N-myc transgene promotes B lymphoid proliferation, elicits lymphomas and reveals cross-regulation with c-myc

Helen Rosenbaum, Elizabeth Webb,
Jerry M. Adams, Suzanne Cory and
Alan W. Harris

The Walter and Eliza Hall Institute of Medical Research, P.O. Royal
Melbourne Hospital, Melbourne 3050, Australia

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To assess the impact of constitutive N-myc expression on lymphocytes, we generated lines of transgenic mice bearing the murine N-myc oncogene coupled to the immunoglobulin heavy chain enhancer ($E\mu$). As in mice carrying an analogous c-myc construct, $E\mu$ -N-myc mice exhibit a limited overgrowth of cycling pre-B cells and eventually succumb to clonal B lymphoid tumours. The endogenous N-myc and c-myc alleles are silent in both $E\mu$ -N-myc and $E\mu$ -myc lymphomas, suggesting that these genes are subject to auto- and cross-regulation. The regulatory interaction and the similar biological effects of N-myc and c-myc imply that the two genes perform interchangeable functions in the promotion of cell proliferation.

Key words: transgenic mice/N-myc oncogene/c-myc oncogene/regulation/myc expression/B lymphocyte/development/tumorigenesis

Introduction

Considerable evidence associates activity of the c-myc proto-oncogene with cell proliferation (reviewed by Kelly and Siebenlist, 1986), but relatively little is known about the function of its structural homologues N-myc and L-myc. All three genes encode nuclear phosphoproteins and similarities in their amino-acid sequence (Legouy *et al.*, 1987) suggest shared biochemical function(s). However, while c-myc expression is almost ubiquitous in proliferating cells, expression of the other myc genes is restricted; N-myc transcription, for example, appears to be confined to early stages of brain, kidney and lymphoid development (Zimmerman *et al.*, 1986).

The c-myc gene has been implicated in a range of neoplasms (see Cory, 1986; Alitalo *et al.*, 1987), particularly in lymphoid tumours such as human Burkitt's lymphoma and murine plasmacytoma, where c-myc is translocated to an immunoglobulin locus (reviewed in Klein and Klein, 1985; Cory, 1986). N-myc has been implicated only in certain tumours of neuroendocrine and embryonic origin, primarily neuroblastoma, retinoblastoma, small cell lung carcinoma and Wilms' tumour (Kohl *et al.*, 1983; Lee *et al.*, 1984; Nau *et al.*, 1986; Nisen *et al.*, 1986). These tumours are characterized by high levels of N-myc expression, often due to gene amplification. In neuroblastoma, amplification is associated with tumour progression rather than initiation (Brodeur *et al.*, 1984). More direct evidence for a role in

tumorigenesis is the ability of N-myc, like c-myc, to render a rat fibroblast cell line malignant (Small *et al.*, 1987) and to co-operate with an activated H-ras gene to transform primary rat embryo cells *in vitro* (Schwab *et al.*, 1985; Yancopoulos *et al.*, 1985).

Activation of the oncogenic potential of myc family genes involves deregulated expression rather than an altered coding region (Cory, 1986; Cole, 1986). Normal c-myc expression is postulated to be regulated through a negative feedback loop triggered by excess myc protein (Leder *et al.*, 1983; Rabbitts *et al.*, 1984; Adams *et al.*, 1985). Furthermore, a survey of Wilms' tumours, neuroblastoma cell lines and transfected fibroblasts has revealed that levels of N- and c-myc mRNA tend to be inversely related, raising the possibility that members of the myc family can regulate one another (Alt *et al.*, 1986).

The tumorigenic potential of c-myc for various tissues has been directly demonstrated in transgenic mouse models (Stewart *et al.*, 1984; Adams *et al.*, 1985; Leder *et al.*, 1986; Suda *et al.*, 1987). In particular, transgenic mice bearing c-myc linked to the immunoglobulin heavy chain enhancer ($E\mu$) develop malignant lymphomas as a result of constitutive myc expression within the B-cell lineage (Adams *et al.*, 1985; Alexander *et al.*, 1987; Schmidt *et al.*, 1988). Young $E\mu$ -myc mice exhibit a distinct pre-tumorous phase characterized by a marked expansion of the pre-B cell compartment (Langdon *et al.*, 1986), from which tumorigenic clones arise at low frequency (Harris *et al.*, 1988).

To investigate whether c-myc and N-myc are functionally homologous, we have produced transgenic mice carrying an N-myc gene subjugated to the immunoglobulin heavy chain enhancer. We report here that $E\mu$ -N-myc mice suffer a fate similar to $E\mu$ -myc mice: their B cell development is perturbed and they succumb to lymphoma. Moreover, N-myc transgene expression in the tumours apparently suppresses the endogenous N-myc and c-myc genes. These results directly demonstrate the transforming activity of N-myc *in vivo* and bear upon the regulation and function of myc genes.

Results

N-myc transgene under lymphoid-specific regulation

To promote oncogene expression in lymphocytes, we developed a simple regulatory cassette, p $E\mu$ SV (Figure 1), comprising the immunoglobulin heavy chain enhancer ($E\mu$), the SV40 early region promoter and to aid production of stable transcripts, an intron and polyadenylation sequence from the SV40 early region. As prokaryotic sequences can interfere with transgene expression (Brinster *et al.*, 1985), the cassette is bounded by *NotI* sites, which are rare in eukaryotic DNA and enable removal of the plasmid sequences.

The murine N-myc genomic sequence introduced into p $E\mu$ SV spans the entire coding region and includes part of

Fig. 1. The $E\mu$ - N -myc transgene. N -myc genomic DNA was inserted into the expression vector p $E\mu$ SV. N -myc exons are depicted as raised boxes and coding regions shown solid. The vector consists of the immunoglobulin heavy chain enhancer ($E\mu$), SV40 early promoter (Psv), a polylinker insertion region, SV40 sequences encompassing a splice and polyadenylation signal and a pUC13 plasmid backbone. Restriction sites shown are: N, *Not*I; B, *Bam*HI; HII, *Hinc*II.

Table I. Pathology of advanced disease in two lines of $E\mu$ - N -myc mice

Feature ^a	180 line	184 line
Enlarged lymph nodes and spleen	97%	100%
Enlarged thymus	20%	30%
Vertebral tumour deposit	62%	78%
Subparietal tumour deposit	74%	22%
Invasion of mammary tissue ^b	8%	91%
Haemorrhagic tumours	4%	48%
Hind limb paralysis	4%	4%
Mean tumour cell volume (fl) ^c	219 ± 47	311 ± 52
Tumour onset rate (per week)	14%	4%
Transplantability	76%(10/13)	80%(8/10)
Transplant latent period (days)	14–28	19–61

^aMajor features found upon autopsy of 69 animals of the 180 line and 23 of the 184 line.

^bIn females.

^cMeasured with a Coulter Counter and channelizer, as described (Harris *et al.*, 1988), on tumour samples from 11 180 and 18 184 mice. Mean ± SD.

the 5' and 3' untranslated regions (Figure 1). Microinjection of $E\mu$ SV- N -myc DNA into fertilized eggs yielded four primary transgenic mice, denoted 180, 184, 185 and 179. The first three developed lymphoma (see below) and possessed 5, 2 and 10 intact copies of the transgene, respectively. Mouse 185 died before producing offspring, but breeding lines were established from 180 and 184. Neither 179 nor its transgenic offspring developed tumours during a 6–12 month observation period, so that line was discarded without further analysis.

$E\mu$ - N -myc lymphoma incidence and pathology

Animals of the 180 and 184 $E\mu$ - N -myc lines have stably transmitted the transgene through three generations and exhibited a high incidence of disseminated lymphoma. Major pathological features are listed in Table I. Most lymph nodes in affected animals were enlarged 5- to 50-fold, the mesenteric node being the most prominently affected. The spleen was enlarged 2- to 7-fold. In some animals the thymus was substantially enlarged and this occasionally constituted the dominant disorder. Other features common to both lines were tumour deposits along the thoracic vertebral column and enlargement (up to 2-fold) of the liver. A few animals

suffered hind limb paralysis. Blood cell analysis revealed that leukaemia accompanied the lymphomas in both lines, leukocyte counts being elevated up to 50-fold with lymphoblasts predominating.

Despite the overall similarity of the disease in the two $E\mu$ - N -myc lines, notable quantitative differences emerged (Table I). Tumour masses inside the skull were frequent in 180 but not in 184 animals; they adhered to the parietal and interparietal bones, sometimes deforming them and indenting the cerebrum and cerebellum. In almost all 184 females but few 180 females, flat subcutaneous tumour masses invested the mammary tissue. In nearly half the 184 mice but in very few 180 animals, tumours showed extensive internal haemorrhage. The cells of 184 tumours were usually larger than those of 180 tumours.

Histologic examination of >20 mice with advanced disease revealed that the tumours in 180 and 184 animals were similar in morphology and invasiveness. Medium-sized, mitotically active lymphoblasts occupied all lymphoid tissues to varying extents and infiltrated several other organs. Lymphoblasts in the enlarged lymph nodes almost completely replaced normal cells, obliterated the architecture and extended beyond the capsule. Invasion of the spleen and bone marrow was highly variable and ranged from a slight excess of lymphoblasts to almost complete replacement of normal components. Severe cases of splenic infiltration were characterized by a blurring of the distinction between red and white pulp. Normal haemopoietic elements were never totally displaced from bone marrow or spleen, however, as some megakaryocytes and normoblasts always remained. Infiltration of the thymus was also variable, ranging from a few tumour foci in the cortex, to total replacement of normal lymphocytes. In all animals examined, the liver contained coherent tumour masses in the portal areas and diffusely invaded lobules. The lungs always showed diffuse infiltration of alveolar septa, often sufficient to collapse many of the alveolar air spaces. The fat and muscle adjacent to the thoracic vertebral column and the sternum were almost always extensively invaded by lymphoblasts. Cardiac muscle was penetrated in three of the eight 184 mice, but in none of the 180 animals examined. The kidney cortex was infiltrated in >50% of the 184 mice but in only one 180 mouse.

The rate of tumour onset differed markedly between the two lines, as is apparent from cumulative incidence curves (Figure 2). Essentially all of the 180 animals became ill with lymphoma between 6 and 50 weeks of age, the median being 13 weeks. In contrast, no tumours appeared in the 184 line before 12 weeks, and only ~50% of the mice had developed advanced tumours by 30 weeks. As calculated from semi-log 'survival' curves, the rate of appearance of new tumours after an initial lag period, was 4% per week for the 184 population and 14% per week for the majority of 180 animals.

As a test of malignancy, cells from primary tumours were transferred into normal histocompatible mice. Over 75% of the 180 and the 184 tumours were transplantable (Table I). The transplanted 184 tumours generally exhibited a longer and more variable latent period than 180 tumours. The spleen, thymus and lymph nodes of transplant recipients were usually enlarged and replete with lymphoblasts, while the thymus was less frequently invaded by tumour cells. Tumours often developed at the site of injection. No gross

Fig. 2. Cumulative incidence of advanced-stage tumours in $E\mu$ -N-myc 180 and 184 mice. The plots were generated from 76 $E\mu$ -N-myc 180 animals and 60 $E\mu$ -N-myc 184 animals of the first and second generations.

Table II. Cell surface phenotypes and Ig locus rearrangements of $E\mu$ -N-myc tumours

Tumour ^a	Cell surface marker ^b				Rearrangement ^c		Tumour type
	Ig	B220	Mac-1	Ly1	J _H	J _κ	
180 Line							
180 (1°)	—	+	—	—	R1,R2	G	Pre-B
A-1	+	+	—	—	R1,R2	G,R1	B
A-2	—	+	—	—	R1-R4	G	Pre-B
B-1	+	+	—	—	R1,R2 ^d	R1,R2	B
B-2	—	+	—	—	R1	G	Pre-B
C-1	—	—	—	—	R1,R2	G	Pre-B
C-2	+/- ^c	+	—	—	ND	G(R1,R2)	Pre-B/B
C-3	—	+	—	—	ND	ND	Pre-B
C-4	+/- ^f	+	—	—	ND	G	Pre-B/B
184 Line							
B-1	+	+	—	—	R1,R2	G,R1	B
B-2	+	+	+ ^g	+ ^g	R1	R1	Ly1 B
B-3	+	+	—	—	R1,R2	G ^h	B
B-4	+	+	—	—	R1,R2	R1-R4 ^d	B
B-5	+	+	—	—	R1,R2 ^d	R1-R3	B
B-6	+	+	+ ^g	—	R1,R2 ^d	G ^h	Ly1 B
B-7	—	+	—	—	ND	ND	Pre-B
C-1	+	+	—	—	ND	ND	B
185 (1°)	—	+	—	—	R1,R2	G(minor R)	Pre-B

^aThe tumours analysed were enlarged mesenteric lymph nodes, except for 180 C-4 and 184 B-7, which were thymomas. A, B and C refer to first, second and third generation descendants of the primary founder.

^bThe proportion of cells bearing the indicated markers as determined by immunofluorescence and flow cytometry. +, >95% positive; -, <5% positive.

^cR1-R4 denote rearranged fragments distinguished by electrophoretic mobility. G, germline configuration; ND, not determined.

^dOne rearranged fragment dominant.

^e70% Ig⁺.

^f84% Ig⁺.

^gAll cells weakly expressed the antigen.

^hSince the cells were sIg⁺, the lack of discernible rearranged kappa bands may indicate that the clone had undergone multiple kappa rearrangements.

sub-parietal deposits were observed but tumour growth at this location could sometimes be seen histologically.

Monoclonal B lymphoid tumours

In order to identify their cellular origins, lymphomas from $E\mu$ -N-myc mice were examined for cell surface antigens

Fig. 3. Immunoglobulin heavy and light chain gene rearrangements in $E\mu$ -N-myc 184 mice. Digests of tumour DNA were fractionated on 0.7% agarose gels, transferred to nitrocellulose and hybridized with ³²P-labelled DNA probes. Tumours are designated as in Table II, while C57 and SJL indicate DNA from normal C57BL/6 and SJL liver. (A) J_H rearrangements were detected in *Eco*RI digests with a 670-bp *Pst*I/*Nae*I DNA fragment from a region immediately 3' of J_{H3} and spanning J_{H4}. (B) J_κ rearrangements were detected in *Bam*HI digests with a 1.5-kb *Hind*III/*Bgl*II fragment from cloned genomic C_κ DNA.

and rearrangement of immunoglobulin (Ig) and T cell receptor loci. The results (Table II) showed that all derived from the B lymphoid lineage. None expressed the T cell marker Thy-1, not even two tumours (180 C-4 and 184 B-7) which predominantly involved the thymus, nor exhibited rearrangement of the T cell receptor γ locus (data not shown). All except one tumour displayed the B lineage marker Ly5(B220) and all had at least one allele of the *Igh* locus rearranged. The simplicity of the *Igh* rearrangement patterns, illustrated for 184 lymphomas in Figure 3A, indicated that the tumours were monoclonal or at most biclonal; the 6.5 kb germline J_H fragment was replaced by two to four other fragments. In several cases tested, tumours dissected from different sites within a single animal exhibited identical rearrangements and phenotypes.

The frequency of Ig-bearing B cell tumours in the two $E\mu$ -N-myc lines differed notably. Seven of the eight tumours examined from the 184 line comprised Ig⁺ cells. In contrast, only two out of nine 180 tumours were Ig⁺. Five were entirely Ig⁻ (Table II), while two others (C-2 and C-4) had significant numbers of both Ig⁻ and Ig⁺ cells, probably reflecting differentiation from pre-B to B cells. Thus a higher proportion of 180 than of 184 tumours present as the pre-B type. Many of the 184 tumours had one or two

Fig. 4. *N-myc* and *c-myc* expression in $E\mu$ -*N-myc* and $E\mu$ -*myc* tumours and B lymphoid cell lines. Poly(A)⁺ RNA isolated by proteinase K digestion and oligo(dT) cellulose chromatography was analysed by Northern blot hybridization. (A) Endogenous and transgene *N-myc* transcripts detected with a *PvuII/XhoI* fragment spanning exon 2 of the murine *N-myc* gene. (B) Endogenous *N-myc* detected with a 0.87-kb *HincII/EcoRI* fragment from the 3' untranslated region of the murine *N-myc* gene, a region absent from the $E\mu$ -*N-myc* transgene. (C) Endogenous (2.3 kb) and transgene (2.9 kb) *c-myc* transcripts detected with a 1.7-kb *XhoI* fragment from a murine cDNA clone containing exon 2 and 3 sequences. (D) Hybridization to a glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) probe (Piechaczyk *et al.*, 1984), indicating the relative quantities and integrity of the mRNA loaded. LYH7 is a pro-B cell line (Palacios *et al.*, 1987), ABL8-8 and 18-81 are pre-B cell lines derived from tumours induced by Abelson murine leukaemia virus and WEHI-279 is a B lymphoma cell line.

kappa rearrangements, but others had a greater number (Figure 3B and Table II), implying that transformation sometimes preceded kappa gene rearrangement and acquisition of the B cell phenotype. Interestingly, two of the 184 tumours (B-2 and B-6) appeared to derive from the 'Ly-1' B cell subpopulation, members of which bear a low level of Mac-1 and (sometimes) Ly-1 (Herzenberg *et al.*, 1987).

N-myc and *c-myc* expression

Tumours from the 180 and 184 lines, as well as from the 185 primary mouse, all displayed the mature 2.9 kb mRNA and the 3.7, 4.9 and 5.6 kb precursor species expected from the $E\mu$ -*N-myc* transgene (Figure 4A). The abundance of transcripts was comparable in tumours from both lines and was at least 20-fold higher than the level of *N-myc* RNA

in the pre-B and pro-B non-transgenic cell lines, ABL8-8, 18-81 and LYH7.

Since the endogenous *c-myc* genes are silent in $E\mu$ -*myc* tumour cell lines (Adams *et al.*, 1985), we wondered if the $E\mu$ -*N-myc* transgene would suppress expression of the normal *N-myc* gene. Indeed, as shown in Figure 4B, an *N-myc* probe from a 3' untranslated region not present in the transgene revealed no endogenous *N-myc* transcripts in pre-B lymphomas of $E\mu$ -*N-myc* mice, six of which were analysed, nor in several B lymphomas. However, readily detectable levels were present in the non-transgenic pre-B and pro-B cell lines. Since *N-myc* expression has also been detected in normal pre-B cells and in all other pre-B cell lines examined (Zimmerman *et al.*, 1986), these data imply that *N-myc* is subject to some form of feedback inhibition.

Evidence of cross-regulation between *c-myc* and *N-myc* was also obtained. For example, no *N-myc* expression could be detected in the three $E\mu$ -*myc* pre-B tumours tested (Figure 4B). Even more striking, none of the 11 $E\mu$ -*N-myc* tumours examined expressed *c-myc*, whereas abundant *c-myc* mRNA was evident in the Abelson pre-B lines ABL8-8 and 18-81 and the B cell line WEHI-279 (Figure 4C). The factor-dependent, non-tumorigenic pro-B cell line LyH7 (Palacios *et al.*, 1987) expressed high levels of *N-myc* but low levels of *c-myc* relative to the Abelson lines (Figure 4B,C), perhaps reflecting its more immature status.

Perturbed B cell development precedes tumorigenesis

Prior to tumour onset, young $E\mu$ -*N-myc* mice of both lines exhibited a distinct prelymphomatous condition, characterized histologically (in all of 14 such animals examined) by an excess of large lymphoid cells in the bone marrow, spleen and lymph nodes. These cells, normally <10% of the nucleated cells in bone marrow, increased variably to 20–70% at the expense of erythroid and granulocytic elements. Lymph nodes and splenic white pulp contained frequent large accumulations of lymphoblasts resembling germinal centres, while in the splenic red pulp many such cells were apparent amongst the normal erythrocytes, normoblasts and megakaryocytes. No notable increase in lymphoblasts occurred in the thymus, but the ratio of cortical to medullary areas was variably reduced by up to fourfold. Although the lymphoblasts in the pre-tumour tissues resembled those in tumours, they were not malignant by transplantation tests. Injection of spleen, bone marrow and lymph node cells (5 – 10×10^6) from >20 pre-tumorous animals into histocompatible recipients initiated no tumour growths in a three-month observation period.

To identify the nature of the perturbation in lymphoid populations during the pre-lymphomatous phase, we determined the cellular composition of various lymphoid organs by flow cytometry with fluorescent antibodies against characteristic cell surface antigens (Table III).

Bone marrow was the most affected tissue. The total number of cells was unaltered, but there was an increase in B lineage ($B220^+$) cells, particularly in mice of the 180 line, and a reduction in Mac-1⁺ cells of the granulocyte-macrophage lineage. The frequency of cells bearing Ig was near normal. Hence, the pre-B compartment ($B220^+$, Ig[−]) had expanded at the expense of myeloid cells.

A notable feature of $E\mu$ -*N-myc* bone marrow was the increased number of large $B220^+$ cells. Figure 5 correlates fluorescence and low angle forward light scatter, an index

Table III. Cellular composition of lymphoid tissues of pre-lymphomatous $E\mu$ -N-myc mice

Tissue		Cell surface markers ^a			
		Ig	B220	Thy-1	Mac-1
Marrow	Normal	13 ± 2	33 ± 8	ND	34 ± 10
	180	11 ± 3	54 ± 8	ND	21 ± 6
	184	11 ± 3	40 ± 5	ND	29 ± 2
Spleen	Normal	49 ± 8	42 ± 7	40 ± 2	7 ± 2
	180	41 ± 7	43 ± 8	29 ± 9	7 ± 2
	184	33 ± 9	36 ± 7	23 ± 6	ND
Lymph node	Normal	27 ± 7	23 ± 6	77 ± 10	<2
	180	17 ± 5	15 ± 5	ND	<2
	184	10 ± 3	9 ± 2	85 ± 8	ND

^aThe mean % ± SD of cells bearing the indicated markers. The normal values derive from 10 non-transgenic littermate or B6JF1 animals, except for Thy-1⁺ cells in the spleen (four mice) and mesenteric lymph node (two mice). The 180 values derive from 10–13 mice, apart from Thy-1⁺ cells in the spleen (two mice), and the 184 values from six animals. ND, not determined.

Fig. 5. Flow cytometric dot-plots comparing Ly5(B220) expression and forward light scatter in pre-lymphomatous and normal bone marrow and spleen. Data were collected on 10⁴ viable cells.

of size and shows that many B lineage cells were larger than normal, while the size of the predominantly myeloid (B220⁻) cells was unchanged.

Large B220⁺ cells also appeared in other lymphoid tissues of prelymphomatous mice. The spleen was normal in size and in total cell number, but showed somewhat reduced numbers of B cells (Table III) and a shift toward

Fig. 6. Transgene expression in tissues of pre-lymphomatous $E\mu$ -N-myc mice. Northern blot analysis of poly(A)⁺ RNA probed with the terminal SV40 sequence of the transgene. Tissues from six animals were pooled for each of the bone marrow (BM) samples, from two animals for each of the thymus (THY) and spleen (SPL) samples, while the liver (LIV), kidney (KID) and brain (BR) samples derive from a single animal. A tumour sample (180 1°) is shown for comparison.

large cells that expressed low levels of B220 (Figure 5). In the lymph nodes, B cells were more severely reduced in number (Table III) and were also larger than normal (data not shown). T cells, on the other hand, were not greatly affected and no abnormalities were detected in the pre-lymphomatous thymus. The size profiles of thymocytes and the proportions of the four thymic T cell subpopulations (Ly2⁺ L3T4⁺, Ly2⁺ L3T4⁻, Ly2⁻ L3T4⁺ and Ly2⁻ L3T4⁻) were normal (data not shown).

Increased cell cycle activity of $E\mu$ -N-myc B lineage cells

The large size characteristic of many $E\mu$ -N-myc B lineage cells suggested increased proliferative activity. We therefore performed cell cycle analysis by flow cytometry on B220⁺ cells FACS-sorted from bone marrow and stained with the DNA-binding dye propidium iodide. Results were averaged from four such analyses of 180 mice, five of 184 mice, and three of non-transgenic animals. About 29% of such cells from 180 mice and 37% of those from 184 mice were in the S and G₂/M phases of the cycle, versus 21% in normal marrow. These are minimum values for the proportion of cells in cycle, because the analysis does not distinguish between non-cycling (G₀) cells and those traversing G₁. It is possible that most of the $E\mu$ -N-myc B220⁺ cells were cycling, as the nuclei of the G₀/G₁ population were somewhat larger than normal (data not shown). Thus N-myc, like c-myc, promotes cell proliferation.

Transgene expression in pre-lymphomatous mice

To investigate the tissue-specificity of transgene activity, tissues from pre-lymphomatous animals were surveyed by Northern blot analysis of poly(A)⁺ RNA (Figure 6). As expected from their cellular composition, bone marrow and spleen expressed significant levels of the transgene. Thymocytes, on the other hand, probably do not express the transgene. Three of four 184 mice and two of four 180 mice exhibited no detectable $E\mu$ -N-myc RNA in the thymus and the low level in other animals (e.g. Figure 6) was probably

due to RNA of B lineage origin, since an Ig κ light chain probe hybridized to these samples (data not shown). No transgene RNA was detected in the brain, kidney or liver (Figure 6) nor in the heart or lung (data not shown). Thus, transgene expression in E μ -N-myc mice appears to be restricted to haemopoietic cells and may be confined to the B lymphoid compartment.

Discussion

N-myc is a potent tumorigenic agent for B lymphoid cells

The N-myc gene has previously been implicated in the progression of human neuroblastomas, retinoblastomas and small cell lung carcinomas by its elevated expression and frequent amplification, most often in late-stage tumours. Using transgenic mice, we have now demonstrated the oncogenic potential of N-myc for B lymphoid cells. Linking the transgene to the IgH enhancer ensured its constitutive expression in B lymphoid cells, increasing their proliferation and susceptibility to lymphomagenesis. Hence N-myc can trigger the tumorigenic process as well as contribute to progression, and its oncogenic action is not restricted to neuroendocrine and embryonic cell types. Based on previous results with a series of c-myc transgenics (Adams *et al.*, 1985), we assume that the Ig enhancer played the major role in deregulating N-myc expression, but cannot rule out the possibility that the absence of certain 5' and 3' untranslated sequences had an influence.

The disease profile in E μ -N-myc transgenic mice is highly reminiscent of that previously described for mice harbouring an analogous c-myc construct (Adams *et al.*, 1985; Langdon *et al.*, 1986; Harris *et al.*, 1988). Two distinct phases are evident. The first is a benign lymphoproliferative syndrome in which pre-B cells accumulate in the bone marrow and to a lesser extent, in the spleen. Most pre-B and B cells in the animals are large and the number in cycle is substantially elevated. Thus N-myc, like c-myc, can drive cell proliferation. The transgenic animals subsequently develop a disseminated clonal B lymphoid malignancy (Tables I and II), presumably through genetic changes within a single member of the cycling pre-neoplastic population.

The two E μ -N-myc lines analysed in detail differ in the kinetics of lymphoma development. Most of the 180 mice succumb at the rate of 14% per week, almost as high as the 17% per week observed for the E μ -myc 292-1 line (Harris *et al.*, 1988), while the rate of N-myc 184 is only 4% per week. Interestingly, the tumour onset rates correlate with the degree to which the pre-B cell compartment expands during the pre-tumour phase, the order for both being E μ -myc > E μ -N-myc 180 > E μ -N-myc 184. A similar correlation was previously reported for subpopulations of E μ -myc mice (Langdon *et al.*, 1986; Harris *et al.*, 1988) and probably reflects the number of cells vulnerable to genetic change. Since such changes usually occur during DNA replication, the risk of malignant conversion increases with the number of vulnerable cycling cells.

Tumours from 180 and 184 E μ -N-myc mice tended to differ in presentation, latent period on transplantation and cell size (Table I). Furthermore, while 180 tumours were predominantly pre-B in nature, almost all 184 neoplasms were composed entirely of B cells (Table II). Two independently generated lines of transgenic mice bearing

c-myc coupled to E μ also differ in the proportion of pre-B and B cell tumours (Harris *et al.*, 1988; Schmidt *et al.*, 1988). Such differences may reflect an effect of the chromosomal location of the transgene on the level or timing of its expression. However, no significant difference was apparent in the amount of transgene RNA in the 180 and 184 tumours. Furthermore, the predominance of B cell tumours in the 184 lineage cannot be due to transformation being confined to the B cell stage; at least two 184 tumours were oligoclonal for κ rearrangements, but clonal for J_H rearrangement, implying that transformation has occurred in a pre-B cell. Since two 184 tumours bear Mac-1 (Table II), which is characteristic of the 'Ly1' minor B cell population (Herzenberg *et al.*, 1987), it is conceivable that the 184 transgene is expressed more predominantly within that sublineage.

Implications for myc gene regulation

The silence of the untranslocated c-myc gene in Burkitt lymphomas and plasmacytomas (Bernard *et al.*, 1983; Nishikura *et al.*, 1983) prompted the hypothesis that c-myc expression is regulated by a negative feedback loop to which the translocated myc allele is insensitive (Leder *et al.*, 1983; Rabbitts *et al.*, 1984). This hypothesis received strong support from the observation that the endogenous c-myc alleles in E μ -myc tumour cells are silent (Adams *et al.*, 1985; Alexander *et al.*, 1987). The absence of detectable endogenous N-myc expression in E μ -N-myc tumour cells now extends this model to N-myc regulation. Furthermore, E μ -N-myc pre-B tumours do not express c-myc, and conversely E μ -myc pre-B tumours do not express N-myc (Figure 4), even though normal pre-B cells express both N-myc and c-myc (Zimmerman *et al.*, 1986). These data provide persuasive evidence that the myc genes can cross-regulate one another, as proposed by Alt *et al.* (1986). If this is a normal regulating mechanism, c-myc must dominate in most cell types and hold N-myc in check. N-myc suppression of c-myc may occur only in the rare or abnormal cell types in which N-myc expression is elevated, as it is in the E μ -N-myc B lymphoid cells.

Functional homology of myc genes

The cross-regulation observed between N-myc and c-myc and the similar effects elicited by their enforced expression, both on proliferation of B lineage cells as well as predisposition to lymphoma, argues strongly for functional homology of the two genes. Indeed, since E μ -N-myc lymphoma cells contain no detectable c-myc mRNA (Figure 4C), it follows that N-myc can completely supplant c-myc in the proliferation of pre-B and B cells.

Our demonstration that N-myc can induce B lymphoid tumours is complemented by recent evidence that N-myc is activated in certain retrovirus-induced T lymphomas (A. Berns, personal communication). Why then has N-myc not been implicated in naturally occurring B lymphoid tumours? The timing of N-myc expression during B cell ontogeny suggests an explanation. Untranscribed genes appear to be less subject to DNA rearrangement (Alt *et al.*, 1987) and N-myc would therefore not be prone to translocation in B cells or plasma cells, where it is not expressed (Zimmerman *et al.*, 1986). By the same argument, however, its transcriptional activity in earlier B lineage cells

might make it a target for deregulation by translocation or amplification. Some cases of pre-B acute lymphoblastic leukaemia might therefore prove to involve an altered N-myc gene.

Materials and methods

Construction of lymphoid-specific expression vector and N-myc transgene

To target oncogene expression to lymphoid cells, we constructed a vector, pE μ SV, containing the IgH enhancer (E μ). The 680-bp *EcoRI/XbaI* fragment is opposite in orientation to that found in the *Igh* gene to prevent transcription from cryptic promoters within this sequence (Lennon and Perry, 1985). Adjacent to E μ is the SV40 early region promoter (a 200-bp *SphI/HindIII* fragment), chosen because it is well-characterized and can be separated from SV40 enhancer sequences. The vector includes the RNA splice signals of the SV40 small tumour antigen intron (*SauIII*A fragment 4700–4100) and the termination and polyadenylation sequence for SV40 early transcripts (*SauIII*A/*BamHI* fragment 2771–2533). The polylinker derives from pUC13 but has an additional *ApaI* site after the *EcoRI* site. The entire cassette is bounded by *NotI* restriction sites.

The murine genomic N-myc clone was a gift from F.Alt. A 4578-bp *BamHI/HincII* fragment from clone p7.7 (DePinho *et al.*, 1986) was inserted at the *BamHI* site in the polylinker (see Figure 1). The construct was digested with *NotI* endonuclease, and the transgene separated from plasmid sequences by electrophoresis through low-melting point agarose in Tris–acetate buffer. The gel slice was dissolved in 8 M NaClO₄ at 37°C and the DNA applied to a glass fibre (GFC) filter, washed with 6 M NaClO₄ in 10 mM Tris pH 7.5, 1 mM EDTA and then with 95% ethanol. The fragment was eluted from the air-dried filter with 1 mM Tris pH 7.5, 0.1 mM EDTA. For microinjection, the DNA was diluted to 2 µg/ml with 10 mM Tris pH 7.5/0.1 mM EDTA and centrifuged at 20 000 g for 20 min to remove particulate matter.

Mice

Transgenic mice were produced essentially as described by Brinster *et al.* (1985). Single-cell fertilized eggs were obtained from superovulated (C57BL/6Wehi × SJL/JWehi)F1 hybrid (BSJF1) females mated with BSJF1 males. Eggs that survived pronuclear microinjection with the E μ –N-myc construct and cleaved during overnight culture were transferred to the oviducts of pseudopregnant females. Transgenic mice were identified by dot-blot hybridization of tail DNA to a ³²P-labelled fragment spanning the terminal SV40 sequence. Each primary mouse was an F2 animal and the transgenic lines have been maintained as FE2 stocks through matings of transgenic males with BSJF1 females.

Mice analysed for pre-tumour changes were 4–8 weeks of age, displayed no overt signs of disease and did not yield tumours in BSJF1 recipients within 12 weeks of lymphoid tissue transplantation.

Antibodies and flow cytometry

Phycoerythrin-conjugated anti-mouse L3T4 (CD4) and fluorescein-conjugated anti-mouse Thy 1.2 and Ly2 (CD8) antibodies were obtained from Becton-Dickinson (Mountain View, CA, USA). Other antibodies against cell surface antigens and flow cytometric analysis of stained cell suspensions were as described by Langdon *et al.* (1986).

To determine the proliferative status of B lineage cells, fluorescent B220⁺ cells were sorted from bone marrow using a FACS II and then stained with propidium iodide (Taylor, 1980). The percentage of cells at each stage of the cell cycle was determined from the single-parameter profile of red fluorescence obtained in a FACScan flow cytometer, using the statistical analysis software package SFIT (Becton-Dickinson).

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