MOLECULAR AND CELLULAR BIOLOGY, Feb. 1988, p. 884–892
0270-7306/88/020884-09$02.00/0

Vol. 8, No. 2

Constitutive Expression of a c-*myb* cDNA Blocks Friend Murine Erythroleukemia Cell Differentiation

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Received 20 July 1987/Accepted 13 November 1987

A full-length human c-*myb* cDNA clone has been isolated from a CCRF-CEM leukemia cell cDNA library. The plasmid vector contains simian virus 40-derived promotor, splice, and polyadenylation sequences as well as a transcription unit for a dihydrofolate reductase cDNA. We have introduced this construct into Friend erythroleukemia (F-MEL) cells and have isolated a number of clones which contain intact and transcriptionally active human c-*myb* sequences. F-MEL clones expressing the highest levels of the human c-*myb* mRNA differentiate poorly in response to dimethyl sulfoxide. Two clones which initially expressed low levels of human c-*myb* transcripts and which differentiated normally were subsequently inhibited in their ability to differentiate when grown in successively higher concentrations of methotrexate, due to amplification and enhanced expression of plasmid sequences. The inhibitory effect on F-MEL differentiation appeared to be independent of the early decline in c-*myc* transcripts which were normally regulated in all cases examined. Our results indicate that constitutive expression of a nontruncated human c-*myb* cDNA can exert profound effects on erythroid differentiation and argue for a causal role of c-*myb* in the F-MEL differentiation process.

v-*myb* is the transforming oncogene of avian myeloblastosis virus and E26 leukemia virus, both of which cause hematopoietic malignancies in chickens (3, 4, 24, 25, 36, 42). A conserved cellular protooncogene sequence, c-*myb*, is present in the genomes of all metazoan species thus far examined (2, 11, 18). The v-*myb* oncogene is truncated relative to c-*myb*, and certain animal leukemias appear to involve retroviral insertion into and truncation of the c-*myb* locus (19, 35, 37–39). These data have led to the suggestion that the loss of either c-*myb* N- or C-terminal sequences unmasks a cryptic transforming potential of the cellular gene (2, 35). Other data, however, suggest that the unrearranged c-*myb* gene might participate in leukemogenesis and hematopoietic differentiation. For example, c-*myb* mRNA is expressed at increased levels in thymocytes and leukemias of immature, but not mature, phenotypes and has recently been shown to be cell cycle regulated in cells of nonthymic origin (44). One allele of the gene is deleted in many tumors, whereas c-*myb* gene amplification has been shown in some leukemia cell lines and fresh tissues (30, 41, 44–46). While we and others have shown that c-*myb* transcript levels decrease during the terminal differentiation of leukemic cell lines (13, 33, 45), the actual significance of this has not been established.

To examine directly the role of c-*myb* on cell differentiation, we have studied the Friend murine erythroleukemia (F-MEL) cell line which may be induced to terminally differentiate with a number of chemical agents such as dimethyl sulfoxide (DMSO) (23). During this process, both c-*myb* and c-*myc* transcript levels decrease drastically (21, 33; E. V. Prochownik and M. F. Clarke, unpublished data). The system thus lends itself to an analysis of the role of these cellular protooncogenes by perturbing their regulation during the differentiation process. We and others have recently shown that the constitutive expression of transfected c-*myc* sequences into F-MEL cells inhibits their chemically induced differentiation whereas the expression of c-*myc* antisense transcripts accelerates this process (7, 8, 20, 31; E. V. Prochownik and J. F. Kukowska, submitted for publication). We have now studied the effect of constitutively expressed human c-*myb* sequences. We have molecularly cloned a 3.4-kilobase (kb) human c-*myb* cDNA which corresponds to the 3.4-kb transcript present in hematopoietic tissues (45). This cDNA has been positioned downstream of a simian virus 40 (SV40) promotor, which allows for its constitutive expression and thus for a molecular definition of the function of a nontruncated c-*myb* transcript in F-MEL differentiation. F-MEL transfectants have been obtained which express high levels of human c-*myb* transcripts that are unaffected by DMSO addition. This results in an inhibition of F-MEL differentiation in a manner directly correlated with the levels of exogenous c-*myb* transcripts. Our results are consistent with the notion that the decline in endogenous c-*myb* transcripts is causally related to F-MEL differentiation and that the continuous unregulated expression of c-*myb* may play a role in the development of certain leukemias.

MATERIALS AND METHODS

cDNA cloning and construction of pMbm1-dhfr. A cDNA library was made from mRNA isolated from the human acute lymphocyte leukemia cell line CCRF-CEM, using a modification of the method of Okayama and Berg (27, 28). The first strand was synthesized by using dT-tailed (30 to 50 T tails) pCDV1 in a reaction mixture containing 50 mM Tris (pH 8.3), 50 mM NaCl, 8 mM $MgCl_2$, 1.1 mM dithiothreitol, 1 mM spermidine, 40 μg of actinomycin D per ml, 2.2 μg of dT-tailed pcDV1, 4 μg of poly(A)-selected RNA, 10 U of placental RNase inhibitor, and 20 U of avian myeloblastosis virus reverse transcriptase. Second-strand synthesis and plasmid circularization were done as described before (5), except RNase H was added only for the last 0.5 h of the reaction. The repaired plasmid was transfected into *E. coli*

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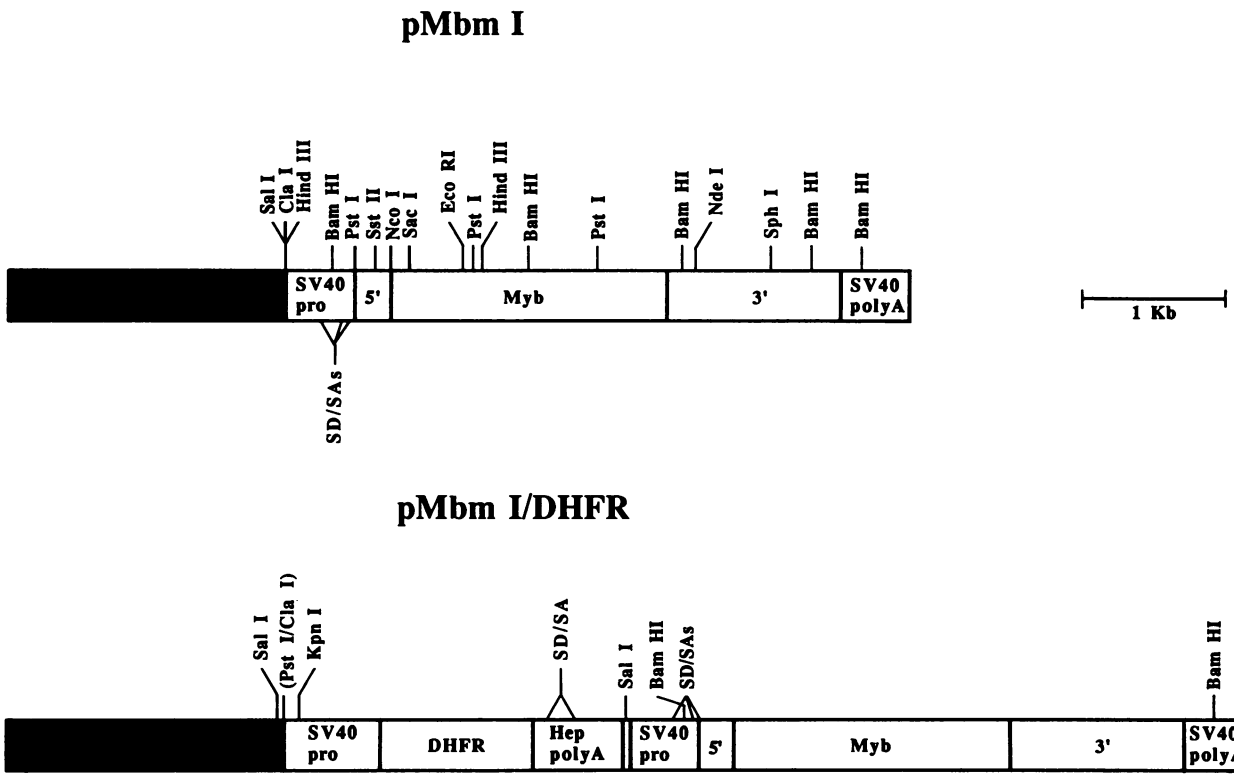

FIG. 1. c-*myb* clone pMbm1 and the pMbm1-dhfr vector are shown schematically. Plasmid pMbm1-dhfr was linearized at the *Kpn*I site 5′ to the dhfr SV40 promotor for electroporation. Relevant restriction endonuclease sites are shown as well as splice donors and accepters in the vectors (SD/SA). Solid bars represent pBR322 sequences. The c-*myb* 5′- and 3′-untranslated region and coding sequence are shown (open bar). SV-40 promotors (SV40 pro), SV40 polyadenylation (SV40 polyA), and hepatitis B polyadenylation (Hep polyA) signals are shown. Both 5′ and 3′ SV40-*myb* borders contain *Xho*I and *Bam*HI restriction sites. The entire c-*myb* cDNA can therefore be excised with *Xho*I as a single 3.4-kb fragment. Digestion with *Bam*HI produces *myb*-specific fragments of 1.4, 1.2, 0.7, and 0.2 kb. For S1 nuclease protection studies, we used an end-labeled 1.5-kb *Hin*dIII restriction fragment derived from the 5′ end of the SV40-*myb* transcription unit.

DH1, using the method of Hanahan (17). A total of 2.5×10^6 recombinants were screened, and 50 individual cDNAs were isolated with a nonreiterated human genomic c-*myb* probe (11). Several clones contained inserts of 3.4 kb, and one of these (pMbm1) was sequenced at its 5′ end by the dideoxy technique. A 2.8-kb dihydrofolate reductase (dhfr) transcription unit from plasmid pFR400 (40) was excised with *Pst*I and *Sal*I, blunt ended with T4 DNA polymerase, and ligated into the blunt-ended *Cla*I site upstream of the pMbm1 SV40 promotor (Fig. 1). This plasmid was designated pMbm1-dhfr.

Cell culture and DNA transfections. F-MEL cells (clone 745) were grown in Dulbecco modified minimal essential medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. DNA transfections were performed by electroporation as previously described (31). Transfection mixes consisted of 2×10^7 F-MEL cells in phosphate-buffered saline, 40 μg of *Kpn*I-linearized pMbm1-dhfr DNA, and 4 μg of *Pvu*I-linearized pHNEO (a gift of Chris Simonsen). The latter is a pSV2neo derivative in which several out-of-phase ATG initiation codons in the neo-5′-untranslated region have been deleted. Transfected clones were grown sequentially in 1 mg of geneticin (G418; GIBCO Laboratories) per ml and 0.25 μM methotrexate (MTX; Lederle Laboratories). Single-cell clones were obtained by limited dilution in 96-well microtiter plates.

Benzidine staining. Logarithmically growing cells were plated at a density of 5×10^4/ml in 35-mm tissue culture dishes. DMSO was added to a final concentration of 1.5%. All cultures were refed with fresh DMSO-containing medium every other day. Cells (100 μl) were removed at daily intervals for benzidine staining as previously described (29, 31). A minimum of 200 cells were counted in duplicate samples of each culture. Variation between duplicate plates and repeat experiments was usually ≤10%.

Nucleic acid analyses. DNA and RNA were prepared as previously described (31). Southern and Northern (RNA) blotting were as described previously, with hybridizations being performed at 42°C in 50% formamide–6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt solution–100 μg of salmon sperm DNA per ml–10 mM phosphate buffer (pH 7.0)–1 mM EDTA–0.1% sodium dodecyl sulfate (31). To detect exogenous c-*myb* sequences in F-MEL cells, we used the entire 3.4-kb human c-*myb* cDNA as a probe, labeled by nick translation with [α-^{32}P] dCTP (Amersham Corp.) to a specific activity of 1×10^8 to 4×10^8 dpm/μg. *myb* copy numbers were estimated from parallel lanes containing control genomic DNAs and serial dilutions of the pMbm1 plasmid corresponding to 1 to 200 copies per haploid genome.

Analysis of endogenous c-*myb* kinetics. The kinetics of endogenous c-*myb* mRNA in response to DMSO were analyzed by Northern (RNA) blot analysis. To detect endogenous mouse c-*myb* transcripts, we synthesized a synthetic oligodeoxynucleotide complementary to bases 3271 to 3305 of the 3′-untranslated sequences of the mouse c-*myb* gene (1) (Applied Biosystems DNA synthesizer). This region is only

approximately 50% homologous to the corresponding human sequences (22, 41). A 10-μg portion of total RNA was electroblotted to GeneScreen (New England Nuclear Corp.) or nitrocellulose and hybridized to the ^{32}P-end-labeled oligomer at 37°C in a buffer containing 50% formamide, 5× SSC, 5× Denhardt solution, 250 μg of tRNA per ml, 1% sodium dodecyl sulfate, and 50 mM $NaPO_4$ (pH 6.5). The filter was washed three times at room temperature with 40 mM sodium phosphate (pH 7.2)–1 mM EDTA–1% sodium dodecyl sulfate and at 37°C in the same buffer and then in 2× SSC–0.5% sodium dodecyl sulfate at 65°C for 30 min. To allow for a comparison of endogenous murine and exogenous human *myb* levels, a 35-nucleotide-long oligonucleotide was synthesized that was complementary to the same region of 3′-untranslated sequence of human c-*myb* RNA as was the murine-specific probe. Both probes were of equivalent G+C content. Each was labeled to the same specific activity and was hybridized to blots containing identical amounts of RNAs from the HL60 human promyelocytic cell line (6) and control F-MEL plus various pMbm1-transfected F-MEL clones.

RESULTS

Cloning and analysis of the c-*myb* cDNA clone pMbm1. To analyze the role of c-*myb* in leukemia and hematopoietic differentiation, we elected to place a full-length c-*myb* cDNA under the regulation of a constitutive promotor, choosing not to use a genomic clone because of its large size and potential negative regulation. We prepared cDNA libraries by using the plasmid primer method with mRNA from the CCRF-CEM and HL-60 cell lines (5, 27, 28). Approximately 50 cDNA clones were isolated from each library, using a human genomic c-myb probe containing nonrepetitive sequences. Several clones contained inserts of 3.4 kb. One of these clones, from the CCRF-CEM library, was designated pMbm1 and used in further analyses. Its size is in good agreement with the 3.4-kb mRNA which is the predominant transcript seen in CCRF-CEM and HL-60 cells (M. F. Clarke and E. Westin, unpublished data) (Fig. 1).

The pMbm1 cDNA insert was further characterized by restriction mapping and partial DNA sequencing. These results were in exact accord with those of Majello et al. (22), who have previously isolated and fully characterized a human c-*myb* cDNA from a T-cell lymphoma cell line. Our sequence, however, is 98 base pairs longer than the one previously reported and begins at one of the known murine transcription initiation sites (1). Our sequence was also in agreement with other human c-*myb* cDNA clones previously reported by Slamon et al. (41).

The transcription unit for a mutant dhfr cDNA was introduced into pMbm1 at a point upstream of the SV40 promotor (31, 40). This allowed for selection of exogenous c-*myb* sequences as a result of their coamplification with dhfr during the growth of cells in MTX (31).

Integration and expression of exogenous myb sequences in F-MEL cells. F-MEL cells were transfected by electroporation with linearized pMbm1-dhfr and pHNEO DNAs. Cells stably expressing both constructs were selected by sequential growth in G418 and 0.25 μM MTX (31).

Individual F-MEL clones, resistant to 0.25 μM MTX, were examined for the presence of pMbm1 sequences by Southern blotting, with the entire 3.4-kb c-*myb* cDNA being used as a probe. According to the map presented in Fig. 1, *Bam*HI-digested plasmid DNAs should contain exogenous *myb* fragments of approximately 1.4, 1.2, 0.7, and 0.2 kb,

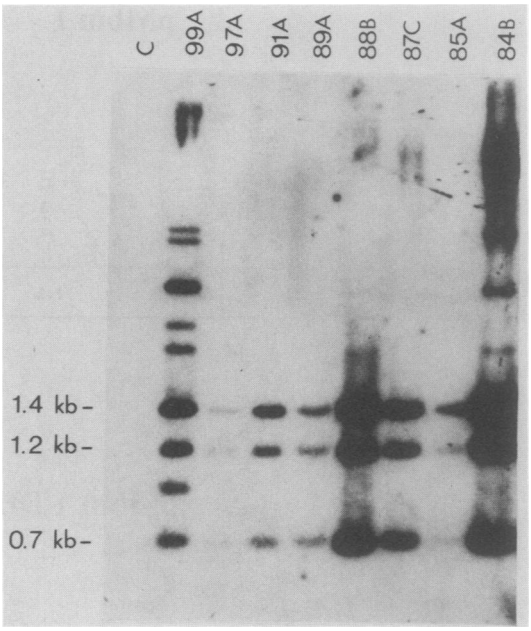

FIG. 2. Southern blots of DNAs from representative individual pMbm1-dhfr-transfected F-MEL clones. All transfected clones were maintained in medium containing 0.25 μM MTX. Purified DNAs (10 μg) were digested with *Bam*HI before Southern blotting. Blots were hybridized with the full-length 3.4-kb *Xho*I cDNA fragment depicted in Fig. 1 followed by stringent washings down to 0.2x SSC, 68°C. The three unrearranged human *myb*-specific fragments (1.4, 1.2, and 0.7 kb) are indicated. C, Control untransfected F-MEL DNA.

assuming no rearrangements have occurred. The first three fragments were detected in DNAs of each clone examined (Fig. 2). We assume that our failure to detect the 0.2-kb fragment is a result of its not being retained by nitrocellulose. Copy numbers ranged from only 1 to 2 per haploid genome as in the cases of clones 85A, 89A, and 97A to as many as 20 to 100 with clones 84B and 88B. We were unable to detect endogenous murine c-*myb* genomic sequences despite the high degree of evolutionary conservation between human and mouse genes (1, 12, 22, 41). This presumably reflects the high stringency of our hybridization and posthybridization washes (0.2× SSC, 68°C). The use of *Bam*HI in this experiment did not clearly allow us to prove clonal uniqueness in most cases. The exceptions to this were seen with clones 84B and 99A, with which additional bands were detected, presumably the result of plasmid rearrangements during the integration or amplification processes or both. Repeat Southern blots, using EcoRI-digested DNAs, revealed unique *myb*-specific junctional bands in each case, however (not shown).

RNAs from individual clones were also examined for the presence of exogenous human c-*myb* transcripts. Total cellular RNAs were purified from each clone and hybridized with an end-labeled 1.5-kb *Hin*dIII restriction fragment spanning the SV40-*myb* 5′ junction. This fragment did not detect endogenous murine c-*myb* sequences in RNA from control untransfected F-MEL cells (Fig. 3). However, each transfected clone expressed human c-*myb* as evidenced by the expected 1.15-, 0.98-, and 0.92-kb protected fragments which represent the unspliced and mature cleaved transcripts (Fig. 1). An additional 0.72-kb S1-protected fragment was also seen. We are uncertain whether this represents simply an artifact of the nuclease S1 digestion procedure or

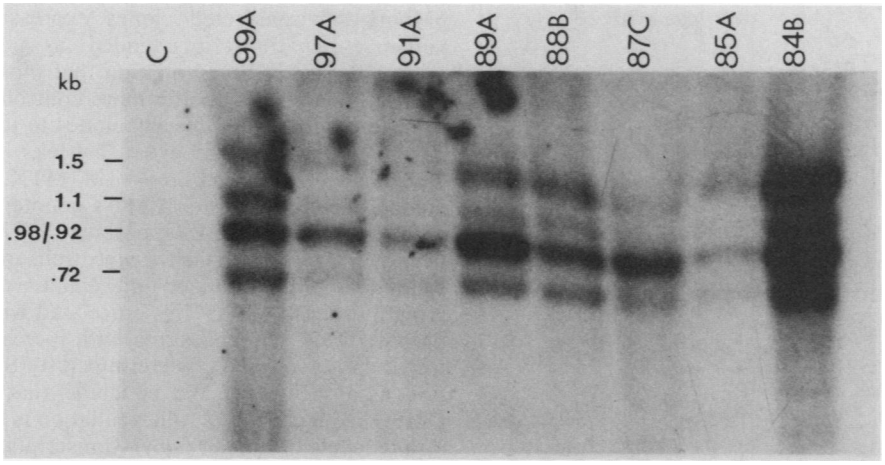

FIG. 3. S1 nuclease protection assays of RNAs from representative pMbM1-dhfr-transfected F-MEL clones. All transfected clones were grown in medium containing 0.25 μM MTX. Total RNAs were purified, and 10 μg of each was hybridized with 2×10^5 dpm of the end-labeled 1.5-kb *Hin*dIII fragment depicted in Fig. 1 (specific activity, $\sim 10^7$ dpm/μg). Hybridizations were performed for 16 h at 51°C in 10 μl of 80% formamide–0.4 M NaCl–40 mM PIPES [piperazine-*N*, *N'*-bis (2-ethanesulfonic acid)] (pH 6.4)–1 mM EDTA (10). Reactions were digested with 1,000 U of S1 nuclease per ml, and half the reaction was subsequently analyzed on a 1% agarose gel. The protected fragments seen here represent the 1.5-kb input probe, the 1.15-kb unspliced primary transcript, and the 0.98- and 0.92-kb spliced transcripts. The latter two are unresolved in this particular run. An additional transcript of 0.72 kb was detected in most cases examined. This may reflect an additional splice acceptor sequence within the c-*myb* 5'-untranslated region.

the presence of an alternate splice accepter approximately 100 nucleotides upstream of the c-*myb* translation start site. In the latter case, this should result in a functional transcript lacking a majority of 5'-untranslated sequences.

It was not possible with the above analysis to determine the human/murine *myb* transcript ratio in our clones. Therefore, we synthesized two 35-nucleotide-long oligonucleotides each of which was perfectly complementary to the equivalent region of the human or *myb* 3'-untranslated region but only 50% complementary to the heterologous transcript. Each oligonucleotide was labeled to the same specific activity and was used to probe Northern (RNA) blots containing equal amounts of RNAs from the human HL60 promyelocytic cell line (6), control F-MEL cells, or a pMbm1-dhfr-transfected F-MEL clone. The murine-specific *myb* oligonucleotide probe detected an appropriately sized transcript in F-MEL cells, whereas the human-specific probe detected transcripts in HL-60 cells and a pMbm1-transfected F-MEL clone (Fig. 4). A comparison of band intensities among pMbm1-dhfr transfectants indicated that most clones contained levels of human transcripts that were at least as high as endogenous murine levels and generally higher than HL60 levels.

F-MEL differentiation is inhibited following transfection with pMbm1-dhfr. We selected 19 individual MTX-resistant clones expressing pMbm1 transcripts and tested each for its ability to differentiate in the presence of DMSO. Figure 5 shows that, with the majority of these clones, benzidine staining following a 5-day exposure to DMSO was significantly reduced. Fourteen of 19 clones showed ≤40% benzidine staining and 4 showed <10%. In contrast, control untransfected F-MEL cells or F-MEL cells transfected with plasmids containing only neo and dhfr sequences showed high-level induction (Fig. 5; unpublished observations). The presence of MTX in the medium of pMbm1-transfected clones during DMSO induction did not influence their ultimate level of differentiation (not shown). The inhibition of F-MEL differentiation we observed was also not due to a nonspecific effect of exogenous nuclear c-*onc* gene expression. Transfection of similar plasmids containing full-length murine c-*fos* cDNAs failed to alter the differentiation pattern of F-MEL cells despite high levels of c-*fos* mRNA transcription (E. V. Prochownik, M. Smith, and J. Kukowska-Latallo, unpublished data).

We observed a general correlation between the levels of c-*myb* transcripts and the degree of inhibition of F-MEL

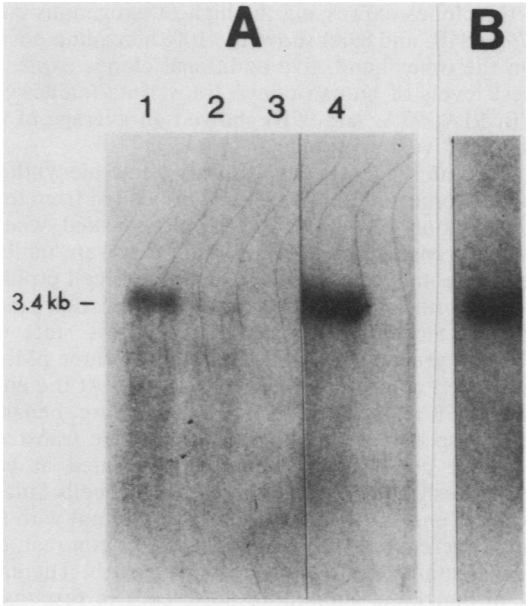

FIG. 4. Comparative levels of human and murine c-*myb* transcripts. (A) A 10-μg amount of total RNA from human HL-60 cells (lane 1), HL-60 cells treated for 5 days with DMSO (lane 2), control F-MEL cells (lane 3), or pMbm1-transfected F-MEL clone 76A (lane 4) was electrophoresed in an agarose gel, transferred to nitrocellulose, and hybridized with an end-labeled human c-*myb*-specific synthetic oligonucleotide (see Materials and Methods). (B) A 10-μg amount of control F-MEL cell RNA was hybridized in parallel with a murine c-*myb*-specific probe labeled to the same specific activity as the probe used in panel A. RNA from HL-60 cells did not hybridize the murine probe (not shown; Fig. 8B).

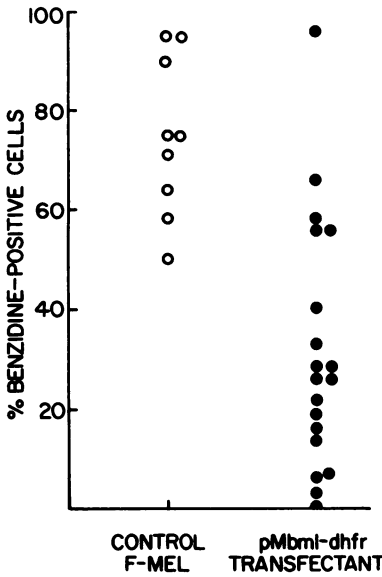

FIG. 5. Induction of heme in control and pMbM1-dhfr-transfected F-MEL clones. Cells were plated in six-well dishes in the presence of 1.5% DMSO and fed with fresh DMSO-containing medium every other day. At the end of 5 days, samples were stained with the benzidine reagent (29, 31) and the percentage of positive cells was determined. A minimum of 200 cells were counted. Each point represents the average of duplicate experiments.

differentiation. Six clones expressing moderate to high levels of exogenous transcripts (76A, 79A, 79D, 84B, 84C, and 89A) showed an average of 23% benzidine-positive cells following 5 days of exposure to DMSO (range, 0 to 54%). Indeed, the clones expressing the highest exogenous c-*myb* levels (76A, 84B, and 89A) showed ≤10% benzidine-positive cells. On the other hand, five additional clones expressing the lowest levels of human c-*myb* transcripts (clones 85A, 87A, 87B, 91A, 97A, and 99B) showed an average of 44% benzidine-positive cells (range, 7 to 69%).

The benzidine reagent measures only heme biosynthesis, which under some conditions can be dissociated from terminal differentiation (15, 16). We therefore asked whether pMbm1-dhfr-transfected F-MEL clones also were inhibited in other differentiated functions. Cessation of cell proliferation occurs with F-MEL terminal differentiation and is probably the most sensitive indicator of this state (23). Therefore, we grew control F-MEL cells and three pMbm1-dhfr-transfected clones in DMSO for 1 week. At the end of this time, 84% of control F-MEL cells were benzidine positive as opposed to 0 to 7% of cells in the transfected clones. These cells were washed and replated at equal densities in fresh MTX-free medium. Control cells failed to grow over the ensuing 4 days, a finding consistent with their terminally differentiated character (Fig. 6). In contrast, each of the transfectants demonstrated rapid growth. Therefore, the lack of benzidine staining in these cells in response to DMSO reflects a more global inhibition of terminally differentiated F-MEL functions.

Finally, in additional experiments, using clones which showed <10% benzidine-positive cells after a 5-day exposure to DMSO, we have been unable to detect any β^{major}-globin transcripts. This contrasts to control F-MEL cells, which contain high levels of this message by 3 days (E. V. Prochownik, unpublished observations).

Inhibition of F-MEL differentiation is directly correlated with human c-*myb* transcript levels. As demonstrated above, pMbm1-dhfr-transfected clones expressing low levels of human c-*myb* transcripts tended, as a group, to show a greater degree of differentiation that those expressing high levels. To verify this under more controlled conditions, we subjected two low-expressing clones to fourfold increments in MTX concentrations every 2 weeks. At the end of the 2-week growth period in 4 μM MTX, each clone was compared with its 0.25 μM MTX counterpart for its degree of differentiation in DMSO, pMbm1-dhfr plasmid copy number, and levels of human c-*myb* transcripts. In each case examined, DMSO-induced differentiation was less following growth in 4 μM MTX. This correlated with amplification of pMbm1 DNA sequences and with increased human c-*myb* transcript expression as determined by S1 nuclease protection assay (Fig. 7). We conclude that the inhibition of DMSO-induced F-MEL differentiation is directly dependent on the levels of human c-*myb* transcripts.

Kinetics of *myb* and *myc* transcripts in F-MEL cells. In subsequent experiments, we asked what effect DMSO exerted upon the kinetics of pMbm1-dhfr c-*myb* transcripts. Two transfected clones (76A and 84B) were exposed for various periods of time to 1.5% DMSO. Total RNAs were purified and hybridized with the previously described S1 probe (Fig. 3). Analysis of the S1 nuclease-protected products is shown in Fig. 8A. It is apparent from this analysis that DMSO had no effect on the levels of exogenous c-*myb* transcripts in these cells.

We next examined the kinetics of endogenous murine c-*myb* transcripts with the above RNAs. Northern (RNA) blots were hybridized with end-labeled, murine c-*myb*-specific, 35-nucleotide-long synthetic oligodeoxynucleotide. Both control F-MEL cell RNA and RNA from clones 76A and 84B showed a dramatic decrease in c-*myb* mRNA at 4 h (Fig. 8B). Unlike the results of a previous report (33), there was a return of c-*myb* mRNA at 24 h. In the control F-MEL cells, these levels again declined between 3 and 5 days, but in clones 76A and 84B there was continued low-level expression of the endogenous c-*myb* mRNA. These experiments

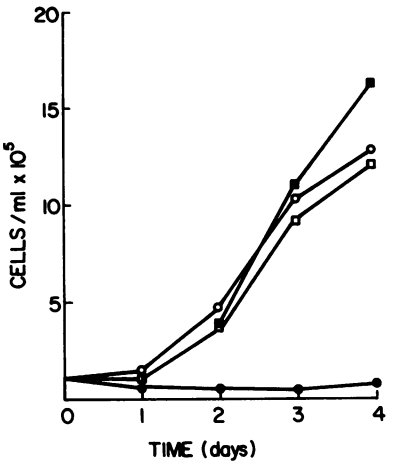

FIG. 6. pMbM1-dhfr-transfectants continue to grow following exposure to DMSO. Control F-MEL cells (●) or pMbm1-dhfr-transfected clones 76A (○), 84B (□), and 88B (■) were plated at a density of 10^5 cells per ml in minimal essential medium containing 1.5% DMSO. Fresh medium with DMSO was added every 2 to 3 days, and cells were diluted to maintain the cell number at approximately 10^5/ml. At the end of 7 days, cells were washed free of DMSO and replated in fresh minimal essential medium without MTX at 10^5 cells per ml. Viable cells were counted daily.

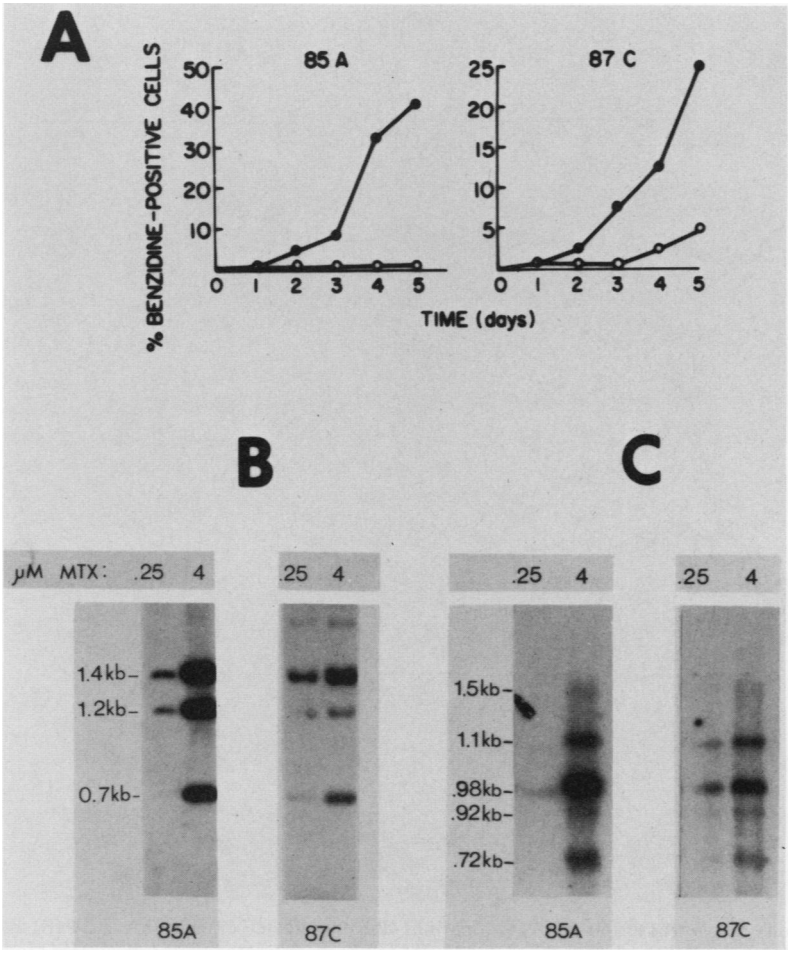

FIG. 7. Amplification of human c-*myb* sequences inversely correlated with F-MEL differentiation. (A) The indicated clones were initially grown in 0.25 μM MTX followed by sequential 2-week intervals in 1 and 4 μM MTX. At the end of this time, cells were replated in medium containing 1.5% DMSO and benzidine-positive cells were scored daily (○). Cells grown only in 0.25 μM MTX were induced in parallel (●). (B) DNAs from 0.25 or 4 μM clones were digested with *Bam*HI and subjected to Southern blotting as described in the legend to Fig. 3. (C) S1 nuclease analysis of 0.25 or 4 μM clones showing human c-*myb*-specific nuclease S1-protected fragments (Fig. 3).

show that the early regulation of endogenous c-*myb* transcripts is unaffected in clones constitutively expressing human c-*myb* sequences. The persistence of endogenous c-*myb* transcripts 5 days after DMSO addition is analogous to what we have observed with c-*myc* (31) and is consistent with the nonterminally differentiated nature of the cells at this point (Fig. 6).

We also examined endogenous c-*myc* transcripts with the same RNAs as for the above S1 analyses. Northern (RNA) blots were hybridized with a full-length nick-translated murine c-*myc* cDNA. Both control RNAs and those from clones 76A and 84B demonstrated the previously described early decline in c-*myc* transcripts followed by a return to preinduction levels by 24 h (Fig. 9) (21, 31). The most notable feature of these experiments, however, was the continued expression of c-*myc* transcripts after 5 days in DMSO. This was consistent with the DMSO-resistant nature of these two clones and presumably mirrors their continued high proliferative potential (31). These results demonstrate that the inhibitory influence of human c-*myb* sequences on F-MEL differentiation is occurring via a mechanism that appears to be independent of the initial changes in c-*myc* transcript levels.

DISCUSSION

In this report, we have investigated the role of the decline in c-*myb* levels which normally accompanies F-MEL differentiation. We have shown that an exogenously introduced full-length human c-*myb* cDNA, driven by an SV40 viral promotor, is expressed constitutively in DMSO-treated F-MEL cells despite an early rapid decline of endogenous c-*myb* transcripts. The net result is that of continuous c-*myb* expression and, in most cases examined, a complete to partial inhibition of erythroid differentiation. This seems to occur through a mechanism independent of the early fall in c-*myc* mRNA levels since the early kinetics of endogenous c-*myc* transcripts closely mimic those seen in control F-MEL cells (Fig. 9). In addition, two pieces of data support the argument that the inhibition seen was dependent upon actual *myb* levels: (i) primary F-MEL clones which were most inhibited in their ability to differentiate expressed higher levels of exogenous c-*myb* transcripts; (ii) in two cases examined, the differentiation of individual F-MEL clones could be further inhibited by the MTX-mediated amplification of c-*myb* sequences (Fig. 7). Despite the fact

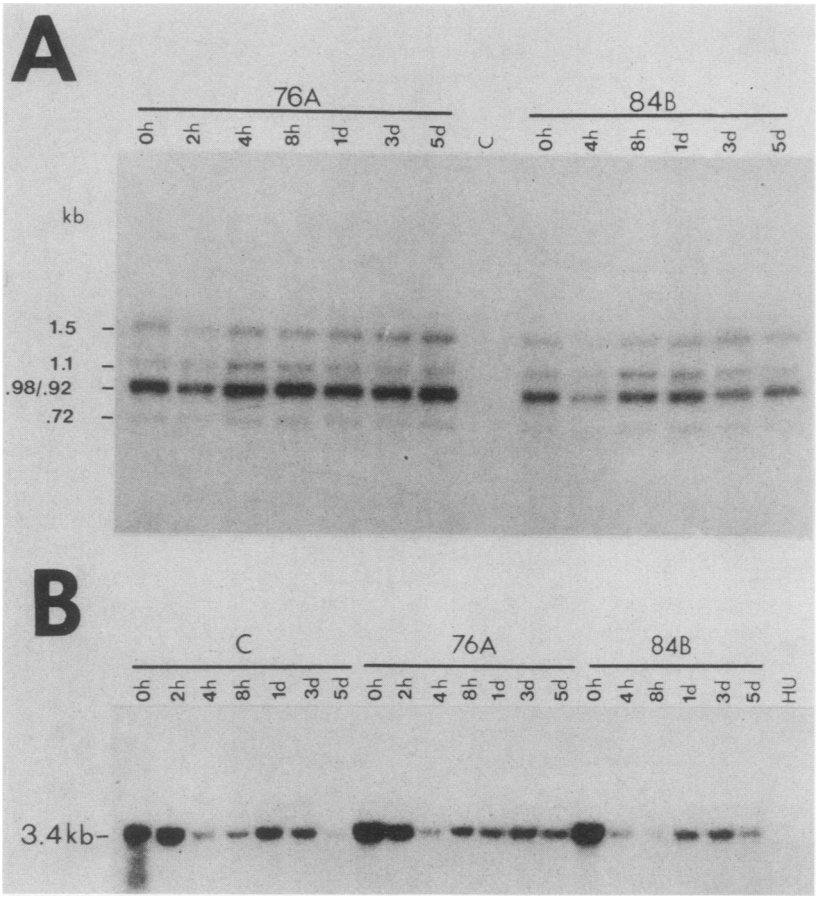

FIG. 8. Kinetics of exogenous c-*myb* transcripts in (A) two pMbm1-dhfr-transfected clones (76A and 84B) and control F-MEL cells and clones 76A and 84B (B). (A) Logarithmically growing cells (in 0.25 μM MTX) were treated with 1.5% DMSO for the times indicated. RNAs were hybridized as described in the legend to Fig. 3, and the S1-protected products were analyzed on a 1% agarose gel. The middle lane (c) shows the S1-protected products when control F-MEL RNA was used in hybridizations. d, Days. (B) The same RNAs as above were resolved in 1% agarose-formaldehyde gels, blotted, and hybridized with an end-labeled murine-specific c-*myb* oligomer. Hu indicates a lane containing 10 μg of ccRF-CEM human promyelocytic leukemia RNA showing no hybridization with this probe.

that higher basal levels of c-*myb* transcripts correlated inversely with F-MEL differentiation, it is not clear at this time whether it is these higher levels, their deregulated expression, or both that are ultimately responsible for the undifferentiated phenotype. In any case, it is quite apparent that a small difference in the level of *myb* expression can exert profound influence over the ultimate degree of cellular differentiation.

The v-*myb* gene product causes hematopoietic neoplasms in chickens (24, 25), whereas disruption of the c-*myb* locus by murine leukemia virus sequences has been reported in certain myelomonocytic tumors (34, 37–39). In both cases, abnormal c-*myb* transcripts have been detected. The E26 leukemia virus causes erythroleukemias in vivo and can transform cells of both myeloid and erythroid origin in vitro (24, 25, 32). In addition to v-*myb*, this virus contains other

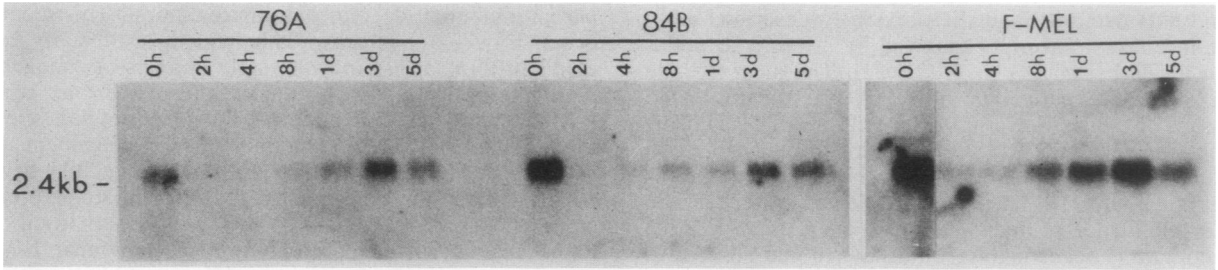

FIG. 9. c-*myc* kinetics in clones 76A and 84B. The same RNAs used in Fig. 8 were resolved in 1% agarose-formaldehyde gels, blotted, and hybridized with a nick-translated murine c-*myc* cDNA fragment (31). F-MEL indicates control untransfected F-MEL RNA. d, Day(s).

unrelated viral oncogene sequences termed v-*ets* (26). It has been proposed that the transformation of cells of myeloid origin is a function of v-*myb* whereas v-*ets* sequences cause transformation of erythroid cells (9, 24, 43). Thus, it has been generally assumed that the transforming function of v-*myb* is confined to cells of myelomonocytic lineage. This interpretation must be made cautiously when extrapolating to c-*myb* for at least two reasons, however. (i) Recent studies have shown an epistatic interaction between v-*myb* and v-*myc* in the transformation of cells of myelomonocytic origin (43). This has suggested that the phenotypic manifestations of v-*myb* transformation can at least in part be determined by interactions with other v-*onc* or c-*onc* gene products. (ii) It is not yet certain what role v-*myb* truncation plays in the genesis of myelomonocytic neoplasms or whether expression of full-length c-*myb* sequences, as is the case here, may alter the spectrum of neoplasia that can be elicited. We have shown that the overexpression of intact human c-*myb* sequences can block erythroid differentiation. Furthermore, our results argue that a disrupted c-*myb* locus is not a prerequisite for this phenomenon since the c-*myb* cDNA used in our experiments is a nearly full-length clone that contains a complete coding sequence. One caveat, however, is that since our clone originated from a leukemia cell line, we cannot exclude the possibility that, despite its intactness, it contains point mutations which serve to impart to it properties similar to those of truncated v-*myb* genes. Indeed, in comparison to the murine c-*myb* gene (1, 12), the pMbm1 clone shows differences in the 5'-untranslated region as well as a few amino acid differences (M. F. Clarke and E. Westin, unpublished data). However, most of these mouse-human differences are not seen with a chicken-human comparison (35). Thus, it seems unlikely that the effect of the pMbm1-dhfr clone on F-MEL differentiation can be attributed to these changes. Since the pMbm1 clone has yet to be fully sequenced, however, such a conclusion must be deemed tentative.

Our data argue that, under the conditions used in this report, c-*myb* may exert phenotypic effects similar to those of v-*ets* of E26 avian virus or v-*erb*-A of avian erythroblastosis virus, both of which have been implicated in blastic transformation of erythroid cells (14, 25).

One interesting observation which can be made from these experiments is that the pMbm1-dhfr-transfected F-MEL cells do not permit the late fall of c-*myc* or c-*myb* mRNA which occurs with terminal differentiation. There is a possibility that c-*myb* is inhibiting differentiation by preventing this decrease. However, this decline normally begins at about 3 days, a point at which F-MEL differentiation is well under way (Fig. 8B). Alternatively, a coordinate decline of endogenous c-*myc* and c-*myb* levels in the first hours of chemical induction may be required for terminal differentiation. The availability of F-MEL clones constitutively expressing c-*myc* and c-*myb* constructs may now provide us with the necessary means to distinguish between these possibilities and to isolate and characterize as yet unidentified genes mediating erythroid differentiation.

These experiments plus previous ones using constitutive c-*myc* expression vectors have begun to elucidate the link between normal cellular oncogene function and F-MEL differentiation (7, 8, 21, 31). The regulation of c-*myc* and c-*myb* seems to be an integral part of a common pathway ultimately leading to F-MEL commitment and differentiation. Both genes appear to be regulated in a similar manner, and inappropriate expression of either one is sufficient to prevent differentiation. The similarities between the *myc* and *myb* gene products suggest that they may also mediate their effects on differentiation through similar, albeit distinct mechanisms.

ACKNOWLEDGMENTS

We thank R. T. Simpson, in whose lab this work was begun, Diane Boman for excellent technical assistance, Steven Emerson for critical review of the manuscript, and Terry Oliver for careful preparation of the manuscript.

M.F.C was supported by grants from the American Cancer Society and Children's Leukemia Foundation of Michigan. E.W. was aided by grant IN-105J from the American Cancer Society. E.V.P. was supported by grants from the Public Health Service National Institutes of Health, the Children's Leukemia Foundation of Michigan, a Basil O'Connor Starter Grant from the March of Dimes, and an Established Investigator Award from the American Heart Association.

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