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Cyclin D1/*bcl-1* cooperates with *myc* genes in the generation of B-cell lymphoma in transgenic mice

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The chromosomal translocation t(11:14) is associated with human lymphoid neoplasia affecting centrocytic B-cells of intermediate differentiation. As a consequence the cyclin D1 (*bcl-1*) gene is juxtaposed to the immunoglobulin heavy chain enhancer Eμ. To show that transcriptional activation of cyclin D1 is causally involved in the generation of B-cell neoplasia we have generated transgenic mice that carry a cyclin D1 gene under the transcriptional control of the Eμ element. Eμ cyclin D1 transgenic mice show only very subtle alterations in the cycling behaviour of B-cell populations in the bone marrow compared with normal mice and do not develop lymphoid tumours. However, Eμ-directed coexpression of cyclin D1 and N-MYC or L-MYC in double transgenic mice reveals a strong cooperative effect between MYC and cyclin D1 provoking the rapid development of clonal pre-B and B-cell lymphomas. Interestingly, crossing of cyclin D1 transgenic mice with Eμ L-*myc* transgenics that express their transgene in both B- and T-cells but predominantly develop T-cell tumours leads in double transgenics exclusively to B-cell neoplasia. The data presented here demonstrate that transcriptional activation of cyclin D1 can oncogenically transform B-cells in concert with a *myc* gene. They establish cyclin D1 as a proto-oncogene whose activity appears to depend on a specific cell type as well as on a specific cooperating partner and link disturbances in the regulation of cell cycle progression to the development of human malignancies.

Key words: B-cell lymphoma/cyclin D1/*myc* family genes/oncogene cooperation/transgenic mice

Introduction

Tumorigenesis is now widely accepted to involve a multistep process in which several genetic lesions have to accumulate in order to produce a fully malignant phenotype (for review see Hunter, 1991). Activation of proto-oncogenes or inactivation of tumour suppressor genes have been recognized to represent these genetic lesions. Activation of proto-oncogenes and the subsequent generation of active oncoproteins can be achieved by a number of different mechanisms such as gene amplification, point mutations and chromosomal translocations or deletions. In most cases these alterations will affect signal transduction pathways that regulate the cellular response to various growth stimuli and thereby modulate cell proliferation and differentiation processes. The fact that several complementary events have to interact synergistically to transform a cell has been attributed to oncogene cooperation, the best known example being the transformation of primary fibroblasts by RAS and MYC (Land *et al.*, 1983; Ruley, 1983). One of the critical endpoints of a signal transduction cascade initiated after a mitogenic stimulus is the regulation of cell cycle progression. It seems obvious that uncontrolled proliferation and subsequent malignant transformation of a cell depends strongly on mechanisms and factors that are responsible for transitions within the cell cycle. Regulation of cell cycle progression is managed in the cell by a series of cyclins and cyclin dependent kinases (for review see Müller *et al.*, 1993; Sherr, 1993; Xiong and Beach, 1991). To date, eight cyclins are known that either regulate G_1/S or G_0/G_1 transitions i.e. cyclin D1–3 and cyclin C, or S phase transitions, such as cyclins A and E, or are involved in G_2/M transitions, such as cyclins B1 and B2. Each individual cyclin has to associate with a specific cyclin dependent kinase, of which six distinct types (CDK1–6) have been identified so far, to gain enzymatic activity and to become functionally active.

It is conceivable that deregulated expression of cyclins or their appropriate CDKs that are involved in the regulation of either G_1/S and G_0/G_1 progression can lead to aberrant growth behaviour and proliferation rates of cells and may be a prerequisite of malignant transformation. The first evidence that altered cyclins may be involved in tumorigenesis was provided by the analysis of genomic integration sites of hepatitis B virus (HBV) DNA in cells of a human hepatocellular carcinoma (Wang *et al.*, 1990). The integration of HBV DNA produced a fusion between the pre-S open reading frame that is able to encode part of the HBV surface protein and the gene for cyclin A. Cyclin A is normally expressed during S phase and associates with CDK1 and CDK2. The expression of the chimeric pre-S–cyclin A protein was immediately suspected to contribute substantially to liver carcinogenesis (Wang *et al.*, 1990). Although this remained an isolated case and did not seem to represent a general mechanism of HBV induced liver carcinogenesis, cyclin A may be involved in cellular transformation by another mechanism. In adenovirus infected cells cyclin A can associate with the viral protein E1A as a member of the multiprotein complex consisting of cyclin A, E1A, p107 and E2F (Giordano *et al.*, 1989; Pines and Hunter, 1990; Nevins, 1992). Cyclin A might therefore play a critical role in adenovirus mediated cellular transformation.

The most direct evidence for the implication of cell

cycle regulators in tumorigenesis is provided by studies with cyclins D1 and D2. The cyclin D1 gene is amplified in human breast carcinoma, squamous cell carcinoma and mouse skin carcinoma (Keyomarsi and Pardee, 1993; Buckley *et al.*, 1993; Bianchi *et al.*, 1993; Schuuring *et al.*, 1992), and it is rearranged in human parathyroid adenomas (Motokura *et al.*, 1991; Seto *et al.*, 1992; Rosenberg *et al.*, 1993). In addition, cyclin D1 represents the product of the *bcl-1* gene which is located in the vicinity of the breakpoint in the chromosomal translocation t(11:14) typical of low malignant human B-cell lymphoma affecting centrocytic B-cells and B-cells of intermediate differentiation (Tsujimoto *et al.*, 1985; Meeker *et al.*, 1989; Medeiros *et al.*, 1990; Raffeld and Jaffe, 1991; Withers *et al.*, 1991). This t(11:14) translocation juxtaposes the cyclin D1 gene to the locus encoding the immunoglobulin μ heavy chain. As a consequence the cyclin D1 gene is under the transcriptional control of the immunoglobulin enhancer Eμ and is consitutively expressed in B-cell lymphomas bearing this translocation (Withers *et al.*, 1991; Palmero *et al.*, 1993). Furthermore, the genes for cyclin D1 and cyclin D2 are both targets for proviral insertion of murine non-acute transforming retroviruses of the Friend or Moloney type (Lammie *et al.*, 1991; Hanna *et al.*, 1993). Similar to the situation in the t(11:14) translocation occurring in human B-cell tumours where the Eμ enhancer must act at several hundred kilobases distance, the transcriptional activation of cyclin D genes through elements in retroviral LTRs must take place at comparable distances as the proviral insertion sites are located 50–75 kb from the genes encoding cyclins D1 and D2 (Lammie *et al.*, 1991; Hanna *et al.*, 1993).

Several lines of experimentation have been undertaken to show directly the oncogenic potential of exogenous cyclin D1, mostly involving transfection of established rat or mouse fibroblast lines. Fibroblasts expressing high levels of cyclin D1 are smaller in size and accumulate more cells that are in S and G_2/M phase than normal fibroblast populations, probably owing to a faster G_1/S transition (Jiang *et al.*, 1993; Quelle *et al.*, 1993). However, these cells did not show a morphologically transformed phenotype and remained anchorage dependent. In one case rat fibroblast lines transfected with cyclin D1 provoked tumour growth in nude mice albeit after a long latency period (Jiang *et al.*, 1993). These experiments strongly implicated the need for cooperating events that would help cyclin D1 to reveal its oncogenic potential. Direct experimental evidence for a transforming activity of cyclin D1 was provided by cotransfection of primary rat embryonic fibroblasts with activated c-*myc* or Ha-*ras* genes. Together with Ha-*ras* cyclin D1 has been shown to be able to transform primary cells, to abrogate their anchorage dependence and to enable them to grow rapidly as fibrosarcomas in nude mice (Lovec *et al.*, 1994). In another experiment cyclin D1 was shown to transform BRK cells in the presence of activated Ha-*ras* and an altered E1A protein (Hinds *et al.*, 1994). Experiments with osteosarcoma cells deficient for the retinoblastoma suppressing protein pRB have provided strong evidence that all three D-type cyclins may exert their function as a member of a multiprotein complex involving physical interaction with pRB (Dowdy *et al.*, 1993; Ewen *et al.*, 1993; Hall *et al.*, 1993; Kato *et al.*, 1993). In this respect D-type cyclins would behave very similarly to the viral oncoproteins E1A, E7 or SV40 large T antigen which also bind to pRB (deCaprio *et al.*, 1988; Whyte *et al.*, 1988; Ewen *et al.*, 1989; Dyson *et al.*, 1989) and modulate its growth suppressive function.

To investigate further the role of a transcriptionally activated cyclin D1 gene in the development of human B-cell lymphoma we generated transgenic mice bearing a cyclin D1 gene under the transcriptional control of the Eμ element. We show here that ectopic expression of cyclin D1 alone in the lymphoid compartment does not predispose transgenic animals to the development of lymphoid malignancies. However, Eμ *myc*–cyclin D1 double transgenic mice very rapidly develop pre-B and B lymphomas. These results clearly attribute a role to cyclin D1 in the generation of human B-cell lymphoid malignancies but also show an indispensable requirement for cyclin D1 to cooperate with partners in order to realise its oncogenic capacity.

Results

Production of transgenic mice carrying a Eμ cyclin D1 construct

The gene encoding human cyclin D1 was isolated via RT-PCR from the Ewings sarcoma cell lines RDES. The sequence was determined and found to be identical to the normal human cyclin D1 sequence from placenta (Lovec *et al.*, 1994). We could show that this cDNA was able to express the correct functional cyclin D1 protein in transfection experiments with primary rat embryo fibroblasts. Here, cyclin D1 was able to cooperate with an activated Ha-*ras* gene to transform REFs into a tumorigenic phenotype (Lovec *et al.*, 1994). This cyclin D1 cDNA was then inserted into the vector pc2δβ where it is under the transcriptional control of a 700 bp fragment containing the immunoglobulin intron enhancer element Eμ and the promotor from a variable segment gene (Möröy *et al.*, 1990). At the 3′ end of the vector the second and third β-globin exons provide a polyadenylation signal and an intron to assure proper termination after transcription (Figure 1) (Pirchner *et al.*, 1989). Eight independent founder animals were obtained that contained one to 10 copies of this construct and two animals were used to establish the transgenic lines 554 and 546.

Expression of the Eμ cyclin D1 transgene

To assess transgene expression in the established lines, RNA from different tissues was analysed by Northern blotting experiments (Figure 1B). Specific transcripts for the Eμ cyclin D1 transgene of the expected length of 1.7 kb were detected in lymphoid tissue of mice from the lines 546 and 554 (Figure 1B) but not in other non-lymphoid organs with the exception of kidney in the line 554 (Figure 1B) indicating a successful targeting of cyclin D1 expression to lymphoid cells. As the size of the endogenous murine cyclin D1 transcript is 4.2 kb, the 1.7 kb transcript seen in spleen and thymus of line 546, in spleen and in the kidney of line 554 clearly stems from the transgene. The origin of the longer transcript seen in thymus of line 546 (Figure 1B) is not clear but could represent mRNA molecules that terminate in the flanking genomic sequence or initiate within the Eμ enhancer

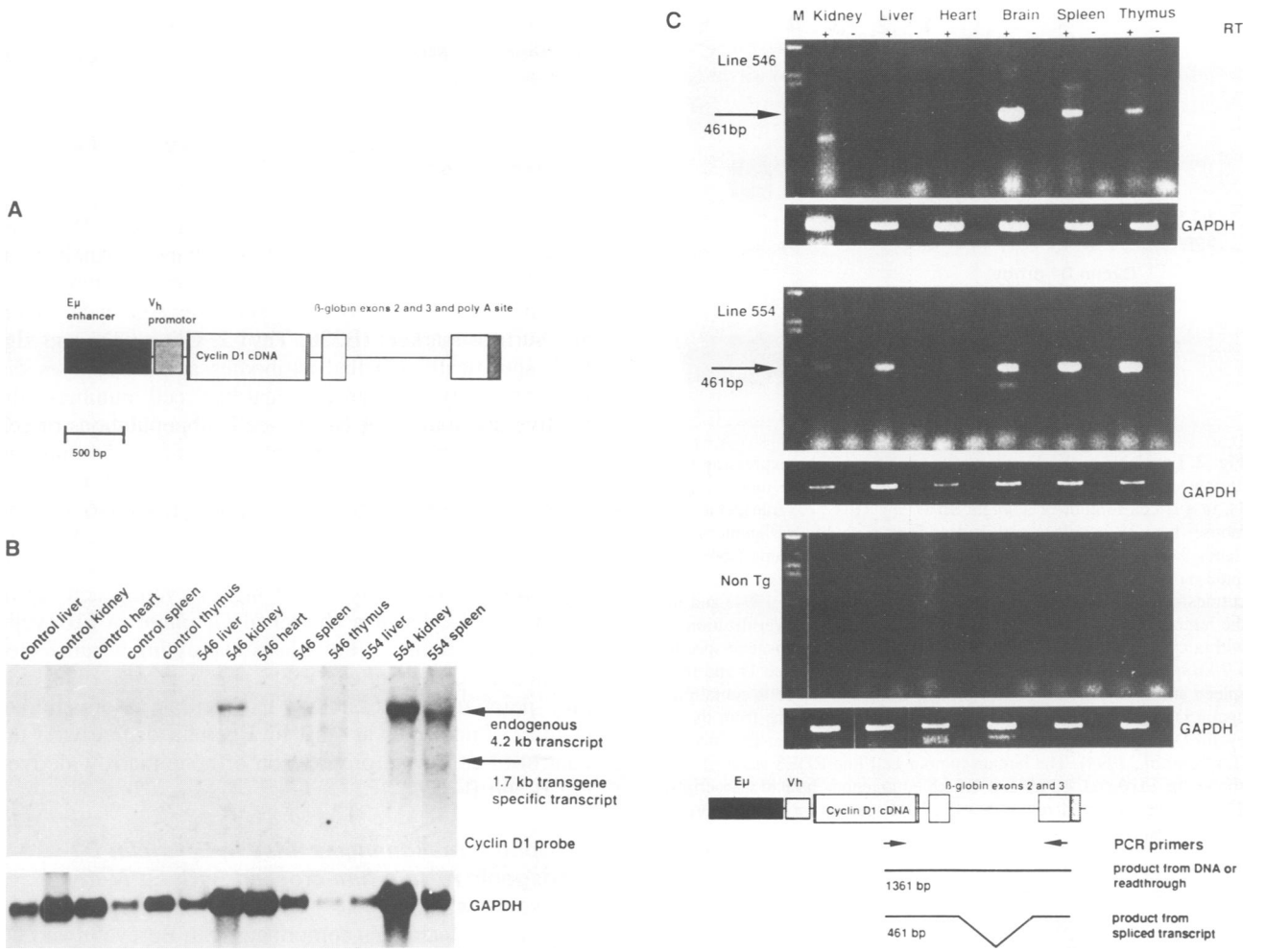

Fig. 1. (**A**) Schematic representation of the construct used to generate Eμ cyclin D1 transgenic mice. (**B**) Northern analysis of cyclin D1 expression in different tissues of control animals and animals of the Eμ cyclin D1 transgenic lines 546 and 554. Approximately 10 μg of total RNA from the indicated sources were separated on a formaldehyde–agarose gel, transferred onto nitrocellulose membranes (Hybond C, Amersham) and hybridized with the 1 kb human cyclin D1 cDNA fragment. Rehybridization with a GAPDH specific probe was performed to control for loading. (**C**) RT-PCR analysis of transgene expression in different tissues from the Eμ cyclin D1 transgenic mouse lines 554 and 546 as well as from a non-transgenic control. To ensure the intactness of first strand cDNA molecules that served as a template for the RT-PCR control experiment with oligonucleotide primers specific for the GADPH gene was performed.

element used here which may contain sites for transcriptional initiation.

To confirm that the transgene produces a properly processed message, expression analysis via RT-PCR with primers that reside in the cyclin D1 cDNA and in the β-globin exon 3 were performed. This allowed specific amplification of transcripts from the transgene and helped to distinguish from amplifications that may arise from unspliced mRNA molecules, from endogenous cyclin D1 transcripts or through contamination of the RNA preparation with genomic DNA. Mice from lines 554 and 546 showed specific expression of the transgene in spleen and thymus (Figure 1C) but also in other tissues. The presence of a 461 bp fragment in the RT-PCR (Figure 1C) clearly indicates that the transgene is expressed as a correctly processed mRNA molecule. Expression was also detected in brain (line 546) and liver and kidney (line 554) indicating again that the Eμ–V_H combination used here is able to target transgene expression to the lymphoid lineage but allows some ectopic expression. However, the level of this ectopic expression is, at least for liver and brain, very low as it was not detectable in the Northern analysis (Figure 1B). Ectopic expression of transgenes has been observed in other experiments with transgenic mice and may depend on the integration site of the transgenic construct (Möröy *et al.*, 1990).

The level of cyclin D1 transgene expression in spleen and thymus of transgenic animals was compared with the level of cyclin D1 expression in fibrosarcomas of nude mice that had received a fibroblast cell line oncogenically transformed with cyclin D1 and Ha-*ras* (Lovec *et al.*, 1994) and the human Ewings sarcoma cell line RDES that abundantly expresses endogenous cyclin D1 specific transcripts (Figure 2). Levels of the transgene specific 1.7 kb mRNA in spleen and thymus are clearly lower than the level of cyclin D1 transcripts in the nude mouse tumour or in the human tumour cell line (Figure 2) indicating that the Eμ–V_H vector used here in the cyclin D1 transgenic construct (Figure 1A) is less efficient in driving high level cyclin D1 expression than the LTR vector used for cyclin D1 in the Ha-*ras* cotransfection assay in fibroblasts (Lovec *et al.*, 1994).

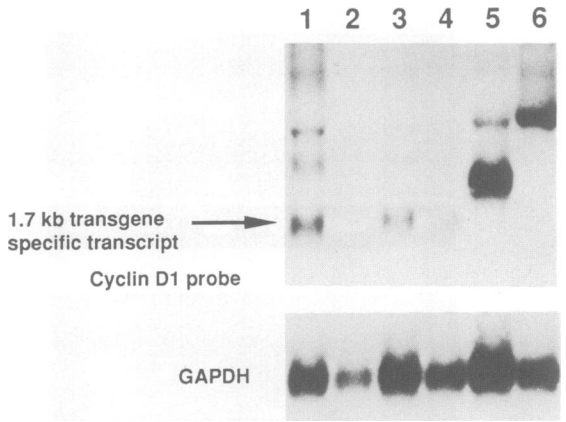

Fig. 2. Levels of cyclin D1 transgene specific mRNA expression in a tumour of a double transgenic Eμ N-*myc*–Eμ cyclin D1 mouse (lane 1), in a B-cell tumour of a single Eμ N-*myc* (EN 177) transgenic mouse (lane 2), in spleen and thymus from a double transgenic mouse (lanes 3 and 4) with early tumorigenesis, in a fibrosarcoma from a nude mouse (lane 5) that had received cyclin D1–Ha-*ras* cotransformed primary rat fibroblast cells (Lovec *et al.*, 1994) and in the human Ewings sarcoma cell line RDES (lane 6). Hybridization with a cyclin D1 specific probe shows the expected transgene specific 1.7 kb transcript (see also Figure 1B) in the tumour (lane 1) and in spleen and thymus (lanes 3 and 4). The fibrosarcoma cells contain a cyclin D1 specific transcript of 2.4 kb (lane 5) that stems from the cyclin D1 expression construct used in the Ha-*ras* cotransfections (Lovec *et al.*, 1994). The human tumour cell line RDES (lane 6) shows the expected 4.0 kb and 1.7 kb endogenous human transcripts. The blot was rehybridized with a GAPDH specific probe to ensure uniform loading.

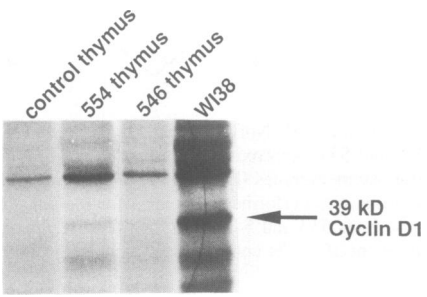

Fig. 3. Level of cyclin D1 protein synthesis in primary thymocytes from transgenic mice, normal mice and in the human fibroblast cell line WI38. Radioimmunoprecipitation assays with anti-cyclin D1 antiserum (Sewing *et al.*, 1993) shows the expected 39 kDa cyclin D1 protein in WI38 fibroblasts (endogenous expression) and in thymocytes of transgenic mice from lines 554 and 546 but not in thymocytes of a non-transgenic control mouse.

To ensure that the expression of the transgene also leads to the synthesis of the correct cyclin D1 protein, thymocytes of Eμ cyclin D1 transgenic mice from lines 546 and 554 were analysed by radio immunoprecipitation experiments. Single cell suspensions from thymi of normal and transgenic mice were prepared and labelled with a [^{35}S]methionine/cysteine mixture (Amersham) *in vivo* for 2 h and lysed in radio immunoprecipitation buffer (RIPA). Cyclin D1 protein was precipitated with an anti-cyclin D1 antiserum (Sewing *et al.*, 1993) and protein A–Sepharose and analysed via gel electrophoresis and fluorography. Thymocytes from Eμ cyclin D1 transgenic mice of lines 546 and 554 and cells from the control cell line WI38, which produces high levels of cyclin D1 (Sewing *et al.*, 1993), allowed the precipitation of the expected 39 kDa cyclin D1 protein (Figure 3). Thymocytes from a non-transgenic control did not show synthesis of cyclin D1 protein (Figure 3).

Phenotypical consequences of deregulated cyclin D1 expression

Histological examination of thymus, spleen, lymph nodes and other non-lymphoid organs from Eμ cyclin D1 transgenic mice did not reveal any disturbances. Analysis of T- and B-cell subsets in thymus and spleen by fluorescence activated cell sorting (FACS) for size, cell cycle status and surface markers (B220, Thy1.2, CD4, CD8 and sIg) with specifically labelled antibodies and DNA dyes did not show any differences regarding cell numbers, the relative distribution of B- or T-cell subpopulations or cell size between transgenic animals from lines 554 and 546 and age matched control mice (not shown). However, in bone marrow of both transgenic mouse lines 546 and 554 the percentage of B220 positive cells in S and G_2/M phase was slightly higher compared with non-transgenic controls (not shown). This may reflect higher cycling activity of pre-B and B-cells or a shortened G_1 phase in Eμ cyclin D1 animals similar to fibroblasts expressing a transfected cyclin D1 (Quelle *et al.*, 1993; Jiang *et al.*, 1993) and may point to the possibility that ectopic expression of cyclin D1 interferes at least to a certain degree with the control of cell cycle progression of bone marrow derived pre-B and B-cells.

Lymphoid malignancies arise in Eμ cyclin D1 transgenic mice when crossed with Eμ N-myc transgenics

A total of 126 animals comprising both Eμ cyclin and D1 transgenic lines 554 and 546 were observed for a period of 12 months without the occurrence of lymphoid malignancies. Thus, we attempted to explore whether any oncogenic potential of cyclin D1 could be unravelled in concert with a cooperating partner. As MYC is well known to play a pivotal role in a variety of B-lymphoid malignancies (for a review see Zimmerman and Alt, 1990) we set out to test its capacity to cooperate with cyclin D1. It has been described before that N-*myc* and L-*myc* can fully replace c-*myc* in the process of malignant transformation but that both have a lower oncogenic potential (Dildrop *et al.*, 1989; Rosenbaum *et al.*, 1989; Möröy *et al.*, 1990). Therefore, we set up crossing experiments with Eμ cyclin D1 animals and Eμ N-*myc* transgenics instead of Eμ c-*myc* transgenic mice to be able to monitor a possible synergistic effect between MYC and cyclin D1 through accelerated tumour formation even after longer time periods. Eμ N-*myc* mice are clearly predisposed to develop B-cell tumours (Dildrop *et al.*, 1989; Rosenbaum *et al.*, 1989; Möröy *et al.*, 1991) and have been derived as several lines with different tumour incidences and latency periods (Dildrop *et al.*, 1989). Two different lines were chosen for the crossing experiments described here; the line 172 that develops tumours very rarely in the C57Bl/6 genetic background although it expresses clearly a functional transgene (Dildrop *et al.*, 1989; Wang *et al.*, 1992) and the line 177 that develops tumours quicker with a mean latency period of 3–4 months (Dildrop *et al.*, 1989).

Thymic and peripheral T-cells of double Eμ cyclin D1–

Table I. Summary and statistical analysis of the crossing experiments between Eμ cyclin D1 and Eμ N- and L-*myc* transgenic mice

Crossing experiments	Transgenic lines	No. of animals in cohort	No. of animals with tumours	Tumour incidence (%)	Average latency period[a] (days)
A	ED 546	32	0	0	–
	EN 172	36	2	6	161 (±2.5)
	ED 546 × EN 172	38	28	74	81 (±6.2)
B	ED 554	15	0	0	–
	EN 177	20	13	65	105 (±7.6)
	ED 554 × EN 177	22	18	82	88 (±5.6)
C	ED 546	79	0	0	–
	EL 27.3	137[b]	11[b]	8[b]	267 (±30.5)[b]
	ED 546 × EL 27.3	18	7	38	135 (±18.5)

See also Figure 4. ED 546 and ED 554 are Eμ cyclin D1 lines, EN 172 and EN 177 are Eμ N-*myc* lines, EL 27.3 is a Eμ L-*myc* line.
[a]The average latency period is given in days with a standard error of the mean in parenthesis.
[b]The data are representative for Eμ L-*myc* transgenics taken from Möröy *et al.* (1990).

Eμ N-*myc* transgenics seemed to be unaffected by the expression of both transgenes, i.e. their numbers, size and cycling behaviour were not changed with regard to non-transgenic control animals from the same litter (not shown). However, pre-B and B-cells from bone marrow and B-cells from spleen were larger in size according to their forward scatter values in the FACS analysis compared with normal controls (not shown) and the relative percentage of bone marrow derived B220-positive cells in S and G_2/M phases appeared to be slightly elevated in double transgenics compared with normal controls as was the case for single Eμ cyclin D1 transgenics (not shown). Interestingly, the coexpression of cyclin D1 and N-MYC provoked a very rapid development of pre-B and B-cell neoplasia in double transgenic animals arising from matings between mice from the Eμ cyclin D1 line 546 and the Eμ N-*myc* line 172 (Table I and Figure 4A). Within an observation period of 30 weeks the death rate in the double transgenic population was dramatically accelerated demonstrating a strong synergistic effect between cyclin D1 and N-MYC (Table I and Figure 4A). The average latency period dropped from 161 days in single Eμ N-*myc* transgenics to 81 days in double transgenics. The incidence rose sharply from 6% in single Eμ N-*myc* animals to 74% in double transgenics (Table I). The efficient cooperation of cyclin D1 and N-MYC was also evident in the crossing experiment with the Eμ N-*myc* line 177 and the Eμ-cyclin D1 line 554 (Table I and Figure 4B). Even in Eμ N-*myc* transgenics of the line 177 where tumours develop quickly without cooperating partners (Dildrop *et al.*, 1989) cyclin D1 was still able to accelerate tumorigenesis. Here, the incidence rose from 65% to 82% and the average latency period was shortened from 105 days to 88 days (Table I). This suggests that cyclin D1 has oncogenic potential but requires a suitable cooperating partner to develop its full efficiency.

Cyclin D1 mediated transformation is confined to the B-cell lineage

To determine the cell type of the tumours that arose in the double transgenic animals the presence of surface markers (B220, Thy1.2), the expression of cell type specific genes (V_{preB}, λ-5) and the rearrangement status of immunoglobulin and T-cell receptor loci were determined. The results demonstrated that all tumours were of either pre-B or B-cell type (Table II) and, in addition, distinct rearrangements of the Ig heavy chain locus indicated in all cases the clonality of the tumours (Table II) very similar to tumours found in single Eμ N-*myc* transgenics (Dildrop *et al.*, 1989). Both transgenes were expressed in all tumour samples analysed although the levels of cyclin D1 transgene expression were somewhat variable between particular samples (Figure 5) the reason for which remains to be determined. The level of cyclin D1 expression in tumours of double transgenic animals appeared to be higher than in spleen and thymus (Figure 2) but did not reach the levels observed in cyclin D1–Ha-*ras* transformed fibroblasts or in the human tumour cell line RDES (Figure 2). No cyclin D1 expression was observed in a B-cell tumour that arose in a Eμ N-*myc* single transgenic animal of line 177 (Figure 2). The expression of the pre-B cell markers V_{preB} and λ-5 was in some cases not concordant with the rearrangement status indicating that occasionally transformed B-cells maintain markers from an earlier differentiation status (Table II and Figure 5). However, all tumours that arose from crossings between Eμ N-*myc* and Eμ cyclin D1 transgenic animals appeared to be confined to the B-lymphoid lineage.

To investigate further whether cyclin D1 would also be able to cooperate with a *myc* gene in the generation of T-cell tumours we set up a crossing experiment with Eμ cyclin D1 mice and Eμ L-*myc* transgenics. Eμ L-*myc* mice express the transgene in both B- and T-cells but highest in thymocytes and predominantly develop T-cell lymphomas with a long latency period of ~7–9 months (average 267 days) thereby providing an excellent tool to monitor cooperating events that lead to accelerated tumorigenesis and shortened latency periods (Möröy *et al.*, 1990, 1991). Indeed, with an average latency period of 135 days double transgenic mice develop tumours much faster than single Eμ L-*myc* mice (267 days) indicating efficient cooperation between cyclin D1 and L-MYC (Table I and Figure 4C). Furthermore, the tumour incidence rose from 8% in single Eμ L-*myc* animals to 38% in double transgenic mice (Table I). Interestingly, these malignancies were exclusively confined to the B-lymphoid lineage similarly to the tumours that arose from crossings with Eμ N-*myc* transgenics (Table II) although Eμ L-*myc* mice show clear pre-malignant changes in their thymi and T-cell areas of the spleen (Möröy *et al.*, 1990). This

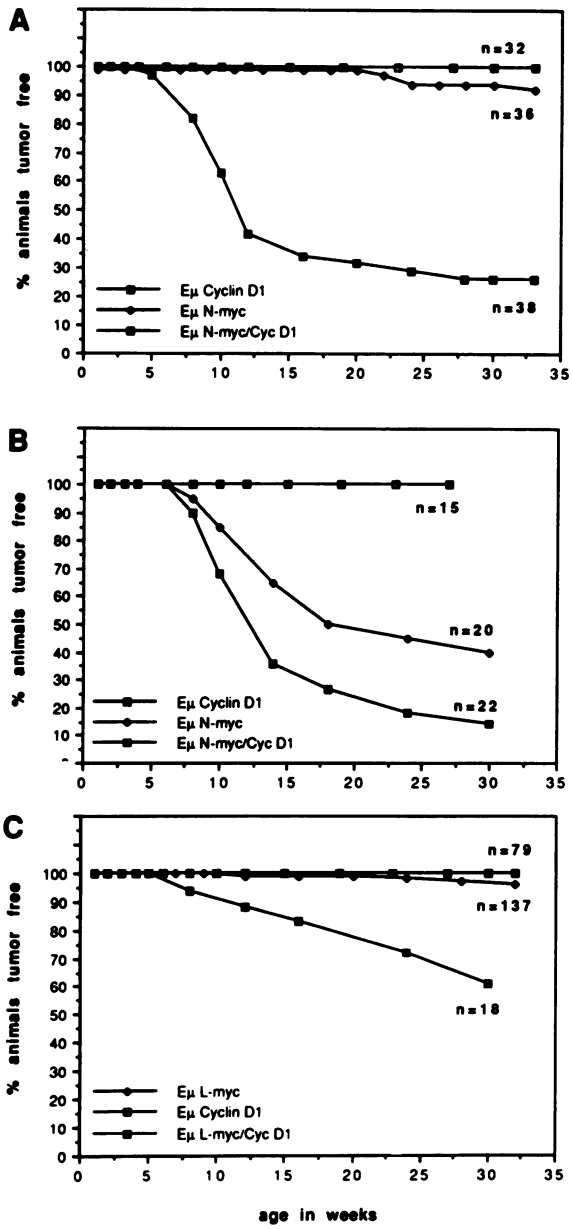

Fig. 4. (**A**) Death rates for double transgenic Eμ N-*myc*–Eμ cyclin D1 animals, using the Eμ N-*myc* line 172 and the Eμ cyclin D1 line 546. The percentage of tumour-free animals is given against the age in weeks. (**B**) Death rates resulting from crossings between the lines 554 (Eμ cyclin D1) and 177 (Eμ N-*myc*). (**C**) Death rates resulting from crossings between Eμ cyclin D1 line 546 and the Eμ L-*myc* line 27.3.

suggests that the oncogenic potential of cyclin D1 depends not only on the cooperating partner but also on the cell lineage and indicates that cyclin D1 may be more efficient at inducing B-cell than T-cell tumours.

Discussion

Transgenic mice have been an invaluable tool in demonstrating the tissue specific transforming activity of oncogenes and have provided insight into the degree of their oncogenic potential and their requirements for cooperating events. Deregulated expression of a given oncogene predisposes transgenic mice to develop a certain type of tumour. In almost all cases these tumours are clonal or oligoclonal clearly indicating the need for additional events in the tumour formation process (reviewed in Adams and Cory, 1991). Examples for the predisposition of transgenic mice to develop malignancies were provided by animals bearing a c-*myc* gene under the transcriptional control of the Eμ immunoglobulin enhancer recapitulating the chromosomal translocation t(8:14) frequently occurring in human Burkitt lymphoma (Adams *et al.*, 1985; Schmidt *et al.*, 1988; Suda *et al.*, 1987). Other members of the *myc* family such as N- and L-*myc* have been shown to be able to replace c-*myc* in its transforming activity and equally predispose transgenic mice to develop lymphoma (Dildrop *et al.*, 1989; Rosenbaum *et al.*, 1989; Möröy *et al.*, 1990). However, Eμ N- or Eμ L-*myc* transgenics develop tumours with a lower incidence and a longer latency period compared with Eμ c-*myc* animals indicating a lower transforming potential of these members of the *myc* family (Dildrop *et al.*, 1989; Rosenbaum *et al.*, 1989; Möröy *et al.*, 1990). The lower transforming potential of Eμ N- and L-*myc* animals makes them ideal tools in crossing experiments with other transgenic mice bearing potential cooperating oncogenes, whereas Eμ c-*myc* transgenic mice would have a very drastic effect rendering double transgenic animals unavailable for further experimentation due to premature death (Möröy *et al.*, 1991; Verbeek *et al.*, 1991).

The data presented here demonstrate that cyclin D1 can act as an oncogene and can induce B-cell lymphoma confirming the notion that the transcriptional activation of the cyclin D1 (*bcl-1*) gene through the t(11:14) translocation indeed plays a key role in the development of human centrocytic B-cell lymphoma. However, it appears that the oncogenic potential of cyclin D1 only comes to light when other pre-lymphomatous changes are manifest. In this case the constitutive expression of cyclin D1 has a dramatic effect on tumour growth when coexpressed with a deregulated *myc* family gene. In contrast to cyclin D1, the deregulated expression of a *myc* gene alone can provoke the outgrowth of tumours in transgenic mice with varying latency periods and incidences very probably because cooperating events i.e. the activation of cellular proto-oncogenes or the loss of tumour suppressor genes occurs with a certain frequency at random in the animals. The question why this is not the case in single transgenic Eμ cyclin D1 animals remains unclear and could simply be due to a weak oncogenic potential of cyclin D1 itself so that randomly occurring cooperating events are not sufficient to provoke tumour development within the life span of a transgenic mouse. Alternatively, it is possible that the expression levels of the cyclin D1 transgene achieved in the experimental setting described here were not high enough for transformation. It could therefore be interesting to test if any activation of *myc* genes occurs in human B-cell neoplasias carrying t(11:14) translocations.

A cell lineage specific requirement of cyclin D1 for cooperating partners could represent a third possibility explaining the inability of cyclin D1 to act alone as a transgene in mice to bring about tumour formation. We and others have previously shown that cyclin D1 alone cannot transform primary embryo fibroblasts from rat but that a full tumorigenic transformation is achieved upon cotransfection with activated Ha-*ras* (Hinds *et al.*, 1994; Lovec *et al.*, 1994) again suggesting that cyclin D1 requires a specific partner to develop its oncogenic activity.

Table II. Characterization of tumours arising from crossings of Eμ cyclin D1 (ED) and Eμ N-*myc* (EN) and Eμ L-*myc* (EL) transgenic lines

Lines	Tumour No.	Igh^R	$Ig\kappa^R$	$TCR\beta^R$	$B220^\alpha$	Thy1.2	Cell type	Localization of tumour	ΔT (days)
ED 546 × EN 172	1319	R	R	G	n.t.	n.t.	B	sp, ln	89
	1320	R	G	G	n.t.	n.t.	pre B	ln	81
	1324	R	R	G	n.t.	n.t.	B	ln	106
	1574	R	G	G	n.t.	n.t.	pre B	ln	77
	1577	R	R	G	+	–	B	ln	111
	1954	R	R	G	+	–	B	ln	80
	1957	R	G	G	+	–	pre B	sp	117
	2266	R	R	G	+	–	B	sp, ln	127
	2267	R	G	G	+	–	pre B	sp, thy	82
	2354	R	G	G	+	–	pre B	ln	55
	2383	R	G	G	+	–	pre B	ln	77
	2384	R	R	G	n.t.	n.t.	B	sp, ln	192
	2395	R	R	G	+	–	B	ln	65
	2732	R	G	G	+	–	pre B	ln	72
	2734	R	R	G	+	–	B	sp	70
	3185	R	G	G	+	–	pre B	ln	57
	3797	R	R	G	n.t.	n.t.	B	sp, ln	83
ED 554 × EN 172	2051	R	R	G	+	–	B	ln	152
	2568	R	R	G	n.t.	n.t.	B	ln	108
ED 554 × EN 177	2274	R	G	G	+	–	pre B	ln	114
	2366	R	R	G	+	–	B	sp	105
	2560	R	G	G	+	–	pre B	ln, thy	86
	2562	R	G	G	+	–	pre B	ln	77
	3486	R	R	G	+	–	B	sp, ln	101
	3498	R	G	G	+	–	pre B	sp, ln	101
	3500	R	R	G	+	–	B	ln	69
	3578	R	G	G	+	–	pre B	sp	73
ED 546 × EL 27.3	2052	R	R	G	+	–	B	ln	107
	2057	R	G	G	+	–	pre B	sp	105
	2576	R	R	G	+	–	B	sp, ln	183
	2997	R	G	G	+	–	pre B	sp, ln	165
	2999	R	G	G	+	–	pre B	ln	60
	3551	R	G	G	+	–	pre B	sp, ln	88

The crossing between Eμ N-*myc* line 172 and the Eμ cyclin D1 554 line also yielded tumours but was not expanded to a sizeable cohort. The rearrangements of the Ig heavy chain locus, the T-cell receptor β locus and the Ig κ locus were determined by Southern blot analysis of DNA directly prepared from tumour tissue using the $J_HE\mu^-$, Jβ2 and the cκ probes, respectively. Preparation of genomic DNA from tumour samples, the blotting procedures and the probes used have been previously described (Möröy *et al.*, 1990, 1991). R, rearranged, G, germline configuration, sp, spleen, ln, lymph node, thy, thymus, n.t., not tested.

[a]Single cell suspension from tumour samples were stained with directly labelled anti-Thy1.2 or anti-B220 antibodies according to standard procedures. The latency period for tumour appearance (ΔT) is given in days.

However, cotransfection of primary fibroblasts with cyclin D1 and c-*myc* did not lead to a transformed phenotype (Lovec *et al.*, 1994), but as shown here, cyclin D1 is clearly able to cooperate with *myc* genes to generate B-cell neoplasia. This suggests that the oncogenic activity of cyclin D1 as well as the efficiency of cyclin D1 to cooperate with other oncogenes depends on the cell type. Such a cell lineage dependence of oncogenic activity and cooperating partners has already been described for other oncogenes including *bcl-2* and c-*abl* (Alexander *et al.*, 1989; Lugo and Witte, 1989; Reed *et al.*, 1990) and could also apply to cyclin D1.

How can the cooperation of cyclin D1 with activated RAS in primary rat cells be reconciled with the cooperation of cyclin D1 with MYC in B-cells observed here? It has recently been shown that cyclin D1 can indirectly stimulate the transcriptional activity of the c-*myc* promoter, which contains an E2F binding site (Oswald *et al.*, 1994). This cyclin D1 dependent transcriptional activation is thought to occur through the liberation of the transcription factor E2F-1 from an inhibitory complex between E2F-1 and the retinoblastoma suppressor protein pRB (Nevins, 1992; Flemington *et al.*, 1993; Oswald *et al.*, 1994) because cyclin D1 is suspected to disrupt protein complexes between pRB and E2F possibly via pRB hyperphosphorylation through cyclin dependent kinases (Sherr, 1994). Thus, it is possible that the cooperation of cyclin D1 with activated RAS in fibroblasts occurs through the E2F-1 dependent transcriptional activation of the c-*myc* gene. In contrast, high level expression of MYC down-regulates cyclin D1 expression significantly (Jansen-Dürr *et al.*, 1993; Lovec *et al.*, 1994). Thus, constitutive expression of both cyclin D1 and a *myc* gene as in the transgenic animals described here would ensure action of MYC and cyclin D1 at the same time leading then to the transcriptional activation of other E2F dependent genes through

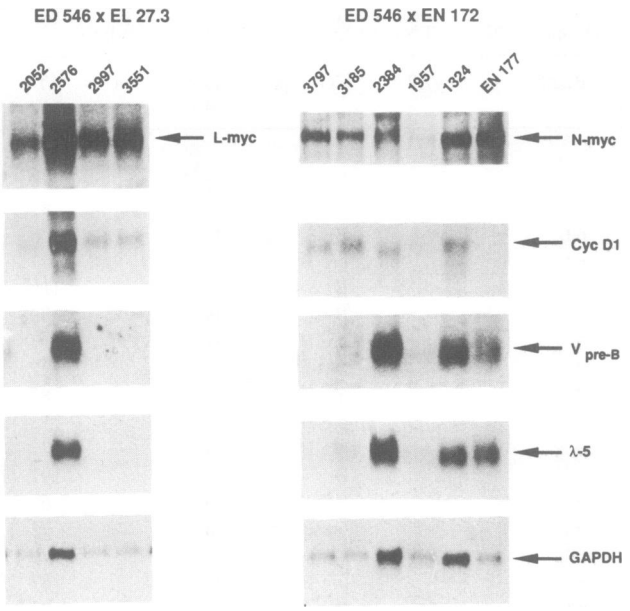

Fig. 5. Expression of the transgenes and specific markers in tumours arising from the different crossings. 10 μg of total RNA from the indicated tumours were analysed by Northern blotting using the indicated probes. EN 177 represents a B-cell tumour that arose in a single transgenic Eμ N-*myc* mouse. Expression of V_{preB} and λ-5 was not detected in the samples 2052, 2997, 3551, 3797 and 1957 even after prolonged exposure.

cyclin D1 mediated liberation of E2F-1, which would be able to cooperate with MYC in tumorigenesis.

Recently a number of inhibitors of cyclin dependent kinases have been discovered (Hunter, 1993) that are able to block with G_1/S transition probably by binding to cyclin–cdk complexes. It is possible that constitutive overexpression of cyclin D1 compromises these inhibitors or their activity and thereby allows unscheduled G_1/S progression as a prerequisite of malignant transformation.

As the data presented here clearly establish cyclin D1 as an oncogene *in vivo* and demonstrate its potential as a key factor in the development of B-cell neoplasia and as a cooperating partner for *myc* oncogenes, both models could provide a first hypothetical basis to understand how the derailment of the regulation of cell cycle progression is involved in the multistep process of neoplastic transformation.

Materials and methods

Constructs and transgenic mice

A schematic representation of the human cyclin D1 cDNA (Lovec *et al.*, 1994) and the construct used to generate Eμ cyclin D1 transgenic mice are given in Figure 1A. The construct was obtained by inserting a 1 kb *Eco*RI–*Xba*I fragment containing the Eμ enhancer and the promotor of a V_H variable gene segment (Möröy *et al.*, 1990) directly 5′ to the cyclin D1 cDNA. At the 3′ end the second and third exon of the human β-globin gene was inserted, which also provided a polyadenylation site (Pirchner *et al.*, 1989). The construct was freed from vector sequences, purified on agarose gels and injected into fertilized mouse oocytes essentially as described (Hogan *et al.*, 1986). The fertilized mouse oocytes were derived from matings between (C57Bl/6 × C3H) F1 animals. Successful integration of the injected DNA was monitored by Southern analysis of tail tip DNA as described (Hogan *et al.*, 1986). All transgenic mouse lines were maintained by breeding the obtained founders for three or more generations with inbred C57Bl/6 animals.

Molecular analyses and probes

Preparation of genomic DNA from tail tips, tumour samples and cell lines was performed as previously described (Hogan *et al.*, 1986). DNA blotting procedures were performed as described elsewhere (Reed and Mann, 1985; Yancopoulos *et al.*, 1984, 1985; Sambrook *et al.*, 1989). The J_H probe used for Southern analysis is a 2 kb fragment covering J_H1–4 (Perry *et al.*, 1980; Gough and Bernhard, 1981), the $J_HE\mu^-$ probe is a *Xba*I–*Eco*RI fragment covering J_H3–4, the Jβ2 probe that was used to detect TCRβ rearrangements is a 1.9 kb *Hin*dIII–*Bam*HI fragment containing the Jβ locus (Malissen *et al.*, 1984). The cκ probe is described in Lewis *et al.* (1982). Preparation of RNA was as described (Auffray and Rougeon, 1980) and Northern blotting was performed according to Sambrook *et al.* (1989). The probes for N-*myc*, L-*myc*, GADPH and cyclin D1 have already been described (Yancopoulos *et al.*, 1985; Dildrop *et al.*, 1989; Möröy *et al.*, 1990, 1991; Lovec *et al.*, 1994). The probes for λ-5 and V_{preB} were 0.5 and 1 kb fragments as described (Ma *et al.*, 1992).

Antibody staining procedures

Single cell suspensions were made at the time of autopsy from spleen, thymus, bone marrow or tumour tissue in PBS supplemented with 0.5% BSA (staining solution). Cells were washed in this solution and were incubated on ice for 30 min either with directly FITC conjugated antibodies or with biotin labelled antibodies. Cells were washed twice in staining solution after the incubation and if necessary counterstained with FITC or phycoerythrin labelled streptavidin and examined under a fluorescent microscope or a FACS analyser (Beckton-Dickinson). Cell cycle analyses were done with cells fixed overnight in 70% ethanol. These cells were treated with RNase A and stained with propidium iodide as described (Möröy *et al.*, 1990).

Immunoprecipitation

For radio immunoprecipitation assays (RIPA), cells from the cell line WI38 or from thymi of normal or Eμ cyclin D1 transgenic mice were seeded onto 5 cm^2 dishes in 1 ml of cysteine/methionine-free RPMI medium containing 20% FCS 30 min prior to labelling. Cells were labelled with 250 μCi of a [^{35}S]methionine/cysteine mix (Amersham) for 2 h, collected by centrifugation, resuspended in 100 μl PBS and lysed for 5 min in 1 ml RIPA buffer (Sewing *et al.*, 1993). The lysate was passed six times through a 25 gauge needle to shear DNA and spun for 15 min at 45 000 r.p.m. The supernatant was then incubated with 1/100 vol of anti-cyclin D1 antiserum (Sewing *et al.*, 1993) for 2 h at 4°C. The samples were then incubated with 30 μl of protein A–Sepharose CL-4B 8 (Pharmacia) that had been previously equilibrated with RIPA buffer. Immune complexes were centrifuged, washed four times in RIPA buffer containing 500 mM NaCl, resuspended, boiled in SDS sample buffer and analysed on discontinuous 12.5% SDS–polyacrylamide gels by fluorography.

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