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v-Myb DNA binding is required to block thrombocytic differentiation of Myb–Ets-transformed multipotent haematopoietic progenitors

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The E26 avian leukaemia virus encodes a fusion oncoprotein consisting of truncated versions of the c-Myb and c-Ets-1 transcription factors. When used to infect embryonic chicken haematopoietic cells two types of self-renewing progenitors are obtained, namely myeloblasts and 'MEPs' (Myb–Ets progenitors). In earlier work we have shown that myeloblasts transformed by the *ts*21 mutant of E26, which has a lesion in v-Myb, can be induced to differentiate into macrophages following shift to the non-permissive temperature. Here we show that the *ts*21 v-Myb is temperature sensitive for DNA binding in band shift experiments and that its inactivation in transformed MEPs induces their maturation into thrombocytes. The MEP transforming capacity of v-Myb is not confined to its fusion with v-Ets, as it is also seen with a virus that co-expresses *ts*Myb with v-ErbB. As with wild-type E26-transformed MEPs, *ts*21-transformed MEPs are multipotent, differentiating into eosinophils and myeloblasts following treatment with 12-*O*-tetradecanoylphorbol-13-acetate. In addition, *ts*21-transformed myeloblasts differentiate into macrophages when shifted to the non-permissive temperature. This shows that v-Myb blocks haematopoietic differentiation at two distinct stages. In contrast, v-Ets inactivation in MEPs transformed by a *ts* E26 mutant with a lesion in the corresponding oncoprotein leads to their differentiation into erythrocytes, myeloblasts and probably eosinophils. These data show that the two domains of Myb-Ets selectively affect decision making processes in different types and stages of haematopoietic cells.

Key words: Myb oncogene/haematopoietic transformation/temperature sensitivity/thrombocyte differentiation

Introduction

Considerable evidence suggests that c-Myb is important for the growth and commitment of early haematopoietic progenitors. c-Myb is abundantly expressed in immature blood cell types and is down-regulated as they differentiate (Westin *et al.*, 1982; Kastan *et al.*, 1989). Furthermore, c-Myb over-expression can prevent terminal differentiation of erythroid cells (Clarke *et al.*, 1988), while ablation of its RNA with antisense oligonucleotides inhibits the formation of haematopoietic colonies (Gewirtz and Calabretta, 1988). Mice homozygous for an inactivated c-*myb* gene die *in utero*, due to an impairment of definitive haematopoiesis in the fetal liver (Mucenski *et al.*, 1991). c-*myb* mRNA levels vary during the cell cycle (Thompson *et al.*, 1986) and experiments with antisense c-*myb* oligonucleotides, leading to growth arrest, indicate that growth is inhibited as the result of a block at the end of the G_1 phase (Gewirtz and Calabretta, 1988; Furukawa *et al.*, 1990; Valtieri *et al.*, 1991). The domains of Myb involved in specific DNA binding, transactivation and intramolecular regulation have been defined in some detail (for reviews see Lüscher and Eisenman, 1990; Graf, 1992). However, although *mim*-1 was identified as a first direct target (Ness *et al.*, 1989), no target genes involved in proliferation have yet been identified.

The c-*myb* gene has been oncogenically activated twice by recombination with avian retroviruses (Graf and Beug, 1978). The E26 leukaemia virus encodes a truncated v-Myb protein that is fused at its C-terminus to residues derived from the c-Ets1 proto-oncogene (Leprince *et al.*, 1983; Nunn *et al.*, 1983). This fusion between the Myb and Ets transcriptional activators has been shown to be essential for the ability of the E26 virus to induce leukaemia (Metz and Graf, 1991a,b).

When used to infect 2 day-old chick embryo (blastoderm) cells *in vitro*, E26 virus induces the clonal expansion of two types of cells. The first type corresponds to immature myelomonocytic cells resembling myeloblasts, while the second type represent multipotent progenitors which can differentiate spontaneously into erythrocytes or into myeloblasts and eosinophils in the presence of high or low concentrations of phorbol esters respectively (Graf *et al.*, 1992). In its potential to generate at least four haematopoietic lineages, this E26-transformed cell type, which has been called MEP (for Myb–Ets-transformed progenitor), resembles murine CFU-Mix (Dexter and Spooncer, 1987).

Analysis of the mechanism of transformation by the E26 fusion protein has been facilitated by the use of *ts* mutants identified in biological screens for loss of the differentiation block at the non-permissive temperature (Beug *et al.*, 1984; Golay *et al.*, 1988). Two classes of *ts* mutants have been isolated. The first class, represented by the *ts*1.1 mutant, contains an amino acid substitution in the DNA binding domain of the Ets portion of the fusion protein (Golay *et al.*, 1988) which results in loss of the Ets DNA binding capacity at 42°C (Kraut *et al.*, 1994). MEP-type cells transformed by the mutant differentiate into erythrocytes and to a lesser extent into myeloid and probably eosinophilic cells when shifted to the non-permissive temperature (Golay *et al.*, 1988; Kraut *et al.*, 1994). The other class of mutant contains a lesion in the DNA binding domain of Myb (Frykberg *et al.*, 1988)

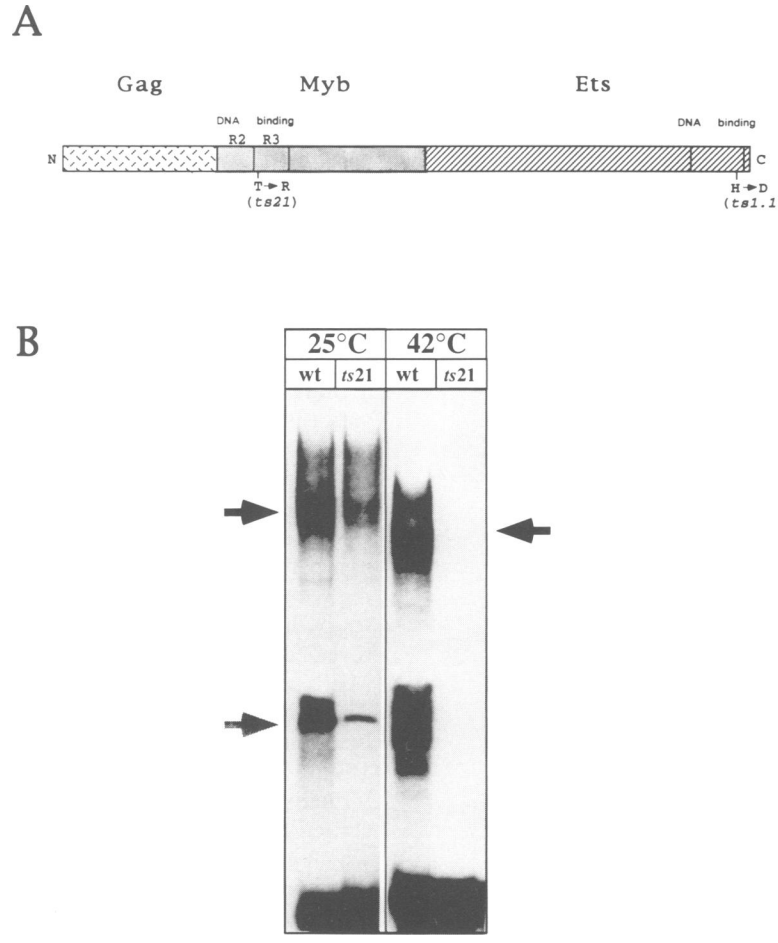

Fig. 1. Temperature sensitivity of *ts*21 v-Myb for specific DNA binding. (**A**) Schematic representation of the Myb–Ets fusion protein showing the location of the Myb DNA binding domain and the position of the *ts*21 Thr→Arg mutation in repeat 3. Also indicated is the *ts*1.1 mutation in the Ets DNA binding domain. (**B**) Band shift experiment with *ts*21 v-Myb. Bacterially expressed wild-type (v-Myb) and *ts*21 (v-Myb^{ts}) proteins were incubated with ^{32}P-labelled Myb recognition site A (MRE-A) oligonucleotide at either 25 or 42°C. Protein–DNA complexes were then resolved on 6% non-denaturing polyacrylamide gels at the same temperature as used in the binding reactions. Free probe can be seen at the bottom. The top arrows indicate complexes of full-length v-Myb with MRE-A (the apparent higher mobility of the Myb complex at 42°C can be explained by the more 'open' polyacrylamide gel matrix at the elevated temperature). The lower arrow shows complexes containing a v-Myb degradation product which has retained the DNA binding domain.

and was isolated by screening for temperature-sensitive myeloid transformation (Beug *et al.*, 1984). Mutant-transformed myeloblasts differentiate terminally into macrophages (Beug *et al.*, 1987) and occasionally into granulocytes (unpublished) following shift to the non-permissive temperature (42°C).

Here we show that Myb^{ts}–Ets-transformed MEPs differentiate along the thrombocytic lineage. Similar results were obtained with MEP-type cells transformed with a virus encoding Myb^{ts} in conjunction with v-ErbB and shifted to the non-permissive temperature. Control of thrombocyte differentiation by the conditional v-Myb should make it possible to further probe the action of v-Myb, such as through the isolation of direct target genes. This system should also be suited to the study of terminal differentiation processes in the thrombocyte lineage.

Results

Specific DNA binding by ts21 Myb protein is temperature sensitive

An Arg→Thr substitution in repeat 3 of the v-Myb DNA binding domain (Figure 1A) is responsible for the temperature-sensitive phenotype of the *ts*21 E26 mutant in myelomonocytic cells (Frykberg *et al.*, 1988) and affects non-specific binding to DNA (Mölling *et al.*, 1985). To test the effect of the *ts*21 mutation on recognition of specific DNA sequences, a gel retardation assay was performed at 25 and 42°C using bacterially expressed Myb. As shown in Figure 1B, both E26 and *ts*21 v-Myb bound to the Myb recognition site derived from the promoter of the *mim*1 gene (MRE-A; Ness *et al.*, 1989) to a comparable extent at 25°C, while at 42°C *ts*21 Myb^{ts} bound very weakly relative to the wild-type protein. To control for possible destabilization of the Myb^{ts} protein at the non-permissive temperature, samples of wild-type and mutant proteins were treated as in the binding reactions at either 25 or 42°C and were then run on a SDS–polyacrylamide gel; no differences were seen in the stability of either protein (data not shown).

Inactivation of Myb in Myb^{ts}–Ets-transformed MEPs induces thrombocytic differentiation

The *ts*21 mutation in the E26 Myb–Ets fusion protein has been shown to relieve the v-Myb-induced block of

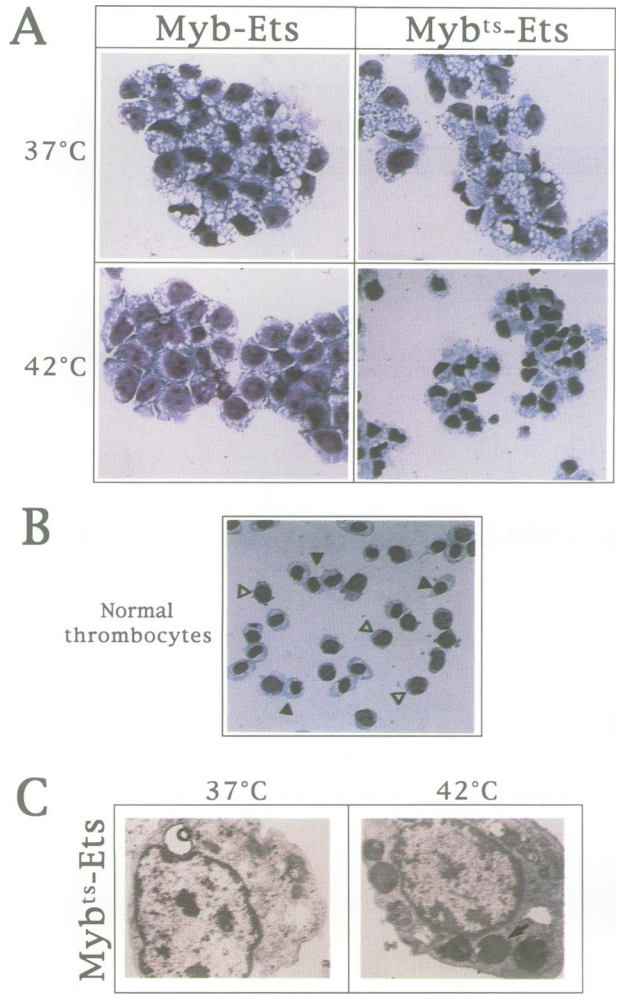

Fig. 2. Morphological changes in Myb^{ts}–Ets-transformed MEPs upon inactivation of Myb. (**A**) May-Grünwald-Giemsa stained cytospin preparations of Myb–Ets (wt E26) and Myb^{ts}–Ets (*ts*21) progenitor cells grown at 37 or shifted to 42°C for 5 days. Note that the Myb^{ts}–Ets cells become smaller, exhibiting a more condensed nucleus and a clearer cytoplasm. (**B**) Stained preparations of thrombocytes (black arrows) purified from the blood of chickens, shown for comparison; the cells with darker cytoplasm and larger nuclei are lymphocytes (open arrows). (**C**) Electron micrographs of sections of Myb^{ts}–Ets MEPs at 37 and after shift to 42°C for 5 days. Note the appearance of large, darkly staining 'dense granules' in the cytoplasm of cells grown at 42°C (arrow).

differentiation in transformed myeloblasts when the cells were shifted to 42°C (Beug *et al.*, 1984, 1987). To assess its role in mutant-transformed MEPs, chick blastoderm cells were infected with the *ts*21 mutant (Myb^{ts}–Ets) followed by plating in methylcellulose at 37 and 42°C. We found that *ts*21 has a similar MEP- and myeloblast-transforming efficiency at 37°C as the wild-type virus (Myb–Ets), while at 42°C essentially no colonies were obtained (data not shown). A number of Myb^{ts}–Ets-transformed MEPs were isolated, expanded in liquid medium and shifted to 42°C. Drastic morphological alterations could be observed within 2 days: the cells appeared to be smaller, more oval in shape, more dispersed and, depending on the particular transformed clone examined, they became more or less adherent. Cytospins of the shifted cells showed marked nuclear condensation and clearing of the cytoplasm (Figure 2A), characteristic of normal chicken thrombocytes (Figure 2B). Cell sections examined under the electron microscope (Figure 2C) showed that Myb^{ts}–Ets MEPs at 42°C exhibited ultrastructural features of normal thrombocytes, including cell shape, the pattern of dense staining in the nucleus and the presence of electron dense cytoplasmic granules (Dieterlen-Lièvre, 1988).

Staining with a panel of monoclonal antibodies against lineage-specific cell surface antigens detected no expression of lymphoid, erythrocytic, myelomonocytic or eosinophilic markers (Table I) in cells grown at either 37 or 42°C (data not shown). No change in expression was seen for the MEP17 antigen (integrin VLA-2; K.McNagny and T.Graf, in preparation) or for the MEP21 and MEP26 antigens, which are expressed on MEPs and normal thrombocytes (McNagny *et al.*, 1992, in preparation). However, the K1 antibody, which detects an antigen expressed on mature chicken thrombocytes (Kaspers *et al.*, 1993), stained Myb^{ts}–Ets MEPs only after shift to the non-permissive temperature (Figure 3A). In addition, staining with 11C3, which recognizes the thrombocyte-specific $\alpha_{IIb}\beta_3$ integrin complex (Lacoste-Eleaume *et al.*, 1994), revealed that this integrin was already expressed in all MEPs at 37°C and increased ~2-fold after shift to 42°C. The antibody LIBS6, which recognizes an epitope on a ligand-activated form of human $\alpha_{IIb}\beta_3$ (Frelinger *et al.*, 1991), showed a more marked increase, from being virtually undetectable at 37°C to expression on most cells shifted to 42°C (Figure 3A). Monoclonal antibody 23C6, which recognizes the human $\alpha_V\beta_3$ integrin (vitronectin receptor) complex (Cheresh and Spiro, 1987), revealed a similar expression to that of the $\alpha_{IIb}\beta_3$ integrin. That these changes were specific to the mutant and not dependent on a particular transformed cell clone could be demonstrated by analysis of a total of 24 MEP clones, each transformed by either Myb^{ts}–Ets or Myb–Ets. Thus, taking LIBS6 expression as a marker of mature thrombocytes, a dramatic increase in expression was observed for most of the 12 Myb^{ts}–Ets-transformed MEPs shifted to 42°C (14.6 ± 8.4-fold), but not for the 12 Myb–Ets-transformed clones (<2-fold). The fluorescence data on integrin expression were confirmed by immunoprecipitation of ^{125}I surface-labelled proteins. As shown in Figure 3B, both 11C3 and 23C6 co-immunoprecipitated the α and β chains of the $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_3$ integrins (the $GP_{IIbIIIa}$ and vitronectin receptors respectively). LIBS6 also detected the $\alpha_{IIb}\beta_3$ complex (the α chain being too faint to be seen in this exposure). With all antibodies the β_3 chain was significantly up-regulated in cells shifted to 42°C, while no change was seen in the α_2 and β_1 chains precipitated by the MEP 17 antibody.

MEP-derived thrombocytes store and release serotonin, produce TGFβ and express high levels of c-Src

To test for functional maturity of the MEP-derived thrombocytes, a number of assays were performed. A characteristic property of vertebrate thrombocytes is the ability to store 5-hydroxytryptamine (serotonin) in granules, from which it can subsequently be released by agents such as thrombin (Hawiger, 1992). Therefore, after growing Myb–Ets and Myb^{ts}–Ets MEPs for 3 days at either 37 or at 42°C cells were briefly incubated with

Table I. Monoclonal antibodies recognizing lineage-specific markers of avian haematopoietic cells

Monoclonal antibody	Specificity[a]	Antigen	Reference
LIBS6	Thr (mature)	$\alpha_{IIb}\beta_3$ integrin	Frelinger *et al.*, 1991
11C3	Thr	$\alpha_{IIb}\beta_3$ integrin	Lacoste-Eleaume *et al.*, 1994
LM609	Thr/MΦ	β_3 integrins	Cheresh and Spiro, 1987
23C6	Thr/MΦ	β_3 integrins	Horton *et al.*, 1985
MEP17	All haematopoietic except late Ery and G	$\alpha_2\beta1$ integrin	McNagny *et al.*; 1992; unpublished
MEP21	P/Thr	?	McNagny *et al.*, 1992
MEP26	P/Thr/Ery (early)	?	McNagny *et al.*, 1992
K1	Thr, MΦ	Integrin?[b]	Kaspers *et al.*, 1993
51/2	M	?	Kornfeld *et al.*, 1983
EOS47	Eos	Melanotransferrin	McNagny *et al.*, 1992; unpublished
JS3	Ery	?	Schmidt *et al.*, 1986
JS4	Ery (late)	?	Schmidt *et al.*, 1986
M1	B	sIgM	Chen *et al.*, 1982
CT3	T	CD3	Chen *et al.*, 1986
CIa	M, B, T	MHC class II	Ewert *et al.*, 1984

[a]P, progenitors; M, myelomonocyte; MΦ, macrophage; G, granulocyte; Thr, thrombocyte; Ery, erythrocyte; Eos, eosinophil; B, B cell; T, T cell.
[b]J.Frampton, unpublished observation.

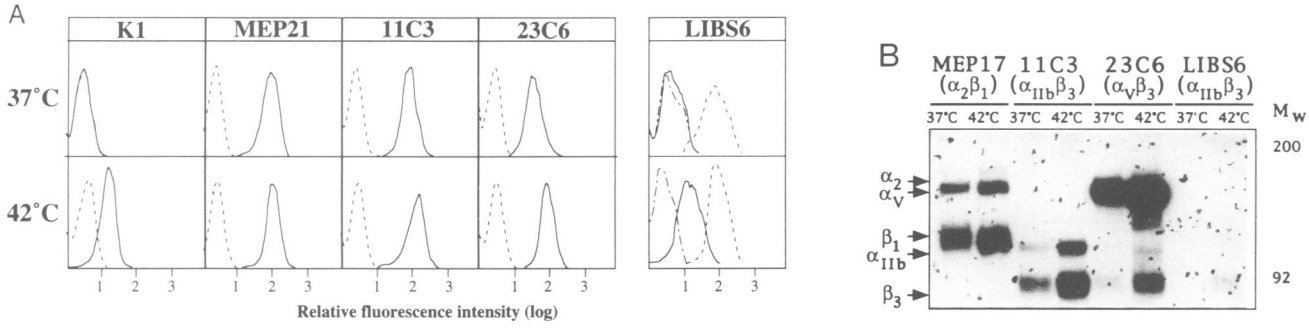

Fig. 3. Up-regulation of thrombocyte-specific cell surface antigens. (**A**) Flow cytometric profiles of Myb^{ts}–Ets MEPs grown at 37 or shifted to 42°C for 4 days using K1, MEP21, 11C3 and 23C6 monoclonal antibodies (unbroken lines). The profiles corresponding to the negative control (monoclonal antibody 327 directed against c-Src) are depicted by the dashed lines. The separate panel on the right shows profiles of the same cells stained by MEP21 (dashed lines) and LIBS6 (unbroken lines). The negative controls are traced with dashed/dotted lines. (**B**) Detection of ^{125}I-labelled cell surface antigens on Myb^{ts}–Ets MEPs. Myb^{ts}–Ets MEPs grown at 37 or 42°C for 5 days were surface-labelled with ^{125}I, lysates immunoprecipitated with the indicated monoclonal antibodies and run on a 7.5% SDS gel. The positions of individual α and β integrin chains are shown by arrows.

[^{14}C]serotonin. The total amount of serotonin stored and the amount released by thrombin were then determined. As shown in Figure 4A, Myb^{ts}–Ets MEPs shifted to 42°C took up ~1.7-fold more serotonin as the same cells before shift or the Myb–Ets control cells at either temperature. In addition, they released almost all of the specifically retained serotonin within 5 min upon addition of thrombin.

Transforming growth factors β (TGFβs) are found in large quantities in mammalian platelets and are secreted by megakaryocytes. To determine the secreted TGFβ activity during the MEP to thrombocyte conversion we employed a sensitive bioassay measuring growth inhibition of Mv1lu cells (CCL64). Large amounts of TGFβ activity could be detected in the supernatants of Myb–Ets and Myb^{ts}–Ets MEPs (equivalent to ~50–70 ng human recombinant TGFβ-1). A 5 day temperature shift resulted in an approximate doubling of TGFβ production in the Myb^{ts}–Ets, but not the Myb–Ets, cells (Figure 4B). No additional activity could be released upon treatment with thrombin. The activity required acid activation and could be inhibited by a pan-specific anti-TGFβ antibody. In contrast, the supernatant of *ts*1.1 (Myb–Ets^{ts})-transformed MEPs contained no TGFβ activity (Figure 4B), which, as will be seen below, is consistent with their inability to produce cells of the thrombocytic lineage.

Finally, mammalian platelets are known to exhibit a high expression of c-Src tyrosine kinase (Golden *et al.*, 1986). We therefore compared Myb–Ets and Myb^{ts}–Ets MEPs before and after shift to 42°C for expression of c-Src protein by immunoprecipitation from extracts of [^{35}S]methionine-labelled cells. As shown in Figure 4C, c-Src is significantly increased in Myb^{ts}–Ets MEPs shifted to 42°C.

v-Myb blocks haematopoiesis at distinct stages of differentiation

The results presented demonstrate that v-Myb is not only capable of blocking differentiation from myeloblasts to macrophages, as had been shown previously, but specifically blocks differentiation of MEP-type cells to thrombocytes. This is surprising, in view of the fact that earlier studies have demonstrated that MEPs are multipotent, having the option to differentiate along the eosinophilic and myelomonocytic lineages following treatment with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Graf *et al.*, 1992). To determine whether Myb^{ts}–Ets-transformed MEPs are likewise multipotent, four MEP clones transformed by *ts*21 E26 at 37°C were treated with 20 or 80 nM TPA for 7 days. As shown in Figure 5A, TPA induced down-regulation of MEP21 antigen and expres-

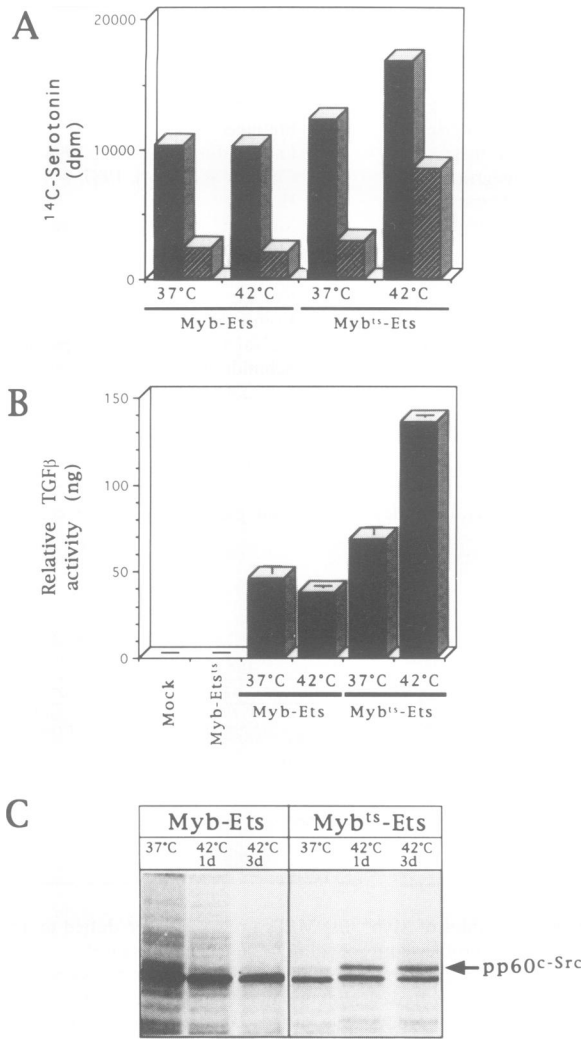

Fig. 4. Up-regulation of thrombocyte-specific functional markers. Myb^{ts}–Ets- and Myb–Ets-transformed MEPs were grown at 37°C and an aliquot shifted to 42°C for 2 days (if not indicated otherwise) before being tested for various biochemical features. (**A**) Uptake and release of 5-hydroxytryptamine (serotonin). Cells were loaded with [^{14}C]serotonin and treated with thrombin (10 U/ml bovine thrombin) or a phosphate-buffered saline control for 5 min. Black bars, total d.p.m. taken up by the cells; cross-hatched bars, ^{14}C d.p.m. released into the supernatant. (**B**) TGFβ production. Cell supernatants were harvested and tested in duplicate for their growth inhibitory activity on an epithelial cell line. Activity is expressed as ng equivalents human recombinant TGFβ-1 measured in the same assay. 'Mock' represents a medium control, 'Myb–Ets^{ts}' represents medium conditioned by *ts*1.1-transformed MEPs. (**C**) Expression of c-Src protein. Cells grown at 37 or shifted to 42°C for either 1 or 3 days were labelled with [^{35}S]methionine and extracts immunoprecipitated with anti-c-Src monoclonal antibody. Immunoprecipitates were then run on a 7.5% SDS gel and radioactivity determined by autoradiography.

sion of predominantly EOS 47 antigen in cells treated with 20 nM TPA or MYL 51/2 in a sub-population of cells treated with 80 nM TPA. Similar results were obtained with the three other Myb^{ts}–Ets-transformed MEP clones. Although all Myb^{ts}–Ets clones tested appeared to be temperature sensitive for thrombocytic differentiation, as well as multipotent, we next wished to determine whether the differentiation of a single Myb^{ts}–Ets transformed clone is blocked by Myb at two distinct stages. Analysis of clone 16 shows that this is the case. As shown in Figure 5B, the clone could be converted qualitatively into thrombocyte-like cells within 2 days after shift to 42°C. The same clone could also be induced to differentiate into predominantly myeloblasts after treatment for 7 days with 80 nM TPA at 37°C. Finally, when these myeloblasts were shifted to 42°C for another 4 days, most of the cells acquired a macrophage-like morphology. No such differentiation could be observed with myeloblasts derived from Myb–Ets-transformed MEPs (data not shown). Similar results were observed with another five clones tested, although the yield of adherent macrophages obtained from myeloblasts after shift was variable.

MEPs transformed by a temperature-sensitive mutant with a lesion in Ets cannot be induced to differentiate along the thrombocytic lineage

To determine whether inactivation of the Ets part of the fusion protein can likewise induce thrombocytic differentiation we examined the effect of shifting Myb–Ets^{ts} (*ts1.1*)-transformed MEPs to the non-permissive temperature. As shown in Figure 6, the thrombocytic marker MEP21 decreased after shift, with a concomitant slight increase in the expression of the erythroid cell surface antigen JS4. In addition, cells lacked expression of the K1 antigen and the integrin $\alpha_{IIb}\beta_3$ (detected by 11C3), while levels of integrin $\alpha_v\beta_3$ (detected by 23C6) decreased. Together with the observed expression of the JS4 antigen in cells at 35°C, these results indicate that the phenotype of the bulk of *ts*1.1 Myb–Ets^{ts}-transformed MEPs more closely resembles erythroblasts than thromboblasts and that these cells are unable to differentiate into thrombocytes.

Myb^{ts} can also control commitment of progenitors to thrombocytes in the absence of v-Ets

Until recently we believed that v-Myb is only capable of transforming haematopoietic progenitors when fused to v-Ets (Graf *et al.*, 1992). However, further analysis of a number of v-Myb-containing viruses revealed that a virus expressing Myb^{ts} as a separate protein in combination with v-ErbB is also capable of transforming MEP-like cells. Typically, an infection of blastoderm cells with a Myb^{ts}/ErbB virus results in ~95% colonies with an immature phenotype resembling MEPs and exhibiting staining with MEP21. The remaining colonies are either of a mature erythroid or promyelocytic phenotype. To determine whether Myb^{ts}/ErbB MEPs can likewise be induced to differentiate along the thrombocyte lineage the expression of thrombocyte-specific cell surface antigens in cells shifted to 42°C was determined. As shown in Figure 7, Myb^{ts}/ErbB-transformed cells shifted to 42°C for 2 days became >85% positive for expression of the thrombocyte-associated K1 antigen, showed a 10-fold increase in levels of integrin $\alpha_{IIb}\beta_3$ (stained by 11C3) and a corresponding elevation in the proportion of its activated form (stained with LIBS6). In contrast, no decrease in MEP21 antigen expression could be observed. Similar changes were not seen in Myb/ErbB-transformed cells. These results demonstrate that the specific influence of v-Myb on thrombocytic differentiation is not confined to its role within the Myb–Ets fusion protein.

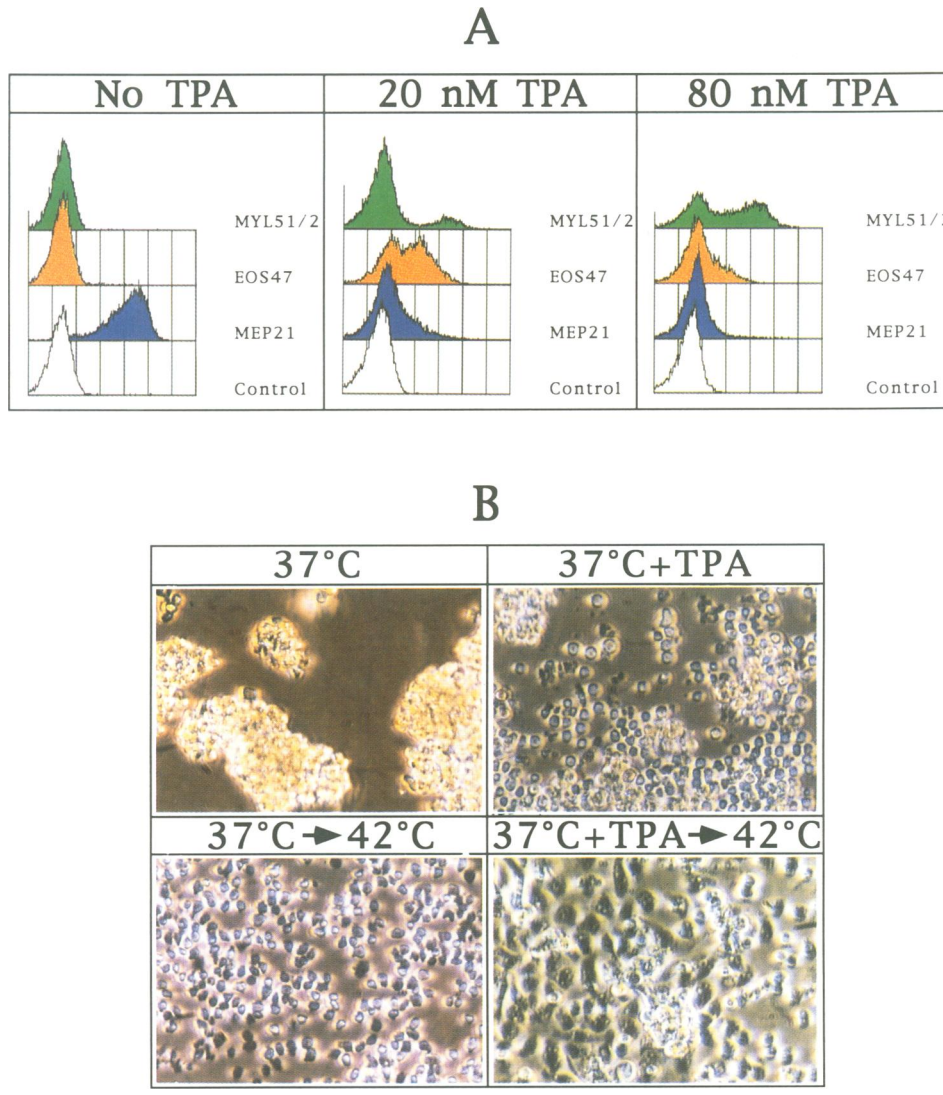

Fig. 5. Multipotency of Myb^{ts}–Ets-transformed MEPs and temperature sensitivity of myeloblast derivatives. (**A**) FACS profiles of TPA-treated MEPs. Myb^{ts}–Ets-transformed MEPs (clone 16) were cultured at 37°C for 7 days in the presence of 20 or 80 nM TPA or left untreated and then assayed for expression of MEP21, EOS47 and MYL51/2 cell surface antigens by immunofluorescence and flow cytometry. The horizontal axis indicates fluorescence intensity on a logarithmic scale. The white peak corresponds to staining with negative control antibody. (**B**) Phase micrographs of Myb^{ts}–Ets MEPs subjected to various protocols. The picture on the upper left shows Myb^{ts}–Ets-transformed clone 16 MEPs grown at 37°C; that on the upper right shows the same clone grown at 37°C for 7 days with 80 nM TPA, now containing predominantly myeloblasts. The picture on the lower left shows thrombocyte-like cells obtained 2 days after shift to 42°C of the 37°C cells (upper left); the picture on the lower right shows macrophage-like cells obtained by shifting TPA-induced myeloblasts (upper right) to 42°C for 4 days.

Discussion

Our results have shown that inactivation of v-Myb in Myb^{ts}–Ets-transformed MEPs by ablation of its DNA binding activity leads to the induction of thrombocytic maturation in the vast majority of the cells. Since it is known that v-Myb also blocks the differentiation of myeloblasts into mature macrophages (Beug *et al.*, 1984) and into granulocytes (T.Ramqvist, J.Frampton and T.Graf; unpublished), this adds a second discrete point in haematopoiesis that can be blocked by the oncogene (Figure 8). In contrast, we have previously shown that the Ets part of the fusion protein inhibits the commitment of Myb–Ets^{ts}-transformed progenitors along the erythroid, myelomonocytic and, possibly, eosinophilic lineages (Kraut *et al.*, 1994; Figure 8) without blocking myelomonocytic differentiation (Golay *et al.*, 1988). A possible reason why we have only now been able to observe the influence of v-Myb on thrombocytic differentiation is the lack of appropriate thrombocytic markers and the use of bone marrow-derived MEPs in earlier studies.

The differences in biological effects resulting from selective inactivation of either of the two DNA binding domains in Myb–Ets suggests that at least part of the fusion protein's transforming capacity results from the independent activities of the v-Myb and v-Ets DNA binding domains. For the following reasons we do not think that the lesions in a given DNA binding domain influence the ability of the other domain to recognize DNA through intramolecular conformational changes. First, the *ts* mutation in the Myb DNA binding domain is effective in releasing the block to thrombocyte differentiation at 42°C in both the context of the Myb^{ts}–Ets fusion protein and as a Myb^{ts} protein expressed in conjunction with v-ErbB. Second, the *ts* mutation in v-Ets does not inhibit

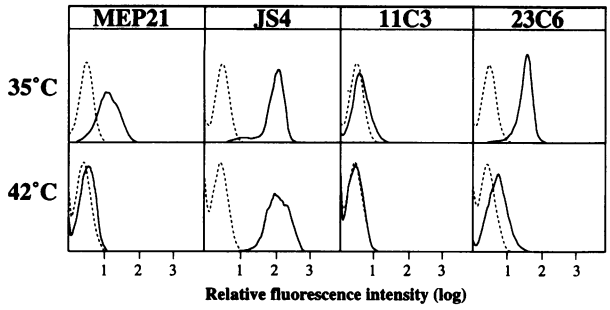

Fig. 6. Cell surface antigens of *ts*1.1 (Myb–Ets^{ts})-transformed MEPs. Cells were grown at 35 or shifted to 42°C for 3 days and analysed by immunofluorescence/flow cytometry with MEP21, JS4, 11C3 and 23C6 antibodies. Specific staining is indicated by the profiles traced with a thick line; the profile corresponding to the negative control antibody is depicted by the dashed line.

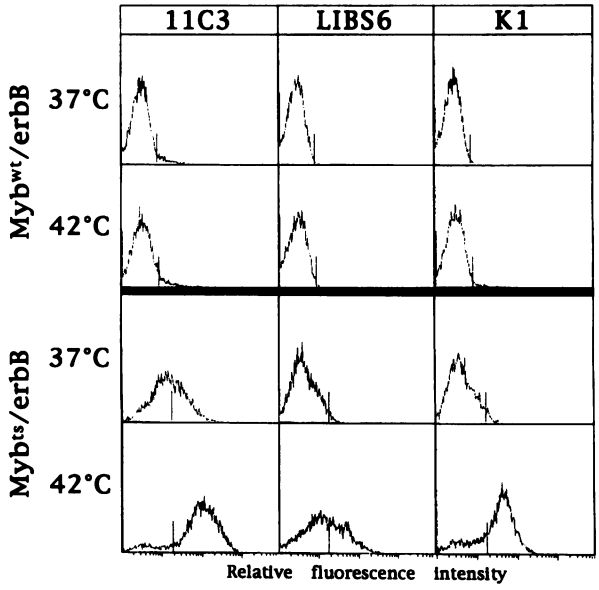

Fig. 7. Expression of cell surface antigens on Myb/ErbB and Myb^{ts}/ErbB cells. Cells were grown at 37 or shifted to 42°C for 2 days and analysed by immunofluorescence/flow cytometry using integrin-specific antibodies. The vertical line indicates the maximum fluorescence intensity observed with negative control antibody stained cells.

the myeloid cell-transforming capacity of v-Myb (Golay *et al.*, 1988). Third, the Myb-regulated gene *mim*-1, whose expression can be abolished in myeloid cells transformed by Myb^{ts}–Ets shifted to 42°C, is not decreased in Myb–Ets^{ts}-transformed myeloid cells at the non-permissive temperature (J.Frampton, unpublished).

The present studies have shown that both Myb–Ets- and Myb^{ts}–Ets-transformed MEPs closely resemble thromboblasts (the avian equivalents of mammalian megakaryoblasts) in a number of antigenic and functional features. Interestingly, Myb–Ets^{ts}-transformed MEPs resemble erythroblasts rather than thromboblasts (see also Kraut *et al.*, 1994) and can be induced to mature into erythrocyte-like cells when Ets is inactivated by a shift to the non-permissive temperature (Golay *et al.*, 1988; Kraut *et al.*, 1994) without giving rise to cells of the thrombocytic lineage. However, both MEP cell types, which we now call thrombocytic MEPs (T-MEPs) and erythroid MEPs (E-MEPs) respectively (Figure 8), share the capacity to

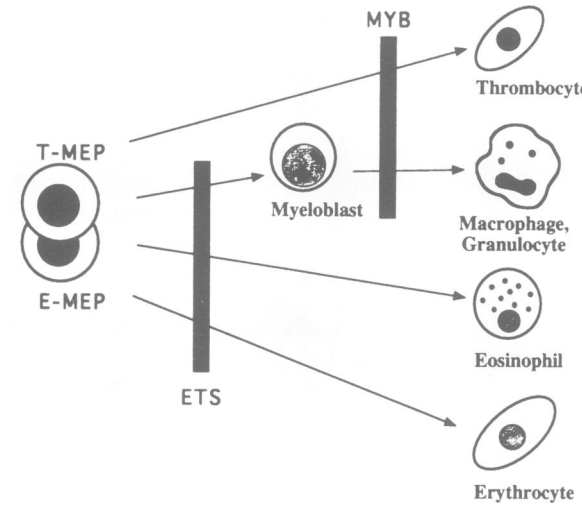

Fig. 8. Summary diagram. Positions at which the v-Myb and v-Ets moieties of the Myb–Ets fusion protein block differentiation are indicated by solid bars. The Myb–Ets-transformed progenitors are represented as two similar, but distinct, cell types, with Myb–Ets- and Myb^{ts}–Ets-transformed cells resembling predominantly thromboblasts (T-MEP) and Myb–Ets^{ts}-transformed cells resembling predominantly erythroblasts (E-MEP). The v-Ets effects depicted are based on an earlier study (Kraut *et al.*, 1994), which showed that inactivation of Ets in Myb–Ets^{ts}-transformed MEPs induces the formation of erythrocytes and to a lesser extent myeloblasts and probably also eosinophils.

differentiate along the eosinophilic and myelomonocytic lineages following TPA treatment (Graf *et al.*, 1992; Kraut *et al.*, 1994; this study).

One interpretation of the observed low or absent thrombocytic marker expression in Myb–Ets^{ts}-transformed MEPs is that the Ets domain is already partly disabled at the permissive temperature (Kraut *et al.*, 1994) and that v-Ets in the context of the fusion protein is required to regulate transcription of genes involved in thrombocytic differentiation. Indeed, several megakaryocyte/thrombocyte-specific gene promoters appear to be regulated by c-Ets in cooperation with GATA-1 (Ravid *et al.*, 1991; Lemarchandel *et al.*, 1993; Pan and McEver, 1993).

There is an intriguing resemblance between Myb–Ets T-MEPs and cells transformed by the murine MPLV virus, which encodes an activated form of the thrombopoietin receptor c-Mpl (de Sauvage *et al.*, 1994; Wendling *et al.*, 1994). MPLV-transformed clones are capable of differentiating into several lineages, including megakaryocytes, erythrocytes and myelomonocytic cells (Souyri *et al.*, 1990). In addition, the finding that expression of c-Mpl RNA in normal haematopoietic cells is highest in megakaryocytes (Methia *et al.*, 1993) suggests that the murine target cells for v-Mpl correspond to multipotent progenitors with some characteristics of megakaryocytes.

Previous studies have shown that neither v-Myb nor v-Ets (or their respective *ts* alleles) on their own are sufficient to transform MEPs (Metz and Graf, 1991a). This capacity is restricted to the fusion of v-Myb (or v-Myb^{ts}) with v-Ets or, as we have described here, to a virus co-expressing v-Myb (or v-Myb^{ts}) with v-ErbB. In view of the fact that constructs co-expressing v-Myb and v-Ets as separate proteins transform cells that resemble erythroblasts (Metz and Graf, 1991a), it is surprising that co-expression of v-Myb with v-ErbB leads to the

transformation of T-MEPs. The ability of v-ErbB to cooperate with v-Myb in transforming T-MEPs suggests that the tyrosine kinase oncoprotein modifies the activity of v-Myb. This could be either direct, such as by phosphorylation of v-Myb, or indirect, such as by modification or induction of the expression of an unknown cooperating factor, perhaps an Ets family member. Taken together with our failure to observe transformed progenitors expressing a combination of thrombocytic and erythroid markers, these observations imply that v-Myb and v-Ets influence cell fate decisions in a mutually exclusive but complex manner.

The biological function of v-Myb in the system described may reflect, at least in part, the function of the corresponding cellular protein. Thus it has been shown that c-Myb can prevent the G-CSF-induced differentiation of a transformed myelomonocytic cell line towards macrophages (Yanagisawa *et al.*, 1991; Selvakumaran *et al.*, 1992). In addition, constitutive expression of c-Myb has been shown to block the chemically induced differentiation of murine erythroid leukaemia cells (Clarke *et al.*, 1988; McMahon *et al.*, 1988; Todokoro *et al.*, 1988). The observation that mice lacking c-Myb expression are not deficient in the formation of mature megakaryocytes (Mucenski *et al.*, 1991) is compatible with our finding that v-Myb acts to block differentiation of thromboblasts to mature thrombocytes.

The finding that Myb–Ets-transformed T-MEPs are functionally multipotent raises the possibility that they consist of two populations of cells; the vast majority representing thromboblasts and a minor fraction being committed to differentiate along the eosinophilic and myelomonocytic lineages. However, we favour the alternative possibility that a sub-population of T-MEPs, which expresses thrombocytic markers, exhibits a high degree of plasticity and can be reprogrammed to acquire new phenotypes. Such an interpretation would be in accord with our earlier finding that following TPA treatment, Myb–Ets-transformed T-MEPs down-regulate the thrombocyte-specific markers MEP21 and integrin $\alpha_{IIb}\beta_3$ before converting into eosinophils or myeloblasts (Graf *et al.*, 1992, unpublished results). In addition, we have recently found that fully committed myelomonocytic cell lines can be reprogrammed to resemble thromboblasts, erythroblasts or eosinophils if they ectopically express GATA-1 (H.Kulessa, J.Frampton and T.Graf, unpublished results). Taken together, these observations imply that even after commitment, haematopoietic cells exhibit a remarkable degree of phenotypic plasticity.

Studies of megakaryocyte/thrombocyte differentiation have been limited by their relative scarcity in bone marrow. This has been partly overcome by the isolation of human cell lines from leukaemia patients which are capable of undergoing some aspects of differentiation, for example polyploidization and up-regulation of thrombocyte-specific genes (reviewed in Hoffman, 1989). However, these immortalized cell lines carry a number of karyotypic abnormalities and their differentiation can only be induced with chemicals such as phorbol esters, which probably have pleiotropic effects. The *ts* mutant-transformed T-MEP system described here offers the advantage that thrombocytic differentiation can be studied in primary cells transformed by a transcription factor-type oncogene which can be inactivated by temperature shifts. This system might become particularly useful in the search for v-Myb target genes in multipotent progenitors resembling thromboblasts.

Materials and methods

Plasmids and bacterial expression of Myb

A pET construct for the bacterial expression of Myb was derived from the pET3b vector (Novagen). Because the full-length fusion protein is very difficult to produce in bacteria, we opted to express the EEA Gag–Myb derivative of v-Myb. Sequences were cloned from the Gag ATG into the *NdeI* site of pET3b to the *XbaI* site at the termination codon of EEA Myb. Protein was expressed following transformation into the bacterial strain BL21(DE3). Crude protein preparations (containing 20–50% Myb protein) were made from freshly picked colonies which had been grown to exponential phase and then induced for 3 h by the addition of 1 mM IPTG. The bacterial pellet was resuspended and sonicated in 1 vol 10 mM HEPES, pH 7.9, 100 mM KCl. After centrifugation, the pellet material was resuspended in 1 vol STED (40 mM Tris–HCl, pH 7.4, 0.25 mM EDTA, 1 mM dithiothreitol (DTT), 25% w/v sucrose). Proteins in the pellet were denatured by adding urea to a concentration of 4 M at 4°C for 30 min and following centrifugation the solubilized denatured proteins were renatured by dialysis against STED, 1 M urea and then STED.

Gel mobility shift assays

These were performed essentially as described in Frampton *et al.* (1991). Binding reactions in 25 μl 20 mM Tris–HCl, pH 6.8, 20% glycerol, 50 mM Kcl, 1 mM DTT contained 2 ng ^{32}P-end-labelled Myb recognition site MRE-A (Ness *et al.*, 1989), 1 μg poly(dI–dC)·poly(dI–dC) and 10 mM EDTA (to inhibit contaminating DNases in the bacterial proteins). Bacterial protein (5 μl) was initially added to binding reactions in the absence of the labelled oligonucleotide and pre-incubated at the temperature of binding and gel separation for 1 h. Labelled MRE-A was then added and incubation continued for a further 20 min. Samples were resolved on 6% polyacrylamide, 0.5× TBE gels at the same temperature as the binding reaction. Gels had been equilibrated at the relevant temperature for 3 h.

Cell culture

All normal chicken cells were obtained from commercially produced Valo eggs. Our standard growth medium, 'blastoderm medium', contained Dulbecco's modified Eagle's medium (DMEM), 10% fetal calf serum (FCS), 2.5% chicken serum, 0.2% $NaHCO_3$, 56 μg/ml conalbumin, 80 mM β-mercaptoethanol and 0.9 μg/ml insulin. Unless otherwise indicated, all cells were maintained at 37°C in 5% CO_2.

Preparation of peripheral blood thrombocytes

Normal peripheral blood thrombocytes were enriched by a combination of density separation and adherence. Briefly, 10 ml heparinized blood was centrifuged for 5 min at 1000 r.p.m. to remove the bulk of the erythrocytes. The supernatant was then layered over Ficoll-Paque (density 1.077; Eurobio, Paris, France) and spun at 2500 r.p.m. for 20 min; cells collecting at the interface were washed in Hank's balanced salts solution (HBSS; Gibco) and incubated at 39°C on tissue culture plates for 2 h in the same medium. Adherent cells were eluted at 4°C using HBSS containing 0.5 mM EDTA.

Blastoderm transformation assay

Blastoderm cells were obtained from 2 day-old chick embryos essentially as described by Moscovici *et al.* (1983) and Graf *et al.*, (1992). They were infected and seeded in blastoderm medium containing 0.8% methylcellulose (Fluka). These conditions allow outgrowth of transformed MEPs, myelomonocytic cells and eosinophils. Individual transformed colonies were isolated after 10 days using drawn-out Pasteur pipettes and seeded for expansion into blastoderm medium supplemented with 1–2% concanavalin A conditioned medium containing cMGF (Leutz *et al.*, 1984) in microtitre wells.

Cell surface and cytoplasmic characterization of cells

Surface immunofluorescent staining of cells was performed essentially as described in Radke *et al.* (1982). Cells were incubated with the appropriate dilution of first antibody at 4°C for 10 min, followed by washing and incubation with fluorescein (FITC)-conjugated goat anti-

mouse second antibody at 4°C for 20 min. The antibodies employed are listed in Table I. Stained cells were analysed on a Becton Dickinson FACScan flow cytometer using Consort software. To visualize the morphology of individual transformed colonies, cells were subjected to cytocentrifugation and stained with May Grünwald-Giemsa (Diff Quick; Harleco).

Labelling of proteins in live cells with [^{35}S]methionine and ^{125}I

Biosynthetic labelling with [^{35}S]methionine and labelling of cell surface proteins with ^{125}I (Amersham) by lactoperoxidase catalysed iodination were performed as described in McNagny *et al.* (1992).

Serotonin uptake/release assay

Cells were pelleted and resuspended in apyrase (1 U/ml in blastoderm medium) at $>10^7$/ml. [^{14}C]Serotonin was added at a concentration of 1 μM to 50 μl aliquots of cells and incubated at 37°C for 10 min to allow uptake. The cells were then washed twice with medium and imipramine was added to a concentration of 2 μM. Thrombin, or an equivalent volume of phosphate-buffered saline, was added to a final concentration of 10 U/ml and incubated at 37°C for 5 or 20 min. The release of serotonin was stopped by the addition of 15 μl 0.6 M formaldehyde, 0.05 M EDTA. The cell supernatant was cleared by centrifugation and aliquots were counted in scintillation fluid in a β-counter. Cell pellets were lysed in 1% Triton X-100 in order to determine the remaining radioactivity.

TGFβ assay

TGFβ activity was assessed in medium conditioned by cells grown at either 37 or 42°C; cells were suspended in HBSS at 37°C for 10 min with or without the addition of 10 U/ml thrombin. Conditioned cell supernatants were acid activated by addition of 25 μl/ml 1.8 N HCl for 5 min at room temperature in the presence of proteinase inhibitors and 1 mg/ml BSA. They were subsequently neutralized by addition of 35 μl/ml 1 M HEPES, pH 7.4, 5 N NaOH (5:2 v/v). For the assays, Mv1lu mink lung epithelial cells grown in DMEM, 10% FCS were plated in 0.2% FCS medium at 5×10^4 cells/24-well dish. Starting with 2.5 μl conditioned medium, serial 1:1 dilutions were added to the cells. After 22 h the cells were labelled for 2 h with 0.5 mCi/well [^{3}H]thymidine (Amersham) and fixed for a minimum of 1 h in methanol:acetic acid (3:1 v/v). After two washes with 80% methanol they were digested for 1 h at room temperature with 0.5 ml of a 0.2 mg/ml trypsin solution (Gibco) and solubilized with 0.5 ml 1% SDS for 5 min. After addition of 9 ml scintillation fluid (Readysafe; Beckman) radioactivity was measured in a β-counter. Human recombinant TGFβ-1 was used for standardization and pan-specific anti-TGFβ polyclonal rabbit IgG (R&D systems) for neutralization.

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References

Beug,H., Leutz,A., Kahn,P. and Graf,T. (1984) Ts mutants of E26 leukemia virus allow transformed myeloblasts, but not erythroblasts or fibroblasts to differentiate at the nonpermissive temperature. *Cell*, **39**, 579–588.

Beug,H., Blundell,P. and Graf,T. (1987) Reversibility of differentiation and proliferative capacity in avian myelomonocytic cells transformed by *ts*E26 leukemia virus. *Genes Dev.*, **1**, 277–286.

Chen,C.L., Lehmeyer,J.E. and Cooper,M.D. (1982) Evidence for an IgD homologue on chicken lymphocytes. *J. Immunol.*, **129**, 2580–2585.

Chen,C.L., Ager,L.L., Gartland,G.L. and Cooper,M.D. (1986) Identification of a T3/T cell receptor complex in chickens. *J. Exp. Med.*, **164**, 375–380.

Cheresh,D.A. and Spiro,R.C. (1987) Biosynthetic and functional properties of an Arg-Gly-Asp-directed receptor involved in human melanoma cell attachment to vitronectin, fibrinogen, and von Willebrand factor. *J. Biol. Chem.*, **262**, 17703–17711.

Clarke,M.F., Kukowska-Latallo,J.F., Westin,E., Smith,M. and Prochownik,E.W. (1988) Constitutive expression of a c-*myb* cDNA blocks Friend murine erythroleukemia cell differentiation. *Mol. Cell. Biol.*, **8**, 884–892.

de Sauvage,F.J. *et al.* (1994) Stimulation of megakaryocytopoiesis and thrombopoiesis by the c-Mpl ligand. *Nature*, **369**, 533–538.

Dexter,T.M. and Spooncer,E. (1987) Growth and differentiation in the hemopoietic system. *Annu. Rev. Cell Biol.*, **3**, 423–441.

Dieterlen-Lièvre,F. (1988) Birds. In Rowley,A.F. and Ratcliffe,N.A. (eds), *Vertebrate Blood Cells*. Cambridge University Press, pp. 257–336.

Ewert,D.L., Munchus,M.S., Chen,C.L. and Cooper,M.D. (1984) Analysis of structural properties and cellular distribution of avian Ia antigen using monoclonal antibody to monomorphic determinants. *J. Immunol.*, **132**, 2524–2530.

Frampton,J., Gibson,T.J., Ness,S.A., Döderlein,G. and Graf,T. (1991) Proposed structure for the DNA binding domain of the Myb oncoprotein based on model building and mutational analysis. *Protein Engng*, **4**, 891–901.

Frelinger,A.L., Du,X., Plow,E.F. and Ginsberg,M.H. (1991) Monoclonal antibodies to ligand-occupied conformers of integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb-IIIa) alter receptor affinity, specificity, and function. *J. Biol. Chem.*, **266**, 17106–17111.

Frykberg,L., Metz,T., Brady,G., Introna,M., Beug,H., Vennström,B. and Graf,T. (1988) A point mutation in the DNA binding domain of the v-*myb* oncogene of E26 virus confers temperature sensitivity for transformation of myelomonocytic cells. *Oncogene Res.*, **3**, 313–322.

Furukawa,Y., Piwnica-Worms,H., Ernst,T.J., Kanakura,Y. and Griffin,J.D. (1990) *cdc2* gene expression at the G1 to S transition in human T lymphocytes. *Science*, **250**, 805–808.

Gewirtz,A.M. and Calabretta,B. (1988) A c-*myb* antisense oligonucleotide inhibits normal human hematopoiesis *in vitro*. *Science*, **242**, 1303–1306.

Golay,J., Introna,M. and Graf,T. (1988) A single point mutation in the v-*ets* oncogene affects both erythroid and myelomonocytic cell differentiation. *Cell*, **55**, 1147–1158.

Golden,A., Nemeth,S.P. and Brugge,J.S. (1986) Blood platelets express high levels of the pp60$^{c\text{-}src}$-specific tyrosine kinase activity. *Proc. Natl Acad. Sci. USA*, **83**, 852–856.

Graf,T. (1992) Myb: a transcriptional activator linking proliferation and differentiation in hematopoietic cells. *Curr. Opin. Genet. Dev.*, **2**, 249–255.

Graf,T. and Beug,H. (1978) Avian leukaemia viruses: interaction with their target cells *in vivo* and *in vitro*. *Biochim. Biophys. Acta Rev. Cancer*, **516**, 269–299.

Graf,T., McNagny,K.M., Brady,G. and Frampton,J. (1992) Chicken 'erythroid' cells transformed by the Gag-myb-ets encoding E26 leukemia virus are multipotent. *Cell*, **70**, 201–213.

Hawiger,J. (1992) Repertoire of platelet receptors. In Hawiger,J. (ed.), *Methods in Enzymology*, Vol. 215. Academic Press, New York, NY, pp. 131–136.

Hoffman,R. (1989) Regulation of megakaryocytopoiesis. *Blood*, **74**, 1196–1212.

Horton,M.A., Lewis,D., McNulty,K., Pringle,J.A.S. and Chambers,T.J. (1985) Monoclonal antibodies to osteoclastomas (giant cell bone tumours): definition of osteoclast-specific antigens. *Cancer Res.*, **45**, 5663–5669.

Kaspers,B., Lillehoj,H.S. and Lillehoj,E.P. (1993) Chicken macrophages and thrombocytes share a common cell surface antigen defined by a monoclonal antibody. *Vet. Immunol. Immunopathol.*, **36**, 333–346.

Kastan,M.B., Stone,K.D. and Civin,C.I. (1989) Nuclear oncoprotein expression as a function of lineage, differentiation stage, and proliferation status of normal human hematopoietic cells. *Blood*, **74**, 1517–1524.

Kornfeld,S., Beug,H., Döderlein,G. and Graf,T. (1983) Detection of avian hematopoietic cell surface antigens with monoclonal antibodies to myeloid cells: their distribution on normal and leukemic cells of various lineages. *Exp. Cell Res.*, **143**, 383–394.

Kraut,N., Frampton,J., McNagny,K.M. and Graf,T. (1994) A functional Ets DNA-binding domain is required to maintain multipotency of hematopoietic progenitors transformed by Myb-Ets. *Genes Dev.*, **8**, 33–44.

Lacoste-Eleaume,A.-S., Bleux,C., Quéré,P., Courdert,F., Corbel,C. and Kanellopoulos-Langevin,C. (1994) Biochemical and functional characterization of an avian homolog of the integrin GPIIb-IIIa present on chicken thrombocytes. *Exp. Cell. Res.*, **213**, 198–209.

Lemarchandel,V., Ghysdael,J., Vignotte,V., Rahuel,C. and Romeo,P.-H. (1993) GATA and Ets *cis*-acting sequences mediate megakaryocyte-specific expression. *Mol. Cell. Biol.*, **13**, 668–676.

Leprince,D., Gegonne,A., Coll,J., deTaisne,C., Schneeberger,A., Lagrou,C. and Stehelin,D. (1983) A putative second cell-derived oncogene of the avian leukemia retrovirus E26. *Nature*, **306**, 395–397.

Leutz,A., Beug,H. and Graf,T. (1984) Purification and characterization of cMGF, a novel chicken meyelomonocytic growth factor. *EMBO J.*, **3**, 3191–3197.

Lüscher,B. and Eisenman,R.N. (1990) New light on MYC and MYB. Part II. MYB. *Genes Dev.*, **4**, 2235–2241.

McMahon,J., Howe,K.M. and Watson,R.J. (1988) The induction of Friend erythroleukaemia differentiation is markedly affected by expression of a transfected c-*myb* cDNA. *Oncogene*, **3**, 717–720.

McNagny,K.M., Lim,F., Grieser,S. and Graf,T. (1992) Cell surface proteins of chicken hematopoietic progenitors, thrombocytes and eosinophils detected by novel monoclonal antibodies. *Leukemia*, **6**, 975–984.

Methia,N., Louache,F., Vainchenker,W. and Wendling,F. (1993) Oligodeoxynucleotides antisense to the proto-oncogene c-*mpl* specifically inhibit *in vitro* megakaryocytopoiesis. *Blood*, **82**, 1395–1401.

Metz,T. and Graf,T. (1991a) v-*myb* and v-*ets* transform chicken erythroid cells and cooperate in *trans* and in *cis* to induce distinct differentiation phenotypes. *Genes Dev.*, **5**, 369–380.

Metz,T. and Graf,T. (1991b) Fusion of the nuclear oncoproteins v-Myb and v-Ets is required for the leukemogenicity of E26 virus. *Cell*, **66**, 95–105.

Mölling,K., Pfaff,E., Beug,H., Beimling,P., Bunte,T., Schaller,H.E. and Graf,T. (1985) DNA-binding activity is associated with purified *myb* proteins from AMV and E26 viruses and is temperature-sensitive for E26 *ts* mutants. *Cell*, **40**, 983–990.

Moscovici,M.G., Jurdic,P., Samarut,J., Gazzolo,L., Mura,C.V. and Moscovici,C. (1983) Characteristics of the hemopoietic target cells for the avian leukemia virus E26. *Virology*, **129**, 65–78.

Mucenski,M.L., McLain,K., Kier,A.B., Swederlow,S.H., Schreiner,C.M., Miller,T.A., Pietryga,D.W., Scott,W.J.,Jr and Potter,S.S. (1991) A functional c-*myb* gene is required for normal murine fetal hepatic hematopoiesis. *Cell*, **65**, 677–689.

Ness,S.A., Marknell,Å. and Graf,T. (1989) The v-*myb* oncogene product binds to and activates the promyelocytic-specific *mim-1* gene. *Cell*, **59**, 1115–1125.

Nunn,M., Seeburg,P.H., Moscovici,C., and Duesberg,P.H. (1983) Tripartite structure of the avian erythroblastosis virus E26 transforming gene. *Nature*, **306**, 391–395.

Pan,J. and McEver,R.P. (1993) Characterization of the promoter for the human P-selectin gene. *J. Biol. Chem.*, **268**, 22600–22608.

Radke,K., Beug,H., Kornfeld,S. and Graf,T. (1982) Transformation of both erythroid and myeloid cells by E26, an avian leukemia virus that contains the myb gene. *Cell* **31**, 643–653.

Ravid,K., Doi,T., Beeler,D.L., Kuter,D.J. and Rosenberg,R.D. (1991) Transcriptional regulation of the rat platelet factor 4 gene: interaction between an enhancer-silencer domain and the GATA site. *Mol. Cell. Biol.*, **11**, 6116–6127.

Schmidt,J.A., Marshall,J., Hayman,M.K., Döderlein,G. and Beug,H. (1986) Monoclonal antibodies to novel erythroid differentiation antigens reveal specific effects of oncogenes on the leukemic phenotype. *Leukemia Res.*, **10**, 257–272.

Selvakumaran,M., Liebermann,D.A. and Hoffman-Liebermann,B. (1992) Deregulated c-*myb* disrupts interleukin-6- or leukemia inhibitory factor-induced myeloid differentiation prior to c-*myc*: role in leukemogenesis. *Mol. Cell. Biol.*, **12**, 2493–2500.

Souyri,M., Vigon,I., Penciolelli,J.-F., Heard,J.-M., Tambourin,P. and Wendling,F. (1990) A putative truncated cytokine receptor gene transduced by the myeloproliferative leukemia virus immortalizes hematopoietic progenitors. *Cell*, **63**, 1137–1147.

Thompson,C.B., Chaloner,P.B., Neiman,P.E. and Groudine,M. (1986) Expression of the c-*myb* proto-oncogene during cellular proliferation. *Nature*, **319**, 374–380.

Todokoro,K., Watson,R.J., Higo,H., Amanuma,H., Kuramochi,S., Yanagisawa,H. and Ikawa,Y. (1988) Down-regulation of c-*myb* gene expression is a prerequisite for erythropoietin-induced erythroid differentiation. *Proc. Natl Acad. Sci. USA*, **85**, 8900–8904.

Valtieri,M. *et al.* (1991) Antisense Myb inhibition of purified erythroid progenitors in development and differentiation is linked to cycling activity and expression of DNA polymerase α. *Blood*, **77**, 1181–1190.

Wendling,F. *et al.* (1994) c-Mpl ligand is a humoral regulator of megakaryocytopoiesis. *Nature*, **369**, 571–574.

Westin,E.H., Gallo,R.C., Arya,S.K., Eva,A., Souza,L.M., Baluda,M.A., Aaronson,S.A. and Wong-Staal,F. (1982) Differential expression of the *amv* gene in human hematopoietic cells. *Proc. Natl Acad. Sci. USA*, **79**, 2194–2198.

Yanagisawa,H., Nagasawa,T., Kuramochi,S., Abe,T., Ikawa,Y. and Todokoro,K. (1991) Constitutive expression of exogenous c-*myb* gene causes maturation block in monocyte-macrophage differentiation. *Biochim. Biophys. Acta*, **1088**, 380–384.

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