

The Avian Transcription Factor c-Rel Is Expressed in Lymphocyte Precursor Cells and Antigen-Presenting Cells During Thymus Development

C. HUGUET^a, F. BOUALI^a, P.J. ENRIETTO^b, D. STEHELIN^a, B. VANDENBUNDER^a and C. ABBADIE^{a*}

^aLaboratoire d'Oncologie Moléculaire, URA1160, Institut de Biologie de Lille, 1 rue Calmette, 59019 Lille Cedex, France; ^bDepartment of Microbiology, State University of New York at Stony Brook, New York 11794

(Received 6 January 1997; Revised 5 June 1997; In final form 18 June 1997)

Transcription factors of the Rel/NF- κ B family are widely involved in the immune system. In this study, we investigate the *in vivo* expression of the avian protein c-Rel in the T-cell lineage during thymus development. The majority of thymocytes do not express the c-Rel protein. However, lymphocyte precursor cells that colonize the thymus express the c-Rel protein shortly after their homing in the organ and before they begin to differentiate. c-Rel is also detected in different subsets of antigen-presenting cells such as epithelial cells, dendritic cells, and macrophages. *In vitro* studies have shown that Rel/NF- κ B proteins are sequestered in an inactive form in the cytoplasm by interaction with the I κ B α inhibitory protein. By immunocytochemistry, we show that *in vivo* c-Rel is localized in the cytoplasm of antigen-presenting cells but in both the cytoplasm and nucleus of lymphocyte precursor cells. The cytoplasmic localization of c-Rel in antigen-presenting cells correlates with a high expression of I κ B α , whereas the nuclear localization of c-Rel in lymphocyte precursor cells correlates with a much lower expression of I κ B α . These results suggest that c-Rel might be constitutively activated in lymphocyte precursor cells.

Keywords: Rel/NF- κ B, thymus, lymphocyte precursor cells, antigen-presenting cells, avian development

INTRODUCTION

The proto-oncogene *c-rel* encodes a transcription factor of the Rel/NF- κ B family. The members of this family share a highly conserved 300 amino acid N-terminal domain, the Rel Homology Domain (RHD), involved in DNA binding, dimerization, nuclear localization, and interaction with inhibitory proteins

of the I κ B family. Rel/NF- κ B proteins associate as homo- or heterodimers and bind a specific DNA sequence, the κ B motif, in the promoter of their target genes. Inside the Rel/NF- κ B family, two subfamilies of proteins can be defined based on the function of their C-terminal domains. The first one includes c-Rel, RelA, RelB, Dorsal, and Dif, which contain a transactivating domain in their C terminus. The

*Corresponding author.

second family is composed of the precursor proteins NF- κ B1 and NF- κ B2, which contain I κ B-like ankyrin repeats in their C terminus. NF- κ B1 and NF- κ B2 undergo a proteolytical processing that eliminates their ankyrin motifs and generates proteins p50 and p52, respectively. These mature p50 and p52 are able to homo- or heterodimerize with other members of the Rel/NF- κ B family, thus becoming active transcription factors (reviewed in Miyamoto and Verma, 1995; Verma *et al.*, 1995).

In most cell types, Rel/NF- κ B dimers are found in an inactive form sequestered in the cytoplasm by an ankyrin repeat protein of the I κ B family. The I κ B family is composed of I κ B α , I κ B β , and Bcl3, as well as NF- κ B1 and NF- κ B2 precursors in their unprocessed form. The activation of the Rel/NF- κ B proteins is regulated by a posttranslational mechanism, which includes the phosphorylation of the I κ B inhibitor and its subsequent degradation, thus liberating the Rel/NF- κ B dimer, which can translocate into the nucleus (reviewed in Verma *et al.*, 1995; Miyamoto and Verma, 1995). The inhibitor degradation occurs within minutes after stimulation, making the Rel/NF- κ B transcription factors very efficient when rapid responses are needed. The immune system takes advantage of this property and extensively uses the Rel/NF- κ B proteins for regulating cytokine, growth factor, acute phase protein, and immunoreceptor gene expression (review in Bauerle and Henkel, 1994; Kopp and Ghosh, 1995). The Rel/NF- κ B proteins are also involved in pathogenesis of the immune system. In humans, alterations at the *rel* locus have been found in follicular and diffuse large cell lymphomas (Lu *et al.*, 1991) and amplifications of the *c-rel* gene have been detected in primary mediastinal thymic B-cell lymphoma (Joos *et al.*, 1996) and in extranodal diffuse large cell lymphoma (Houldsworth *et al.*, 1996).

The expression of c-Rel has been widely described in the hematopoietic organs during mouse development. In the fetal liver, the c-Rel protein expression is restricted to hematopoietic precursor cells of the erythroid and B-cell lineage. In the spleen, c-Rel expression is also found in B cells. In the embryonic thymus, c-Rel is essentially detected in medullary

epithelial cells and in some B cells, whereas thymocytes do not express any c-Rel protein. In the T-cell lineage, only mature helper and cytotoxic T cells of the lymph nodes express c-Rel (Carrasco *et al.*, 1994). A better understanding of the mouse c-Rel functions in hematopoiesis and immune system was obtained with the establishment of null mice (Köntgen *et al.*, 1995). In these mice, no developmental defect in any hematopoietic lineage could be evidenced, indicating that c-Rel is not essential for embryonic hematopoiesis. However, *c-rel*-deficient mice displayed defects in humoral immunity and unresponsiveness of mature B and T cells to most mitogenic stimuli.

In the avian embryo, such exhaustive studies have not yet been performed. Nevertheless previous *in vitro* analysis suggested a potential involvement of some Rel/NF- κ B members in avian hematopoiesis (reviewed in Huguet *et al.*, 1994). Northern blot analysis revealed that *c-rel* is predominantly expressed in chick hematopoietic organs such as the bursa of Fabricius, the thymus, the spleen, and the bone marrow (Moore and Bose, 1989). Moreover, the avian retrovirus Rev-T, bearing the v-Rel oncogene, causes fatal lymphomatosis in chicken (Sevoian *et al.*, 1964; Olson, 1967). The *in vivo* preferential target cells for Rev-T are immature and mature lymphoid cells expressing B- and T-cell determinants as well as myeloid cells (Lewis *et al.*, 1981; Barth *et al.*, 1990; Zhang *et al.*, 1991). *In vitro* studies also showed that the majority of v-Rel transformed hematopoietic cells bore lymphoid markers (Morrison *et al.*, 1991). More recently, a v-RelER fusion protein has been shown to transform an early progenitor of dendritic and neutrophil cells (Boehmelt *et al.*, 1995).

Altogether, these results prompted us to further investigate the relationships between c-Rel and hematopoiesis in chicken. We focused our study on the embryonic thymus that constitutes the primary site of T-cell development. Since the activity of the avian c-Rel is tightly dependent on interactions with the I κ B α inhibitor (Morrison *et al.*, 1989; Davis *et al.*, 1990; Kerr *et al.*, 1991; Kochel *et al.*, 1991; Hrdlickova *et al.*, 1995; Schatzle *et al.*, 1995), we studied in parallel the expression of both genes and searched for the

nucleocytoplasmic localization of the c-Rel protein, which gives evidence about its activation.

RESULTS

High Expression of *c-rel* in the Thymus Correlates with the Differentiation of the Medulla

The analysis of *c-rel* expression was performed at three stages representative of the avian thymus development: (1) at E9.5, the thymus is an epithelio-mesenchymal rudiment filled up with very immature precursor cells; (2) at E15, small medullary areas are separating from the cortex, figuring the maturation of the thymic stroma; (3) at E19, the avian thymic structure is almost completed with well-defined cortical lobules and large medullary areas. The cortex is essentially composed of double-positive CD4⁺CD8⁺ immature thymocytes enclosed in an epithelial meshwork, whereas the medulla is mainly composed of single-positive mature thymocytes among antigen-presenting cells and epithelial cells.

In situ hybridization experiments performed at E9.5 show that *c-rel* mRNAs are homogeneously expressed throughout the thymic organ [Figures 1(A) and 1(B)]. At E15, *c-rel* expression becomes heterogeneous with higher levels in the developing medulla than in the cortex [Figures 1(C) and 1(D)]. At E19, this heterogeneous distribution of *c-rel* mRNAs is even more accentuated [Figures 1(E) and 1(F)]. At both E15 and E19 stages, high magnifications show that inside the medulla the areas of highest expression correspond to cells with large cytoplasm and nucleus that could be clusters of dendritic or epithelial cells and macrophages, according to their morphology and localization. In contrast, cortical and medullary thymocytes, recognizable by a small round nucleus and a low cytoplasmic volume, express lower levels of *c-rel* mRNAs (Figure 2). Thus, during the thymus development, the high expression of *c-rel* correlates with the establishment of mature medullary areas, whereas the expression in developing thymocytes is continuous and at low levels.

The c-Rel Protein Is Expressed in Endothelial, Epithelial, and Antigen-Presenting Cells of the Thymus

To investigate the *c-rel* expression at the protein level, Western blot experiments were performed with an immuno-purified anti-c-Rel antibody on hematopoietic organs at different developmental stages. One major band at 68 kD, corresponding to the avian c-Rel protein molecular mass (Simek and Rice, 1988), is detected in the thymus at E15 and E19, although less intensely than in the spleen and the bursa of Fabricius (Figure 3). Immunocytochemistry experiments were performed with this same anti-c-Rel antibody on serial thymus sections to identify the c-Rel-expressing cells. At E15 and E19, consistent with its mRNA distribution, the c-Rel protein is predominantly expressed inside the medulla in large clusters of cells (Figure 4). Some isolated cells scattered in the cortex also express c-Rel. Cortical as well as medullary thymocytes, identified with anti-CD3, -CD4, and -CD8 antibodies (data not shown), do not express any detectable amounts of c-Rel. At least four different types of cells were found to express c-Rel. The most easily identifiable are endothelial cells bordering the thymic vessels [Figure 5(A)]. Epithelial cells of different morphology and localization, identified with an anti-cytokeratin antibody (data not shown), also express c-Rel: Some line the cortical lobules [Figure 5(B)] and others form large clusters inside the central medulla [Figure 5(C)]. Other medullary clusters, containing two to three large cells with large vacuoles, also express c-Rel. In these cells, the protein is clearly localized in the cytoplasm [Figure 5(D)]. These cells displayed the violet color characteristic of myeloid cells when stained by the panoptic method of Pappenheim (data not shown). According to their morphology, localization and staining properties, they could be dendritic cells. At the corticomedullary junction, inside the medulla and scattered inside the cortex, isolated large round cells also express c-Rel in their cytoplasm [Figure 5(E)]. These cells possess a characteristic eccentric nucleus that is clearly negative. We assume that these cells are macrophages because of their morphology, pink staining by the

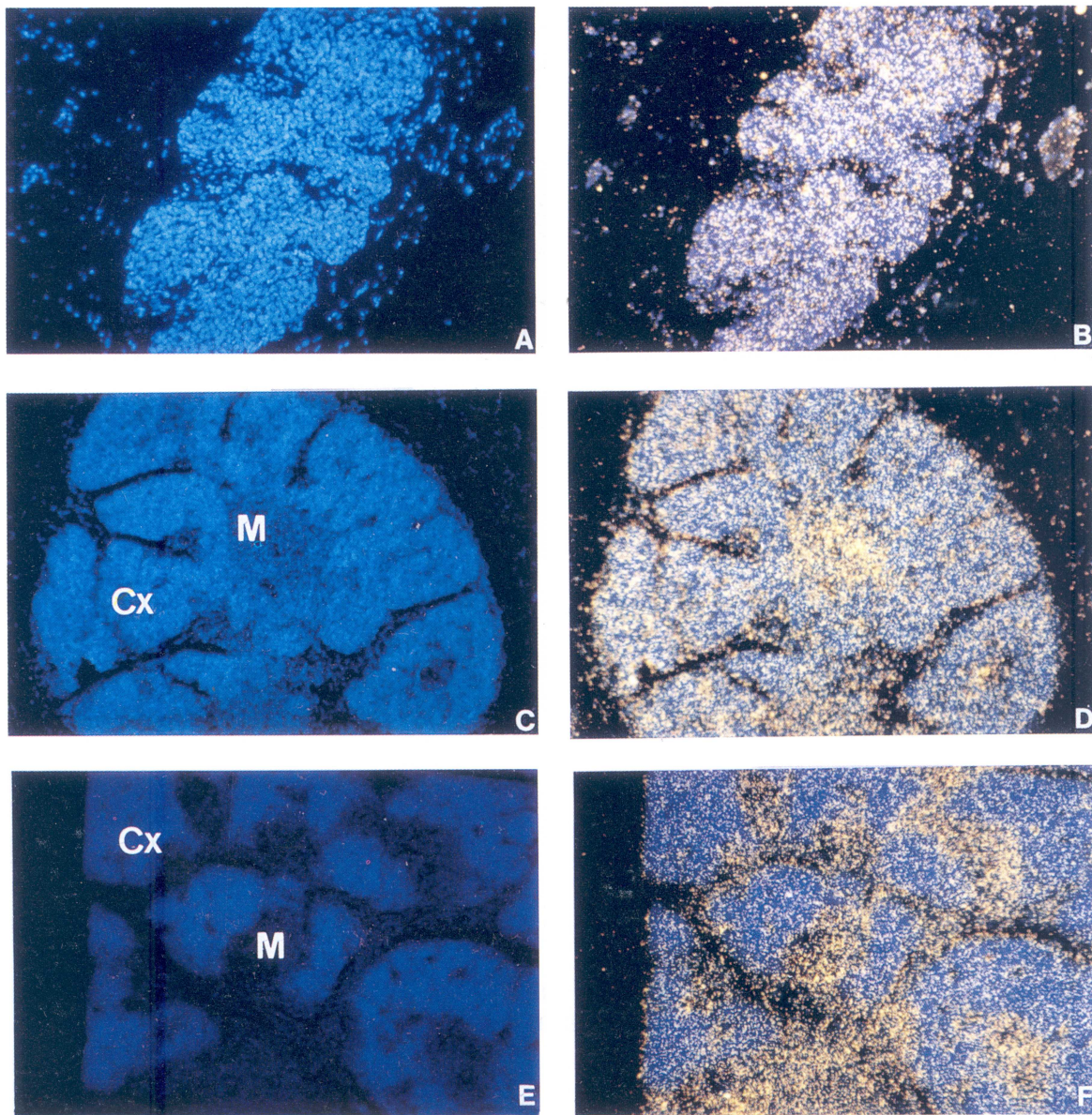


Figure 1 (See Color Plate at back of issue.) *In situ* hybridization analysis of *c-rel* expression pattern in E9.5, E15, and E19 thymuses. In order to visualize the nuclei, sections were counterstained with the DNA intercalant Hoechst 33258, which fluoresces in blue under UV illumination. Sections were observed under UV illumination alone (A, C, and E) or UV and dark field illumination (B, D, and F). (A and B) At E9.5, *c-rel* is homogeneously expressed. (C and D) At E15, *c-rel* expression becomes heterogeneous with higher expression in the medulla (M) than in the cortex (Cx). (E and F) At E19, the heterogeneous expression of *c-rel* is even more obvious with the highest levels in the medulla. Magnification: $\times 100$.

Pappenheim method (data not shown), and because some of them, probably the most mature, display a nonspecific esterase activity detectable *in situ* characteristic of the monocytic lineage (Duncan and McArthur, 1978) (data not shown).

The *c-rel* expression in dendritic cells and macrophages was further characterized in primary cell culture. These cells were isolated from E19 thymuses by fractionation on a bovine serum albumin gradient and cultured on the basis of their glass-adherence properties (Oliver and Le Douarin, 1984). In culture, dendritic cells appear as long bipolar cells with the nucleus at one pole and large vacuoles. The *c-Rel* protein is clearly observed in the cytoplasm, whereas there is no detectable protein in the nucleus [Figure 5(F)]. Macrophages appear as large round cells with an eccentric nucleus. In these cells, *c-Rel* is also restricted to the cytoplasm [Figure 5(G)].

Lymphocyte Precursor Cells Express the *c-Rel* Protein Both in Their Cytoplasm and Nucleus

In the cortex at E19, a few round cells, larger than most of the cortical thymocytes, also express *c-Rel*. They are usually located in the marginal cortex and often observable in only one lobule [Figure 6(A)]. Unlike the majority of cortical thymocytes, numerous cells of the marginal cortex do not express any of the CD3, CD4, or CD8 cell-surface markers (data not shown), indicating that they are very immature (Dieterlen-Lièvre, 1994). Moreover, the Pappenheim coloration reveals that the large round cells of the marginal cortex are stained in dark blue compared to more mature thymocytes that appear pale blue [Figure 6(C)]. This dark blue staining indicates that they are highly basophilic, a characteristic of lymphocyte precursor cells (LPCs). Therefore, to further identify

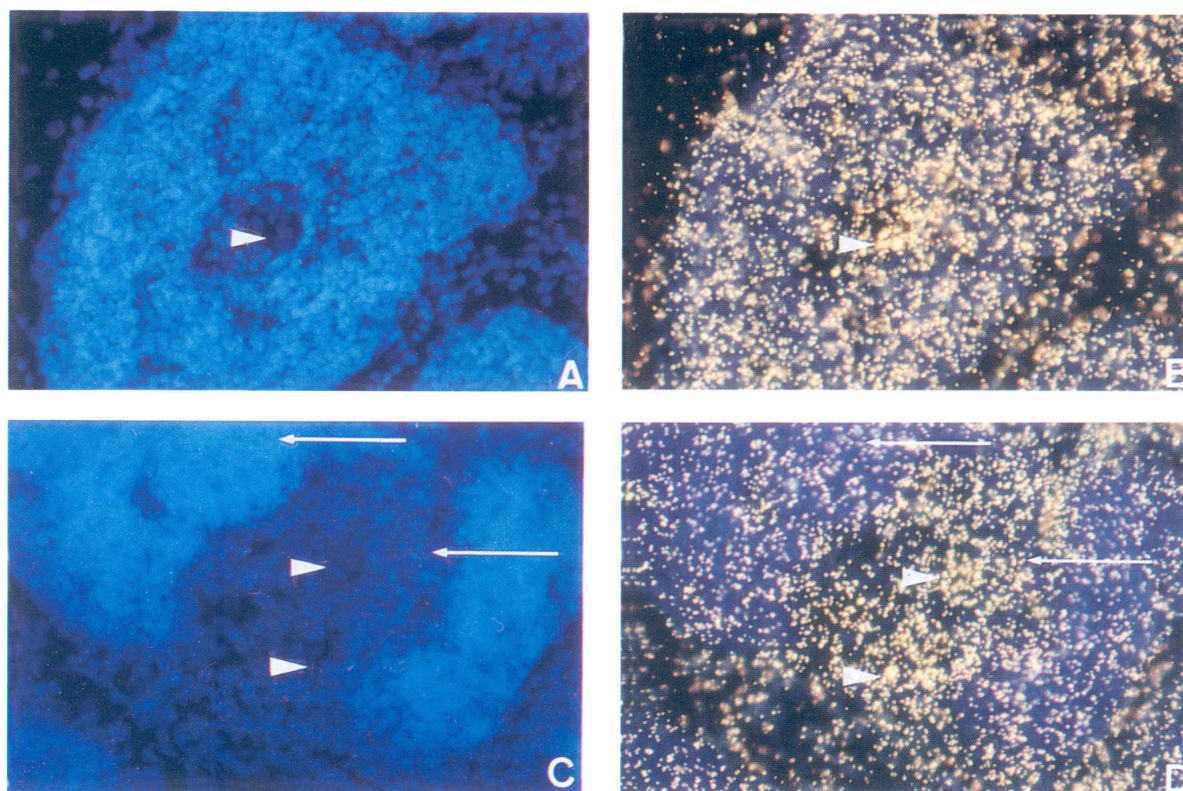


Figure 2 (See Color Plate at back of issue.) Higher magnification of *c-rel* expression in the medulla of E15 and E19 thymuses. (A and C) Hoechst staining. (B and D) *In situ* hybridization. At both stages E15 and E19, the highest expressions are found inside the medulla in clusters of large cells with large nuclei appearing as dark areas (arrowheads). Cortical and medullary thymocytes express low levels of *c-rel* (arrows). Magnification: $\times 300$.

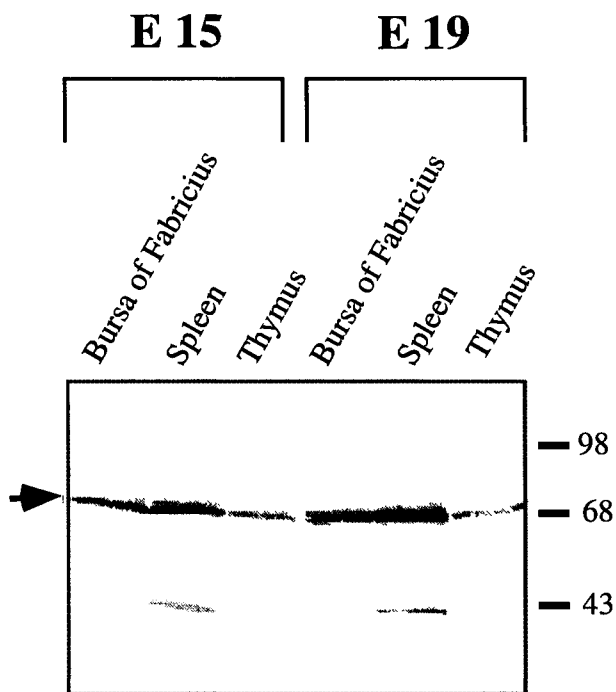


FIGURE 3 Western blot of protein extracts of E15 and E19 hematopoietic organs. The p68^{c-rel} protein is indicated by an arrow. The lower band seen in spleen extracts was shown to be a breakdown product (data not shown).

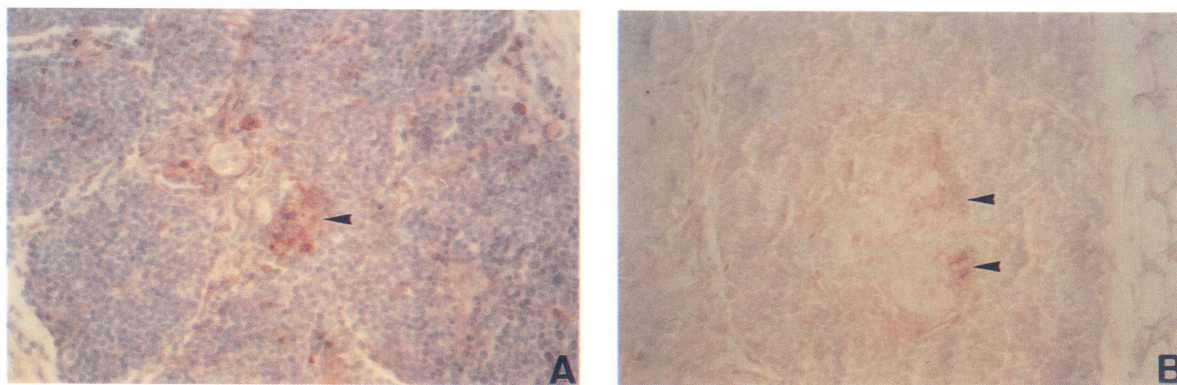


Figure 4 (See Color Plate at back of issue.) Immunocytochemical detection of c-Rel in E15 and E19 thymuses. Cells expressing c-Rel are stained in red in contrast to negative cells, which appear in blue due to hematoxylin counterstaining. At both stages (A) E15 and (B) E19, c-Rel is predominantly expressed in cell clusters in the medullary areas (arrowheads). Magnification: $\times 200$.

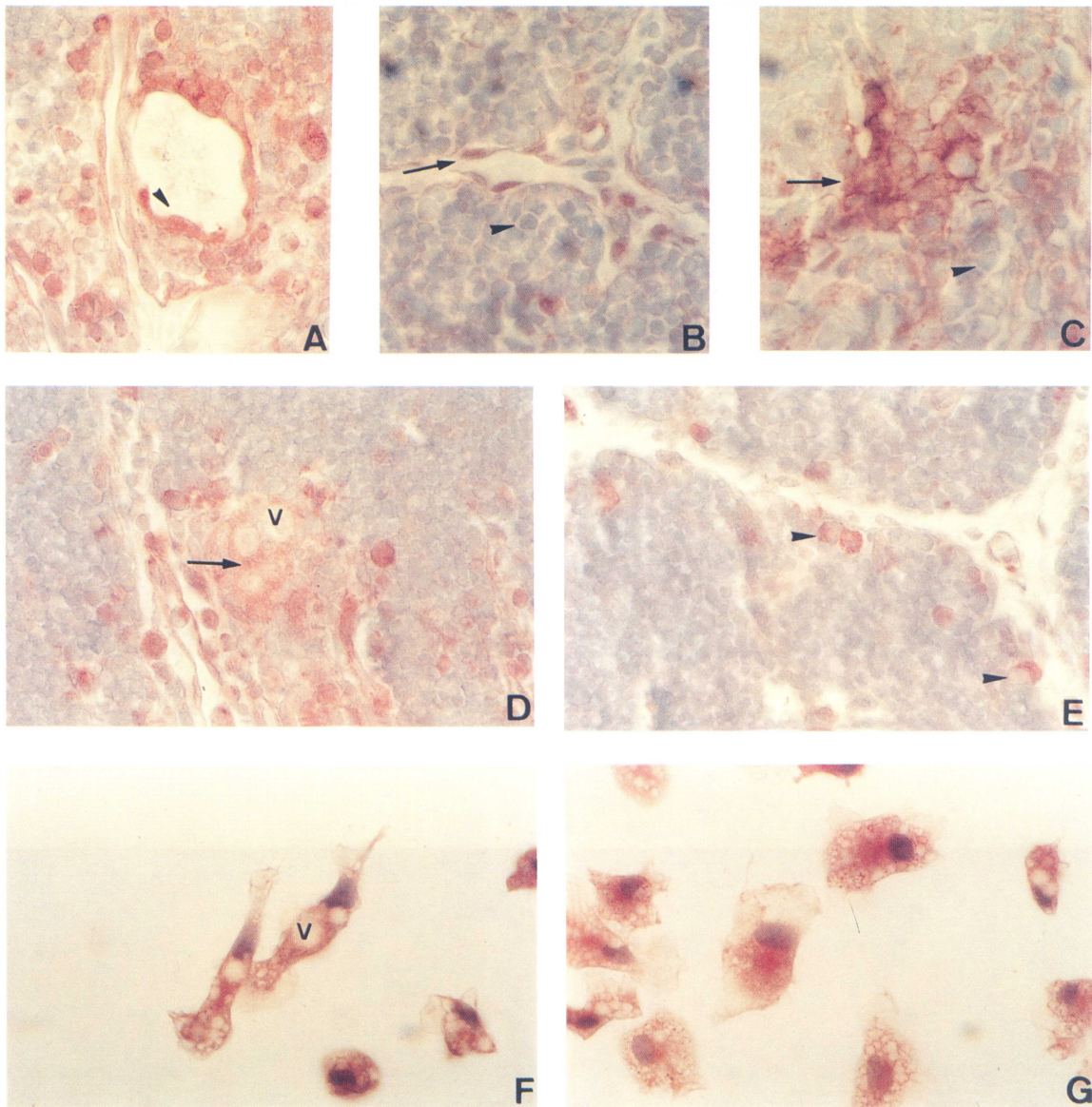


Figure 5 (See Color Plate at back of issue.) High magnification of the immunocytochemical detection of c-Rel in E19 thymus and in primary cell culture. (A) c-Rel is detected in endothelial cells (arrowhead). (B) Epithelial cells lining the cortical lobules express c-Rel (arrow), whereas cortical thymocytes are clearly negative (arrowhead). (C) c-Rel is also detected in clusters of medullary epithelial cells (arrow); medullary thymocytes are negative (arrowhead). (D) Clusters of dendritic cells with vacuoles (V) express c-Rel in their cytoplasm (arrow). (E) Isolated macrophages with an eccentric nucleus also express c-Rel in their cytoplasm (arrowheads). (F and G) Detection of c-Rel in primary culture of dendritic cells and macrophages: (F) long bipolar dendritic cells with large vacuoles (V) and (G) macrophages with an eccentric nucleus express c-Rel in their cytoplasm. In both type of cells, the nuclei are clearly negative and appear stained in blue by the hematoxylin. Magnification: $\times 550$.

these cells, we investigated the expression of c-Rel during the first wave of thymus colonization by LPCs.

The thymus rudiment is colonized by three consecutive waves of LPCs: from E6.5 to E8, from E12

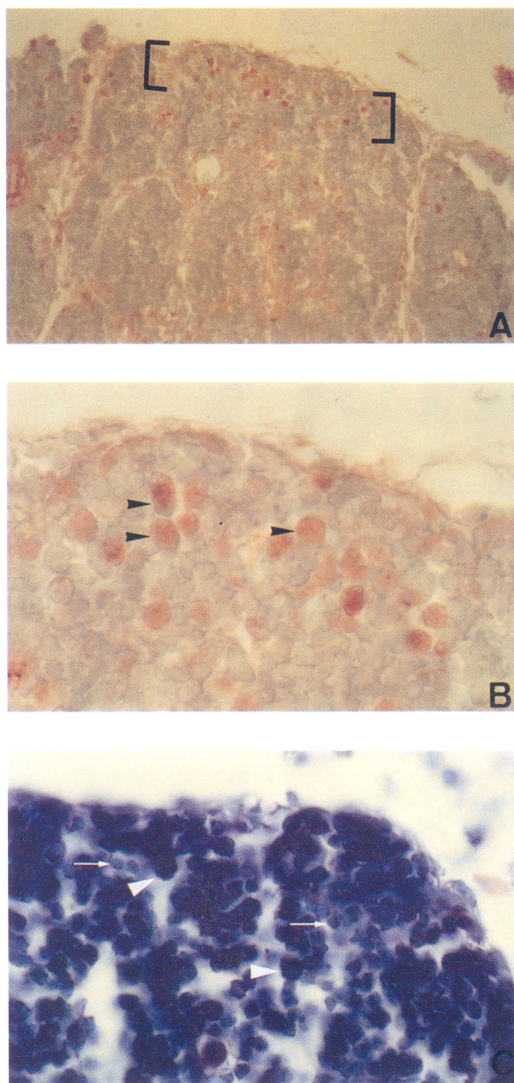


Figure 6 (See Color Plate at back of issue.) Immunocytochemical detection of c-Rel in lymphocyte precursor cells (LPCs) in E19 thymus. (A) In the marginal cortex (brackets), some LPCs express c-Rel. (B) High magnification showing that c-Rel is localized in the nucleus and cytoplasm of these cells (arrowheads). (C) LPCs are stained in dark blue by the Pappenheim coloration (arrowheads) compared to small cortical thymocytes that are pale blue (arrows). Magnification: $\times 200$ in (A) and $\times 750$ in (B) and (C).

to E14, and from E18 to hatching. The first wave of LPCs gives rise to the myeloid and lymphoid cells of the thymus; they are renewed in the successive waves of colonization. Between the three colonization periods, the thymus is refractory to the entry of precursor cells (Coltey *et al.*, 1987). To estimate the number and localization of LPCs in thymus sections, we used the panoptic Pappenheim coloration. At E8.5, just after the first wave of colonization, round dark blue cells are detected [Figure 7(A)]. Comparison with sections treated with the anti-c-Rel antibody indicates that no cells express c-Rel at this stage [Figure 7(B)]. At E9.5, the thymus is filled up with dark blue stained LPCs. the majority of these cells express high levels of c-Rel [Figures 7(C) and 7(D)]. At E11, nearly no large round cells stained in dark blue are detected; the majority of round cells are small and stained in pale blue, indicating that they are already differentiated thymocytes [Figure 7(E)]. These cells do not express c-Rel [Figure 7(F)]. Thus, c-Rel is expressed in LPCs during a limited period between the homing and the beginning of the differentiation. Observation at high magnification of sections at E9.5 and E19 reveals that c-Rel is localized in both the cytoplasm and nucleus of LPCs [Figures 6(B) and 7(D)].

Nuclear or Cytoplasmic Localization of c-Rel Correlates Respectively with Low and High Levels of *ikba* Gene Expression

Since the nucleocytoplasmic localization of c-Rel in antigen-presenting cells and lymphocyte precursor cells differs, we investigated the expression of *ikba*, the gene that encodes $I\kappa B\alpha$, in these cells at E9.5 and E19.

At E19, the *ikba* gene shows high levels of expression in the medulla, whereas weaker signals are found in the cortex [Figures 8(A) and 8(B)]. Within the medulla, intense signals are detected in clusters of large cells with large nucleus as well as in thymocytes [Figures 8(C) and 8(D)]. The expression of *ikba* is also detected in round isolated macrophages located at the cortico-medullary junction and in the interlobular spaces (data not shown). The expression in macrophages and dendritic cells was confirmed by *in*

situ hybridization on primary cell cultures [Figure 8(E)]. Additionally, *ikba* is highly expressed in endothelial cells [Figures 8(F) and 8(G)] and in epithelial cells lining the cortex [Figures 8(H) and

8(I)]. At E9.5, the *ikba* expression is heterogeneous. It is highly expressed in a few isolated cells, probably corresponding to the first differentiating antigen-presenting cells, while it is expressed at low levels in

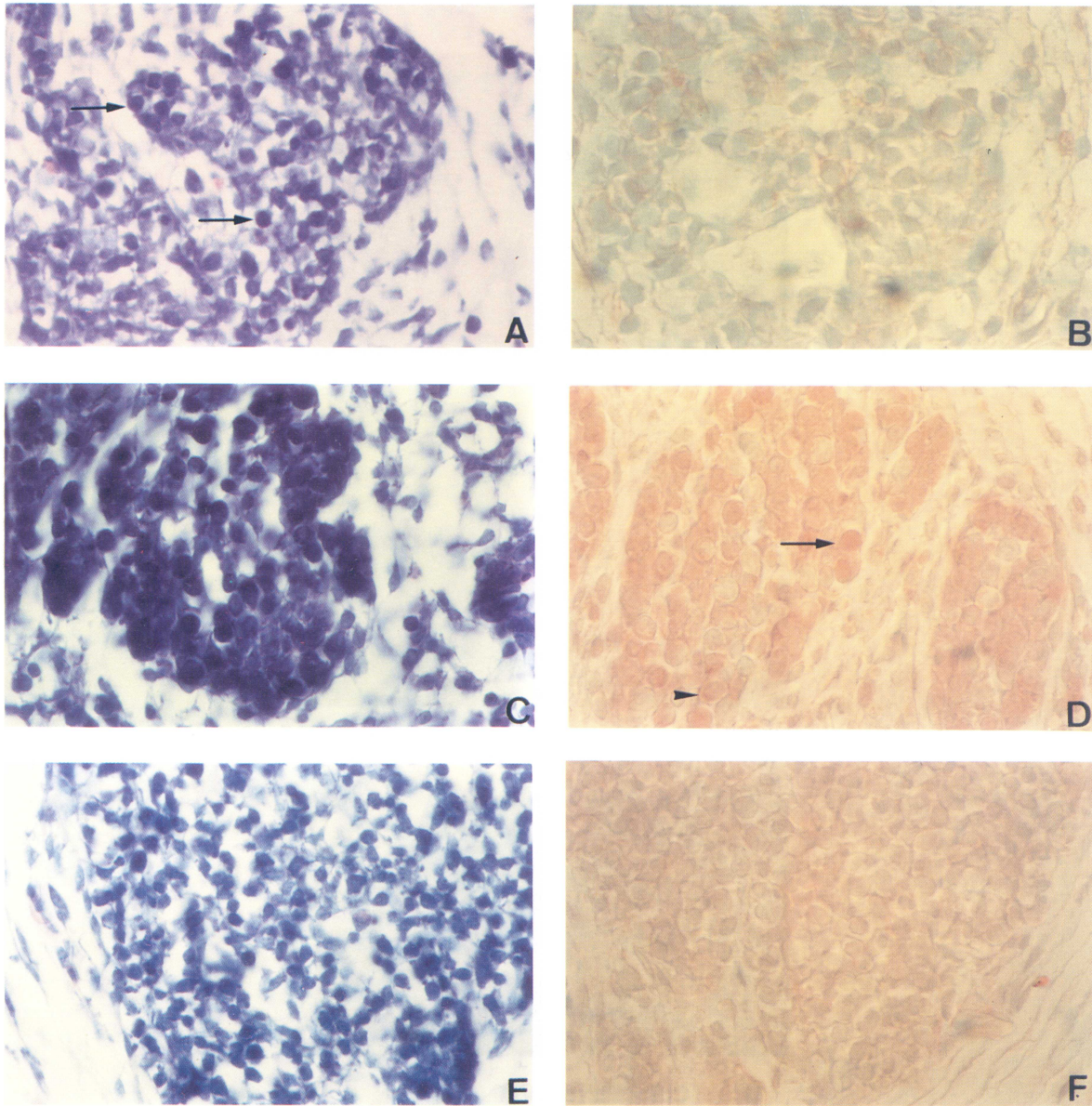


Figure 7 (See Color Plate at back of issue.) Immunocytochemical detection of c-Rel in LPCs during the first wave of colonization. Pappenheim coloration (A, C, E) and immunocytochemistry (B, D, F). At E8.5: (A) a few large round LPCs are stained dark blue by the Pappenheim coloration (arrows); (B) c-Rel is not detected in these cells. At E9.5: (C) the thymus is filled up with dark blue stained LPCs; (D) the majority of these cells express c-Rel and the protein is detected in the cytoplasm (arrowhead) or both in the nucleus and cytoplasm (arrows). At E11: (E) the thymus mainly contains differentiating thymocytes that are small cells stained in pale blue; (F) they do not express c-Rel. Magnification: $\times 550$.

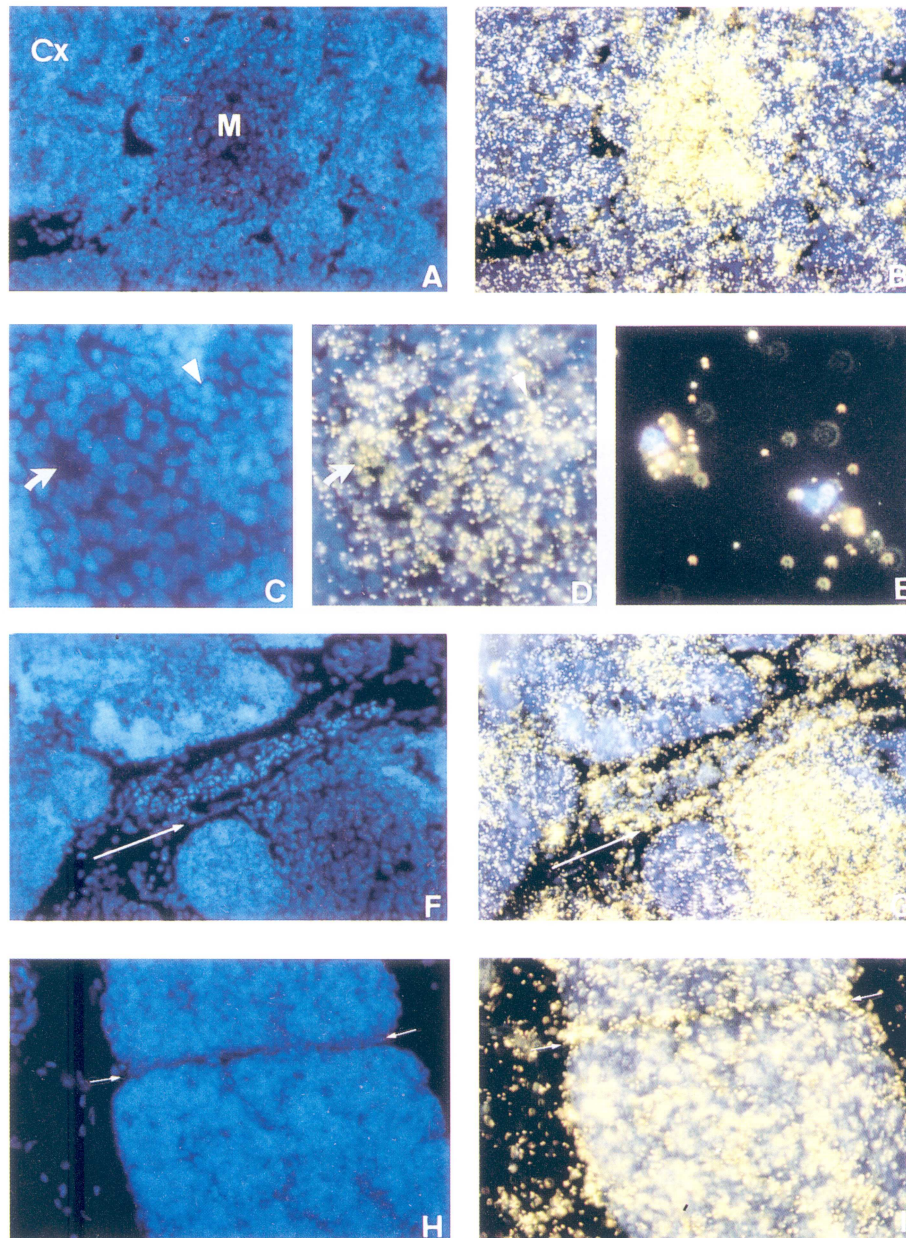


Figure 8 (See Color Plate at back of issue.) *In situ* hybridization analysis of *ikba* expression in E19 thymus and primary cell culture. (A, C, F, and H) Hoechst staining. (B, D, E, G, and I) *In situ* hybridization. (A and B) At E19, *ikba* is highly expressed in the medulla (M). (C and D) Higher magnification of the medulla shows *ikba* expression in clusters of large cells appearing as dark areas (arrows) and in medullary thymocytes (arrowheads). (E) *ikba* expression in primary culture of macrophages and dendritic cells. (F and G) *ikba* is also expressed in endothelial cells (arrow) and (H and I) in epithelial cells lining the cortical lobules (arrows). Magnification: $\times 200$ in (A) and (B), $\times 550$ in (C to E), $\times 300$ in (F to I).

the majority of lymphocyte precursor cells (Figure 9). In summary, *ikba* expression is high in all of the stromal cells, where c-Rel protein is cytoplasmic. In contrast, low levels of *ikba* are detected in LPCs, where c-Rel is detected in both the cytoplasm and nucleus.

DISCUSSION

Previous studies have shown that the mouse proto-oncogene *c-rel* is involved in mature T-cell activation and in T-cell education via an indirect role in thymic epithelial cells (Carrasco et al., 1994; Köntgen et al., 1995). In this study, we investigated the expression of the avian proto-oncogene *c-rel* and its I κ B α inhibitor during the T-cell development occurring in the chick embryonic thymus.

c-Rel Is Expressed in the Nucleus of Proliferating Lymphocyte Precursor Cells

Immunocytochemistry experiments pointed out the expression of c-Rel in a specific subset of T cells, the lymphocyte precursor cells (LPCs), where it had never been detected so far. Colonization of the avian thymus by LPCs has been widely studied through the construction of quail-chick chimeras (Le Douarin and Jotereau, 1975; Coltey et al., 1987). The thymus

rudiment is seeded by three waves of LPCs from E6.5 to E8, from E12 to E14, and from E18 onwards. Between these three periods, the thymus is refractory to hematopoietic cell entry. LPCs of the second wave stay dormant about 3 days before starting proliferation (Jotereau and Le Douarin, 1982). Differentiation of LPCs gives birth to three T-cell sublineages. The first fully differentiated cells appear at E12; they express the TCR1/CD3 complex. Cells expressing the TCR2/CD3 complex appear at E14-15. The TCR3/CD3 cells constitute the last T-cells to differentiate from E17 (reviewed in Dieterlen-Lièvre, 1994). At E8.5, after the end of the first wave of colonization, LPCs in the thymus rudiment do not express c-Rel. At E9.5, the number of LPCs in the thymus has greatly increased. Most of them express c-Rel. This expression is no longer detected at E11 when differentiation of the first T-cell sublineage begins. Thus, the expression of c-Rel in LPCs seems to occur during their proliferation and to be induced at E9.5. c-Rel-expressing LPCs were also observed at E19. At this stage, temporal correlations are more difficult to establish since the duration of the third wave of colonization is not well defined.

It is worth to notice that in LPCs, c-Rel is expressed both in the cytoplasm and nucleus. Therefore, c-Rel might be constitutively activated in proliferating LPCs and that way may sustain their proliferation. A constitutive activation of c-Rel has already been

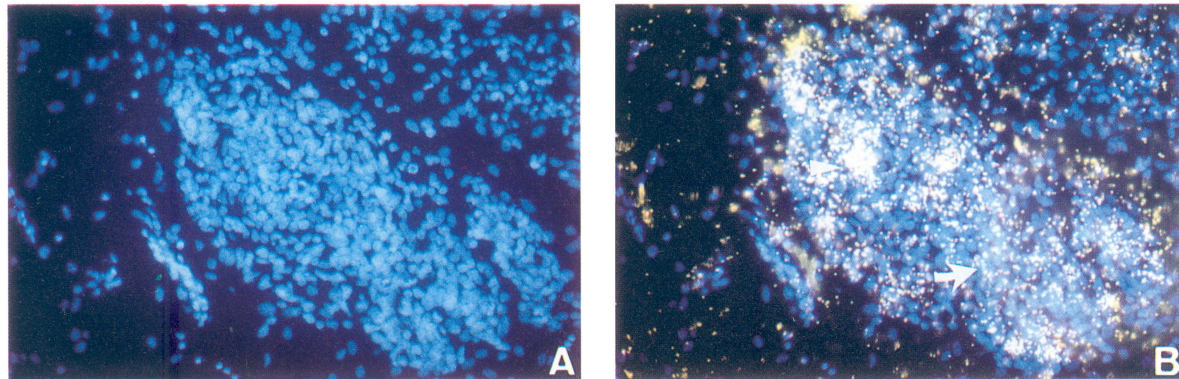


Figure 9 (See Color Plate at back of issue.) *In situ* hybridization analysis of *ikba* expression in E9.5 thymus. (A) Hoechst staining. (B) *In situ* hybridization. At E9.5, *ikba* is heterogeneously expressed throughout the organ. LPCs, which represent the majority of cells, only express low levels of *ikba* (arrows). High expressing cells (arrowheads) cannot be clearly identified. Magnification: $\times 200$.

described in a mature mouse B-cell line and was explained by a high and continuous degradation of $\text{I}\kappa\text{B}\alpha$ in the absence of external signals (Miyamoto *et al.*, 1994). Here, we show that avian LPCs express low levels of $\text{I}\kappa\text{B}\alpha$, suggesting that the constitutive nuclear localization of c-Rel might be due to a low expression of the inhibitor. This hypothesis requires further investigation of $\text{I}\kappa\text{B}\alpha$ expression at the protein level.

The involvement of c-Rel in proliferation has already been suggested by *in vitro* experiments showing that overexpression of c-Rel in chicken embryo fibroblasts led to morphological transformation, life-span extension, and proliferation (Abbadie *et al.*, 1993; Kralova *et al.*, 1994). The constitutive nuclear localization of c-Rel in LPCs documented in this paper represents the first *in vivo* argument in favor of a participation of this transcription factor in the proliferation of very immature progenitor of T cells.

c-Rel Is Expressed in the Cytoplasm of Thymic Antigen-Presenting Cells

In avian thymuses from E15 to E19, c-Rel is expressed in epithelial cells, dendritic cells, and macrophages. Avian thymic epithelial cells, dendritic cells, and macrophages share common surface antigens and particularly MHC molecules (Peck *et al.*, 1982; Guillemot *et al.*, 1984; Boyd *et al.*, 1992); they constitute the three main types of antigen-presenting cells (APCs) of the thymus. These cells govern positive and negative selection of T cells in the thymus, via interactions between their MHC and the T-cell receptor (TCR) (reviewed in von Boehmer, 1994; Nossal, 1994).

In vitro studies have shown that avian MHC class I and II genes are induced when c-Rel or v-Rel are overexpressed (Hrdlickova *et al.*, 1994). Therefore, a potential function of c-Rel in APCs could be to regulate the expression of MHC genes. However, in macrophages, besides genes involved in T-cell maturation, c-Rel could regulate genes involved in basic antimicrobial functions, since in HD11 cells, a chicken myelomonocytic cell line, RelA, and c-Rel

are able to activate the transcription of the lysozyme gene (Phi Van, 1996).

In vitro experiments have demonstrated that in chicken, $\text{I}\kappa\text{B}\alpha$ is able to inactivate c-Rel by sequestering it in the cytoplasm (Davis *et al.*, 1990; Kerr *et al.*, 1991). In accordance with these studies, our *in situ* hybridization analysis revealed that *in vivo*, high expression of *ikba* is detected in all of the stromal cells that express the c-Rel protein in the cytoplasm, that is, in endothelial cells, epithelial cells, dendritic cells, and macrophages.

The expression pattern of c-Rel is more widespread in chick than in mouse APCs, where c-Rel appears restricted to medullary epithelial cells. Moreover, in mouse, the expression of c-Rel in progenitor cells is restricted to the erythroid and B-cell lineages (Carrasco *et al.*, 1994). Our results on *ikba* are also different from those obtained in mouse. In the mouse thymus, *ikba* expression is higher in the cortex than in the medulla (Weih *et al.*, 1994).

In conclusion, both avian and murine c-Rel proteins might be indirectly involved in T-cell maturation via their expression in antigen-presenting cells, but this expression seems restricted to epithelial cells in mouse, whereas it also extends to dendritic cells and macrophages in chicken. In contrast, the sole avian c-Rel protein might be also involved in early lymphopoiesis via its expression in proliferating lymphocyte precursor cells.

MATERIAL AND METHODS

Animals

Fertilized White Leghorn chicken eggs were incubated at 39°C in a humidified chamber. The age of embryos is indicated as E1, E2, ..., E1 corresponding to 24 hr of incubation.

Histological Sections

Chick embryo thymuses at different developmental stages were dissected in PBS and fixed at 4°C for 18 hr in 4% paraformaldehyde in PBS (Na_2HPO_4 - NaH_2PO_4 , 0.1 M, pH 7.4), then dehydrated in ethanol

and toluene, embedded in paraffin, and processed for 5-6- μ m histological sections.

Isolation and Primary Culture of Macrophages and Dendritic Cells

The protocol was adapted from Oliver and Le Douarin (1984). E19 thymuses were dissected, minced with scissors, and crushed through a fine nylon gauze, which retains connective tissue and epithelial cells. The collected cell suspension was rinsed in culture medium (RPMI 1640, 10% FCS, 1000 IU/ml penicillin, 1000 μ g/ml streptomycin) and resuspended in 10% BSA (Sigma) in RPMI 1640 buffered with Hepes pH 7.3. A discontinuous gradient of 22%, 27%, and 29% BSA in RPMI 1640, pH 7.3, was prepared in a 5-ml polyallomer tube and the cell suspension was layered on top of the gradient. After centrifugation at 35,000 g for 30 min, the fraction located above the 22% layer was collected. Cells enriched in macrophages and dendritic cells were rinsed in culture medium and plated on glass coverslips for 4 hr at 37°C in 5% CO₂ humidified incubator. Contaminating thymocytes were eliminated by hard pipetting. Adherent cells were fixed in 4% paraformaldehyde in PBS overnight at 4°C and processed for *in situ* hybridization or immunocytochemistry.

In Situ Hybridization

Synthesis of ³⁵S RNA Probes

The chicken full-length *c-rel* c-DNA was cloned in Bluescript SK⁻ (Stratagene) (Abbadie et al., 1993). A 0.9-kb EcoRI fragment from the chicken *ikba* cDNA, kindly provided by H. Bose, was cloned in Bluescript SK⁻ phagemid (Stratagene). ³⁵S RNA probes were transcribed from 2 μ g of linearized plasmids by 20 U of either T7, T3, or SP6 RNA polymerase for sense and antisense probes in a 20- μ l reaction mixture containing 200 μ Ci ³⁵S-CTP (1300 Ci/mMol), 200 μ M UTP, ATP, and GTP for 1 hr at 39°C. To facilitate their penetration into cells, probes were submitted to a limited alkaline hydrolysis generating

fragments of approximately 150 bases as recommended by (Cox et al., 1984).

In Situ Hybridization

In situ hybridization was adapted from the method of Cox et al., (1984), as described by Quéva et al. (1992). Briefly, after being deparaffinized and rehydrated, sections were incubated in 0.1 M glycine, 0.2 M Tris-HCl, pH 7.4, for 10 min at 20°C, treated with 1 μ g/ml proteinase K (Boehringer Mannheim) for 15 min at 37°C, postfixed in 4% paraformaldehyde, washed in PBS, acetylated 10 min with 0.25% acetic anhydride in 0.1 M triethanolamine, washed in 2X SSC (0.3 M NaCl, 30 mM sodium citrate), and dehydrated in ethanol. ³⁵S RNA probes were denatured at 80°C and diluted in the hybridization buffer at a concentration of 50 pg/ μ l. Hybridization was performed at 60°C for 18 hr. After a wash in 4X SSC at 20°C, slides were treated with 10 μ g/ml of RNase A (type III A, Sigma) for 30 min at 37°C, subsequently washed in 0.1X SSC at 60°C, dehydrated by ethanol, and dipped in nuclear track emulsion (Kodak NTB2). The sections were exposed at 4°C for 2 weeks. After developing, sections were stained with a DNA intercalating fluorescent dye (Hoechst 33258), and mounted and observed under dark-field and UV illumination. Both antisense and sense probes were used, and the sense probes never gave any signal.

Immunocytochemistry and Western Blot

Antibodies

A rabbit immuno-purified anti-c-Rel serum (SB146) raised against the 15 carboxy terminal amino acids specific of the chicken c-Rel protein (Abbadie et al., 1993) was used both in Western blot and immunocytochemistry experiments. A normal rabbit immunoglobulin fraction (Dako) was used as negative control. A rabbit polyclonal anti-keratine serum (Dako) was used in immunocytochemistry to identify epithelial cells. Mouse monoclonal anti-chicken-CD3, -CD4,

and -CD8 antibodies (Southern Biotechnology Associates) were used in immunocytochemistry to identify the different types of thymocytes.

Immunocytochemistry

After being deparaffinized and rehydrated, sections were incubated in 0.5 mg/ml Saponine (Sigma) in PBS for 30 min; then endogenous peroxidase activity was quenched by incubation in 80% methanol, 20% PBS, 0.6% H₂O₂ for 30 min followed by 30 min saturation in 5% dry defatted milk in PBS. Sections were incubated with the primary antibody overnight at 4°C. After three washes in PBS, the EXTRA-3 kit (Sigma Immunochemicals) and the AEC substrate system (Dako) were used to detect the primary antibody. Sections were counterstained with hematoxylin (Sigma) and mounted in Glycergel (Dako).

Western Blot of Hematopoietic Organs

Hematopoietic organs at various developmental stages were dissected in PBS, transferred into a solution containing 0.06 M tris-HCl, pH 6.8, 5% β -mercaptoethanol, 2% SDS, 10% glycerol, 0.002% bromophenol blue, homogenized in a Dounce, sonicated and centrifuged at 10,000 g. In order to measure protein concentration in each sample, 20- μ l aliquots were separated on a 10% SDS-PAGE; proteins were then stained with Coomassie-blue and the gel scanned lane by lane. Equicharged 10% SDS-PAGE were run and transferred onto nitrocellulose sheets. Blots were first incubated 30 min with 5% dry defatted milk in PBS, then 2 hr at room temperature with SB146. After three washes in PBS, the sheets were incubated with horseradish-peroxidase-conjugated swine anti-rabbit immunoglobulin (Dako) for 2 hr at room temperature. After washing, the peroxidase activity was revealed by incubation in diaminobenzidine (30 mg in 100 ml 0.05 M tris-HCl, pH 7.4, 0.01% H₂O₂).

Panoptic Coloration of Pappenheim

This protocol of hematological coloration is described in Gabe (1968). Briefly, after being deparaffinized

and rehydrated, sections were stained 3 min in May-Grünwald, 3 min in May-Grünwald/Sörensen buffer (Na₂HPO₄-KH₂PO₄, 0.1 M, pH 6.8) V/V, washed in Sörensen buffer, stained in Giemsa/Sörensen buffer 1V/75V for 15 min, washed in Sörensen buffer, differentiated in 0.15% acetic acid, washed in Sörensen buffer, and dehydrated in two washes of acetone and one wash of toluene. Sections were then mounted in Eukitt (O. Kindler GmbH, Germany).

Acknowledgements

We are grateful to Eric Maire and Virginie Mattot for their technical help and advice. We thank Jean Coll, Pierre-Antoine Desfossez, Véronique Fafeur, Corine Glineur, and Fabrice Soncin for critical reading of the manuscript. This work was supported by grants from the Institut Pasteur de Lille, the Centre National de la Recherche Scientifique, and the Association pour la Recherche sur le Cancer. C.H. was supported by a training grant from the Association pour la Recherche sur le Cancer.

References

- Abbadie C., Kabrun N., Bouali F., Smardova J., Stéhelin D., Vandebunder B. and Enrietto P. (1993). High levels of *c-rel* expression are associated with programmed cell death in the developing avian embryo and in bone marrow cells *in vitro*. *Cell* **75**: 899-912.
- Barth C.F., Ewert D.L., Olson W.C. and Humphries E.H. (1990). Reticuloendotheliosis virus REV-T (REV-A)-induced neoplasia: Development of tumors within the T-lymphoid and myeloid lineages. *J. Virol.* **64**: 6054-6062.
- Bauerle P.A. and Henkel T. (1994). Function and activation of NF- κ B in the immune system. *Annu. Rev. Immunol.* **12**: 141-179.
- Boehmelt G., Madruga J., Dörfler P., Briegel K., Schwarz H., Enrietto P.J., and Zenke M. (1995). Dendritic cell progenitor is transformed by a conditional v-Rel Estrogen Receptor fusion protein v-RelER. *Cell* **80**: 341-352.
- Boyd R.L., Wilson T.J., Bean A.G. and Ward H.A. (1992). Phenotypic characterization of chicken thymic stromal elements. *Dev. Immunol.* **2**: 51-66.
- Carrasco D., Weih F. and Bravo R. (1994). Developmental expression of the mouse *c-rel* proto-oncogene in hematopoietic organs. *Development* **120**: 2991-3004.
- Coltey M., Jotereau F.V. and Le Douarin N.M. (1987). Evidence for a cyclic renewal of lymphocyte precursor cells in the embryonic chick thymus. *Cell Diff.* **22**: 71-82.
- Cox K.H., DeLeon D.V., Angerer L.M. and Angerer R.C. (1984). Detection of mRNAs in sea urchin embryos by *in situ*

- hybridization using asymmetric RNA probes. *Dev. Biol.* **101**: 485-502.
- Davis J.N., Bargmann W. and Bose H.J. (1990). Identification of protein complexes containing the *c-rel* proto-oncogene product in avian hematopoietic cells. *Oncogene* **5**: 1109-1115.
- Dieterlen-Lièvre F. (1994). Hemopoiesis during avian Ontogeny. *Poultry Sci. Rev.* **5**: 273-305.
- Duncan R.L. and McArthur W.P. (1978). Partial characterisation and the distribution of chicken mononuclear cells bearing the Fc receptor. *J. Immunol.* **120**: 1014-1020.
- Gabe M. (1968). *Techniques Histologiques* (Paris: Masson et Cie).
- Guillemot F.P., Oliver P.D., Peault B.M. and Le Douarin N. (1984). Cells expressing Ia antigens in the avian thymus. *J. Exp. Med.* **160**: 1803-1819.
- Houldsworth J., Mathew S., Rao P.H., Dyomina K., Louie C.D., Parsa N., Offit K. and Chaganti R.S.K. (1996). *REL* proto-oncogene is frequently amplified in extranodal diffuse large cell lymphoma. *Blood* **87**: 25-29.
- Hrdlickova R., Nehyba J. and Humphries E.H. (1994). *v-rel* induces expression of three avian immunoregulatory surface receptors more efficiently than *c-rel*. *J. Virol.* **68**: 308-319.
- Hrdlickova R., Nehyba J., Roy A., Humphries E.H. and Bose H.R., Jr. (1995). The relocalization of *v-rel* from the nucleus to the cytoplasm coincides with induction of expression of *Ikb* and *nfkb* and stabilization of *IκB-α*. *J. Virol.* **69**: 403-413.
- Huguet C., Enrietto P., Vandebunder B. and Abbadie C. (1994). C-Rel: A multifunctional transcription factor? *Cell Death Diff.* **1**: 71-76
- Joos S., Otano-Joos M.I., Ziegler S., Brüderlein S., du Manoir S., Bentz M., Möller P. and Lichter P. (1996). Primary mediastinal (thymic) B-cell lymphoma is characterized by gains of chromosomal material including 9p and amplification of the *REL* gene. *Blood* **87**: 1571-1578.
- Jotereau F.V. and Le Douarin N.M. (1982). Demonstration of a cyclic renewal of the lymphocyte precursor cells in the quail thymus during embryonic and perinatal life. *J. Immunol.* **129**: 1869-1877.
- Kerr L.D., Inoue J., Davis N., Link E., Baeuerle P.A., Bose H.J. and Verma I.M. (1991). The *rel*-associated pp40 protein prevents DNA binding of Rel and NF-κB: Relationship with IκBβ and regulation by phosphorylation. *Genes Dev.* **5**: 1464-1476.
- Kochel T., Mushinski J.F. and Rice N.R. (1991). The *v-Rel* and *c-Rel* proteins exist in high molecular weight complexes in avian and murine cells. *Oncogene* **6**: 615-626.
- Köntgen F., Grumont R.J., Strasser A., Metcalf D., Li R., Tarlinton D. and Gerondakis S. (1995). Mice lacking the *c-rel* proto-oncogene exhibit defects in lymphocyte proliferation, humoral immunity, and interleukin-2 expression. *Genes Dev.* **9**: 1965-1977.
- Kopp E.B. and Ghosh S. (1995). NF-κB and Rel proteins in innate immunity. *Adv. Immunol.* **58**: 1-27.
- Kralova J., Schatzle J.D., Bargmann W. and Bose H.R.J. (1994). Transformation of avian fibroblasts overexpressing the *c-rel* proto-oncogene and a variant of *c-rel* lacking 40 C-terminal amino acids. *J. Virol.* **68**: 2073-2083.
- Le Douarin N.M. and Jotereau F.V. (1975). Tracing of cells of the avian thymus through embryonic life in interspecific chimeras. *J. Exp. Med.* **142**: 17-40.
- Lewis R.B., McClure J., Rup B., Niesel D.W., Garry R.F., Hoelzer J.D., Nazerian K.A. and Bose H.R. (1981). Avian reticuloendotheliosis virus: Identification of the hematopoietic target cell for transformation. *Cell* **25**: 421-431.
- Lu D., Thompson J.D., Gorski G.K., Rice N.R., Mayer M.G. and Yunis J.J. (1991). Alterations at the *rel* locus in human lymphoma. *Oncogene* **6**: 1235-1241.
- Miyamoto S., Chiao P.J. and Verma I.V. (1994). Enhanced IκB degradation is responsible for constitutive NF-κB activity in mature murine B-cell lines. *Mol. Cell. Biol.* **14**: 3276-3282.
- Miyamoto S. and Verma I.M. (1995). *Rel/NF-κB/IκB* story. *Adv. Cancer Res.* **66**: 255-292.
- Moore B.E. and Bose H.R. (1989). Expression of the *c-rel* and *c-myc* proto-oncogenes in avian tissues. *Oncogene* **4**: 845-852.
- Morrison L.E., Boehmelt G., Beug H. and Enrietto P.J. (1991). Expression of *v-rel* in a replication competent virus: Transformation and biochemical characterization. *Oncogene* **6**: 1657-1666.
- Morrison L.E., Kabrun N., Mudri S., Hayman M.J. and Enrietto P.J. (1989). Viral *rel* and cellular *rel* associate with cellular proteins in transformed and normal cells. *Oncogene* **4**: 677-683.
- Nossal G.J.V. (1994). Negative selection of lymphocytes. *Cell* **76**: 229-239.
- Oliver P. and Le Douarin N.M. (1984). Avian thymic accessory cells. *J. Immunol.* **132**: 1748-1755.
- Olson L.D. (1967). Histopathologic and hematologic changes in moribund stages of chicks infected with T-virus. *Am. J. Vet. Res.* **28**: 1501-1507.
- Peck R., Murthy K.K. and Vainio O. (1982). Expression of B-L (Ia-like) antigens on macrophages from chicken lymphoid organs. *J. Immunol.* **129**: 4-5.
- Phi Van L. (1996). Transcriptional activation of the chicken lysozyme gene by NF-κBp65 (RelA) and c-Rel, but not by NF-κBp50. *Biochem J.* **313**: 39-44.
- Quéva C., Ness S.A., Graf T., Vandebunder B. and Stéhelin D. (1992). Expression patterns of *c-myb* and of *v-myb* induced myeloid-1 (*mim-1*) gene during the development of the chick embryo. *Development* **114**: 125-133.
- Schatzle J.D., Kralova J. and Bose H.R. (1995). Avian IκBα is transcriptionally induced by c-Rel and v-Rel with different kinetics. *J. Virol.* **69**: 5383-5390.
- Sevoian M., Larose R.N. and Chamberlain D.M. (1964). Avian lymphomatosis. VI. A virus of unusual potency and pathogenicity. *Avian Dis.* **8**: 336-347.
- Simek S. and Rice N.R. (1988). Detection and characterization of the protein encoded by the chicken *c-rel* protooncogene. *Oncogene Res.* **2**: 103-119.
- Verma I.M., Stevenson J.K., Schwarz E.M., Van Antwerp D. and Miyamoto S. (1995). *Rel/NF-κB/IκB* family: Intimate tales of association and dissociation. *Genes Dev.* **9**: 2723-2735.
- von Boehmer H. (1994). Positive selection of lymphocytes. *Cell* **76**: 219-228.
- Weih F., Carrasco D. and Bravo R. (1994). Constitutive and inducible *Rel/NF-κB* activities in mouse thymus and spleen. *Oncogene* **9**: 3289-3297.
- Zhang J.Y., Olson W., Ewert D., Bargmann W. and Bose H.J. (1991). The *v-rel* oncogene of avian reticuloendotheliosis virus transforms immature and mature lymphoid cells of the B cell lineage in vitro. *Virology* **183**: 457-466.