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The upstream regulatory region of the human papilloma virus-16 contains an E2 protein-independent enhancer which is specific for cervical carcinoma cells and regulated by glucocorticoid hormones

B.Gloss[1], H.U.Bernard[1], K.Seedorf[2] and G.Klock

German Cancer Research Center, Institute for Cell and Tumor Biology, Im Neuenheimer Feld 280, 6900 Heidelberg, FRG

[1]Present address: Institute of Molecular and Cell Biology, National University of Singapore, Singapore
[2]Present address: Genentech Inst., San Bruno Blvd, San Francisco, CA, USA

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The upstream regulatory region of the human papilloma virus-16 (HPV-16) genomic DNA contains a sequence element with a large degree of homology to the partially palindromic sequence GGTACANNNTGTTCT, which is the consensus sequence of the glucocorticoid responsive elements of known genes regulated by this steroid hormone. DNase I and dimethylsulfate protection experiments reveal the binding of this sequence by rat glucocorticoid receptor protein. A 400-bp DNA segment centrally containing this sequence confers strong inducibility by dexamethasone to the promoter p97 of HPV-16 and to the Herpes simplex virus thymidine kinase promoter, as judged by chloramphenicol acetyltransferase activity and RNase protection assays. The same DNA segment, that does not contain the consensus sequences of all papilloma viruses relevant for E2 protein-mediated transcription enhancement, functions in an enhancer-like fashion in addition to its glucocorticoid responsive action. This hormone-independent transcription enhancement is absent in human MCF7 cells, but is strong in human HeLa cells where the combined activity of the constitutive and the steroid hormone-dependent enhancer elements stimulate transcription by a factor of 500. This cell type specificity of the HPV-16 enhancer may be responsible for the tissue tropism of the virus. These observations and the presence of numerous homologies to known enhancers of cellular and viral genes suggest a complex pattern of activation of the human papilloma virus-16 promoters.

Key words: cervical carcinoma/enhancer/glucocorticoids/human papilloma virus/steroids

Introduction

More than 40 types of human papilloma viruses (HPVs) have been isolated (Giri and Danos, 1986). Many of them cause benign neoplasias of mucosal epithelia, and the DNA of a subgroup of these HPVs is frequently associated with malignant tumors of the cervix. This suggests a causative role of these HPVs in the etiology of these neoplasias (for review see Pfister, 1984; Peto and zur Hausen, 1986). HPV-16, the primary isolate (Dürst *et al.*, 1983) and in many studies the most frequently encountered member of this group, is becoming a model system to investigate the biology and pathology of these viruses. The genome of HPV-16 consists—in homology to all papilloma viruses—of a 7905-bp, circular double-stranded DNA. The 10 major open-reading frames (ORFs) are concentrated in ~90% of the genome and spare a 735-bp segment located between ORF-L1 and E6 (Seedorf *et al.*, 1985). In case of the similarly organized bovine papilloma virus-1 (BPV-1) this segment has been termed 'upstream-regulatory region' (URR) (Haugen *et al.*, 1987) or 'non-coding region' (NCR) (Sarver *et al.*, 1984). Transcription starts have been mapped to the very 3′ end of the URR of HPV-16, namely 14 bp downstream of the ATG of the first ORF E6, 3′ of the URR (Smotkin and Wettstein, 1986). This promoter, termed p97, is at a position comparable with the major 'early' promoter found in cells containing the closely related HPV-18 (Schneider-Gädicke and Schwarz, 1986), or CRPV (Danos *et al.*, 1985) and BPV-1 (Heilman *et al.*, 1982; Stenlund *et al.*, 1985). Three additional transcription starts and elements relevant for replication have been mapped within the URR of BPV-1 (Baker and Howley, 1987). Also, upstream transcription starts seem to exist in the HPV-18-URR (Schneider-Gädicke and Schwarz, 1986). All sequenced papilloma virus DNAs exhibit a highly homologous genome organization and many aspects of the biology of different papilloma viurses are similar (Pfister, 1984; Giri and Danos, 1986). These homologies suggest that *cis*-acting elements comparable with those of BPV-1 have to be expected in the URR of HPV-16. Beyond this, it can be hypothesized that relevant aspects of HPV-16 biology, such as species tropism, tissue tropism and autoregulation ('early—late switch') are accounted for by sequences in its URR as it has been shown for the promoter enhancer regions of different tumor viruses (Rio *et al.*, 1980; Hansen *et al.*, 1981; Brady *et al.*, 1984; Keller and Alwine, 1984; Amati, 1985; Rochford *et al.*, 1987). In addition, several clinical and epidemiological observations might be explained through the action of extracellular factors on HPV promoters, e.g. the correlation of viral lesions of the genitals with cortisone treatment or cigarette-smoking (for reviews see Peto and zur Hausen, 1986).

As part of a broader study towards these questions we have concentrated our interest in this report on a region that is centered ~300 bp upstream of the most frequently used 'early' promoter, p97. This region caught our attention since it contains in close proximity several consensus sequences of the target sites of known DNA binding proteins, among them a site that could bind the receptor protein for glucocorticoid hormones. So far, no papovaviruses have been shown to be regulated by steroid hormones, while the occurrence of consensus sequences of glucocorticoid-responsive elements (GREs) is a common phenomenon in different retroviruses (Miksicek *et al.*, 1986). In particular, the long terminal repeat promoter of the mouse mammary tumor virus (MMTV LTR) has been shown to be dependent on glucocorticoids, progesterone and androgens (Darbre *et al.*, 1986; Cato *et al.*, 1986, 1987), and the closely related promoter sequences of the mouse sarcoma virus LTR and the Moloney murine leukemia virus LTR are inducible by or at least bind glucocorticoid receptor (Ucker and Yamamoto, 1984; De Franco and Yamamoto, 1986; Miksicek *et al.*, 1986; Speck and Baltimore, 1987). The mechanism of hormonal induction of transcription

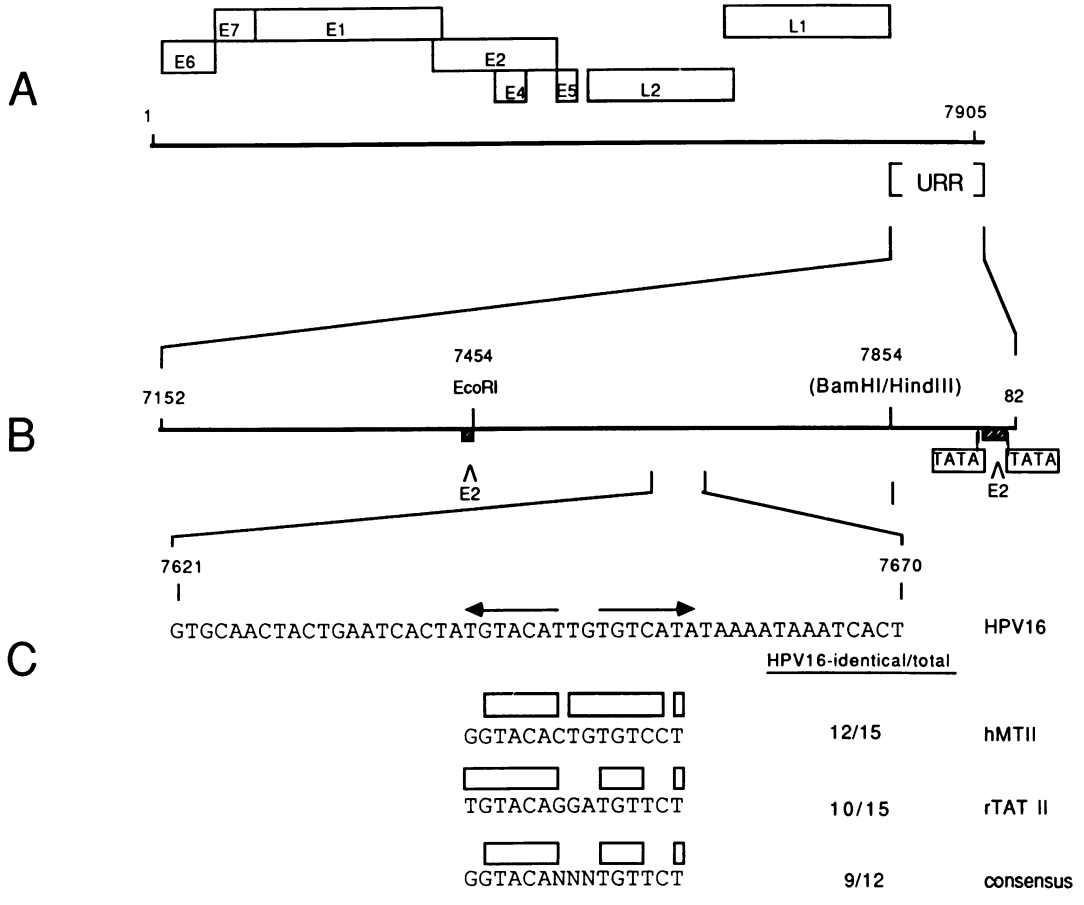

Fig. 1. Sequence organization of the genome and of subgenomic segments of the human papilloma virus-16. (**A**) Localization of major open reading frames (E1–E6, L1, L2) and of the upstream regulatory region (URR) on the circular DNA of HPV-16, linearized at position 7905/1. (**B**) Enlargement of the URR. The location of the two TATA-boxes associated with the natural promoter p97 is indicated, also the three consensus sequences of E2 protein binding sites, two of which are located between the two TATA-homologies. The *Eco*RI site is a natural restriction site of the HPV-16 genome, the *Bam*HI/*Hin*dIII motif terminates the URR in the clone pHPV-16-40.4, and constitutes a linker inserted to the end of a fragment that was created during a *Bal*31 deletion (Seedorf, 1986). (**C**) Comparison of the glucocorticoid-responsive element in the URR of HPV-16 to two natural GREs and a GRE consensus sequence (Jantzen *et al.*, 1987).

involves binding of the glucocorticoid receptor protein to recognition sites on the DNA (Payvar *et al.*, 1983; Scheidereit *et al.*, 1983), which can be identified independently by deletion analysis of promoter regions (Buetti and Diggelmann, 1983; Hynes *et al.*, 1983; Majors and Varmus, 1983). These glucocorticoid-responsive elements (GREs) can act on heterologous promoters independently of their position and orientation (Chandler *et al.*, 1983; Ponta *et al.*, 1985), and on homologous promoters over long distances from positions upstream or downstream of the transcription start site (Moore *et al.*, 1985; Jantzen *et al.*, 1987). The comparison of numerous GREs has led to the identification of a 15-bp consensus sequence, which includes an incomplete palindrome of the hexanucleotide 5′-TGTTCT-3′ (Scheidereit *et al.*, 1983; Jantzen *et al.*, 1987). In this report we describe the functional analysis of a sequence in the URR of HPV-16, which shows a large degree of homology to the GRE consensus sequence as well as to the functionally tested GREs of the rat tyrosine aminotransferase gene (TAT) (Jantzen *et al.*, 1987) and the human metallothionein IIa gene (Karin *et al.*, 1984) (Figure 1).

The glucocorticoid receptor protein binds to this HPV-16 sequence *in vitro* and protects it from DNase I cleavage and dimethylsulfate (DMS) methylation. A fragment from the HPV-16 URR region containing this element stimulates transcription from the TK promoter after addition of the synthetic glucocorticoid dexamethasone to transfected HeLa cells (a human cervix carcinoma-derived cell line), or MCF-7 cells (a human breast tumor-derived cell line). Similarly, the natural promoter p97 is regulated by dexamethasone in HeLa cells. Beyond this regulatory effect, we describe a strong constitutive enhancement of the TK promoter activity by this HPV-16-DNA segment in HeLa cells, but not in MCF-7 cells. These observations suggest that the upstream regulatory region of the HPV-16 genomic DNA contains a glucocorticoid-responsive element and a cell type-specific constitutive enhancing element, which can work independently or together.

Results

The upstream regulatory region of HPV-16 contains a binding site for glucocorticoid receptor

During the sequencing of the genome of the HPV-16 (Seedorf *et al.*, 1985; Seedorf, 1986), we noticed a DNA sequence in the URR of this virus with a large degree of homology to glucocorticoid-responsive promoter elements, namely the sequence 5′-TGTACATTGTGTCAT-3′ between positions 7641 and 7655. To initiate a functional analysis of this potential GRE of HPV-16, we subjected its DNA sequence to a computer search for homologies to the three sequences shown in Figure 1, which represent two natural GREs and a consensus sequence of all

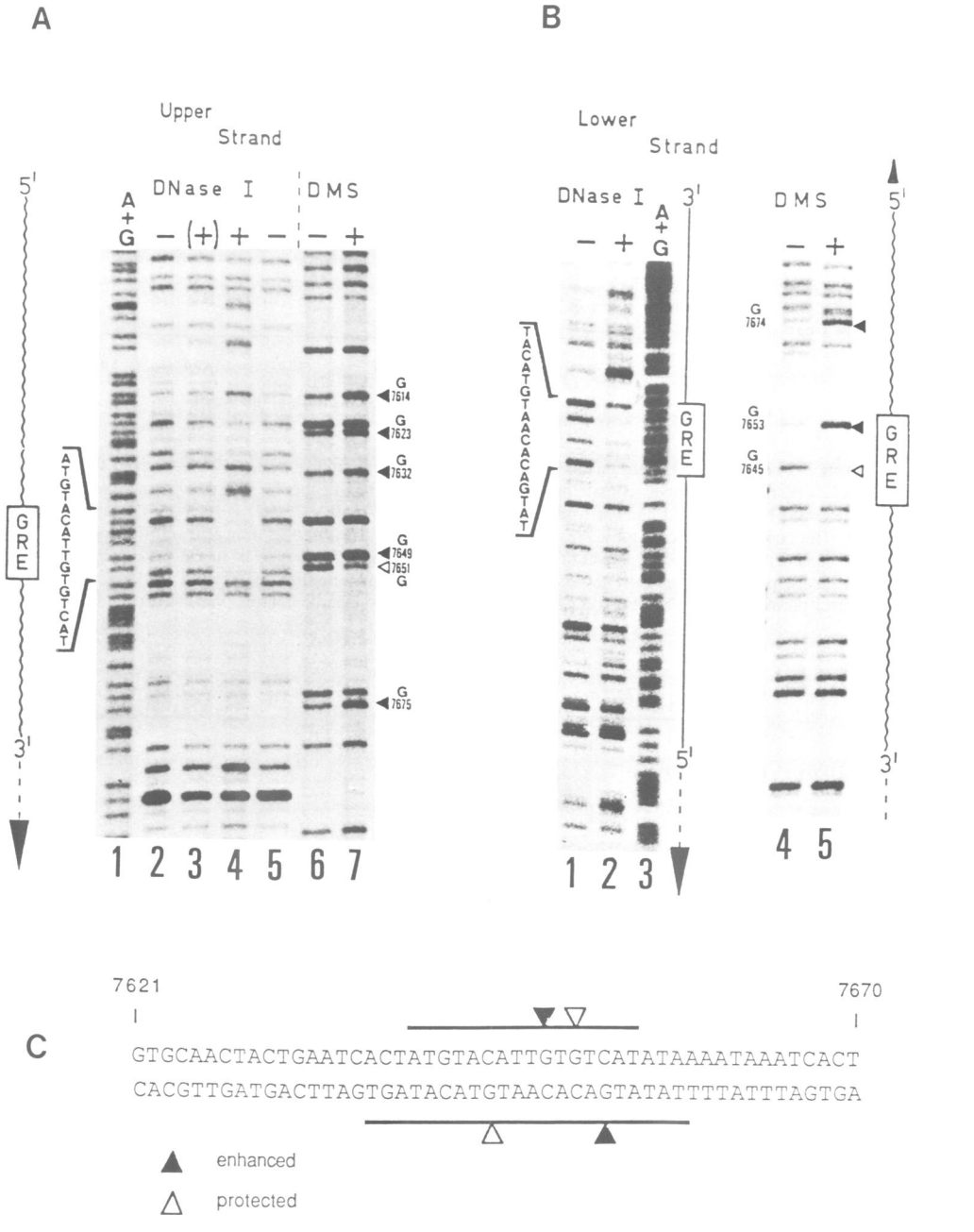

Fig. 2. DNase I and DMS protection of the glucocorticoid-responsive element of HPV-16 by a glucocorticoid receptor protein shown for the upper strand (**panel A**) and for the lower strand (**panel B**). Alongside the DNase I cleavage products, A + G Maxam–Gilbert sequencing reactions (Maxam and Gilbert, 1980) of the same DNA fragment define the 5′ and 3′ borders of the protected region. Lanes designated with a – contained only BSA in the binding and cleavage assay; lanes designated with a + contained partially purified glucocorticoid receptor in the binding and cleavage assay. In **panel A** the lane designated with (+) contained in the binding and cleavage assay the partially purified glucocorticoid receptor which was inactivated at 42°C for 10 min prior to the binding assay. The DNA sequence of the region protected by the glucocorticoid receptor is given alongside each panel. Protected G residues are indicated with an open triangle, enhanced G residues are marked with a closed triangle.

published GREs (Jantzen *et al.*, 1987). The sequence in the HPV-16 genome with maximal homology to these three elements was the sequence indicated above, which is located ~300 bp upstream of the most frequently used transcription start site of HPV-16 (Smotkin and Wettstein, 1986). A comparison of these sequences is shown in Figure 1, which also illustrates the location of the element in the URR of the HPV-16 genome. Within the 12-bp incomplete palindrome of the sequence 5′-TGTTCT-3′, this 15-bp segment has 9, 10 and 11 bp in common with the consensus sequence, the tyrosine aminotransferase gene motif II and the GRE in the human metallothionein IIA gene promoter respectively. This 12-bp palindrome is considered solely to be relevant for glucocorticoid receptor binding (Becker *et al.*, 1986; Strähle *et al.*, 1987). These similarities have encouraged us to initiate a functional analysis of this HPV-16 sequence. In order to find out whether the HPV-16 motif is able to bind the glucocorticoid receptor, we have made use of the genomic subclone pHPV-16-40.4 (Seedorf, 1986), which contains a single *Eco*RI site ~190 bp upstream and single *Bam*HI and *Hin*dIII sites ~100 bp downstream of the sequence elements (Figure 1). These restriction sites were used to label radioactively the DNA and to perform DNaseI footprinting and DMS protection assays in

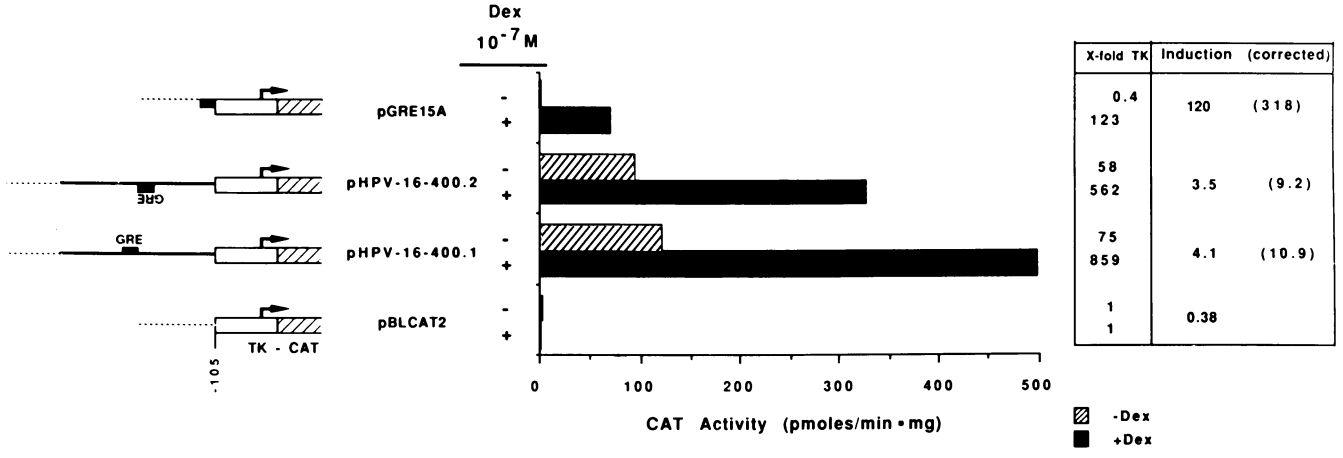

Fig. 3. Constitutive and glucocorticoid hormone dependent enhancement of the HSV-TK promoter by a HPV-16-derived fragment in human HeLa cells. The structure of the plasmid pBLCAT2 and three derivatives containing inserts at position −105 of the TK promoter (open bar) is schematically outlined in the left part: hatched bar, CAT gene; solid line, 400-bp fragment from HPV-16-URR in *syn*-(pHPV-16-400.1) or *anti*-orientation (-400.2); solid bars, binding sites for glucocorticoid receptor. The central part shows the CAT activities in HeLa cell extracts after transient transfection of the plasmid DNAs in the absence (−) or presence (+) of dexamethasone respectively. CAT values relative to pBLCAT2 are given in the boxed table (×-fold TK); the induction numbers give the CAT activities in the presence divided by those in the absence of hormone, either uncorrected or corrected for the reduction of CAT expression observed for the control plasmid pBLCAT2. For details about pGRE15A, the positive control, see the text and Strähle *et al.* (1987).

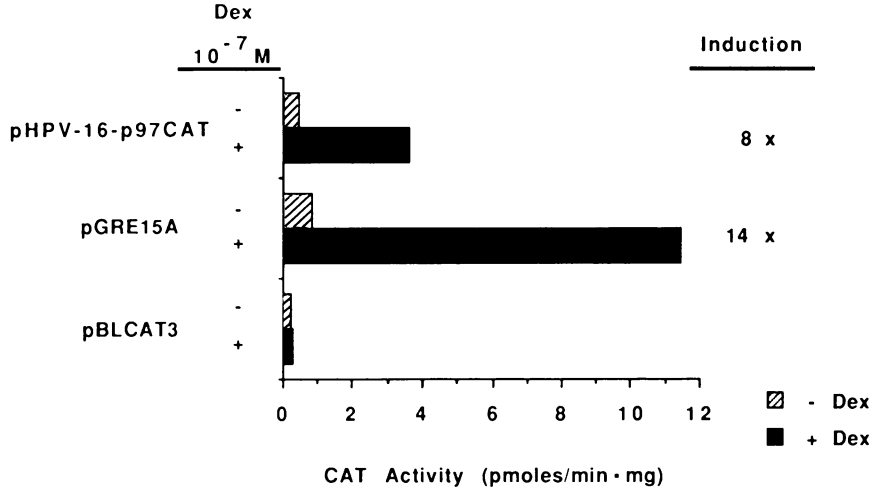

Fig. 4. Stimulation by glucocorticoids of CAT gene expression under the control of the p97 promoter of HPV-16. The three indicated plasmids were transiently transfected into HeLa cells in the absence or presence of 10^{-7} M dexamethasone. The construction of pHPV-16-p97CAT is described in Materials and methods. pBLCAT3 is a promoterless vector (Luckow and Schütz, 1987).

the presence or absence of partially purified glucocorticoid receptor from rat liver (Danesch *et al.*, 1987).

Figure 2 demonstrates specific binding of the glucocorticoid receptor protein to the sequence discussed above. On the upper strand (Figure 2A) a region of 16 bp is protected in the presence of receptor (lane 4) as compared with lanes 2 and 5, where no receptor was present. In lane 3 the receptor protein was heat inactivated prior to addition of DNA and DNase I, and no protection is seen. On the lower strand a sequence of 20 bp is protected in the presence of receptor (Figure 2B, lane 1 and 2). The protected DNA is indicated in Figure 2C, revealing that both protected strands overlap. We have complemented the DNase I protection data by a DMS methylation protection experiment (Figure 2), since the glucocorticoid receptor is known to protect guanines from methylation by DMS on several recognition sites (Scheidereit and Beato, 1984). One guanine on the upper strand (Figure 2) is protected by the glucocorticoid receptor (open triangle) and one adjacent guanine is slightly stronger methylated. Both Gs lie within the region protected by DNase I (Figure 1C). On the lower strand one G within the binding region is protected from methylation and one G is enhanced in the presence of receptor. In both strands several enhancements at G residues outside of the region protected from DNase I occurred, which may be due to secondary changes of the DNA structure, or due to changes stimulated by DNA binding proteins contaminating the glucocorticoid receptor preparation. Taken together these results demonstrate specific binding of the glucocorticoid receptor to a sequence on HPV-16 that shows similarity with known glucocorticoid-responsive elements.

A 400-bp-long DNA fragment from the URR of HPV-16 contains a constitutive enhancing activity, that can be stimulated in HeLa cells by glucocorticoids

The detection of a binding site for the glucocorticoid receptor in the URR of HPV-16 raises the question whether this sequence

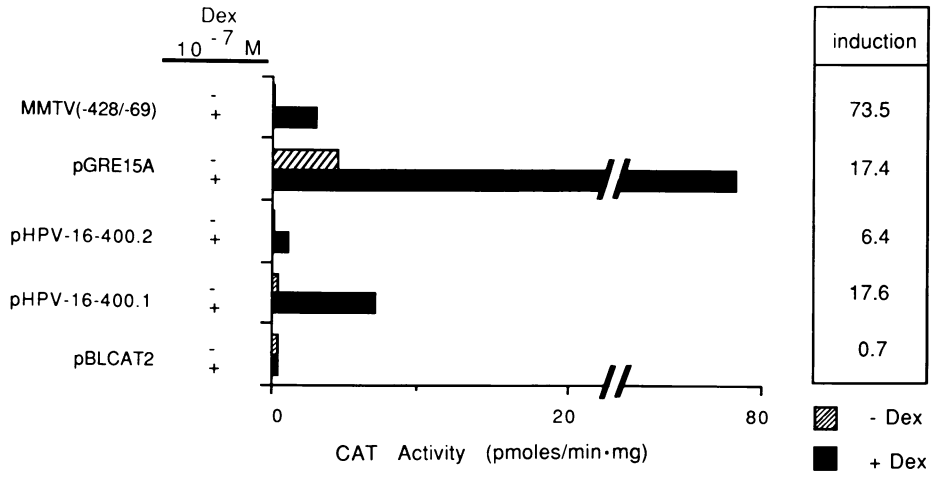

Fig. 5. Glucocorticoid hormone-dependent transcriptional stimulation in human MCF7 cells. The five plasmids indicated [MMTV(−428/−69): see Miksicek *et al.*, 1986] were transiently transfected into MCF7 cells. The bars show the relative CAT activities in the absence or presence of 10^{-7} M dexamethasone (Dex).

element can function to stimulate the transcriptional activity of a linked promoter after hormone administration. To address this question we have decided to separate the DNA region of interest from its natural promoter elements and to link it to a heterologous promoter. We have chosen a 400-bp fragment, spanning the DNA region from position 7454 to 7854, which was cloned from pHPV-16-40.4 as described in Materials and methods. The fragment does not contain the two TATA box regions and the binding site (Androphy *et al.*, 1987) for the virus-encoded protein E2 between the two TATA homologies (Seedorf *et al.*, 1985), and only one-half of the palindromic E2 recognition site at position 7451−7462.

As a test promoter for glucocorticoid responsiveness we have chosen the Herpes simplex virus TK promoter, which is known to respond to steroid hormone regulatory elements linked in *cis* (Chandler *et al.*, 1983; Ponta *et al.*, 1985; Miksicek *et al.*, 1986). The 400-bp fragment from HPV-16 was inserted into the polylinker region of pBLCAT2 upstream of the TK promoter (Klein-Hitpass *et al.*, 1986; Luckow and Schütz, 1987). The bacterial chloramphenicol acetyltransferase (CAT) gene, which is transcribed by the TK promoter in this vector, can be monitored as an indicator gene after transient transfection of plasmid DNA into mammalian cells (Gorman *et al.*, 1982).

The structure of two test plasmids containing the HPV-16 fragment is as follows: pHPV-16-400.1 harbors the fragment in the *syn*-orientation relative to TK-CAT as compared to the HPV transcription direction (see Figure 1), whereas pHPV-16-400.2 contains it in the *anti*-orientation. We have chosen HeLa cells for the transfection experiment, since this cell line was derived from a human cervical carcinoma and may still exhibit some of the differentiation specificites of cells that are found infected by HPV-16 (Seedorf *et al.*, 1987). In particular, by expression of its endogenous HPV-18 copies, it still exhibits its competence for recognizing papilloma virus promoters (Schwarz *et al.*, 1985).

Figure 3 shows the result of a transient transfection of HeLa cells with the two HPV-derived plasmids. pBLCAT2, the recipient vector without an enhancer, serves as negative control. The positive control is pGRE15A, which contains a 15-bp long oligonucleotide representing the rat TAT gene GRE inserted immediately upstream of the TK promoter (Strähle *et al.*, 1987). In the absence of hormone, both control plasmids, pBLCAT2 and pGRE15A, show a comparable low expression of the CAT gene. However, the two plasmids pHPV-16-400.1 and pHPV-16-400.2 give rise to 75- and 58-fold stimulation of the expression of the CAT gene. We conclude from these results that even in the absence of steroid hormones the inserted segment from HPV-16 can stimulate the function of an heterologous promoter. This occurs independently of orientation, i.e. in an enhancer-like fashion (for a review see Khoury and Gruss, 1983).

In the presence of the synthetic glucocorticoid dexamethasone pBLCAT2 shows no increased CAT expression. Instead, the CAT activity is slightly but reproducibly reduced, which represents a general inhibitory effect of glucocorticoids encountered in many but not all cell types (our unpublished observations). The CAT expression of pHPV-16-400.1 and pHPV-16-400.2, which is strong already under the influence of the enhancer-like element, is even further increased by the hormone (Figure 3). The induction values are 10.9- and 9.2-fold, when corrected for the suppression of pBLCAT2. This represents typical induction rates found in the analysis of GREs of genes known to be regulated by glucocorticoids *in vivo* (Danesch *et al.*, 1987; Jantzen *et al.*, 1987). The GRE in pGRE15A leads to a very strong dexamethasone dependent induction of >300-fold.

In conclusion, the accumulation of the enhancing effect and of the glucocorticoid response conferred by the HPV-16 fragment allows a TK promoter-dependent CAT expression, stimulated by factors of >500.

To test whether the GRE of HPV-16 regulates the homologous promoter p97, we inserted an *Sph*I−*Ava*II fragment with the HPV-16 sequences from position 7464 to 115 into plasmid pBLCAT3, a promoterless vector that contains the complete CAT gene leading to the plasmid pHPV-16−p97CAT. This fusion brings CAT transcription under the control of p97. CAT translation would occur from an ATG at position 104, which is in frame to the CAT-ATG leading to a CAT protein extended at its N terminus by 18 amino acids. In independent unpublished experiments, however, we have found that CAT enzymes with significant extensions at their N termini are active, sometimes at a significantly reduced rate. The control by dexamethasone of p97-dependent CAT expression from plasmid pHPV-16−p97CAT after transfection into HeLa cells is shown in Figure 4. CAT activity is induced 8-fold, while pGRE15A is 14-fold inducible by the hormone. This result demonstrates that the GRE of HPV-16 is active in the original context of the HPV-16 promoter.

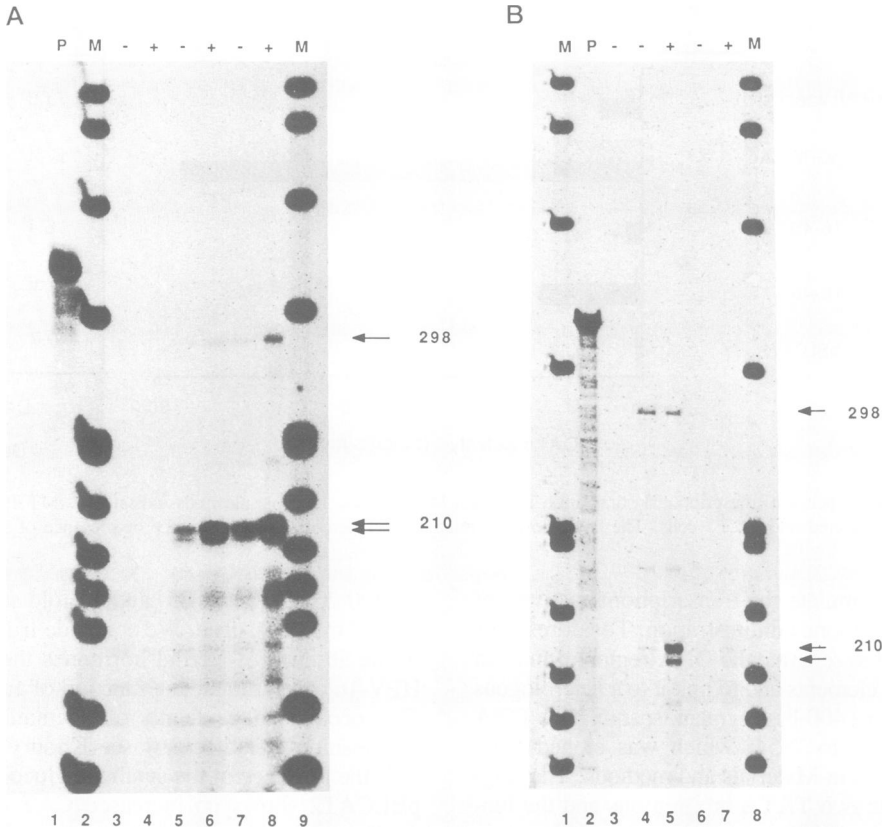

Fig. 6. RNase protection assays to demonstrate an increase of CAT mRNA concentration dependent on the constitutive enhancer effect and on glucocorticoid hormones. RNA was isolated from HeLa (**A**) or MCF7 cells (**B**) after transient transfection of various plasmids and subjected to an RNase protection assay using a probe specific for CAT mRNA transcribed from the TK promoter (see Materials and methods). The protected RNAs and the RNA probe used for the protection (**P**, **lanes A1** and **B2**) were run on a 6% sequencing gel together with 5′-labeled DNA fragments resulting from a *Hpa*II digest of pBR322 (M; **lanes A2** and **9**; lanes **B1** and **8**). The cells were grown either in the absence (−) or presence (+) of 10^{-7} M dexamethasone, as indicated above each lane. Plasmids used for the transfection: **A** (HeLa): **lanes 3** and **4**; pBLCAT2; **5** and **6**, pHPV-16-400.1; **7** and **8**, pHPV-16-400.2 (**lanes 3, 5** and **7**, −Dex; **lanes 4, 6** and **8**; +Dex). **B**, (MCF7): **lane 3**, no DNA; **4** and **5**, pHPV-16-400.1; **6** and **7**, pHPV-16.400.2 (**lanes 3, 4** and **6**, −Dex; **5** and **7**, +Dex). RNA (20 μg) was used in all cases, except for **lanes B6** and **B7**, where 8 μg were used. The arrows point to the bands that result from correct starts at the TK promoter (210 nucleotides), or from readthrough transcripts (298) respectively (Miksicek *et al.*, 1986).

The enhancing element is inactive, but the glucocorticoid-responsive element is active in MCF-7 cells

In order to test whether the observed enhancer-like properties of the HPV-16 fragment on the TK promoter are cell type independent, we decided to test our constructs in a second human cell line, which does not originate from cervical epithelium. We chose the human breast tumor cell line MCF-7 (Horwitz *et al.*, 1975). Also, this cell line permits a better quantification of the GRE effect, since glucocorticoids do not have the inhibitory effect observed with HeLa cells (our unpublished data). In transfections of MCF-7 cells, we included the plasmid MMTV (−438/−69), which contains the GRE from the MMTV-LTR cloned upstream of the TK-CAT sequence (Figure 5A) (Miksicek *et al.*, 1986). Unexpectedly, the strong constitutive increase of the TK promoter activity, which was observed with pHPV-16-400.1 and pHPV-16-400.2 in HeLa cells is absent in MCF-7 cells, as can be seen from Figure 5; CAT expression in the absence of dexamethasone is about the same for all constructs, except for pGRE15A, which has a higher basal level than pBLCAT2 (Figure 5). This finding argues in favor of a cell-type-specific enhancer activity contained within the 400-bp fragment from HPV-16.

Both plasmids, pHPV-16-400.1 and pHPV-16-400.2, however, give rise to a 17.6- and 6.4-fold stimulation of CAT expression through treatment with dexamethasone (Figure 5), suggesting that the GRE within the HPV-16 fragment does not functionally require cell-type-specific transcription factors. The two control plasmids pGRE15A and MMTV(−428/−69) give rise to a 17.4- and 73.5-fold induction by glucocorticoids. pBLCAT2 does not respond to dexamethasone.

We wished to determine whether the induction of CAT activity conferred by the cell-type-specific as well as the glucocorticoid-dependent enhancer is caused by an increase of transcription at the natural start site of the HSV TK promoter. This was done with an RNase protection assay (Zinn *et al.*, 1983; Melton *et al.*, 1984). Figure 6 shows the analysis of RNA from MCF-7 and HeLa cells transfected with pHPV-16-400.1 and pHPV-16-400.2. In both cell lines, both plasmids give rise to transcripts originating from correct starts at the TK promoter cap site as can be see from the appearance of the 210-nucleotide-long protected RNA (Cato *et al.*, 1986; Miksicek *et al.*, 1986). In the presence of dexamethasone the intensity of this band increased in all cases. This proves that the segment from the HPV-16-URR stimulates transcription initiation at the authentic start site of the TK promoter in a cell-type-specific and glucocorticoid dependent way.

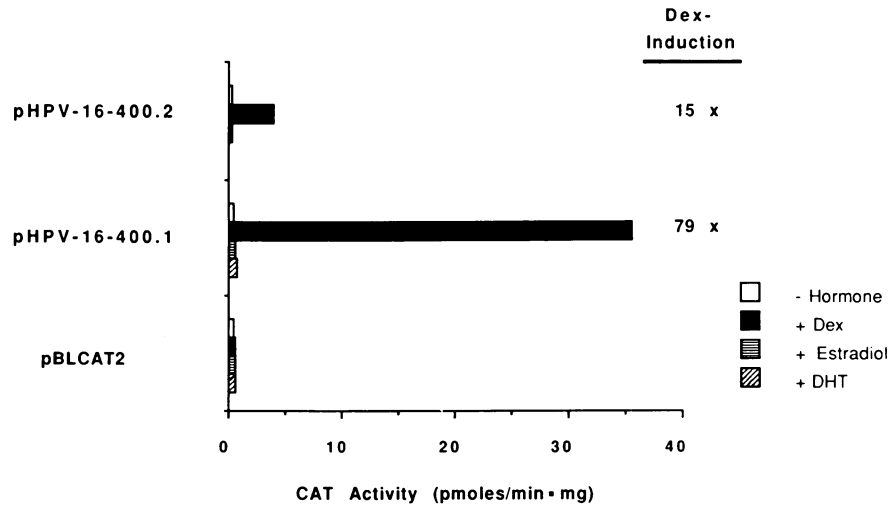

Fig. 7. Steroid hormone induction by the HPV-16-URR is specific for glucocorticoid hormones. The two HPV-16-derived plasmids were transfected parallel with the vector pBLCAT2 into MCF7 cells. The cells were treated with either dexamethasone (Dex), estradiol, or dihydrotestosterone (DHT), as indicated. The induction numbers (dexamethasone) for pHPV-16-400.1 and pHPV-16-400.2 are uncorrected (see Figures 3 and 4). All hormones were applied at 10^{-7} M.

The steroid hormone-dependent induction occurs only after treatment with glucocorticoids, but not with estrogens or androgens

The human breast tumor cell line MCF-7 contains hormone receptors for glucocorticoids, androgens and estrogens (Horwitz *et al.*, 1975). In addition, the responsiveness of several genes and DNA fragments to glucocorticoids (Strähle *et al.*, 1987) and estrogens (Klein-Hitpass *et al.*, 1986; Klock *et al.*, 1987) after transient transfection into this cell line has been demonstrated. Therefore, we tested whether or not steroids other than glucocorticoids can induce CAT expression after transfection of the pHPV-16-400 plasmids into MCF-7 cells. Figure 7 gives the results of such an experiment. The data demonstrate that only dexamethasone, but not estradiol or dihydrotestosterone, lead to increased CAT enzyme activities. Therefore we conclude that the fragment from the HPV-16-URR does not contain a hormone-responsive element for estrogen and androgen induction.

Discussion

We have chosen HPV-16, one of the medically most relevant members of the large group of human papilloma viruses, as a model system to analyze *cis*-acting elements relevant for promoter function. In analogy to BPV-1, 704 bp of its 7905-bp genome can be named URR. Analysis of transcripts from cells that contain human or animal papilloma virus genomes suggests that most viral transcripts initiate in this URR. This occurs most frequently at the 3′ boundary of the URR close to the first ATG of the E6 open reading frame (Heilman *et al.*, 1982; Danos *et al.*, 1984; Schwartz *et al.*, 1985; Smotkin and Wettstein, 1986; Stenlund *et al.*, 1985), but additional starts have been observed at least for BPV-1 and HPV-18 (Schneider-Gädicke and Schwarz, 1986; Baker and Howley, 1987). In analogy to all eukaryotic and viral promoters, it is likely that transcription initiation at these starts is under control of distal elements and enhancers. The four promoters mapped in the URR of BPV-1 (Baker and Howley, 1987), the autoregulation by E2 protein binding sequences in all sequenced papilloma viruses (Spalholz *et al.*, 1985; Androphy *et al.*, 1987; Haugen *et al.*, 1987; Moskaluk and Bastia, 1987), the 'early−late switch' (Baker and Howley, 1987) and the tissue specificity of papilloma viruses (Pfister, 1984) make it likely that a complex system of transcription-controlling elements will be found in the URR of these viruses, that interact in a complex way. Also, *cis*-acting elements related to transcriptional activities have been localized in BPV-1 outside the URR (Lusky *et al.*, 1983; Lusky and Botchan, 1984; Baker and Howley, 1987). To initiate research on transcription elements in the URR of HPV-16, we have found by computer search with known consensus sequences, and by DNase I footprinting with HeLa cell nuclear extracts, numerous protein binding sites spread throughout the URR (our unpublished observations). To separate some of these elements, we have described in this paper a transcriptional enhancer which is associated wtih a glucocorticoid responsive element and functions differentially in two cell lines.

It is now a generally accepted model that the members of different groups of steroid hormones bind different receptor proteins (Green and Chambon, 1986). These receptor proteins have a binding specificity for particular target sequences on the DNA, which may differ for at least some receptor types (Klock *et al.*, 1987). For binding of the glucocorticoid receptor, consensus sequences involving the motif 5′-TGTTCT-3′ have been postulated (Payvar *et al.*, 1983; Scheidereit *et al.*, 1983; Jantzen *et al.*, 1987). More recently, it has become clear that GREs involve an at least partial palindrome of this motif (Strähle *et al.*, 1987; Klock *et al.*, 1987), the perfect palindrome being represented by the sequence 5′-AGAACANNNTGTTCT-3′.

In naturally occurring GREs, a consensus sequence has been formulated to be 5′-GGTACANNNTGTTCT-3′ (Jantzen *et al.*, 1987). In this paper, we have described the identification of a sequence in the URR of HPV-16, with a good homology to this consensus GRE, comparable to other functional GREs. This motif binds rat hepatocyte glucocorticoid receptor protein, and stimulates transcription from TK promoter linked in *cis*. The observed rate of stimulation is in the range of the data obtained wtih genes known to be regulated by glucocorticoids in a physiologically relevant way, e.g. the genes coding for tryptophane oxygenase (Danesch *et al.*, 1986) and tyrosine aminotransferase (Jantzen *et al.*, 1987).

At the moment it is unclear what biological relevance this transcription signal has for HPV-16, just as this question has not

been answered for the GREs of the Moloney murine sarcoma virus (Miksicek *et al.*, 1986) and the MMTV (Ucker and Yamamoto, 1984). It is relevant to emphasize in this context that we did not observe a general effect by steroid hormones, and that this enhancer of HPV-16 (which is found in more genital tumors in women than in men), is not a target site of estrogen receptors. The sequence of progesterone-responsive elements, however, appears to be closely related to GREs (Cato *et al.*, 1986; Strähle *et al.*, 1987). Since we do not have a cell system that allows us to differentiate *in vitro* between glucocorticoid and progesterone induction, we did not include such an experiment into our analysis.

Only one promoter at position 97 of the HPV-16 genome is known so far, and it is in a similar location to the major 'early' transcription start of other papillomaviruses (Smotkin and Wettstein, 1986). Fusion of a promoterless CAT gene to this promoter shows glucocorticoid-dependent CAT expression after transfection into HeLa cells proving an activation of the homologous promoter by the GRE. To extend this analysis we used CaSki cells, which contain and express endogenous HPV-16 genomes (Smotkin and Wettstein, 1986). We quantified HPV-16-RNA of dexamethasone-treated CaSki cells in a Northern blot analysis after dexamethasone treatment of CaSki cells. Since we did not find a difference with and without dexamethasone (data not shown), we transfected into these CaSki cells pGRE15A DNA, the positive control plasmid described above. CAT expression from this plasmid in CaSki cells was not stimulated by dexamethasone. This makes it clear that we can transfect CaSki cells and that the TK promoter functions, but not in a dexamethasone-regulated way, which may be due to a glucocorticoid receptor level too low to give a hormone response.

A second, independent effect exerted by the 400-bp HPV-16 segment is a strong constitutive stimulation of TK promoter transcription. This occurs only in HeLa and not in MCF-7 cells, though both lines are epithelial cells of human origin. In contrast to this observation, different enhancers, such as the SV40 72-bp repeat, function strongly in MCF-7 cells (Matthias *et al.*, 1982; R.Miksicek and W.Ankenbauer, unpublished observation). This HPV-16-specific effect may be due to the transcription apparatus making this virus functional in its most frequently infected target cells, namely epithelial cells of the cervix. We have initiated research to identify the exact location of the element responsible for this differential activity, and we will compare this element in numerous different human and animal cell lines.

Some papilloma virus enhancers including an enhancer from the URR of HPV-16 (Phelps and Howley, 1987) are under the influence of the virus-encoded DNA binding E2 protein. However, it is not known whether the E2 enhancer acts on all papilloma virus promoters, and whether it plays a role in every part of the viral life-cycle. For example, in a freshly infected cell viral promoters may have to be active in the absence of E2 protein, unless this is incorporated in the virus particle. Also, the enhancer element that we have started to characterize may play a role independently of the E2 enhancer or in co-operation with this element (Phelps and Howley, 1987). It should be emphasized that the effects we describe do not occur under the influence of the E2 protein, since (i) HeLa cells do not have an intact E2 gene (Schwarz *et al.*, 1985), and (ii) only the 3′ half of the consensus sequence 5′-ACCGNNNNCGGT-3′ recognized by the E2 protein (Androphy *et al.*, 1987) exists on the analyzed HPV-16 fragment.

Materials and methods

Plasmids

pHPV-16-40.4 contains a segment from position 7454 to 7854 of HPV-16 in pUC8. Its left border is the natural *Eco*RI site in HPV-16, and its right border was created by *Bal*XXXI deletion and ligation to a *Bam*HI linker (Seedorf *et al.*, 1985; Seedorf, 1986). From pUC8, it was isolated as an *Eco*RI−*Bam*HI fragment for DNase I and DMS protection experiments. For insertion into pBLCAT2 (Luckow and Schültz, 1987), it was isolated as an *Eco*RI−*Hin*dIII fragment, the sticky ends were filled in with Klenow-Polymerase, and the fragment ends were ligated to a *Bam*HI linker. After cleavage with *Bam*HI, it was inserted into *Bam*HI cleaved pBLCAT2, where its right end is positioned 6 bp upstream of the distal SPI box of the Herpes simplex virus TK promoter (McKnight and Kingsbury, 1986). The two orientations of the HPV-16 fragment were determined with the help of an internal *Hin*fI site. pHPV-16−p97CAT contains a 556-bp *Sph*I/*Ava*II fragment from position 7464 to 115 of HPV-16. It has the p97 promoter followed by the ATG codon of the E6-ORF at position 104 plus three codons fused in frame to the CAT gene on pBLCAT3 (Luckow and Schütz, 1987) 14 codons upstream of the ATG start codon of the gene. pHPV-16−p97CAT was constructed from pBLCAT3, which was cut at the unique *Xho*I site, and after filling in with Klenow-Polymerase was recut with *Sph*I. The 556-bp HPV-16 fragment was isolated by cutting pHPV-16-14 (Seedorf *et al.*, 1987) with *Ava*II, filling in the Klenow-Polymerase, recutting with *Sph*I and preparative PAG electrophoresis and was ligated with the cut pBLCAT3 vector. After ligation the DNA was recut with *Sal*I in order to increase the ratio of recombinant clones. pHPV-16−p97CAT contains a *Xho*I site resulting from the fusion of the filled in *Xho*I and *Ava*II sites.

Cell culture and transfection

MCF7 and HeLa cells were cultured as described elsewhere (Cato *et al.*, 1986). Transfection by the DEAE Dextran procedure was performed as described (Cato *et al.*, 1986) with the modification that the plasmid concentration in the DEAE Dextran mixture was reduced to 1 μg/ml DNA and that after chloroquine diphosphate treatment the cells were kept in DMEM supplemented with 10% charcoal-stripped fetal calf serum. After 14−18 h the medium was replaced by serum-free DMEM and the incubation was continued for another 20 h before harvesting. Immediately after chloroquine diphosphate treatment hormones were administered in ethanol solution to a final concentration of 0.1% ethanol, control plates contained 0.1% ethanol. All hormones were applied at 10^{-7} M.

CAT assays

CAT assays were performed as described (Gorman *et al.*, 1982). After exposure to X-ray film, radioactive spots on the thin-layer chromatogram were cut out and quantitated by liquid scintillation counting. All numbers given represent the average of CAT activity (pmol/min/mg protein) from two parallel transfections.

RNA start mapping

Total RNA was extracted with guanidinium-thiocyanate followed by centrifugation through a CsCl cushion (Chirgwin *et al.* 1979). Transcription start sites were determined by the RNase protection procedure using uniformly labeled antisense RNA probes (Zinn *et al.*, 1893; Melton *et al.*, 1984; Cato *et al.*, 1986). Twenty-microgram aliquots of total cellular RNA, if not indicated otherwise, were hybridized with the probe and further processed as described (Cato *et al.*, 1986), with the modification that the concentration of RNase T1 was reduced to 20 U/ml.

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