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Identification of Androgen Response Elements in Mouse Mammary Tumour Virus and the Rat Prostate C3 Gene

Malcolm G. Parker, Paul Webb, Maurice Needham, Roger White, and Jonathan Ham

Molecular Endocrinology Laboratory, Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, England

The effect of steroid hormones on mouse mammary tumour virus (MMTV) promoter activity has been analysed in the breast cancer cell lines ZR-75-1 and T-47D 11. Both androgens and progestins were stimulatory in both cell lines, whereas glucocorticoids were stimulatory in only ZR-75 cells. A glucocorticoid response was restored to T-47D cells by the transient expression of a glucocorticoid receptor expression vector, suggesting that endogenous receptors were limiting. The effect of androgen was mediated by a region of MMTV previously shown to be essential for glucocorticoid and progestin responses between nucleotides −201 and −69 upstream of the cap site.

Similarly, the effect of androgens on the rat prostate C3(1) gene promoter was analysed in the rat prostate cell line T5. Although these cells had functional androgen receptors which could stimulate MMTV-promoter activity, they were incapable of interacting with androgen response elements to stimulate transcription from the C3(1) promoter.

Key words: androgen response elements, hormone response elements, testosterone

Steroid hormones regulate the expression of specific genes as a steroid-receptor complex by binding to DNA sequences, termed hormone response elements, that are associated with certain genes. The hormone response elements of one such gene, encoding mouse mammary tumour viral (MMTV) RNA, has been identified in the long terminal repeat of the virus upstream of the promoter. The interaction of this element with glucocorticoid-receptor complexes has been well characterized by binding studies in vitro [1,2] and shown to be functional in vivo by gene transfer studies [3–6]. Further characterization of the binding sites showed that they were also able to bind progesterone receptors [7], and subsequently they too were shown to be functional in vivo in a human breast cancer cell line, T-47D [8]. It has been shown that testosterone is capable of stimulating the expression of MMTV [9] by acting on

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sequences within the long terminal repeat [10]. Clearly the hormone response elements of MMTV are activated by a variety of steroid hormones in different cell lines. In this paper, we have investigated the function of the hormone response element in two human breast cancer cell lines, ZR-75 [11] and T-47D 11 [12] cancer cell lines, that contain receptors for four classes of steroid hormone—namely, androgen, glucocorticoid, estrogen, and progestin. Briefly, we find that androgens as well as glucocorticoids and progestins act on the hormone response element to stimulate MMTV promoter activity, whereas estrogens are without effect. Since androgens also stimulate the expression of specific genes such as the prostatic steroid binding protein gene [13,14], we have attempted to identify androgen response elements within the C3(1) gene but without success.

MATERIALS AND METHODS

Construction of Recombinant Plasmid Vectors

A prototype CAT vector containing simian virus 40 (SV40) splice and polyadenylation signals [15], but lacking promoter sequences was constructed in the polylinker of pSP65 to give pCAT (Fig. 4). Upstream DNA sequences from MMTV, herpes simplex viral thymidine kinase (TK) gene, or the rat ventral prostate C3(1) gene [13,14], were then inserted in the polylinker to provide promoter sequences. The MMTV long terminal repeat was cloned as a 1.5-kilobase (Kb) Pst-1 DNA fragment extending from −1,225 to +268 relative to the cap site (−1,225 CAT), and 5′ deletion mutants were derived as follows. The −107 CAT mutant was produced by Sst-1 digestion and self-ligation of −1,225 CAT. The −201 CAT mutant was produced by subcloning the 463 bp Dral-Pst-1 fragment into pCAT which had been digested with Sma and Pst-1. The −504 CAT was constructed in two steps. Firstly, a 521-base pair (bp) Pst-1 fragment produced by Mbo2 digestion of MMTV, repair, and addition of Pst-1 linkers was subcloned into pSP65. This plasmid was then digested with Sst-1 to generate a 430-bp fragment comprising MMTV sequences between −107 and −504 which was cloned into the Sst-1 site of p107. The thymidine kinase promoter was inserted as a 250-bp Pst-1 DNA fragment to produce pTK CAT. MMTV sequences from −428 to −69 as a Hinf1 fragment were then ligated using Xba linkers in both orientations in front of the TK promoter. The C3(1) upstream sequences from −750 to −110 and −750 to +20 were cloned as EcoRI-BAM H1 DNA fragments and from −7,000 to +3,000 as an EcoRI DNA fragment [13].

Transfection Procedures

ZR-75 and T-47D cells were maintained in Dulbecco's modified Eagle's (DMEM) and RPMI medium, respectively, each supplemented with 10% fetal calf serum (FCS). Prior to transfection cells were withdrawn from endogenous steroid by growth overnight in 2% serum which had been treated with dextran-coated charcoal. Cells (8×10^5) were transfected for 6 hr with 10 μg DNA using the $CaPO_4$ coprecipitation method [16], washed in DMEM and then with 25% glycerol in DMEM for 30 sec and incubated overnight in 2% charcoal-treated serum. The following day steroids were added to the 2% serum as follows: dexamethasone, 10^{-8} M; progesterone, 10^{-6} M; testosterone, 3×10^{-8} M; or estradiol, 3×10^{-8} M, and cells were harvested after 48 hr for CAT assays [15].

Steroid Receptor Determination

Receptor determinations in cytosol of cell lines were determined in triplicate by the dextran-coated charcoal method [8].

RESULTS

We investigated the effect of androgen, glucocorticoid, progestin, and estrogen on MMTV-promoter activity in two human breast cancer cell lines, Zr-75-1 [11] and T-47D 11 [12], both of which contain receptors for each class of steroid hormone (Table I). The concentration of receptors for glucocorticoid and estrogen are similar to that in many target tissues, whereas the concentration of receptors for androgen and progestin are elevated severalfold [11,12].

MMTV-Promoter Activity in ZR-75-1 Cells

MMTV-promoter activity was initially examined in ZR-75-1 cells and shown to be stimulated by progesterone, dexamethasone, and testosterone but not by estradiol (Table II). Dose-response curves (Fig. 1) indicate that each steroid is acting at physiological concentrations with half-maximal induction observed with 8×10^{-9} M for testosterone, 8×10^{-8} M for dexamethasone, and 5×10^{-8} M for progesterone.

Since it has been established that the receptors for glucocorticoid and progesterone bind to the long terminal repeat (LTR) between nucleotides -190 and -70 [8], we investigated whether this region was sufficient to act as an androgen response element as well as a glucocorticoid and progesterone response element. Analysis of 5′ deletions of the long terminal repeat (Fig. 2) indicated that stimulation of promoter activity was abolished for all three steroids by deleting DNA sequences between nucleotides -201 and -107. The hormone response element was further delineated by testing a Hinf1 DNA fragment from nucleotide -428 to -69 that had been ligated upstream of the TK promoter fused to CAT. It was shown that testosterone as well as glucocorticoid and progesterone stimulated promoter activity irrespective of its orientation relative to the TK promoter (Table II). Thus MMTV DNA sequences

TABLE I. Steroid Hormone Receptor Levels

	fmoles/mg protein			
	AR	GR	PR	ER
ZR-75	590	40	260	55
T-47D 11	230	15	510	90

Receptors for androgen (AR), glucocorticoid (GR), progestin (PR), and estrogen (ER) were assayed in cytosols.

TABLE II. MMTV Promoter Activity

	Induction ratio[a]	
Steroid	ZR-75	T-47D
Testosterone	85	52
Dexamethasone	48	2
Progesterone	60	145
Estrogen	1	1

[a]The induction ratio represents the ratio of CAT activity obtained with the full-length LTR as promoter in the presence and absence of steroid.

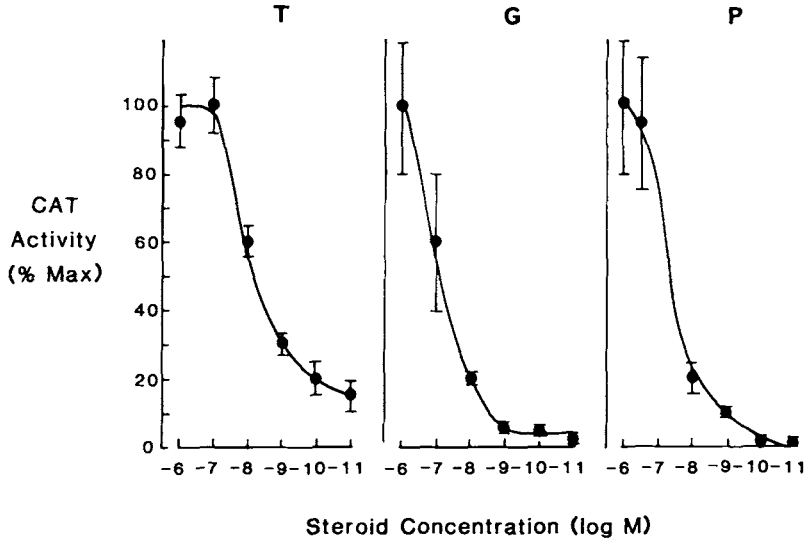


Fig. 1. Dose-response curves of MMTV-promoter activity in ZR-75 cells. LTR-CAT was transiently expressed in ZR-75 cells, and the dependence of CAT activity on steroid concentration was determined. The steroids were testosterone (T), dexamethasone (G), and progesterone (P).

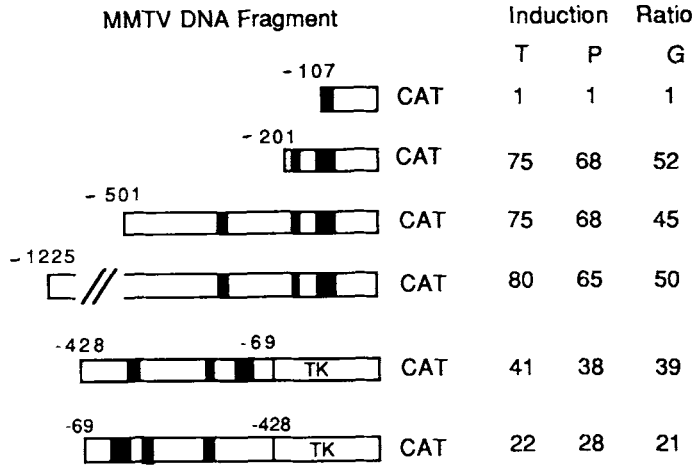


Fig. 2. Deletion analysis to identify hormone response element in MMTV. MMTV DNA fragments containing the homologous viral promoter or fused to the heterologous TK promoter were ligated to CAT and expressed transiently in ZR-75 cells. The induction ratio represents the CAT activity obtained in the presence of testerosterone (T), progesterone (P), or dexamethasone (G), compared with no steroid. The MMTV fragments extend from nucleotides −107, −201, −501, and −1,225 to +268 or from −425 to −69 relative to the cap site. Solid black boxes represent regions of DNA which bind glucocorticoid receptors in vitro.

between −201 and −107 are able to act as an androgen-dependent enhancer as well as a glucocorticoid- and progesterone-dependent enhancer [4–6,8].

MMTV Promoter Activity in T-47D Cells

In T-47D cells, testosterone and progesterone stimulated MMTV promoter activity by more than 100-fold, but, in contrast to ZR-75 cells, stimulation by dexamethasone was markedly reduced, ranging from one- to fivefold in ten experiments. This reduction may be accounted for by the difference in glucocorticoid receptor contents in the two cell lines' being threefold higher in ZR-75-1 cells (Table I), but an alternative possibility is that inhibitory factors may exist in T-47D cells. We have distinguished these possibilities by cotransfecting LTR-CAT with the gene for mouse glucocorticoid receptor in an expression vector using SV40 promoter, splicing, and termination sequences [17]. It was found that transient expression of the glucocorticoid receptor was sufficient fully to restore dexamethasone stimulation to the MMTV-hormone-response elements (Fig. 3). We conclude that low levels of glucocorticoid receptor rather than inhibitory factors are more likely to be responsible for the failure of glucocorticoids to stimulate MMTV-promoter activity.

C3 Promoter Activity in Prostate Cell Lines

Androgen receptors bind selectively to the C3 gene adjacent to the promoter between nucleotides −225 and +80 and within the first intervening sequences [18,19]. We have fused different C3 DNA fragments to the CAT gene utilising either the homologous C3 promoter or the heterologous TK promoter (Fig. 4). Promoter activity was then examined in the prostate cell lines T5 [20] with or without testosterone. Testosterone stimulates MMTV promoter activity approximately 10-fold showing that it contains functional androgen receptors (Fig. 4). Preliminary experiments

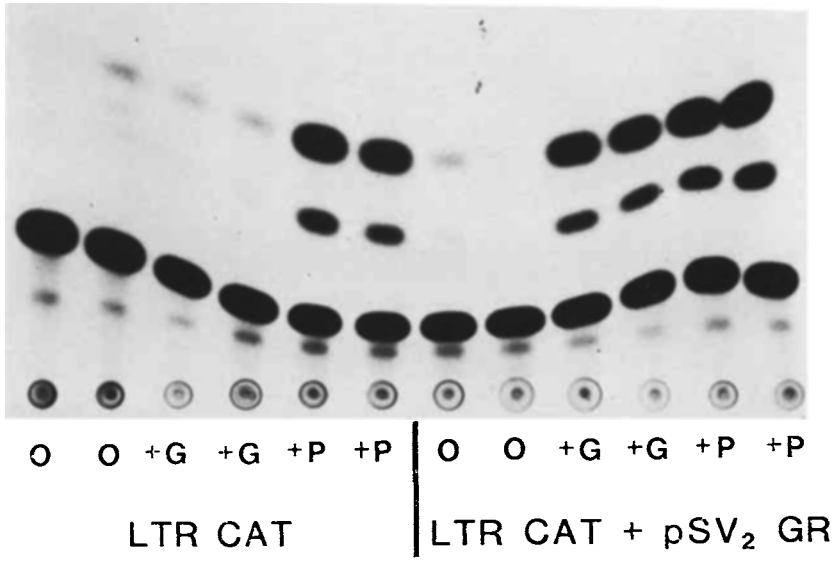
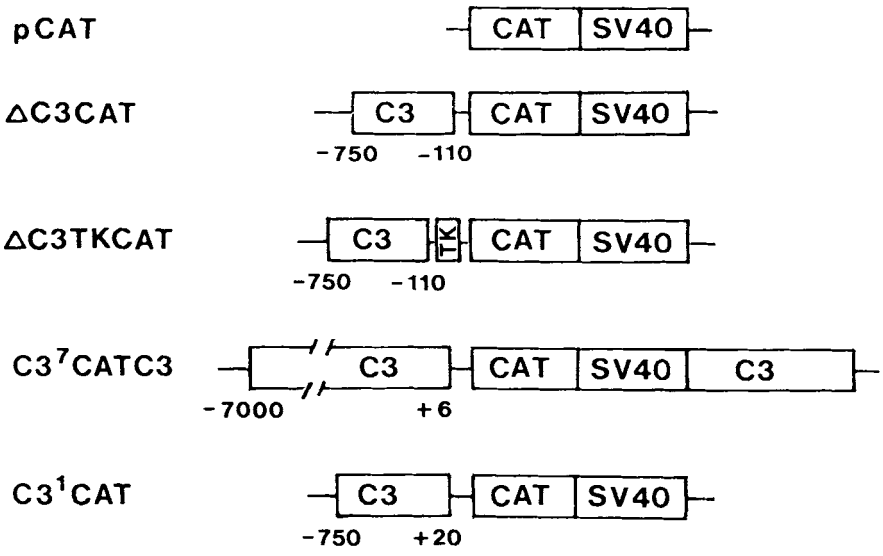


Fig. 3. Effect of steroids on MMTV-promoter activity in T47-D cells. LTR-CAT, with or without the glucocorticoid expression vector pSV2GR, was transiently expressed in T47-D cells and CAT activity determined by autoradiography. The steroids were dexamethasone (G) and progesterone (P).



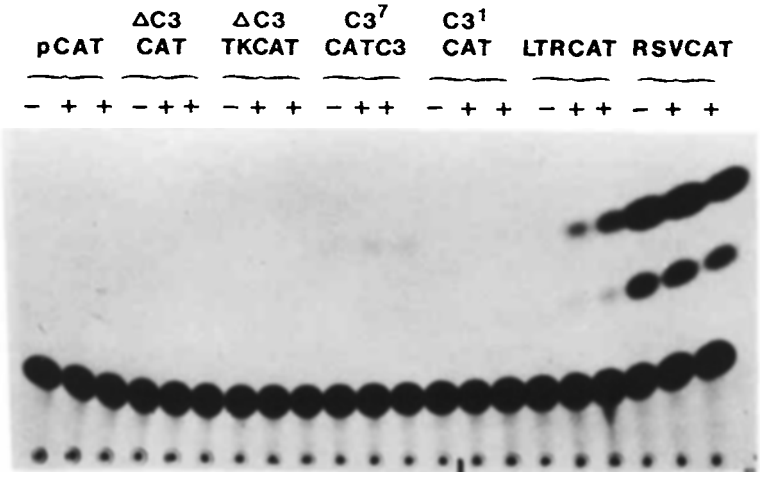


Fig. 4. Effect of testosterone on C3 promoter activity. A variety of C3 CAT vectors were constructed which contained either the intact C3 promoter, ($C3^1$ CAT), the upstream sequences from −750 to −110 lacking a promoter (C3 CAT), or fused to the TK promoter (C3 TK CAT). The $C3^7$ CAT C3 vector contains 7 Kb 5′ flanking DNA and 2.8 Kb 3′ flanking DNA. These vectors as well as LTR CAT and RSV CAT were transiently expressed in prostate cells T5 [20] with (+) or without (−) testosterone and CAT activity detected by autoradiography.

with the chaemeric genes shown in Figure 4 established that the C3 and TK promoter were functional by using prostate cells C10 [20] that transfect efficiently but which show no androgen regulation. In T5 cells, however, C3 promoter activity was poor; $C3^7$ CAT C3, containing 7 Kb DNA upstream and 2.8 Kb DNA downstream of the C3 gene expressed detectable CAT activity, but the stimulatory effect of testosterone was twofold or less. We conclude that C3(1) sequences which selectively bind androgen receptors in cell-free systems do not function as androgen response elements in these prostate cell lines.

DISCUSSION

The stimulation of MMTV-promoter activity by glucocorticoid and progestins is achieved by the interaction of the steroid receptor complex with specific DNA sequences termed hormone-response elements which are adjacent to the promoter [1–8]. Our results demonstrate that the androgen regulation of MMTV expression [9,10] is also mediated by this response element and that glucocorticoid, progestin, and androgen are equally effective as transcriptional activators in cells with sufficient concentration of steroid receptor.

It has been found that the DNA binding regions of the glucocorticoid and progesterone receptors are highly conserved, with 86% amino acid sequence homology [21,22], and bind to similar overlapping DNA sequences within the hormone response element [7]. In contrast, the estrogen receptor shares only 60% amino acids with the glucocorticoid and progesterone receptors in the DNA binding region [23], which may account for its failure to interact with the MMTV-hormone-response element and stimulate the rate of transcription from the viral promoter. Since androgens have now been shown to have a receptor-mediated interaction with the hormone-response element of MMTV, the DNA binding region of the androgen receptor may be related more to those of the glucocorticoid and progesterone receptors than to that of the estrogen receptor.

Mouse mammary tumour virus appears to induce mammary carcinomas by activating specific cellular genes such as int-1 [24] and int-2 [25]. In certain strains of mice, tumour growth is initially pregnancy-dependent, although eventually it becomes autonomous. The hormone dependence of tumour growth might be explained by the effect of progesterone, which is elevated during pregnancy, acting on the hormone response element to stimulate either viral expression, the oncogenes int-1 and int-2, or both.

Finally, our failure to demonstrate androgen response elements within the gene for C3 despite selective binding in vitro could be explained in a number of distinct ways. First, although the administration of testosterone increases the steady-state levels of C3 nuclear RNA [26], run-off assays in nuclei indicate that transcription rates are stimulated by approximately fivefold, so that the predominant effect of the hormone may be via post-transcriptional events [27]. Second, since the endogenous C3 gene is not expressed in the T5 and C10 prostate cell lines (unpublished data), it is conceivable that specific transcription factors are absent which may be necessary to demonstrate a hormone response. Attempts to overcome these problems by culturing prostate cells on matrices or feeder layers of cells have been unsuccessful. Finally it is possible that androgen response elements exist within the C3 gene, but either their activity is masked by flanking repressor DNA sequences or that androgen

receptor is limiting in the prostate cells. Since MMTV induction is 5–10-fold in contrast to 85-fold in ZR-75 cells, androgen receptors appear to be suboptimal for MMTV induction.

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