Temperature-dependent Modification and Activation of B-MYB

IMPLICATIONS FOR CELL SURVIVAL*

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B-MYB is a ubiquitous transcription factor with an essential role in mouse development. Because cells with a disrupted B-MYB gene cannot be obtained, it is still unknown what is the critical function(s) exerted by B-MYB in mammalian cells. In this study we have observed that reducing B-MYB expression in primary human fibroblasts by using RNA interference results in a partial block of the cells in the G2 phase of the cell cycle and cell death. Surprisingly, suppressing B-MYB transcriptional activity with a dominant-negative molecule is without effect, suggesting that its transactivating function is not essential. Only human or murine fibroblasts exposed to high temperature are sensitized to cell death in the presence of dominant-negative B-MYB. This correlates with temperature-dependent binding of endogenous B-MYB to transcriptional regulatory elements of the stress-related gene ApoJ/clusterin. We find that regulation of ApoJ/clusterin by B-MYB is a pro-survival response to thermal stress. Thus, B-MYB is regulated by temperature to activate genes required for cell survival.

B-MYB is a transcription factor that has been implicated in regulation of cell growth, differentiation, and transformation (1). It is homologous to the prototype member of the MYB family, the protooncogene c-MYB, which is prevalently expressed in hematopoietic cells where it controls their differentiation and proliferation (2). All MYB family members bind to the same consensus sequence (C/T)AACNG in in vitro assays, reflecting their high homology in the DNA-binding domain region (2). It is possible, however, that the different MYB proteins regulate different genes in vivo after post-translational modifications or interaction with specific cofactors (3, 4).

B-MYB can be considered the most intriguing member of the MYB family because it seems to exert a fundamental function in mammalian development. Firstly, unlike c-MYB and A-MYB, it is expressed ubiquitously. Secondly, B-MYB inactivation in mice causes an early block of development, without formation of the inner cell mass and normal blastocyst (5). This absolute requirement mirrors the high levels of B-MYB, but not of c-MYB and A-MYB, mRNA observed in mouse embryonal stem cells (5). B-MYB expression is detected in virtually all mammalian cancer cell lines, and its locus at 20q13 is often amplified or overexpressed in a wide variety of human neoplasias (6–9). In contrast, the B-MYB gene is not transcribed in differentiated adult mammalian tissues. Although B-MYB mRNA expression is found in highly proliferating human progenitor cells induced to differentiate along the erythroid or granulocytic/monocytic lineage in vitro (10), B-MYB protein expression is barely detectable in the spleen and totally absent in the liver, lungs, kidney, adrenal gland, and sympathetic ganglia of adult mice. Initial characterization in cancer cell lines or in immortalized fibroblasts suggested that B-MYB may be required for cell proliferation or survival. B-MYB expression in fibroblasts is growth-regulated and peaks at the G1/S transition phase of the cell cycle (11). Ectopic expression of B-MYB has been shown to increase the number of cells in the S phase (12). On the other hand, antisense inhibition of B-MYB results in suppression of clonogenesis (13–15). Whether or not this latter effect is due to a block of cell proliferation or enhanced cell death has not been investigated. Overexpression of B-MYB often promotes cell survival; it has been shown to counteract apoptosis of lymphoid cell lines in culture and to up-regulate the antiapoptotic BCL2 gene (16). Ectopic B-MYB expression confers protection to doxorubicin killing in neuroblastoma cells (17). In monocytic cells, B-MYB suppresses apoptosis and sustains proliferation in the presence of the terminal differentiating cytokine interleukin 6 (18). In one recent study, Liu and Greene have observed that MYB proteins are induced by DNA damage to promote neuronal cell death (19). However, in our laboratory we have found no evidence of c-MYB, A-MYB, or B-MYB activation in DNA-damaged neuronal cancer cell lines suggesting that more studies are needed to fully understand the role of MYB genes in neuronal demise (20).

MYB consensus sequences have been found in promoter or enhancer elements of a number of genes. B-MYB can directly or indirectly transactivate ApoJ/clusterin, MYC, B-MYB itself, HIV LTR, collagen, FGF, UCHL1, IGFBP5, BCL2, and a plethora of potential B-MYB, c-MYB, and A-MYB target genes have been identified by microarrays (4, 16, 17, 21–28). B-MYB could also induce cell proliferation and survival through protein-protein interactions. The retinoblastoma family member p107 is found associated with B-MYB in vitro and in vitro, and these proteins behave like mutual antagonists (10, 22, 29). More recently, B-MYB has been shown to bind to and suppress the cyclin-dependent kinase inhibitor p57kip2 (30). These studies unveil a high level of complexity of B-MYB functions and support a model in which B-MYB regulates cell homeostasis by modulating both gene transcription and the activity of other

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proteins. One outstanding question is whether B-MYB transcriptional activity is essential or dispensable for its positive effects in the cell cycle. Many of the putative B-MYB target genes have been shown to regulate cell growth and survival, and it is reasonable to assume that at least part of B-MYB biological function is dependent on its transcriptional activity. However, potential target genes were studied in largely non-physiological contexts, and binding of B-MYB to putative regulatory regions was almost exclusively studied in vitro. The absence of viable mouse embryonic cells with a disrupted B-MYB gene has hampered more rigorous studies of its functions in vivo.

In the study presented here we have used different approaches to investigate the role of B-MYB in normal mammalian fibroblasts. Our results highlight its unsuspected role in the stress response.

**MATERIALS AND METHODS**

**Cell Cultures, Transfections, and Fluorescence-activated Cell Sorter Analysis**—WI38 and NIH3T3 cells were purchased from ATCC (Manassas, VA) and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Transfections were carried out with Lipofectamine 2000 following the manufacturer’s instructions. Infections were carried out as described previously (31). In brief, Phoenix A/E cells were grown to 80% confluency and transfected with the MLV retroviral constructs by the Profection system following the manufacturer’s instructions (Promega). Retrovirus-containing supernatants were collected 48 h after transfections and used to infect cells. To obtain a homogenous population of transduced cells, GFP+positive cells were isolated by FACS sorting. FACS analysis was carried out by fixing the cells with 70% ethanol at 4 °C overnight. Cells were stained with 0.5 μg/ml propidium iodide in PBS containing 0.5% Nonidet P-40. Luciferase assays were carried out by transecting 500 ng of the reporter vector-pGL2-ApoJ/clusterin promoter or its relative with mutated MYB-binding site (17) with or without empty pCDNA3 vector or pCDNA3 vector containing the B-MYB-DNA-binding domain. For heat shock experiments, subconfluent cells in sealed flasks or plates were transferred into water baths at 42, 45, or 47 °C for 30 min and then dissolved in Laemmli buffer containing 10 mM DTT for electrophoresis in SDS-containing gels. For tryptic digests, nuclear extracts were prepared from NIH3T3 cells and digested with 1 μg of Lipofectamine 2000 using a ratio of 1 μg of Lipofectamine for 2 μg of siRNAs. In these conditions we achieved a 70–80% transfection efficiency.

**Protein Electrophoresis and Western Blotting**—Total cellular proteins were extracted in PBS containing 500 mM NaCl and 1% Triton X-100 and a tablet containing a mixture of protease inhibitors (Complete-mini, Roche Applied Science). After incubation in ice, cell lysates were cleared by centrifugation, diluted with 2 volumes of PBS, and incubated at different temperatures with or without DTT. Lysates were then dissolved in a buffer containing glycerol and 0.001% SDS for electrophoresis in native gels or dissolved in Laemmli containing 1 mM DTT for electrophoresis in SDS-containing gels. For tryptic digests, nuclear extracts were prepared from NIH3T3 cells transfected with a B-MYB expression vector. Extracts were incubated at 45 °C for 1 h or at room temperature with or without DTT, diluted with water, and digested with 1 μg of trypsin for 30 min. Samples were boiled prior to fractionation by SDS-polyacrylamide gel electrophoresis. After transfer, membranes were blocked with 5% dry nonfat milk and 0.5% Nonidet P-40 in PBS. Antibodies were incubated for 1 h in blocking buffer. We have generated and used a B-MYB monoclonal antibody that recognizes the carboxyl terminus of human and murine B-MYB. Actin and ApoJ/clusterin antibodies were purchased from Santa Cruz Biotechnology. In most cases the same nitrocellulose membrane was stripped and used with different antibodies. Parallel blots were also used to avoid excessive stripping and reprobing.

**Reverse Transcription (RT)-PCR**—For semiquantitative RT-PCR, preliminary experiments were carried out to determine the linear range of amplification. Total cellular RNA (1 μg) from MEFs or NIH3T3 cells was reverse-transcribed using a reverse transcription kit (Invitrogen) in a total volume of 25 μl. PCR was carried out using 5 μl of the reverse transcriptase mixture in 1× polymerase buffer, 0.4 μM upstream primer, 0.4 μM downstream primer, 0.2 mM dNTPs, and 2 units of Taq polymerase (Roche Applied Science) in a total volume of 50 μl.

PCR conditions for ApoJ/clusterin amplification were as follows: 45 s at 94 °C, 45 s at 58 °C, and 1 min at 72 °C for 28 cycles, followed by a final extension step of 7 min at 72 °C. The primers used were: ApoJ forward, 5′-ATGAAGATCTCTCGTGC-3′; ApoJ reverse, 5′-CTT-ACACCAACCTCTAGT-3′.

**Cell Shift**—An oligonucleotide containing the B-MYB-binding site of the murine ApoJ promoter was end-labeled with T4 kinase and 32P. After purification, the probe was annealed to an excess of the minus strand. Oligonucleotides used were: + strand, 5′-ATCCGGTTAGCAGTTATTTACAGAAA-3′; − strand, 5′-TTCTGTAAATAACTGCTC-ACCGCAT-3′.

For the binding reaction, 100 ng of glutathione S-transferase protein (GSTM1) of 1 μg/ml poly(dI-dC), and 1 μg of cold competitor were mixed with 10 × 105 cpm of the probe in 1× binding buffer at RT. The samples were loaded onto a non-denaturing 5% acrylamide mini gel (Bio-Rad), run at 100 V, dried, and exposed to an x-ray film.

**Chromatin Immunoprecipitation (CHIP) Assays**—CHIP assays were carried out as described with minor modifications (32). In brief, fibroblasts were cultured in 10-cm2 dishes for 48 h in a humidified incubator. After heat shock following the manufacturer’s instructions (Promega), chromatin was isolated by digestion with micrococcal nuclease for 15 min at RT. After the reaction was stopped with glycine, cell pellets were resuspended in 550 μl of radiolabeled precipitation assay buffer, syringed up and down, and left on ice for 20 min. Chromatin was sheared by 6 cycles of sonication, 2 min each, at 50% power, on ice. After pre-clearing with 40 μl of protein A-Sepharose, 1 μg of antibodies (B-MYB (N-19) or an IgG-matched TRAF antibody, both from Santa Cruz Biotechnology) was added and incubated 2 h to overnight at 4 °C. After washes, beads were eluted with 1% SDS, 0.1 mM NaHCO3. Protein-DNA complexes were de-cross-linked at 65 °C for 5 h and ethanol-precipitated overnight. Pellets were resuspended in TE, digested with proteinase K, and phenol-extracted. Digested samples were ethanol-precipitated in the presence of glycogen, and DNA was resuspended in 30 μl of water. 5 μl of each sample was amplified with ApoJ/clusterin promoter-specific primers encompassing the B-MYB-binding site.

**RESULTS**

**siRNA Inhibition of B-MYB Expression in Normal Human Fibroblasts Causes Cell Death and a Partial Block in the G2 Phase of the Cell Cycle**—We previously showed that antisense inhibition of B-MYB expression causes suppression of the clonogenic potential of BALB/c 3T3 fibroblasts (13). In that study we did not establish whether the decrease of colony formation was due to a defect in cell proliferation or survival. RNA interference is thought to be more effective than antisense methods and has been now widely applied to knockdown gene expression. We decided to study the effect of suppressing B-MYB expression with siRNAs in human embryonal fibroblasts. WI38 cells are normal human fibroblasts with a finite lifespan. B-MYB expression in this cell line is low compared with cancer cells or with immortalized murine fibroblasts such as NIH or BALB/c 3T3 cells (data not shown). Therefore, WI38 cells are potentially useful for RNA interference experiments aiming to assess the biological consequences of inhibiting B-MYB expression.

Cells were exposed to a scrambled control or two independent siRNA molecules targeting different regions of the B-MYB mRNA. Both sequences caused down-regulation of B-MYB protein, although sequence 1 (seq 1) was slightly more efficient than sequence 2 (seq 2) (Fig. 1). Ablation of B-MYB expression caused a marked increase in sub-G1 DNA, suggesting that targeted cells may undergo apoptosis (Fig. 1). Observation under the microscope confirmed that cells were rounding-up and
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FIG. 1. B-MYB is required for cell cycle progression and survival of human fibroblasts. WI38 cells were transfected in duplicate wells with control (scr) or B-MYB (seq 1 and seq 2) synthetic siRNAs. Cells were harvested 48 h later and subjected to Western blot analysis with the indicated antibodies (upper left panel) and FACS assay (right panel). Quantification and statistical analysis (S.D.) of FACS results are shown in the center of the figure.

detaching from the plate (data not shown). Notably, cell death was accomplished at the expense of G1, suggesting that a pool of cells did not re-enter the cell cycle after G2 and mitosis. In agreement with this notion, more cells were arrested in the G2/M phase in the presence of sequence 1 (Fig. 1). These phenotypic effects were proportional to the degree of B-MYB down-regulation achieved with the different siRNAs. The interferon response gene OAS1 or the growth suppressor gene p53 was not induced by siRNA treatment, suggesting that off-target effects were unlikely (data not shown).

A Transcriptional Dominant-negative B-MYB Construct Is without Effect in Unstressed Fibroblasts but Promotes Cell Death in Cells Subjected to Heat Shock—The RNAi experiments have clearly established that B-MYB is required for the progression and survival of human fibroblasts during the cell cycle. We hypothesized that B-MYB could transcriptionally regulate the expression of critical target genes. If this were the case, inhibiting its transcriptional activity should result in a phenotype similar to that obtained with RNAi experiments. The B-MYB DNA-binding domain can behave as a transcriptional dominant-negative molecule to inhibit MYB-dependent gene expression (Fig. 2). We have infected human WI38 or murine NIH3T3 fibroblasts with a bicistronic retroviral vector (MIG-R1) containing GFP and the B-MYB DNA-binding domain (MIG-DBD). Surprisingly, fibroblasts infected with the MIG-DN-BMYB construct proliferated normally (Table 1). We hypothesized that subjecting fibroblasts to stress could disclose the prosurvival function of B-MYB transcriptional activity. Although control or dominant-negative expressing cells were viable at physiological temperature, they were more prone to die at high temperature, with WI38 cells being especially sensitive to temperature stress (Fig. 3, A and B). The expression of the dominant-negative protein was confirmed by Western analysis (Fig. 3C). These experiments suggest that

B-MYB transcriptional activity is dispensable for proliferation but required for stress survival.

B-MYB Transactivating Activity Is Enhanced by Thermal Stress and Is Essential for Temperature-dependent Transcription of the B-MYB-Target Gene ApoJ/clusterin in Mammalian Fibroblasts—The B-MYB requirement in heat-shocked cells suggests that it could be activated by thermal stress. We exposed fibroblasts to heat shock and observed B-MYB protein by Western blot. B-MYB protein levels did not consistently change with temperature in WI38 or NIH3T3 cells (data not shown). We observed, however, in several independent assays that the molecular weight of B-MYB increased slightly after 30 min of heat shock in NIH 3T3 cells (Fig. 4). This preceded induction of the stress-activated protein ApoJ/clusterin that occurred 2 h after heat shock (Fig. 4). Expression of the related members c-MYB and A-MYB was undetectable in fibroblasts (not shown). To monitor transcriptional activity of endogenous B-MYB in different conditions, we transfected WI38 fibroblasts with the human ApoJ/clusterin promoter, linked to the luciferase gene, with or without a mutation in the B-MYB-binding site. We have previously shown that exogenous B-MYB can efficiently transactivate only the ApoJ/clusterin promoter with an intact B-MYB-binding site. In fact, mutation of the B-MYB-binding site causes a 70% reduction of promoter transactivation (17). Interestingly, the ApoJ/clusterin promoter is activated by high temperatures, and mutation of the B-MYB-binding site caused a significant reduction of promoter activation (Fig. 5A). To prove that B-MYB, and not another MYB family member, is involved in thermal regulation of the ApoJ/clusterin promoter, we have monitored its activity in the presence of the siRNA sequence 1. Heat-shock activation of the ApoJ/clusterin promoter is substantially abolished in the presence of B-MYB, but not control, siRNA, suggesting that thermal regulation of the ApoJ/clusterin promoter is, at least in
part, dependent on the presence of endogenous B-MYB (Fig. 5). To further confirm these results, NIH3T3 cells were infected with the dominant-negative B-MYB construct or its corresponding empty vector. Cells were FACS-sorted, GFP-positive cells were exposed to high temperature where indicated (HS), and the percentage of cell death was scored 24 h later by trypan blue dye exclusion assay. Error bars indicate the S.D. from the counts of four independent wells. These assays were repeated twice with similar results. C, NIH3T3 cells infected with the MIGR1 or the dominant-negative B-MYB vector (MIG-DN) were subjected to Western blot analysis with a B-MYB antibody. Expression of the truncated B-MYB protein was observed in MIG-DN-infected cells only.

Modification of the B-MYB Protein by Temperature—We have observed that in NIH3T3 cells B-MYB protein is modified as soon as 30 min after heat shock (Fig. 4). Many research groups, including our own, have shown that B-MYB can be phosphorylated and thereby activated by the cyclin-dependent kinase cdk2 (22, 33–35). A reasonable explanation for our observations was that B-MYB could be phosphorylated and activated during heat shock. Nevertheless, using different approaches we could not find increased phosphorylation, or acetylation, of B-MYB protein after heat shock. We next investigated whether B-MYB could undergo a temperature-dependent redox modification. B-MYB contains several cystins that have been shown to be sensitive to redox conditions (36). Interestingly, redox modification induced by heat causes a change in electrophoretic mobility of heat shock factor 1 (HSF1) (37). To assess whether B-MYB could be modified by heat, whole NIH3T3 cell extracts were incubated at different temperatures. Samples were then run onto native or denaturing SDS-PAGE gels under reducing or non-reducing conditions. When subjected to electrophoresis in native conditions only non-reduced B-MYB can enter the gel and be detected by the antibody. In fact, we have observed that preincubation of cell lysates with 10 mM DTT completely blocks B-MYB electrophoretic mobility. This is probably due to the formation of a high molecular weight complex that is blocked in the loading well and does not enter the gel (Fig. 7A, upper panel). Loss of epitope reactivity or protein degradation is ruled out because DTT treatment does not prevent detection of B-MYB in denaturing conditions (Fig. 7A, lower panel). Preincubation of cell lysates at 45 °C inhibits B-MYB electrophoretic mobility in a manner similar to that of DTT (Fig. 7A, upper panel). Again, this is not due to loss of protein because B-MYB is detectable in SDS-PAGE gels (Fig. 7A, lower panel). To further investigate whether heat shock can induce a conformational change of B-MYB protein, we carried out tryptic digestion of nuclear extracts after heat shock or DTT treatment. The digestion
FIG. 5. Suppression of B-MYB causes reduction of ApoJ/clusterin promoter response to thermal injury. A, WI38 cells were transfected with the ApoJ/clusterin reporter vector in its wild type or mutant conformation and 12 h later were exposed to heat shock. Numbers in the y axis indicate the ratio between luciferase activity from the reporter vector and Renilla luciferase activity from the normalization plasmid. Error bars indicate S.D. B, WI38 cells were transfected with the ApoJ/clusterin reporter vector in the presence of control or B-MYB siRNAs. 12 h later, cells were exposed to heat shock. Luciferase activity was quantified as described in A. C, NIH3T3 cells were transfected with the ApoJ/clusterin reporter or a MYC-derived promoter containing T cell factor (TCF)-binding sites with or without CMV-driven dominant-negative B-MYB (DN). Cells were exposed to heat shock, and luciferase activity was quantified as described above. All experimental points were performed in triplicate, and transfections were repeated at least three times.

FIG. 6. Dominant-negative B-MYB suppresses heat-regulated transcription of the ApoJ/clusterin gene. NIH3T3 fibroblasts were infected with the MIG-DN-BMYB (Δ) or empty (−) retroviral vectors. Homogeneous populations of transduced cells were obtained by FACS sorting the GFP-positive cells. Transduced cells were exposed to heat shock and collected at the indicated times. After reverse transcription-PCR amplification of the ApoJ/clusterin or actin cDNAs was carried out for the indicated cycles. This experiment was repeated three times with similar results.

FIG. 7. B-MYB electrophoretic mobility in native gels is modified, and its digestion profile is altered in response to increased temperature and in the presence of the reducing agent DTT. A, NIH3T3 cell lysates were exposed to the indicated temperatures and run in native conditions with or without preincubation with DTT (top panel) or in reducing, denaturing conditions (bottom panel). After transfer to nitrocellulose, filters were incubated with a B-MYB antibody. B, NIH3T3 nuclear extracts were exposed to DTT or heat shock and digested with trypsin before being loaded onto a 15% SDS gel. B-MYB full-length and digestion products were visualized by Western analysis with a B-MYB antibody. C indicates control, untreated extracts. Arrow indicates full-length B-MYB protein. These assays were repeated twice with similar results.

profile of B-MYB was clearly altered after heat shock treatment and to a lesser extent by DTT (Fig. 7B). Overall these results suggest that the B-MYB structure is changed by temperature in a manner, at least partially, compatible with a redox modification. However, other events could also be involved in this phenomenon.

Endogenous B-MYB Binds to the Stress-related Genes ApoJ/clusterin in a Temperature-dependent Fashion—We investigated whether the mouse ApoJ/clusterin promoter contains functional B-MYB-binding sites. Among others, a potential MYB-binding site is located in a position similar to that of the human sequence. Gel shift analysis confirmed that B-MYB can bind to this sequence (Fig. 8A). A cold excess of the MYB-binding site from the MIM-1 promoter competed the probe, suggesting that the interaction is MYB-specific (Fig. 8A). More-
Fig. 9. CHIP assays reveal temperature-dependent binding of B-MYB onto the human ApoJ/clusterin promoter. WI38 cells were exposed to heat shock and fixed for CHIP assays at different times. Lane 1, cells cultured at normal temperature immunoprecipitated with control antibody; lane 2, cells collected 30 min after heat shock, control antibody; lane 4, cells at normal temperature, B-MYB antibody; lane 5, cells collected 30 min after heat shock, B-MYB antibody; lane 6, cells collected 2 h after heat shock, B-MYB antibody; lane 7, chromatin input from control cells; lane 8, chromatin input from cells collected 30 min after heat shock; lane 9, chromatin input from cells collected 2 h after heat shock. This assay was repeated twice with the same result.

over, expression of ectopic B-MYB can induce transcription of endogenous ApoJ/clusterin in mouse embryo fibroblasts (Fig. 8B), suggesting that B-MYB regulation of ApoJ/clusterin is conserved between species. We speculated that the heat-induced modification could lead to the formation of a protein complex competent to bind to target sequences. We monitored the occupancy of the human promoter, before or after heat shock, by CHIP assays. Binding of endogenous B-MYB to the ApoJ/clusterin promoter is difficult to detect in normal conditions (Fig. 9, compare lanes 1 and 4). Exposure of cells to heat shock causes a remarkable increase of chromatin immunoprecipitated with the B-MYB, but not control, antibody (Fig. 9, compare lanes 2 and 3 and lanes 5 and 6). This increase is even more remarkable if one takes into consideration that substantially less chromatin input is obtained after 2 h of heat shock compared with normal conditions (Fig. 9, compare lanes 7 and 9).

ApoJ/clusterin Protects Primary Fibroblasts from Thermal Injury—The experiments described above depict a model in which B-MYB may be switched on by rising temperature to drive expression of ApoJ/clusterin. Evolutionary conservation of B-MYB regulation suggests that ApoJ/clusterin might exert an important role in thermal injury. ApoJ/clusterin is inducible by heat (Fig. 4), but whether it is required for the cell to survive or to die is still undetermined. To elucidate its role in thermal injury we exposed ApoJ/clusterin knock-out MEFs, or their wild type counterparts, to a temperature of 47 °C for 30 min. We then returned the cells to normal temperature, and 24 h later we assessed cell viability by propidium iodide staining and FACS analysis. Heat shock caused some cells to detach from the substrate, suggesting that it had provoked some degree of cell death. Quantification of sub-G₁ DNA by FACS analysis showed that cell death was significantly higher in cells with a disrupted ApoJ/clusterin gene (Table II). Thus, induction of ApoJ/clusterin expression after thermal injury is a protective response mechanism in mammalian fibroblasts.

**DISCUSSION**

Although the key role of B-MYB in mammalian development has been clearly established, there is a remarkable gap in our knowledge of the biological processes regulated by it. Perhaps many approaches that have been used in the past were not adequate because they were based mainly on analyses of the phenotypic effects of ectopic B-MYB overexpression in cell lines. Experiments with antisense constructs have not been particularly informative because it was impossible to obtain a cell line stably expressing B-MYB antisense transcripts. Antisense oligonucleotides have been used with some success in hematopoietic cells (38), but they are not very effective in fibroblasts or other adherent cell lines.³ To make the matter worse, cells or mice with homologous deletion of the B-MYB gene cannot be obtained (5). Conditional gene knock-out would be required to rigorously assess the role of B-MYB in vivo. However, the recent advent of RNAi technology has made inactivation of gene expression more affordable and easy.

In this study, we used siRNAs to investigate the effect of B-MYB ablation in normal human fibroblasts. To further characterize B-MYB function in mammalian fibroblasts, we generated a dominant-negative B-MYB molecule that interferes with its transcriptional activity. Differently from another widely used dominant-negative construct, c-MYB-engrailed (39), it is not fused to a transcriptional repressor. Our construct should inhibit MYB-driven, but not basal, gene expression. B-MYB is detectable in WI38 human fibroblasts, and its expression is low compared with human cancer cell lines or immortalized murine fibroblasts. Thus, we thought that WI38 cells were particularly suitable for targeting experiments. We synthesized two different siRNA molecules that suppressed B-MYB protein expression with different efficacy. Significantly, spontaneous cell death and accumulation of cells in the G₂ phase of the cell cycle was proportional to the reduced B-MYB levels. B-MYB has been shown to increase during the G₁/S transition of the fibroblast cell cycle and to be phosphorylated throughout S phase (40). It is possible that accumulation of phosphorylated B-MYB renders cells competent to accomplish the G₂ phase. This hypothesis is consistent with studies in flies where it has been shown that Drosophila-MYB (dMYB), which is considered the equivalent of mammalian B-MYB, is required for the G₂/M transition of eye imaginal disc cells (41). Another interesting parallel between our results and those obtained in Drosophila is that abnormal cell cycle activity caused by suppression of dMYB is accompanied by apoptosis in the eye disc (41). Thus, the accumulation of sub-G₁ DNA observed in WI38 cells in the absence of B-MYB could be caused by apoptosis following de-regulated cell cycle activity.

Having shed some light on the role of B-MYB in the biology of fibroblasts, we next asked whether its transcriptional activity was involved. To our surprise, WI38 and NIH3T3 cells grew well when infected with a retroviral vector expressing high levels of dominant-negative B-MYB. This demonstrates that transactivation of B-MYB target genes is not required in normal conditions. Indeed, Watson and co-workers (29) have shown that B-MYB rescues the G₁ block imposed by the retinoblastoma family member p107 independently from its transcriptional activity. Even though WI38 or NIH3T3 cells expressing dominant-negative B-MYB grew similarly to control cells, they became sensitized to thermal stress. In the light of these results, one could hypothesize that transactivation of B-MYB target genes is only required under specific circum-

³ A. Sala, unpublished observations.
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In conclusion, we have investigated the role of B-MYB in mammalian fibroblasts. We have shown that B-MYB can be modified by thermal stress and recruited onto the stress-activated gene ApoI/clusterin to direct cell survival. Other stress-regulated genes are likely to be controlled by B-MYB. We have observed that B-MYB can bind in a temperature-dependent manner to the first intron of the ATF3 gene, although its role in thermal injury is still to be investigated.4 Global analysis of gene expression in cells subjected to different types of damage will be implemented to fully understand the role of B-MYB in the stress response.

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