

# Cooperation between structural elements in hormone-regulated transcription from the mouse mammary tumor virus promoter

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## ABSTRACT

The mouse mammary tumor virus (MMTV) promoter is under the control of several types of regulatory agents. The proximal promoter within the long terminal repeat (LTR), from -200 to the CAP site and its regulation by steroid hormones have been extensively studied. However the precise role of sequences located upstream of this region remain unclear. We have constructed MMTV LTR deletion mutants coupled to the luciferase reporter gene and assayed their activities after transient transfection into transformed mammary epithelial cells (34i) and immortalized fibroblasts (NIH-3T3). In the absence of hormone, the MMTV promoter is almost silent, and deletions in the LTR have no significant effect on basal activity. In the presence of hormone, deletions spanning from the 5'-end to -455 have only slight effects on luciferase levels. In contrast, deletion of the region spanning from -450 to -201 leads to a dramatic decrease in transcription. A substantial decrease, more marked in 34i cells, is also clear when 90bp between -290 and -201 are deleted. At least one element cooperating positively with the glucocorticoid response element (GRE) is present between -223 and -201, as supported by the results of substitution mutation experiments. In 34i cell line, dexamethasone stimulates the MMTV LTR transcriptional activity to a level comparable to that of SV40. In contrast, in NIH-3T3 cells, MMTV promoter inducibility is weak. This results from a glucocorticoid receptor content 10-fold lower in NIH-3T3 cells than in 34i cells. Transfection of a glucocorticoid receptor expression plasmid allows recovery of a high inducibility of the MMTV promoter. This was true with all the MMTV LTR mutants studied here and suggests that NIH-3T3 cells possess all the factors necessary to cooperate with the steroid hormone in order to achieve a high transcriptional activity.

## INTRODUCTION

Mouse mammary tumor virus (MMTV) is a slow-transforming retrovirus that causes mammary carcinomas in female susceptible mice (for reviews see ref. 1,2). MMTV is expressed mainly in the mammary gland, but a few copies are transcribed in other tissues, especially salivary gland and testis epithelial cells, and lymphoid cells (3). In the mammary gland, high viral expression is found only during pregnancy and lactation. In lactating mammary glands, integration of newly acquired proviruses causes transformation by insertional activation of the *int* proto-oncogenes. Activation of at least, four unrelated genes (*int-1*, *int-2*, *int-3* and *int-4*) leads to phenotypical changes and transformation of mammary epithelial cells into adenocarcinoma cells (4-7). In mammary tumor cell lines, transcription of the viral genome is dependent on steroids such as glucocorticoids and progestins (8-11), hormones implicated in the proliferation and differentiation of the mammary gland. Growth factors and prolactin also contribute to a high viral expression but only in the presence of steroid hormones (12,13). The role of the MMTV long terminal repeat (LTR) in the hormonal control of viral transcription has been demonstrated (14,15). The target elements involved in this process, termed glucocorticoid responsive elements (GREs) have been localized in the U3 part of the LTR between positions -202 and -79 upstream of the RNA start site (16,17), and a consensus sequence for the glucocorticoid-receptor complex target has been deduced from *in vitro* footprinting experiments (18,19). In addition to hormonal regulation, modulation of MMTV expression also involves the binding of ubiquitous and probably tissue-specific factors to *cis*-acting elements. The participation, in a hormone-dependent fashion, of NF-   and TFIID in the transcription complex has been demonstrated (20,21). Three negative regulatory elements have been described: two, located upstream of the GREs at positions -631/-560 (22) and -455/-364 (22,23) and one located between the proximal and the distal GREs at position -162/-156 (24). The involvement of the 5'-end of the LTR in the tissue-specific expression of MMTV has been suggested by transgenic

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mice experiments (25). In addition, a regulatory element at position  $-1094/-739$ , able to enhance a foreign promoter in mouse mammary cells when placed in a reverse orientation, has been identified (22). In rare cases, MMTV can induce renal adenocarcinomas (26) and lymphomas (27–30) in old mice. In these lymphoid cells, newly acquired MMTV proviruses are highly expressed, compared to the endogenous copies, and always display internal deletions within the LTR-U3 region (between  $-670/-160$ ). It has been suggested that they have lost elements involved in the mammary gland tissue-specific expression (27).

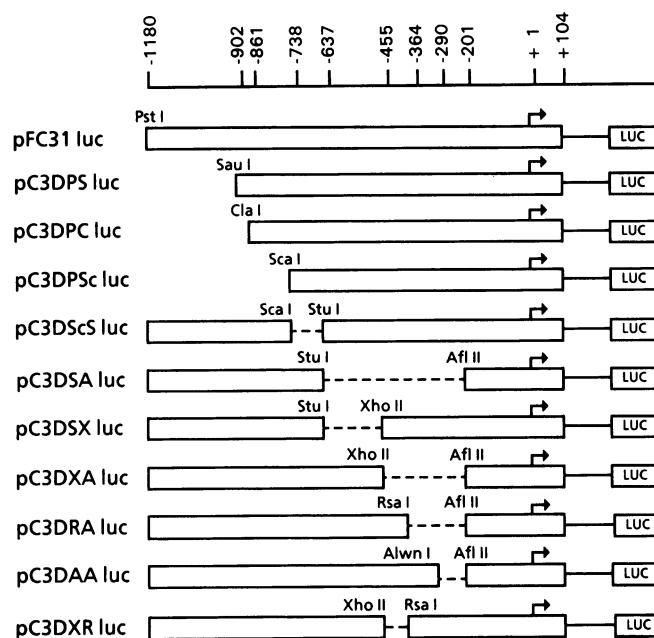
To study the role of *cis*-acting elements on both basal and hormone-induced transcriptional activity of the MMTV promoter, we have constructed plasmids in which the luciferase reporter gene (31) is under the control of either the entire MMTV LTR from the C3H strain (32) or mutants with 5'-end or internal deletions. Two target cell lines were chosen for transient transfection experiments: NIH-3T3 fibroblasts and 34i, an epithelial mammary carcinoma cell line (33).

Here we present data indicating that, in these two cell lines, the MMTV promoter is almost silent unless activated by hormone, and that no particular region upstream of the GRE is able to dramatically change its basal level of activity. In contrast, we found a region, containing at least two positive regulatory elements, able to cooperate with the GREs in the presence of hormone. These elements are probably binding sites for ubiquitous factors, since they are functionally active in both epithelial and fibroblastic cells.

## MATERIAL AND METHODS

### Plasmids description

All MMTV LTR deletion mutants were constructed in the cloning vector pSP72 (Promega), using standard procedures (34). Plasmid pSVD5'Luc contains the PvuII/HindIII fragment of pSV2CAT (35) (SV40 promoter) followed by the HindIII/BamHI fragment of pSV0AL-delta5'Luc (31) (i.e. the luciferase coding sequences, the SV40 splice and polyadenylation sites). Plasmid plucDSS contains the HindIII/BamHI fragment of pSVOALdelta5'Luc (promoterless luciferase gene). Plasmid pAplucDSS is plasmid plucDSS with a polyadenylation site inserted upstream of the gene to prevent the use of a cryptic promoter present in the vector (36). The HpaI/BglII fragment from plucDSS (SV40 polyadenylation site) was inserted into the NdeI/XhoI sites of plucDSS, after a partial filling of BglII and XhoI sites that made them cohesive. Plasmid pC3H contains the PstI/HpaII ( $-1184/+105$ ) fragment of the C3H MMTV LTR inserted at the PstI/ClaI sites of pSP71 (Promega). Plasmid pFC31luc contains the MMTV LTR driving luciferase. The PstI/HindIII fragment of pC3H (MMTV LTR) was ligated to the HindIII/BglII fragment of plucDSS, and inserted at the PstI/BglII sites of pSP72. Plasmid pC3mCACluc was obtained by substituting a polylinker fragment (GGGTACCGAGCTCGAATTCATCG-ATGAT) for the sequence spanning from  $-223$  to  $-201$  (CCC-AACCTTGCGGTTCCCAAGGC). The other plasmids, were pFC31luc with the following deletions: pC3DPSluc, 5'-end to  $-902$  (PstI to SauI); pC3DPCluc, 5'-end to  $-861$  (PstI to ClaI); pC3DPScluc, 5'-end to  $-738$  (PstI to ScaI); pC3DScSluc,  $-737$  to  $-637$  (ScaI to StuI); pC3DSXluc,  $-636$  to  $-455$  (StuI to XhoII); pC3DSAluc,  $-636$  to  $-201$  (StuI to AflII); pC3DXAluc,  $-450$  to  $-201$  (XhoII to AflII); pC3DRAluc,  $-365$  to  $-201$  (RsaI to AflII); pC3DAAluc,  $-294$  to  $-201$  (AlwNI to AflII); pC3DXRluc,  $-450$  to  $-364$  (XhoII to RsaI). MMTV LTR



**Figure 1.** Schematic representation of the MMTV LTR deletion mutants driving the luciferase reporter gene. For a detailed description of the constructs see material and methods.

deletion mutant plasmids are depicted in Fig.1. For those deletions which could not be verified by restriction analysis, the double-stranded plasmid DNA was sequenced. Plasmid pRSVhGR $\alpha$ , used in cotransfection experiments, contains the cDNA encoding the human glucocorticoid receptor gene under the control of the Rous sarcoma virus promoter (37). Plasmid pCH110 contains the  $\beta$ -galactosidase gene under the control of the SV40 promoter (Pharmacia).

### Cell lines and transfection

Two cell lines were used in this work: NIH-3T3 fibroblasts and 34i, derived from a C3H mouse mammary epithelial carcinoma (33). Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. For transfection experiments, cells were plated to a density of  $5 \times 10^5$  cells/10cm dish (NIH-3T3) or  $10^6$  cells/10cm dish (34i). Medium was changed 3 hours before transfection. Each dish was transfected with:  $5 \mu\text{g}$  of the plasmid to be tested,  $5 \mu\text{g}$  of control plasmid (pCH110), when indicated  $5 \mu\text{g}$  of pRSVhGR $\alpha$ , and 5 or  $10 \mu\text{g}$  of pSP72, to adjust total DNA to  $20 \mu\text{g}$  final. DNA samples were mixed and coprecipitated with calcium phosphate according to standard procedure (38). Sixteen hours after transfection, plates were rinsed twice with DMEM without serum, and the medium was changed. For hormonal induction experiments, this medium contained dexamethasone at a final concentration of  $5 \times 10^{-7}$  M. Cells were collected 24 hours later. Each plasmid construction was assayed in at least 3 independent transfections.

### Luciferase and $\beta$ -galactosidase assays

Cell extracts for luciferase and  $\beta$ -galactosidase assays were prepared according to Van Trung Nguyen et al. (39) with the following modification: cells were lysed by addition of  $600 \mu\text{l}$  of 25mM glycyl-glycine buffer pH7.8 containing 8mM MgSO $_4$ , 1mM EDTA, 1mM DTT, 1% Triton X100 and 15% glycerol. For luciferase assays,  $50 \mu\text{l}$  of cell extracts were mixed with  $350 \mu\text{l}$

of a solution containing glycyl-glycine 25mM pH7.8, 15mM MgSO<sub>4</sub> and 5mM ATP. Luciferase activity of each sample was determined by measuring luminescence after injection of 100 $\mu$ l of 1mM luciferine (Berthold Biolumat LB 9501 luminometer, integration time 30sec.). For  $\beta$ -galactosidase assays, 200 $\mu$ l of cell lysate were mixed with 200 $\mu$ l of Z buffer (60mM Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, 40mM NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 10mM KCl, 1mM MgSO<sub>4</sub>, 50mM  $\beta$ -mercaptoethanol, pH7.0) and 80 $\mu$ l of ONPG solution (4mg/ml ONPG in 100mM phosphate buffer pH7.0). The samples were incubated at 37°C until the yellow color developed. The reaction was stopped by addition of 200 $\mu$ l of 1M Na<sub>2</sub>CO<sub>3</sub> and the absorption at 420nm measured. To correct for differences in transfection efficiencies, the luciferase data were normalized to the  $\beta$ -galactosidase data in each individual sample. Normalized results are expressed in arbitrary units and represent the ratio: RLU (integration time 30 sec, 50 $\mu$ l sample)/OD<sub>420</sub> (reaction time 2 hours, 200 $\mu$ l sample).

### Receptor measurement

Nine T-175 flasks were grown to confluence. The cells were scraped, collected by centrifugation, and the drained pellet was frozen in liquid nitrogen. The frozen cells were resuspended in 1ml of cold TEGWD buffer (20mM Tris pH7.4, 1mM EDTA, 20mM sodium tungstate, 1mM DTT, 10% glycerol), homogenized in a glass-teflon potter, and 1.5ml of cold TEGWD buffer was added. The homogenate was centrifuged for 45min at 45000rpm in a Ti50 rotor. The resulting cytosol was incubated for 4hours at 4°C with increasing concentrations (3–100nM) of <sup>3</sup>H-labelled dexamethasone (specific radioactivity: 40–45 Ci/mmmole). Ligand binding to the receptor was determined by the dextran-charcoal technique according to ref. 40. The total number of binding sites (N) and the dissociation constant (Kd) were determined by Scatchard analysis using SCAT 3.03, an IBM compatible version of the software originally used in ref. 41.

## RESULTS

### Influence of MMTV LTR deletions on promoter basal activity

Constructions containing a mutated MMTV LTR, with either 5' deletions up to –738 or internal deletions across the region from –738 to –201, driving the luciferase reporter gene were transiently transfected in NIH-3T3 or 34i in the absence of hormone. The results presented in Table 1 suggest that the basal luciferase activity is not significantly different for the full-length and the MMTV LTR deletion mutants. However, in NIH-3T3 cells, constructs pC3DXAluc, and at a lesser extent, pC3DPScluc seem to have a higher luciferase level than the other constructs in NIH-3T3 cells. These activities are very low compared to the positive control plasmid (pSVD5'luc) containing the SV40 promoter ( $\leq 2.5\%$ ). They are also slightly lower than the values obtained for the promoterless luciferase construct plucDSS. The lower luciferase level observed for MMTV constructs could result from the use of the LTR polyadenylation signal located upstream of the CAP site, suggesting the presence of a cryptic promoter in the vector. It was important to be able to rule out such a possibility, since this phenomenon might obliterate or attenuate differences in basal transcription levels for the MMTV LTR mutants. The low level of luciferase (or any other reporter gene) expression in the absence of hormone made accurate RNA measurements in transient assay experiments very difficult. Several reports in the literature present experiments in which stable transfectants, obtained by using selectable markers,

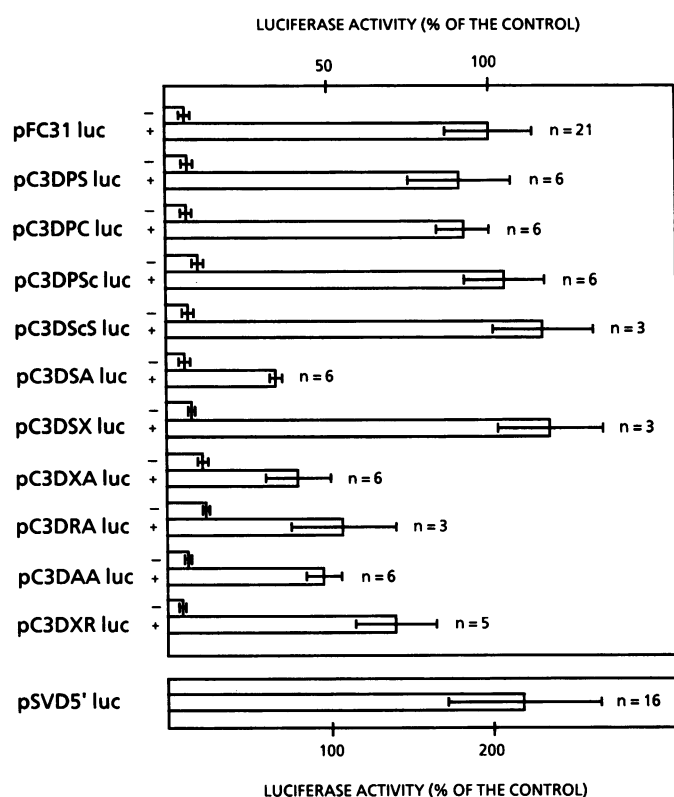
**Table 1.** Luciferase activity of mutated MMTV LTR constructs in NIH-3T3 and 34i cells, in the absence of hormone. Luciferase activities were normalized for transfection efficiency using a  $\beta$ -galactosidase expression plasmid, and are expressed in arbitrary units (see material and methods); n indicates the number of independent transfection experiments

	Luciferase activity (Arbitrary Units)			
	34i		NIH-3T3	
pFC31luc	18.3 $\pm$ 3.0 (n=21)	10.1 $\pm$ 1.9 (n=15)		
pC3DPSluc	20.7 $\pm$ 4.1 (n=6)	11.7 $\pm$ 2.7 (n=3)		
pC3DPCLuc	19.6 $\pm$ 2.3 (n=6)	12.8 $\pm$ 3.5 (n=3)		
pC3DPScluc	24.6 $\pm$ 2.1 (n=6)	15.0 $\pm$ 0.7 (n=3)		
pC3DScSluc	17.2 $\pm$ 2.0 (n=3)	9.2 $\pm$ 1.0 (n=3)		
pC3DSXluc	17.2 $\pm$ 1.9 (n=3)	10.4 $\pm$ 0.4 (n=3)		
pC3DSAluc	18.2 $\pm$ 3.0 (n=6)	10.0 $\pm$ 3.3 (n=3)		
pC3DXAluc	22.6 $\pm$ 4.2 (n=6)	17.6 $\pm$ 0.8 (n=3)		
pC3DAAluc	21.3 $\pm$ 4.2 (n=12)	9.0 $\pm$ 2.7 (n=6)		
pC3DXRluc	19.6 $\pm$ 3.5 (n=11)	9.6 $\pm$ 2.4 (n=9)		
plucDSS	32.8 $\pm$ 5.1 (n=6)	19.8 $\pm$ 3.4 (n=5)		
pAplucDSS	40.1 $\pm$ 10.0 (n=6)	33.5 $\pm$ 6.9 (n=5)		
pSVD5'luc	578.3 $\pm$ 120.0 (n=18)	651.0 $\pm$ 106.8 (n=4)		

produce detectable levels of mRNA in the absence of hormone (42,43). However we have not retained such an approach to compare basal levels of expression, since the selection pressure will necessarily bias the results making it impossible to interpretate. Thus to determine if the luciferase is transcribed from a cryptic promoter, we decided to inactivate this putative promoter in plasmid plucDSS, by inserting a polyadenylation signal upstream of the luciferase gene (36). The results obtained with this plasmid (pAplucDSS) indicate that no cryptic promoter was driving the luciferase gene in the constructs. These data strongly suggest that, in the absence of hormone, the MMTV promoter is almost silent, the measured activities corresponding to the background of the system. None of the deletion described here can be at the origin of changes in basal transcription level, of significant physiological or pathological relevance in mammary gland.

### Effects of MMTV LTR deletions on hormone-induced transcription

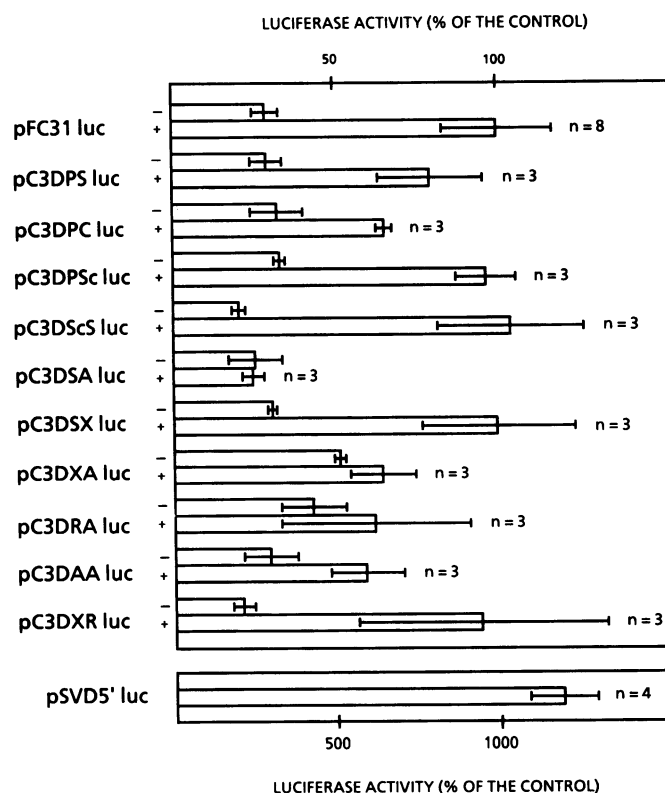
The same constructs were transfected in 34i and NIH-3T3 cells, in the presence or absence of  $5 \times 10^{-7}$ M dexamethasone. Results are summarized in figures 2 and 3. In 34i cells (Fig. 2) after hormonal induction, the full-length MMTV construct (pFC31luc) exhibits a luciferase activity  $\approx 20$ -fold higher than in the untreated sample. This induced luciferase level is  $\approx 50\%$  of the level obtained for the control plasmid pSVD5'luc. Thus, in the presence of hormone the MMTV LTR behaves as a strong promoter. Deletions between PstI and StuI sites (5'-end to –635: pC3DPSluc, pC3DPCLuc, pC3DPScluc and pC3DScSluc) have little effect on the luciferase level in the presence of hormone. In contrast, deletion of the region spanning from –636 to –201 (pC3DSAluc) leads to a dramatic decrease in activity (70%). Comparison of pC3DSXluc (deletion from –636 to –455) and pC3DXAluc (deletion from –450 to –201) hormone-induced activities clearly demonstrates that the region between –636 and –455 does not contribute to the maximal level of transcription in the presence of hormone and indicates that one or several positive regulatory elements are present between –450 and –201. Shorter deletions in this last region, –450/–364 (pC3DXRluc), –363/–201 (pC3DRAAluc), and –294/–201 (pC3DAAluc), all lead to a significant decrease in the luciferase



**Figure 2.** Transfection of the MMTV LTR deletion mutants in 34i cells. Results are expressed in % of the luciferase activity obtained for the full-length MMTV LTR (pFC31luc) in the presence of hormone, in each set of transfections. All the results were normalized for transfection efficiency using plasmid pCH110 (see material and methods). Cells were treated (+) or not (-) with  $5.10^{-7}$  M dexamethasone; n indicates the number of independent transfections. On the bottom of the figure, on a different scale, is the positive control plasmid pSVD5'luc. The mean luciferase activity values for the positive controls pFC31luc (in the presence of hormone) and pSVD5'luc were 262 and 535 arbitrary units, respectively.

level. The identical luciferase activities for pC3DRA luc and pC3DAA luc suggest that the region located between RsaI and AlwNI (-363/-294) does not contain regulatory elements, and that at least two regions (-450/-364 and -294/-201) are involved in this enhancement of the GRE-mediated hormone-induced transcription. Since an effect of these LTR deletions is observed only in the presence of hormone, we conclude that these positive regulatory elements can influence the MMTV LTR promoter activity and act synergistically with the GRE.

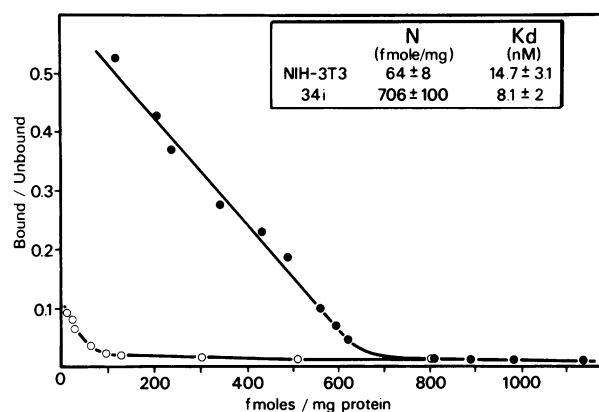
The same mutants were assayed in NIH-3T3 cells. The results are presented in Fig. 3. After hormonal induction, the level of expression of the full-length MMTV LTR luciferase construct (pFC31luc) is much lower than in 34i cells, representing  $\approx 8\%$  of the activity of the positive control pSVD5'luc plasmid, with a hormonal induction of  $\approx 4$ -fold. The difference in hormone-inducibility of the MMTV promoter in 34i and NIH-3T3 was intriguing. The most obvious explanation was a difference between the two cell lines, in either receptor content or receptor-ligand binding properties. Using cytosol from untreated cells, we measured the receptor concentration (N) and its dissociation constant for dexamethasone (Kd). Results plotted according to Scatchard are presented in Fig. 4. They clearly indicate that NIH-3T3 cells contain  $\approx 10$ -fold less glucocorticoid receptors



**Figure 3.** Transfection of the MMTV LTR deletion mutants in NIH-3T3 cells. Results are expressed as in Fig. 2. The mean luciferase activity values for the positive controls pFC31luc (in the presence of hormone) and pSVD5'luc were 52 and 616 arbitrary units, respectively.

than do 34i cells, and that the binding properties of the receptor are the same in both cell lines ( $N=64 \pm 8$  fmoles/mg proteins,  $K_d=14.7 \pm 3.1 \times 10^{-9}$  M for NIH-3T3 cells and  $N=706 \pm 100$  fmoles/mg proteins,  $K_d=8.1 \pm 2 \times 10^{-9}$  M for 34i cells). This suggests that the low luciferase activity in NIH-3T3 cells is due to a limiting amount of receptor in the cells. The low glucocorticoid receptor content was not particular to the NIH-3T3 cell line carried in our laboratory. The same results were obtained with NIH-3T3 cells maintained in another laboratory (data not shown).

In NIH-3T3 cells, MMTV LTR deletions affect hormonally induced luciferase activity, roughly in the same way as in 34i cells (Fig. 3). The effect of the deletion of the 5'-end of the LTR (pC3DPS luc and pC3DPC luc), is more marked than in 34i cells ( $\approx 20-30\%$  less activity than with the full length LTR). Further deletion (pC3DPSc luc) restore the full-length MMTV LTR luciferase level. This could suggest the presence of both a negative element, between -861/-738, and a positive one, between -1180/-902. However, the variations are weak and do not allow a conclusion regarding their relevance. Removal of the region -636 to -201 (pC3DSA luc) not only decreases the induced luciferase level, but leads to a complete lack of response to hormone. In this cell line too, the data suggest that elements cooperating positively with the GREs are present in the region spanning from -450 to -201, but, as a result of the low level of luciferase induction upon hormone treatment, it is difficult to accurately determine the locations of the elements. The higher luciferase activity obtained for construct pC3DXA luc, when



**Figure 4.** Dexamethasone binding to the glucocorticoid receptor in 34i and NIH-3T3 cytosol: Scatchard plot. Open circles: NIH-3T3 cells, closed circles: 34i cells.

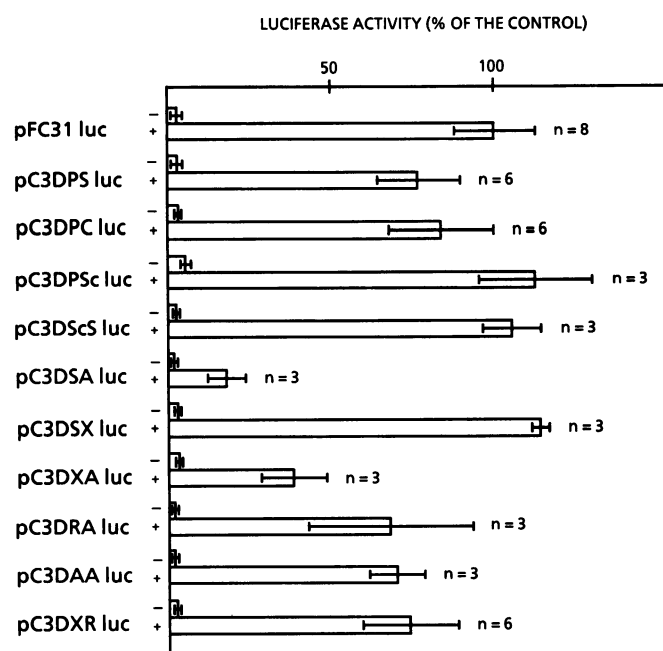
compared to pC3DSAluc may result from an increase in basal expression for construct pC3XAluc.

#### Effect of the co-transfection of the glucocorticoid receptor gene on transcriptional activity of MMTV-LTR mutants in NIH-3T3 cells

We conducted a new set of experiments in which the MMTV LTR constructs and a plasmid expressing the glucocorticoid receptor were cotransfected in NIH-3T3 cells, in the presence or absence of dexamethasone. Results are summarized in Fig. 5. The increase in glucocorticoid receptor content in NIH-3T3 cells considerably enhances the luciferase activity of the full-length MMTV LTR plasmid (pFC31luc) in the presence of hormone. The induction was  $\approx 40$ -fold compared to the cells untreated with hormone. This luciferase activity was 100–150% of the level obtained with the positive control plasmid pSVD5'luc. The influence of the different MMTV LTR mutations on luciferase expression paralleled the results obtained in 34i cells. Plasmids pC3DPSluc and pC3DPCluc displayed a  $\approx 20\%$  decrease in luciferase activity, compared to the full-length LTR. Deletion of the  $-636/-201$  region (pC3DSAluc) results in a decrease in luciferase activity even more dramatic than in 34i cells ( $\geq 80\%$ ). The  $-636/-455$  and  $-363/-295$  regions, again, do not seem to be involved in the transcription level modulation and the higher luciferase activity of construct pC3DXAluc, compared to pC3DSAluc, may be explained by a change in basal expression. These results, again suggest that, in NIH-3T3 cells, the regions comprised between  $-450/-364$  and  $-294/-201$  exert a positive effect on MMTV promoter activity, in synergy with the GRE region.

#### Effect of the substitution mutation of a 23bp MMTV LTR region located immediately upstream of the GREs on luciferase activity

In the experiments presented here, plasmid pC3DAAluc, containing a 94bp deletion in the region just upstream of the GREs, always displayed a luciferase activity markedly lower than the full-length MMTV LTR construct. This effect was more pronounced in 34i than NIH-3T3 cells. Sequence analysis of the  $-290/-201$  region reveals the presence of two interesting motifs: a CArG box (CCTTTATTGG) at position  $-232/-223$  and a CAC box (CCAACC) at position  $-222/-217$ . In order to



**Figure 5.** Cotransfection of the MMTV LTR deletion mutants with a glucocorticoid receptor expression plasmid in NIH-3T3 cells. Results are expressed as in Fig. 2.

determine if these regions play a role in the positive cooperation described above, we substituted, in pFC31luc, a 22bp fragment containing the CAC box but not the CArG box with a synthetic DNA fragment. Results of an experiment in which this plasmid (pC3mCACLuc) was transfected in 34i and NIH-3T3 cells is presented in Table 2. The transfections were performed with and without hormone treatment, and the glucocorticoid receptor expression plasmid was cotransfected or not in NIH-3T3 cells. In both cell lines, the pC3mCACLuc luciferase activity was lower than for the control (pFC31luc). The values compare to those obtained for constructs pC3DRAAluc and pC3DAAluc (see Fig. 2, 3 and 5), suggesting that one of the regulatory elements is located between  $-223/-201$ . This result also suggests that the effect observed with construct pC3DAAluc does not result from the creation of a new sequence resulting from the deletion, but from the presence of one or several regulatory targets in this region.

## DISCUSSION

We have investigated the role in transcriptional activity of the MMTV LTR regions located upstream of the GREs activity, in the presence or absence of hormone. The proximal MMTV LTR region ( $-200$  to the CAP site) contains the GREs and targets for ubiquitous factors as NF-I, TFIID, and an OTF-1 (23,43–47). The organization of this region has been studied in detail by several groups, and these 200bp are sufficient to promote hormone-dependent transcription (16,17). Investigations in transgenic animals (25,48), the analysis of rearranged MMTV proviruses in lymphomas (27–29), and the coupling of MMTV LTR sequences to heterologous promoters (22,23) have highlighted the importance of regions located between the 5'-end of the LTR and position  $-200$ , for the control of both transcription level and for tissue-specific expression of the promoter.

**Table 2.** Influence of a substitution mutation in the -223/-201 region on the luciferase activity. Luciferase activities are expressed in % of the value obtained for the construct containing the intact MMTV LTR (pFC31luc), in the presence of hormone, in the corresponding experiment. Each value is the mean of the indicated number (n) of independent transfections

Dex	Receptor	Luciferase activity (%)				
		pFC31luc		pC3mCACluc		
		-	+	-	+	
34i	-	6.8 ± 1.7	100 ± 12.0	8.7 ± 1.7	66.8 ± 15.4	(n=6)
NIH-3T3	-	38.9 ± 18.7	100 ± 28.9	37.7 ± 5.9	77.2 ± 10.6	(n=3)
	+	3.1 ± 0.8	100 ± 19.0	3.8 ± 0.8	65.8 ± 7.0	(n=3)

In this paper, we report studies on the mutagenesis of the 5'-end/-200 region of MMTV LTR. We have constructed two main types of mutants. The first type contains LTRs with progressive deletions from the 5'-end up to -738. They were designed to investigate the role of the LTR 5'-end in tissue specificity, since such a role has been proposed from transgenic animal experiments (25,48), and to better analyze the region -1094/-739, in which an enhancer has been described (22). The second type of mutants contains internal deletions spanning from -738 to -201. They were designed in order to keep intact the proximal promoter and GREs, to preserve as much as possible the LTR integrity, and to avoid simultaneous deletion of multiple regulatory elements. We used the luciferase gene as a reporter (31), instead of the CAT gene, since the MMTV LTR is known to be a very weak promoter in the absence of hormone, making the sensitivity of the reporter assay critical for studies on promoter basal activity. To investigate a possible involvement of these regions in tissue-specific expression, the mutated constructs were transfected into fibroblasts and transformed epithelial mammary cells. Our data on basal level of expression show that none of the LTR deletions described here result in a significant change in luciferase activity, and therefore cannot account for physiological or pathological effects in the absence of hormone in mammary gland.

Clear differences among the mutants are visible only in the presence of hormone (induced activity). Progressive deletions of the 5'-end of the LTR have little effect on luciferase activity. Deletion from -1180 to -861 resulted, in both 34i and NIH-3T3 cells, in a slight but reproducible decrease in the activity ( $\approx 10-20\%$  decrease), while deletion from -861 to -738 led to a restoration of the full length LTR activity. These results, although the observed effects are weak, suggest the presence of a positive element, located between -1180 and -902, followed by a negative one between -861 and -738. There were no noticeable differences between fibroblasts (NIH-3T3) and transformed epithelial mammary cells (34i). This contrasts with conclusions drawn from transgenic mice experiments, in which two types of constructs, either a large MMTV fragment, containing a part of the viral envelope gene and the full length LTR, or a LTR deleted at the 5'-end up to -861 (ClaI site), both driving the *int-2* gene were used (48). In these experiments, the authors have shown the inability of the truncated-LTR constructs to target the transgene expression into epithelial cells. Our data suggest that sequences responsible for such a phenomenon are not located in the LTR between -1180 and -861, but rather upstream, possibly in the envelope sequences. Our experiments did not show the tissue-specific enhancer recently described as located between -1094/-739 (22). This

was not surprising, since the activity of this element has been visualized only when coupled, in a reverse orientation, to the thymidine kinase heterologous promoter.

We constructed LTR mutants with internal deletions, encompassing various regions between -738 and -201. In this region two negative regulatory elements have been described. One is located between -631/-560 (22) and the other between -455 and -364 (22,23). The negative effect of these elements have been visualized by their coupling either to a heterologous promoter or to a proximal MMTV promoter, partially disabled in its hormone inducibility. The first region (-637/-455) is a part of the sequence deleted in pC3DSXluc and the second (-455/-364) is the one deleted in pC3DXRluc. Our studies do not show any negative effect of these two regions, and, in our assay, deletion of region -450/-364 even suggests a positive effect, more marked in NIH-3T3 than 34i cells. This result is not surprising, since an increasing number of reports in the literature describe dual behaviour of regulatory regions, depending on the environment (49,50). An interesting parallel can be drawn with results described in the literature. A negative element located between -162 and -156 has been characterized by coupling this region to a heterologous promoter (24). Point mutations have been performed in the same region. When such a mutant was assayed, in transient or stable transfections, no effect of the mutation was observed on the reporter gene activity (44). Thus, if these negative elements can repress either a heterologous promoter or an active minimal MMTV promoter, they apparently do not modulate the transcription from an intact MMTV LTR. However, they may play a role when rearrangements of the GRE region of the promoter occur.

More interesting are the data obtained from deletion mutations of the region -450/-201, always deleted in MMTV proviruses causing lymphomas. In the absence of hormone, no noticeable effect of the mutations can be detected. In contrast, in the presence of hormone the deletion of this region has a dramatic effect in both NIH-3T3 and 34i cells, with a decrease in luciferase levels of  $\approx 80\%$ . A modulation of the glucocorticoid responsiveness of the MMTV promoter by a sequence located upstream of the GRE has been described (51). However, this sequence cannot account for the effect described here, since the proposed target TTTAAA, is CTAAAG in the C3H MMTV variant that we have used. The -450/-201 region probably contains several regulatory elements. At least one of them must be located between -290/-201 (pC3DAAluc), and seems to be more efficient in 34i cells than in NIH-3T3 cells. Preliminary *in vitro* DNaseI footprinting results, obtained with crude nuclear extracts, show partial protection and increased hypersensitivity to DNaseI in a region between -230 and -200. Sequence analysis reveals the

presence of two neighboring consensus sequences for known binding factors, a CarG box (serum responsive element) (52) and a CAC box (53,54) at positions -233 and -222, respectively. The homologous region of the MMTV LTR GR strain (55) displays a point mutation of the last 3'-G of the CarG consensus (CC(A/T)<sub>6</sub>GG) and a conservation of the CAC box. The comparison of serum responsive factor binding sites described in the literature shows a large heterogeneity in the (A/T)<sub>6</sub> content, but no modification of one of the two Cs or Gs in functional elements has been reported. CAC boxes have been described as cooperating with the GRE and enhancing the level of hormone-induced transcription (53,54). We have substituted the 23bp region containing the CAC box, but not the CarG box, with a synthetic piece of DNA. Mutants pC3DAALuc (90bp deletion) and pC3mCACLuc (23bp substitution) give similar results. Therefore we conclude that at least one of the regulatory targets is located within these 20bp.

In 34i cells, dexamethasone stimulates the MMTV LTR transcriptional activity to a level comparable to that of the SV40 promoter. In contrast, in NIH-3T3 cells, MMTV promoter inducibility was weak. We demonstrate here that this results from a very low glucocorticoid receptor content in NIH-3T3 cells (10-fold less than in 34i cells). Transfection of a glucocorticoid receptor expression plasmid allows recovery of a high inducibility of the MMTV promoter. This was true with all the LTR mutants described and suggests that NIH-3T3 cells possess all the factors necessary to transactivate the MMTV LTR promoter. Thus, it is possible to envisage a control of tissue-specific expression of the MMTV promoter through steroid hormone cooperation with ubiquitous factors.

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