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The avian transcription factor c-Rel is induced and translocates into the nucleus of thymocytes undergoing apoptosis

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Abstract

This study investigates the involvement of the avian transcription factor c-Rel in thymocyte apoptosis occurring either *in vivo* or in organotypic culture. *In vivo*, only a few cortical thymocytes express the c-Rel protein. Their number, localization and morphology resemble that of apoptotic cells evidenced by TUNEL staining. In organotypic culture, the expression of c-Rel is induced in medullary thymocytes as apoptosis is triggered. This induction would be post-transcriptional since no increase in the c-*rel* gene expression is detected. Moreover, c-Rel translocates into the nucleus of medullary thymocytes during the time course of apoptosis. This translocation is preceded by a decrease in *ikba* expression, the gene which encodes the avian homologue of IκBα. Altogether these results suggest that the proto-oncogene c-*rel* could take an active part in apoptosis of cortical thymocytes occurring *in vivo* during T-cell selection as well as in experimentally-induced apoptosis of medullary thymocytes.

Keywords: Rel/NF-κB; transcription factor; apoptosis; avian thymus

Abbreviations: NF-κB: Nuclear factor-κB; TUNEL: TdT-mediated dUTP nick end labelling; RHD: Rel homogy domain; AEC: 3-amino-9-ethylcarbazole

Introduction

Cell fate depends on informations addressed by the extracellular environment via the cell membrane and through various signalling cascades to the nucleus. In response, a wide array of genes are switched 'on' and 'off' leading to cell proliferation, differentiation or death. Switch of gene expression is driven by transcription factors which are usually classified in families, where individual members perform specific or similar tasks. Rel/NFκB transcription factors form one of these families; every member shares a highly conserved 300 amino acids N-terminal domain, the Rel Homology Domain (RHD), which contains sequences necessary for DNA binding, dimerization, nuclear localization and interaction with inhibitory proteins of the IκB family. Rel/NF-κB proteins associate as homo- or heterodimers and bind a specific DNA sequence, the κB motif, in the promoter of their target genes. In most cell types, Rel/NF-κB dimers are found in an inactive form sequestered in the cytoplasm by an ankyrin-repeat protein of the IκB family. Upon stimulation, the IκB inhibitor is phosphorylated and degraded, thus allowing the transcription factor to enter the nucleus. The Rel/NF-κB family can be subdivided into two classes based on differences in the C-terminal part of the proteins. The first class includes c-Rel, RelA (p65) and RelB as well as the *drosophila* proteins Dorsal and Dif. These proteins contain in their C-terminal part a transactivating domain. The second class consists of p50 and p52, two proteins devoid of transactivating domain and proteolytically maturated from precursor proteins, p105 (or NFKB1) and p100 (or NFKB2) respectively. p50 or p52 do not generally activate transcription as homodimers, but they become potent transactivators when they heterodimerize with RelA or c-Rel. Interestingly, p105 and p100 precursors contain IκB-like ankyrin-repeats at their C-terminus and behave as inhibitory proteins by complexing and retaining Rel/NF-κB factors in the cytoplasm. These ankyrin-repeats are eliminated by proteolytic maturation (for reviews see Miyamoto and Verma, 1995; Gilmore *et al*, 1996). Rel/NF-κB transcription factors are ubiquitously expressed, activated by a great variety of stimuli and able to regulate the expression of genes as different as genes encoding immunoreceptors, cytokines or cell-adhesion molecules (for review see Bauerle and Henkel, 1994). Altogether these data suggest that Rel/NF-κB transcription factors could be involved in numerous physiological processes. Investigation of Rel/NF-κB physiological functions began with the study of the proto-oncogene c-*rel* which was the first Rel/NF-κB gene cloned as the cellular homologue of the oncogene v-*rel* (for review see Huguet *et al*, 1994). Northern blot analysis has revealed that the expression of c-*rel* is restricted to hematopoietic organs such as the bursa of Fabricius, thymus, spleen and bone-marrow (Moore and Bose, 1989). However, *in situ* hybridization experiments performed in young embryos from 3 to 9.5 days of development have indicated that c-*rel* is ubiquitously expressed at low levels while high levels of transcripts were detected in cells exhibiting an apoptotic morphology (Abbadie *et al*, 1993). These expression patterns suggested that c-*rel* may function in hematopoiesis and apoptosis. To evaluate the

causal link between the expression of c-*rel* and the occurrence of apoptosis, the protein was overexpressed in two different types of primary cells. Chick embryo fibroblasts overexpressing c-Rel acquired a transformed phenotype and an extended life span in culture. In contrast, bone marrow cells overexpressing c-Rel failed to grow in culture and underwent programmed cell death (Abbadie *et al*, 1993). The c-*rel* functions in mammals were investigated more recently by the description of its expression pattern in embryonic and adult mice and by the establishment of null mice. In contrast to chicken c-*rel*, mouse c-*rel* transcripts were detected late in the embryonic development and restricted to hematopoietic organs. This pattern was conserved in the post-natal period. The c-Rel protein was found in erythroid precursors of the fetal liver, in mature splenic B cells and in thymic epithelial cells (Carrasco *et al*, 1994). c-*rel*-deficient mice exhibited an impaired immunity attributable to defects in the activation of mature B and T-cells but surprisingly no developmental defect in the hematopoietic system. In addition, no change in the occurrence of apoptosis was noticed (Köntgen *et al*, 1995). Thus, whereas a clear *in vivo* function of mouse c-*rel* is now established in the specific immune response, the significance of c-*rel* expression during embryonic development of hematopoietic organs remains elusive. Moreover, discrepancies between mouse and chicken were evidenced concerning the expression and function of c-*rel* in apoptosis. This prompted us to further study the proto-oncogene c-*rel* in the avian system and to focus on its involvement in apoptosis. The thymus was chosen as model since T-cell maturation and selection include the elimination of numerous non-functional thymocytes by apoptosis (for reviews see von Boehmer, 1994; Nossal, 1994).

We describe here the expression of c-*rel* during normal thymocyte apoptosis occurring *in vivo* and after induction of massive apoptosis in thymus organ culture. Since the activity of Rel/NF-κB proteins depends on interactions with IκB proteins that determine their nuclear or cytoplasmic localization, we have described the subcellular distribution of the c-Rel protein during the process of apoptosis together with the expression of *ikba*, the gene encoding the avian homologue of IκBα (Davis *et al*, 1991; Kerr *et al*, 1991). Our results indicate that the c-Rel protein is expressed in thymocytes undergoing apoptosis through natural selection and moreover that c-Rel is induced and translocates into the nucleus of thymocytes during the time course of experimentally-induced apoptosis. This nuclear translocation of c-Rel is preceded by a decrease in *ikba* expression.

Results

c-Rel is expressed during thymocyte apoptosis occurring through natural selection

To evaluate the involvement of c-*rel* in apoptosis of thymocytes, we have studied the expression of the c-Rel protein by immunocytochemistry with an immunopurified anti-cRel antibody during thymus development. Since no data was available about the occurrence of apoptosis in the avian thymus, we have first performed a study by TUNEL, a method

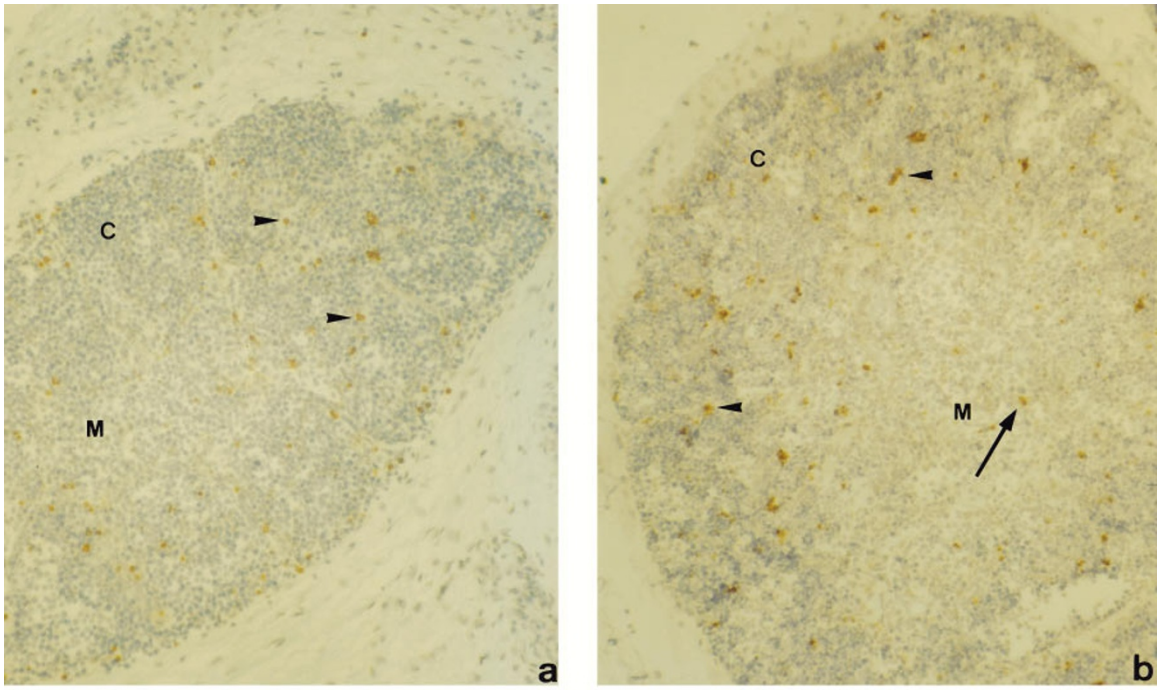

Figure 1 *In situ* detection of apoptotic cells during thymus development. E15 and P1 thymus sections were processed for TUNEL staining of apoptotic cells. Cortical and medullary areas are indicated as (C) and (M). Apoptotic cells are stained in brown compared to negative cells which are counterstained in blue by hematoxylin. At E15 (**a**) and P1 (**b**), apoptotic thymocytes are scattered in the cortex (arrowheads). Only a few cells are stained in the medulla (arrow). The number of apoptotic cells increases from E15 to P1 × 200.

which reveals apoptotic cells by detecting DNA strand breaks (Gavrieli *et al*, 1992). Experiments were done at four representative stages of the chick thymus development: at E9.5 when the thymic rudiment mainly contains thymocyte precursors; at E15 when cortical and medullary areas begin to separate indicating that thymocyte maturation is engaged and at E19 or P1 when the thymic structure is completed. At E9.5, almost no apoptotic cells are detected (data not shown). At E15, E19 and P1, apoptotic cells are mainly found in the cortex scattered among numerous healthy thymocytes whereas only a few apoptotic thymocytes are detected in the medulla (Figure 1). The major difference between E15, E19 and P1 is the amount of apoptotic thymocytes which increases from E15 to P1. It should be noticed that only a low number of apoptotic thymocytes are detected *in situ* in spite of the high number of thymocytes that are eliminated through natural selection (Egerton *et al*, 1990). This apparent paradox can be explained considering that apoptosis is a rapid phenomenon and that dead cells are efficiently engulfed. In view of these results, we have focused our analysis of the expression of c-Rel in the cortex of thymuses from E15 to P1. The vast majority of cortical thymocytes do not express c-Rel. However, a few c-Rel-expressing thymocytes can be detected scattered all over the cortex. They are generally isolated among negative thymocytes but they can also form clusters (Figure 2d and e). A few c-Rel-expressing thymocytes appear condensed or even fragmented. The cellular fragments are round and grouped by two or three or have an irregular and damaged morphology (Figure 2f and g). TUNEL-positive cells also appear isolated or in clusters, condensed or fragmented in apoptotic bodies more or less damaged (Figure 2a, b and c). From these morphological similarities between c-Rel-expressing cells and TUNEL-positive cells, we assumed that the cortical thymocytes that express c-Rel undergo apoptosis.

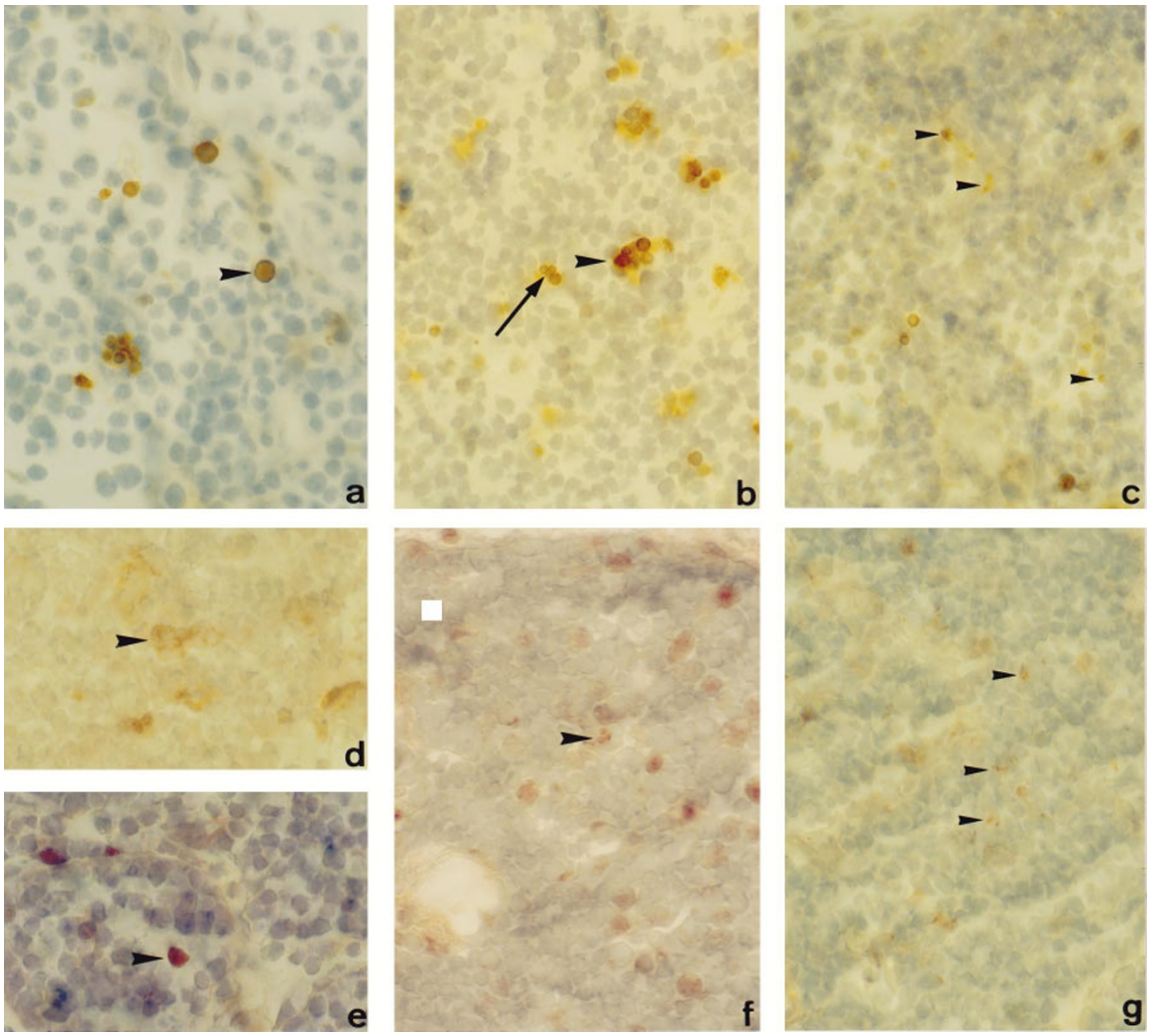

Figure 2 High magnifications of apoptotic and c-Rel-expressing thymocytes of the cortex. Thymus sections were processed for TUNEL staining (**a, b** and **c**) or anti-c-Rel immunocytochemistry (**d, e, f** and **g**). Apoptotic cells are stained in brown, c-Rel-expressing-cells in red and negative cells in blue. TUNEL-positive thymocytes are either isolated (**a**, arrowhead) or in cluster (**b**, arrowhead). The TUNEL staining also detects apoptotic bodies (**b**, arrow) or cell debris (**c**, arrowheads). Similarly, c-Rel-expressing thymocytes appear in cluster (**d**, arrowhead) or as isolated cells (**e**, arrowhead). c-Rel is also detected in apoptotic bodies-like elements (**f**, arrowhead) and cell debris (**g**, arrowheads). $\times 600$.

c-Rel translocates into the nucleus of medullary thymocytes undergoing experimentally-induced apoptosis

The scarceness of apoptotic thymocytes observable in a thymus section makes their *in situ* study difficult. To bypass this limitation and to check whether c-Rel is activated when thymocytes undergo apoptosis, we have developed a protocol for the induction of massive apoptosis inside the thymus. Dissection and organotypic culture of E19 or P1 thymuses in complete RPMI medium, at 40°C, with 10% CO_2 create a stress sufficient to induce thymocyte apoptosis. TUNEL staining revealed that apoptosis occurs first in the medulla after 2 h in culture. The number of TUNEL-positive thymocytes increases in the course of the culture and at 4 h the majority of medullary thymocytes are apoptotic (Figure 3b, and d). Interestingly, 4 h in culture do not affect the level of apoptosis occurring in the cortex. However, when the culture is maintained over 24 h, cortical thymocytes also undergo apoptosis (data not shown). Stromal cells remain unaffected by the culture until 5 h (data not shown). According to these results, we have studied the expression of c-Rel in medullary thymocytes of thymuses maintained in organotypic culture up to 4 h. Thymuses were fixed at different times of culture and serial sections were processed either for immunocytochemistry or TUNEL staining. Experiments were done both with E19 and P1 thymuses and gave identical results. In control thymuses, medullary thymocytes do not express c-Rel and are TUNEL-negative (Figure 3a and b). Beside thymocytes, some medullary cells express c-Rel; they were previously identified as dendritic cells, epithelial cells and macrophages (unpublished observation). After 1 h in culture, no change appears either in the expression of c-Rel or in the occurrence of apoptosis (data not shown). After 2 h in culture, c-Rel is faintly detected in numerous medullary thymocytes which still display a healthy morphology (data not shown). The TUNEL staining reveals only a few positive cells, indicating that most thymocytes are still healthy but that apoptosis has already begun (data not shown). After 3 h in culture, a great number of medullary thymocytes are TUNEL-positive. Numerous thymocytes express c-Rel and among them some are highly stained (data not shown). After 4 h in culture, almost all medullary thymocytes are apoptotic; they are highly positive in immunocytochemistry (Figure 3c and d). These results indicate that, in thymocytes, c-Rel expression is induced when apoptosis is induced.

To evaluate the activation of c-Rel during the process of apoptosis, we have determined its subcellular localization by observing the sections at high magnification. From 2 to 4 h in culture, the c-Rel protein is generally localized in the cytoplasm of thymocytes (Figure 4a). However, at 3 and 4 h in culture, several thymocytes display a staining of both

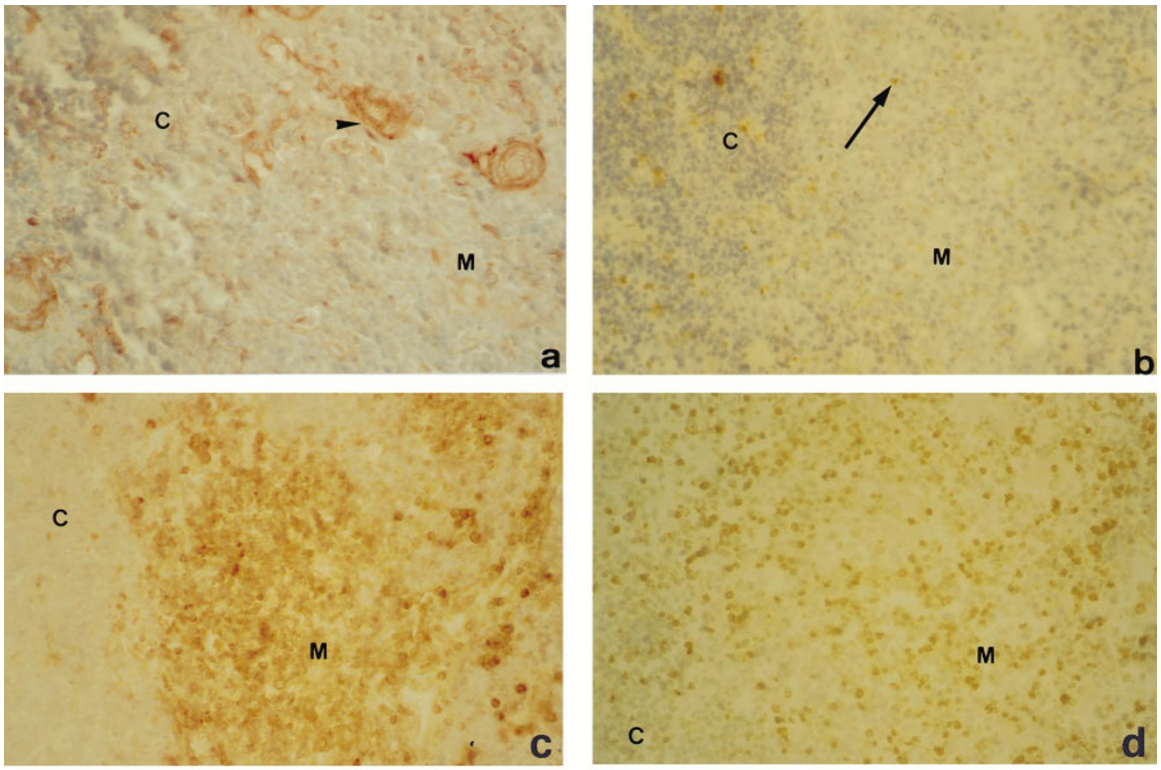

Figure 3 Detection of c-Rel expression after induction of apoptosis in thymus organ culture. P1 thymus sections were processed either for TUNEL staining (**b** and **d**) or anti-c-Rel immunocytochemistry (**a** and **c**) at different times of culture. Cortical and medullary areas are indicated as (C) and (M). Apoptotic cells are stained in brown, c-Rel-expressing-cels in red and negative cells in blue. In control thymuses (**a** and **b**), cortical and medullary thymocytes do not express c-Rel in contrast to stromal cells that are highly positive (**a**, arrowhead); only very few medullary thymocytes are TUNEL positive (**b**, arrow). After 4 h in culture (**c** and **d**), a majority of medullary thymocytes are highly stained in immunocytochemistry (**c**) and almost all medullary thymocytes are apoptotic as revealed by their TUNEL staining (**d**). × 250.

the cytoplasm and the nucleus, with however a higher staining of the cytoplasm (Figure 4b and e). c-Rel is also detected in condensed thymocytes and in apoptotic bodies with an homogenous distribution, suggesting a localization in both the condensed cytoplasm and nucleus (Figure 4d). Therefore, c-Rel could be activated to translocate into the nucleus during apoptosis. To better address this point, we have performed Western blot analysis on nuclear and cytoplasmic extracts of thymus prepared at different times of culture. In control thymuses and until 2 h in culture, the c-Rel protein is mainly found in the cytoplasmic fraction; very few amounts of protein are detected in nuclear extracts. At 3, 4 and 5 h in culture, c-Rel amounts in the nuclear fraction are clearly higher than in control thymuses (Figure 5) indicating that c-Rel has translocated into the nucleus from 3 h of culture onwards. Taken together, immunocytochemistry and Western blot experiments show that c-Rel is induced and activated to translocate into the nucleus during thymocyte apoptosis.

The *ikba* gene expression decreases in thymocytes during apoptosis

In order to investigate the mechanism of c-Rel induction and nuclear translocation during experimentally-induced apoptosis, we have examined the expression of the c-*rel* and *ikba* genes by *in situ* hybridization. *ikba* encodes the avian homologue of IκBα which was shown to be able to sequester the avian c-Rel protein in the cytoplasm and to inhibit its binding to DNA (Diehl *et al*, 1993; Kerr *et al*, 1991; Davis *et al*,

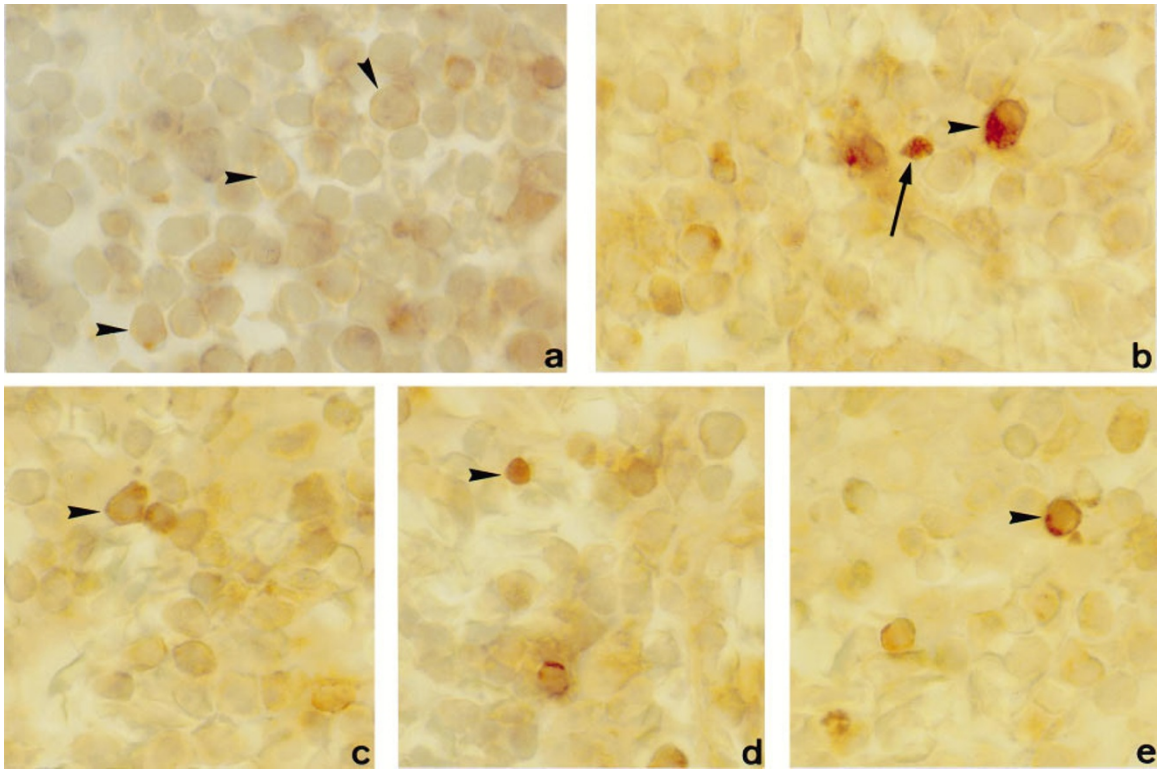

Figure 4 Subcellular localization of c-Rel during apoptosis of medullary thymocytes. P1 thymus sections were processed for anti-c-Rel immunocytochemistry and observed at high magnification. c-Rel is faintly detected in the cytoplasm of thymocytes after 2 h in culture (**a**, arrowheads). After 3 h in culture, some medullary thymocytes are highly stained either in the cytoplasm (**c**, arrowhead) or both in the cytoplasm and the nucleus, although less intensely in the latter (**b** and **e**, arrowheads). c-Rel is also highly expressed in condensed thymocytes (**d**, arrowhead) and in apoptotic bodies (**b**, arrow). × 1250.

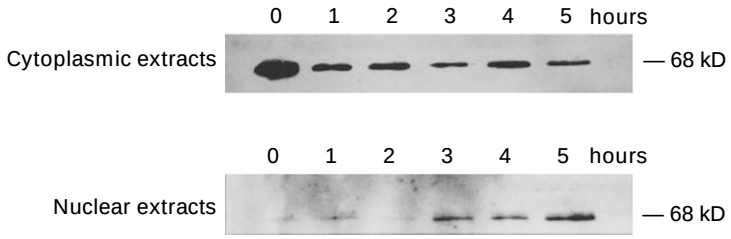

Figure 5 Western-blot analysis of cytoplasmic and nuclear extracts from E19 thymuses in organotypic culture. The anti-c-Rel antibody detects a protein at 68 kD, the molecular mass of the avian c-Rel protein (Capobianco *et al*, 1990). In thymus control and until 2 h in culture, the c-Rel protein is mainly found in cytoplasmic extracts: only few amounts are detected in nuclear extracts. After 3 h of culture, c-Rel amounts increase in nuclear extracts.

1991). Experiments were done with E19 and P1 thymuses and gave similar results. Serial sections were used for immunocytochemistry, TUNEL staining and *in situ* hybridization experiments and thus any changes in either gene expression, protein expression or occurrence of apoptosis were directly correlable. In control thymuses, c-*rel* mRNAs are detected in both the cortex and the medulla (Figure 6a and c) with a higher level in medullary stromal cells (Huguet *et al*, 1997). In contrast, *ikba* is highly expressed in the medulla and more faintly in the cortex (Figure 6e and g). Inside the medulla, stromal cells give the most intense signal but it has to be noticed that thymocytes also highly express *ikba* (Huguet *et al*, 1997). After 1 h in culture, no change is observed in the expression of the c-*rel* and *ikba* genes. After 2 h in culture, c-*rel* mRNA expression remains stable (Figure 6b and d) although, as described above, the c-Rel protein expression is already induced. In contrast, *ikba* expression decreases in thymocytes whereas it remains unchanged in stromal cells (Figure 6f and h). It has to be reminded that at this stage the c-Rel protein is mainly localized in the cytoplasm of thymocytes and that its nuclear translocation is evidenced especially from 3 h in culture. Therefore, the decrease in *ikba* expression precedes and could account for the translocation of c-Rel into the nucleus of thymocytes undergoing apoptosis. After 3 and 4 h in culture, *ikba* expression still decreases but c-*rel* expression decreases too (data not shown). According to the high number of apoptotic bodies in the medulla at 3 and 4 h of culture, we estimated that decreases in gene expression are not due to specific mechanisms of regulation but rather to a too far engagement of the cells in the death process.

Discussion

Previous studies have shown that the mouse proto-oncogene c-*rel* is involved at two different levels in T-cell development: 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). No relationship between the expression of mouse c-*rel* and apoptosis could be evidenced in these experiments, thus contrasting with results obtained in the avian system where c-*rel* and its viral counterpart v-*rel* have been involved in apoptosis of hematopoietic cells as well as of cells of different origins during embryonic development (Neiman *et al*, 1991; White *et al*, 1995; White and Gilmore, 1996; Abbadie *et al*, 1993). In this study, we have investigated the involvement of the avian proto-oncogene c-*rel* in thymocyte apoptosis.

c-Rel is induced in apoptotic thymocytes

Considering the high amounts of c-*rel* mRNAs described in apoptotic cells in the avian embryo (Abbadie *et al*, 1993), we expected such an expression in the thymus cortex where a majority of thymocytes undergo apoptosis (Egerton *et al*, 1990). Surprisingly, only low levels of c-*rel* transcripts were detected in cortical thymocytes, slightly higher levels in medullary thymocytes and the highest levels were found in non-lymphoid cells (Huguet *et al*, 1997). Since apoptotic thymocytes are buried among numerous healthy cortical thymocytes, it was not possible to identify them in the *in situ* hybridization experiments and therefore to evaluate their level of c-*rel* expression. By contrast, we were able to show by immunocytochemistry that the c-Rel protein is only expressed in a few isolated cortical thymocytes that could be apoptotic since their number, localization and morphology resemble that of TUNEL-positive cells. This result suggesting that c-Rel could be induced during apoptosis, we have developed an assay to test whether c-Rel is induced when apoptosis is triggered experimentally. The organ culture protocol used for mouse thymus has been adapted to the very young E10 chick thymus (Davidson *et al*, 1992). When we applied this technique to fully developed E19 or P1 thymuses, medullary thymocytes underwent massive apoptosis within a few hours. This event may be induced by hypoxia as well as lack of cytokines or hormones, all stresses known to induce apoptosis. However, in these conditions, the deepest cortical thymocytes did not undergo apoptosis, suggesting that medullary thymocytes are the most sensitive to stress-induced apoptosis. Our results show that the c-Rel protein became detectable, as soon as 2 h in culture, in the cytoplasm of most medullary thymocytes. At this stage, these thymocytes still appeared healthy, although apoptosis has already begun as evidenced by the presence of a few TUNEL-positive cells. Most thymocytes progressed into fully apoptotic cells from 3 h in culture and the expression of c-Rel became conspicuous. We therefore consider that after 2 h in culture most thymocytes were committed to die. Thus, the c-Rel protein would be induced in medullary thymocytes committed to die upon apoptotic stimulation. This induction would occur by a post-transcriptional mechanism since no increase in c-*rel* mRNA expression was detected before or during apoptosis. In conclusion, our results suggest that c-Rel is involved in naturally-occurring apoptosis of cortical thymocytes as well as in stress-induced apoptosis of medullary thymocytes.

c-Rel translocates into the nucleus of thymocytes during apoptosis

As shown above, during organotypic culture c-Rel was first detected in the cytoplasm of thymocytes committed to die. During the time course of apoptosis, the subcellular localization of c-Rel changes: from 3 h in culture onwards, c-Rel can be found either in the cytoplasm or in both the cytoplasm and the nucleus of thymocytes still healthy in appearance or already condensed. This change in subcellular localization was confirmed by Western blot experiments. Therefore, c-Rel seems to translocate from the cytoplasm into the nucleus of thymocytes during apoptosis. Once in the nucleus, c-Rel might modulate the transcription of some target genes involved in the mechanism of apoptosis. However, the transcriptional activity of c-Rel during thymocyte apoptosis remains to be established and target genes have to be identified.

Mechanism triggering the c-Rel nuclear translocation

In many cell types, Rel/NF-κB proteins are expressed in an inactive form sequestered in the cytoplasm by an IκB protein.

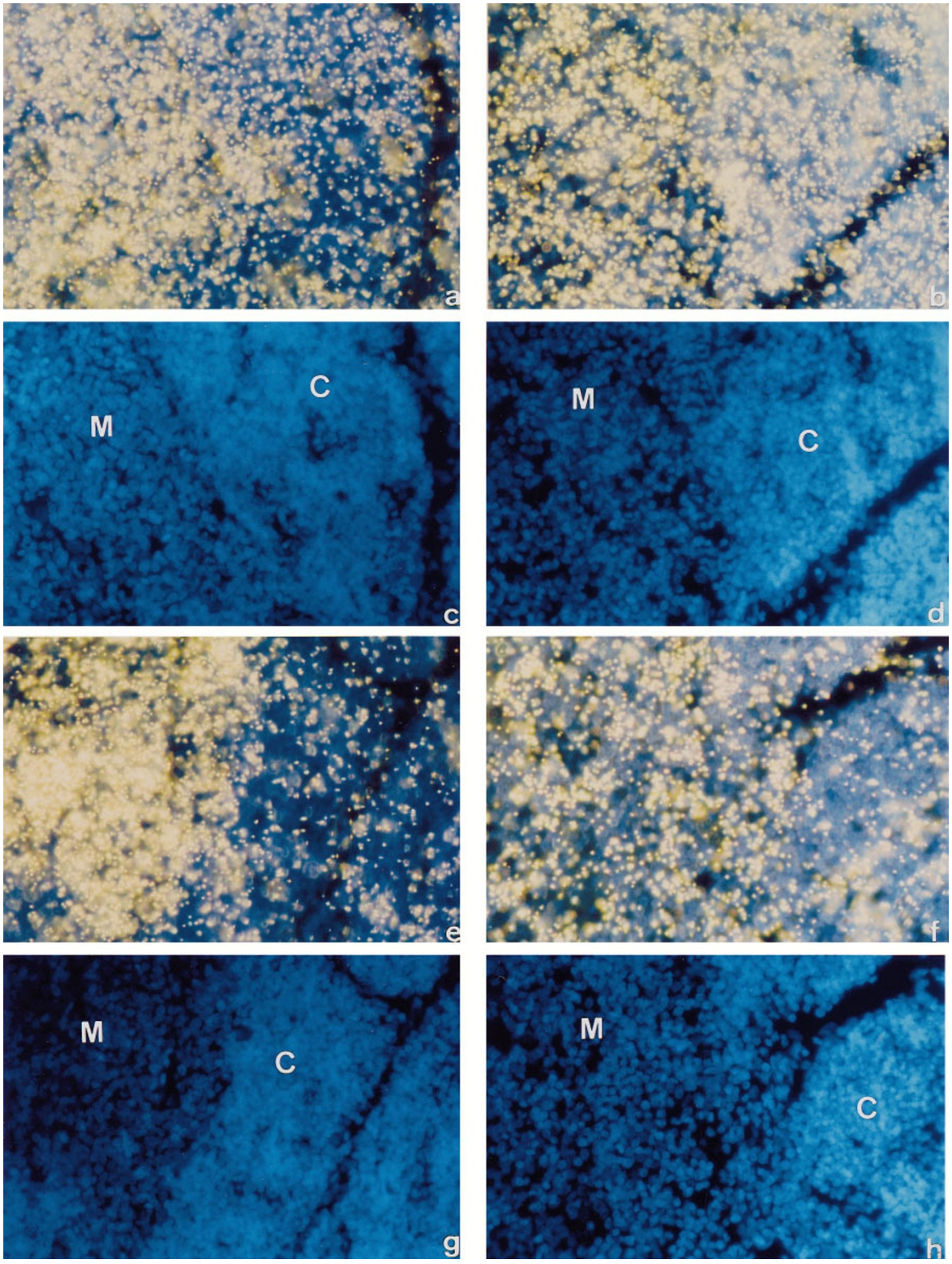

Figure 6 Detection of *c-rel* and *ikba* gene expression during experimentally-induced apoptosis. Sections of P1 thymuses maintained in organ culture were processed for *in situ* hybridization and counterstained with a fluorescent DNA intercalant. Sections are observed in dark field and UV illumination (**a, b, e** and **f**) or under UV illumination alone (**c, d, g** and **h**). In control thymus, c-*rel* is expressed both in the cortex (C) and the medulla (M) with a higher expression in the latter (**a** and **c**). After 2 h in culture, this pattern of expression does not change (**b** and **d**). In control thymus, *ikba* is highly expressed in all medullary cell types and at lower level in cortical cells (**e** and **g**). After 2 h of culture, *ikba* expression decreases in medullary thymocytes. The remaining expression is mainly in stromal cells.

The major pathway allowing the activation of Rel/NF-κB proteins involves the phosphorylation of IκBα and its subsequent ubiquitination and degradation by the proteasome (review in Verma *et al*, 1995). Our results show that there is no detectable preexisting c-Rel proteins in medullary thymocytes before the apoptotic stimulation, indicating that the molecules which enter the nucleus are neo-synthesized. Moreover, we have previously shown that the *ikba* gene is highly expressed in medullary thymocytes of control thymuses (Huguet *et al*, 1997). Therefore, to investigate the mechanism of avian c-Rel nuclear translocation in apoptotic thymocytes, we examined *ikba* gene expression. From 2 h in culture, i.e. before the beginning of the nuclear translocation of c-Rel, *ikba* expression decreases. Thus, the c-Rel protein would not be sequestered as its synthesis proceed and could be able to enter the nucleus. Although the *ikba* gene expression was shown to be upregulated by overexpressed c-Rel in avian fibroblasts (Schatzle *et al*, 1995), we did not observe such an upregulation in medullary thymocytes from 3 h of culture. These results are the first example of an activation of c-Rel that would occur through a decrease in the transcriptional activity of *ikba*. However, whether this decrease is alone sufficient for a complete c-Rel activation or whether it occurs together with an active degradation of the IκBα protein remains to be established.

Involvement of different Rel/NF-κB members in apoptosis

The involvement of the avian c-Rel protein in apoptosis was previously shown for a variety of non-lymphoid cells of the avian embryo as well as for bone marrow cells (Abbadie *et al*, 1993). Here, we present evidence for a role of c-Rel in the apoptosis of cortical thymocytes occurring *in vivo* during selection as well as in stress-induced apoptosis of medullary thymocytes. Other Rel/NF-κB members were shown to be able to induce or repress apoptosis. In a mouse T-cell hybridoma, overexpression of Bcl-2 blocked dexamethasone-induced apoptosis and led to a decrease in RelA/p50 heterodimers and an increase in p50/p50 homodimers by a post-transcriptional mechanism (Ivanov *et al*, 1995). Likewise, disruption of the mouse RelA gene induced severe hepatocyte apoptosis (Beg *et al*, 1995). Treatment with TNF-α of embryonic fibroblasts and macrophages derived from these RelA-deficient mice resulted in a dramatic decrease in viability, thus suggesting that RelA protects cells from apoptosis (Beg and Baltimore, 1996). Similar conclusions were drawn from studies using human fibrosarcoma cells, human primary fibroblasts and human Jurkat T-cells overexpressing a super-repressor form of IκBα. When treated by TNF-α, these cells were more susceptible to apoptosis than control cells (Wang *et al*, 1996, Van Antwerp *et al*, 1996). However, in human 293 kidney cells, overexpression of a dominant-negative RelA protein inhibited apoptosis suggesting that in that case RelA induces cell death (Grimm *et al*, 1996). Finally, one experiment done with Hela cells has evidenced the ability of c-Rel to protect against TNF-induced apoptosis, even with a more efficiency than RelA (Liu *et al*, 1996). Taken together, these conflicting results illustrate the possibility that different Rel/NF-κB members may be activated upon apoptotic stimulation resulting in stimulation or inhibition of apoptosis depending on cell type or species.

Materials and Methods

Animals

Fertilized white Leghorn chicken eggs were incubated at 39°C in a humidified air chamber. The age of embryos is indicated as E1, E2..., E1 corresponding to 24 h of egg incubation and as P1 corresponding to 24 h post-hatching.

Histological sections

E9.5, E15, E19 and P1 chick embryo thymuses were dissected in PBS and fixed at 4°C for 18 h in 4% paraformaldehyde in PBS (Na_2HPO_4-NaH_2PO_4 0.1 M pH7.4), embedded in paraffin and processed for histological sections.

Thymus organ culture

E19 and P1 thymuses were rapidly dissected in culture medium (RPMI 1640, 10% FCS, 2% chicken serum, 1000 IU ml^{-1} penicillin, 1000 μg ml^{-1} streptomycin, 2 mM glutamine) on ice. Organs were set to float on top of millicell chambers in 6 well culture plates (Millipore) filled up with culture medium. The culture was carried out from 1 to 24 h, at 40°C, in 10% CO_2, 95% humidity.

In situ hybridization

(a) Synthesis of ^{35}S RNA probes: The chicken full length c-*rel* cDNA was cloned in Bluescript SK^- (Stratagene) as described in (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). ^{35}S RNA probes were transcribed from 2 μg of linearized plasmids by 20 U of either T7, T3 or SP6 RNA polymerase for sense and antisense probes in a 20 μl reaction mixture containing 20 μCi ^{35}S-CTP (1300 Ci/mmole), 200 μM UTP, ATP and GTP for 1 h 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). (b) *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 μg ml^{-1} proteinase K (Boehringer Mannheim) for 15 min at 37°C, post-fixed in 4% paraformaldehyde, washed in PBS, acetylated 10 min with 0.25% acetic anhydride in 0.1 M triethanolamine, washed in 2 × SSC (0.3 M NaCl, 30 mM sodium citrate) and dehydrated in ethanol. ^{35}S RNA probes were denatured at 80°C and diluted in the hybridization buffer at a concentration of 50 pg μl^{-1}. Hybridization was performed at 60°C for 18 h. After a wash in 4 × SSC at 20°C, slides were treated with 10 μg ml^{-1} of RNase A (type III A, Sigma) for 30 min at 37°C, subsequently washed in 0.1 × SSC at 60°C, dehydrated by ethanol and dipped in nuclear track emulsion (Kodak NTB2). Slides were exposed at 4°C for 2 weeks. After developing, sections were stained with a DNA intercalating fluorescent dye (Hoechst 33258), mounted and observed under dark-field and UV illumination with a Zeiss microscope. Both antisense and sense probes were used and the sense probes never gave any signal.

Immunocytochemistry and Western-blot

(a) 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. Normal rabbit immunoglobulin fraction (Dako) was used as negative control. (b) Immunocytochemistry: After being deparaffinized and rehydrated, sections were incubated in 0.5 mg ml^{-1} saponine (Sigma) in PBS for 30 min; then endogenous peroxidase activity was quenched by incubation in 80% methanol, 20% PBS, 0.6% H_2O_2 for 30 min followed by 30 min saturation in 5% 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). (c) Western blot of cytoplasmic and nuclear extracts: To separate nuclear from cytoplasmic proteins, cultured thymuses were collected in a solution containing 0.015 M Hepes pH 7.9, 0.014 M β-mercaptoethanol, 0.3 M Saccharose, 0.060 M KCl, 0.015 M NaCl, 0.15 mM spermine, 0.5 mM spermidine, 0.5 mM EGTA, 0.002 M EDTA. After suspension in a Dounce homogenizer on ice, 3% Nonidet P40 was added and samples incubated for 5 min on ice, then centrifuged 2 min at 10,000 g at 4°C. The supernatants containing cytoplasmic proteins were stored in aliquots at −80°C. The pellets of nuclei were resuspended in nine volumes of NUN 1.1× buffer (1.1 M Urea, 0.33 M NaCl, 1.1% Nonidet P40, 0.27 M Hepes pH 7.6), incubated for 15 min on ice, and centrifuged 15 min at 10,000 g at 4°C (Schibler *et al*, 1993; Lavery and Schibler, 1993). The supernatants containing nuclear proteins were stored in aliquots at −80°C. Protein concentration was measured by optic density at 260 nm. Equiloaded 10% SDS-polyacrylamide gels were run and blotted onto nitrocellulose sheets. Nitrocellulose sheets were saturated 1 h with 5% milk in PBS and then incubated overnight at 4°C with SB146. After three washes in PBS, the sheets were incubated with an anti-rabbit antibody for 2 h at room temperature. After washing, the peroxidase activity was revealed by incubation with a chemiluminescent substrate (Enhanced Chemiluminescence system, Amersham).

TUNEL staining

The TUNEL protocol was adapted from the Apoptag kit instructions (APOPTAG™ PLUS, Oncor). Briefly, after being deparaffinized and rehydrated, sections were incubated in 0.1 M glycine, 0.2 M Tris buffer pH 7.4 for 10 min at room temperature, then digested by 20 μg ml^{-1} proteinase K in 0.05 M EDTA, 0.1 M Tris buffer pH 8 for 15 min at 37°C and finally incubated in 80% methanol, 20% PBS, 0.6% H_2O_2 for 30 min to quench endogenous peroxidase activity. The following steps were carried out as described in the kit. Sections were finally counterstained with hematoxylin and mounted in Glycergel (Dako).

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