present on S1 mapping or RNase mapping autoradiograms and were corrected for the levels of β- and γ-actin mRNA, measured as an internal control.

strated recently that receptor-mediated activation of the MTV transcription unit results in the recruitment of two factors, NF1 and TFIID, to the promoter (7). These proteins thus represent one set of factors that potentially interact with the receptor in the MTV promoter and which may restrict hormone inducibility to this transcription unit. As for glucocorticoid inhibition of POMC-Tag transcription, the absence of any long-distance effect is consistent with a model for glucocorticoid inhibition in which the glucocorticoid receptor acts as a repressor (10).

In summary, we have shown that opposite regulation of two transcription units can occur independently even if the promoters of these units are physically linked and juxtaposed on relatively small episomes. This strict specificity of regulation mimics the specificity of gene regulation observed in vivo and suggests that the activity of hormone-responsive elements may be restricted by specific interactions within highly organized eucaryotic promoters, in contrast to their apparent position independence when they are tested as isolated regulatory elements by insertion in the vicinity of a heterologous reporter gene.

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