Drastically increased expression of *MYC* and *FOS* protooncogenes during *in vitro* differentiation of chronic lymphocytic leukemia cells

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ABSTRACT Chronic lymphocytic leukemia cells, representing a clonal population of resting B lymphocytes, were induced to differentiate into immunoglobulin-secreting lymphoblasts and plasmablasts by phorbol 12-myristate 13-acetate. The induction resulted in a rapid increase in the molar ratio of secreted/membrane-bound μ -chain mRNA. Immunoglobulin secretion was preceded by a transition of the cells from the G₀ to G₁ phase of the cell cycle, as indicated by an increase in RNA and protein synthesis, and an overall increase in cellular RNA. The cells, however, became blocked in G₁ and did not enter S phase. The expression of *MYC* and *FOS* was rapidly induced by the phorbol 12-myristate 13-acetate treatment. The induction of *FOS* preceded the shift in secreted/membrane-bound μ -chain mRNA molar ratio, while that of *MYC* occurred concomitantly with the shift, but prior to induction of total RNA synthesis and immunoglobulin secretion. *MYC* expression remained at a relatively high level during the whole differentiation process. It is thus concluded that a decline of *MYC* expression is not a prerequisite for differentiation of the chronic lymphocytic leukemia cells. This suggests that *MYC* expression may play a different role during differentiation of nonproliferating B cells than in the myelomonocytic cell lines HL-60 and U-937, where *MYC* expression has been reported to decrease during induced differentiation. The results also show that the expression of the *MYC* and *FOS* genes does not result in the transition of these cells into the S phase of the cell cycle.

It has been suggested that protooncogenes have a role in the control of growth and differentiation of normal and malignant cells (1), a view which is supported by the characteristics of the protooncogenes *MYC* and *FOS* in various cell types. The expression of these two genes is rapidly induced during the G₀ to G₁ transition by several growth factors and mitogens (2–5). Moreover, a high constitutive expression of the *c-myc* or *v-myc* genes under the influence of strong promoters has been reported to reduce the requirement for growth factors in various cell types (6–8). These results suggest that the expression of *MYC* and *FOS* may be part of the signal pathway used by growth factors.

Normal hematopoietic cells appear to be regulated by two coupled, but inversely related, genetic programs—one for proliferation and one for differentiation—operating during the stepwise maturation process resulting in terminally differentiated cells (9). Established malignant hematopoietic cell lines, blocked in their differentiation but capable of undergoing partial or terminal differentiation when exposed to various inducers, have been used as models for investigating the relationship between protooncogenes and differentiation. A decrease in *MYC* expression has been reported during induced differentiation of the human cell lines HL-60

and U-937 (10–12). The view has, therefore, been put forward that *MYC* expression needs to be down-regulated to allow the differentiation program to be carried out (13). *FOS*, on the other hand, is rapidly induced and, thereafter, continuously expressed after phorbol 12-myristate 13-acetate (PMA) induction of HL-60 and U-937 cells and has been suggested to be a prerequisite for macrophage differentiation (14, 15).

Few studies have been performed concerning the relationship between the expression of *MYC* and *FOS* and human B-cell differentiation (16). In the present study we have employed a human chronic lymphocytic leukemia (CLL), I-73, with phenotypic features similar to those of resting normal B lymphocytes. I-73 cells may be induced by PMA to differentiate into immunoglobulin-(Ig) secreting lymphoblasts and plasmablasts without undergoing proliferation (17, 18). Using flow cytometry to quantitate RNA, DNA, cell volume, and some cell cycle-related antigens, Nilsson *et al.* (19) have shown that the I-73 cells represent a homogeneous population of resting G₀ cells that synchronously enter G₁ only to become blocked in late G_{1A} upon induction by PMA.

This system enables us to study the relationship between the expression of *MYC* and *FOS* and the differentiation of nonproliferative B lymphocytes. The present results suggest that (i) *MYC* and *FOS* expression is drastically increased during the G₀ to G₁ transition, (ii) continuous expression of *MYC* mRNA and protein does not prevent differentiation in contrast to what has been suggested for the HL-60 and U-937 cell lines, (iii) the increased expression of *MYC* and *FOS* does not result in proliferation.

MATERIALS AND METHODS

Cells and Cell Culture. Leukemic, monoclonal (IgM κ) lymphocytes were obtained from a CLL patient (I-73). Mononuclear cells, purified over a one-step Ficoll/Hypaque density gradient, consisted of monoclonal B-CLL cells contaminated with 5% OKT3 monoclonal antibody-positive T cells and <0.5% monocytes. I-73 cells were cultured in RPMI 1640 medium supplemented with glutamine, antibiotics, and 10% (vol/vol) newborn calf serum at a cell density of $1\text{--}2 \times 10^6$ cells per ml in the presence or absence of 1.6×10^{-7} M PMA (Sigma).

RIA for IgM. An inhibition RIA, using purified antibodies against human μ chains (Dakopatts, Copenhagen, Denmark) was performed to quantitate the amount of IgM in supernatants, as described (20).

[³H]Uridine and [³H]Thymidine Labeling. Triplicates of 2×10^6 I-73 cells were labeled for 2 hr with 10 μ Ci of [³H]uridine (specific activity, 44 Ci/mmol; 1 Ci = 37 GBq; Amersham) and [³H]thymidine (specific activity, 25 Ci/mmol; Amer-

sham), respectively. The incorporation of acid-insoluble radioactive material was then measured by liquid scintillation counting.

RNA Isolation and Analysis. Total cellular RNA was isolated by the guanidine thiocyanate/cesium chloride procedure (21). Denatured total RNA samples (15 μ g per well) were fractionated on a 1.2% formaldehyde/agarose gel, transferred to a nitrocellulose filter, and hybridized with a 32 P-labeled probe as described (22). The filters were subsequently washed in 15 mM NaCl/1.5 mM sodium citrate/0.1% NaDodSO₄ at 53°C for 2 hr prior to autoradiography. Autoradiograms were analyzed by densitometric scanning (Shimadzu CS-930, Kyoto, Japan).

Probes. For *MYC* a 1.5-kilobase (kb) *EcoRI*–*Cla* I fragment spanning the third exon of the human *MYC* gene, prepared from a human *MYC* genomic clone (23), a gift from M. Jansson and B. Vennström, EMBL, Heidelberg, was used. For *FOS* a 2.7-kb *Xho* I–*Nco* I fragment from a human *FOS* clone (24), provided by R. Müller, EMBL, Heidelberg, was used. For actin a 1.2-kb *Pst* I fragment from a mouse α -actin cDNA clone, p91 (25), provided by M. Buckingham, Pasteur Institute, Paris, was used. For μ chains a mixture of three *EcoRI* fragments, each approximately 1.5 kb, from a human μ chain probe, CH4-38 (26), supplied by P. Leder, Harvard Medical School, Boston, was used. The purified fragments were 32 P-labeled by nick-translation (27) to a specific activity greater than 10^8 cpm/ μ g of DNA.

35 S-Labeling and Immunoprecipitation. I-73 cells were labeled with [35 S]methionine (50 μ Ci/ml; specific activity, 1100 Ci/mmol; Amersham) for 3 hr. Prepared cell extracts were immunoprecipitated and analyzed by electrophoresis in NaDodSO₄/polyacrylamide gels followed by autoradiography as described in detail (28). Films were exposed for 4–6 days and scanned densitometrically. Two anti-*MYC* peptide antisera ("anti-c-myc N-ter I" and "anti-c-myc N-ter II") were used for the immunoprecipitation of *MYC* proteins from the CLL cells. Both antisera are directed against residues 24–40 of the human *MYC* protein as described in detail for anti-c-myc N-ter I (29). Anti-c-myc N-ter I recognizes a relatively stable, nonphosphorylated p65 *MYC* protein in human cells, whereas anti-c-myc N-ter II detects a short-lived phosphorylated pp64 *MYC* protein in human cells (28, 29). Anti-c-myc N-ter I was provided by H. Persson, Department of Medical Genetics, University of Uppsala, Uppsala, Sweden, and anti-c-myc N-ter II was generated and provided by T. P. Chow, W. F. De Grado, and E. Knight, Jr., Central Research and Development Department, DuPont, Wilmington, DE.

RESULTS

TPA-Induced Activation and Differentiation of I-73 CLL Cells in the Absence of Proliferation. The activation of the CLL cells by PMA was analyzed by measuring [3 H]uridine and [35 S]methionine incorporation as well as the total RNA content per cell (Fig. 1 A and B). The [3 H]uridine incorporation into trichloroacetic acid-precipitable material began to increase between 4 and 12 hr after induction, yielding a 13-fold increase, peaking at 48 hr. The protein synthesis was enhanced already after 4 hr, peaking at a 25-fold higher level, between 24 and 72 hr after PMA treatment. The elevated RNA synthesis resulted in an increase in total RNA content per cell between 12 and 24 hr after induction. At 72 hr, the RNA content per cell was 3-fold higher when compared to uninduced cultures (Fig. 1B). The morphological changes, consisting of an increase in cellular adherence and an acquisition of lymphoblastoid characteristics in the majority and plasmablast features in a small minority of the cells, were clearly demonstrable at 24 hr and progressed during the following 72 hr. The secretion of IgM was detectable 24 hr

FIG. 1. PMA-induced activation and differentiation of I-73 CLL cells. (A) Kinetics of [3 H]uridine (●) and [35 S]methionine incorporation (○). Aliquots of 2×10^6 CLL cells per ml were labeled with 10 μ Ci/ml of [3 H]uridine for 2 hr and 50 μ Ci/ml [35 S]methionine for 3 hr, respectively, at each time point, samples were precipitated with trichloroacetic acid, and radioactivity was measured in a liquid scintillation counter. (B) Kinetics of IgM secretion (○) and total RNA content (●). Supernatants were collected at different time intervals, and the concentration of secreted IgM was measured by a competitive RIA. Total RNA from equal amounts of cells was prepared by the guanidine isothiocyanate/cesium chloride method (21), and the concentration of RNA was measured spectrophotometrically at 260 nm. (C) Cell count (●) and kinetics of [3 H]thymidine incorporation (○). [3 H]Thymidine labeling was carried out as described for [3 H]uridine above. Exponentially growing HL-60 cells were used as controls.

after induction, reaching a level of 2.5 μ g per ml after 72 hr (Fig. 1B).

The activation of the I-73 cells was accompanied by minimal DNA synthesis between 24 and 72 hr (Fig. 1C). The level of [3 H]dThd incorporation was, however, only 6% of that observed in exponentially growing reference HL-60 cells. In the HL-60 cultures $\approx 50\%$ of the cells became labeled by a 2-hr [3 H]dThd pulse (unpublished observation), we, therefore, estimate that 2–3% of the I-73 cell population were in S phase. The cell recovery at 72 hr was 60%. The viability gradually declined from 95% to 65%.

Increase in the Ratio Between Secreted and Membrane-Bound Forms of μ -Chain mRNA During PMA-Induced Differentiation of I-73 CLL Cells. The relationship between μ -chain mRNA expression and *in vitro* differentiation was studied by RNA gel blot analysis (Fig. 2A) of RNA extracted at different time intervals after the addition of PMA. Two closely migrating mRNA species were found, ≈ 2.4 and 2.7 kb in size, corresponding to the secreted (μ_s) and membrane-bound (μ_m) forms of μ chains, respectively (31). The total amount of μ -chain mRNA was relatively constant during the first hour and then decreased during the next 3 hr to reach a

FIG. 2. RNA gel blot analysis of μ -chain and actin expression in I-73 cells at the times indicated during PMA-induced differentiation. Total cellular RNA was purified from I-73 cells at different times of PMA treatment and fractionated in a 1.2% formaldehyde/agarose gel. Each slot contains 15 μ g of total cellular RNA. After blotting, hybridization was carried out at 42°C in hybridization buffer for 24 hr. (A) Hybridization was carried out using 32 P-labeled fragments of the human μ -chain gene (26) as a probe. (B) The probe was a mouse α -actin cDNA clone (25).

minimum level that was 35% of the value in uninduced cells at 4 hr. In parallel with this alteration, the molar ratio between μ_s and μ_m mRNA changed gradually from $\approx 1:1$ to 4:1. The amount of μ -chain mRNA subsequently increased reaching a maximum between 12 and 24 hr (data not shown), coinciding with the initial detection of IgM secretion (Fig. 1B), and finally approaching the control level 72 hr after induction. Actin expression increased after 12 hr, concomitantly with the blast transformation, and reached a 4.5-fold higher level compared with uninduced cells at 72 hr (Fig. 2B).

Drastically Increased Levels of MYC mRNA After PMA Induction. To investigate whether MYC mRNA is expressed during activation and differentiation of the nonproliferative CLL cells, the same samples as above were analyzed using a human MYC-specific probe (Fig. 3A). Hybridization analysis revealed a 2.3-kb mRNA species. Fresh, noninduced I-73 cells, and samples collected early after induction (15–60 min) contained barely detectable amounts of MYC mRNA. The level began to increase 2 hr after induction and reached a peak at 4 hr. Samples collected later contained less MYC mRNA, and a plateau level was reached 12 hr after induction. The MYC expression between 12 and 72 hr was relatively constant (data not shown). Analysis of mRNA from cells of the human cell line HL-60, known to contain amplified MYC genes (32, 33), demonstrated approximately the same relative amount of MYC mRNA as is present in the I-73 cells 4 hr after induction. Densitometric scanning of the autoradiogram revealed an ≈ 70 -fold increase in relative amounts of MYC

FIG. 3. Analysis of MYC and FOS mRNA expression in I-73 cells after PMA induction. The analysis was carried out as described in Fig. 2. Two RNA samples from HL-60 cells, prepared from uninduced cells and from cells after 48 hr of PMA treatment, were used as controls. (A) A 32 P-labeled fragment derived from the third exon of the human MYC gene (23) was used as a probe. (B) A human FOS probe (24) was used.

mRNA during the first 4 hr after PMA induction. The plateau level was estimated to be 20-fold higher than in noninduced fresh CLL cells.

The FOS Gene Is Rapidly Induced After PMA Treatment. It has been demonstrated that the FOS gene is expressed at elevated levels during PMA-induced monocytic differentiation of HL-60 and U-937 cells, and in several cell types in response to growth factors (3–5, 14, 15). To examine whether FOS expression is also activated during PMA-induced differentiation of I-73 cells, an RNA gel blot analysis was performed, using a fragment from the human FOS gene as a probe. The results (Fig. 3B) revealed a 2.2-kb mRNA hybridizing with the FOS probe. A detectable increase of FOS mRNA was noticed already at 15 min after the induction, reaching its maximum after 1 hr. The level then declined, but the sample collected at 4 hr still contained $\approx 50\%$ of the peak level. At 12 hr and, thereafter, FOS transcripts were barely detectable. A comparison of the FOS gene expression in fresh, uninduced CLL cells and in PMA-induced HL-60 cells revealed that the level of FOS mRNA was 2-fold higher in the CLL cells than in PMA-stimulated HL-60 cells.

Densitometric scanning of the autoradiogram showed an ≈ 35 -fold difference between FOS mRNA in the uninduced sample and in the sample collected 1 hr after PMA induction. Fig. 3B also shows the presence of a possible full-length nuclear transcript of about 3.5-kb increasing drastically up to 1 hr after induction, indicating that the FOS gene transcription reached a maximum during this period. Finally, an RNA

species of ≈ 750 nucleotides was detected, which may represent the excised first intron of the full-length mRNA.

Neither the *MYC* nor the *FOS* expression in the I-73 cell population was significantly altered after proliferating T cells were depleted (from 5% to 0.1%), using OKT3 antibody plus two rounds of a 1:3 dilution of baby rabbit complement, demonstrating that the elevated oncogene expression indeed occurred in the B cells (data not shown).

Increased Levels of MYC Proteins After PMA Induction. As shown above, *MYC* expression in the CLL cells is rapidly increased after PMA stimulation, similar to the effect of mitogens or growth factors in other cell systems (2–5, 29, 30, 34). As the I-73 cells never enter S phase, and as *MYC* expression is down-regulated in other differentiating systems, it was important to rule out the possibility of a specific block in the translation of the *MYC* mRNA. Immunoprecipitations of [35 S]methionine labeled proteins at different times after induction, using the *MYC* protein-specific antibody anti-c-myc N-ter II (28), showed that a 64-kDa *MYC* protein was present in very low amounts in uninduced I-73 cells (Fig. 4), increased drastically after 4 hr of PMA stimulation, and then decreased to a minimum value at 24 hr and remained constant at 72 hr after induction.

By densitometric scanning we found the stimulation at 4 hr to be ≈ 18 -fold and ≈ 3 -fold at 24 hr. The changes in the *MYC* protein levels were similar to those observed for the *MYC* mRNA although the degree of stimulation was less pronounced. Immunoprecipitation with another *MYC*-specific antibody (anti-c-myc N-ter I) (29), detected the accumulation of a 65-kDa *MYC* protein in PMA-stimulated cultures, which was hardly detectable in uninduced cells (data not shown).

DISCUSSION

MYC gene expression has been studied in normal human lymphocytes that were induced to proliferate (29, 30, 34, 35), but none of these studies have examined whether differentiation was simultaneously induced. However, the expression of *MYC* and *FOS* in differentiating human hematopoietic cells has been investigated using established cell lines (10–12, 16). We chose to study the expression of these two oncogenes in the B-CLL system, which is a clonal population of resting

FIG. 4. Immunoprecipitation with anti-human *MYC* peptide antisera. I-73 cells were incubated in the presence of PMA and labeled with [35 S]methionine for 3 hr at the different times after induction indicated. Uninduced cells were also labeled as a control. Cell extracts were prepared and immunoprecipitated with anti-c-myc N-ter II antisera. The samples analyzed were adjusted to contain equal amounts of incorporated radioactivity. Washed immunoprecipitates were analyzed on a 10% NaDodSO₄/polyacrylamide gel followed by fluorography. NRS, normal rabbit serum.

G₀ B cells, that can be induced to differentiate without proliferating by PMA.

Our findings concerning the PMA-induced activation of RNA and protein synthesis, IgM secretion, morphological differentiation, and minimal [3 H]dThd incorporation in the I-73 CLL cells, agree with reports (17–19, 36). The results reveal that differentiation is also reflected at the mRNA level by a rapid increase in the molar ratio of μ_s/μ_m mRNA. The early transient decrease in μ -chain expression after induction has also been observed in another CLL population (37) and may contribute to the transient down-regulation of surface IgM reported to occur after activation of normal B cells (38).

MYC expression is drastically increased after PMA treatment and peaks concomitantly with the shift in μ_s/μ_m mRNA molar ratio, but prior to the induction of RNA synthesis, actin mRNA expression and Ig-secretion. The kinetics and level of *MYC* mRNA expression in the I-73 cells appear to be similar to those observed in normal B cells activated to proliferation by antibodies to μ chain plus B-cell growth factor (34). A noticeable difference was, however, that in the case of I-73 cells a continued high level of *MYC* mRNA expression could be observed during the whole 72-hr test period, while in the normal B cells a drop in *MYC* mRNA to control levels could be seen after 21 hr.

Our results concerning *FOS* mRNA expression agree with observations in other cell systems after growth factor or PMA stimulation (3–5, 14, 15) in that the gene is rapidly and transiently induced to a very high level of expression. This occurs prior to the induction of *MYC* expression and the shift in μ_s/μ_m mRNA molar ratio. The elevation of *FOS* mRNA persists several hours longer than in platelet-derived growth factor-stimulated fibroblasts, but in contrast to monocytic differentiation of HL-60 cells, *FOS* mRNA disappears from the I-73 cells 12 hr after induction. An interesting finding is that in uninduced I-73 cells *FOS* is expressed at a level higher than in PMA-induced HL-60 cells. Constitutive expression of the *FOS* mRNA may, therefore, not be restricted to macrophages, amnion cells, and the few other cell types reported (14).

The present finding that B-cell differentiation may occur without the down-regulation of *MYC* gene expression conflicts with the hypothesis that the *MYC* product might prevent differentiation of many hematopoietic cell types. The latter view is supported by several studies on induced differentiation in the HL-60 and U-937 cell lines (10–12) and by theoretical assumptions based on the deregulated *MYC* expression in Burkitt lymphoma (13). We, therefore, tested the possibility that the translation of the *MYC* RNA was defective in the I-73 cells. Immunoprecipitation, however, using *MYC*-specific antibodies clearly demonstrated that the *MYC* mRNA was translated into the expected protein products. Thus B-cell differentiation may indeed proceed without down-regulation of *MYC*. A similar conclusion was drawn by Benjamin *et al.* (16) studying PMA-induced differentiation of the established B-lymphoma cell line JD 38, containing a 8;14 translocation. In contrast to JD 38, the *MYC* locus in I-73 does not appear to be rearranged as judged by Southern blot analysis (data not shown).

There seem to be at least two major hypotheses to explain our findings, not necessarily excluding each other. (i) An important difference between induced differentiation of HL-60/U-937 cells and that of normal B cells is that the former is coupled to an inhibition of growth, whereas in the latter case proliferation is often found during the early phase of maturation into plasma cells. It is possible that the reduction of *MYC* expression observed in HL-60/U-937 cells is primarily linked to the growth arrest as suggested (12), while the induction of *MYC* and *FOS* expression during differentiation of I-73 cells may be part of a proliferation program, which for unknown reasons is abortive. Smeland *et al.* (34) have shown

that the induction of *MYC* expression is not sufficient for the proliferation of normal B cells, which implies that other factors are also required. The lack of proliferation in I-73 could be due to the absence of such factors. Alternatively I-73 cells may represent B cells programmed to rapidly convert into Ig-secreting cells without proliferation (memory cells?). Finally I-73 cells may have a malignancy-associated defect in respect to proliferation.

(ii) An alternative view is that the increased *FOS* and *MYC* expression in I-73 may promote differentiation. The maturation of I-73 cells to Ig secretion is preceded by a G_0 to G_1 transition during which *FOS* and *MYC* expression is induced. It is possible that *MYC* and *FOS* play a role in the regulation of some of the events that take place during this transition; i.e., the enhancement of several metabolic processes including RNA and protein synthesis, cellular enlargement and competence to respond to growth and differentiation factors (38). This activation of the cellular machinery appears to be a prerequisite for Ig secretion. *FOS* and *MYC* expression in B cells may thus promote competence to respond not only to growth factors, as suggested for normal B cells (34), but also to differentiation factors. Moreover, the distinction between growth and differentiation factors for B cells may be an oversimplification, since a report suggests that a single cloned factor will promote both proliferation and differentiation (39).

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