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The v-*rel* oncogene promotes malignant T-cell leukemia/lymphoma in transgenic mice

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The oncogene product from the avian reticuloendotheliosis virus strain T, v-Rel, is a member of the Rel/NF-κB family of transcription factors. The mechanism by which v-Rel induces oncogenic transformation remains unclear. Several attempts to transform mammalian cells with v-Rel have failed, suggesting that v-Rel transformation may be a species-specific event. However, here we demonstrate that v-Rel, but not a truncated c-Rel, expressed under the control of the *lck* promoter, efficiently induced malignancies in transgenic mice. Most of the animals died before 10 months of age and developed immature, multicentric aggressive T-cell leukemia/lymphomas. Most tumors contain $CD4^+CD8^+$ cells or $CD4^-CD8^+$ cells, which have an immature rather than a mature peripheral phenotype. No tumor development was observed in control littermates and transgenic mice expressing a truncated form of c-Rel. Tumor formation was correlated with the presence of constitutive p50/v-Rel DNA binding activity and overexpression of several κB-regulated genes in v-*rel* transgenic thymocytes. However, v-Rel is also transforming in transgenic thymocytes lacking p50, indicating that p50/v-Rel heterodimer formation is not essential for the transforming activity of v-Rel. The transforming activity of v-Rel in p50 null mice has been identified as v-Rel/v-Rel homodimers. Since tumors represent immature T-lymphocytes, constitutive v-Rel expression appears to be leukemogenic at earlier stages of T-cell development. These v-Rel mice should aid in the study of lymphoma development, T-cell development and NF-κB regulation.

Keywords: lymphoma/transgenic/v-*rel*

Introduction

The Rel/NF-κB family of transcription factors represents a group of homo- and heterodimeric complexes that plays an important role in the expression of cellular genes involved in immune and inflammatory responses. In addition, these proteins bind to some viral regulatory sequences, including the enhancers of human immunodeficiency virus (HIV) and cytomegalovirus (for review see Liou and Baltimore, 1993; Baeuerle and Henkel, 1994; Siebenlist *et al.*, 1994). Rel/NF-κB proteins are related through a common N-terminal domain known as the Rel homology domain (RHD), which consists of ~300 amino acids and contains sequences important for dimerization, DNA binding and nuclear localization. The C-terminal halves of Rel proteins have little sequence similarity, but have been used to distinguish two classes of Rel proteins. One class includes NF-κB1 (p105/p50) and NF-κB2 (p100/p52), which by proteolytic processing generates the mature DNA binding subunits p50 and p52, respectively. The second class of Rel proteins, which in vertebrates includes c-Rel, RelA and RelB, does not undergo proteolytic processing and does harbor transcriptional activation domains in the C-terminal half (reviewed by Liou and Baltimore, 1993). The Rel/NF-κB members are differentially expressed during mouse embryonic development and in adult lymphoid tissues (Carrasco *et al.*, 1993, 1994; Weih *et al.*, 1994), and recent studies using knockout mice have demonstrated that p50, RelA, RelB and c-Rel have distinct biological functions (Beg *et al.*, 1995; Burkly *et al.*, 1995; Konteng *et al.*, 1995; Sha *et al.*, 1995; Weih *et al.*, 1995a).

The different NF-κB activities are modulated by interactions with the IκB family of inhibitory proteins, which contain multiple copies of ankyrin repeats and includes IκBα (Haskill *et al.*, 1991), IκBβ (Thompson *et al.*, 1995), IκBγ (Hatada *et al.*, 1992; Liou *et al.*, 1992) and Bcl-3 (Ohno *et al.*, 1990; Bours *et al.*, 1993; Franzoso *et al.*, 1993). The current model suggests that in unstimulated cells, NF-κB dimers remain in the cytoplasm as inactive complexes through association with IκB molecules that mask their nuclear localization signals. Upon cell stimulation, IκB molecules are rapidly phosphorylated and degraded, allowing the NF-κB dimer to translocate into the nucleus and regulate transcription through binding to κB sites (reviewed by Finco and Baldwin, 1995; Verma *et al.*, 1995). The precursors p105 and p100 show significant homology to the IκB molecules in their C-terminal region, and it has been suggested that they also trap NF-κB complexes in the cytoplasm (Inoue *et al.*, 1992; Mercurio *et al.*, 1992; Naumann *et al.*, 1993; Scheiman *et al.*, 1993; Sun *et al.*, 1994; Dobrzanski *et al.*, 1995).

Analogous to various other transcription factors, several NF-κB gene products are also believed to be involved in tumorigenesis. For example, amplification and rearrangement of the c-*rel* gene has been found in human B-cell lymphomas (Lu *et al.*, 1991); the Bcl-3 gene has been implicated in the development of chronic lymphocytic leukemia (Ohno *et al.*, 1990); altered expression of p50 has been observed in non-small cell lung carcinoma (Mukhopadhyay *et al.*, 1995), and chromosomal translocation of the NF-κB2 gene has been described in human lymphomas (Neri *et al.*, 1991; Fracchiolla *et al.*, 1993; Zhang *et al.*, 1994; Chang *et al.*, 1995). The mechanism by which various structural alterations of NF-κB genes contribute to tumorigenesis is not presently understood.

The oncogene v-*rel* was originally identified as the

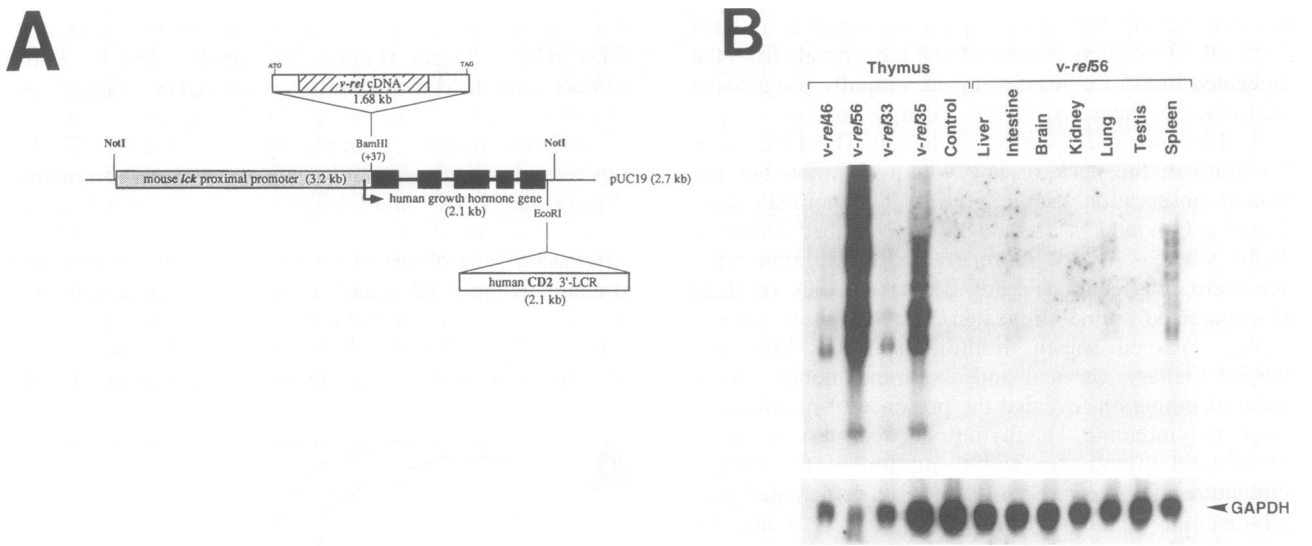

Fig. 1. Expression of the v-*rel* transgene. (**A**) Construct used for generating v-*rel*-tissue-specific transgenic mice. The complete v-*rel* (1.68 kb) was placed under control of the mouse *lck* proximal promoter (3.2 kb); the human growth hormone gene sequences (2.1 kb) were added to confer stability to the transcripts and the 3′ flanking sequences (3′-LCR) of the human CD2 gene (2.0 kb) were added for copy-dependent position-independent expression of the transgene. (**B**) Northern blot analysis of total RNA from thymus of control animals and different transgenic lines, and from different tissues from the transgenic line 56. Blots were hybridized with probes specific for v-*rel* and GAPDH.

transforming component of the avian retrovirus reticuloendotheliosis virus-T (Theilen *et al.*, 1966). An extensively altered form of avian c-Rel, the oncogenic v-Rel, efficiently induces a variety of neoplastic diseases in chicken. The mechanism by which v-Rel induces oncogenic transformation is unknown. The finding that v-Rel and the corresponding proto-oncogene c-Rel are members of the Rel/NF-κB transcription factor family led to the suggestion that transformation resulted from v-Rel-induced changes in normal transcriptional activity, perhaps by v-Rel acting as a dominant negative mutant of c-Rel (Bose, 1992; Gilmore, 1992; Kabrun and Enrietto, 1994). The major difference between c-Rel and v-Rel involves a truncation of the carboxy-terminus of c-Rel that eliminates both a large part of the transactivation domain and a cytoplasmic anchoring sequence (Capobianco *et al.*, 1990; Kamens *et al.*, 1990; Richardson and Gilmore, 1991; Hrdlickova *et al.*, 1994a). Furthermore, v-Rel differs from c-Rel by additional 14 single-amino-acid changes and three small in-frame deletions (Wilhelmsen *et al.*, 1984). The majority of these small mutations contribute to the transformation potential of v-Rel (Bhat and Temin, 1990). Mutations in the carboxy-distal end of the RHD appear to be responsible for decreased inhibition by IκBα of v-Rel DNA binding (Diehl *et al.*, 1993). It was believed that v-Rel could only transform avian cells, since v-Rel, c-Rel and chimeric proteins failed to cause transformation of mammalian cells (Bose, 1992; Gilmore, 1992; Kabrun and Enrietto, 1994).

To further understand the molecular mechanisms involved in v-Rel transformation, we have generated transgenic mice expressing v-Rel in thymocytes. Here, we show that v-Rel is able to transform mammalian cells and, although young animals appear normal, adult transgenic mice develop aggressive T-cell lymphoma/leukemia. v-Rel tumor induction was correlated with a constitutive p50/v-Rel DNA binding activity. However, by generating v-*rel* transgenic mice in a p50 null background, we have demonstrated that p50 is not necessary for v-Rel transformation. The use of transgenic mice provides a unique *in vivo* approach to study the molecular mechanisms involved in v-Rel transformation in a specific cell type.

Results

Generation of transgenic mice expressing v-Rel in the thymus

To study the effect of v-Rel expression in mammalian T-lymphoid cells, we have generated a transgenic mouse model that places v-*rel* under the control of the mouse *lck* proximal promoter and the human CD2 3′-locus control region (Figure 1A). These regulatory sequences confer copy number-dependent and integration site-independent expression of the transgene to all thymocyte subsets beginning at the earliest stages of T-cell development (Allen *et al.*, 1992; Weih *et al.*, 1995b). Several independent transgenic mouse lines were generated, and tissue expression of the v-*rel* transgene was determined by Northern blot analysis (Figure 1B). All the lines expressed the transgene in the thymus and spleen (Figure 1B and data not shown). Lines 35 and 56 presented the highest levels of transgene expression. v-Rel corresponds to a mutated version of c-Rel in which, besides other mutations, the last 118 carboxy-terminal amino acids are deleted (Wilhelmsen *et al.*, 1984). In order to address the function of this deletion in the transforming ability of v-Rel, we also generated transgenic mice expressing a mouse c-Rel protein that lacked 117 carboxy-terminal amino acids (Δc-Rel). Several transgenic lines were generated and high levels of Δc-Rel protein were detected in the thymus by Western blot analysis (data not shown).

Constitutive v-Rel expression induces T-cell lymphoma in transgenic mice

Young transgenic mice, between 3 and 8 weeks old, appeared normal as assessed by habit, weight, posture and histologic and flow cytometric analysis of lymphoid cells

(data not shown). However, at between 4 and 10 months of age, all 10 founders generated and the animals from the propagated lines (35, 56) developed a rapidly progressive lethal disease characterized by lethargy, loss of weight, hunched posture and distended abdomen. The appearance of a lump in the neck or jaw was a common but not constant observation. Some 50% of the animals were affected at 6 months of age and 95% succumbed before 10 months, whereas control littermates and Δc-Rel transgenic mice were unaffected (Figure 2A). Autopsies of dead and euthanized animals revealed, in most cases, thymic atrophy, enlarged spleen, infiltration of the liver and enlarged axillary, cervical and abdominal nodes. Histological examination revealed the presence of multicentric malignant lymphoma. The thymus showed massive reduction of total thymocytes and disruption of the normal architecture of the organ with a poorly demarcated cortical–medullary junction (Figure 2B, panels a and b). Splenic involvement ranged from focal areas of tumor, in minimally enlarged spleens, to total replacement of the normal splenic architecture in massively enlarged spleens (Figure 2B, panels c and d). Lymph node involvement was similar, as a massive infiltration of a monotonous population of cells and disruption of the normal structure of the organ was observed (Figure 2B, panels e and f). Extensive infiltration with tumor cells was also observed in bone marrow (Figure 2B, panels g and h), liver (Figure 2B, panel i) and lung (Figure 2B, panel j). In bone marrow, a reduction of immature and mature forms of hematopoietic cell lineages such as granulocytes, red cells and megakaryocytes was also observed in severely diseased animals (Figure 2B, panels g and h and data not shown).

Lymphoblasts appeared in the peripheral blood of some but not all sick animals (Figure 3A, panel a) and often only for a short period of time during the entire evolution of the disease. Histologically, the tumors were composed of a heterogeneous population of intermediate and large-sized mononuclear cells with scant cytoplasm, round nuclei and open chromatin (Figure 3A, panels a and b). The histologic appearance of these tumors resembles that seen in T-ALL (Crist *et al.*, 1991), where the patients present leukemic blood profiles, and that seen in lymphoblastic lymphoma, where individuals usually present a mediastinal mass that disseminates to the bone marrow and peripheral blood (Noltenius, 1988).

Flow cytometric analysis of tumor-bearing spleens showed a paucity of B-cells with an expanded T-cell population and the presence of large numbers of immature $CD4^+/CD8^+$ T-cells (Figure 3B, panels a and b). Some tumors contained cells with a $CD4^-/CD8^+$ phenotype, rendering a continuous range of CD4 expression on cells of different tumors. Remarkably, most T-cells (Thy1.2 positive) co-expressed markers associated with immature thymocytes: HSA^{hi} and $CD3^{low}$ (Figure 3B, panels c and d and data not shown). Intermediate and low levels of expression were observed for T-cell receptor (TCR) α and β chains (Figure 3B, panels e and f). A graded reduction in the B-cell compartment was also detected in spleen tumors (Figure 3A, panels b and c and B, panels g and h). This may be due to a dilutional effect caused by the

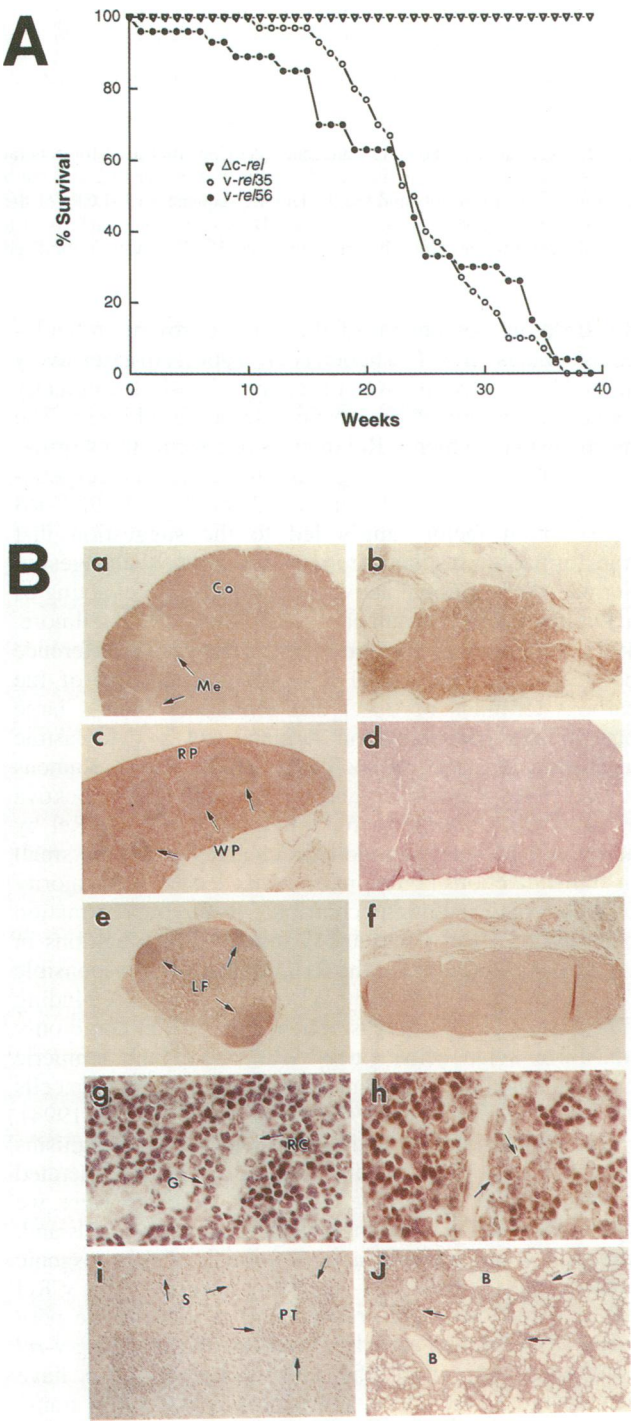

Fig. 2. (**A**) Mortality curve. Two lines of v-*rel* and one Δc-*rel* transgenic mice were used. Δc-*rel* transgenic mice behaved similarly to normal littermates during the entire period of observation. Deaths in transgenic animals were the result of malignant lymphoma/leukemia confirmed by autopsy or histologic examination. $n = 29$ for v-*rel*35; $n = 28$ for v-*rel*56; $n = 15$ for Δc-*rel*; and $n = 30$ for non-transgenic littermate controls. (**B**) Tumor pathology in v-*rel* transgenic mice. Tissue blocks were fixed in 10% neutral buffered formalin and processed for paraffin embedding. 10 μm sections were stained with hematoxylin and eosin. Sections from control (panels a, c, e and g) and tumor tissues (panels b, d, f, h, i and j) are shown. Thymus (a and b); spleen (c and d); lymph node (e and f); bone marrow (g and h); liver (i) and lung (j). B, bronchiole; Co, cortex; G, granulocyte; LF, lymphatic follicle; Me, medulla; RC, red cells; RP, red pulp; PT, portal triad; S, sinusoids; WP, white pulp. Photomicrographs were taken at ×12.5 (c, d and f); ×25 (a, b and e); ×50 (i and j) and ×250 (g and h) magnifications. Arrows indicate normal structures and sites of cellular infiltration in transgenic animals.

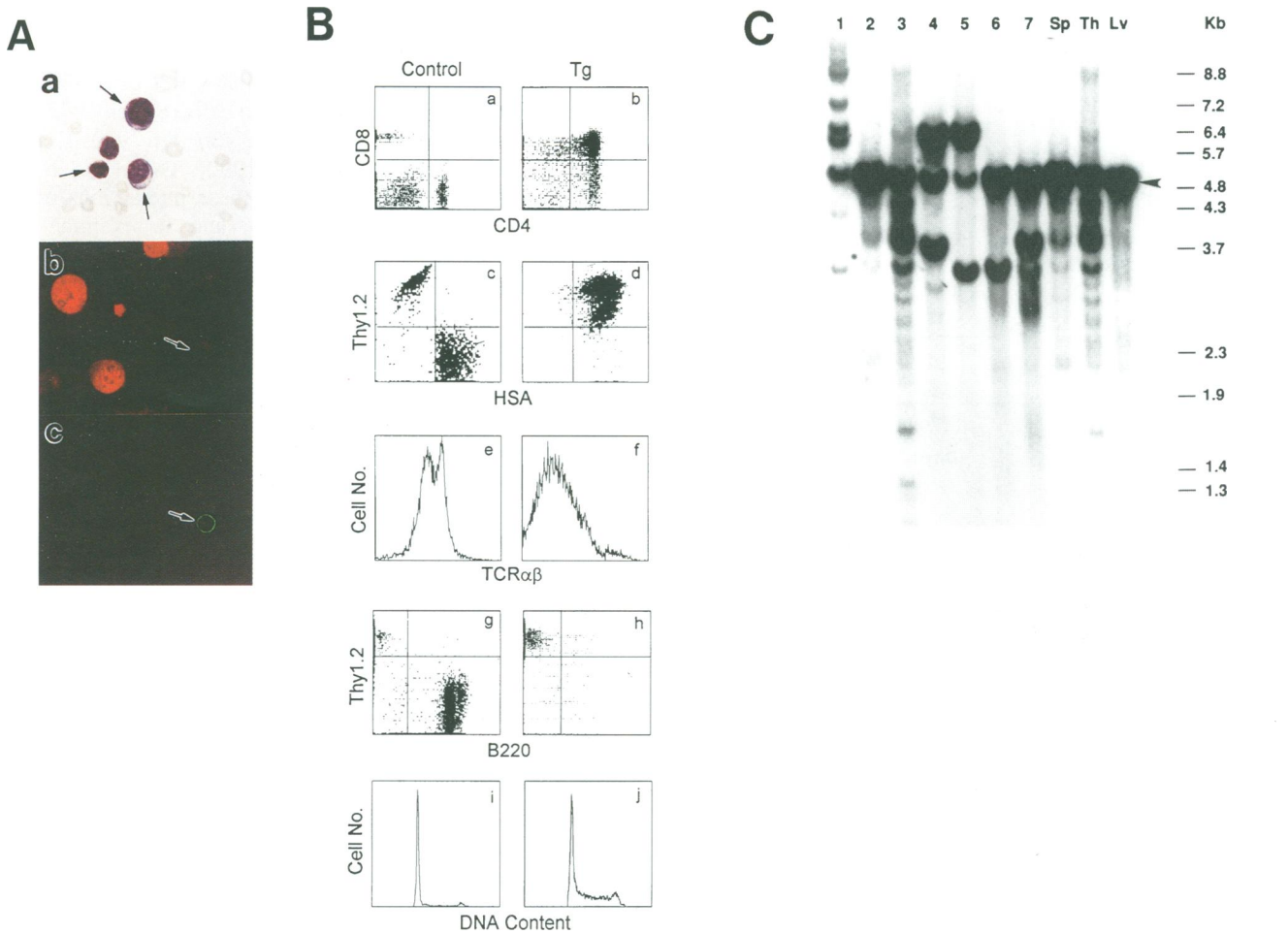

Fig. 3. Phenotype and genotype of v-*rel*-transformed T-cells. (**A**) a, Wright's stain of peripheral blood smear; the presence of T-cell blastic forms is observed (×250 magnification); b and c, immunofluorescence of v-*rel*-transformed T-cells; splenic tumor cells were spun onto slides and incubated with a mixture of antibodies against v-Rel and CD45R/B220 (clone RA3-6B2 from Gibco–BRL). Anti-v-Rel antibody was visualized with donkey anti-rabbit IgG-Texas red (Amersham) and anti-CD45R antibody with goat anti-rat IgG-FITC (Boehringer). (**B**) Flow cytometric analysis of lymphoid cell surface markers. Flow cytometry was performed using a Coulter Epics Profile II flow cytometer and cell sorter. Splenocytes from control and tumor spleens were prepared and stained as previously described in Materials and methods. (**C**) Analysis of T-cell receptor gene rearrangements in tumors. DNA prepared from tumors (lanes 1, 2, 4, 5, 6 and 7), 3-week-old transgenic mouse thymus (lane 3) and control spleen (Sp), thymus (Th) and liver (Lv) were digested with *Hin*dIII, and analyzed by Southern blot hybridization. The position of the germline band is indicated by an arrow.

large number of immature thymocytes and/or a secondary effect of bone marrow failure from infiltrating tumor cells (Figure 2B, panels g and h). The large size of transformed thymocytes is suggestive of increased cell proliferation. To determine whether more of these cells were progressing through the cell cycle than their normal counterparts, their DNA content was determined by flow cytometry using propidium iodide. Tumor cells from transgenic mice showed an increased proportion of cells in S phase (Figure 3B, panels i and j).

The clonality of some of the v-Rel tumors was examined by Southern hybridization with TCR-β probes, since in almost all T-cells this locus is rearranged on both alleles in a cell-specific manner. DNA was made from spleen tumors and hybridized with a DNA probe specific for TCR β chain gene joining region Jβ1. As expected, spleens and thymuses from control and young transgenic mice showed a polyclonal pattern of rearranged bands, while a discrete set of specific rearranged bands was evident in all tumor samples analyzed, indicating that the tumor cells are oligoclonal in origin (Figure 3C).

v-Rel tumor cells grew in soft agar, but attempts to serially transplant them (intraperitoneally) into syngeneic recipients was unsuccessful. These data indicate the presence of neoplastic cells with low malignant potential.

v-Rel-transformed T-cells are interleukin-2 dependent

Spleen tumor cells cultured in medium enriched with cytokines presented a sustained but slow growth rate. In contrast, control spleen cells rapidly decreased in number (Figure 4A and B, panels a and b). The slow growth rate of tumor cells is consistent with only a subpopulation of the cells being in the S phase of the cell cycle (Figure 3B, panels i and j). When cytokines were removed from the medium, tumor cells stopped growing and their viability was dramatically impaired (Figure 4A, insert). Assessment of cell viability with trypan blue staining showed a significantly increased proportion of dead or dying tumor cells in the absence of cytokines (not shown). This result shows that the survival of v-Rel-transformed T-cells is cytokine-dependent and prompted us to investi-

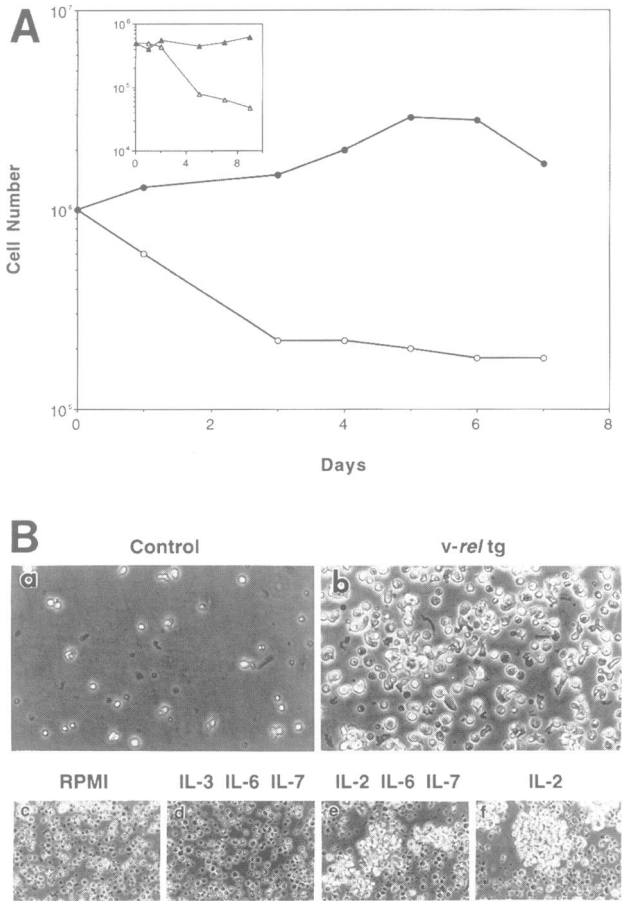

Fig. 4. Cell growth of v-Rel-transformed T cells. (**A**) Low, but sustained growth rates of v-Rel-transformed T-cells. 10^6 tumor spleen cells (●) or control thymocytes (○) were incubated during a period of 7 days in liquid media supplemented with 10% FCS and containing a combination of IL-2, IL-3, IL-6 and IL-7, and the number of viable cells was determined every 24 h. Insert: 10^6 tumor cells were incubated during a period of 7 days in the absence (△) or presence (▲) of cytokines and viable cells were counted every 24 h. (**B**) Cell growth of v-Rel-transformed T-cells is IL-2-dependent. 10^6 control splenocytes (a) or spleen tumor cells (b) were grown in medium supplemented with 10% FCS containing cytokines for 7 days; cells were then disaggregated for cell count and photography. 10^5 tumor spleen cells were grown for 7 days in medium supplemented with 10% FCS in the absence of cytokines (c), or in the presence of IL-3, IL-6 and IL-7 (d), IL-2, IL-6 and IL-7 (e), or IL-2 (f).

gate whether a specific factor was required. When the growth of v-Rel-transformed T-cells was tested in media containing different combinations of cytokines, a selective requirement for interleukin-2 (IL-2) was observed (Figure 4B, panels c–f). In the absence of cytokines (Figure 4B, panel c) or in the presence of a combination of IL-3, IL-6 and IL-7 (Figure 4B, panel d), no cellular growth was observed. However, in medium supplemented with a combination of IL-2, IL-6 and IL-7 (Figure 4B, panel e) or IL-2 alone (Figure 4B, panel f), slow, but continuous growth rates were observed. In the presence of IL-2, the cells grew in clumps, but in its absence, the cell clumps disaggregated and lost their ability to proliferate. These results indicate that v-Rel-transformed T cells have a selective requirement for IL-2 and are in agreement with the observation that IL-2 is expressed at very low levels in transgenic thymocytes (Figure 5D).

v-Rel associates with other Rel/NFκB proteins in transgenic thymocytes

Several reports have described that v-Rel can associate with other NF-κB proteins in avian cells (Simek and Rice, 1988; Morrison *et al.*, 1989; Davis *et al.*, 1990; Kochel *et al.*, 1991; Capobianco *et al.*, 1992). Therefore, it was of interest to determine whether these interactions occur in v-Rel transgenic thymocytes, and to examine the relevance of these associations with the transforming capability of v-Rel. Isolated thymocytes from transgenic mice were labeled for 3 h in medium containing [^{35}S]-methionine, lysed under non-denaturing conditions and total protein extracts were immunoprecipitated with v-Rel or p50 antisera (Figure 5A). In the left panel, total protein extract from transgenic thymocytes were immunoprecipitated with anti-vRel-specific antibodies. The immunoprecipitate was denatured to dissociate the v-Rel-containing immune complexes and then reprecipitated, first with p50 and subsequently with IκBα antisera. As shown in Figure 5A (left panel), v-Rel immunoprecipitates contained a significant amount of p50 and IκBα, demonstrating the existence of p50/v-Rel/IκBα complexes in v-Rel transgenic thymocytes. In the right panel the v-Rel immunoprecipitate was denatured and then reprecipitated with v-Rel antiserum to determine the total amount of v-Rel, while that from anti-p50 was denatured and then sequentially reprecipitated with v-Rel, IκBα, RelA, p52, RelB and p50 antibodies. Interestingly, v-Rel and p50 immunoprecipitates contained a comparable amount of v-Rel protein, indicating that most v-Rel is complexed with p50. These findings suggest that this association may be important for the transforming activity of v-Rel in mouse T-cells. The other protein associated with the p50/v-Rel complex, at a significant amount, is the inhibitory protein IκBα (Figure 5A and data not shown). A minor fraction of v-Rel is associated with RelA and p100, and no association was detected with RelB or p52 (data not shown). Since the v-Rel antibody cross-reacts with the mouse c-Rel protein (data not shown), the absence of c-Rel protein in the immunoprecipitate indicates that c-Rel is absent in thymocytes and confirms our previous observations (Carrasco *et al.*, 1994).

v-Rel localizes in the nucleus of transgenic thymocytes in spite of increased cytoplasmic levels of IκBα and p105

The transcriptional activity of the Rel/NF-κB family of proteins is regulated, in part, by their association with inhibitory molecules (IκBα, IκBβ, p100 and p105) which sequester them as inactive complexes in the cytoplasm (Nolan and Baltimore, 1992; Beg and Baldwin, 1993; Gilmore and Morin, 1993). It has been suggested that v-Rel transforms cells through its association with IκBα in the cytoplasm, allowing the inappropriate translocation of other Rel/NF-κB proteins to the nucleus (Bose, 1992; Gilmore, 1992; Kabrun and Enrietto, 1994; Hrdlickova *et al.*, 1995). Therefore, it was of interest to determine the nuclear/cytoplasmic distribution of v-Rel and the endogenous Rel/NF-κB proteins by Western blot analysis in control and transgenic thymocytes (Figure 5B). In order to rule out possible cross-contamination during the subcellular fractionation procedure, we used the cytoplasmic enzyme lactate dehydrogenase (LDH) as a control.

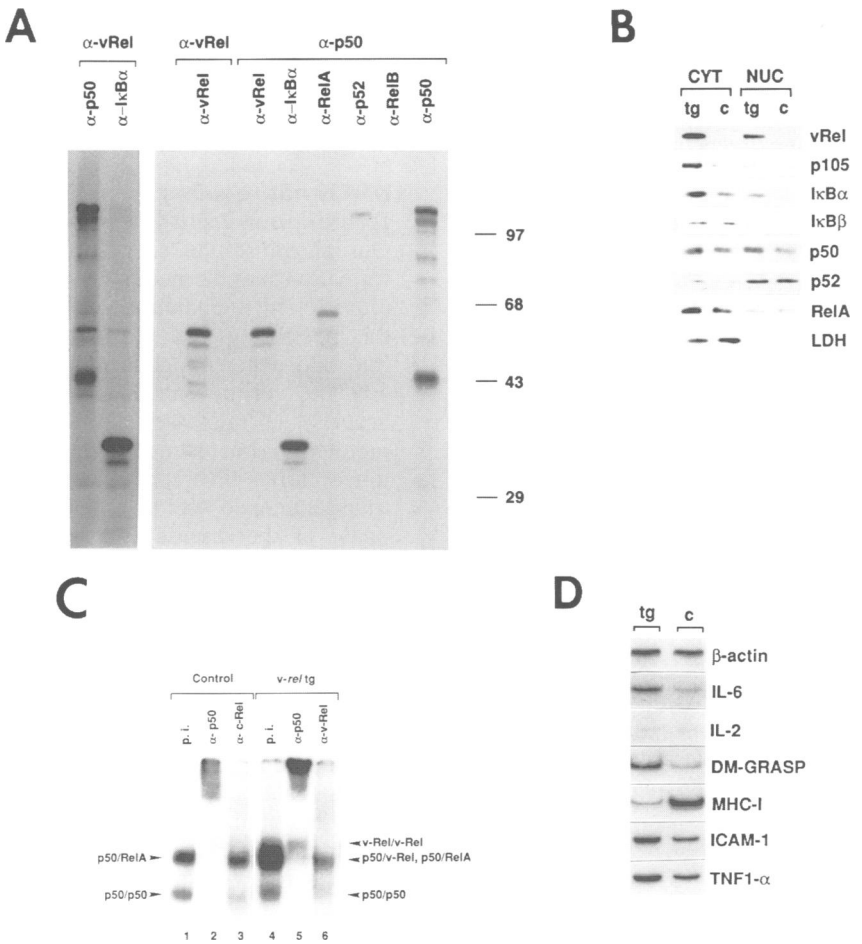

Fig. 5. Biochemical analysis of prelymphomatous v-*rel* transgenic thymocytes. (**A**) v-Rel immune complexes in transgenic thymocytes. Cell lysates from v-*rel* transgenic thymocytes were first immunoprecipitated with anti-v-Rel or anti-p50 antibodies, the complexes were denatured, then diluted 4-fold and reprecipitated with the indicated specific antisera. (**B**) Rel/NF-κB, IκBα and IκBβ protein levels in v-Rel transgenic thymocytes. Western blot analysis of cytoplasmic (CYT) and nuclear (NUC) extracts (20 μg) from thymocytes of control (c) and transgenic (tg) mice. Specific antisera against v-Rel, IκBα, IκBβ, p105-p50, p52, and RelA were used. Anti-LDH antiserum was used to rule out cross-contamination during the preparation of nuclear and cytoplasmic protein extracts. (**C**) p50/v-Rel heterodimers are the major κB-DNA binding activity. EMSAs using a palindromic κB-binding site were performed using nuclear protein extracts from control and v-Rel transgenic thymocytes. (**D**) p50/v-Rel DNA binding activity correlates with changes in the expression of several NF-κB-regulated genes. Total RNA was prepared from control and transgenic thymocytes. After cDNA synthesis, one-tenth of the reaction mixture was amplified in the presence of 0.1 mCi of [^{32}P]dCTP using *Taq* polymerase and sequence-specific primers. One-fifth of the amplified products were separated on 6% non-denaturing polyacrylamide gels. Gels were dried and exposed to X-ray film.

In thymocytes from transgenic mice, v-Rel was localized predominantly in the cytoplasm, but a significant amount (~30% of the total) was also detected in the nuclear fraction. Similar distribution of v-Rel protein was detected by immunofluorescence (data not shown). Our immunoprecipitation studies indicated that p50/v-Rel also binds to IκBα (Figure 5A). Western blots revealed that prelymphomatous transgenic thymocytes present increased cytoplasmic levels of IκBα and p105 (Figure 5B). Since we have not detected augmented levels of *ikba* mRNA in v-Rel transgenic thymocytes (data not shown), the increase in IκBα is most likely due to stabilization through its association with p50/v-Rel, as has been described for RelA (Rice and Ernst, 1993; Scott *et al.*, 1993; Perez *et al.*, 1995). We have also found a significant fraction of IκBα in the nucleus of v-Rel-expressing thymocytes. This nuclear IκBα is unlikely to result from contamination between subcellular fractions as no LDH was detected in the nuclear fraction. In addition, our data show that nuclear levels of c-Rel, RelA and p52 were not increased in thymocytes from transgenic animals (Figure 5B). The increase in nuclear p50 is likely due to the augmented synthesis of its precursor, p105.

No changes in protein levels and nuclear/cytoplasmic distribution of IκBβ were detected in v-Rel transgenic thymocytes (Figure 5B), suggesting that this inhibitor may play a minor role in v-Rel inactivation.

p50/v-Rel is the major κB-binding activity in v-Rel transgenic thymocytes

To investigate whether the κB-binding activity was altered in thymus of transgenic mice as a consequence of v-Rel expression, we analyzed thymocyte protein extracts by electrophoretic mobility shift assays (EMSAs), using a palindromic κB site (Figure 5C). Nuclear extracts from thymocytes of control mice revealed the presence of two complexes (lane 1). Anti-p50 antiserum eliminated both κB-binding activities (lane 2) while anti-RelA antiserum removed only the slower-migrating complex (not shown), indicating that it consisted of p50/RelA heterodimers,

while the faster-migrating complex consisted of p50 homodimers. Anti-c-Rel antiserum did not alter the pattern of complexes (lane 3), indicating that c-Rel is absent from these dimers, and confirming our previous observations (Weih *et al.*, 1994). Nuclear extracts from v-Rel-expressing thymocytes contained two additional complexes absent in control cells, one weaker and slower-migrating complex that co-migrates with p50/RelA heterodimers and one stronger and faster-migrating complex that co-migrates with p50/RelA heterodimers (lane 4); p50 (lane 5) and v-Rel (lane 6) antisera removed the faster-migrating one, demonstrating that it consists of p50/v-Rel heterodimers. The other complex is removed only by v-Rel antibodies (lane 6), demonstrating that it consists of v-Rel homodimers. Antibodies against RelA, RelB and p52 similarly affected the nuclear DNA binding pattern of transgenic thymocytes compared with control cells (data not shown), indicating that v-Rel does not cause inappropriate nuclear translocation of other NF-κB complexes. These results demonstrate that p50/v-Rel is the major DNA binding activity in transgenic thymocytes and suggest that this heterodimeric complex formation may be important in the transforming activity of v-Rel in these cells.

EMSA performed with nuclear protein extracts from tumor cells derived from older mice that exhibit overt disease did not differ from that described in transgenic thymocytes derived from young apparently healthy mice (data not shown).

Expression of NF-κB responsive genes in v-Rel transgenic thymocytes

To correlate the altered κB-binding activity in the pre-lymphomatous transgenic thymocytes, with changes in the expression of NF-κB-responsive genes such as major histocompatibility complex class I (MHC I), IL-2, IL-6, tumor necrosis factor-α (TNF1-α), intercellular cell adhesion molecule I (ICAM-I), β-interferon (IFNβ) and granulocyte macrophage colony-stimulating factor (GM-CSF), which may mediate the transforming capability of v-Rel, we analyzed their mRNA levels by reverse transcription–polymerase chain reaction (RT–PCR) (Figure 5D). cDNA was prepared from total mRNA extracted from control and v-Rel transgenic thymocytes and then amplified with 25 PCR cycles in the presence of [^{32}P]dCTP and the appropriate pairs of specific oligonucleotides. The same amount of total mRNA was present in all the samples, as demonstrated by the equal amounts of β-actin PCR products. By comparing the amount of PCR products detected in control and transgenic thymocytes, it is evident that v-Rel can have a positive or a negative effect on the expression of target genes, confirming previous observations (Gelinas and Temin, 1988; Walker *et al.*, 1992). For instance, the adhesion molecule DM-GRASP, potentially involved in the growth of v-Rel-transformed tumor cells (Zhang *et al.*, 1995), is over-expressed in transgenic thymocytes. It remains to be determined whether there are κB sites in the DM-GRASP gene (Zhang *et al.*, 1995). On the other hand, MHC class I gene is down-regulated, a mechanism that may allow tumor cells to escape immune surveillance. This result differs from what has been observed in avian retroviral v-*rel*-infected cells, where an increase in MHC class I gene expression was detected (Boehmelt *et al.*, 1992; Hrdlickova *et al.*, 1994b). Other molecules up-regulated in v-Rel transgenic thymocytes include IL-6, TNF1-α and ICAM-1. No differences were observed in the expression of IL-2, IFNβ and GM-CSF genes (Figure 5D and data not shown).

p50 is not required for v-Rel-induced tumor formation in transgenic thymocytes

Our above results suggest that p50/v-Rel heterodimer formation may be necessary in the transforming activity of v-Rel in mammalian cells (Figure 5). We examined this possibility further by crossing the v-*rel* transgenic line into a homozygous p50$^{-/-}$ background (Sha *et al.*, 1995) to generate mice with a v-Rel/p50$^{-/-}$ genotype. A group of 15 mice were bred and their health status was monitored. Interestingly, these mice became sick at a much earlier time than what was observed in the v-*rel* transgenic mice with a normal p50 background. The acute phase of the disease appeared to be more severe and animals succumbed at much earlier times; 50% of v-Rel/p50$^{-/-}$ mice died by 11 weeks while 50% of v-*rel* transgenic mice died by 25 weeks (Figure 6A). As observed with v-*rel* transgenic mice, in v-Rel/p50$^{-/-}$ mice that died at earlier times, we did not detect obvious histologic abnormalities which suggested the presence of tumor development. In contrast, in mice that died at later times, tumor infiltration was detected in lung, liver and lymph nodes (data not shown). In order to identify the form of v-Rel that transforms thymocytes in v-Rel/p50$^{-/-}$ mice we analyzed the κB-binding activity by EMSA. Nuclear extracts from v-*rel*/p50$^{-/-}$ thymocytes revealed that the only detectable κB-binding complexes were v-Rel homodimers (Figure 6B). As expected, anti-p50 antiserum did not alter this migrating complex (compare lanes 2 and 3). However, anti-v-Rel antiserum completely eliminated the complex (lane 4), indicating that it consisted of v-Rel/v-Rel homodimers. These results indicate that if p50/v-Rel heterodimers are responsible for T-cell transformations, this heterodimeric formation is not essential and that v-Rel homodimers are sufficient for transformation in a mammalian system. In addition, the longer latency in the appearance of the transformed phenotype in v-*rel* transgenic thymocytes as compared with the latency in a v-*rel*/p50$^{-/-}$ genotype indicate that p50/v-Rel heterodimers are less active than v-Rel homodimers in transformation.

Because of the suggestion that v-Rel transforms cells through its association with IκBα in the cytoplasm, it was of interest to study the interaction of v-Rel and IκBα in conjunction with the v-Rel/p50$^{-/-}$ genotype. When cytoplasmic protein extracts from control, v-Rel transgenic and v-Rel/p50$^{-/-}$ transgenic thymocytes were first immunoprecipitated with anti-v-Rel antibodies and the v-Rel-containing immune complex then split up and probed with IκBα-specific antibodies, very little IκBα associated with v-Rel was detected in the v-rel/p50$^{-/-}$ thymocytes (data not shown). This result suggests that the presence of p50 modulates the interaction of v-Rel and IκBα and indicates that v-Rel does not transform cells through its association with IκBα/pp40 (Kabrun and Enrietto, 1994; Hrdlickova *et al.*, 1995).

Discussion

The oncogene v-*rel* was originally identified as the transforming component of the avian retrovirus reticuloendo-

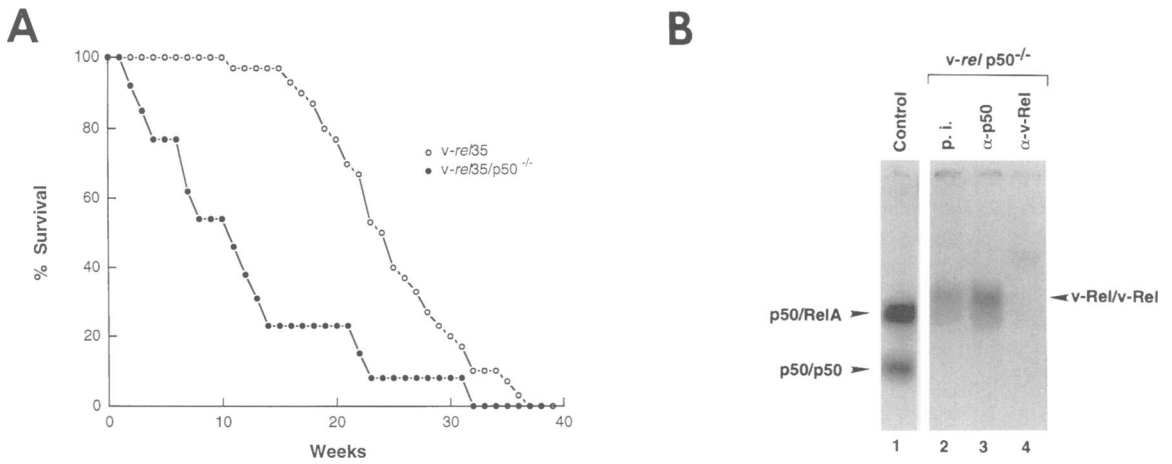

Fig. 6. (**A**) p50 is not required for v-Rel tumor promotion. Mortality curve for v-*rel* transgenic mice in a p50 null background. v-*rel* transgenic and p50 knockout ($p50^{-/-}$) mice were bred to generate a colony of v-*rel* transgenic mice in a p50 null background (v-*rel*/$p50^{-/-}$). A colony of 15 mice was monitored during a period of 32 weeks and the cause of death studied by autopsy or histologic examination. Tumor formation was confirmed in 13 v-*rel* $p50^{-/-}$ mice. The other two animals that succumbed at the earlier time showed no obvious indications of tumor formation. $n = 29$ for v-*rel*35; $n = 15$ for v-*rel*/$p50^{-/-}$. (**B**) v-Rel/v-Rel homodimer is the transforming activity in v-*rel*/$p50^{-/-}$ mice. Nuclear protein extracts of thymocytes from control and v-*rel*/$p50^{-/-}$ mice were analyzed by EMSA as described in Materials and methods.

theliosis virus-T. A mutated homologue of the proto-oncogene, c-*rel*, v-*rel* induces a rapidly fatal hematopoietic malignancy in young birds. The mechanism by which v-Rel induces oncogenic transformation is unclear, although the identification of v-Rel as a member of the Rel/NF-κB transcription factor family led to the suggestion that transformation resulted from v-Rel-induced changes in NF-κB-regulated genes. The focus of this study was to investigate *in vivo* the potential effects of the constitutive expression of v-Rel on T-cell development and the significance of this expression in endogenous NF-κB activity. This transgenic system provides an opportunity to test some of the current models proposed for v-Rel transformation.

We have demonstrated for the first time that v-Rel is able to induce transformation in mammalian cells. Previous attempts to transform murine cells have failed because of the cytopathic effect of v-Rel (Gelinas and Temin, 1988; Schwartz and Witte, 1988; Hannink and Temin, 1989). In our transgenic system, transformed cells expressed high levels of v-Rel protein, as determined by immunofluorescence. Despite a decreased number of total thymocytes, the chronic exposure to v-Rel in a protected microenvironment may have allowed for the selection of cells that can overcome v-Rel toxicity. The v-Rel-induced tumor formation in our transgenic system is particularly important as it demonstrates that avian-specific factors or events are not required for v-Rel transformation.

The true target cell for v-*rel* transformation in the avian system remains obscure. Initial evidence suggested that v-*rel* contained within the REV-TREV-A virus induced reticuloendotheliosis (Theilen *et al.*, 1966; Olson, 1967), affecting cells associated with endothelia of blood vessels and with sinusoids of spleen, kidney, liver and lymphoid organs. Cells derived from liver or spleen tumors of v-*rel* REV-T/REV-A-infected chickens exhibited either T-lymphoid or myeloid determinants (Barth *et al.*, 1990). The *in vitro* target cell for v-*rel* transformation was also classified as lymphoid (Beug *et al.*, 1981; Lewis *et al.*, 1981; Barth and Humphries, 1988; Benatar *et al.*, 1991). By using a replication-competent v-*rel* virus, it has been demonstrated that v-*rel*-transformed chicken cells co-express surface antigens specific to both lymphoid and myeloid cells (Morrison *et al.*, 1991). Moreover, with the use of conditional v-Rel variants it has been proposed that v-Rel transforms a common progenitor for neutrophils and dendritic cells (Boehmelt *et al.*, 1995). In our transgenic system, by directing v-Rel expression to thymocytes, we demonstrated that v-Rel is able to transform and stabilize immature double positive $CD4^+CD8^+$ or single positive $CD4^-CD8^+$ T-cells. v-Rel-transformed T-cells displayed only surface markers specific for T-cells. Thus, v-Rel transgenic mice constitute a powerful tool to study T-cell lymphoma development and T-cell differentiation. By using a similar transgenic approach with a different cell-specific promoter it will be interesting to see whether B-cells or non-hematopoietic cells are susceptible to v-Rel transformation.

The latency in the appearance of the transformed phenotype, including alterations in T-cell clonality and phenotypic abnormalities, suggests that v-Rel would promote tumor formation while other events are necessary for tumor progression. The existence of a prelymphomatous period during the evolution of the disease in the transgenic mice will help to identify key elements in the transition to the transformed phenotype. This latency and the inability to reproduce tumor formation in syngeneic mice indicated a low malignant behavior of v-Rel-transformed T-cells. This low malignant potential is supported by our findings that a small fraction of the tumor cells are in the S phase of the cell cycle, have slow growth rates, and are dependent on IL-2 for their proliferation.

Our observation that Δc-*rel* does not induce tumor formation in transgenic thymocytes suggests that the deletion of the C-terminal end of c-Rel does not play a major role in the transforming activity of v-Rel. This is in agreement with previous studies demonstrating that v-Rel/c-Rel hybrid proteins that contain an intact C-terminus are highly transforming (Hannink and Temin, 1989). Since v-Rel lacks a strong transcriptional activation domain, it is believed that v-Rel does not induce transformation by affecting directly the expression of NF-κB

responsive genes. Instead, v-Rel is thought to act either as a dominant negative regulator of the endogenous NF-κB activity through the formation of inactive heterodimers with Rel/NF-κB proteins or by the titration of IκBα and other inhibitory molecules, allowing the inappropriate translocation of Rel/NF-κB family members to the nucleus. However, these models cannot explain why low levels of v-*rel* expression such as those detected in lines 46 and 33 are still transforming. Also, since neither c-Rel expression (Weih *et al.*, 1994) nor c-Rel-dependent DNA binding activity (Carrasco *et al.*, 1994) have been detected in thymocytes, it seems unlikely that v-Rel induces oncogenic transformation by being a dominant inhibitor of c-Rel. Furthermore, although significant amounts of the v-Rel complexes are associated with IκBα, the nuclear levels of c-Rel, RelA or p52 were unchanged in v-Rel transgenic thymocytes. Moreover, transgenic animals overexpressing RelA, RelB, p52 or the truncated c-Rel, show no transformed phenotype (Perez *et al.*, 1995; Weih *et al.*, 1995b; P.Perez, unpublished results).

Although v-Rel was found in a complex with p50 and the p50/v-Rel heterodimer was the major DNA binding activity detected in transgenic thymocytes, the formation of tumors in p50-deficient mice indicates that the heterodimerization of v-Rel with p50 is not essential for v-Rel tumorigenesis. On the contrary, our observation that tumor development is faster in mice lacking p50 indicates that v-Rel/v-Rel homodimers are more active in transforming activity than the heterodimer p50/v-Rel. This would explain in part why in the avian system, where no p50 is detected in v-Rel immunoprecipitates (Capobianco *et al.*, 1992), tumor development is very rapid (Bose, 1992). In p50 null mice, the only κB-binding activity was identified as v-Rel homodimers.

Thus, our studies support a model in which v-Rel transformation involves a direct mechanism where v-Rel binds DNA as p50/v-Rel heterodimer or v-Rel/vRel homodimer and alters gene expression rather than indirect alterations in the composition of preformed cytoplasmic NF-κB complexes (Walker *et al.*, 1992). This is in agreement with the observation that mutations introduced in the DNA binding domain of v-Rel abolish its transforming ability (Ballard *et al.*, 1990; Mosialos *et al.*, 1991) and with recent data demonstrating that v-Rel can transform chicken lymphoid cells as a homodimer or heterodimer with other Rel/NF-κB molecules like p52 and that a v-Rel mutant that does not interact efficiently with IκBα can still transform spleen cells (White *et al.*, 1996). In v-Rel transgenic thymocytes it seems that v-Rel transforms T-cells by disrupting the normal transcriptional balance, by acting as both a transactivator and a transrepressor. We have demonstrated that v-Rel may up-regulate DM-GRASP and ICAM-1 genes required for tumor development. DM-GRASP was found to be important for proliferation of v-*rel*-transformed avian cells (Zhang *et al.*, 1995), while ICAM-1 is involved in the interaction of T- and B-cells (Sanders *et al.*, 1986) and is essential in cell-mediated cytotoxicity (Schmidt *et al.*, 1985). As a repressor, v-Rel may be involved in the down-regulation of tumor supressor genes or MHC class I genes. The down-regulation of MHC class I expression in v-*rel* transgenic thymocytes is particularly interesting in light of the observation that down-regulation of MHC class I complex is associated with low immunogenicity of tumors (De Batselier *et al.*, 1980; Eisenbach *et al.*, 1984).

To bind DNA, v-Rel must translocate to the nucleus. In transgenic thymocytes and transformed T-cells, a significant amount of v-Rel was nuclear, based on Western blots and immunofluorescence. This nuclear v-Rel was enough to disturb dramatically the endogenous NF-κB activity as measured by DNA binding and expression of NF-κB-regulated genes. v-Rel was able to translocate to the nucleus in spite of increased levels of two inhibitory proteins, IκBα and p105. Since we have not detected increased levels of *ikba* mRNA in v-*rel* transgenic thymocytes (data not shown), the augmentation in IκBα is most likely due to stabilization through its association with v-Rel, as described for RelA (Rice and Ernst, 1993; Scott *et al.*, 1993; Perez *et al.*, 1995). It will be interesting to determine whether overexpression of IκBα in v-Rel transgenic mice would block v-Rel-induced transformation by trapping it in the cytoplasm.

We believe that the mouse transgenic system provides new opportunities over transient transfection (Abbadie *et al.*, 1993) and viral infection of cells (Boehmelt *et al.*, 1995) for the understanding of v-Rel function. The availability of knockout mice for different members of the Rel/NF-κB family will permit a genetic analysis of the transforming mechanisms of v-Rel.

Materials and methods

Plasmid construction and generation of transgenic mice

The chicken v-*rel* cDNA (Kumar *et al.*, 1992) and the mouse Δc-*rel* (Bull *et al.*, 1990) were cloned into the *Bam*HI site of the pTLC vector. The mouse c-*rel* cDNA corresponds to a PCR product that is missing a guanine at nucleotide 1421 compared with the original cDNA (Bull *et al.*, 1990). This mutation induces a change in frame shift and a premature stop codon with the generation of a shorter form of c-Rel protein that is missing 117 amino acids at the carboxy-terminus. The pTLC vector contains a 3.2 kb fragment of the mouse *lck* promoter and a 2.1 kb fragment of the human growth hormone (GH) gene which provides introns and the polyadenylation signal sequence. The mouse proximal *lck* promoter drives transcription of the transgene to all thymocyte subsets from the earliest stages of T-cell development (Allen *et al.*, 1992). A 2.1 kb fragment of the 3′-locus control region (LCR) of the human CD2 gene was subsequently inserted downstream of the human GH sequence to obtain copy number-dependent and insertion site-independent levels of expression (Greaves *et al.*, 1989). The transgene-encoding sequences were released from the vector sequences by *Not*I digestion and purified by zonal sucrose gradient centrifugation as previously described (Mann and McMahon, 1993). The microinjection of the transgene and the screening of the transgenic mice using PCR analysis were performed as previously described (Perez *et al.*, 1995).

RNA and DNA analyses

Total RNA from tissues of 4- to 5-week-old mice was isolated using RNAzol according to the manufacturer's instructions (Cinna/Biotecx). 20 μg of total RNA were denatured and separated by electrophoresis in a 0.8% agarose gel containing 2.2 M formaldehyde according to standard procedures (Sambrook *et al.*, 1989). The gels were blotted onto Nytran membranes and hybridized against full-length v-*rel* cDNA, c-*rel* cDNA or 1.2 kb rat GAPDH cDNA gene probes labeled by random priming (Sambrook *et al.*, 1989).

High molecular weight DNA was isolated from fresh or snap-frozen tumor tissues or non-tumorous thymus, spleen and liver, digested with *Hin*dII restriction enzyme and analyzed by Southern blot hybridization using previously described procedures (Cleary *et al.*, 1986). As a radiolabeled probe, a 2.9 *Hin*dIII–*Bam*HI genomic fragment upstream and adjacent to the mouse TCR Jβ1 gene segment was used (Malissen *et al.*, 1984).

Histopathology, blood smears and immunofluorescence

Mice were sacrificed and tissues immersion-fixed in 10% buffered formalin. Tissues were embedded in paraffin blocks and processed by routine methods, sectioned at 5–10 μm thickness, stained with hematoxylin–eosin and examined by light microscopy. Peripheral blood smears were prepared with a drop of blood from mouse tails and stained with May–Grunwald stain.

Single cell suspensions from tumor-bearing spleens were prepared according to standard procedures (Coligan *et al.*, 1992) and spun onto slides as previously described (Carrasco *et al.*, 1994). For double immunofluorescence, the cytospins were incubated at room temperature for 1 h with anti-v-Rel antibodies together with anti-CD45R (clone RA3-6B2) (Pharmingen). Anti-v-Rel antibodies were visualized with a donkey anti-rabbit immunoglobulin conjugated with Texas red (TR, Amersham). Anti-CD45R was visualized with a goat anti-rat IgG conjugated with FITC (Boehringer).

Flow cytometric analysis

Flow cytometry was performed with a Coulter Epics Profile II flow cytometer and cell sorter. Single cell suspensions from control spleens and tumor-bearing spleens were prepared and analyzed for surface expression of CD4 (clone H129.19), CD8 (clone 7D4), TCRαβ (clone H57-597), Thy-1.2 (clone 53-2.1), B220 (clone RA3-6B2) and HSA (clone J11d) as previously described (Weih *et al.*, 1995a). Monoclonal antibodies were obtained from Gibco–BRL and Pharmingen.

Cell culture analysis

Single cell suspensions of tumors derived from spleens were placed in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 4 mM glutamine, 50 mM 2-mercaptoethanol, 50 U/ml penicillin and 50 mg/ml streptomycin. Growth factors were added individually or in combinations at the following concentrations: 25 U/ml IL-2, 50 U/ml IL-3, 200 U/ml IL-6, and 2.5 ng/ml IL-7. Cell counts of viable cells were determined by trypan blue exclusion. Cells were fed with fresh medium at 3 days.

Western blot assays

Cytoplasmic and nuclear fractions from thymocytes were prepared as previously described (Schreiber *et al.*, 1989). Aliquots of cytoplasmic and nuclear extracts (20 μg) were boiled in Laemmli buffer and run overnight on a 12.5% acrylamide–bisacrylamide (200:1) gel at 12 mA. For Western blotting procedures and antibody description, see Weih *et al.* (1994). The proteins were efficiently transferred onto nitrocellulose membranes as assessed by Ponceau S staining. Possible contamination of the nuclear extracts was checked by incubating the membranes with an antiserum specific for the cytosolic enzyme LDH.

Cell labeling, lysis and immunoprecipitation

Isolated thymocytes were labeled for 2 h with 500 mCi/ml of [^{35}S]-methionine (1000 Ci/mmol) in DMEM medium lacking methionine and containing 10% heat-inactivated and dialyzed FCS. The labeling medium was removed and the cells were washed with cold phosphate-buffered saline (PBS) and lysed on ice by adding RIPA buffer (10 mM Tris–HCl pH 7.5, 0.5% Nonidet P-40, 150 mM NaCl). Cell lysates were first cleared with a preimmune serum (3 μl) for 3 h, then immunoprecipitated with specific antisera as previously described (Perez *et al.*, 1995).

Electrophoretic mobility shift assay (EMSA)

The palindromic κB site used for these assays has been previously described (Dobrzanski *et al.*, 1993). Nuclear extracts (3 μg) were incubated with 20 000 c.p.m. labeled probe, 3 μg poly(dI:dC) in buffer containing 20 mM HEPES pH 7.9, 60 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 0.5 mM PMSF and 17% glycerol in 25 μl final volume for 15 min on ice. Complexes were separated on 5.5% native polyacrylamide gels run in 0.25× TBE buffer, dried and exposed to Kodak X-Omat AR film at –70°C.

Semi-quantitative RT–PCR analyses

Semi-quantitative PCR was performed as described previously (Mumberg *et al.*, 1991). The PCR conditions were established such that amplification of the cDNAs was linearly dependent on the concentration of the corresponding mRNAs. This was achieved by performing the reactions in the presence of [^{32}P]dCTP, so that owing to the greater sensitivity of autoradiography as opposed to ethidium bromide staining, a relatively low number of PCR cycles was sufficient to detect the amplified product. In this way, the cDNA concentration remained the rate-limiting factor throughout the amplification procedure.

Total RNA from control, Δc-*rel* and v-*rel* transgenic thymocytes was prepared as described above and quantified by absorbance at 260 nm. 10 μg of total RNA were used for cDNA synthesis using 1000 U of reverse transcriptase (Gibco–BRL). After cDNA synthesis, each sample was diluted five times and 5 μl were taken for amplification with 25 cycles by using *Taq* polymerase in the presence of [^{32}P]dCTP and sequence-specific primers. One-tenth of the PCR products were separated on 8% native polyacrylamide gels run in TBE buffer, dried and exposed to Kodak X-Omat AR film at –70°C. Specific oligonucleotides were obtained from Stratagene. For DM-GRASP amplification the following pair of oligonucleotides were used: 5′-CTGCCACCAAAAAATGCC-3′ and 5′-CCTTCATCCACACCACAGTC-3′.

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