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Murine myeloid cell lines derived by *in vitro* infection with recombinant c-*myb* retroviruses express *myb* from rearranged vector proviruses

Thomas J.Gonda, Robert G.Ramsay and Gregory R.Johnson[1]

The Ludwig Institute for Cancer Research, Melbourne Tumour Biology Branch and [1]The Walter and Eliza Hall Institute of Medical Research, Post Office, Royal Melbourne Hospital, Victoria 3050, Australia

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To date, cellular transformation *in vitro* by the *myb* oncogene has been described for avian haemopoietic cells only. In order to exploit the well-characterized murine haemopoietic system to study transformation by *myb*, we have infected fetal liver cells with retroviral vectors carrying cDNAs that encode either complete or carboxy-terminally truncated c-*myb* proteins. We describe four cell lines which, despite our ability to efficiently infect haemopoietic target cells, were generated at low frequency. This was due, as least in part, to the requirement for a rearrangement within the vector that allowed expression of *myb* sequences. Three of the lines express a truncated *myb* protein while the fourth apparently expresses a normal c-*myb* protein, and thus constitutes an exception to the general association of truncation with transformation by *myb*. All four cell lines resemble immature cells of the myelomonocytic lineage and are dependent on colony-stimulating factors (CSFs) for their growth *in vitro*. One representative line could be converted to CSF-independence by infection with either Abelson murine leukaemia virus or a recombinant granulocyte-macrophage-CSF-encoding retrovirus; unlike the parental line, the resultant sublines were highly tumorigenic when injected into syngeneic mice.

Key words: myb/retroviral vector/CSF-dependence/leukaemogenicity

Introduction

Two lines of evidence suggest that the c-*myb* proto-oncogene plays a major role in regulating the proliferation and differentiation of haemopoietic cells. First, high levels of c-*myb* expression are associated primarily with immature cells of the various haemopoietic sublineages (Gonda *et al.*, 1982; Westin *et al.*, 1982), and in each case, the level of expression decreases markedly on differentiation (Craig and Bloch, 1984; Gonda and Metcalf, 1984; Bender and Kuehl, 1986; Ramsay *et al.*, 1986). While the precise mode of action of the *myb* proteins has not been elucidated, both viral and cellular *myb* genes encode nuclear proteins (Klempnauer *et al.*, 1983, 1984) that bind to DNA (Moelling *et al.*, 1985; Klempnauer and Sippel, 1986), possibly in a sequence-specific manner (Bading *et al.*, 1987; Biedenkapp *et al.*, 1988). Moreover, recent data have demonstrated that the c-*myb* gene product can function as an activator of transcription (Nishina *et al.*, 1989).

Second, deregulated expression combined with structural alteration of c-*myb* is associated primarily with haemopoietic neoplasias, which are predominantly of the myeloid lineage. For example, the v-*myb* oncogenes of the avian acute leukaemia viruses AMV and E26, which have arisen by transduction of part of c-*myb* (Klempnauer *et al.*, 1982; Nunn *et al.*, 1983), are able to transform myeloid cells *in vitro* and *in vivo* (Beug *et al.*, 1979; Moscovici and Gazzolo, 1982; Radke *et al.*, 1982). *Myb* has also been implicated in the transformation of murine myeloid cells since a number of tumours and transformed cell lines have been described in which c-*myb* appears to be activated by retroviral insertion (Mushinski *et al.*, 1983; Shen-Ong *et al.*, 1986; Weinstein *et al.*, 1986; Gonda *et al.*, 1987). In all of these cases, nucleotide sequence comparisons have predicted that the viral or rearranged cellular *myb* genes encode proteins that are truncated at either their amino (N)- and/or carboxy (C)-termini; in many cases, this has been confirmed at the protein level (Klempnauer *et al.*, 1983; Ramsay *et al.*, 1989). Thus, these studies have lead to the suggestion that truncation may be required for the oncogenic activation of c-*myb*.

To date, cellular transformation *in vitro* by the *myb* oncogene has been described in the avian system only (reviewed in Moscovici, 1975). We have therefore attempted to utilize retroviral vectors in combination with the well-characterized murine haemopoietic system to examine aspects of transformation by *myb*. In particular, we wanted to directly test the effect of truncation of the c-*myb* gene product on its transforming capacity, and to examine biological properties, e.g. lineage, differentiation state, dependence on colony-stimulating factors (CSFs) and tumorigenicity, of murine cells transformed *in vitro*.

In this, the first of two reports, we describe the properties of CSF-dependent myeloid cell lines which were generated, at low frequency, following infection of primary cells with recombinant *myb* retroviruses. Each of these lines carries a rearranged vector provirus which, unlike the parental vector, efficiently expresses the introduced *myb* sequences. We have characterized and molecularly cloned (one of) the rearranged proviruses and examined the mode by which they express the truncated *myb* proteins detected in these cells. We also assessed the tumorigenicity of a representative cell line. While these cells did not give rise to tumours in syngeneic mice, we could generate highly tumorigenic CSF-independent sublines by superinfection with retroviruses that express granulocyte-macrophage (GM)-CSF or v-*abl* sequences. In the accompanying paper (Gonda *et al.*, 1989), we describe the use of retroviral vectors derived from the rearranged provirus described here, to study the effect of truncation of c-*myb* on its biological activity.

Results

Generation of cell lines by infection with myb retroviruses

In order to examine the effects of aberrant *myb* expression on haemopoietic cells, we inserted cDNAs encoding either full-length or C-terminally truncated *myb* proteins into the M3Neo vector (see Figure 3A). We could readily derive Ψ2 lines producing M3Neo-based viruses which gave high titres (10^5-10^6 G418^r c.f.u./ml) when assayed on 3T3 fibroblasts and which could convert 30–100% of haemopoietic progenitor cells from murine fetal liver to G418 resistance. However, despite this efficient infection, no obvious effects on soft agar cultures of infected cells were observed: the numbers, composition and CSF-dependence of colonies obtained were indistinguishable from those of control cultures. Although several hundred infected colonies were examined in a number of experiments, a single colony with abnormal morphology (reminiscent of the compact colonies formed by many transformed myeloid cells) was observed in a culture infected with the M3Neo(CTmyb) virus. When isolated and placed in liquid culture in the presence of a source of CSFs, the cells from this colony continued to proliferate and gave rise to the T59 cell line.

Table I. Properties of M3Neo(myb)-derived cell lines

Cell line	Virus	Morphology[a]	Astra blue staining	Surface markers[c]		% Phagocytic cells[d]	CSF dependence[e]
				Mac-1	Gm3.2		
T59	CT *myb*	blast plus myelomoocytic	+	+	+	1.5	+
U16.6	CT *myb*	blast plus myelomonocytic	±	+	+	1.2	+
U22.3	CT *myb*	blast plus monocyte/macrophage	−	++	+++	12	+
U22.4	FL *myb*	monocyte/macrophage plus metamyelocytes	−	+	++	4.8	+

[a]Assessed by May–Gruenwald Giemsa staining of cytocentrifuge preparations. Classifications are to designate similarity to indicated normal cell types only.

[b]Arbitrary scale; IL-3-dependent primary mast cells score '+++'.

[c]Determined by antibody staining and flow cytometry. Symbols indicate relative intensity of fluorescence only; '+' indicates fluorescence at least 3-fold over control.

[d]Percentage of cells taking up latex beads over 24–48 h.

[e]See Figure 3 and text for details.

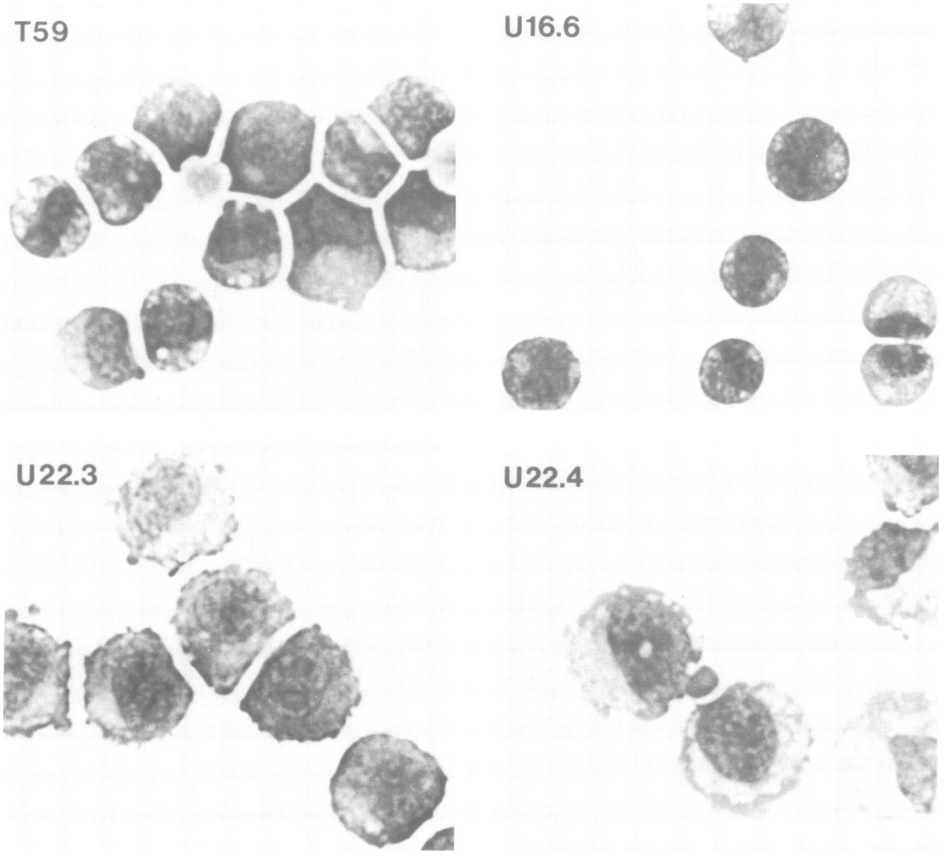

Fig. 1. Morphology of M3Neo(myb)-transformed cell lines. Shown are micrographs of cytocentrifuge preparations of the indicated cell lines stained with May–Gruenwald Giemsa.

Since the growth of T59 cells in liquid and agar cultures was dependent on and stimulated by GM-CSF (see below), we also placed infected fetal liver cells in liquid cultures containing this factor and selected for continued growth of loosely adherent or non-adherent cells. In this way, over the course of several experiments, three more clonal, CSF-dependent lines—U16.6, U22.3 and U22.4 (see below)—were derived following infection with M3Neo(myb) viruses. No lines were derived from cultures infected with the M3Neo virus alone or from fetal liver cells co-cultivated with non-producing Ψ2 cells.

Biological properties of the M3Neo(myb) cell lines

All four lines resembled immature cells of the myelomonocytic lineage. We base this conclusion on the properties shown in Table I and Figures 1 and 2. Specifically, they were positive for the Mac-1 (Springer *et al.*, 1979) and Gm 3.2 (Hibbs *et al.*, 1985) surface markers found on cells of the monocytic and granulocytic lineages, and were dependent on the presence of CSFs for proliferation (see below).

The observation that only a small percentage of the cells could phagocytose latex beads suggested that the majority of the cells of each line were functionally immature but capable of differentiation to more mature macrophages. This is consistent with the morphology of the cells (Figure 1); although the T59 and U16.6 lines are more blast-like than either U22.3 or U22.4, the latter do not have the appearance of fully mature macrophages. Despite the weak staining with Astra blue—typically a mast cell characteristic—displayed by T59 and to a lesser extent, U16.6 cells, the other properties of these lines place them in the monocytic lineage.

The pattern of responses of the four cell lines to the various CSFs revealed differences between these lines. All four lines were initially selected in GM-CSF-containing media, to which they continued to show the greatest proliferative response as determined by colony formation in agar (Figure 2). The GM-CSF dose-response curves for these lines approximate those of normal colony-forming cells, with U16.6 and U22.4 being slightly more sensitive than the others. Responses to the other CSFs varied. At high levels, interleukin-3 (IL-3) stimulated colony formation by T59, U16.6 and U22.4, while GM-CSF could stimulate only U16.6 cells (Figure 2). Only U22.4 cells formed colonies in response to stimulation with CSF-1 (Figure 2); however, this factor could stimulate a lesser degree of proliferation to generate clusters (clones of <50 cells) from T59, U16.6 and, very efficiently, from U22.3 cells (data not shown).

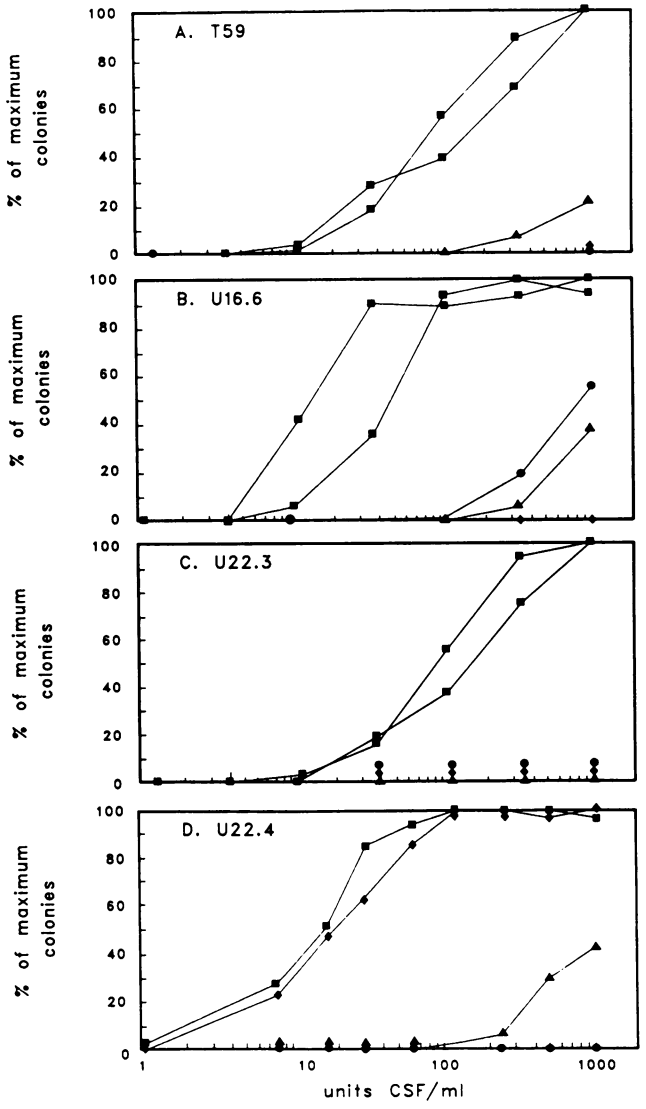

Fig. 2. Response of T59 (**A**), U16.6 (**B**), U22.3 (**C**) and U22.4 (**D**) cells to CSFs. The number of colonies (>50 cells) obtained at the indicated concentrations of each CSF is shown as percentage of the maximum number of colonies obtained with the most effective stimulus, GM−CSF. Symbols: ■,GM-CSF; ▲,IL-3; ●, G-CSF; ◆, CSF-1. The results of two independent GM-CSF titrations are shown in (A), (B) and (C).

Structure and expression of the vector proviruses in the M3Neo(myb)-infected cell lines

Southern blotting analysis of *Bam*HI-digested DNA from the T59 and U16.6 cell lines using a *neo*^{r} probe (Figure 3B) revealed the presence of 10−13 discrete fragments, suggesting that these lines contained multiple copies of viral DNA, since *Bam*HI cuts only once within the M3Neo(myb) provirus (Figure 3A). These bands were of the expected size

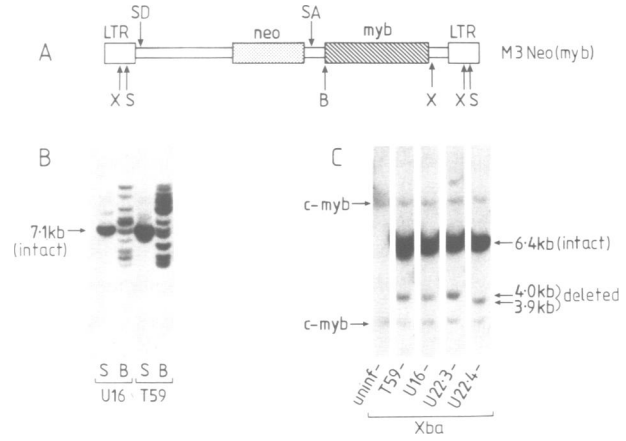

Fig. 3. (**A**) Structure of the M3Neo(myb) provirus. SD and SA indicate the positions of splice donor and acceptor sites; X, S and B indicate positions of cleavage sites for *Xba*I, *Sac*I and *Bam*HI respectively. (**B**) Southern blotting analysis, using a *neo*^{r} probe, of T59 and U16.6 DNA after digestion with *Sac*I (S) or *Bam*HI (B). The positions of the expected 7.1 kb band corresponding to the intact M3Neo(myb) provirus is indicated. (**C**) Southern analysis of *Xba*I-digested DNA from the four cell lines and from uninfected cells using a c-*myb* probe. The positions of bands corresponding to the endogenous c-*myb* gene are indicated as are the sizes of bands corresponding to intact and 'deleted' M3Neo(myb) proviruses.

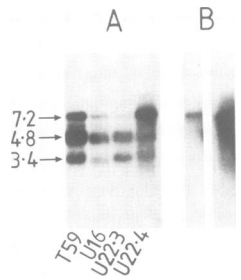

Fig. 4. Northern analysis of RNA from M3Neo(myb)-infected cells. One microgram of poly(A)$^+$ RNA, from (**A**) the indicated transformed cell lines and (**B**) a pool of NIH3T3 cells infected with M3Neo(CTmyb) and selected for resistance to 400 μg/ml of G418, was hybridized with a c-*myb* probe. The left lane in (**B**) shows an exposure equivalent to that of (**A**), while the right lane shows a 15-fold longer exposure. The sizes of the major RNA species detected are indicated at left.

(7.1 kb) after digestion with *Sac*I (Figure 3B), which cleaves the provirus once within each long terminal repeat (LTR) (Figure 3A). The clonality of these lines was demonstrated by the identity of these patterns in several subclones of each (data not shown). Similarly, the intensity of hybridization of a *myb* probe demonstrated multiple copies of the expected proviral fragments in all four lines following digestion with *Xba*I (Figure 3C), which cuts within each LTR and also 0.6 kb upstream of the site in the 3′ LTR (see Figure 3A). However, this probe revealed additional *Xba*I fragments of 4.0 kb in DNA from T59, U16.6 and U22.3, and of 3.9 kb in U22.4. This observation, and the presence of corresponding *Sac*I fragments [i.e. 0.6 kb larger than the 3.9 or 4.0 kb *Xba*I fragments (data not shown)], suggested that a provirus bearing a deletion encompassing the *neo*^{r} sequences was present in each of the four cell lines.

Northern analysis of poly(A)$^+$ RNA from the cell lines, followed by hybridization to a *myb* probe (Figure 4A) initially revealed the presence of variable levels of the expected 7.2 kb genomic transcript of the complete M3Neo(myb) provirus, although in RNA isolated from later passages of T59 and U16.6, this species could not be detected (see Figure 7B below; and data not shown). However, all four lines also contained high levels of a 4.8 kb transcript and somewhat lower levels of a 3.4 kb species; both these species and the 7.2 kb RNA also hybridized to a virus-specific probe [an oligonucleotide representing the U5 region of the LTR (data not shown)]. To determine whether this pattern of transcripts could be generated by the complete M3Neo(myb) provirus, we also examined RNA from a pool of 3T3 fibroblasts which had been infected with M3Neo(CTmyb) and selected for G418 resistance (Figure 4B). The structure of the M3Neo(myb) provirus (Figure 3A) would predict the presence, in addition to the 7.2 kb genomic transcript, of a 3.2 kb subgenomic *myb* mRNA generated by splicing between the sites indicated; however, even after prolonged autoradiography, only the 7.2 kb species could be detected.

Identification of myb-encoded proteins

Translation products of the viral RNAs were identified by Western blotting with a monoclonal antibody (5.1) directed against bacterially expressed c-*myb* protein (Ramsay *et al.*, 1989) or antiserum (4:3) raised against a synthetic peptide corresponding to the C-terminus of murine c-*myb* (G.Evan and T.J.Gonda, unpublished data). As expected, both

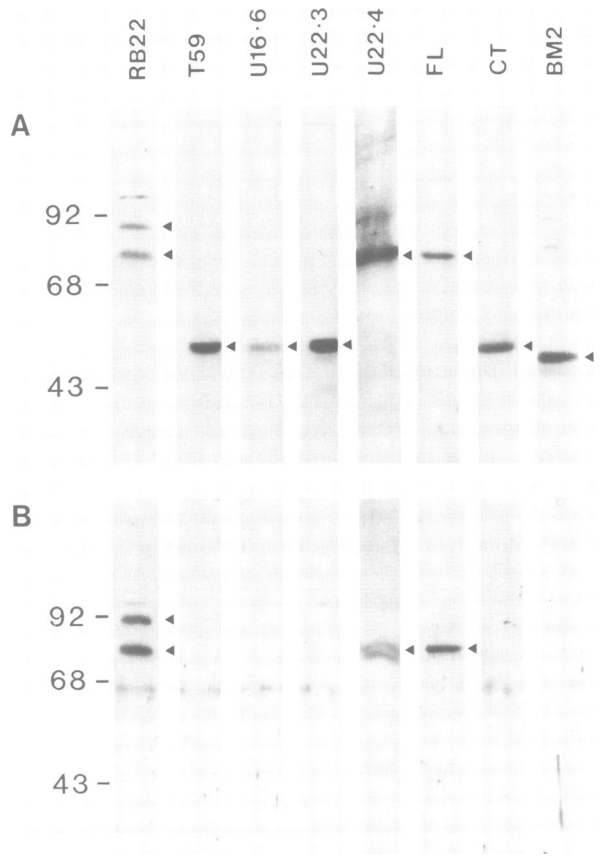

Fig. 5. Identification of *myb* proteins by immunoblotting. Cell lysates of the RB22, T59, U16.6, U22.3, U22.4 and BM2 cell lines (as indicated), and *in vitro* translated complete (FL) and C-terminally truncated (CT) murine c-*myb* proteins were analysed using (**A**) the monoclonal antibody 5.1 and (**B**) the anti-C-terminal peptide antiserum 4:3 (see Materials and methods). Arrowheads indicate *myb* proteins, and the positions and mol. wts of size markers are shown at the left.

reagents detected the normal murine c-*myb* proteins of 75 kd (Boyle *et al.*, 1986) and 90 kd (Ramsay *et al.*, 1989), in the RB22 thymoma cell lines (Cook, 1985) as well as *in vitro* synthesized 75 kd c-*myb* protein (Figure 5A and B). Additionally, Figure 5A shows that the monoclonal antibody 5.1 recognized a C-terminally truncated *myb* protein synthesized *in vitro* and the v-*myb* protein p45$^{v\text{-}myb}$ in the AMV-transformed chicken BM2 cell line (Moscovici *et al.*, 1982); also as expected, these latter species were not detected by the peptide antiserum (Figure 5B).

The three lines derived by infection with M3Neo(CTmyb)—T59, U16.6 and U22.3—contained a ~50 kd protein which was recognized by the anti-c-*myb* monoclonal antibody (Figure 5A) [and by a polyclonal anti-v-*myb* antiserum (Boyle *et al.*, 1986); data not shown] but not by an antiserum directed against the C-terminus of murine c-*myb* (Figure 5B). Thus, these proteins are, as expected, truncated at the C-terminus and in fact correspond closely to the predicted size (but see below). By contrast, the U22.4 cell line (derived by infection with M3Neo(FLmyb) expressed a 75 kd protein that co-migrated with authentic c-*myb* protein and reacted with both the monoclonal antibody 5.1 (Figure 5A) and with the antiserum against the C-terminus of c-*myb* (Figure 5B).

These observations can be interpreted as follows: (i) the M3Neo(myb) viruses do not direct the expression of *myb* since the subgenomic *myb* mRNA is not produced in

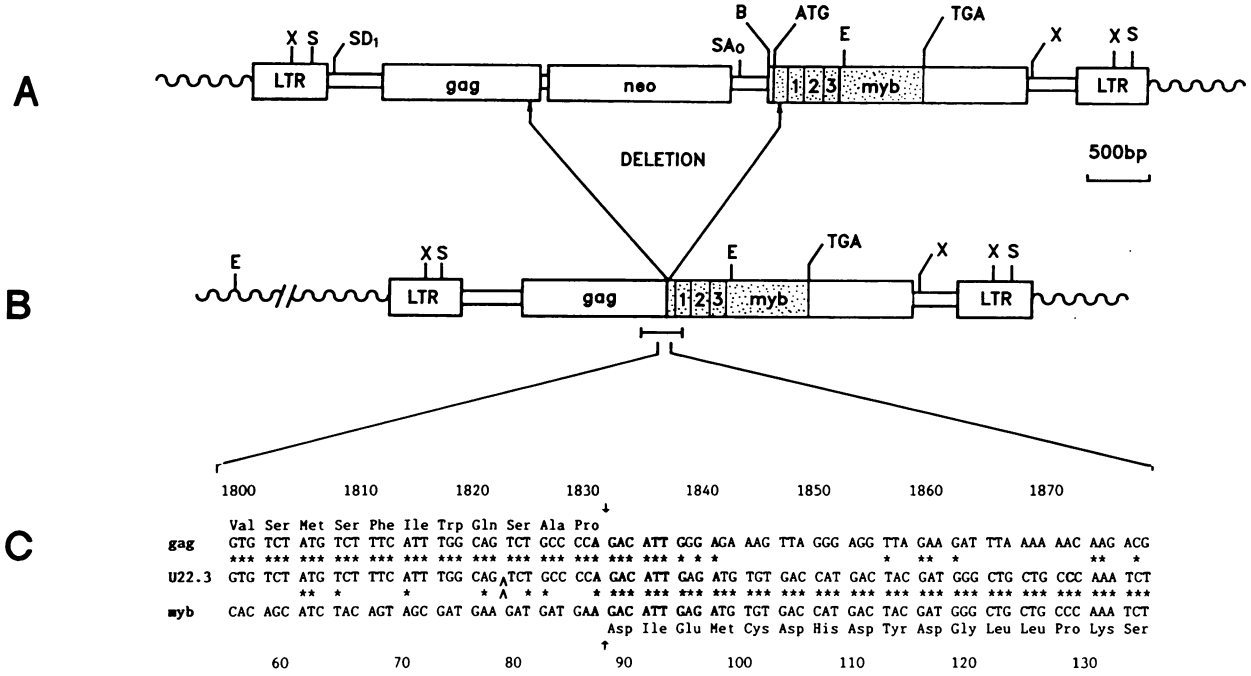

Fig. 6. Generation and structure of the rearranged ('deleted') M3Neo(CTmyb) provirus present in U22.3 (**A**) Structure of the parental M3Neo (CTmyb) provirus. Large boxes represent LTRs or *gag*, *neo*^{R} and *myb*, as indicated; the stippled region within *myb* indicates the coding sequences bounded by the normal c-*myb* initiation (ATG) codon and the CT*myb* termination codon (TGA). Segments labelled '1', '2' and '3' represent the three 51–52 amino acid repeats in c-*myb*. SD_1 and SA_0 indicate the locations of splice donor and acceptor sites respectively, and X, S, B, and E show cleavage sites for *Xba*I, *Sac*I, *Bam*HI and *Eco*RI respectively. Wavy lines represent flanking cellular DNA. (**B**) Structure of the 'deleted' provirus. The horizontal bar indicates the region that was sequenced. Symbols are as above. (**C**) Nucleotide sequence across the *gag*–*myb* junction (indicated by vertical arrows) of the 'deleted' provirus. The top three lines are nucleotide and amino acid sequences from Mo-MLV (Shinnick *et al.*, 1981), and the bottom three lines are from the sequence of murine c-*myb* (Gonda *et al.*, 1985). the middle line is the experimentally determined sequence; asterisks indicate sequence identity and the area in bold shows the homology between *gag* and *myb* at the junction. The chevron 2 indicates the location of a potential splice acceptor site (SA_2 in Figure 7).

detectable amounts (see also Bowtell *et al.*, 1988); (ii) the four cell lines derived by infection with M3Neo(myb) viruses each carry a provirus which has undergone a deletion that includes the *neo*^{r} gene; (iii) this 'deleted' provirus (of ~4.6 kb) is expressed, giving rise to a 4.8 kb genomic transcript and a 3.4 kb subgenomic RNA, one of which encodes the *myb* protein detected in each of the lines.

Molecular cloning of a fragment of the 'deleted' provirus

Further characterization of the 'deleted' proviruses required molecular cloning to define more precisely their structure and coding capacity. Since the deletion involved loss of *neo*^{r} sequences (see above), and retention of *myb* sequences both 3′ and 5′ of the *Eco*RI site within *myb* (see Figures 3A and 6A) (data not shown), we cloned a 3.8 kb *Eco*RI fragment from U22.3 DNA that extends 5′ from this *Eco*RI site to an *Eco*RI site in adjacent cellular sequences (Figure 6B). Two positive recombinant phage were isolated and found to contain inserts of identical size; these were subcloned, mapped with restriction endonucleases and subjected to partial nucleotide sequence analysis. The results (Figure 6) confirmed our interpretation of the Southern analysis by revealing that the deletion had removed all of *neo*^{r} as well as the 3′ portion of the viral *gag* sequence; surprisingly, the viral splice acceptor region and 86 nucleotides of the 5′ end of the *myb* cDNA, including the first 17 codons, had also been deleted. Moreover, the juxtaposition of *myb* and *gag* sequences maintained the authentic reading frames of both (Figure 6C) and, thus, we would predict that this 'deleted' provirus could encode a *gag*–*myb* fusion protein (Figure 7C) of ~88 kd. This is at variance with the size of the protein detected (Figure 5) in U22.3 (and in T59 and U16.6, which have a similarly rearranged provirus—see below); this discrepancy is addressed below. Interestingly, the sequence analysis also suggested the rearrangement may have occurrred via homologous recombination, since the *myb* and *gag* sequences surrounding the junction share 10 of 11 nucleotides (Figure 6C).

M3Neo(myb) proviruses in U22.3, T59 and U16.6 have undergone identical rearrangements, distinct to that in U22.4

We next wished to determine whether the 'deleted' proviruses in the other three lines had undergone similar rearrangements to U22.3, and to show that the 4.8 and 3.4 kb transcripts did indeed correspond to the 'deleted' proviral genomes. An oligonucleotide probe was prepared that represented the 12 nucleotides at each side of the *gag*–*myb* junction in U22.3 (Figure 7A), and hybridized with RNA from the four cell lines. After washing at high stringency, only the 4.8 and 3.4 kb RNAs in T59, U16.6 and U22.3 were detected (Figure 7B), implying that the 'deleted' proviruses in these three lines, which were all derived by infection with the M3Neo(CTmyb) virus, had the same *gag*–*myb* junction. As a control, a replicate filter was hybridized with a *myb* cDNA probe which revealed the expected 7.2, 4.8 and 3.4 kb RNAs (Figure 7B). (Note that the U16.6 and U22.3 RNA preparations used here were from late passages of these lines which, like later passages of T59, no longer expressed the unrearranged proviruses.) The

failure of the 7.2 kb species in either U22.4 or T59 RNA to react with the oligonucleotide probe demonstrated its specificity, since this transcript contains unrearranged *gag* and *myb* sequences. Furthermore, these data imply that the rearrangement in U22.4 is different to that in the other three lines. To confirm this point, we determined the sequence around the deletion breakpoints of the rearranged provirus in U22.4 following amplification of this region using the polymerase chain reaction (Saiki *et al.*, 1988). We found that in contrast to the U22.3, T59 and U16.6 rearrangements, the deletion did not remove the c-*myb* initiation codon and adjacent sequences (data not shown).

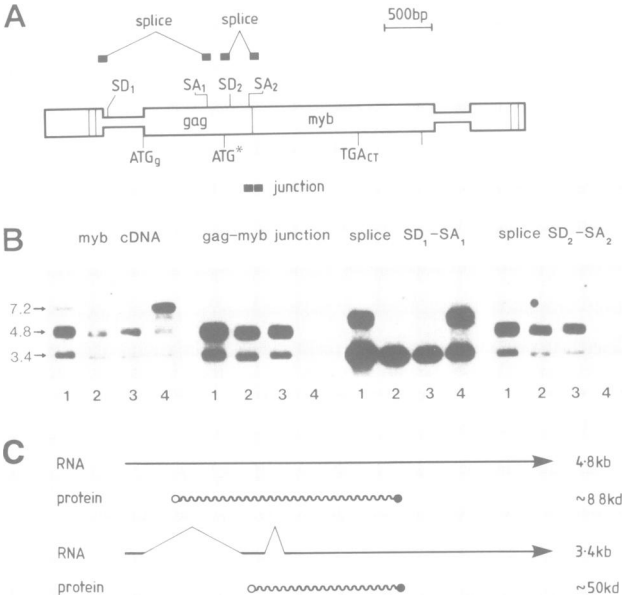

Fig. 7. Structure and coding potential of transcripts of the 'deleted' M3Neo(CTmyb) provirus. **(A)** Structure of the provirus and oligonucleotide probes used to analyse transcripts. SD_1 and SD_2 are potential splice donor sites, SA_1 and SA_2 are potential acceptor sites; ATG_g is the initiation codon for the viral *gag* gene, while ATG* is a potential initiation codon in another reading frame, which can be transferred by splicing from SD_2 (see text). Closed rectangles represent the two 12-base 'halves' of the each of the three oligonucleotide probes used in the Northern analysis. **(B)** Northern analysis of poly(A)$^+$ RNA from T59 (**lane 1**), U16.6 (**lane 2**), U22.3 (**lane 3**), and U22.4 (**lane 4**) cells. The hybridization probes used are indicated at the top of each panel; the sequences represented by the oligonucleotide probes are illustrated in (A) above. Sizes of the three major transcripts are shown at left. **(C)** Coding potential of the 4.8 kb genomic transcript and the doubly spliced 3.4 kb transcript. The predicted proteins both terminate at the TGA_{CT} stop codon in *myb* (see part A); the ~88 kd protein starts at ATG_g while the ~50 kd protein starts at ATG* (see part A).

This is consistent with the immunoblotting data (Figure 5) which revealed an apparently normal c-*myb* protein in U22.4 cells.

Splicing and coding potential of transcripts of the rearranged proviruses

Because the 50 kd *myb* proteins in T59, U16.6 and U22.3 were much smaller than the ~88 kd *gag*−*myb* fusion proteins that the rearranged proviruses were predicted to encode, we considered the possibility that these proteins were translated from a spliced mRNA lacking most of the *gag* sequence. The 3.4 kb RNA was an obvious candidate as it carried the same *gag*−*myb* junction as the 4.8 kb transcript of the rearranged proviruses. We therefore looked for potential splice donor and acceptor sites which could explain both the size of the smaller RNA and the synthesis of a 50 kd *myb* protein. The rearranged proviruses retain the original splice donor (SD_1) but have lost the acceptor site (SA_0) (Figure 6A). However, analysis of cell lines with rearranged Mo-MLV proviruses has revealed a cryptic splice acceptor site (SA_1 in Figure 7A) in *gag* (Gonda *et al.*, 1987) and a donor site (SD_2) further downstream that can transfer a functional initiation codon (ATG* in Figure 7A) (Shen-Ong *et al.*, 1986; Gonda *et al.*, 1987). Moreover, inspection of the sequence near the *gag*−*myb* junction of the rearranged provirus revealed a second potential acceptor site (SA_2 in Figure 7A; see also Figure 6C). We therefore prepared oligonucleotide probes complementary to the junctions formed by each pair of splice sites (SD_1/SA_1 and SD_2/SA_2). Northern analysis using these probes showed that, indeed, the 3.4 kb RNA contained molecules exhibiting both these splices (Figure 7B). While at least some of the 4.8 kb RNA had also undergone the SD_2/SA_2 splice, use of a probe complementary to sequences removed by this splice indicated that some RNA of each class remained unspliced (data not shown). Figure 7C illustrates the splicing patterns and the coding potential of the unspliced and doubly spliced RNAs; note that translation of the latter species accounts for the observed 50 kd protein.

Isolation and tumorigenicity of CSF-independent variants of a myb-transformed cell line

Because of the correlation between CSF-independence and tumorigenicity of murine myeloid cell lines (reviewed in Dunn, 1987), we decided to generate a number of CSF-independent sublines from one of the *myb*-transformed lines, T59. Infection with a GM-CSF-expressing retrovirus (GMV) (Lang *et al.*, 1985) and with Abelson murine leukaemia virus (Ab-MLV) (Cook *et al.*, 1985) have been shown to abrogate

Table II. Generation and tumorigenicity of CSF-independent sublines of T59

T59 cells infected with	No. of CSF-independent lines	CSF secretion[a]	Tumorigenicity	
			No. of lines tested[d]	No. of mice with tumours
−	0	NT[b]	1	0/3
Zip Neo	0[c]	NT	2	0/6
GMV	7	+	3	9/9[e]
Ab-MLV	1	−	1	3/3

[a]Assayed on agar cultures of murine fetal liver cells.
[b]NT = not tested. It is not feasible to measure GM-CSF production by cells maintained in its presence.
[c]It was possible to establish three G418-resistant (CSF-dependent) lines in this case.
[d]Mice were injected with $1-3 \times 10^6$ cells.
[e]Tumours were apparent between 2 and 8 weeks post-injection.

the requirement of the GM-CSF-dependent cell line FDC-P1 for exogenous CSFs, and concomitantly, to render these cells tumorigenic. Therefore, we attempted to derive CSF-independent lines from T59 by infection with these same viruses. Following infection, T59 cells were plated in agar medium in the presence or absence of added GM-CSF. While no colonies of parental T59 cells or cells infected with a virus carrying only the *neo*^{r} gene [ZipNeoSV(X)] (Cepko *et al.*, 1984) were obtained in cultures lacking added CSF, colonies were obtained from cells infected with GMV and, to a lesser extent, with Ab-MLV (data not shown). Seven cell lines were derived from GMV-infected colonies and one from an Ab-MLV infected colony (Table II); as expected, only the former secreted a detectable level of CSF. The clonality and presence of the expected exogenous GM−CSF or *abl* sequences in these lines was demonstrated by Southern analysis (data not shown).

Tumorigenicity of the cell lines was assessed by injection into syngeneic (CBA) mice. Table II shows that while neither the parental T59 cells nor two ZipNeoSV(X)-infected sublines gave rise to tumours, all three GMV-infected lines tested and the Ab-MLV line did so. Subcutaneous tumours were apparent as early as 10 days after injection in some cases. Autopsy (6 weeks after injection) revealed that in addition to large (2−4 cm) tumours at the site of injection, the mice variably showed enlargement of the spleen, pale bone marrow, and secondary tumours on the peritoneal wall. CSF-independent cells could readily be cultured from single-cell suspensions of tumour masses, spleens or bone marrow of the tumour-bearing mice. These cells were clearly derived from the injected T59 cells, since Southern analysis of DNA from these cells showed the same 'fingerprint' of *neo*^{r}-containing fragments as the parental T59 line and the CSF-independent sublines (data not shown). Thus we concluded that the CSF-independent sublines of T59, but not the parental line, were highly tumorigenic.

Discussion

Viral myb expression in cell lines derived by infection in vitro

The cell lines described here represent the first examples of mammalian cells transformed *in vitro* by introduction of exogenous *myb* sequences. Although the cell lines were generated at low frequency from M3Neo(myb)-infected fetal liver cells, the following observations and arguments, when taken together, strongly imply that viral *myb* expression was required for the generation of these cell lines. (i) No cell lines were derived from uninfected fetal liver cells or cells infected with the parental M3Neo virus, even though the titre of the latter was at least as high as that of the *myb*-containing derivatives. (ii) Each of the lines carries a rearranged vector provirus which, unlike the parental virus, directs efficient expression of *myb* sequences. The simplest explanation for this is that the experimental protocols used have selected for the expression of virally encoded *myb* proteins. Furthermore, the requirement for a rearrangement to enable expression of *myb* explains, at least in part, the low frequency at which the lines were obtained, compared with the high frequency at which cells were infected with the M3Neo(myb) viruses. (iii) We have shown in the accompanying report (Gonda *et al.*, 1989) that a *myb* virus reconstructed from a molecular clone of the rearranged provirus from U22.3 is capable of stimulating the proliferation of immature, GM-CSF-dependent myeloid cells, and indeed can generate cell lines similar to those described here.

We can therefore conclude from both retrospective and functional analyses that the *myb* proteins expressed in the four cell lines are at least in part responsible for their transformed phenotype. Surprisingly, one of the lines (U22.4) was generated by infection with a virus that encodes a normal c-*myb* protein, and the rearrangement in this line has apparently left the coding sequence intact. Thus, the U22.4 line represents an exception from the general association of truncation with transformation by *myb*. The relationship between truncation and transformation is addressed in detail in the accompanying report (Gonda *et al.*, 1989).

Properties of the myb-transformed cell lines

In common with other *myb* transformants, e.g. chicken cells transformed by v-*myb* (Beug *et al.*, 1979; Durban and Boettiger, 1981), and murine cell lines that carry rearranged c-*myb* genes (Warner *et al.*, 1982; Shen-Ong *et al.*, 1986, 1987; Weinstein *et al.*, 1986, 1987; Gonda *et al.*, 1987) all four cell lines described here represent immature cells of the myelomonocytic lineage. Furthermore, like both chicken v-*myb* transformants (Beug *et al.*, 1982) and the murine NFS-60 cell line (Weinstein *et al.*, 1986), our cell lines are dependent for proliferation on exogenous CSFs. Nevertheless there are clear differences between the murine cell lines (see Table I, Figures 1 and 2 and Weinstein *et al.*, 1986), e.g. in their responsiveness to the various CSFs, levels of expression of surface markers and in morphology, even though three of the lines express identical *myb* proteins. It is possible that the lines represent cells 'trapped' at different stages of differentiation by expression of the truncated *myb* gene product. Alternatively, the differences may reflect additional genetic changes which may have occurred following (or prior to) transformation by *myb*; in the accompanying paper (Gonda *et al.*, 1989) we suggest that such events may be necessary for the establishment of *myb*-transformed cells as permanent cell lines.

Although we have referred to these cell lines as 'transformed', they are not overtly tumorigenic, a property also shared with the NFS-60 cell line and with an AMV-transformed non-producer cell line (Moscovici *et al.*, 1982). This suggests that expression of an activated *myb* gene may not suffice to generate malignant cells. By contrast, the tumour-derived cell lines W265 and W274, and the ABPL/ABML tumours which express N-terminally truncated *myb* proteins are clearly malignant but these may all have undergone additional oncogenic changes, as has been shown for W274 (Vousden and Marshall, 1984; K.Leslie and J.Schrader, personal communication, cited in Gonda *et al.*, 1987). Indeed, we have shown here that expression of exogenous GM-CSF or v-*abl* sequences in one of the *myb*-transformed lines, T59, renders these cells tumorigenic. The conversion of the T59 cell line to CSF-independence and tumorigenicity closely parallels previous studies with the GM-CSF-dependent FDC-P1 cell line (Lang *et al.*, 1985; Cook *et al.*, 1985). However, none of the transforming events that gave rise to this latter line are known. The present study thus demonstrates co-operativity between *myb* and GM-CSF or v-*abl* genes in generating fully transformed, i.e tumorigenic cell lines.

Materials and methods

Retroviral vectors

The M3Neo vector (Laker *et al.*, 1987) was kindly provided by W.Ostertag (Heinrich Pette Institute, Hamburg). cDNA constructs that encode complete or C-terminally truncated *myb* proteins were excised as *Bam*HI–*Bgl*II fragments from the plasmids pMB96 and pMB95 respectively and inserted into the *Bam*HI site of pM3Neo. The pMB96 plasmid was constructed by joining the *myb* cDNA clones pMM46 and pMM49 via their common *Ssp*I site at position 1721; in pMB95, the join is via the *Sma*I site a position 1011 (Gonda *et al.*, 1985). The *Bam*HI site is derived from the pJL3 vector and is immediately upstream of nucleotide 1 in the published sequence (Gonda *et al.*, 1985). Because of a 17 bp deletion present in the pMM46 insert at position 1740, the pMB95 insert contains a premature termination codon and encodes a protein of ~50 kd. Ψ2 lines (Mann *et al.*, 1983) producing these viruses were generated and titred as described previously (Lang *et al.*, 1985).

Infection and culture of haemopoietic cells

Procedures for the isolation, infection and culture in soft agar medium of haemopoietic cells from murine fetal liver have been described in detail elsewhere (Johnson and Metcalf, 1978; Bowtell *et al.*, 1988). In the studies reported here, 2×10^5 fetal liver cells were co-cultivated with the Ψ2 cells which had been plated at 2×10^5/60 mm Petri dish 24 h previously in Dulbecco's modified Eagle's medium (DME) containing 20% fetal calf serum (FCS) and 10% Pokeweed-mitogen-stimulated spleen-cell-conditioned medium (SCM) as a source of CSFs. After 2 days, non-adherent cells were harvested and either plated in soft agar medium (DME/20% FCS/SCM/0.3% agar) or placed in liquid culture in DME/20% FCS containing 800 U/ml of GM-CSF (kindly provided by N.Nicola, Walter and Eliza Hall Institute) or both. As described in Results, the T59 line was isolated from a single colony in agar, while the U16.6, U22.3 and U22.4 lines were obtained from liquid cultures. Cell lines were maintained in DME/20% FCS containing 800 U/ml GM-CSF (T59 and U16.6) or 20% L-cell-conditioned medium (kindly provided by J.Hamilton, Department of Medicine, University of Melbourne) (U22.3 and U22.4).

Biological properties of myb cell lines

Response to the various CSFs was determined by culturing cells in soft agar medium at 500–5000 cells/ml in the presence of varying concentrations of CSF. Purified native or recombinant GM-CSF, IL-3, CSF-1 and G-CSF were kindly provided by N.Nicola, Walter and Eliza Hall Institute. Colonies or clusters were scored after 7 days. Staining of cells with monoclonal Mac-1 (Springer *et al.*, 1979) and Gm 3.2 (Hibbs *et al.*, 1985) antibodies, and flow cytometric analysis was performed as described previously (Langdon *et al.*, 1986).

Phagocytic capacity was determined by incubating cells in 1 ml of medium in 24-well tissue-culture trays in the presence of 3 μm latex beads; cells were photographed after 24–48 h and the percentage of cells ingesting the beads determined from the photographs.

Generation of CSF-independent sublines of T59 and assessment of tumorigenicity

T59 cells were infected with the GM-CSF-encoding retroviral vector GMV (Lang *et al.*, 1985), the parental ZipNeoSV(X) vector (Cepko *et al.*, 1984) or with Ab-MLV by co-cultivating 4×10^5 virus-producing Ψ2 cells with a similar number of T59 cells for 2 days in the presence of SCM as a source of CSFs. After washing, cells were plated in the presence or absence of SCM and/or 1 mg/ml G418 as described for the FDC-P1 cell line (Lang *et al.*, 1985). Colonies growing in the absence of SCM and/or the presence of G418 were picked and expanded to generate cell lines (see Table II).

Tumorigenicity was assessed by s.c. or i.p. injection of $1.5–3 \times 10^6$ cells into syngeneic (CBA) mice. Following autopsy of tumour-bearing animals, cell suspensions were prepared from tumour masses, bone marrow and spleen, and cultured in DME/20% FCS in the absence of added CSFs. Conditioned medium was assayed by its ability to stimulate colony formation in agar cultures of 2×10^4 day-13 CBA fetal liver cells, and DNA from the tumour cells was analysed by Southern blotting.

Analysis of cellular RNA and DNA

DNA and poly(A)$^+$ RNA was isolated from cell lines as described by Hughes *et al.* (1979) and Gonda *et al.* (1982) respectively. The procedures used for Southern and Northern blotting, and hybridization to ^{32}P-labelled nick-translated probes have also been described previously (Gonda *et al.*, 1987; Botwell *et al.*, 1988). The *myb* cDNA probe was the 5′ (~0.7 kb) *Eco*RI fragment of pMM49 (Gonda *et al.*, 1985). The *neo*^{r} probe was the ~1.4 kb *Hin*dIII–*Sma*I fragment of pSV2Neo (Southern and Berg, 1982). Conditions for labelling and hybridization with oligonucleotide probes were as in Gonda *et al.* (1987). All oligonucleotides were 24 bases in length and RNA-complementary; their sequences are indicated or referenced in the text.

Immunoblotting of myb proteins

Total cell lysates were pepared by disrupting 10^8 cells/ml in 2% SDS, 10% 2-mercaptoethanol, 20 mM Tris–HCl pH 8.0, 2 mM PMSF by sonication. Samples equivalent to 2×10^6 cells/lane were fractionated by electrophoresis on 10% polyacrylamide gels and transferred to nitrocellulose by electroblotting. Filters were blocked with 10% non-fat skim milk powder in 25 mM Tris–HCl pH 8.2, 144 mM NaCl, 0.1% Tween 20 (TBS) then incubated with the anti-myb monoclonal antibody or with an anti-peptide antiserum raised to the C-terminal 14 amino acids of murine c-*myb* (a gift from G.Evan). After washing with TBS, antibodies were detected with alkaline phosphatase-conjugated anti-immunoglobulin antisera (Biorad). The production, use and properties of these antibodies will be described elsewhere, as will the *in vitro* synthesized *myb* proteins (Ramsay *et al.*, 1989).

Molecular cloning and sequencing

The 5′ portion of the rearranged provirus from U22.3 was cloned as follows. U22.3 DNA (60 μg) was digested with *Eco*RI plus *Bcl*I and fractionated by agarose gel electrophoresis. Since southern blotting analysis had previously revealed that the rearranged provirus gave rise to a 3.8 kb *Eco*RI fragment (data not shown), DNA from the corresponding region of the gel was electro-eluted and collected by ethanol precipitation. [Cleavage of each of the unrearranged M3Neo(CTmyb) proviruses gave rise to a 2.4 kb *Eco*RI–*Bcl*I fragment.] Two hundred nanograms of this DNA was ligated to 1 μg of *Eco*RI-digested, phosphatase-treated λgt10 DNA (Promega) and packaged ('Gigapack', Stratagene) to generate a library of ~350 000 recombinants on infection of *Escherichia coli* C600hfl. The library was screened by standard procedures (Maniatis *et al.*, 1982) using a c-*myb* cDNA probe and an Mo-MLV LTR probe on duplicate filters. Two double-positive phage were isolated which contained identical inserts of 3.8 kb as expected. These were subcloned into pGEM3 (Promega) and analysed by restriction endonuclease digestion and subsequently by nucleotide sequencing using c-*myb*- and *gag*-specific primers on denatured supercoiled plasmid DNA (Kraft *et al.*, 1988, and references therein).

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