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The hormone response element of the mouse mammary tumour virus DNA mediates the progestin and androgen induction of transcription in the proviral long terminal repeat region

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Mouse mammary tumour virus (MMTV) gene expression has been shown to be regulated by glucocorticoids. A hormone response element (HRE) located between −202 and −59 upstream of the start of transcription in the long terminal repeat (LTR) region of the proviral DNA is required for this induction. We have investigated the role played by the HRE in the induction of MMTV LTR transcription by other classes of steroid hormones. Chimaeric constructs containing the HRE and the authentic LTR promoter linked to an indicator gene or the HRE linked to an otherwise hormone insensitive promoter directing the transcription of an indicator gene, were transfected into the human mammary tumour cell line T47D. Transcription at the MMTV LTR promoter or at the previously hormone-insensitive promoter was induced by progestins and androgens but not by oestradiol in transfected cells that contained functional receptors for these hormones. These results identify the HRE as the *cis*-acting element that mediates the progestin and androgen induction of MMTV LTR transcription. The HRE is therefore a DNA element that is required not just for glucocorticoid but also for progesterone and androgen induction of MMTV LTR transcription.

Key words: Dihydrotestosterone response/progesterone response/human breast cancer cell line/gene transfer/hormone response element

Introduction

Transcription of the proviral DNA of the mouse mammary tumour virus (MMTV), an endogenous murine retrovirus, is induced by glucocorticoids in infected tissues or in cells derived from mammary carcinoma (Parks *et al.*, 1974; Lasfargues *et al.*, 1976; Pauley *et al.*, 1979).

The increase in transcription of the MMTV DNA occurs rapidly after glucocorticoid treatment and it is mediated by the glucocorticoid receptor (Ringold *et al.*, 1975; Young *et al.*, 1975). The DNA sequence element required for the glucocorticoid induction of transcription has been mapped to position −202 and −59 upstream of the start site of transcription of the MMTV long terminal repeat (LTR) region (Hynes *et al.*, 1983; Buetti and Diggelmann, 1983; Majors and Varmus, 1983) and has been termed the hormone response element (HRE) (Ponta *et al.*, 1985). This HRE contains preferential binding sites for the glucocorticoid receptor (Scheidereit *et al.*, 1983 Payvar *et al.* 1983; Pfahl *et al.*, 1983) and induces the transcription of its own promoter or of heterologous promoters relatively independent of its position or orientation (Chandler *et al.*, 1983; Ponta *et al.*, 1985). This transcriptional activating property of the HRE is reminiscent of the activity of enhancer elements that occur in viral and cellular genomes (Khoury and Gruss, 1983). The enhancer elements studied so far, such as the SV40, the immunoglobulin heavy chain and the insulin gene enhancers all require for their activity the binding of *trans*-acting factors (Schöler and Gruss, 1984; Mercola *et al.*, 1985; Ohlsson and Edlung, 1986). The HRE therefore belongs to a specific class of transcription enhancer elements whose enhancer property is dependent on binding by the *trans*-acting glucocorticoid receptor complex.

Although glucocorticoids regulate the transcription of the MMTV proviral DNA through the HRE in a number of mouse cell lines and in cells transferred with MMTV LTR chimaeric constructs (Ringold *et al.*, 1975; Hynes *et al.*, 1981; Lee *et al.*, 1981; Buetti and Diggelmann, 1981), in the mouse, the transcription of proviral sequences occurs predominantly in mammary gland cells (Varmus *et al.*, 1973). This tissue-specific expression would suggest that steroid hormones other than glucocorticoids may be involved in controlling MMTV proviral transcription. Evidence to support this suggestion comes from recent studies that show that progesterone and androgens can induce the transcription of MMTV LTR chimaeric constructs transfected into either the human mammary tumour cell line T47D or the mouse mammary tumour cell line S115 (Cato *et al.*, 1986; Darbre *et al.*, 1986).

For the progesterone induction of MMTV LTR transcription, Cato *et al.* (1986) have demonstrated that sequences between −428 and −69 upstream of the MMTV LTR promoter are required. These sequences contain binding sites for the progesterone receptor (von der Ahe *et al.*, 1985). In contrast, no information regarding the DNA sequence requirement for the androgen induction or androgen receptor binding in the MMTV LTR is available. Differences in the kinetics of induction of MMTV LTR transcription by androgens and glucocorticoids in the transfected S115 cells and the partial inhibitory effect of cycloheximide on the androgen but not glucocorticoid induction of MMTV LTR transcription might argue against a direct androgen effect (Darbre *et al.*, 1986). Nevertheless Darbre *et al.* (1986) stressed that their results are compatible with a direct effect of both glucocorticoids and androgen an MMTV LTR transcription.

Here we show by the determination of receptor levels and by the use of receptor-specific antagonists that the stimulation of MMTV LTR transcription by progestin and androgens in T47D cells transfected with MMTV LTR chimaeric constructs is dependent on steroid–receptor interactions. The induction of transcription at the MMTV LTR promoter after treatment of the transfected cells with progestin and androgen is a primary response to the two hormones. Oestradiol does not specifically induce transcription at the MMTV LTR promoter. As in the case of the glucocorticoid stimulation of transcription in the MMTV LTR, the HRE mediates the progestin and androgen responsiveness of MMTV LTR transcription. The HRE is therefore a DNA element required for glucocorticoid, progestin or androgen induction of MMTV LTR transcription and must contain binding sites for all three hormone receptors.

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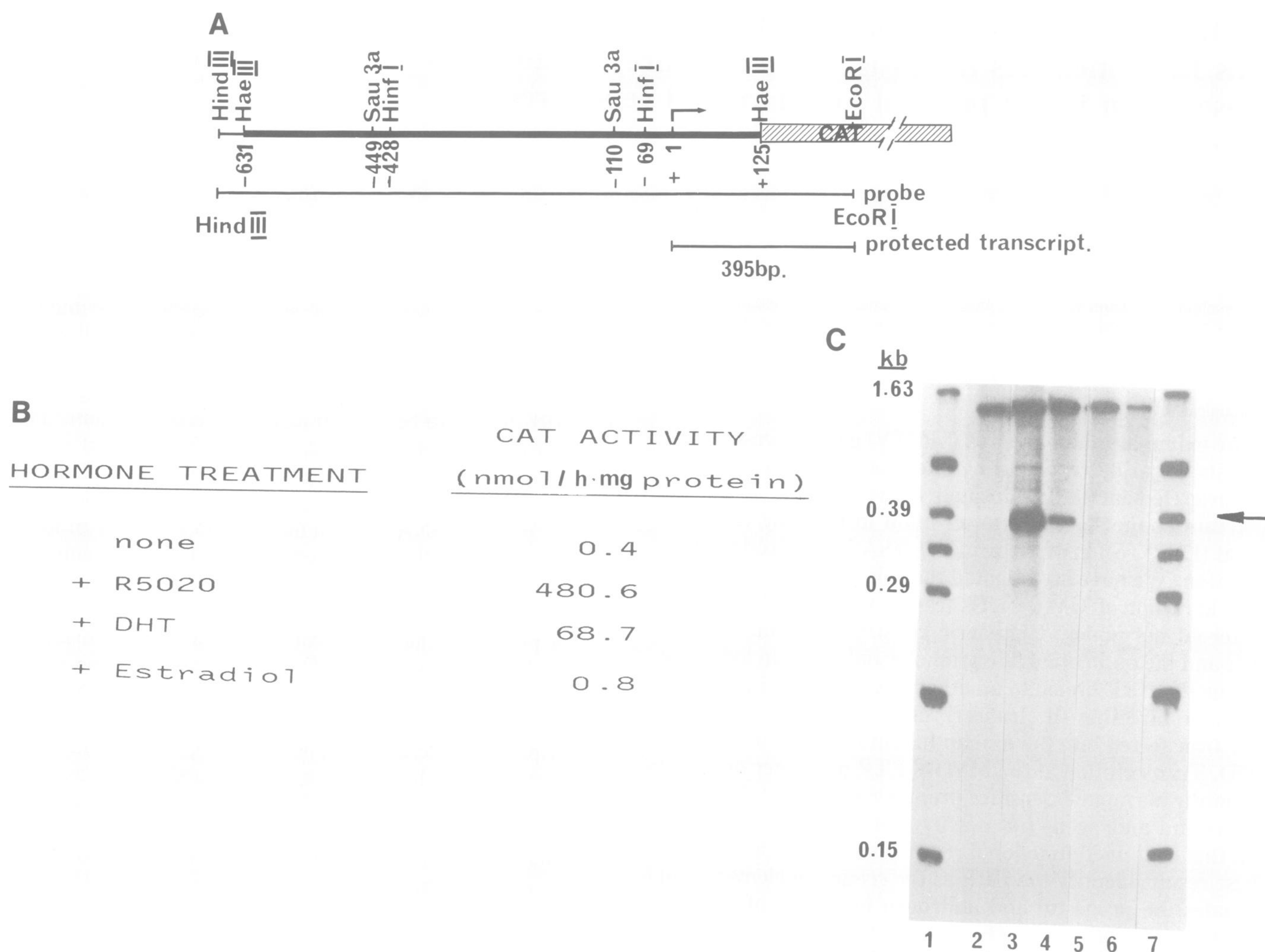


Fig. 1. The induction of expression by R5020, DHT and E_2 of an MMTV.CAT fusion gene stably transfected into T47D cells. (**A**) The pMMTV.CAT construct contains MMTV LTR sequences (−631/+125) linked to the CAT gene (Cato *et al.*, 1986). (**B**) CAT activity in a clone of T47D cells transfected with pMMTV CAT. The transfected cells were treated with and without hormones for 40 h. The concentration of the hormones used were 30 nM R5020 and 0.1 μM DHT and 0.1 μM E_2. (**C**) autoradiograph of S1 nuclease mapping experiment (Weaver and Weissmann, 1979) with RNA extracted from the above transfected T47D cells in the presence and absence of steroid hormones. The cells were treated under identical conditions as in (b). RNA was extracted and S1 nuclease mapping was performed using the 1.0-kb *Hin*dIII−*Eco*RI fragment shown in Figure 2a. The arrow indicates the size of the DNA fragment protected by the correctly initiated MMTV LTR transcript. **Lanes 1** and **7**, labelled *Alu*I pBR322 fragments as size markers. S1 nuclease mapping with 30 μg RNA from cells treated without hormone (**lane 2**); with 30 nM R5020 (**lane 3**); with 0.1 μM DHT (**lane 4**); with 0.1 μM E_2 (**lane 5**); with 30 μg yeast RNA (**lane 6**).

Results

Progestin and androgen induce MMTV LTR transcription via their corresponding receptors

To study the induction of MMTV transcription by steroid hormones other than glucocorticoids, the constructs pMMTV.CAT (Figure 1A) and pHRE.tk.CAT (Figure 3A) were used to transfect the human mammary tumour cell line T47D. The transcription in stably or transiently transfected cells and the influence of progestin, androgen and oestradiol on the transcription of the different constructs was measured.

The construct pMMTV.CAT (Figure 1A) and the plasmid pSV_2neo (Southern and Berg, 1982) were co-transfected into T47D cells. Transfected clones were isolated based on their resistance to the antibiotic G418, and their expression of CAT activity. In one such clone, the levels of receptors measured per milligram protein were 4.4 pmol progesterone, 88.9 fmol androgen and 67.3 fmol oestradiol receptors. No detectable level of glucocorticoid receptor was measured in this clone. The CAT activity in this clone could be induced from 0.4 to 480.6 nmol/h/mg protein by R5020 (17,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione), from 0.4 to 68.7 nmol/h/mg protein by dihydrotestosterone (DHT) but only from 0.4 to 0.8 nmol/h/mg protein by E_2 (Figure 1B). The induction of CAT activity correlated with the increase in RNA transcription initiating at the correct MMTV LTR start site, as judged by S1 nuclease mapping studies (Figure 1B and C).

R5020 induces CAT activity half maximally at 0.1 nM (Figure 2A), a value close to the dissociation constant for the R5020−receptor complex (0.5 nM, Chalbos and Rochefort, 1984). This induction by R5020 could be reversed in a concentration-dependent manner by the antiprogestins RU38486 [11β-(4-dimethylaminophenyl)-17β-hydroxy-17α-(prop-1-ynyl)-estra-4,9-dien-3-one] (Philibert, 1984) or [11β-(4-dimethylamino-phenyl)-17β-hydroxy-17α-(3-hydroxy-1(z)-propenyl)-estra-4,9-diene-3-one] (ZK98734) (Henderson, 1987) (Figure 2B). The CAT activity induced by 0.1 nM R5020 is completely abrogated by an equimolar concentration of RU38486 and >60% inhibited by an equimolar concentration of ZK98734 (Figure 2B). In contrast, the antiandrogen, hydroxyflutamide [2-hydroxy-2-

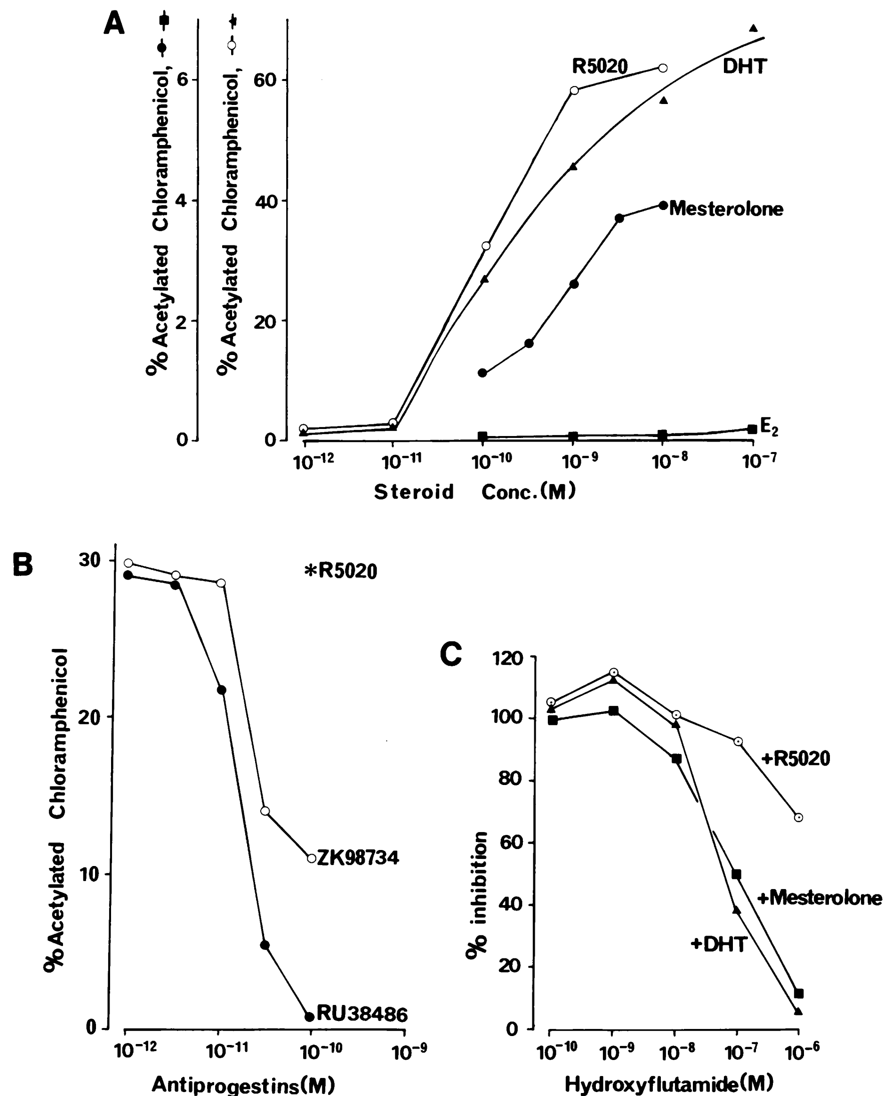
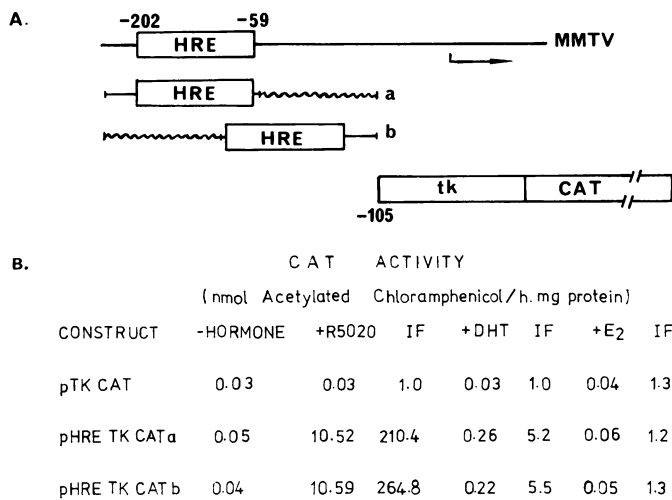


Fig. 2. The dose-dependent induction of CAT activity by hormone agonists in T47D cells transfected with the pMMTV.CAT construct and the effect of antiprogestins and antiandrogen on this induction. (**A**) A clone of T47D cells containing the pMMTV.CAT construct (see Figure 1b) was treated with the indicated concentration of R5020 (○-----○), DHT (▲-----▲), Mesterolone (●-----●) or oestradiol (■-----■). The CAT activity induced by these hormones was determined as described in Materials and methods. The amounts of protein extract used for the CAT assays were 3 μg for R5020 effect, 20 μg for DHT effect, 5 μg for Mesterolone effect and 20 μg for E_2 effect. (**B**) The clone of transfected T47D cells described in (a) was treated with 0.1 nM R5020 and the indicated concentrations of the antiprogestins RU38486 (●-----●) or ZK98734 (○-----○) and CAT activity was determined (Gorman *et al.*, 1982). The amount of protein in the cellular extract used for this assay was 2 μg. The asterisk represents the CAT activity (% acetylation of chloramphenicol) produced by 0.1 nM R5020 in the absence of anti progestins. (**C**) The T47D clone (b) was treated with the indicated concentration of the antiandrogen, hydroxyflutamide and 0.1 nM R5020 (⊙-----⊙), 0.3 nM DHT (▲-----▲) or 1 nM Mesterolone (■-----■) and CAT activity was determined. The amounts of protein used for the CAT assays were 2 μg for R5020 effect, 10 μg for DHT effect and 5 μg for Mesterolone effect. The calculation of the percentage inhibition was made relative to the induction by R5020 (14% acetylation), DHT (17.1% acetylation) or Mesterolone (11.6% acetylation) in the absence of the antiandrogen.

methyl-*N*(4-nitro-3(trifluoromethyl)phenyl) propanamide] (Neri *et al.*, 1979) at a 1000-fold excess concentration (0.1 μM) over R5020 (0.1 nM), does not show any significant inhibition of the R5020 response (Figure 2C), indicating that R5020 is functioning through the progesterone and not the androgen receptor. Like R5020, the androgens DHT and Mesterolone (1-α-methyl DHT) showed a dose-dependent induction of CAT expression (Figure 2A). The half maximal dose of induction by both steroids was 0.3 nM, a value close to the dissociation constant for the DHT–androgen receptor complex (0.45 nM, Asselin *et al.*, 1980). The CAT activity induced by 0.3 nM DHT or 1 nM Mesterolone was inhibited 65% in both cases by 1000-fold excess concentration of the antiandrogen hydroxyflutamide (Figure 2C) indicating that these compounds are acting through the androgen receptor. Although hydroxyflutamide is a relatively specific androgen antagonist, higher concentrations of this non-steroidal compound over the concentration of the agonist are required for complete inhibition of androgen response. In contrast, the steroidal compounds such as RU38486 or ZK98734 that inhibit progestin action are relatively potent and their complete inhibitory activities are manifested in an equimolar concentration range of antagonist to agonist. Oestradiol concentrations ($10^{-12}-10^{-7}$M) have no significant effect on CAT activity in the transfected T47D cells (Figure 2A) indicating a lack of 17β-oestradiol (E_2) effect on MMTV LTR transcription.

Taken together the results with the hormone agonists and an-



B.

CAT ACTIVITY

(nmol Acetylated Chloramphenicol/h.mg protein)

CONSTRUCT	-HORMONE	+R5020	IF	+DHT	IF	+E_2	IF
pTK CAT	0.03	0.03	1.0	0.03	1.0	0.04	1.3
pHRE TK CATa	0.05	10.52	210.4	0.26	5.2	0.06	1.2
pHRE TK CATb	0.04	10.59	264.8	0.22	5.5	0.05	1.3

Fig. 3. The HRE of the MMTV LTR mediates the progestin and androgen inductions of CAT activity in T47D cells transfected with pHRE.*tk*.CAT constructs. (**A**) Schematic representation of the pHRE.*tk*.CATa and pHRE.*tk*.CATb constructs. The open boxes marked HRE represent the hormone response elements of the MMTV LTR. This sequence has been cloned in two possible orientations in front of the *tk* start site of transcription at position −105 in the ptkCAT construct (Cato *et al.*, 1986) The wavy line represents mouse DNA sequence and the horizontal line stands for MMTV LTR sequence. (**B**) The constructs ptkCAT, pHRE.*tk*.CATa and pHRE.*tk*.CATb were transfected transiently into T47D cells. The transfected cells were treated with R5020, DHT or E_2 and CAT activity was determined as described in Materials and methods. The hormone concentrations used were 3 nM R5020, 0.1 μM DHT and 0.1 μM E_2. IF stands for induction factor and it is derived by dividing the CAT activity from cells treated with hormone by the CAT activity from non-treated cells.

tagonists show that progestins and androgens induce the transcription of the MMTV LTR in T47D cells via their corresponding receptors.

The R5020 or DHT induction of MMTV LTR transcription is rapid, occurring within 60 min after hormone treatment and it is maximal by 2 h (data not shown). The effect of the two hormones on MMTV LTR transcription was not abolished in the presence of 20 μg/ml cycloheximide, indicating that *de novo* protein synthesis is not required for the increase in transcription (data not shown). As in the case of the induction of MMTV LTR transcription by glucocorticoids (Groner *et al.*, 1983), we conclude that the stimulation of MMTV LTR transcription by R5020 or DHT represents a genuine primary response of these steroids.

Progestin and androgen induction of MMTV LTR transcription is mediated by the HRE

DNA sequences within the HRE bind the glucocorticoid receptor (Scheidereit *et al.*, 1983; Payver *et al.*, 1983; Pfahl, 1983) and confer glucocorticoid inducibility to hormone-insensitive promoters (Chandler *et al.*, 1983; Ponta *et al.*, 1985). As the HRE has also recently been shown to bind the progesterone receptor (von der Ahe *et al.*, 1985), we determined whether it can mediate the androgen and progestin responses we have described. The 330-bp fragment containing the sequence from −236 to −52 of the 5′ upstream region of the MMTV LTR transcriptional start site, was linked in two possible orientations to the *tk* promoter, directing the transcription of the CAT gene (Figure 3A). One of the resulting constructs pHRE.tk.CATa, contains the HRE in the orientation as it occurs in the MMTV LTR and it is located at −279 bp from the tk start site of transcription. In the other construct pHRE.tk.CATb, where the orientation of the HRE is reversed, the HRE is −135 bp from the tk start site of transcription. These two constructs together with the vector ptkCAT (as control) were transiently transfected into T47D cells. The transfected cells were treated with either R5020, DHT or E_2, and CAT activity was determined.

CAT activity in cells transfected with the ptkCAT vector alone was insensitive to R5020 or DHT treatment. CAT activity in transfected cells containing the HRE linked to the ptkCAT vector in either orientation was however increased 250-fold by R5020 and 5-fold by DHT (Figure 3B). As with the results from the T47D cells transfected with the pMMTV CAT construct (Figure 1B and C), induction by R5020 was substantially greater than by DHT. Whether this reflects a higher affinity of the progesterone receptor for the HRE or it is simply due to the lower concentration of androgen receptor compared to the progesterone receptor in these transfected T47D cells is not yet known.

Oestradiol has no significant effect on CAT activity in T47D cells transfected with either the ptkCAT vector or the pHRE.tk.CAT constructs (Figure 3B).

These results identify the HRE as the *cis*-acting element that is required for the progestin and androgen induction of MMTV LTR transcription. The position and orientation independent stimulation of transcription at the tk promoter only in the presence of R5020 or DHT, agree with the data that led to the description of the HRE as a hormone inducible enhancer (Chandler *et al.*, 1983; Ponta *et al.*, 1985).

Discussion

We have demonstrated that the HRE present in the MMTV LTR confers progestin and androgen responses on its own promoter and on the *tk* promoter independent of the orientation or distance it is from this promoter. These results are consistent with the concept that the HRE acts as a hormonally regulated enhancer (Chandler *et al.*, 1983; Ponta *et al.*, 1985). By demonstrating the requirement of progesterone−receptor or androgen−receptor interaction for the transcriptional activation, we have identified these receptors as *trans*-acting factors that, in addition to the glucocorticoid receptor, interact with the HRE to mediate its activity.

The sequence comparison of the cloned glucocorticoid and progesterone receptor cDNAs shows a conserved region (86% amino acid sequence identity) in the postulated DNA binding domains of these receptors (Jeltsch *et al.*, 1986; Conneely *et al.*, 1986). This might explain why both receptors could recognise similar sequences in the HRE. It is conceivable that like the glucocorticoid and progesterone receptors, the androgen receptor has also such a conserved region that would make it recognize sequences in the HRE. This interaction of different steroid hormone receptors with the same DNA element is compatible with the view that the steroid receptors share some identity and arose from a family of DNA binding proteins that may be derived from a common primordial gene (Jeltsch *et al.*, 1986; Conneely *et al.*, 1986).

The DNA sequences in the HRE of the MMTV LTR contain four repeats of the hexanucleotide 5′-TGTTCT-3′ which are bound by both the glucocorticoid (Scheidereit *et al.*, 1983; Payver *et al.*, 1983) and progesterone (von der Ahe *et al.*, 1985) receptors. Results from *in vitro* binding studies using the androgen receptor and the C3(1) subunit gene of rat prostatein, show that the region bound by the androgen receptor on this gene contains sequences homologous to the above hexanucleotide, e.g. 5′-TGTTC-3′ or 5′ TGATTG-3′ (Perry *et al.*, 1984). Thus the receptors for glucocorticoid, progesterone and androgens have recognition sequences that encompass the hexanucleotide motif or closely related variants of this sequence.

DNA sequences bound by the oestrogen receptor are however different. Recently, oestrogen receptor preparation from human MCF-7 cells have been shown to bind preferentially to an 18-bp sequence in a DNA fragment from the *Xenopus laevis* vitellogenin A2 gene (A.C.B.Cato, G.U.Ryffel and L.Klein-Hitpass, in preparation) which mediates oestradiol response (Klein-Hitpass *et al.*, 1986). The sequence bound by the oestrogen receptor is found in other oestrogen-regulated genes but not in the HRE or the entire LTR of the MMTV DNA (A.C.B.Cato, unpublished results). Furthermore MMTV LTR transcription is not induced by oestradiol in either transfected T47D cells (Figure 1B, C and 2A) or in the oestrogen-positive MCF-7 cells (data not shown). The amino acid sequence of the region of the oestrogen receptor that binds DNA is less conserved (61.5% amino acid sequence identity) when compared to similar regions of the gluccorticoid receptor (Krust *et al.*, 1986; Kumar *et al.*, 1986). It is therefore not surprising that oestradiol does not selectively induce MMTV LTR transcription. Oestradiol may nevertheless have an indirect influence on MMTV LTR transcription. In a number of different tissues and species such as the chick oviduct, the rat uterus or the human mammary tumour cell line MCF-7, oestradiol treatment leads to an increased progesterone receptor level (Scherman *et al.*, 1970; Faber *et al.*, 1972; Horwitz and McGuire, 1978). This so-called priming action of oestradiol can lead indirectly to stimulation of MMTV LTR transcription in cells that contain the MMTV proviral DNA, oestrogen and progesterone receptors and a source of both hormones.

Apart from the priming effect of oestradiol, no sufficient information is at present available to decide exactly how specificity of hormone control is achieved *in vivo* in cells containing the MMTV LTR and more than one class of steroid hormone receptors. One possibility is that the different steroid receptors may have different affinities for the HRE. This suggestion stems from experiments with the chicken lysozyme gene, a gene whose transcription is induced by glucocorticoids and progesterone. On this gene although both hormone receptors bind to the same region, they do so with different affinities (von der Ahe *et al.*, 1985; Renkawitz *et al.*, 1984). The introduction of specific point-mutations into the sequence of the HRE and/or the DNA binding domains of the receptor genes followed by *in vitro* DNA–receptor binding and functional analyses, would provide some information on how specificity of hormone action is achieved in the MMTV LTR.

Whether there is any biological meaning to the induction of MMTV LTR transcription by androgens is not known. The demonstration that progestins induce MMTV LTR transcription, however, provides a mechanistic explanation for the role progestins and MMTV play in the progression of mouse mammary tumours. This is especially obvious in the case of the GR mouse strain where treatment of ovariectomized animals with progestins led to the development of mammary tumours which were not observed in control non-treated animals (Van Nie and Hilgers, 1976). However, the progestin induction of MMTV LTR transcription on its own cannot explain the tissue-specific expression of MMTV in mammary gland cells. Other cells, for example, cells in the uterus with high progesterone receptor levels do not express MMTV. It is therefore likely that tissue specific factors may play a decisive role in the tissue-specific expression of this virus.

Materials and methods

Hormones and antihormones

Non-radioactive DHT was purchased from Sigma and R5020 from New England Nuclear. 5α-Dihydro[1,2,4,5,6,7,-^{3}H] testosterone 148 Ci/mmol and [17α-methyl-^{3}H]R5020 85.0 Ci/mmol were purchased from Amersham and New England Nuclear respectively. All other hormones and antihormones were supplied by Schering AG, Berlin.

Constructs of plasmids

Plasmids ptkCAT and pMMTV CAT. The construction of the plasmids ptkCAT and pMMTV CAT has been previously described (Cato *et al.*, 1986).

Plasmid pHRE.tk.CAT. The 754-bp env-HRE *Eco*RI fragment (Ponta *et al.*, 1985) was cleaved with the restriction enzyme *Bam*HI to generate a 330-bp *Eco*RI–*Bam*HI fragment containing the sequence from −236 to −52 of the MMTV LTR. The ends of this fragment were blunted by filling in with Klenow polymerase I and tailed with *Bam*HI linkers. After digestion with *Bam*HI, this fragment was inserted in two possible orientations into the single *Bam*HI site in the plasmid ptkCAT (Cato *et al.*, 1986) 'a' denotes the orientation as it occurs in the MMTV LTR whereas 'b' stands for the reverse orientation.

Cell culture and transfection of cells

T47D cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 0.6 μg/ml insulin. Transient transfection of T47D cells was carried out as previously described (Cato *et al.*, 1986). Stable transfections were carried out using the calcium phosphate precipitation method (Wigler *et al.*, 1979). The pMMTV CAT construct was co-transfected with the plasmid pSV$_2$neo (Southern and Berg, 1982). The transfected T47D clones were selected in culture medium supplemented with 300 μg/ml G418. Studies of the effect of hormones on the isolated clones were carried out on cells cultured for 1 week in media supplemented with charcoal stripped FCS (FCS/DCC) (Westley and Rochefort, 1980) according to the procedure of Chalbos and Rochefort, 1984.

CAT assay

Transiently transfected cells or cells stably transfected with CAT constructs were disrupted by freezing three times in a dry-ice/ethanol bath and thawing at 37°C. CAT assay was performed as described by Gorman *et al.* (1982). Protein concentration was determined according to the procedure of Lowry *et al.* (1951).

Hormone receptor measurements

The steroid hormone receptor levels in the cytosol of transfected T47D cells were determined in duplicates by the dextran-coated charcoal procedure (Beato and Feigelson, 1972) using saturating concentrations of ligand (10 nM [^{3}H]DHT or 30 nM [^{3}H]R5020) and competing with a 500-fold excess of non-radioactive hormone.

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