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Down-regulation of AP1 activities after polarization of vas deferens epithelial cells correlates with androgen-induced gene expression

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Abstract

Vas deferens epithelial cell subcultures were used to study the sequential regulation of jun/fos proto-oncogene expression and AP1 activities during cell proliferation, polarization and androgen-induced expression of a terminal differentiation marker, i.e. the *mvdp* gene. Proliferation of epithelial cells is associated with a high expression in the nucleus of most Jun and Fos oncoproteins. After cell seeding on an extracellular matrix which allows polarization and expression of the *mvdp* gene in response to androgens, AP1 protein accumulation is greatly altered and consists in a loss of JunB, Fra1, FosB and a decrease in c-Fos, c-Jun and Fra2, while JunD remained at the same level. This was correlated with a drop in AP1 binding activity as evaluated by gel shift assay using either AP1 consensus sequence or AP1 binding sites of the *mvdp* gene promoter region, and in AP1 transactivating activity, as estimated by stable transfection experiments using an AP1 responsive promoter (TRE-TK-luc). Androgens did not significantly influence AP1 activities. On the contrary, stimulation of AP1 proteins by the tumor-promoting phorbol ester caused a decrease in androgen-induced *mvdp* mRNA accumulation, and this effect was reversed by staurosporine, a potent inhibitor of PKC. Our data suggest that a down-regulation of AP1 activities induced by epithelial cell differentiation is a prerequisite to androgen-induced *mvdp* gene expression. The high AP1 activities observed during proliferative state or induced in TPA-treated polarized cells, exert a repressive effect on androgen action.

Keywords: Jun Fos oncoproteins; Differentiation; Androgens; Epithelial cells; Mouse vas deferens

1. Introduction

The development and maintenance of differentiated functions of the male genital tract are highly dependent on androgens. They act via a specific receptor of the superfamily of ligand-dependent transcription factors, the androgen receptor. When complexed to its hormone, the receptor is able to bind to specific DNA sequences and interact with different transcription factors, thus enhancing gene expression (see reviews [1,2]). Cell proliferation and differentiation are complex processes requiring the coordinate participation of multiple gene products that can also modulate androgen action. Proteins of the jun and fos families which are components of the AP1 complex play an important role in the control of the balance between proliferation, differentiation and apoptosis (see reviews [3,4]), and there is increasing evidence that these proto-oncogene products and steroid hormone receptors can influ-

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ence their mutual expression and function [5–7]. Although there are less evidence for the androgen receptor, its interaction with AP1 complex can lead to activation or inhibition of their transacting activity depending on the promoter and the cell line [1,8–11]. Mouse vas deferens protein (MVDP) is an androgen-regulated marker of epithelial cell differentiation. MVDP is mainly expressed in the vas deferens epithelium [12] and to a lesser extent in the adrenal gland [13,14]. It belongs to the superfamily of aldose reductases [15]. In vivo studies have demonstrated that androgens are the major regulator of *mvdp* gene expression in the vas deferens at puberty; however until 10 days after birth, MVDP basal expression is not influenced by androgen status suggesting that other factors are also of critical importance in the programming of MVDP expression during postnatal development [16,17]. Moreover, in subculture, the expression of MVDP in response to androgens only occurs when cells are polarized on an extracellular matrix [18] and depends on a continuous protein synthesis [19]. Thus, other factors associated with cell polarization probably contribute to *mvdp* gene activation. Our cell culture system which allows to monitor the proliferation and cell polarization independently of androgen action, provides a good tool to study the mechanism by which some genes are selectively and sequentially activated at specific stages during cell differentiation. Our work aims to explore the influence of AP1 protein content on the hormonal sensitivity of the androgen-regulated *mvdp* gene.

The present work shows that Jun/Fos proteins are down-regulated following cell polarization and that the androgenic induction of *mvdp* gene transcription occurs when the expression of jun and fos proteins and AP1 binding and transacting activities are very low. The changes in AP1 activities are independent of androgens. Together with the fact that tumor-promoting phorbol ester partly inhibits the androgen-induced *mvdp* gene, these data suggest that AP1 proteins would not participate in androgen-induced gene transcription in differentiated cells but could rather repress *mvdp* gene expression during cell proliferation. Possible interactions in the signaling pathways of androgen receptors and AP1 complexes in the expression of a differentiation marker are discussed.

2. Materials and methods

BioWhitakker (Fontenay sous bois) supplied the Dulbeccos modified Eagles (DMEM) and Ham F12 medium, glutamine, HEPES, transferrin, PBS 10X. Penicillin, streptomycin, bovine insulin, collagen S, epidermal growth factor were from Boehringer, Mannheim (Meylan, France). Cholera toxin, mitomycin C, dibutyril cAMP, selenium, hydrocortisone, phorbol 12-myristate 13-acetate (TPA), dihydrotestosterone were from Sigma (St. Louis, MO). Costar transwell-Coll culture chambers, precoated with collagen type I and III were from Corning Costar Corporation (Cambridge) and non-coated membranes were from Falcon (Becton Dickinson Labware, USA). Matrigel was provided by Collaborative Biomedical Products (Becton Dickinson, USA). RNAzol was from Bioprobe. Antibodies, c-Jun (sc45 and KM-1 sc 822X) and c-Fos (sc-52X) were from Santa Cruz Biotechnology (Santa Cruz, CA). The other antibodies against Jun and Fos proteins were prepared using recombinant fusion proteins [20]. All other chemicals were of reagent grade.

2.1. Culture conditions

Epithelial cell cultures, derived from primary cultures of vas deferens explants were propagated on a NIH 3T3 feader layer as previously described [18]. Cells used were between 20 and 25 passages. They were cultured in a basal mixture of DMEM/Ham F12 (1/1 vol/vol) containing transferrin (10 μg/ml), EGF (10 ng/ml), cholera toxin (10 ng/ml), selenium (17.3 ng/ml), cAMP (1.5 μg/ml), glutamine (2 mM), ethanolamine (0.6 μg/ml), insulin (10 μg/ml), penicillin (100 U/ml), streptomycine (100 μg/ml), Hepes (4.76 μg/ml). For proliferation, hydrocortisone 10^{-6} M was added in the medium. When proliferating epithelial cells reached confluency, the cultures were devoid of 3T3 fibroblast feeder layer which had been pushed away by growing epithelial cells. After trypsinization, epithelial cells were seeded at confluency (1.5×10^6 cells/well) onto collagen-coated microporous membranes, which allow cell polarization and expression of *mvdp* gene. They were cultured in the same medium; the next day, the medium was replaced by the basal medium containing only hydrocortisone at 10^{-8} M to prevent the cells to go on proliferating. After three days, when the whole cell population was homogeneously polarized (observable under microscope), MVDP expression was induced by adding dihydrotestosterone (DHT: 10^{-6} M) in the medium. The medium was changed every two days and the day before trypsinization for analysis. NIH 3T3 fibroblastic cells used as control were cultured in DMEM medium containing 10% fetal calf serum.

2.2. RNA extraction and Northern blot analysis

Before trypsinization, cells cultured with or without DHT (10^{-6} M) were treated with cycloheximide (a translation inhibitor) in the absence or in the presence of TPA (100 ng/ml) for 30 min. Total RNAs were extracted with RNAzol solution, according to the protocol of the manufacturer. After RNA precipitation

with isopropanol, RNA pellets were washed in 75% ethanol and dissolved in EDTA (1 mM). Total RNAs of each sample (30 μg/lane) were subjected to electrophoresis in 1% denaturing formaldehyde–formamide agarose gels and transferred to a Hybond N filter (Amersham, France). The Northern blots were prehybridized overnight at 42°C in a solution containing 50% formamide, 5× SSC, 5× Denhart's solution, 0.5% SDS and 200 μg/ml salmon sperm DNA carrier. Hybridization was performed for 24 h at 42°C in an identical solution containing 10^6 cpm/ml of different cDNA probes. Murine c-fos, c-jun, *mvdp* and 18S cDNA probes were labelled with α^{32}P dCTP (3000 Ci/mmol) using the Megaprime labelling system (Amersham), according to the recommendations of the manufacturer. Membranes were washed twice in 1× SSC, then in 1× SSC, 0.1% SDS and finally in 0.1× SSC, 0.1% SDS for 10 min at 65°C. Northern blots were subjected to autoradiography for 1–3 days to have various signal intensities allowing the best quantitation. Each signal was measured by densitometric analysis using a specifically developed software (Grafteck, Aix en Provence, France). Each mRNA signal intensity for *mvdp*, c-jun and c-fos was reported to the corresponding value measured for 18S mRNA signal, which serves as control for loading. mRNA accumulation from the various conditions was calculated in comparison with the condition giving the highest value (100%).

2.3. Immunocytochemistry

Vas deferens epithelial cells, in proliferation on plastic or differentiated on matrigel-coated Falcon membrane, were fixed for 5 min with methanol containing 5% acetic acid; then, they were permeabilized with 0.2% triton for 4 min. After washing with phosphate buffer (PBS), cells were incubated overnight with the various immune sera against Fra1 (1/400), Fra2 (1/400), FosB (1/100), JunD (1/400), JunB (1/100) or with c-Jun (sc45 or sc822 × , 1/100) or c-Fos (sc52, 1/100). On some samples, double staining was performed with antibodies against proteins and the monoclonal antibody against MVDP (B263, 1/100; [21]). The detections were made using the anti-rabbit IgG TRITC conjugate (1/400) or the biotin conjugated anti mouse or anti-rabbit IgGs (1/200) followed by strepavidin–fluorescein (1/200) system (Amersham, France) as second antibodies. The double staining was examined under a fluorescence microscope with the appropriate filters.

2.4. Protein analysis

The same protein extracts were used for western blot analysis and gel shift assay. Attached cells were rinsed once with PBS and then scraped in 0.7 ml PBS containing 3.5 mM beta-mercaptoethanol. After centrifugation at 1500 rpm, the pellets were resuspended in one volume of buffer C (20 mM Hepes, pH 7.6, 0.42 M NaCl, 1.2 mM $MgCl_2$, 0.2 M EDTA, 25% glycerol plus 1 mM PMSF, aprotinin 2.5 μg/ml, leupeptin 2.5 μg/ml and 2 mM beta-mercaptoethanol). After mixing the suspension, NaCl concentration was adjusted to 0.42 M with a 5 M NaCl solution. The suspension was quickly frozen in liquid nitrogen and stocked at −80°C until analysis. Protein extracts were prepared just before analysis to avoid protein degradation; cell lysis was achieved by thawing on ice; then the extracts were centrifuged at 20,000 ***g*** for 20 min at 4°C; the supernatants were saved and after estimation of protein concentration (Bio-Rad protein assay kit), the samples were prepared on one-dimensional electrophoresis and transferred to nitrocellulose. MVDP was detected either using the monoclonal antibody B263 (1/700) or the polyclonal immune serum (1/5000); proto-oncogene products were detected using rabbit polyclonal antibodies against c-Fos (sc52, 1/1000) c-Jun (sc45, 1/500) or rabbit polyclonal antibodies against Fra1 (1/1000), Fra2 (1/1000), FosB (1/500), JunB (1/500), JunD (1/1000). Specific complexes were detected using the Amersham ECL detection system.

2.5. Electrophoretic mobility shift assay

Nuclear extract preparation was performed as follows: cell pellets were resuspended in buffer A containing 20 mM hepes, 0.5 M EDTA, 230 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine, 0.5 M sucrose, 1 mM PMSF, 1% aprotinin and 1 mM DTT. After centrifugation at 2500 rpm for 5 min, the pellet was resuspended in the same buffer with 0.5% NP40 for 10 min on ice. Nuclei were isolated by centrifugation at 5000 rpm for 10 min and then washed in the same bufffer A, without NP40. Nuclei were lysed in 25–50 μl of the buffer A containing 0.45 M NaCl. The supernatant was collected after centrifugation at 12,000 rpm for 20 min.

The AP1 double-stranded oligonucleotide (Hotfoot TM footprinting Kit, Stratagene, Cambridge) or oligonucleotide sequences from *mvdp* promoter synthesized by MWG Biotech (Les Ulis, France) were labelled with T4 polynucleotide kinase and γ^{32}P dATP (110 TBq nmol, Amersham, France). 6–12 μg whole protein extracts or 8 μg of nuclear protein extracts were incubated 1 h on ice, in gel shift buffer (10 mM HEPES, pH 8, 0.1 mM EDTA, 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 2 mM DTT, 17% glycerol) with 2 μg poly (dI-dC) and 30,000 cpm of probe. Unlabelled AP1 or AP3 oligonucleotides were used at 100–200 fold molar excess. For supershift analysis, specific antibodies were added to the samples before addition of

the probe. Complexes were resolved by electrophoresis through 5–7% polyacrylamide gels (acryl/bisacryl: 29/1) in 0.5× TBE. Oligonucleotides corresponding to consensus AP1 sequence and AP1 sites within the *mvdp* gene promoter are indicated in Fig. 5. The unrelated oligonucleotide was AP3 consensus sequence: 5′CTAGTGGGACTTTCCACAGATC-3′.

2.6. Stable transfection

Vas deferens epithelial cells were stably transfected at the proliferative state with the plasmid carrying the reporter gene encoding the protein luciferase under the control of the thymidine kinase promoter regulated by three repeated AP1 binding consensus sequences (plasmid TRE3-TK-luc). This vector has been demonstrated to be sensitive to activators or repressors of the PKC activity [22]; lipofection was performed with DOTAP (Boehringer, Mannheim). The clone selection was allowed by the cotransfection of plasmid, which confer the resistance to the drug neomycin (G418). Among the TRE3-TK-luc clones, two of those showing different activities were chosen for AP1 activity measurements: clone 12 (AP1 activity = 60,600 units light intensity/μg protein) and clone 98 (AP1 activity = 1,325,000 units light intensity/μg protein). Luciferase activities were evaluated using the luciferase assay system from Promega (Madison, USA), according to the protocol of the manufacturer.

Statistical analyses were made using the test of Mann and Whitney.

3. Results

3.1. Changes in the Jun and Fos protein expression during differentiation and androgenic stimulation of vas deferens epithelial cells

Subcultures of vas deferens epithelial cells provide a useful model to study changes in gene expression during cell differentiation because cell proliferation and polarization occur independent of androgenic stimulation, and once polarized, cells are able to express specific genes in response to an androgenic stimulation. After proliferation, cells seeded at confluency onto collagen-coated membranes stop to divide and establish cell–cell contacts. Polarization of the whole cell population is reached within three days (observable under inverse microscope). From that

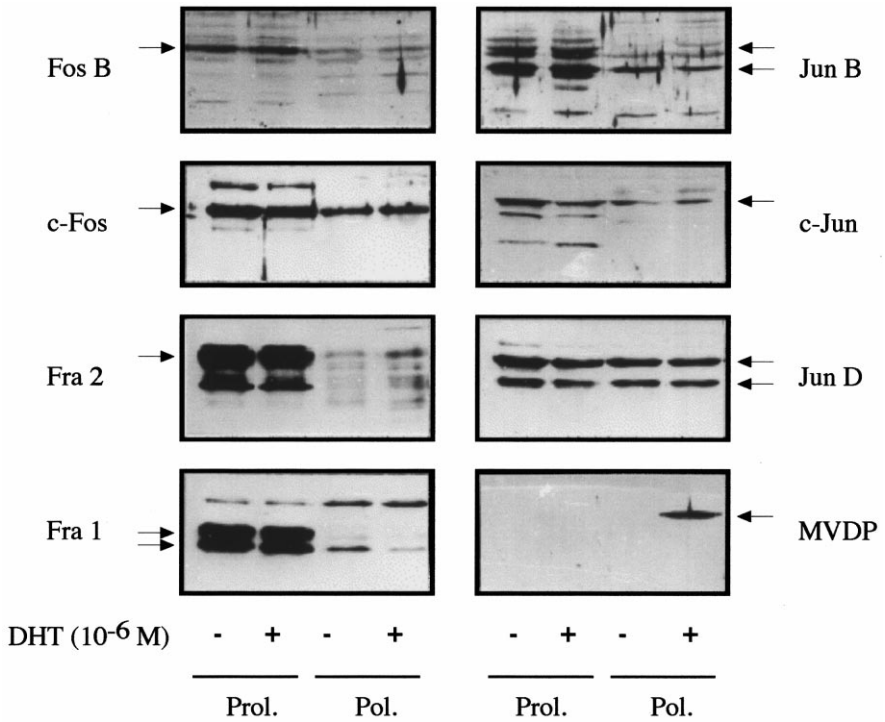

Fig. 1. Down-regulation of Jun and Fos proteins expression during cell differentiation. 30 μg whole cell extracts from epithelial cells at different states of the culture were submitted to SDS page electrophoresis and transferred onto nitrocellulose. Proteins were immunodetected using monoclonal antibody B263 against MVDP (1/700), rabbit polyclonal antibodies against Fra1 (1/1000), Fra2 (1/1000), FosB (1/500), JunB (1/500) and JunD (1/1000) and rabbit antibodies against c-Fos (SC 52 1/1000) or c-Jun (SC 45, 1/1000). The specific complexes were detected using ECL system as described in Section 2. Extracts from NIH 3T3 cells served as positive controls (not shown). Western blots are representative of five independent experiments using either normal vas deferens epithelial cells or stably transfected epithelial cells (clones 12 and 98) used to estimate the AP1 activity. Arrows indicate the position of the specific bands detected by the antibodies.

time, cells are able to respond to androgen stimulation for expressing the *mvdp* gene [18].

AP1 proteins detected by western-blotting were differentially expressed in function of the culture state (Fig. 1). In proliferative cells, c-Fos, FosB, Fra1 and Fra2 were coexpressed with Jun proteins, JunD, c-Jun and JunB. After cell polarization, only JunD was easily detectable. The others were greatly reduced or lost. The c-Jun and c-Fos proteins were still detectable in polarized cells. Androgen status had no influence on the Jun and Fos protein expression, neither during cell proliferation nor during differentiation. The results from five independent experiments were highly reproducible.

Since the transcriptional activity of Jun and Fos proteins is partly controlled by their nuclear transloca-

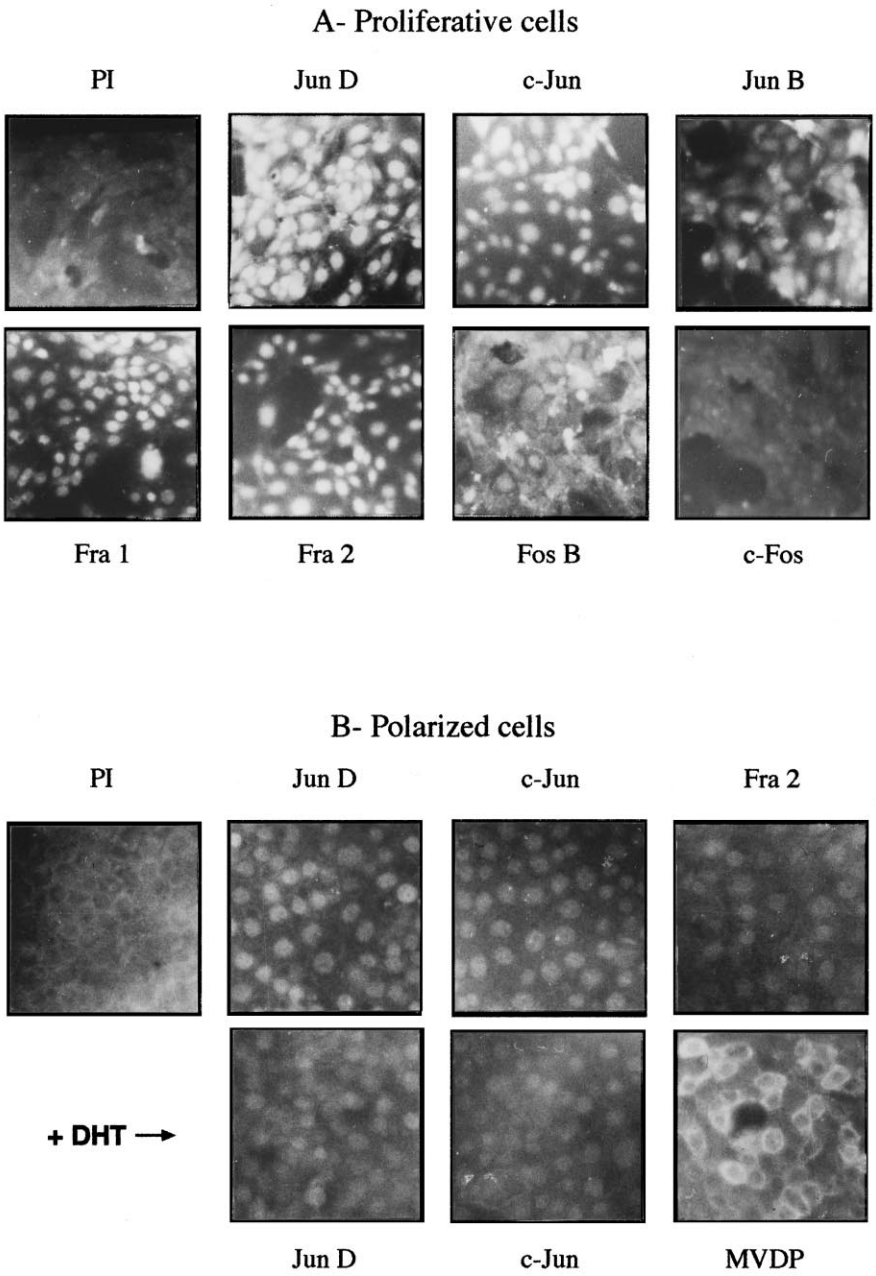

Fig. 2. Immunocytochemical detection of Jun and Fos protein expression in vas deferens epithelial cells at (A) proliferative or (B) differentiated states. Proliferative cells cultured on glass or cells polarized on matrigel-coated Falcon membranes and cultured in the absence (upper part) or in the presence of dihydrotestosterone (DHT, lower part) were fixed and then permeabilized, as described in Section 2. The antibodies used were: B263 monoclonal antibody against MVDP (1/200); rabbit antibodies against Fra1 (1/400), Fra2 (1/400), FosB (1/100), Jun D (1/400), JunB (1/400) or mouse monoclonal anti c-Jun (KM-1: Sc 822 × 1/100) and rabbit polyclonal anti c-Fos (Sc 52 1/100). Detection was made using either fluorescein or TRITC conjugates as described in Section 2 (magnitude: 340).

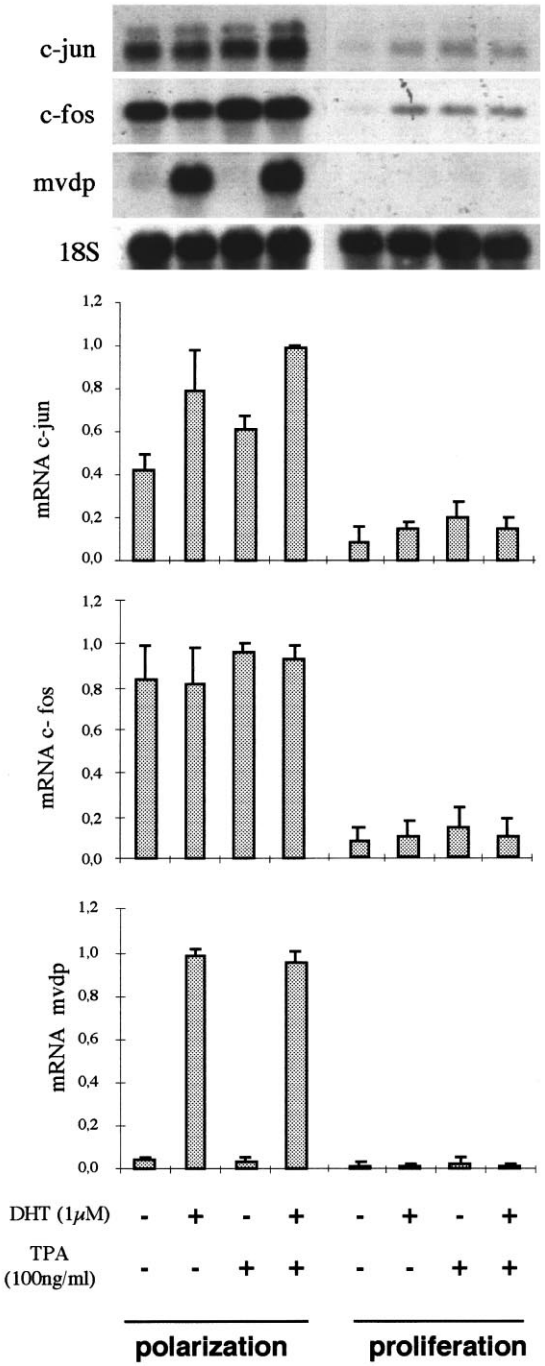

Fig. 3. Transcriptional activity of c-fos and c-jun genes during epithelial cells polarization: modulation by androgens and TPA. Proliferative or polarized cells were cultured without or with DHT (1 μM) for three days; then they were incubated for 30 min with cycloheximide in presence or absence of TPA (100 ng/ml). 30 μg of total RNA were separated on agarose gel and then transferred onto hybond N membrane. Northern blots were hybridized with the murine c-fos, c-jun, *mvdp* and 18S cDNA probes as described in Section 2. Upper part: representative northern blot autoradiography; lower part: cumulative data shown as the mean SE of at least three individual experiments.

tion and that it can be modulated by cytosolic inhibitors [23,24], immunocytochemistry was used to determine their precise intracellular localization (Fig. 2). Fra1, Fra2, c-Jun and JunD were clearly expressed in the nucleus during proliferation. JunB and FosB staining was low and diffused in the nucleus and cytoplasm. No staining was obtained for c-Fos probably because the antibody is inefficient in immunochemistry conditions while it works well in Western-blot (Fig. 1). After cell polarization, a nuclear staining was observed specifically with antibodies against c-Jun, JunD and Fra2. After androgenic stimulation for three days, only JunD and c-Jun were clearly detected in the nucleus of every cell (Fig. 2); JunB, FosB, Fra1 and Fra2 were not detected any more (not shown).

3.2. Changes in c-jun and c-fos mRNA accumulation during cell differentiation and androgenic stimulation

Thereafter, northern-blot analysis was done to test whether the decrease in c-Jun and c-Fos protein levels after cell polarization was due to a transcriptional event.

Proliferative cells and cells polarized for three days were cultured with or without DHT (1 μM) for three additional days and then treated in presence or absence of TPA (100 ng/ml) for 30 min with cycloheximide. Cycloheximide is known to induce an accumulation of c-jun and c-fos mRNAs due to inhibition of both mRNA degradation and translation [3]. As shown in Fig. 3, c-jun mRNA (3.4 and 2.6 kb) and c-fos mRNA (2.2 kb) accumulation were increased four fold ($p < 0.05$) and eight fold ($p < 0.005$), respectively, after cell polarization. In polarized cells, addition of DHT in the medium induced a two-fold increase ($p < 0.05$) in c-jun mRNA levels and did not change c-fos mRNA levels. TPA induced a similar increase ($p < 0.05$) in c-jun mRNA accumulation as did DHT. In the presence of DHT, the values obtained in response to TPA were the highest but not significantly different from that with DHT alone. Thus, the decrease in c-Jun and c-Fos protein expression was not due to a decrease in gene transcription.

3.3. Change in AP1 binding activity during cell differentiation and androgenic stimulation on the mvdp promoter

The *mvdp* promoter contains several putative sites for AP1 complexes. We scanned the 1.8 kb 5′-flanking region of the *mvdp* gene to localize AP1 binding sequences using the TESS-String-based Search (Transfac v3.2). Three of them located at positions -1791/-1767 (AP1a), -1459/-1435 (AP1b), -378/-356

(AP1c) as indicated in Fig. 4(a) were tested for their ability to bind AP1 complexes from proliferative epithelial cell extracts. Only sequences AP1b (Fig. 4b) and to a lower extent AP1c (not shown) were able to specifically retain AP1 complexes. Intensities of retarded complexes with the AP1b oligonucleotide were much higher in proliferation than after cell polarization (Fig. 4b). To test the relative affinity of each binding site, the corresponding oligonucleotides (in 100–200 molar excess) were tested for competition with the AP1 consensus sequence (AP1cs). Fig. 4(c) shows that the AP1a oligonucleotide was unable to compete with AP1 consensus oligonucleotide while there was a strong and low competition with the AP1b and AP1c oligonucleotides, respectively.

3.4. Change in AP1 transactivating activity during cell differentiation and androgenic stimulation

AP1 activity was estimated from stably transfected cells with an expression vector carrying the luciferase coding region under the control of the herpes simplex virus thymidine kinase promoter associated with three TPA-responsive elements able to bind the AP1 complexes (vector TRE3-TK-luc). This promoter construct was previously shown to be inducible by TPA [22]. As observed in Fig. 5, TRE3-TK-luc activity from two independent cell clones was dramatically decreased after cell polarization (85–95%, $p < 0.05$). Addition of DHT (1 μM) in the culture medium did not change luciferase activity.

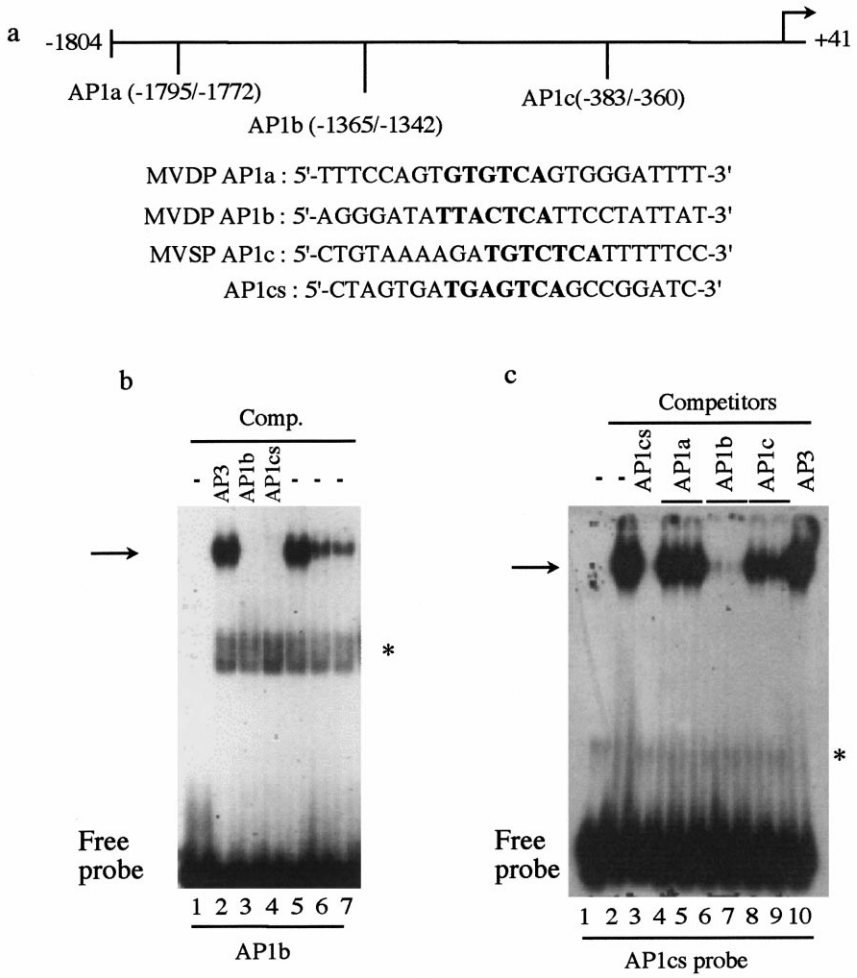

Fig. 4. Specific AP1 binding activity of AP1 sequences within the *mvdp* gene promoter in proliferative epithelial cells. (a) Sequences and localization of three potential AP1 binding sequences in the *mvdp* promoter. (b) Specific binding capacity of the AP1b sequence: 8 μg of nuclear extracts from proliferative cells (lanes 2–5) or polarized cells cultured without DHT (lane 6) or with 1 μM DHT (lane 7) were incubated with labelled double-stranded AP1b and competed with a 100-fold molar excess of double-stranded unlabelled AP1b (lane 3) or 100-fold molar excess of either consensus AP1 oligonucleotide (lane 4) or unrelated AP3 oligonucleotide (lane 2). (c) Relative affinity of *mvdp* gene AP1 binding sites: 10 μg whole cell extracts from proliferative epithelial cells were incubated with labelled AP1 consensus oligonucleotide and competed with a 100-fold molar excess of unlabelled AP1 consensus oligonucleotide (lane 3), with a 100- and 200-fold molar excess of unlabelled AP1a (lane 4–5), AP1b (lane 6–7), AP1c (lane 8–9) oligonucleotides and with a 100-fold excess of AP3 unrelated oligonucleotide (lane 10). The autoradiograms are representative of two series of experiments. Specific and non specific complexes are indicated by arrows and asterics, respectively.

3.5. Effect of the tumor-promoting phorbol ester (TPA) on the androgenic induction of mvdp mRNA accumulation

The Jun and Fos proteins are components of the PKC pathway which enhances Jun and Fos proteins at the post-translational level by modification of their phosphorylation status as well as their synthesis. To test whether Jun and Fos proteins are involved in androgen-induced *mvdp* mRNA accumulation, polarized cells were incubated for 12 h with 1 μM DHT in the presence or absence of TPA (100 ng/ml). The results show that activation of expression and transcriptional activity of AP1 proteins by phorbol ester strongly inhibits androgen-induced transcription of the *mvdp* gene (Fig. 6). Addition of DHT in the medium caused a 150% increase in *mvdp* mRNA accumulation. Simultaneous treatment with TPA significantly ($p < 0.05$) reduced by 46% the DHT-induced mRNA levels. This effect was reversed when an inhibitor of PKC, staurosporine at 40 nM, was added with TPA. In the absence of DHT, the incubation of cells with DMSO (the vehicle) or TPA or TPA plus Staurosporine did not significantly change the basal levels of *mvdp* mRNA. Note that a treatment for 30 min with TPA at the end of DHT incubation period, as described previously to study proto-oncogene transcription stimulation (Fig. 3), is too short to influence the levels of *mvdp* mRNA, already accumulated for 72 h after DHT induction.

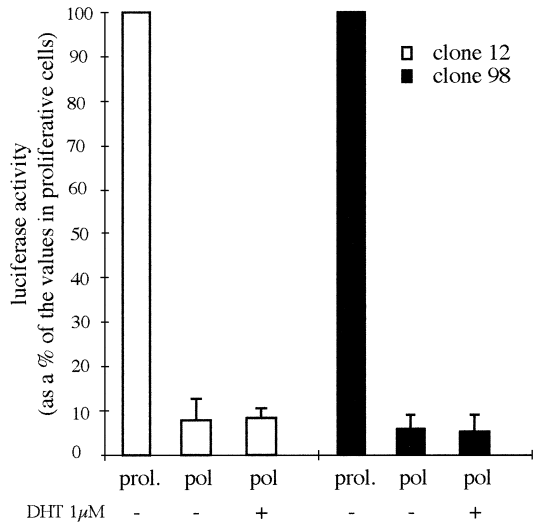

Fig. 5. Changes in AP1 transactivating activity during epithelial cell differentiation. Increasing concentrations of protein extracts (1–10 μg) from stably transfected epithelial cell clones (clones 12 and 98), expressing the TRE-TK-luc construct, were analyzed for change in luciferase activity at different culture states (in proliferation or after differentiation on microporous membranes, with or without DHT). For comparison, the histogram represents mean values (standard deviation) expressed as percentage of the luciferase activity in proliferative cells.

At the protein level, the inhibitory influence of TPA was of the same order of magnitude; addition of TPA with DHT for 24 h lowered by 36% the MVDP levels (results not shown).

4. Discussion

Using an appropriate cell culture system, in which cell proliferation and polarization can be monitored easily and independently of androgen action, we have shown that cell differentiation and polarization are marked by a dramatic decrease in the accumulation of most AP1 proteins (excepted a constitutive expression of JunD). This drop of Jun and Fos protein concentrations is associated both to a decrease in AP1 binding activity, evaluated by EMSA, and to a decrease in AP1 transacting activity, evaluated by stable transfection of AP1 responsive promoter construct. Experiments using cycloheximide and TPA have

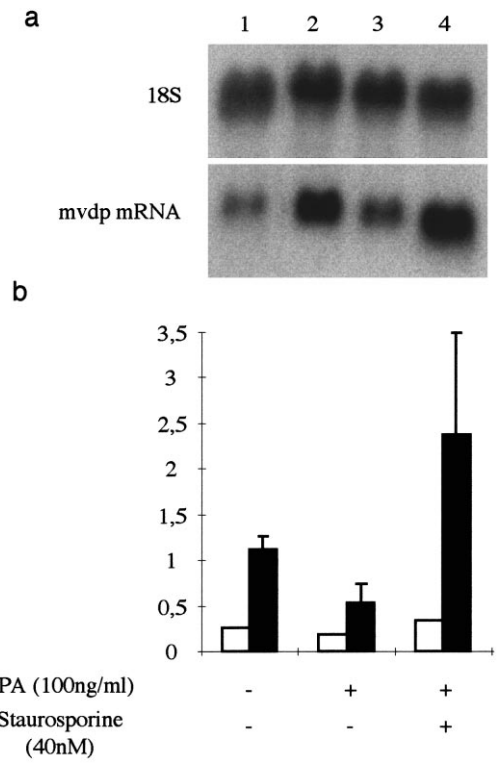

Fig. 6. Effect of TPA and staurosporine on DHT-induced *mvdp* mRNA accumulation in polarized epithelial cells. Cells were incubated for 12 h with 1 μM DHT in the presence or absence of TPA (100 ng/ml) or TPA plus staurosporine (40 nM). The northern blots were hybridized with *mvdp* and 18S cDNA probes. (a) Representative northern blot autoradiograms. Cells cultured without DHT (lane 1) or with DHT (lanes 2–4). They were also treated with the DMSO (as vehicle, lanes 1 and 2) or with TPA (lane 3) or with TPA plus staurosporine (lane 4). (b) Histograms showing cumulative data as the mean (±SE) from three individual experiments. Cells cultured without DHT (white colums) or with DHT (black colums) were treated with DMSO or with TPA or with TPA plus staurosporine.

demonstrated that the differentiation-induced decreased expression of c-Jun and c-Fos proteins was not due to alteration in gene transcription but rather to post-transcriptional modifications leading to a greater instability of mRNA and/or proteins.

As there was no change in the medium composition, the low AP1 activity in differentiated cells is the direct result of cell growth arrest, which is induced by seeding cells at confluency on an extracellular matrix, and this event is independent of androgen action. As in our cell culture system, the down-regulation of AP1 protein levels (especially c-Jun and c-Fos) and the decrease in AP1 DNA binding activity observed in hepatocytes cultured on laminin rich matrix was due to growth inhibition [25]. Cell density, which promote E cadherin cell–cell adhesion, is known to induce in various cell line changes in the binding capacity of various growth factor receptors such as those of FGF, PDGF [26] or cytokine receptors [27]. This can result in prevention of receptor activation (i.e. EGFR, [28]) and modification of phosphorylation status of nuclear factors able to activate Jun and Fos expression and AP1 transacting activities. More generally, growth arrest associated down-regulation of AP1 transcription factors is a prerequisite to cell differentiation of adipocytes [29], myocytes [30], osteoblastes [31], and keratinocytes [32,33]. Conversely, long term overexpression of oncoproteins such as c-FOS induces cell depolarization and epithelial-fibroblastoid cell conversion [34].

There are at least two specific AP1 binding sites within the 5′-flanking region of the *mvdp* gene (AP1b,c) which bind Jun/Fos complexes mainly at the proliferative state as observed in EMSA. The core sequence is similar to those of some AP1 binding sites (TGTGTCA and TTACTCA) which have been shown to regulate the E6/E7 oncogenes of the human papillomavirus. This study also showed a down-regulation of E6/E7 oncoprotein expression after epithelial cell differentiation [33]. The presence of these sequences has raised the question whether AP1 complexes could interfere with androgen receptor signaling pathway in *mvdp* gene transactivation. Our data provide evidence that AP1 activity is inversely correlated to MVDP expression. Moreover, the tumor-promoting phorbol ester (TPA) is able to decrease MVDP expression in response to DHT. This effect is fully reversed by staurosporine (a potent PKC inhibitor) which in addition seems to potentiate DHT action. Thus, AP1 proteins could have a repressive effect on *mvdp* gene transactivation. They could either repress through AP1 binding sites or indirectly through interactions with the androgen receptor. The last hypothesis seems more likely since AP1 protein interactions with androgen receptors have been recently reported [9,11,35] and thus could explain the repressive effect of TPA observed on different promoters: (i) the 0.5 kb flanking region of the *mvdp* gene promoter (which does not contain AP1 binding sequence) in heterologous CV1 cells [35], (ii) the PSA promoter in LNCap cells [11,36]. DNA binding independent-action of AP1 proteins is conforted by the fact that opposite effects of TPA on the *mvdp* promoter have been previously observed using transfection experiment in the heterologous T47D cell line: TPA enhanced the androgen-induced activation of *mvdp* promoter constructs containing either the 1.8 kb 5′ flanking region (containing AP1 binding sites) or the 0.16 kb 5′ flanking region devoid of AP1 binding site [37,38]. All these data suggest that AP1 binding sites within the *mvdp* gene promoter are poorly active and that AP1 repressive activity is likely mediated by protein–protein interactions. This could result from direct binding to the androgen receptor but one cannot exclude that AP1 complex and the androgen receptor could compete for common coactivators such as CBP or F-SRC-1, thus inducing a decrease in *mvdp* gene transcription [39–41]. Furthermore, combinatory protein interactions with the transcription machinery would also explain cell-specific modulations of the androgen receptor activity even in the absence of the ligand [42,43,44].

In vas deferens epithelial cells, androgen-induced *mvdp* gene transcription is dependent on cell polarization and this expression is inversely correlated with AP1 protein expression and transacting activity. Contrary to previous experiments using heterologous T47D tumoral cells [37,38], this study shows that, in vas deferens epithelial cells ex vivo, the AP1 complex does not participate to androgen-induced *mvdp* gene transcription but that its down-regulation is a prerequisite to androgen action. Indeed during cell proliferation, high levels of AP1 would block androgen action by interacting with the androgen receptor. Inversely after cell polarization which is required for androgenic stimulation of *mvdp* gene transcription, the AP1/androgen receptor ratio is reversed: AP1 protein levels become very low while androgen receptor levels are upregulated by androgens [45]. To assess the role of AP1 proteins in the repression of the *mvdp* promoter during cell proliferation, further experiments in which AP1 protein expression would be inhibited by stable expression of antisens or negative dominant oncoprotein cDNAs would be necessary.

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References

[1] P.J. Kallio, T. Ikonen, A. Moilanen, H. Poukka, P. Reinikainen, J.J. Palvimo, A.O. Jänne, Androgen receptor in transcriptional regulation, in: V. Hanssoz, F.O. Levy, F. Tasken, Ernst Shering Research Foundation Workshop, 1996, vol. 2, pp. 167–184.

[2] M. Beato, A. Sanchez-Pacheco, Interaction of steroid hormone receptors with the transcription initiation complex, Endocrine Rev. 17 (1996) 587–609.

[3] P. Angel, M. Karin, The role of Jun, Fos and the AP- complex in cell-proliferation and transformation, Biochim. Biophys. Acta 1072 (1991) 129–157.

[4] M. Karin, Z.G. Liu, E. Zandi, AP function and regulation, Curr. Opin. Cell. Biol. 9 (1997) 240–246.

[5] W.V. Vedeckis, Nuclear receptors, transcriptional regulation and oncogenesis, Proc. Soc. Exp. Biol. Med. 199 (1992) 1–12.

[6] A. Weisz, F. Bresciani, Estrogen regulation of proto-oncogenes coding for nuclear proteins, Crit. Rev. Oncog. 4 (1993) 361–388.

[7] M. Schuchard, J.P. Landers, N. Punkay-Sandhu, T.C. Spelsberg, Steroid hormone regulation of nuclear proto-oncogenes, Endocrine Rev. 14 (1993) 659–669.

[8] L. Shemshedini, R. Knauthe, P. Sassone-Corsi, A. Pornon, H. Gronemeyer, Cell-specific inhibitory and stimulatory effects of Fos and Jun on transcription activation by nuclear receptors, Embo. J. 10 (1991) 3839–3849.

[9] A. Bubulya, S.C. Wise, X.-Q. Shen, L.A. Burmeister, L. Shemshedini, c-Jun can mediate androgen receptor-induced transactivation, J. Biol. Chem. 271 (1996) 24583–24589.

[10] P.E. Murtha, W. Zhu, J. Zhang, S. Zhang, C.Y.F. Young, Effect of Ca^{++} mobilization on expression of androgen-regulated genes: interference with androgen receptor-mediated transactivation by AP-1 proteins, Prostate 33 (1997) 264–270.

[11] N. Sato, M.D. Sadar, N. Bruchovsky, F. Saatcioglu, P.S. Rennie, S. Sato, P.H. Lange, M.E. Gleave, Androgenic induction of prostate-specific antigen gene is repressed by protein–protein interaction between the androgen receptor and AP-1/c-Jun in the human prostate cancer cell line LNCaP, J. Biol. Chem. 272 (1997) 17485–17494.

[12] C. Taragnat, M. Berger, Cl. Jean, Preliminary characterization, androgen-dependence and ontogeny of an abundant protein from mouse vas deferens, J. Reprod. Fertil. 83 (1988) 835–842.

[13] E.T. Lau, D. Cao, C. Lin, S.K. Chung, S.S. Chung, Tissue-specific expression of two aldose reductase-like genes in mice: abundant expression of mouse vas deferens protein and fibroblast growth factor-regulated protein in the adrenal gland, Biochem. J. 312 (1995) 609–615.

[14] C. Aigueperse, A. Martinez, A.M. Lefrançois-Martinez, G. Veyssiere, C. Jean, Cyclic AMP regulates expression of the gene coding for the mouse vas deferens protein related to the aldo–keto reductase superfamily in human and murine adrenocortical cells, J. Endocrinol. 160 (1999) 147–154.

[15] E.A. Pailhoux, A. Martinez, G.M. Veyssiere, Cl.G. Jean, Androgen-dependent protein from mouse vas deferens cDNA cloning and protein homology with the aldo–keto reductase superfamily, J. Biol. Chem. 265 (1990) 19932–19936.

[16] A. Martinez, E. Pailhoux, M. Berger, Cl. Jean, Androgen regulation of the mRNA encoding a major protein of the mouse vas deferens, Mol. Cell. Endocrinol. 72 (1990) 201–211.

[17] A. Martinez, M. Manin, E. Pailhoux, Cl. Jean, Characterization, molecular cloning, developmental and hormonal regulation of an abundant mouse vas deferens protein related to the aldo-keto reductase superfamily, Curr. Trends Exp. Endocr. 1 (1993) 19–26.

[18] M. Manin, G. Veyssière, D. Cheyvialle, M. Chevalier, P. Lécher, C. Jean, In vitro androgenic induction of a major protein in epithelial cell subcultures from mouse vas deferens, Endocrinology 131 (1992) 2378–2386.

[19] A. Dassouli, M. Manin, G. Veyssire, Cl. Jean, Androgens regulate expression of the gene coding for a mouse vas deferens protein related to the aldo–keto reductase superfamily in epithelial cell subcultures, J. Steroid Biochem. Molec. Biol. 48 (1994) 121–128.

[20] D. Lallemand, G. Spyrou, M. Yaniv, C.M. Pfarr, Variations in Jun and Fos protein expression and AP-1 activity in cycling resting and stimulated fibroblasts, Oncogene 14 (1997) 819–830.

[21] C. Taragnat, M. Berger, Cl. Jean, Tissue and species specificity of mouse ductus deferens protein, J. Androl. 11 (1990) 279–286.

[22] M.E. Astruc, C. Chabret, P. Bali, D. Gagne, M. Pons, Prolonged treatment of breast cancer cells with antiestrogens increases the activating protein-1-mediated response involvement of the estrogen receptor, Endocrinology 136 (1995) 824–832.

[23] P. Roux, J.M. Blanchard, A. Fernandez, N. Lamb, P. Jeanteur, M. Prechaczyk, Nuclear localization of c-Fos but not v-Fos proteins, is controlled by extracellular signals, Cell 63 (1990) 341–351.

[24] J. Auwerx, P. Sassone-Corsi, IP-1: a dominant inhibitor of Fos/Jun whose activity is modulated by phosphorylation, Cell 64 (1991) 983–993.

[25] B. Rana, D. Mischoulon, Y. Xie, N.L. Bucher, S.R. Farmer, Cell-extracellular matrix interactions can regulate the switch between growth and differentiation in rat hepatocytes reciprocal expression of C/EBP alpha and immediate-early growth response transcription factors, Mol. Cell. Biol. 14 (1994) 5858–5869.

[26] A. Rizzino, P. Kazakoff, E. Ruff, C. Kuszynski, J. Nebelsick, Regulatory effects of cell density on the binding of transforming growth factor beta epidermal growth factor platelet-derived growth factor and fibroblast growth factor, Cancer Res. 48 (1988) 4266–4271.

[27] E. Pocsik, R. Mihalik, F. Ali-Osman, B.B. Aggarwal, Cell density-dependent regulation of cell surface expression of two types of human tumor necrosis factor receptors and its effect on cellular response, J. Cell. Biochem. 54 (1994) 453–464.

[28] K. Takahashi, K. Suzuki, Density-dependent inhibition of growth involves prevention of EGF receptor activation by E-cadherin-mediated cell–cell adhesion, Exp. Cell. Res. 226 (1996) 214–222.

[29] H. Wang, R.E. Scott, Adipocyte differentiation selectively represses the serum inducibility of c-jun and junB by reversible transcription-dependent mechanisms, Proc. Natl. Acad. Sci. USA 91 (1994) 4649–4653.

[30] S.K. Lehtinen, P. Rahkila, M. Helenius, P. Korhonen, A. Salminen, Down regulation of transcription factors AP-1, Sp-1 and NF-kappa B precedes myocyte differentiation, Biochem. Biophys. Res. Commun. 229 (1996) 36–43.

[31] L.R. McCabe, C. Banerjee, R. Kundu, R.J. Harrison, P.R. Dobner, J.L. Stein, J.B. Lian, G.S. Stein, Developmental expression and activities of specific Fos and Jun proteins are functionally related to osteoblast maturation role of Fra-2 and JunD during differentiation, Endocrinology 137 (1996) 4398–4408.

[32] P. Briata, F. D'Anna, A.T. Franzi, R. Gherzi, AP-1 activity during normal human keratinocyte differentiation: evidence for a cytosolic modulator of AP-1/DNA binding, Exp. Cell. Res. 204 (1993) 136–146.

[33] S. Kyo, D.J. Klumpp, M. Inoue, T. Kanaya, L.A. Laimins, Expression of AP1 during cellular differentiation determines

human papillomavirus E6/E7 expression in stratified epithelial cells, J. Gen. Virol. 78 (1997) 401–411.
[34] E. Reichmann, H. Schwarz, E.M. Deiner, I. Leitner, M. Eilers, J. Berger, M. Busslinger, H. Beug, Activation of an inducible c-Fos ER fusion protein causes loss of epithelial polarity and triggers epithelial–fibroblastoid cell conversion, Cell 71 (1992) 1103–1116.
[35] J.M. Lobaccaro, N. Poujol, B. Terouanne, V. Georget, S. Fabre, S. Lumbroso, C. Sultan, Transcriptional interferences between normal and mutant androgen receptors nad the protein 1-dissection of the androgen receptor functional domain, Endocrinology 140 (1999) 350–357.
[36] P.E. Andrews, C.Y. Young, B.T. Montgomery, D.J. Tindall, Tumor-promoting phorbol ester down-regulates the androgen induction of prostate-specific antigen in a human prostatic adenocarcinoma cell line, Cancer Res. 52 (1992) 1525–1529.
[37] S. Fabre, C. Darne, G. Veyssiere, Cl. Jean, Characterization of the promoter of the gene for a mouse vas deferens protein related to the aldo–keto reductase superfamily: effect of steroid hormones and phorbol esters, J. Steroid Biochem. Molec. Biol. 55 (1995) 315–325.
[38] S. Fabre, C. Darne, G. Veyssiere, Cl. Jean, Protein kinase C pathway potentiates androgen-mediated gene expression of the mouse vas deferens specific aldose reductase-like protein (MVDP), Mol. Cell. Endocrinol. 124 (1996) 79–86.
[39] T. Ikonen, J.J. Palvimo, O.A. Janne, Interaction between the amino- and carboxyl-terminal regions of the rat androgen receptor modulates transcriptional activity and is influenced by nuclear receptor coactivators, J. Biol. Chem. 272 (1997) 29821–29828.
[40] P. Aarnisalo, J.J. Palvimo, O.A. Janne, CREB-binding protein in androgen receptor-mediated signalling, Proc. Natl. Acad. Sci. USA 95 (1998) 2122–2127.
[41] S.A. Onate, S.Y. Tsai, M.J. Tsai, B.W. O'Malley, Sequence and characterization of a coactivator for the steroid hormone receptor superfamily, Science 270 (1995) 1354–1357.
[42] P.J. Kallio, H. Poukka, A. Moilanen, O.A. Jänne, J.J. Palvimo, Androgen receptor-mediated transcriptional regulation in the absence of direct interaction with a specific DNA element, Mol. Endocrinol. 9 (1995) 1017–1028.
[43] P.E. de Ruiter, R. Teuwen, J. Trapman, R. Dijkema, A.O. Brinkmann, Synergism between androgens and protein kinase C on androgen-regulated gene expression, Mol. cell. Endocrinol. 110 (1995) R1–R6.
[44] Ch. Darne, G. Veyssière, Cl. Jean, Phorbol ester causes ligand-independent activation of the androgen receptor, Eur. J. Biochem. 265 (1998) 541–549.
[45] A. Dassouli, Ch. Darne, S. Fabre, M. Manin, G. Veyssière, Cl. Jean, Vas deferens epithelial cells in subculture: a model to study androgen regulation of gene expression, J. Mol. Endocrinol. 15 (1995) 129–141.