

Structural and functional characterization of the promoter regions of the NFKB2 gene

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Received December 20, 1994; Revised April 12 and Accepted May 2, 1995

EMBL accession no. X83768

ABSTRACT

In order to clarify the transcriptional regulation of the NFKB2 gene (*I κ B-10*, NF- κ Bp100), we have characterized the structure and function of its promoter regions. Based on the nucleotide sequence of cDNA clones and the 5' flanking genomic region of the NFKB2 gene, RT-PCR analysis in a number of human cell lines demonstrated the presence of two alternative non-coding first exons (1a and 1b). Two distinct promoter regions, P1 and P2, were identified upstream of each exon, containing multiple sites of transcription initiation, as shown by RNase protection analysis. Sequence analysis of these regions showed a CAAT box upstream of exon 1a and high G-C content regions within both P1 and P2. Consensus binding sites for transcription factors, including SP1, API and putative NF- κ B (κ B sites), were found upstream of each exon. In particular, six κ B sites were identified, all but one of them capable of binding NF- κ B complexes *in vitro*. Transfection in HeLa cells of plasmids containing P1 and P2 sequences linked to a chloramphenicol acetyltransferase reporter gene indicated that both P1 and P2 can act independently as promoters. Co-transfection of NF- κ B effector plasmids (NF- κ Bp52 and RelA) with a reporter gene linked to P1 and P2 showed that the NFKB2 promoter regions are regulated by NF- κ B factors. RelA transactivates the NFKB2 promoter in a dose-dependent manner, whereas NF- κ Bp52 acts as a repressor, indicating that the NFKB2 gene may be under the control of a negative feedback regulatory circuit.

INTRODUCTION

The transcription factor NF- κ B is a pleiotropic protein complex which exerts its activity via the *cis*-acting κ B DNA binding motif present in a wide variety of cellular genes, including those involved in the immune response and acute phase reactivity, as well as viral genes such as those of human immunodeficiency

virus and other viruses (1–3). A number of structurally and functionally heterogeneous NF- κ B complexes have been identified. It is now evident that NF- κ B activity is strictly dependent upon protein–protein interactions among various NF- κ B factors and among these and inhibitory molecules (I κ Bs) which control the subcellular (nuclear/cytoplasmic) localization of the active complexes. In response to extracellular stimuli, including cytokines, mitogens and viruses, the NF- κ B complexes are released from I κ Bs and translocated to the nucleus (4–6). Among the NF- κ B transcription factors, the high structural homology between the NFKB1 (7–9) and NFKB2 proteins (10–13) allowed the identification of a subfamily of NF- κ B factors. Both proteins are synthesized as precursor proteins (p105 and p100 respectively) containing: (i) an N-terminal DNA binding *rel* domain homologous to the one present in RelA (p65) (14,15), *c-rel* (16–18), RelB (19,20), *v-rel* (21,22) and the product of the *Drosophila dorsalis* morphogen gene (23); (ii) a stretch of glycine residues proposed as the site of proteolytic cleavage in NFKB1 protein; (iii) a C-terminal domain containing *ankyrin* repeats (24), which has also been found in the inhibitory proteins I κ B α /MAD-3 (25), I κ B β /pp40 (26), I κ B γ (27,28), bcl-3 (29,30) and ECI-6 (31). Regarding the function of the NFKB2 protein, recent data indicate that the active form, NFKB2p52, derives from post-translational processing of p100, is localized in the nucleus and binds DNA with an affinity for κ B sequences which is different from that of NFKB1 (32,33). NFKB2p52 is capable of regulating κ B-dependent transcription activity when present in heterodimers with RelA or when complexed with bcl-3, while it acts as a negative transcriptional regulator in the form of a p52/p52 homodimer (12,33,34).

Transcription regulation of NF- κ B genes, including NFKB2, NFKB1 and I κ B α , may also be an important mechanism in regulating NF- κ B activity. Furthermore, a number of observations indicate that NF- κ B activity is involved in the self-regulation of various NF- κ B coding genes, suggesting the presence of inducible autoregulatory pathways within the NF- κ B system (35–40). RelA, NFKB1 and the heterodimer RelA/NFKB1 transactivate the NFKB1 promoter, indicating that NFKB1 also participates in its own regulation (35). Negative autoregulation of *c-rel* and modulation of *c-rel* by *v-rel* has been shown in chicken

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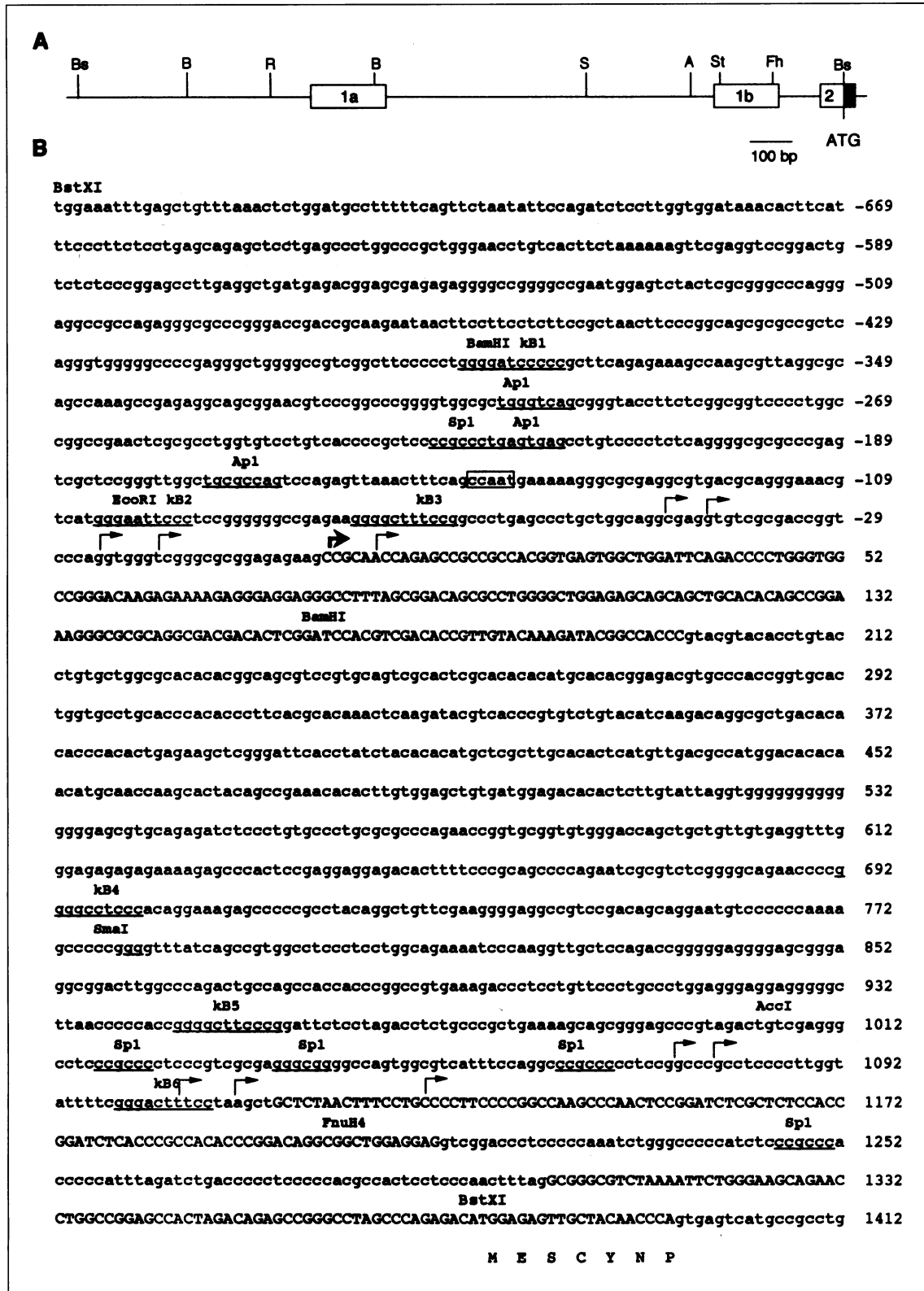


Figure 1. Structure of the 5' flanking region of the NFKB2 gene. (A) Partial restriction map of the genomic *BstXI*-*BstXI* clone including the untranslated exons 1a and 1b. (B) Nucleotide sequence of the 5' flanking region of the NFKB2 gene. The italic letters correspond to the transcribed sequences; the arrows indicate the positions of the transcription initiation sites according to the results shown in Figure 3, and '0' indicates the major transcription initiation site upstream of exon 1a. The start of exon 1b corresponds to the first nucleotide of our longest cDNA NFKB2 clone (A. Neri, unpublished results) The CAAT box is boxed and the putative binding sites for transcription factors are underlined. Restriction enzymes sites: Bs, *BstXI*; B, *BamHI*; R, *EcoRI*; S, *SmaI*; A, *AccI*; F, *FnuH4*.

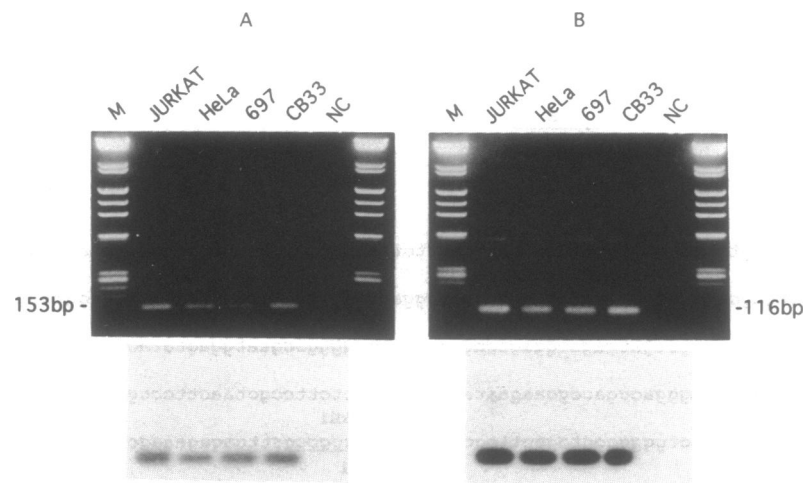


Figure 2. Alternative transcription of exons 1a and 1b of the NFKB2 gene. RT-PCR analysis of RNA from different cell lines stimulated with PMA (50 ng/ml). (A) Primers specific for exons 1a and 2; (B) primers specific for exons 1b and 2. M, Markers; NC, negative control. The lower parts of the panels show Southern blot analysis using probes specific for exons 1a and 1b. The larger and faint PCR-amplified product detectable in (B) could represent non-specific PCR product.

cells (36,37), while the murine *c-rel* promoter is transactivated by the *c-rel* protein in B cells (38). The genes encoding for the inhibitory proteins are also regulated by NF- κ B activity, as shown by the presence of a κ B motif within the promoter region of the I κ B α gene, which is specifically controlled by RelA (39,40). These observations indicate that characterization of the regulatory/promoter region of other NF- κ B genes, particularly of those that are inducible, could improve our understanding of the overall regulatory network of the NF- κ B system. The present paper reports a structural and functional analysis of the NFKB2 gene promoter region and shows that RelA enhances the activity of the NFKB2 promoters, while NFKB2p52 is capable of negatively regulating its own promoter.

MATERIALS AND METHODS

Cell lines

The lymphoblastoid cell line CB33, the pre-B cell line 697 and the Jurkat T cell line were maintained in RPMI 1640 containing glutamine, antibiotics and 10% heat-inactivated fetal calf serum (FCS). The HeLa cell line was grown in Dulbecco's modified Eagle's medium supplemented with 10% FCS. In some experiments the cells were treated for 12 h with 50 ng/ml phorbol myristate acetate (PMA) (Sigma, St Louis, MO).

Genomic cloning

Genomic clones representative of the NFKB2 locus were isolated from a genomic library constructed by cloning partially *Sau*3A-digested human placental DNA into the EMBL3 phage vector (Stratagene, La Jolla, CA). Screening was performed by plaque hybridization using the full-length NFKB2 cDNA clone as probe (10), 32 P-labelled by the random priming method (41). Isolated plaques were grown and phage DNA obtained according to established procedures (42). Inserts were analysed by restriction enzyme mapping and then subcloned into plasmid vector pGEM3 (Promega, Madison, WI) for further analysis.

DNA sequencing

DNA sequencing was performed on restriction fragments cloned into the pGEM3 plasmid by dideoxy chain termination analysis using the Sequenase sequencing kit (USB, Cleveland, OH).

cDNA amplification

First-strand cDNA was synthesized in 20 μ l reactions containing 1 μ g total RNA extracted from various human cell lines, 1 mM each dNTP, 0.1 mM DTT, 5 \times reverse-transcription buffer, 1 U RNasin (Promega), 200 U Super-Script reverse transcriptase (Gibco-BRL, Gaithersburg, MD) and 20 pM primer (5'-TAG-CAACTCTCCATGTCTCT-3') specific for the second exon of the NFKB2 gene. The reaction mixes were incubated for 1 h at 37°C and stored at -20°C. PCR amplifications were performed by diluting 10 μ l of first-strand cDNA from each cell line into 50 μ l mixtures containing 200 mM each dNTP, 50 mM KCl, 10 mM Tris, pH 8.3, 1.5 mM MgCl₂, 0.01% (w/v) gelatin, 2.5 U *Taq* polymerase (Boehringer Mannheim, Germany) and 20 pM each specific primer. As 3' primer an internal primer specific for the second exon was used (5'-GCTCTGTCTAGTGGCTCC-3'). The 5' primers were the following: 5'-AGAGCAGCAGCTGCACACAG-3' (specific for exon 1a); 5'-AACTCCGGATCTCGCTCTC-3' (specific for exon 1b). Amplification reactions (30 cycles) were performed using a Perkin-Elmer-Cetus (Norwalk, CT) DNA thermal cycler under the following conditions: denaturation at 94°C for 15 s, annealing at 65°C for 30 s and extension at 72°C for 30 s. The amplified fragments (153 bp for exons 1a/2, 116 bp for exons 1b/2) were resolved in agarose gel and further analyzed by Southern blot using specific internal probes.

RNA extraction and RNase protection analysis

RNA was extracted using the guanidine isothiocyanate method. The RNase protection assay was performed as previously described (43). The *Eco*RI-*Pvu*II (217 bp) and the *Acc*I-*Bgl*III (256 bp) fragments, respectively containing parts of exons 1a and 1b and their 5' regions, were cut from the genomic clone

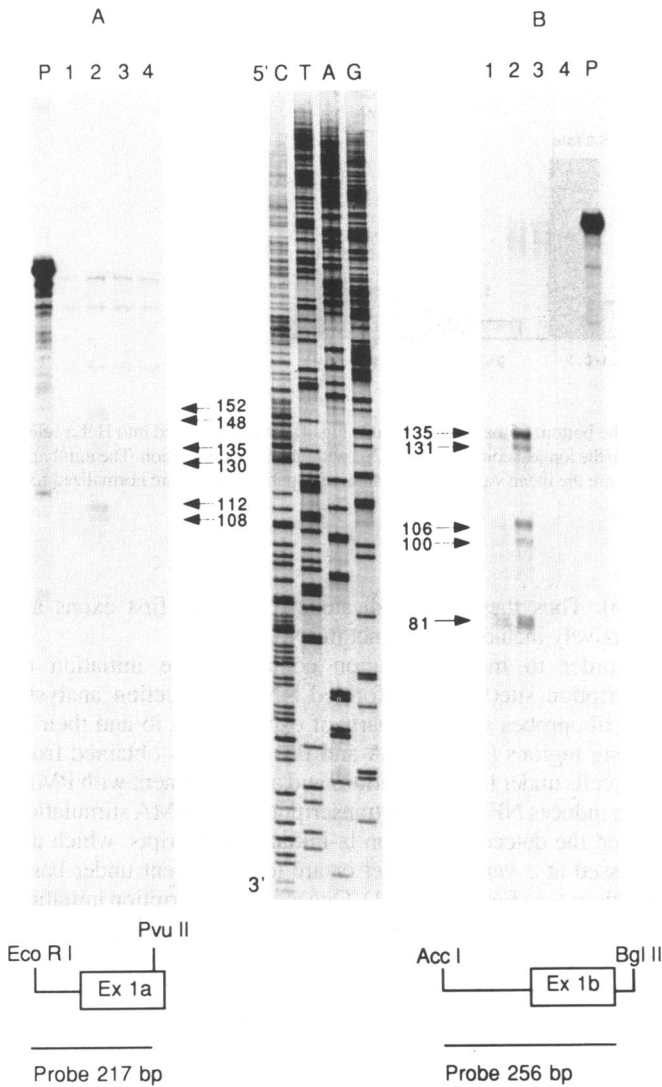


Figure 3. Determination of the transcription initiation sites of the NFKB2 gene by RNase protection analysis. Total RNA (30 µg) from HeLa cells unstimulated (lane 1) and stimulated with 50 ng/ml PMA (lane 2) was analysed using riboprobes specific for exon 1a (A) and exon 1b (B). The riboprobes are schematically described at the bottom of the figure. Mouse RNA (lane 3) and tRNA (lane 4) were used as negative controls. The size of the protected fragments was determined from the sequencing ladder in the middle of the figure, which represents the sequence of the *Bst*XI 2.1 kb fragment using as sequencing primer a reverse primer specific for the 3' border of exon 1b (Fig. 1, positions 1192–1211). P, probes.

*Bst*XI–*Bst*XI fragment of the NFKB2 gene (see below), cloned into pGEM3 vector (Promega) and transcribed using SP6 RNA polymerase. The antisense ³²P-labelled riboprobes were hybridized with 30 µg total RNA for 16 h at 65°C, followed by RNase digestion. The protected fragments were resolved in 6% denaturing polyacrylamide gels and analysed by autoradiography.

Expression vectors and plasmid construction

The entire 5' region of the NFKB2 gene containing exons 1a and 1b and part of the second exon (a *Bst*XI–*Bst*XI fragment of 2113 bp, shown in Figure 1, originally cloned in the *Hinc*II site of

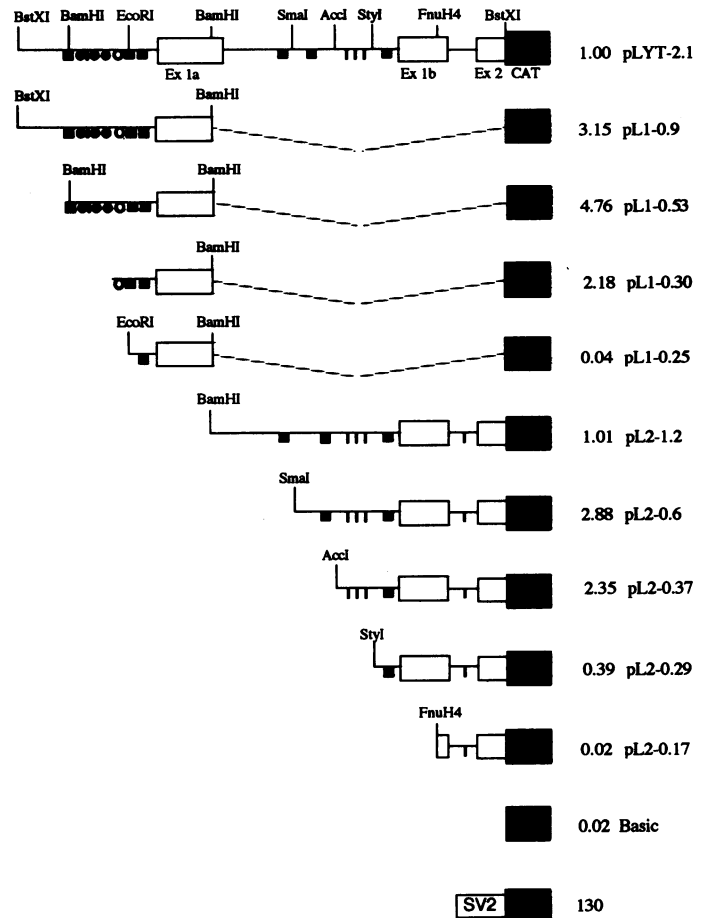


Figure 4. Functional analysis of the NFKB2 promoters. A series of deleted plasmids was prepared from the pLyT-2.1 construct using the indicated restriction enzyme sites and tested for their CAT activity in HeLa cells (0.5 pmol). The putative NF-κB binding sites are indicated by filled squares, the CAAT consensus sequence by an open circle, the AP1-like sites by filled circles and the SPI box by small bars. The relative CAT activity and the nomenclature of the plasmids are indicated on the right of the figure. The results are representative of at least three experiments and are normalized for transfection efficiency.

plasmid pGEM3) was inserted in the *Hind*III and *Xba*I sites of the pCAT-basic plasmid (Promega). Several deletions in the cloned fragment generated a series of plasmids whose restriction mapping is presented in Figure 4 in the context of the analysis of reporter constructs. The plasmid pL1-0.3 was constructed by cloning a PCR-amplified product of 318 bp from –159 to +159 into the same expression vector.

The full-length cDNA of RelA (obtained from C. A. Rosen) was inserted in the pCEP4 expression vector (Invitrogen, San Diego, CA) at the *Pvu*II site. The same expression vector was used for cloning the *Eco*RI–*Xho*I fragment of NFKB2 cDNA, containing the *rel* domain and lacking the *ankyrin* domain (10).

Transfection and chloramphenicol acetyltransferase (CAT) assay

HeLa cells were plated at 3 × 10⁵/100 mm Petri dish 24 h prior to the experiment. The plasmids were transfected at the doses indicated in the legends to the figures using a modified CaPO₄ precipitation procedure (42). Approximately 48 h after transfection

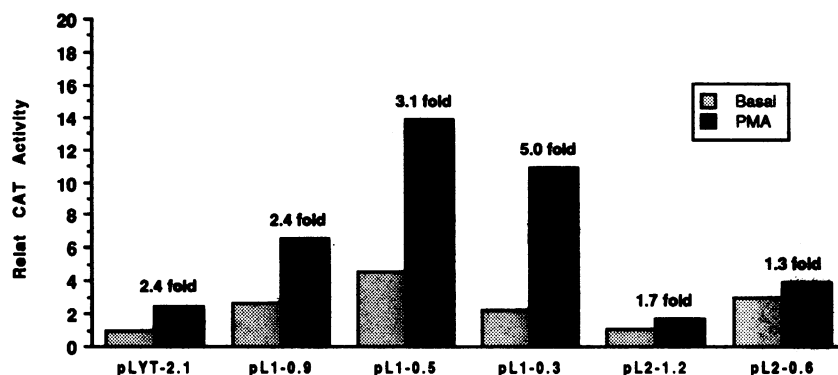


Figure 5. PMA stimulation of the NFkB2 promoters. The plasmids (0.5 pmol) indicated at the bottom of the figure (see also Fig. 4) were transfected into HeLa cells with or without PMA stimulation (50 ng/ml for 12 h). The relative CAT activities are referred to the longest construct, pLyt-2.1, without PMA stimulation. The numbers above the bars indicate fold transactivation versus the basal value of the plasmid. The results are the mean values of at least three experiments and are normalized for transfection efficiency.

the cells were harvested and lysed by several rounds of freeze-thaw in 25 mM Tris, pH 7.5. The transfections in HeLa cells also included 0.5 pmol CMV- β -gal plasmid, which was used as an internal control to normalize transfection efficiency. When NF- κ B2p52 and p65 expressing vectors were co-transfected with NFkB2-CAT plasmids the values of acetylation were referred to the protein content, because of the interference of RelA protein with the promoter driving the β -gal gene. CAT and β -gal activities were determined as described by Gorman *et al.* (44) using 20–50 μ l cell extracts. CAT activity was expressed as a percentage of the acetylated form of chloramphenicol as compared with the total number of counts in the acetylated and non-acetylated forms.

Electrophoretic mobility shift assays (EMSA)

The preparation of nuclear extracts has been previously described (45), as well as the EMSA analysis (46). The six κ B oligonucleotide probes include each κ B site and the surrounding 5 nt at the 5' and 3' borders. For antibody-induced supershift assays 0.5 μ l undiluted antiserum was incubated with 2–5 μ g nuclear extract on ice for 30 min, followed by 20 min incubation with labelled probes.

RESULTS

Characterization of the NFkB2 promoter regions

In our previous report (47) we described the cloning and intron/exon organization of the NFkB2 gene. One of the clones isolated from a genomic library constructed from human placental DNA (the *Bst*XI–*Bst*XI subclone) was shown to contain 2113 bp of DNA (Fig. 1A) including two alternative first exons (1a and 1b) identified in distinct cDNA clones (10,12). To confirm the alternative transcription of exons 1a and 1b, RT-PCR analysis was performed in different cell lines. Figure 2 shows that two distinct PCR-amplified fragments can be obtained using a combination of primers specific for exon 2 and exon 1a or 1b. These fragments represented the linkage between exons 1a/2 (Fig. 2A) and exons 1b/2 (Fig. 2B), as demonstrated by their length, hybridization to specific probes and direct DNA sequencing (data not shown). Moreover, cDNA amplification using exon 1a/1b-specific primers did not generate fragments containing these sequences (data not

shown). Thus these data indicate that the two first exons are alternatively included in distinct mRNA species.

In order to map the region containing the initiation of transcription site(s) we performed RNase protection analysis, using riboprobes spanning parts of exons 1a and 1b and their 5' flanking regions (see Fig. 3A and B). RNA was obtained from HeLa cells under basal conditions and after treatment with PMA, which induces NFkB2 gene transcription (12). PMA stimulation allowed the detection of exon 1a-initiated transcripts, which are expressed at a very low level or are totally absent under basal conditions (see Fig. 3A, lane 1). One major transcription initiation site was identified in the 5' region flanking exon 1a (Fig. 1A) and this was arbitrarily designated site 0 in relation to the rest of the sequence. Five other minor start sites were localized at approximate positions –45, –41, –23, –16 and +5. Multiple start sites were also responsible for transcription through exon 1b; within this region two major start points were localized at positions +1074 and +1079 and minor start points were identified at positions +1105, +1111 and +1130. The sequence analysis of the *Bst*XI–*Bst*XI clone (Fig. 1B) showed a CAAT box located 144 bp upstream of exon 1a and CpG islands upstream of both exons (75% G+C-rich within 100 bp). A number of potential binding sites for transcription factors were identified in this region (Fig. 1B). Five SP1 consensus sequences were found, one 229 bp upstream of exon 1a and four interspersed among the transcription start sites upstream of exon 1b. Furthermore, the 5' region flanking exon 1a also contains three AP1-like elements. Interestingly, six NF- κ B consensus sequences were identified (κ B 1–6): κ B3 has been found in TNF α and vimentin promoters (48–50); κ B5 is present in the TNF β and also the NFkB1 promoter (35,51–52); κ B6 is common to various viruses, such as HIV, SV40, CMV and adenovirus (53–56). The remaining κ B sites (1, 2 and 4) are slightly different from the κ B motifs previously described in the regulatory region of other genes.

Functional analysis of the NFkB2 promoter regions

In order to demonstrate that the 5' region of the NFkB2 gene contains a functional promoter a series of chimeric plasmids containing various segments of this region linked upstream of the CAT reporter gene was prepared and transfected into HeLa cells

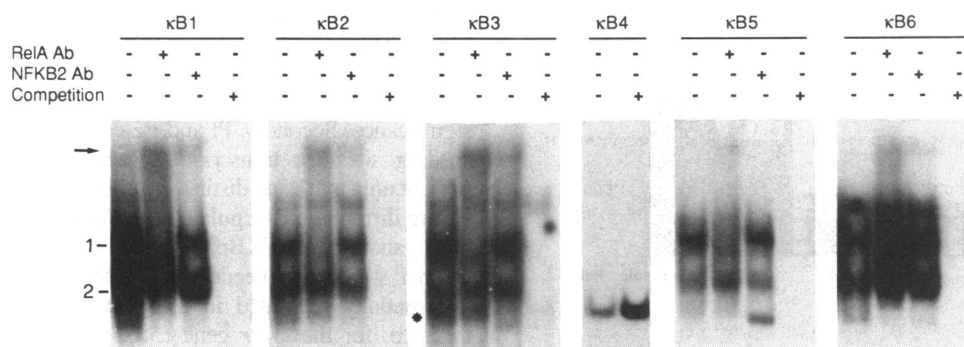


Figure 6. Characterization of the functional activity of the κ B sites of the NFKB2 gene promoter regions. The six putative κ B sites were used as probes in EMSA with HeLa cell nuclear extracts. The arrow indicates the supershifted bands; 1 and 2 indicate the two major NF- κ B complexes; the asterisk indicates a possible homodimeric form of p52.

(Fig. 4). The longest construct (pLYT-2.1, a *Bst*XI–*Bst*XI fragment containing exons 1a, 1b and the untranslated part of exon 2) displayed a promoter activity which is ~35-fold higher than the parental CAT vector (pCAT basic). Since the first two exons are alternatively transcribed, we analysed the 5' regions spanning exon 1a and 1b separately. The activity of pL1-0.9 was 3-fold higher than that of the pLYT-2.1 construct, whereas the activity of pL2-1.2 was similar to pLYT-2.1. This indicates that two distinct regions (P1 and P2) can act independently as promoters with comparable strength. To further characterize the active promoter regions a series of 5' deletions was performed in the constructs pL1-0.9 and pL2-1.2. As shown in Figure 4, the sequences from –159 to –101 are essential for transcriptional activity of P1, as shown by the dramatic reduction (>50-fold) observed when this fragment is deleted (see pL1-0.3 versus pL1-0.25). The smallest region sufficient for P2 promoter activity (20-fold above basal level) can be identified within the *StyI*–*Fnu*H4 fragment (pL2-0.29). The evidence that constructs pL2-0.37, pL2-0.6 and pL2-1.2 have higher activity than pL2-0.29 indicates that these regions may contain positive regulatory sequences in addition to promoter sequences. The same sequences did not display any activity when used in inverted orientation. PMA treatment has a slight effect in inducing P1 and P2 CAT activity, even if P1 seems to be the preferential target for such an induction (Fig. 5). The pL1-0.3 construct, which contains two κ B sites but no AP1-like sequences, is still induced by PMA treatment, suggesting that the κ B sites are responsible for PMA induction.

Regulation of NFKB2 promoters by RelA and NFKB2 proteins

The presence of NF- κ B consensus sequences in the promoter region of the NFKB2 gene prompted us to analyse both the capability of the various κ B sites to bind NF- κ B complexes *in vivo* and the effect of overexpression of RelA and/or NFKB2 proteins on the activity of the promoters. Figure 6 shows the EMSAs performed with nuclear extracts from PMA-stimulated HeLa cells and double-stranded nucleotides encompassing the sequences of all six κ B sites. NF- κ B complexes were detectable when κ B1-3, 5 and 6 were used as probes, indicating that the non-canonical κ B1 and κ B5 are also functionally active in binding NF- κ B complexes. Using specific antibodies we observed that all of the active κ B sites are capable of binding

complexes containing RelA; in addition, κ B1-3 and 6 are also able to bind complexes containing NFKB2p52. Similar results were obtained with Jurkat T cells (data not shown).

To investigate the effect of RelA and NFKB2p52 on the NFKB2 promoter, transient co-transfection experiments were carried out in HeLa cells using 0.5 pmol pLYT-2.1 CAT construct (see Fig. 4) as a target for the RelA and/or NFKB2p52 products expressed under control of the CMV promoter. As demonstrated by Western blot analysis (data not shown), the levels of RelA expression in these experiments and in those represented in Figure 8 were similar when transfected alone or in combination with NFKB2p52. As shown in Figure 7A, RelA is capable of transactivating the promoter of the NFKB2 gene in a dose-dependent manner, indicating that RelA may be involved in the positive regulation of NFKB2 expression. Using the same experimental approach we analysed the activity of NFKB2 on its own promoter (Fig. 7B). A progressive dose-dependent repression was achieved, reaching 70% at high (2 pmol) inputs of effector. NFKB2 was also capable of strongly inhibiting the transactivation activity of RelA on the NFKB2 promoter (Fig. 7C). These data indicate that NFKB2 p52 can negatively control the activity of its own promoter.

We analysed further the activity of the RelA and NFKB2p52 proteins on P1 and P2 separately (Fig. 8). pL1-0.9, which contains P1 and its 5' flanking region, is transactivated by RelA (Fig. 8B) at a level comparable with the value observed in the construct pLYT-2.1, representative of the entire promoter region. Conversely, NFKB2p52 does not significantly reduce either the activity of the P1 promoter (Fig. 8A) or the activity of RelA on P1 (Fig. 8B). We found similar results when the upstream κ B site (pL1-0.53) and the AP1 and SP1 sites (pL1-0.3) were deleted. The P2 promoter region (pL2-1.2) is transactivated by RelA at a similar level to P1, but is negatively regulated by NFKB2 (~3-fold reduction with respect to the basal value). Deletion of the 600 bp upstream sequence (pL2-0.6) slightly reduces the activity of RelA, which is even more reduced in pL2-0.37 (only ~2-fold induction) when κ B5, which is able to bind RelA-containing complexes, is deleted.

DISCUSSION

This study reports the identification and initial characterization of the NFKB2 promoter regions and provides evidence for the

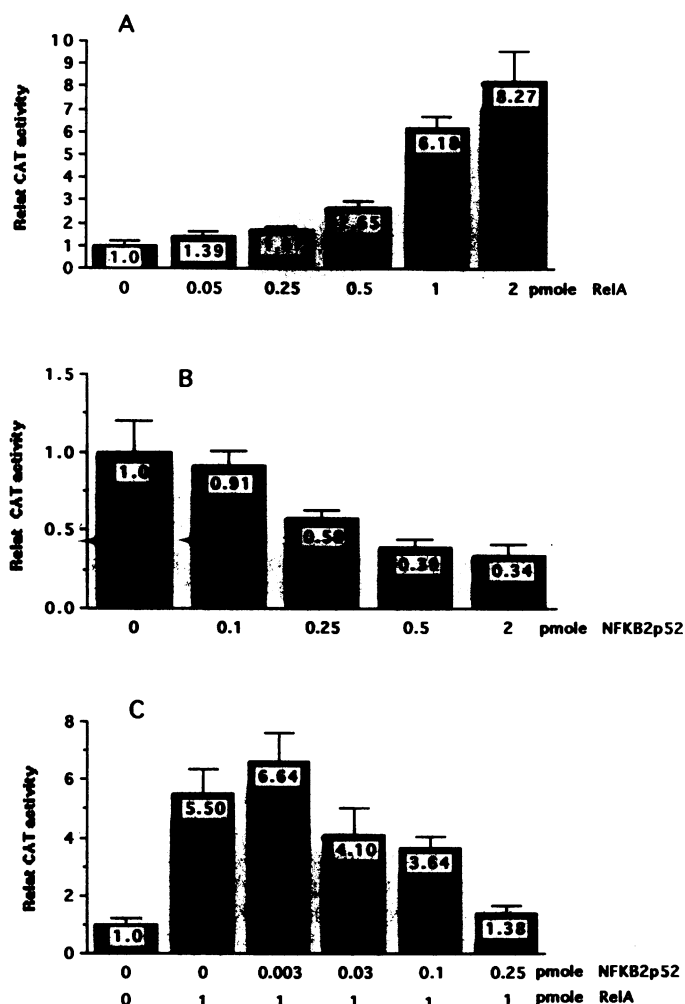


Figure 7. Activity of the RelA and NFKB2 products on the NFKB2 promoters. HeLa cells were co-transfected with 0.5 pmol NFKB2 promoter CAT plasmid pLyt-2.1 plus (A) increasing amounts of pCMV-RelA, (B) increasing amounts of pCMV-NFKB2p52 or (C) 1 pmol RelA and increasing amounts of pCMV-NFKB2p52. The values of relative CAT activity are referred to the activity of the NFKB2 promoter in the absence of exogenous factors. Each column represents the mean of three or more independent experiments after normalization to the protein concentration of cellular extracts.

involvement of the NFKB2 protein in its own regulation. These results have implications for the mechanisms regulating NFKB2 expression and, in general, for the mechanism regulating overall NF- κ B activity.

In a previous paper (47) we showed that two non-coding exons are present in the 5' region of the NFKB2 locus. The results herein indicate that exons 1a and 1b are alternatively transcribed, generating two species of mRNA transcripts of similar length that, although differing in their 5' sequences, maintain the same coding sequences. Furthermore, our results indicate that transcription of these two exons is under the control of two distinct promoters, which can be differentially regulated, based on several observations. First, sequence analysis revealed that although both promoter regions have typical features of promoters containing multiple transcription initiation sites surrounded by GC-rich regions (75%), they differ in the fact that P1 has a CAAT box and no TATA box.

Although the influence of CAAT sequences on promoter activity has not been clearly defined as yet, a similar arrangement has been found in other human genes, such as the genes encoding interferon regulatory factors 1 and 2 (56), and may have regulatory significance. Secondly, P1 and P2 contain distinct sets of putative binding sites for transcription factors: AP1-like binding sites present only in P1 and distinct κ B sites in P1 and P2. Finally, P1 and P2 are differentially regulated by PMA, which acts preferentially on P1, and by the NFKBp52 protein, which negatively controls P2. Further studies are needed to determine whether P1 and P2 are differentially regulated in different tissues, as is the case, for instance, for the *c-fgr* gene (58), or in different stages of cell differentiation, as has been shown for the *p53* gene (59).

These features of the NFKB2 promoter regions distinguish the NFKB2 gene from the NF- κ B and I κ B genes whose promoter regions have been characterized so far, namely NFKB1 (35), murine and avian *c-rel* (36,38), RelA (60), I κ B α (40) and ECI-6 (31). Conversely, the promoter regions of the NFKB2 gene are similar to those of other NF- κ B and I κ B genes (31,35,36,38,40), in that they contain several functional κ B sites, supporting the notion that the NF- κ B transcription factor represents a highly autoregulated system. In particular, our results show that the NFKB2 gene can be positively regulated by RelA, as well as negatively by itself. These findings are consistent with recent studies (33) demonstrating that NFKB2p52 has no intrinsic transactivation capability and that its homodimeric form can repress the transactivating activity of RelA. It has been shown that the diversity of the sequences of the κ B sites reflects a preferential affinity for different NF- κ B subunits (3). We observed that the P2 promoter is the target for NFKB2p52 negative autoregulation and the κ B6 site is probably responsible for this regulatory mechanism. Further studies should aim to understand the activity of other members of the NF- κ B family on the NFKB2 promoter and to verify whether the same pattern of response is present in different cells or under different stimuli. In fact, a recent paper (61) reporting the characterization of the NFKB2 promoter showed that a positive autoregulation is exerted by the NFKB2 protein on P1 in cells stimulated with PMA, while in the absence of NF- κ B activity a mechanism of repression is mediated by κ B sites through a repressor protein termed Rep- κ B.

Finally, it is possible that the NF- κ B autoregulatory mechanism may be critical to maintain a physiological level of NF- κ B function in the presence of a multitude of activating stimuli and that disruption of this mechanism may be involved in tumorigenesis. In fact, it is known that the NFKB2 locus is involved in lymphoma-associated chromosomal rearrangements (10,62,63), which lead to truncations within the *ankyrin* domain and the consequent production of abnormal, constitutively active *rel*-containing proteins.

ACKNOWLEDGEMENTS

We are grateful to Dr C. A. Rosen for providing us with the pMT2T/RelA expression vector and to Dr R. Dalla-Favera for his critical reading of the manuscript. This work was supported by a grant from the Associazione Italiana Ricerca sul Cancro (AIRC) to A.N. and by a grant 'Progetto Finalizzato 1993' from the Italian Ministry of Health to Ospedale Maggiore IRCCS. D.T. is a recipient of an AIRC fellowship.

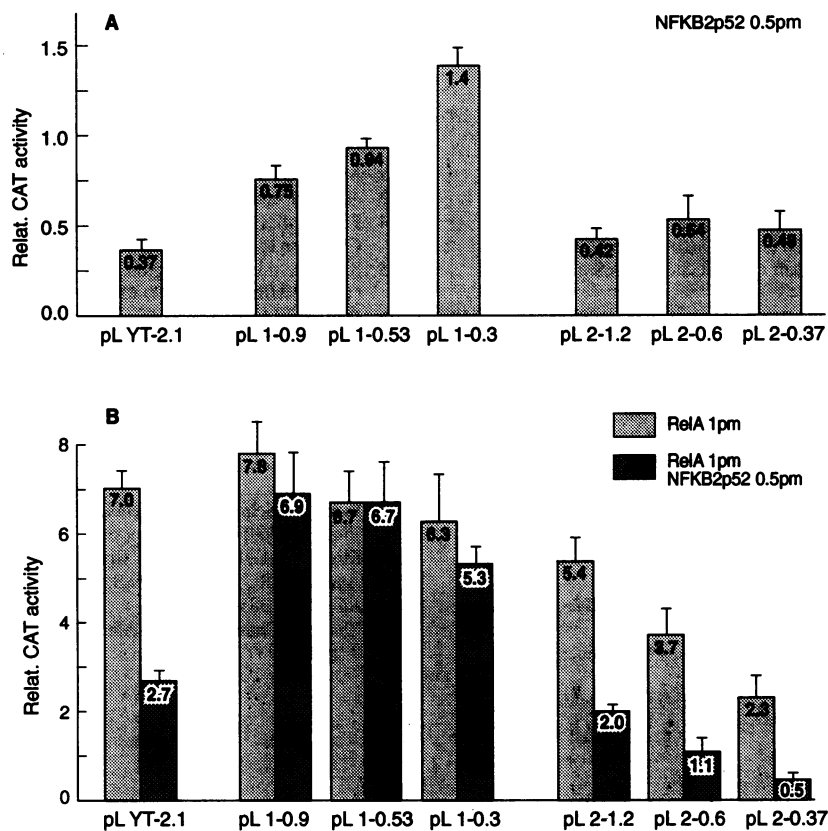


Figure 8. Activity of the RelA and NFKB2 products on the P1 and P2 promoters. pL1-0.9 and pL2-1.2 and their respective 5' deletion constructs (see Fig. 4) were transfected (0.5 pmol) into HeLa cells with NFKB2p52 (A), RelA or a combination of both (B). The relative CAT activity is referred to the value expressed by each construct in the absence of exogenous proteins. The data are the mean of three independent experiments.

REFERENCES

- Bauerle, P.A. (1991) *Biochim. Biophys. Acta*, **1072**, 63–80.
- Bauerle, 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, The Netherlands, pp. 409–432.
- Grilli, M., Chiu, J.S. and Lenardo, M.J. (1993) *Int. Rev. Cytol.*, **143**, 1–62.
- Blank, V., Kourilsky, P. and Israel, A. (1991) *EMBO J.*, **10**, 4159–4167.
- Henkel, T., Zabel, U., VanZee, K., Muller, J.M., Fanning, E. and Bauerle, P.A. (1992) *Cell*, **68**, 1121–1133.
- Zhang, Q., Didonato, J.A., Karin, M. and McKeithan, T.W. (1994) *Mol. Cell Biol.*, **14**, 3915–3926.
- Bours, V., Villalobos, J., Burd, P.R., Kelly, K. and Siebenlist, U. (1990) *Nature*, **348**, 76–80.
- Ghosh, S., Gifford, A.M., Riviere, L.R., Tempst, P., Nolan, G.P. and Baltimore, D. (1990) *Cell*, **62**, 1019–1029.
- Kieran, M., Blank, V., Logeat, F., Vandekerckhove, J., Lottespeich, F., Le Bail, O., Urban, M.B., Kourilsky, P., Bauerle, P.A. and Israel, A. (1990) *Cell*, **62**, 1007–1018.
- Neri, A., Chang, C.-C., Lombardi, L., Salina, M., Corradini, P., Maiolo, A.T., Chaganti, R.S.K. and Dalla-Favera, R. (1991) *Cell*, **67**, 1075–1087.
- Schmid, R.M., Perkins, N.D., Duckett, C.S., Andrews, P.C. and Nabel, G.J. (1991) *Nature*, **352**, 733–736.
- Bours, V., Burd, P.R., Brown, K., Villalobos, J., Park, P., Ryseck, R.P., Bravo, R., Kelly, K. and Siebenlist, U. (1992) *Mol. Cell Biol.*, **12**, 685–695.
- Mercurio, F., Didonato, J., Rosette, C. and Karin, M. (1992) *Cell Biol.*, **11**, 523–537.
- Nolan, G., Ghosh, S., Liou, H.C., Tempst, P. and Baltimore, D. (1991) *Cell*, **64**, 961–969.
- Ruben, S.M., Dillon, P.J., Schreck, R., Henkel, T., Chen, C.-H., Maher, M., Bauerle, P.A. and Rosen, C.A. (1991) *Science*, **251**, 1490–1493.
- Brownell, E., Mittereder, N. and Rice, N.R. (1989) *Oncogene*, **4**, 935–942.
- Grumont, R.J. and Gerondakis, S. (1988) *Oncogene Res.*, **4**, 1–8.
- Hannink, M. and Temin, H.M. (1989) *Mol. Cell Biol.*, **9**, 4323–4336.
- Ryseck, R.P., Bull, P., Takamiya, M., Bours, V., Siebenlist, U., Dobrzansky, P. and Bravo, R. (1992) *Mol. Cell Biol.*, **12**, 674–684.
- Ruben, S.M., Klement, J.F., Coleman, T.A., Maher, M., Chen, C.H. and Rosen, C.A. (1992) *Genes Dev.*, **6**, 745–760.
- Stephens, R.M., Rice, N.R., Hiebsch, R.R., Bose, H.R. and Gilden, R.V. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 6229–6232.
- Wilhelmsen, K.C., Eggleton, K. and Temin, H.M. (1984) *J. Virol.*, **52**, 172–182.
- Steward, R. (1987) *Science*, **238**, 692–694.
- Lux, S.E., John, K.M. and Bennett, V. (1990) *Nature*, **344**, 36–42.
- Haskill, S., Beg, A.A., Tompkins, S.M., Morris, J.S., Yurochko, A.D., Sampson-Johannes, A., Mondal, K., Ralph, P. and Baldwin, A.S. (1991) *Cell*, **65**, 1281–1289.
- Davis, N., Ghosh, S., Simmons, D.L., Tempst, P., Liou, H., Baltimore, D. and Bose, H.R. (1991) *Science*, **253**, 1268–1271.
- Inoue, J.I., Kerr, D., Kakizuka, A. and Verma, I. (1992) *Cell*, **68**, 1109–1120.
- Liou, H.C., Nolan, G.P., Ghosh, S., Fujita, T. and Baltimore, D. (1992) *EMBO J.*, **12**, 3003–3009.
- Ohno, H., Takimoto, G. and McKeithan, T.W. (1990) *Cell*, **60**, 991–997.
- Hatada, E.N., Nieters, A., Wulczyn, F.G., Naumann, M., Meyer, R., Nucifora, G., McKeithan, T.W. and Scheiderei, C. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 2489–2493.

- 31 De Martin,R., Vanhove,B., Cheng,Q., Hofer,E., Csizmadia,V., Winkler,H. and Bach,F.H. (1993) *EMBO J.*, **7**, 2773-2779.
- 32 Mercurio,F., Didonato,J., Rosette,C. and Karin,M. (1993) *Genes Dev.*, **7**, 705-718.
- 33 Chang,C.C., Zhang,J., Lombardi,L., Neri,A. Chaganti,R.S.K. and Dalla-Favera,R. (1994) *Oncogene*, **9**, 923-933.
- 34 Bours,V., Franzoso,G., Azarenko,V., Park,S., Kanno,T., Brown,K. and Siebenlist,U. (1993) *Cell*, **72**, 729-739.
- 35 Ten,R.M., Paya,C.V., Israel,N., Le Bail,O., Mattei,M.G., Virelizier,J.L., Kourilsky,P. and Israel,A. (1992) *EMBO J.*, **11**, 195-203.
- 36 Hannink,M. and Temin,H.M. (1990) *Oncogene*, **5**, 1843-1850.
- 37 Capobianco,A.J. and Gilmore,T.D. (1991) *Oncogene*, **6**, 2203-2210.
- 38 Grumont,R.J., Richardson,I.B., Gaff,C. and Gerondakis,S. (1993) *Cell Growth Differentiation*, **4**, 731-743.
- 39 Sun,S.C., Ganchi,P.A., Ballard,D.W. and Greene,W.C. (1993) *Science*, **259**, 1912-1915.
- 40 Le Bail,O., Schmidt-Ullrich,R. and Israel,A. (1993) *EMBO J.*, **12**, 5043-5049.
- 41 Fainberg,A.P. and Vogelstein,B. (1983) *Anal. Biochem.*, **132**, 6-13.
- 42 Sambrook,J., Fritsch,F.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- 43 Lombardi,L., Newcomb,E.W. and Dalla-Favera,R. (1987) *Cell*, **49**, 161-170.
- 44 Gorman,C.M., Moffat,L.F. and Howard,B.H. (1982) *Mol. Cell. Biol.*, **2**, 1044-1051.
- 45 Dignam,J.D., Lebovitz,R.M. and Roeder,R.G. (1983) *Nucleic Acids Res.*, **11**, 1475-1489.
- 46 Hansen,S., Nerlov,C., Zabel,U., Verde,P., Jhonsen,M., Baeuerle,P. and Blasi,F. (1992) *EMBO J.*, **11**, 205-213.
- 47 Fracchiolla,N.S., Lombardi,L., Salina,M., Migliazza,A., Baldini,L., Berti,E., Cro,L., Polli,E., Maiolo,A.T. and Neri,A. (1993) *Oncogene*, **8**, 2839-2845.
- 48 Shakhov,A.N., Collart,M.A., Vassalli,P., Nedospasov,S.A. and Jongeneel,C.V. (1990) *J. Exp. Med.*, **171**, 35-47.
- 49 Lilienbaum,A., Duc Dodon,M., Alexandre,C., Gazzolo,L. and Paulin,D. (1990) *J. Virol.*, **64**, 256-263.
- 50 Anisowicz,A., Messineo,M., Lee,S.W. and Sager,R. (1991) *J. Immunol.*, **147**, 520-527.
- 51 Paul,N., Lenardo,M.J., Novak,K.D., Sarr,T., Tang,W.-L. and Ruddle,N.H. (1990) *J. Virol.*, **64**, 5412-5419.
- 52 Messer,G., Weiss,E.H. and Baeuerle,P.A. (1990) *Cytokine*, **2**, 1-9.
- 53 Nabel,G. and Baltimore,D. (1987) *Nature*, **326**, 711-713.
- 54 Kanno,M., Fromental,C., Staub,A., Ruffenach,F., Davidson,I. and Chambon,P. (1989) *EMBO J.*, **8**, 4205-4214.
- 55 Sambucetti,L.C., Cherrington,J.M., Wilkinson,G.W.G. and Mocarski,E.S. (1989) *EMBO J.*, **8**, 4251-4258.
- 56 Williams,J.L., Garcia,J., Harrich,D., Pearson,L., Wu,F. and Gaynor,R. (1990) *EMBO J.*, **9**, 4435-4442.
- 57 Cha,Y. and Deisseroth,A.B. (1994) *J. Biol. Biochem.*, **7**, 5279-5287.
- 58 Link,D.C., Gutkind,S.J., Robbins,K.C. and Ley,T.J. (1992) *Oncogene*, **7**, 877-884.
- 59 Reisman,D. and Rotter,V. (1989) *Oncogene*, **4**, 945-953.
- 60 Ueberla,K., Lu,Y., Chung,E. and Haseltine,W.A. (1993) *J. AIDS*, **6**, 227-230.
- 61 Liptay,S., Schimd,R.M., Nabel,E.G. and Nabel,G.J. (1994) *Mol. Cell. Biol.*, **14**, 7695-7703.
- 62 Migliazza,A., Lombardi,L., Rocchi,M., Trecca,D., Chang,C.C., Antonacci,R., Fracchiolla,N.S., Ciana,P., Maiolo,A.T. and Neri,A. (1994) *Blood*, **84**, 3850-3860.
- 63 Zhang,J., Chang,C.C., Lombardi,L. and Dalla Favera,R. (1994) *Oncogene*, **9**, 1931-1937.