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Calcyclin Gene Expression Modulation by Medroxyprogesterone Acetate

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ABSTRACT. Calcyclin is a cell-cycle–related gene corresponding to a calcium-binding protein whose expression is mainly controlled by platelet-derived growth factor. This paper illustrates medroxyprogesterone acetate (MPA) inhibition of endogenous calcyclin RNA expression of both estrogen-dependent human mammary carcinoma cells and estrogen-independent hamster fibroblasts. Transfection of fragments of the calcyclin promoter driving the chloramphenicol-acetyl-transferase (CAT) gene into hamster fibroblasts was used to evaluate the hormone sensitivity of different promoter regions by considering calcyclin expression at both the RNA and protein level, as evaluated by the CAT assay. A 164 bp promoter fragment showed a good activity that was inhibited by MPA, thereby confirming the results of the observation of endogenous calcyclin gene: smaller fragments, however, required cotransfection of progestin receptor to show full activity, with MPA displaying a stimulatory effect. These findings show that progestin modulation of calcyclin gene expression may be independent of progestin receptors, and that MPA has opposite effects on different promoter regions. BIOCHEM PHARMACOL **54**;2:299–305, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. calcyclin; fibroblasts; mammary cells; MPA; PCR; reverse transcription

Calcyclin is a cell-cycle gene with a structure typical of a calcium-binding protein [1]. Its function has not been completely elucidated. Functional analysis of the calcyclin promoter has shown that epidermal growth factor exerts a negative effect on a 1371 bp promoter fragment upstream from the cap site not conserved in smaller fragments, whereas platelet-derived growth factor stimulates various fragments down to the smallest (42 bp) [2]. Zinc and calcium ions, annexin family proteins, prolactin receptor, glyceraldehyde-3-phosphate dehydrogenase, and sialic acid are related in different ways to calcyclin, whose expression has been described in normal tissues: skeletal and cardiac muscle, lung, kidney, spleen, and skin [3–7]. Nonetheless, it seems to play a role in the pathogenesis of some malignancies. Overexpression of the calcyclin gene, in fact, has been observed in some human tumors, such as leukemia, melanoma, and skin tumors [8–10]. It has been suggested that this overexpression stems from genomic rearrangement of enhancer/promoter sequences, resulting in increased accessibility to transcriptional factors [11].

This paper investigates the sex steroid hormone control of calcyclin gene expression following the treatment of the estrogen-dependent human mammary carcinoma cell line CG5 [12] with medroxyprogesterone acetate (MPA),† a potent progestin with low estrogenic and no androgenic activity used in human neoplasia therapy [13]. The hormone sensitivity of CG5 cells was assessed by comparison with calcyclin expression in the estrogen-independent hamster fibroblast line ts13 [14], which had also been transfected with different calcyclin promoter fragments driving the chloramphenicol-acetyl-transferase (2.3.1.28, CAT) gene [15] and progestin receptor cDNA under the control of the simian virus 40 (SV40) promoter [16]. Polymerase chain reaction (PCR) and CAT assay were employed to test both RNA expression and RNA transduction mechanisms.

MATERIALS AND METHODS

Reverse Transcription and PCR

Total RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction [17] and 1.5 μg was transcribed into specific cDNA by using reverse transcriptase 15 U (Amersham, Little Chalfont, UK), placental RNAse inhibitor 20 U (Amersham) [18], and the following antisense oligonucleotide primers: CATTGTAGATCAAAG CCAAG for calcyclin [1], ATGATGAACCTGAATCG CCA for CAT [19], ATGATGGAGTTGAAGGTAGT

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† *Abbreviations:* CAT, chloramphenicol-acetyl-transferase (2.3.1.28); MPA, medroxyprogesterone acetate (17-acetate-6α-methylpregn-4-ene-3,20-dione); PCR, polymerase chain reaction; PDU, pixel density units; SV40, simian virus 40.

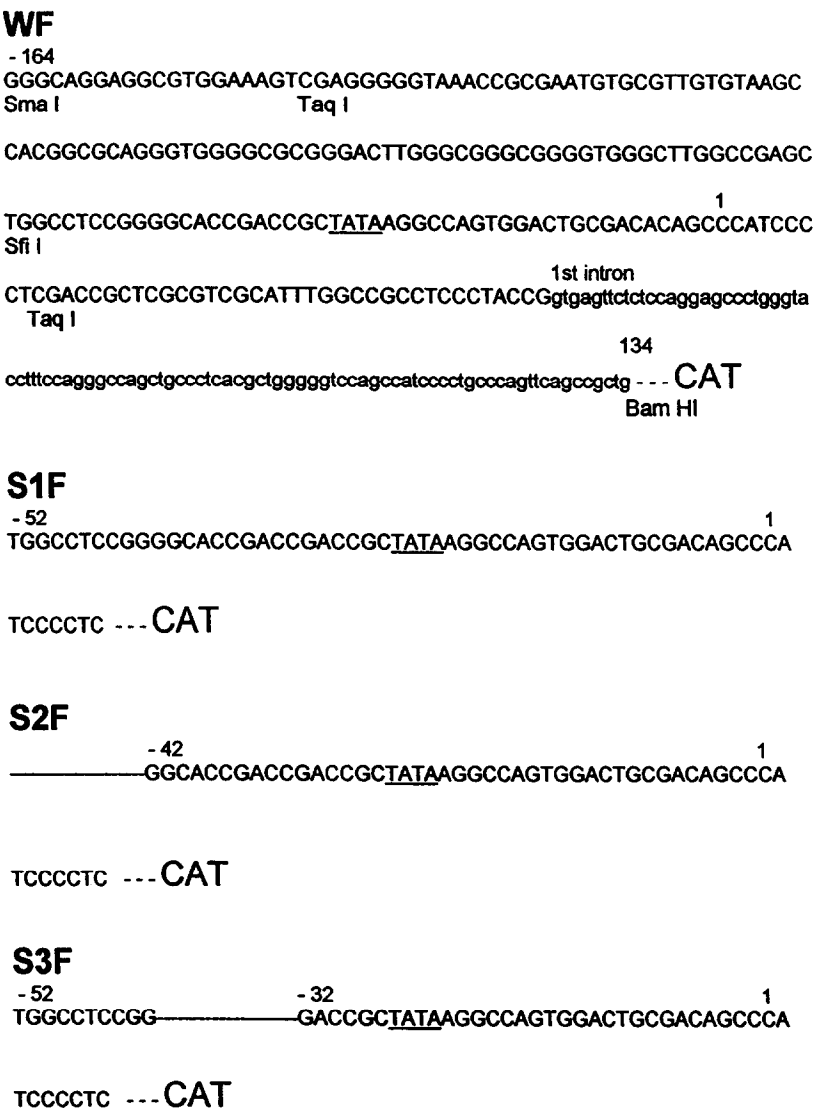

FIG. 1. Calcyclin promoter fragments driving the CAT gene employed in the experiments. Negative numbers denote bases positioned 5′ the cap site, positive numbers denote bases positioned 3′ the cap site. Continous line represents missed bases, broken line represents connection between calcyclin promoter and CAT cDNA. TATA box is underlined, principal restriction sites are indicated, and lower case letters indicate first intron bases. WF: wild fragment containing 164 bp of the promoter sequence. S1F, S2F, S3F: wild-type synthetic fragments containing 52 and two combinations of the 42 bp of the promoter sequence, respectively.

TTCGTGGAT for β-actin [20], and GTAGGCCTCAC AIGCCTCCT for H3-histone [21]. After 90 min at 37°C, 5 μL out of 20 of the incubation mixture were employed for the PCR [18, 22] by using 1 U of Taq polymerase (Biochemia, Milan, Italy) with the amplification protocol: one cycle of 5 min at 94°C, 2 min at 50°C, and 3 min at 72°C followed by 25 cycles each consisting of 1 min at 94°C, 2 min at 50°C, and 3 min at 72°C. The last cycle was programmed with an annealing time of 10 min at 72°C. The same antisense primers were employed for the PCR along with the following sense oligonucleotides: GCCCC CTGGATCAGGCCATT for calcyclin, ATTGAGAAT ATGTTTTTCGT for CAT, ACACTGTGCCCATCTC GAGGGG for β-actin, and CGCAAGCAGCTGGCIAC CAA for H3-histone. The reaction products for calcyclin, CAT, β-actin, and H3-histone were 248, 165, 249, and 367 bp fragments, respectively. In the PCR, 2.5 μCi for each sample of [^{32}P]dCTP (Amersham) were mixed with the other bases [23] to form the incubation mixture for each sample. Because the sense oligonucleotide of calcyclin corresponds to a sequence of the second exon and the antisense to one of the third, any DNA contamination of RNA preparation was promptly revealed by the presence of an anomalous heavier band. After amplification, 10 μL of the amplification products were run in 2% agarose gel. After electrophoresis, the gel was dried, sealed in a plastic bag, and incubated for 1 hr on a screen before scanning in the Phosphor Imager system (Bio-Rad, Richmond, CA) to obtain an autoradiograph, which was then processed. Image plotting was performed with system arbitrary pixel density units (PDU) vs. spot profile length (mm). Each RNA sample was reverse transcribed and amplified three times in different experiments, all of which gave similar results.

Plasmids

Preparation of plasmids containing fragments of calcyclin promoter (the wild 164 bp fragment and the three synthetic wild-type fragments of 52 and 42 bp, Fig. 1) was performed as previously described [1, 2]. Briefly, the Sma I-Bam HI calcyclin deletion mutant (−164/134) was cloned in the pUC9-CAT plasmid cut with Hind III and Acc I after

filling the ends, while the synthetic oligonucleotides were cloned directionally in this plasmid after hybridization of the oligomers. The plasmid containing progestin receptor cDNA under the control of the SV40 promoter [16] was generously supplied by Prof. Chambon (Faculty of Medicine, Strasbourg, France).

Cell Culture

CG5 human mammary tumor cells (a gift from Prof. Sica of the Sacro Cuore University of Rome, Italy) [12] and ts13 fibroblasts (a gift from Dr. Baserga of the Jefferson University of Philadelphia, PA) [19] were cultured in 5% activated charcoal-treated fetal calf serum [24] Dulbecco's modified Eagle's medium without phenol red with the addition of 10^{-8} M estradiol (17β-estradiol, Sigma, St. Louis, MO). In each experiment, cells were incubated for 48 hr. MPA (Sigma) was generally employed at the pharmacological concentration of 10^{-7} M. Cycloheximide (Sigma) was employed in culture at the final concentration of 25 mg/mL [25].

Proliferation Assay

As CG5 cells form syncytial islets during their growth, tetrazolium salt reduction assay [26] was performed by using a 96-well microplate in order to evaluate the amount of vital cells more precisely than with the simple cell count: after a 6-day culture of a starting amount of 3×10^2 and 1.5×10^2 cells/well for CG5 and ts13, respectively, 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT, Sigma) at a final concentration of 50 mg/100 mL was incubated for 4 hr at 37°C. The supernatant was discarded, 150 μL of dimethylsulfoxide were added to each well, and the absorbance was evaluated with a microplate reader (model 450, Bio-Rad).

Transient Expression

Cells were transfected following the protocol of Shen *et al.* [27]: 10 μg of plasmid DNA were used for 2×10^6 cells and 30% polyethyleneglycol 8,000 (Sigma) was employed for the osmotic shock. Fibroblasts were detached after 48 hr for RNA extraction by guanidinium thiocyanate, or by scraping to test CAT activity [15]: this was measured, after thin layer chromatography, by scanning acetylated [^{14}C]chloramphenicol (Amersham) in the Phosphor Imager. Experiments performed at least three times always produced similar results.

Progestin Receptor

Chemical determination of cytosolic progestin receptor was performed as previously described [13]. Briefly, cells were detached from the incubation plate by scraping and homogenized in TEG buffer (50 mM Tris-HCl pH 7.4, 1 mM EDTA, 12 mM thioglycerol, 10% glycerol). Cytosol obtained from the supernatant after centrifugation at 105,000 × *g* for 60 min at 4°C was incubated overnight at 4°C with (0.07–2.8 nM) radioactive ORG 2058 (16 α-ethyl-21-hydroxy-19-nor [6,7-3H] pregn-4-en-3,20-dione, 42 Ci/mmol, Amersham). Nonspecific binding was determined by parallel incubation in presence of a 250-fold excess of unlabelled ORG 2058. Unbound hormone was removed by treatment with dextran-charcoal. Results obtained by Scatchard analysis were expressed as fmol of hormone specifically bound/mg of cytosol protein.

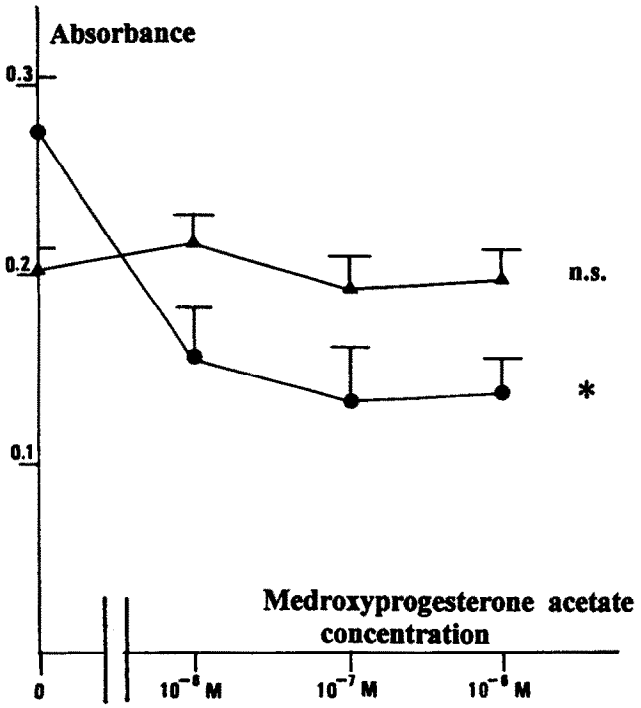

FIG. 2. Effect of MPA on proliferation of CG5 and ts 13 cells after 6 days of incubation, evaluated by tetrazolium salt reduction. The first point was obtained from cultures containing only estradiol, the other three from cultures containing 10^{-8} M estradiol plus increasing concentrations of MPA. ● CG5 cells. ▲ Ts13 cells. Mean + SD of three independent experiments. Statistics, analysis of variance: CG5 (*: $P < 0.005$) and ts13 (n.s.: not significant) slopes.

RESULTS

Inhibition of CG5 cell proliferation by MPA (Fig. 2) was accompanied by reduced expression of the calcyclin gene (Fig. 3: X, Y), which remained in the presence of cycloheximide, whereas MPA inhibition of the expression of H3-histone expression was absent in presence of cycloheximide and much less marked in the case of β-actin. In addition, MPA did not inhibit ts13 cell growth (Fig. 2). In contrast with CG5, these cells did not have progestin receptors, although they managed to express them after transfection with cDNA of progestin receptor during transient expression (Fig. 4). Figure 3Z shows that calcyclin expression was also inhibited by MPA in ts13 cells. This was true of both the endogenous gene and the transfected gene composed of the wild 164 bp fragment controlling the CAT gene. This phenomenon was equally observable at the

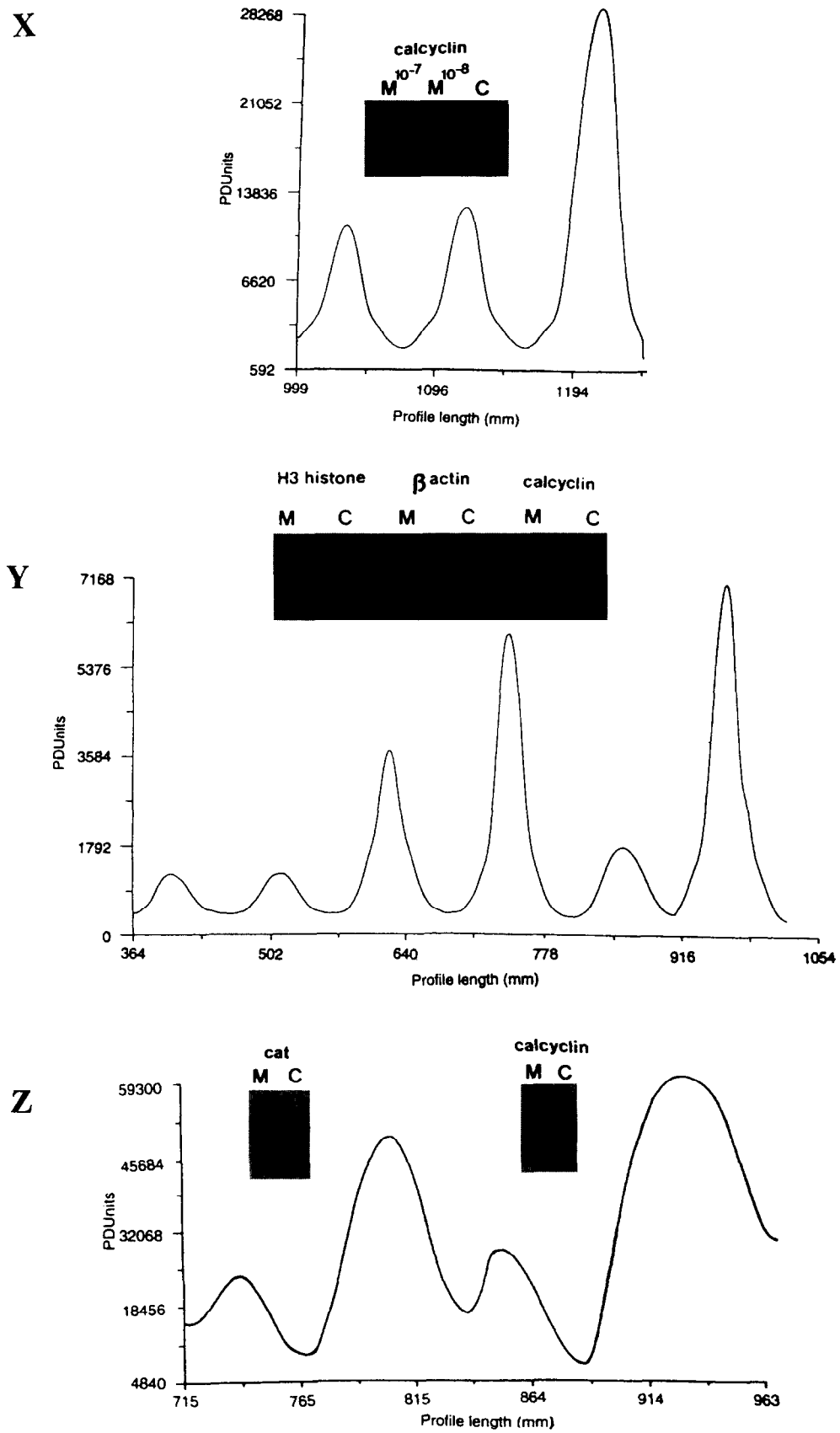

FIG. 3. PCR after mRNA reverse transcription. Spots are obtained with 1 hr autoradiograph of dried agarose gel after running of PCR amplification products that incorporated [^{32}P]dCTP by scanning in the Phosphor Imager system. Plots: PDU vs. profile length (mm). 2×10^6 cells after 2 days of culture. X: CG5 cell calcyclin expression in culture without (C) and with two concentrations of MPA: 10^{-8} M (M^{-8}), and 10^{-7} M (M^{-7}). Y: CG5 cells cultured with cycloheximide, without (C) and with 10^{-7} M MPA (M), from right to left: calcyclin, β-actin, and H3-histone. Z: ts13 cells transfected with CAT gene driven by the 164 bp wild calcyclin promoter. From right to left: endogenous calcyclin gene expression and transfected CAT gene expression, without (C) and with 10^{-7} M MPA (M).

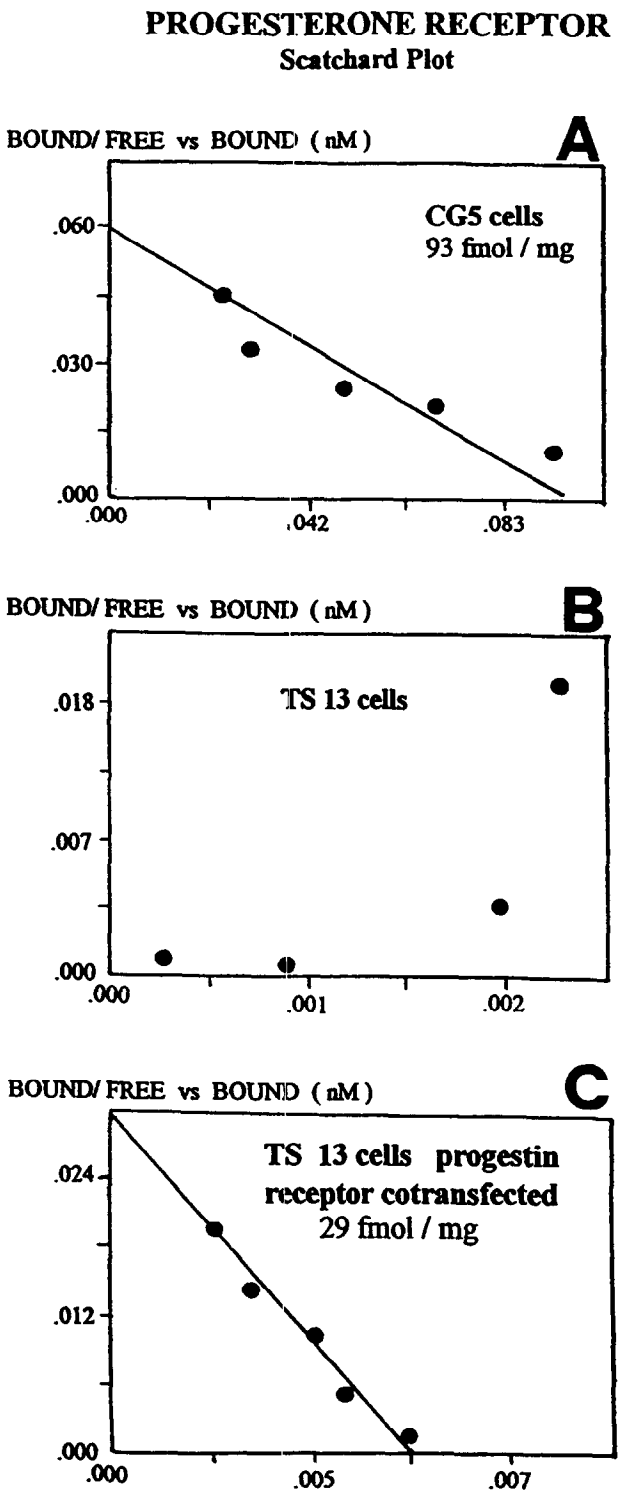

FIG. 4. Progestin receptor, Scatchard plot. A: CG5 cells. B: ts13 cells. C: ts13 2 days after transfection with progestin receptor cDNA driven by the SV40 promoter (transient expression). Bound/free vs. bound (nM), fmol of hormone specifically bound/mg cytosol protein.

protein level in the CAT assay (Fig. 5: WF), and was not significantly influenced by cotransfection of the progestin receptor. When smaller fragments were transfected, however, expression was greatly reduced and solely accentuated by cotransfection with the progestin cDNA (Fig. 5: S1F, S2F, S3F), while MPA had a stimulating effect in these cases.

DISCUSSION

Our data show that MPA inhibits both the growth of estrogen-dependent CG5 cells and their calcyclin gene expression. This phenomenon is not influenced by the addition of cycloheximide, and hence, cannot be attributed to a simple inhibition of protein synthesis, nor to a decrease in calcyclin mRNA resulting from its consumption due to enhanced protein synthesis secondary to possible activation of the gene. Surprisingly, MPA inhibited the gene expression, but not the growth of estrogen-independent ts13 cells devoid of specific receptors. This suggests either that MPA can act even if the receptor concentration is below the sensitivity threshold of the measurement method employed, or that its mechanism of action does not require binding to its receptor. In any case, inhibition of calcyclin expression does not seem to be sufficient to enable MPA to exert an antiproliferative effect. It could, however, have a point of action common to sex hormone-dependent and -independent cells. Transient expression experiments have demonstrated that the 164 bp promoter fragment behaves in the same way as the endogenous promoter, while examination of its sequence shows an absence of onsensus sequences for steroid hormones [28], and it is not significantly influenced by cotransfection with the progestin-specific receptor gene. It is not known for certain whether specific sequences can be the target of steroid hormone inhibition in the same way as the consensus sequences [29]. It is even more difficult to explain why MPA stimulates fragments shorter than 52 bp. The 32 bases upstream from the first exon seem to be sufficient for the positive response to MPA to be revealed. The promoter would appear in this case to be liberated from an inhibiting influence that prevails over the stimulating effect and appears even in the absence of consensus sequences. There have been reports of steroid hormones acting on gene promoters in the absence of the classic consensus sequences [30]. No clear alternative mechanisms have been formulated thus far. Further work will be directed to securing a more precise identification of the DNA zones responsible for the hormonal effect, bearing in mind that additional deletions would be likely to impair the operation of the TATA box. At this stage, it seems reasonable to assert that MPA inhibition of calcyclin expression does not depend on the presence of measurable concentrations of specific receptors. In contrast, if progestin receptors are present, MPA stimulates the activity of the proximal fragments of the calcyclin promoter. It can, therefore, be concluded that calcyclin gene expression is inhibited in both sex steroid hormone-dependent CG5 cells and in sex steroid-independent ts13 cells and that calcyclin gene 164 bp promoter is inhibited by MPA, while the smaller fragments (less than 52 bp) are stimulated if progestin receptor cDNA is cotransfected. This indicates

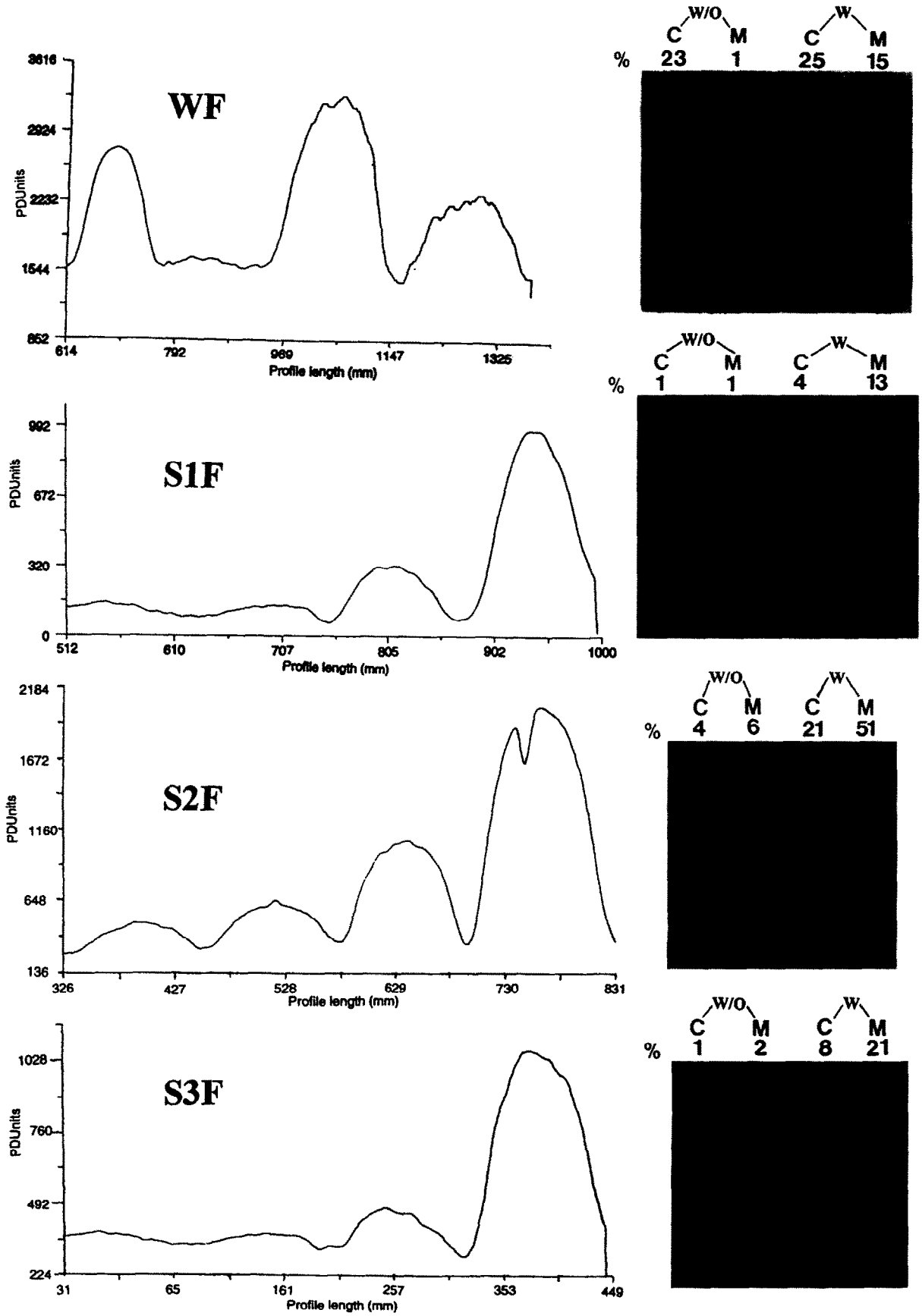

FIG. 5. CAT assay with [^{14}C]chloramphenicol 2 days after transfection. Right, overnight autoradiograph of thin layer chromatography (acetylated chloramphenicol as % of total chloramphenicol) plotted by the Phosphor Imager system on the left (PDU vs. profile length). Ts13 cells (2×10^6) were transfected with different calcyclin promoter fragments driving the CAT gene without (W/O) and with (W) progestin cDNA driven by SV40 promoter cotransfection, cultured in absence (C) or in presence (M) of 10^{-7} M MPA. WF, S1F, S2F, S3F: as in Fig. 1.

that MPA acts on the calcyclin promoter as both an inhibitor, when it does not require the presence of the progestin receptor, and a stimulator, when its presence is essential. The inhibitory mechanism seems to reside in the promoter sequence between base −164 and base −52, the stimulatory mechanism between base −32 and 1. The final result seems to be MPA inhibition of the expression of the cell-cycle gene calcyclin. This finding helps to explain the antiproliferative effect of this hormone on sex steroid-dependent cells. The fact that MPA decreases calcyclin expression, but not proliferation, in sex steroid-independent cells suggests that hormonal inhibition of calcyclin expression is not enough to block cell proliferation.

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