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Surface IgM mediated regulation of *RAG* gene expression in Eμ-N-*myc* B cell lines

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Transgenic mice carrying either the c-*myc* or N-*myc* oncogene deregulated by the immunoglobulin heavy chain enhancer element (Eμ) develop both pre-B and B cell lymphomas (Eμ-c-*myc* and Eμ-N-*myc* lymphomas). We report here that B cell lines derived from these tumors, as well as a line derived from v-*myc* retroviral transformation, simultaneously express surface immunoglobulin (a hallmark of mature B cells) as well as a common subset of genes normally restricted to the pre-B stage of development—including the recombinase activating genes *RAG-1* and *RAG-2*. Continued *RAG-1* and *RAG-2* expression in these lines is associated with VDJ recombinase activity detected with a VDJ recombination substrate. Cross-linking of the surface immunoglobulin on these lines with an anti-μ antibody leads to rapid, specific and reversible down-regulation of *RAG-1* and *RAG-2* gene expression. We also find that a small but significant percentage of normal surface immunoglobulin bearing bone marrow B cells express the *RAG-1* gene. These findings are discussed in the context of their possible implications for the control of specific gene expression during the pre-B to B cell transition.
Key words: down-regulation/Eμ-N-*myc* B cells/pre-B genes/ recombinase activating genes

Introduction

The c-, N- and L-*myc* genes encode a family of nuclear oncoproteins (reviewed in Cole, 1986; Zimmerman and Alt, 1990). Deregulated *myc* gene expression can contribute to cell transformation *in vitro* and in transgenic animals (e.g. Land *et al.*, 1983; Schwab *et al.*, 1985; Adams *et al.*, 1985; Birrer *et al.*, 1988; Dildrop *et al.*, 1989; Moroy *et al.*, 1990). The normal function of *myc* gene products is unknown, but structural homologies between the three *myc* proteins and known DNA binding and transcriptional activator proteins suggest that *myc* proteins may regulate transcription (reviewed in Collum and Alt, 1990). In this regard, the c-*myc* protein was found to bind to a specific DNA sequence (Blackwell *et al.*, 1990); the N-*myc* and L-*myc* proteins also bind to this sequence (A.Ma, T.Moroy, R.Collum, H.Weintraub, F.W.Alt and T.K. Blackwell, submitted). A transient inhibition of *myc* gene expression follows the induction of differentiation in various cultured cell lines (Westin *et al.*, 1982; Reitsma *et al.*, 1983; Lachman and Skovitchi, 1984; Jakobovits *et al.*, 1985; Thiele *et al.*, 1985) and constitutive expression of an exogenous *myc* gene can prevent such differentiation (Coppola and Cole, 1986; Dmitrovsky *et al.*, 1986; Thiele and Israel, 1988). Thus, *myc* proteins may influence differentiation via transcriptional regulation of stage-specific genes.

Transgenic mice bearing *myc* oncogenes deregulated by the immunoglobulin heavy chain enhancer (Eμ-N-*myc* and Eμ-c-*myc* mice) provide models to study the effects of deregulated *myc* gene expression on B cell development *in vivo*. During B cell development, immunoglobulin heavy and light chain genes are rearranged and expressed in a developmentally regulated and tissue-specific fashion (reviewed in Alt *et al.*, 1987). Assembly of the immunoglobulin heavy chain variable region gene leads to its expression as cytoplasmic μ heavy chain protein in precursor B cells. Subsequent assembly and expression of immunoglobulin light chain genes leads to the formation of complete immunoglobulin molecules (H plus L chains) which are expressed on the cell surface ($sIgM^+$ B cells)—thus defining the B lymphoycyte differentiation stage. VDJ recombinase activity, which mediates the assembly of immunoglobulin H and L chain variable region genes, is found in cell lines that represent the pre-B, but not mature, B cell stage of development (Blackwell *et al.*, 1986; Lieber *et al.*, 1987; Schatz *et al.*, 1989; Yancopoulos *et al.*, 1990a). Expression of the *RAG-1* and *RAG-2* genes that together confer VDJ recombinase activity to non-lymphoid cells is believed to be restricted to pre-B cells and not to occur in more mature cells of this lineage (Schatz *et al.*, 1989; Oettinger *et al.*, 1990). Down-regulation of VDJ recombinase activity at the pre-B/B cell juncture would ensure allelically excluded expression of immunoglobulin genes and has been postulated to be signalled via the expression of surface immunoglobulin (Alt *et al.*, 1980). To date, however, no adequate models for studying this process have existed.

Bone marrow pre-B cell populations are selectively expanded in both Eμ-c-*myc* and Eμ-N-*myc* mice, suggesting that deregulated *myc* gene expression delays or interferes with the maturation of pre-B cells into B cells (Langdon *et al.*, 1986; Dildrop *et al.*, 1989; Rosenbaum *et al.*, 1989). Functional studies also indicate retarded but grossly intact B cell differentiation in Eμ-*myc* mice (Vaux *et al.*, 1987). Preliminary characterization of Eμ-N-*myc* tumors revealed some cell lines which possessed rearranged κ genes in the absence of the μ protein (Dildrop *et al.*, 1989). Other cell lines possessed λ gene rearrangements despite producing κ proteins (R.Dildrop, unpublished data). Such aberrancies

suggested deregulation of VDJ recombinase activity in these lines. To characterize further potentially novel aspects of Eμ-N- and c-*myc* tumors, we have assayed for expression of VDJ recombinase activity and various pre-B specific genes in sIgM$^+$ B cell lines derived from spontaneously arising tumors in Eμ-N- and Eμ-c-*myc* transgenic mice. We find that both Eμ-N- and Eμ-c-*myc* lines, as well as a v-*myc* transformed line, express a common subset of pre-B-specific markers including *RAG-1* and *RAG-2*. Modulation of the immunoglobulin receptors on Eμ-N-*myc* B cell lines with anti-IgM antibodies leads to a rapid and specific down-regulation of *RAG* gene expression.

Results

Characterization of sIgM positive Eμ-N-myc and Eμ-c-myc lymphoid cell lines

Surface IgM bearing (sIgM$^+$) B cell tumors appear infrequently compared with pre-B tumors in Eμ-N-*myc* transgenic mice (Dildrop *et al.*, 1989; Rosenbaum *et al.*, 1989; A.Ma and F.W.Alt, unpublished); however, we have established several cultured cell lines (Dildrop *et al.*, 1989). Normal murine B cells do not express the N-*myc* gene (Zimmerman *et al.*, 1986; Smith *et al.*, 1992). To assay for potential effects of continued N-*myc* expression at the B cell stage, we characterized two sIgM$^+$ Eμ-N-*myc* B cell lines (A2 and B3) in detail. Surface staining of A2 and B3 with antibodies to murine IgM and to κ light chain confirmed that they were uniformly sIgM/Igκ$^+$ (Figure 1). Furthermore, staining with antibodies to murine κ and λ light chains revealed that a significant subset of A2 cells were double producers of both κ and λ light chains (data not shown). The simultaneous expression of two light chain isotypes indicated that mechanisms normally responsible for isotype exclusion were not operative in these lines.

As described below, we found unexpected expression of several pre-B genes in these sIgM$^+$ Eμ-N-*myc* cell lines. To determine whether this phenomenon represented a specific effect of deregulated N-*myc* expression or was a more general property of tumors that arise in the context of deregulated *myc* gene expression, we extended our analyses to three Eμ-c-*myc* sIgM$^+$ B lymphoma cell lines (WEHI 411, WEHI 404B and WEHI 405B; Adams *et al.*, 1985) and to a v-*myc/raf* retrovirus-induced sIgM$^+$ plasmacytoma cell line, Balb 14.27 (U.Rapp, unpublished data). These lines all fail to express endogenous *myc* genes, suggesting that they share a common phenotype related to deregulated *myc* gene expression (A.Harris, unpublished data; Mushinski *et al.*, 1987; Dildrop *et al.*, 1989; Ma *et al.*, 1991). For convenience, we will describe the results of our analyses of all these lines simultaneously.

Expression of RAG-1 and RAG-2 in Eμ-N-myc, Eμ-c-myc and v-myc B cell lines

The double isotype expression in Eμ-N-*myc* B cell lines suggested the possibility of continued expression of VDJ recombinase activity. To test this possibility, we assayed for expression of *RAG-1* and *RAG-2* genes; these genes have been found to have a highly pre-B-specific expression pattern within previously assayed transformed lines derived from the B cell lineage (Schatz *et al.*, 1989; Oettinger *et al.*, 1990). For these analyses, total RNA from Eμ-N-*myc*, Eμ-c-*myc*, Balb 14.27 and non-transgenic cell lines was

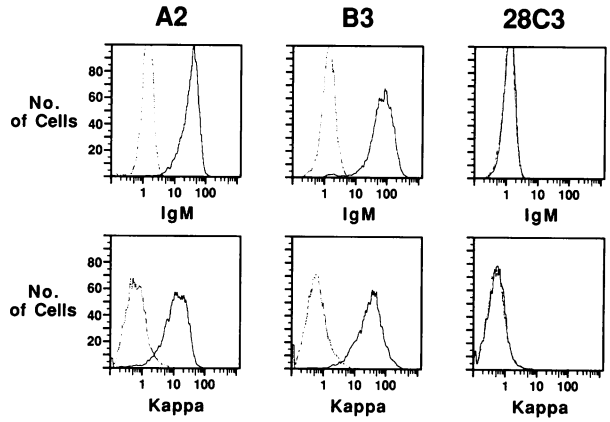

Fig. 1. Surface immunoglobulin staining of Eμ-N-*myc* B cell lines. 5×10^5 cells from various cell lines were simultaneously stained with FITC-conjugated 331 monoclonal rat anti-mouse μ antibody and with biotin-conjugated 187 monoclonal rat anti-mouse κ antibody. After visualization of κ protein with Texas red–avidin, cells were analyzed by flow cytometry (see Materials and methods). A2 and B3 are Eμ-N-*myc* B cells and 28C3 is an A-MuLV pre-B cell line lacking surface immunoglobulin.

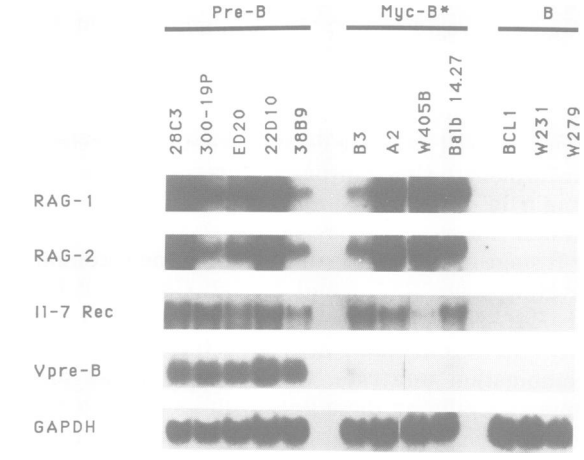

Fig. 2. Northern analysis of pre-B gene expression in non-transgenic and Eμ-N- and Eμ-c-*myc* B cell lines. 20 μg of total RNA from various cell lines was analyzed by Northern blotting for hybridization to probes for various pre-specific genes. *GAPDH*-specific signal indicates the relative amounts of RNA present.

assayed by Northern blotting for hybridization to *RAG-1*- and *RAG-2*-specific probes. Significant levels of *RAG-1* and *RAG-2* RNA expression was observed in all non-transgenic pre-B lines (i.e. 28C3, 300-19P, ED-20, 22D10 and 38B9; Figure 2). Variation in these *RAG* expression levels among individual pre-B cell lines is, at least in part, related to the duration the lines have been grown in culture (Alt *et al.*, 1992; G.Rathbun, unpublished). We observed a comparable range of *RAG* gene expression levels in the Eμ-c-*myc*-, Eμ-N-*myc*- and v-*myc*-transformed B cell lines to those observed in pre-B lines (Figure 2; Table I). However, as found previously (Schatz *et al.*, 1989; Oettinger *et al.*, 1990) no *RAG* gene expression was detected in non-transgenic sIgM$^+$ B cell lines (BCL1, WEHI 231 and W279) (Figure 2). Thus, both *RAG-1* and *RAG-2* are expressed in all six Eμ-N-*myc*, Eμ-c-*myc* and v-*myc* B cell lines.

To test whether Eμ-N-*myc* B cell lines actually possessed VDJ recombinase activity, we utilized a V-*gpt*–J-*neo*

Table I. Recombinase activity and pre-B gene expression in B lineage cell lines

	Pre-B[a]					*Myc*-B[b]						B[c]		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Recombinase activity[d]	++	++	+	++	+	+	++	ND	ND	ND	ND	−	−	−
RAG-1	++	+	+	++	+	+	++	++	++	+/−	+	−	−	−
RAG-2	++	+	+	++	+	+	++	++	++	+/−	+	−	−	−
myb	++	++	++	+	+	++	+	++	++	ND	ND	−	−	−
IL7-R	++	++	++	++	+	++	+	+	+/−	++	++	−	−	−
PB-74	++	++	++	++	+	++	+	+	++	ND	ND	−	−	−
V-pre-B	++	++	++	++	++	+/−	+/−	+/−	+	+/−	+/−	−	−	−
λ-*5*	++	++	++	++	++	+/−	+/−	+/−	+	+/−	+/−	−	−	−
PB-99	++	++	++	++	++	−	−	−	−	−	−	−	−	−

[a]Pre-B cell lines: 1, 28C3; 2, 330-19; 3, 1881-A20; 4, 22D10; 5, 38B9.
[b]*Myc*-transformed B cell lines: 6, B3; 7, A2; 8, W405B; 9, W411; 10, W404B; 11, Balb 14.27.
[c]B cell lymphomas: 12, BCL1; 13, WEHI231; 14, WEHI279.
[d]VDJ recombinase activity in the indicated cell lines was assayed as described by Yancopoulos *et al.* (1990a).
The expression levels of the various genes in the different lines are roughly estimated relative to those of an A-MuLV-transformed cell line (28C3; line 1) that expresses the highest levels of all genes assayed. ++, expression level similar to that of 28C3. +, expression level ~5–20% that of 28C3 line. +/−, expression detectable but substantially <5% that of 28C3. −, expression undetectable. ND, not determined.

recombination construct to assay for VDJ recombinase activity in the A2 and 1810 (B3) cell lines as described previously (Yancopoulos *et al.*, 1990a). Both pre-B (171.732) and sIgM$^+$ (84.27) Eμ-N-*myc* lines had significant levels of VDJ recombinase activity (Table I). Previous studies have confirmed VDJ recombinase activity in the Balb 14.27 line (Lieber *et al.*, 1987). In contrast, none of the non-transgenic B cell lines tested (except Balb 14.27) had detectable VDJ recombinase activity (Table I; Lieber *et al.*, 1987; Yancopoulos *et al.*, 1990a). Therefore expression of *RAG-1* and *RAG-2* in Eμ-N-*myc* B cell lines is correlated with continued expression of VDJ recombinase activity.

Expression of pre-B cell specific genes in myc-transformed B cell lines

The expression of the pre-B-specific *RAG-1* and *RAG-2* genes in Eμ-N-*myc*, Eμ-c-*myc* and v-*myc* B cell lines could be due to the specific deregulation of these genes or a more general deregulation of multiple pre-B-specific genes. To distinguish these possibilities, total RNA from Eμ-N-*myc*, Eμ-c-*myc* and non-transgenic cell lines was assayed by Northern blotting for hybridization to a large panel of probes specific for pre-B-specific genes. Expression of the c-*myb*, *IL-7 receptor*, λ-*5* and *V-pre-B* genes has been found predominantly in pre-B but not B cells (Sakaguchi and Melchers, 1986; Bender and Kuehl, 1987; Kudo and Melchers, 1987; E.Oltz, unpublished). The functions of these genes have been partially elucidated. The *myb* gene product is a DNA binding protein with transcriptional activating activity (Weston and Bishop, 1989); c-*myb* expression is greatly reduced (relative to pre-B cells) in most sIgM$^+$ B cell lines (Bender and Kuehl, 1987); the *IL-7 receptor* confers responsiveness to interleukin 7 and is thought to be confined to cell lines representing the pre-B cell stage (Goodwin *et al.*, 1990; E.Oltz, unpublished). The products of the λ-*5* and *V-pre-B* genes are associated with immunoglobulin heavy chain proteins in pre-B cells prior to the assembly of light chain proteins (Kudo *et al.*, 1989). Expression of the *PB-99* and *PB-74* genes is similarly restricted to pre-B cells (Yancopoulos *et al.*, 1990b), but the functions of these genes are unknown.

Representative Eμ-N-*myc* pre-B cell lines expressed a similar set of pre-B-specific genes as non-transgenic pre-B cells (e.g. A-MuLV transformants; data not shown). However, Eμ-N-*myc*, Eμ-c-*myc* and v-*myc* B cell lines differed from conventional B cell lines by expressing a common subset of pre-B-specific genes at levels comparable to those found in pre-B cell lines (Figure 2; Table I). In particular, c-*myb*, *IL-7 receptor* and *PB-74* genes (in addition to *RAG-1* and *RAG-2*) were expressed in the six Eμ-N-*myc*, Eμ-c-*myc* and v-*myc* B cell lines at levels generally comparable to those of non-transgenic pre-B lines (Figure 2; Table I). However, λ-*5*, *V-pre-B* and *PB-99* transcripts were markedly down-regulated in the transgenic B cell lines compared with pre-B lines (Figure 2; Table I). Thus, *myc*-transformed B cell lines appear to have a common phenotype with respect to expression of a subset of pre-B-specific genes.

Expression of other genes in Eμ-N-myc lines

The Ly-1 surface marker has been found on some B cell tumors and in certain sub-populations of B lineage cells; a Ly-1$^+$ B cell line, NSF-5, has been reported to rearrange immunoglobulin genes and express VDJ recombinase activity (Kleinfeld *et al.*, 1986). However, Northern blotting analyses of total RNA from Eμ-c-*myc* and Eμ-N-*myc* B cells failed to detect a *Ly-1*-specific transcript under conditions in which *Ly-1* gene expression was clearly detected in the BCL1 and WEHI 231 B cell lines (data not shown). Thus the preservation of pre-B-specific gene expression in Eμ-N-*myc* B cell lines is not associated with the selection of an Ly-1$^+$ subset of cells.

Anti-μ treatment of surface IgM-positive Eμ-N-myc lines down-regulates RAG expression

The appearance of complete surface immunoglobulin molecules on B cells has been postulated to result in the down-regulation of VDJ recombinase activity (Alt *et al.*, 1980). How such down-regulation might be signalled has not been an easily answered question. The simultaneous expression of surface IgM and VDJ recombinase activity, and *RAG* gene expression, by Eμ-N-*myc* B cells suggested that these lines might serve as a model for characterizing potential regulatory links between them (see also Discussion). To test this hypothesis, we utilized an affinity purified,

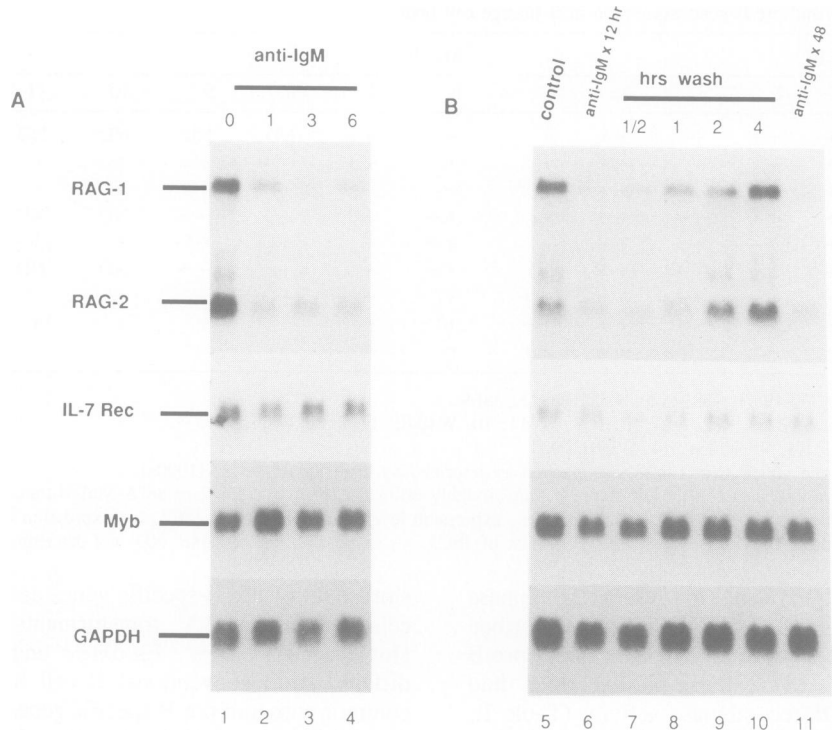

Fig. 3. Cross-linking surface immunoglobulin down-regulates *RAG* gene expression. 15 μg of total RNA from A2 cells that had been treated with various agents were analyzed by Northern blotting for hybridization to probes specific for *RAG-1*, *RAG-2*, *myb*, *IL-7 receptor* and *GAPDH*.
A. Down-regulation of *RAG* by anti-IgM antibody. 20×10^6 A2 cells were treated with anti-μ antibody for 1, 3 or 6 h and harvested for RNA. Control cells grew without antibody for 12 h. Lane 1, medium alone after 12 h; lanes 2–4, anti-μ antibody after 1, 3 and 6 h, respectively.
B. Recovery of *RAG* expression after removal of antibody. 20×10^6 A2 cells were treated with anti-μ for 12 h, washed and replated for various intervals. Lane 5, medium after 12 h; lane 6, anti-μ antibody after 12 h; lanes 7–10, anti-μ after 12 h, washed and replated in fresh media for 30 min, 1, 2 and 4 h, respectively; lane 11, anti-μ after 48 h.

FITC-conjugated rabit anti-mouse μ antibody to cross-link surface immunoglobulin on these cell lines. Anti-μ antibody was added to a final concentration of 10 μg/ml to 20×10^6 A2 and G1 (another subclone of 84.27) cells. Cross-linking of surface immunoglobulin was confirmed by fluorescence microscopy after 1 h. Cells were harvested for RNA at various times and total RNA was analyzed by Northern blotting for hybridization to *RAG-1*, *RAG-2* and other probes (see below). Expression of both *RAG-1* and *RAG-2* mRNAs was dramatically reduced by treatment of cells with anti-μ; levels declined >10-fold compared with the control over 6 h, with a substantial decrease occurring within 1 h of antibody treatment (Figure 3A). Treatment of A2 cells with affinity purified rabbit anti-γ antibody (10 μg/ml) did not down-regulate *RAG* expression, thus controlling for F_c receptor-mediated effects (data not shown). In addition, anti-μ treatment of $sIgM^-$ cells (e.g. 28C3, an A-MuLV pre-B cell line) did not affect *RAG* expression, thus controlling for non-specific effects of the anti-μ antibody (data not shown). Similar results were obtained for both the G1 and the A2 cell lines (data not shown). Therefore the A2 line was used for subsequent experiments.

To determine whether the IgM-mediated down-regulation of *RAG* expression was specific, we assayed whether other pre-B-specific genes deregulated in Eμ-N-*myc* B cell lines were simultaneously down-regulated by cross-linking of surface immunoglobulin. Thus, duplicate blots were hybridized with probes specific for *myb*, *IL-7 receptor* and *PB-74* genes. Cross-linking of surface immunoglobulin did not affect the levels of these other pre-B-specific transcripts (Figure 3A; data not shown for *PB-74*). This specific down-regulation of *RAG-1* and *RAG-2* was maintained when anti-μ antibody was left in the medium for up to 48 h, during which time no significant loss of cell viability (assayed by trypan blue exclusion) was observed (Figure 3B, lane 11). To test whether *RAG* down-regulation by surface immunoglobulin cross-linking was a reversible event (as opposed to an irreversible differentiation event), cells were treated with anti-μ antibody for 12 h, washed and replated in fresh medium without antibody for various periods prior to extracting RNA. *RAG-1* and *RAG-2* mRNA levels increased within 30 min after removal of anti-μ antibody and returned to pre-treatment levels at ~4 h (Figure 3B).

If *RAG* mRNA turned over more rapidly than the other pre-B mRNA sequences expressed in these lines, we could underestimate relative effects of the various treatments on the different genes by quantifying steady-state levels on Northern blots. To evaluate the relative half-lives of the different pre-B-specific mRNA sequences in these lines, we measured steady-state levels of the transcripts following treatment of the cells with actinomycin D (Figure 4). Densitometric and linear regression analyses of these data demonstrated that the *RAG-1*, *RAG-2*, *myb*, *IL-7 receptor* and *GADPH* (glyceraldehyde phosphate dehydrogenase) mRNAs all display apparent half-lives of ~20–30 min. As the anti-μ treatment periods lasted 6–12 h, mRNA stability differences cannot explain the observed differences in steady-state levels. Therefore cross-linking of surface

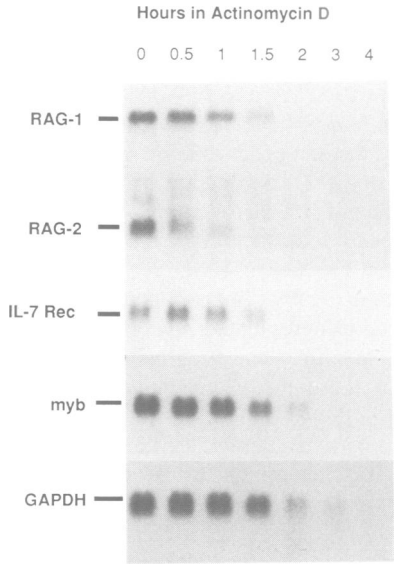

Fig. 4. Actinomycin D treatment of Eμ-N-*myc* cells. Actinomycin D was added at a final concentration of 5 μg/ml to arrest transcription in multiple 10 ml cultures containing 20 × 10^6 A2 cells each. Cells were harvested for RNA at 0, 30, 60, 90, 120, 180 and 240 min after addition of actinomycin D. 15 μg of total RNA was analyzed by Northern blot and hybridized to probes specific for *RAG-1*, *RAG-2*, *IL-7 receptor*, *myb* and *GAPDH*.

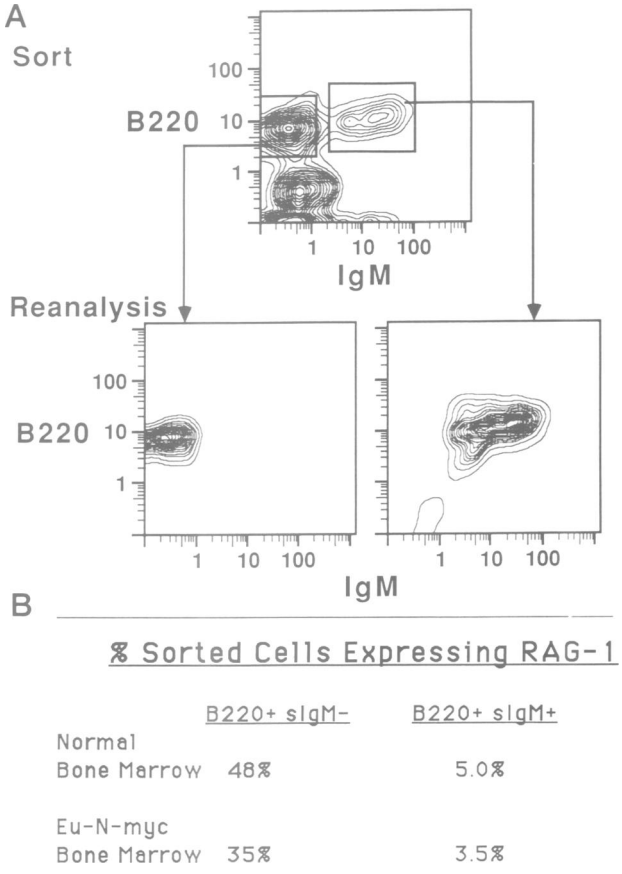

Fig. 5. Detection of *RAG-1* expression in surface immunoglobulin bearing bone marrow B cells. **A.** FACS sorting of normal bone marrow cells into (1) $B220^+$, $sIgM^+$ and (2) $B220^+$, $sIgM^-$ populations. $B220^+$, $sIgM^+$ cells represented 3.7% of total bone marrow cells, while $B220^+$, $sIgM^-$ cells represented 6.3% of total bone marrow cells. Reanalysis of sorted populations indicated that $B220^+$, $sIgM^+$ sorted B cells were contaminated with no more than 0.6% $B220^+$, $sIgM^-$ (pre-B) cells. **B.** *In situ* hybridization of cells from various populations with *RAG-1* antisense riboprobe. Numbers reflect the percentage of each population of cells covered with >25 grains of silver after hybridization with the antisense *RAG-1* probe. Approximately 1000 cells were counted for each population. Based on the same criteria, no positive cells were detected following hybridization to a sense strand *RAG-1* probe.

immunoglobulin molecules on Eμ-N-*myc* B cell lines leads to a highly specific and reversible down-regulation of *RAG-1* and *RAG-2* expression levels.

Expression of RAG-1 in a sub-population of normal sIgM± B cells

Peripheral splenic B and T lymphocytes apparently do not express significant levels of *RAG-1* (Shatz *et al.*, 1989). Therefore in order to determine whether aspects of the phenotype exhibited by the *myc*-transformed B cell lines was shared by a sub-population of normal $sIgM^+$ B cells, we assayed for *RAG-1* expression in $sIgM^+$ B cells from normal or pre-lymphomatous Eμ-N-*myc* murine bone marrows. For these analyses, $B220^+$, $sIgM^+$ cells were isolated and assayed *in situ* for hybridization to antisense and sense *RAG-1* riboprobes. As a control for specificity, the probes and conditions employed yielded hybridization patterns to thymus sections essentially identical to those described previously (Boehm *et al.*, 1991; Chun *et al.*, 1991; data not shown). *In situ* hybridization analyses of the $sIgM^+$ B cell population sorted from bone marrow indicated that 3.5−5% expressed transcripts that hybridized to antisense *RAG-1* probes while none hybridized to sense probes (Figure 5B). Importantly, re-analysis of the same $sIgM^+$ sorted cells demonstrated that significantly <1% represented contaminating $B220^+$, $sIgM^-$ cells (pre- or pro-B cells; data not shown). Therefore a distinct sub-population of bone marrow $sIgM^+$ B cells expresses *RAG-1* in the absence of either tumor formation or *myc* transgenes; however, this population represents <0.1% of bone marrow cells and could only be detected by the sensitive cell sorting−*in situ* hybridization assay employed. Similarly, *in situ* analyses of the $B220^+$, $sIgM^-$ (pre- and pro-B) population revealed that a substantial yet unexpectedly low proportion (35−50%) of these cells expressed levels of *RAG-1* transcripts detectable by our assay (Figure 5B); the potential significance of this finding is discussed below.

Discussion

Expression of pre-B-specific genes in Eμ-N-myc, Eμ-c-myc and v-myc B cell lines

We have shown here that six independent $sIgM^+$ B cell lines derived from three different sources—Eμ-c-*myc* transgenic mouse lymphomas, Eμ-N-*myc* transgenic mouse lymphomas and a v-*myc*/*raf* induced plasmacytoma—express a set of genes previously thought to be expressed only in the pre-B stage of B lymphoid development. Our finding that the six separate *myc*-transformed B cell lines analyzed share a phenotype that has never been described before argues that it is of general significance. The genes unexpectedly expressed by these lines include *RAG-1*, *RAG-2* and the associated VDJ recombinase activity. This is a remarkable phenotype because almost all previously tested $sIgM^+$ B cell lines and normal B lymphocytes lack

expression of *RAG* genes and VDJ recombinase activity (Lieber *et al.*, 1987; Shatz *et al.*, 1989; Oettinger *et al.*, 1990; Yancopoulos *et al.*, 1990a; this study). Down-regulation of VDJ recombinase activity at the B cell stage is generally considered an important aspect of B cell differentiation related to maintenance of allelic exclusion (Alt *et al.*, 1980).

Deregulated *myc* gene expression may contribute to the unusual expression patterns we observe either directly through transcriptional activation of pre-B gene expression or indirectly by disturbing differentiation and/or contributing to transformation. In the latter context, deregulated expression of *myc* genes in the lymphoid lineage has been shown to selectively expand pre-B cell populations in both Eμ-c-*myc* and Eμ-N-*myc* transgenic mice (Langdon *et al.*, 1986; Dildrop *et al.*, 1989; Rosenbaum *et al.*, 1989). Since deregulated *myc* gene expression can interfere with the differentiation of various cell lines *in vitro*, this *in vivo* expansion probably reflects interference with the maturation of pre-B cells to B cells. Cells at the pre-B/B cell juncture would be likely targrets for this interference. Thus, the simultaneous expression of pre-B and B cell markers in *myc*-transformed B cell lines may reflect expression patterns of a normal stage of B cell development which is selected for transformation.

Although the vast majority of normal peripheral B cells do not express the subset of genes described here (Yancopoulos *et al.*, 1990b; Oettinger *et al.*, 1991), the set of novel markers expressed by Eμ-*myc* tumors should facilitate a search for normal B lineage counterparts (e.g. surface immunoglobulin and *IL-7 receptor* bearing). Our demonstration that normal animals possess a small population of $sIgM^+$ B cells which express *RAG-1* reinforces the idea that Eμ-*myc* tumors may represent a previously undescribed stage of normal B cell development (see below). In addition, we find that only 35−50% of the $B220^+$, $sIgM^-$ population of bone marrow cells (pre- and pro-B cells) express significant levels of *RAG-1* as detected by our *in situ* hybridization assay. Cells within this population have generally been considered to be in a state of active Ig heavy or light chain gene rearrangement (reviewed by Rolink and Melchers, 1991). The existence of a significant portion of pre- or pro-B cells that have very low levels of *RAG* expression might be explained in several contexts including variations in *RAG* gene expression (possibly VDJ recombination activity) with respect to cell cycle or stage of differentiation. More detailed analyses of *RAG* expression in pre-and pro-B sub-populations (Hardy *et al.*, 1991) will be necessary to resolve this issue further.

Our finding that pre-B-specific genes apparently fall into two differentially regulated groups in *myc*-transformed B cell lines may also provide insight into normal genetic regulatory pathways involved in the transition from pre-B to B cells. For example, the pre-B-specific λ-*5* and *V-pre-B* genes are significantly down-regulated in these cells. The products of these genes bind to μ heavy chain proteins in pre-B cells prior to the expression of immunoglobulin light chain proteins; expression of complete IgM on the cell surface has been proposed to functionally exclude λ-5 and V-pre-B proteins (Kudo *et al.*, 1989). Therefore the presence of complete surface immunoglobulin molecules on Eμ-N-*myc*, Eμ-c-*myc* and v-*myc* B cell lines may directly down-regulate the expression of λ-*5* and *V-pre-B* genes by a pathway not susceptible to the effects of deregulated *myc* expression. In support of this notion, $sIgM^-$ variants of 84.27 express both λ-*5* and *V-pre-B* transcripts at levels similar to normal pre-B cell lines (A.Ma and F.W.Alt, unpublished data).

The c- and N-*myc* genes are evolutionarily conserved in vertebrates as distinct genes that are differentially expressed during development. Elimination of the N-*myc* is lethal in early development (J.Charron and F.Alt, unpublished). This finding argues for a distinct function for this gene. However, the finding that Eμ-N-*myc*, Eμ-c-*myc* and v-*myc* B cell lines express a common subset of pre-B genes indicates a common phenotype due to deregulated *myc* gene expression, rather than effects specific to N-*myc* deregulation. The shared phenotype of these three *myc*-induced tumor types also includes the absence of endogenous *myc* expression, a common finding when deregulated expression of one of the *myc* genes is implicated in the transformation process (Leder *et al.*, 1983; Adams *et al.*, 1985; Dildrop *et al.*, 1989; Rosenbaum *et al.*, 1989; Moroy *et al.*, 1990; Ma *et al.*, 1991). Common effects of deregulated *myc* expression during tumorigenesis have also been observed in other systems. A common feature of such tumors is the deregulated and usually very high level expression of the involved *myc* gene. In this context, it is important to note that c-, N- and L-*myc* proteins can all bind to the same DNA sequence when assayed at high concentrations (Blackwell *et al.*, 1990; A.Ma, T.Moroy, R.Collum, H.Weintraub, F.W.Alt and T.K.Blackwell, submitted). Therefore high level expression of these genes may result in overlapping activities (due to direct binding of a common DNA sequence or interaction with common partners) such as the activation of common target genes by transcriptional activation.

Regulation of RAG gene expression by cross-linking of surface immunoglobulin

It has been proposed that expression of immunoglobulin molecules on the cell surface might play a role in the down-regulation of VDJ recombinase activity during the pre-B to B cell transition (Alt *et al.*, 1980). Because *myc*-transformed B cell lines express surface IgM and *RAG* genes simultaneously, we could assay directly whether surface immunoglobulin is linked to pathways that regulate *RAG* expression. We find that cross-linking of surface IgM molecules with antibody clearly leads to the prompt and specific down-regulation of *RAG* expression in these lines. Significantly, other workers have found that normal cortical thymocytes expressed both T cell receptor (TCR)−CD3 complex and *RAG* genes. Cross-linking of this TCR−CD3 complex with anti-CD3 antibody caused down-regulation of *RAG* expression (Turka *et al.*, 1991). Together, these results are in accord with the possibility that signalling through newly acquired functional antigen receptors on immature T or B cells can initiate a signal transduction pathway that leads to the down-regulation of *RAG* expression.

The down-regulation of *RAG* expression in Eμ-N-*myc* B cell lines suggests that a population of B cells from normal mice might also express both surface immunoglobulin and *RAG* genes. This prediction is supported by our *in situ* hybridization studies which identify $sIgM^+$ B cells expressing *RAG-1* mRNA in both normal mice and pre-lymphomatous Eμ-N-*myc* mice. The number of these cells in a normal bone marrow is quite small (~0.1% of the total bone marrow cells; Figure 6), making detailed analysis and

manipulation difficult. One possible explanation for the limited size of this population is that these cells represent B cells with newly acquired surface immunoglobulin, and that they exist only transiently in the bone marrow before encountering an extracellular signal which down-regulates *RAG* gene expression.

Materials and methods

Cell lines

84.27 and 1810.15 are $sIgM^+$ B cell lines derived from spontaneously arising tumors from Eμ-N-*myc* transgenic mice and have been described previously (Dildrop *et al.*, 1989). A2 and G1 are $sIgM^+$ subclones of 84.27 and B3 is a $sIgM^+$ subclone of 1810.15. Eμ-c-*myc* B cell lines WEHI 411, WEHI 404B and WEHI 405B are $sIgM^+$ lines established in culture from Eμ-c-*myc* transgenic mice by A.Harris as described (Adams *et al.*, 1985). Balb 14.27 is a $sIgM^+$ B cell line derived from a plasmacytoma induced by (i) pristane priming and (ii) injection of J-2 murine recombinant v-*myc*/*raf* retrovirus (Rapp *et al.*, 1985; U.Rapp, unpublished data).

Detection of surface immunoglobulin

The presence of surface μ was detected with FITC-conjugated 331 monoclonal rat anti-mouse μ antibody (Kincade *et al.*, 1981), while surface κ was detected using a biotin-conjugated 187 rat anti-mouse κ antibody (Yeltin *et al.*, 1981) and avidin–Texas red (Molecular Probes). 5×10^5 cells from various cell lines were washed and stained by standard techniques. Subsequent analysis was performed by flow cytometry using a FACStar Plus.

Molecular analyses

RNA was prepared from cell lines as described previously (Auffrey and Rougeon, 1980). Northern blotting and hybridization to specific probes was performed as described (Yancopoulos *et al.*, 1984). *RAG-1* mRNA was detected using a 1.4 kb *Eco*RI cDNA fragment and *RAG-2* mRNA was detected using a 2.0 kb *Eco*RV–*Not*I cDNA fragment. Both fragments were isolated from cDNA clones kindly provided by Drs D.Schatz and M.Oettinger (Schatz *et al.*, 1989; Oettinger *et al.*, 1990). The 1.2 kb pRGAPDH cDNA probe was used to detect mouse *GAPDH* mRNA (Fort *et al.*, 1985).

PB-99, *PB-74*, λ-*5* and *V-pre-B* represent previously described pre-B-specific genes. Partial cDNA clones were isolated by subtractive hybridization (Sakaguchi and Melchers, 1986; Kudo and Melchers, 1987; Yancopoulos *et al.*, 1990b). A 700 bp partial cDNA clone corresponding to the murine *IL-7 receptor* gene was isolated by PCR using oligonucleotide primers based on the human *IL-7 receptor* nucleotide and protein sequences and murine thymus cDNA (E.Oltz and F.Alt, unpublished data; Goodwin *et al.*, 1990). *myb*-specific mRNA was detected using a 2.4 kb partial cDNA clone, #634, kindly provided by Dr M.Kuehl (Bender and Kuehl, 1986).

Recombinase function analysis

The V-*gpt*–J-*neo* recombination substrate vector and its use in studying the recombinase potential of cell lines are described in detail elsewhere (Yancopoulos *et al.*, 1990a). Briefly, cell lines were infected with the V-*gpt*–J-*neo* virus. G418-resistant cells were frozen for DNA analysis and simultaneously transferred to medium containing mycophenolic acid to select for cells that had performed an inversional rearrangement of the recombination construct and thus expressed *gpt*. Mycophenolic acid-resistant cells were then frozen for DNA analysis.

Cross-linking of Eμ-N-myc B cell lines with anti-IgM antibody

Eμ-N-*myc* B cell subclones A2 and G1 were derived from the 84.27 cell line described above. For cross-linking experiments, 20×10^6 cells were washed and plated in fresh media containing 10 μg/ml of rabbit anti-μ polyclonal antibody (Zymed) or no antibody. Other treatments included: 10 μg/ml of rabbit anti-λ antibody (Zymed) and 5 μg/ml actinomycin D (Sigma). Cells were harvested at various times for isolation of total RNA.

In situ hybridization of sorted bone marrow cells

To isolate $sIgM^+$ B cells from bone marrows of adult mice, animals were killed and femoral bone marrow cells were harvested, washed and stained in deficient RPMI (Irving Scientific) with 3% fetal calf serum and 0.1% sodium azide for 15 min on ice. Bone marrow cells were stained with fluorescein-conjugated 331.12 (anti-IgM; Kincade *et al.*, 1981) and biotin-conjugated RA3-6B2 monoclonal antibodies (Coffman and Weissman, 1981). Biotin antibodies were revealed with avidin–Texas red and cells were analyzed and sorted with a FACStar Plus. Cells were sorted at 4°C in the presence of 0.1% sodium azide since antibody-mediated down-regulation of *RAG* expression in Eμ-N-*myc* cell lines did not occur under these conditions (data not shown).

Detection of *RAG-1* transcripts in individual cells was performed essentially as described (Zeller, 1989). Briefly, a 959 bp *Bgl*II fragment (bp 878–1837) of the *RAG-1* cDNA was subcloned into Bluescript (Stratagene) and *in vitro* transcription using T3 and T7 polymerases was used to generate ^{35}S-labelled sense and antisense riboprobes. Sorted cells were fixed in 0.5% paraformaldehyde, cytospun onto siliconized slides and hybridized with either sense or antisense *RAG-1* riboprobes. Other details are in the legend to Figure 6.

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