The EMBO Journal vol.12 no.4 pp.1333-1341, 1993

Influence of the v-Myb transactivation domain on the oncoprotein's transformation specificity

J.Frampton[3], T.Kouzarides[1], G.Döderlein, T.Graf and K.Weston[2]

Differentiation Programme, European Molecular Biology Laboratory, Postfach 10.2209, 6900 Heidelberg, Germany, [1]Wellcome/CRC Institute, Cambridge CB2 2QQ and [2]Institute of Cancer Research, Chester Beatty Laboratories, 237 Fulham Road, London SW3 6JB, UK
[3]Corresponding author

Communicated by T.Graf

The *v-myb*-containing viruses AMV and E26 induce the proliferation of myelomonocytic cells. The E26 Myb protein, by virtue of its fusion to Ets, is also able to transform multipotent haematopoietic cells (MEPs). We have examined the biological effects of substituting the v-Myb transactivation domain with the strong acidic activator domain from the C-terminus of the HSV-1 VP16 protein. In the absence of Ets, deletion of the transactivation domain destroyed the ability of v-Myb to stimulate transcription and to transform cells, whilst the substitution of the VP16 transactivation domain into v-Myb resulted in a greatly enhanced transactivation potential and altered TATA box binding protein (TBP) binding properties. In spite of these functional differences, the v-MybVP16 protein regained the ability to transform myeloid cells with the same characteristics as wild type v-Myb. A construct encoding v-MybVP16 fused to v-Ets was still capable of inducing leukaemia and of transforming both myeloid cells and MEPs *in vitro*, although the latter cells exhibited an altered phenotype. Our results demonstrate that the transformation of myeloid cells by v-Myb is largely independent of the type and potency of the transactivation domain it contains, whereas transformation of MEPs by the Myb-Ets fusion protein has more stringent transactivation requirements of Myb.

Key words: haematopoietic transformation/Myb/transcriptional regulation/VP16

Introduction

The v-*myb* oncogene is contained in two acutely leukaemogenic avian retroviruses, avian myeloblastosis virus (AMV) and E26 (for reviews see Graf and Beug, 1978; Graf, 1992). AMV transforms myeloid cells and causes a monoblastic leukaemia, whereas E26 transforms myeloid and erythroid cells *in vitro* and causes predominantly an erythroleukaemia (Radke *et al.*, 1982; Moscovici *et al.*, 1983). The 'erythroid' cells have recently been shown to be multipotent and have been designated as 'Myb-ets transformed progenitors' or MEPs (Graf *et al.*, 1992). Truncation of c-Myb has been demonstrated to be sufficient to activate its myeloid transforming potential (Gonda *et al.*, 1989; Grässer *et al.*, 1991; Hu *et al.*, 1991). In both AMV and E26, the v-Myb sequences have lost N- and C-terminal residues relative to c-Myb. In addition, in E26 the C-terminus of v-Myb is fused to sequences related to the c-*ets1* proto-oncogene; these latter are largely responsible for the progenitor cell transforming properties of the E26 virus (Leprince *et al.*, 1983; Nunn *et al.*, 1983; Metz and Graf, 1991a,b).

The c-*myb* proto-oncogene is expressed predominantly in immature haematopoietic cells; its product is located in the nucleus and like v-Myb can act as a sequence-specific transcription factor (for reviews see Lüscher and Eisenman, 1990; Graf, 1992). c-Myb function can apparently be regulated in haematopoietic cells by lineage-specific components, since *mim1*, the only cellular gene known to be regulated by Myb is only expressed in a restricted compartment of the myeloid lineage (Ness *et al.*, 1989).

At least three functional domains have been characterized in c-Myb (for a review see Graf, 1992): (i) a DNA binding domain near the N-terminus, which recognizes the consensus $^{T}/_{C}AAC^{T}/_{G}G$ (Biedenkapp *et al.*, 1988; Weston, 1992), and consists of three repeats, each of which contains a helix–turn–helix type motif (Frampton *et al.*, 1989, 1991); (ii) a centrally located transactivation domain (Sakura *et al.*, 1989; Weston and Bishop, 1989); and (iii) a domain near the C-terminus which appears to regulate negatively the ability of Myb to function in transactivation and DNA binding (Gonda *et al.*, 1989; Sakura *et al.*, 1989; Hu *et al.*, 1991; Ramsay *et al.*, 1991). The N-terminal sequences lost in both the AMV and E26 Myb proteins include most of the first repeat of the DNA binding domain, whilst the C-termini are truncated such that the negative regulatory domain is lost. Generally, the integrity of both the DNA binding and transactivation domains is necessary for Myb to function in transactivation and transformation (Ibanez and Lipsick, 1988; Weston and Bishop, 1989; Lane *et al.*, 1990); however, transactivation independent of DNA binding has also been described (Klempnauer *et al.*, 1989).

The transactivation domain of Myb appears to be different from the major defined classes of transactivation domains (for a review see Mitchell and Tjian, 1989). Members of the most studied class of transactivation domains contain acidic motifs which are often α helical. A comparison of the Myb activator sequences with the paradigmatic acidic activator domain of the herpes simplex virus 1 VP16 protein (Triezenberg *et al.*, 1988) reveals both similarities and differences (Figure 1): (i) the Myb sequences are highly charged, but unlike the typical acidic activators, have approximately equal numbers of positively and negatively charged residues; (ii) many acidic activators are characterized by segments of predicted α helical structure with an amphipathic distribution of the negatively charged residues; in contrast, although the activator domain of Myb is predicted to be an α helix, it contains a cluster of both positively and negatively charged residues which are arranged around, rather than on one side of, the helix.

Given the apparent differences between the Myb transactivation domain and typical acidic activators, we have asked if an example of the latter is capable of functioning in the context of Myb, and if so, whether substitution of the unique Myb transactivation structure affects its transformation phenotype. As a substitute, we chose the activator domain of the VP16 protein since it can function in heterologous proteins (for example, see Sadowski *et al.*, 1988; Cousens *et al.*, 1989; Elliston *et al.*, 1990; Ibanez and Lipsick, 1990), and its structure and interactions with the basic transcription machinery have been well studied (for recent reviews see Greenblatt, 1991, and Struhl, 1991). In this paper, we show that the Myb transactivation domain, whether in the context of Myb alone or in combination with Ets, can be replaced by the activator domain of VP16 with full restoration of function in terms of transactivation, transformation and leukaemogenicity. Interestingly, the phenotype of the Myb-Ets transformed MEPs but not that of Myb or Myb-Ets transformed myeloid cells was altered. We further demonstrate that the Myb and VP16 transactivation domains are structurally distinct, as determined by their differential binding to the TATA box binding protein, TBP. We have therefore manufactured a synthetic Myb protein, which, although apparently distinct in terms of its mechanism of transactivation, produces a transformation phenotype similar, but not identical, to that produced by wild type v-Myb.

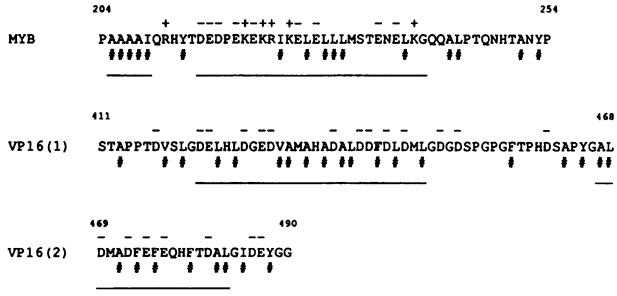

Fig. 1. Comparison of the transactivation domain of v-Myb with the α helical acidic activator domain from VP16. The VP16 sequence is shown in two segments; that designated VP16(1) is the shorter sequence present in the MybVP16ΔC-Ets construct, and segment VP16(2) is additionally present in the longer chimeras. Charged and hydrophobic residues are indicated by +/− and # respectively. Regions predicted to be α helical are underlined.

Results

The transactivation domain of v-Myb can be functionally replaced by sequences from the HSV-1 VP16 activator motif

To determine the effects of substituting the Myb transactivator domain, we first constructed a deletion mutant known to abolish the protein's activity (Weston and Bishop, 1989). We employed a deletion mutant in which 17 amino acids (residues 215−231) of the AMV v-Myb activator domain were removed, and reconstructed this deletion into the EAA retroviral background [a construct which expresses a chimeric E26-AMV Gag-Myb protein (Introna *et al.*, 1990)]. As shown in Figure 2, this deletion mutant (hereafter referred to as MybΔ) completely ablated transactivation of the *mim1* promoter attached to a luciferase marker gene (Ness *et al.*, 1989). Next, we inserted the activator domain sequences of the HSV-1 VP16 protein (residues 411−490, Triezenberg *et al.*, 1988) into MybΔ, to give MybVP16. The MybVP16 chimera was found to be ~10-fold more active than the wild type Myb in its ability to transactivate *mim1* (Figure 2).

The VP16 transactivating motif also functions in the background of the Myb-Ets fusion protein of the E26 virus

To determine the effect of substituting the transactivation domain of v-Myb in the context of the E26 Gag-Myb-Ets fusion protein, we next constructed MybΔ-Ets, containing a deletion in the transactivation domain of Myb, and MybVP16-Ets, containing the VP16 activator sequences. When compared with the wild type Myb-Ets for ability to transactivate the *mim1* promoter, MybΔ-Ets had a low but significant activity above background, whilst the VP16 sequences in MybVP16-Ets completely restored function (Figure 3A); however, unlike substitution into Myb, the VP16 sequences in the Myb-Ets background did not lead to an enhanced transactivation potential. The observed low transactivation potential of MybΔ-Ets is consistent with the possibility that the Ets transactivation domain (Wasylyk

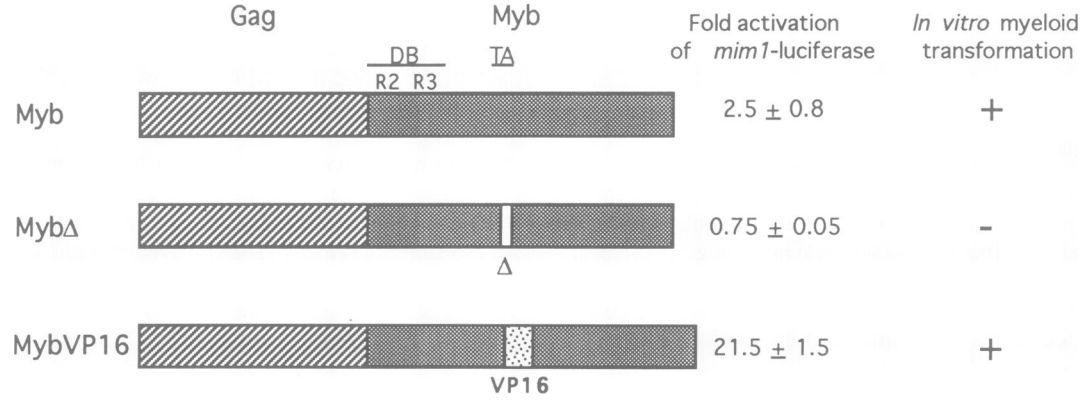

Fig. 2. Transactivation and transformation by Myb and its derivatives. The structures of Myb and the deleted and substituted variants are illustrated schematically. DB, DNA binding domain; TA, transactivator domain. The corresponding activity on the *mim1* promoter in a co-transfection assay in HD11 macrophages and ability to transform myeloid cells *in vitro* is indicated. The activation data is expressed as the ratio of luciferase activities with and without the respective Myb construct. The ability to transform myeloid cells in the bone marrow co-cultivation assay, that is, whether 'outgrowth' of cells was seen before senescence of the uninfected control (see Table I), is indicated by a '+'.

et al., 1990; Schneikert *et al.*, 1992) is weakly active in conferring activation potential to Myb.

The activity of the substituted VP16 transactivation motif is abolished by a mutation which perturbs the interaction with components of the transcription initiation complex

Mutagenesis of the VP16 transactivation domain has shown that substitution of a phenyalanine residue at position 442 for a proline leads to a loss of transactivating activity and binding capacity to components of the RNA polymerase II initiation complex (Cress and Triezenberg, 1991; Ingles *et al.*, 1991; Lin *et al.*, 1991). We therefore introduced this mutation into the MybVP16-Ets protein. Since the effect of the position 442 mutation was originally determined in the context of a half-maximally active VP16 transactivation domain deleted at its C-terminus (Cress and Triezenberg, 1991), we constructed a shorter VP16 sequence into the Myb-Ets context to yield MybVP16ΔC-Ets (VP16 residues 411−468). The corresponding position 442 mutant was designated MybVP16ΔC^{F442P}-Ets. The activity of MybVP16ΔC-Ets was essentially the same as that of MybVP16-Ets (Figure 3B); however, introduction of the F442P mutation resulted in a reduction in the transactivation of the *mim1* promoter to the basal level seen with MybΔ-Ets. We therefore conclude that the VP16 transactivator motif behaves normally within the MybVP16ΔC-Ets protein.

The MybVP16-Ets chimeras also activate endogenous mim1

We next asked if the activities of the Myb-Ets and MybVP16-Ets chimeras in transactivating the *mim1* promoter construct are paralleled in terms of stimulation of the endogenous *mim1* gene in myeloid cells. HD11 macrophages were transiently transfected with Myb-Ets and its derivatives, and activation of the endogenous *mim1* gene was assayed after 2 days by extraction of total cellular RNA and analysis by Northern blot hybridization as described in Ness *et al.* (1989). As can be seen from Figure 3C, transactivation of the endogenous *mim1* promoter parallels stimulation of the *mim1*-luciferase construct (Figure 3A and B). No expression of *mim1* RNA was detected in the untransfected control whilst MybVP16ΔC-Ets and MybVP16-Ets induced expression to levels comparable to the wild type Myb-Ets (the relative levels of expression of *mim1* RNA as determined from the Northern blot by densitometric scanning were 1.0, 0.64 and 0.88 for Myb-Ets, MybVP16ΔC-Ets and MybVP16-Ets respectively). The constructs carrying the deleted Myb transactivator domain or the inactivating mutation in the VP16 sequences did not stimulate *mim1* RNA transcription.

MybVP16, but not Myb or MybΔ, interacts strongly with TBP in vitro

As discussed above, the Myb activator domain appears to differ structurally from the classical acidic activator domain of VP16. *In vitro* analysis of the interactions of the VP16 activator domain with the basic transcriptional apparatus has shown that it binds directly to TBP, the TATA box binding protein (Stringer *et al.*, 1990). To determine whether the Myb activator domain also binds TBP, we employed a glutathione S-transferase (GST) 'pull down' assay (Hagemeier *et al.*, 1992). Briefly, GST-TBP fusion proteins attached to glutathione−Sepharose beads are incubated with radiolabelled test protein, and any TBP bound material is resolved by SDS−PAGE. Myb, MybΔ and MybVP16 proteins containing the Myb activator domain, the 17 amino acid deletion within the Myb activator domain or the VP16 activator domain, respectively, were transcribed and translated *in vitro* in the presence of [^{35}S]methionine. Equal amounts of the three proteins (Figure 4A, lanes 1−3) were then incubated with either GST-TBP linked to beads or, as a negative control, GST-vimentin linked to beads. Figure 4B, lane 3 shows that MybVP16 bound strongly and specifically to TBP; ~50% of input MybVP16 protein was bound to TBP. This contrasted markedly with Myb and MybΔ, both of which bound very weakly (Figure 4B, lanes 1 and 2). This weak binding activity must be contributed by a region of the Myb protein that lies outside the activation domain since Myb and MybΔ showed identical binding potential. The background binding observed with all three proteins

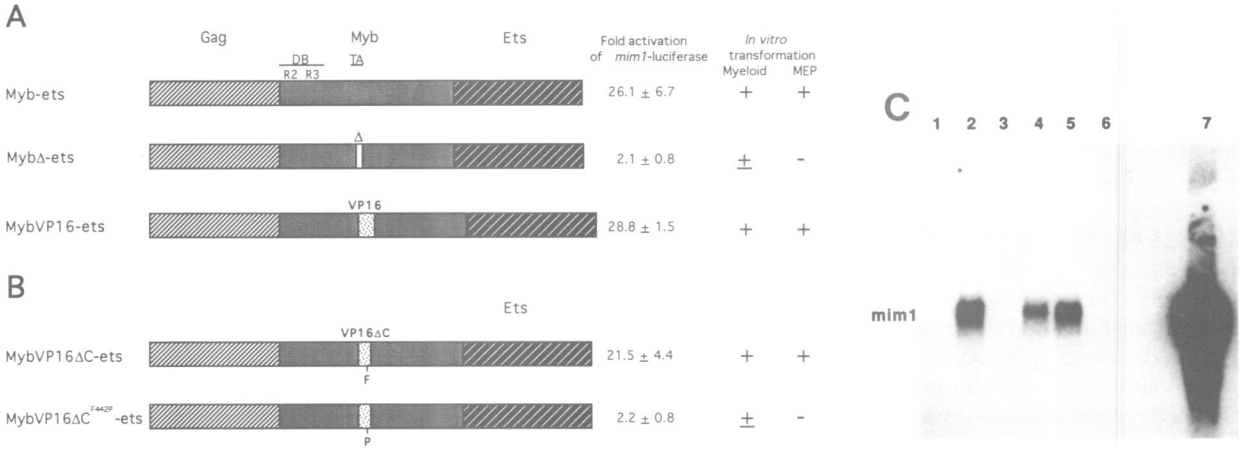

Fig. 3. Transactivation and transformation by Myb-Ets and its derivatives. (**A**) and (**B**) are as for Figure 2 with the addition that ability of each derivative to transform MEPs in the *in vitro* blastoderm assay is indicated by a '+' (these results are presented quantitatively in Table II). (**C**) Northern blot hybridization of *mim1* RNA expressed from the endogenous gene in transfected HD11 macrophages. 2×10^6 HD11 cells were transfected with 2 μg of the viral constructs expressing the indicated version of Myb-Ets. Each lane contains 20 μg of total cell RNA prepared 2 days after transfection. The RNAs were as follows: mock-transfected control (lane 1); Myb-Ets (lane 2); MybΔ-Ets (lane 3); MybVP16ΔC-Ets (lane 4); MybVP16-Ets (lane 5); MybVP16ΔC^{F442P}-Ets (lane 6); and 3 μg of Myb-Ets transformed myeloblast RNA as a positive control (lane 7).

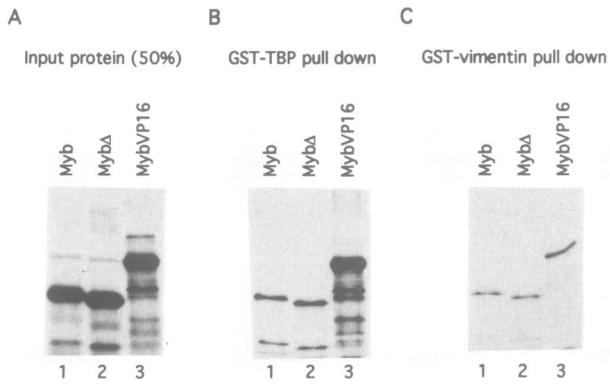

Fig. 4. Interaction with TBP *in vitro*. Denaturing gel electrophoresis of *in vitro* translated proteins. Lanes in panel **A** contain an equivalent amount of protein to that applied to lanes in panels **B** (GST-TBP) and **C** (GST-vimentin).

(Figure 4C) is due to the propensity of the Myb protein to bind non-specifically to the glutathione-Sepharose beads (data not shown). We conclude that the VP16 and Myb activator domains have distinct TBP binding properties: VP16, but not Myb, being capable of direct interaction with TBP.

MybVP16 and MybVP16-Ets are still capable of transforming myeloid cells

Wild type Myb, MybΔ and MybVP16 were compared for their ability to transform myeloid cells by co-cultivation of chick bone marrow cells with chick embryo fibroblasts expressing these proteins in a retroviral background. As indicated in Figure 2, only the wild type Myb and MybVP16 viruses caused an outgrowth of rapidly proliferating, transformed myeloid cells. Immunoprecipitation of proteins labelled by incorporation of [^{35}S]methionine showed that both transformed populations expressed the expected proteins (Figure 6A). The chimeric protein containing the VP16 sequences had a greater than calculated apparent molecular weight; however, this was anticipated since the particular structure of the VP16 domain is known to affect migration in denaturing gels (Pellet *et al.*, 1985). Characterization of the transformed cells by cytochemical staining (Figure 5A) and immunofluorescence staining of the myeloid surface markers MYL 51/2 (pan-myeloid; Kornfeld *et al.*, 1983) and IC3 (granulocyte-specific) revealed no difference in their phenotypes (data not shown).

The derivatives of Myb-Ets were also tested for transformation of myeloid cells in chick bone marrow. MybVP16ΔC-Ets- and MybVP16-Ets-expressing viruses caused a rapid outgrowth of transformed myeloid cells with similar kinetics to the wild type virus. Interestingly, the very weakly transactivating MybΔ-Ets and MybVP16ΔC^{F442P}-Ets constructs also led to myeloid transformation, but the latency of transformation was longer and the resulting myeloid cells were phenotypically distinct (Table I, Figure 5B and description below). Labelling and immunoprecipitation analysis showed the transformed cells contained proteins of the expected sizes expressed at equivalent levels (Figure 6B).

Myeloid cells transformed by each of the viruses showed little difference in myeloid-specific surface antigen expression, with the exception of some minor changes on MybΔ-Ets and MybVP16ΔC^{F442P}-Ets transformed cells which were quite variable from experiment to experiment (Table I). The most pronounced differences were seen with regard to the growth properties of the cells. MybΔ-Ets and MybVP16ΔC^{F442P}-Ets transformed myeloblasts had a doubling time approximately twice that of the wild type Myb-Ets cells, and a small decrease compared with wild type was noted in the doubling time of MybVP16-Ets myeloblasts (Table I). In addition, myeloid cells transformed by the VP16 chimeras showed a slight but consistent reduction in their dependency on chicken myelomonocytic growth factor (cMGF, Leutz *et al.*, 1984; data not shown). The surprising observation that the MybΔ-Ets and MybVP16ΔC^{F442P}-Ets were able to cause the outgrowth of myeloid cells presumably indicates that the low transactivation activity of these proteins observed in transient transfection assays is sufficient for weak myeloid transformation to occur.

MybVP16-Ets is able to transform multipotent haematopoietic cells

Since the conditions of the bone marrow co-cultivation assay preclude assessment of erythroid transforming capacity, we tested viruses expressing the mutant derivatives of Myb-Ets for their ability to transform MEPs in our *in vitro* blastoderm assay. Two day-old embryos were infected and transformed colonies were picked from the semi-solid medium 10 days later and expanded in liquid culture, and their phenotypes were assessed. This was done by staining the cells with monoclonal antibodies against various lineage-specific markers and with histochemical reagents which detect haemoglobin and eosinophilic myeloperoxidase. When using the viruses expressing MybΔ-Ets and MybVP16ΔC^{F442P}-Ets only myeloid colonies were produced. In contrast, viruses expressing proteins with an intact or substituted transactivation domain (Myb-Ets, MybVP16-Ets and MybVP16ΔC-Ets) gave rise not only to myeloid colonies, but in also to MEP and mixed colonies. However, the preponderance of MEP colonies which is observed with the wild type Myb-Ets was not seen for the two viruses expressing proteins with substituted transactivation domains from VP16 (Table II).

We did not expect to observe transformed mature erythroid cells with any of the viruses under the assay conditions used. The work of Metz and Graf (1991a) has previously shown that Myb and Ets individually or in combination are capable of transforming mature erythroid cells in a bone marrow co-cultivation under very specific conditions which were not employed here. Domenget *et al.* (1992) have demonstrated that an E26 mutant, which is analogous to our MybΔ-Ets, being deleted in the transactivation domain of Myb, can also transform such cells, but again under specific bone marrow cultivation conditions. It has never been possible in the *in vitro* blastoderm assay to detect mature erythroid cells transformed by Ets alone or by Myb-Ets proteins defective in either the DNA binding or transactivation domains of Myb (T.Metz, T.Graf and J.Frampton, unpublished observations).

MEP cells transformed by MybVP16-Ets have an altered phenotype but still differentiate in response to TPA

Detailed examination of the MEP clones transformed by MybVP16-Ets and MybVP16ΔC-Ets revealed some phenotypic differences when compared with Myb-Ets

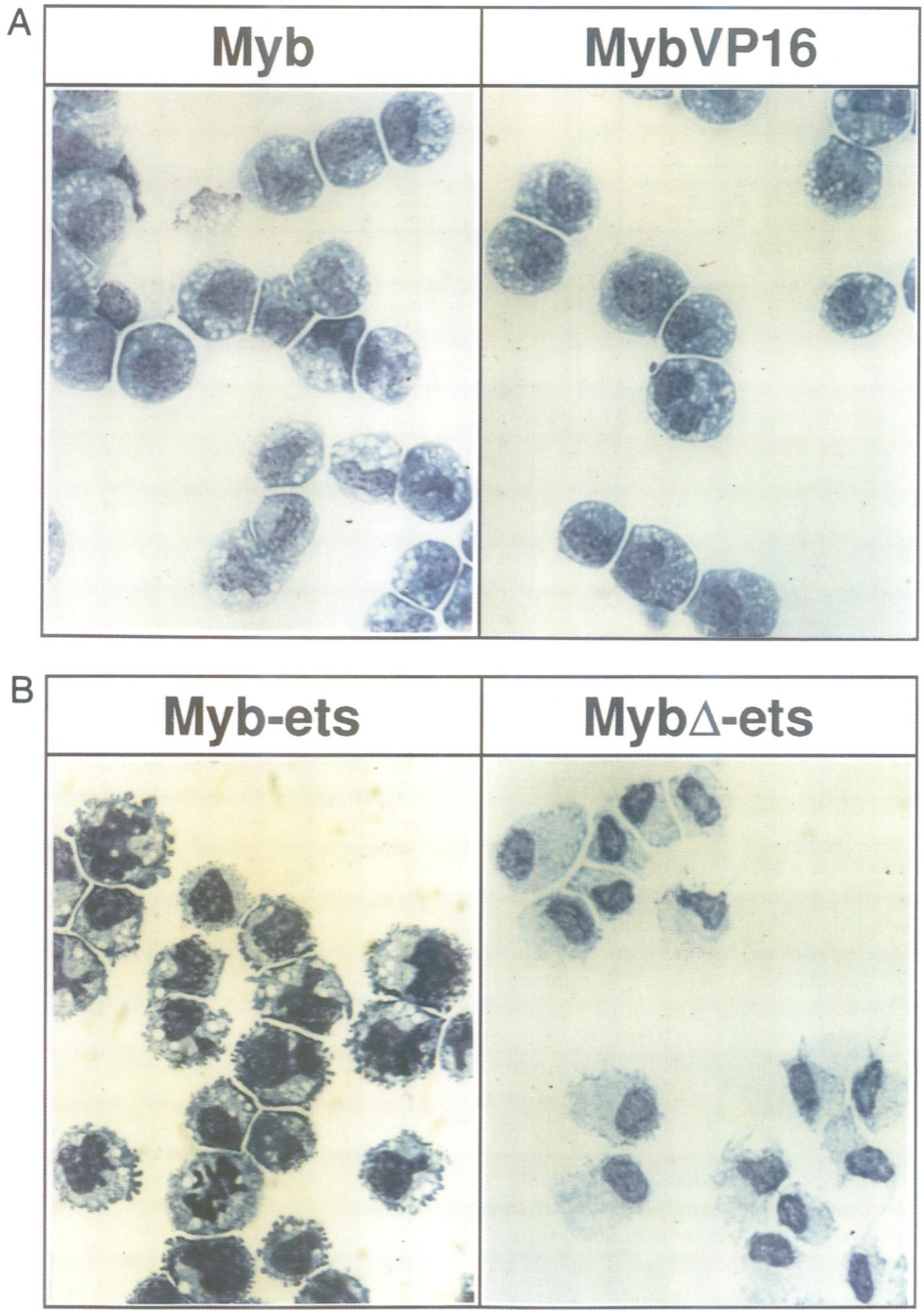

Fig. 5. Transformation of myeloid cells in bone marrow co-cultivation assays by Myb and Myb-Ets derivatives. (**A**) Cytospin of cells transformed by Myb and MybVP16. (**B**) Cytospin of cells transformed by Myb-Ets and MybΔ-Ets.

Table I. Properties of myeloid cells transformed by E26 derivatives

Virus	Latency of transformation (days)	Doubling time of transformed cells (h)	Percent marker positive cells	
			MYL 51/2	IC3
Myb-Ets	7–10	23	100	100
MybΔ-Ets	15–20	38	100	30–60
MybVP16-Ets	7–10	17	100	70
MybVP16ΔC-Ets	7–10	24	100	95
MybVP16ΔC^{F442P}-Ets	15–20	37	100	<5

Data are shown for transformation of bone marrow-derived cells which were grown as mass cultures. The latency of transformation, i.e. the time taken to observe clear 'outgrowth' of transformed cells, was taken as the time required before the first passage of cells from the 3 cm assay dish to a 5 cm plate.

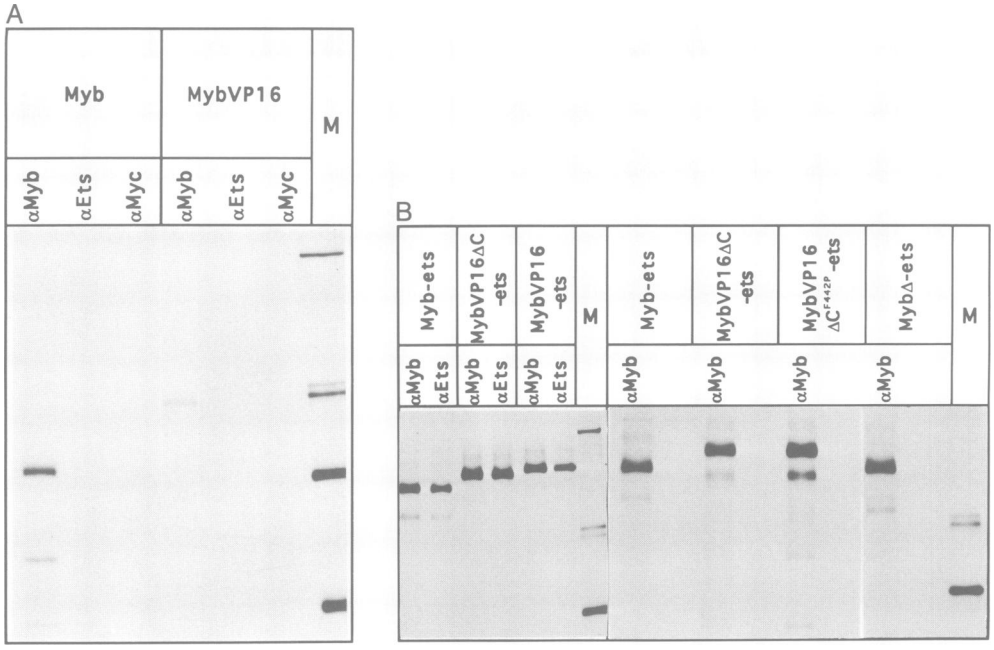

Fig. 6. Analysis of v-Myb proteins in transformed myeloid cells. [^{35}S]methionine-labelled cells were immunoprecipitated with the indicated antibodies. (**A**). Molecular weight size markers (M) are, in descending order, 200, 92.5, 69 and 46 kDa. (**B**) Markers visible are 200, 92.5 and 69 kDa in the left-hand panel and 92.5 and 69 kDa in the right-hand panel.

transformed MEPs. Although all clones expressed MEP21 antigen, they differed with respect to the relative expression of the eosinophil-specific cell surface marker EOS47 and the erythroid/thrombocytic marker histone H5; MEP cells transformed by the wild type virus expressed high levels of histone H5 and essentially no EOS47. In contrast, colonies transformed by MybVP16-Ets and MybVP16ΔC-Ets viruses were often EOS47$^+$ and histone 5$^-$ (Figure 7) with some clones resembling those of the wild type virus. However, MEP clones representing the two extreme phenotypes transformed by MybVP16-Ets differentiated into the myeloid lineage after TPA treatment (Graf *et al.*, 1992), expressing the myeloid surface marker MYL 51/2 after 10–14 days.

MybVP16-Ets but not MybΔ-Ets causes leukaemia

To test for their leukaemogenic capacity, MybΔ-Ets and MybVP16-Ets viruses were injected intravenously into 12 day embryos. Blood smears were then prepared, stained and examined periodically after hatching of the chicks. Myb-Ets, but not the deletion construct, induced leukaemias with incidences and latencies comparable to that of the wild type virus (Table III). Like wild type infected animals, MybVP16-Ets animals exhibited enlarged spleens and their stained blood smears revealed a predominantly erythro-leukaemic phenotype. Interestingly, in the case of one chick, a mixed phenotype consisting of erythroid, myeloid and possibly eosinophilic cells was seen; the possibility that these phenotypic differences resulted from a rearrangement in the fusion protein was excluded since immunoprecipitation of labelled leukaemic cells with both Myb- and Ets-specific antisera revealed protein of the expected size (data not shown). Post mortem examination of this latter chick revealed a large number of disseminated tumours in the gut, heart, liver and abdominal cavity. Such tumours have never been seen with the wild type virus (T.Graf, unpublished observations). Cells cultured from the effusate of tumours taken from the liver and abdominal cavity had high levels of peroxidase positive eosinophils and myeloid cells (data not shown).

Table II. *In vitro* transformation capacity of E26 derivatives

Virus	Numbers of colonies			
	Myeloid	MEP	Mixed	ratio myeloid/MEP
Myb-Ets	34	90	11	0.4
MybVP16-Ets	20	14	10	1.4
MybVP16ΔC-Ets	17	7	3	2.4
MybΔ-Ets	35	0		>10
MybVP16ΔC^{F442P}-Ets	60	0	0	>10

The phenotype of the blastoderm-derived Methocel colonies was determined by morphology. This was confirmed for a selection of clones by surface staining with MYL 51/2 and MEP21.

Thus, the induction of leukaemia by Myb-Ets and its derivatives reflected, to a large extent, the transformation observed in the blastoderm assay. The weak capacity of MybΔ-Ets to transform myeloid cells *in vitro* was obviously insufficient to produce a leukaemic phenotype. Both Myb-Ets and MybVP16-Ets viruses induced erythroleukaemia; however, the occurrence of disseminated myelo-eosinophilic tumours in at least one chick infected by MybVP16-Ets suggested a different *in vivo* phenotype.

There were no indications that the inclusion of the VP16 activator domain had in any way extended the range of transformation of the E26 Myb beyond the erythroid–myeloid compartment of haematopoiesis. However, since chicks became leukaemic very rapidly after hatching, it would have been impossible to observe any more slowly developing neoplasms.

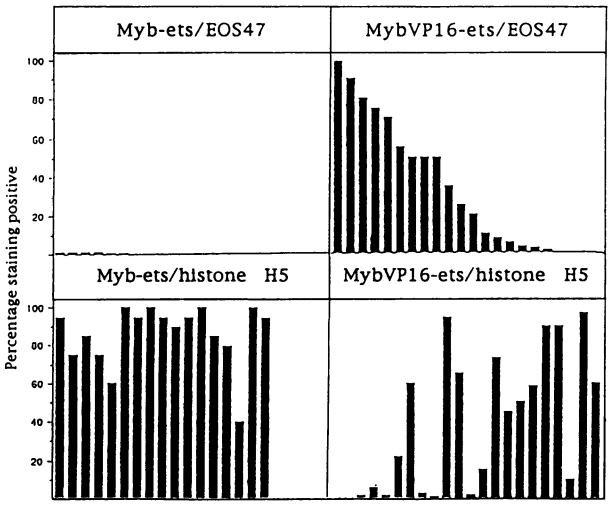

Fig. 7. Immunofluorescence analysis of EOS47 and histone H5 expression in MEP cells transformed by Myb-Ets and MybVP16-Ets. Individual transformed MEP clones expressing the EOS47 antigen are ranked as far as possible in descending order of its abundance. The corresponding level of histone H5 for each clone is indicated in the lower histogram.

Table III. Incidence of leukaemia induced by E26 derivatives

Virus	Leukaemic animals/animals infected	Latency (days)
Myb-Ets	8/10	13–26
MybΔ-Ets	0/10	NR
MybVP16-Ets	9/9	13–22

Virus was injected intravenously into 12 day-old embryos and leukaemia was assessed by periodic examinations of blood smears. Dead animals were autopsied. The experiment was terminated after 3 months. NR, not relevant.

Discussion

We have investigated the link between the functioning of Myb as a transactivator of gene expression and its capacity to transform cells of several haematopoietic lineages. In agreement with previous studies (Sakura *et al.*, 1989; Weston and Bishop, 1989; Lane *et al.*, 1990), we observed that deletion of the transactivation domain of v-Myb resulted in loss of the ability of a retrovirally expressed Myb construct to activate the promoter of the *mim1* gene in a transient transfection assay. This loss of function can be restored by the insertion of the 80 amino acid transactivation domain of the HSV-1 VP16 protein; indeed, the ability of the chimeric MybVP16 protein to stimulate expression of the *mim1* promoter in a transient transfection assay is enhanced 10-fold relative to the wild type.

Analogous experiments using retrovirally expressed Myb-Ets fusion protein showed a number of differences when compared with Myb. Myb-Ets is a much stronger transcription factor in transient transfection assays than Myb, having approximately similar activity to MybVP16. Deletion of the Myb activator domain, in the MybΔ-Ets protein, does not completely ablate transactivation as shown for MybΔ; instead, there is some residual transactivation activity, presumably resulting from the presence of the Ets activator motif (Wasylyk *et al.*, 1990; Schneikert *et al.*, 1992). It therefore appears that in our assay, the weak activator domains of Myb and Ets, when present in one protein, can act synergistically to stimulate transcription. This synergism is not observed between the VP16 and Ets activator domains since the Myb transactivation domain in Myb-Ets could be substituted by that of VP16, regaining the wild type transactivation potential of the protein, but not enhancing it. The synergism between Myb and Ets when fused together has been reported before in the context of transformation; a Myb-Ets fusion protein is a far more potent inducer of transformation than is either protein alone (Metz and Graf, 1991a,b). It seems possible that this enhanced transforming capacity is mediated at least in part by the increase in transcriptional activity we have observed.

Are the Myb and VP16 activator domains functionally distinct?

The Myb activation domain works only very weakly when tested on a minimal β globin promoter in which only the TATA box is present, whereas an acidic activator such as VP16 works efficiently under these conditions. In contrast, a longer β globin promoter, containing binding sites for other transcription factors in addition to the added Myb sites and TATA box, is activated strongly by Myb proteins (Weston and Bishop, 1989). This result led us to speculate that unlike canonical acidic activators, the Myb activator domain was unable to contact the TFIID protein complex efficiently, and therefore must interact with the basic transcription machinery in a different way. Here we provide further evidence for this assertion, showing that the Myb activator domain has no apparent TBP binding capacity, whereas the VP16 activator domain does. These observations fit with the secondary structural differences in the activator domains (Figure 1). The *in vivo* mechanistic significance is unclear; it is currently not known whether direct TBP–VP16 interactions are important for activated transcription (Dynlacht *et al.*, 1991; for review see Lewin, 1990); however, it seems likely that the difference reflects an important structural divergence between the two activators. Therefore, based on their functional and structural dissimilarity, we propose that replacement of the Myb activator domain by that of VP16 imposes an altered mechanism of transcriptional activation on the MybVP16 chimeric proteins to that normally employed by Myb.

How does the transcriptional activation potential of Myb relate to its capacity to transform haematopoietic cells?

Despite its increased transactivation capacity and structurally distinct activator domain, the MybVP16 protein transforms myeloid cells in an identical fashion to wild type Myb, showing that the strength or nature of the transactivation domain must contribute little to the transformation specificity of Myb, at least in this cell system. Assuming that the lower activity of Myb compared with MybVP16 in a transient transfection assay reflects the *in vivo* situation, an implication of this result is that a threshold of transactivation function is required for myeloid transformation, and that any elevation of this activity on transformation target genes is of little consequence. The MybVP16 chimera also did not appear to affect regulation of the genes responsible for the differentiation program of the transformed cells, which were

apparently frozen at the same stage of differentiation as wild type transformants.

Although both myeloid and MEP cells were transformed by the MybVP16-Ets virus, the ratio of MEP to myeloid colonies was reversed, a large number of colonies contained peroxidase-positive eosinophils (data not shown), and the phenotype of the MEP cells altered. The discovery that the Myb-Ets-transformed MEPs are capable of differentiating along the erythroid, myeloid and eosinophilic lineages (Graf *et al.*, 1992) might provide an explanation. Thus, the MybVP16-Ets protein could still be able to transform MEPs but these cells might be more predisposed to differentiate towards the myeloid/eosinophilic pathways. Indeed, the EOS47$^+$ cells transformed by MybVP16-Ets might represent a differentiation intermediate between MEPs and myeloid cells or a precursor on the pathway towards eosinophils. It has recently been described that the action of Oct-1 and Oct-2 on specific promoters is dictated by their respective activator domains (Tanaka *et al.*, 1992) and it will be interesting to determine whether the changes that we have observed in the MEP phenotype can be linked to qualitative or quantitative effects on the expression levels of specific genes; this awaits the identification of genes which are regulated by the wild type E26 fusion protein. Although the apparent structural and functional differences between the Myb and VP16 activator domains could be the determinant of the phenotypic alterations seen in the transformed MEPs, it remains a possibility that the effect is more indirect; that is, the insertion of heterologous sequences into the Myb-Ets fusion protein results in subtle structural effects on the whole molecule. One argument against this latter possibility is that fusion proteins isolated *in vivo* and containing large deletions were unaffected in their MEP transformation phenotype (Metz and Graf, 1991b; T.Graf, unpublished observations).

Our work has shown that replacement of the activation domain of the v-Myb oncoprotein with the acidic activation domain from HSV-1 VP16 selectively influences its transformation specificity, in that the MEP but not the myeloid phenotype was affected. This indicates that domains other than the DNA binding motif can determine the transformation specificity of v-Myb, probably doing so by interaction with cell-type-specific co-factors. Indeed, it has already been observed that single point mutations in the DNA binding domain which do not affect DNA recognition *per se* are sufficient to alter the phenotype of v-Myb-transformed myeloid cells (Introna *et al.*, 1990). Factors regulating binding to and/or transcription of v-Myb-activated genes have yet to be defined; their isolation, and the identification of target genes regulated by v-Myb will tell us much about the mechanism by which this transcription factor can cause cell type specific transformation.

Materials and methods

Construction of viruses

The retroviral constructs which express the Myb and Myb-Ets proteins have been described previously (Introna *et al.*, 1990; therein described as EAA and EEE respectively). MybΔ: Myb residues 830−330 (numbering as in Klempnauer *et al.*, 1982) were removed by site-directed mutagenesis. MybVP16 and MybVP16ΔC: pSGVPΔ490 and pSGVPΔ468 [kindly given by Dr I.Sadowski, numbering as in Triezenberg *et al.* (1988)] were cut with *Eco*RI and *Hin*cII, and the resulting 259 and 200 bp fragments were end-repaired and ligated into blunted, *Sal*I-cut MybΔ, to give MybVP16 and MybVP16ΔC respectively. The 5′ Myb−VP16 junctions encode the amino acid sequence .myb..MS/KFPGI/S...VP16, where the sequence KFPGI is an artificial linker. 3′ myb-VP16 junctions encode the sequence VP16 (position 490 or 468)/LDRSV/ST...myb, where the sequence LDRSV is an artificial linker. MybΔ-Ets, MybVP16-Ets, MybVP16ΔC-Ets: the activation domains detailed above from Myb, MybVP16 and MybVP16ΔC were reconstructed into Myb-Ets. Details of virus construction are available upon request. MybVP16ΔC^{F442P}-Ets was generated by PCR mutagenesis using oligonucleotides from Myb sequences flanking the VP16 insert in MybVP16ΔC-Ets and a specific mutagenesis oligonucleotide (5′-GTCCAGATCGGGATCGTCTAGCGC-3′) in which the underlined base results in conversion of codon 442 from phenylalanine to proline. A fragment from the *Eco*RI site 5′ to the *Dsa*I site 3′ of the insert was replaced into MybVP16ΔC-Ets and checked by sequencing throughout.

Cotransfection and transactivation assays

The reporter gene construct used consisted of 1.6 kbp of the *mim-1* promoter attached to the luciferase gene (Ness *et al.*, 1989). The v-Myc-transformed macrophage cell line HD11 (Beug *et al.*, 1979) was cotransfected with 0.1−1 μg of a viral plasmid expressing the v-Myb derivative, 1 μg of the luciferase reporter construct and 0.5 μg of pRSV-β-gal construct for control of transfection efficiency. The transfection and assay procedures were as detailed in Ness *et al.* (1989).

RNA extraction and Northern blotting

Total cellular RNA was prepared by the method of Chomczynski and Sacchi (1987) and blotting and hybridization were conducted according to standard protocols (Maniatis *et al.*, 1982).

GST 'pull down' assay

Plasmids used for *in vitro* transcription/translation: Myb: pT7βV (Weston, 1992); MybΔ: pT7βVSD11 contains the same 50 bp deletion as described for EAA MybΔ in a pT7βV background; MybVP16: pT7βV490 contains the same VP16 insertion as described for the MybVP16 virus in a pT7βV background. Details of construction are available upon request. All three plasmids were linearized with *Bam*HI, and transcribed and translated in the presence of [^{35}S]methionine by standard methods (Jackson and Hunt, 1983; Melton *et al.*, 1984). Translated proteins were quantified by SDS−PAGE and their concentrations adjusted to be roughly equivalent. GST pull down assays and subsequent SDS−PAGE analyses were performed as described by Hagemeier *et al.* (1992), using 2 μl of *in vitro* translated protein. GST-TBP and GST-vimentin fusion proteins were prepared and bound to glutathione−Sepharose beads as described by Hagemeier *et al.* (1992).

Cells

All cells were derived from a flock of White Leghorn Spafas chickens maintained in Heidelberg. Chicken embryo fibroblasts (CEFs) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 8% fetal calf serum, 2% chicken serum and 10 mM HEPES. Transformed myeloid cells were grown in the same medium but containing approximately 10 U/ml crude cMGF (Leutz *et al.*, 1984). MEPs were grown in 'blastoderm medium' (Graf *et al.*, 1992).

Production of infectious virus stocks and bone marrow transformation

Primary CEFs were transfected with 10 μg of viral DNA plus 1 μg of RAV-1 helper virus DNA (Frykberg *et al.*, 1988). Cells were selected for neomycin resistance starting 2 days after transfection using 800 μg/ml G418 (GIBCO). G418-resistant, virus-producing cells were then used to infect bone marrow cells from 1- to 2-week old chicks under conditions which favour the outgrowth of myeloid cells (Metz and Graf, 1991a). The supernatants of transformed cultures were collected, filtered through a 0.45 μm filter and frozen at −70°C as a source of virus.

Blastoderm transformation assay

Blastoderm cultures were obtained from 2-day-old chick embryos (stages 10−12) essentially as described by Moscovici *et al.* (1983). Cells were infected with virus and then seeded in 'blastoderm medium' (277 ml DMEM, 38 ml H_2O, 40 ml fetal calf serum, 10 ml chicken serum, 14 ml 5.6% $NaHCO_3$, 22.5 mg conalbumin, 320 μl 0.1 M β-mercaptoethanol, 220 μl insulin (1.7 mg/ml) and a standard complement of antibiotics) containing 1% anaemic chicken serum, 5−10 U/ml crude cMGF and 0.8% methocel as described earlier (Graf *et al.*, 1981, 1992). Transformed erythroid or myeloid colonies were isolated after ~10 days using a drawn-out Pasteur pipette and were seeded in microtitre wells for expansion.

Infection of chickens and growth of leukaemic cells

A suspension of 300 μl of virus stock was injected into the chorioallantoic veins of 12 day embryonated eggs. Beginning 10 days after injection animals were monitored twice weekly for the onset of leukaemia by Diff Quick (Harleco) staining of blood smears. Buffy coat cells were prepared from peripheral blood of leukaemic chicks by washing once in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline and diluted to twice the original volume. The cells were then layered on top of Ficoll-containing lymphocyte separation medium (density 1.077, Eurobio) and centrifuged at 800 *g* for 20 min. Cells collecting at the interface were grown in 'blastoderm medium' containing 5–10 U/ml crude cMGF to allow the growth of both myeloid and erythroid cells.

Immunoprecipitation of viral proteins

Proteins were analysed by radioimmunoprecipitation using anti-Myb (Ness *et al.*, 1987) and anti-Ets sera (Ghysdael *et al.*, 1986). Cells (15×10^6) were labelled for 1 h with [^{35}S]methionine according to Radke *et al.*(1982). Lysis of cells and immunoprecipitation of proteins were performed essentially as described by Ghysdael *et al.* (1986).

Cytochemical assays for differentiation markers

To visualize the morphology, granule content and haemoglobin expression of individual transformed colonies from the blastoderm assay, cells were subjected to cytocentrifugation, stained with benzidine at neutral pH, and counterstained with Diff Quick (Harleco) as described by Beug *et al.* (1982). A more sensitive determination of the proportion of erythroid cells expressing haemoglobin was achieved by staining cell suspensions with benzidine at acid pH (Orkin *et al.*, 1975). Cells positive for the eosinophil-specific peroxidase were detected by a modification of the method described by Kaplow (1965): briefly, $\sim1\times10^4$ cells in 50 μl of medium were mixed with 10 μl of the staining solution (30% ethanol; 0.3% benzidine–HCl; 0.038% $ZnSO_4\cdot7H_2O$; 0.73% sodium acetate; 0.21% H_2O_2; 0.015 M NaOH; 0.2% safranin O) and incubated at room temperature for 5–30 min. Peroxidase-positive eosinophils contain golden-brown to black granules.

Immunofluorescence assays of differentiation markers

Surface immunofluorescence staining of cells was performed essentially as described in Radke *et al.* (1982). Cells were incubated with the appropriate dilution of primary antibody at 4°C for 10 min followed by washing and incubation with fluorescein isothiocyanate (FITC) conjugated goat anti-mouse secondary antibody at 4°C for 20 min. Myeloid cells were characterized with the monoclonal antibody 51/2 (Kornfeld *et al.*, 1983) and, in order to identify differentiation stage-specific cell surface markers, the monoclonal antibodies IC3 (granulocyte-specific; Mandi *et al.*, 1987), MEP21 (erythroid progenitor-specific), MEP26 (progenitor and later erythroid cell-specific) and Eos47 (eosinophil-specific) were employed (McNagny *et al.*, 1992). The presence of nuclear histone H5 was detected by immunofluorescence staining of fixed cells as described by Beug *et al.* (1979).

Acknowledgements

The authors would like to thank Norbert Kraut, Scott Ness, Martin Eilers, Achim Leutz and other members of the Graf lab for helpful comments and suggestions. We also thank Susan Weston and Samantha O'Loughlin for synthesis of oligonucleotides. K.W. and T.K. are supported by the Cancer Research Campaign.

References

Beug,H., von Kirchbach,A., Döderlein,C., Conscience,J.F. and Graf,T. (1979) *Cell*, **18**, 375–390.
Beug,H., Döderlein,C., Freudenstein,C. and Graf,T. (1982) *J. Cell. Physiol. Suppl.*, **1**, 195–207.
Biedenkapp,H., Borgmeyer,U., Sippel,A.E. and Klempnauer,K.H. (1988) *Nature*, **335**, 835–837.
Chomczynski,P. and Sacchi,N. (1987) *Anal. Biochem.*, **162**, 156–159.
Cousens,D.J., Greaves,R., Goding,C.R. and O'Hare,P. (1989) *EMBO J.*, **8**, 2337–2342.
Cress,W.D. and Triezenberg,S.J. (1991) *Science*, **251**, 87–90.
Domenget,C., Leprince,D., Pain,B., Peyrol,S., Li,R.P., Stehlin,D., Samarut,J. and Jurdic,P. (1992) *Oncogene*, **7**, 2231–2241.
Dynlacht,B.D., Hoey,T. and Tjian,R. (1991) *Cell*, **66**, 563–576.
Elliston,J.F., Tsai,S.Y., O'Malley,B.W. and Tsai,M.J. (1990) *J. Biol. Chem.*, **265**, 11517–11521.
Frampton,J., Leutz,A., Gibson,T.J. and Graf,T. (1989) *Nature*, **342**, 134.
Frampton,J., Gibson,T., Ness,S.A., Döderlein,G. and Graf,T. (1991) *Protein Engng*, **4**, 891–901.
Frykberg,L., Metz,T., Brady,G., Introna,M., Beug,H., Vennström,B. and Graf.T. (1988) *Oncogene Res.*, **3**, 313–322.
Ghysdael,J., Gegonne,P., Pognonec,P., Dernis,D., Leprince,D. and Stehelin,D. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 1714–1718.
Gonda,T.J., Buckmaster,C. and Ramsay,R.G. (1989) *EMBO J.*, **8**, 1777–1783.
Graf,T. (1992) *Curr. Opin. Gen. Dev.*, **2**, 249–255.
Graf,T. and Beug,H. (1978) *Biochim. Biophys. Acta*, **516**, 269–299.
Graf,T., von Kirchbach,A. and Beug,H. (1981) *Exp. Cell Res.*, **131**, 331–343.
Graf.T., McNagny,K. Brady,G. and Frampton,J. (1992) *Cell*, **70**, 201–213.
Grässer,F., Graf,T. and Lipsick,J. (1991) *Mol. Cell. Biol.*, **11**, 3987–3996.
Greenblatt,J. (1991) *Cell*, **66**, 1067–1070.
Hagemeier,C., Walker,S., Caswell,R., Kouzarides,T. and Sinclair,J. (1992) *J. Virol.*, **66**, 4452–4456.
Hu,Y., Ramsay,R.G., Kanei-Ishii,C., Ishii,S. and Gonda,T.J. (1991) *Oncogene*, **6**, 1549–1553.
Ibanez,C. and Lipsick,J. (1988) *J. Virol.*, **62**, 1981–1988.
Ibanez,C.E. and Lipsick,J.S. (1990) *Mol. Cell. Biol.*, **10**, 2285–2293.
Ingles,C.J., Shales,M., Cress,W.D., Triezenberg,S.J. and Greenblatt,J. (1991) *Nature*, **351**, 588–590.
Introna,M., Golay,J., Frampton,J., Nakano,T., Ness,S.A. and Graf,T. (1990) *Cell*, **63**, 1287–1297.
Jackson,R.J. and Hunt,T. (1983) *Methods Enzymol.*, **96**, 50–74.
Kaplow,L.S. (1965) *Blood*, **26**, 215.
Klempnauer,K.-H., Gonda,T.J. and Bishop,J.M. (1982) *Cell*, **31**, 453–463.
Klempnauer,K.-H., Arnold,H. and Biedenkapp,H. (1989) *Genes Dev.*, **3**, 1582–1589.
Kornfeld,S., Beug,H., Döderlein,G. and Graf,T. (1983) *Exp. Cell Res.*, **143**, 383–394.
Lane,T.N., Ibanez,C.E., Graf,T. and Lipsick,J.S. (1990) *Mol. Cell. Biol.*, **10**, 2591–2598.
Leprince,D., Gegonne,A., Coll,J., de Taisne,C., Schneeberger,A., Lagrou,C. and Stehelin,D. (1983) *Nature*, **306**, 395–397.
Leutz,A., Beug,H. and Graf,T. (1984) *EMBO J.*, **3**, 3191–3197.
Lewin,B. (1990) *Cell*, **61**, 1161–1164.
Lin,Y.-S., Ha,I., Maldonado,E., Reinberg,D. and Green,M.R. (1991) *Nature*, **353**, 569–571.
Lüscher,B. and Eisenman,R.N. (1990) *Genes Dev.*, **4**, 2235–2241.
Mandi,Y., Veromaa,T., Baranji,K., Miczak,A., Beladi,I. and Toivanen,P. (1987) *Immunobiology*, **174**, 292–299.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
McNagny,K., Lim,F., Grieser,S. and Graf,T. (1992) *Leukemia*, **6**, 975–984.
Melton,D.A., Krieg,P.A., Rebagliati,M., Maniatis,T., Zinn,K. and Green,M.R. (1984) *Nucleic Acids Res.*, **12**, 7035–7056.
Metz,T. and Graf,T. (1991a) *Genes Dev.*, **5**, 369–380.
Metz,T. and Graf,T. (1991b) *Cell*, **66**, 95–105.
Mitchell,P.J. and Tjian,R. (1989) *Science*, **245**, 371–378.
Moscovici,M.G., Jurdic,P., Samarut,J., Gazzolo,L., Mura,C.V. and Moscovici,C. (1983) *Virology*, **129**, 65–78.
Ness,S.A., Beug,H. and Graf,T. (1987) *Cell*, **51**, 41–50.
Ness,S.A. and Marknell,Å and Graf,T. (1989) *Cell*, **59**, 1115–1125.
Nunn,M., Seeburg,P.H., Moscovici,C. and Duesberg,P.H. (1983) *Nature*, **306**, 391–395.
Orkin,S.H., Harosi,F.I. and Leder,P. (1975) *Proc. Natl. Acad. Sci. USA*, **72**, 98–102.
Pellet,P.E., McKnight,J.L.C., Jenkins,F.J. and Roizman,B. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 5870–5874.
Radke,K., Beug,H., Kornfeld,S. and Graf,T. (1982) *Cell*, **31**, 643–653.
Ramsay,R.G., Ishii,S. and Gonda,T.J.(1991) *Oncogene*, **6**, 1875–1879.
Sadowski,I., Ma,J., Triezenberg,S. and Ptashne,M. (1988) *Nature*, **335**, 563–564.
Sakura,H., Kanei-Ishii,C., Nagase,T., Nakagoshi,H., Gonda,T.J. and Ishii,S. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 5758–5762.
Schneikert,J., Lutz,Y. and Wasylyk,B. (1992) *Oncogene*, **7**, 249–256.
Stringer,K.F., Ingles,C.J. and Greenblatt,J. (1990) *Nature*, **345**, 783–786.
Struhl,K. (1991) *Curr. Biol.*, **1**, 188–191.
Tanaka,M., Lai,J.-S. and Herr,W. (1992) *Cell*, **68**, 755–767.
Triezenberg,S.J., Kingsbury,R.C. and McKnight,S.L. (1988) *Genes Dev.*, **2**, 718–729.
Wasylyk,B., Wasylyk,C., Flores,P., Begue,A., Leprince,D. and Stehelin,D. (1990) *Nature*, **346**, 191–193.
Weston,K. (1992) *Nucleic Acids Res.*, **20**, 3043–3049.
Weston,K. and Bishop,J.M. (1989) *Cell*, **58**, 85–93.

Received on November 11, 1992; revised on December 4, 1992