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# The p65 subunit is responsible for the strong transcription activating potential of NF-κB

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**The nuclear form of the NF-κB transcription factor binds to DNA as a heterodimer of a 50 kDa (p50) and 65 kDa (p65) polypeptide. The two polypeptides are encoded by different genes but share a long region of homology, the NRD motif, encompassing domains required for DNA binding and dimerization. In this study we have analysed the contribution of the two subunits to the strong transactivating potential of NF-κB. Transient expression of the p65 subunit alone resulted in a potent transactivation of a CAT reporter construct under the control of two NF-κB binding sites in monkey COS and mouse L cells. The strongly DNA binding p50 subunit showed only very weak, if any, induction of gene expression. Co-expression of p50 suppressed the transactivation by p65 presumably by competitive DNA binding of transcriptionally inactive p50 dimers (KBF1). Fusion of p65 sequences to DNA binding domain of the yeast GAL4 transcription factor allowed detection of the principal transactivation domain of p65 ($TA_1$) in the C-terminal 30 amino acid sequence. $TA_1$ is likely to adopt an amphipathic α-helical structure which clusters serine residues on the hydrophilic surface, a structural feature conserved between human, mouse and *Xenopus* p65. The unique C-terminal third of p65 contained at least one more activation domain, $TA_2$, within a 90 amino acid sequence directly adjacent to $TA_1$. In two mammalian cell lines, $TA_1$ and $TA_2$ acted separately, while in an insect cell line, the two domains were inactive after their separation. Our study suggests that the p50 subunit in NF-κB might only serve a helper function in DNA binding whereas the p65 subunit is responsible for initiating transcription. Homodimers of p50 seem to have the potential of down-regulating κB-specific gene expression.**

*Key words:* amphipathic helix/NF-κB/p50 and p65 subunits of NK-κB/transactivation

## Introduction

Nuclear factor κB (NF-κB) is a pleiotropic transcription activator (for review, see Baeuerle and Baltimore, 1991; Baueuerle, 1991). A hallmark of the factor is its multi-subunit composition and the existence of an inducible cytoplasmic form. A protein subunit called IκB is responsible for inhibition of DNA binding and cytoplasmic retention of NF-κB (Baeuerle and Baltimore, 1988; for review, see Schmitz *et al.*, 1991). Dissociation of IκB upon stimulation of cells with viruses or cytokines releases a nucleophilic DNA-binding heterodimer composed of a 50 (p50) and 65 kDa (p65) subunit. p50 and p65 subunits can also form homodimers which bind with distinguishable affinity and specificity to κB sequence motifs in DNA (Urban *et al.*, 1991).

The capability of p50 and p65 subunits of NF-κB to form homo- and heterodimers and to recognize the same binding motifs has its molecular basis in a large homology domain shared by p50 and p65 (Bours *et al.*, 1990; Ghosh *et al.*, 1990; Kieran *et al.*, 1990; Meyer *et al.*, 1991; Nolan *et al.*, 1991; Ruben *et al.*, 1991). This domain of ~300 amino acids is necessary for DNA binding and dimerization (Ghosh *et al.*, 1990; Kieran *et al.*, 1990; Nolan *et al.*, 1991). It is also found in proteins of the *rel* oncogene family and the *Drosophila melanogaster* morphogene *dorsal* (for review, see Gilmore, 1990). Subsequently, it was shown that Rel and dorsal proteins also have DNA binding activity and recognize motifs identical or related to those bound by NF-κB (Kieran *et al.*, 1990; Ip *et al.*, 1991). We will refer to this novel DNA binding and dimerization motif shared by NF-κB, Rel and dorsal proteins as the NRD motif. Moreover, there is evidence that also Rel and dorsal are associated with IκB-like molecules that control their nuclear/cytoplasmic distribution and DNA binding activity (Kerr *et al.*, 1991; for reviews see Gilmore, 1990; Govind and Steward, 1991; Schmitz *et al.*, 1991).

Apart from the DNA binding/dimerization domain, the primary structures of p50 and p65 proteins are completely divergent (Nolan *et al.*, 1991; Ruben *et al.*, 1991). Whereas p50 has very few additional sequence information, p65 has ~200 amino acids of unique sequence in its C-terminal portion suggesting that the two proteins might be functionally distinct. One distinction is that only p65 can interact with IκB proteins (Baeuerle and Baltimore, 1989; Urban and Baeuerle, 1990). Two variant forms of IκB, the α- and β-form (Zabel and Baeuerle, 1990), inhibit the DNA binding of a p65 dimer or a p50–p65 heterodimer, but not that of a p50 dimer (Urban *et al.*, 1991). Because IκB-β also inhibits the DNA binding of v- and c-Rel (Kerr *et al.*, 1991), the IκB binding domain is likely to be within the conserved NRD motif rather than in the unique C-terminus of p65 (Nolan *et al.*, 1991). The study by Kerr *et al.* (1991) indeed showed that the presumed IκB-β homologue from chicken, pp40, binds to the N-terminal 51 amino acids of c-Rel.

A function that has not yet been investigated separately for the p50 and p65 DNA binding subunits of NF-κB is their transactivating potential. The most direct evidence that NF-κB can initiate transcription comes from an *in vitro* transcription study using purified NF-κB containing both subunits (Kawakami *et al.*, 1988). In the present study, we analysed the transactivating capacities of human p50 and p65 subunits transiently expressed in monkey kidney epithelial cells (COS), mouse fibroblasts ($Ltk^-$) and *Drosophila melanogaster* Schneider cells. The p65 subunit alone could transactivate a chloramphenicol acetyl transferase (CAT)

reporter construct under the control of two κB binding motifs. Despite its stronger DNA binding activity, p50 could barely transactivate the κB reporter construct. Co-expression of p50 suppressed the transactivation by p65 and NF-κB presumably by competitive binding of transcriptionally inactive p50 dimers to the NF-κB sequence motif. At least two distinct transactivation domains, $TA_1$ and $TA_2$ were present in the unique C-terminal third of p65. Strongest was $TA_1$ which was confined to the C-terminal 30 amino acid sequence of p65. It presumably formed a serine-rich, amphipathic α-helical structure which was highly conserved between human, mouse and *Xenopus* p65. $TA_2$ encompassed amino acids between positions 441 and 518. The activities of $TA_1$ and $TA_2$ domains were remarkably different in monkey COS, mouse L and insect cells.

## Results

### *Transient expression of NF-κB subunits in COS cells*

The p50 subunit of NF-κB is produced in form of a larger precursor of 110 kDa (p110) from which it is presumably released by a proteolytic event from the N-terminal portion (Kieran *et al.*, 1990; Ghosh *et al.*, 1990). Because the C-terminus of p50 has not yet been identified, we constructed two forms of p50 encompassing either the N-terminal 399 or 502 amino acids of p110. These two forms of 'p50' should cover all possible C-terminal sequences of a 50 kDa polypeptide. While in mock-transfected COS cells only very small amounts of endogenous NF-κB (p50–p65) and no p50 DNA binding activity were detected in EMSAs (Figure 1, lane 1), transient expression of p65 under control of the CMV enhancer gave rise to a weak κB-specific complex migrating more slowly than that of the p50 forms (Figure 1, lane 3, compare with lanes 4 and 5). The weak DNA binding and slow electrophoretic mobility of the p65–DNA complex is consistent with characteristics described for the p65 protein isolated from human placenta (Urban *et al.*, 1991) and obtained with *in vitro* translated p65 (Ruben *et al.*, 1991). Expression of $p50^{1-399}$ or $p50^{1-502}$ in COS cells gave rise to novel protein–DNA complexes binding to a κ enhancer DNA probe (lanes 4 and 5). The complexes were κB-specific (data not shown) and their distinct mobilities reflected the size difference of the two p50 forms. Co-expression of the p50 forms with p65 created complexes with mobilities intermediate to those of p50 and p65 complexes (Figure 1, lanes 6 and 7). The novel complexes had a mobility indistinguishable from that of purified NF-κB (data not shown) and most likely contained heterodimers of p50 and p65. In complex with p65, the size differences of the two p50 forms were barely detectable (Figure 1, compare lanes 6 and 7). Identical results were obtained if the NF-κB subunits were expressed in the presence of a κB reporter plasmid (data not shown). No novel κB-specific complexes were induced if a CMV-driven expression vector without an insert was transfected (Figure 1, lane 2).

From the EMSA analysis, it appears that neither p50 nor p65 can recruit significant amounts of endogenous p50 or p65 subunits to form activated NF-κB because there was no striking increase in a protein–DNA complex with the mobility of NF-κB (Figure 1, compare lane 3 to 5). Apparently, most if not all of the NF-κB which is formed from the transiently expressed p50 and p65 subunits (Figure 1, lanes 6 and 7) escaped inhibition of DNA binding by endogenous IκB proteins because the protein–DNA complex of NF-κB was as abundant as that of p50. The latter was shown to be unable to react with IκB (Baeuerle and Baltimore, 1989).

We have raised antisera against various peptides representing N-terminal and internal sequences unique to p50 and p65 proteins. An indirect immunofluorescence study using the affinity-purified anti-peptide antibodies showed that in Vero, COS and HeLa cells the endogenous p50 (and p110) and p65 were barely detectable (K.van Zee, U.Zabel, E.Fanning and P.Baeuerle, manuscript in preparation). After microinjection of CMV–p50 and CMV–p65 plasmids, a strong, predominantly nuclear immunofluorescence staining was obtained with all cell lines tested (data not shown). The staining was specific for the anti-p50 and anti-p65 peptides, respectively. In agreement with the data from the EMSA analysis, this indicates that p50 and p65 subunits (i) can be expressed to a much higher level than the endogenous proteins, (ii) can translocate to the nucleus independently from each other, and (iii) escape cytoplasmic retention by endogenous IκB proteins.

### *The p65 subunit is sufficient for transactivation of a κB reporter construct*

Expression vectors encoding the two p50 forms and p65 under control of the CMV enhancer were transfected into cells together with the CAT reporter construct J16 containing two κB binding sites linked to a truncated c-*fos* promoter (Pierce *et al.*, 1988). In order to test whether the induction of CAT activity was dependent on NF-κB binding motifs, a CAT reporter construct with two mutated κB sites (J32; Pierce *et al.*, 1988) was used in parallel transfection experiments. Upon expression of the long and short version of p50, only very low levels of CAT activity were detected with κB wild-type and mutant CAT reporter constructs (Table I). While there was virtually no κB-specific CAT induction by CMV–$p50^{1-339}$ in L and COS cells, we consistently observed that the longer version of p50 caused a very weak κB-dependent increase in CAT activity in both cell lines tested. This increase was at most 2-fold. It is possible that the acidic C-terminal sequence between amino acids 399 and 502 of p50 contains a weak transactivation domain. However, as long as the C-terminus of p50 is not identified, the physiological relevance of this finding remains unclear. It appears that a dimer of p50 has only very weak, if any, transactivating capacity on its own.

Transient expression of the p65 subunit in COS and L cells caused a strong induction of CAT activity from the construct J16 (Table I). Depending on the cell line, transfection of the plasmid CMV–$p65^{1-550}$ induced an increase in CAT activity between 18- (COS) and 13-fold (L cells) over the CAT activity obtained with the κB mutant construct J32 (Table I). The absence of substantial amounts of a p50–p65 heterodimer (NF-κB) in EMSAs following transfection of CMV–$p65^{1-550}$ (see Figure 1), suggests that over-expressed p65—most likely as homodimer—can initiate gene expression independently of the p50 subunit.

Transfection of the same amounts of CMV–$p50^{1-399}$ and CMV–$p65^{1-550}$ plasmids caused also a strong induction of CAT activity (Table I). In both cell lines, the induction was however slightly less than that obtained with CMV–$p65^{1-550}$ alone (see below) and was most likely caused by a heterodimer of p50 and p65 (NF-κB) which

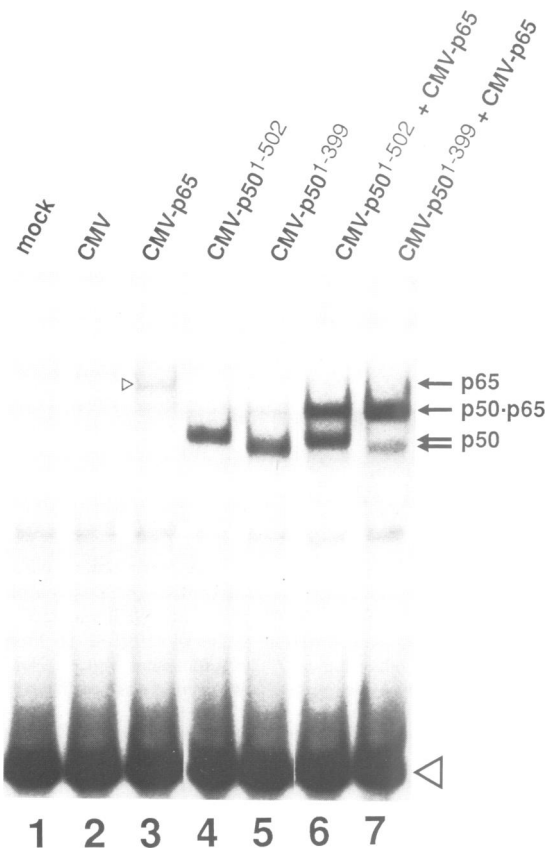


**Fig. 1.** Expression of NF-κB subunits in COS cells. The constructs indicated at the top of the lanes were transfected into COS7 cells, total cell extracts prepared and analysed by EMSA for DNA binding to a $^{32}$P-labelled oligonucleotide containing the κB motif from the IRF-1 promoter (see Materials and methods). Very similar results were obtained if a κB motif from the mouse κ enhancer was used (data not shown). A fluorogram of a native gel is shown. The small open triangle in lane 3 indicates the position of the protein–DNA complex formed by the p65 subunit. Arrows at the right show positions of this and other κB-specific complexes. The open arrowhead indicates the position of unbound DNA probe.

**Table I.** Analysis of the κB dependent transcription activation by the p50 and p65 subunits of NF-κB.

| | κB reporter | | | | | |
|---|---|---|---|---|---|---|
| | COS | | | Ltk$^-$ | | |
| | wt | mu | wt/mu | wt | mu | wt/mu |
| κB reporters alone | 0.11 | 0.12 | 0.9 | 0.46 | 0.17 | 2.7 |
| CMV–p50$^{1-399}$ | 0.65 | 0.74 | 0.9 | 0.43 | 0.43 | 1.0 |
| CMV–p50$^{1-502}$ | 1.4 | 1.0 | 1.4 | 0.79 | 0.43 | 1.8 |
| CMV–p65$^{1-550}$ | 47.9 | 2.65 | 18 | 5.6 | 0.42 | 13.3 |
| CMV–p50$^{1-399}$ + CMV–p65$^{1-550}$ | 10.5 | 0.65 | 16.2 | 6.0 | 0.64 | 9.4 |

CAT reporter plasmids bearing wildtype (wt) and mutant NF-κB binding sites (mu) were transfected alone or together with expression plasmids encoding the p50 and p65 subunits into COS7 and Ltk$^-$ cells. The conversion of [$^{14}$C]chloramphenicol into acetylated forms by the reporter enzyme CAT is given as a percentage. The names of the plasmids used for the cotransfection experiments are shown on the left. The κB-dependent induction of CAT activity is expressed as the ratio of CAT activity obtained with wildtype and mutant reporter constructs. Mean values from two transfection experiments are shown. The deviation was <10%.

constituted under these conditions the predominant protein–DNA complex in EMSAs (see Figure 1, lane 7).

### *Coexpression of the p50 subunit suppresses κB-dependent transcription*

The findings that a p50 dimer is fairly inactive in transcription activation (Table I) but can bind with high affinity to the κB motif 5′-GGGACTTTCC-3′ (Urban and Baeuerle, 1990; Urban *et al.*, 1991) prompted us to test whether overexpression of p50 relative to p65 can suppress transcription initiation from the κB-controlled reporter construct J16. The amount of κB reporter plasmid was reduced in order to increase effects coming from competitive DNA binding. Transfection of 0.75 pmoles CMV–p65$^{1-550}$ into COS cells strongly induced CAT activity from the construct J16 (Figure 2, compare lanes 1 and 2). Cotransfection of only 0.25 pmoles of the vector CMV–p50$^{1-399}$ reduced the induction of CAT activity by 40% (lane 3) and 0.5 pmoles by 80% (lane 4). (It is possible that p50 is more efficiently expressed than p65.) One to 3 pmoles of the p50 plasmid reduced the CAT activity by >90% (lanes 5, 6 and 7). The strong inhibitory effect from cotransfection of one pmol of p50 expression plasmid (lane 5) was dose-dependently relieved if increasing amounts of CMV–p65$^{1-550}$ vector were used for transfection (lanes 8–10). Also a plasmid harbouring two NF-κB binding sites (pUC-κB-Bend; Schreck *et al.*, 1990) could to a low extent relieve the suppression by p50 perhaps by absorbing some of the p50 dimers (Figure 2, lane 11 and 12). Transfection of 1.5 pmoles of a control plasmid (J32) instead of CMV–p50$^{1-399}$ had no effect on the induction of CAT activity by CMV–p65$^{1-550}$ (lane 13) indicating that the inhibitory effect of CMV–p50$^{1-399}$ did not come from overloading the cell with DNA. The observed effects could come from competition of transcriptionally inactive p50 dimers for binding of p65 dimers and NF-κB to limited κB sites on the CAT reporter plasmid. This idea is strongly supported by an experiment with a GAL4–p65 fusion protein displaying a dual DNA binding specificity (see Figure 4C).

### *Characterization of GAL4–p65 and –p50 fusion proteins*

To explore further the transactivating potential of p50 and p65 NF-κB subunits and to map domains required for transactivation, we constructed chimaeric p50 and p65 proteins that contain at their N-termini the DNA binding domain of the yeast GAL4 transcription factor. This sequence of GAL4 also contains a nuclear translocation signal (Silver *et al.*, 1984) and a dimerization domain (Carey *et al.*, 1989).

The correct nucleotide sequence of all GAL4 constructs was verified by sequence analysis. Plasmid constructs encoding the GAL4 fusion proteins were transfected into COS7 cells and aliquots of high salt extracts from transfected cells analysed by EMSA for both κB- and GAL4-specific DNA binding of fusion proteins. With the exception of GAL4–p50$^{18-230}$ and GAL4–p65$^{1-77}$, all proteins were functional in DNA binding to either a $^{32}$P-labelled GAL4 or κB oligonucleotide probe (Table II). κB specific binding was only observed with GAL4 constructs that preserved the NRD motif (Table II). Consistent with data from mouse p65 (Nolan *et al.*, 1991), deletion of C-terminal sequences from

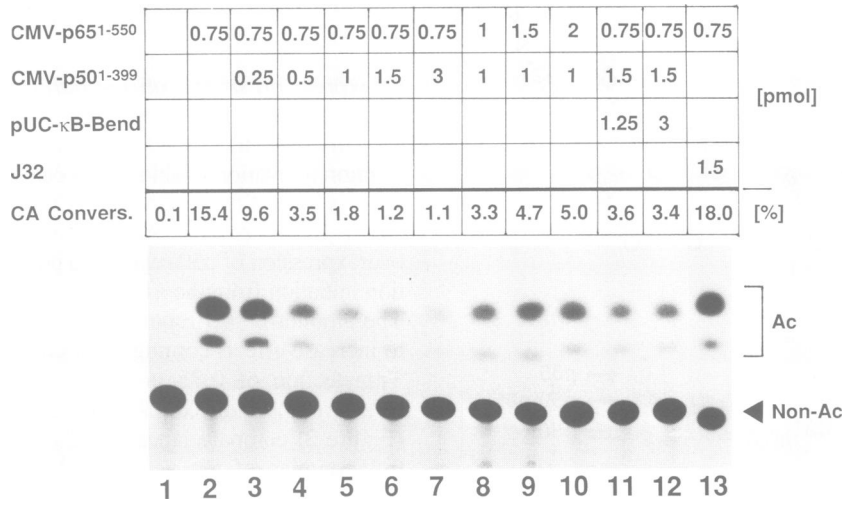


**Fig. 2.** The effect of p50 coexpression on the transactivation by p65. All COS7 cells received 1.5 pmoles of the κB-dependent reporter construct J16. The indicated amounts (in pmol) of p50 and p65 expression vectors were cotransfected with the reporter construct. pUC-κB-Bend is a plasmid containing two κB binding sites and was used previously in a DNA bending study (Schreck *et al.*, 1990). J32 (Pierce *et al.*, 1988) was used as control plasmid because it does not contain intact κB binding sites and gives a basal CAT conversion comparable to that of J16 (lane 1). Empty boxes indicate no addition. An autoradiogram from a thin-layer chromatography is shown. The conversion of [$^{14}$C]chloramphenicol (CA; Non-Ac) into acetylated forms (Ac) is given as a percentage. Sample number 13 was run on a different thin layer plate. A typical result out of three independent sets of experiments is shown.

p65 improved the κB-specific DNA binding of GAL4−p65 constructs. Two fusion proteins, GAL4−p50$^{1-502}$ and GAL4−p65$^{1-285}$, showed a strong κB-specific DNA binding but barely any binding to the GAL4 probe. Fusion proteins showing a weaker binding to the κB probe, such as GAL4−p65$^{1-550}$ and GAL4−p65$^{1-519}$, bound significantly better to the GAL4 probe (Table II). All fusion proteins with no detectable κB-specific binding activity could form prominent complexes with the GAL4 probe. These correlations suggest that dimerization of fusion proteins via the NRD motif impaired the dimerization and DNA binding of the GAL4 domain, but not vice versa.

The protein−DNA complexes of the GAL4 fusion proteins fell into two mobility groups (Figure 3). A good correlation between mobility and expected size was seen for fusion proteins binding exclusively to the GAL4 probe (Figure 3B, points 8−11). The complexes of fusion proteins that could bind to the κB site showed altogether an increased mobility (Figure 3B, points 1−6). This was not due to the use of different DNA probes since κB and GAL4 probes showed the same mobility in native gels and their complexes with GAL4−p65$^{1-519}$ were of indistinguishable mobility (point 5). Apparently, complexes of GAL4 fusion proteins harbouring an intact second DNA binding and dimerization domain (the NRD motif) form complexes with a different shape from those dimerizing only via the GAL4 domain. The construct GAL4−p50$^{231-502}$ which can only bind to the GAL4 probe, also seems to belong to the high mobility group, or is at least intermediate. This construct still contains sequences that are sufficient for dimerization but not DNA binding of p50 (Logeat *et al.*, 1991). Fusion protein constructs that exhibited no GAL4- or κB-specific transactivation (see below) were tested for expression and proper localization of the GAL4 fusion proteins in nuclei by indirect immunofluorescence labelling using a GAL4-specific antiserum (Table II). With one exception, all tested constructs gave a GAL4-specific, predominantly nuclear staining.

**Table II.** DNA binding activity and specificity of GAL4 fusion proteins

| No. | Fusion protein | Binding to | | Complete NRD | Anti-GAL4 |
|---|---|---|---|---|---|
| | | GAL4 | κB | motif present | staining |
| 1 | GAL4−p50$^{18-502}$ | −/+ | ++ | + | N |
| 2 | GAL4−p50$^{18-230}$ | − | − | − | − |
| 3 | GAL4−p50$^{231-502}$ | + | − | − | N |
| 4 | GAL4−p65$^{1-550}$ | ++ | −/+ | + | n.d. |
| 5 | GAL4−p65$^{1-519}$ | ++ | + | + | N |
| 6 | GAL4−p65$^{1-285}$ | −/+ | ++ | +[a] | N |
| 7 | GAL4−p65$^{1-77}$ | − | − | − | n.d. |
| 8 | GAL4−p65$^{286-550}$ | ++ | − | − | n.d. |
| 9 | GAL4−p65$^{286-467}$ | ++ | − | − | n.d. |
| 10 | GAL4−p65$^{286-550}$ | ++ | − | − | n.d. |
| 11 | GAL4−p65$^{520-550}$ | ++ | − | − | n.d. |

Plasmids encoding GAL4 fusion proteins with sequences from p50 and p65 subunits were transfected into COS7 cells. EMSAs were performed using $^{32}$P-labelled oligonucleotides containing either a binding site for the GAL4 protein or for NF-κB with the same high salt extracts from cells (see Materials and methods). At least two, in most cases three independent transfection experiments were performed. Native gels were exposed to X-ray films for various periods of time. −/+, complex only detectable upon 3 day exposure; +, complex detectable after overnight exposure; ++, complex detectable after 3 h exposure. [a]This construct is lacking 20 C-terminal residues of the NRD motif. N, predominantly nuclear staining; n.d., not determined.

### *GAL4−p65$^{1-550}$ can transactivate GAL4- as well as κB-dependent reporter constructs*

The transactivating potential of the GAL4 fusions with sequences from p50 and p65 was tested by cotransfection with a CAT reporter construct containing two binding sites for the GAL4 protein (Figure 4A). As expected (see Table II), p50 sequences from position 18−50 fused to GAL4 could not induce a significant increase of GAL4-dependent CAT activity in L or insect Schneider cells (Figure 4A). A GAL4 fusion with the C-terminal portion

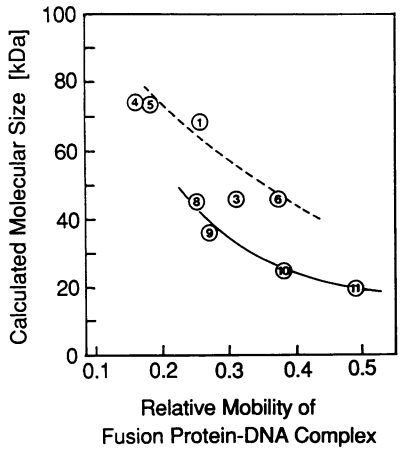


**Fig. 3.** Characteristics of GAL4 fusion protein−DNA complexes. The mobility of GAL4- and κB-specific fusion protein−DNA complexes in relation to the mobility of the free DNA probe is shown plotted against the calculated molecular size of fusion proteins. Unbound GAL4 and κB probes showed almost identical mobility in native gels and could form complexes of identical mobility, for instance with the fusion protein GAL4−p65$^{1-519}$. The numbers refer to the listing of GAL4 fusion proteins shown in Table II. The solid line shows a low mobility group of proteins that can only bind the GAL4 probe; the dashed line shows a high mobility group that can bind the κB probe and, as seen in Table II, also the GAL4 probe.

of p50 from position 231−502, which can strongly bind to GAL4 sites (Table II), showed a 1.4-fold increase in induction in L cells and no induction in Schneider cells (Figure 4A). Cell extracts from Schneider cells exhibited no endogenous κB-specific DNA binding activity in the absence or presence of deoxycholate (data not shown). The *Drosophila* NF-κB homologue dorsal was previously shown to bind to the κ enhancer motif only barely (Ip *et al.*, 1991).

Full-length p65 linked to GAL4 gave a strong induction of CAT activity in L and Schneider cells (Figure 4A). The induction was 106-fold higher in Schneider and 91-fold higher in L cells compared with the CAT activity obtained with the GAL4 domain alone. A GAL4 fusion with the very strong acidic transactivation domain of the Herpes virus protein vp16 (Triezenberg *et al.*, 1988; Sadowski *et al.*, 1988) still gave 2- and 3.5-fold higher activity than the GAL4−p65$^{1-550}$ construct.

The κB-binding GAL4 fusions with p50 and p65 (see Table II) were tested with the κB-dependent CAT reporter construct J16. Transfection of the construct GAL4−p65$^{1-550}$ into Ltk$^-$ cells gave a 10-fold increase in the κB-specific CAT activity (Figure 4B). Apparently, a chimaeric transcription factor was created that can transactivate two completely unrelated reporter constructs. Expression of the strongly κB-binding fusion protein GAL4−p50$^{18-502}$ showed no significant κB-specific induction of CAT activity. Also the κB-binding fusion protein GAL4−p65$^{1-286}$ showed barely any transactivation of the construct J16 indicating that the C-terminal portion of p65 is required for initiating transcription (see below).

The dual DNA binding specificity of GAL4−p65$^{1-550}$ was used to investigate further the mechanism of transcriptional suppression seen upon p50 co-expression (Figure 2). As shown in Figure 4C, p50 can efficiently suppress the κB-dependent (compare lane 1 with lane 2) but barely the GAL4-dependent induction of CAT activity by GAL4−p65$^{1-550}$ (compare lane 3 with lane 4). The κB-specificity of the p50 effect strongly supports the idea that p50 dimers suppress transactivation by competitive DNA binding and not by a mechanism involving protein−protein interaction.

### *Most of the transcription activating potential of p65 resides in the C-terminal 30 amino acids*

Domains of the p65 molecule required for initiating transcription were further analysed by deletion analysis of the construct GAL4−p65$^{1-550}$. Removal of only 30 amino acids from the C-terminus of p65 reduced the induction of CAT activity by >85% in L and 95% in COS cells compared to the full-length p65 sequence (Figure 5A). Removal of 250 amino acids from the C-terminus of p65 completely abrogated the transactivating potential of p65. Expression of GAL4−p65$^{1-285}$ gave the same background CAT activity as the non-DNA-binding construct GAL4−p65$^{1-77}$

In COS cells, the C-terminal 30 amino acids of p65 were sufficient to restore fully the transactivating potential of the full-length p65 protein; the induction of CAT activity by the C-terminal sequence was even higher than that obtained with the complete p65 sequence (Figure 5A). The potential of the C-terminal 30 amino acids to induce transcription from the GAL4-controlled CAT reporter gene was not improved if additional 52 or 234 amino acids from the C-terminus were present. This suggests that a principal transactivation domain of p65 (referred to as $TA_1$) was confined to the C-terminal 30 amino acids of the protein.

In L cells, $TA_1$ could restore only ~50% of the activity of the full-length protein (Figure 5A). In contrast to COS cells, the presence of additional C-terminal sequences increased in L cells the transactivating potential of fusion proteins. The complete C-terminal portion of p65 from position 286−550 induced in L cells a CAT activity which was even higher than that of the full-length p65 sequence (Figure 5A). This indicated the presence of additional transactivating sequences between position 286 and 520. Sequences between position 286 and 467 could indeed induce CAT activity to a level reaching ~20 and 30% of the full-length p65 sequence in COS and L cells, respectively (Figure 5A). The additional transactivating sequences in the C-terminal portion of p65 will be referred to collectively as the $TA_2$ domain.

A fine mapping showed that $TA_2$ encompassed sequences of p65 between positions 441 and 518 (Figure 5B). C-terminal deletion of the $TA_2$ domain caused a gradual loss in transactivating activity suggesting that $TA_2$ was composed of independent subdomains. A strong decrease was seen when sequences between position 518 and 467 were removed. Of some importance for $TA_2$ activity was a region containing a potential leucine zipper structure (position see Figure 8). Two constructs deleting 33 amino acids which contain two of the three heptade-arranged leucine residues (Leu435, -442 and -449) showed a significant loss in transactivating activity when compared to their parental constructs (Figure 5B). A fusion protein containing the sequence between the leucine repeat and $TA_1$ (position 468−519) could weakly induce transcription. Taken together, these results suggest that $TA_2$ contained at least two independent subdomains.

In *Drosophila* Schneider cells, only GAL4 fusion proteins containing the full-length p65 sequence or the complete C-

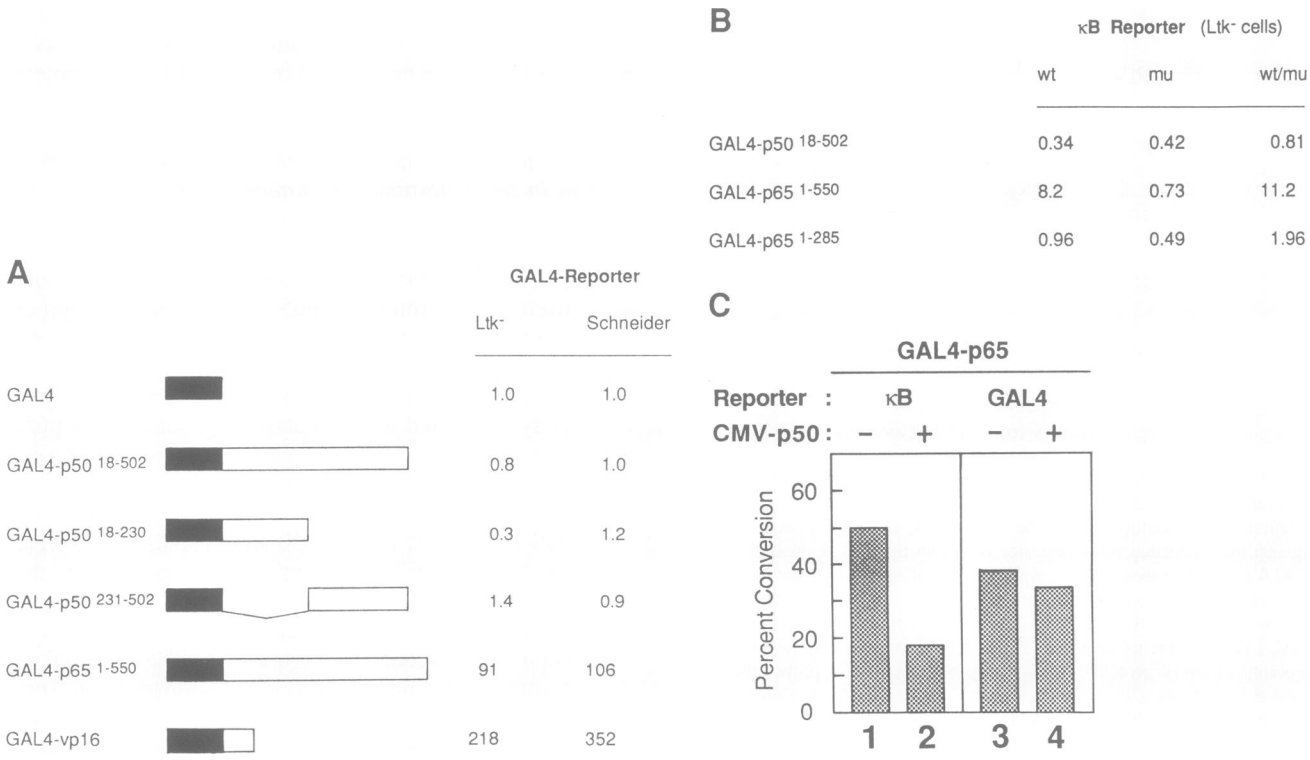


**Fig. 4.** Transcriptional activity of NF-κB subunits fused to the DNA binding domain of GAL4. (**A**) The DNA binding and dimerization domain of GAL4 (amino acids 1−147) are shown as filled bar. The C-terminally fused portions of p50 and p65 subunits are shown as open bars. The names of the constructs with respective sequence positions are indicated at the left. The constructs were cotransfected with a CAT reporter gene construct bearing two GAL4 binding sites into Ltk$^-$ and Schneider cells. The CAT activity obtained with the plasmid harboring only the GAL4 portion was set to 1.0. The CAT activities obtained with the other plasmids are given in relation to this value. Mean values from two to four independent experiments are shown. (**B**) Transcriptional activation of a κB-reporter construct by GAL4−p50 and −p65 fusion proteins. The indicated plasmids were cotransfected with the κB-dependent CAT reporter construct J16 (wt) or the mutant plasmid J32 (mu) into Ltk$^-$ cells. The CAT activity induced by the constructs is given in the percentage conversion of chloramphenicol into acetylated forms. The ratio between the CAT conversion obtained with wild type and mutant κB-reporter construct is shown at the right. (**C**) The effect of p50 co-expression on the activity of the GAL4−p65$^{1-550}$ fusion protein. The fusion protein (0.75 pmol) was expressed in the absence (lanes 1 and 3) and presence (lanes 2 and 4) of 1 pmol CMV−p50$^{1-339}$. Either the κB-dependent reporter construct J16 (lanes 1 and 2) or the GAL4-dependent construct was used (lanes 3 and 4). 1.5 pmol of reporter plasmids were used per dish. Mean values from two independent transfection experiments are shown.

terminal third of p65 from positions 286 to 550 could initiate transcription from the GAL4-driven CAT reporter construct (Figure 5A). Both fusion proteins induced very similar levels of CAT activity. GAL4 fusions with only the C-terminal 30 amino acids of p65 (encompassing $TA_1$) or sequences from position 286 to 486 (encompassing part of $TA_2$) gave virtually no increase of CAT activity in the invertebrate cell line. The deletion of $TA_1$ nevertheless reduced the transactivating potential of p65 by >90% (Figure 5A). This suggests that the $TA_1$ domain is required in insect cells but not sufficient for gene induction. There was also no increased CAT activity observed with the construct GAL4−p65$^{286-466}$ which gave in L and COS cells 20−30% of the activity of GAL4−p65$^{1-550}$ (Figure 5A). It seems that also (part of) the $TA_2$ domain is required but not sufficient for transactivation. Thus we anticipate that in the invertebrate cell line $TA_1$ and $TA_2$ domains could only act in concert.

### *The $TA_1$ domain is likely to form an amphipathic α-helix*

The C-terminus containing the $TA_1$ domain is fully conserved in human and mouse p65 within the last 24 amino acids but shows three diversions in the N-terminally following 8 amino acids (Figure 6A). The conserved stretch of 24 amino acids (aa) are enriched in hydrophobic (7 aa), serine (6 aa) and acidic residues (5 aa). Moreover, there are no prolines and only one glycine at the beginning of the sequence. Analyses by both the method of Chou and Fasman (1978) and Garnier *et al.* (1978) predicted the same stretch of 16 amino acids in the conserved part of the C-terminus to adapt an α-helical structure (Figure 6A). In addition, one method (Chou−Fasman) predicts that the putative α-helix extends by four amino acids to the very C-terminus; the other (Garnier) predicts that the helix ends N-terminally with a turn structure.

The amino acid composition of the putative α-helix prompted us to plot the C-terminal sequence of p65 from amino acid 532 to 550 on a helical wheel. As is shown in Figure 6B, there is a cluster of five hydrophobic amino acids on one face of the hypothetical α-helix, whereas five hydrophilic serine residues are clustered on the opposite face. This arrangement is typical for an amphipathic helix. Acidic (charged) residues are excluded from the clusters of serine and hydrophobic residues and occur at their interfaces. Moreover, charged residues are restricted to the N-terminal half of the putative helix (position 531−540), whereas the C-terminal half of the putative helix (position 541−550) is uncharged and composed of four serine, one glutamine and five hydrophobic residues. Four of the seven serine residues in the $TA_1$ domain (positions 528, 535, 542 and 549) are spaced as a heptade repeat such that they would fall into one line on the surface of an α-helix (Figure 6A).

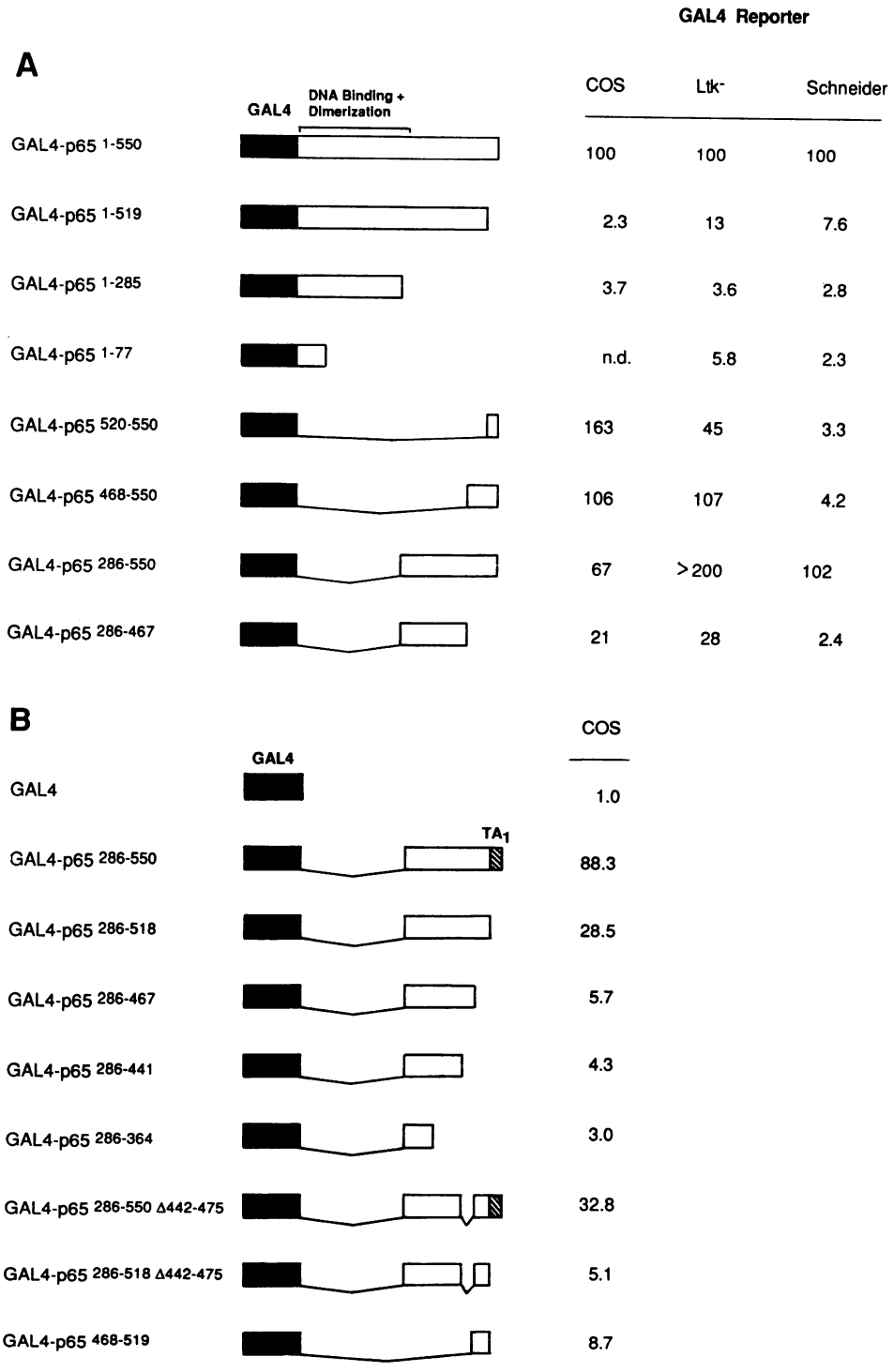


**Fig. 5.** Deletion analysis of p65 linked to the GAL4 domain. (**A**) Identification of the $TA_1$ domain. The DNA binding/dimerization domain of GAL4 is shown as filled bar and the various portions of p65 which were fused to the C-terminus of GAL4 as open bars. The names of the constructs and sequence positions are given on the left. These constructs were cotransfected with a GAL4 reporter construct into COS7, $Ltk^-$ and Schneider (S2) cells. The CAT conversion obtained with the full-length p65 sequence ($GAL4-p65^{1-550}$) was set to 100%. Average values from three to six independent transfection experiments are shown. In one case (indicated as >200), a strong scatter with values between 200 and 800% was obtained. The construct $GAL4-p65^{1-77}$ showed no GAL4-specific DNA binding activity. (**B**) Fine mapping of the $TA_2$ domain. The induction of CAT activity by C-terminal portions of p65 was assayed in COS cells and is given as 'fold increase' over the activity of the GAL4 domain. The $TA_1$ domain is shown as hatched box. All fusion proteins were tested by EMSA and found to be functional in GAL4-specific DNA binding (data not shown).

### *The structural features of the $TA_1$ domain are highly conserved*

Recently, Kao and Hopwood (1991) isolated a v-*rel* homologous cDNA clone from a *Xenopus laevis* oocyte expression library by low-stringency screening. The authors suggested that the encoded protein is the *Xenopus* homologue of the avian and mammalian c-Rel proteins and called the clone Xrel1. We undertook a sequence comparison of Xrel1

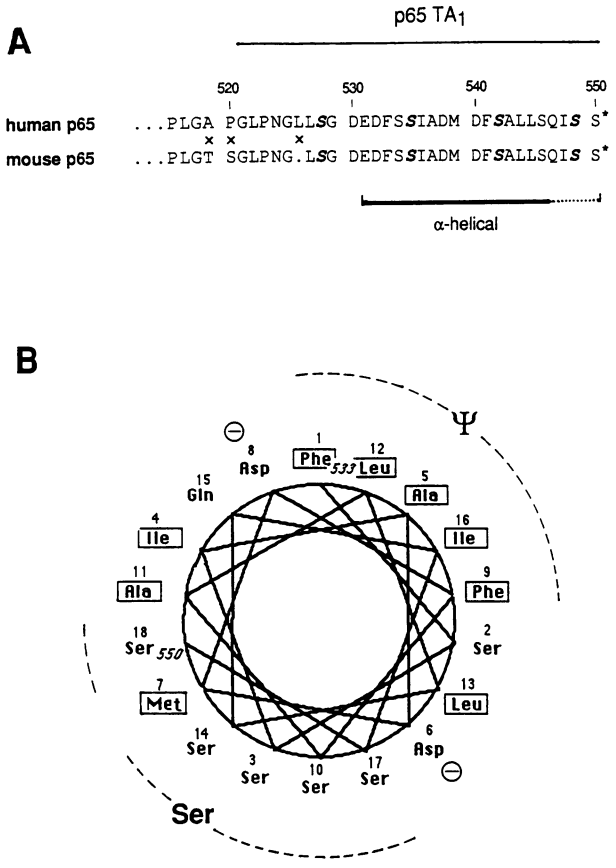


**Fig. 6.** Structural analysis of the $TA_1$ domain. (**A**) The primary structure of the C-terminal 30 amino acids of p65 encompassing the principal transactivation domain $TA_1$. The C-termini from the human (Ruben *et al.*, 1991) and mouse p65 subunits (Nolan *et al.*, 1991) were compared. Crosses indicate diverging residues. The solid part of the lower bracket indicates amino acid residues that by two different programs (Chou and Fasman, 1978; Garnier *et al.*, 1978) were predicted to be present in an α-helical conformation. The dotted line marks residues which were predicted to be α-helical by only one of the two programs (Chou–Fasman). Serine residues in a heptade arrangement are shown in bold. Asterisks mark the C-termini. (**B**) Plotting of the last 18 amino acid residues of human p65 on a helical wheel. The helical wheel plot starts with the Phe533 in position 1 and ends with Ser550 in position 18. All hydrophobic amino acids are boxed. Serine residues located on one face of the presumptive α-helix are indicated by a dashed circle. Hydrophobic residues clustered on the opposite site of the serine residues are marked by a dashed circle and the letter Ψ.

with human c-Rel and human p65 under high stringency using a compare plot (Maizel and Lenk, 1981) (Figure 7A). As indicated by a continuous diagonal line, Xrel1 showed a perfect match with both human p65 and c-Rel sequences within the NRF motif (Figure 7A, compare left and right panels). In the C-terminal portion, however, Xrel1 showed only a striking homology to human p65, but not to c-Rel. Moreover, the size of Xrel1 is more similar to that of p65 (4.7% difference) than c-Rel (17% difference). The homologies in the C-terminal third between human p65 and Xrel1 suggest that Xrel1 encodes the *Xenopus* homologue of the human p65 protein and is not equivalent to human c-Rel.

This finding gave us the opportunity to investigate further the evolutionary conservation of the $TA_1$ domain and to narrow in sequence and structural requirements possibly important for $TA_1$ function. A comparison between human p65 and Xrel1 sequences showed in the C-terminal 21 amino acids 38% identity and 62% similarity (Figure 7B). No conservation is found in the N-terminally following 10 amino acids (compare to Figure 6A). A heptade repeat arrangement of four serine residues was not evident. Instead, there was a Ser-Leu-Ser heptade repeat. Analyses of the secondary structure by the methods of Chou and Fasman (1978) and Garnier *et al.* (1978) both predicted that the same stretch of 10 amino acid residues in the C-terminus of Xrel1 adopts an α-helical structure. The α-helical domain might be longer by seven adjacent residues as predicted by only one of the two structure programs (Figure 7B). Despite the low sequence conservation, the alignment of the C-terminal sequence of Xrel1 on a helical wheel showed an amphipathic arrangement of amino acid residues which was strikingly similar to that of p65 (Figure 7C). The C-terminal third of *Xenopus* p65 contains even a second copy of the $TA_1$ motif between residues 433 and 448 with all the structural features of the C-terminal domain. The extraordinary conservation of this particular structure suggests that a novel type of amphipathic helix is involved in transcription initiation by p65.

## Discussion

By transient high-level expression in two mammalian cell lines and in an apparently NF-κB-deficient insect cell line, we tested in this study the DNA binding p50 and p65 subunits of NF-κB for their potential to initiate transcription from κB- and, as GAL4 fusion proteins, from GAL4-controlled CAT reporter plasmids. These diverse experiments yielded a homogenous picture: in all systems tested, the p65 subunit behaves as a strong transcription activator whereas the p50 subunit was inactive or weakly inducing transcription. As consequence, p65 dimers could initiate transcription independently of the p50 subunit and p50 dimers could suppress the activity of NF-κB. In the following we discuss the implications of our findings in detail.

### *p65—a p50-independent transactivator?*

Originally, the p65 subunit was thought to be a non-DNA binding accessory protein of the NF-κB transcription factor (Baeuerle and Baltimore, 1989). Since its discovery, however, various observations lend increasing support to the idea that not only p50 but also p65 is involved in DNA binding of NF-κB (reviewed in Urban *et al.*, 1991). Very recent data then indeed showed that p65 has a NRD motif (Nolan *et al.*, 1991; Ruben *et al.*, 1991) and directly contacts DNA in a heterodimer with p50 (Urban *et al.*, 1991). Like the p50 subunit, p65 can homodimerize and bind specifically to DNA. The affinity of the p65 dimer for the classical κB motif 5′-GGGACTTTCC-3′ is however considerably lower than that of the p50 dimer and p50–p65 heterodimer, as is evident from the comparatively low amounts of p65–DNA complex formed in mobility shift assays. Nolan *et al.* (1991) showed that C-terminal deletions enhanced the DNA binding activity of p65 and the authors proposed that the C-terminus sterically interferes with p65 homodimerization (and DNA binding) but still allows efficient heterodimerization with p50.

Hence, it is a surprising outcome from the present study that transient expression of p65 alone strongly induces transcription. From the mobility shift analysis, there is good

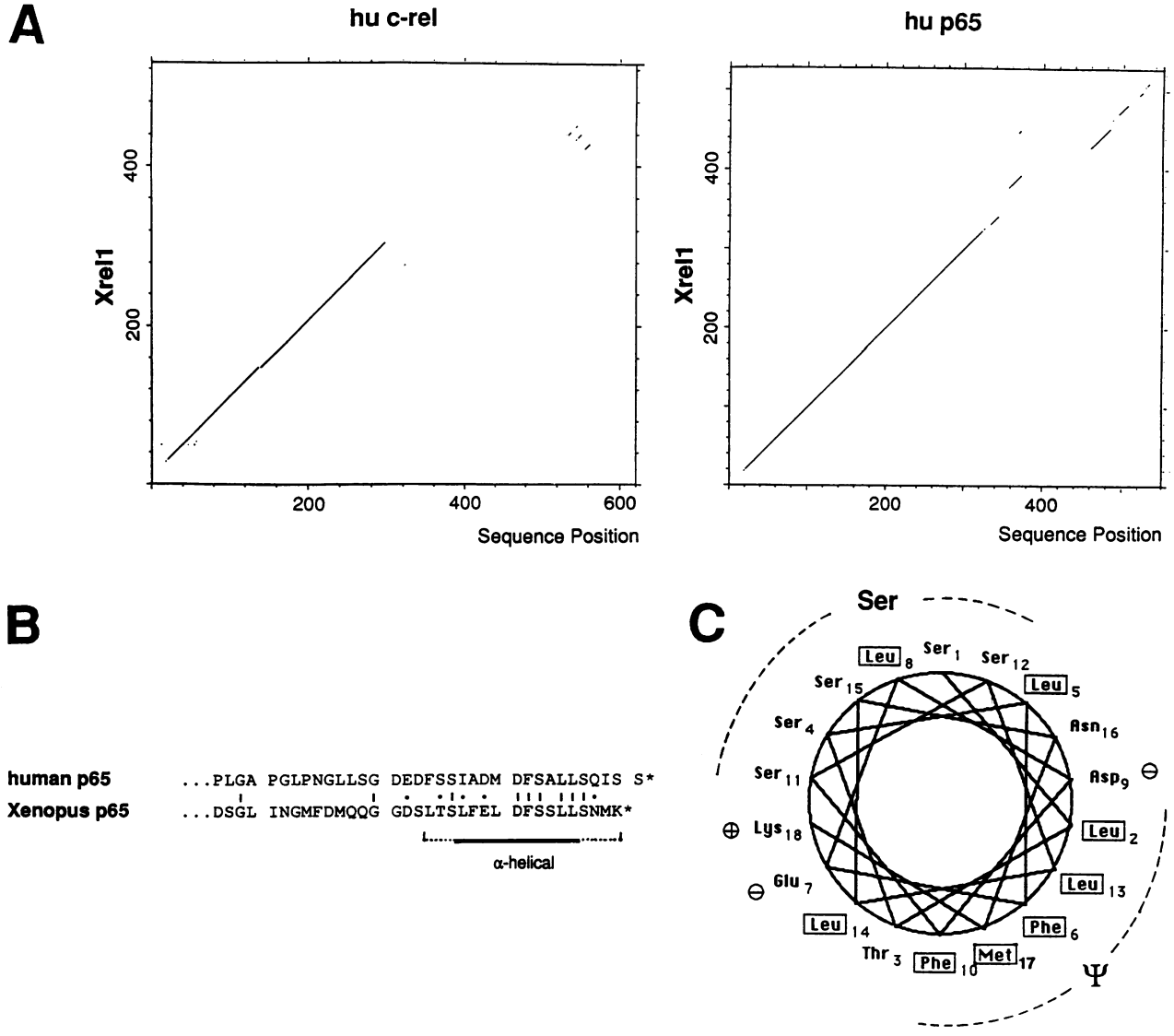


**Fig. 7.** Evolutionary conservation of the $TA_1$ structure. (**A**) The Xrel1 protein from *Xenopus laevis* is the homologue of human p65 rather than human c-Rel. The sequence of Xrel1 (Kao and Hopwood, 1991) was compared to that of human c-Rel (Brownell *et al.*, 1988) (left panel) and human p65 (Ruben *et al.*, 1991) (right panel) by a Dotplot analysis (Maizel and Lenk, 1981). The y-axis shows Xrel1 and the x-axis p65 and c-Rel, respectively. The window of the analysis was set to 35 residues and the stringency to 18. Under these conditions, 317 dots were obtained for c-Rel and Xrel1 and 419 dots for Xrel1 and p65. The diagonal lines indicate sequences of high homology. (**B**) Sequence homologies between the C-terminus of Xrel1 and human p65. Identical amino acids are marked by bars and similar residues are indicated by points. The solid part of the bracket indicates amino acids predicted to be in an α-helical conformation by two distinct programs (Chou and Fasman, 1978; Garnier *et al.*, 1978). The dotted part of bracket marks amino acids predicted to be in an α-helix by only one of the two programs. Asterisks mark the C-termini. (**C**) Helical wheel plot of the last 18 amino acid residues of Xrel1. For details, see legend to Figure 5B.

evidence that p65 acted as a homodimer and not as heterodimer between p65 and endogenous p50. It thus appears that under the given experimental conditions the weak DNA-binding activity of p65 was not limiting for activation of transcription. The physiological situation might however be different. Endogenous p65 protein is barely detectable by indirect immunofluorescence labelling (K.van Zee, U.Zabel, E.Fanning and P.Baeuerle, unpublished) which is consistent with the very low copy number of NF-κB per cell calculated by titration of DNA-binding sites (Baeuerle and Baltimore, 1989; Lenardo *et al.*, 1988). There is no evidence from mobility shift analyses or affinity purifications that p65 dimers are present in detectable amounts in nuclear extracts or cytoplasm. Following transfection of CMV−$p65^{1-550}$ into COS cells, a strong p65-specific nuclear staining is observed indicating a high expression of p65. It is thus possible that a high-level expression of p65 compensated in our experiments for the low DNA binding affinity of the protein and allowed an efficient occupation of binding sites in the κB-reporter plasmid. A physiological role of p65 dimers in gene regulation is at present unclear. At physiological concentrations, the p65 subunit might strongly depend on association with the transcriptionally much less active or inactive p50 subunit in order to bind with high affinity to DNA. One important role of p50 in the p50−p65 heterodimer is then to serve as helper and control subunit in DNA binding for the transactivating p65 subunit.

### *The regulatory potential of the p50 subunit*

A second surprising outcome from our study was that the p50 subunit of NF-κB suppressed the activity of the p65

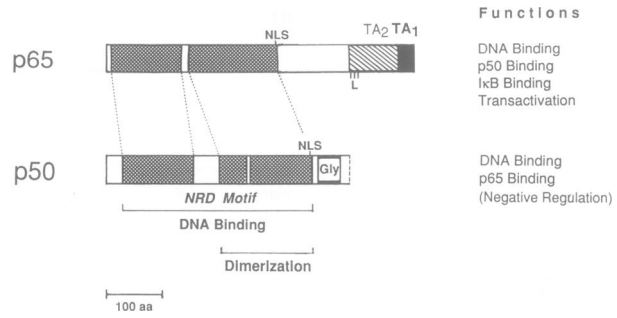


**Fig. 8.** Model summarizing the functional characteristics of p50 and p65 subunits of NF-κB. Shaded areas and connecting dotted lines indicate the region of homology between NF-κB p65, NF-κB p50, Rel and Dorsal proteins, the NRD motif. The NRD motif is required for DNA binding (Kieran *et al.*, 1990; Ghosh *et al.*, 1990). The C-terminal half of the NRD motif of p50 was recently shown to be sufficient for dimerization (Logeat *et al.*, 1991). The dashed line at the C-terminus of p50 indicates that the precise length of p50 is not known yet. The most carboxy-terminal residues of the NRD motif are likely to contain a nuclear location signal (NLS) which is highly conserved in the NRD family of transcription factors. It was identified first in v-Rel (Gilmore and Temin, 1985). A glycine-rich domain is framed. The weakly transactivating domain $TA_2$ in p65 is shaded and the principal transactivation domain $TA_1$ shown as filled box. The position of a potential leucine zipper in p65 is indicated by an L.

subunit. This negative effect was dependent on κB binding sites and could be counteracted by increasing the level of p65 or the number of NF-κB binding sites in the cell. This suggests that overexpression of p50 relative to p65 leads to formation of p50 dimers which occupy limited binding sites for p65 and p50–p65 dimers in the reporter plasmid. By this mechanism, both, p50 with none or a low-level transactivating capacity would suppress the strong p65-induced CAT induction. A puzzling observation was that even the cotransfection of very low amounts of p50 expression plasmid decreased the strong transcription activation by p65. One rather expects that a low-level co-expression of p50 allows formation of p50–p65 which would increase transactivation due to its higher DNA binding affinity than the p65 dimer. We assume, however, that NF-κB containing only one p65 subunit is transcriptionally less active than an overexpressed dimer of two transactivating p65 subunits. If the reporter plasmid is saturated with overexpressed p65, the strongly DNA binding p50–p65 would chase the p65 dimers from binding sites. Only under these conditions, the binding of NF-κB could lead to a decrease of the CAT activity induced by p65 dimers. However, at physiological concentrations of subunits, p65 dimers (if they exist) cannot efficiently bind to κB sites and it would be the strongly DNA binding NF-κB which is responsible for transcription activation.

The activity of p50 seems to be very similar to that of v-Rel. v-Rel was recently shown to suppress the transactivation of c-Rel from κB sites (Inoue *et al.*, 1991). As p50, v-Rel is only a very weak transcriptional activator because a C-terminal domain required for transactivation of c-Rel (see below) is disrupted in the viral protein (Richardson and Gilmore, 1991). v-Rel can transform several avian cell types. It will be interesting to see whether expression of mammalian p50 in avian cells has a similar effect.

Two groups have reported on the presence of a constitutive κB-like factor in nuclear extracts from various cell types. This factor was referred to as KBF1 (Israel *et al.*, 1987; 1989) or EBP1 (Clark *et al.*, 1990) and proposed to mediate a low basal expression of the class I gene H-2K$^b$. Recent cDNA cloning of KBF1 identified the factor as homodimer of the p50 subunit of NF-κB (Kieran *et al.*, 1990). We have recently compared the binding affinity and specificity of p50 dimer and NF-κB (Urban and Baeuerle, 1990; Urban and Baeuerle, 1991). Both forms can bind with high affinity to the κB motif 5′-GGGACTTTCC-3′ present in the reporter plasmid but bind best to palindromic, 11 bp motifs. p50–p65 can bind better than the p50 dimer to 10 bp motifs of reduced symmetry. Because dimers of p50 (KBF1/EBP1) can apparently exist in cells as constitutive nuclear factor independently from the inducible p50–p65 and have DNA binding properties similar to those of p50–p65, p50 dimers might function as negative transcriptional regulators of NF-κB mediated gene activation *in vivo*. The differences in the DNA binding specificity of p50 dimer and NF-κB (see Urban and Baeuerle, 1991) suggest that certain κB motifs are more susceptible to negative regulation of p50 dimers than others. Future studies have to investigate whether NF-κB binding sites which are of high symmetry and allow 11 or 12 bp contacts (listed in Baeuerle, 1991) are under stronger negative control by p50 dimers than 10 bp κB motifs with a low symmetry.

An important question is under what conditions and by what mechanism p50 dimers are generated in the cell. A physiological role as negative regulators implies that their biogenesis must be highly controlled. Such a regulatory step could involve the proteolytic release of p50 from its inactive cytoplasmic precursor (p110). If proteolysis of p110 is triggered and there is not enough p65 available as partner for heterodimerization, p50 dimers should form. The HIV-1 protease was recently shown to cleave the p50 precursor *in vitro* (Rivière *et al.*, 1991). Also *in vivo*, active NF-κB accumulated in nuclei upon HIV-1 infection. This nuclear form of NF-κB was however associated with p65. Another way to increase the amount of p50 dimers in cells would be down-regulation of p65 expression. Only future studies on a possible transcriptional autoregulation of NF-κB subunits (see Meyer *et al.*, 1991) and the characterization of p110-processing enzymes will allow to investigate the origin of p50 dimers and their possible role in negative regulation.

### *The transactivation domains of p65*

p65 has at least two transcriptional activation domains in its unique C-terminal third. $TA_1$ consists of a short but strongly transactivating sequence in the very C-terminus. A highly conserved feature of this sequence is a putative *a*-helix in the last 20–24 amino acids. The α-helix would form a unique amphipathic structure; one face is strictly hydrophobic while the opposite hydrophilic face clusters serine residues. Charged residues are restricted to the interfaces. An amphipathic helix as transcription activator sequence is not without precedent. An artificial acidic peptide of 15 amino acids with a possible α-helical amphipathic structure was shown to initiate transcription in yeast (Giniger and Ptashne, 1987). Also the transcriptional activation domain in the viral vp16 protein is likely to contain a short, acidic, amphipathic helical stretch but mutational analysis indicated that the helix is not an important structure feature (Cress and Triezenberg, 1991). Because the $TA_1$ sequence is shorter than the vp16 domain and mostly in a putative α-helical structure, the situation in p65 might be different.

A novel feature of $TA_1$ appears to be the clustering of serine residues on one side of the putative amphipathic helix. Also the heptade arrangement of four serine residues (in mouse and human p65) deserves attention. It is possible that the serine residues are involved in a zipper-like structure serving a role in protein–protein interaction as has been proposed for leucine repeats (Landschulz *et al.*, 1988). It is assumed that activation domains of transcription factors serve to bind other proteins that function in communicating between the specific and more general factors (Ptashne, 1986, 1988; Struhl, 1987). We anticipate that the $TA_1$ domain can establish an interaction between p65 and such factors.

Additional transactivating sequences of p65 encompass amino acids between position 441 and 518 (referred to as $TA_2$). Although the C-terminal third of p65 including $TA_2$ is enriched in proline residues (18%) there seem to be no proline-rich transactivating sequences as described in other factors (listed in Mitchell and Tjian, 1989). First of all, proline-rich sequences of p65 between position 286 and 441 linked to GAL4 showed only very week induction of the reporter gene. Secondly, proline clusters are not conserved between *Xenopus* and human p65. The conservation seems to be restricted to very C-terminal sequences containing $TA_1$ and $TA_2$ domains (see Figure 7A). Future studies have to investigate the role of a potential leucine zipper within the $TA_2$ domain in transactivation.

The transcriptional activation domains of p65 are confined to the C-terminal part which is not homologous to p50, Rel and dorsal proteins suggesting that the N-terminal NRD motif does not play a direct role in transcription activation. This would explain why p50 does not display significant transactivation potential. The sequence of p50 consists mainly of the NRD motif. Most of its extra sequences are a 100 amino acid insert in the NRD motif which might separate DNA binding and dimerization functions (Logeat *et al.*, 1991). The unique C-terminal sequence of p50 encompasses a glycine-rich domain which might serve as a hinge region in the precursor. As suggested by the apparent molecular size of 'p50' in SDS gels between 45 and 51 kDa, there are at most 30 residues at the C-terminus in addition to the glycine rich region.

In mouse c-Rel, the transactivating sequences are also present in the unique C-terminal sequence (Bull *et al.*, 1990). The NRD motif of c-Rel showed no transcription activation when linked to a GAL4 domain. Only a defined portion in the C-terminal half of the protein was active. Unlike p65, mouse c-Rel (588 aa) has no short transactivating sequence in the very C-terminus but an ~160 amino acid long domain within position 403 and 568. This domain has no apparent sequence similarity with $TA_1$ or $TA_2$, nor a similar amino acid composition. There are stretches in the c-Rel domain that are likely to adopt an α-helical amphipathic structure but there is no clustering of serine residues. The finding that the activation domains of p65 and c-Rel are distinct suggests that the two proteins can interact with different TA-binding factors which could provide the basis for yet unknown differences of the two transactivators.

It appears as if the DNA binding activity of p65 is low in order to make p65 dependent on p50 for DNA binding and transactivation. This dependence has the advantage that mutations of p65 abolishing p50 binding or loss of function mutants of p50 do not create a constitutive activator. A mutation resulting in C-terminal truncation of p65 would increase the DNA binding activity of p65 (Nolan *et al.*, 1991) and render the protein independent of p50. However, as shown in the present study, such a mutant is also unlikely to be a constitutive activator because most of the transactivating potential of p65 would be lost with the very C-terminus. It thus appears that several mechanisms have evolved to prevent p65 from turning into a constitutive transcriptional activator: direct negative control by IκB, dependence on p50 for strong DNA binding and C-terminal arrangement of the strongest transactivating domain. In the case of p50, the cell must prevent generation of a constitutive suppressor. This might be the main reason why p50 is synthesized in form of an inactive precursor.

## Materials and methods

### *Cell culture*

Mouse L cells (thymidine kinase deficient, $tk^-$) and monkey COS7 cells were grown at 37°C in Dulbecco's modified Eagle's Medium (GIBCO) supplemented with antibiotics (Pen-Strep) and 5% and 10% fetal calf serum (GIBCO), respectively. *Drosophila* Schneider cells (S2; kindly provided by Dr Herbert Jäckle, Göttingen) were maintained at room temperature in Schneider's *Drosophila* medium (GIBCO) supplemented with 10% fetal calf serum, amphotericin, penicillin and streptomycin (GIBCO).

### *Transfections and CAT assays*

One day prior to transfection, $10^5$ $Ltk^-$ cells or $10^6$ Schneider cells were plated onto 6 cm dishes. Both cell types were transfected using the calcium phosphate coprecipitation technique as described by Wigler *et al.* (1978). The plasmid DNA samples used for transfections were purified twice by CsCl gradient centrifugation. Each plate received 1 pmol of a particular expression vector, 2 pmoles of CAT reporter plasmid and, in some experiments, 0.5 pmol of an internal control plasmid containing a luciferase reporter gene. The same amount of DNA was used for transfection of $8 \times 10^5$ COS7 cells which were transfected in suspension by the DEAE dextran method as described (Lopata *et al.*, 1984).

Cells were harvested 48 h after transfection and cell extracts prepared after three cycles of freeze–thawing. For the p65 deletion experiments using $Ltk^-$ cells (Figure 5), portions of cell extracts were used for CAT assays that contained the same luciferase activity from a cotransfected luciferase reporter plasmid. Values obtained by this standardization did not significantly differ from values obtained with equal amounts of protein determined by the method of Bradford (Bradford, 1976). In all other assays, cell extracts were therefore normalized for the same amount of protein. The CAT activity was determined as described (Pierce *et al.*, 1988). Quantification of results was performed by liquid scintillation counting of pieces from the thin-layer chromatography plate containing unreacted [$^{14}$C]chloramphenicol and pieces containing the various acetylated forms of chloramphenicol. Assays giving more than 50% conversion of chloramphenicol were discarded and assays repeated for shorter periods of time or with less cell extract. Between two and six independent transfection experiments were performed and CAT activities determined at least in duplicate assays. If not otherwise indicated, deviations between experiments were <20%.

### *Constructs*

The reporter plasmid J16 containing two κB sites (5′-GGGACTTTCC-3′) upstream from a truncated human c-*fos* promoter and the control plasmid J32 with two mutant κB sites (5′-TTCACTTTCC-3′) are described in Pierce *et al.* (1988). The GAL4 reporter plasmid has two GAL4 binding sites upstream from a truncated c-*fos* promoter, and the construct GAL4–vp16 contains amino acids 413–490 of vp16 (both kindly provided by Dr Rainer Renkawitz, Giessen).

The expression plasmid CMV–$p50^{1-339}$ was a kind gift of Dr Alain Israel (Paris) (for details see Kieran *et al.*, 1990). The plasmid CMV–$p50^{1-502}$ was obtained by cutting the human p110 precursor for the p50 NF-κB subunit cloned into the expression vector RcCMV (Invitrogen) with *Xba*I, followed by religation. For the CMV–$p65^{1-550}$ plasmid, the full-length p65 cDNA (Ruben *et al.*, 1991) was excised from a Bluescript vector with *Hin*dIII–*Xba*I and inserted into the *Hin*dIII–*Xba*I-opened expression vector RcCMV. The plasmid pABGal-linker (Baniahmad, 1991) was derived from a construct described by Muller *et al.*, (1989) and contained the DNA binding and dimerization domain of the yeast GAL4

transcription factor (Carey *et al.*, 1989) in context of an upstream RSV promoter/enhancer and a downstream polylinker sequence. GAL4−p50$^{18-502}$ was constructed by opening pABGal-linker with *Bam*HI and inserting the *Bam*HI−*Hin*dIII-fragment of pSK15$^{+}$ containing the p50 subunit (Kieran *et al.*, 1990) by a blunt-end ligation after filling up the sticky ends with the Klenow polymerase (Klenow fill-in). GAL4−p50$^{18-230}$ was obtained by cutting GAL4−p50$^{18-502}$ with *Hin*dIII−*Nar*I, Klenow fill-in and subsequent religation. GAL4−p50$^{231-502}$ was generated by cutting GAL4−p50$^{18-502}$ with *Nar*I, blunting with mung bean nuclease, recutting with *Sma*I followed by religation. GAL4−p65$^{1-519}$ was constructed by inserting a 1.6 kb *Sma*I−*Sma*I fragment from the Bluescript vector p65/3 (a gift of Dr Craig Rosen, Nutley) into the *Pvu*II site of pABGal-linker. In order to obtain the full-length p65 protein coupled to GAL4 (GAL4−p65$^{1-550}$), GAL4−p65$^{1-519}$ was opened with *Bst*EII−*Hin*dIII followed by insertion of the respective fragment from the p65 cDNA from the Bluescript vector p65/3. GAL4−p65$^{1-77}$ was generated by removing the *Bst*EII−*Hin*dIII fragment from GAL4−p65$^{1-519}$. GAL4−p65$^{286-550}$ and GAL4−p65$^{286-467}$ were prepared by digesting GAL4−p65$^{1-550}$ partially with *Eco*RI, isolating the respective restriction fragments by electrophoresis, followed by religation. GAL4−p65$^{520-550}$ and GAL4−p65$^{468-550}$ were obtained by cutting GAL4−p65$^{1-550}$ with *Sma*I or *Sal*I, respectively, followed by religation. GAL4−p65$^{286-467}$ was constructed by cutting GAL4−p65$^{286-550}$ with *Sal*I and *Hin*dIII followed by Klenow fill-in and religation. GAL4−p65$^{286-518}$, GAL4−p65$^{286-441}$ and GAL4−p65$^{286-364}$ were obtained by opening of GAL4−p65$^{286-550}$ with *Eco*RV and subsequent cutting with *Sma*I, *Pvu*II and *Nco*I, respectively. After Klenow fill-in of the 5′ overhang generated by cutting with *Nco*I the vectors were religated. GAL4−p65$^{286-550\Delta442-475}$ and GAL4−p65$^{286-518\Delta442-475}$ were obtained by removing the *Pvu*II fragment from GAL4−p65$^{286-550}$ and GAL4−p65$^{286-518}$, respectively. GAL4−p65$^{468-519}$ was obtained after cutting GAL4−p65$^{1-519}$ with *Sal*I and religation. The correct nucleotide sequence of all constructs was verified by automatic DNA sequencing on a Genesis 2000 (Du Pont).

### *Electrophoretic mobility shift assays*

1−2 × 10$^6$ cells grown on 6 cm dishes were harvested 48 h after transfection. Cells were washed once with PBS and scraped from the dish using a rubber policeman. After 2 min centrifugation with 200 *g* at 4°C, the cell pellet was resuspended in 80 μl of 5 × extraction/binding buffer (EB buffer; 100 mM Tris−HCl, pH 7.5, 500 mM KCl, 25 mM $MgCl_2$, 35% (vol/vol) Glycerol, 5 mM dithiothreitol, 1% aprotinin (Sigma) and 0.5 mM phenylmethylsulfonylfluoride (Sigma). Cells were broken by two cycles of freeze−thawing and particulate material pelleted for 20 min at 100 000 *g*. After preincubation of whole cell extracts (2−6 μg of protein in 4 μl of 5 × EB-buffer) with 2 μg poly(dI-dC) (Pharmacia), 5000−10 000 c.p.m. (Cerenkov) of $^{32}$P-labelled oligonucleotide probe was added and incubation allowed for 15 min in a final volume of 20 μl followed by gel electrophoresis. Oligonucleotides were labelled either using [α$^{32}$P]dNTPs (3000 Ci/mmol; Amersham) with the Klenow polymerase (Boehringer), or using [γ$^{32}$P]ATP (5000 Ci/mmol; Amersham) with T4 polynucleotide kinase (Boehringer). Native electrophoresis was performed with 0.5 × TBE 4% polyacrylamide gels at 4°C for 3 h. After drying, gels were exposed to Kodak XAR5 films at −70°C.

The sequence of the oligonucleotide used to detect GAL4-specific DNA binding activity is (the GAL4 binding motif is underlined):

5′-GGCGGGTCGGAGTACTGTCCTCCGACTGC- 3′
3′ - GCCCAGCCTCATGACAGGAGGCTGACGGAC- 5′

The oligonucleotide used to detect κB-specific DNA binding activity contains a sequence from the promoter of the IRF-1 transcription factor (Miyamoto *et al.*, 1988) with a high-affinity binding site for NF-κB (R.Schreck and P.Baeuerle, in preparation). Its sequence is (the κB motif is underlined):

5′ - AGCTTGGGCCGGGGAATCCCGCTAAG- 3′
3′ - ACCCGGCCCCTTAGGGCGATTCAGCT- 5′

### *Indirect immunofluorescence labelling*

COS7 cells were transfected with plasmid DNA as described above and grown on cover slips for 48 h. After removal of the medium, cells were fixed for 20 min in 3% paraformaldehyde dissolved in PBS/10% FCS. The cells were subsequently incubated in PBS/10% FCS containing 0.2% (v/v) Triton X-100. Fixed cells were then reacted with a rabbit anti-GAL4 serum (a gift of Dr Marc Ptashne, Cambridge) or a control serum diluted 1:100 in PBS/FCS. After 1 h incubation followed by extensive washing in PBS/FCS, the sample was reacted for another hour with a fluorescein isothiocyanate (FITC)-labelled goat anti-rabbit antibody (Sigma) which was diluted 1:80 in PBS/FCS. After several washing steps in PBS/FCS, the cover slips were mounted on a slide covered with 0.1% (w/v) *p*-phenylenediamine, 10% PBS and 90% (v/v) glycerol and analysed by fluorescence microscopy.

### *Computing*

The amino acid sequences of human p65, human c-Rel and *Xenopus* Xrel1 were compared by the program DotPlot designed by Maizel and Lenk (1981). Parameters are given in the legend to Figure 6. For secondary structure predictions, the program PeptideStructure from the Genetic Computer Group was used. The structure of p65 and Xrel1 in their C-termini was analysed by the methods of Chou and Fasman (1978) and Garnier *et al.* (1978).

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