Human Annexin 1 Is Highly Expressed during the Differentiation of the Epithelial Cell Line A 549: Involvement of Nuclear Factor Interleukin 6 in Phorbol Ester Induction of Annexin 1

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Abstract

The role of annexin 1 (Ax 1) in cell differentiation was studied in the A 549 epithelial cell line, a human lung adenocarcinoma line, that responds to phorbol esters and glucocorticoids by induction of differentiated properties. Ax 1 has also been reported to be involved in the control of cell proliferation. We report that Ax 1 synthesis occurs upon phorbol 12-myristate 13-acetate (PMA) treatment of A 549 cells and its appearance is correlated with the presence of dipeptidyl peptidase IV, or CD26, a marker of epithelial cell differentiation. In addition, using transfection experiments and site-directed mutagenesis with the Ax 1 promoter coupled to a reporter gene, we report that a unique region of the Ax 1 promoter confers the response of the reporter gene to PMA and dexamethasone. This response to PMA and/or dexamethasone involves the induction of the synthesis and/or the activity of trans/cis-activating transcriptional factors. Furthermore, we have delineated the mechanism of the transcriptional activation of Ax 1 by PMA and the involvement of a specific transcription factor, nuclear factor interleukin 6 (C/EBP β).

Introduction

Annexins (also known as lipocortins) are a family of structurally related proteins that exhibit Ca2+-dependent binding to anionic phospholipids. They have been identified in many organisms, ranging from mammals to molds and even plants. Many annexins are abundant intracellular proteins, sometimes comprising more than 2% of total cellular protein mass. Clear roles for annexins have not yet been demonstrated, although a wide range of biological functions has been proposed, including anti-inflammatory and anticoagulant activities. The involvement of some annexins in the aggregation and fusion of membranes and in endocytosis and exocytosis is well documented (1) but not yet demonstrated in vivo. Some annexins are expressed in a growth-dependent manner and are targets for cellular kinases in vivo, suggesting that they may be involved in differentiation and mitogenesis (2).

Ax 1, the first member of this family, has been reported to be involved both in differentiation and inflammation. Indeed, Ax 1 has been reported to be the mediator of the anti-inflammatory action of glucocorticosteroids. This effect of GCs on Ax 1 formation requires that cells be initially differentiated so that GCs can induce Ax 1 formation (3), raising the possibility that Ax 1 was involved in the differentiation process. In parallel, it was shown that Ax 1 was abundant in a restricted number of differentiated cell types in adult organs, whereas it was apparently totally absent from undifferentiated tissues (2). The presence of Ax 1 was also observed during the differentiation of neoplastic astrocytes, ependymocytes, and Schwann cells (4). A large number of reports have shown that Ax 1 was induced during the differentiation of the U-937 and HL 60 cells toward the macrophage lineage (5–7). Violette et al. (8) reported that Ax 1 was involved in the GC-induced terminal differentiation of a human cell squamous carcinoma and correlated this effect to the extracellular presence of Ax 1. These results were further supported by those of Kang et al. (9), who demonstrated that PMA-induced differentiation of the U-937 cell line was correlated with the translocation of Ax 1 to the membrane. Ax 1 has also been found to be involved in the control of differentiation and/or proliferation of many different types of epithelial cells: human epidermal keratinocytes (10, 11), cultured bronchial epithelial cells (12), and hepatocytes during hepatocellular carcinoma (13). Taken together, these data argue for a role of Ax 1 in the control of cell proliferation and/or differentiation in cells of the macrophage lineage, as well as in cells of epithelial origin.

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3 The abbreviations used are: Ax 1, annexin 1; GC, glucocorticoid; PMA, phorbol 12-myristate 13-acetate; DPP-IV, dipeptidyl peptidase IV; DEX, dexamethasone-21-phosphate; NFIL-6, nuclear factor interleukin 6; C/EBP, CCAAT enhancer binding protein; FACS, flow cytometric analysis; GRE, glucocorticoid response element; WT, wild-type; GR, GC receptor; EMSA, electrophoretic mobility shift assay.
The A 549 cell line is an epithelial cell line derived from a human lung adenocarcinoma. Epidermal growth factor stimulates its proliferation and dexamethasone induces its growth arrest. Ax 1 has been reported to be involved in the growth arrest of this cell line (14, 15). In this model, the extracellular presence of Ax 1 seems to also be related to the role on cell differentiation. This epithelial cell line appeared, therefore, to be a good model for studying the effect of PMA and GC on cell differentiation, while evaluating Ax 1 at the promoter level. Using this cell line, we have shown that Ax 1 synthesis occurs after PMA treatment and is correlated with the presence of DPP-IV (or CD26), a marker of epithelial cell differentiation. In addition, we have performed transfection experiments with the Ax 1 promoter coupled to a reporter gene. We now report that a unique region of the Ax 1 promoter confers the response of the reporter gene to PMA and DEX and that this response to PMA and/or DEX requires the induction of the synthesis and/or the activity of trans/cis-
activating transcriptional factors. Furthermore, using gel shift assays and site-directed mutagenesis, we have delineated the mechanism of the transcriptional activation of Ax 1 by PMA and the involvement of a specific transcription factor, NFIL-6 (C/EBP β).

Results
Expression of Ax 1 after PMA and DEX Treatment. Fig. 1 shows the expression of Ax 1 protein in A 549 cells and its induction by different stimuli, both in the cytosol (Fig. 1A) and bound to the external membrane (Fig. 1B), as measured by immunoprecipitation. In the cytosol (Fig. 1A), the protein is constitutively abundant in untreated cells (Lane 1). PMA (Lane 3) and DEX (Lane 4) treatments up-regulate the synthesis of the protein. The combined stimuli of PMA and DEX (Lane 5) also show a strong induction of the protein. The stimulation of the low molecular weight forms of the protein is striking, although we worked in the presence of protease inhibitors, but it is known that Ax 1 exists in three different forms, one of which can be phosphorylated. Fig. 1B shows membrane-associated Ax 1. In unstimulated cells (control, Lane 1) or cells stimulated with PMA alone (Lane 2), membrane-associated Ax 1 is not detected. Treatment with DEX alone (Lane 4) or with PMA and DEX (Lane 5) translocates the protein to the membrane and processes it to the external surface of the cell. It is important to note that we have to take into account the total amount of the protein (cytosol and membrane) after each treatment to correctly understand the importance of the induction.

Differentiation Effect of PMA on A 549 Cells. Because it is well documented that the expression of DPP-IV (or CD26) is correlated with the state of epithelial cell differentiation, the differentiating effect of PMA treatment on these cells of epithelial origin was evaluated by FACS using anti-CD26 antibody as a marker. Fig. 1C illustrates A 549 cells, which, after PMA treatment, become positive for CD26 when an anti-CD26 antibody is used. Dexamethasone, alone or added after PMA treatment, did not modify either the constitutive or PMA-regulated expression of CD26 (data not shown).

Expression of the Cloned Ax 1 Promoter in A 549 Cells. As described in "Materials and Methods," a region of 880 bp (−610 to +270 bp) of the Ax 1 promoter (Fig. 2A) was cloned
into an expression vector reporting the luciferase gene (pGL2-basic-Luc). This construct was tested by transient transfection in A 549 cells. Transcriptional activity, measured as luciferase activity, was compared to that of the control plasmid (pSV4O-Luc). A 549 cells showed a marked response to both steroid and PMA treatments with 3- and 6-fold increases, respectively, in luciferase activity (Fig. 2B). The combined stimuli (PMA and DEX) resulted in an 11-fold increase of luciferase activity (Fig. 2B). The cells containing the control plasmid (pSV4O-Luc) responded only to the PMA stimulation, as expected.

**5' and 3' Deletions of the Ax 1 Gene Promoter.** A computer search for canonical consensus sequences of potential regulatory elements is shown in Fig. 3. By sequence analysis two *jun* (AP1) consensus sequences were located at −425 and +177 bp, respectively, the latter lying not far from a simple GRE consensus sequence bearing only a hexamer half-site. Upstream of the consensus for *jun* (+177 bp), we identified a consensus for the P62 TCF involved in the response to serum factors. Two CCAAT sequences (nuclear factor-1) were identified at −77 and −313 bp. To analyze the promoter elements contained in this DNA sequence, eight deletion mutants were constructed as described (Fig. 3). The basal expression was analyzed by transient transfection in A 549 cells (Fig. 3, A and B). The WT promoter containing the entire cloned region showed a basal activity overlapping that of the control construct containing the SV4O promoter. On the other hand, the basal expression of the mutants M1 (−298 to +270 bp), M2 (−180 to +270 bp), and M3 (−106 to +270 bp; Fig. 3A), in the presence of the first intron, was
Table 1: Effect of GR occupancy on Ax1 gene expression

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Luciferase activity (luciferase index)</th>
</tr>
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<tbody>
<tr>
<td>DEX</td>
<td>248 ± 96</td>
</tr>
<tr>
<td>PMA</td>
<td>539 ± 90</td>
</tr>
<tr>
<td>PMA + DEX</td>
<td>1069 ± 16</td>
</tr>
<tr>
<td>DEX-RU-486</td>
<td>112 ± 24</td>
</tr>
<tr>
<td>PMA + DEX + RU-486</td>
<td>280 ± 70</td>
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significantly reduced. However, the results obtained with M5, M6, and M7 (−298 to +20 bp, −180 to +20 bp, and −106 to +20 bp, respectively; Fig. 3B) mutants indicate that this reduction was partially abrogated in the absence of the first intron, except for M8, which has almost totally lost its transcriptional activity.

To verify if these promoter mutants responded to PMA and DEX, A 549 cells were transiently transfected either with the WT promoter or with each individual mutant and stimulated as described above. The results are shown in Fig. 3C. Treatment with DEX, PMA, or PMA and DEX stimulated luciferase activity to a similar extent in the presence of the WT or M1 mutant. The M2 mutant showed a reduction of approximately 20–50% in luciferase activity after PMA and PMA plus DEX treatment. In contrast, a marked reduction of the response to all of the different stimulations was observed in the M3 mutant. These results suggest that the region spanning −180 to −106 bp is important for DEX and PMA responsiveness in the presence of the first intron. The luciferase activity of the M4 mutant was reduced compared to the WT, whereas the luciferase activity of M5 was very similar to that of the WT, suggesting that the region −610 to −298 bp contains negative cis-elements that may be activated in the absence of the first intron. The mutant M6 had a similar profile as the mutant M2. Finally, the very low luciferase activity of mutant M7 confirms the importance of the region from −180 to −106 bp.

Effect of RU-486 on the Induction of Ax1 Promoter. To determine whether GC effects required occupancy and/or activation of the GR, we used the steroid analogue RU-486, which is a potent antagonist of GCs and which binds with high affinity to the GR. A 549 cells were transfected with the WT promoter of Ax1 and treated with different stimuli. Table 1 shows that RU-486 (1 µM) inhibited DEX-mediated luciferase induction by 45%. RU-486 treatment did not affect responses induced by PMA (data not shown). However, RU-486 inhibited the combined action of DEX and PMA by 47%. We conclude that the GC effect requires GR occupancy (at least with 50% efficiency) under these experimental conditions.

EMSA Using Oligonucleotides Designed According to the Sequences of the Ax1 Promoter. According to the results obtained from the reporter gene assay, we performed gel retardation assays on the region spanning −180 to −106 bp of the Ax1 promoter gene to identify potential trans/cis transcriptional factors involved. Three different oligonucleotides of 30 bp were synthesized (oligonucleotides A, B, and C) that covered the entire region. Nuclear proteins from unstimulated or stimulated A549 cells (PMA, DEX, and PMA plus DEX) were incubated with the different labeled oligonucleotides A, B, and C. Because we did not find any induced complex after the treatment with either oligonucleotide A or C and because we observed no effect of DEX alone and no difference between PMA and DEX plus PMA, we concentrated our work on the sequence covered by oligonucleotide B and on PMA. The results are shown in Fig. 4. The incubation of cells with PMA revealed the appearance of a complex (denoted by III in Fig. 4A) that was not present in the unstimulated cells (Fig. 4A, compare Lanes 3 and 2, respectively). The complexes designated I and II that were already present in unstimulated cells were strongly induced by the PMA treatment (Fig. 4A). Preincubation of the extracts with an excess of cold oligonucleotide B resulted in the loss or strong reduction of all of the three complexes as compared to the control or to preincubation with an irrelevant oligonucleotide (Lane 6 versus Lanes 3 and 4). Taken together, these results show that, in the presence of PMA, transcriptional factors that bind to a specific region of the Ax1 promoter spanning a 30-bp region (−160 to −130) are induced. A computer analysis of this region showed a sequence that is NFIL-6-like; examination of the larger region spanning −180 to −106 bp of the Ax1 promoter revealed 60% homology to the regulatory elements of the haptoglobin promoter region, which is regulated by NFIL-6 (C/EBP β). Consequently, we decided to analyze the possibility that NFIL-6 could also participate in the regulation of the PMA-induced transcriptional activation of Ax1. Fig. 4A (Lane 5) demonstrates that, after incubation of nuclear extracts with an oligonucleotide designed to bind NFIL-6, the complex II was partly reverted and the complex III was totally reverted.

The Ax1 Oligonucleotide B Binds Complex C/EBP β. To identify the nuclear protein(s) that could specifically bind to the region covered by oligonucleotide B, we incubated the nuclear extract with different antibodies: one directed against C/EBP β (c-19), which does not cross-react with C/EBP α, C/EBP δ, or C reactive protein 1, and an irrelevant antibody (Ab 14). As shown in Fig. 4B (Lane 4 compared to Lanes 2 and 3), the antibody c-19, which specifically recognized C/EBP β, was able to partly revert the complex I and III, whereas an irrelevant antibody did not (Lane 3). The intensity of the signal measured using the PhosphorImager was reduced by 15 and 30%, respectively (Lane 4 compared to Lane 2). In addition, the presence of a new labeled band (denoted with an asterisk) was noted on the same gel and could be due to a partial supershift of the labeled complex III. The addition of increasing amounts of anti-C/EBP β antibody failed to supershift the entire complex detected in the nuclear extracts from PMA-treated cells (data not shown), indicating the likely presence of an additional protein(s). These results demonstrated that C/EBP β is an important component that contributed to the formation of the DNA–protein complex.
complex between the consensus NFIL-6 sequence present in the Ax 1 promoter and nuclear extracts from PMA-treated A 549 cells.

Site-directed Mutagenesis and C/EBP trans-Activation. To further support the data obtained with the EMSA analysis of the involvement of the NFIL-6(C/EBP β) binding site within the Ax 1 promoter in the response to PMA, transient transfections were performed either with a luciferase gene driven by 880 bp of 5' Ax 1 promoter [pAx (880)-Luc WT] or with a luciferase reporter construct [pAx (880)-Luc NFIL-6 mut] that contained mutations within the NFIL-6-binding site that had been identified by computer analysis (see "Materials and Methods").

Two separate preparations of the mutated constructs were used. In unstimulated A 549 cells, luciferase activity was measured in the cell extracts (Fig. 5) 18 h after transfection with either pAx 1(880)-Luc WT or pAx 1(880)-Luc NFIL-6 mut (colonies 1 and 2). PMA treatment of pAx 1(880)-Luc WT-transfected cells resulted in a 7-fold increase in luciferase activity, whereas cells transfected with pAx 1(880)-Luc NFIL-6 colonies 1 and 2 had only a 1.5-fold increase. When A 549 cells were cotransfected with the expression construct containing the C/EBP β cDNA (pMSV-CEBP/β), the reporter activity of the pAx 1(880)-Luc WT was increased 6-fold. Cotransfection of pAx 1(880)-Luc NFIL-6 mut resulted only in a 0.4–3-fold increase in activity.

PMA treatment of the A 549 cells transfected with pAx 1(880)-Luc WT and pMSV-C/EBP β increased the luciferase activity by 19-fold, whereas PMA treatment of A 549 cells transfected with pAx 1(880)-Luc NFIL-6 mut and pMSV-C/EBP β increased luciferase activity only 0.3- and 3-fold, respectively (Fig. 5). Cotransfection with pMSV-C/EBP β plus PMA treatment resulted in a greater activation of the Ax 1 WT promoter than that observed when PMA was omitted. Transfection with pMSV-C/EBP β demonstrates that PMA treatment resulted in an increased activity of the Ax 1 promoter WT and that this increase can be mediated in part by a C/EBP β (NFIL-6) factor through the element previously identified by EMSA.

Discussion
Here, we show that PMA-induced differentiation of the A 549 epithelial cell line, as measured by expression of DDP-IV, is associated with an increased expression of Ax 1. The addition of the GC dexamethasone after incubation of A 549 cells with PMA augments both the expression of Ax 1 and its translocation to the cellular membrane, whereas dexamethasone does not further induce expression of DPP-IV. These

labeled Ax 1 oligonucleotide B; Lane 4, 200-fold molar excess of nonspecific competitor oligonucleotide; Lane 5, 200-fold molar excess of oligonucleotide NFIL-6; Lane 6, 50-fold molar excess of cold oligonucleotide B. B, antibody analysis. One μg of antibodies directed against C/EBP β (c-19) or Ab 14 nonspecific antibody was added to the nuclear extract (see "Materials and Methods"). Lane 1, unstimulated cells incubated with the Ab c-19; Lane 2, nuclear extract from PMA-treated cells; Lane 3, PMA nuclear cell extract incubated with the antibody Ab 14; Lane 4, PMA nuclear cell extract in presence of the specific Ab C/EBP (c-19), partly reverted complexes are indicated. The results shown are one representative of three independent experiments.

**Fig. 4.** EMSA. A, nuclear protein extracts (10 μg) were made from unstimulated A 549 cells or from cells treated with PMA (10 nm, 24 h) and processed as described in "Materials and Methods." Roman numerals, the different retarded bands formed. Lane 1, probe alone; Lane 2, nuclear extracts from untreated cells incubated with labeled Ax 1 oligonucleotide B; Lane 3, nuclear extracts from PMA stimulated cells incubated with...
results prompted us to analyze the effects of PMA and DEX on Ax 1 gene expression at the promoter level. Using several approaches, including transfection into the A549 cells of the Ax 1 promoter coupled to a reporter gene and EMSA of the nuclear extracts, in control and treated cells, we report that Ax 1 transcription, already present at a high constitutive level, is augmented by PMA and DEX. Using deletion mutants and site-directed mutations of the Ax 1 promoter, we identified a region spanning −180 to −106 bp of the Ax 1 promoter that was responsible for the effect both of PMA and of DEX. In addition, we have identified NFIL-6 (C/EBP β) as the transcription factor that is probably involved in the PMA effect.

The data reported here further confirm and extend the involvement of Ax 1 in proliferation/differentiation that is already reported in various tissues and models. These data shed some new light on the role of DEX in relation to differentiation. Indeed, whereas DEX increases the expression of Ax 1, alone and when combined with PMA, it has no effect on CD26 expression, suggesting that DEX does not up-regulate the latter gene. The role of DEX on expression of Ax 1 protein, as already reported (3, 16), is not only to increase the intracellular pool of the protein but also to permit its translocation to the extracellular leaflet of the membrane. The functions of the augmented intracellular Ax 1 in the differentiation process are still unknown. However, it should be noted that PMA as well as DEX augments the low molecular weight forms of the protein. The identification of the low molecular weight forms is not clear and could reflect cleavage of the NH2-terminal end of Ax 1 or of additional, tyrosine-phosphorylated forms. The extracellular form of Ax 1 is the only high molecular weight form.

Our understanding of the gene regulation of Ax 1 is still very limited. The most extensive study thus far was performed on the regulation of the two Ax 1 genes of the pigeon, cp 35 and cp 37 (17–19). The cp 37 gene is constitutively expressed and is closely related to the mammalian Ax 1 gene, whereas the cp 35 gene is only expressed in response to prolactin. Analysis of the mechanism of prolactin induction of cp 35 gene suggests that tyrosine phosphorylation and subsequent activation of at least one member of the STAT family of transcription factors could lead to direct transcriptional activation of cp 35. These data suggest that, in birds, two genes for Ax 1 may have evolved, one giving rise to a constitutive protein and the other giving rise to a prolactin-inducible protein involved in differentiation. In contrast, the single Ax 1 gene in mammals implies a potentially complex differential regulation of a single protein product: constitutively expressed, similar to the avian cp 37, but specifically up-regulated, similar to the inducible avian cp 35.

The data we have obtained with the human Ax 1 promoter, indeed, demonstrate that there exist at least two systems regulating transcription of Ax 1: one constitutive and the other inducible. Our data on the constitutive regulation of Ax 1 promoter activity demonstrate that the first intron, as well as the last 40 bp at the 5' end of the promoter, are important for the process of transcriptional initiation. This high constitutive level of expression in vitro could be explained by the presence of serum factors. Using DEX and PMA, we have found that the same gene is also inducible. DEX alone in this cell line had a low distinct stimulatory effect, whereas PMA alone had a more pronounced effect, and the two agents together had a greater than additive effect in induction of total Ax 1, as well as in its subcellular localization.

Although our results show that promoter activation by DEX decreases when it is in the presence of the GR antagonist RU-486, the DEX regulation/response of the Ax 1 promoter is independent of the GRE present in the first intron because deletion of this GRE does not change its response to any stimuli (data not shown). The consensus sequence present in the first intron is not palindromic and has been termed a "simple GRE"; it gives a slow response to the endogenous steroids present in the serum. Therefore, the Ax 1 promoter could be considered a slow re-
sponder to DEX treatment. A number of DEX-inducible genes, e.g., phosphoenolpyruvate carboxykinase, albumin, and α1-acid glycoprotein, have been reported to require protein synthesis for DEX induction. This type of steroid response is designated as a secondary GC response, i.e., one that depends on protein synthesis. Indeed, the effect of DEX on Ax 1 promoter activation was diminished by actinomycin D (data not shown). Taken together, these data indicate that DEX-mediated regulation of Ax 1 is a secondary response involving induction of proteins. Transfection experiments using different deletion mutants showed that the response to DEX, similar to the PMA response, was under the control of the region spanning −180 to −106 bp of the Ax 1 promoter. However, when EMSAs were performed using labeled oligonucleotides spanning this region, no complex was found after DEX treatment of the cells. These results suggest that the proteins induced by DEX participate in the promoter regulation in a manner that has not yet been elucidated.

PMA is a strong inducer of the Ax 1 gene. Using deletion mutants and transfection experiments, we show that the full-length promoter responds to PMA; we have mapped a minimal region of 80 bp that is important for the PMA responsiveness. This region, divided into three different sub-regions of 30 bp each and analyzed by EMSA, confirms the mutagenesis data and indicates the involvement of inducible factors that bind DNA or other protein(s) already present. We do not yet know whether these factor(s) are discrete or multiple proteins or even proteolytic fragments of a single protein that share similar DNA-binding domains. The pattern of the complexes formed in PMA-treated cells is highly reproducible and specific because, under the same conditions, control cell extracts did not contain the same complexes. In addition, our data suggest that the transcriptional factor C/EBP β binds the specific region covered by oligonucleotide B after PMA treatment. The site-directed mutagenesis data in the region identified by EMSA and C/EBP transactivation of the Ax 1 promoter support our gel shift data on involvement of a C/EBP β factor in up-regulation of the Ax 1 by PMA.

Recently, it was reported that Ax 1 is involved in embryonic palate development (20). Its striking immunolocalization to the palate epithelium suggests that it plays a role in signaling differentiation of palatal epithelial cells.

The literature indicates that C/EBP (NFIL-6) and other members of this transcriptional factor family are involved in the differentiation of several cellular systems, including bronchial and lung epithelial cells (21). NFIL-6 is involved in phorbol ester induction of p-glycoprotein in U-937 cells (22), and different C/EBP isoforms are active in the differentiation of adipocytes (23).

The conclusion that expression of Ax 1 may be correlated with cell differentiation of is further supported by the finding that certain cells in the central nervous system were only expressed Ax 1 after they had stopped dividing and had undergone terminal differentiation (24). Ax 1 overexpression was also found in well-differentiated hepatocellular carcinoma tissue (13). This last report suggests that Ax 1 overexpression may be one of several factors that contribute to malignant transformation, progression, and differentiation in hepatocellular carcinoma.

In parallel with the results reported here, we have shown, in A 549 cells, that Ax 1 synthesis and secretion are stimulated by interleukin 6 and that the transcription factor implicated in this regulation was also NFIL-6 (25). However, the pattern of the induced complexes was different, suggesting that, although NFIL-6 participated in both cases, other proteins of the complexes may be different.

In conclusion, our data indicate that, although Ax 1 may be expressed at a high constitutive level, in some cells, its expression is further regulated by differentiating agents as well as by the inflammatory cytokine interleukin 6. An anti-inflammatory role of Ax 1 has been well documented, but a role for Ax 1 in differentiation, which now appears highly probable, is not yet well understood. Therefore, differentiation of A 549 cells may offer a good model for studying the human Ax 1 functions in differentiation and may shed light upon the roles of this gene both in normal development and carcinogenesis.

Materials and Methods

Cell Culture and FACS. The human adenocarcinoma cell line A 549 (kindly provided by Dr. Jamie Croxtall, The William Harvey Research Institute, London, England) was cultured in monolayers in DMEM/F-12 (Life Technologies, Inc.) supplemented with 10% FCS (Life Technologies, Inc.). DEX, PMA, ONPG, ATP, and luciferin were from Sigma Chemical Co. RU 486 was from Roussel-Uclaf (Paris, France). Cells were plated at 5 × 10⁴/plate in 10-mm plates the day after they were treated (or not) for 40 h with 10 nM PMA. To assess the state of differentiation induced by this treatment, CD26 expression (DPP-IV) was measured. Briefly, cells were fixed in 2% paraformaldehyde and incubated for 30 min at 4°C, then washed in 25 mM HEPES supplemented with 1 mM CaCl₂ and MgCl₂ and saponin (0.025% from Saponaria species). Nonspecific binding was blocked with human IgG (1 mg/ml) and incubation with a monoclonal antibody against CD26 (Valbiotech) was performed as reported (19). FACS was performed on a Epics-Elite ( Coultronica) Flow Cytometer equipped with an Argon ion laser beam operating at 488 nm using 15 mW of power to excite the FITC and the propidium iodide. Logarithmic fluorescence histograms (256 channels) were obtained from approximately 5000 viable cells for each sample. With the Elite 4.02 Data Analysis System from Coultronics, mean channel number fluorescence was used to assess differences in fluorescence intensity.

Immunoprecipitation and Western Blot Analysis. A 549 cells were treated with or without 10 nM PMA for 24 h, followed by a further 16-h incubation with or without 1 μM DEX. After this incubation, the protein bound to the membrane was EDTA-extracted from the membrane, and the cells, after being washed in PBS supplemented with 1 mM EDTA and a cocktail of protease inhibitors (Boehringer), were scraped in the same buffer without EDTA. After three cycles of freeze-thawing, cells were centrifuged to eliminate nuclei and broken membranes for preparing cytosol. Both the cytosol and the EDTA supernatant were then processed for immunoprecipitation as described. Immunoprecipitated proteins were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad). Immunostaining was performed using a polyclonal antibody directed against the entire protein, and the immunoreactive bands were detected using enhanced chemiluminescence (Amersham).

DNA Transfections and Luciferase Assay. DNA transfections were performed by the calcium phosphate precipitation technique. Typically, a transfection experiment included 4 μg of reporter plasmid and 0.8 μg of CMV-Lac Z plasmid used for controlling transfection efficiency (26). Cells were harvested 48 h after transfection, and the luciferase activity was measured using a Berthold Luminometer (LB 9501), as described by De Wet et al. (27).

Plasmid Constructions and Mutagenesis. An 880-bp fragment containing the first nontranslated exon and 250 bp of the first intron
was amplified by PCR from a J28p11 phage genomic library (Ref. 28; a kind gift from Dr. J. Browning, Biogen, Cambridge, MA). Artificial SaI and HindIII sites were introduced into the primers RPS5 and RPH3 to facilitate cloning. Oligonucleotides with engineered restriction sites at 5’ and 3’ were used as primers for the PCRs used to obtain all of the mutants. Eight different mutants, spanning the regions −610 to +270 bp and −610 to +20 bp were constructed (see Fig. 3, A and B). The mutants were designated by numbers corresponding to the nucleotide length and the letter M followed by a number from 1 to 8, whereas the nonmutated construct is designated WT. The M1 mutant, pAX (1568)-Luc, contains the region spanning −298 to +270 bp; the M2 mutant, pAX (1450)-Luc, contains the region spanning −180 to +270 bp; the M3 mutant, pAX (1376)-Luc, contains the region spanning −106 to +270 bp; the M4 mutant, pAX (1630)-Luc, contains the region spanning −610 to +20 bp; the M5 mutant, pAX (1318)-Luc, contains the region −298 to +20 bp; the M6 mutant, pAX (1200)-Luc contains the region −180 to +20 bp; M7 mutant pAX 1 (126)-Luc, contains the region spanning −106 to +20 bp; the M8 mutant, pAX (176)-Luc, contains the region from −56 to +20 bp. The PCR-amplified 880-bp segments from the AxA promoter region and the mutants obtained were sequenced by the Sanger method using the United States Biochemical Sequenase kit. The promoter region and all of the mutants at the 5’ end were cloned in a commercial mammalian vector containing the luciferase gene (pGL2-basic-Luc; Promega).

The WT pAX (1880)-Luc construct contained the sequence −180 5’-TTTTGAAAGCCAGTTGAGTAGG-3’, whereas the mutant pAX (1880)-Luc NF-6 mut construct contained the sequence −180 5’-TTTTGAAAGCCAGTTGAGTAGG-3’, where the underlined bases were mutated by site-directed mutagenesis according to Kunkel et al. (29). The C/EBP β expression vector (pMSV-C/EBP β) was a generous gift from Dr. Steven L. McKnight (Carnegie Institution of Washington, Baltimore, MD; Refs. 23 and 30–32).

**Nuclear Proteins Extracts and EMSA.** Nuclear proteins for all of the gel shift experiments were prepared according to Schreiber et al. (33). Gel retardation assays were performed as described previously (34). Briefly, cell nuclear extracts (10 μg), as reported previously (10 μg PMA, 1 μg DEX, or PMA and DEX combined) were incubated with cold synthetic oligonucleotides or the competitors (50 and 200-fold molar excess, respectively) for 15 min at room temperature, and then the 32P-labeled double-stranded synthesized DNA oligonucleotide probe (0.1 pmol, 100,000 cpm) was added, and samples were further incubated for 20 min at room temperature. As a control, labeled oligonucleotides were tested for intrinsic gel shift activity by incubation without nuclear proteins. The oligonucleotides used for gel shift assay were synthesized and purified by Genset SA (Paris, France). The probe covering the sequences −298 to −106 bp was obtained by annealing the following oligonucleotides: oligonucleotide A, 5’-GGGCTAAAAACCCTCTAATTTGTTGATGGTTGATG-3’, oligonucleotide C, 5’-GGGCTAAAAACCCTCTAATTTGTTGATGGTTGATG-3’, oligonucleotide NF-6, 5’-ACACCCCTAGCTGCGCCACCCCA-3’.

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**References**


