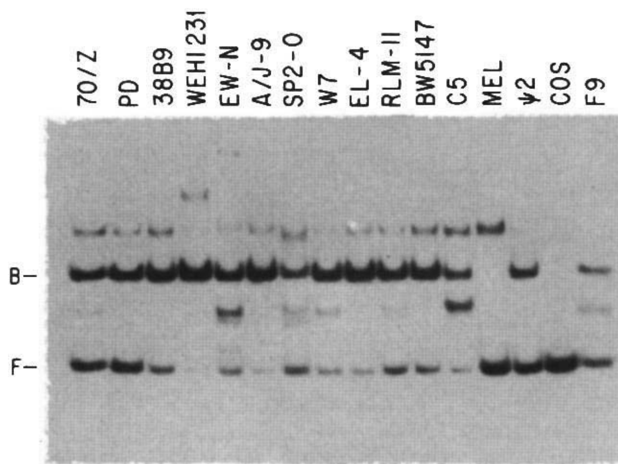

Fig. 4 Analysis of distribution of E1-binding activity in various cell types. Nuclear extracts were prepared[14] from murine or human pre-B-cell lines (70/Z, PD, 38B9), B-cell lines (WEHI-231, EW-N), a myeloma (W7), T-cell lines (EL-4, RLM-11, BW-5147), a primitive B-cell progenitor cell line[15] (C5, a derivative of Ba/F_3, a line like those described in ref. 15), an erythroid line (MEL), a fibroblast line (Ψ2), simian virus 40-transformed African green monkey kidney cell line (COS), and an embryonal cell line (F9). Binding reactions were carried out with the E1 probe as described in Fig. 2 legend. The results of the reaction were analysed by electrophoresis on native polyacrylamide gels. Autoradiography of the dried gels was performed at −70 °C with intensifying screens. Longer exposures of the autoradiogram reveal detectable levels of a species co-migrating with B, the bound complex, in MEL and COS cells.

of action are unknown. The evolutionary conservation of an E1-binding site between mouse and human immunoglobulin genes suggests an important biological function. Deletions of sequences containing the E1 site have been shown to produce severalfold reduction in the transcriptional stimulation activity of the immunoglobulin enhancer element in B cells[2,13]. The E1 element may be a transcriptional control element, with either constitutive or lymphocyte-restricted transcriptional activation. Perhaps the most direct evidence for the involvement of binding to the E1 site is the previous *in vivo* methylation protection analysis. A factor is bound to the E1 site *in situ* which forms contacts with identical guanine residues to those found with the solubilized NF-μE1 factor[3]. This suggests that NF-μE1 is bound to the E1 site in B lymphocytes.

In the above studies, NF-μE1 binding *in vivo* was found only in cells of the B lineage. *In vitro*, however, factors with identical binding specificity are present in extracts from cells of non-lymphocytic lineage. Although similar binding specificities are defined, the proteins found in the various cell types may differ in important aspects that would not be detectd by this assay. An obvious explanation for these apparently inconsistent observations is that the immunoglobulin enhancer is in an inactive conformation in non-B cells and thus inaccessible for binding by NF-μE1. The ubiquitous expression of an NF-μE1 activity suggests that this factor is probably important for expression of genes in non-lymphocytic cells. It is possible, for example, that NF-μE1 binding has completely different consequences in cells of different developmental lineages or stages or in different genomic contexts, including *cis*-dominant transcriptional suppression. These possibilities are being investigated.

Several sites (μE2, μE3 and μE4) with limited sequence homology to the E1-binding site have been identified within the immunoglobulin heavy-chain enhancer. It has been suggested that these four sites bind a common factor. Evidence presented here, in fact, suggests that this is not the case. NF-μE1 clearly does not bind to the μE3 or μE4 sites; conversely, a different factor, NF-μE3, binds with high affinity to E3 but does not bind E1 to a significant degree[9]. Thus, the immunoglobulin heavy-chain enhancer element must be recognized by at least three and probably more different factors. How the combination of these factors sum to a cell-type-specific enhancer is a fascinating problem.

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Deregulated expression of c-*myc* by murine erythroleukaemia cells prevents differentiation

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Friend murine erythroleukaemia (F-MEL) cells are a permanent line of primitive erythroid precursors originally derived from the spleens of mice infected with the Friend strain of murine leukaemia virus[1]. F-MEL cells differentiate *in vitro* in response to various chemical inducers[1,2]. Concomitantly with induction, a biphasic regulation of c-*myc* oncogene transcripts is observed[3]. Within one hour of the addition of dimethyl sulphoxide (DMSO) or hypoxanthine (Hyp), the levels of c-*myc* transcripts fall dramatically and remain virtually undetectable for the next few hours. Between 8 and 24 hours after induction, c-*myc* transcripts return to pre-induction levels and then decline again between 3 and 5 days as most of the cells undergo terminal differentiation. To explore the potential relationship between c-*myc* expression and F-MEL terminal differentiation, we have investigated here whether reversing the early fluctuations in c-*myc* transcript levels affects the ability of F-MEL cells to differentiate. We therefore constructed an amplifiable plasmid vector containing a full-length mouse c-*myc* complementary DNA and introduced it stably into recipient F-MEL cells. The exogenous c-*myc* sequences are transcribed in F-MEL cells and the transcript levels do not change significantly in response to inducing agents. The net result is continued c-*myc* expression following DMSO or Hyp induction and a complete or partial inhibition of F-MEL differentiation.

Figure 1 shows the plasmid used for transfections. The backbone of this vector has been derived from pSV_2neo, in which bacterial neomycin resistance (*neo*) sequences have been replaced with a full-length murine c-*myc* cDNA clone. The simian virus 40 (SV40) early splice and poly(A) sites of pSV_2neo have been retained. Downstream of *myc* and in the opposite transcriptional orientation, we have introduced an expressible

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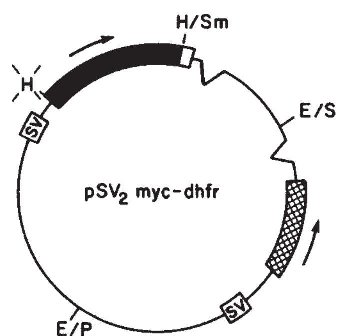

Fig. 1 Structure of an amplifiable mouse c-*myc* expression vector. SV, SV40 promoters; dark box, mouse *myc* cDNA sequences; light box, *neo* 3'-untranslated region sequence; cross-hatched boxes, *dhfr* sequences; V, SV40 splice junctions; H, *Hin*dIII; Sm, *Sma*I; E, *Eco*RI; S, *Sal*I; P, *Pst*I.
Methods. All plasmids were purified by the alkaline lysis method[27]. The plasmid pSV_2neo (ref. 28) was digested with *Hin*dIII and *Sma*I to remove *neo* sequences and then treated with the large fragment of DNA polymerase I. A 1.5-kilobase (kb) *Hin*dIII fragment containing the entire coding region of a mouse c-*myc* cDNA was excised from the plasmid pMc-myc 54 (ref. 29), treated with the large fragment of DNA polymerase and ligated into the pSV_2neo-derived vector. The resultant plasmid, with c-*myc* sequences under the control of the SV40 early promoter, was treated with *Eco*RI and the large fragment of DNA polymerase. Into this site we cloned a 2.8-kb *Pst*I-*Sal*I fragment from the plasmid pFR400 (ref. 4) after treating the fragment with T_4 DNA polymerase and the large fragment of DNA polymerase to render it blunt-ended. This fragment contains the SV40 early promoter, a mutant *dhfr* cDNA and splice-polyadenylation sites derived from the gene encoding hepatitis-B virus surface antigen.

full-length cDNA encoding a mutant form of dihydrofolate reductase (*dhfr*) which facilitates a selective amplification of plasmid sequences in the presence of high concentrations of the antimetabolite methotrexate (MTX)[4]. We linearized pSV_2myc-dhfr along with a selectable plasmid marker encoding neomycin resistance. The two plasmids were introduced by electroporation at a 10:1 molar ratio into recipient F-MEL-745 cells. We selected six clones which demonstrated resistance to both neomycin and MTX. Of these, three expressed the exogenously introduced c-*myc* sequences (not shown).

We first examined the levels of endogenous and exogenous c-*myc* transcripts in response to DMSO using an S_1 nuclease protection assay (Fig. 2). Transfected F-MEL cells were first removed from MTX-containing medium for 3-5 days, then washed and cultured in Dulbecco's minimal essential medium (DMEM) containing 1.5% DMSO for varying periods of time. Total RNAs were extracted and used in the S_1 protection assay. The results confirmed the previously reported changes in endogenous c-*myc* transcripts which occur after DMSO addition[3]. The behaviour of exogenous, pSV_2myc-dhfr-derived c-*myc* transcripts was quite different. Following the addition of DMSO, c-*myc* transcript levels declined by no more than 50%, remaining easily detectable even when endogenous transcripts were not. The net effect was therefore a partial reversal of the early DMSO-induced decline in c-*myc* transcript levels. Of particular interest was the failure of endogenous transcripts in transfected cells to decline significantly by day 5, suggesting that the cells still retained proliferative potential.

The experiments described above indicated that it was possible to reverse at least partially the inhibitory effects of DMSO on c-*myc* transcript levels. We therefore next investigated whether this reversal had any inhibitory effect on the differentiation capacity of F-MEL cells in response to two structurally unrelated inducers of differentiation. F-MEL cells were cultured for varying times in 1.5% DMSO or 5 mM Hyp. The extent of differentiation was monitored daily by determining the fraction of cells that stained with benzidine[5]. The results (Fig. 3) showed that in each of the three clones tested, the extent of benzidine positivity was significantly reduced in relation to non-transfected F-MEL-745 cells or to clones of F-MEL cells which had been

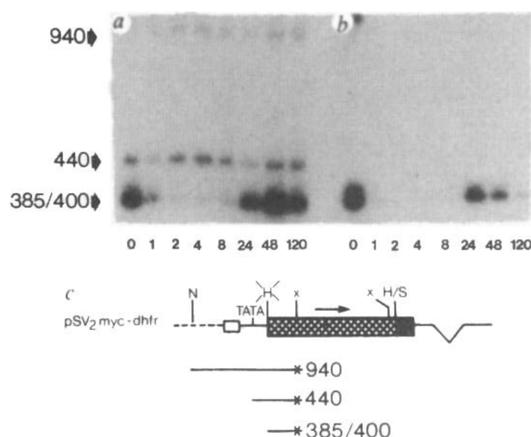

Fig. 2 Levels of mouse c-*myc* transcripts in F-MEL clone 15 (*a*) or 745 (*b*) following the addition of DMSO. The dark arrows indicate the 940-nucleotide reannealed input probe, the 440-nucleotide S_1-protected fragment representing pSV_2myc-dhfr-derived exogenous transcripts and the two, unresolved 400- and 385-nucleotide fragments representing endogenous *myc* transcripts[30]. Numbers below each lane indicate the length of time (in hours) that cells were incubated in the presence of DMSO. *c*, The S_1 probe was a 940-nucleotide long *Nde*I-*Xho*I fragment, derived from pSV_2 myc-dhfr and end-labelled at the *Xho*I site.
Methods. F-MEL cells (clone 745) were grown in DMEM supplemented with 10% fetal calf serum (FCS), 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 2 mM glutamine (Gibco). Transfections were performed by electroporation as described previously[31,32] in 1 ml of phosphate-buffered saline containing $1\text{-}2\times10^7$ cells ml^{-1}, 20 μg of linearized SV_2myc-dhfr and 2 μg of linearized pSV_2neo DNA. Immediately after electroporation, cells were kept on ice for 10 min and then replated in a series of 6-well dishes in fresh DMEM containing dialysed FCS. Two days later, the antibiotic G418 (Gibco) was added to a final concentration of 1 mg ml^{-1}. After ~2 weeks, G418-resistant clones were replated in fresh medium containing 0.25 μM MTX (Lederle) plus dialysed FCS. Surviving cells from each well were cloned by limiting dilution in 96-well microtitre dishes. In this way, we insured that each clone originated from an independent transfection event. MTX-resistant clones were expanded with continuous growth in 0.25 μM MTX. Clone 8, 15 and 21 cells were each derived from single-cell isolates of 745 cells stably transfected with pSV_2myc-dhfr and pSV_2neo. For S_1 nuclease studies, clones were grown for 3-5 days in the absence of MTX. DMSO was added for the times indicated and total cellular RNAs were extracted by the guanidine hydrochloride method[33]. Aliquots (10 μg) of each RNA were hybridized with an end-labelled 940-nucleotide *Nde*I-*Xho*I fragment derived from pSV_2myc-dhfr (*c*)[34]. This probe was labelled with polynucleotide kinase and [γ-^{32}P]ATP (Amersham) to a specific activity of $\sim10^7$ d.p.m. μg^{-1}. Hybridization reactions contained 10^5 d.p.m. of probe and 10 μg of RNA in a total volume of 10 μl of hybridization solution (80% formamide, 300 mM NaCl, 40 mM PIPES *p*H 6.4, 1 mM EDTA). Reactions were placed at 85 °C for 5 min followed by a 16-h incubation at 51 °C, then treated with 300 U of S_1 nuclease (Sigma) at 37 °C for 1 h, precipitated with isopropanol and subjected to analysis on a 1.8% agarose gel.

transfected with a derivative of the plasmid shown in Fig. 1 in which *myc* sequences were not included or were reversed. Control F-MEL cells demonstrated 85-95% benzidine positivity after 5 days of induction whereas pSV_2myc-dhfr clones showed 10-50% staining.

Transfected clones also failed to accumulate β^{major}-globin messenger RNA as determined by Northern blot analysis of cellular RNAs (not shown). Furthermore, unlike control F-MEL cells, all three transfected clones continued to proliferate after they had been incubated for 7 days in medium containing 1.5% DMSO (not shown). We conclude from these studies that the perturbations of *myc* expression seen in these clones are associated with the preservation of features normally associated with the uncommitted, non-terminally differentiated state.

The inability of clone 21 cells to differentiate as well as clones 8 and 15 might have been related to levels of exogenous c-*myc* transcripts. Indeed, we found that these levels are two to three-fold lower in clone 21 cells than in the other clones (not shown). To address this aspect more directly, we grew cells from clone 21 in increasing concentrations of MTX. At each stage, cells were tested for their ability to differentiate in response to DMSO. RNAs were also extracted and the ratios of exogenous to

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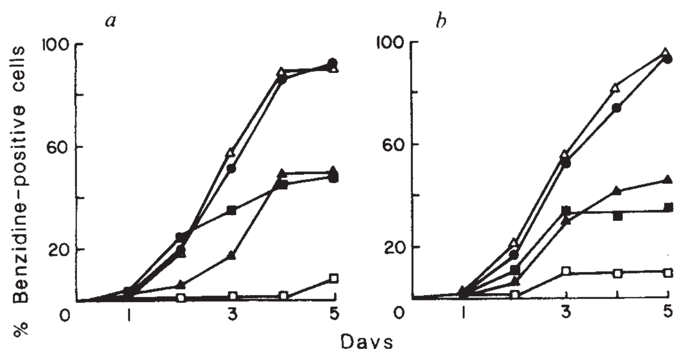

Fig. 3 Benzidine staining of F-MEL clones in response to dimethyl sulphoxide (DMSO) (*a*) or hypoxanthine (Hyp) (*b*). Equivalent numbers of cells from each of the indicated clones were cultured in the presence of 1.5% DMSO or 5 mM Hyp. At the times indicated, aliquots of cells were removed and stained with benzidine as described previously[5]. Clones 8 (■), 15 (□) and 21 (▲) were each derived from single-cell clones of F-MEL clones which had been transfected with pSV_2myc-dhfr. Clone 101 (△) was derived from a transfection with the above plasmid minus the mouse c-*myc* sequences. Clone 745 cells (●) were the non-transfected parental line used for all transfection studies. Each point represents the average of three to five independent experiments.

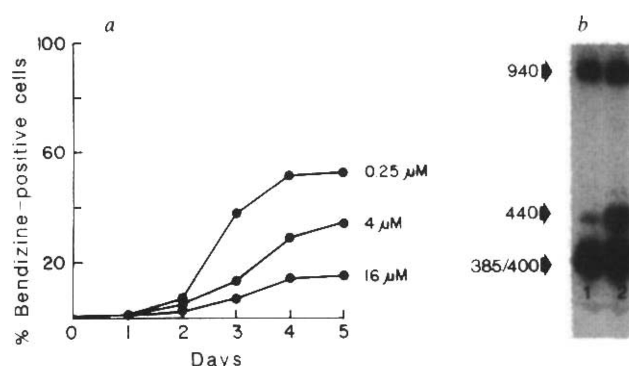

Fig. 4 The degree of F-MEL differentiation is related to the level of exogenous c-*myc* expression. Clone 21 cells were initially grown in 0.25 μM MTX. To amplify pSV_2myc-dhfr sequences, MTX concentrations were increased fourfold for 14-day intervals. At the end of the incubation period, MTX was removed for 3-5 days to allow for recovery of viable cells. Cells were then induced with 1.5% DMSO to monitor benzidine staining (*a*) or were subjected to the next round of MTX selection. *b*, After growth in the indicated concentrations of MTX, cellular RNAs were extracted and the levels of exogenous and endogenous c-*myc* transcripts were determined as described for Fig. 2. The concentrations of MTX in which clone 21 cells were grown was either 0.25 μM (lane 1) or 16 μM (lane 2).

endogenous transcripts examined. As shown in Fig. 4, benzidine staining of clone 21 cells was progressively inhibited as the concentration of MTX was raised. Exogenous c-*myc* transcript levels were also increased, indicating an amplification of plasmid sequences. These observations demonstrated that, at least in the case examined, progressive refractoriness to DMSO induction could be correlated with levels of exogenous *myc* transcripts.

The highly conserved nature of cellular oncogenes suggests that they are intimately associated with growth and/or differentiation processes. Indirect evidence in support of this comes from studies of both fresh and cultured tumour cells where aberrancies such as c-*onc* gene amplification, rearrangement and overexpression have been reported[6-11]. Changes in the levels of transcripts of otherwise normal c-*onc* genes have been shown to accompany the normal differentiation and mitotic process both *in vitro* and *in vivo*[12-15].

c-*myc* is a short-lived nuclear protein possessing DNA-binding properties[16] and is structurally related to the adenovirus E1A gene product which acts to *trans*-activate other viral genes[17]. Indeed, c-*myc* itself can enhance transcription of a chimaeric gene containing the 5'-flanking region of the *Drosophila hsp70* heat shock protein gene[18]. Together with the findings of c-*myc* transcript modulation during differentiation[3,6], these attributes make the c-*myc* gene product an ideal candidate to control developmental events, presumably by positively or negatively influencing the transcription of other cellular genes[19]. This in turn might exert effects on proliferation since, in the case of F-MEL cells, proliferation and terminal differentiation have been shown to be intimately related[20].

The aberrant expression of several c-*onc* genes can stimulate or inhibit differentiation in several *in vitro* systems[21-23]. We are currently investigating the role of other c-*onc* genes in the differentiation of F-MEL cells. A more difficult problem will be that of the possible role of Friend virus complex genes in complementing the action of c-*myc* in this system.

c-*myc* transcripts originating from the SV40 promoter were less responsive to inducing agents than were endogenous c-*myc* transcripts. This is not surprising because the exogenous c-*myc* sequences are under the control of a foreign promoter, and the c-*myc* transcription unit has been substantially altered so as to contain a modification of the 5'-untranslated region and to lack first intron sequences. Both regions have been implicated in contributing to the considerable instability of normal *myc* mRNA[24]. We also note that even high-level overexpression of the exogenous c-*myc* sequences did not affect the levels of endogenous transcripts. This result is consistent with previous observations regarding c-*myc* overexpression in NIH/3T3 and F-MEL cells[25,26].

After submission of this manuscript, Coppola and Cole reported findings similar to ours[26], in addition to demonstrating that some of the events leading to commitment have already occurred in c-*myc*-transfected cells.

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