The EMBO Journal vol.10 no.7 pp.1817–1825, 1991

NF-κB contacts DNA by a heterodimer of the p50 and p65 subunit

Manuela B.Urban, R.Schreck and Patrick A.Baeuerle[1]

Laboratorium für Molekulare Biologie der Ludwig-Maximilians-Universität München, Genzentrum, Am Klopferspitz, D-8033 Martinsried, FRG
[1]Corresponding author

Communicated by E.-L.Winnacker

We recently reported that the apparently non-DNA-binding 65 kd subunit (p65) of the NF-κB transcription factor can modulate the DNA-binding specificity of the 50 kd subunit (p50) of NF-κB. In this study we provide an explanation for this property of p65. In electrophoretic mobility shift assays and upon UV cross-linking to DNA, gel-purified p65 is shown to be a κB-specific DNA-binding protein on its own. The binding activity was only detectable if high amounts of p65 were used for the analyses and after the application of a modified renaturation protocol. DNA-binding of the p65 dimer, in contrast to that of p50, was inhibited by IκB-α and -β. This finding is consistent with a receptor function of p65 for both inhibitory subunits. Direct UV cross-linking of NF-κB to DNA probes which were photoreactive within only one half-site and a binding competition analysis with p65 showed that p65 has a strong preference for binding to the less conserved half site of κB motifs whereas p50 has a moderate preference for the more highly conserved half site. In electrophoretic mobility shift assays and upon sedimentation through glycerol gradients, NF-κB appears to exist as a heterodimer composed of one p50 and one p65 subunit whereas data from gel filtration suggest a higher order complex.

Key words: heterodimer/NF-κB/p50 subunit/p65 subunit

Introduction

Nuclear factor κB (NF-κB) is a protein that inducibly initiates the transcription of a wide variety of genes by binding to decameric and undecameric sequence motifs in enhancer and promoter elements (reviewed in Baeuerle and Baltimore, 1991; Baeuerle, 1991). Most of its target genes fall into three classes encoding immuno-modulatory cytokines, immunologically important cell surface receptors and acute phase response proteins. NF-κB can rapidly activate these genes because the protein resides in a latent form in the cytoplasm of many different cell types and simply requires the release of the inhibitory subunit IκB for its appearance in the nucleus as active DNA-binding protein (Baeuerle and Baltimore, 1988). Within enhancer and promoter elements of genes, NF-κB usually synergizes with other DNA-binding proteins which are likely to control, for instance, a cell type-specific response of NF-κB or a modulation of the kinetic and strength of the gene induction by NF-κB (reviewed in Baeuerle and Baltimore, 1991; Baeuerle, 1991). In contrast to most other inducible transcription factors, NF-κB is not activated by a single hormone or agent but by a great variety of very distinct substances. These include viruses and their transactivator proteins, T cell mitogens, the cytokines TNF and IL-1, lipopolysaccharide, activators of protein kinase C, UV light and parasites (reviewed in Baeuerle and Baltimore, 1991; Baeuerle, 1991).

The inducible cytoplasmic form of NF-κB contains three protein subunits: a 50 kd (p50) and 65 kd protein subunit (p65), both of which are also found in the active nuclear form and a third subunit IκB, which is confined to the cytoplasmic form. IκB is a specific inhibitor of the DNA-binding of NF-κB and is apparently responsible for the cytoplasmic retention of the heterotrimeric complex (Baeuerle and Baltimore, 1988). IκB proteins were recently purified and characterized (Ghosh and Baltimore, 1990; Zabel and Baeuerle, 1990).

Several studies reported on the purification of NF-κB (Kawakami *et al.*, 1988; Baeuerle and Baltimore, 1989; Ballard *et al.*, 1990). In all studies, the DNA-binding was found to be associated only with p50. The copurifying 65–68 kd protein (p65) showed no DNA-binding in electrophoretic mobility shift assays (EMSAs) or in UV cross-linking experiments. In contrast, dimerized p50 binds with high affinity to DNA and shows the same methylation interference pattern with the κB motif 5′-GGGACTTTCC-3′ as the form containing in addition p65 (Baeuerle and Baltimore, 1989). The complex of p50 and p65 elutes upon gel filtration with a Stokes' radius corresponding to a size larger than 200 kd. Based on these data, it was assumed that NF-κB forms a heterotetramer containing a DNA-binding p50 dimer with two p65 molecules attached. The only function found associated with p65 was its requirement for inhibition of NF-κB by IκB (Baeuerle and Baltimore, 1989). While the DNA-binding of NF-κB containing p65 and p50 was rapidly inactivated after addition of IκB, a dimer of p50 was inert. We could recently sustain a receptor function of p65 for IκB (Urban and Baeuerle, 1990). Isolated p65 but not p50 suppressed the inhibitory activity of IκB and excess amounts of p65 were found to activate the inducible form of NF-κB presumably by absorbing occasionally released IκB.

In the meantime, a series of observations have accumulated indicating an involvement of p65 in the DNA-binding of NF-κB and which would be consistent with a model in which both p50 and p65 form a DNA-binding heterodimer. (i) The DNA-binding specificity of the p50 dimer and the complex of p50 and p65 can be distinguished if artificial palindromic κB motifs are used in a binding competition assay (Urban and Baeuerle, 1990). The pentameric half sites in the frequent κB motif 5′-GGGACTTTCC-3′ (AB) were duplicated to yield the motifs 5′-GGGACGTCCC-3′ (AA) and 5′-GGAAATTTCC-3′ (BB). A dimer of p50 recognized the AA motif best followed by AB and BB. NF-κB containing

© Oxford University Press

both p50 and p65 could efficiently recognize only the low-symmetry AB motif. (ii) The affinity constant of the p50 dimer for the AB motif is >two times lower than that of NF-κB. (iii) An analysis of all known physiological NF-κB binding motifs showed that they are usually of low symmetry and that the sequence of their half sites is distinctly conserved (Zabel *et al.*, 1991; Baeuerle, 1991). One half site (A) was in all motifs highly conserved and had the consensus 5'-GGGPuN-3' while the other half site (B) with the consensus 5'-G(GPuPu)N-3' was degenerate. (iv) A mutational analysis of the 11-mer motif 5'-GGGGATTCCCC-3' from the enhancer of the MHC class I gene H-$2K^b$ showed that an alteration of the 3' half site affected the binding of the p50 dimer more than that of NF-κB (Urban and Baeuerle, 1991).

cDNA clones encoding a 110 kd precursor of p50 (Kieran *et al.*, 1990; Ghosh *et al.*, 1990; Bours *et al.*, 1990) and p65 (Ruben *et al.*, 1991) were recently isolated and found to be highly homologous to the product of the proto-oncogene c-*rel* within a large domain required for DNA-binding and dimerization. The homology of p50 to rel proteins was also apparent from its immunoreactivity with antisera raised against v-*rel* (Ballard *et al.*, 1990). In the latter study a novel rel-related protein of 75 kd (p75) was identified. An important outcome from these studies was the finding that rel proteins are DNA-binding proteins which recognize the same sequence motif as NF-κB and have the potential to form heterodimers with the p50 subunit of NF-κB. The homology of p65 with p50 and rel proteins raises the possibility that p65 is a DNA-binding protein as well.

Despite these indications, a participation of p65 in the DNA-binding of NF-κB was not yet observed. In the present study, we tested high amounts of SDS–gel-purified p65 which was subjected to a modified protocol for renaturation and DNA-binding in EMSAs. The modifications allowed us to demonstrate that the p65 subunit of NF-κB is a specific DNA-binding protein on its own which recognizes in the absence of p50 various NF-κB binding motifs apparently as homodimer. In a binding competition assay, the p65 dimer preferred to interact with the less conserved second half site of the κB motif 5'-GGGACTTTCC-3'. Consistent with a receptor function of p65 for IκB, the DNA-binding of p65 was inhibited by two variants of the inhibitory subunit IκB. A UV cross-linking study using two DNA probes which were photoreactive in either half site A or B of the NF-κB binding motif 5'-GGGAAATTCC-3' showed that native NF-κB indeed contacts DNA with the p50 as well as p65 subunit. p50 preferred the highly conserved first half site and p65 the less conserved second half site. The finding that the protein–DNA complex of p65 migrates more slowly than that of NF-κB suggests that in native gels NF-κB binds as a heterodimer of p50 and p65 to DNA.

Results

Detection of a p65–DNA complex

NF-κB was purified from cytosol of human placenta as described (Zabel *et al.*, 1991) and subjected to one more round of DNA affinity purification. p50 and p65 (Figure 1A, lane 2) were efficiently separated from two protein species of 72 and 85 kd molecular size found in fractions of a higher salt concentration (lane 1). The latter proteins were most likely the κB-specific p75 and c-rel DNA-binding proteins described recently (Ballard *et al.*, 1990). These proteins required 0.5 M KCl to elute from the DNA-affinity resin of multimerized NF-κB binding motifs (Figure 1A, lane 1) whereas the majority of NF-κB was eluted with 0.35 M KCl (lane 2) or 0.3 M KCl (Zabel *et al.*, 1991). Most if not all of the p50 and p65 appeared not to be associated with the 72 and 82 kd proteins in placenta tissue.

As reported earlier, renaturation of gel-purified p50 on its own produces a protein–DNA complex migrating faster than that of NF-κB (Figure 1B, lane 1) and an equivalent amount of gel-purified p65 does not show detectable DNA-binding (lane 2) (Baeuerle and Baltimore, 1989; Ballard *et al.*, 1990; Ghosh *et al.*, 1990; Kieran *et al.*, 1990; Urban and Baeuerle, 1990). After combined renaturation of p50 and p65, a protein–DNA complex with the mobility of NF-κB is restored which shows that both p50 and p65 subunits are contained in the complex of NF-κB (data not shown; Baeuerle and Baltimore, 1989; Urban and Baeuerle, 1990).

Despite the absence of detectable DNA-binding of p65, several recent findings made the idea attractive that p65 participates in the protein–DNA interaction of NF-κB. Important ones are that p65 modulates the DNA-binding specificity of NF-κB (Urban and Baeuerle, 1990), that p65 has high homology with the DNA-binding rel proteins (Ghosh *et al.*, 1990; Ruben *et al.*, 1991) and that most physiological NF-κB binding motifs are of low symmetry (Zabel *et al.*, 1991). In an effort to demonstrate DNA-binding of p65, we used in EMSAs much higher amounts of gel-purified p65 than in previous studies and omitted the non-specific competitor poly(dI-dC) (Figure 1B). If a 32-fold excess of gel-purified p65 over a fraction of gel-purified p50 was analyzed by EMSA a protein–DNA complex was indeed obtained with protein from a SDS gel fraction encompassing a molecular size of 65 kd (Figure 1B, lanes 4 and 5). The protein–DNA complex showed a mobility in native gels which was considerably lower than that of the p50 dimer. This would be consistent with the larger size of a p65 dimer. The protein–DNA complex migrated even more slowly than that of NF-κB containing p50 and p65 subunits (Figure 1D) suggesting that NF-κB might bind in native gels as a heterodimer to DNA. The use of higher amounts of saturated urea in the renaturation protocol could significantly increase the DNA-binding activity of p65 (Figure 1B and C, compare lanes 5). A further increase was observed after repeated freeze–thawing of the sample.

To reassure that the DNA-binding activity comigrated with the 65 kd subunit of NF-κB, we also analyzed flanking size fractions one of which encompassed the position where the 72 kd protein would migrate (Figure 1A, lane 2). Also p75 was found to have a protein–DNA complex migrating more slowly than that of p50–p65 (Ballard *et al.*, 1990). However, the novel slow-migrating DNA-binding activity was confined to the molecular size fraction containing p65 (Figure 1C, lane 4) and no activity was found in fractions of a larger molecular size (lanes 5 and 6). We also tried to renature DNA-binding activity from the 72 kd protein contained in the 0.5 M KCl fraction (Figure 1A, lane 1) by the same protocol used for p65. Despite the apparent association of this protein with the DNA-affinity resin, we were unable to obtain any signal in EMSAs with the approriate size fraction (data not shown). This further rules out that the protein–DNA complex seen with 65 kd fraction was a contamination with the 72 kd protein. The slow-

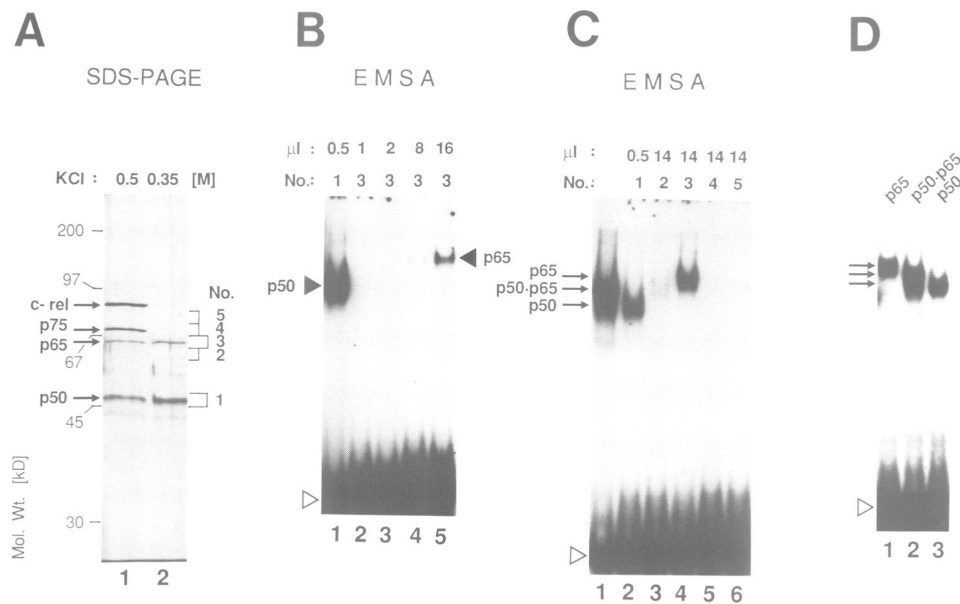

Fig. 1. Detection of the DNA-binding activity of purified p65. (**A**) Separation of NF-κB from p75 and c-rel. Aliquots of the 0.5 M KCl (lane 1) and 0.35 M KCl fractions (lane 2) from the second round of a κB-DNA affinity chromatography were analyzed by 10% SDS–PAGE and silver-staining. Arrows on the left indicate the positions of the two NF-κB subunits p50 and p65 and the positions of the presumed p75 and c-rel proteins. Brackets on the right show molecular size fractions used for renaturation experiments (No. 1–5). The two fractions containing p50 and p65 (No. 1 and 3, respectively) have longer brackets. The positions of molecular size standards (Amersham) are shown on the left. (**B**) DNA binding activity in the 65 kd molecular size fraction. Various amounts from 0.5 to 16 μl (lanes 2–5) of renatured material from the 65 kd size fraction (No. 3) were subjected to electrophoretic mobility shift assays (EMSA) in the absence of poly(dI-dC). In the first lane, 0.5 μl of renatured material from the 50 kd size fraction (No. 1) was analyzed. Filled arrowheads indicate positions of protein–DNA complexes obtained with p50 and p65 and the open arrowhead indicates the position of unbound DNA-probe, an oligonucleotide encompassing the NF-κB binding motif from the murine κ light chain enhancer. A fluorogram of a native gel is shown. (**C**) Analysis of flanking size fractions and improved renaturation of p65 binding activity. Lane 1, native NF-κB; lane 2, 0.5 μl renatured material from size fraction No. 1; lanes 3–6, 14 μl from size fractions No. 2–5. Compared with B, the amount of saturated urea was increased in the renaturation protocol and the H-2K^b DNA probe instead of the κB probe was used in the EMSA. Arrows indicate the positions of protein–DNA complexes of p50, native NF-κB (p50–p65) and p65. (**D**) Comigration of protein–DNA complexes of p65, NF-κB and p50 in native gels.

migrating protein–DNA complex of p65 was not detectable if p65 was renatured together with p50 but the combined renaturation gave rise to a protein–DNA complex migrating with the same mobility as native NF-κB (data not shown). The results show that the p65 subunit of NF-κB has DNA-binding activity on its own and gives rise to a protein–DNA complex migrating more slowly than that of the p50-dimer and that of p50–p65. The affinity of p65 for DNA is presumably lower than that of p50 and, in addition, its activity appears to renature less efficiently after electrophoretic purification.

p65 is a κB-specific DNA-binding protein

The DNA-binding specificity of p65 was tested in a binding competition experiment (Figure 2A). A ^{32}P-labeled DNA probe encompassing the motif 5′-GGGGATTCCCC-3′ from the MHC class I gene H-2K^b was mixed with a 25-fold molar excess of various unlabeled competitor oligonucleotides before the addition of p65. The most efficient competition was seen with the homologous H-2K^b motif (Figure 2A, lane 2). The second strongest competition was seen with the NF-κB binding motif 5′-GGGAAATTCC-3′ from the β-interferon promoter (Figure 2A, lane 4). The mouse κ enhancer motif 5′-GGGACTTTCC-3′ competed less efficiently (Figure 2A, lane 3), and the motif 5′-GGGAATCTCC-3′ from the promoter of the IL-2 receptor α-chain gene showed the weakest competition of the four NF-κB binding motifs tested (lane 5). An unrelated DNA fragment showed no competition effect (Figure 2A, lane 6). These data show that p65 is a κB-specific DNA-binding protein. The observation that all four κB motifs in the competitor oligonucleotides had very similar 5′ half sites to the consensus sequence 5′-GGGAN-3′ but profoundly differed in their 3′ half sites, suggests that the less conserved 3′ half site determined the distinct competition efficiencies of the oligonucleotides.

We also tested the competition effect of the non-specific competitor DNA poly(dI-dC) (Figure 2B). The DNA-binding of p50 (Figure 2B, lanes 6–10) and p65 (lanes 11–15) to a ^{32}P-labeled κB probe was competed more strongly by increasing amounts of poly(dI-dC) than that of p50–p65 (lanes 1–5). While 2 μg poly(dI-dC) completely inhibited binding of p50 and p65 to the radioactive H-2K^b probe (Figure 2B, lanes 10 and 15), the amount of radioactive complex formed with p50–p65 was only reduced by ~70% (lane 5; see also Zabel *et al.*, 1991). The inhibition of p50 and p65 dimers by high poly(dI-dC) concentrations could mean that their amounts in nuclear extracts were highly underestimated so far because microgram amounts of the competitor poly(dI-dC) are usually added to DNA-binding reactions with nuclear extracts.

p65 preferentially binds to the 3′ half site of the κ light chain motif

We recently analyzed the interaction of p50 dimers and NF-κB with artificial palindromic κB motifs created by

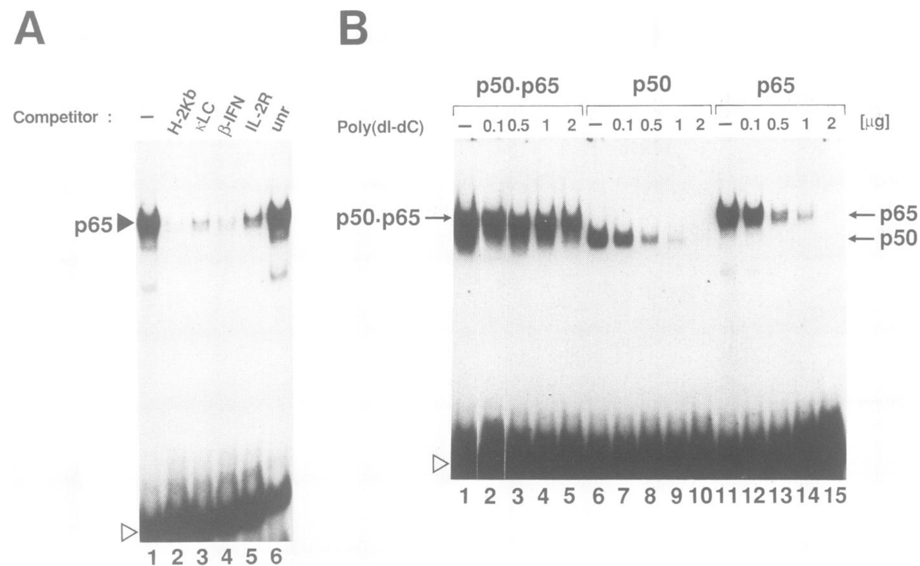

Fig. 2. Binding competition analyses with p65. (**A**) Competition with various NF-κB binding motifs. The labeled H-2K^b probe was mixed with a 25-fold molar excess of oligonucleotides containing the NF-κB binding motifs from enhancers or promoters of the following genes: the H-2K^b MHC class I (lane 2), murine κ light chain (κLC; lane 3), β-interferon (β-IFN; lane 4) and human interleukin-2 receptor α-chain genes (IL-2R; lane 5); lane 6, a 250-fold molar excess of a 56 bp fragment from a bacterial plasmid (unr; described in Urban and Baeuerle, 1990). EMSA reactions were started by the addition of 2 μl p65 fraction. A fluorogram of a native gel is shown. (**B**) Competition with poly(dI-dC). The labeled H-2K^b probe was mixed with 0.1 to 2 μg of poly(dI-dC) prior to the addition of native NF-κB (p50–p65; 0.1 μl), gel-purified p50 (0.5 μl) or p65 (2 μl). For details of illustration see legend to Figure 1.

duplicating the half sites in the motif 5′-GGGACTTTCC-3′ (AB) into 5′-GGGACGTCCC-3′ (AA) and 5′-GGAAATTTCC-3′ (BB) (Urban and Baeuerle, 1990). It was found that p50–p65 showed only a suboptimal recognition of the palindromic AA and BB motifs but preferred the low symmetry AB motif. In contrast, the p50 dimer recognized the palindromic AA motif best. Approximately four times more of the BB oligonucleotide was required to achieve the same competition efficiency as with the AA oligonucleotide and the AB motif was intermediate. This showed a preference of the p50 dimer for the more highly conserved A half site of the κB motif 5′-GGGACTTTCC-3′.

Now, we had the opportunity to test also p65 in a binding competition assay using various amounts of unlabeled AB, AA and BB oligonucleotides (Figure 3). The radioactive DNA probe was an AB oligonucleotide. With the p65 protein, the competition efficiencies of the AB and BB oligonucleotides were very similar (Figure 3, compare lanes 2–4 with 8–10). In sharp contrast to the p50 dimer, the AA oligonucleotide was only a very weak competitor of binding of the p65 dimer to the labeled AB motif; ~50 times more AA oligonucleotide was required to achieve the competition effect seen with the BB oligonucleotide (Figure 3, compare lanes 5–7 with 8–10). A 250-fold molar excess of an unrelated DNA fragment showed no competition (Figure 3, lane 11). These results demonstrate that p65 and p50 homodimers have quite distinct DNA-binding specificities. p65 strongly prefers binding to the BB motif (duplicated less conserved second half site) over binding to the AA motif (duplicated conserved half site) while the p50 dimer has only a slight preference for the AA motif but can also efficiently recognize the BB motif (see Urban and Baeuerle, 1990). Both p50 and p65 homodimers appear to efficiently recognize the physiological AB motif in the binding competition assay suggesting that an optimal interaction of one protein subunit with either half site A or B is sufficient for a high-affinity binding of the homodimers.

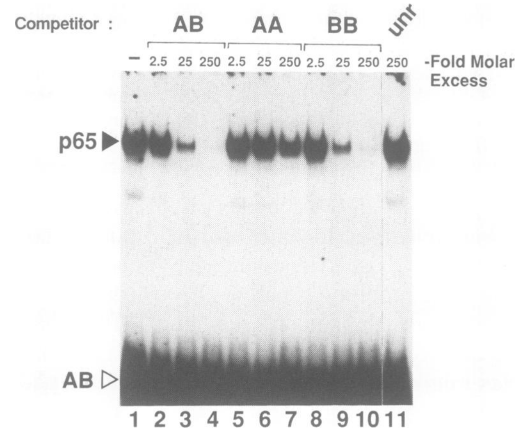

Fig. 3. Binding competition analysis with palindromic NF-κB binding motifs. A labeled κB probe (5′-GGGACTTTCC-3′; AB) was mixed with 2.5-, 25- and 250-fold molar excesses of AB, AA (5′-GGGACGTCCC-3′) and BB (5′-GGAAATTTCC-3′) oligonucleotides prior to the addition of gel-purified p65 protein (2 μl). Lane 11, competition with a 250-fold molar excess of an unrelated DNA fragment (unr; see legend to Figure 2). A fluorogram of a native gel is shown. For details of illustration see legend to Figure 1.

Direct participation of p65 in the DNA-binding of NF-κB

To sustain the idea that NF-κB binds κB motifs with the p50 subunit contacting the highly conserved half site A and the p65 subunit the less conserved half site B, we performed a UV cross-linking study. Thus far, no signal corresponding to a 65 kd polypeptide could be demonstrated in UV cross-

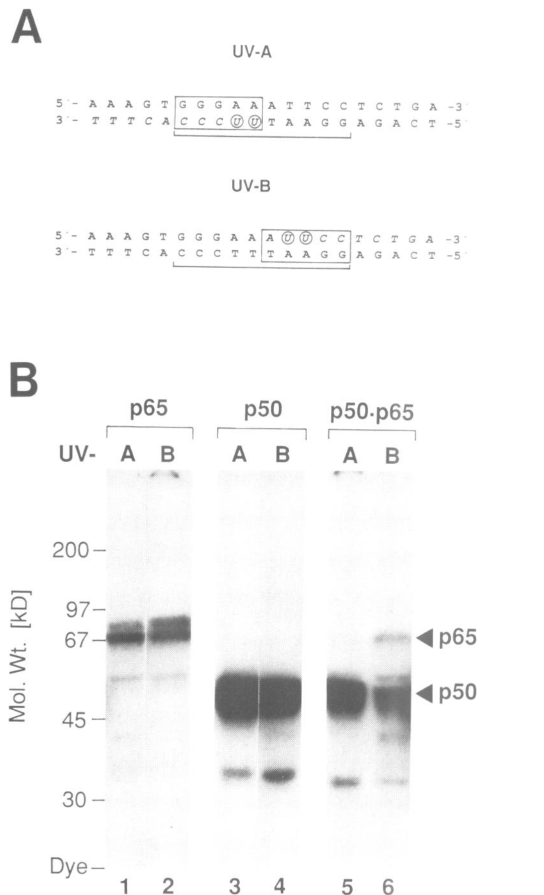

Fig. 4. Direct UV cross-linking of p65 to the NF-κB binding motif from the β-interferon promoter. (**A**) The sequence of the two oligonucleotides used for UV cross-linking. The boxes indicate the pentameric half site containing the photoreactive base BrdU (U in circles) in the UV-A and UV-B probes. The nucleotides shown in italics were filled in by the Klenow polymerase using ^{32}P-labeled dCTP and unlabeled dGTP, dATP and BrdU. The T residues in italics also correspond to BrdU residues. The bracket indicates the decameric NF-κB binding motif (Lenardo *et al.*, 1989; Visvanathan and Goodbourn, 1989). According to the definition by Zabel *et al.* (1991) the first half site is the highly conserved one because it fulfils the consensus 5'-GGGPuN-3'. (**B**) SDS–PAGE analysis of protein–DNA adducts. p65 (lanes 1 and 2; 40 μl), p50 (lanes 3 and 4; 10 μl) and native NF-κB (lanes 5 and 6; 5 μl) were incubated with UV-A and UV-B probes followed by irradiation with UV-light. After removal of overhanging bases with nucleases, samples were analyzed on 10% polyacrylamide gels and autoradiographed. Also the stacking gel is shown. The position of molecular size standards (Amersham) is shown on the left and the position of p65– and p50–DNA adducts on the right. Additional faster migrating bands seen in lane 6 might be breakdown products of p50 and p65.

linking studies using purified NF-κB (Kawakami *et al.*, 1988) or NF-κB contained in nuclear extracts (Ballard *et al.*, 1990; Collart *et al.*, 1990; Ron *et al.*, 1990). For our analysis, we have chosen the NF-κB binding motif 5'-GGGAAATTCC-3' from the β-interferon gene (Visvanathan and Goodbourn, 1989; Lenardo *et al.*, 1989) since it allows the incorporation of the same amount of the photoreactive nucleotide bromodeoxyuridine monophosphate (BrdU) into each half site. Oligonucleotides were designed that allowed us to fill in BrdU and α-^{32}P-labeled dCMP into only one half site by the Klenow polymerase reaction (Figure 4A). UV-A was photoreactive and labeled in the conserved first half site only and UV-B in the less conserved second half site.

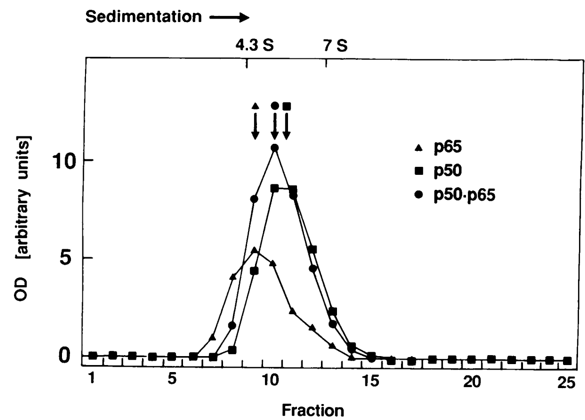

Fig. 5. The behavior of NF-κB, p50 and p65 upon sedimentation through glycerol gradients. p50 and native NF-κB were co-sedimented in one tube. p65 and a mix of the marker proteins bovine serum albumin (4.3 S), immunoglobulin G (7S) and ferritin were sedimented in separate tubes. All samples were analyzed in the same rotor run. Fractions of gradients were analyzed for the specific DNA-binding activities of p50, NF-κB and p65 by EMSA using a labeled H-2K^b probe. Optical density values obtained from a densitometric analysis of protein–DNA complexes seen in fluorograms are plotted.

The two DNA probes were reacted with purified p65, p50 and native NF-κB. After direct UV cross-linking, the DNA was digested by a treatment with *Micrococcus* nuclease and DNase I. As tested by EMSA, the incorporation of BrdU and the UV-treatment did not interfere with the formation of specific protein–DNA complexes of p65, p50 or NF-κB (data not shown). A five-fold excess of gel-purified p65 over p50 was used for the reactions. With both DNA probes, gel-purified p65 gave a prominent 68 kd and a weaker 75 kd band upon SDS–PAGE and autoradiography (Figure 4B, lanes 1 and 2). The bands were not seen in the absence of p65 or if an excess of unlabeled κ enhancer oligonucleotide was present during the cross-linking reaction (data not shown). The covalent linkage of DNA might have increased the apparent molecular size of p65 in SDS gels as observed for p50 in other studies (Kawakami *et al.*, 1988; Ballard *et al.*, 1990). p65 gave similar amounts of protein–DNA adduct with the two DNA probes. The reaction of UV-A and UV-B probes with five times less p50 gel eluate gave a much stronger signal with a molecular size between 50 and 55 kd (Figure 4B, lanes 3 and 4).

When native NF-κB was used for direct UV cross-linking, a strongly asymmetric pattern of adducts with the UV-A and UV-B probes was evident. p50 gave now a stronger signal with the UV-A than with the UV-B probe (Figure 4B, lanes 5 and 6). Quantification of the protein–DNA complexes by Cerenkov counting indicated that five times more p50 signal was cross-linked to the A half site than to the B half site. p65 showed the reciprocal behavior; a single band of 68 kd was obtained with UV-B (Figure 4B, lane 6) and only upon a very long exposure time was a signal seen with UV-A (data not shown). These results show a direct participation of p65 in the protein–DNA interaction of NF-κB. The asymmetric distribution of the p50 and p65 signals between the two half sites strongly supports a preferential binding of the two NF-κB subunits to the two half sites in the NF-κB binding motif from the β-interferon promoter. The distribution is consistent

with the idea that p50 prefers the highly conserved half site A and p65 the less conserved half site B.

The behavior of p65, p50 and NF-κB in glycerol gradients

The finding that p50–p65 elutes upon gel filtration with a Stokes' radius slightly greater than that of α-amylase (200 kd) suggested that NF-κB forms a high molecular weight complex (Baeuerle and Baltimore, 1989). Since p65 showed no detectable DNA-binding in the past and the methylation interference patterns of purified p50 and p50–p65 were identical (Baeuerle and Baltimore, 1989), it was assumed that the high molecular weight complex of NF-κB was a heterotetramer of two p50 and two p65 molecules in which the two p50 molecules contact the DNA. A finding arguing against this model was that the complex of p50 and p65 sedimented similarly as the p50 dimer through a glycerol gradient. The possibility was therefore discussed that the tetramer dissociated during sedimentation by the hydrodynamic pressure. The present data prompted us to reinvestigate this issue and to determine the S values of the dimers of p50 and p65 and that of the p50–p65 complex.

A mixture of p50 and p50–p65 was subjected to sedimentation through a continuous glycerol gradient (Figure 5). The amount of protein complexes in fractions of the gradient was thereafter determined by their DNA-binding activity in EMSAs. The p50–p65 complex (5.1 S) had a slightly lower S value than that of p50 (5.5 S) (Figure 5). This observation was reproducible and confirmed by a co-sedimentation of the two forms in one centrifugation tube. While p50 forms a homodimer of 100 kd (Baeuerle and Baltimore, 1989), p50–p65 was most likely present as a heterodimer of 115 kd. We also tested in glycerol gradients the effects of agents known to promote the stability of multiprotein complexes. No complexes of higher molecular size were observed if 1 mM spermine or 5% polyethylene glycol were present during sedimentation (data not shown).

The slightly lower S value of p50–p65 compared with p50–p50 was in contrast to its higher molecular size of 115 kd. Since the S value is also dependent on the shape of a macromolecule, the discrepancy might indicate that p50–p65 has an unusual perhaps elongated shape caused by p65, while the p50 dimer is more globular. Despite its larger size, a p65 dimer should then have an S value smaller than that of p50–p50 and p50–p65. To test this idea, purified p65 was subjected to glycerol gradient centrifugation. As expected, the DNA-binding form of p65, had a smaller S value (4.5S) than the p50 dimer and p50–p65 heterodimer (Figure 5). This finding would support the idea that a p65 dimer is not ideally globular and therefore p65 decreased the S value of the p50–p65 heterodimer. It is also possible that p65 sedimented as monomer. Future studies have to investigate whether two p50–p65 heterodimers form a labile heterotetramer in solution.

The DNA-binding of p65 is inhibited by IκB-α and -β

Two studies indicated that the inhibiting activity of IκB towards the DNA-binding of NF-κB depends on the p65 subunit (Baeuerle and Baltimore, 1989; Urban and Baeuerle, 1990). One showed that only a form of NF-κB containing p65 but not the p50 dimer is inhibited by IκB; the other

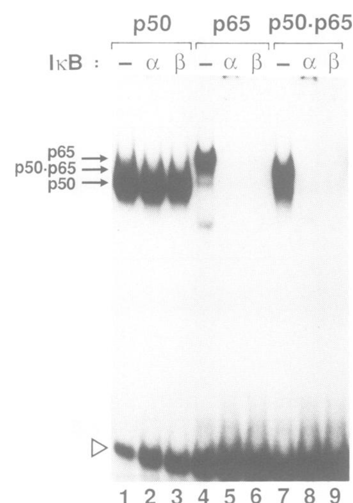

Fig. 6. Reactivity of p65 with IκB-α and -β. p50 (lanes 1–3; 1 μl), p65 (lanes 4–6; 2 μl) and native NF-κB (lanes 7–9; 0.1 μl) were reacted with buffer (lanes 1, 4 and 7), purified IκB-α (lanes 2, 5 and 8) or purified IκB-β (lanes 3, 6 and 9) followed by analysis of the DNA-binding activity in EMSAs using a labeled H-2K^b probe. A fluorogram of a native gel is shown. For details of illustration see legend to Figure 1.

provided evidence for a receptor function of p65 by the finding that isolated p65 neutralizes IκB activity. With the detection of p65 DNA binding activity in EMSA, we could now investigate whether IκB-α and -β can inhibit the DNA-binding of a p65 dimer. As shown in Figure 6 (lanes 5 and 6) purified IκB-α (Zabel and Baeuerle, 1990) as well as purified IκB-β (E.Link, L.Kerr, R.Schreck, U.Zabel, I.Verma and P.Baeuerle, submitted) could very efficiently block the DNA-binding activity of p65 in EMSAs. Also the DNA-binding of p50–p65 was inhibited (Figure 6, lanes 8 and 9) but that of the p50 dimer was not affected by the two IκB forms (lanes 1 to 3). The results suggest that only p65 can interact with IκB and that the inducibility of NF-κB depends on the presence of the p65 subunit.

Discussion

Why was the DNA-binding of p65 not detected earlier?

The only reported way to separate the p65 and p50 subunits of NF-κB is by their dissociation with the ionic detergent SDS followed by an electrophoretic size separation (Baeuerle and Baltimore, 1989). This procedure requires that the isolated subunits are subjected to a renaturation protocol after their elution from polyacrylamide gel pieces and removal of SDS by acetone precipitation. The strong DNA-binding found with gel-purified p50 is an indication that p50 elutes and renatures efficiently with this protocol. If p50 is renatured together with an equivalent amount of a gel eluate containing p65, almost all of the DNA-binding activity of p50 is found in the form of a p50–p65 heteromer (Baeuerle and Baltimore, 1989; Urban and Baeuerle, 1990). This suggests that p65 elutes as efficiently as p50 from the gel and, at least in the presence of p50, can renature to the same extent as p50. However, our present finding that increasing the amount of saturated urea in the renaturation protocol (see Materials and methods) facilitates the detection of a

p65–DNA complex in EMSA, suggests that p65 might not renature efficiently on its own. Another factor that might have handicapped the detection of p65–DNA complexes in the past is the inhibiting effect of poly(dI-dC), an agent routinely used in EMSA reactions.

The weak signals obtained after UV cross-linking of p65 to DNA are not entirely explained by an inefficient renaturation of p65. In UV cross-linking experiments using native NF-κB, the p65 signal has $<5\%$ of the intensity of the p50 signal. If in earlier studies the photoreactive and/or labeled nucleotides were primarily incorporated into the conserved half site of the NF-κB binding motifs or simply, if fluorograms were not overexposed, a p65 signal was easily missed. The weak reactivity of p65 with photo-activated BrdU might be related to the sequence degeneration of its preferred half site 5′-G(GPyPy)N-3′. The only highly conserved bases are G residues in positions 1 and 2 which however, do not allow the incorporation of BrdU on the opposite strand. Apparently, p65 has no amino acids in its DNA-binding domain which are able to react efficiently with the photo-activated BrdU.

The subunit composition of NF-κB

With the present study, our previous model is obsolete that NF-κB contacts the DNA as a p50 dimer with two non-DNA-binding p65 subunits attached. Rather, the half sites of κB motifs are contacted by a heterodimer of one p50 and one p65 subunit, a situation reminiscent of the jun/fos heterodimer (Gentz *et al.*, 1989; Turner and Tjian, 1989). As suggested by gel filtration experiments (Baeuerle and Baltimore, 1989; M.Urban and P.Baeuerle, unpublished), association of two p50–p65 heterodimers to a heterotetramer is still possible. A heterotetrameric complex is however not evident from glycerol gradient analyses where NF-κB appears to sediment as a p50–p65 heterodimer. Also the protein–DNA complex of NF-κB seen in EMSAs is rather a heterodimer than a heterotetramer as suggested by the fact that the protein–DNA complex of p65 migrates more slowly than that of NF-κB in native gels. Theoretically, the complex of p65 could contain a p65 homotetramer but the mobility differences of the three complexes containing p50, p50–p65 and p65 are typical for those of homo- and heterodimers with subunits of different size (see Hope and Struhl, 1987). The present data would be consistent with a model in which two p50–p65 heterodimers are loosely associated in solution. We are now trying to investigate the quaternery structure of NF-κB further by cross-linking and electron microscopy studies.

p65 appears to bind as a homodimer to DNA as has been demonstrated for p50 (Kieran *et al.*, 1990). Evidence for this is as follows: (i) that the protein–DNA complex of p65 migrates more slowly in native gels than that of a p50 dimer and p50–p65 heterodimer; (ii) p65 can heterodimerize with p50 presumably because it posesses the dimerization domain of rel proteins (Ruben *et al.*, 1991); (iii) p65 binds to completely palindromic κB motifs; and (iv) one p65 molecule can be cross-linked to each half site of the β-interferon κB motif. The homodimerization of both p50 and p65 provides an explanation why there is no rapid formation of NF-κB if p50 and p65 are combined after renaturation (Urban and Baeuerle, 1990); the protein–protein interactions of the p50 and p65 homodimers must first dissociate to allow heterodimerization of p50 and p65.

Heterodimerization and the DNA-binding specificity of NF-κB

Differences in the DNA-binding properties of p50 dimers and NF-κB (see Introduction) find now a simple explanation. NF-κB contacts the pentameric half sites of κB motifs by one p50 and one p65 molecule whereas the p50 dimer uses two p50 molecules. Because p50 and p65 have distinct DNA-binding properties, certain NF-κB binding motifs are recognized by p50–p65 with a different affinity than by p50–p50. For instance, p50–p65 prefers to bind to motifs in which the half sites are of different sequence while palindromic motifs with identical half sites are recognized less efficiently (Urban and Baeuerle, 1990). In contrast, the p50 dimer prefers completely palindromic motifs over those with lower symmetry. The finding that most physiologically relevant κB motifs are not perfect palindromes, but show a distinct sequence conservation in their half sites (Zabel *et al.*, 1991; Baeuerle, 1991) suggests that they are predominantly targets for the p50–p65 heterodimer (NF-κB) and not for the p50 dimer. It is at present questionable whether the p50 dimer (also called KBF1) has a physiological role in gene activation (Kieran *et al.*, 1990).

The more than two-fold higher affinity of the p50–p65 heterodimer than the p50 homodimer for the motif 5′-GGGACTTTCC-3′ (Urban and Baeuerle, 1990) could be explained by a higher affinity of p65 for the 3′ half site than p50. While the 5′ half site is occupied in the homodimer only and in the heterodimer predominantly by one p50 subunit, the 3′ half site is in one case bound by a second p50 and in the other case by a p65 subunit. NF-κB-binding motifs were shown to differ significantly in their efficiency to compete binding of the heterodimer to a labeled probe (Zabel *et al.*, 1991). Since all the tested motifs had very similar 5′ half sites, the affinity of NF-κB binding motifs seems to be primarily determined by the interaction of p65 with the less conserved 3′ half site.

The protein–DNA interaction of NF-κB as heterodimer can also explain another property of NF-κB which distinguishes it from the p50 dimer. We recently found that the p50 dimer bends the otherwise linear DNA upon its binding (Schreck *et al.*, 1990). The center of bending was localized to the middle of the κB motif 5′-GGGAC-TTTCC-3′. Compared with the p50 dimer, NF-κB induced a stronger bending angle and the center of bending was localized closer to the 3′ end of the motif. Now, it appears as if the increased bending angle and asymmetric bending by NF-κB is simply due to distinct DNA-bending properties of p50 and p65: p65 which binds preferentially to the 3′ half of the motif appears to bend its half site more strongly than p50.

In this study a novel NF-κB species, a p65 dimer, is described. Although we could not yet detect a protein–DNA complex with the mobility of a p65 dimer in nuclear extracts, it is well possible that the species exists in living cells. The titration experiment with the non-specific competitor DNA poly(dI-dC) has shown that the DNA-binding of both p50 and p65 dimers is strongly inhibited at the poly(dI-dC) concentration required to analyze nuclear extracts in EMSA. Their amounts in cells might therefore be highly underestimated.

The inactivation of the p50–p65 heterodimer by IκB

The present study strongly supports the idea that only the p65 subunit can interact with IκB. As the p50–p65

heterodimer, the DNA-binding of the p65 dimer is efficiently inactivated after addition of two variant forms of IκB, whereas the p50 dimer showed no loss of binding activity. In our previous NF-κB model we had to assume that two IκB molecules dissociate a heterotetramer into two p50−p65-IκB molecules. This mechanism provided an explanation for the loss in DNA-binding activity: the DNA-binding p50 dimer was dissociated. With the present model, the inhibition of NF-κB by IκB appears less complicated. The binding of IκB to p65, which constitutes one of the two DNA-binding subunits of NF-κB, could simply mask the DNA-binding domain of p65. A single p50 molecule is then unable to bind with high affinity to a single half site which would be consistent with the extremely weak competition efficiency of single half sites (Zabel *et al.*, 1991). There is however one observation that argues against a masking of p65 (or p50) by IκB. Even if the p50−p65 heterodimer is bound with high affinity to DNA, IκB can apparently bind p65 and release the complex from DNA (Zabel and Baeuerle, 1990). This was shown by determination of the half-lives of NF-κB−DNA complexes in the absence and presence of IκB. The rapid and non-linear decay of the protein−DNA complex in the presence of IκB showed that IκB did not simply inhibit occasionally-released NF-κB but actively removed the DNA-bound NF-κB by a higher order reaction. It seems therefore, very unlikely that IκB can mask the DNA-binding domain of DNA-bound p65. Rather, we assume that IκB allosterically inhibits the DNA-binding activity of p65. This appears to be sufficient to substantially decrease the affinity of the p50−p65-IκB complex for the cognate DNA.

Materials and methods

Oligonucleotides

Oligonucleotides were synthesized by the phosphoroamidite method on an Applied Biosystems synthesizer 380A and purified on OPC cartridges (Applied Biosystems) as described by the manufacturer. The sequences of the double-stranded oligonucleotides AA, AB (κB probe) and BB are shown elsewhere (Urban and Baeuerle, 1990). As indicated in the Figure legends, either the κB probe (AB) or an oligonucleotide encompassing the motif from the enhancer of the class I gene H-2K^b (H-2K^b probe) was used. Labeling was performed with the Klenow polymerase (Boehringer) using [α-^{32}P]dATP (Amersham, 3000 Ci/mmol) and the three other dNTPs in unlabeled form. The sequence of the H-2K^b probe was as follows:

H-2K^b: 5′- TCGAGGGCTGGGGATTCCCCATCTC-3′
3′-CCCGACCCCTAAGGGGTAGAGAGCT-5′

The two double-stranded oligonucleotides UV-A and UV-B used for UV cross-linking were labeled with the Klenow enzyme using [α-^{32}P]dCTP (Amersham, 3000 Ci/mmol), 50 μM BrdU (Sigma), 50 μM dATP and 50 μM dGTP. The sequences were as follows:

UV-A: 5′-AAAGTGGGAAATTCCTCTGA-3′
3′-TAAGGAGACT-5′

UV-B: 5′-AAAGTGGGAA-3′
3′-TTTCACCCTTTAAGGAGACT-5′

Purification of NF-κB

NF-κB was purified from cytosol of human placenta as described (Zabel *et al.*, 1991). The 350 mM KCl eluate obtained from the first DNA affinity column (Baeuerle and Baltimore, 1989) was diluted to 100 mM KCl with buffer H and subjected to a second round of DNA affinity chromatography. Bound proteins were eluted with 350 and 500 mM KCl in buffer H.

Renaturation of NF-κB subunits

The subunits of double-affinity purified NF-κB were separated by SDS−PAGE and molecular size fractions cut as shown in Figure 1A. Proteins were eluted from gel pieces and precipitated by acetone as described earlier (Baeuerle and Baltimore, 1989). To remove residual SDS, the resulting pellet was dissolved in buffer D containing 1% of the non-ionic detergent Nonidet P-40 (Sigma), precipitated again with acetone and the final pellet washed with methanol. For renaturation, the dry pellet was carefully dissolved in 5 μl saturated urea containing 2 mM dithiothreitol and the solution diluted 50-fold by the addition of renaturation buffer (Baeuerle and Baltimore, 1989). We noted that repeated freeze−thawing of the renatured p65 sample increased the DNA-binding activity such that lower amounts of p65 could be used in the assays.

Electrophoretic mobility shift assays

Binding conditions were described in detail elsewhere (Zabel *et al.*, 1991). For the binding competition assay, the ^{32}P-labeled κB probe (AB; ~0.1 ng) and various amounts (0.25, 2.5 and 25 ng in 1 μl TE) of unlabeled double-stranded competitor oligonucleotides were added to a DNA binding mix containing no poly(dI-dC). The binding reaction (20 μl) was then started by the addition of proteins. The samples obtained equal volumes of the renaturation buffer.

UV cross-linking

Gel-purified p50 and p65 subunits and native NF-κB were incubated for 20 min at room temperature with 10^5 c.p.m. (Cerenkov) of UV-A and UV-B probes, respectively, under the same binding conditions used in EMSAs. Samples were then exposed to a UV-lamp (Herolab; 302 nm) for 30 min on ice at a distance of 5 cm. Excess nucleotides were removed by incubation with 1 U *Micrococcus* nuclease and 30 U DNase I (both Boehringer) in the presence of 20 mM $CaCl_2$ for 30 min at 37°C. Samples were analyzed on a 10% SDS−polyacrylamide gel.

Glycerol gradient centrifugation

Proteins were sedimented for 40 h at 150 000 *g* and 4°C in a SW41-Ti rotor (Beckman) through a continuous 7.5−25% glycerol gradient in buffer D(+) (Baeuerle and Baltimore, 1989). The distribution of proteins was determined by their DNA binding activity using EMSAs. The amount of protein−DNA complex was determined by scanning of pre-flashed fluorograms (Biorad system). Molecular size standards were bovine serum albumin (67 kd; 4.3 S) and immunoglobulin G (158 kd; 7 S). Ferritin (440 kd) sedimented to the bottom of the tube.

Acknowledgements

We are grateful to Andrea Stumpe for help with protein purification, Egenhard Link and Ulrike Zabel for purified IκB-α and -β, Inge Leitner and Dr Georg Arnold for synthesis of oligonucleotides, Cornelia Spetl for help with the densitometric analysis and Professor E.-L.Winnacker for his continuous support. This study was supported by grants from the BMFT and DFG (Ba 957/1−2) and is part of the doctoral thesis of M.B.U and R.S.

References

Baeuerle,P.A. (1991) *Biochim. Biophys. Acta*, in press.

Baeuerle,P.A. and Baltimore,D. (1988) *Science*, **242**, 540−546.

Baeuerle,P.A. and Baltimore,D. (1989) *Genes Dev.*, **3**, 1689−1698.

Baeuerle,P.A. and Baltimore,D. (1991) In Cohen,P. and Foulkes,J.G. (eds) *Molecular Aspects of Cellular Regulation: Hormonal Control Regulation of Gene Transcription*. Elsevier/North Holland Biomedical Press, Amsterdam. Vol. 6. pp. 409−432.

Ballard,D.W., Walker,W.H., Doerre,S., Sista,P., Molitor,J.A., Dixon,E.P., Peffer,M.J., Hannink,M. and Greene,W.C. (1990) *Cell*, **63**, 803−814.

Bours,V., Villalobos,J., Burd,P.R., Kelly,K., Siebenlist,U. (1990) *Nature*, **348**, 76−80.

Collart,M.A., Baeuerle,P.A. and Vassalli,P. (1990) *Mol. Cell. Biol.*, **10**, 1498−1506.

Gentz,R., Rauscher III,F.J., Abate,C. and Curran,T. (1989) *Science*, **243**, 1695−1699.

Ghosh, S. and Baltimore,D. (1990) *Nature*, **344**, 678−682.

Ghosh,S., Gifford,A.M., Riviere,L.R., Tempst,P., Nolan,G.P. and Baltimore,D. (1990) *Cell*, **62**, 1019−1029.

Hope,I.A. and Struhl,K. (1986) *EMBO J.*, **6**, 2781−2784.

Kawakami,K., Scheidereit,C. and Roeder,R.G. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 4700−4704.

Kieran,M., Blank,V., Logeat,F., Vandekerckhove,J., Lottspeich,F., LeBail,O., Urban,M.B., Kourilsky,P., Baeuerle,P.A. and Israel,A. (1990) *Cell*, **62**, 1007−1018.

Lenardo,M.J., Fan,C.-M., Maniatis,T. and Baltimore,D. (1989) *Cell*, **57**, 287−294.

Ron,D., Brasier,A.R., Wright,K.A., Tate,J.E. and Habener,J.E. (1990) *Mol. Cell. Biol.*, **10**, 1023−1032.

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.
Schreck,R., Zorbas,H., Winnacker,E.-L. and Baeuerle,P.A. (1990) *Nucleic Acids Res.*, **18**, 6497–6502.
Turner,R. and Tjian,R. (1989) *Science*, **243**, 1689–1694.
Urban,M.B. and Baeuerle,P.A. (1990) *Genes Dev.*, **4**, 1975–1984.
Urban,M.B. and Baeuerle,P.A. (1991) *The New Biologist*, in press.
Visvanathan,K.V. and Goodbourn,S. (1989) *EMBO J.*, **8**, 1129–1138.
Zabel,U. and Baeuerle,P.A. (1990) *Cell*, **61**, 255–265.
Zabel,U. Schreck,R. and Baeuerle,P.A. (1991) *J. Biol. Chem*, **266**, 252–260.

Received on February 21, 1991; revised on March 18, 1991