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The C terminus of the NF-κB p50 precursor and an IκB isoform contain transcription activation domains

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ABSTRACT

The p50 subunit of the NF-κB transcription complex is derived from the N-terminal half of a larger precursor protein, p105. Although a fair amount is known about functions located within the p50 sequences, less is known about the C-terminal half of p105. In this report, we have identified a potent transcription activation domain located in the C terminus of mouse p105. In addition, the IκBβ proteins chicken p40 and human MAD-3, proteins that are related to the p105 C terminus, strongly activated transcription in chicken cells and yeast when fused to GAL4 DNA-binding sequences. Furthermore, chicken p40 is primarily located in the nucleus of chicken cells when overexpressed from a retroviral vector. Our results suggest novel models for the function and regulation of NF-κB transcription complexes.

INTRODUCTION

The Rel family of proteins includes a number of DNA-binding proteins known to be involved in transcription regulation, including the *Drosophila melanogaster dorsal* gene product, the v-Rel oncoprotein, and several interacting proteins that make up mammalian NF-κB-like transcription complexes (1, 2). These proteins are related through a highly conserved N-terminal domain of approximately 300–350 amino acids (aa) called the Rel homology (RH) domain, but are generally unrelated in their C-terminal halves. The RH domain contains sequences important for DNA binding, nuclear localization and protein-protein interactions.

NF-κB, originally described as a factor involved in the tissue-specific control of transcription of the mouse κ light chain immunoglobulin gene, is a heterodimer consisting of 50 kilodalton (p50) and 65 kilodalton (p65) subunits (2). However, it is now clear that NF-κB-like transcription complexes are involved in the control of a large number of cellular and viral promoters. Active NF-κB-like transcription complexes can be homodimers or heterodimers consisting of any one or two Rel family cellular proteins designated p50, p65, p49, RelB and the c-Rel proto-oncoprotein (1, 2). Biochemical evidence and sequence data indicate that p50 is derived from the proteolytic processing of an approximately 105 kilodalton precursor protein (p105) (3–6). Nevertheless, in many cells, a large amount of the p50/p105 protein is stably present in the nonprocessed p105 form (7–9).

NF-κB-like transcription complexes, such as p50-65 or p50-c-Rel, are generally thought to be retained in an inactive form in the cytoplasm through interaction with a family of inhibitor proteins, the IκB proteins (IkBα, β, and γ) (10, 11). However, neither the subcellular location of these IκB proteins nor the mechanism by which they retain NF-κB complexes in the cytoplasm has been determined. p105 is likely to be retained in the cytoplasm by interaction of C-terminal sequences with the N-terminal RH domain since it has been shown that C-terminal deletions in p105 allow it to enter the nucleus and N-terminal and C-terminal p105 sequences can associate *in trans* (8, 12). Thus, one function for p105 C-terminal sequences has been proposed to be cytoplasmic retention.

IκB proteins also inhibit the binding of NF-κB complexes to κB target DNA sequences *in vitro* (10, 11, 13). Although IκBβ can interact with the RH domain of p65, c-Rel and p50, it inhibits DNA binding only of complexes containing p65 and c-Rel (13–16); it is likely that DNA binding by p50 homodimers is inhibited by C-terminal sequences of p105, derived either by cleavage or as encoded by an alternatively spliced form of p105 (IκBγ) (11, 12). A second function for p105 C-terminal sequences thus appears to be inhibition of DNA binding by certain Rel family proteins.

The C terminus of p105 has striking sequence similarity with the IκBβ proteins MAD-3 (human) and p40 (chicken), in that they all contain 5–7 copies of an ankyrin or cell-cycle repeat (16, 17). Besides IκB proteins and the C terminus of p105 (proteins that inhibit DNA binding of Rel transcription factors), ankyrin-like repeats have also been found in transcription factors such as mammalian GABPβ and the *Saccharomyces cerevisiae* SWI4 and SWI6 proteins (18–20). It is likely that the ankyrin-like repeats are important for mediating protein-protein interactions in these transcription factors (19, 21).

Mouse p50, which is derived from p105 after proteolytic removal of C-terminal sequences (6), is a functional DNA-binding protein that has a limited ability to activate transcription (22). In contrast, the C-terminal halves of most other Rel family proteins are probably not removed, and the C termini of these

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Rel proteins contain potent transcription activation domains (9, 23–27). By analogy, we wished to determine whether the C-terminal half of p105 also contained sequences that could function as a transcription activation domain. In this report, we demonstrate that mouse p105 C-terminal sequences and IκBβ proteins activate transcription when fused to a heterologous DNA-binding protein (GAL4). These results identify a third functional domain in the p105 C-terminal sequences and suggest novel models for the regulation of NF-κB-like transcription complexes.

MATERIALS AND METHODS

Plasmids

All recombinant DNA techniques were performed according to standard procedures (28). Plasmids for the expression of GAL4 fusion proteins in chicken and mammalian cells were made using convenient restriction enzyme sites, and subcloned at the polylinker of pSG424 (29) such that the fusion would be in-frame with GAL4 aa 1–147. The sequence at the fusion point was confirmed by double-stranded DNA sequencing using Sequenase (United States Biochemical). Plasmids for expressing GAL4 fusion proteins in yeast were made by subcloning the sequences encoding the fusion proteins from the SG424 vectors into the *Eco*RI of pMA424 (30). pMA241 and pMA210 are plasmids for the expression of GAL4 1–147 and full-length GAL4, respectively (30).

GM282 is a spleen necrosis virus vector that expresses wild-type v-Rel (31). JDp40 is a spleen necrosis virus vector that expresses wild-type chicken p40; JDp40 was created by subcloning an *Xba*I to *Hin*dIII fragment containing p40 cDNA sequences into a retroviral vector derived from pJD214 (32).

Cell culture, immunofluorescence and CAT assays

Chicken embryo fibroblasts (CEF) were prepared and maintained as described previously (31). All transfections were performed by the DMSO-polybrene method (31, 33).

For CAT assays, CEF were transfected with 5 μg of pG5BCAT reporter plasmid (34), 5 μg of the indicated producer plasmid, and 1 μg of plasmid MSV-βgal, essentially as described previously (25). Cells were lysed 48 hrs later, samples were normalized according to β-galactosidase activity, and assayed for CAT activity. CAT activity is presented as the percentage of radioactivity in acetylated chloramphenicol out of the total radioactivity in the acetylated and nonacetylated forms of chloramphenicol.

For immunofluorescence, CEF were transfected with 10 μg of plasmid DNA; in the cases of the retroviral vectors GM282 and JDp40, 0.1 μg of helper virus DNA SW253 (35) was also included. Two or three days after transfection, CEF were passaged onto glass coverslips and indirect immunofluorescence was performed as described previously (36). Primary antibodies were diluted as described in the legend to Fig. 2. In every case,

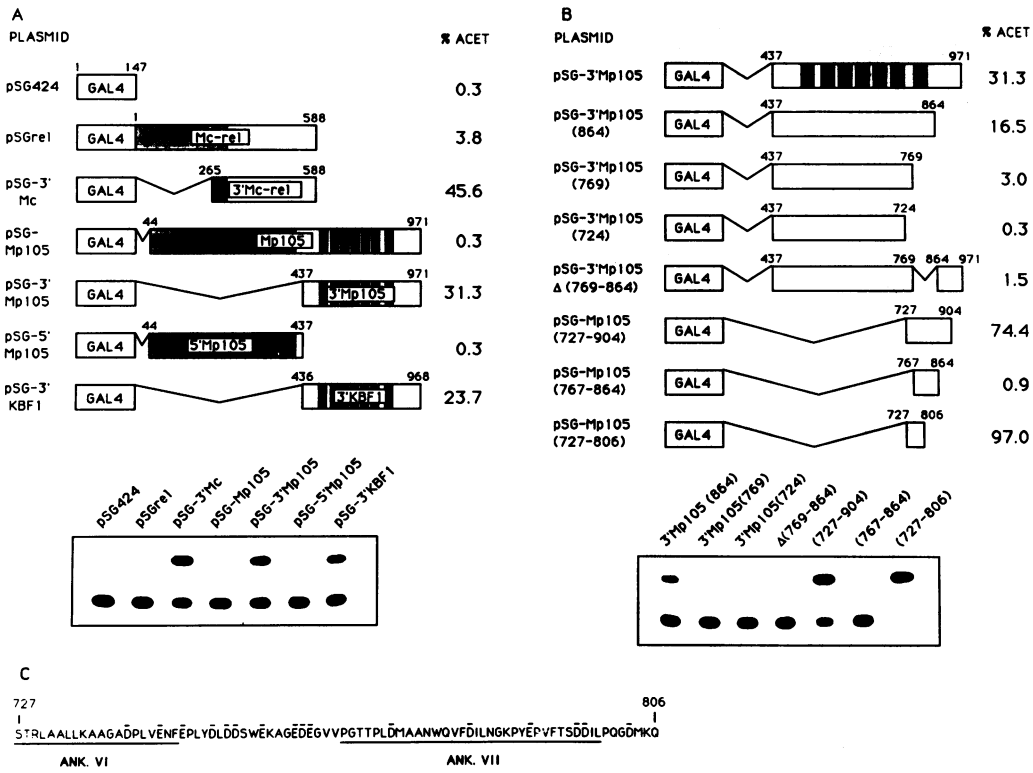

Figure 1. Transcription activation by GAL4-p105 fusion proteins. Structures of the GAL4 fusion proteins encoded by the indicated plasmids are shown in (A) and (B). Numbers above the boxes refer to the aa from the indicated protein that are included in the fusion protein. The approximate positions of the Rel homology domains are indicated by shading; the approximate positions of the ankyrin-like repeats are indicated by black bars. A CAT assay in transfected CEF is shown below each set of constructs. Each fusion protein has been tested at least three times, and percent acetylation (%ACET) for this representative assay is reported. The two values above 50% may be underestimates of the real strength of activation since they are likely to be beyond the linear range of the assay. (C) The sequence of mouse p105 between aa 727 and 806 is shown (3). Sequences that are contained within the sixth and seventh ankyrin repeats are underlined; as indicated, the sequence has an overall negative charge of −11.

a fluorescein-conjugated goat anti-rabbit immunoglobulin secondary antibody (Sigma) was used at a 1:20 dilution.

Transcription assays in yeast

To express GAL4 fusion proteins in yeast, 5 μg of plasmid DNA was transformed into yeast strain YM335:RY171 (37) using the LiCl method (38). Cells were plated on His^- selective plates and incubated at 30°C for 2 days. Three independent colonies were picked for each plasmid (except for two for pMA-Chp40), and yeast were grown in liquid culture to an approximate OD_{600} of 0.4 to 1.0. β-galactosidase activity was determined essentially as described previously (24).

RESULTS AND DISCUSSION

Activation of transcription by C-terminal p105 sequences in avian and mammalian cells

Because higher eukaryotic cells contain a number of proteins that can bind to κB sites and because NF-κB complexes are regulated by subcellular localization, we used hybrid genes encoding aa 1–147 of GAL4 (which contain nuclear localizing and dimerization domains) fused to mouse p105 sequences to assay gene activation. We used a reporter plasmid (pG5BCAT) that contains five copies of a GAL4 target sequence, a TATA box and the chloramphenicol acetyltransferase (CAT) gene (23, 34). GAL4 1–147 sequences by themselves (pSG424; ref. 29) did not activate transcription from reporter plasmid pG5BCAT when plasmids were cotransfected into CEF (Fig. 1A). Consistent with previous results (23, 25), a GAL4 fusion protein expressing full-length mouse c-Rel sequences (SGrel) was weakly activating, and removal of N-terminal RH sequences (SG-3'Mc) created a strongly activating protein. Similarly, a GAL4 fusion protein expressing aa 44–971 of mouse p105 (SG-Mp105) was not activating under these conditions, and a GAL4 fusion protein expressing aa 437–971 of mouse p105 (SG-3'Mp105) was almost as strongly activating as the C-terminal mouse c-Rel sequences in SG-3'Mc. Furthermore, C-terminal sequences from human and chicken p50 precursor proteins (SG-3'KBF1 and SG-3'Chp105) also activated transcription in CEF (Fig. 1A and ref. 39, respectively). A GAL4 fusion protein containing only N-terminal RH sequences from mouse p105 (SG-5'Mp105; aa 44–437 of mouse p105) did not activate transcription in CEF.

Qualitatively identical results were obtained in mouse 3T3 cells, human Hela cells, and monkey Cos-1 cells: that is, SG-3'Mp105 was a strong activator of transcription, and full-length p105 sequences were not activating (Table 1). Likewise, pSG-3'KBF1 (human p105) activated transcription in these mammalian cells (data not shown). These results demonstrate that mouse p105, like mouse c-Rel, contains a strong C-terminal transcription activation domain that can function in a variety of cell types.

Using an anti-GAL4 antibody, we performed immunofluorescence on CEF expressing GAL4-p105 fusion proteins (Fig. 2). GAL4 fusion proteins containing nearly full-length p105 sequences (SG-Mp105) and C-terminal p105 sequences (SG-3'Mp105) were expressed as primarily cytoplasmic proteins in CEF. In contrast, GAL4 1–147 alone and N-terminal p105 aa fused to GAL4 (SG-5'Mp105) were primarily nuclear proteins in CEF. Therefore, the differences in transcription activation by these proteins are unlikely to be due to the amount of each protein in the nucleus. More specifically, SG-3'Mp105 is more strongly activating than SG-5'Mp105 (see Fig. 1A) even though very little of the SG-3'Mp105 protein appears in the nucleus of CEF. We have previously shown that LEXA-chicken c-Rel fusion proteins can function as strong activators of transcription in CEF even though much of the protein appears cytoplasmic by immunofluorescence (25).

Successive C-terminal deletions (Fig. 1B) indicated that most of the C-terminal transcription activation domain is between aa 724 and 864 of mouse p105. Furthermore, a GAL4 fusion protein expressing only aa 727 to 806 of p105 activated transcription even more strongly than the intact p105 C terminus. This may be due to the absence of most of the ankyrin repeats in pSG-Mp105(727–806), and increased nuclear accumulation of the SG-Mp105(727–806) protein in CEF as compared to SG-3'Mp105.

Based on these results, it is likely that most of the transcription activation domain of the C terminus of p105 is contained between aa 727 and 806. This region of p105 is overall negatively charged (Fig. 1C), like many transcription activation domains (40).

Table 1. Transcriptional activation by C-terminal mouse p105 sequences in mammalian cells.

Plasmid	Cell type 3T3	Hela	Cos1
	Relative CAT activity		
pSG424	0.9	0.6	0.9
pSG-3'Mc	100	100	100
pSG-Mp105	0.7	0.6	1.4
pSG-3'Mp105	39	57	18
pSG-Mp105 (727–904)	212	94	55

Producer and reporter plasmids were cotransfected into the indicated cell types and CAT activity was determined as described in the legend to Fig. 1. Values were determined from a representative assay done in each cell type and are normalized to the CAT activity seen in that cell type with pSG-3'Mc (100).

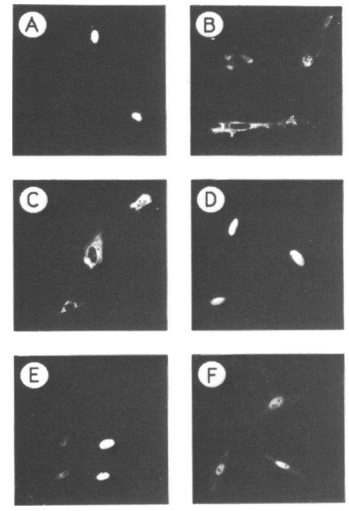

Figure 2. Subcellular localization of proteins in CEF by immunofluorescence. CEF were transfected with plasmid DNA, and indirect immunofluorescence was performed as described in Materials and Methods. (**A**) pSG424 (GAL4 1–147); (**B**) SG-Mp105; (**C**) SG-3'Mp105; (**D**) SG-5'Mp105; (**E**) GM282 (wild-type v-Rel); (**F**) JDp40 (wild-type p40). In panels A–D, the primary antibody was an anti-GAL4 antibody (diluted 1:20); in panel E, it was an anti-v-Rel antibody (diluted 1:40); and in panel F, it was an anti-p40 antibody (diluted 1:80).

Furthermore, the acidic nature of this region is conserved in p105 from humans, mice and chickens (3–5, 39).

Activation of transcription by p40 sequences

Because of the homology between the C terminus of p105 and the IκB family of proteins, we determined whether p40 (a 318 aa chicken IκBβ protein that complexes with the v-Rel oncoprotein and c-Rel [16]) could activate transcription when fused to GAL4 1–147 (Fig. 3). Surprisingly, a GAL4 fusion protein containing p40 aa 49–318 (SG-Chp40), including all five ankyrin-like repeats, activated transcription very strongly in CEF. Furthermore, a GAL4-MAD-3 fusion protein (aa 62–317 of MAD-3) was also strongly activating in CEF (data not shown). The C-terminal 89 aa of p40 (SG-3'Chp40, which contains only part of the fifth ankyrin repeat) did not activate transcription, whereas a GAL4 fusion protein containing aa 49–229 of p40 still activated transcription almost as strongly as mouse c-Rel, even though it was about five times more weakly activating than SG-Chp40 (Fig. 3). Thus, part of the transcription activation domain of p40 is contained between aa 49 and 229, which essentially contains only the ankyrin repeats. A titration experiment showed that p40 sequences activated transcription approximately ten times more strongly than C-terminal sequences from mouse p105 (Fig. 3). In addition, the majority of wild-type p40 is located in the nucleus of CEF when overexpressed from an avian retroviral vector (Fig. 2F), although it is not as fully nuclear as the v-Rel oncoprotein (Fig. 2E).

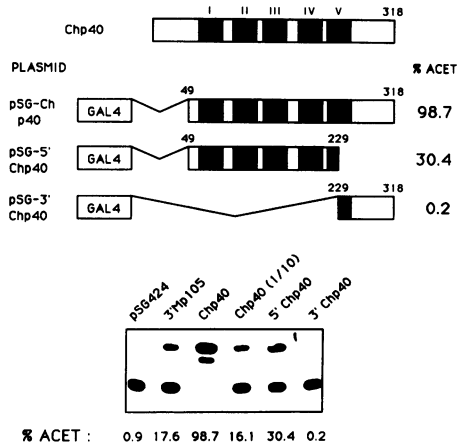

Figure 3. Activation of transcription by chicken p40. GAL4 fusion proteins containing the indicated p40 aa were assayed for transcription activation by measuring CAT activity in lysates from CEF that had been cotransfected with reporter (pG5BCAT) and producer plasmids as described for Fig. 1. Black boxes indicate the approximate positions of the five ankyrin-like repeats. CAT activity (% ACET) is shown for a single representative assay (at bottom of figure); however, each protein has been tested at least four times with similar results. To obtain an estimate of the relative strength of activation by SG-Chp40 as compared to SG-3'Mp105, CAT activity in a ten-fold dilution of the SG-Chp40 lysate was determined (Chp40 [1/10]).

Activation of transcription in yeast

We considered it possible that we were simply mapping domains in p40 or p105 that interacted with endogenous transcription activating Rel family proteins, and that it was these cellular proteins that were providing the transcription activation seen in our assays (see ref. 41). Since yeast are not known to contain Rel-like proteins, we determined whether the transcription activation domains of mouse p105, MAD-3, p40 and mouse c-Rel were functional in *S. cerevisiae*. As shown in Fig. 4, GAL4 fusion proteins expressing the C terminus of p105, the C terminus of mouse c-Rel, p40 and MAD-3 were more strongly activating in *S. cerevisiae* than full-length GAL4 itself. The minimal domain of mouse p105 (aa 727–806) that activated strongly in CEF,

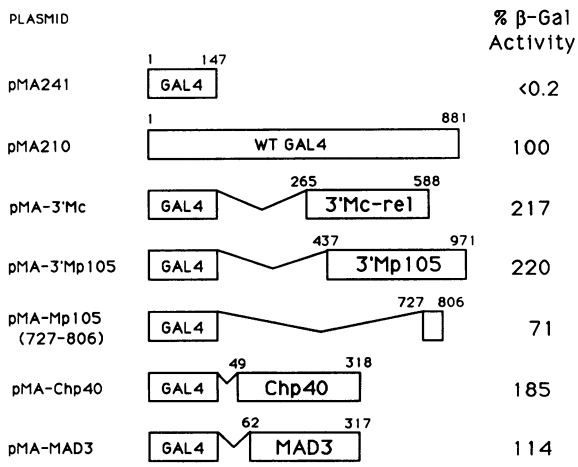

Figure 4. Transcription activation by the C terminus of mouse p105 and by IκBβ proteins in *S. cerevisiae*. Gene activation by the indicated GAL4 fusion proteins was determined by measuring β-galactosidase activity in *S. cerevisiae* strain YM335:RY171 which contains a chromosomal copy of a reporter locus containing a *GAL1-lacZ* fusion gene and which lacks a functional endogenous *GAL4* gene (37). All values were determined as described previously (24) and are relative to the activity (100%) seen with plasmid pMA210 that expresses the full-length GAL4 protein (WT GAL4). GAL4 1–147 alone was expressed from plasmid pMA241. Each value is the average of values obtained from three independent transformants, except pMA-Chp40 which is from two independent transformants. Standard errors ranged from 12 to 28%. (The GAL4-p40 and GAL4-MAD3 fusion proteins were toxic in yeast, and transformants containing these plasmids grew very slowly.)

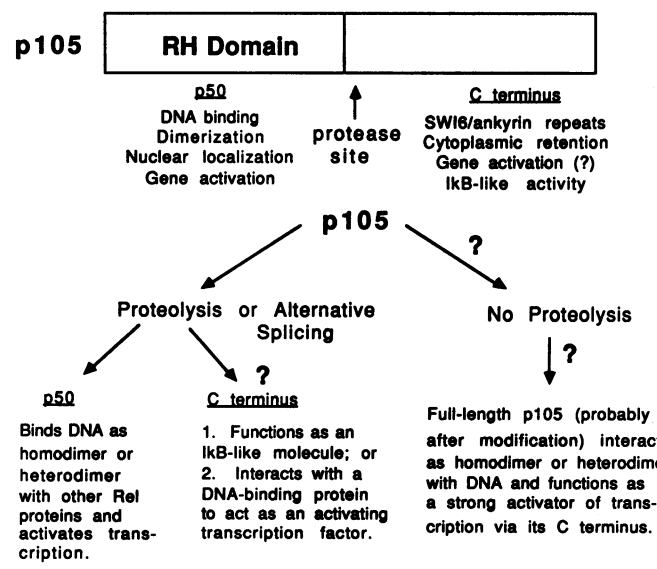

Figure 5. Possible models for regulation and processing of NF-κB p50/p105. The structure of p105, including the indicated functional domains (DNA binding, nuclear targeting, etc.) is shown at top. Known and possible functions of p105 with and without proteolysis are shown in the flow diagram. Possible functions are indicated by '?'. These data are taken from various sources (see refs. 1 and 2) or are suggested by our results in this paper.

although less strongly activating in yeast than the intact C terminus of p105 (aa 437 to 971), was still almost as strongly activating as full-length GAL4.

From our results in yeast, we do not think that the transcription activation by the C terminus of p105 or by p40 that we observed in CEF is simply due to the interaction of these sequences with endogenous activating avian Rel family proteins. Furthermore, Inoue *et al.* (13) have determined that aa that are C-terminal to the last ankyrin repeat in p40 are necessary for interaction of p40 with mouse c-Rel and NF-$\varkappa$B; however, we have shown that a GAL4-p40 protein (SG-5'p40) missing these C-terminal sequences was still capable of activating transcription in CEF (Fig. 3).

Alternate models for the regulation of NF-$\varkappa$B transcription complexes

Based on many results (see refs. 1, 2), the general structure of p105 is as shown in Fig. 5. The N-terminal RH sequences of p105 contain dimerization, nuclear-targeting, DNA-binding, and I$\varkappa$B-binding functions. C-terminal to the RH sequences, in order, there are a glycine-rich region where proteolysis of p105 is likely to occur, a series of six complete and one partial ankyrin-like repeats, and approximately 100 aa of unknown function. Our results (Fig. 1) indicate that there is also a gene activation domain located approximately between the sixth and seventh ankyrin-like repeats.

There are several possible models for the subcellular processing of p105 (Fig. 5). While it has been shown that p50 (which lacks the C-terminal transcription activation domain that we have described here) activates transcription by association with another activation-competent Rel family protein such as p65, c-Rel, and in some cases p50 itself (22), our results suggest that there may be certain situations wherein full-length p105 can bind to DNA and activate transcription. This might occur after post-translational modification of p105, or p105 might bind to DNA sequences that differ from those recognized by p50. Consistent with this hypothesis, the GAL4-full-length p105 fusion protein (SG-Mp105; Fig. 1A) was activating as compared to GAL4 1–147 alone when the CAT assay was performed for an extended period of time (data not shown). Alternatively, after proteolytic cleavage or as I$\varkappa$Bγ, the C-terminal sequences of p105 could associate with another DNA-binding protein to create a transcription activation complex.

Our findings with p40 and MAD-3 were quite unexpected since it has been presumed that these I$\varkappa$B proteins, as known inhibitors of $\varkappa$B site-binding by NF-$\varkappa$B-like complexes and as proteins involved in cytoplasmic retention of Rel family proteins, would not be involved in activation of transcription (Fig. 3) or located in the nucleus (Fig. 2). Our results suggest that p40 and MAD-3, under certain circumstances, might form part of a transcription activation complex. The association of p40 or MAD-3 with other proteins could occur by means of the ankyrin-like repeats, since the GABPβ transcription factor associates with GABPα via ankyrin-like repeats (21) and the yeast SWI4 and SWI6 proteins (which contain ankyrin-like repeats) form a complex that binds DNA (42). It is interesting that p40 can bind to p50 but does not inhibit DNA binding by p50 (13); we are currently designing experiments to test transcription activation by p50-p40 complexes.

It is, of course, possible that the C terminus of p105 or p40 and MAD-3 fortuitously activated transcription when fused to GAL4 1–147. However, since sequences from these proteins activated transcription as strongly as sequences in known transcription activating proteins (such as mouse c-Rel and GAL4) and are present in proteins (p105 and I$\varkappa$Bβ) known to be involved in the regulation of gene expression, we consider this unlikely. More specifically, even though random sequences from bacteria can act as transcription activating domains in yeast when fused to GAL4 (43), they are generally quite weak as compared to full-length GAL4, and we know of no cases where eukaryotic proteins have fortuitous activating domains that are conserved through evolution. Furthermore, the C-terminal 89 aa of p40 have a net negative charge of −11 and yet do not activate transcription when fused to GAL4 (Fig. 3).

Our results strongly suggest that certain aspects of the model for the activity and regulation of NF-$\varkappa$B complexes may need to be reassessed or expanded. It will likely be of interest to identify proteins that can bind to the ankyrin-like repeats and to the activation domains of p105 and p40.

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