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# Cytoplasmic retention, DNA binding and processing of the NF-κB p50 precursor are controlled by a small region in its C-terminus

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**The transcription factor NF-κB binds to DNA as a heterodimer composed of two subunits of 50 kDa (p50) and 65 kDa (p65). p50 contains a DNA binding and dimerization domain and represents a truncated form of a 105 kDa (p105) precursor molecule. We show here that in different cell types the p105 precursor as well as the processed p50 coexist in the cytoplasm, but that only the latter enters the nucleus. The cytoplasmic retention of the precursor molecule is controlled by a small region in its C-terminal part. We show that this region is responsible for the observed lack of DNA binding of the p50 precursor and controls the extent of processing of the precursor to the mature form. We also present evidence that a stretch of four basic amino acids, similar to a sequence found in the other proteins belonging to the rel/NF-κB family, is required for translocation of the processed p50 protein into the nucleus and thus could be the target for the retention mechanism.**

*Key words:* ankyrin/KBF1/NF-κB/p50/p105

## Introduction

The transcription factor NF-κB is involved in the regulation of expression of a variety of cellular and viral genes (for review see Lenardo and Baltimore, 1989; Baeuerle, 1991). It contacts its decameric DNA recognition sequence as a heterodimer consisting of two DNA binding subunits of 50 kDa (p50) and 65 kDa (p65) (for review see Baeuerle, 1991). A homodimer consisting of two p50 subunits called KBF1 binds to the same DNA sequences and is constitutively present in the nucleus of a variety of cell types (Israël *et al.*, 1987; Yano *et al.*, 1987). The cDNAs encoding the p50 (Bours *et al.*, 1990; Ghosh *et al.*, 1990; Kieran *et al.*, 1990; Meyer *et al.*, 1991) as well as the p65 (Nolan *et al.*, 1991; Ruben *et al.*, 1991) subunit have been cloned recently.

p50 is most probably a truncated form of a 105 kDa (p105) precursor molecule (Ghosh *et al.*, 1990; Kieran *et al.*, 1990; see also Rivière *et al.*, 1991). It has been shown that the HIV-1 protease is able to process p105 into a protein slightly smaller than p50, and to increase the level of active NF-κB complex (Rivière *et al.*, 1991). No cellular protease involved in this processing event has been characterized to date.

The p105 precursor does not bind to DNA. p50, which is included in its N-terminal portion, contains DNA binding and dimerization domains as well as a potential nuclear localization signal. These domains are highly homologous to the N-terminal portions of the products of the *rel* (proto-) oncogene and the *Drosophila* maternal effect gene *dorsal* (Bours *et al.*, 1990; Ghosh *et al.*, 1990; Kieran *et al.*, 1990; Meyer *et al.*, 1991). The C-terminal part of p105 contains a repeated motif (Kieran *et al.*, 1990; Bours *et al.*, 1990, Meyer *et al.*, 1991), found also in human erythrocyte ankyrin, the DNA binding protein GABPβ and several proteins involved in control of the cell cycle or tissue differentiation (Lux *et al.*, 1990; Thompson *et al.*, 1991).

p50 can also form heterodimers with other members of the NF-κB/rel family, like products of the v-*rel* and c-*rel* (proto-)oncogenes (Kieran *et al.*, 1990; Ballard *et al.*, 1990). A transdominant mutant of p50 (ΔSP), which is unable to bind DNA but able to form homo- and heterodimers, has been shown to reduce the binding activity of wild-type rel/NF-κB family proteins *in vitro* and *in vivo* (Logeat *et al.*, 1991).

NF-κB is nuclear only in a restricted set of cell types, including mature B cells, some T cell lines and differentiated monocytes (for review see Baeuerle, 1991). In most other cell types p50 is complexed in the cytoplasm via the p65 subunit with an inhibitory protein, called IκB, which prevents its translocation into the nucleus (Baeuerle and Baltimore, 1988a,b). Mammalian and avian cDNAs encoding IκB-like activities have been cloned recently (Haskill *et al.*, 1991; Davis *et al.*, 1991). Activation of cells by various agents leads to the dissociation of IκB, releasing an active heterodimer, which is translocated into the nucleus (Baeuerle *et al.*, 1989). Similar cytoplasmic-nuclear translocation events have also been reported for other proteins of the rel/NF-κB family (Gilmore and Temin, 1986; for review see Govind and Steward, 1991).

We thus investigated the subcellular localization of p50 and its p105 precursor. Immunoprecipitation as well as immunocytochemical analysis of various cell types showed that p105 is exclusively found in the cytoplasm, whereas processed p50 is present in both the cytoplasm and the nucleus. By deletion analysis and site-directed mutagenesis we identified several elements involved in cytoplasmic retention and/or inhibition of DNA binding. These elements are apparently also able to control the extent of processing of the p105 precursor.

## Results

### *Subcellular localization of p105 and p50*

[$^{35}$S]methionine-labelled cytoplasmic and nuclear extracts from various cell types were immunoprecipitated using an anti-p50 polyclonal antiserum raised against the N-terminal half of p105 (antiserum 3, Kieran *et al.*, 1990). Its specificity has been demonstrated previously (Rivière *et al.*, 1991). In addition, by immunoprecipitation of *in vitro* translated products we could show that the antiserum does not cross-react with c-rel or p65 (Kieran *et al.*, 1990; data not shown). Most of the additional bands seen in the immunoprecipation

experiments (Figures 1, 3, 5B and 6C) are non-specific and are also detected using preimmune serum (data not shown).

As seen in Figure 1 and Figure 3 lane 1, p105 was detected only in the cytoplasm of the human, monkey and mouse cell lines tested. In some cases multiple (up to three) bands of p105 are seen (Figure 1, lanes 2, 3 and 5; Figure 3, lane 1), which may correspond to post-translationally modified forms of the precursor molecule. The amount of p105 varies in different cell lines; it is more abundant in mature B lymphocytes (Figure 1, lanes 3, 4 and 5) than in pre-B (lane 2), embryonal or epithelioid carcinoma cells (lanes 1 and 6). No p105 was detected in the nucleus of any cell line.

In contrast, p50 was found in both cell compartments. The amount of p50 in the nucleus correlates well with the nuclear DNA binding activities (KBF1 and NF-κB) found in different cell lines. In particular, F9 embryonal carcinoma (EC) cells (Figure 1, lane 1), in which no NF-κB or KBF1 binding activity can be detected in the nucleus (Israël *et al.*, 1989b) but where inactive NF-κB is sequestered in the cytoplasm (Macchi *et al.*, 1989), display as much p105 and p50 in their cytoplasm as HeLa cells (lane 6), but no processed form is found in the nucleus (lane 1′).

### *Analysis of truncated forms of the p105 precursor*

The p105 precursor is not only confined to the cytoplasm (as shown above), but also unable to bind DNA (Kieran *et al.*, 1990; Ghosh *et al.*, 1990). We asked whether the C-terminal moiety of p105 that is absent in processed p50 confers these properties. This part of the molecule contains seven motifs similar to those found in human erythrocyte ankyrin, which have been reported to act as sites of attachment to the cytoskeleton or to integral membrane proteins (Lux *et al.*, 1990). These motifs are referred to as ankyrin-like repeats and numbered 1 to 7, from the N- to the C-terminus (see Figure 4).

We constructed various derivatives of the cDNA coding for the p105 protein, each containing a different number of ankyrin-like repeats. They were transfected into monkey COS7 cells, in which plasmids containing the origin of replication of SV40 can replicate autonomously, thus yielding high levels of the protein of interest. Then the subcellular localization of the proteins was assayed by immunofluorescence with the antibody described above. The full-length protein as well as slightly shorter constructs (Figure 2, panels 2–4) show exclusively cytoplasmic staining. The product of the Bal construct, which contains six of the seven ankyrin-like repeats, was found either in the cytoplasm or in the nucleus or in both compartments (Figure 2, panel 5). Deletion of 64 more amino acids, leaving almost five complete ankyrin-like repeats, resulted in a predominantly nuclear localization of the protein (Figure 2, panel 6). Shorter forms of the protein, with the exception of the Tth truncation (Figure 2, panel 7), which showed staining similar to that of the Bal construct (Figure 2, panel 5), were exclusively nuclear (Figure 2, panels 8–10).

The antibody used for fluorescence experiments was raised against the N-terminal half of the protein and thus does not discriminate between the full-length (or truncated forms of) p105 and the processed p50. To clarify the distribution of the different protein species, we prepared cytoplasmic and nuclear extracts of [$^{35}$S]methionine-labelled transfected COS7 cells and assayed them by immunoprecipitation. The results are shown in Figure 3 and summarized in Figure 4.

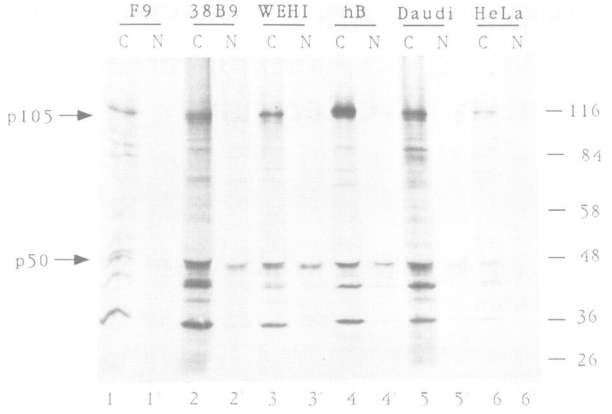


**Fig. 1.** Immunoprecipitation analysis of different cell lines. Cells were metabolically labelled with [$^{35}$S]methionine and cytoplasmic (C) and nuclear (N) extracts were prepared and subjected to immunoprecipitation using anti-p50 polyclonal antiserum 3 (Kieran *et al.*, 1990). Equivalent amounts of cytoplasmic (75 μg) and nuclear (15–35 μg) protein were used. Mouse F9 embryonal carcinoma (lanes 1 and 1′), 38B9 mouse pre-B lymphoblasts (lanes 2 and 2′), WEHI-231 murine B cell lymphoma cells (lanes 3 and 3′), human B lymphocytes transformed with EBV (lanes 4 and 4′), Daudi human Burkitt lymphoma cells (lanes 5 and 5′), HeLa epithelioid carcinoma cells (lanes 6 and 6′). Mol. wt markers (kDa) are on the right.

The transfected full-length cDNA gave rise to a band corresponding to p105, which can be detected over the background of the endogenous monkey protein (Figure 3, lanes 1 and 2). The full-length p105 is found in the cytoplasmic fraction and not in the nucleus of the transfected cells, as previously observed for the endogenous protein (Figure 3, lanes 2 and 2′). A small amount of processed p50 is present in the cytoplasm, but only a fraction of this processed species enters the nucleus. Slightly shorter constructs coding for proteins which retain all the ankyrin-like repeats are also detected only in the cytoplasm of the transfected cells (Figure 3, lanes 3 and 3′; 4 and 4′). The product of the Bal construct containing six of the seven repeats is still found predominantly in the cytoplasm (Figure 3, lane 5 and 5′). In contrast, shorter products (Figure 3, lanes 6–10′) are located almost exclusively in the nucleus. These results are in agreement with those obtained by immunofluorescence.

All constructs, except for Rsa, the product of which is shorter than the naturally processed form (Figure 3, lanes 10 and 10′), gave rise to a processed p50 protein. Thus, the C-terminal part of the protein is not necessary for the correct processing of the precursor. Previously, we showed that p50 is slightly larger than the product obtained upon cleavage by the HIV-1 protease (amino acids 1–412; Rivière *et al.*, 1991). Here we show that the construct containing 436 amino acids (Figure 3, lane 9′) is still processed to give rise to p50. Thus, the cleavage site is located between amino acids 413 and 436 of the p105 molecule.

In summary (Figure 4), cytoplasmic retention of p105 is controlled by either the length or a particular sequence of the C-terminal part of the precursor. In contrast, 24 amino acids (413–436) C-terminal to the cleavage site are sufficient for processing to take place.

### *Fusion of ankyrin-like repeats 6 and 7 to the Rsa construct*

The significant switch from the essentially cytoplasmic Bal product (amino acids 1–779; Figure 3, lanes 5 and 5′) to

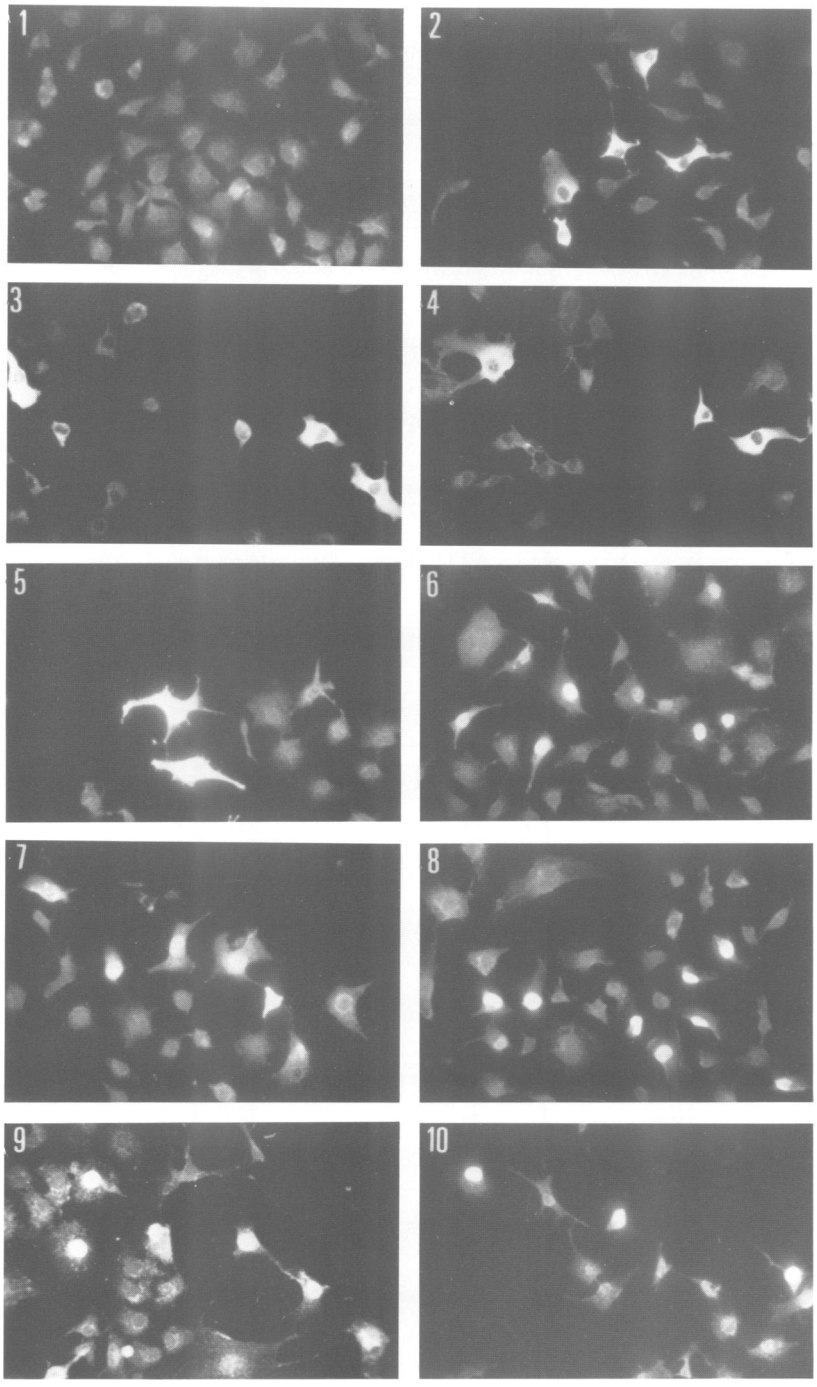


**Fig. 2.** Immunofluorescence analysis of COS7 cells transfected with expression vectors coding for the full-length and C-terminal truncated versions of the human p105 precursor. **Panel 1**, non-transfected cells; **panels 2–10**, cells transfected with a construct containing amino acids: (2) 105: 1–969; (3) 14: 1–901(+9); (4) Pst: 1–819(+29); (5) Bal: 1–779(+37); (6) Sca: 1–715(+53); (7) Tth: 1–544/924–969; (8) Xba: 1–502(+7); (9) Nco: 1–436(+25); (10) Rsa: 1–400(+26). The constructs are described in detail in Figure 4. The amino acid coordinates of p105 are indicated according to the published sequence (Kieran *et al.*, 1990); the number of additional amino acids derived from the linker region of the cloning vectors is given in brackets.

the predominantly nuclear Sca product (amino acids 1–715; lanes 6 and 6′), led us to investigate in detail the small region unique to the longer Bal product. This region contains ankyrin-like repeats 6 and 7, as well as a highly acidic region (eight acidic residues out of 17) located between these repeats. We asked whether this part of the molecule would confer cytoplasmic localization on a smaller construct which is normally exclusively nuclear. We constructed proteins containing amino acids 1–400 (the Rsa construct; see Figure 3, lanes 10 and 10′ and Figure 5B, lanes 1 and 1′) plus either 716–777 (RsaSX) or 716–819 (RsaSP). Immunoprecipitation analysis of COS7 cells transfected with these constructs was performed. Both hybrid proteins containing either ankyrin-like repeat 6 plus the adjacent acidic region (Figure 5B, lanes 2 and 2′) or both ankyrin-like repeats 6 and 7 (lanes 3, 3′), are still found predominantly in the nucleus. This was confirmed by immunofluorescence (data not shown). We thus conclude that regions 716–777

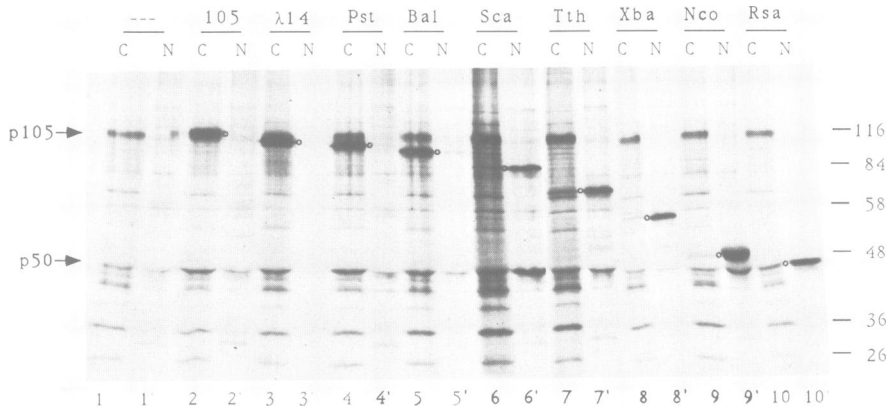


**Fig. 3.** Immunoprecipitation analysis of COS7 cells transfected with expression vectors coding for the full-length and truncated versions of the human p105 precursor. The endogenous monkey p105 and p50 are indicated by arrows. The transfected unprocessed protein species are indicated by open circles. Numbering of lanes 1–10 corresponds to panels 1–10 in Figure 2 (--- indicates untransfected cells). Cells were metabolically labelled with [$^{35}$S]methionine and cytoplasmic (C) and nuclear (N) fractions were prepared. To account for the different transfection efficiencies of the various constructs, different amounts of cytoplasmic (20–60 μg) and nuclear (2–7 μg) extracts were used for immunoprecipitation. Mol. wt markers (kDa) are on the right.

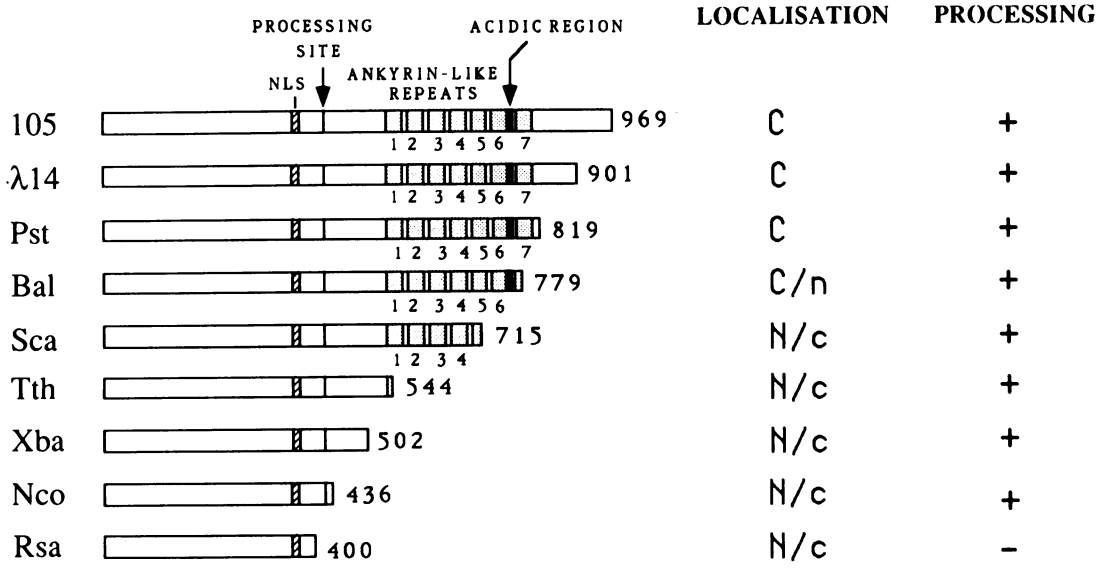


**Fig. 4.** Localization and processing of p105 and various C-terminal truncations. The names of the constructs are on the left. The processing site indicates the probable cleavage area of p105. Ankyrin-like repeats are shown in light stippling, the acidic region in black. (NLS, nuclear localization sequence; C, Cytoplasm; N, Nucleus; C/n indicates predominantly cytoplasmic localization, N/c predominantly nuclear)

or 716–819 alone outside of their natural context are unable to confer cytoplasmic localization to a shorter nuclear product.

***Internal deletion analysis of the full-length precursor***

We next prepared deleted versions of the full-length precursor as follows. We deleted either ankyrin-like repeat 6 (p105ΔA6; lacking amino acids 719–751) or 7 (p105ΔA7; lacking amino acids 772–804) or the highly acidic region (p105ΔDE; lacking amino acids 752–768) (Figure 6A). Immunofluorescence analysis of transfected CoS7 cells showed that the mutant proteins lacking the acidic region or ankyrin-like repeat 7 are found in the cytoplasm, as seen for the wild-type protein (Figure 6B, panels 2, 4 and 5). However, the protein lacking ankyrin-like repeat 6 was located both in the cytoplasm and nucleus of transfected cells (Figure 6B, panel 3). Immunofluorescence using an antibody specific for the 15 most C-terminal amino acids of p105 (antiserum 1140, kindly given by Nancy R.Rice) also demonstrated the presence of full-length p105ΔA6 product in the nucleus (data not shown). This observation was confirmed by immunoprecipitation experiments using [$^{35}$S]methionine-labelled cytoplasmic and nuclear extracts of transfected cells. As shown in Figure 6C lanes 5 and 5′, deletion of ankyrin-like repeat 7 has no significant effect on the localization of the precursor, which is still exclusively found in the cytoplasm. Deletion of the acidic region resulted in ~10% nuclear localization of the mutant p105 (lanes 4 and 4′). The deletion of ankyrin-like repeat 6 had a more drastic effect, resulting in ~30% of the mutant precursor molecules being found in the nucleus (lanes 3 and 3′). Unlike p105, p105ΔDE and p105ΔA7, much of the mutant p105ΔA6 is processed and a large amount of the processed product is present in the nucleus (lane 3′). We conclude that deletion of ankyrin-like repeat 6 and to a lesser extent that of the adjacent acidic region may largely account for the

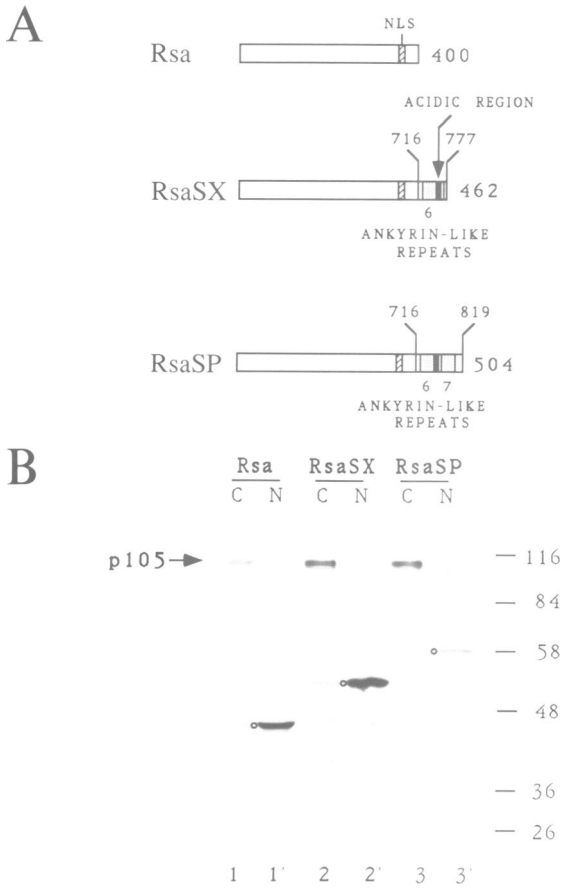


**Fig. 5.** (**A**) Maps of fusion constructs. Ankyrin-like repeats from the C-terminal part of p105 (RsaSX, amino acids 717−777; RsaSP, amino acids 716−819) were fused to the Rsa product encoding amino acids 1−400 of p105. The total number of amino acids of each construct are as follows: Rsa, 400(+26); RsaSX, 462(+6); RsaSP, 504(+27). Amino acid coordinates are indicated according to the published sequence of p105 (Kieran *et al.*, 1990); the number of additional amino acids derived from the linker region of the cloning vectors is given in the brackets. Ankyrin-like repeats are shown in light stippling, the acidic region in black. NLS, nuclear localization sequence. (**B**) Immunoprecipitation analysis of COS7 cells transfected with expression vectors coding for the hybrid products described in panel A. The endogenous monkey p105 is indicated by an arrow. The transfected protein species are indicated by open circles. Construct Rsa, lanes 1 and 1′; RsaSX, lanes 2 and 2′; RsaSP, lanes 3 and 3′. Cells were metabolically labelled with [$^{35}$S]methionine and cytoplasmic (C) and nuclear (N) fractions were prepared. Equivalent amounts of cytoplasmic (100 μg) and nuclear (10−17 μg) protein were used for immunoprecipitation. Mol. wt markers (kDa) are on the right.

predominantly nuclear localization of the Sca product (Figure 3, lanes 6 and 6′).

In order to determine the effect of these internal deletions on the DNA binding ability of the p105 protein, we assayed cytoplasmic and nuclear extracts derived from the various transfected COS7 cells by bandshift. As shown in Figure 7 transfection with the plasmid encoding the full-length p105 protein (panel A, lanes 6 and 7) showed a pattern very similar to untransfected cells (lanes 4 and 5), confirming the lack of DNA binding activity of the p105 protein; transfection with the Rsa construct (lanes 2 and 3) gave rise to an almost exclusively nuclear DNA binding activity, in accordance with the immunoprecipitation and immunofluorescence experiments. The results obtained with the internally deleted constructs were unexpected. Transfection with the p105ΔA6 construct gave rise to an almost exclusively nuclear complex, migrating like bona fide KBF1 (lanes 8 and 9; compare with lane 2). Thus deletion of ankyrin 6 results in a much more extensive processing of the precursor, with the processed product (p50) being translocated to the nucleus, as indicated previously by the immunoprecipitation experiments shown in Figure 6C, lane 3′. Surprisingly, transfection with the p105ΔDE and p105ΔA7 constructs gave rise to two predominantly cytoplasmic complexes (C1 and C2 in Figure 7, lanes 10−13) migrating slower than bona fide NF-κB (lane 1). In order to characterize these complexes further, we used two anti-p105 antibodies (kindly given by Nancy R.Rice), recognizing either the N-terminus (1141 antibody) or the C-terminus (1140 antibody) of the p105 precursor. As can be seen in panel B, addition of either antibody led to the disappearance of the two complexes, indicating that they both contain the p105ΔDE or ΔA7 protein. The use of anti-c-rel antibodies had no effect, indicating that the two complexes do not contain this proto-oncogene product (data not shown).

### *p50 contains a functional nuclear localization sequence (NLS)*

A possible mechanism for the cytoplasmic retention of the precursor is by masking of the potential nuclear localization signal (NLS) located at the end of the region of homology to *rel* and *dorsal* (Kieran *et al.*, 1990; Ghosh *et al.*, 1990). This stretch of basic amino acids is also present in the other members of the rel/dorsal family of proteins (for review see Govind and Steward, 1991) and has been shown to be a functional NLS in the v-*rel* oncogene product (Gilmore and Temin, 1988). We introduced three different types of mutations of the NLS (Figure 8A) in the context of the Xba construct, whose product was found exclusively in the nucleus of transfected COS7 cells (Figure 2, panel 8; Figure 3, lanes 8 and 8′). We assayed the mutant constructs by transfection followed by immunofluorescence. As seen in Figure 8B, in contrast to the wild-type Xba product (panel 1), all three mutated proteins were found in the cytoplasm (panels 2−4). Thus the three mutations inhibited the nuclear translocation of the protein, indicating that the proposed nuclear localization sequence is functional and therefore could be the target of the C-terminal region conferring cytoplasmic localization.

## Discussion

In this study we examined the subcellular localization of the p105 precursor of p50 as well as that of truncated and mutated forms of p105. We showed that in different cell types the p105 precursor is confined to the cytoplasm, whereas processed p50 molecules are found both in the cytoplasm and in the nucleus. C-terminal truncations of the precursor defined a small region that is necessary for cytoplasmic localization and controls the extent of proteolytic processing and the DNA binding activity of the molecule. A detailed analysis of this region showed that deletion of ankyrin-like repeat 6 and of the adjacent acidic region results in the nuclear localization of a subset of the molecules. We also demonstrated that the putative NLS located in the N-terminal part of the molecule is indeed functional and is required for the nuclear localization of p50. Finally we showed that the inability of p105 to bind DNA can be

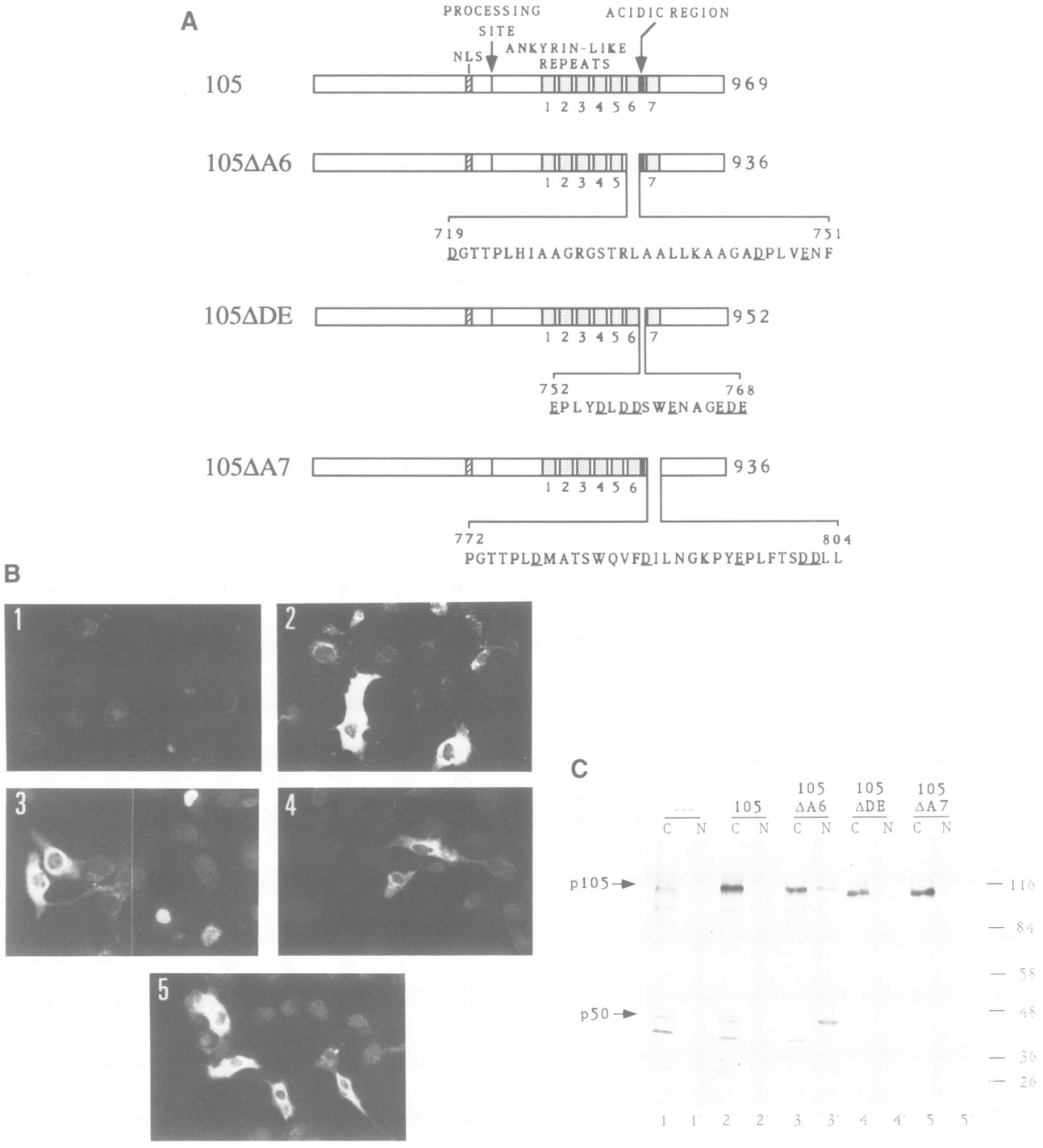


**Fig. 6.** (**A**) Maps of internal deletion constructs of p105. The deleted amino acids are shown. Acidic residues are underlined. The total number of amino acids is indicated on the right. The processing site indicates the probable cleavage area of p105. Ankyrin-like repeats are shown in light stippling, the acidic region in black. NLS, nuclear localization sequence. (**B**) Immunofluorescence analysis of COS7 cells transfected with expression vectors coding for the deletion mutants of human p105 precursor described in panel A. (1) Non-transfected cells; (2–5) cells transfected with constructs: (2) 105; (3) 105ΔA6; (4) 105ΔDE; (5) 105ΔA7. (**C**) Immunoprecipitation analysis of CoS7 cells transfected with expression vectors coding for the deleted mutants of the human p105 precursor described in panel A. The endogenous monkey p105 and p50 are indicated by arrows. Numbering of lanes 1–5 corresponds to panels 1–5 in Figure 6B. Cells were metabolically labelled with [$^{35}$S]methionine and cytoplasmic (C) and nuclear (N) fractions were prepared. To account for the different transfection efficiencies of the various constructs different amounts of cytoplasmic (20–200 μg) and nuclear (2–12 μg) extracts were used for immunoprecipitation.

abolished by deletion of either ankyrin-like repeat 7 or the highly acidic region between repeats 6 and 7.

### *Possible mechanisms involved in cytoplasmic retention of the precursor*

Our results strongly suggest that the region of ankyrin-like repeats 6 and 7 found in the C-terminal part of the precursor molecule is involved in inhibiting DNA binding and preventing nuclear localization. In human erythrocyte ankyrin, such motifs act as binding sites for cytoskeleton components, such as tubulin, and for integral membrane proteins, such as the erythrocyte anion exchanger (Lux *et al.*, 1990). Recently, it has been shown that the four ankyrin-like repeats located in the N-terminal part of the β subunit

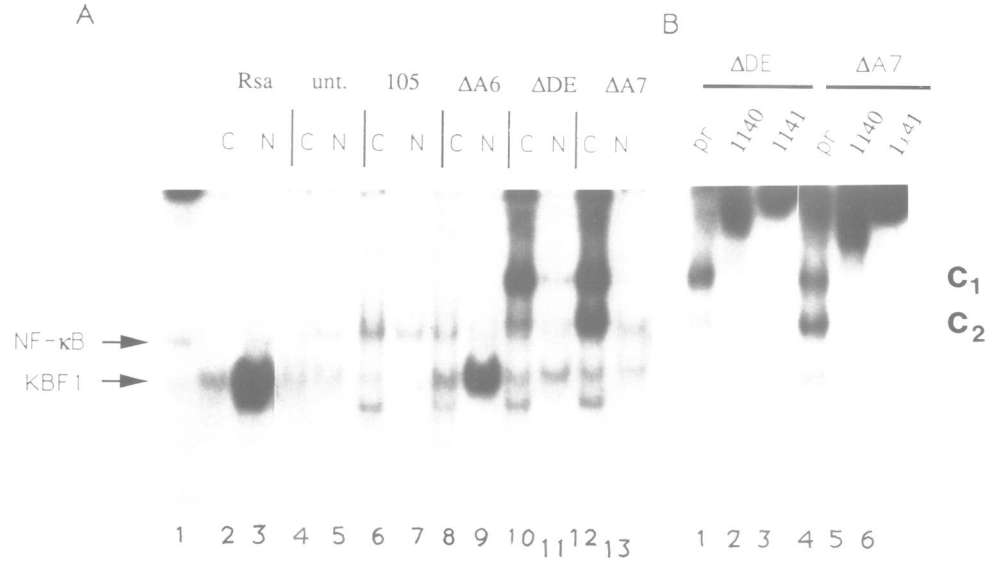


**Fig. 7.** Bandshift analysis of cytoplasmic (C) or nuclear (N) fractions derived from transfected COS7 cells. (**A**) The names of the construct are on the top, unt indicates untransfected cells. Cytoplasmic and nuclear fractions were prepared as described in Materials and methods, and used for bandshift with no further treatment. 3 μg of nuclear and 15 μg of cytoplasmic proteins were used. In lane 1, purified KBF1 (Kieran *et al.*, 1990) and NF-κB (kindly given by P.Baeuerle) were used. The probe used was the KBF1/NF-κB site derived from the H-2 $K^b$ MHC class I gene promoter. (**B**) pr indicates preimmune serum; antiserum 1140 is directed against the C-terminal last 15 amino acids of p105; antiserum 1141 is directed against the first N-terminal 15 amino acids of p105. C1 and C2 correspond to the two complexes described in the text.

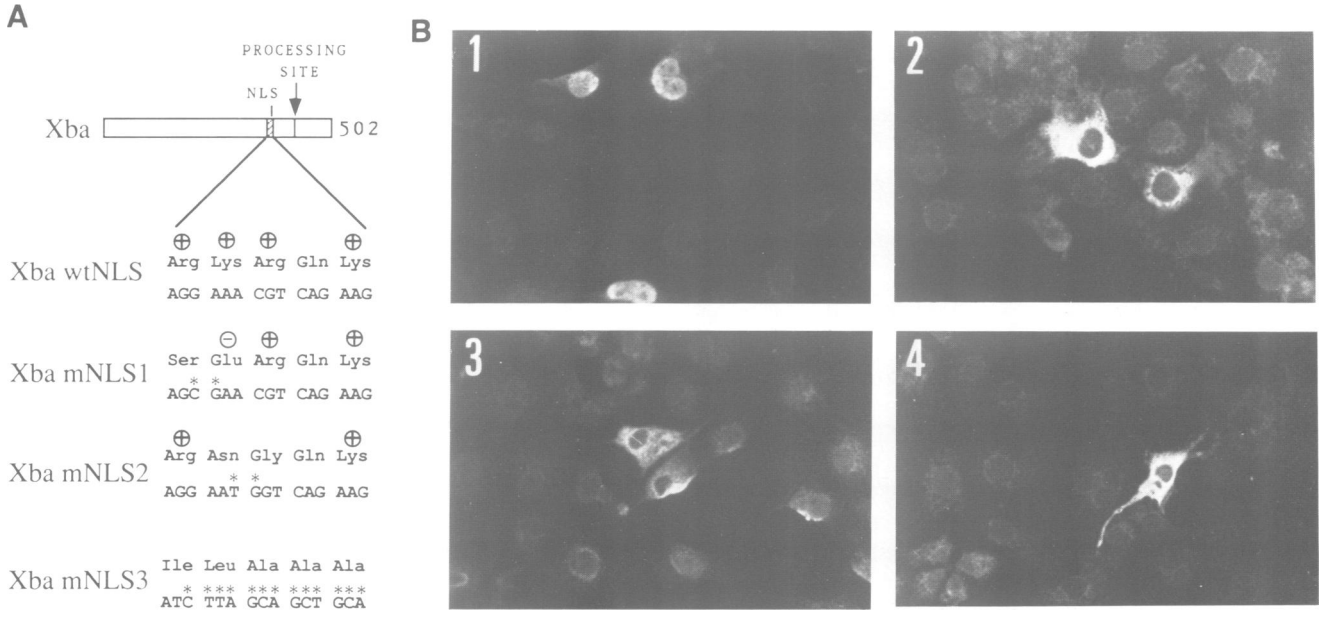


**Fig. 8.** (**A**) Mutations of the nuclear localization sequence (NLS) of the Xba construct (for amino acid coordinates see Figure 4). Mutated nucleotides (*), basic (+) and acidic (−) residues are indicated. The processing site indicates the probable cleavage area of p105. (**B**) Immunofluorescence analysis of COS7 cells transfected with expression vectors encoding the mutated Xba products described in panel A. Cells transfected with constructs (1) Xba; (2) Xba mNLS1; (3) Xba mNLS2; (4) Xba mNLS3.

of the DNA binding protein GABP mediate the stable interaction with the GABPα subunit and, when complexed with GAPBα directly contact DNA (Thompson *et al.*, 1991). However, in other proteins containing different numbers of such motifs, the function of these domains has not been determined. These proteins are involved in tissue-differentiation [e.g. the products of the genes *Notch* (*Drosophila melanogaster*), *lin-12* and *glp-1* (*Caenorhabditis elegans*)] or in cell cycle control [e.g.the yeast proteins encoded by *cdc10*, *SWI6* and *SWI4* (Lux *et al.*, 1990)].

In transfected COS7 cells, where the replicating vector is expected to give rise to large amounts of expressed proteins, the precursor is exclusively found in the cytoplasm (Figure 2, panel 2 and Figure 3, lanes 2 and 2′). This indicates that the 'anchor' responsible for cytoplasmic retention of p105 cannot be titrated under those conditions. One possible explanation could be that ankyrin-like repeat 6 found in the precursor, and the acidic region just downstream, might anchor the protein to some abundant component of the cytoskeleton or of the plasma membrane, thereby inhibiting its translocation to the nucleus. However, attachment of p105 to the plasma membrane was not observed in a confocal microscopy study and preliminary subcellular fractionation experiments showed that the precursor is present in the

cytosol but not in the membrane fraction of HeLa cell extracts (data not shown). In addition, as mentioned above, the results obtained for the DNA binding protein GABP clearly show that these motifs can be also involved in functions other than attachment to the cytoskeleton, such as mediating interactions with other subunits or with DNA (Thompson *et al.*, 1991).

A more attractive possibility is that cytoplasmic localization of p105 is a property of the molecule *per se*, as would be the case if the nuclear localization sequence (NLS) were masked by a portion of the C-terminal region. For this reason, we introduced mutations in the putative NLS sequence (Bours *et al.*, 1990; Ghosh *et al.*, 1990, Kieran *et al.*, 1990; Meyer *et al.*, 1991), a stretch of four basic amino residues also present in the rel and dorsal proteins (see Figure 8A). As seen in Figure 8B, various mutations changing the net charge of the NLS all abolish nuclear translocation. Thus, this sequence, as was previously shown for its v-rel homologue (Gilmore and Temin, 1988), is functional and might be the target for control of subcellular localization. One might speculate for example that the acidic region directly interacts with the basic residues of the NLS, or that the ankyrin-like repeat 6 directly or indirectly prevents access to the NLS. However, the situation may be more complicated: deletion of ankyrin-like repeat 6 has a more pronounced effect (3-fold higher) than deletion of the acidic region and none of these deletions results in a predominantly nuclear localization of the precursor as observed for the C-terminal truncated Sca product (Figure 2, panel 6 and Figure 3, lanes 6 and 6′). Thus other parts of the protein may also be important for its correct subcellular localization.

### *Inhibition of DNA binding by the acidic region and ankyrin-like repeat 7 in the wild-type precursor*

By gel-shift experiments with extracts of transfected COS7 cells we have shown that, in contrast to the full-length p105 (Bours *et al.*, 1990; Ghosh *et al.*, 1990; Kieran *et al.*, 1990; Meyer *et al.*, 1991) constructs lacking either the acidic region or ankyrin-like repeat 7 are able to bind DNA. Thus these regions may block DNA binding by the wild-type precursor. In cells transfected with p105ΔDE and p105ΔA7, in addition to the band corresponding to KBF1, two retarded bands with slower mobility (C1 and C2) were seen. Since both bands can be shifted with antiserum specific for either the N- or C-terminus of p105, they both contain the p105 mutant precursor. In a parallel experiment we observed that the complex due to the homodimer formed by *in vitro* translated c-rel migrates slightly faster than C1 (not shown). These observations suggests that C1 is formed by a homodimer of the ΔDE or ΔA7 product. The origin of C2 is more difficult to assess, but the most likely possibility is that it is formed of a 'heterodimer' between the full-length ΔDE or ΔA7 products and their processed derivative, p50. The presence of p50 in C2 is difficult to demonstrate since all antibodies against p50 would also recognize the precursor. An alternative, although less likely, possibility is that C2 is due to binding of a monomer of ΔDE or ΔA7. If the first hypothesis is correct, this would imply that in the wild-type precursor the region of ankyrin-like repeats 6 and 7 could interact with the dimerization domain and thus prevent DNA binding.

It is likely from the above data that the p105 precursor is folded in such a way that the region containing ankyrin-like repeats 6 and 7 interacts with at least part of the dimerization domain (Logeat *et al.*, 1991), the NLS and the processing site (this region corresponds to the C-terminal part of mature p50). Various deletions of the region of repeats 6–7 result in a more extensive processing, a partial nuclear localization and the ability to bind DNA, probably through unmasking of the dimerization region. However, as suggested by the data shown in Figure 5, the integrity of the C-terminal region is probably required for this interaction to take place correctly. More extensive mutagenesis as well as structural studies will be necessary to fully understand these complex interactions.

## Materials and methods

#### *Plasmids*

C-terminal truncated derivatives of p105 described in Figure 4 were obtained by cloning fragments of the complete p105cDNA into the polylinker of either pRc/CMV (Invitrogen) or pKC3/4 (Van Doren *et al.*, 1984) expression vectors (details of the constructs can be obtained upon request). The construct λ14 corresponds to an incomplete cDNA clone containing nucleotides 1–2876 of the published sequence (Kieran *et al.*, 1990). The hybrid constructs described in Figure 5 were obtained by fusing the *ScaI–XbaI* (nucleotides 2319–2501) and *ScaI–PstI* (nucleotides 2319–2629) fragments to the *RsaI* site located at position 1373 (Kieran *et al.*, 1990) and subcloning into the pRc/CMV expression vector (Invitrogen).

#### *Site-directed mutagenesis*

Site-directed mutagenesis was performed using the Muta-Gene M13 *in vitro* Mutagenesis Kit (Bio-Rad) according to the manufacturer's instructions. Base substitutions and deletions were introduced into the full-length p105 cDNA cloned in pBluescript vector (Stratagene) and appropriate fragments were subcloned into pRc/CMV expression vector (Invitrogen). The oligonucleotides (Applied Biosystems 381A synthesizer) used are listed below: ΔA6 5′-GTGGACAGTACTACCTACGAGCCTCTCTATGAC-CTG-3′; ΔDE, 5′-CCCCTGGTGGAGAACTTTGGAGTTGTGCCTGG-AACC-3′; ΔA7, 5′-GAGGATGAAGGAGTTGTGGCACAAGGAGAC-ATGAAA-3′; mNLS1, 5′-AGAAGTGCAGAGCGAACGTCAGAAGC-3′; mNLS2, 5′-AGTGCAGAGGAATGGTCAGAAGCTCA-3′; and mNLS3, 5′-GAAGAAGTGCAGATCTTAGCAGCTGCCACTCATGCCCAAT-3′

#### *Cell cultures*

Monkey COS7, human HeLa and F9 embryonal carcinoma cells were grown in DMEM supplemented with 10% new-born calf serum or 10% fetal calf serum in the case of F9 cells. The mouse pre-B cell lines WEHI, 38B9, human Daudi cells and the human B cell line transformed with EBV were grown in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum and 5 μM β-mercaptoethanol.

#### *Transfection*

COS7 cells were grown to 40% confluency in DMEM medium supplemented with 10% newborn calf serum in 100 mm Petri dishes and transfected with 10 μg of plasmid DNA using the calcium phosphate co-precipitation procedure (see Kimura *et al.*, 1986). After 24 h the cells were rinsed twice with PBS and supplied with fresh medium. 48 h after transfection, labelling and extraction of the cells were performed as described below.

#### *Indirect immunofluorescence*

Cells were attached to cover slips and then transfected as described above. After 48 h the cells were rinsed once with PBS and fixed by incubating for 4 min in a mixture of methanol and acetone (1:1). After rinsing twice with PBS, anti-p50 polyclonal antiserum 3 (Kieran *et al.*, 1990) was added at a 50- or 100-fold dilution in 200 μl PBS with 3% BSA and incubated for 1 h at 4°C. After washing three times with PBS the cells were incubated with goat anti-rabbit antibodies conjugated to fluorescein (Pasteur) at a 100-fold dilution in 200 μl PBS with 3% BSA for 1 h at 4°C. Fixed and stained cells were washed again three times with PBS and were mounted in Mowiol. Fluorescence microscopy was performed with a Zeiss photomicroscope III.

#### *In vivo labelling and immunoprecipitation*

COS7 cells in logarithmic phase were washed twice with PBS and incubated in RPMI medium depleted of methionine and supplemented with 2% dialysed

fetal calf serum for 30 min at 37°C. 100 μCi/ml of [$^{35}$S]methionine were added to the culture for 3–5 h at 37°C. After labelling, the cells were washed twice with PBS and nuclear and cytoplasmic extracts were prepared as described previously (Israël *et al.*, 1987). Cytoplasmic and nuclear extracts containing equivalent amounts of protein were boiled for 5 min in the presence of 1% SDS, and RIPA buffer (0.15 M NaCl, 1% Triton, 0.1% SDS, 0.5% DOC, 50 mM Tris pH.7.5, 0.5 mM PMSF, 1 mg/ml BSA) was then added, followed by preimmune serum and protein A–Sepharose beads, and incubated for several hours to overnight at 4°C. After centrifugation, the supernatant was mixed with protein A–Sepharose beads plus anti-p50 polyclonal antiserum 3 (Kieran *et al.*, 1990) and incubated for several hours to overnight at 4°C. The beads were washed three times with modified RIPA (see above, without BSA and PMSF), boiled in 15 μl of Laemmli buffer (5% mercaptoethanol) and then analysed on a 10% SDS–polyacrylamide gel.

### *Gel-shift assays*

These were performed as described in Israël *et al.* (1987). The probe used was the KBF1/NF-κB binding site located in the enhancer region of the H-2 $K^b$ promoter. 3 μg of nuclear proteins or 15 μg of cytoplasmic proteins were used.

### *Antisera*

Antiserum 3 has been described in Kieran *et al.* (1990). Rabbit polyclonal antiserum 1140 (directed against the C-terminal last 15 amino acids of the p105 precursor) and 1141 (directed against the N-terminal first 15 amino acids of p105) were kindly given by Nancy R.Rice.

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## References

Baeuerle,P.A. (1991) *Biochim. Biophys. Acta*, **1072**, 63–80.
Baeuerle,P.A. and Baltimore,D. (1988a) *Cell*, **53**, 211–217.
Baeuerle,P.A. and Baltimore,D. (1988b) *Science*, **242**, 540–546.
Baeuerle,P.A. and Baltimore,D. (1989) *Genes Dev.*, **3**, 1689–1699.
Baeuerle,P.A., Lenardo,M., Pierce,J.W. and Baltimore,D. (1988) *Cold Spring Harbor Symp. Quant. Biol.*, **53**, 789–798.
Ballard,D.W., Walker,W.H., Doerre,S., Sista,P., Molitor,J.A., Dixon,E.P., Peffer,N.J., Hannink,M. and Greene,W.C. (1990) *Cell*, **63**, 803–814.
Bours,V., Villabos,J., Burd,P.R., Kelly,K. and Siebenlist,U. (1990) *Nature*, **348**, 76–78.
Davis,N., Ghosh,S., Simmons,L.D., Tempst,P., Liou,H.-C., Baltimore,D. and Bose,H.R.Jr (1991) *Science*, **253**, 1268–1271.
Ghosh,S., Gifford,A.M., Riviere,L.R., Tempst,P., Nolan,G.P. and Baltimore,D. (1990) *Cell*, **62**, 1019–1029.
Gilmore,T.D. (1990) *Cell*, **62**, 841–843.
Gilmore,T.D. and Temin,H.M. (1988) *J. Virol.*, **62**, 703–714.
Govind,S. and Steward,R. (1991) *Trends Genet.*, **7**, 119–125.
Hannink,M. and Temin,H.M. (1989) *Mol. Cell. Biol.*, **9**, 4323–4336.
Haskill,D., Beg,A.A., Tompkins,S.M., Morris,J.S., Yurochko,A.D., Sampson-Johannes,A., Mondal,K., Ralph,P. and Baldwin,A.S.,Jr (1991) *Cell*, **65**, 1281–1289.
Israël,A., Kimura,A., Kieran,M., Yano,S., Kanellopoulos,J., Le Bail,O. and Kourilsky,P. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 2653–2657.
Israël,A., Yano,S., Logeat,F., Kieran,M. and Kourilsky,P. (1989a) *Nucleic Acids Res.*, **17**, 5245–5257.
Israël,A., Le Bail,O., Hatat,D., Piette,J., Kieran,M., Logeat,F., Wallach,D., Fellous,M. and Kourilsky,P. (1989b) *EMBO J.*, **8**, 3793–3800.
Kieran,M., Blank,V., Logeat,F., Vandekerckhove,J., Lottspeich,F., Le Bail,O., Urban,M.B., Kourilsky,P., Baeuerle,P. and Israël,A. (1990) *Cell*, **62**, 1007–1018.
Kimura,A., Israël,A., Le Bail,O. and Kourilsky,P. (1986) *Cell*, **44**, 261–272.
Lenardo,M.J. and Baltimore,D. (1989) *Cell*, **56**, 227–229.
Logeat,F., Israel,N., Ten,R., Blank,V., Le Bail,O., Kourilsky,P. and Israël,A. (1991) *EMBO J.*, **10**, 1827–1832.
Lux,S.E., John,K.M. and Bennett,V. (1990) *Nature*, **344**, 36–42.
Macchi,M., Borner,J-M., Davidson,I., Kanno,M., Rosales,R., Vigneron,M., Xiao,J-H., Fromental,C. and Chambon,P. (1989) *EMBO J.*, **8**, 4215–4227
Meyer,R., Hatada,E. N., Hohmann,H.-P., Haiher,M., Bartsch,C., Röthlisberger,U., Lahm,H.-W., Schlaeger,E.J., Van Loon,A.P.G.M. and Scheidereit,C. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 966–970.
Nabel,G. and Baltimore,D. (1987) *Nature*, **326**, 711–713.
Nolan,G.P., Ghosh,S., Liou,H.-C., Tempst,P. and Baltimore,D. (1991) *Cell*, **64**, 961–969.
Rivière,Y., Blank,V., Kourilsky,P. and Israël,A. (1991) *Nature*, **350**, 625–626.
Ruben,S., Dillon,P.J., Schreck,R., Henkel,T., Chen,C.-H., Maher,M., Baeuerle,P.A. and Rosen,C. (1991) *Science*, **251**, 1490–1493.
Sen,R. and Baltimore,D. (1986) *Cell*, **47**, 921–928.
Thompson,C.C., Brown,T. and McKnight,S.L. (1991) *Science*, **253**, 762–768.
Van Doren,K., Hanahan,D., Gluzman,G. (1984) *J. Virol.*, **50**, 606–614.
Yano,O., Kanellopoulos,J., Kieran,M., Le Bail,O., Israël,A. and Kourilsky,P. (1987) *EMBO J.*, **6**, 3317–3324.

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## Note added in proof

The IκB-like molecules MAD (human) and pp40 (avian) also contain five ankyrin-like repeats (Haskill *et al.*, 1991; Davis *et al.*, 1991). These motifs may interact with p65 or c-rel by masking the NLS, as described here for the C-terminus of the p105 precursor molecule.