Glucocorticoid inhibition of transcription from episomal proopiomelanocortin gene promoter

(bovine papilloma virus/steroid hormone action/gene regulation)

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ABSTRACT Glucocorticoid hormones alter transcription of specific genes. Glucocorticoid-stimulated genes have been especially useful in unraveling molecular events responsible for positive gene regulation in mammals. The gene encoding proopiomelanocortin (POMC), which is under feedback inhibition by glucocorticoids, provides a model system to study negative gene regulation. Using an episomal bovine papilloma virus vector, we now demonstrate that a 769-base-pair fragment containing the rat POMC promoter is sufficient to confer glucocorticoid inhibition. Transcription from the episomal POMC promoter starts at the same site and is inhibited by glucocorticoids to the same extent as POMC transcription in the anterior pituitary. Glucocorticoid inhibition is specific for POMC transcripts; neither bovine papilloma virus nor cellular actin mRNAs are affected by glucocorticoids. Thus, the episomal bovine papilloma virus/POMC system can be used to study the relationship between negative regulation of POMC transcription and chromatin structure.

Steroid hormone-responsive genes are good model systems to study transcriptional regulation of mammalian genes. In particular, many steps involved in the glucocorticoid stimulation of mouse mammary tumor virus transcription are now understood (for review, see ref. 1). Glucocorticoid stimulation of transcription requires binding of the glucocorticoid receptor–hormone complex to the same DNA sequences that are necessary for transcriptional activation (2–8). These well-defined sequences confer hormone inducibility to heterologous transcription units and behave as glucocorticoid-dependent enhancer elements (9, 10). While they stimulate transcription of some genes, steroid hormones also inhibit transcription of other genes, but the mechanism or DNA sequences involved in this inhibition has not yet been defined. The gene encoding proopiomelanocortin (POMC) offers a model system to study inhibition of transcription by steroid hormones (11–13). Indeed, in the anterior pituitary glucocorticoids specifically inhibit transcription of the POMC gene (14–16). In addition, pituitary POMC gene transcription is stimulated by corticosteroids and cyclic AMP (16; unpublished data). In vivo, glucocorticoid inhibition and corticosteroid stimulation of POMC transcription result in lower and higher, respectively, POMC mRNA levels (17, 18).

POMC is the precursor to a variety of hormones [corticotropin, β-endorphin, and the melanotropins (19)]. It is expressed in the pituitary (anterior and intermediate lobes), certain areas of the brain (hypothalamus, amygdala, and cortex), the testes, the ovaries, and the placenta (20–23). Processing of POMC results in the secretion of a specific set of peptides in each tissue. The release of POMC peptides is also differentially regulated in each tissue and under multihormonal control (24).

We have used a bovine papilloma virus (BPV) vector (25) to study the control of POMC transcription. BPV replicates as an autonomous episome in rodent cells and thus offers the possibility to characterize regulatory mechanisms in a well-defined chromosomal environment. This system was successfully used to study glucocorticoid-stimulation of mouse mammary tumor virus transcription (26). We have found that a hybrid transcription unit constituted of a rat POMC promoter fragment linked to simian virus 40 (SV40) tumor (T) antigen coding sequences is transcribed from the correct site of initiation when present on BPV episomes. Furthermore, transcription from this promoter is specifically inhibited by glucocorticoids.

MATERIALS AND METHODS

Plasmid Construction. Recombinant plasmids were constructed in pBR327 using standard cloning procedures (27). The rat POMC promoter fragment was subcloned from a genomic DNA fragment isolated in this laboratory (13). SV40 early region was excised from pSV3neo (28). The cloned BPV genome (BPV 100%) or its 69% transforming fragment (BPV 69%) were kindly provided by N. Sarver and M. Lusky, respectively. The 1500-base-pair (bp) rat brain β-actin cDNA clone used as control in RNA gel blots was obtained from Stephen Farmer.

Cell Culture and Transfection. Mouse C127t cells (ATCC CRL 1616) were obtained from American Type Culture Collection and maintained in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum and, in short-term experiments, with penicillin (100 μg/ml) and streptomycin (200 μg/ml). Dexamethasone treatment of cells was performed as follows: 24 hr before the addition of dexamethasone, media were changed to DMEM containing 10% (vol/vol) dextran-coated charcoal-absorbed fetal calf serum to remove endogenous steroids (29). Transfections were performed as described (25) and transformed foci were isolated using glass cylinders or trypsin-impregnated filter paper.

Analysis of Cellular DNA. Episomal and total cellular DNA were isolated as described by Ostrowski et al. (26) and Gross-Bellard et al. (30), respectively. Cellular DNAs were analyzed on 0.75% agarose gels using standard blotting and hybridization procedures (27).

RNA Extraction and Analysis. Cytoplasmic RNA was isolated as follows: trypsinized cells were washed once in cold phosphate-buffered saline (137 mM NaCl/3 mM KCl/8

Abbreviations: BPV, bovine papilloma virus; POMC, proopiomelanocortin; T, tumor; SV40, simian virus 40; Tag, fragment encoding the SV40 large and small T antigens; bp, base pair(s); nt, nucleotide(s).

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mM Na$_2$HPO$_4$/1.5 mM KH$_2$PO$_4$ and once in RBS (10 mM Tris-HCl, pH 7.4/5 mM NaCl/1 mM MgCl$_2$). The cells were resuspended in RBS, and 0.1 vol of 4% (vol/vol) Nonidet P-40/0.6% deoxycholate was added. After 5 min on ice, nuclei and cell debris were removed by centrifugation at 1750 × g for 5 min, and RNA was extracted from the supernatant by phenol extraction and ethanol precipitation. The precipitates were dissolved in 10 mM Tris-HCl pH 7.4/10 mM MgCl$_2$ and digested with RNase-free DNase I at 5 μg/ml ( Worthington). After phenol extraction and ethanol precipitation, the RNA was quantitated by spectrophotometry at 260 nm. RNA gel blots were performed as described (31). S1 nuclease and RNase mapping were performed as described (27, 32).

RESULTS

Plasmid Construction. A hybrid transcription unit was constructed from a 769-bp Xmn I rat POMC (rPOMC) DNA fragment joined to a 2657-bp Stu I–BamHI SV40 DNA fragment encoding small and large T antigens (Tag). The fragments were joined to each other within their respective 5'-untranslated sequences. The rPOMC fragment contains the 706-bp 5'-flanking sequences and the first 63 bp of the first exon; it was subcloned using Sal I and synthetic linker sequences. This fragment was joined by blunt-end ligation to the Stu I end of the SV40 fragment after digestion of the Sal I sticky ends with S1 nuclease. This rPOMC-Tag fragment was inserted into the Sal I–BamHI site of a modified pBR327 on which the only Cla I site was changed to an Xho I site using synthetic linkers. BPV sequences were inserted in both orientations in the resultant plasmids at the only BamHI site; the entire BPV genome, linearized at the BamHI site (25), and the BPV 69% transforming fragment (HindIII–BamHI fragment with BamHI linker added at HindIII site, ref. 33) were used. The structures of the plasmids used in this study are shown in Fig. 1.

BPV-Transformed Cells. The four POMC-Tag/BPV plasmids contained POMC-Tag and BPV sequences (69% or 100%) in the two possible orientations. All four plasmids were transfected in mouse C127I cells as supercoiled plasmids or deleted of their pBR327 sequences. Fewer transformed foci were obtained by transfection of the supercoiled plasmids and none of these foci contained only unrearranged episomal BPV DNA (data not shown). For this reason, these cells were not studied further. Transfection data from plasmids with pBR327 sequences deleted are summarized in Table 1. pBR sequences were removed before transfection by digestion with Sal I and Xho I and religation of the complementary cohesive ends at low DNA concentration. The four plasmids transform C127I cells at similar efficiency. For each transfection, 6–10 foci were isolated and propagated for analysis. Episomal and total DNA were isolated from all transformed cell lines thus established; BPV sequences were characterized by Southern blot analysis using appropriate probes as shown in Fig. 2. Complete data are only presented for clones that were later used for transcription and regulation studies.

All clones transformed with pJA66SX examined contained integrated and/or rearranged BPV DNA in addition to episomal DNA; for example, clone C66SX22 mostly contains a deleted episome (Fig. 2A). Only one clone transformed with a complete BPV genome was found to contain only unrearranged episomal DNA (C67SX4) as judged from Southern blots of episomal (Fig. 2A) and total DNA (Fig. 2B and C). Hybrid BPV genomes containing only the 69% transforming fragment yielded such clones more frequently: 2/6 for pJA79SX and 5/10 for pJA80SX. The average episomal DNA copy number for these cell lines varies from 10 to >350 copies per cell (Table 1, Fig. 2C). These values could be underestimated because foci were not cloned from single cells. However, we have checked the homozygosity of a Poisson number of cell lines by immunoperoxidase detection of nuclear T antigen (data not shown). These data indicate that cell lines C67SX4, C79SX5, and C80SX9 were 92, 64, and 95% positive for nuclear T antigen at passages 18, 15, and 14, respectively.

Transcription and Regulation. Three representative cell lines transformed by different POMC-Tag/BPV episomes were selected for RNA analysis and to test glucocorticoid sensitivity. POMC-Tag, BPV, and cellular actin transcripts were detected by RNA gel blotting of cytoplasmic RNA extracted from these cell lines (Fig. 3). All three cell lines contain the two expected POMC-Tag mRNAs of 2322 and 2602 nucleotides encoding large and small T antigens, respectively. Other clones transformed with the same BPV episomes predominantly contain these POMC-Tag transcripts whether they contain integrated BPV DNA or not (data not shown). Incubation of these cells overnight in media containing 0.1 μM dexamethasone decreases POMC-Tag cytoplasmic mRNA levels by a factor of 3 in various cell lines as calculated by densitometric analysis of the autoradiograms (Fig. 3). A similar decrease is observed at 10 nM dexamethasone (data not shown) and in cell lines transformed with every BPV construct. Cellular actin mRNA measured in the same samples is not affected by dexamethasone and serves as internal standard (Fig. 3). Similarly, BPV transcripts are not affected by dexamethasone in cell lines transformed by BPV 100% (line C142-21) or by POMC-Tag/BPV constructs (Fig. 3).

Cells transformed with BPV constructs in which BPV early and POMC-Tag transcription are in opposite directions (e.g., line C80SX9 shown in Fig. 3) contain an additional, very abundant BPV transcript of about 1650 nucleotides (nt) (Fig. 4). In contrast, cells transformed with episomes in which early BPV transcription is in the same direction as POMC-Tag (e.g., lines C67SX4 and C79SX5) or with wild-type BPV...
in pML2d (e.g., line C142-21), contain BPV transcripts similar to those described (34–36). Transcripts of 2100–2300 nt are also found only in C80SX9 cells whereas transcripts of 1900 nt are present at similar levels in all cell lines. The level of the 1200-nt transcripts is much lower in C80SX9 cells than in cells transformed with BPV 100%, pJA67SX4, or pJA79SX5 where these 1200-nt transcripts are the most abundant (Fig. 4). The 1650-nt and 2300-nt transcripts are present in all lines transformed with pJA80 (10 examined) or pJA66 (8 examined) whether BPV DNA is episomal or integrated. This transcript is not detected by hybridization with nick-translated POMC or Tag probes or by RNase mapping using SP6 RNA probes for each strand of the POMC promoter fragment. The 1650-nt transcript hybridizes only with BPV fragments previously shown to encode BPV early transcripts (34, 35), namely, the EcoRI-Kpn I 1342-bp and Kpn I-BamHI 995-bp fragments (data not shown).

The precise 5' end of POMC-Tag transcripts detected by RNA gel blot was determined by RNase (32) and nuclease S1 mapping (27). RNA and DNA probes used for mapping are schematically illustrated at the bottom of Fig. 5. The 787-nt single-stranded RNA probe was prepared using an SP64 (32) recombinant template containing the Sal I-HindIII fragment and linearized with Sal I. The same DNA fragment was 5' labeled at the HindIII site and digested with Sac II for S1

**Table 1.** Transformation of C127I cells with POMC-Tag/BPV plasmids

<table>
<thead>
<tr>
<th>Transfected DNA</th>
<th>% BPV DNA</th>
<th>Orientation of POMC-Tag relative to BPV early transcription</th>
<th>Focus-forming transcripts of BPV per μg of DNA per 5 × 10^3 transfected cells</th>
<th>Foci with unrearranged episomes</th>
<th>Copy number of episomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>pJA66SX*</td>
<td>100%</td>
<td>Opposite</td>
<td>360</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>pJA67SX</td>
<td>100%</td>
<td>Same</td>
<td>150</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>pJA79SX</td>
<td>69%</td>
<td>Same</td>
<td>83</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>pJA80SX</td>
<td>69%</td>
<td>Opposite</td>
<td>180</td>
<td>10</td>
<td>5</td>
</tr>
</tbody>
</table>

*SX indicates that purified recombinant plasmids were digested with Sal I and Xho I and circularized with T4 DNA ligase before transfection.

**Fig. 2.** Southern blot analysis of episomal and total DNA isolated from BPV transformed cell lines. (A) Episomal DNA extracted from 2 × 10^6 cells of different clones was linearized with Kpn I, separated on a 0.7% agarose gel, blotted, and hybridized with 32P-labeled pJA66. (B) Total DNA (10 μg) isolated from selected clones was digested with BamHI, and transacted sequences were analyzed as above. (C) The episome copy number was measured by digestion of 2 μg of total DNA with EcoRI and by analysis as above. Increasing amounts of plasmid pJA79 are used as standards.

**Fig. 3.** RNA gel blot analysis of total cytoplasmic RNA present in BPV-transformed cell lines. Selected BPV-transformed clones were grown overnight with (+) or without (−) 0.1 μM dexamethasone. Total cytoplasmic RNA (25 μg per lane) isolated from these cultures was separated on a 1.2% glyoxal/dimethyl sulfoxide/agarose gel, blotted, and hybridized with specific nick-translated probes to detect POMC-Tag transcripts (A), BPV transcripts (B), and cellular β-actin (C) mRNA. Molecular size markers are indicated in bp on the left.
mapping. In all cell lines, both probes protect only a fragment of the size (87 nt) expected of transcripts initiating in POMC.

**DNA at the same site as in rat pituitary (13). As revealed by RNA gel blot (Fig. 3), the amount of protected fragment is decreased by a factor of ~3 by dexamethasone treatment (Fig. 5). Therefore, we conclude that transcription of the POMC-Tag transcription unit present on the BPV episomes initiates at the correct site and is inhibited by glucocorticoids.**

**DISCUSSION**

POMC gene transcription is inhibited by glucocorticoids: this gene, therefore, offers a model system to study negative control of transcription in eukaryotes. We have used the BPV genome as a vector to study transcription and regulation from the rat POMC promoter in the hope of developing a system to correlate glucocorticoid inhibition of transcription with chromatin structure. This report describes an episomal BPV system where transcription from the POMC promoter initiates correctly and is inhibited by glucocorticoids.

BPV has been used as episomal vector to study transcriptional regulation in a number of systems with varying degrees of success (26, 37–40). In most cases, as in ours, removal of plasmid sequences was necessary for maintenance of episomal state. We also found a higher frequency of clones containing only unarranged episomal DNA (Table 1, Fig. 2) when using the BPV 69% transforming fragment (almost 50% of clones examined) rather than the entire BPV genome (only 1 of 16 clones examined). However, all cell lines derived from POMC-Tag/BPV transformed foci contain similar POMC-Tag transcripts as judged from RNA gel blots (e.g., Fig. 3) whether or not these cell lines contain only episomal DNA. The level of these transcripts roughly correlates with episomal DNA copy number in cell lines where BPV DNA is entirely episomal.

The two POMC-Tag cytoplasmic transcripts present in our cell lines correspond to the size expected from alternate splicing of SV40 early transcripts; they appear to be correctly transcribed starting at the POMC initiation site as assessed by RNase and nuclease S1 mapping (Fig. 5). The same POMC-Tag transcripts are found regardless of the orientation of POMC-Tag transcription relative to BPV transcription (as in lines C79S5X and C80XS9, Fig. 3). In contrast, two additional BPV transcripts (about 1650 and 2300 nt) are found in all cell lines transformed with epismes where BPV early and POMC-Tag transcription are in opposite orientations. These transcripts do not appear to change the properties of the transformed C1271 cells. The 1650-nt BPV transcript is the most abundant and appears to be entirely transcribed from BPV sequences: it does not contain any POMC sequences as judged by hybridization and RNase mapping with continuously labeled probes (data not shown). It hybridizes to BPV fragments (*EcoRI*–*Kpn I* and *Kpn I–BamHI*) encoding the 3' region common to many BPV early transcripts (34, 35). The 1650-nt transcript could correspond to the type 3 or 4 mRNAs described by Stenlund *et al.* (35), but we have not determined its precise structure, and its size does not correspond exactly to any BPV transcript reported (35). Since they do not contain POMC or Tag sequences, the newly identified BPV transcripts most probably initiate somewhere in BPV. The appearance of a different pattern of BPV transcription appears to depend solely on the orientation of the POMC-Tag segment. It would be interesting to find out if other genes carried on BPV vectors have similar effects on BPV transcription or if a specific sequence present in our plasmids is responsible for this orientation-dependent effect.

Transcription of the POMC-Tag transcription unit carried on BPV episomes is sensitive to glucocorticoid regulation to the same extent (Figs. 3 and 5) as the cellular POMC gene in anterior pituitary cells (16). We observed a similar glucocorticoid-dependent inhibition of transcription from the POMC promoter stably reintroduced into POMC-expressing cells in eukaryotes.
culture; the strictly tissue-specific POMC promoter is active in these pituitary POMC-expressing cells (Art-20) while it is not in fibroblasts (L cells) or in C127I cells (unpublished data). In this context, it is interesting to note that Israel and Cohen (41) observed a 40% glucocorticoid inhibition of transcription from a human POMC promoter fusion transfected into L cells. Since the POMC promoter is inactive in L cells, these authors needed to insert an SV40 enhancer fragment in their construct to detect transcription from the POMC promoter. Either the insertion of this enhancer to drive transcription or species differences might account for the decreased glucocorticoid inhibition observed in this system.

Inhibition of POMC-Tag transcription by glucocorticoids does not affect BPV transcription (Fig. 3), indicating that the POMC glucocorticoid-sensitive element is only active on the downstream proximal promoter in our epibmes. Similarly, glucocorticoid stimulation of mouse mammary tumor virus transcription from BPV episomes does not affect BPV transcription (26).

We have localized glucocorticoid receptor binding sites in the POMC promoter fragment used in these experiments and shown that one of these is sufficient for glucocorticoid inhibition of POMC transcription (unpublished data). A detailed analysis of this glucocorticoid receptor binding site will be published elsewhere. The POMC-Tag/BPV transformed cell lines obtained in this work may now be used to study the relationship between chromatin structure and glucocorticoid inhibition of POMC transcription.

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