

## ORIGINAL ARTICLE

## Alteration of C-MYB DNA binding to cognate responsive elements in HL-60 variant cells

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**Aims:** To establish whether the MYB protein expressed in HL-60 variant cells, which are cells resistant to 12-O-tetradecanoylphorbol-13-acetate (TPA) induced differentiation, is able to bind MYB recognition elements (MREs) involved in the transcriptional regulation of myb target genes. In addition, to determine whether alterations in the binding of the MYB protein to MREs affects HL-60 cell proliferation and differentiation.

**Methods:** Nuclear extracts of HL-60 variant cells exhibiting different degrees of resistance to TPA induced monocytic differentiation were used in electrophoretic mobility shift experiments (EMSA), bandshift experiments performed with labelled oligonucleotides containing the MYB consensus binding sequences.

**Results:** The MYB protein contained in nuclear extracts from HL-60 variant cells did not bind efficiently to the MYB recognition elements identified in the mim-1 and PR264 promoters. Molecular cloning of the myb gene and analysis of the MYB protein expressed in the HL-60 variant cells established that the lack of binding did not result from a structural alteration of MYB in these cells. The lack of MRE binding did not abrogate the ability of variant HL-60s to proliferate and to undergo differentiation. Furthermore, the expression of the PR264/SC35 splicing factor was not affected as a result of the altered MYB DNA binding activity.

**Conclusions:** Because the MYB protein expressed in HL-60 variant cells did not appear to be structurally different from the MYB protein expressed in parental HL-60 cells, it is possible that the HL-60 variant cells contain a MYB binding inhibitory factor (MBIF) that interferes with MYB binding on MREs. The increased proliferation rate of HL-60 variant cells and their reduced serum requirement argues against the need for direct MYB binding in the regulation of cell growth.

The MYB family of transcriptional regulators includes the structurally related A, B, and C MYB proteins, which are thought to play pivotal roles in controlling cellular proliferation and differentiation. Whereas A and B myb genes are expressed in various cell types, such as fibroblasts, neural cells, cells of the reproductive tissues, and B cells,<sup>1–6</sup> c-myb is predominantly expressed in normal and tumoral immature cells of different haemopoietic lineages.<sup>7–10</sup> The C-MYB protein is a transcription factor that contains an N-proximal highly conserved DNA binding domain, a central transactivation domain, and a C-proximal domain responsible for the negative regulation of transcription.<sup>11–12</sup> The negative regulatory domain contains a leucine zipper and the DNA binding domain comprises three imperfect repeats designated R1, R2, and R3, with these last two domains being sufficient for sequence specific binding.<sup>13</sup>

Early studies established that C-MYB proteins bind DNA in a specific way<sup>14–15</sup> via MYB recognition elements (MREs), with a consensus core sequence originally defined as YAACNG or YAACGN,<sup>16</sup> and later extended to YAACBGYCR or YAACKGHH.<sup>17</sup> The first half site of the MRE is absolutely required for the binding of MYB, whereas the second half accommodates some flexibility.<sup>16</sup>

The c-myb gene is primarily expressed in immature haemopoietic cells of the lymphoid, erythroid, and myeloid lineages (reviewed in Oh and Reddy<sup>18</sup>). Although many tumour cells of T and B lymphoid origins express increased amounts of C-MYB, in normal developmental conditions, c-myb is preferentially expressed in the T but not the B cell lineage.<sup>19–20</sup>

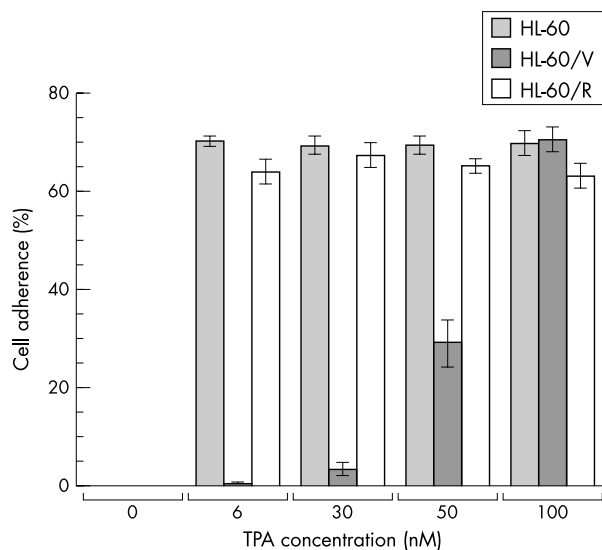
Numerous studies have suggested that C-MYB acts as a key regulator of the haemopoietic proliferation and differentiation processes. Indeed, for many years the expression of c-myb was reported to decrease greatly as normal and leukaemic cells

terminally differentiate.<sup>21</sup> Constitutive expression of an exogenous c-myb gene was also shown to block chemically induced differentiation in several leukaemia cell lines and antisense c-myb oligonucleotides inhibited the proliferation of normal human haemopoietic precursors in vitro (reviewed in Ness<sup>22</sup>). Murine fetuses homozygous for a null allele of c-myb displayed a strikingly abnormal phenotype, consistent with severe anaemia, and died by day 15 of gestation.<sup>23</sup> Analysis of the peripheral blood of these mutants has highlighted the importance of c-myb expression in maintaining adult-type but not embryonic erythropoiesis. Furthermore, altered thymopoiesis and inhibition of T cell proliferation were reported in transgenic mice carrying dominant interfering myb alleles.<sup>24</sup>

“Numerous studies have suggested that C-MYB acts as a key regulator of the haemopoietic proliferation and differentiation processes”

Studies aimed at understanding the mechanisms by which C-MYB controls both proliferation and differentiation processes have permitted the characterisation of several genes representing potential C-MYB targets. These include genes encoding proteins that regulate the cell cycle, proliferation, splicing, and chromatin assembly, and proteins that are

**Abbreviations:** EMSA, electrophoretic mobility shift experiment; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MBIF, MYB binding inhibitory factor; MRE, MYB recognition element; TPA, 12-O-tetradecanoylphorbol-13-acetate



**Figure 1** Cellular adherence of TPA induced HL-60 and HL-60 variant cells. Cells in exponential growth were seeded at a density of  $4 \times 10^5$  cells/ml of complete medium containing either 6, 30, 50, or 100nM TPA. After a 48 hour incubation period, the percentage of adhesive cells was determined. Values are the mean of three experiments.

inhibitors or activators of the MYB binding and transcriptional activation of *mim-1*.<sup>25-35</sup>

The human myelomonocytic HL-60 cell line<sup>36</sup> has been extensively studied as a model to investigate the molecular events underlying haemopoietic differentiation.<sup>37</sup> Upon addition of 12-O-tetradecanoylphorbol-13-acetate (TPA), these cells undergo a monocytic type of differentiation, giving rise to

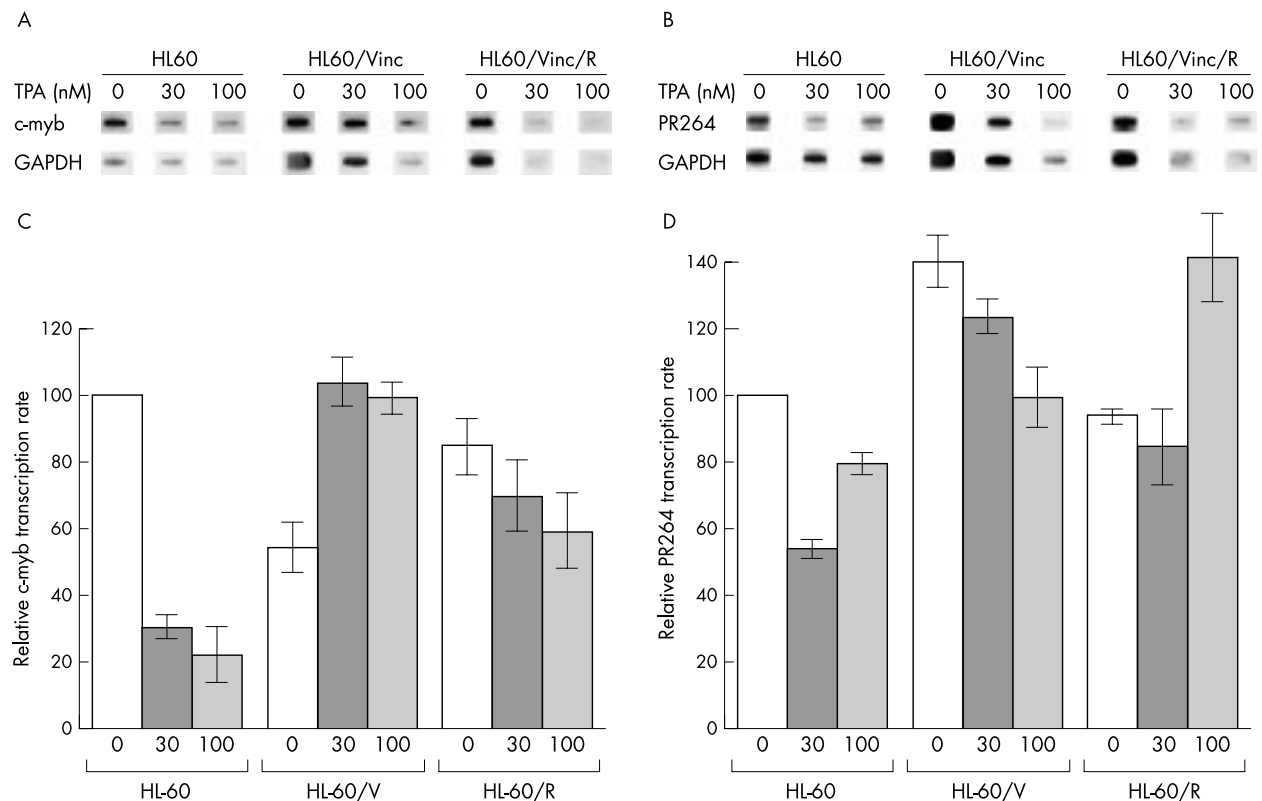
adherent macrophage-like cells.<sup>38</sup> A concomitant decrease of *c-myb* expression has been associated with the differentiation of HL-60 cells.<sup>21</sup> Two variant HL-60 cell lines (HL-60/V and HL-60/R) were selected for their different degrees of resistance to vincristine.<sup>39, 40</sup> HL-60/V cells overexpress the multidrug resistance gene product. These cells also exhibit alteration in chromatin structure<sup>41</sup> and several quantitative differences in cell protein expression when compared with the parental cells.<sup>42</sup> HL-60/V cells are highly defective in TPA induced differentiation to macrophage-like cells<sup>43</sup> and do not form the TPA inducible AP-1 complex. By contrast, HL-60/R revertant cells, which are spontaneously derived from HL-60/V cells, have partially regained sensitivity to vincristine, express relatively low amounts of P-glycoprotein, and differentiate into monocyte/macrophage cells after TPA induction. Both cell lines are defective in signalling events that confer TPA inducibility of the *c-jun* gene.<sup>44</sup>

The results of our study indicate that the C-MYB protein expressed in the HL-60 variant cells is unable to bind MREs *in vitro*. However, the lack of MYB binding to the MREs did not interfere with the ability of variant HL-60 cells to proliferate and undergo differentiation. Furthermore, we also show that in these cells the variations of SC35 splicing factor expression were not dependent upon the MYB protein.

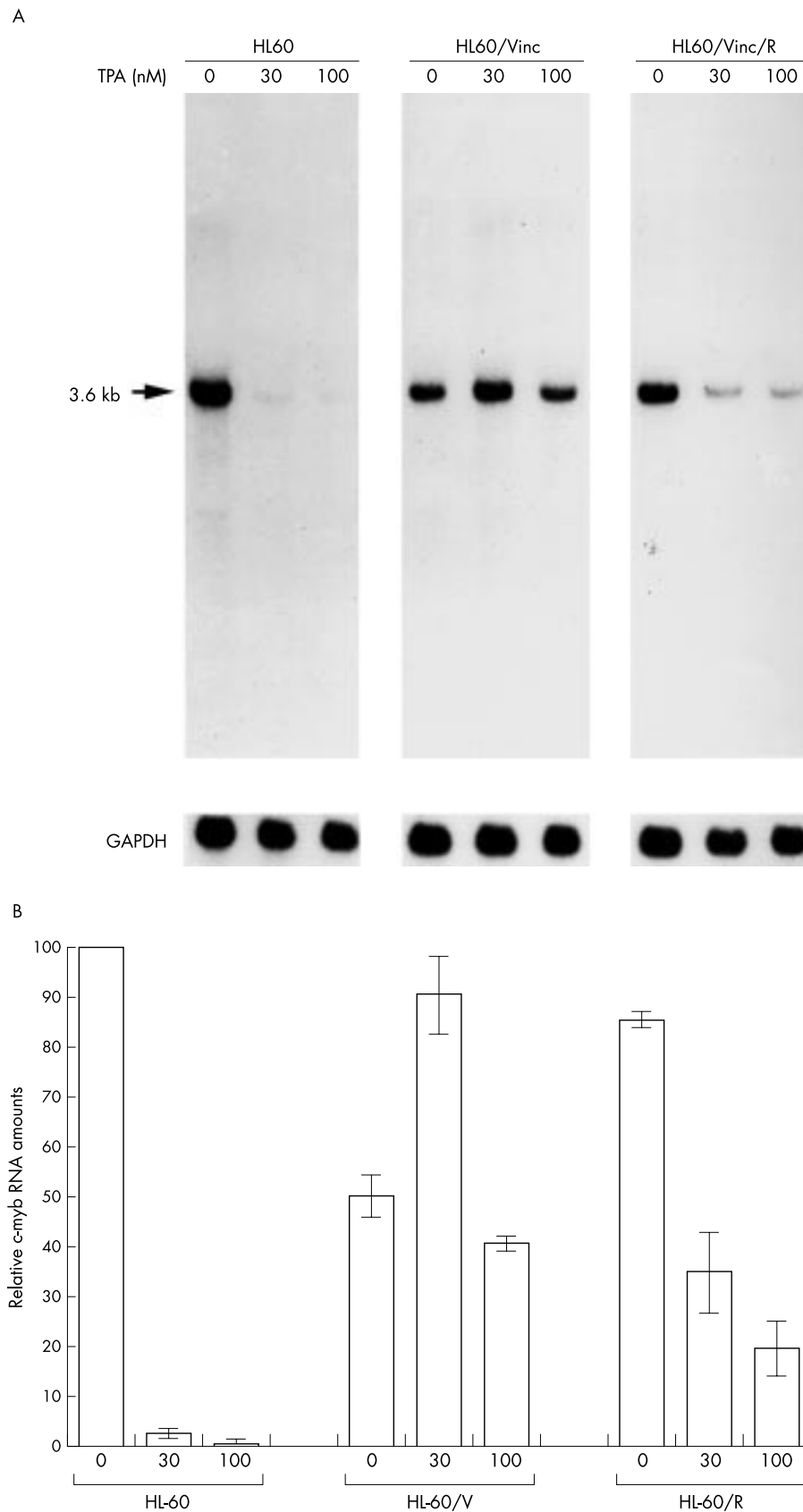
## MATERIALS AND METHODS

### Cell lines and culture conditions

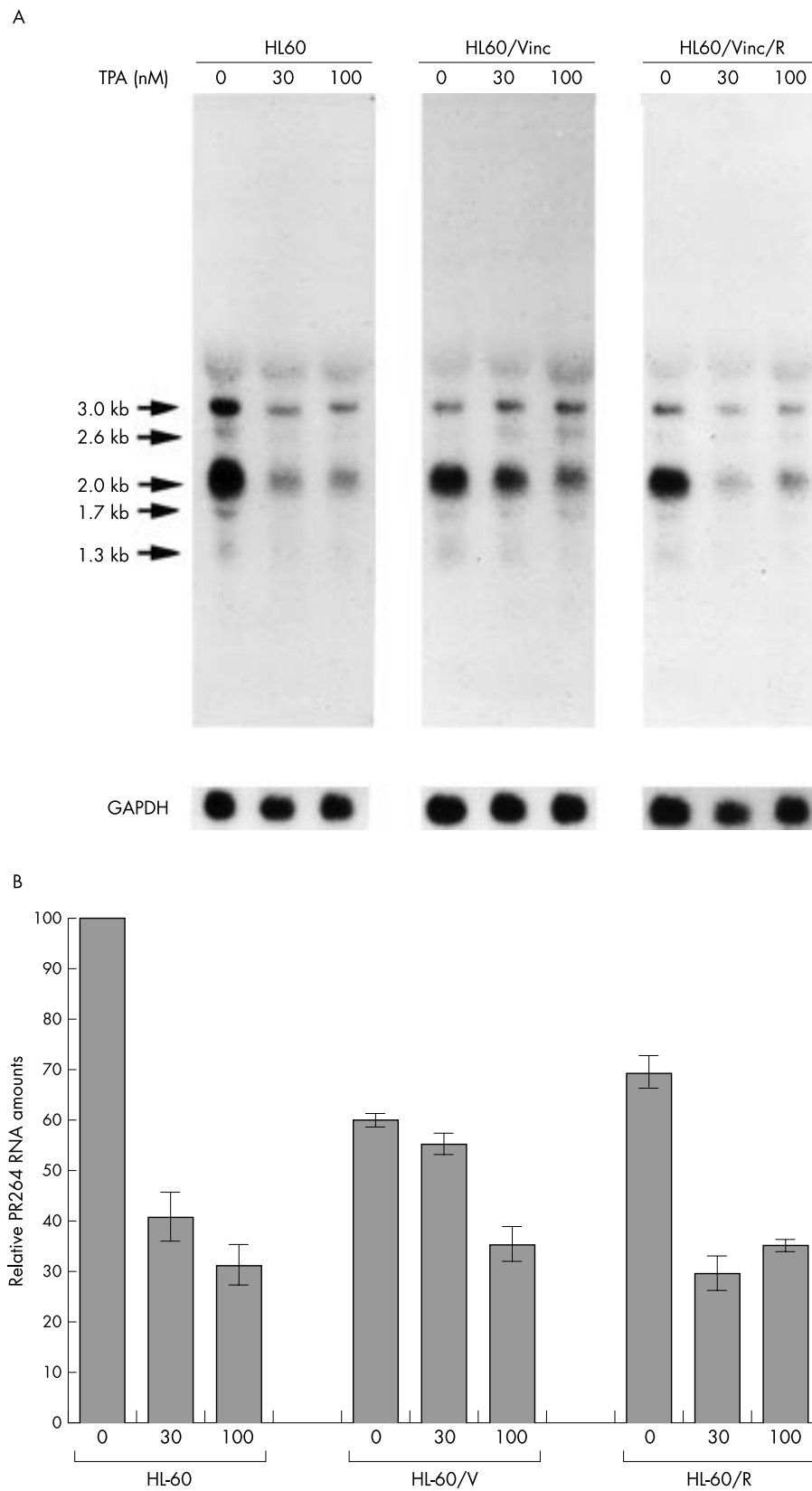
The human myelomonocytic HL-60 (240-CCL) cell line was obtained from the American Type Culture Collection (ATCC; Rockville, Maryland, USA). Variant HL-60 cell lines, HL-60/Vinc and HL-60/Vinc/R, have been described previously,<sup>39, 40</sup> and are referred to as HL-60/V and HL-60/R herein. Cells were seeded in suspension at an initial density of  $0.4 \times 10^6$ /ml in RPMI 1640 medium (Bio Whittaker, Emerainville, France) supplemented with 2mM glutamine and 10% fetal calf serum



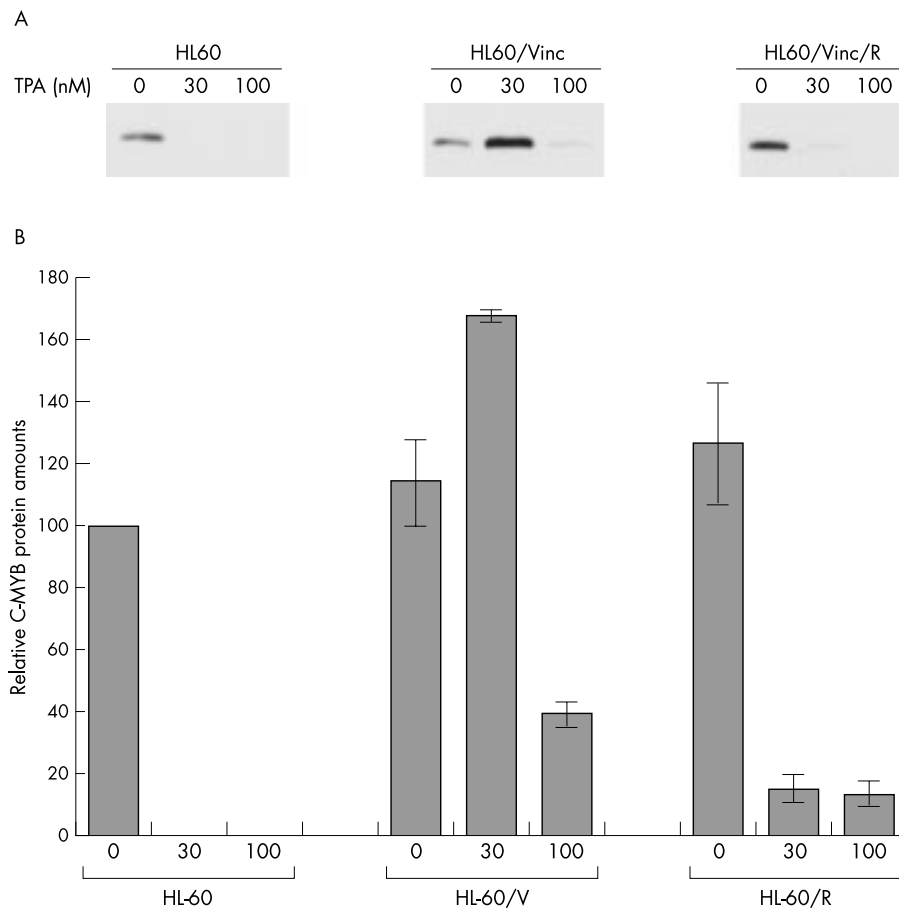
**Figure 2** Nuclear run-on analysis of (A) *c-myb* and (B) PR264/SC35 transcription in control and TPA induced HL-60 and HL-60 variant cells. Histograms representing the relative transcription rate of the (C) *c-myb* and (D) PR264 genes were normalised with respect to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcription rate.



**Figure 3** The detection of c-myb mRNA in HL-60 and HL-60 variant cell lines. (A) Northern blotting was performed with 15  $\mu$ g of total RNA from control and TPA induced (30 and 100nM) HL-60 and HL-60 variant cells. Blots were sequentially hybridised with the c-myb and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes. GAPDH hybridisation signals were used as an internal control to normalise for variations in RNA amounts. (B) Hybridisation signals were quantified with a phosphorimager and values reported in the histogram.



**Figure 4** PR264 mRNA values in HL-60 and HL-60 variant cell lines. (A) Total RNA (15 µg) from control and TPA induced (30 and 100nM) HL-60 and HL-60 variant cells was analysed by northern blot hybridisation. Blots were sequentially probed with PR264 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes. GAPDH hybridisation signals were used as an internal control to normalise for variations in RNA amounts. (B) Hybridisation signals were quantified with a phosphorimager and values reported in the histogram.



**Figure 5** C-MYB protein expression in control and TPA treated HL-60 and HL-60 variant cell lines. (A) Cellular lysates corresponding to  $10^6$  cells were separated on a 9% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel and transferred on to a PVDF membrane. The membrane areas containing proteins ranging from 200 kDa to 50 kDa were probed with the anti-MYB ( $\alpha$ R2R3) antibody. Proteins were visualised using the enhanced chemiluminescence kit. Only the regions corresponding to the C-MYB proteins are shown. The membranes were subsequently stained with Coomassie blue reagent to control for varying amounts of transferred protein. (B) Variations in C-MYB protein amounts were estimated from densitometric analysis of the autoradiograms and normalised values reported in histograms (mean  $\pm$  SD of three experiments).

(Bio Whittaker). Cells were passaged when they reached a density of  $1.2\text{--}1.6 \times 10^6/\text{ml}$  at  $37^\circ\text{C}$  in a humidified 5%  $\text{CO}_2$  atmosphere. The vincristine resistance of HL-60/V cells was assayed at regular intervals. The cell viability was determined by trypan blue exclusion.

#### Induction of differentiation

Cells in logarithmic growth phase were resuspended in RPMI 1640 medium ( $0.4 \times 10^6/\text{ml}$ ) before incubation with the differentiating agent. TPA (Sigma, L'isle d'Abeau Chesne, France) was stored as a 1.6mM stock solution in DMSO. The induction of differentiation by TPA was performed over a 48 hour period at a final concentration of 6, 30, 50, or 100nM. As a solvent of TPA, DMSO was at a final concentration of 0.006% in the culture medium and did not affect TPA induced differentiation.<sup>45</sup> Monocytic differentiation was assessed using established morphological criteria<sup>46</sup> and by determining the proportion of macrophage-like cells adhering to the plastic culture dishes. Cell adherence and cell growth inhibition were used as markers of monocytic differentiation.<sup>43, 44</sup>

#### Origin and labelling of probes

The L755 human *c-myb* cDNA (J Soret and B Perbal, unpublished, 1995) was used to isolate the EcoRI–EcoRI 0.68 kb DNA fragment (EE 0.68), which contains the sequences of the first six *c-myb* exons. The HPR5 human PR264 cDNA clone<sup>47</sup> was used to isolate the EcoRI–BglII 0.65 kb DNA fragment (EB 0.65), which contains the PRE1

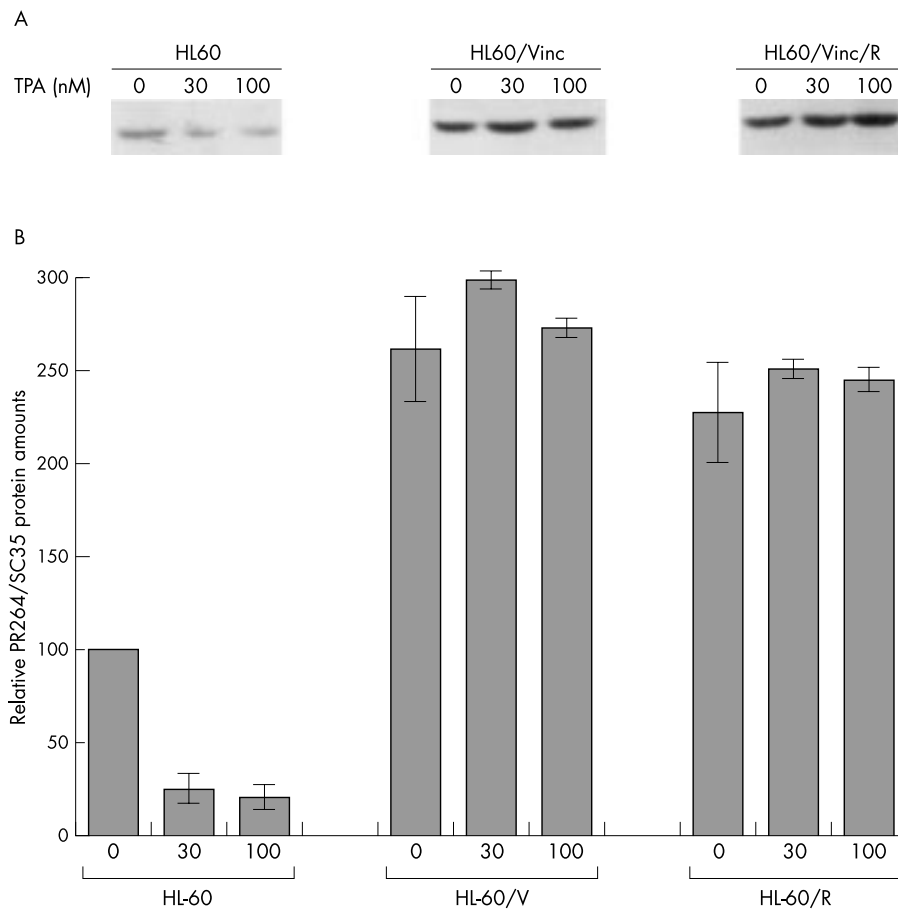
(PR264 exon 1) and the 5' proximal sequences of the PRE2 exon. The HPR5 cDNA was also used to isolate a 200 bp RsaI DNA fragment (RR 0.2), which contains the PRE1 exon. For run on experiments, the EE 0.68 and RR 0.2 DNA fragments were cloned into the M13mp18 vector (New England Biolabs, Beverly, Massachusetts, USA). A full length human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (Clontech, Palo Alto, California, USA) was used for normalisation of hybridisation signals. Double stranded probes were nick translated with the BRL labelling kit in the presence of [ $\alpha$ - $^{32}\text{P}$ ]dCTP.

#### RNA purification and northern blot analyses

Total cellular RNA was purified as described previously.<sup>48</sup> Following electrophoresis in formaldehyde/agarose gels, total RNA species (15  $\mu\text{g}/\text{lane}$ ) were transferred on to a Nytran plus membrane (Schleicher and Schuell, Ecquevilley, France) and fixed by baking for one hour at  $80^\circ\text{C}$ . Hybridisation of filters was carried out as recommended by the supplier. Hybridised filters were exposed to Kodak AR X-Omat films at  $-70^\circ\text{C}$  with intensifying screens. Densitometric analyses of autoradiograms were performed with a Molecular Dynamics (Boston, Massachusetts, USA) phosphorimager.

#### Nuclear run on transcription assays

Nuclei were purified essentially as described.<sup>49</sup> After incubation with lysis buffer, nuclei were pelleted by centrifugation and directly resuspended in nuclear storage buffer. In vitro



**Figure 6** PR264/SC35 protein expression in control and TPA treated HL-60 and HL-60 variant cell lines. (A) Proteins were analysed by western blotting as described in fig 4. The membrane areas containing proteins ranging from 40 kDa to 20 kDa were probed with the PR264 polyclonal antibodies. Proteins were revealed with the enhanced chemiluminescence system. Only the regions corresponding to the PR264/SC35 proteins are shown. The membranes were subsequently stained with Coomassie blue reagent to control for varying amounts of transferred proteins. (B) Variations in protein amounts were estimated from densitometric analysis of the autoradiograms. Histograms indicate the variations of C-MYB protein values. The values were the mean  $\pm$  SD of three experiments.

elongation of nascent RNA chains was carried out using  $2 \times 10^7$  nuclei incubated in the presence of [ $\alpha^{32}$ P]ATP and [ $\alpha^{32}$ P]CTP (50  $\mu$ Ci each). Labelled RNA was purified as described previously.<sup>30</sup>

Target DNA sequences (10  $\mu$ g/slot) to be probed with labelled RNA species were denatured by incubation with 0.2M NaOH, neutralised with 10 volumes of 6 $\times$  saline sodium citrate and then transferred on to nitrocellulose (BAS-85; Schleicher and Schuell) using a Bio-Rad slot blot apparatus. Linearised plasmid DNA containing GAPDH cDNA sequences was used to normalise the hybridisation signals. Filters were hybridised at 50°C for three days and washed as described previously.<sup>31</sup>

#### Western blot immunoassays

Control and TPA treated cells were harvested and lysed directly by boiling for five minutes in 2 $\times$  sample buffer.<sup>32</sup> The proteins derived from 10<sup>6</sup> cells were fractionated on a sodium dodecyl sulfate polyacrylamide gel, transferred on to a PVDF membrane (Immobilon P; Millipore, Saint Quentin en Yvelines, France), and western blot analysis was carried out by standard procedures.<sup>48</sup> Detection of the immune complexes was performed with the enhanced chemiluminescence system according to the supplier's instructions (Amersham, Les Ulis, France). A recombinant MYB protein (gift of Dr O Gabrielsen, University of Oslo, Norway) consisting of the R2R3 repeats of the DNA binding domain was used to raise rabbit anti-R2R3 polyclonal antisera ( $\alpha$ R2R3). The rabbit anti-PR264 polyclonal

antisera, raised against a synthetic peptide of 15 amino-acids (NH<sub>2</sub>-KSPPKSPPEEGAVSS-COOH) localised in the C-terminal region of the PR264/SC35 protein, was provided by Dr J Stévenin (Institute of Genetics and Molecular & Cellular Biology, Strasbourg, France).

#### Electrophoretic mobility shift assays (EMSA)

Nuclear extracts of HL-60 and variant cells were prepared as described previously.<sup>33</sup> EMSAs were carried out using labelled duplex oligonucleotide probes containing either mim-1 MRE-A (5'-gCATTATAACGGTTTTTTCAGCgc-3')<sup>26</sup> or PR264 MRE-C (5'-CCACCGGCAGTTAGGATACTCC-3').<sup>33</sup> Negative controls were performed with mutated mim-1 MRE mut (5'-CACATTATATGCCATTTTTTTCAGC-3')<sup>33</sup> and PR264 MRE Cm (5'-CCACCGGGAGAAAGGATACTCC-3'). Single stranded oligonucleotides were labelled using T4 polynucleotide kinase (New England Biolabs) and [ $\gamma$ -<sup>32</sup>P]ATP (3000 Ci/mmol; ICN Pharmaceuticals, Orsay, France), annealed, and purified in a non-denaturing 10% polyacrylamide gel. EMSAs were performed by incubating 10  $\mu$ g of nuclear extracts with 15–20 fmol of end labelled double stranded oligonucleotides for 15 minutes at 0°C in 25  $\mu$ l of 20% (vol/vol) glycerol, 50mM KCl, 20mM HEPES, pH 7.9, 5mM MgCl<sub>2</sub>, 0.1mM EDTA, 1mM dithiothreitol, and 40  $\mu$ g/ml poly(dI-dC) (Pharmacia). For the identification of MYB specific complexes, the rabbit anti-MYB polyclonal antiserum ( $\alpha$ R2R3) and corresponding preimmune serum were preincubated for 15 minutes at 0°C with nuclear extracts before the binding reaction. Competition studies were

performed in the presence of a 500 and 1000 fold molar excess of unlabelled competitor oligonucleotides MRE mut and MRE Cm. Complexes were resolved either in a 5% or 7% polyacrylamide gel and revealed by autoradiography.

The HL-60/V (MYB) nuclear extracts were prepared from HL-60/V cells transfected with the L755 full length c-myb cDNA cloned in pCDNA3 (Invitrogen, Cergy Pontoise, France).

## RESULTS

### TPA induced monocytic differentiation of HL-60 variant cell lines

About 70% of the HL-60 and HL-60/R cells had morphological criteria characteristic of macrophage-like cells after 48 hours of incubation with 6–100nM TPA. As previously reported,<sup>43</sup> HL-60/V cells were resistant to monocytic differentiation, as measured by the lack of cell adherence (fig 1) and cell growth inhibition in the presence of 6nM TPA. However, 4% and 30% of the HL-60/V cells adhered to the support when treated with 30nM and 50nM TPA, respectively. Upon incubation with 100nM TPA, the proportion of HL-60/V adhesive cells was similar to that obtained with the HL-60 and HL-60/R cells (fig 1). These results indicated that the previously reported resistance of HL-60/V cells to monocytic differentiation<sup>43</sup> could be challenged by increasing the concentration of TPA.

### Expression of c-myb and PR264/SC35 in HL-60 variant cells

Because HL-60 variant cells have an altered capacity to undergo TPA induced differentiation, we have examined whether the expression of the c-myb proto-oncogene and PR264/SC35 was impaired in these cells.

Nuclear run on analysis revealed that the c-myb transcription rate in untreated HL-60/V and HL-60/R cells was 55% and 85% (respectively) of that seen in HL-60 cells (fig 2A). When measured under the same conditions, the variations of PR264/SC35 expression in the HL-60 variant cells were not found to match those seen for c-myb (fig 2B). In TPA induced HL-60 cells (30nM and 100nM), the PR264 transcription rate decreased by 20% and 45%, respectively. In TPA treated HL-60/V cells, the PR264 transcription rate was reduced by 10% and 30%, respectively. After incubation of HL-60/R cells in the presence of 30 nM TPA, the PR264 transcription rate was decreased by 10%. In contrast, PR264 transcription was increased by 50% in the presence of 100 nM TPA.

These results were in agreement with the amounts of c-myb and PR264/SC35 mRNA detected in northern blotting experiments (figs 3,4)

Western blotting experiments revealed that in untreated HL-60/V and HL-60/R cells, the amount of p75<sup>c-myb</sup> was 15–25% higher than that seen in HL-60 cells (fig 5). Densitometric analysis of northern and western blots indicated that the ratio of c-myb (protein/mRNA) was 1.5 to 2.3 fold higher in HL-60 variant cells than in HL-60 cells. These observations suggested that the C-MYB protein expressed in HL-60/V and HL-60/R cells was more stable than that expressed in the parental HL-60 cells.

Nucleotide sequencing of c-myb cDNA clones isolated from HL-60, HL-60/V, and HL-60/R cells (data not shown) indicated that the C-MYB protein expressed in the variant cells contained 640 amino acids and was not different from that of the MYB protein expressed in parental HL-60 cells.<sup>34</sup>

Incubation of HL-60 cells in the presence of TPA led to an 80% decrease in the PR264/SC35 protein concentration (fig 6). After incubation of HL-60/V and HL-60/R cells in the presence of various concentrations of TPA, the PR264/SC35 protein concentration was increased by 5–15%.

These results established that: (1) the differentiation of HL-60 variant cells can proceed, irrespective of the levels of c-myb and PR264 transcription; (2) variations in the concentrations of C-MYB proteins did not affect PR264 transcription

in untreated and TPA treated HL-60 variant cells; and (3) the regulation of PR264 transcription probably involves transcription factors, the expression of which may be modulated by TPA.

### MYB proteins expressed in HL-60 variant cells do not efficiently bind to mim-1 and PR264 MYB responsive element in vitro

To determine whether the C-MYB proteins expressed in HL-60 variant cells interact with MREs, EMSA were performed with the mim-1 high affinity MRE<sup>26</sup> and nuclear protein extracts purified from HL-60 variant cells.

As shown in fig 7A, the HL-60 nuclear extracts gave rise to several complexes designated  $\alpha$  (1–5) and  $\gamma$  (1–3). Because the  $\alpha$  complexes were no longer seen when the MYB protein was depleted from the HL-60 nuclear extracts, either by pre-incubation of nuclear extracts with anti-MYB antibody or with nuclear extracts purified from TPA treated HL-60 cells, they probably resulted from the binding of MYB proteins to the mim-1 MRE-A. Competition assays performed with the unlabelled mutated form of the mim-1 MRE-A (MRE mut), in addition to the use of the labelled MRE mut probe, previously reported not to bind the MYB R2R3 polypeptide,<sup>33</sup> confirmed that the  $\alpha$  complexes were MYB specific. The  $\gamma$  complexes obtained with HL-60 nuclear extracts did not result from the binding of MYB proteins to the mim-1 MRE-A because they were neither challenged by anti-R2R3 antibodies, nor eliminated by the use of the MRE mut probe.

Nuclear extracts from HL-60/V and HL-60/R cells did not allow the formation of MYB-specific  $\alpha$ 1,  $\alpha$ 2, or  $\alpha$ 4 complexes with mim-1 MRE-A. The low mobility of  $\alpha$ 3,  $\alpha$ 5, and the additional  $\alpha$ 6 MYB specific complexes, which were only weakly detected, might result from the binding of a high molecular weight MYB related protein or from the formation of a multi-protein complex involving the C-MYB polypeptide and other regulatory factors specifically expressed in variant cells.

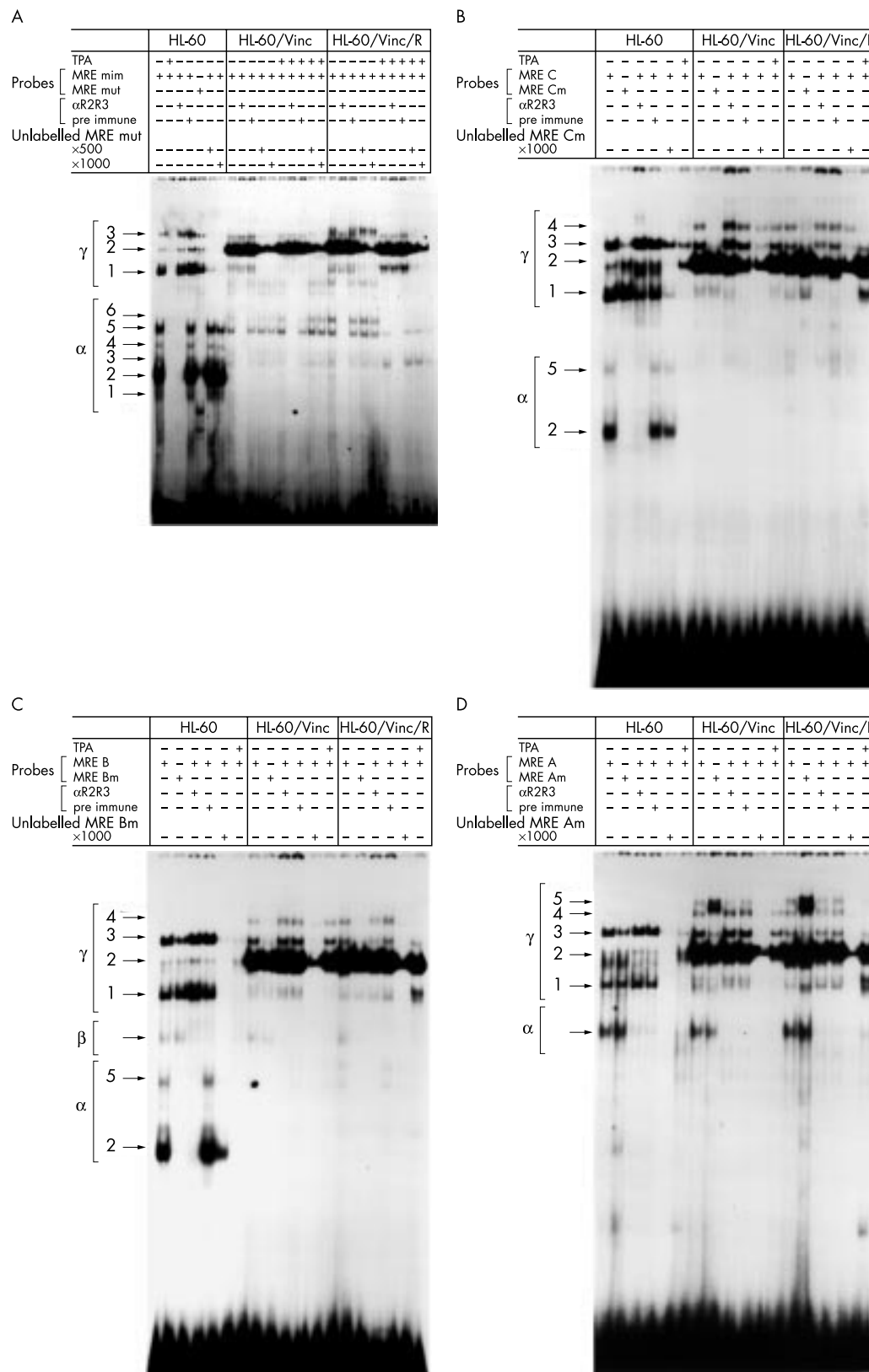
By mixing extracts from HL-60 and HL-60 variant cells, we could establish that the lack of these complexes did not result from large amounts of an inhibitory factor present in the nuclear extracts of variant cells (data not shown). Furthermore western blotting experiments allowed us to check for the presence and the integrity of the MYB product in nuclear extracts from variant cells (data not shown).

We had established previously that the PR264 promoter contained 11 putative MREs showing different affinities for MYB.<sup>33</sup> Nuclear extracts from HL-60 variant cells did not permit the formation of MYB specific complexes with PR264 MRE-C, MRE-B, or MRE-A (fig 7B–D).

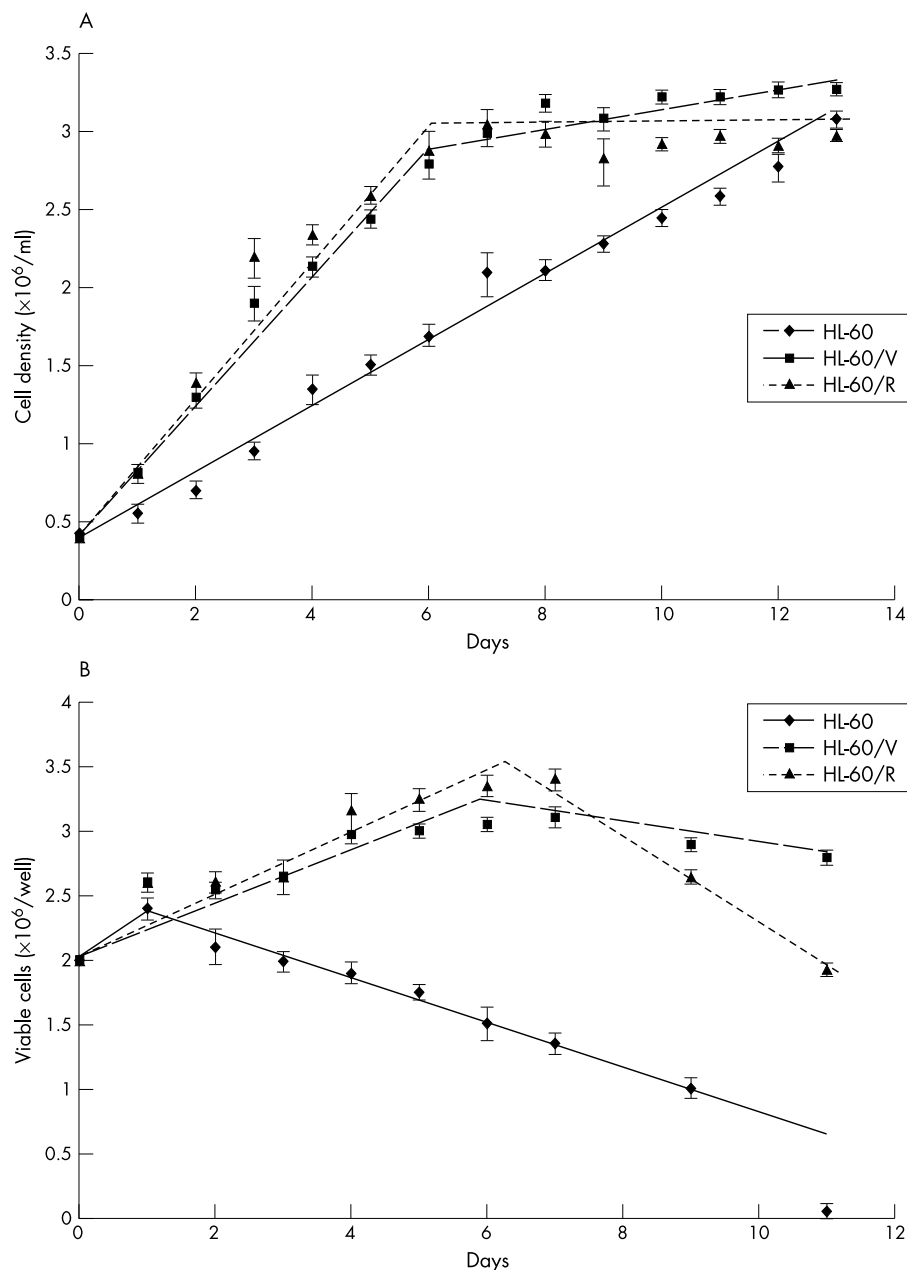
The fact that the C-MYB proteins expressed in HL-60 and HL-60 variant cells are identical suggests that inefficient binding results from inappropriate interactions of MYB with the MREs.

### Cell growth of HL-60 variant cells

HL-60/V and HL-60/R cells proliferated more rapidly than HL-60 over a six day period after seeding. Doubling times were 24 hours for both HL-60/V and HL-60/R cells and 55 hours for HL-60 cells (fig 8A). HL-60 variant cells reached a plateau at day 7, whereas HL-60 cells grew until day 13, when they reach the same plateau as HL-60 variant cells. In standard RPMI 1640 medium, the minimum seeding density that could be used to obtain viable cultures varied from 2.10<sup>4</sup> cells/ml for HL-60 to 10 cells/ml for HL-60 variant cells (data not shown). When cell cultures were shifted in a serum deprived RPMI 1640 medium, the number of viable HL-60 variant cells increased slightly over the six day period after seeding and then decreased regularly until day 11. In the HL-60 culture, the number of viable cells decreased from day 1 to 11. These results suggested that HL-60 variant cells had a lower serum requirement than HL-60 cells (fig 8B).



**Figure 7** Band shift pattern of MYB recognition element (MRE) associated complexes obtained with HL-60 and HL-60 variant nuclear extracts. 10  $\mu$ g nuclear extracts purified from either untreated or TPA treated (30 nM for 48 hours) HL-60 and HL-60 variant cells were incubated in the presence of (A)  $^{32}$ P labelled mim-1 MRE-A, (B) PR264 MRE-C, (C) PR264 MRE-B, and (D) PR264 MRE-A oligonucleotides. Resulting complexes were resolved at 4°C in a 0.5 $\times$  TBE 7% polyacrylamide gel (40 cm long) at 400 V. Nuclear extracts were incubated with labelled MRE, corresponding mutated probes or an excess of mutated MRE competitor oligonucleotide as indicated. Anti-MYB ( $\alpha$ R2R3) or preimmune serum was preincubated with the nuclear extracts 15 minutes before the addition of labelled probe.



**Figure 8** Growth of HL-60 and HL-60 variant cells in liquid culture suspension. (A) Cells ( $0.4 \times 10^6$ /ml) were seeded and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. (B) Cells were seeded in serum deprived RPMI 1640 medium. The percentage of viable cells was determined by trypan blue exclusion. Values are the mean  $\pm$ SD of three experiments.

## DISCUSSION

By performing EMSAs, we have established that the C-MYB proteins expressed in HL-60 variant cells were altered in their capacity to interact with the MREs previously identified in the promoter region of the *mim-1* and *PR264* genes.<sup>26 33</sup>

Several possibilities may account for the altered DNA binding activity of C-MYB in HL-60 variant cells, namely: (1) the C-MYB protein is structurally altered, (2) the HL-60 variant cells lack a crucial factor needed for binding, (3) a MYB binding inhibitory factor (MBIF) is expressed in HL-60 variant cells.

Because the predicted primary sequence of HL-60/V C-MYB was identical to that of HL-60 and the HL-60/V C-MYB was detected as a single protein of 75 kDa (data not shown), with an isoelectric point the same as wild-type C-MYB, we concluded that the lack of C-MYB DNA binding did not result from the expression of a structurally altered MYB protein form in HL-60 variant cells.

“Our present working hypothesis is that in HL-60 variant cells the C-MYB protein is stably combined with or altered by MYB binding inhibitory factor, which interferes with binding to MYB recognition elements under physiological conditions”

Two sets of experiments ruled out the possibility that HL-60 cells lack an essential factor needed for binding. First, mixtures of nuclear extracts from HL-60 and HL-60 variant cells gave rise to a MYB specific band shift pattern, the intensity of which was proportional to the amount of HL-60 nuclear extract added to the mix (data not shown). Second, incubation of nuclear extracts from HL-60/V cells transfected with full length *c-myb* cDNA gave rise to a band shift pattern similar to that seen with HL-60 nuclear extracts when incubated in the presence of the *mim-1* MRE-A probe (data not shown). This last result also suggested that nuclear

### Take home messages

- The MYB protein found in HL-60 variant cells resistant to TPA does not bind Myb recognition elements and does not appear to be structurally different from that expressed in the parental HL-60 cells
- Thus, it is possible that the HL-60 variant cells contain a MYB binding inhibitory factor that interferes with MYB binding to the MYB recognition element
- The increased proliferation rate of HL-60 variant cells and their reduced serum requirement argues against the need for direct MYB binding in the regulation of cell growth
- HL-60 variant cells provide a unique system in which to study the interactions of C-MYB with other regulators

extracts do not contain raised concentrations of MBIF, but did not exclude the possibility that a limiting amount of MBIF might account for the altered binding of MYB to MREs.

Our present working hypothesis is that in HL-60 variant cells the C-MYB protein is stably combined with MBIF, which interferes with binding to MREs under physiological conditions. Increased expression of recombinant wild-type MYB protein in transfected HL-60 variant cells would allow residual binding as visualised by band shift assays.

Among the various proteins interacting with C-MYB (reviewed by Ness<sup>55</sup>), the c-Maf, ATBF1, RARa, p160/p67, ZEB, and nucleolin proteins were reported to repress the transcriptional activity of MYB, whereas others such as C/EBP $\alpha$  interact with the DNA binding domain of MYB to stimulate its activity.<sup>56–59</sup> It would be interesting to determine whether MBIF is related to either normal or mutated versions of these factors in HL-60 variant cells and whether their physical interaction with MYB may be responsible for its inability to bind to MREs. One likely candidate for MBIF in variant HL-60 cells is Cyp40 (a cyclophilin with peptidyl-prolyl isomerase activity that can alter protein conformation and catalyse protein folding) or a Cyp40-like factor. With other co-chaperones (FKBP51, FKBP52), cyp40 belongs to a group of immunophilins that modulate steroid receptor. Upon heat shock, a redistribution of cytoplasmic and nucleolar Cyp40 can occur in response to stress.<sup>60</sup> Of interest with respect to our results, it has been reported that the tetratricopeptide (TRP) repeats contained within the C-terminal protein binding domain of Cyp40 can physically interact with the DNA binding domain of C-MYB and therefore inhibit its binding activity.<sup>61</sup>

Our previous results obtained with HL-60 cells suggested that a shutdown of PR264 expression was associated with monocytic differentiation and resulted from reduced synthesis of C-MYB in differentiated cells.<sup>33</sup> We took advantage of the lack of C-MYB DNA binding activity in the HL-60 variant cells to examine whether transcription of the PR264 putative target gene was also altered. Because the rate of PR264 transcription in HL-60 variant cells varied irrespective of C-MYB protein concentrations, we concluded that C-MYB did not directly regulate PR264 expression in these cells. The presence of a TPA responsive element in the PR264 promoter (C Gaillard and B Perbal, unpublished observations, 1997), the alteration of TPA signalling in HL-60 variant cells,<sup>44</sup> and the results reported here therefore raise the possibility that the decreased PR264 expression seen in TPA treated HL-60 cells does not result from the concomitant decrease of C-MYB, but rather from TPA mediated downregulation of PR264.

In summary, our results established that the proliferation and differentiation of HL-60 variant cells is not dependent upon the direct binding of C-MYB to cognate MREs. They also suggest that HL-60 variant cells express low amounts of a factor that inhibits C-MYB binding. The HL-60 variant cells therefore provide a unique system in which to study the interactions of C-MYB with other regulators.

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### REFERENCES

- 1 **Arsura M**, Introna M, Passerini F, *et al*. B-myb antisense oligonucleotides inhibit proliferation of human hematopoietic cell lines. *Blood* 1992;**79**:2708–16.
- 2 **Reiss K**, Travalì S, Calabretta B, *et al*. Growth regulated expression of B-myb in fibroblasts and hematopoietic cells. *J Cell Physiol* 1991;**148**:338–43.
- 3 **Trauth K**, Mutschler B, Jenkins NA, *et al*. Mouse A-myb encodes a trans-activator and is expressed in mitotically active cells of the developing central nervous system, adult testis and B lymphocytes. *EMBO J* 1994;**13**:5994–6005.
- 4 **Golay J**, Erba E, Bernasconi S, *et al*. The A-myb gene is preferentially expressed in tonsillar CD38+, CD39-, and slgM- B lymphocytes and in Burkitt's lymphoma cell lines. *J Immunol* 1994;**153**:543–53.
- 5 **Golay J**, Luppi M, Songia S, *et al*. Expression of A-myb, but not c-myb and B-myb, is restricted to Burkitt's lymphoma, slg+ B-acute lymphoblastic leukemia, and a subset of chronic lymphocytic leukemias. *Blood* 1996;**87**:1900–11.
- 6 **Piccinini G**, Golay J, Flora A, *et al*. C-myb, but not B-myb, upregulates type I collagen gene expression in human fibroblasts. *J Invest Dermatol* 1999;**112**:191–6.
- 7 **Shen-Ong GL**. The myb oncogene. *Biochim Biophys Acta* 1990;**1032**:39–52.
- 8 **Luscher B**, Eisenman RN. New light on Myc and Myb. Part II. Myb. *Genes Dev* 1990;**4**:2235–41.
- 9 **Introna M**, Luchetti M, Castellano M, *et al*. The myb oncogene family of transcription factors: potent regulators of hematopoietic cell proliferation and differentiation. *Semin Cancer Biol* 1994;**5**:113–24.
- 10 **Thompson MA**, Ramsay RG. Myb: an old oncoprotein with new roles. *Bioessays* 1995;**17**:341–50.
- 11 **Lipsick JS**. One billion years of Myb. *Oncogene* 1996;**13**:223–35.
- 12 **Sakura H**, Kanei-Ishii C, Nagase T, *et al*. Delineation of three functional domains of the transcriptional activator encoded by the c-myb protooncogene. *Proc Natl Acad Sci U S A* 1989;**86**:5758–62.
- 13 **Gabrielsen OS**, Sentenac A, Fromageot P. Specific DNA binding by c-Myb: evidence for a double helix-turn-helix-related motif. *Science* 1991;**253**:1140–3.
- 14 **Nakagoshi H**, Nagase T, Kanei-Ishii C, *et al*. Binding of the c-myb proto-oncogene product to the simian virus 40 enhancer stimulates transcription. *J Biol Chem* 1990;**265**:3479–83.
- 15 **Howe KM**, Watson RJ. Nucleotide preferences in sequence-specific recognition of DNA by c-myb protein. *Nucleic Acids Res* 1991;**19**:3913–19.
- 16 **Ordin E**, Bergholtz S, Brebdeford EM, *et al*. Flexibility in the second half-site sequence recognised by the c-Myb R2 domain—in vitro and in vivo analysis. *Oncogene* 1996;**13**:1043–51.
- 17 **Weston K**. Extension of the DNA binding consensus of the chicken c-Myb and v-Myb proteins. *Nucleic Acids Res* 1992;**20**:3043–9.
- 18 **Oh IH**, Reddy EP. The myb gene family in cell growth, differentiation and apoptosis. *Oncogene* 1999;**18**:3017–33.
- 19 **Akashi K**, Traver D, Miyamoto T, *et al*. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature* 2000;**404**:193–7.
- 20 **Allen RD**, Ill, Bender TP, Siu G. c-Myb is essential for early T cell development. *Genes Dev* 1999;**13**:1073–8.
- 21 **Westin EH**, Gallo RC, Arya SK, *et al*. Differential expression of the amv gene in human hematopoietic cells. *Proc Natl Acad Sci U S A* 1982;**79**:2194–8.
- 22 **Ness SA**. The Myb oncoprotein: regulating a regulator. *Biochim Biophys Acta* 1996;**1288**:F123–39.
- 23 **Mucenski ML**, McLain K, Kier AB, *et al*. A functional c-myb gene is required for normal murine fetal hepatic hematopoiesis. *Cell* 1991;**65**:677–89.

- 24 **Badiani P**, Corbella P, Kioussis D, *et al*. Dominant interfering alleles define a role for c-Myb in T-cell development. *Genes Dev* 1994;**8**:770–82.
- 25 **Deng QL**, Ishii S, Sarai A. Binding site analysis of c-Myb: screening of potential binding sites by using the mutation matrix derived from systematic binding affinity measurements. *Nucleic Acids Res* 1996;**24**:766–74.
- 26 **Ness SA**, Marknell A, Graf T. The v-myb oncogene product binds to and activates the promyelocyte-specific mim-1 gene. *Cell* 1989;**59**:1115–25.
- 27 **Siu G**, Wurster AL, Lipsick JS, *et al*. Expression of the CD4 gene requires a Myb transcription factor. *Mol Cell Biol* 1992;**12**:1592–604.
- 28 **Melotti P**, Calabretta B. Ets-2 and c-Myb act independently in regulating expression of the hematopoietic stem cell antigen CD34. *J Biol Chem* 1994;**269**:25303–9.
- 29 **Plaza S**, Turque N, Dozier C, *et al*. C-Myb acts as transcriptional activator of the quail PAX6 [PAX-QNR] promoter through two different mechanisms. *Oncogene* 1995;**10**:329–40.
- 30 **Evans JL**, Moore TL, Kuehl WM, *et al*. Functional analysis of c-Myb protein in T-lymphocytic cell lines shows that it trans-activates the c-myc promoter. *Mol Cell Biol* 1990;**10**:5747–52.
- 31 **Ku DH**, Wen SC, Engelhard A, *et al*. c-myb transactivates cdc2 expression via Myb binding sites in the 5'-flanking region of the human cdc2 gene. *J Biol Chem* 1993;**268**:2255–9.
- 32 **Nicolaides NC**, Gualdi R, Casadevall C, *et al*. Positive autoregulation of c-myb expression via Myb binding sites in the 5' flanking region of the human c-myb gene. *Mol Cell Biol* 1991;**11**:6166–76.
- 33 **Sureau A**, Soret J, Vellard M, *et al*. The PR264/c-myb connection: expression of a splicing factor modulated by a nuclear protooncogene. *Proc Natl Acad Sci U S A* 1992;**89**:11683–7.
- 34 **Wang Q-F**, Lauring J, Schlissel MS. c-Myb binds to a sequence in the proximal region of the RAG-2 promoter and is essential for promoter activity in T-lineage cells. *Mol Cell Biol* 2000;**20**:9203–11.
- 35 **Lutz PG**, Houzel-Charavel A, Moog-Lutz C *et al*. Myeloblastin is a Myb target gene: mechanisms of regulation in myeloid leukemia cells growth-arrested by retinoic acid. *Blood* 2001;**97**:2449–56.
- 36 **Collins S J**, Gallo RC, Gallagher RE. Continuous growth and differentiation of human myeloid leukaemic cells in suspension culture. *Nature* 1977;**270**:347–9.
- 37 **Collins SJ**. The HL-60 promyelocytic leukemia cell line: proliferation, differentiation and cellular oncogene expression. *Blood* 1987;**70**:1233–44.
- 38 **Rovera G**, Santoli D, Damsy C. Human promyelocytic leukemia cells in culture differentiate into macrophage-like cells when treated with a phorbol diester. *Proc Natl Acad Sci U S A* 1979;**76**:2779–83.
- 39 **McGrath T**, Center MS. Adryamycin resistance in HL-60 cells in absence of detectable P-glycoprotein. *Biochem Biophys Res Commun* 1987;**145**:1171–6.
- 40 **McGrath T**, Center MS. Mechanisms of multidrug resistance in HL-60 cells: evidence that a surface membrane protein distinct from P-glycoprotein contributes to reduced cellular accumulation of drug. *Cancer Res* 1988;**48**:3959–63.
- 41 **Dufer J**, Millot-Broglio C, Oum'Hamed Z, *et al*. Nuclear DNA content and chromatin texture in multidrug-resistant human leukemic cell lines. *Int J Cancer* 1999;**60**:108–14.
- 42 **Sedláčik J**, Hunakova L, Sulikova M, *et al*. Protein kinase inhibitor-induced alterations of drug uptake, cell cycle and surface antigen expression in human multidrug-resistant (Pgp and MRP) promyelocytic leukemia HL-60 cells. *Leuk Res* 1997;**21**:449–58.
- 43 **Ma L**, Krishnamachary N, Perbal B, *et al*. HL-60 cells isolated for resistance to vincristine are defective in 12-O-tetradecanoylphorbol-13-acetate induced differentiation and the formation of a functional AP-1 complex. *Oncol Res* 1992;**4**:291–8.
- 44 **Slapak C**, Kharbanda S, Saleem A, *et al*. Defective translocation of protein kinase C in multidrug-resistant HL-60 cells confers a reversible loss of phorbol ester-induced monocytic differentiation. *J Biol Chem* 1993;**268**:12267–73.
- 45 **Huberman E**, Callahan MF. Induction of terminal differentiation in human promyelocytic leukemia cells by tumor-promoting agents. *Proc Natl Acad Sci U S A* 1979;**76**:1293–7.
- 46 **Gallagher R**, Collins S, Trujillo J, *et al*. Characterization of the continuous differentiation myeloid cell line (HL-60) from a patient with acute promyelocytic leukemia. *Blood* 1979;**54**:713–33.
- 47 **Sureau A**, Perbal B. Several mRNAs with variable 3' untranslated regions and different stability encode the human PR264/SC35 splicing factor. *Proc Natl Acad Sci U S A* 1994;**91**:932–6.
- 48 **Perbal B**. *A practical guide to molecular cloning*, 2nd ed. New York: John Wiley & Sons, 1988.
- 49 **Srivastava RAK**, Schonfeld G. Measurements of rate of transcription in isolated nuclei by nuclear "run-off" assay. *Methods Mol Biol* 1994;**31**:281–8.
- 50 **Linial M**, Gunderson N, Groudine M. Enhanced transcription of c-myc in bursal lymphoma cells requires continuous protein synthesis. *Science* 1985;**230**:1126–32.
- 51 **Cereghini S**, Yaniv M, Cortese R. Hepatocyte dedifferentiation and extinction is accompanied by a block in the synthesis of mRNA coding for the transcription factor HNF1/LFB1. *EMBO J* 1990;**9**:2257–63.
- 52 **Laemmli UK**. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;**227**:680–5.
- 53 **Osborn L**, Kunkel S, Nabel GJ. Tumor necrosis factor alpha and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor kappa B. *Proc Natl Acad Sci U S A* 1989;**86**:2336–40.
- 54 **Majello B**, Kenyon LC, Dalla-Favera R. Human c-myb protooncogene: nucleotide sequence of cDNA and organization of the genomic locus. *Proc Natl Acad Sci U S A* 1986;**83**:9636–40.
- 55 **Ness SA**. Myb binding proteins: regulators and cohorts in transformation. *Oncogene* 1999;**18**:3039–46.
- 56 **Verbeek W**, Gombart AF, Chumakov AM, *et al*. C/EBPepsilon directly interacts with the DNA binding domain of c-myb and cooperatively activates transcription of myeloid promoters. *Blood* 1999;**93**:3327–37.
- 57 **Kaspar P**, Dvorakova M, Kralova J, *et al*. Myb-interacting protein, ATBF1, represses transcriptional activity of Myb oncoprotein. *J Biol Chem* 1999;**274**:14422–228.
- 58 **Sano Y**, Ishii S. Increased affinity of c-Myb for CREB-binding protein (CBP) after CBP-induced acetylation. *J Biol Chem* 2001;**276**:3674–82.
- 59 **Ying GG**, Proost P, van Damme J, *et al*. Nucleolin, a novel partner for the Myb transcription factor family that regulates their activity. *J Biol Chem* 2000;**275**:4152–8.
- 60 **Mark PJ**, Ward BK, Kumar P, *et al*. Human cyclophilin 40 is a heat shock protein that exhibits altered intracellular localization following heat shock. *Cell Stress Chaperones* 2001;**1**:59–70.
- 61 **Leverson JD**, Ness SA. Point mutations in v-Myb disrupt a cyclophilin-catalyzed negative regulatory mechanism. *Mol Cell* 1998;**1**:203–11.

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## Alteration of C-MYB DNA binding to cognate responsive elements in HL-60 variant cells

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