The EMBO Journal vol.8 no.4 pp.1121 - 1128, 1989

IgH enhancer-mediated deregulation of N-*myc* gene expression in transgenic mice: generation of lymphoid neoplasias that lack c-*myc* expression

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Communicated by K.Rajewsky

We have generated transgenic mouse lines that carry one of three different constructs in which the murine N-*myc* gene is expressed under the control of the immunoglobulin heavy chain transcriptional enhancer element (Eμ-N-*myc* genes). High-level expression of the Eμ-N-*myc* transgenes occurred in lymphoid tissues; correspondingly, many of these Eμ-N-*myc* lines reproducibly developed pre-B- and B-lymphoid malignancies. The Eμ-N-*myc* transgene also appeared to participate in the generation of a T cell malignancy that developed in one Eμ-N-*myc* mouse. These tumors and cell lines adapted from them expressed exceptionally high levels of the Eμ-N-*myc* transgene; the levels were comparable to those observed in human neuroblastomas with highly amplified N-*myc* genes. In contrast, all of the Eμ-N-*myc* cell lines had exceptionally low or undetectable levels of the c-*myc* RNA sequences, consistent with the possibility that high-level N-*myc* expression can participate in the negative 'cross-regulation' of c-*myc* gene expression. Our findings demonstrate that deregulated expression of the N-*myc* gene has potent oncogenic potential within the B-lymphoid lineage despite the fact that the N-*myc* gene has never been implicated in naturally occurring B-lymphoid malignancies. Our results also are discussed in the context of differential *myc* gene activity in normal and transformed cells.

Key words: enhancer/gene expression/immunoglobulin/N-*myc*/transgenic mice

Introduction

The *myc*-family of cellular oncogenes is a dispersed multigene family that includes the c-, N- and L-*myc* genes (Alt *et al.*, 1986); these genes encode related but distinct nuclear proteins (DePinho *et al.*, 1986, 1987; Kohl *et al.*, 1986; Stanton *et al.*, 1986; LeGouy *et al.*, 1987; Kaye *et al.*, 1988). The three genes also co-operate similarly with an activated Ha-*Ras* oncogene to transform primary rat embryo fibroblasts (Schwab *et al.*, 1985; Yancopoulos *et al.*, 1985; DePinho *et al.*, 1987; Birrer *et al.*, 1988). However, the three genes are conserved as distinct sequences in multiple vertebrate species, suggesting unique functional roles (DePinho *et al.*, 1987; LeGouy *et al.*, 1987; R.C.Collum and F.W.Alt, in preparation). This possibility is supported by observations that the genes are differentially expressed in a stage- and tissue-specific manner during human and murine development (Jackobovits *et al.*, 1985; Zimmerman *et al.*, 1986; Mugrauer *et al.*, 1988). In addition, deregulated c-*myc* expression has been implicated in the genesis of a wide variety of different tumor types and occurs by various mechanisms; whereas deregulation of the N- and L-*myc* genes, to date, has been implicated only in a few naturally occurring tumors (e.g. human neuroblastomas and small cell lung carcinomas) and only by the mechanism of gene amplification (reviewed by Alt *et al.*, 1986). Together, these findings suggest that the *myc* genes play related but distinct roles in growth and/or differentiation of mammalian cells.

myc-family genes are differentially expressed during the progression of cells through the B-lymphocyte differentation pathway (Zimmerman *et al.*, 1986). Expression of both c-*myc* and N-*myc* occurs in normal and transformed precursor (pre)-B cells—which have rearranged and express heavy (H) but not light (L) immunoglobulin (Ig) chains; but N-*myc* expression ceases while c-*myc* expression continues when pre-B cells differentiate to B cells—which express complete (H plus L chains) Ig molecules on the cell surface. *myc*-gene expression shows similar patterns during the differentiation of T lymphocytes; N-*myc* expression declines once cells acquire surface T cell receptor (R.A.DePinho and F.W.Alt, in preparation). These findings suggest that differential expression of *myc* family genes may be important for progression of lymphoid cells through their respective lineages (Zimmerman *et al.*, 1986). Deregulated c-*myc* expression is a common feature of human B cell lymphomas and murine plasmacytomas (reviewed by Klein and Klein, 1985; Cory, 1986). Furthermore, introduction of a c-*myc* gene deregulated by the presence of an IgH chain transcriptional enhancer element (Eμ-c-*myc* gene) into the germline of transgenic mice generates lines that reproducibly inherit a dramatic predisposition to pre-B and B cell malignancies (Adams *et al.*, 1985; Harris *et al.*, 1988). However, despite significant N-*myc* expression during pre-B cell development, deregulated expression of this gene has not been implicated in any B-lymphoid neoplasia. The lack of evidence for involvement of deregulated N-*myc* expression in B-lymphoid tumors examined thus far may reflect a limited transforming potential of the N-*myc* gene or a relative insusceptibility of the gene to appropriately deregulated expression in these cells.

Various analyses suggested that transcription of the c-*myc* gene may be negatively regulated through an autoregulatory feedback mechanism (e.g. Kelly *et al.*, 1983; Adams *et al.*, 1985; Bentley and Groudine, 1986). Similarly, c-*myc* expression is very low or absent in cell lines with very high N-*myc* expression levels including neuroblastomas, Wilm's tumors and fibroblasts neoplastically transformed by introduction of an activated Ha-*ras* gene plus an N-*myc* expression vector (Nau *et al.*, 1985; Alt *et al.*, 1986; Nisen

et al., 1986; Cleveland *et al.*, 1987). However, the N- and c-*myc* genes can be expressed simultaneously at 'baseline' levels in a variety of different cell types, including pre-B cells (Zimmerman *et al.*, 1986). Together, these findings suggest that high-level N-*myc* expression may, in certain contexts, lead to negative 'cross-regulation' of c-*myc* expression (Alt *et al.*, 1986; Nisen *et al.*, 1986).

The Eμ-c-*myc* mice have provided a rich system for studying the role of the c-*myc* gene in normal B cell development and in B cell neoplasia (reviewed by Cory and Adams, 1988). To elucidate common and differential functions of the c- and N-*myc* genes in normal and transformed cells and to assess further the transforming potential of the N-*myc* gene, we now have generated N-*myc* transgenic mouse lines that respectively carry one of three separate constructs designed to deregulate N-*myc* expression in the B-lymphoid lineage.

Results

Production of transgenic mice carrying Eμ-N-myc constructs

Three separate constructs were used to deregulate N-*myc* expression in transgenic mice (Figure 1). All contained the lymphoid-specific heavy chain enhancer (Eμ), an element that is active throughout B cell differentiation and also is active in T cells (Bangerji *et al.*, 1983; Gillies *et al.*, 1983; Neuberger, 1983; Grosschedl *et al.*, 1984; Gerster *et al.*, 1986); incorporation of Eμ into the three constructs was expected to force N-*myc* expression beyond the pre-B/B cell transition. The EN construct contained a complete copy of the N-*myc* gene including the promoter linked to an upstream copy of Eμ. The ESN construct contained the second and third exons of the N-*myc* gene (with the complete exon 2/3 open reading frame) downstream of Eμ and an SV40 promoter. The third construct (EVN) contained the second and third N-*myc* exons downstream of Eμ and an Ig V_H gene promoter. All constructs were made from an N-*myc* genomic clone into which an *Xba*I linker was inserted into the 3′-untranslated region (Figure 1); this allowed endogenous and transgenic N-*myc* transcripts to be distinguished (see below). DNA of the various constructs was microinjected into either fertilized (C57BL/6 × CBA)F2 or inbred C57BL/6 eggs which were transferred into the oviducts of pseudo-pregnant females—leading to the generation of 14 independent primary transgenic mice: five animals carrying the EN construct, four carrying the ESN construct and five carrying the EVN construct (Table I). Transgenic lines were established by breeding transgenic animals with normal C57BL/6 mice. The numbers of integrated transgene copies ranged from 1 to >20 (Table I) and integration occurred at a single site in a tandem array with the exception of line 171 which had two independent integration sites (data not shown).

Lymphoid neoplasias in Eμ-N-myc transgenic mice

All three N-*myc* constructs employed were capable of provoking B-lymphoid malignancies in affected founder animals and their progeny (Figure 2; Table 1). In 7 of the 14 primary transgenic animals carrying the Eμ-deregulated N-*myc* constructs, lymphoid malignancies developed within 9–43 weeks of age. Four independent lines of control mice carrying the complete N-*myc* gene including its normal controlling elements (the 7.7-kb genomic N-*myc* clone shown in Figure 1, but *Xba*I linker-tagged) showed no predisposition to any neoplasia (not shown)—clearly implicating deregulation by the IgH enhancer element as fundamental to the

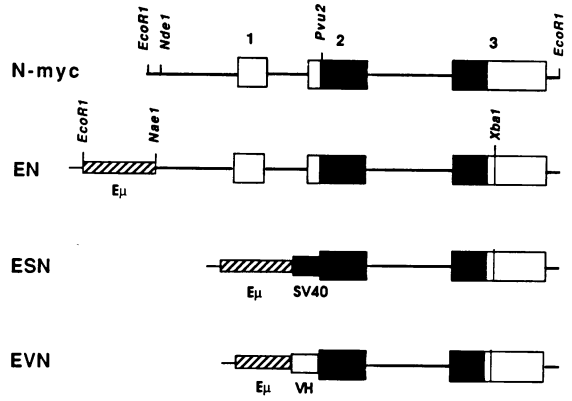

Fig. 1. Eμ-N-*myc* constructs. N-*myc* depicts the genomic *Eco*RI fragment (insert 277; DePinho *et al.*, 1986) carrying the three exons of the murine N-*myc* gene with coding regions represented by black boxes and non-coding regions by white boxes (the position of the N-*myc* promoter is just upstream of the beginning of exon 1). In the three constructs displayed below, the position of the Eμ element (hatched boxes) and the SV40 and V_H gene promoters (shaded boxes) are indicated. All constructs were marked with an *Xba*I-linker fragment inserted into the 3′-non-coding region of exon 3 (indicated by a vertical line). For details of construction see Materials and methods.

Table I. Summary of transgenic lines produced with Eμ-N-*myc* constructs

Founder mouse	Mouse strain[a]	Line established	Copy number[b]	tumor incidence[c]	T_{50}[f] (weeks)[d]
EN 71	(B6 × CBA)F2	–[e]	2	0/1	–
EN 82	(B6 × CBA)F2	+	10	26/32	16
EN 84	(B6 × CBA)F2	+	10	44/48	13
EN 211	B6	sterile	10	1/1	[33]
EN 212	B6	sterile	>20	1/1	[13]
ESN 184	B6	+	1	0/12	–
ESN 1812	B6	+	1	0/18	–
ESN 186	B6	+	5	7/13	≥33
ESN 1810	B6	–[e]	>20	2/2	[10]
EVN 107	(B6 × CBA)F2	+	5	0/27	–
EVN 172	B6	+	5	0/12	–
EVN 171[f]	B6	+ (177)[f]	10	18/26	18
		+ (175)[f]	15	16/20	16
EVN 108	(B6 × CBA)F2	+	>20	8/16	≥38
EBN 109	(B6 × CBA)F2	+	>20	19/39	≥39

[a]Genetic background of primary founder animals, which were subsequently backcrossed to inbred C57BL/6 (B6) mice to establish heterozygous transgenic lines.
[b]The number of integrated transgene copies was determined in F1 transgenic progeny or in original founder mice (founders 71, 211, 212).
[c]Frequency of transgenic animals (progeny up to the third generation) developing lymphoid malignancies between 5 and 40 weeks of age.
[d]T_{50} = time point (in weeks) at which 50% of transgenics were affected by tumor development. Numbers in brackets indicate age of individual animals at tumor onset. Animals which are still healthy are between 6 and 12 months old.
[e]Founder 71 did not transmit the transgene. Line 1810 could not be maintained since both the founder animal and the one transgenic offspring developed tumors early in life and stopped breeding.
[f]Founder 171 had two integrants which segregated independently giving rise to transgenic lines 175 and 177.

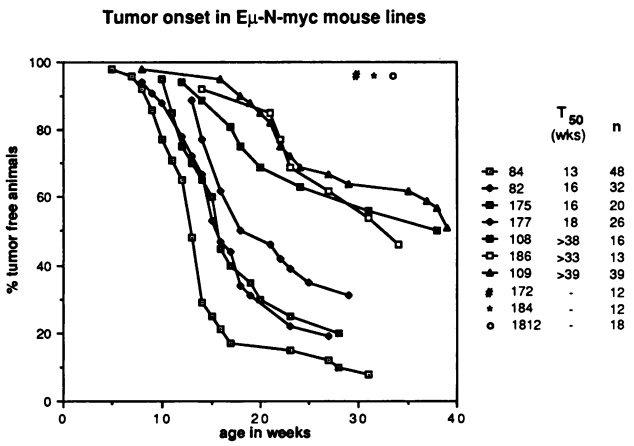

Fig. 2. Kinetics of tumor occurrence in Eμ-N-*myc* mouse lines. T_{50} = time point by which 50% of transgenics developed tumors; n = total number of transgenics monitored.

genesis of these tumors. Transgenic progeny of 11 established transgenic lines were monitored at 2-week intervals for onset of tumor formation—which usually first manifested as enlarged lymph nodes (see below). In four lines (82, 84, 175 and 177), lymphoid tumors developed frequently with 50% of these animals developing tumors by 13–18 weeks of age (Figure 2). In comparison, in three other lines (186, 108 and 109) detectable tumor formation appeared to be delayed (Figure 2). Mice of four additional transgenic lines (184, 1812, 107 and 172) thus far have not developed tumors; notably, these mice have the lowest copy number of their respective constructs (Table I). In affected lines, susceptibility to lymphoid neoplasia has been a stable characteristic for the three generations analyzed; furthermore, as with Eμ-c-*myc* mice (Adams *et al.*, 1985; Cory and Adams, 1988), onset of tumor formation appears random with respect to given individuals (Figure 2).

The general pathological diagnosis for the disease in Eμ-N-*myc* transgenic mice was lymphoma with involvement of all major lymphoid organs and infiltration of other non-lymphoid tissues. Tumor development usually first manifested itself by enlargement of the lymph nodes and progressed (in general) within a few weeks to a stage where animals became terminally ill. In animals that were killed after marked onset of malignancy, most lymph nodes were drastically enlarged due to infiltration of tumor cells. Involvement of the spleen and, in many cases, the thymus was also observed. Another frequent manifestation was deformation of the head due to involvement of the skull bone and adjacent tissues by tumor cells. Tumor deposits also frequently ocurred along the spinal column and in several cases the disease first manifested itself by a hind limb paralysis. In advanced-stage disease, gross or microscopic involvement of various other tissues including liver and lung often was detected; infiltration of the marrow and adjacent tissue of long bones also was observed. In one case (see Discussion), both kidneys were massively invaded by tumor cells (the 8.4 tumor; see below). Tumor cells generally were lymphoblastoid in gross morphology; more detailed staging indicated that they predominantly represented pre-B and B cell stages of the B cell pathway (see below). After transplantation of 10^5-10^6 tumor cells from 10 independent tumor samples into the peritoneal cavity of normal (C57BL/6 × CBA)F1 mice, tumors were generated in the recipients within a few weeks (data not shown). In addition, tumor cells isolated from either transgenic or recipient animals were usually readily adaptable to growth in culture (see below).

Table II. Staging of Eμ-N-*myc* cell lines

Cell line	DNA rearrangements			RNA expression		Ig production			Cell type[c]
						Lysate[a]		Surface[b]	
	IgH	Igκ	TCRβ	μ[d]	κ	μ	κ	SIgM	
8.4	–	–	+	+	–	–	–	NT	T
82.3	+	–	–	+	–	–	NT	NT	pre-B
171.7–32	+	–	–	+	–	–	NT	NT	pre-B
10.9	+	+[e]	NT	+	+	–	+	NT	pre-B*
171.1–3	+	+	NT	+	–	–	–	NT	pre-B*
21.2	+	+	NT	+	+	–	+	NT	pre-B*
1810.15	+	+	NT	++	+	+	+	+	B
84.27	+	+	NT	++	+	+	+	+	B

NT = not tested.
[a]Determined by immunoprecipitation of extracts from biosynthetically labeled cells and/or Western blot analysis.
[b]Determined by cell surface staining.
[c]Staging of cells according to rearrangement and expression data; pre-B* = B-lineage cells with heavy and light chain rearrangements, but no heavy chain protein.
[d]Cμ-bearing transcripts detected: (+) germline and/or abberant μ-mRNA transcripts; (++) predominant μ-mRNA transcripts.
[e]Tumor 10.9 started to rearrange its κ-loci in culture.

Immunoglobulin gene rearrangement and expression in Eμ-N-myc tumors

Various analyses indicated that all tumors and derived cell lines except one were B-lymphoid in origin (Table II; see below); preliminary analyses of Ig expression in primary Eμ-N-*myc* tumors suggested that individual tumors usually represented either the pre-B (μ only) or B cell (μ plus L chain) stages (not shown). To characterize these tumors further, we analyzed the rearrangement patterns of Ig genes in primary tumors and derived cell lines. To assay the rearrangements of the IgH chain J_H region, DNA was digested with *Eco*RI and assayed by Southern blotting for hybridization to a ^{32}P-labeled probe (J_H-Eμ-probe) that hybridizes to a portion of the J_H-containing genomic *Eco*RI fragment lying just upstream of the Eμ-containing restriction fragment employed in N-*myc* constructs (Figure 3A). Utilization of this probe allowed the endogenous J_H alleles to be distinguished from the transgenic Eμ-containing fragment—although a low-level contamination of the downstream fragment resulted in faint hybridization to the construct in higher copy number lines (Figure 3A; see legend). In 15 of 16 primary tumor samples, rearrangements of the J_H locus were directed; usually only two prominent rearrangements were evident, but occasionally additional faint hybridizing fragments were observed (Figure 3A, lanes T; representative data is shown). Rearrangement at the κ light chain locus was assayed by probing *Bam*HI-digested DNA for hybridization to a Jκ-specific probe (Figure 3B; lanes T). Eleven of the 15 primary tumors exhibiting J_H rearrangements also had rearrangements of their Jκ loci. Unlike the other tumors, the 8.4 tumor had neither J_H nor Jκ rearrangement (Figure 3A and B). However, Southern blotting assays of 8.4 (cell line) and appropriate control DNAs, after digestion with appropriate restriction enzymes, for hybridization to a T cell receptor (TCR) Cβ1-probes (Figure 3C, lanes 1–4) or a Jβ2-probe (Figure 3C, lane 5–8), demonstrated that the line had rearranged both TCRβ

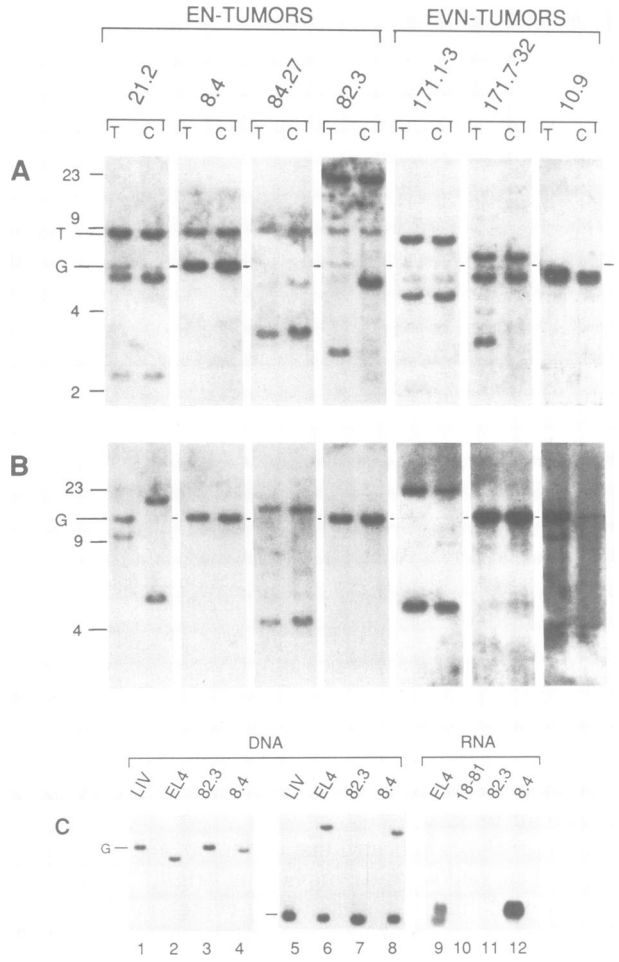

Fig. 3. Analysis of immunoglobulin and T cell receptor gene rearrangements in Eμ-N-*myc* cells. (**A**) DNA (10 μg) from various Eμ-N-*myc* tumors (T) or cell lines (C) was digested with *Eco*RI, electrophoresed through an agarose gel, transferred to a nylon membrane and assayed for hybridization to the $J_H-E\mu^-$ probe. In EN tumors, the transgene (T) was also detected in this particular experiment due to a probe contamination with $E\mu^+$ DNA. The position of the unrearranged germline fragment (G) and mol. wt markers (in Kb) are indicated on the left. (**B**) DNA (10 μg) from the indicated sources was digested with *Bam*HI, processed as described above and assayed for hybridization to the 3′ Jκ probe. (**C**) **Lanes 1–4**: DNA (10 μg) from the indicated sources was digested with *Hin*dIII and assayed as described above for hybridization to TCR-Cβ1 probe); **Lanes 5–8**: DNA (10μg) from the indicated sources was digested with *Eco*RI and assayed for hybridization to the TCR-Jβ2 probe; the positions of germline fragments are indicated by a dash in the respective panels. **Lanes 9–12**: Total RNA (10 μg) from the indicated sources was assayed by Northern blotting procedures for hybridization to the TCR-α probe; the band in the 8.4 lane co-migrates with the band corresponding to mature TCR-α mRNA in the EL4 lane. LIV, BALB/c liver control. EL4, T cell lymphoma; 82.3, Eμ-N-*Myc* pre-B line; 18–81, Abelson murine leukemia virus transformed pre-B cell line; 8.4, Eμ-N-*myc* T cell line.

loci. In addition, Northern blotting analysis of RNA from 8.4 and control cell lines for hybridization to a TCR cα-probe, demonstrated that the 8.4 line produced hybridizing transcripts that corresponded in size to mature TCR α-mRNA (Figure 3C, lanes 9–12). Finally, this cell line stained positively with an anti-Thy1.2 monoclonal antibody (A.Ma and N.Braunstein, unpublished data). Together, these analyses clearly indicate that the 8.4 tumor represents a T cell lymphoma.

All tested Eμ-N-*myc* cells lines, including the 8.4 line, produced RNA transcripts that hybridized to a probe specific for the μ heavy chain constant region (Table II). These sequences could be categorized as germline transcripts of normal-sized μmRNA transcripts depending on the line (Table II); expression of germline Cμ-hybridizing transcripts as observed in the 8.4 T cell line is a common characteristic of normal and transformed T-lineage cells (Kemp *et al.*, 1980; Alt *et al.*, 1982). Cells that had κ gene rearrangements, but not the others, also expressed Cκ-hybridizing transcripts corresponding in size to that of normal κ mRNA (Table II). Two Eμ-N-*myc* cell lines had J_H but no Jκ rearrangements (82.3 and 171.7–32) but did not produce Ig chains that were detectable by immunoprecipitation or Western blotting analyses (Table II), indicating that their rearrangements were either incomplete DJ_H rearrangements or aberrant V_HDJ_H rearrangements. Thus, these cells represent null pre-B cell tumors. Two of the five lines that had both J_H and Jκ rearrangements (1810.15 and 84.27) produced both μ- and κ-protein; immunofluorescent analyses confirmed that these lines carried IgM on their surface—indicating that they represented tumors of the B-lymphocyte stage (Table II). In the other three cell lines (10.9, 171.1–3 and 21.2) that had rearranged both J_H and Jκ loci, no μ-protein was detected (Table II). However, assay for expression of a set of novel pre-B-specific sequences suggested that all three of these lines were at the pre-B stage of differentiation (G.D.Yancopoulos, A.Ma, R.Dildrop and F.W.Alt, unpublished data). Two of these lines (21.2 and 10.9) produced κ-protein in the absence of detectable μ-protein. This relatively unusual phenotype was also observed for some Eμ-c-*myc* tumors (Adams *et al.*, 1985) and warrants further examination.

Tumor samples usually had only two J_H rearrangements (corresponding to the two J_H alleles) and identical patterns were observed for tumors taken from different sites in the same animal. These results indicate that the major population of tumor cells was of clonal origin, as previously noted for B cell neoplasias in Eμ-c-*myc* (Adams *et al.*, 1985). Cell culture lines established from several primary tumor samples usually had Ig-gene rearrangements patterns identical to those of parent tumors with the occasional loss of J_H- or Jκ-hybridizing restriction fragments that was often accompanied by the appearance of new fragments (Figure 3; compare lanes T and C). The latter phenomenon could be explained either by outgrowth of a subset of cells in the original tumor sample or by changes due to recombination events that occurred during growth of the cells (e.g. the 10.9 line; see Table II). Continued rearrangement of Ig loci during growth in culture is a common characteristic of pre-B lines derived by Abelson murine leukemia virus transformation (Alt *et al.*, 1986) and also of pre-lines derived from tumors that arise in Eμ-c-*myc* transgenic mice (Adams *et al.*, 1985).

Tissue distribution of N-myc expression in Eμ-N-myc mice

To assay for endogenous and transgenic N-*myc* expression, RNA prepared from tissues of 5-week-old mice (free of detectable signs of tumor development) was assayed for ability to differentially protect a ^{32}P-labeled N-*myc* probe from digestion by S1-nuclease. The probe derived from exon 3 of the marked N-*myc* gene was employed for the transgenic constructs and contained the inserted *Xba*I linker. Therefore, hybridization to N-*myc* RNA derived from the transgene

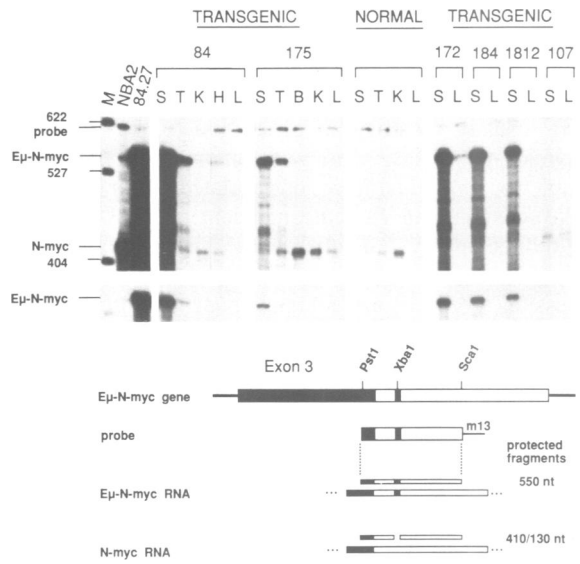

Fig. 4. Tissue distribution of Eμ-N-*myc* and endogenous N-*myc* expression. **Upper panel**: a *PstI*–*ScaI* N-*myc* probe was uniformly labeled with ^{32}P, hybridized to RNA from the indicated sources, subjected to digestion with S1 nuclease, and protected fragments resolved by polyacrylamide gel electrophoresis. As diagrammed in the **bottom panel** of the figure, hybridization to Eμ-N-*myc* RNA will yield a protected fragment of 550 nt while hybridization to endogenous N-*myc* RNA yields a protected fragment of 410 nt. The **middle panel** is a shorter exposure of the same autoradiograph shown in the upper panels for better comparison of transgene expression levels in different tissues. The position of the probe, Eμ-N-*myc*-specific fragment, endogenous N-*myc*-specific fragment (N-*myc*) and markers (in bp) are indicated to the left of the panels. RNA was derived from the following tissues and cell lines: S, spleen; T, thymus, K, kidney; H, heart, B, brain; L, liver. Mouse neuroblastoma NBA2 RNA that expresses baseline N-*myc* levels (Zimmerman *et al.*, 1986) was used as a control for endogenous N-*myc* expression and RNA from the 84.27 Eμ-N-*myc* tumor was used as a control for Eμ-N-*myc* expression.

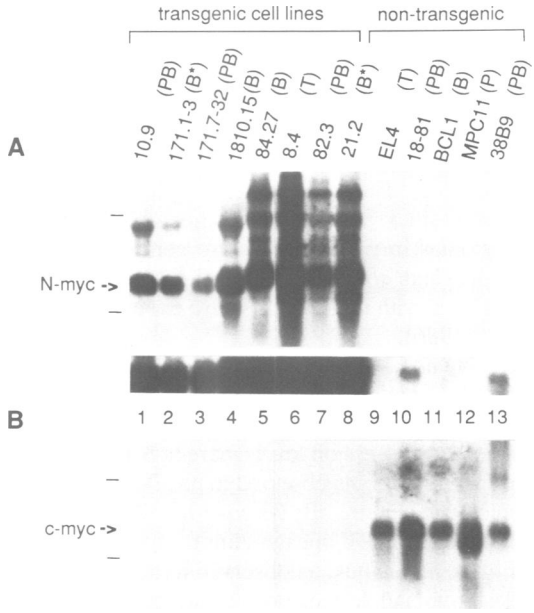

Fig. 5. Expression of *myc* genes in Eμ-N-*myc* cell lines. (**A**) Total RNA (10 μg) from the indicated cell lines was assayed by Northern blot procedures for hybridization to a ^{32}P-labeled N-*myc* probe; the lower panel is a 10-fold longer exposure of the autoradiograph depicted in the top panel. (**B**) A duplicate Northern blot to that used in (A) was assayed for hybridization to a ^{32}P-labeled c-*myc* probe. Transgenic cell lines were derived from Eμ-N-*myc* tumors indicated in Table I and are further described in the text. The non-transgenic cell line MPC 11 is a plasmacytoma and BCL1 is a B cell lymphoma; the other non-transgenic lines are described in the legend to Figure 3. Abbreviations: T, T cell. PB, pre-B cell; B, B cell; B*, Eμ-N-*myc* line that has rearranged H and L chain genes but appears to be at the pre-B differentation stage; P, Plasmacytoma. Positions of 28S and 18S RNAs are indicated.

results in the generation of an S1-resistant fragment of 550 nt while hybridization to RNA derived from the endogenous N-*myc* gene generates two smaller protected fragments, one of which is 410 nt (Figure 4). Analyses of progeny from transgenic lines that displayed a high incidence of tumor development indicated that expression of the Eμ-N-*myc* transgenes occurred in a tissue-specific fashion that was consistent with dominant control being exercised by the associated IgH transcriptional enhancer element; thus, highest expression levels occurred in the spleen with lower, but still very high expression occurring in the thymus (Figure 4). Transgene expression was low in all other (non-lymphoid) tissues analyzed and probably derived from circulating lymphocytes. As previously described for normal mice (Zimmerman *et al.*, 1986), endogenous N-*myc* expression in the Eμ-N-*myc* mice or their non-transgenic siblings was highest in the brain and kidney and low in the spleen (Figure 4). Notably, the level of endogenous N-*myc* expression, even in the brain, is lower relative to the level of transgene expression in the spleen—indicating that the transgenic constructs lead to the accumulation of very high levels of N-*myc* RNA in some spleen cells. High-level expression of the N-*myc* constructs was observed in the spleens of mice from several different lines carrying, respectively, either the EN or EVN constructs (Figure 4; other data not shown), indicating that the IgH enhancer element (the only common known transcriptional control element among the constructs) is responsible for high-level expression.

As indicated above, a number of the transgenic mouse lines did not develop detectable malignancies; these were also lines that generally carried lower copy numbers of the respective transgenic N-*myc* constructs (Table I). Therefore, it seemed likely that tumor development in the various lines may depend on copy number and, as a result, relative expression levels of the transgenic constructs. To test this possibility, we assayed transgenic and endogenous N-*myc* expression in selected tissues derived from four of the transgenic lines (172, 184, 1812 and 107) that, thus far, have not developed tumors. One of these lines (107) does not express detectable levels of the transgene-consistent with absence of tumor development; however, surprisingly the other three transgenic lines expressed splenic levels of the transgene comparable to those observed in spleens derived from lines that have a high incidence of tumor development.

Expression of the c- and N-myc genes in Eμ-N-myc cell lines

To assay relative levels of N- and c-*myc* expression, total RNA prepared from Eμ-N-*myc* and appropriate control cell lines was assayed for hybridization to ^{32}P-labeled c- and N-*myc*-specific probes by Northern blotting methods (Figure 5). Relative expression of endogenous- and transgene-derived N-*myc* transcripts also was quantitated for all lines by the S1 nuclease assay as well (data not shown). All transformed cell lines generated from the Eμ-N-*myc* transgenics expressed very high levels of the N-*myc* transgene (Figure 5A)—expression levels that were at least 50-fold greater than those

of A-MuLV-transformed pre-B lines (Figure 5A; N-*myc* expression in the non-transgenic pre-B line is barely detectable in the longer exposure shown in the lower panel of Figure 5A but is still >20-fold greater than the undetectable levels of more mature B-lymphoid lines and the representative T cell tumor line derived from non-transgenic mice). The levels of N-*myc* expression in the Eμ-N-*myc* cell lines are more comparable to those of neuroblastomas in which the N-*myc* gene is amplified 50- to 100-fold and to those found in N-*myc*-transformed fibroblast lines with high-level N-*myc* expression (Figure 5; Alt *et al.*, 1986).

Strikingly, none of the Eμ-N-*myc* cell lines representing any B cell differentiation stage or the Eμ-N-*myc* T cell tumor expressed readily detectable levels of endogenous c-*myc* transcripts; the expression levels were at least 50-fold lower than the 'baseline' levels observed in pre-B tumors from non-transgenic mice (Figure 5B; Nisen *et al.*, 1986) or the levels found in other B and T lineage tumors from non-transgenic mice (Figure 5B). Thus, as observed in naturally occurring neuroblastomas and derived neoplastic fibroblast lines whose transformation was associated with high-level N-*myc* expression, the Eμ-N-*myc*-transformed B lineage tumor cell lines lacked readily detectable c-*myc* expression. Endogenous N-*myc* expression is found in pre-B cells and tumors (Zimmerman *et al.*, 1986; Figure 5); yet S1 nuclease analyses failed to detect significant levels of endogenous N-*myc* expression in any Eμ-N-*myc* pre-B tumours examined. These analyses would have detected N-*myc* expression levels at least 20-fold lower than those found in pre-B cell tumors from normal mice or in unamplified neuroblastomas (not shown), indicating that the Eμ-N-*myc* pre-B tumor lines also have decreased endogenous N-*myc* levels compared to those of previously described pre-B tumors that derive from normal mice.

Discussion

Eμ-N-myc lymphoid neoplasia

A complete N-*myc* transgene without the IgH enhancer did not elicit any malignant disease; but all three transgenic Eμ-N-*myc* constructs utilized in this study were capable of generating an inheritable predisposition to develop lymphoid malignancies. These three constructs employed different transcriptional promoters (the normal N-*myc* promoter, the SV40 early region promoter and an Ig V_H promoter) with the only known transcriptional regulatory element common to all three being the IgH enhancer; one construct contained the complete N-*myc* gene while the others lacked exon 1. Based on the current analyses, the three promoter elements employed appear equally capable of promoting tumor development. Together, these results suggest that the major components of the various Eμ-N-*myc* constructs involved in the generation of the disease were the N-*myc* exon 2/3 coding region and the IgH enhancer element. These results also implicate the IgH enhancer element as the dominant genetic regulatory element in the generation of the Eμ-N-*myc* disease.

Deregulated expression of the Eμ-N-*myc* transgene effected by the IgH enhancer element appeared to occur specifically in lymphoid tissues, with highest expression levels in the spleen and significant levels in the thymus. Correspondingly, all analyzed tumors in transgenic lines were of lymphoid origin and predominantly derived from B-lineage cells. The Eμ-N-*myc* disease had similar manifestations in different mouse lines and generally resembled the Eμ-c-*myc* disease (reviewed by Cory and Adams, 1988)—usually resulting in generation of pre-B- and B-lymphoid noeplasias. Onset of tumor development was variable for individual mice, and isolated tumor specimens displayed simple Ig J_H rearrangement patterns consistent with monoclonality. Together, these findings indicate that genetic events in addition to the deregulated N-*myc* expression are necessary for malignant transformation. In this regard, it is of interest that several Eμ-N-*myc* lines that expressed high levels of the transgene did not show a predisposition to B cell neoplasia. The transcripts generated from the transgene in these mice appeared of normal size, although we could not rule out point mutations or small deletions based on our analyses. Of note, these lines also had the lowest copy numbers of the Eμ-N-*myc* construct (Table I); but additional analyses would be necessary to assess any potential role of copy number in the development of the disease.

Although B-lineage tumors were predominant in the Eμ-N-*myc* mice, we have identified one T cell tumor. The appearance of additional T cell tumors in the Eμ-N-*myc* lines will be necessary to establish unequivocally a causal link between Eμ-N-*myc* expression and T cell malignancy; however, this relationship seems likely for several reasons. First, Eμ-N-*myc* expression levels were very high in the thymus, consistent with the known activity of the IgH enhancer in T cells. Second, the 8.4 T cell tumor had extremely high levels of the Eμ-N-*myc* transgene expression and no detectable c-*myc* expression. To date, all previously analyzed T cell tumors express the endogenous c-*myc* gene and do not express N-*myc* at high levels (Zimmerman *et al.*, 1986; R.A.DePinho *et al.*, in preparation).

Deregulation of c-*myc* expression by various mechanisms has been strongly implicated in B-lymphoid neoplasias (Klein and Klein, 1985; Cory, 1986). However, although our current studies demonstrate that deregulated N-*myc* expression can be strikingly potent in generating B-lymphoid malignancies in transgenic animals, deregulated expression of the N-*myc* gene has thus far not been implicated in the development of any naturally occurring B cell tumors. In this context, a potentially significant difference between the Eμ-N-*myc* and Eμ-c-*myc* diseases is that all Eμ-N-*myc* tumors expressed exceptionally high levels of the Eμ-N-*myc* gene (see above); but Eμ-c-*myc* expression levels in Eμ-c-*myc* tumors were found to be only modestly elevated (Adams *et al.*, 1985). Thus it is possible that two events—deregulated expression and greatly increased expression—may be required for N-*myc* but not c-*myc* transforming activities. In addition, as we have previously suggested (Yancopoulos *et al.*, 1985), it is possible that the expression characteristics of *myc* genes may target their activation; thus, in correspondence to its endogenous expression pattern, deregulated endogenous N-*myc* (as opposed to c-*myc*) expression might be expected to contribute only to very early stage B (or T) lymphoid tumors.

Cross-regulation of myc-gene expression

Previous studies have suggested that deregulated c-*myc* expression may down-regulate c-*myc* expression from normal c-*myc* alleles; in support of this possibility B cell

tumor lines generated from Eμ-c-*myc* transgenics did not express the endogenous c-*myc* gene (reviewed by Cory and Adams, 1988). Consistent with a potential cross-regulatory activity with respect to *myc* gene expression, all Eμ-N-*myc* lymphoid tumors examined, unlike all naturally occurring lymphoid tumors described to date, did not express the endogenous c-*myc* gene at significant levels. Yet, we have previously observed coordinate *baseline* expression levels (defined for N-*myc* as the expression level in a neuroblastoma with a single copy number of the N-*myc* gene; Nisen *et al.*, 1986) of two or in some cases all three *myc* genes in normal and transformed cells including normal and transformed pre-B cells (eg. Zimmerman *et al.*, 1986; Nisen *et al.*, 1986; Figure 5). One possibility consistent with the findings of cross-regulation in some cells (e.g. the Eμ-N-*myc* pre-B tumors) and coordinate expression in others (e.g. A-MuLV-transformed pre-B cells) is that high-level expression of N-*myc* (i.e. >20-fold above baseline levels) is necessary to lead to down-regulation (cross-regulation) of c-*myc* expression (Nisen *et al.*, 1986). Support for this idea comes from findings that N-*myc* expression above a similar threshold level is necessary to down-regulate MHC class I gene expression in neuroblastomas (Bernards *et al.*, 1986). The lack of detectable endogenous N-*myc* expression in the Eμ-N-*myc* pre-B lines might also suggest an 'autoregulatory' activity of high-level N-*myc* expression as well.

It should be emphasized that the putative cross-regulatory activity of the N-*myc* gene has not been shown to be a direct effect of high-level N-*myc* expression and may be secondary to other associated (perhaps differentiative) events. Almost all examples of cross-regulation, to date, have been observed in tumors or cell lines in which high-level N-*myc* expression was implicated in their neoplastic transformation. However, numerous 3T3 fibroblast cell lines that expressed >20-fold baseline levels of N-*myc* from an introduced construct, but which were not selected in the context of neoplastic transformation, all expressed endogenous c-*myc* (K.A.Zimmerman and F.W.Alt, unpublished). Thus, it is possible that factors in addition to high level N-*myc* expression, possibly selected by the neoplastic transformation process, may be necessary to contribute to the cross-regulatory phenomenon.

B cell differentiation in Eμ-N-myc transgenic mice

A major goal in the generation of Eμ-N-*myc* transgenics is to determine how disruption of normal N-*myc* expression affects the B cell pathway. All Eμ-N-*myc* mice generate mature B cells and an immune system that appears to function normally. Whether this implies that the decline in N-*myc* expression at the pre-B/B cell stage has no consequence with respect to normal differentiation in this pathway will require many additional analyses. Our current analyses of Eμ-N-*myc* tumors clearly indicate that transformed high-level N-*myc*-expressing cells can exist at the B cell stage in the absence of significant endogenous c-*myc* expression. However, we have not yet determined the expression levels of the transgenic N-*myc* gene or of the c-*myc* gene in normal, transgenic B lineage cells; it is possible that Eμ-N-*myc* expression is extinguished in normal cells that progress beyond the pre-B stage or that deregulated N-*myc* expression accompanied by perturbations in c-*myc* expression levels allow apparently normal differentiation to occur.

Materials and methods

DNA constructs

To discriminate transgenic transcripts from those of the endogenous gene, an *Xba*I-linker fragment was inserted into a unique *Hin*cII site in the 3′-untranslated region of the p277 genomic N-*myc* clone (DePinho *et al.*, 1986); the resulting clone (p277X) was used for all vector constructions. The EN-construct contains a 7.1-kb *Nde*I−*Eco*RI DNA fragment carrying a complete copy of the murine N-*myc* gene excised from genomic clone p277X (DePinho *et al.*, 1986) and linked at the 5′ end to a 1.1-kb *Eco*RI−*Nae*I fragment carrying the mouse Ig heavy chain enhancer element Eμ (Banerji *et al.*, 1983). The Ig enhancer-containing fragment was linked in opposite orientation to the N-*myc* gene relative to its orientation in the Ig heavy chain gene. To make the ESN construct, we removed an internal 2.9-kb *Stu*I−*Pvu*II fragment of the EN construct that contained the entire 5′ genomic region of the N-*myc* gene (including promoter, exon 1 and extending 5 bp 3′ of the first ATG codon in exon 2) and substituted in its place a 208-bp *Rsa*I−*Hin*dIII fragment derived out of plasmid XS-13 (Fromm and Berg, 1982) that contained the SV40 early region promoter but not the SV40 enhancer. To make the EVN construct a DNA segment comprised of a 682-bp *Eco*RI−*Xba*I Eμ-containing fragment linked to a 360-bp *Hin*cII−*Hin*dIII fragment containing a V_H promoter (Blankenstein *et al.*, 1988) was linked to the 5′ end of the same N-*myc* fragment (containing exons 2 and 3) that was used in the ESN construct. The SV40 and V_H gene fragments were joined to the N-*myc* fragment at the *Pvu*II site that lies just within the N-*myc* coding region; these segments were linked via a 61-bp synthetic oligonucleotide that restored the missing N-*myc* codons.

All constructs were cloned into the *Eco*RI site of pUC19 and were excised for microinjection *Nde*I−*Hin*cII (EN) or *Nde*I−*Kpn*I (ESN and EVN) linear fragments retaining 213 bp of pUC19 DNA.

Microinjection and mice

Microinjection of fertilized mouse eggs was performed essentially as described by Hogan *et al.* (1986). (C57BL/6 × CBA)F1 and C57BL/6 mice were purchased from Jackson Laboratories. Transgenic lines were maintained by backcrossing heterozygous transgenic males to normal C57BL/6 females.

Establishment of cell lines from Eμ-N-myc tumors

Single-cell suspensions prepared from primary tumor samples were either directly grown in RPMI or DME medium both supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 μM 2-mercaptoethanol and antibiotics at a concentration of $10^5-2\times10^6$ cells/ml, or alternatively 10^5-10^6 cells were propagated intraperitoneally in normal (C57BL/6 × CBA)F1 mice for several weeks and subsequently removed and used to inoculate cultures as described above.

Molecular analyses

Preparation of genomic DNA from tail tips, primary tumor samples and cell lines was performed as previously described (Hogan *et al.*, 1986). DNA blotting procedures were performed as described elsewhere (Reed and Mann, 1984; Yancopoulos *et al.*, 1984, 1985). The following probes were used for Southern blot analysis: $E\mu^+$, a 1150-bp genomic *Nae*I−*Eco*RI fragment 3′ of J_H1-4 (Gough and Bernard, 1981) for detection of the transgene; $J_H-E\mu^-$, a 850-bp genomic *Bam*HI−*Nae*I fragment covering J_H3-4 (Gough and Bernard, 1981) for detection of Ig J_H gene rearrangements; 3′Jκ, the 1-kb genomic *Xba*I−*Hin*dIII fragment lying 3′ of $J_\kappa1-5$ (Perry *et al.*, 1980) for detection of Ig Jκ gene rearrangements; Cβ1, a 730-bp cDNA copy of Cβ1 (Caccia *et al.*, 1984) for detection of rearrangements of the TCR Jβ1 locus or deletion of the TCR Cβ1 locus; Jβ2, a 1.9-kb genomic *Hin*dIII−*Bam*HI fragment containing the Jβ2 locus (Malissen *et al.*, 1984) for detection of rearrangements of the TCR Jβ2 locus.

Preparation of total RNA was performed as previously described (Auffray and Rougeon, 1979) and Northern blotting procedures were performed as described elsewhere (Yancopoulos *et al.*, 1984; 1985). The N-*myc* cDNA probe contained part of murine N-*myc* exon 1 and exons 2 and 3 and was kindly provided by Dr C.Cepko (Harvard University). The cDNA probe specific for murine c-*myc* was described previously (Zimmerman *et al.*, 1986). The TCR-α probe is a 370-bp genomic *Eco*RI fragment covering the Cα gene (Iwamoto *et al.*, 1987).

Immunoprecipitations and Western blotting analyses

Western analyses and immunoprecipitations were performed as previously described (Reth *et al.*, 1985; Dildrop *et al.*, 1987) using affinity-purified rabbit anti-mouse antibodies specific for mouse μ and κ chains and ^{125}I-labeled protein A (for Western analyses).

S1 analysis

A *PstI–ScaI* restriction fragment containing the inserted *XbaI*-linker sequence was excised from p277X and subcloned into an m13 phage vector, uniformly labeled with ^{32}P, and a single-stranded probe prepared as previously described (Biggin *et al.*, 1984). Hybridization to RNA, digestion with S1 nuclease and anlaysis on denaturing polyacrylamide gels were performed as described by Kohl *et al.* (1986).

Acknowledgements

This work was supported by the Howard Hughes Medical Organization and NIH grant CA42335. R.D. was supported by a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft. E.H. is a postdoctoral fellow of the H.H.M.I., A.M. and R.A.D. were recipients of Physician Scientists Awards, and R.A.D. is a Cancer Research Institute scholar.

References

Adams,J.M., Harris,A.W., Pinkert,C.A., Corcoran,L.M., Alexander,W.S., Cory,S., Palmiter,R.D. and Brinster,R.L. (1985) *Nature*, **318**, 533–538.

Alt,F.W., Rosenberg,N., Enea,V., Siden,E. and Baltimore,D. (1982) *Mol. Cell. Biol.*, **2**, 386–400.

Alt,F.W., DePinho,R., Zimmerman,K., Legouy,E., Hatton,K., Ferrier,P., Tesfaye,A., Yancopoulos,G. and Nisen,P. (1986) *Cold Spring Harbor Symp. Quant. Biol.*, **51**, 931–941.

Auffray,C. and Rougeon,F. (1980) *Eur. J. Biochem.*, **107**, 303–314.

Banerji,J., Olson,L. and Schaffner,W. (1983) *Cell*, **33**, 729–740.

Bentley,D.L. and Groudine,M. (1986) *Nature*, **321**, 702–706.

Bernard,O., Cory,S., Gerondakis,S., Webb,E. and Adams,J.M. (1983) *EMBO J.*, **2**, 2375–2383.

Bernards,R., Dessain,S.K. and Weinberg,R.A. (1986) *Cell*, **47**, 667–674.

Biggin,M., Farrell,P. and Barrell,B. (1984) *EMBO J.*, **3**, 1083–1090.

Birrer,M.J., Segal,S., DeGreve,J.S., Kaye,F., Sauville,E.A. and Minna,J.D. (1988) *Mol. Cell. Biol.*, **8**, 2668–2673.

Blankenstein,T., Winter,E. and Müller,W. (1988) *Nucleic Acids Res.*, **22**, in press.

Caccia,N., Kronenberg,M., Saxe,D., Haars,R. Bruns,G.A.P., Goverman,J., Malissen,M., Willard,H., Yoshikai,Y., Fitch,F., Mak,T.W. and Hood,L. (1984) *Cell*, **37**, 1091–1099.

Cleveland,J.L., Huleihel,M., Eisenman,R., Siebenlist,U., Ihle,J.N. and Rapp,U.R. (1987) *Proc. Alfred Benzon Symp.*, **24**, 339.

Cory,S. (1986) *Adv. Cancer Res.* **47**, 189–234.

Cory,S. and Adams,J.M. (1988) *Annu. Rev. Immunol.*, **6**, 25–48.

DePinho,R.A., Legouy,E., Feldman,L.B., Kohl,N.E., Yancopoulos,G.D. and Alt,F.W. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 1827–1831.

DePinho,R.A., Hatton,K.S., Tesfaye,A., Yancopoulos,G.D. and Alt,F.W. (1987) *Genes Dev.*, **1**, 1311–1326.

Dildrop,R., Gause,A., Müller,W. and Rajewsky,K. (1987) *Eur. J. Immunol.*, **17**, 731–734.

Fromm,M. and Berg,P. (1982) *J. Mol. Appl. Genet.*, **1**, 457–481.

Gerster,T., Picard,D. and Schaffner,W. (1986) *Cell*, **45**, 45–52.

Gillies,S.D., Morrison,S.L., Oi,V.T. and Tonegawa,S. (1983) *Cell*, **33**, 717–728.

Gough,N.M. and Bernard,O. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 509–513.

Grosschedl,R., Weaver,D., Baltimore,D. and Costantini,F. (1984) *Cell*, **38**, 674–658.

Harris,A.W., Pinkert,C.A., Crawford,M., Langdon,W.Y., Brinster,R.L. and Adams,J.M. (1988) *J. Exp. Med.*, **167**, 353–371.

Hogan,B., Costantini,F. and Lacy,E. (1986) *Manipulating the Mouse Embryo*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Iwamoto,A., Chaski,P.S., Pircher,R., Walker,C.L., Michalopoulos,E.E., Rupp,F., Hengartner,H. and Mak,T.W. (1987) *J. Exp. Med.*, **165**, 591–600.

Jakobovits,A., Schwab,M., Bishop,J.M. and Martin,G.R. (1985) *Nature*, **318**, 188–191.

Kaye,F., Battey,J., Nau,M., Brooks,B., Seifter,E., De Greve,J., Birrer,M., Sausville,E. and Minna,J. (1988) *Mol. Cell. Biol.*, **8**, 186–195.

Kelly,K., Cochran,B., Stiles,C. and Leder,P. (1983) *Cell*, **35**, 603–610.

Kemp,D.J., Harris,A.W., Cory,S. and Adams,J.M. (1980) *Proc. Natl. Acad. Sci., USA*, **77**, 2876–2880.

Klein,G. and Klein,E. (1985) *Immunol. Today*, **6**, 208–215.

Kohl,N.E., Legouy,E., DePinho,R.A., Nisen,P.D., Smith,R.K., Gee,C.E. and Alt,F.W. (1986) *Nature*, **319**, 73–77.

LeGouy,E., DePinho,R., Zimmerman,K., Collum,R., Yancopoulos,G., Mitsock,L., Kriz,R. and Alt,F. (1987) *EMBO J.*, **6**, 3359–3366.

Malissen,M., Minard,K., Mjolsness,S., Kronenberg,M., Goverman,J., Hunkapiller,T., Prystowsky,M.B., Yoshikai,Y., Fitch,F., Mak,T.W. and Hood,L. (1984) *Cell*, **37**, 1101–1110.

Mugrauer,G., Alt,F.W. and Ekblom,P. (1988) *J. Cell Biol.*, **107**, in press.

Nau,M.M., Brooks,B.J., Battey,J., Sausville,E., Gazdar,A.F., Kirsch,I.R., McBride,O.W., Bertness,V., Hollis,G.F. and Minna,J.D. (1985) *Nature*, **318**, 69–73.

Neuberger,M.S. (1983) *EMBO J.*, **2**, 1373–1378.

Nisen,P.D., Zimmerman,K.A. Cotter,S.V., Gilbert,F. and Alt,F.W. (1986) *Cancer Res.*, **46**, 6217–6222.

Perry,R.P., Kelley,D.E., Coleclough,C., Seidman,J.G., Leder,P., Tonegawa,S., Matthyssens,G. and Weigert,M. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 1937–1941.

Reed,K.C.and Mann,D.A. (1985) *Nucleic Acids Res.*, **13**, 7207–7221.

Reth,M.G., Ammirati,P., Jackson,S. and Alt,F. (1985) *Nature*, **317**, 353–355.

Schwab,M., Varmus,H.E. and Bishop,J.M. (1985) *Nature*, **316**, 160–162.

Stanton,L.W., Schwab,M. and Bishop,J.M. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 1772–1776.

Yancopoulos,G.D. and Alt,F.W.(1985) *Cell*, **40**, 271–281.

Yancopoulos,G.D., Desiderio,S.V., Paskind,M., Kearney,J.F., Baltimore,D. and Alt,F.W. (1984) *Nature*, **311**, 727–733.

Yancopoulos,G.D., Nisen,P.D., Tesfaye,A., Kohl,N.E., Goldfarb,M.P. and Alt,F.W. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 5455–5459.

Zimmerman,K.A., Yancopoulos,G.D., Collum,R.G., Smith,R.K., Kohl,N.E., Denis,K.A., Nau,M.M., Witte,O.N., Toran-Allerand,D., Gee,C.E., Minna,J.D. and Alt,F.W. (1986) *Nature*, **319**, 780–783.

Received on November 28, 1988; revised on January 5, 1989