The transcription factor B-Myb is essential for S-phase progression and genomic stability in diploid and polyploid megakaryocytes

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Summary

The cell-cycle-regulated Myb-family transcription factor B-Myb is crucial during S phase in many diploid cell types. We have examined the expression and function of B-Myb in megakaryocytic differentiation, during which cells progress from a diploid to a polyploid state. In contrast to terminal differentiation of most haematopoietic cells, during which B-*myb* is rapidly downregulated, differentiation of megakaryocytes is accompanied by continued B-*myb* RNA and protein expression. Overexpression of B-Myb in a megakaryoblastic cell line resulted in an increase in the number of cells entering S phase and, upon induction of differentiation, the fraction of cells actively endoreplicating increased. By contrast, reduction of B-Myb levels using short interfering (si)RNA resulted in a decline in S-phase progression during both normal and endoreplicative DNA synthesis. This effect correlated with aberrant localisation of initiation of DNA replication within the nucleus and an increased fraction of cells in mitosis. Chromosomal fragmentation and other aberrations, including shorter, thicker chromatids, end-to-end fusion, and loss of a chromatid, suggest that reduced B-Myb activity is also associated with structural chromosomal instability.

Key words: B-Myb, Megakaryocytes, S phase, Endoreplication, Transcriptional regulation, Replication, Genome stability, Chromosome condensation, Chromosome instability

Introduction

B-Myb belongs to a family of transcription factors that is implicated in regulatory decisions affecting cell proliferation, differentiation and apoptosis (Oh and Reddy, 1999; Weston, 1998). B-Myb and the other family members c-Myb and A-Myb each comprise an N-terminal DNA-binding domain made up of three tandem repeats containing a helix-turn-helix motif and a C-terminal domain containing regulatory and transactivation domains (Klempnauer and Sippel, 1987; Biedenkapp et al., 1988; Nomura et al., 1988). B-Myb is ubiquitously expressed in proliferating cells (Latham et al., 1996), exhibiting maximal expression in S phase of the cell cycle (Lam et al., 1992). The essential function of B-Myb, at least during embryonic development, is highlighted by the death of mouse embryos homozygous for an inactivated B-*myb* allele at about E4.5 (Tanaka et al., 1999). The presumed importance of B-Myb during S phase is reflected in the mechanisms that ensure maximal expression at this stage of the cell cycle. Hence, B-*myb* RNA expression has been reported to be limited to proliferating cells by an E2F-dependent mechanism, whereas activity of the B-Myb protein is stimulated by the cdk2-cyclin A complex in S phase (Robinson et al., 1996). B-Myb also appears to have a role in mitosis, as suggested from the observation that zebrafish embryos carrying the mutation *crash&burn* (*crb*), which represents a loss-of-function mutation in *bmyb*, have defects in mitotic progression and spindle formation and exhibit genome instability (Shepard et al., 2005). The function of B-Myb in mitosis might relate at least partly to its ability to regulate cyclin B1 gene expression.

Of the vertebrate Myb proteins, B-Myb appears to be the one most related to the single *Drosophila* Myb protein (Dm-Myb) (Ganter et al., 1999; Simon et al., 2002). Dm-Myb has been shown to be essential for development (Katzen and Bishop, 1996; Fitzpatrick et al., 2002; Manak et al., 2002). Studies of Dm-Myb mutants have suggested roles in the transcriptional regulation of cyclin B (Okada et al., 2002) and the regulation of S phase and genomic stability (Beall et al., 2002; Manak et al., 2002; Beall et al., 2004). Interestingly, Dm-Myb appears to have a controlling influence on some processes of endoreplication, that is, cell cycles in which replication of all or part of the genome occurs without completion of mitosis or even, in some instances, the end of the normal S phase (reviewed by Edgar and Orr-Weaver, 2001). Dependent upon the cell type, Dm-Myb can act either as a repressor or as a promoter of endoreplication. In normal mitotic cells, Dm-Myb serves to maintain diploidy by repressing endoreplication (Katzen et al., 1998; Fitzpatrick et al., 2002). By contrast, Dm-Myb is expressed in endocycling tissues, being most concentrated in nuclear structures that contain recently replicated DNA, implying a functional role during DNA replication (Manak et al., 2002). Consistent with this, Dm-Myb has been shown to form a complex with four other proteins involved in site-specific DNA replication at the chorion loci in *Drosophila* ovarian follicle cells (Beall et al., 2002).

Endoreplication also occurs in specialised cell types in vertebrates, such as trophoblast giant cells (Varmuza et al., 1988) and megakaryocytes (Hoffman et al., 1988). In megakaryocytes, endoreplication involves exit from mitosis during anaphase B prior to nuclear division and cytokinesis, and has a key role in terminal differentiation by enhancing their ability to produce blood platelets, largely as a result of the consequent increase in cell size (Zimmet and Ravid, 2000). Mechanistically, endoreplication in megakaryocytes appears to be the consequence of maintained activity of G1-S cyclins and reduced cdc2-cyclin B kinase activity (Datta et al., 1996; García and Calés, 1996). We have also shown that cyclin E is necessary for endoreplication to occur (García et al., 2000) and, consistent with this, megakaryocytes from double-knockout mice lacking both cyclin E1 and E2 have a lower degree of ploidy compared with cells derived from wild-type animals (Geng et al., 2003). *Drosophila* endocycles also require cyclin E, which is transcribed prior to the onset of each S phase (Knoblich et al., 1994; Duronio and O'Farrell, 1995; Sauer and Lehner, 1995); however, the importance of an oscillating cycle of cyclin E levels is suggested by the observation that its continuous overexpression inhibits endocycle progression (Follette et al., 1998; Weiss et al., 1998).

As for other commitment and differentiation processes occurring in haematopoiesis, it is believed that the regulation of gene expression underlies megakaryocytopoiesis, presumably including the switch from normal cell cycling to the endoreplicative phase. Gene-knockout and overexpression studies have identified several transcription factors that are essential in megakaryocytopoiesis, affecting one of three steps: (1) commitment [e.g. Meis1 and FOG-1 (Hisa et al., 2004; Tsang et al., 1998)], (2) the proliferating megakaryoblast [e.g. GATA-1 and MafG (Shavit et al., 1998; Vyas et al., 1999)] and (3) terminal maturation leading to platelet release [e.g. NF-E2 (Shivdasani et al., 1995)]. However, no factor has yet been linked mechanistically to the onset and maintenance of endoreplication.

Here, we have investigated the involvement of the B-Myb protein in megakaryocytic endoreplication. We show that expression of B-Myb is maintained during megakaryocytic differentiation of megakaryoblastic cell lines and in mature primary megakaryocytes. By overexpression or reduction of B-Myb levels, we show that B-Myb regulates S-phase progression of both mitotic and endoreplicating cell cycles during megakaryocyte differentiation. The defects caused by reduced B-Myb levels lead to improper chromosome condensation, chromosome fragmentation and mitotic arrest in diploid and polyploid cells, and we propose that this results largely because B-Myb is essential for proper progression of S phase. We discuss whether this role of B-Myb is a reflection of its proposed evolutionary relationship to *Drosophila* Myb.

Results

B-Myb is expressed throughout megakaryocytic differentiation

Given the association between B-Myb and normal S phase of the cell cycle, we asked how B-Myb expression is regulated during the successive endoreplicative S phases in megakaryocytes.

Several human megakaryoblastic cell lines have been characterised that can be induced to undergo differentiation in the presence of phorbol esters such as 12-O-tetradecanoylphorbol 13-acetate (TPA) (Roth et al., 1988), and this is associated with inhibition of cell proliferation, nuclear polyploidisation, and an increase in the expression of platelet proteins (Tabilio et al., 1984; García and Calés, 1996; Berlanga et al., 2000).

HEL and CMK cells treated with 10^{-8} M TPA for 24, 48 and 72 hours were examined to determine DNA content by flow cytometry after permeabilisation and staining with propidium iodide. Exponentially growing HEL and CMK cells had a normal distribution between G1-S and G2-M, whereas cells treated with TPA exhibited a progressive increase in ploidy (Fig. 1A). After 72 hours of treatment with TPA, HEL and CMK cells reached maximum ploidy values of 16C and 8C, respectively. As a control, we used U937 cells that differentiate in response to TPA, and in so doing exit the cell cycle. As expected, the population of U937 cells treated with TPA for 24 hours showed a decreased proportion in S phase and by 48 hours 90% of cells were arrested in G1 (Fig. 1A).

Total protein extracts from HEL, CMK and U937 cells treated with TPA were analysed by western blotting with an anti-B-Myb antibody. HEL and CMK cells treated with TPA continued expressing B-Myb even after 72 hours, whereas B-Myb levels in TPA-treated U937 cells were reduced as early as 24 hours after TPA was added (Fig. 1B).

To determine whether the B-Myb detected after TPA stimulation was associated with cells undergoing endoreplication, we performed a flow cytometric analysis of cells stained with an antibody against B-Myb and with propidium iodide to measure DNA content. This revealed that B-Myb is expressed throughout the cell cycle in exponentially growing HEL cells (Fig. 1C). When HEL cells were treated with TPA, the continued expression of B-Myb detected by western blotting was reflected in positive staining of cells with both normal and polyploid DNA contents (Fig. 1C). Moreover, not only were all cells positive for B-Myb expression, but an increase in the level of B-Myb expression was detected in those cells with an active endoreplicating S phase, as has been described for diploid cells (Fig. 1C, enlarged panel, arrow). The specificity of the anti-B-Myb antibody was confirmed using a blocking peptide (Fig. 1D).

Reflecting the important role of B-Myb during the cell cycle, the level of active protein is regulated by both an E2F-dependent transcriptional mechanism and by post-translational modification (Lam and Watson, 1993). To examine whether the continued expression of B-Myb protein during megakaryocytic endoreplication is paralleled at the level of B-*myb* gene transcription, we performed semi-quantitative RT-PCR analysis on RNA extracted from exponentially growing and TPA-treated HEL and CMK cells, with TPA-treated U937 cells as a control. Hypoxanthine guanine phosphoribosyl transferase (HPRT) RNA expression was measured to standardise samples. B-*myb* RNA levels changed dramatically in U937 cells, with an approximate 50% decrease between 24 and 48 hours of treatment with TPA (Fig. 1E). By contrast, B-*myb* RNA levels changed little in the population of HEL and CMK cells during treatment with TPA (Fig. 1E), suggesting that B-*myb* transcription is maintained during megakaryocytic differentiation.

Next we wanted to know whether our observations on B-Myb expression during induced differentiation of

megakaryoblastic cell lines also holds true for primary megakaryocytes derived from either foetal liver or bone marrow. As summarised in the scheme (Fig. 2A), E15 foetal livers were cultured in vitro for four days in the presence of thrombopoietin (TPO), a regulatory factor in platelet production, to stimulate megakaryocytic differentiation. Myeloid progenitors, macrophages, granulocytes, erythrocytes, neutrophils and B cells were then removed by immunomagnetic depletion and the resulting population subjected to fractionation on a discontinuous bovine serum albumin (BSA) gradient. Two fractions were collected and their DNA content analysed by flow cytometry (Fig. 2B). Fraction 4 represented highly enriched, fully mature polyploid megakaryocytes based on DNA content (85% greater than 8C), acetylcholinesterase staining and surface antigen expression (Fig. 2B,C, and data not shown).

RT-PCR and western blot analysis indicated the presence of B-*myb* RNA (data not shown) and protein (Fig. 2D) in the

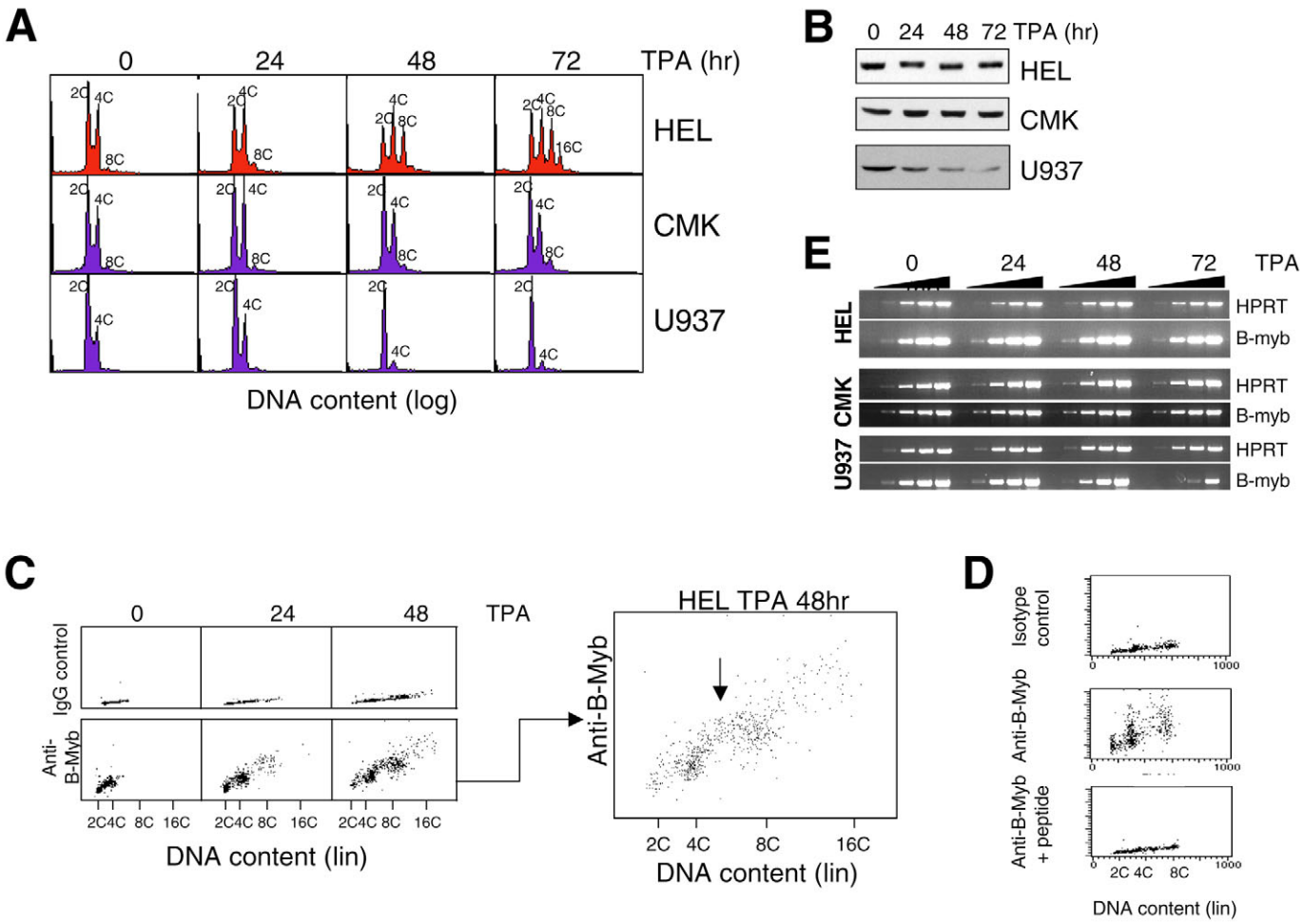

Fig. 1. B-Myb is expressed in endoreplicating cells during megakaryocytic differentiation. Cells were cultured from an initial density of 1.5×10^5/ml in the absence or presence of 10^{-8} M TPA for 24, 48 or 72 hours. (A) Propidium iodide staining of TPA-treated and untreated HEL, CMK and U937 cells. The vertical axis indicates the relative number of cells and the horizontal axis indicates the relative red fluorescence (FL2) on a logarithmic scale as a measure of DNA content. The positions of peaks representing cells with DNA content of 2, 4, 8 and 16C are indicated. (B) 50 μg of total protein extract from cells exponentially growing (0) or treated with TPA for 24, 48 and 72 hours were subjected to SDS-PAGE, transfered to a membrane and probed with antibodies against B-Myb. Coomassie Blue staining was performed with the upper part of the gel as loading control. (C) Flow cytometric analysis of HEL cells that were either exponentially growing (0) or treated with 10^{-8} M TPA for 24 and 48 hours. Expression of B-Myb (lower panels) or IgG control (upper panels) was detected by indirect immunofluorescence using anti-IgG or anti-B-Myb in conjunction with FITC-conjugated goat anti-rabbit IgG (FL1, vertical axis, linear scale), and total DNA content was monitored by propidium iodide staining (FL2, horizontal axis, linear scale). In the right panel is shown an enlargement of the profile for HEL cells treated with TPA for 48 hours, the arrow indicating cells with active endoreplicating S phase. (D) Flow cytometric analysis of B-Myb expression in HEL cells treated with 10^{-8} M TPA for 48 hours as described in (C) except that blocking peptide was included in the sample in the lower panel. (E) HEL, CMK and U937 cells were cultured from an initial density of 1.5×10^5/ml in the absence or presence of 10^{-8} M TPA for 24, 48 or 72 hours. cDNA was prepared from 3 μg RNA and semi-quantitative PCR analysis was performed to measure the relative expression of B-*myb*. HPRT was used to standardise loading of equal amounts of cDNA. The upper and lower panels show the RT-PCR analysis of HPRT and B-*myb* RNA, respectively. The size of PCR products was 620 bp for HPRT and 310 bp for B-*myb*. The PCR reactions were sampled at cycles 25, 28, 31, 34 and 37 for reactions using HEL cDNA and at cycles 22, 25, 28, 31 and 34 for reactions using CMK and U937 cDNA.

enriched population of mature primary megakaryocytes. To determine the relative expression of B-Myb in cells with different DNA contents, we stained cells from foetal liver treated with TPO for 4 days with an antibody against B-Myb and with propidium iodide. Flow cytometric analysis showed that B-Myb is expressed in mature polyploid megakaryocytes at a level that increases in proportion to the DNA content of the cell (Fig. 2E). Those cells with lower DNA content (2C and 4C) that stained negative for B-Myb are probably mature haematopoietic cells that have downregulated B-Myb as part of their differentiation program, or are cells that cannot replicate in the presence of TPO as the sole stimulus. Similar results were obtained with mature megakaryocytes derived from adult bone marrow (data not shown). The high expression of B-Myb in mature polyploid cells suggested that it could either be involved in continuing DNA replication or alternatively some other late differentiation-associated function. Labelling of mature fraction 4 megakaryocytes with 5-bromo-2-deoxyuridine (BrdU) indicated that these cells, including those that are in the final process of cytoplasmic maturation before platelet release, are still actively synthesising DNA and, therefore, that B-Myb could still be performing an S-phase-related function (Fig. 2F).

Collectively, the results with cell lines and primary mouse cells demonstrate that B-Myb is present throughout both normal and endoreplicative cell cycles in megakaryocytes.

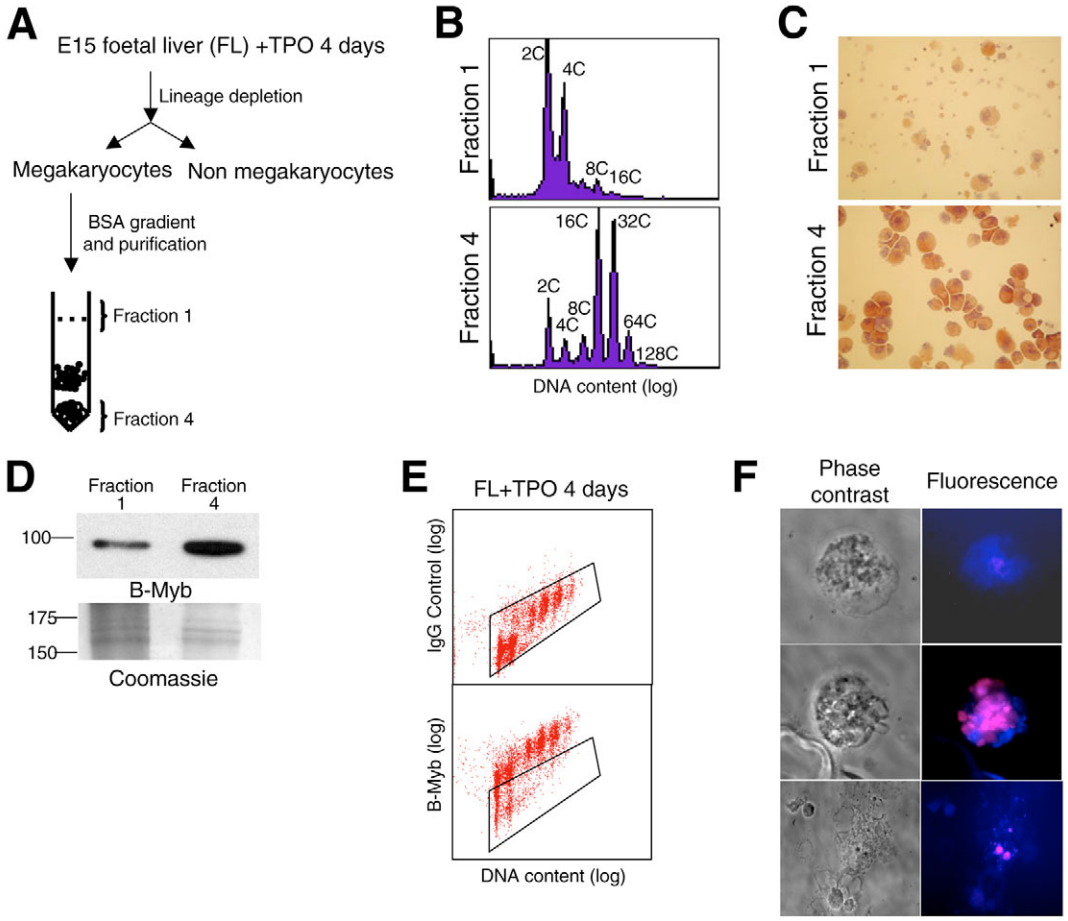

Fig. 2. B-Myb protein is expressed in primary mature megakaryocytes. (A) E15 foetal liver cells were cultured in vitro for 4 days in the presence of TPO, then immunodepleted and subjected to fractionation on a discontinuous BSA gradient. (B) Propidium iodide staining of cells from Fractions 1 and 4. The vertical axis indicates the relative number of cells and the horizontal axis indicates the relative red fluorescence (FL2) on a logarithmic scale as a measure of DNA content. Peaks representing each ploidy class are labelled. (C) Megakaryocytes from Fractions 1 and 4 were cytospun and stained for acetylcholinesterase. (D) 50 μg of total protein extract from cells in Fractions 1 and 4 were subjected to SDS-PAGE and detected by western blot with antibody against B-Myb. The position of relevant molecular weight standards is shown on the left side of the blot. Coomassie Blue staining was performed with the upper part of the gel as a loading control. (E) Expression of B-Myb was detected in E15 foetal liver (FL) cells cultured in the presence of TPO for 4 days, by indirect immunofluorescence using a B-Myb polyclonal antibody or IgG control and FITC-conjugated goat anti-rabbit IgG (FL1, vertical axis, logarithmic scale), and total DNA content was monitored by propidium iodide staining (FL2, horizontal axis, logarithmic scale). The polygon shows the position of cells stained in parallel with the isotype control. (F) DNA synthesis in mature megakaryocytes. Cells that had differentiated for 5 days were labelled with BrdU for 16 hours. Incorporation of BrdU was detected by indirect immunofluorescence using a mouse anti-BrdU antibody and PE-conjugated goat anti-mouse Ig secondary antibody (red). Nuclei were stained with DAPI (blue). The upper panels are of a mature megakaryocyte that had not been labelled with BrdU. The middle and lower panels are examples of cells that have incorporated BrdU, representing, respectively, a mature polyploidy megakaryocyte and a terminally differentiated, platelet-producing cell.

Overexpression of B-Myb enhances both normal and endoreplicative S phase in megakaryocytic cells

Overexpression of B-Myb has previously been shown to overcome G1 arrest in SAOS-2 cells induced by either the retinoblastoma-related protein p107 or the cell-cycle inhibitor p57 (KIP2) (Joaquin et al., 2002; Joaquin and Watson, 2003). Similarly, Dm-Myb can induce proliferation in imaginal discs (Manak et al., 2002), whereas overexpression in salivary glands inhibits endoreplication (Fitzpatrick et al., 2002). Our observations on the expression of B-Myb during megakaryocytic differentiation suggest that, in these mammalian cells, B-Myb continues to perform a role related to entry into, or progression through, endoreplicative S phases.

To gain some insight into the function of B-Myb during megakaryocytic differentiation, we set out to alter the levels of B-Myb protein. First, we overexpressed B-Myb in HEL cells by introduction of a plasmid containing human B-*myb* cDNA under the control of a cytomegalovirus (CMV) promoter. The construct was also capable of expressing enhanced green fluorescent protein (EGFP) by virtue of the inclusion of EGFP sequences linked to an IRES element placed downstream of the B-*myb* cDNA (Fig. 3A). The transfected HEL cell population was sorted 18 hours after transfection into GFP$^-$ and GFP$^+$ fractions. Western blot analysis of extracts obtained from the GFP$^-$ and GFP$^+$ fractions using anti-B-Myb antibody revealed high levels of exogenous B-Myb only in the GFP$^+$ fraction (Fig. 3B). Interestingly, 24 hours after transfection, the levels of B-Myb protein were reduced to normal levels, reflecting its tight regulation (data not shown).

Next we determined the effect that B-Myb overexpression has when cells have established endoreplicative cycles. Owing to the negation of the effects of ectopic expression of B-Myb on overall protein levels 24 hours after transfection, HEL cells were transfected as described above with control and B-Myb expression vectors and, two hours later, treated with TPA. This strategy ensured that the transfected cells had entered into differentiation prior to accumulation of exogenous B-Myb, and that they would experience at least 18 hours of high levels of B-Myb during their differentiation. GFP$^+$ cells were sorted 18 hours after transfection and culture was then continued for a further 48 hours. After transfection and sorting, the resulting population became more homogeneous because bigger cells were lost as a result of the size of the nozzle. Analysis of DNA content by flow cytometry showed a difference between GFP$^+$ transfected cells containing the control vector or the B-Myb expression plasmid. Hence, B-Myb overexpression resulted in

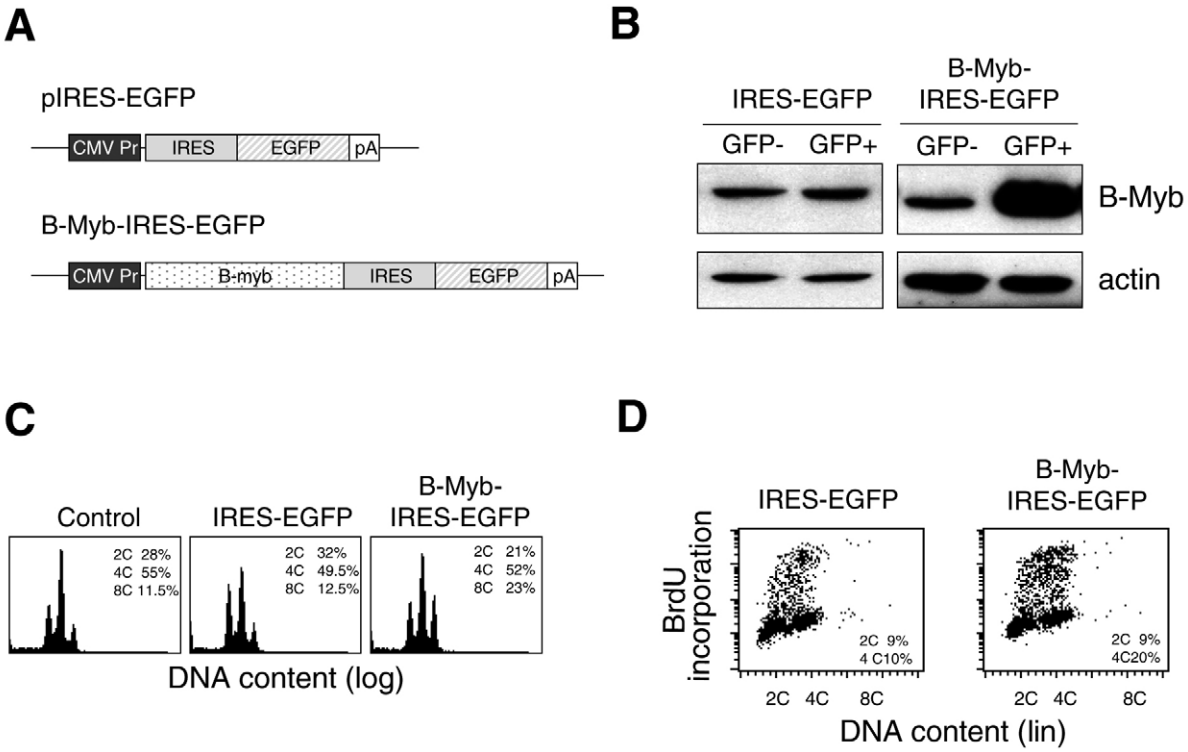

Fig. 3. Overexpression of B-Myb influences endoreplication in megakaryoblastic cells. (A) Diagram of the pIRES-EGFP and B-Myb-IRES-EGFP plasmids. HEL cells were transfected with pIRES-EGFP control plasmid or B-Myb-IRES-EGFP and sorted for GFP expression after 18 hours. CMV Pr, cytomegalovirus promoter. (B) 50 μg of total protein from GFP$^+$ and GFP$^-$ populations were subjected to SDS-PAGE and detected by western blot with antibodies against B-Myb and β-actin. (C) Cell-cycle distribution of sorted GFP$^-$ cells and GFP$^+$ cells treated with TPA for 72 hours; the middle and right panels correspond to GFP$^+$ cells from transfections with the control vector and B-Myb-IRES-EGFP, respectively. The vertical axis indicates the relative number of cells and the horizontal axis indicates the relative red fluorescence (FL2) on a logarithmic scale as a measure of DNA content. (D) Flow cytometric analysis of GFP$^+$ cells that were in the presence of TPA for 18 hours, followed by 3 hours in the presence of TPA and BrdU. Incorporation of BrdU was detected by indirect immunofluorescence using a FITC-conjugated mouse anti-BrdU monoclonal antibody and FITC-conjugated mouse IgG1 isotype control (FL1, vertical axis, logarithmic scale), and total DNA content was monitored by propidium iodide staining (FL2, horizontal axis, linear scale). The left and right panels correspond to GFP$^+$ cells from transfections with the control vector and B-Myb-IRES-EGFP, respectively.

a decrease in the percentage of cells with DNA content of 2C and an approximate doubling in the percentage of cells with DNA content of more than 4C (Fig. 3C). GFP^- cells from the population transfected with B-Myb-IRES-EGFP exhibited a profile very similar to the GFP^+ cells containing the control vector.

We also determined what effect B-Myb overexpression has on DNA synthetic activity by measurement of BrdU incorporation. HEL cells were transfected with control and B-Myb expression vectors and 2 hours later treated with TPA. After 18 hours, BrdU was added to the cultures and incubation continued for a further 3 hours before sorting GFP^+ cells. Assessment of the incorporation of BrdU revealed that the cells overexpressing B-Myb were exhibiting an increased rate of DNA synthesis in both normal (S1) and endoreplicative (S2) S phases (Fig. 3D). Hence, in the population of cells transfected with the B-Myb expression vector, 11% and 17% had incorporated BrdU in S1 and S2 respectively, compared with only 6% and 9% when cells contained the control vector.

Together, these data indicate that B-Myb acts to potentiate normal and endoreplicative S phases in megakaryocytic cells.

B-Myb downregulation leads to defects in megakaryocytic cell-cycle progression and chromosomal instability

We next tested the effect of reduction in B-Myb levels using short interfering (si)RNAs directed against B-*myb* RNA. HEL cells treated with TPA for 16 hours were transfected with FITC-conjugated double-stranded siRNAs corresponding to two distinct sequences within the B-Myb coding region or with a control siRNA with no match to B-*myb*. Flow cytometric analysis of cells after 60 hours revealed that the transfection efficiency was more than 80% (Fig. 4A). Western blotting revealed a drastic decrease in the levels of B-Myb when cells had been transfected with either siRNA1 or siRNA2 compared with cells treated with the control, although the extent of knockdown was most effective using siRNA1 (Fig. 4B).

To investigate whether decreased B-Myb levels had any profound effect on megakaryocytic differentiation, we performed RT-PCR analysis to determine the level of expression of key differentiation-associated genes. Such semi-quantitative examination of RNAs encoding GPIIb, GPIIIa and GPVI did not reveal any significant changes following B-*myb* knockdown (Fig. 4C).

Flow cytometric analysis of cells labelled with BrdU between 48 and 64 hours after transfection with the siRNAs directed against B-*myb* revealed a dramatic decrease in the extent of BrdU incorporation per cell (Fig. 5A). Taking, for example, the population of cells with DNA content of 4C, it can be seen that roughly 40% of these incorporated BrdU. Applying the same analysis to cells treated with B-*myb* siRNAs, the percentage of cells that were labelled was similar to that seen using the control siRNA, however the intensity of fluorescence in the positive peak was considerably reduced. Whereas the ratio between the mean fluorescence intensities comparing the negative and positive peak was 1:7 for the control siRNA, this ratio was reduced to 1:3 in the case of siRNA1 and siRNA2 (see Fig. 5A right panel), and was the same in populations of cells with a DNA content of 2C and 8C. This implies that, although the number of cells that were capable of incorporating BrdU after treatment with B-*myb* siRNA oligoribonucleotides was the same, there was a clear reduction in overall DNA synthesis, possibly as a result of a deficiency in completion of S phase. The fact that both siRNA1 and siRNA2 had a similar effect on BrdU incorporation, and yet siRNA1 resulted in a greater degree of knockdown of B-Myb protein, suggests that both siRNAs had nevertheless decreased expression below a critical minimal threshold.

To address whether the knockdown of B-Myb levels leads to a reduction in the rate of DNA synthesis, we performed labelling with a short pulse of BrdU and then observed the cells by fluorescent microscopy. As expected from the flow cytometric analysis, the percentage of cells incorporating BrdU was the same, indicating that there was not a decrease in the

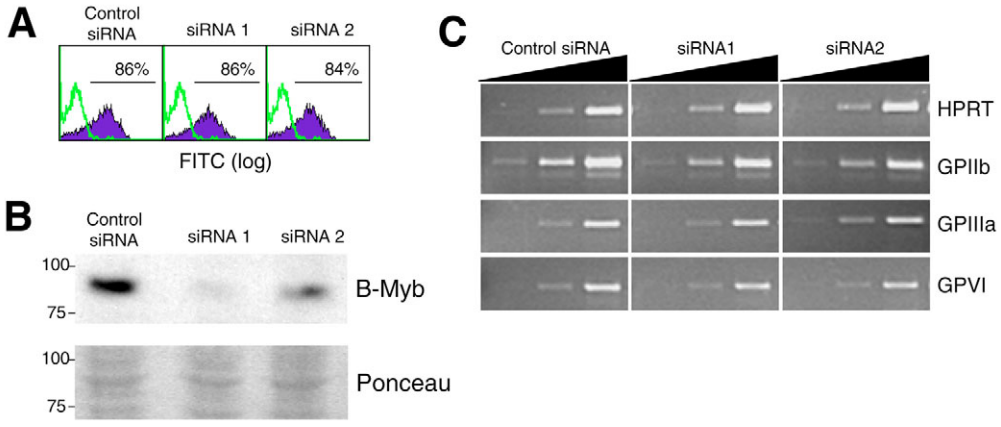

Fig. 4. Downregulation of B-Myb by RNA interference. HEL cells treated with 10^{-8} M TPA for 16 hours were subjected to three rounds of transfection with 50 nM of the different FITC-conjugated siRNAs for three hours at one-day intervals. (A) 80 hours after TPA treatment, transfection efficiency was determined by flow cytometry to detect FITC-positive cells. The histograms indicate green fluorescence (FL1) on the horizontal logarithmic axis. The controls are mock-transfected cells. (B) 40 μg of total protein extract from siRNA-treated cells was used for a western blot analysis of B-Myb protein levels. The upper panel shows the signal obtained after probing with anti-B-Myb, whereas the lower panel is an image of the Ponceau Red staining of the same region of the filter after transfer. (C) RT-PCR analysis of gene expression following siRNA knockdown of B-Myb. cDNA was prepared from 1 μg RNA extracted from cells treated as described in A. Semi-quantitative RT-PCR was performed for the indicated genes, sampling reactions at 22, 25 and 28 cycles, using HPRT to normalise the cDNA input.

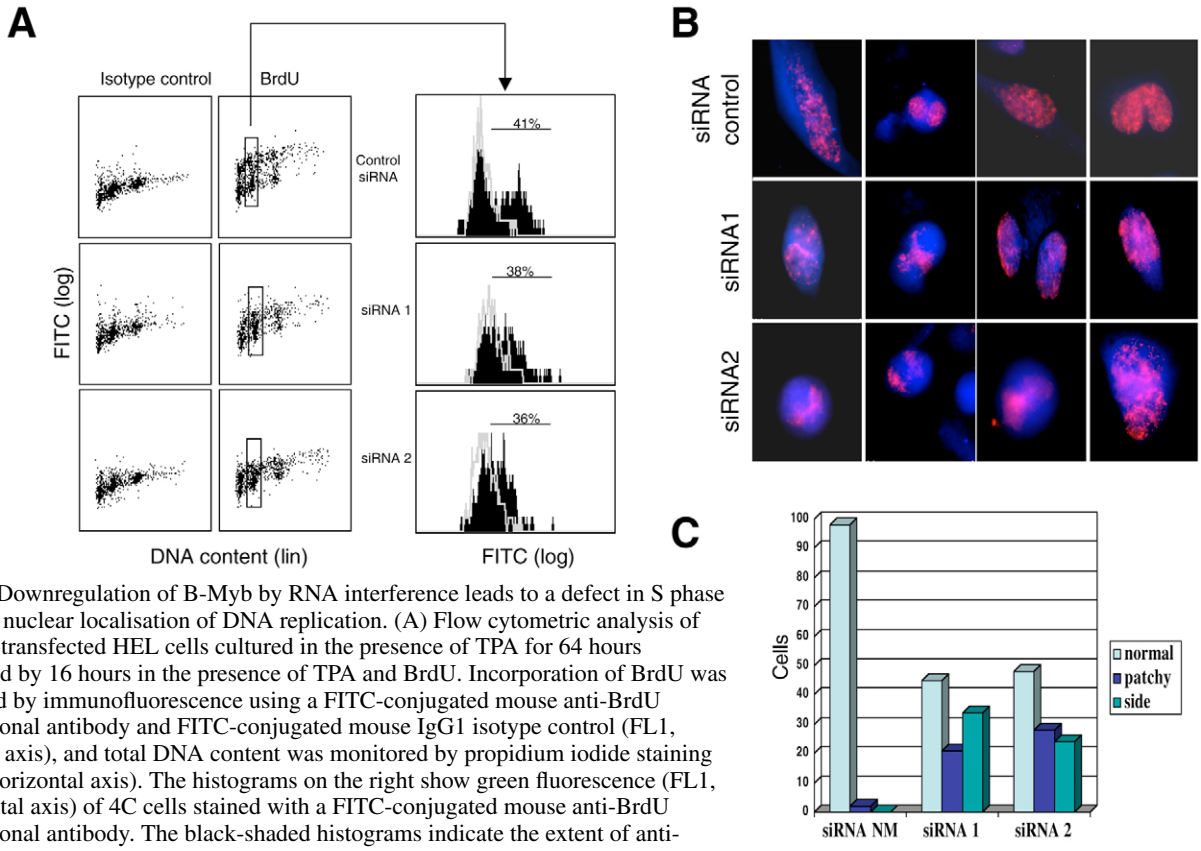

Fig. 5. Downregulation of B-Myb by RNA interference leads to a defect in S phase and the nuclear localisation of DNA replication. (A) Flow cytometric analysis of siRNA-transfected HEL cells cultured in the presence of TPA for 64 hours followed by 16 hours in the presence of TPA and BrdU. Incorporation of BrdU was detected by immunofluorescence using a FITC-conjugated mouse anti-BrdU monoclonal antibody and FITC-conjugated mouse IgG1 isotype control (FL1, vertical axis), and total DNA content was monitored by propidium iodide staining (FL2, horizontal axis). The histograms on the right show green fluorescence (FL1, horizontal axis) of 4C cells stained with a FITC-conjugated mouse anti-BrdU monoclonal antibody. The black-shaded histograms indicate the extent of anti-BrdU staining, whereas the grey lines show the staining obtained with the isotype control. (B) Immunofluorescence analysis of siRNA-transfected HEL cells grown in the presence of TPA for 64 hours and then pulse-labelled with BrdU for 45 minutes. Incorporation of BrdU was detected by indirect immunofluorescence using a mouse anti-BrdU antibody and PE-conjugated goat anti-mouse Ig secondary antibody (red). Nuclei were stained with DAPI (blue). Upper panels: control siRNA. Middle panels: B-*myb* siRNA1. Lower panels: B-*myb* siRNA2. (C) Histogram representing the pattern of incorporation of BrdU in 100 cells treated with each of the siRNAs. NM, no match.

initiation of DNA replication. However, the distribution of BrdU incorporation was strikingly different; those cells transfected with control siRNA showed discrete foci of replication equally distributed throughout the nucleus (Fig. 5B). By contrast, more than 50% of cells transfected with the B-*myb* siRNA that had incorporated BrdU showed an aberrant localisation with a heterogeneous and sometimes diffuse accumulation of BrdU (Fig. 5B). To gain a semi-quantitative representation of these results, we classified the BrdU labelling as 'normal', 'patchy' or 'side'. Cells transfected with the control siRNA were almost entirely in the normal category, whereas B-*myb* siRNA1 and siRNA2 resulted in the patchy pattern of BrdU incorporation in 20% and 28%, respectively, and the side pattern in 33% and 23%, respectively (Fig. 5C).

Overall, the BrdU incorporation results following B-*myb* knockdown indicate that B-Myb is essential for proper and complete S-phase DNA synthesis in both normal and endoreplicative cycles in megakaryocytes. To look more closely at cell-cycle progression, TPA-treated HEL cells were transfected with siRNAs, cytospun in the absence of hypotonic treatment and then scored for the presence of mitotic cells by immunofluorescent detection of phospho-histone 3 (H3) (see Table 1). The percentage of phospho-H3-positive cells was the same in TPA-treated HEL cells that were mock transfected or transfected with the siRNA control. However, TPA-treated HEL cells transfected with siRNAs 1 and 2 exhibited an unexpected three-fold increase in mitotic cells compared with the control, suggesting that they were arrested at a mitotic checkpoint.

To define the mitotic arrest further, 4′,6-diamidino-2-phenylindole (DAPI)-stained chromosome spreads were prepared using a hypotonic treatment to define the degree of condensation better. Both cells transfected with siRNA1 and cells transfected with siRNA2 showed abnormally condensed mitotic chromosomes, resulting in thicker, shorter chromatids. This effect could be seen in both diploid (Fig. 6, upper panels) and polyploid cells (Fig. 6, lower panels). Such aberrantly condensed chromosomes were found in more than 62% and 45% of mitotic cells after treatment with siRNA1 and siRNA2, respectively (Fig. 6B,C). No abnormal condensed chromosomes were found in either HEL cells treated with siRNA control (Fig. 6A), HEL cells treated with TPA, or HEL cells that were exponentially growing (data not shown). Consistent with the idea that these aberrations result from incomplete S phase, we observed frequent chromosome fragmentation in all well-spread metaphases containing condensed chromosomes (Fig. 6, arrows).

We also stained chromosome spreads from B-*myb* siRNA-treated cells with an antibody against the centromere protein CENPa in order to highlight any chromosomal abnormalities

Table 1. Histone H3 phosphorylation in HEL cells

Treatment	Mitotic cells (%)	Fold increase
TPA	2.0±0.09	–
TPA + siRNA control	1.8±0.17	0.9
TPA + siRNA1	5.6±0.32	3.1
TPA + siRNA2	5.1±0.21	2.8

The mitotic frequency was determined by averaging the percentage of mitotic cells observed in three independent immunofluorescence stainings using anti-phospho-H3.

that might be indicative of chromosome instability (Fig. 7). More than 80% of well-spread metaphases from siRNA1- or siRNA2-treated cells exhibited a combination of end-to-end fusion, chromosome fragmentation, loss of one chromatid, centromeric duplication or loss of chromosome segments (Fig. 7B,C). Such abnormalities were not seen in TPA-treated HEL cells with or without transfection of the control siRNA.

Collectively, our results indicate that reduction of B-Myb levels directly or indirectly affects the cell cycle, leading to several defects that might be linked or independent. These defects include perturbed S-phase progression, arrest in mitosis, aberrant chromosome condensation and structural integrity, and signs of chromosome fragmentation.

Discussion

The process of endoreplication is key to the ability of the megakaryocyte to generate several thousand platelets for release into the circulation. It is assumed that the mechanisms that regulate endoreplication are determined ultimately by changes in transcriptional regulation, but to date no single transcription factor has been linked directly to the process.

Here, we demonstrate that the transcription factor B-Myb is expressed throughout megakaryocytic differentiation and that modulation of its level can influence the extent of both normal and endoreplicative DNA synthesis. This contrasts with the rapid drop in B-Myb expression seen during the differentiation of most cell types, a feature that appears to be a necessary prerequisite since differentiation can be blocked by ectopic overexpression in a variety of cell systems (Raschella et al., 1995; Bies et al., 1996; Engelhard et al., 2000). In non-mammalian vertebrates, the equivalent process leading to the generation of platelet-like nucleated thrombocytes does not involve endoreplication but instead exhibits nuclear condensation accompanying terminal differentiation. Interestingly, using avian multipotential progenitors transformed by a temperature-sensitive derivative of the Myb oncoprotein that can be induced to differentiate towards thrombocytes upon shift of cultures from 37°C to 42°C (Frampton et al., 1995), we have shown that B-*myb* expression is rapidly lost during thrombopoiesis (data not shown). Since avian thrombocytes share many of the functional characteristics of mammalian platelets, this observation of B-Myb downregulation during thrombopoiesis implies that B-Myb is not required for aspects of terminal megakaryocytic differentiation apart from endoreplication. It is also consistent with the idea that B-Myb is a major determinant of the ability of the megakaryocyte to become polyploid.

Given the effect we observed upon overexpression of B-Myb, it could have been expected that B-*myb* knockdown would reduce the overall ploidy of the population. That we actually observed similar ploidy values in the control and siRNA-treated populations could be a technical issue. Hence, although BrdU incoporation indicated that B-*myb* knockdown affected DNA synthesis, this was assessed in the last 16 hours of the 80-hour experiment. If there was some lag in the effectiveness of the reduction in B-Myb levels, then the population as a whole might have been able to progress through endoreplication normally until the latter stages when BrdU was added. An alternative explanation is that B-Myb overexpression has an artificial direct influence on endoreplication in cells in which c-Myb serves to stimulate cell-cycle progression. Circumstantial evidence in support of this idea comes from a comparison of the effects of loss of B-Myb in cells that do or do not express c-Myb. Loss of B-Myb from the haematopoietic cell line DT40, which like HEL cells express c-Myb, suggests that B-Myb is not required for proliferation (Ahlbory et al., 2005), whereas our analysis of mouse embryonic fibroblasts (MEFs), which express little or no c-Myb, demonstrate that these cells can no longer enter S phase after undergoing conditional deletion of B-*myb* (García et al., 2005). Perhaps overexpression of B-Myb in c-Myb-expressing cells might artificially enhance DNA replication by acting upon c-Myb targets.

On the basis of our observations from knockdown of B-Myb activity, we hypothesise that B-Myb has a principal role in vertebrates that facilitates correct progression through S phase and concomitant chromosome condensation during mitosis. As a direct or indirect consequence of B-Myb downregulation, replication and chromosome condensation are affected, possibly leading to the observed chromosome fragmentation,

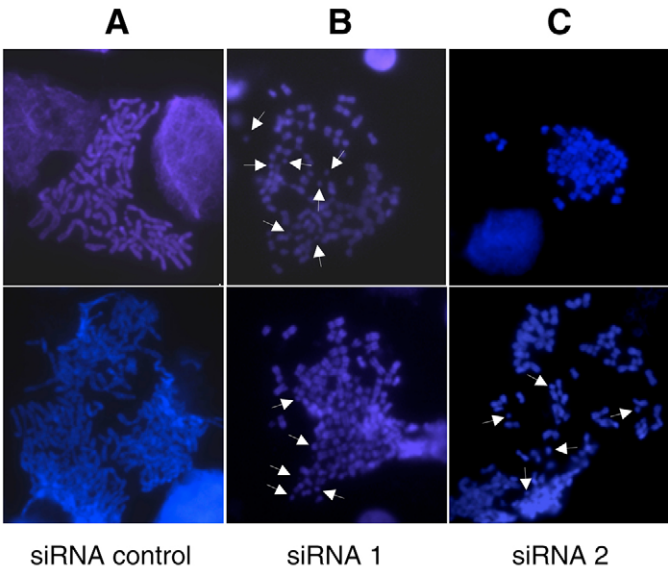

Fig. 6. Downregulation of B-Myb by RNA interference leads to a defect in chromosome condensation. HEL cells treated with 10^{-8} M TPA for 16 hours were subjected to three rounds of transfection with 50 nM of the different siRNAs for 3 hours at intervals of one day. At 2 hours after the final treatment, chromosome spreads were prepared in the presence of hypotonic buffer and stained with DAPI. (A) Control siRNA, (B) siRNA 1, (C) siRNA 2. The upper and lower panels show diploid and polyploid cells, respectively. The arrows indicate fragmented chromosomes.

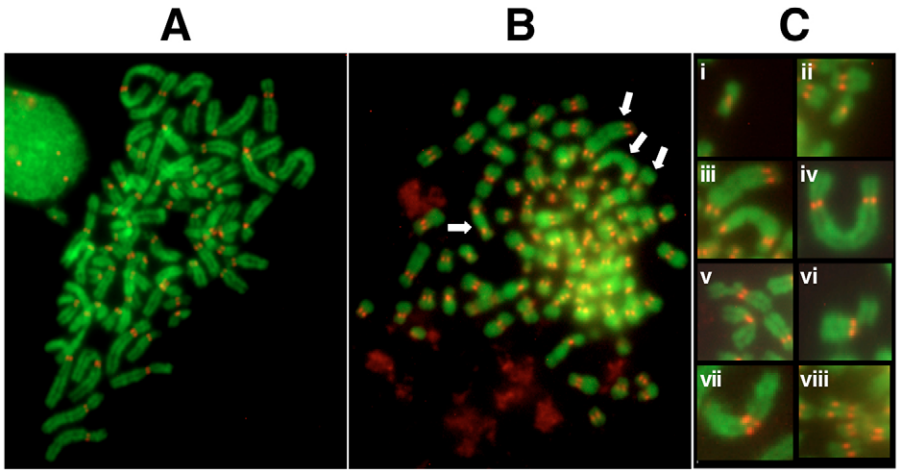

Fig. 7. Downregulation of B-Myb by RNA interference leads to chromosome instability. HEL cells treated with 10^{-8} M TPA for 16 hours were subjected to three rounds of transfection with 50 nM of the different siRNAs for 3 hours at intervals of one day. At 2 hours after the final treatment, chromosome spreads were prepared and stained using anti-CENPa (orange) and DAPI (green). Representative chromosome spreads are shown for cells treated with (A) control siRNA and (B) siRNA1 targeted against B-*myb*. The arrows in B indicate examples of aberrant chromosomes. (C) Expanded images of the types of chromosome aberration resulting from B-*myb* knockdown, including single chromatids (i,ii), chromosomes joined end-to-end (iii,iv), loss of chromosome segments (v,vi), and greater than two centromeres (vii,viii).

chromosomal instability and arrest in mitosis. It is known that normal cell-cycle progression through S phase might have profound consequences upon the structure of the mitotic chromosome. For example, when G2 mammalian HeLa cells are fused to G1 cells, both nuclei enter mitosis in synchrony. However, the DNA of the G1 cell passes through S phase somewhat more rapidly and results in mitotic chromosomes that are less condensed (Rao and Johnson, 1970). Most importantly, premature chromosomal condensation detected by fusion of a G1 nucleus with a mitotic nucleus results in extended chromatin fibres much longer than normal chromatids (Johnson and Rao, 1970; Gollin et al., 1984). These experiments indicate that activities present in mitotic cells are able to condense interphase chromatin but that the morphology of the condensed chromosomes is incomplete. It has been suggested that there is a correlation between apparent chromosome fibre loop lengths and mean distances between replication origins (Paulson and Laemmli, 1977; Marsden and Laemmli, 1979) and therefore that there is a possibility that the positions and density of DNA replication origins on the DNA fibre might influence chromosome folding. In eukaryotes, the origin recognition complex (ORC) serves as the platform upon which replication initiation complexes assemble (Lee and Bell, 1997), and thus the distribution of ORC along the DNA in part determines the sites at which replication might start. Mutant versions of several genes directly involved in DNA synthesis in *Drosophila* (encoding Orc2, Orc5, PCNA, Mcm4 and Dup/Cdt1) result in a characteristic aberrant regulation of the cell cycle, with an improper S phase leading to arrest at M phase with inappropriate chromosome condensation (Pflumm and Botchan, 2001; Whittaker et al., 2000). The effects of these *Drosophila* mutants on the cell cycle also resemble the results obtained with B-*myb* siRNA described here, as well as recent preliminary findings we have made following conditional deletion of B-*myb* in embryonic fibroblasts (García et al., 2005), and observations of others on zebrafish embryos containing a loss-of-function mutation in the B-Myb-encoding *crb* gene (Shepard et al., 2005).

At this time, we can only speculate on the mechanism(s) by which B-Myb allows proper completion of S phase and maintenance of chromosome stability. As a transcriptional regulator, B-Myb could regulate the expression of any of a number of genes whose products are involved in S-phase progression. For example, B-Myb has recently been described to be active on the promoter of the gene encoding cyclin A1 (Bartusel et al., 2005). The proposed effect of B-Myb on the gene encoding cyclin B1 might also be relevant to later effects of B-Myb on mitotic arrest (Shepard et al., 2005). The possible evolutionary and functional homology between B-Myb and *Drosophila* Dm-Myb suggests that B-Myb could be part of a multiprotein complex that determines origin of replication use and/or the activity of genes linked to DNA replication. Hence, Dm-Myb has been shown to be part of a complex that operates at the replication origins of chorion gene amplification in follicle cells (Beall et al., 2002; Beall et al., 2004), whereas Lewis et al. have extended these observations and suggested that the Dm-Myb complex can act as an origin selector or as an activator or repressor of gene expression depending on the inclusion of different coactivators or corepressors (Lewis et al., 2004). The chromosome instability that we observe following B-*myb* knockdown suggests several possible mechanistic defects, including effects on cell-cycle checkpoints but also involving the DNA damage response or the maintenance of telomere integrity (Gollin, 2003). Interestingly in this context, the recent study by Ahlbory et al. (Ahlbory et al., 2005) points to a role for B-Myb in the DNA damage response.

The inherent tight cell-cycle-phase-related regulation of B-Myb activity by a variety of post-transcriptional mechanisms has made it experimentally difficult to manipulate B-Myb levels. It is hoped that conditional deletion of the B-*myb* gene, currently under development in our laboratory (García et al., 2005), will enable a more detailed analysis of the function of B-Myb at specific stages of the cell cycle that leads to maintenance of normal S-phase progression and chromosomal integrity through normal and endoreplicative S phase.

Materials and Methods

Cell culture

HEL and CMK megakaryocytic and U937 monocytic cell lines were cultured in RPMI 1640 medium (Gibco) supplemented with 10% (vol/vol) foetal calf serum (Bioclear), 2 mM L-glutamine (Gibco) and penicillin/streptomycin (Gibco). Cells were maintained at 37°C in 5% CO_2 in a humidified incubator. To induce differentiation, $1.5\text{-}2.0\times10^5$ cells per ml were cultured in the presence of 10^{-8} M 12-O-tetradecanoylphorbol 13-acetate (TPA) (Sigma).

Foetal liver culture

Foetal livers from E15 embryos were disrupted in PBS. Mature red blood cells were lysed by incubation in ACK lysis buffer at room temperature for 10 minutes. Cells

were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% foetal calf serum, 2 mM L-glutamine, penicillin/streptomycin and 1% of culture supernatant from a murine thrombopoietin (TPO)-producing cell line. After 4 days, immunomagnetic depletion was performed using antibodies against the lineage markers ERMP-20, Mac-1, Gr-1, Ter119, CD71, B220 and FcγRII/III. The remaining cells were fractionated on a discontinuous bovine serum albumin (BSA) gradient (0%/1.5%/3% BSA in PBS). The upper fraction (Fraction 1) contained predominantly (92%) diploid cells, whereas fully mature polyploid megakaryocytes were mainly in the lower fraction (Fraction 4). At least 85% of Fraction 4 was polyploid, and all cells were acetylcholinesterase positive.

Acetylcholinesterase assay

Cells were cytospun onto glass slides and incubated for 2 hours in a solution of 100 mM sodium phosphate buffer (pH 6) containing 0.66 mg/ml acetylthiocholine iodide, 5 mM sodium citrate, 3 mM copper sulphate and 0.5 mM potassium ferricyanide, then washed with sodium phosphate buffer, fixed with 95% ethanol for 5 minutes and counter stained for 20 seconds with Harris' Haematoxylin. Pictures were taken using an Olympus microscope at 20× magnification.

Semi-quantitative RT-PCR determination of mRNA levels

RT-PCR was conducted as described previously (Moore et al., 1993). Total RNA was isolated using TRIzol (BioRad) and contaminating genomic DNA removed by treatment with RNAse-free DNAseI (Pharmacia). Single-stranded cDNA was prepared using MoMuLV reverse transcriptase (Life Technologies) with first-strand synthesis primed using oligo(dT). HPRT was used as a control to standardise samples. For a given PCR reaction, samples were removed at intervals of three cycles to enable comparison of the linear phases of amplification. PCR products were analysed by agarose gel electrophoresis. The PCR primer pairs used were: human B-myb: 5′-ATGAGCTGCACTACCAGG-3′ (forward), 5′-TTCAGGTGCT-TGGCAATCA-3′ (reverse); human HPRT: 5′-AGTGATGATGAACCAGGT-3′ (forward), 5′-GGCTTTGTATTTTGCTTTTC-3′ (reverse); human gpIIb: 5′-TGG-TGCAAGATTCACTGAAT-3′ (forward), 5′-CGCCAGCTCTGCTTCATA-3′ (reverse); human gpIIIa: 5′-AGATGCGAAAGCTCACCA-3′ (forward), 5′-TGAG-CTCACTATAGTTCTG-3′ (reverse); human gpVI: 5′-ATGTCTCCATCCCCG-ACC-3′ (forward), 5′-AAGACTTTGTTTGTGAATGAG-3′ (reverse).

Flow cytometric analysis of DNA content

DNA content was determined by staining with 50 μg/ml propidium iodide (Sigma) as previously described (García and Calés, 1996). Cell-cycle analysis was performed with a FACScalibur analyser and CellQuest software (Becton Dickinson).

Immunofluorescent detection of B-Myb

For the simultaneous analysis of cell cycle and B-Myb, cells were treated as previously described (García et al., 2000). Cells were washed in PBS and fixed in suspension in 10 ml cold 75% ethanol. After 30 minutes at 4°C, the fixed cells were washed once in PBS, 0.1% NaN_3, 5% FCS (wash buffer) and then resuspended in 5 ml cold 0.25% Triton X-100 in wash buffer and incubated at 4°C for 5 minutes. Permeabilised cells were pelleted after addition of 10 ml wash buffer. Cells in wash buffer were then incubated with anti-human B-Myb rabbit polyclonal IgG antibody (Santa Cruz), or normal rabbit IgG control. In controls involving the use of the blocking peptide, anti-B-Myb antibody was incubated with a fivefold excess of the peptide at room temperature for 1 hour prior to addition to the cells. B-Myb was detected by incubating for 30 minutes with goat anti-rabbit IgG-FITC (Sigma) in wash buffer. Labelled cells were washed again and resuspended in 5 μg/ml propidium iodide in PBS. The analysis was performed using a FACScan analyser using CellQuest Software (Becton Dickinson).

BrdU labelling

Pulse labelling for flow cytometric analysis of DNA synthesis was performed by culturing cells in the presence of 20 μM 5-bromo-2-deoxyuridine (BrdU) (Sigma). Cells were harvested, fixed in 75% ethanol for 15 minutes at room temperature and incubated for 15 minutes in 1N HCl, 0.5% Tween-20 at room temperature. After washing with PBS/0.1% BSA, cells were incubated for 30 minutes with 100 μg/ml of RNAseA at 37°C and then for 30 minutes with FITC-conjugated mouse anti-BrdU antibody (Pharmingen) or FITC-conjugated mouse IgG1 monoclonal isotype control (Pharmingen). After washing with PBS/0.1% BSA, cells were resuspended in 5 μg/ml propidium iodide in PBS for flow cytometric analysis.

For microscopic examination of DNA synthesis, cells were cultured in the presence of 20 μM BrdU (Sigma) for 45 minutes, cytospun onto glass cover slips and fixed in ethanol/acetic acid for 20 minutes and then incubated with 1N HCl at 60°C for 30 minutes. After several washes in water, cells were incubated with mouse anti-BrdU antibody (Dako) for 1 hour at 37°C followed by secondary anti-mouse-Ig-phycoerythrin (PE) (Sigma). Cells were mounted in medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories).

Immunofluorescent detection of histone H3 phosphorylation

For microscopic examination of histone H3 phosphorylation, cells were cytospun onto glass cover slips and fixed with 1% paraformaldehyde for 5 minutes. After permeabilising with 0.5% Triton X-100 for 2 minutes, cells were incubated for 1 hour with anti-phospho-histone 3 (H3) (Abcam) diluted 1:50. After several washes in PBS, cells were incubated for 1 hour with secondary anti-rabbit-Ig-TRITC (Dako). Cells were mounted in medium containing DAPI (Vector Laboratories).

Chromosome spreads

Cells were collected, resuspended in 75 mM KCl, and maintained at room temperature for 10 minutes. Cells were then cytospun and immersed immediately in KCM buffer (120 mM KCl, 20 mM NaCl, 10 mM Tris-HCl pH 8, 0.5 mM EDTA, 0.1% v/v Triton X-100) for 5 minutes. Cells were then fixed in 4% paraformaldehyde in KCM buffer for 10 minutes at room temperature, washed in distilled water and mounted in medium containing DAPI (Vector Laboratories).

Immunofluorescent detection of centromeres

Cells were collected, resuspended in 75 mM KCl, and maintained at room temperature for 10 minutes. Cells were then cytospun and immersed immediately in KCM buffer (120 mM KCl, 20 mM NaCl, 10 mM Tris-HCl pH 8, 0.5 mM EDTA, 0.1% v/v Triton X-100) for 5 minutes. Cell were then incubated for 45 minutes on ice with human anti-mouse CENPa antibody (Abcam) diluted 1:100 in KCM buffer containing 1% BSA. After two washes in KCM buffer, cells were incubated for 45 minutes on ice with biotinylated anti-mouse antibody (Vector Laboratories) in KCM buffer with 1% BSA followed by incubation for 45 minutes on ice with streptavidin-PE in KCM/1% BSA (eBioscience). Cells were then fixed in 4% paraformaldehyde in KCM buffer for 10 minutes at room temperature, washed in distilled water and mounted in medium containing DAPI (Vector Laboratories).

Western blot analysis

50 μg of total protein extract (Lysis Buffer: 20 mM Tris-HCl, pH 7.4; 10 mM EDTA; 100 mM NaCl; 1% Triton X-100 containing protease and phosphatase inhibitors) was subjected to SDS-PAGE and proteins transferred to BioTrace PVDF membranes (Bio-Rad) for 2 hours at 15 V using a semi-dry transfer apparatus (Amersham). Ponceau Red staining was routinely performed on membranes to check sample loading and transfer efficiency. After blocking overnight in TBS containing 0.1% Tween 20 (T-TBS) and 10% BSA, filters were incubated at room temperature with the appropriate primary antibody diluted in T-TBS. Antibodies used were as follows: anti-human B-Myb mouse monoclonal at a 1:1000 dilution; anti-mouse actin-HRP monoclonal (Santa Cruz) at 1:400 dilution. After washing and incubation with an appropriate secondary antibody conjugated to horseradish peroxidase (Amersham), signals were detected using the enhanced chemiluminescence system (Pierce).

Plasmid construction and transfection

The human B-Myb expression vector B-Myb-pIRES-EGFP was generated by cloning a 2.6 kb *Hin*dIII fragment encoding the complete B-*myb* cDNA from pKC4-HuBmyb into the *Sma*I site of pIRES2EGFP (Clontech). 5×10^6 HEL cells were transfected by electroporation with 15 μg of B-Myb-pIRES-EGFP or control pIRES-EGFP using Cytomix (Van den Hoff et al., 1992) at 280 V, 950 μF in a 0.4 cm cuvette. After 18 hours, transfected cells were harvested and sorted on the basis of GFP expression using a Cytomation MoFlo FACS machine. GFP-positive and -negative fractions were collected with a purity of sorting in excess of 99%.

RNA interference

siRNA oligoribonucleotides were designed with a 5′ FITC modification and TT overhangs at the 3′ end. The sequence of the upper strands were as follows: siRNA control: 5′-CUUCAGUUCGCGUGACCAATT-3′; siRNA1: 5′-GAUCUGGAUG-AGCUGCACUTT-3′; siRNA2: 5′-GUUAAGAAGUAUGGCACAATT-3′. 5×10^5 HEL cells treated with TPA for 16 hours and transfected using jetSI cationic transfection reagent (Eurogentec) as described in the manufacturer's protocol with 50 nM siRNA on three consecutive days. After 80 hours of transfection, cells were harvested and efficiency was assayed by flow cytometry; in all the cases, this was more than 85%.

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