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Selection of down-regulated sequences along the monocytic differentiation of leukemic HL60 cells

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Abstract In order to dissect the molecular mechanisms of monocytic differentiation we have developed a subtractive hybridisation method based on a simplified 'representational difference analysis'. We have selected 16 sequences and confirmed their down-regulation along the TPA-induced monocytic differentiation of HL60 cells. Among these sequences we have identified the α-tubulin, the TaxREB protein and two ribosomal protein sequences which had not been previously described as differentially expressed. These results add to our knowledge about the molecules implicated along the monocytic differentiation and growth arrest of leukemic cells and provide a first step in the study of their respective roles.

Key words: Subtractive hybridization; Monocytic differentiation; Leukemia; α-Tubulin; Ribosomal protein; TaxREB

1. Introduction

The monocyte-macrophages are key components of the immune system. They are multifunctional cells and participate in both specific and non-specific immune responses. When challenged by pathological agents or neoplastic growth, the monocyte-macrophages are able to be activated and develop specialised functions, such as phagocytosis, cytokine secretion, T-cell antigen presentation process and stimulate the cell-mediated immunity. They also actively participate in acute and chronic inflammation processes by infiltrating inflammation sites and secreting the inflammatory cytokines, interleukin-1, interleukin-6 and tumour necrosis factor, which mediate the recruitment and activation of other immune cells, such as neutrophils (reviewed in [1]).

The monocytes develop in the bone marrow from multipotent hematopoietic precursors, under the influence of soluble growth factors, such as GM-CSF, M-CSF, interleukin-3, and physical interactions with stromal cells (reviewed in [2]). Acquisition of the mature phenotype is accompanied by growth arrest and passage from the bone marrow to the bloodstream. Blood monocytes provide a common source of tissue macrophages. Monocytes infiltrate tissues and body cavities in response to yet unknown signals and develop into resident macrophages, depending on the local microenvironment. They acquire specialised phenotypes and functions, such as those exhibited by Kupffer cells in the liver, osteoclast in the bone or microglia in the brain [3].

The cell differentiation process needs an ordered gene expression regulation including both activation and repression of lineage-specific and ubiquitous genes, leading to growth arrest and acquisition of novel functions. The molecular mechanisms involved in this process have been partially understood, using in vitro models of monocytic differentiation such as the human myelomonocytic cell lines HL60 or U937 induced by a variety of chemical agents including phorbol ester, 1,25-dihydroxyvitamin D3 or cytokines such as GM-CSF [4]. Numerous reports focused on the transcription regulation by *c-myc*, *c-fos* and *c-jun* oncogenes or the PU.1, NF-IL6 transcription factors, in order to define their key roles in monocytic differentiation [5–8]. The isolation of several HL60 and U937 TPA-resistant variants allowed the characterisation of essential regulators such as different protein kinase C isoforms [9,10]. Furthermore, analyses of gene expression modulations by cDNA differential screening have lead to the identification of some molecules including the transcription factor Erg-1 [11], the Mpg-1 protein [12] or the monocyte serine esterase-1 [13], the expressions of which are enhanced along monocytic differentiation.

Recently, 'representational difference analysis' (RDA) was developed to isolate genomic sequences containing mutations [14] and subsequently adapted to cDNA difference analysis by Hubank and Schatz [15]. To isolate the sequence differences between two nucleic acid populations, these methods use PCR amplification of DNA populations, DNA hybridisation in solution, enzyme digestion to remove shared sequences and target sequence selection by PCR. The subtracted product containing the target sequences can be directly cloned or labelled to screen cDNA libraries. By adapting the ratio between driver and tester amounts, this method has proved to efficiently isolate sequences present at different levels in driver and tester cDNA representations, as well as sequences present only in the tester representation [15]. We developed a similar procedure involving fewer steps, based on the simplified genomic RDA procedure described by Zhu [16] and adapted to cDNA populations. Moreover, our method presents the advantage of isolating qualitative and quantitative differences between mRNA populations in a single subtraction experiment.

Here we report the results obtained by the selection of sequences down-regulated along the monocytic differentiation of HL60 cell line induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA). For cDNA subtraction, the driver cDNA was

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Abbreviations: RDA, representational difference analysis; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; MPO, myeloperoxidase; CREB, cAMP-responsive element-binding protein; UP, universal primer; FUSE-BP, far upstream binding protein; RBP, ribosomal protein

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prepared from differentiated cells and the tester cDNA was from uninduced cells. Sixteen clones were selected by a cDNA library screen with the subtracted product. They were subsequently confirmed to be differentially expressed and then, sequenced. Two of these sequences encode proteins already known as down-regulated along monocytic pathway whereas, five sequences encode proteins that have not been described as repressed along monocytic differentiation, among them, the α-tubulin protein, the TaxREB transcription factors, and ribosomal proteins. The last one corresponds to sequences not found in data bases. These results add to our knowledge about the molecules the expression of which is modulated after TPA-induced monocytic differentiation and provide the first step of the study of their respective roles along the monocytic differentiation.

2. Materials and methods

2.1. Cell culture and differentiation

The human promyelocytic cell line HL60 and the promonocytic cell line U937 were cultured in RPMI-1640 medium supplemented with 10% foetal calf serum (Gibco, BRL), 4 mM L-glutamine, and 100 μg/ml gentamicine. Cells were grown to a density of 10^6 cells per ml and then, induced to differentiate with 10 nM of TPA (Sigma). The macrophage differentiation was controlled by morphologic assessment after May Grünwald Giemsma staining and cytofluorimetry using a fluorescein-CD11c antibody (Sigma).

2.2. RNA preparations

Total and poly(A)$^+$ RNA were respectively prepared by guanidine isothiocyanate method [17] and polyAttract System 1000 kit (Promega).

2.3. Oligonucleotides

UPdN6	GCCGGAGCTGCAGAATTCNNNNNN
UP	GCCGGAGCTGCAGAATTC
AD1	CGGGAATTCGCTCGACATG
NlaIII(dT)15(V)	CGGGAATTCGCTCGACATGTTTTTTTTTTTT-TTTV
T3	ATTAACCCTCACTAAAGGGA
T7	TAATACGACTCACTATAGGG

2.4. Subtractive cDNA hybridisation in solution

For driver cDNA synthesis, 2 μg of poly(A)$^+$ RNA was used to synthesise random double-stranded cDNA, as described by P. Froussard [18] with a few modifications. Briefly, the first-strand cDNA synthesis reaction was set up combining the mRNA template, 100 ng of UPdN$_6$ primer, 5× RT-buffer, 1 U of RNAse inhibitor (RNAsin, Promega), 100 U of M-MLV reverse transcriptase (Promega, France) in a final volume of 12.5 μl; the mixture was incubated at 37°C for 2 h then heated at 99°C for 2 min. Second-strand cDNA synthesis was achieved by adding DNA polymerase buffer, 0.3 mM of each dNTP, 5 U of Klenow fragment (Promega, France) in a final volume of 50 μl. The mixture was incubated at 37°C for 20 min. The double-stranded cDNA was purified on a Sephacryl S400 HR spin column (Pharmacia). Multiple PCR reactions were set up combining 5 μl of purified cDNA, 1 μM of UP primer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 10× *Taq* polymerase buffer and 0.4 U of *Taq* Polymerase (Goldstar, Eurogentec) in a final volume of 100 μl, then 30 cycles were performed (94°C for 1 min, 55°C for 1 min, 72°C for 3 min). The amplifiates were purified on Sephacryl S400 HR spin columns and the quantification was performed by spectrophotometry at 260 nm.

Double-stranded tester cDNA synthesis was performed as described previously [19]. The first strand was primed with an anchored oligo(dT) carrying a *Nla*III restriction site at the 5′ end. The cDNA was purified on a Sephacryl spin column and precipitated with 1 volume of isopropanol and 0.1 volume of sodium acetate (3 M, pH 4). Then *Nla*III digestion was performed with 10 U of *Nla*III enzyme (Biolabs, New England) at 37°C for 6 h. After cDNA precipitation, the AD1 oligonucleotide was ligated in a reaction mixture combining the digested cDNA, 200 ng of AD1, the ligation buffer and 0.5 mM ATP in a final volume of 9 μl. Heating at 50°C and cooling to 10°C over a period of 50 min performed the annealing of AD1 to the 3′ protruding cDNA ends. Then, 1 U of T4 DNA ligase (Promega, France) was added and the medium was incubated at 14°C for 12 h. After enzyme inactivation (70°C for 10 min) the 3′ ends were filled in by adding 0.3 mM of each dNTP, DNA polymerase buffer and 5 U of Klenow fragment, in a final volume of 20 μl and the mixture was incubated at 37°C for 10 min, then the enzyme was heat inactivated at 70°C for 10 min. Excess AD1 was removed by purification on a Sephacryl spin column and quantification of the remaining tester cDNA was performed by staining with colloidal gold (Genogold, Tebu), according to the manufacturer's instructions.

For subtractive hybridisation, 50 μg of driver cDNA and 250 ng of tester cDNA were co-precipitated. The pellet was ethanol-washed and resuspended in 4 μl of hybridisation buffer (30 mM HEPES, pH 8, at 20°C, 1 mM EDTA). After denaturation (99°C for 7 min), the salt concentration was adjusted to 1 M NaCl with 1 μl of 5 M NaCl and the cDNA was allowed to anneal for 24 h at 68°C. Then the reaction mixture was 5-fold diluted and the DNA was precipitated with isopropanol. The S1 nuclease digestion was carried out in a final volume of 50 μl of 1× nuclease buffer with 25 U of S1 nuclease (Promega) at 22°C for 30 min. The amount of enzyme must be adjusted for each batch. The pH of the reaction was adjusted to 7.0 with 0.2 μl of 2 M NaOH and the reaction was phenol/chloroform extracted, isopropanol precipitated in the presence of an additional 25 μg of driver cDNA. After centrifugation and ethanol wash, the pellet was resuspended in 4 μl of hybridisation buffer for a second and a third round of subtraction in the same conditions as above. After purification on a Sephacryl spin column, the selective amplification was performed with 2 μl of subtraction mixture, 1 μM of AD1, 1.5 mM $MgCl_2$, 1× *Taq* DNA polymerase buffer and 0.4 U of *Taq* DNA polymerase (Goldstar, Eurogentec).

2.5. cDNA library screen

An HL60 cDNA library was constructed using the lambda ZAPII cloning kit (Stratagene). The mRNA prepared from uninduced, TPA-induced cells and DMSO-induced cells were pooled to obtain a representative library of the HL60 phenotypes. The cDNA library screen was performed as described [19]. The subtracted probe was labelled by PCR in the same conditions as above, including 0.2 mM of digoxigenin-dUTP in the dNTP mix and decreasing dTTP to 0.5 mM.

2.6. Sequencing and database searches

The sequencing reactions were performed with the Autoread sequence kit (Pharmacia) according to the manufacturer's instructions, using the T3 and T7 fluorescein end labelled primers. The sequence gels were run on an ALF automatic sequencer (Pharmacia). The comparisons to Genbank and EMBL databases were performed with Blast program [20].

2.7. Northern blot and dot-blot analyses

Five micrograms of total RNA per lane were size fractionated on 1.2% agarose gel containing 2.5% formaldehyde and transferred on a nylon membrane positively charged (Appligene). Hybridisations and chemiluminescent detection of digoxigenin-labelled probes were performed as previously described [21]. The filters were exposed to XAR-Biomat film (Kodak) for times ranging from 6 to 24 h. The amount of transferred RNA was controlled by staining the blots with colloidal gold (Genogold, Tebu). The digoxigenin-labelled probes used in Northern and dot-blots analyses were synthesised by PCR using the vector T3 and T7 primers in the same conditions as above.

Random cDNAs were synthesised from poly(A)$^+$ RNA of differentiated and undifferentiated cells as above for driver synthesis [18]. Hybridisation and revelation steps were conducted as for Northern blots.

3. Results

3.1. Subtractive hybridisation

The promyelocytic cell line HL60 was stimulated by 10 nM of TPA for 48 h and the macrophage phenotype was controlled by microscopic observations of cell adherence, cell ag-

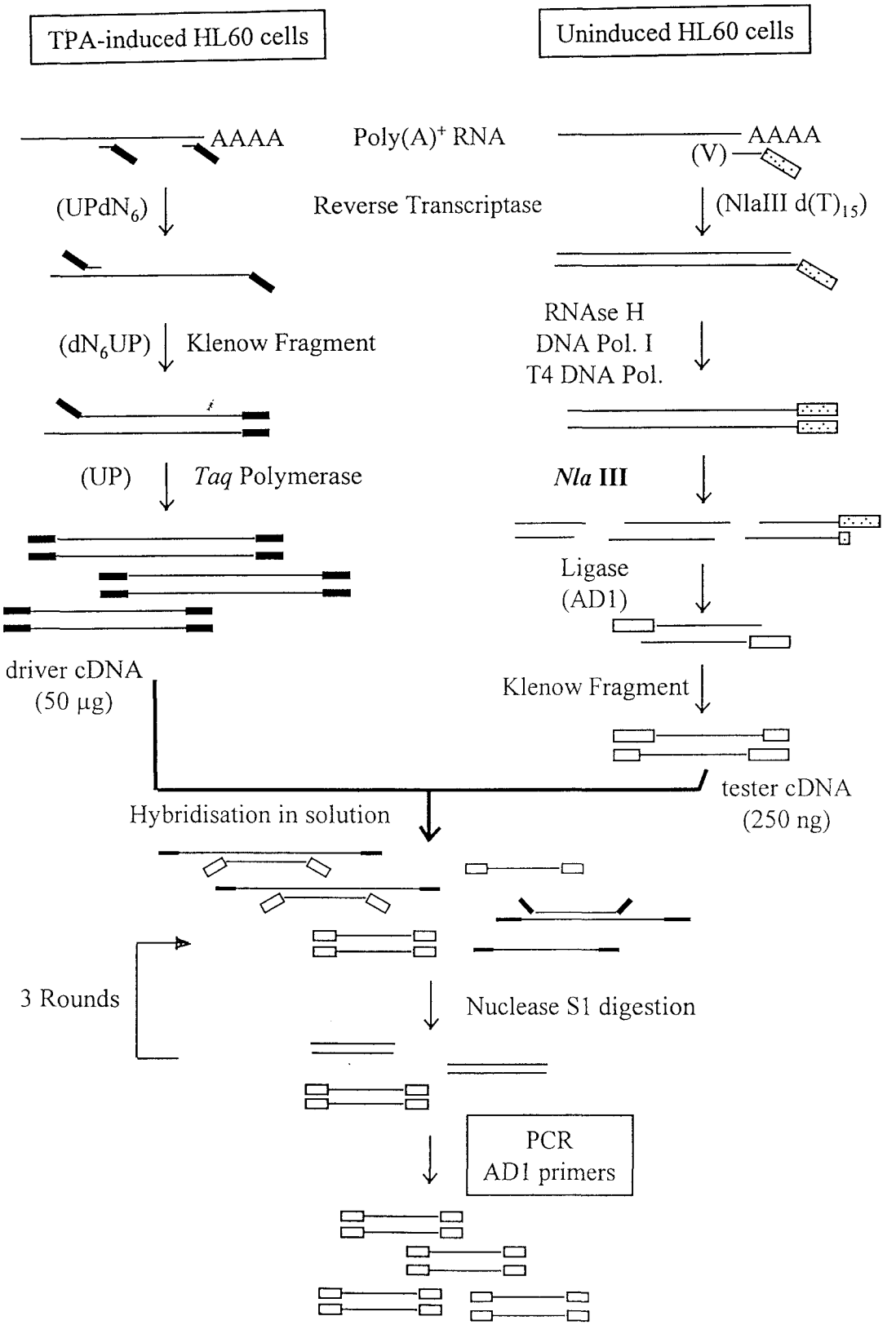

Fig. 1. Outline of the cDNA subtractive procedure. For details see text.

gregation and morphology after MGG staining, as well as FACS analysis of the CD11c expression [22] (data not shown).

To isolate the sequences down-regulated during the maturation process, a cDNA subtraction procedure was performed (Fig. 1). The driver cDNA was randomly synthesized with a degenerated hexanucleotide carrying an 'universal-primer' (UP) at the 5′ end [18]. The length of the second-strand

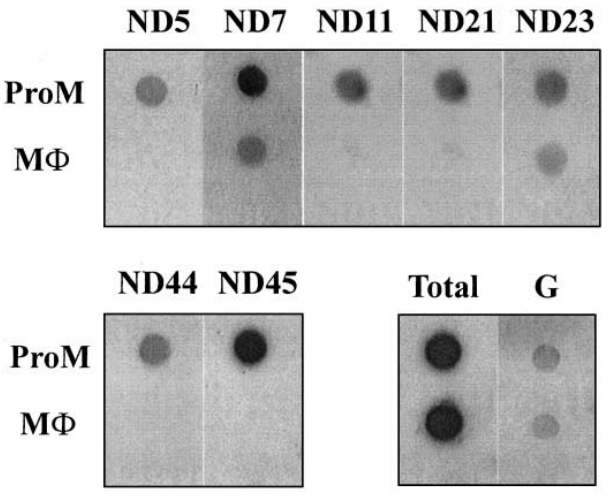

Fig. 2. Differential expression of seven clones isolated by the cDNA subtraction procedure. Random-PCR cDNAs from undifferentiated (ProM) and TPA-differentiated (MΦ) HL60 cells were dotted on nylon membranes. Hybridisations were performed with digoxigenin-labelled probes corresponding to the repressed sequences. The amounts of dotted cDNA were controlled either by hybridisation with a total cDNA probe (Total) or by direct staining with colloidal gold (G).

cDNA ranged from 200 to 800 bp, with an average of 500 bp, making these molecules amplifiable with the same efficiency using UP. Parallel amplification reactions were run to obtain enough driver cDNA for an efficient subtraction.

For the tester preparation, the cDNA synthesis was initiated with an oligo(dT) carrying a 3′ degenerated nucleotide (to avoid getting a long poly(dT) stretch) and a *Nla*III restriction site at the 5′ end. The double-stranded cDNA was restricted with this 4-cutter enzyme and the AD1 oligonucleotide was ligated at the 5′ ends. Then the 3′ ends were filled in to generate amplifiable blunt end molecules. We choose *Nla*III enzyme to ensure that most cDNA molecules would be cut at least once; the length of restricted cDNA molecules was quite homogeneous, with an average of 300 bp, so all these fragments can be expected to be amplified with the same efficiency.

The tester and the driver were mixed in a ratio of 1:200 and allowed to hybridise at 68°C for 24 h. Then, the S1 nuclease digestion removed the non-hybridised ends of the tester/driver hetero-hybrids, making these molecules non-amplifiable. For the second and third round of subtraction an additional amount of driver was added to compensate the loss of driver during S1 nuclease digestion because of the random nature of these cDNA molecules. After three rounds of hybridisation/S1 nuclease digestion the specific tester sequences still carrying the AD1 sequence were selectively amplified.

The specificity of the subtracted product was checked by hybridisation on dot-blots of tester and driver cDNA generated by random-PCR. As expected, the subtracted product gave a hybridisation signal higher with the cDNA obtained from undifferentiated cells than with the cDNA obtained from differentiated cells (data not shown).

3.2. cDNA library screen

The resulting subtracted product was digoxigenin-labelled and used to screen 25000 clones of a HL60 cDNA library. Forty-five positive clones were selected and cross-hybridised to eliminate redundant sequences. As a first control for differential expression of the isolated sequences, each of them was digoxigenin-labelled and hybridised on dot-blots of cDNA amplifiates prepared from differentiated and undifferentiated cells (Fig. 2). The amounts of dotted cDNA were controlled either by hybridisation with a total cDNA probe or by direct DNA staining with colloidal gold. Both methods gave similar results. All the tested sequences showed a decrease in their expression levels but some of them are still expressed in differentiated cells.

3.3. Sequencing of the selected clones

Sixteen clones were further characterised by sequencing and comparison to Genbank and EMBL databases. The results are summarised in Table 1. The myeloperoxidase (MPO) and the far upstream sequence element-binding protein (FUSE-BP) were previously described as differentially expressed along monocytic differentiation [31–33]. The ND46 sequence matched equally the CREB-2 and TaxREB67 sequences, which are two members of the ATF/CREB transcription factor family.

3.4. Kinetic of expression of the selected clones

The decrease in α-tubulin, RBP P0, TaxREB107, and MPO mRNA amounts were investigated along macrophage differentiation. The HL60 cells were collected at various times after the TPA induction (0, 30 min, 1 h, 24 h, and 48 h) and total RNA were submitted to Northern blot analyses. The *c-myc* and *c-fos* mRNA levels were also determined as controls. The decrease in *c-myc* expression correlates with the growth arrest of the mature cells, and the increase in *c-fos* expression correlates with the increase in activity of the AP1 transcription factor [34].

Fig. 3 shows a rapid 2-fold decrease in α-tubulin mRNA

Table 1
Sequencing results of the 16 selected clones

Clone numbers	Identification	References
ND51, ND 35	Myeloperoxidase	[23]
ND45	FUSE-binding protein	[24]
ND46	CREB-2 and TaxREB67	[25,26]
ND22, ND25, ND26	α-Tubuline	[27]
ND11	TaxREB107	[28]
ND5, ND28, ND34, ND 39, ND40	RBP P0	[29]
ND21	RBP L17	[30]
ND38	Non-identified	–
BqND5	Alu sequence repeat	–

Comparisons to Genbank and EMBL databases were performed with Blast softwares. The sequences were submitted to Genbank database; the accession numbers are AF004537 to AF004552.

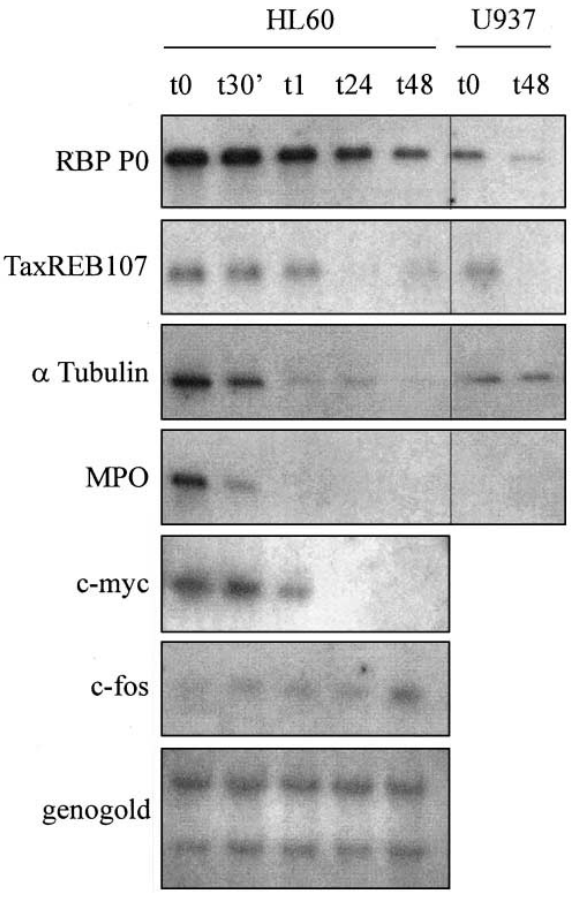

Fig. 3. mRNA levels of selected clones along the differentiation process. Five micrograms of total RNA per lane were size fractionated on a 1.2% agarose formaldehyde gel and transferred on nylon membranes. Probes corresponding to RBP P0, α-tubulin, MPO, *c-myc*, *c-fos* mRNAs were digoxigenin-labelled. Hybridisation and revelation were performed as described in Section 2. The loading and transfer of the RNAs were checked by direct staining with colloidal gold.

within 30 min after the TPA stimulation and after 48 h, the signal was barely visible with non-radioactive Northern blot detection. TaxREB107 and RBP P0 mRNA levels decrease significantly after 24 h of TPA stimulation. The results obtained for MPO mRNA were consistent with those previously described [30,31].

We also investigated the mRNA levels along the TPA-induced differentiation of the myelomonocytic cell line U937. These cells are more mature than HL60 cells and do not express the MPO mRNA. We observed comparable down-regulations for TaxREB107 and RBP P0 mRNAs, but not for α-tubulin mRNA.

So the subtraction-selected sequences showed different kinetic decreases suggesting that different mechanisms are involved in controlling their mRNA levels. Some may be related to the cell proliferative state and other may be related to the maturation stage.

4. Discussion

The aim of this work was to isolate and characterise molecules implicated in macrophage differentiation, in order to dissect the molecular mechanisms of this physiological process. A subtractive cDNA hybridisation was performed to isolate the sequences down-regulated after TPA-induced differentiation of the HL60 cell line.

Our results show that the simplified RDA procedure described here efficiently generates cDNA enriched for differentially expressed genes of both high and low abundance. Housekeeping genes as well as transcription factors have been identified, with no false-positives among the 16 clones analysed. Moreover, qualitative and quantitative changes in mRNA levels can be detected in a single subtractive procedure. Indeed, we identified either sequences present only in uninduced cells, or sequences down-regulated but still expressed in differentiated cells. The previously described cDNA RDA and suppression subtractive hybridisation (SSH) procedures need multiple subtractions with various driver/tester ratios to select the sequences presenting quantitative differences between the two cDNA representations [15,35].

We have identified five down-regulated species, among which two encode ribosomal proteins, RBP L17 and RBP P0. Our results add information to the previously reported observations of the down-regulation of several other ribosomal proteins after the chemical-induced differentiation of leukemic cells [36,37]. Some ribosomal proteins are often overexpressed in cancer cells and the growth arrest of the cells is often associated with the normalisation of their expression. This over-expression of ribosomal proteins is not correlated to an over-expression of all the transcriptional machinery: there is a tissue and stage specificity of the over-expressed species, suggesting a specific role for each ones in the proliferation and/or differentiation. The specialised functions of the ribosomal proteins remain to be understood. Some of them may differently affect the translation of certain mRNA as do the acidic ribosomal proteins P1 and P2 in *Saccharomyces cerevisiae* [38]. The fact that the amount of RBP P0 mRNA is weaker in undifferentiated U937 than in undifferentiated HL60 cells suggests that the mRNA level of this ribosomal protein is rather correlated to the maturation stage of the cells than to the proliferative state.

The acquisition of the mature macrophage phenotype is characterised by morphological changes including cell adherence, pseudopodia formation and phagocytosis. These changes are dependent on the reorganisation of the cytoskeletal networks, in particular the microtubules and the microfilaments [39,40]. The chemical-induced depolymerisation of the microtubule network potentiates the TPA-induced monocytic differentiation of leukemic cell lines, suggesting a role of the microtubules in some signal transduction pathways [41]. The protein kinase C activation induced by TPA leads to α-tubulin and microtubule-associated protein phosphorylations and, as a consequence, to the destabilisation of the microtubules architecture and an increase in the microtubule depolymerisation [42]. It has been recently reported that this depolymerisation is crucial for the early stages of the U937 cell differentiation induced by TPA. U937 variants lacking cytoskeletal reorganisation and β2-integrins translocation to the cell membrane after TPA-stimulation, fail to acquire a mature macrophage phenotype [10]. So, this cytoskeletal reorganisation appears to be a prerequisite for complete monocytic maturation and transcriptional activation of genes such as *CD11b* and *c* genes. Furthermore, it has been reported that the modification of soluble to polymeric tubulin ratio directly

regulates the expression of some genes such as the urokinase-type plasminogen activator gene [43,44] and regulates the activities of the NF-κB transcription factor and c-Myc protein [45,46]. Our Northern blot analysis indicates a rapid decrease in the α-tubulin mRNA level after 30 min of TPA-stimulation of HL60 cells, suggesting that the mechanism involved in the early stage would rather be a decrease in the mRNA stability than a modification of the transcription rate. An autoregulation of the α-tubulin mRNA stability by the soluble protein has been suggested by several reports as a way of controlling the polymeric to soluble tubulin ratio [47]. Surprisingly, no decrease in mRNA level was observed between unstimulated and 48 h TPA-stimulated U937 cells, but a transient decrease after TPA-addition cannot be excluded.

Taken together, these results indicate that the cytoskeleton does not have only a mechanic role in monocyte biology: the microtubules participate actively to the regulation of gene expression and our results show that the regulation mechanisms involving microtubules seem to occur not only at the post-translational but also at the synthesis levels.

The TaxREB107 protein is a leucine zipper transcription factor that binds specifically the Tax responsive enhancer element in the long terminal repeat of the human T-cell leukemia virus type I. It was isolated from the human T-cell line Jurkat, but significant levels of TaxREB107 mRNAs were observed in all cell lines and tissues analysed [27]. This factor acts together with other cellular factors such as ATF and CREB to mediate trans-activation of the viral protein Tax [48]. The down-regulation of both CREB and TaxREB107 mRNAs suggests a possible cooperation between these transcription factors in some gene expression regulations even in the absence of HTLV-1 Tax protein. The physiological function of TaxREB107 in non-infected cells, is not yet known, however, its constitutive and ubiquitous expression argues for an important and biologically conserved function. Here we have shown that the TaxREB107 mRNA level dramatically decreases along monocytic differentiation of HL60 and U937 cell lines. Moreover, the TaxREB107 mRNA level is comparable in both proliferating cell lines and so, it seems to be related to the proliferative state rather than to the maturation stage. It has been reported previously an increase in TaxREB107 mRNA level in Jurkat cells after TPA stimulation which leads to an increase in growth rate [49]. In all cases the maximal expression of this factor is associated with the proliferative stage of the cells. It will be interesting to identify the consensus recognition sequence of this transcription factor and its target genes to appreciate its function in macrophage differentiation and T-cell activation.

As a conclusion, we developed a subtractive hybridisation procedure which can be applied to very low amounts of starting material and seems to give very few false-positive clones, which is a major drawback in many subtractive procedures. This procedure allowed us to identify several sequences down-regulated during HL60 leukemic cell differentiation. The data reported here may provide new approaches in the study of myeloid differentiation and proliferation arrest.

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