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Detection of c-*rel*-Related Transcripts in Mouse Hematopoietic Tissues, Fractionated Lymphocyte Populations, and Cell Lines

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A portion of the human cellular homolog of v-*rel*, the transforming gene of the leukemogenic retrovirus reticuloendotheliosis virus, strain T, was used to survey RNAs from several mouse tissues, selected lymphocyte populations, and hematopoietic cell lines for c-*rel* expression. Relatively high levels of a high-molecular-weight transcript were observed in peripheral B and T cells, whereas lower levels were detectable in functionally immature thymocytes. These results suggested that, unlike c-*myb* and c-*ets*, the c-*rel* proto-oncogene plays a role in later stages of lymphocyte differentiation.

REV-T, a highly leukemogenic virus of galliform birds, is believed to have arisen by recombination between a replication-competent type C retrovirus and a portion of a turkey cellular gene designated c-*rel* (30, 31). Evolutionary comparisons between cloned segments of the turkey and chicken c-*rel* homologs indicate that the *rel* locus resembles many other cellular proto-oncogenes in being both large and complex (6, 30). The nine exons from which v-*rel* was derived, for example, are dispersed over more than 20 kilobase (kb) pairs of DNA and range in size from ~60 to over 500 base pairs (30). Very little is known about the normal expression of this cellular gene except that elevated levels of *rel*-related transcripts can be detected in avian hematopoietic tissues, suggesting a potential role for c-*rel* in the differentiation of lymphoid and myeloid cell lineages (6, 14).

Recently, we presented evidence that unique c-*rel* loci could also be identified in humans, mice, and cats, all of which provide potentially useful genetic model systems for studying the function of this proto-oncogene (3, 4). To date, however, no clear picture has emerged regarding c-*rel* transcription in any mammalian group, a problem based partly on the fact that the *rel*-specific probes used in previous studies were derived from the distantly related avian viral *rel* oncogene (19, 23, 25, 28). In this study, we used a recently described cloned DNA fragment containing two exons of the human c-*rel* proto-oncogene (4) to screen RNAs from various postnatal mouse tissues, fractionated lymphocytes, and cell lines. We detected large (~7.5 kb) *rel*-homologous transcripts in thymic and splenic RNAs from 9-, 18-, and 28-day-old mice, whereas RNAs from kidney and liver tissues from the same mice were essentially negative in comparison. Analyses of RNA samples from fractionated lymphocytes showed high levels of *rel* transcripts in surface immunoglobulin-positive splenic B cells, followed closely by Lyt2$^-$ L3T4$^+$ and Lyt2$^+$ L3T4$^-$ splenic T lymphocytes. RNA samples from thymocyte populations obtained by differential agglutination with peanut lectin (PNA) were weakly positive, although the more mature, medullary-type PNA$^-$ fraction showed a slightly higher level of expression. We corroborated this observation by analyzing RNAs from thymocyte subsets obtained by positive selection and from various established murine lymphocyte cell lines. Judging from the elevated levels found in peripheral (i.e., functionally mature) B and T cells, we hypothesized that c-*rel* functions in later stages of murine lymphocyte differentiation.

c-*rel* transcripts show tissue-specific distribution. Previous investigators have detected c-*rel* transcripts in a variety of uninfected chicken cell types. The highest levels of RNA are found in hematopoietic tissues, such as bursa, liver, bone marrow, spleen, and thymus, with little or none in muscle or brain (6, 14). A 4-kb c-*rel* mRNA has also been detected in cultured chicken T cells (MSB-1) and, at various levels, in REV-T-transformed chicken spleen cell lines (14). The observation that the avian c-*rel* gene is expressed in a tissue-specific manner thus prompted us to ask whether the mouse homolog shows a similar transcriptional pattern. For these experiments, we used a molecularly cloned fragment of the human c-*rel* proto-oncogene (Fig. 1A; 4) as a probe to screen total cellular RNA samples from several murine tissues (7, 10, 20). The human probe, pPHHS*rel*-1, carries two highly conserved c-*rel* exons (4) and hybridizes under moderately high stringency to a unique c-*rel* genetic locus on mouse chromosome 11 (3). The human probe detected a 7.5-kb RNA in thymus and spleen RNA preparations (Fig. 1B). A similarly sized RNA was also observed with a v-*rel*-specific probe at much lower stringency (data not shown). Faint bands of the same molecular weight were evident in RNAs from livers of 9- and 18-day-old mice, whereas RNAs from livers of 4-week-old mice and all three kidney samples were negative. The decreasing levels and ultimate loss of c-*rel* expression in liver may correlate with the age-related conversion of this organ from a hematopoietic to a nonhematopoietic function. In contrast, expression of the 7.5-kb RNA showed a slight increase in thymic and splenic tissues from older (28-day-old) mice. Rehybridization of the same filter with a chicken β-actin cDNA probe revealed that these results could not be attributed to gross differences in RNA amounts (data not shown).

c-*rel* transcripts in PNA-separated cortical and medullary thymocytes. We next asked whether the *rel* transcripts observed in mouse thymus RNA preparations (Fig. 1B) exhibited further cell type specificity. Murine thymus is composed of two major histological parts, the outer cortex and the

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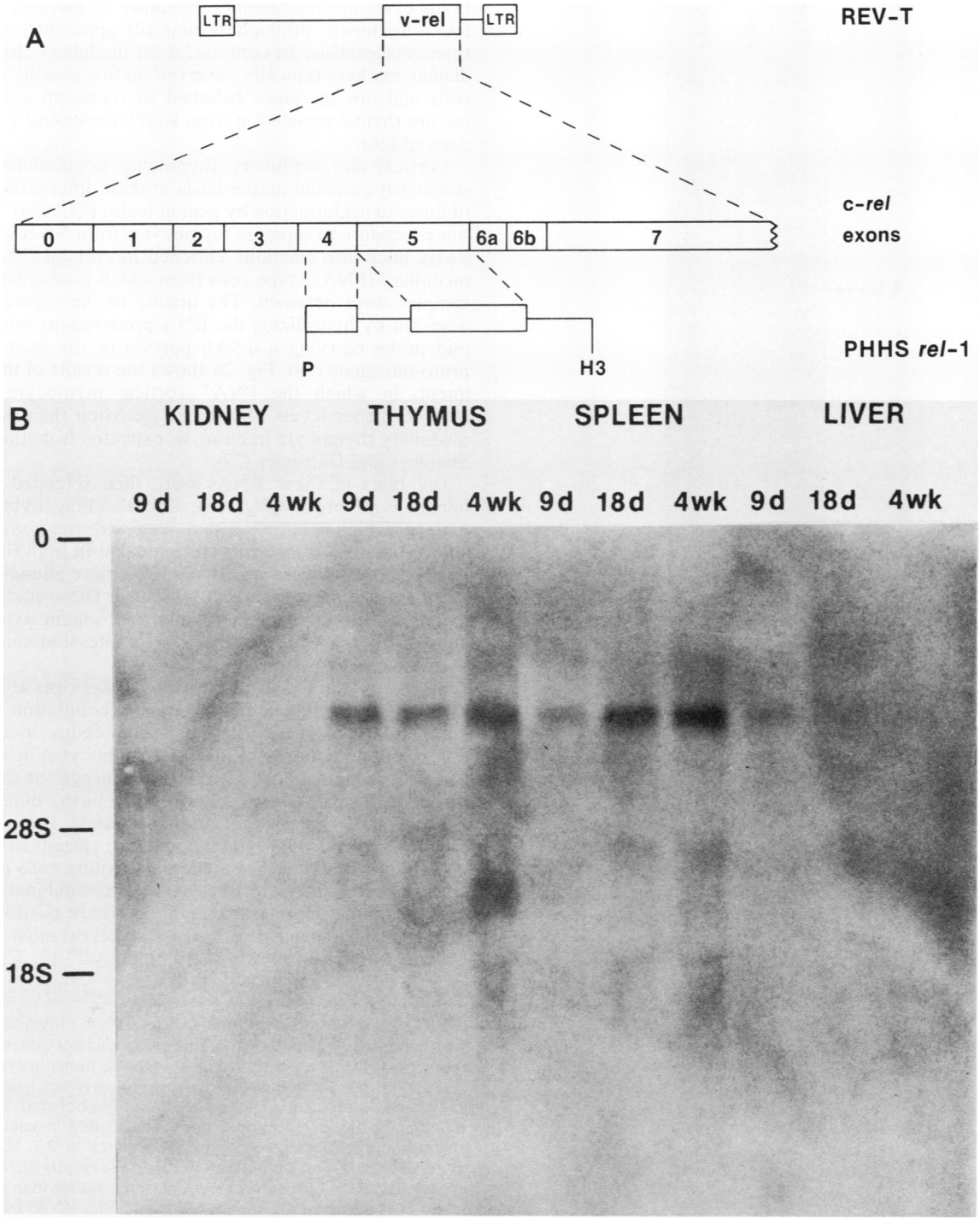
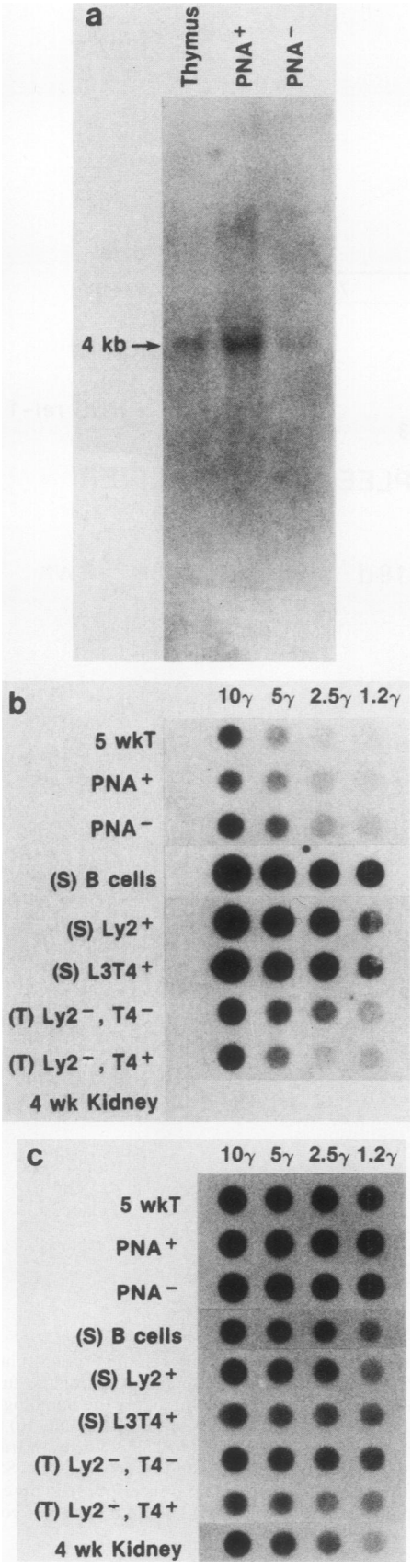


FIG. 1A. Derivation of the human c-*rel* probe. The genome of REV-T is ~5.7 kb long and contains a 1.4-kb substitution relative to its replication-competent helper virus. The substitution, termed v-*rel*, is believed to be derived from nine cellular exons of the turkey c-*rel* homolog (middle line) (30). The human genomic c-*rel* probe (4) used in this study is a *Pst*I-*Hin*dIII fragment containing the homologs of exons 4 and 5 (bottom line). (B) Survey of NIH Swiss mouse tissues for c-*rel* transcripts. Samples (50 μg) of total cellular RNAs (7, 10) from each of the designated mouse tissues were separated on 1% agarose gels in the presence of 2.2 M formaldehyde (20). The Northern transfer was hybridized with the cloned human c-*rel* insert as previously described (3), except that the final wash consisted of 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate and was performed at 65°C. The 7.5-kb transcripts seen here were also detected in poly(A)$^+$ mRNA preparations (data not shown), whereas the two faint bands at approximately 4 and 2 kb were not consistently observed.



centrally located medulla which, represent ~85 and ~15% of the total cell population, respectively (21, 26). Most cortical thymocytes are functionally immature or inactive and their fate is unknown, although the majority apparently die during thymocytopoiesis. In contrast, most medullary thymocytes display markers typically observed on functionally mature T cells and are therefore believed to represent a relatively mature thymic population from which peripheral T cells are derived (26).

Cortical and medullary thymocyte populations can be somewhat enriched on the basis of their differential susceptibilities to agglutination by peanut lectin PNA (24). We used this procedure to separate thymocytes from 5-week-old NIH Swiss mice into fractions enriched in cortical (PNA^+) and medullary (PNA^-) type cells from which total cellular RNA samples were obtained. The quality of the separation was assessed by hybridizing the RNA preparations with a plasmid probe carrying a 0.5-kb portion of the mouse c-*myb* proto-oncogene (29). Fig. 2a shows the results of this experiment, in which the PNA^+ cortical thymocyte fraction showed higher levels of c-*myb* expression than the PNA^- medullary thymocyte fraction, as expected from the work of Sheiness and Gardinier (27).

Dot blots of these RNAs were then screened with the human c-*rel* probe (Fig. 2b). The resulting hybridization pattern, which was unlike that seen with the c-*myb* probe, showed that *rel* transcripts were present in both thymocyte populations and were slightly (~50%) more abundant in the more mature medullary (PNA^-) cells. These findings next led us to survey various thymic and splenic lymphocyte subsets to determine whether c-*rel* expression showed further lineage restriction.

Splenic B and T cells express c-*rel* transcripts at relatively high levels. During hematopoietic differentiation in mice, committed B- and T-cell progenitors acquire many of the characteristic features of mature lymphocytes in either the mammalian bursal equivalent (B-cell lineage) or the thymic medulla (T-cell lineages). Soon after birth, differentiated cells of both lineages begin to populate the secondary lymphoid organs (i.e., lymph nodes and spleen), where they constitute a reservoir of functionally mature cells exhibiting cell surface markers in lineage-specific combinations. For example, most peripheral T helper cells are positive for the marker L3T4 and negative for Lyt2, whereas most cytotoxic and suppressor T cells are $L3T4^-$ but $Lyt2^+$ (5, 22). Splenic

FIG. 2A. Hybridization of PNA^+ and PNA^- thymocyte RNAs with a mouse c-*myb* probe (29). This proto-oncogene encodes a 4-kb transcript (11) that is expressed most highly in immature thymocytes (11, 27), in contrast to the pattern shown by c-*rel* (see below). About 25 μg of total cellular RNAs from unfractionated and PNA-fractionated thymocyte populations was loaded in each lane and electrophoretically separated in the presence of 2.2 M formaldehyde. Hybridization conditions were as previously described (3), except that the whole recombinant plasmid, rather than an isolated insert, was labeled. (B) Dot blot of total cellular RNAs from various fractionated mouse lymphocyte populations hybridized with the human c-*rel* probe. (C) The same filter rehybridized at reduced stringency with a chicken β-actin cDNA probe. The hybridization solution contained 35% formamide, and the experiment was performed at 37°C. The final wash contained 0.1× SSC and 0.1% sodium dodecyl sulfate and was done at 37°C. Abbreviations: S, Splenic lymphocyte populations; T, thymocyte populations; PNA, peanut agglutinin; 5 wkT, thymus RNA samples from 5-week-old mice. All samples were included on the same filter, but the resulting exposure was rearranged in composing the final photographs for B and C.

B cells are distinguished from their more immature precursors by the presence of surface immunoglobulin molecules (sIg^+). We used antibodies to these different cell surface markers to isolate highly enriched lymphocyte populations from the thymuses and spleens of young adult BALB/c or C57BL/6 mice. Purified lymphocyte subsets (containing approximately 10^8 cells) were obtained by sequential positive selection. Cell suspensions of disrupted splenic tissues were first incubated on nylon wool (15) and the adherent population (containing sIg^+ cells and macrophages) was discarded. The nonadherent cells were then incubated on petri dishes coated with goat anti-mouse immunoglobulin antibody (18), and the adherent cells (sIg^+) were processed for RNA. The nonadherent cells from this step were then treated with rat anti-L3T4 antibody (22) to remove $L3T4^+$ T cells by adherence to anti-rat immunoglobulin-coated petri dishes. The nonadherent cells, now primarily sIg^- $L3T4^-$, were further treated with mouse anti-Lyt2.2 monoclonal antibody (13) to permit adherence of $Lyt2^+$ cells by exposure to goat anti-mouse immunoglobulin-coated petri dishes. Total cellular RNAs were then extracted from cell pellets of these peripheral T-cell populations (about 10^8 cells per sample), which represented the $Lyt2^-$ $L3T4^+$ helper and the $Lyt2^+$ $L3T4^-$ cytotoxic-suppressor cell lineages. Thymocyte subsets were similarly prepared without the initial steps for removal of sIg^+ cells. In addition, the final nonadherent population (primarily sIg^- $L3T4^-$ and $Lyt2^-$) was treated in two steps to remove any remaining contaminating $L3T4^+$ and $Lyt2^+$ cells and to yield a highly enriched pool of precursor $L3T4^-$ $Lyt2^-$ thymocytes (5, 21). First, the nonadherent population was treated with mouse anti-rat immunoglobulin monoclonal antibody MAR18.5 (obtained from Lewis Lanier through T. Chused, National Institutes of Health, Bethesda, Md. [17]) to identify the contaminating $L3T4^+$ cells previously coated with rat anti-L3T4 antibody. Second, mouse complement was added to lyse the mouse anti-rat immunoglobulin-coated $L3T4^+$ cells and any remaining $Lyt2^+$ cells that were already coated with mouse anti-Lyt2 antibody.

Dot blot analyses of total cellular RNAs prepared from these lymphocyte populations showed extremely high levels of c-*rel* expression in sIg^+ splenic B cells, with notable levels in both $Lyt2^+$ and $L3T4^+$ splenic T cells (Fig. 2b). Low signals were observed in RNAs from the immature thymocyte population displaying the $Lyt2^-$ $L3T4^-$ phenotype. $Lyt2^-$ $L3T4^+$ thymocytes, which can be considered the immediate thymic precursors of peripheral T helper cells (5), showed a slightly elevated level of c-*rel* expression, relative to the β-actin control, when compared with the immature $Lyt2^-$ $L3T4^-$ subset (compare Fig. 2b and c). These results are consistent with those obtained with the PNA-agglutinated subsets, and they suggest that c-*rel* plays a role in the later stages of lymphocyte differentiation.

Cultured cell lines express the *rel* transcript. In our last series of experiments, we asked whether c-*rel* was expressed in established murine lymphocyte cell lines and, if so, whether its expression showed the same distribution as expected from our in vivo results. For this purpose, total cellular RNAs were extracted from three established murine cell lines, NS-1, EL-4, and RL-12. The NS-1 line was derived from a mouse plasmacytoma (16) and displays the characteristics of mature B cells. EL-4 was established from a chemically induced thymoma of C57BL/6 mice (12), its phenotype corresponding most closely to that of mature medullary thymocytes. In contrast, RL-12, which was derived from a radiation-induced thymoma of C57BL/Ka mice (8), resembles cells of the thymic cortex. Northern analyses of RNAs from these different cell lines (Fig. 3) were consistent with the results of the PNA experiment, showing higher levels of c-*rel* transcripts in EL-4 than in RL-12. NS-1 also displayed significant levels of the 7.5-kb transcript, consistent with the results shown in Fig. 2b. Taken together, these observations suggested that c-*rel* expression increases during lymphocyte differentiation, perhaps playing an important role in the acquisition of B- and T-cell function.

Possible roles for c-*rel* in hematopoietic differentiation. This is the first study in which a mammalian c-*rel* probe was used to examine the expression of this proto-oncogene in laboratory mice. This probe (4) offers distinct advantages over the available avian v-*rel* probes, since it anneals with mouse DNA and RNA at much higher stringencies than can be used with the avian sequences, although both *rel* probes detect identical bands in mouse DNA (3) or RNA preparations. We surveyed a number of mouse tissues, fractionated cell populations, and cultured lymphoid cells to determine whether the avian and mammalian c-*rel* homologs exhibit, at least superficially, the same transcriptional profiles. In both organisms, c-*rel* expression was pronounced in hematopoietic tissues, with little or no expression detectable in avian brain or muscle or mammalian kidney (6, 14). One potentially interesting difference between c-*rel* expression in birds and mammals is that transcripts are abundant in the livers of 2-week-old chickens (second only to bursal tissue) (14), whereas few or none are seen in the livers of 9-day-, 18-day-, or 4-week-old mice. One plausible explanation for this observation is that livers of postnatal mice no longer serve as important sites of hematopoietic differentiation as they do in fetal mice and young birds. A second difference concerns the fact that the most abundant avian transcript is approximately

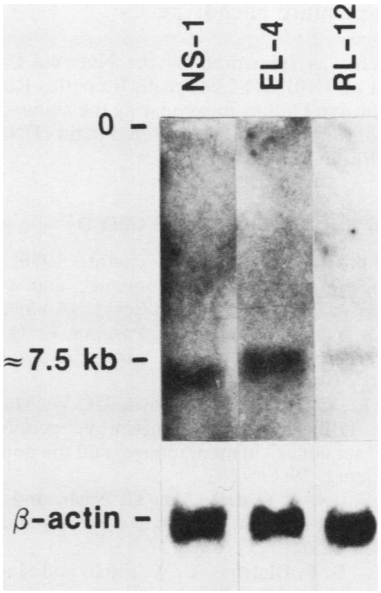


FIG. 3. Northern transfer of total cellular RNA samples from three established murine cell lines hybridized with the human c-*rel* probe (7.5-kb transcript) or a chicken β-actin cDNA probe. NS-1 and EL-4 displayed more mature B- and T-cell phenotypes, respectively, whereas RL-12 represented a more immature thymocyte lineage. The apparent size difference between the NS-1 and EL-4 transcripts was an artifact of this particular gel. The bands detected by the β-actin probe were aligned with each other in composing the final photograph, although they exhibited the same distorted migration pattern in the original autoradiograph.

4 kb long (6, 14), about half the apparent size of the newly described mouse transcript. The molecular basis for this size difference is unknown, but it may result from species-specific variations in the number of exons, the lengths of 5′ and 3′ untranslated regions, the locations and lengths of poly(A) tails, or patterns of splicing. Variations of this sort are not unprecedented (1, 2), but the magnitude of the size difference between the avian and mammalian transcripts makes the c-*rel* gene unusual.

No information on potential age-related transcriptional differences is available for the avian c-*rel* gene, but data presented here suggests that a slight elevation of c-*rel* expression is detectable in the spleens and thymuses of more mature mice. This observation correlates well with the finding that mouse c-*rel* transcripts are most abundant in the more mature B and T cells that are ready to leave or have already left the primary sites of lymphopoiesis to seed the periphery. Peripheral B and T cells, although phenotypically and functionally similar to their immediate predecessors in bone marrow and thymic medulla, nonetheless have been found to display a few new markers upon emigration from the primary lymphoid tissues. For example, peripheral T lymphocytes, but not medullary thymocytes, display the cell surface antigens Qa4 and Qa5 (13). Our results suggest that c-*rel* expression might represent a new marker for distinguishing these cells from their less mature counterparts.

Finally, the observation that c-*rel* expression is most abundant in more mature lymphocytes contrasts directly with the pattern shown by two other cellular proto-oncogenes, c-*myb* (Fig. 3; 11, 27) and chicken c-*ets* (9), both of which have been implicated in the earlier stages of hematopoietic differentiation. Future studies using mutant B- or T-cell lines that are blocked at specific stages of differentiation will help us to define further the contribution of c-*rel* to the mature phenotype.

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