Changes in FOS/JUN binding activity on a negative regulatory element of c-*myc* during differentiation of mouse erythroleukemic cells

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Abstract. The binding activity of the AP-1 FOS/JUN complex to a negative regulatory element on the c-*myc* promoter was investigated and correlated with events characteristic of terminal differentiation of HMBA-induced mouse erythroleukemic cells. It is suggested that the altered AP-1 activity that was found upon induction of the erythroid differentiation may contribute to the late decline in c-*myc* mRNA.

Introduction

The c-*myc* proto-oncogene encodes for two highly conserved nuclear phosphoproteins p62 and p64 involved in cellular proliferation and differentiation (1). c-*myc* expression may be controlled at at least four levels (reviewed in ref. 2). First, at the level of transcription initiation through four separate RNA start sites (P0, P1, P2 and P3). Furthermore, a block in transcriptional elongation has been observed during differentiation of HL60 human promyelocytic leukemia (3) and MEL cells (4), while mRNA stability may be another important mechanism of regulation as demonstrated for murine plasmacytomas (5). Finally, expression of the murine c-*myc* gene appears also to be controlled at the level of mRNA translation (6).

Transcriptional regulation of c-*myc* is extremely complex and is modulated by both positive and negative *cis*-acting regulatory elements. The role of regulatory elements located within 5' flanking sequences and within exon I of c-*myc* are not only important for the regulation of transcription from P0, P1 and P2 (7-11) but also for premature termination of transcripts (12) and translational control (6).

Friend leukemic cells (FLC) are mouse erythroid precursor cells blocked at an early stage of erythroid differentiation (13). When these cells are exposed to HMBA or a variety of other chemicals, they reinitiate a program of erythroid differentiation, culminating in hemoglobin synthesis and terminal cell division (13). During the differentiation process, a biphasic change in c-*myc* and c-*myb* mRNAs is observed (14,15). Alterations in c-*fos*, K-*ras* and p53 gene expression have also been reported in some but not all FLC lines (15,16).

The c-*fos* oncogene is known to participate in transcriptional regulation through formation of a c-*fos*:c-*jun* heterodimer protein known as the AP-1 complex (for a review see ref. 17). The AP-1 complex has been reported to participate in the process of differentiation (18) and may also control the expression of genes involved in cellular proliferation (17). Since an AP-1 binding site has been found within the c-*myc* promoter (19), it was of interest to study the levels of AP-1 complex bound to that region during HMBA-induced erythroid differentiation. Our data suggests that differential AP-1 binding may represent a possible mechanism (or part of a mechanism) which regulates c-*myc* expression during this process.

Materials and methods

Cell lines and culture conditions. The F412B2TK$^-$ mouse erythroleukemic (Friend) cells and the HeLa cell line used in this study, were continuously maintained in RPMI-1640 (Gibco) with 50 μg/ml streptomycin and 300 μg/ml penicillin, supplemented with 10% Fetal Calf Serum (Flow), at 37°C in a 5% CO_2 atmosphere.

Erythroid differentiation was achieved by exposing FLC to 3 mM HexaMethyleneBisAcetamide (HMBA, Sigma) dissolved in water.

Hemoglobin quantitation. Hemoglobin content of induced and non-induced cells was estimated using the method of Rutherford (20), as modified.

10^7 cells exposed to 3 mM HMBA for various time intervals (0, 24, 48, 72, 96 and 120 h) were centrifuged (700 x g, 5 min, 4°C) and washed with PBS twice. After removing the supernatant, the pellet was lysed in 1 ml distilled water and two cycles of freeze (-70°C) and thaw (37°C). Centrifugation at 12000 x g for 5 min at 4°C gave a clear

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supernatant which was used immediately for photometric estimation of hemoglobin at wavelength range of 405-420 nm and quantitation at 414 nm, in a LKB 4054 spectrophotometer.

Extraction of total RNA and Northern blot analysis. RNA extracts from differentiated and non-differentiated cells were prepared as described by Sambrook *et al* (21).

Briefly, cells were harvested (700 x g, 5 min, 4°C), washed with PBS and the pellet was then lysed in guanidinium thiocyanate solution. After phenol/chloroform/isoamylalcohol extraction, the aqueous phase was removed, an equal volume of isopropanol was added and left for 1 h at -20°C. Centrifugation at 12,000 x g for 25 min at 4°C gave a RNA pellet which was rinsed in 70% ethanol, dried in vacuum and resuspended in RNase-free water. We estimated the RNA concentration photometrically at 260 nm.

12 µg of each RNA sample were incubated with a solution containing 2 µl 5 x MOPS, 3.5 µl formaldehyde and 10 µl formamide in a final volume of 20 µl, at 65°C for 15 min.

Samples were then loaded in a 1% formaldehyde gel and run for approximately 4 h at 80V in a MOPS/formaldehyde running buffer.

For Northern blotting we followed the Hybond-N+ protocol (Amersham) using 20 X SSPE buffer. After blotting, RNA was fixed by baking the membrane at 80°C for 2 h.

Pre-hybridization with 5 X SSPE, 5 x Denhardt's solution 50 % formamide and 0.5% (w/v) SDS for 6 h in 42°C, was followed by overnight hybridization. The probes used were the 7.0 Kb EcoRI/EcoRI fragment of plasmid pBRMßG-6 carrying the mouse ß-globin gene, the 1.4 Kb ClaI/EcoRI fragment of plasmid pMC41-3RC, carrying the third exon (EIII) of the human c-*myc* gene and the 3 Kb XhoI/NcoI fragment of plasmid pc-*fos*, carrying the human c-*fos* gene. Mouse ß-actin DNA was a gift from Dr E. Gonos (Ludwig Institute for Cancer Research, London, UK). All DNA-inserts were labelled with ^{32}P dCTP (Amersham) using the random primed DNA labelling kit of Boehringer Mannheim.

The membranes were finally washed twice in 2 X SSPE, 0.1% SDS in room temperature for 30 min, twice in 1 X SSPE, 0,1% SDS for 20 min at 42°C and once in 0.5 X SSPE, 0.1% SDS in 42°C for 15 min, followed by exposure to X-ray film (Fuji) with an intensifying screen.

Preparation of nuclear extracts. Nuclear extracts from F412B2TK$^-$ and HeLa cells were prepared as previously described (22). Briefly, cells were harvested (700 x g, 5 min, 4°C), washed with PBS and dried. The cell pellets were homogenised with a B-type dounce homogenizer in hypotonic buffer (25 mM Tris.HCl pH=7.5, 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT) and 0.5 mM PMSF), centrifuged at 2,000 x g, washed three times with isotonic buffer (25 mM Tris.HCl pH=7.5, 5 mM KCl, 0.5 mM $MgCl_2$, 0.1 M surcose, 0.5 mM DTT and 0.5 mM PMSF), and resuspended in nuclei extraction buffer (25 mM Tris.HCl pH=7.5, 1 mM EDTA, 0.1% Triton X-100, 0.5 m M DTT, 0.5 mM PMSF and 0.6 M KCl). Nuclei were inverted slowly for 30 min and the extract clarified by centrifugation at 2.5 x 10^4 g for 60 min at 4°C. Nuclear extracts were stored at -70°C. Protein estimations were performed using Biorad Protein Assay (Biorad).

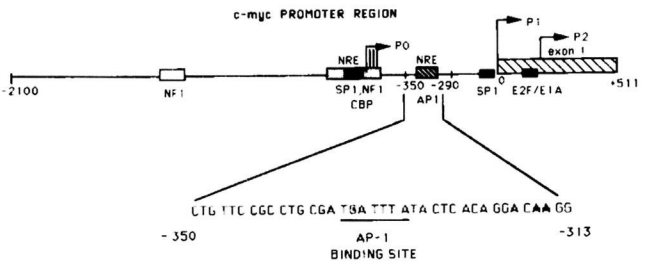

Figure 1. Regulatory elements and transcription factor binding sites on the c-*myc* promoter (2,8-11). The AP-1 binding site is located whithin an NRE domain of c-*myc* promoter (19).

Synthesis of oligonucleotides and gel retardation assays. The E3AP1 oligonucleotide 5'-CCGAAGTTCAGATGA CTAACTCAGGG-3' representing the region between nucleotides -81 and -103 of the EIA inducible E_3 promoter (23) was used as control for AP-1 binding activity. The *myc*AP-1 oligonucleotide 5'-CCTGCGATGATTTATAC TCACAGG-3' contains the AP-1 binding site of the NRE domain of the human c-*myc* promoter (-342 to -313) (19) (Fig. 1). Both oligonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer.

Single stranded complementary oligonucleotides were annealed, end-labelled using γ-^{32}P-ATP (Amersham) and T4 polynucleotide kinase (Boehringer) and run on a 8% polyacrylamide gel to test that the annealing had worked.

The binding reaction mixture contained 10 mM Hepes (pH=7.9), 0.2 mM EDTA, 94 mM NaCl, 0.1 mM PMSF, 0.1 ng/ml BSA, 4% glycerol, 3 µg poly(dI-dC) (Pharmacia), 0.2-1.0 ng ^{32}P labelled DNA and 20 µg nuclear extracts (exept when otherwise indicated). Incubation was on ice for 30 min. Then, reaction mixtures were loaded onto a 5% polyacrylamide gel (29:1 acrylamide: N,N'-methylene bisacrylamide, Serva). Gels were run at 4°C in 0.55 X TBE, dried and exposed to X-ray film for autoradiography (Fuji).

FOS 388 and JUN 948 antibodies used in this study were a generous gift of Dr D.A.F. Gillespie (Beatson Institute for Cancer Research, Glasgow, UK).

Assay for cell proliferation. Cell proliferation was estimated using the rapid colorimetric assay of Mossman (24) as previously described (25). In brief, 10 µl of MTT [3-(4,5-dimethylthiazol 2-yl)-2,5 - diphenyl tetrazolium bromide] (Sigma) in PBS were added to 100 µl complete medium in a Costar 96 well plate containing F412B2TK$^-$ cells exposed to 3 mM HMBA for various lengths of time (0, 24, 48, 72, 96 and 120 h). After incubation for 4 h at 37°C, 110 µl of 0.04 HCl in isopropanol were added. Optical Density (O.D) was recorded at 540 nm using a Titertek Flow Micro ELIZA reader.

Results

Exposure of mouse erythroleukemic cells to HMBA results in alteration of expression of several genes, the most characteristic of which are the globin genes. In order to examine whether the differentiated process had occurred in our system, we performed Northern blot analysis of total

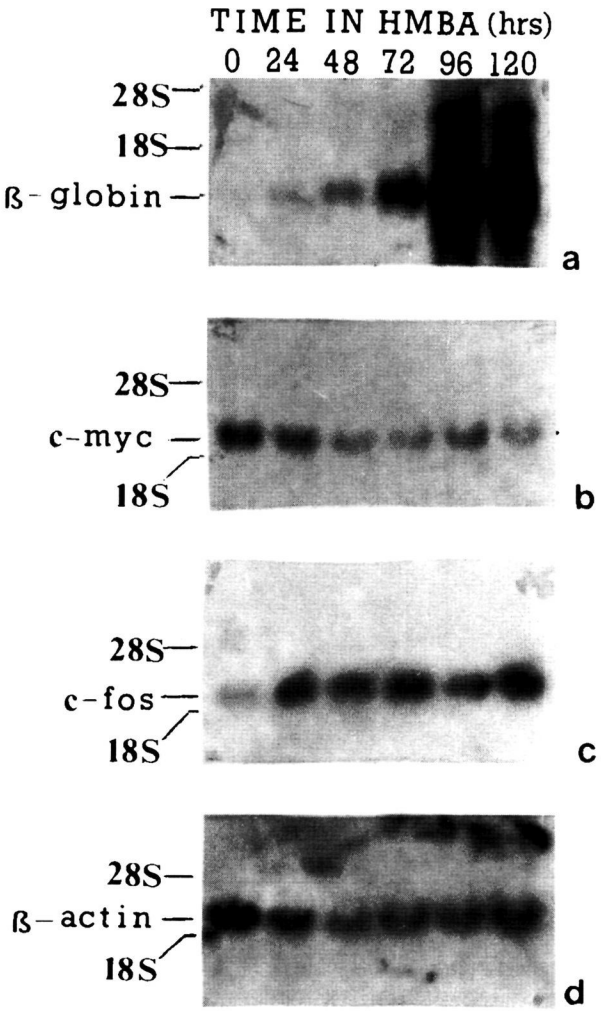

Figure 2. Relative levels of ß-globin (a), c-*myc* (b), c-*fos* (c) and ß-actin (d) mRNAs during HMBA induction of F412B2TK$^-$ cells. Total RNA isolation, Northern blot analysis and hybridization probes are described in Materials and methods. The same filter was used for the four hybridizations. Autoradiographic exposure time was approximately 36 h. The positions of 18S and 28S ribosomal RNAs in the gel, visualized by ethidium bromide staining, are indicated in the figure. Densitometric analysis of the autoradiograms, normalized to ß-actin, verified the continuous down-regulation of c-*myc* and the increased levels of ß-globin and c-*fos* mRNA. Two independent experiments were performed.

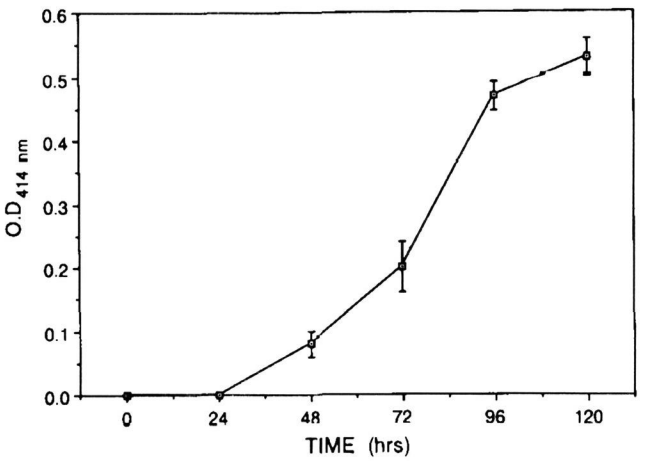

Figure 3. Photometric estimation of hemoglobin accumulation during differentiation of FL cells. Absorption values at 414 nm after exposure of F412B2TK$^-$ cells to 3 mM HMBA for 0, 24, 48, 72, 96 and 120 h. Mean values from 4 independent experiments are given. Standard deviation is depicted as vertical bars.

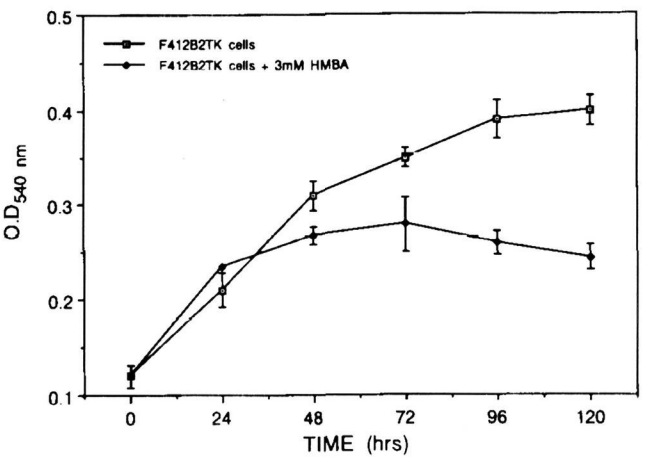

Figure 4. Cell proliferation of differentiating and control F412B2TK$^-$ cells. Mean values from 3 independent experiments are given. Standard deviation of 9 values for each point is depicted as vertical bars.

RNA isolated from differentiated (days 1 to 5) and non-differentiated cells. As depicted in Fig. 2a, a dramatic increase in ß-globin expression was observed during the differentiation process, followed by hemoglobin production. Hemoglobin gives a characteristic peak in 413-414 nm (20) which becomes obvious as early as the second day of differentiation (Fig. 3). No significant differences in globin gene expression were observed between days 4 and 5 after the initiation of drug exposure. Growth arrest, which is another characteristic of finally differentiated cells was demonstrated by a rapid cell proliferation assay for various times intervals (0, 24, 48, 72, 96 and 120 h) (Fig. 4).

c-*myc* is one of the oncogenes that respond early to induction of differentiation and presents a marked decrease in its expression during the final steps of differentiation of FLC cells, as depicted in Fig. 2b. The c-*fos* oncogene is up-regulated as early as 24 h post-induction but its levels remain constant thereafter (Fig. 2c).

We further investigated the alteration in AP-1 levels bound to AP-1 site of c-*myc* promoter. When crude extracts from HeLa cells were incubated with labelled *myc*AP-1 oligo (Fig. 1), three protein-DNA complexes formed (a, b and c, Fig. 5, lanes 5,6) which is consistent with previous reports (19,26). On the contrary, when the same extracts were incubated with E3AP1 oligo, only the two, slower migrating a, b complexes were formed (lanes 1 and 2, Fig. 5). A similar pattern of protein binding was observed with extracts from Friend leukemic cells (FLC) F412B2TK$^-$ (Fig. 5, lanes 3,4 and 7,8).

Among the three protein-*myc* oligo complexes, only the b complex has been shown to be an AP-1 (FOS/JUN) (19). Indeed, in gel retardation experiments, FOS and JUN antibodies eliminated the b-band in extracts of both differentiated and finally differentiated (day 4) FLC (Fig. 6,

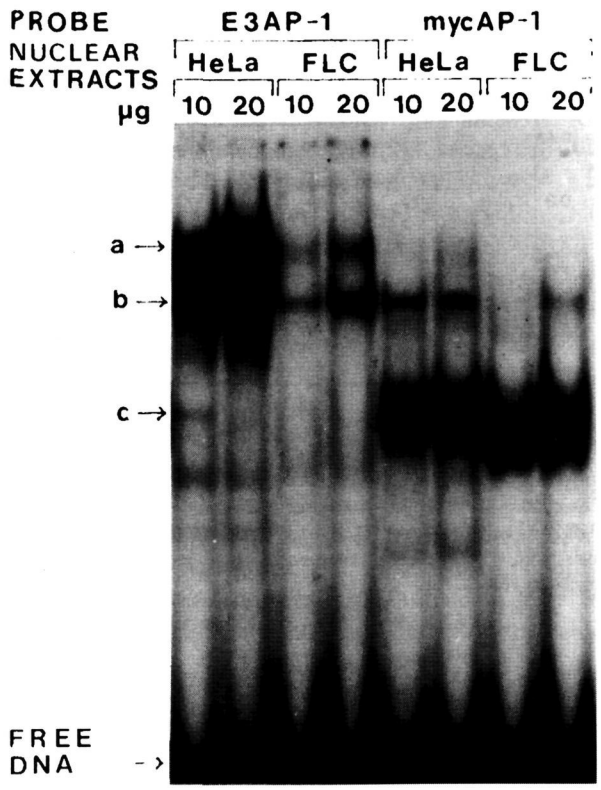

Figure 5. Binding of nuclear factors from HeLa and F412B2TK$^-$ cells to E_3AP1 and *myc*AP-1 oligo. Only the b complex has been shown to be a FOS/JUN (AP-1) protein (see text).

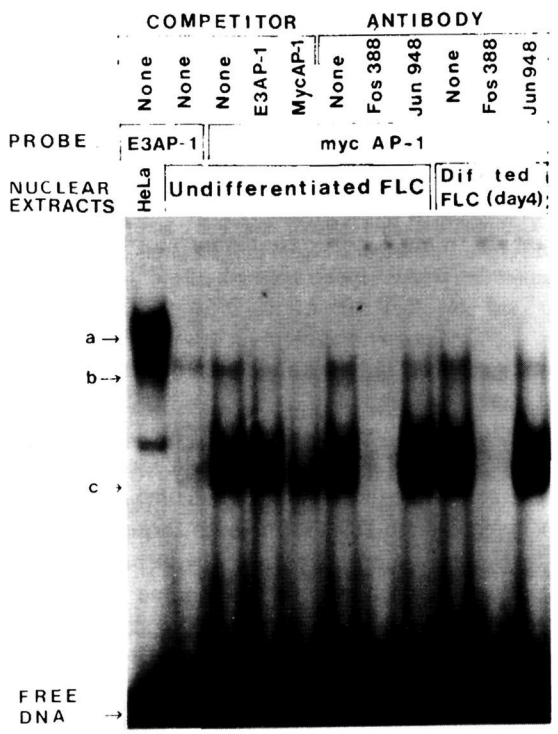

Figure 6. Effect of competitors (lanes 3-5) and FOS and JUN antibodies (lanes 6-11) on c-*myc* DNA-protein complexes in a gel retardation assay. Extracts from both undifferentiated and finally differentiated (day 4) FL cells were used for the study of AP-1. FOS 388 antibody eliminates both b and c bands while JUN 948 inhibits only the binding of the b (AP1) complex.

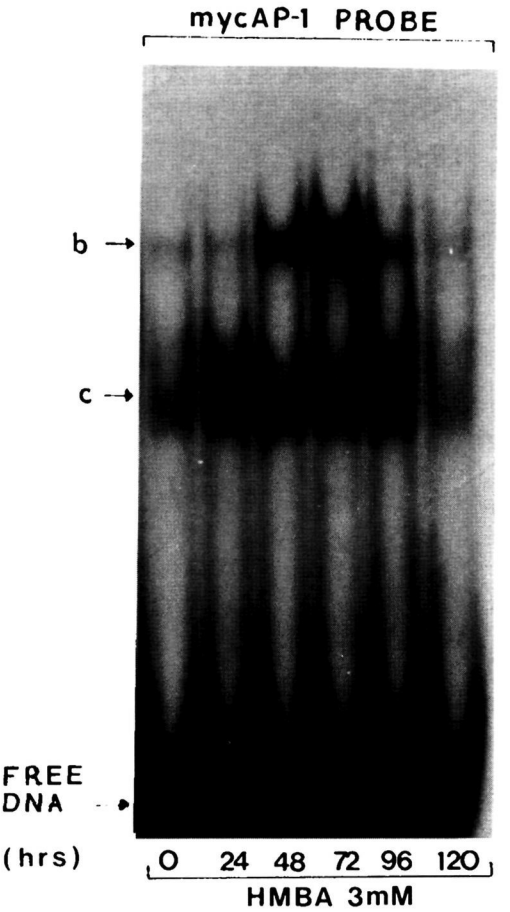

Figure 7. Altered protein-binding activity on *myc*AP-1 oligo was observed during differentiation of F412B2TK$^-$ cells with 3 mM HMBA.

lanes 6-11). Competition assay with excess (200X) of E_3AP1 showed a decrease in the formation of protein-DNA complex b, while both protein-DNA complexes (b and c) were inhibited in the presence of 200X excess of *myc*AP-1 oligo (Fig. 6, lanes 3-5).

Finally, we used end-labelled *myc*AP-1 oligo to study the binding activity of AP-1 during HMBA-induced differentiation of FLC. Cell extracts from FLC induced with 3 mM HMBA or non-induced were incubated with the *myc*AP-1 probe. As shown in Fig. 7, a dramatic increase in AP-1 binding is observed during the first 72 h, followed by a drop at 96 and 120 h post-induction. Similar results were obtained in two other independent experiments, while in another, the peak of binding was observed on day 2 (48 h) after induction, probably reflecting cell-cycle effects.

In order to further certify that the altered protein binding was indeed AP-1, we repeated the experiment described above, using E_3AP-1 probe instead of *myc*AP-1. As shown in Fig. 8, the same binding pattern was observed, except that there was no formation of the complex c.

Discussion

HMBA-induced differentiation of mouse erythroleukemic (Friend) cells is a multiple-stage process involving an early latent period during which metabolic changes can be detected,

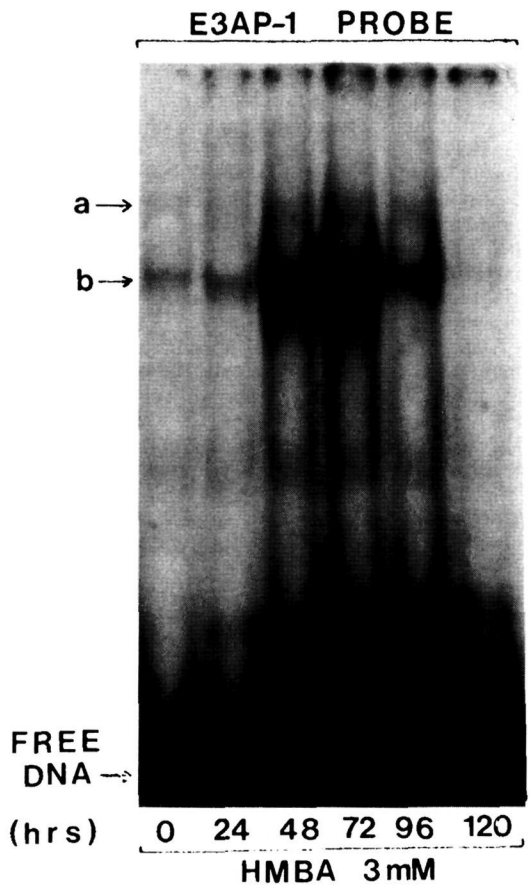

Figure 8. Protein extracts from differentiated and undifferentiated Friend cells were incubated with E_3AP-1 and run in a gel retardation assay. A similar binding effect, compared with *myc*AP-1 oligo, was observed except that the c-complex did not bind.

although cells are not yet committed irreversibly to differentiate, that is, they can not undergo the program of terminal cell division and gene expression upon removal of HMBA from the culture. In the presence of HMBA more than 90% of the cells are committed after 48-60 h exposure to HMBA (15). Induction of globin genes is observed after 24 h (Fig. 2a and 3) while irreversible cessation of proliferation occurs 72 to 96 h post-induction (Fig. 4).

Nuclear oncogenes, especially c-*myc* and c-*myb*, show a biphasic change in their expression, which may be correlated with the commitment of FLC to differentiate. Constitutive c-*myc* expression blocks differentiation but not commitment (27), probably by interfering at a point prior to or close to the commitment (28). Taking also into account that the late but not the early decline in mRNA, coincides well with the change of protein level (29), commitment may require regulatable expression of c-*myc* whereas induction to differentiation may require only the late decline in c-*myc* mRNA (28). The mechanism of c-*myc* down regulation in both cases is complicated and both transcriptional and post-transcriptional control have been reported (4,16,30). Since a promoter-driven regulation has been suggested for c-*myc* expression during the differentiation of F412B2(TK$^-$) Friend erythroleukemia cells (16), it was of interest to study the binding activity of FOS/JUN (AP-1) complex on a c-*myc* negative regulatory element found to contain an AP-1 binding site (19). The NRE domain of c-*myc* has been previously shown to be important for c-*myc* expression after anthracycline (25) and platinum compound (31) exposure of Friend cells.

Among the three factors bound to the NRE domain of c-*myc*, the AP-1 complex and the ubiquitous octamer-binding factor have been identified (Fig. 5, bands b and a, respectively) (19,26). These two complexes bind to highly overlapping segments of the c-*myc* sequence but do not cross-compete (26). In agreement with these experimental data, we observed no alteration in binding activity of either octamer-binding factor or the unidentified complex c (Fig. 5 and 7) on *myc*AP-1 oligo, indicating that any effect exerted from *myc* NRE during differentiation of F412B2TK$^-$ cells could be explained in terms of FOS/JUN altered levels bound to the AP-1 site.

In previous studies, FOS antibodies and *in vitro* translated FOS and JUN proteins were used to certify that the specific factor which binds to the AP-1 site of the NRE of c-*myc* is a FOS/JUN complex (19,26). We showed that JUN antibody also inhibits the binding of AP-1 complex to *myc* NRE (Fig. 6, lanes 8 and 11), further supporting that the components of the nuclear factor with AP-1 binding capacity are the FOS and JUN proteins.

FOS and JUN proteins have been proposed as possible contributors to the repression of c-*myc*, through binding to *myc*-NRE (19). Reciprocal regulation of c-*myc* and c-*fos* has been observed during differentiation of a murine myeloid leukemia cell line; c-*myc* expression ceases as those cells differentiate, whereas c-*fos* is activated (32). It has also been shown that induction of c-*myc* by mitogens begins only after expression of c-*fos* begins to decline (33). Similarly, we showed that c-*myc* is down-regulated while c-*fos* mRNA levels become higher in differentiated than non-differentiated FL cells (Fig. 2, lanes b and c). Nevertheless, regulation of *fos* and *jun* expression is complex and both transcriptional and post-transcriptional control could contribute to the final AP-1 activity (17). Since there is a clear increase in AP-1 binding to the c-*myc* AP-1 site (Fig. 7) we propose that FOS/JUN complex may contribute to down regulation of c-*myc* during the HMBA-induced differentiation of FL cells. Furthermore, since the increase of binding is observed during the first 3 days of induction, at or before cellular terminal division and gene expression (Figs. 2, 4 and 7) we conclude that the increased binding of AP-1 followed by a drop on days 4 and 5 may contribute to repression of c-*myc*, which is most prominent during the last days of exposure to HMBA, and that repression may, in turn, be correlated with the terminal differentiation steps. Recent results, suggesting that induction to differentiation but not commitment may require the late decline in c-*myc* mRNA, are in agreement with such a correlation (28).

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