# Differences in expression of transcription factor AP-1 in human promyelocytic HL-60 cells during differentiation towards macrophages versus granulocytes

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Commitment of HL-60 cells to macrophage or granulocytic differentiation was achieved by incubation with 4$\beta$-phorbol 12-myristate 13-acetate (PMA) for 30–60 min or with dimethyl sulphoxide (DMSO) for 24 h respectively. The commitment stage towards PMA-induced macrophage differentiation was associated with increases in *jun* B and c-*fos* mRNA levels, as well as with an increase in the binding activity of transcription factor AP-1. Nevertheless, gel retardation analysis indicated that the AP-1 activity detected in untreated cells was drastically reduced during the commitment stage of DMSO-induced HL-60 differentiation towards granulocytes. When HL-60 cells were treated with sodium butyrate, which induced monocytic differentiation, a remarkable increase in AP-1 binding activity was detected. Treatment of HL-60 cells with 1$\alpha$,25-dihydroxyvitamin $D_3$, another monocytic differentiation agent, induced a weak, but appreciable, increase in AP-1 activity. Furthermore, addition of sodium butyrate or 1$\alpha$,25-dihydroxyvitamin $D_3$ to HL-60 cells induced the expression of c-*fos*, c-*jun*, *jun* B and *jun* D proto-oncogenes. In contrast, when HL-60 cells were treated with retinoic acid, a granulocytic differentiation inducer, no enhanced AP-1 binding activity was observed, and only a weak increase in *jun* D mRNA level was detected. These data indicate that formation of AP-1 is not required for the induction of HL-60 differentiation towards granulocytes, whereas induction of monocytic differentiation is correlated with an increase in AP-1 activity. The differential expression of AP-1 activity may be critical in the differentiation of HL-60 cells towards monocytic or granulocytic lineages.

## INTRODUCTION

Mature blood cells originate from a self-renewing population of multipotential haemopoietic stem cells, localized mainly in the bone marrow, which generate progenitor cells irreversibly committed to originate different haemopoietic lineages. Understanding of the molecular mechanisms that control differentiation and maturation of haemopoietic cells is crucial for the clinical manipulation of blood-cell production. The human promyelocytic HL-60 cell line has been used to study gene expression during the differentiation of myeloid cells. HL-60 cells can be induced to differentiate into granulocyte-like cells by treatment with dimethyl sulphoxide (DMSO) or retinoic acid [1–4], whereas treatment of HL-60 cells with 4$\beta$-phorbol 12-myristate 13-acetate (PMA), sodium butyrate or 1$\alpha$,25-dihydroxyvitamin $D_3$ results in differentiation towards the monocytic lineage [2,5–7]. PMA induces HL-60 to differentiate to cells exhibiting macrophage characteristics, while sodium butyrate and 1$\alpha$,25-dihydroxyvitamin $D_3$ induce HL-60 cells to differentiate towards cells showing monocyte characteristics [2,5–7].

Expression of nuclear proto-oncogenes is thought to be involved in the induction of cellular proliferation and differentiation [8,9]. Previous studies in embryonal carcinoma cells have suggested a role for c-*jun* in cell differentiation [10]. The nuclear proto-oncogene c-*jun* encodes for a component of the transcription factor AP-1 [11,12], which binds to regulatory sequences upstream of numerous viral and cellular promoters and is important in regulating the transcriptional activation of these genes [11–14]. c-Jun/AP-1 homodimer binds weakly to the heptameric DNA consensus sequence TGA(C/G)TCA, but the binding affinity of AP-1 for its cognate sequence dramatically increases when heterodimerization with c-Fos occurs [13–19]. Thus, the c-Fos/c-Jun heterodimer has a much higher affinity to the AP-1 enhancer sequence than the c-Jun homodimer, whereas c-Fos is not able to form homodimers [13–19]. As dimer formation, mediated through the 'leucine zipper' domains, is a prerequisite for high-affinity binding to the AP-1 binding site, c-Fos does not bind DNA by its own [13–19] and therefore does not stimulate transcription in c-Jun's absence [20,21]. Two related proteins, named Jun B and Jun D, have similar structures to c-Jun and also form nuclear complexes with c-Fos or Fos-related proteins [22–24]. However, in spite of these structural similarities, the three members of the *jun* family are differently expressed during development [25] as well as in distinct tissues [24,26,27] and cell lines [26,28]. Furthermore, Jun proteins have been shown to have different biochemical and physiological properties, displaying distinct DNA binding and transactivating activities [20,29]. Thus Jun B is an efficient activator of artificial promoters which contain multimeric AP-1 sites, but inhibits transactivation by c-Jun on promoters containing only one AP-1 site [30]. These findings suggest that Jun proteins play distinct roles in regulating gene expression and might be used differently by various cells in response to external or internal environmental stimuli.

Induction of c-*fos* mature transcripts has been detected during monocytic differentiation of HL-60 cells induced by PMA or 1$\alpha$,25-dihydroxyvitamin $D_3$ [31,32]. However, other studies suggest that c-*fos* expression is not sufficient to commit cells towards monocytic differentiation [33,34]. On the other hand,

Abbreviations used: DMSO, dimethyl sulphoxide; PMA, 4$\beta$-phorbol 12-myristate 13-acetate.

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differentiation of HL-60 cells towards granulocytic lineage by DMSO does not induce appreciable levels of c-*fos* transcripts [31]. Induction of c-*jun* mature transcripts has been recently reported during monocytic differentiation of HL-60 cells [28,35,36]. In contrast, c-*jun* mRNA expression is not detected when HL-60 cells are induced to differentiate towards granulocyte-like cells by treatment with DMSO [28,36]. We have previously reported a distinct expression of c-*jun*, *jun* B and *jun* D proto-oncogenes during PMA-induced HL-60 differentiation [28], and an increase in *jun* B and *jun* D mRNA levels during DMSO-induced HL-60 differentiation [28]. However, the AP-1 binding activity was not examined in those studies.

As expression of distinct *jun* proto-oncogenes can be induced during monocytic and granulocytic differentiation [28,36], we have analysed in the present study whether the expression of transcription factor AP-1 activity was correlated with the time required to commit HL-60 cells to differentiate towards macrophages and granulocytes. By examining the expression of AP-1 binding activity, we report here that the basal AP-1 activity observed in untreated HL-60 cells is increased during monocyte/macrophage differentiation and decreased, or not affected, during granulocytic differentiation. Furthermore, induction of HL-60 differentiation towards the monocytic or granulocytic lineages was associated with a distinct expression of *fos* and *jun* proto-oncogenes, the levels of c-*fos* and *jun* B mRNA being rapidly increased during induction of monocyte/macrophage differentiation. The present studies suggest that qualitative and quantitative differences in AP-1 expression occur during monocytic and granulocytic differentiation.

## MATERIALS AND METHODS

### Materials

RPMI 1640 culture medium, fetal-calf serum and L-glutamine were purchased from Flow Laboratories (Irvine, Ayrshire, Scotland, U.K.). Antibiotics were from Laboratorios Llorente (Madrid, Spain). BSA, retinoic acid (all-*trans*), sodium butyrate and PMA were from Sigma (St. Louis, MO, U.S.A.). PMA was dissolved in DMSO at a concentration of 2 mg/ml and used at a final concentration of 20 ng/ml. Controls containing equivalent amounts (0.05%, v/v) of DMSO were run in parallel, and no effect on either cell differentiation or gene expression was observed. Retinoic acid was prepared as a 1 mM stock in ethanol and used at a final concentration of 1 $\mu$M in culture. 1$\alpha$,25-Dihydroxyvitamin $D_3$, generously given by Dr. M. Uskokovic (Hoffmann–La Roche, Nutley, NJ, U.S.A.), was prepared as a $10^{-5}$ M stock in ethanol and used at a final concentration of $10^{-8}$ M in culture. Sodium butyrate was prepared as a 1 M stock solution in phosphate-buffered saline and used at a final concentration of 3 mM in culture. The retinoic acid, PMA and 1$\alpha$,25-dihydoxyvitamin $D_3$ stock solutions were stored at $-20$ °C. Sodium butyrate was freshly prepared and stored refrigerated at 4 °C. In control experiments carrier blanks failed to affect gene expression or induce differentiation. Fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG was purchased from Dakopatts (Glostrup, Denmark). Acrylamide, bisacrylamide, ammonium persulphate and *NNN'N'*-tetramethylethylenediamine were from Bio-Rad (Richmond, CA, U.S.A.). Guanidine thiocyanate and formamide were from Fluka (Buchs, Switzerland). Formaldehyde was from J. T. Baker Chemicals B. V. (Deventer, Holland). All other chemicals were from Sigma and Merck (Darmstadt, Germany).

The anti-Mol alpha subunit (CD11b) Bear-1 monoclonal antibody was generously given by Dr. J. E. De Vries (Unicet, Lyon, France) [37]. P3X63 myeloma culture supernatant was used as a negative control. The anti-Fos antibody, against the M peptide [38] was generously given by Dr. M. J. Iadarola (NIDR, NIH, Bethesda, U.S.A.).

### Cells

The human cell line HL-60 was grown at 37 °C in a humidified atmosphere ($CO_2$/air, 1:19) in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal-calf serum, 2 mM L-glutamine, 100 units/ml penicillin and 24 $\mu$g/ml gentamicin. Cell differentiation was induced by adding 20 ng/ml PMA, 3 mM sodium butyrate, $10^{-8}$ M 1$\alpha$,25-dihydroxyvitamin $D_3$, 1 $\mu$M retinoic acid or 1.3% (v/v) DMSO for the times indicated in the respective Figure legends. Cell differentiation was monitored by CD11b antigen expression, cell-cluster formation, adhesion to culture plates, non-specific esterase activity and cell morphology [2]. Adherent macrophage-differentiated HL-60 cells were scraped off with a rubber policeman and washed with sterile phosphate-buffered saline before the isolation of either total RNA or nuclei. Cell viability was evaluated by Trypan Blue exclusion and was always higher than 98%.

### Immunofluorescence flow cytometry

Fluorescence-flow-cytometry analysis was performed with an EPICS-C cytofluorimeter (Coulter Científica, Móstoles, Spain) as described in [39]. Fluorescence data were collected on a logarithmic scale. Negative controls were obtained using P3X63 myeloma supernatant instead of the anti-CD11b monoclonal antibody.

### Northern-blot analysis

Total RNA was isolated by the guanidine thiocyanate/CsCl method [40]. Samples of 20 $\mu$g of RNA were electrophoresed on 1.1% (w/v) agarose–formaldehyde gels, followed by transfer to nitrocellulose membranes (Schleicher und Schuell, Dassel, Germany) as previously described [41]. $^{32}$P-labelled cDNA probes were prepared using the random hexanucleotide priming method [42] (Oligo-Labelling Kit; Pharmacia, Uppsala, Sweden) to a specific radioactivity equal to, or higher than, $7 \times 10^8$ c.p.m./$\mu$g of cDNA. cDNA probes for c-*fos* [43], c-*jun* [44], *jun* B [26] and *jun* D [24] were kindly provided by Dr. P. Sassone-Corsi (LGME, CNRS, Strasbourg, France) and Dr. R. Bravo (Squibb Institute, Princeton, NJ, U.S.A.). Under the experimental conditions used in the Northern-blot analysis, there was no cross-reactivity among the above probes [28,45]. Northern blots were prehybridized at 42 °C for 6–24 h in a buffer containing 40% formamide, 4 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate), 4 × Denhardt's solution (1 × Denhardt's solution is 0.02% BSA/0.02% Ficoll-400/0.02% polyvinylpyrrolidone) and salmon sperm DNA (80 $\mu$g/ml). Then blots were hybridized in the above buffer containing 1 × Denhardt's solution, 10% (w/v) dextran sulphate and about $5 \times 10^7$ c.p.m. of probe at 42 °C for 16–24 h. After hybridization, blots were washed once in 2 × SSC/0.1% SDS for 30 min at 42 °C, once in 0.1 × SSC/0.1% SDS for 1 h at 55 °C, and once more with the same buffer for 15 min at 55 °C. Subsequently, blots were air-dried and exposed to Kodak X-Omat film with enhancing screens at $-80$ °C.

$^{32}$P-labelled probes were removed from nitrocellulose membranes by immersion in boiling 2% (v/v) glycerol for 2–3 min. Before hybridization to a second probe, removal of radioactivity was verified by autoradiography.

### Nuclear extracts and gel retardation assays

Mini-nuclear extracts were obtained from about $3 \times 10^6$ cells, essentially as described in [46], with some modifications. Briefly, buffer A was supplemented with 0.75 mM spermidine, 0.15 mM spermine, 2 $\mu$g/ml aprotinin, 2 $\mu$g/ml leupeptin and 10 mM $Na_2MoO_4$. Buffer C was also supplemented with 2 $\mu$g/ml aprotinin, 2 $\mu$g/ml leupeptin and 10 mM $Na_2MoO_4$. Protein concentration was determined in 5 $\mu$l of each sample by the Bradford method [47], using a Bio-Rad kit and BSA as standard. The protein concentration was about 1 $\mu$g/$\mu$l for nuclear extracts (final volume, 50 $\mu$l). Binding reactions were performed in a total volume of 20 $\mu$l containing 0.5 × buffer C, 4 $\mu$g of poly(dI-dC) (Pharmacia), 6 mM $MgCl_2$, 0.45 pmol of $^{32}$P-labelled double-stranded oligonucleotide and 4 $\mu$g of nuclear protein. After 15 min of incubation on ice, 1.5 $\mu$l of loading buffer [10 mM Hepes (pH 7.6)/10% glycerol/0.01% Bromophenol Blue] were added, and 14 $\mu$l of each reaction mixture were run through 4.5% acrylamide/bisacrylamide (29:1)/0.4 × TBE (1 × TBE is 0.089 M Tris base/0.089 M boric acid/2 mM EDTA) non-denaturing gels as described in [48]. Polyacrylamide gels were run at 200 V in 0.4 × TBE, dried under vacuum at 80 °C and subjected to autoradiography with enhancing screens overnight at −80 °C. The double-stranded oligonucleotide used in the gel-retardation assays was a synthetic 22 bp oligonucleotide containing the AP-1 consensus sequence (5′-CTAGTGATGAGTCAGCCGGATC-3′) [49]. Oligonucleotides were synthesized in a Cyclone Plus DNA synthesizer (Milligen, Novato, CA, U.S.A.) and purified by electrophoresis through denaturing acrylamide gels. After elution, complementary oligonucleotides were annealed, and double-stranded products were end-labelled by fill-in reaction using reverse transcriptase. In order to block binding of AP-1 to the consensus sequence, nuclear extracts (4 $\mu$g of protein) were preincubated with 1 $\mu$l of anti-(Fos M peptide) antiserum for 12 h at 4 °C, and then samples were subjected to the gel-retardation assay as described above. Controls preincubated with 1 $\mu$l of preimmune rabbit serum for 12 h at 4 °C were run in parallel.

## RESULTS

### Induction of c-*fos* and *jun* B expression during commitment of PMA- and DMSO-induced HL-60 cell differentiation

Complete differentiation of HL-60 cells towards macrophage- or granulocyte-like cells was rendered after treatment with 20 ng/ml PMA for 24 h or with 1.3% (v/v) DMSO for 7 days respectively. It had been previously reported that commitment of the myeloid human cell line HL-60 to the macrophage differentiation pathway occurred during the first 30–60 min of phorbol ester treatment [31]. We found that addition of DMSO for 24 h, followed by its removal from the medium, was sufficient to commit the HL-60 cell line to a granulocytic differentiation pathway (Figure 1), as assessed by expression of the differentiation antigen CD11b, which is a marker of myeloid differentiation. Figure 1 shows the percentages of positive cells for CD11b antigen in untreated control HL-60 cells (Figure 1a, left) and in cells treated with DMSO for the times indicated (Figure 1b, left), as well as the percentage of CD11b-positive cells in HL-60 cells treated for 72 h with DMSO (Figure 1a, right). Other cells were treated with DMSO for 6, 24 or 48 h, and then thoroughly washed with phosphate-buffered saline and cultured in the absence of DMSO up to 72 h (Figure 1b, right). We found that HL-60 cells treated with 1.3% (v/v) DMSO for 24 h, followed by successive washing in phosphate-buffered saline, and then cultured in DMSO-free culture medium for additional 48 h, showed a percentage of CD11b-positive cells similar to that obtained with HL-60 cells treated with 1.3% (v/v) DMSO for 72 h (Figure 1). This indicates that preincubation of HL-60 cells with 1.3% (v/v) DMSO for 24 h is sufficient to commit cells towards granulocytic differentiation (Figure 1).

We found accumulation of c-*fos* specific 2.2 kb transcripts and *jun* B specific 2.1 kb transcripts after 30 min of PMA addition (Figure 2), in agreement with the results of previous studies [28,31]. The sizes of 2.2 kb and 2.1 kb for c-*fos* and *jun* B transcripts respectively, were similar to those already reported [26,43,45,50]. In a previous study [28] we found by Northern-blot analysis a small, transient and rapid increase of c-*jun* mRNA levels after 15–30 min after PMA addition, returning to basal levels after 1 h of treatment. However, maximal induction of c-*jun* mRNA level was achieved 24 h after PMA addition [28]. On the other hand, no increase in the c-*jun* and c-*fos* mRNA levels has been detected during granulocytic HL-60 differentiation induced by DMSO [28,31,36]. Corroborating these reports, we found that treatment of HL-60 cells with 1.3% (v/v) DMSO for 10 min, 30 min, 1 h, 6 h and 24 h did not induce accumulation of c-*fos* transcripts (results not shown). Nevertheless, we have

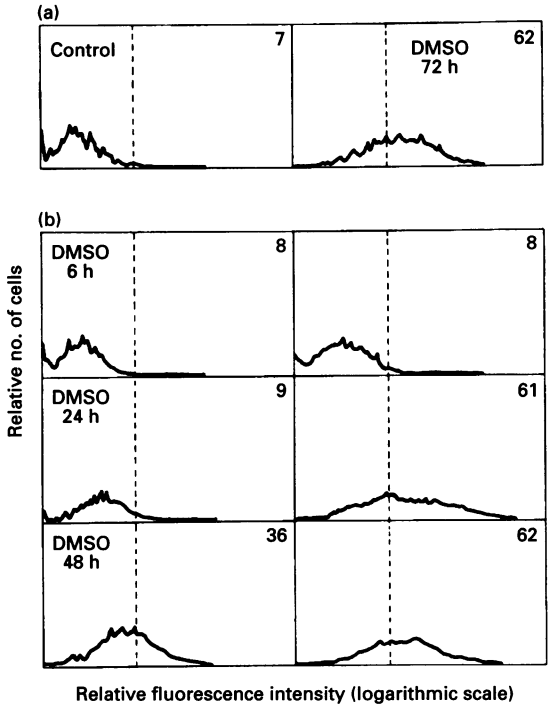


**Figure 1 Commitment of HL-60 cell differentiation towards granulocytic lineage upon DMSO treatment**

Profiles represent the cell-surface expression of CD11b differentiation antigen during DMSO-induced cell differentiation. HL-60 cells were cultured as described below and then chilled at 4 °C, labelled with anti-CD11b monoclonal antibody and fluorescein isothiocyanate-conjugated anti-mouse IgG and subjected to immunofluorescence flow cytometry as described in the Materials and methods section. (**a**) Profiles of untreated HL-60 cells (left) and HL-60 cells treated with 1.3% (v/v) DMSO for 72 h (right). (**b**, left) Profiles of HL-60 cells treated with 1.3% (v/v) DMSO for 6 h, 24 h and 48 h and then labelled with anti-CD11b monoclonal antibody. (**b**, right) Profiles of HL-60 cells treated with 1.3% (v/v) DMSO for 6, 24 and 48 h, followed by removal of DMSO through successive washes with phosphate-buffered saline and cultured for up to 72 h in the absence of DMSO in the culture medium, and then labelled with anti-CD11b monoclonal antibody. The percentages of CD11b-positive cells are shown in the upper right part of each profile. Data correspond to a representative experiment out of three performed. The vertical broken lines represent the threshold to calculate the percentage of positive cells.

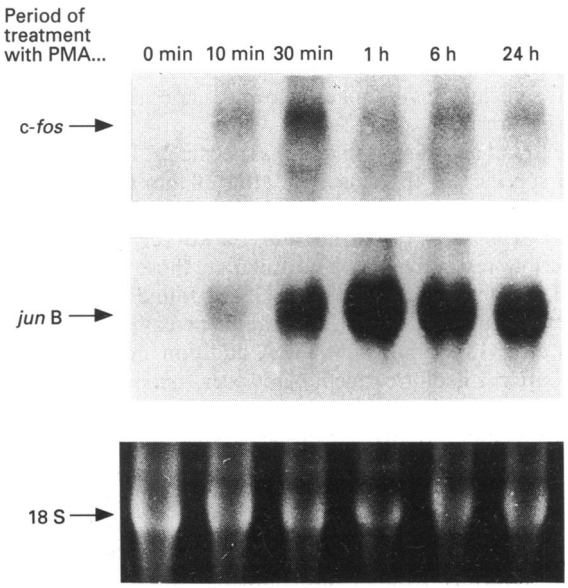


**Figure 2 Expression of c-*fos* and *jun* B proto-oncogenes in PMA-treated HL-60 cells**

Northern-blot analysis of mRNA levels after cell treatment with PMA (20 ng/ml) for 10 min, 30 min, 1 h, 6 h and 24 h. Basal control levels in uninduced HL-60 cells are also shown (first lane, left). Ethidium bromide staining of the same gel reveals equivalent amounts of ribosomal RNA (18 S). Autoradiograms were developed after 24 h of exposure. For further details, see the Materials and methods section.

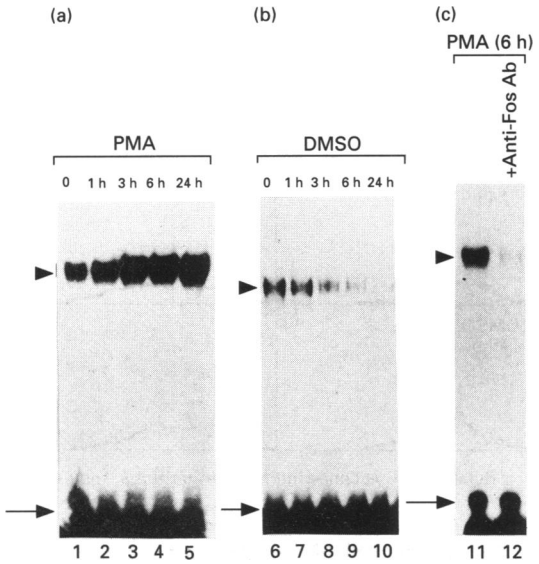


**Figure 3 Comparison of binding to an AP-1 sequence of nuclear extracts from PMA- and DMSO-treated HL-60 cells**

(**a**) Gel-retardation analysis of nuclear extracts from uninduced HL-60 cells (lane 1) and from HL-60 cells treated with 20 ng/ml PMA for 1 h (lane 2), 3 h (lane 3), 6 h (lane 4) and 24 h (lane 5). (**b**) Gel-retardation analysis of nuclear extracts from uninduced HL-60 cells (lane 6) and from HL-60 cells treated with 1.3% (v/v) DMSO for 1 h (lane 7), 3 h (lane 8), 6 h (lane 9) and 24 h (lane 10). (**c**) Binding inhibition using the specific anti-(Fos M) antibody ('Anti-Fos Ab'). Nuclear extracts from HL-60 cells treated with 20 ng/ml PMA for 6 h were incubated with preimmune serum (lane 11) or with anti-(Fos M) antibody (lane 12), and then tested for AP-1 binding capacity. The arrowhead points to the specific binding complex to the AP-1 site. The arrow points to the free oligoprobe containing the consensus AP-1 site. For further details, see the Materials and methods section.

recently described an increase in the levels of *jun* B and *jun* D mRNA after 6–24 h after DMSO addition [28].

## Binding to the AP-1 recognition site during PMA- and DMSO-induced HL-60-cell differentiation

We detected an appreciable AP-1 activity in untreated HL-60 cells by gel-retardation analysis (Figure 3). This AP-1 activity was rapidly increased along the incubation time of HL-60 cells with PMA (Figure 3a). In contrast, the AP-1 activity detected in untreated cells disappeared progressively during DMSO treatment (Figure 3b). Competition experiments were performed to identify the specificity of the binding complexes. Addition of a 50-fold molar excess of an unlabelled competitor oligonucleotide containing the AP-1 site to the binding reaction resulted in the absence of bound labelled oligonucleotide, whereas competition with an oligonucleotide containing the CRE (cyclic AMP-responsive element) site [51], identical with the AP-1 site except for one nucleotide, showed no effect (results not shown). We confirmed with specific anti-(Fos M) antibody that the DNA-binding complex required the presence of Fos protein (Figure 3c).

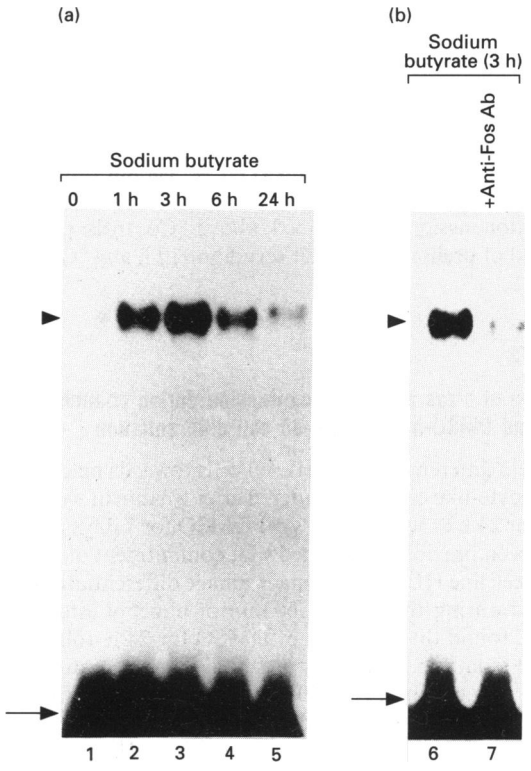


**Figure 4 Binding to an AP-1 sequence of nuclear extracts from sodium butyrate-treated HL-60 cells**

(**a**) Gel-retardation analysis of nuclear extracts from untreated HL-60 cells (lane 1) and from HL-60 cells treated with 3 mM sodium butyrate for 1 h (lane 2), 3 h (lane 3), 6 h (lane 4) and 24 h (lane 5). (**b**) Binding inhibition using the specific anti-(Fos M) antibody ('Anti-Fos Ab'). Nuclear extracts from HL-60 cells treated with 3 mM sodium butyrate for 3 h were incubated with preimmune serum (lane 6) or with anti-(Fos M) antibody (lane 7), and then tested for AP-1 binding capacity. The DNA-binding complex (arrowhead) and the free oligoprobe containing the consensus AP-1 site (arrow) are indicated. For further details, see the Materials and methods section.

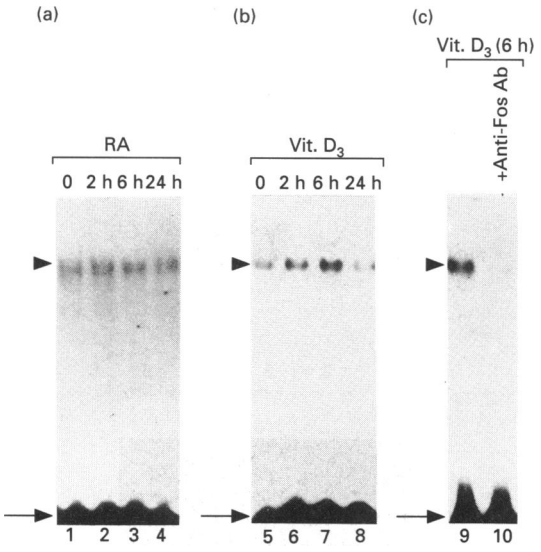


**Figure 5 Comparison of binding to an AP-1 sequence of nuclear extracts from retinoic acid- and 1α,25-dihydroxyvitamin $D_3$-treated HL-60 cells**

(**a**) Gel-retardation analysis of nuclear extracts from uninduced HL-60 cells (lane 1) and from HL-60 cells treated with 1 μM retinoic acid (RA) for 2 h (lane 2), 6 h (lane 3) and 24 h (lane 4). (**b**) Gel-retardation analysis of nuclear extracts from uninduced HL-60 cells (lane 5) and from HL-60 cells treated with $10^{-8}$ M 1α,25-dihydroxyvitamin $D_3$ (Vit. $D_3$) for 2 h (lane 6), 6 h (lane 7) and 24 h (lane 8). (**c**) Binding inhibition using the specific anti-(Fos M) antibody ('Anti-Fos Ab'). Nuclear extracts from HL-60 cells treated with $10^{-8}$ M 1α,25-dihydroxyvitamin $D_3$ for 6 h were incubated with preimmune serum (lane 9) or with anti-Fos M antibody (lane 10), and then tested for AP-1 binding capacity. The DNA-binding complex (arrowhead) and the free oligoprobe containing the AP-1 site (arrow) are indicated. For further details, see the Materials and methods section.

### Effect of other inducers of myeloid differentiation on AP-1 binding activity

Treatment of HL-60 cells with 3 mM sodium butyrate, an inducer of monocytic HL-60 cell differentiation [2,6], resulted in a rapid and transient increase in AP-1 binding activity (Figure 4a). Using the specific anti-(Fos M) antibody, we confirmed the presence of Fos/AP-1 complex binding to the AP-1 site (Figure 4b). Furthermore, treatment of HL-60 cells with $10^{-8}$ M 1α,25-dihydroxyvitamin $D_3$, another monocytic HL-60 differentiating agent [2,7], induced a weak, but appreciable, increase in AP-1 binding activity (Figure 5b), which required Fos protein (Figure 5c). However, when HL-60 cells were treated with 1 μM retinoic acid, an inducer of granulocytic HL-60 cell differentiation [2,4], no increase in AP-1 binding activity was observed (Figure 5a).

### Expression of *fos* and *jun* proto-oncogenes during treatment of HL-60 cells with sodium butyrate, 1α,25-dihydroxyvitamin $D_3$ or retinoic acid

As sodium butyrate induced a rapid and remarkable increase in AP-1 binding activity (Figure 4), we analysed the effect of this monocytic differentiation agent on the expression of *fos* and *jun* proto-oncogenes in HL-60 cells. Addition of sodium butyrate to HL-60 cells induced a rapid and sustained increase in the 2.2 kb c-*fos* mRNA level (Figure 6). A detailed time course showed that the c-*fos* mRNA level was elevated as early as 30 min after the addition of sodium butyrate, reaching its maximum at 24 h (Figure 6). In addition to the major 2.2 kb band, a weak hybridization band of about 2.0 kb was visible, presumably related to r-*fos* gene transcripts [31]. The faint band at about 3.5 kb has been suggested to represent an unprocessed transcript [31]. Addition of sodium butyrate to HL-60 cells resulted in a

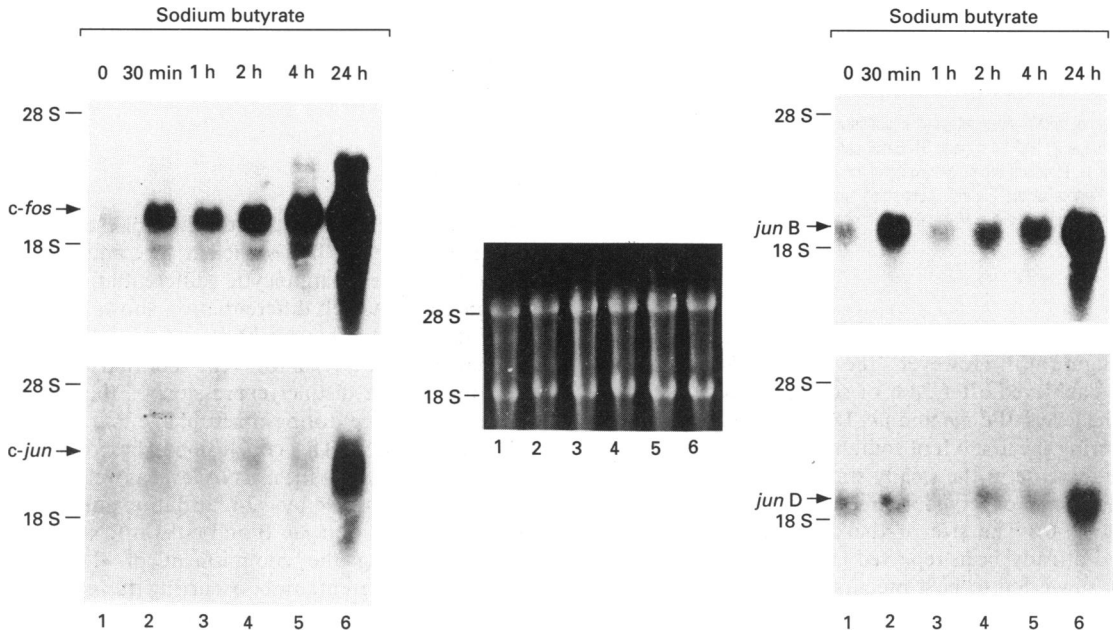


**Figure 6 Expression of c-*fos*, c-*jun*, *jun* B and *jun* D proto-oncogenes in sodium butyrate-treated HL-60 cells**

Northern-blot analysis of mRNA levels after cell treatment with sodium butyrate (3 mM) for 30 min (lane 2), 1 h (lane 3), 2 h (lane 4), 4 h (lane 5) and 24 h (lane 6). Basal control levels in uninduced HL-60 cells are also shown (lane 1). Ethidium bromide staining of the same gel reveals equivalent amounts of ribosomal RNA (middle of the Figure). Autoradiograms were developed after 24–48 h of exposure. For further details, see the Materials and methods section.

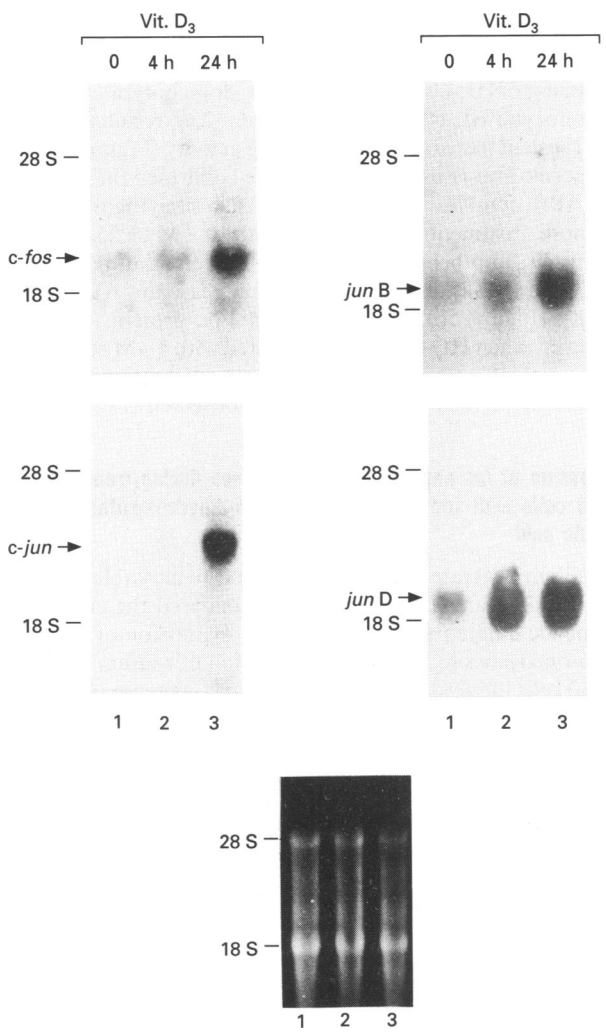


**Figure 7 Expression of c-*fos*, c-*jun*, *jun* B and *jun* D proto-oncogenes in 1α,25-dihydroxyvitamin $D_3$-treated HL-60 cells**

Northern-blot analysis of mRNA levels after cell treatment with $10^{-8}$ M 1α,25-dihydroxyvitamin $D_3$ (Vit. $D_3$) for 4 h (lane 2) and 24 h (lane 3). Basal control levels in uninduced HL-60 cells are also shown (lane 1). Ethidium bromide staining of the same gel reveals equivalent amounts of ribosomal RNA (bottom of the Figure). Autoradiograms were developed after 24–48 h of exposure. For further details, see the Materials and methods section.

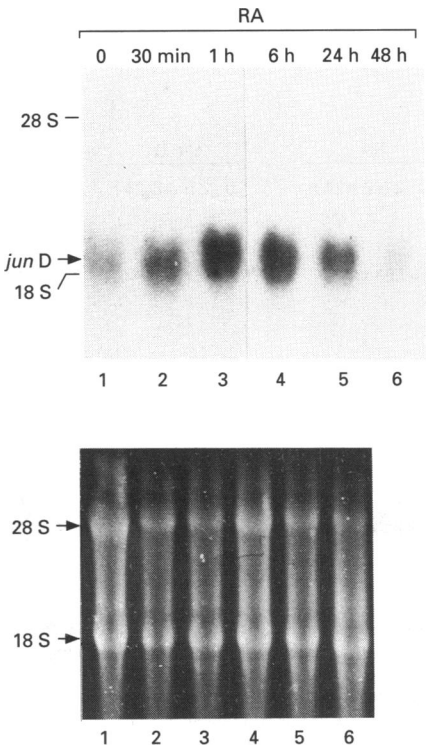


**Figure 8 Expression of *jun* D proto-oncogene in retinoic acid-treated HL-60 cells**

Northern-blot analysis of mRNA levels after cell treatment with 1 μM retinoic acid (RA) for 30 min (lane 2), 1 h (lane 3), 6 h (lane 4), 24 h (lane 5) and 48 h (lane 6). Basal control level of *jun* D mRNA in uninduced HL-60 cells is also shown (lane 1). Ethidium bromide staining of the same gel reveals equivalent amounts of ribosomal RNA. The autoradiogram was developed after 48 h of exposure. For further details, see the Materials and methods section.

rapid increase in the 2.1 kb *jun* B mRNA level after 30 min treatment (Figure 6). However, the highest *jun* B mRNA induction was achieved after 24 h of sodium butyrate addition (Figure 6). The levels of c-*jun* and *jun* D mRNA were practically unchanged during the first 4 h of sodium butyrate treatment, but a remarkable increase in the steady-state mRNA levels of both proto-oncogenes was observed after 24 h after sodium butyrate addition (Figure 6). The size of the c-*jun* mRNA was about 2.7 kb, as had already been reported [11,12]. A minor band of about 3.4 kb, suggested to be a precursor molecule [11,12], was also evident after overexposure of the autoradiogram. The size of *jun* D transcript was about 2.0 kb, also as already reported [24].

Addition of 1α,25-dihydroxyvitamin $D_3$ to HL-60 cell cultures resulted also in an increase in the steady-state mRNA levels of c-*fos*, c-*jun*, *jun* B and *jun* D proto-oncogenes (Figure 7). However, exposure of HL-60 cells to retinoic acid did not induce any accumulation of c-*fos*, c-*jun* or *jun* B transcripts (results not shown). Only the *jun* D mRNA level was weakly increased by retinoic acid treatment (Figure 8).

## DISCUSSION

Incubation of HL-60 cells with phorbol esters for 30–60 min or with DMSO for 24 h is sufficient to commit the cells towards macrophage or granulocytic differentiation respectively, although complete cell differentiation shows up much later (24 h and 7 days for full macrophage and granulocytic differentiation respectively). The results herein reported demonstrate for the first time that a distinct expression of the AP-1 transcription factor takes place during macrophage and granulocytic HL-60 cell differentiation. This conclusion is based on the examination of AP-1 expression at the mRNA level as well as on the functional AP-1 activity assessed by gel-retardation analysis. Northern-blot analysis showed that *jun* B and c-*fos* mRNA levels were highly increased during the commitment of PMA-induced HL-60 macrophage differentiation, occurring during the first 30–60 min of PMA treatment. After that time, the level of c-*fos* transcripts diminished, but appreciable levels of c-*fos* transcripts could be detected, even after 24 h of PMA treatment (see Figure 2). The content of *jun* B mRNA remained at high levels throughout the PMA incubation time assayed (from 30 min to 24 h) (see Figure 2). The finding that PMA treatment induces expression of *jun* B in conjunction with c-*fos* suggests that both Jun–Jun and Jun–Fos

complexes can be formed during the commitment stage of PMA-induced HL-60 cell differentiation. The AP-1 binding activity rapidly increased after PMA addition, and a remarkable AP-1 activity was observed after 24 h of PMA treatment. Thus we observed the formation of a nucleoprotein complex able to bind to the AP-1 site during the commitment of macrophage differentiation as well as in fully differentiated HL-60 macrophages. This suggests that the presence of transcription factor AP-1 can be important in the commitment to the PMA-induced macrophage differentiation and in the regulation of gene expression in mature macrophages. On the other hand, it has been reported that in spite of an early, though small and transient, increase in the level of c-*jun* transcripts during PMA-induced HL-60 cell differentiation, maximal induction of c-*jun* mRNA level was observed in terminally PMA-differentiated HL-60 macrophages [28,35,36]. We have recently reported the late expression of both c-*jun* and *jun* D proto-oncogenes during PMA-induced HL-60 macrophage differentiation [28]. These data suggest that c-*jun* and *jun* D proto-oncogenes can be mainly involved in the regulation of gene expression in the late stages of macrophage differentiation or in terminally differentiated macrophages rather than in the commitment of macrophage differentiation, when c-*fos* and *jun* B are actively expressed.

The results herein reported indicate for the first time the disappearance of the AP-1 activity detected in untreated cells during the commitment of granulocytic HL-60 differentiation induced by DMSO. Previous studies have indicated that no accumulation of c-*jun* and c-*fos* transcripts was observed during granulocytic HL-60 differentiation induced by DMSO [28,31,36]. Interestingly, we have found an increase in Jun-immunoreactive protein during DMSO treatment (F. Mollinedo and J. R. Naranjo, unpublished work). This increase in Jun-immunoreactive protein is likely due to the induction of Jun B and Jun D proteins, since we have previously reported [28] that the *jun* B and *jun* D, but not c-*jun*, mRNA levels are increased during DMSO treatment of HL-60 cells [28]. The disappearance of the basal AP-1 binding activity during DMSO treatment of HL-60 cells could be consistent with our previous data describing the induction of *jun* B and *jun* D proto-oncogenes during DMSO treatment [28]. Jun B and Jun D, in the absence of c-Fos, bind to the AP-1 oligonucleotide very poorly [29]. Therefore, the increased levels of Jun proteins, other than c-Jun, could 'squelch' the formation of functional c-Fos/c-Jun heterodimers and contribute to reducing the basal AP-1 binding activity observed in untreated HL-60 cells by gel-retardation analysis. This DMSO-induced inhibitory effect on AP-1 activity could also be explained by the degradation of the transcription factor AP-1 already present in undifferentiated HL-60 cells. Taken together, these data indicate that the induction of the transcription factor AP-1 is not involved in the differentiation of HL-60 cells towards granulocytes upon DMSO treatment. Accumulating evidence indicates that protein dimerization plays an important role in transcriptional regulation [52]. It has been reported that the three members of the Jun family are able to form homodimers as well as heterodimers with each of the other Jun proteins, leading to binding to an AP-1 site in DNA, and that this AP-1 binding is highly increased by interaction of each Jun protein with Fos [23,29]. In the present study we did not detect any increase in c-*fos* mRNA level during DMSO treatment. Thus during DMSO-induced HL-60 cell differentiation, the induced *jun* B and *jun* D proto-oncogenes might form heterodimers with other nuclear proteins, showing DNA-binding specificities different from the AP-1 site. In this context it has been reported that members of the Fos/Jun and ATF/CREB families of transcription factors can form selective cross-family heterodimers with distinguishable DNA-binding specificities from each other and from their parental homodimers [53].

Unlike DMSO-induced HL-60 cell differentiation, macrophage cell differentiation induced by PMA resulted in a rapid increase in AP-1 binding activity, suggesting a putative role for transcription factor AP-1 in macrophage differentiation. It has been recently reported that 1$\alpha$,25-dihydroxyvitamin $D_3$ treatment of HL-60 cells increases the c-*jun* mRNA level [35,36], whereas HL-60 cell treatment with DMSO or retinoic acid does not alter c-*jun* expression [28,36]. Furthermore, the treatment of HL-60 cells with cyclic AMP analogues or with agents that increase intracellular cyclic AMP levels, inducing the appearance of a differentiated monocytic phenotype, is associated with a rapid increase in c-*fos* and *jun* B mRNA levels [54,55]. Moreover, we herein report that treatment of HL-60 cells with sodium butyrate, an inducer of monocytic HL-60 differentiation, promotes rapid increases in AP-1 binding activity as well as in the c-*fos* and *jun* B mRNA levels. The levels of c-*jun* and *jun* D mRNA were also elevated after 24 h of sodium butyrate addition. These effects of sodium butyrate on AP-1 activity as well as on *fos* and *jun* proto-oncogene expression in HL-60 cells resemble those induced by PMA (the present work; [28]). Nevertheless, a noteworthy difference resides in the high and sustained elevation of c-*fos* mRNA level induced by sodium butyrate (cf. Figures 2 and 6). In this regard it has been reported that treatment of PC12 phaeochromocytoma cells with sodium butyrate results in stable high levels of c-*fos* mRNA [56]. We have also found that treatment of HL-60 cells with the monocytic differentiating agent 1$\alpha$,25-dihydroxyvitamin $D_3$ induced an increase in AP-1 activity as well as an elevation in the levels of c-*fos*, c-*jun*, *jun* B and *jun* D mRNA. We also found that an anti-Fos antibody blocked the AP-1 binding activity present in nuclear extracts from HL-60 cells treated with the three monocytic differentiating agents used (PMA, sodium butyrate and 1$\alpha$,25-dihydroxyvitamin $D_3$), thus demonstrating that the AP-1 binding activity observed was caused by Fos/Jun heterodimers, probably c-Fos/Jun B. We observed that there was a correlation between the increased expression of c-*fos* and *jun* B proto-oncogenes and an enhancement of the AP-1 binding activity present in nuclear extracts from HL-60 cells treated with the three monocytic differentiation inducers used in the present study at early times of incubation. However, this correlation was lost at later times of incubation, suggesting that a post-translational control mechanism could be additionally involved in the regulation of AP-1 activity, as previously suggested [49]. In contrast, treatment of HL-60 cells with retinoic acid, a granulocytic differentiation inducer, neither enhances AP-1 binding activity nor modifies the basal levels of c-*fos*, c-*jun* or *jun* B transcripts. It is noteworthy that retinoic acid behaved similarly to DMSO [28] in inducing an increase of the *jun* D mRNA level when added to HL-60 cells. As no increase in AP-1 binding activity was detected in either retinoic acid- or DMSO-treated cells, it could be envisaged that transcriptional complexes involving *jun* D gene product and lacking AP-1 activity could be formed during granulocytic HL-60 differentiation.

On these grounds it could be envisaged that the differential induction of AP-1 activity could play an important role in the induction of HL-60 cell differentiation towards monocytic or granulocytic lineages. Thus an increase in AP-1 activity could be correlated with the induction of monocytic differentiation, whereas no induction, or even inhibition, of AP-1 activity could be correlated with granulocytic differentiation. In this regard it is tempting to suggest that inhibition of AP-1 activity could favour granulocytic differentiation. The data herein reported also suggest that specific regulatory programmes, involving distinct *jun*

proto-oncogenes, are activated during differentiation to specific myeloid-cell lineages in order to maintain the expression of specific cellular genes.

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