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Promoter analysis of the gene encoding the IϰB-α/MAD3 inhibitor of NF-ϰB: positive regulation by members of the rel/NF-ϰB family

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In order to characterize the regulation of the gene encoding the IϰB-α/MAD3 inhibitor of the transcription factor NF-ϰB, we have isolated a human genomic clone and sequenced the promoter of this gene. The *MAD3* promoter exhibits a potential TATA element upstream of one of the two major transcription sites, and contains several potential NF-ϰB binding sequences, suggesting that the gene is positively regulated by members of this family. Transfection experiments demonstrate that the *MAD3* promoter can be activated by various combinations of members of the rel/NF-ϰB family, as well as by phorbol esters and tumor necrosis factor. Specific deletion of one of the ϰB motifs, located 37 bp upstream of the TATA box, abolishes responses to PMA and TNF. This ϰB motif binds NF-ϰB (p50/relA), p50/c-rel and relA/c-rel heterodimers as well as KBF1 (p50 homodimer). These results help to explain the previously observed transient nature of the NF-ϰB response: following NF-ϰB activation, the expression of the inhibitor is increased, therefore the extent of nuclear translocation of the active complex is reduced, resulting in a decreased activation of its target genes.

Key words: IϰB-α/MAD3/NF-ϰB transcription factor/ transcriptional regulation

Introduction

Nuclear factor ϰB (NF-ϰB) is a transcription factor implicated in the regulation of numerous cellular genes as well as some viruses (reviewed in Blank *et al.*, 1992; Grilli *et al.*, 1993 and references therein). It is made of hetero- or homodimeric combinations between several proteins belonging to the same family. The most common species are heterodimers between a 50 kDa subunit (p50, encoded by the *NFKB1* locus) and either a 65 kDa (relA, formerly p65) protein or the product of the c-*rel* proto-oncogene. These complexes are constitutively expressed in an active form in the nuclei of mature B cells, monocytes and some T cell lines. In most other cell types NF-ϰB is found as an inactive cytoplasmic form, bound to the inhibitor IϰB. Upon cell activation by phorbol esters, mitogens, cytokines or some viruses, IϰB undergoes specific modifications (most likely involving phosphorylation), which induce release of NF-ϰB which then translocates to the nucleus. The active nuclear form of NF-ϰB binds to specific decameric sequence motifs (ϰB sequences) located in the regulatory regions of numerous genes involved in immune, inflammatory and acute phase responses, as well as some viral enhancers, including HIV-1. New NF-ϰB synthesis can be induced by phorbol 12-myristate 13-acetate (PMA) and tumor necrosis factor (TNF), which increase the steady state level of the mRNAs encoding p50 (Meyer *et al.*, 1991; Ten *et al.*, 1992) as well as c-rel (Grumont and Gerondakis, 1990; Capobianco and Gilmore, 1991). Therefore, both phorbol esters and TNF not only activate the cytoplasmic forms of NF-ϰB, but also have been proposed as inducers of the genes encoding two members of this family.

Several IϰB proteins have been purified (reviewed in Schmitz *et al.*, 1991). IϰB-α and IϰB-β have been isolated from human placenta (Zabel and Baeuerle, 1990; Link *et al.*, 1992), a 35 kDa IϰB from rabbit lung (Ghosh and Baltimore, 1990) and pp40 from avian cells (Kerr *et al.*, 1991). All these molecules were shown to be inhibitors of the DNA binding activity of NF-ϰB, through their interaction with either relA or c-rel (Kerr *et al.*, 1991; Beg *et al.*, 1992; Tewari *et al.*, 1992a). cDNA clones encoding pp40 and MAD3 have been isolated recently (Davis *et al.*, 1991; Haskill *et al.*, 1991; Tewari *et al.*, 1992b). MAD3 most likely represents IϰB-α and pp40 is probably its avian homolog. It was then demonstrated that MAD3 can also interact with p50, but is unable to dissociate a complex between homodimeric p50 and DNA. The cDNA encoding IϰB-β has not been isolated yet.

Activation of NF-ϰB is probably due to phosphorylation of MAD3 and its subsequent degradation (Ghosh and Baltimore, 1990; Kerr *et al.*, 1991; Link *et al.*, 1992; Beg *et al.*, 1993; Brown *et al.*, 1993; Cordle *et al.*, 1993; Sun *et al.*, 1993), resulting in rapid translocation of the active complex to the nucleus. However, this activation state is transient. This is in part due to an increase in the expression of *MAD3* following induction, detectable both at the level of mRNA and protein. We present here the characterization of the promoter of the *MAD3* gene and demonstrate that it contains two ϰB sites, one of them being responsible for induction following all stimuli that are known to activate NF-ϰB, resulting in a feedback loop which helps to explain the transient nature of this response.

Results

Structure of the MAD3 promoter

To analyze the promoter region of the *MAD3* gene we screened ~10^6 clones of a human genomic library with a probe containing a 5′ fragment of the *MAD3* cDNA and isolated two clones containing sequences located upstream of the published cDNA. The positive clones all contained a 3 kb *Pvu*II fragment that hybridized to the probe. Sequence analysis of this fragment demonstrated that it contained ~400 bp of sequence, upstream of the published cDNA sequence. This sequence is presented in Figure 1. To determine the localization of the transcription start site, we

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                                         κB2
-385                                CGGATCTGGGTCGACCTGCAGGTCAACGGATCT
-352   CAGCAGCCGACGACCCCAATTCAAATCGATCGTGGGAAACCCCAGGGAAA
-302   GAAGGCTCACTTGCAGAGGGACAGGATTACAGGGTGCAGGCTGCAGGGAA
                                   κB3
-252   GTACCGGGGGGAGGGGGCCTGGTCGGAAGGACTTTCCAGCCACTCGGCGC
-202   TCATCAAAAAGTTCCCTGTCCGTGACCCTAGTGGCTCATCGCAGGGAGTT
-152   TCTCCGATGAACCCCAGCTCAGGGTTTAGGCTTCTTTTTCCCCCTAGCAG
                                                κB1
-102   AGGACGAAGCCAGTTCTCTTTTTCTGGTCTGACTGGCTTGGAAATTCCCC
-52    GAGCCTGACCCCGCCCCAGAGAAATCCCCAGCCAGCGTTTATAGGCGGCG
-2     GCGGCTGCAGAGCCCACAGCAGTCCGTGCCGCCGTCCCGCCCGCCAGCGC
+49    CCCAGCGAGGAAGCAGCGCGCAGCCCGCGGCCCAGCGCACCCGCAGCAGC
+99    GCCCGCAGCTCGTCCGCGCCATG
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Fig. 1. Nucleotide sequence of the *MAD3* promoter. The nucleotide sequence of the 5′ flanking region of the *MAD3* gene is shown. The ATG start codon is in bold. The two major transcription start sites are in bold with an arrow on top. The three κB sites (κB1, 2 and 3) are boxed. Two other putative κB sites which fit poorly with the consensus κB sequence are underlined. The putative TATA box is underlined twice. The coordinates on the left are relative to nucleotide G of the downstream major transcription start site, taken arbitrarily as +1.

performed primer extension using total cellular RNA isolated from PMA-treated THP1 cells and a primer located in the published cDNA sequence (Figure 2). Two start sites were identified. The most upstream site does not seem to be associated with a canonical TATA or CCAAT box, nor to be surrounded by a very GC-rich region. The downstream one is located 9 bp downstream of a putative TATA box and is arbitrarily designated as +1 relative to the rest of the sequence. In addition, three potential κB sites are boxed: κB1 (−63 to −53), κB2 (−319 to −310) and κB3 (−225 to −216) (other potential κB sites, located at −159 to −150 and −34 to −24, underlined in Figure 1, are discussed below).

Functional analysis of the promoter

To analyze this promoter further, we cloned a fragment corresponding to coordinates −385 to +22 in front of the luciferase gene into the pGL2-basic vector. This construct, 0.4SK-luc, exhibited a weak basal activity in a T cell line (J.Jhan, a subclone of Jurkat) and in a leukemia cell line, K562 (Figure 3A). This basal level could be induced by PMA or TNF in both cell lines. Interestingly, in J.Jhan cells the stimulation by TNF was more efficient than that by PMA, while in K562 cells the opposite was true. However, this was not a property of the *MAD3* promoter since a control plasmid containing the HIV LTR cloned upstream of the luciferase gene exhibited the same type of differential response (not shown).

The response of the *MAD3* promoter to stimulation by PMA and TNF as well as the existence of κB-like sites led us to investigate the involvement of members of the rel/NF-κB family in these stimulations. We first cotransfected the 0.4SK-luc construct with various combinations of members of the NF-κB family. The results are presented in Figure 3B. While p50 was unable to transactivate the promoter, cotransfection of relA, c-rel, relA + p50, c-rel + p50 and relA + c-rel resulted in transactivation, although its extent varied for the various combinations. Interestingly, the strongest response was obtained with relA alone. To confirm the involvement of the rel/NF-κB family in the inductions observed following treatment with PMA or TNF we

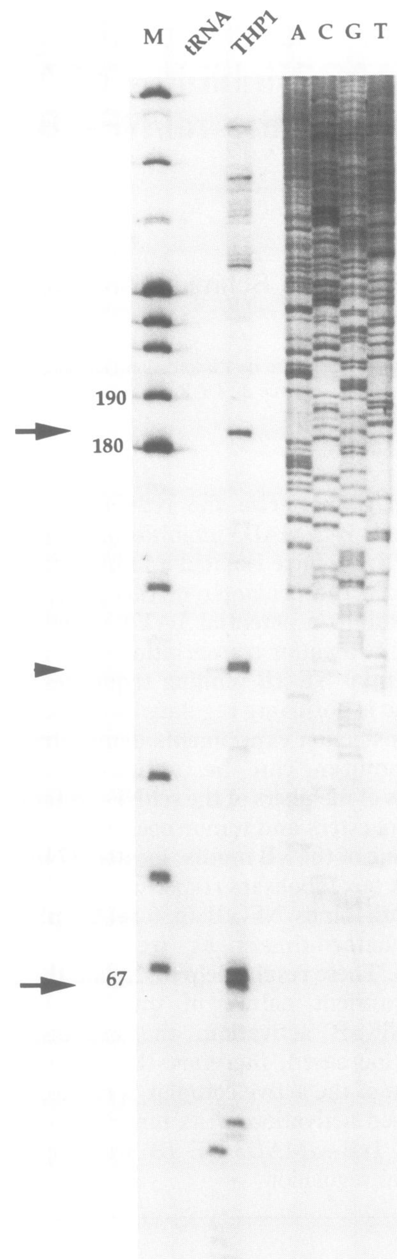

Fig. 2. Determination of the transcription intiation sites of the *MAD3* gene by primer extension. Total cellular RNA from PMA-treated THP1 cells or tRNA (as a negative control) were extended with a radiolabelled primer corresponding to nucleotides 51−68 of the sequence shown in Figure 1. M: molecular weight markers (pBR322 digested with *Hpa*II). The arrows correspond to the two major transcription start sites. The arrowhead indicates a non-specific extension product which is also visible in the tRNA lane, although its intensity varies from experiment to experiment. The signals observed in the upper part of the gel have not been found reproducibly. The four right-hand lanes (ACGT) correspond to the sequencing reactions of the genomic clone obtained by using the same primer and allow the precise localization of the start sites.

cotransfected the *MAD3* promoter construct and an expression vector encoding a p50 mutant that is able to dimerize with other members of the family but does not bind DNA (ΔSP in Figure 3C; Logeat *et al.*, 1991). This mutant behaves as a transdominant negative mutant, since it has been shown to block transcriptional activation by NF-κB, probably by forming inactive non-DNA binding heterodimers with other members of the rel/NF-κB family.

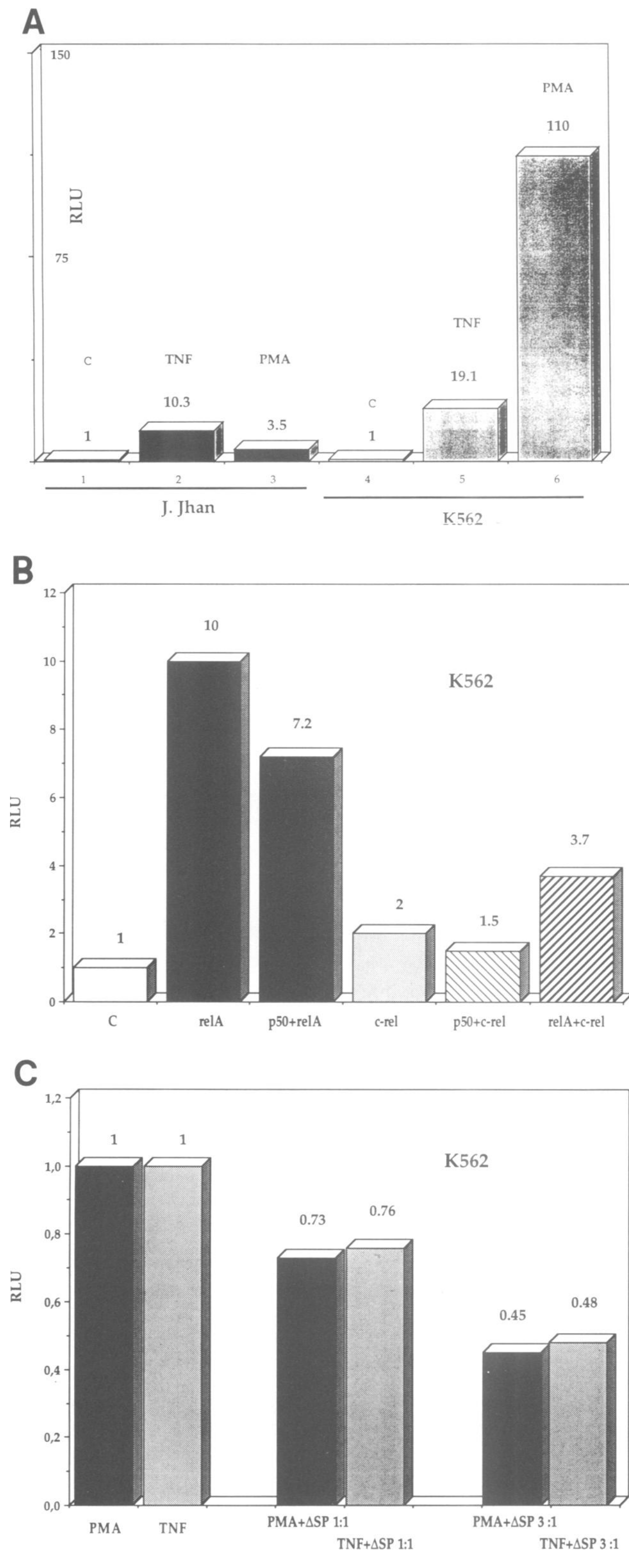

Fig. 3. (**A**) Stimulation of the *MAD3* promoter by PMA or TNF in K562 or J.Jhan cells. The 0.4SK-luc plasmid containing a 0.407 kb fragment (coordinates −385 to +22) of the *MAD3* promoter cloned upstream of the luciferase gene was transfected into K562 leukemia cells or J.Jhan T cells which were stimulated as described in Materials and methods. Each point was done in quadruplicate for each experiment. The numbers above the bars indicate the relative luciferase activities (indicated as RLU on the left axis), the value obtained in non-treated cells being taken as 1. These stimulation values are the average of at least four experiments. Experimental variation never exceeded 20%. C: untreated cells. (**B**) Activation of the *MAD3* promoter by members of the rel/NF-κB family. 50 ng of the 0.4SK-luc construct were cotransfected into K562 cells together with either 150 ng (for relA or c-rel alone) or 75 ng + 75 ng (for combinations of two proteins), and the luciferase activity was measured in quadruplicate as described in Materials and methods. Numbers above the bars indicate 'fold transactivation' over the value obtained with the 0.4SK-luc construct transfected with 150 ng of the Rc-CMV vector without insert (shown as 'C' under the first bar). Results shown here are representative of at least five experiments. (**C**) Inhibition of PMA or TNF activation of the *MAD3* promoter by increasing amounts of the ΔSP mutant. 50 ng of the 0.4SK-luc construct was transfected into K562 cells together with 50 ng (1:1 ratio) or 150 ng (3:1 ratio) of the ΔSP mutant. The total amount of transfected DNA was kept at 200 ng with Rc-CMV. Cells were treated with PMA or TNF and luciferase activities were measured as in panel A. Numbers above the bars indicate 'fold stimulation', the value obtained with PMA- or TNF-treated cells without cotransfected ΔSP being arbitrarily taken as 1.

ΔSP decreased by >50% the activation of the *MAD3* promoter by PMA or TNF in K562 cells, in a dose-dependent manner. The same result was obtained in J.Jhan cells (not shown).

Characterization of the proteins binding to the putative κB motifs

Since NF-κB seems to be involved in controlling the activity of the *MAD3* promoter, we focused our attention on the putative κB sites described above. We performed bandshift assays and competitions using double-stranded oligonucleotides corresponding to the various sites and nuclear extracts derived from various lymphoid cells. As controls we used oligonucleotides corresponding to the canonical κB sites located in the intronic enhancer of the immunoglobulin κ light chain gene (Igκ site) or in the promoter of the MHC class I gene H-2 K^b (see Materials and methods). Figure 4A shows that when using extracts from stimulated J.Jhan cells, four complexes binding to the Igκ site can be detected, and three to the κB1 site. To identify the proteins responsible for the formation of these complexes we used specific antisera directed against p50, relA and c-rel. The specificity of these antisera has been demonstrated previously (Rice *et al.*, 1992). The anti-p50 serum induced disappearance or reduced the intensity of complexes 2, 3 and 4 (the complexes are numbered 1 to 4 from top to bottom for J.Jhan cells, and 2 to 4 for Daudi cells). The anti-relA serum inhibited formation of complexes 1 and 2. The anti-c-rel serum inhibited formation of complexes 1 and 3. From the results obtained we can conclude that the most slowly migrating complex corresponds to a heterodimer of relA with c-rel, which is apparently absent from Daudi B cells. Then from top to bottom we find complexes made of p50/relA, p50/c-rel and p50 homodimers (this last complex is more easily detectable when using the κB site from the MHC class I H-2 K^b promoter; see panel B). The most prevalent complex seems to be p50/relA in J.Jhan cells, while nuclei from Daudi B cells contain more of the p50/c-rel complex. These various heterodimers bind to the two sites with similar affinities, while the p50 homodimer seems to bind more weakly to the κB1 site. However, it can be seen in panel B that when using extracts derived from TNF-stimulated K562 cells, binding of the p50 homodimer to the κB1 site can clearly be detected.

Two other sites in the *MAD3* promoter, κB2 and κB3 (boxed in Figure 1), more or less closely fit the consensus κB sequence (GGGRNNYYCC), while the sites at −159 to −150 (GGGAGTTTCTC) and −34 to −24

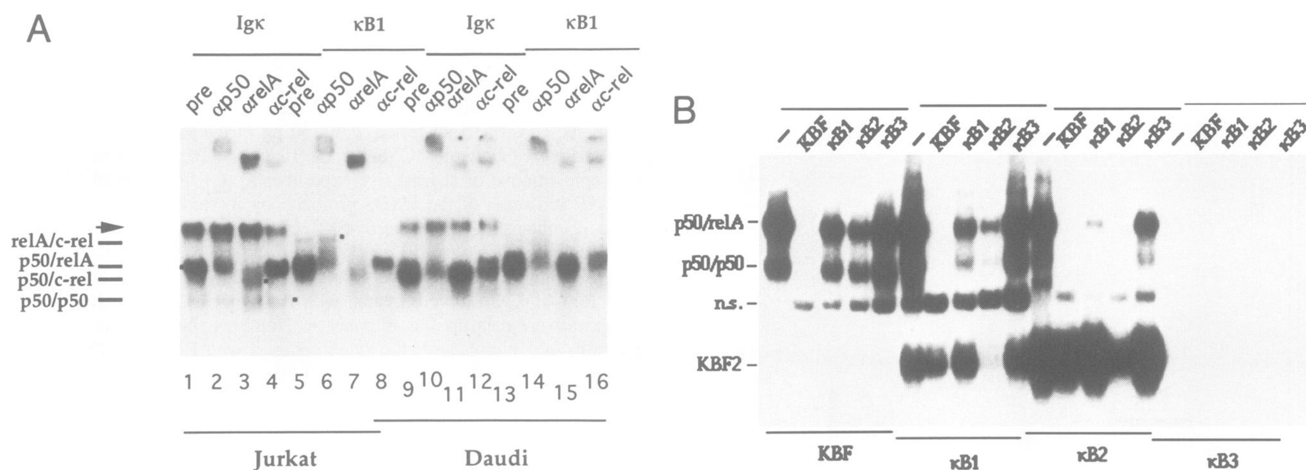

Fig. 4. Characterization of the proteins binding to the κB sites. Binding assays were performed using double-stranded oligonucleotides corresponding to the immunoglobulin intronic enhancer κB site (Igκ) or the MAD3 κB1 site in panel A, to the MHC class I H-2 K^b κB site (KBF) or to the three κB sites of the *MAD3* promoter (κB1, 2 and 3) in panel B. The extracts were obtained from nuclei of Jurkat cells (the J.Jhan clone) that had been treated with 40 ng/ml of PMA for 15 h or from Daudi B cells (panel A) and from nuclei of K562 cells that had been treated with 500 U/ml of TNF for 15 h (panel B). (**A**) The 10 μl binding reaction contains 0.3 μl of preimmune serum (lanes 1, 5, 9 and 13), anti-p50 serum 2 (Kieran *et al.*, 1990) (lanes 2, 6, 10 and 14), anti-relA serum 1207 (lanes 3, 7, 11 and 15) or anti-v-rel serum 8542 (lanes 4, 8, 12 and 16) (Rice *et al.*, 1992). The various complexes are indicated on the left of the figure, and by square dots on the picture. The arrow indicates a non-specific complex, which is formed only with the Igκ site. Complex 1 (relA/c-rel), migrating slightly faster than the non-specific complex, is indicated by a square on the right of lane 6; complex 2 (p50/relA) is indicated by a dot on the left of lane 1; complex 3 (p50/c-rel) is just under complex 2 in lane 1 and is more visible in lane 3, where it is marked by a dot on its right; complex 4 (p50/p50) is visible only with the Igκ probe using Jurkat extracts (lanes 1–4) and is marked in lane 4 by a dot on its right. (**B**) Cross-competition between the different κB sites: 0.25 ng of each of the labelled probes (indicated under the lanes) was incubated with a nuclear extract derived from TNF-treated K562 cells in the presence of a 40-fold excess of each of the unlabelled oligonucleotides (indicated above the lanes). The identity of the complexes is indicated on the left of the figure. n.s. corresponds to a non-specific band; KBF2 indicates binding of the KBF2 protein (see text).

(GGGGATTTCTC on the other strand) fit poorly with this consensus sequence and do not bind NF-κB at all (data not shown). We therefore carried out cross-competitions using the KBF site of H-2 K^b and the MAD3 sites κB1, κB2 and κB3. We labelled each individual binding site and performed competition with a 40-fold excess of each unlabelled binding site, in the presence of nuclear extract derived from K562 cells that have been treated with TNF for 15 h. Figure 4B shows that specific binding can be obtained using sites κB1 and κB2. On the other hand the κB3 site does not bind any member of the NF-κB family nor does it compete binding to the KBF, κB1 and κB2 sites. The affinity of the κB1 and κB2 sites for p50/relA or p50/p50 seems to be quite similar, while being lower than that of the KBF site. It can also be seen in this figure that the κB1 and κB2 sites strongly bind the KBF2 protein, which we recently showed to belong to the RBP-Jκ family (F.Logeat *et al.*, submitted).

Therefore the κB1 and κB2 sequences behave like *bona fide* κB sites, suggesting that they might be responsible for the PMA and TNF inducibility of the *MAD3* promoter.

The κB1 site is responsible for PMA and TNF inducibility of the MAD3 promoter

To analyze further the functional role of the κB binding sites in the activation of the *MAD3* promoter, we first constructed a shorter version of the promoter (−198 to +22) which includes the κB1 but not the κB2 site. As can be seen in Figure 5A, this truncated promoter still responds to PMA and TNF and the extent of stimulation is similar to that observed with the larger (−385 to +22) construct. This result suggests that the κB1 site is functionally important while κB2 does not seem to be involved in the inducibility of the promoter. To confirm this point we deleted the κB1 motif by site-directed mutagenesis in the context of the 0.4 kb promoter (construct $\Delta\kappa$B-0.4SK-luc). As shown in Figure 5B, deletion of this κB motif greatly diminished activation by PMA (by 95%) and completely abolished the activation by TNF in K562 cells. These results indicate that the κB1 motif mediates TNF inducibility of the *MAD3* promoter and is the main element responsible for the PMA response (similar results have been obtained in J.Jhan cells; data not shown).

Discussion

Recent data have provided new insights into the mechanism of action of IκB-α/MAD3. It has first been shown that the MAD3 molecule can interact with relA, p50 and c-rel (Baeuerle and Baltimore, 1988a,b; Kerr *et al.*, 1991; Beg *et al.*, 1992; Tewari *et al.*, 1992b). In cotransfection experiments it is able to retain these three molecules in the cytoplasm, probably by preventing access to their nuclear localization signal. It can also cause the dissociation of complexes of DNA with most of the possible homo- or heterodimers (except p50 homodimers). In addition, the MAD3 molecule can be found not only in the cytoplasmic but also in the nuclear compartment of the cell (Baeuerle and Baltimore, 1988a; Davis *et al.*, 1990; Beg *et al.*, 1992; Morin and Gilmore, 1992; Zabel *et al.*, 1993). This led to the suggestion that MAD3 might be able to enter the nucleus and dissociate DNA-bound NF-κB complexes, therefore terminating κB-dependent transcription stimulation. The transient nature of the NF-κB response was observed several years ago (Sen and Baltimore, 1986; Baeuerle *et al.*, 1988; Hohmann *et al.*, 1991), when it was shown that the active complexes found in the nucleus following stimulation could be detected under an inactive form in the cytoplasm a few hours later. This process requires protein synthesis. In

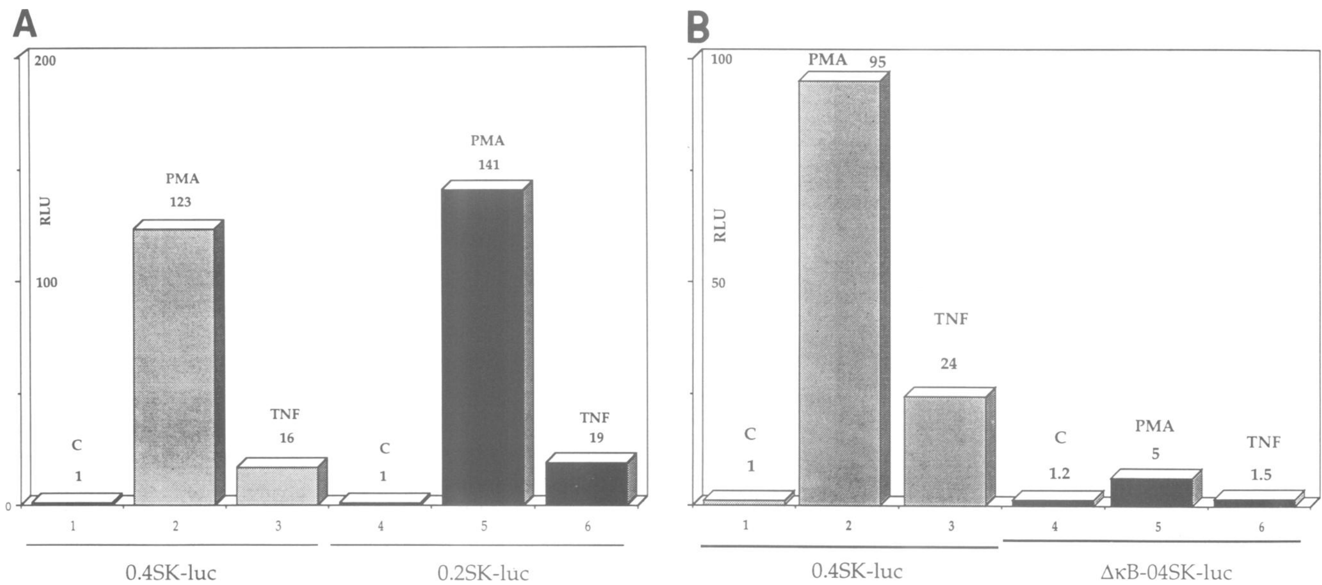

Fig. 5. The κB1 element is responsible for TNF and PMA inducibility of the *MAD3* promoter. (**A**) K562 cells were transfected with 0.4SK-luc (columns 1–3) or with 0.2SK-luc (columns 4–6) and were treated with PMA (columns 2 and 5) or with TNF (columns 3 and 6). Luciferase activity was measured as described in Materials and methods. Values given are representative of three experiments, where variation never exceeded 20%. The numbers above the bars indicate the relative luciferase activities, the value obtained with untreated cells transfected with 0.4SK-luc being arbitrarily taken as 1. (**B**) Same as in panel A, except that K562 cells were transfected with either 0.4SK-luc (columns 1–3) or with ΔκB-0.4SK-luc (columns 4–6).

addition it has been shown more recently that following stimulation of T or monocytic cell lines, MAD3 is rapidly degraded, but its disappearance is followed by a rapid replenishment (Beg *et al.*, 1993; Brown *et al.*, 1993; Cordle *et al.*, 1993; Sun *et al.*, 1993). This is best explained by an increase in the transcription rate of the *MAD3* gene, a hypothesis which is supported by the results presented here.

The analysis of the *MAD3* promoter has revealed the presence of two major transcription start sites, one of them being preceded by a potential TATA box. The most remarkable feature of this promoter is the presence of two κB motifs (κB1 and κB2) with similar affinities for members of the NF-κB family, one of them (κB1) being responsible for the inducibility of the promoter. One of the transcription start sites is located 52 bp downstream of the κB1 site, while the second one is located upstream of this site. In general κB sites are located upstream of the cap sites of the genes they control, or inside introns. The location of a κB site inside the transcribed 5′ non-coding region of a gene is more unusual, although this is probably the case for the c-*myc* gene (Kessler *et al.*, 1992). By gel shift assay we have shown that both κB1 and κB2 sites can bind the same complexes as the canonical Igκ or H-2 K^b sites. The use of a mutant of p50 (ΔSP) which inhibits specifically the activity of the members of the rel/NF-κB family confirmed the involvement of these proteins in PMA and TNF stimulation of the promoter. Specific deletion of the κB1 sequence resulted in suppression of the response to PMA and TNF, confirming the functional relevance of this motif. The reason why the κB2 motif does not seem to be required for PMA or TNF inducibility is unclear at the moment (although it might be involved in the residual response to PMA which is visible after mutagenesis of the κB1 site). It could be involved in induction in response to stimuli that have not been assayed here, or in some tissues different from the ones analyzed here. It is also possible that the κB2 site is not occupied *in vivo*. We have started a genomic footprinting analysis in order to clarify this point. The stimulation of the *MAD3* promoter by both PMA and TNF suggests that, whatever the differences in the modes of action between these two types of stimuli, they both induce an increase in the amount of MAD3 following the initial NF-κB activation phase.

Therefore the NF-κB response could be regulated as follows: following a given stimulus MAD3 would be degraded (maybe following a modification like phosphorylation), thus leading to the nuclear translocation of NF-κB. This would stimulate the expression of several target genes, including *MAD3*. Part of these newly synthesized molecules would translocate to the nucleus (either by an active or a passive mechanism) and retrieve DNA-bound NF-κB complexes. Apparently the most active species able to induce *MAD3* expression is relA. This in turn raises the question of the functional relevance of relA homodimers. Full-length relA has been observed to bind with a very low affinity (if at all) to various κB sites *in vitro*. However, activation of target genes by cotransfection of relA has been observed repeatedly. The lack of apparent binding activity could be due to an artifact of the bandshift assay, or the transfected relA molecules could be able to recruit an endogenous partner in order to bind DNA efficiently.

This induction of an inhibitory molecule by the same stimuli which result in the NF-κB response ensures that this activation is transient, and that activators and inhibitors are kept in constant equilibrium.

Very recently, de Martin *et al.* (1993) have cloned what is most likely the pig homolog of *MAD3* and have analyzed its promoter. A few differences can be found between the two species. In human there are two transcription start sites, the most downstream one being almost identical to the unique start site seen in the pig. In addition, only one of the two κB sites analyzed in the pig seems to be functional in humans. The second site shows a single difference in the 5′-flanking nucleotide [GAGGACTTTCC for the BS1 site in the pig (de Martin *et al.*, 1993) versus AAGGACTTTCC

for the κB3 site] which might possibly be responsible for the difference observed. In addition, the κB2 site of human is perfectly conserved in the pig and its absence in the p140 construct (see Figure 7 in de Martin *et al.*, 1993) might be responsible for the decreased response to cotransfected relA. In any case, the main conclusion that IκB-α/MAD3 is upregulated by its target NF-κB (and more specifically relA) is identical for both species.

Materials and methods

Cloning and sequencing of the MAD3 promoter

A genomic library, derived from human liver and constructed in λEMBL3, was screened by hybridization with a 500 bp PCR-generated fragment corresponding to the 5′ region of the published cDNA sequence (Haskill *et al.*, 1991), and labelled by random priming (Amersham). Two positive clones were digested with different restriction enzymes and probed with the same labelled fragment, which hybridized with a 7 kb *Sca*I fragment or a 3 kb *Pvu*II fragment. These fragments were analyzed further by subcloning into Bluescript SK+ (Stratagene), followed by sequencing using the Sequenase kit (USB).

Primer extension

Total cellular RNA was isolated from THP1 monocytic cells treated with 20 ng/ml of PMA for 18 h until they were adherent. MAD3 expression was verified by Northern blotting with a *MAD3* cDNA probe using 10 μg of total RNA. For primer extension, an 18mer oligonucleotide derived from the 5′ end of the *MAD3* cDNA (coordinates 51–68 in Figure 1) was end-labelled using T4 polynucleotide kinase. Hybridization between 10^5 c.p.m. of this primer and 30 μg of total THP1 RNA or tRNA (as a negative control) was performed overnight at 30°C in 40 mM PIPES buffer pH 6.4, 1 mM EDTA, 0.4 M NaCl and 80% formamide after an initial treatment at 80°C for 10 min. After ethanol precipitation, primer extension was carried out at 42°C for 1 h in 50 mM Tris pH 7.6, 60 mM KCl, 10 mM $MgCl_2$, 1 mM of each deoxynucleotide, 1 mM dithiothreitol, 40 U RNAsin (Promega) and 30 U AMV reverse transcriptase (Promega). After heat inactivation of reverse transcriptase for 10 min at 52°C, samples were treated with 0.25 mg/ml of pancreatic RNase at 37°C for 30 min, extracted with phenol–chloroform, precipitated with ethanol and analyzed on a denaturing 8% polyacrylamide gel. Klenow-labelled fragments from *Hpa*II-digested pBR322 were used as molecular weight markers. A sequencing reaction of the *MAD3* promoter region utilizing the same primer was electrophoresed in adjacent lanes.

Plasmid constructs

A 3 kb *Pvu*II fragment containing sequences upstream of the published cDNA was subcloned into Bluescript SK+. A 0.38 kb *Sac*II–*Hin*dIII fragment was then subcloned into Bluescript, and was excised using the *Sac*I and *Kpn*I sites in the polylinker of this vector, allowing cloning in the same sites of the pGL2-basic vector (Promega), upstream of the luciferase gene, yielding the plasmid 0.4SK-luc. ΔκB-0.4SK-luc represents the 0.4SK-luc plasmid with a deletion of the κB motif. The 0.2SK-luc construct was obtained by amplification of the −198 to +22 fragment of the promoter (using primers including *Kpn*I and *Bgl*II sites) followed by cloning into the same sites of the pGL2-basic vector. As positive controls for PMA and TNF stimulation we used the (Igκ)3-cona-luc construct, which contains three copies of the Igκ enhancer κB site cloned upstream of the conalbumin promoter, followed by the luciferase gene. Expression vectors for p50 and relA (formerly p65) consisted in the full-length cDNA encoding each protein (Kieran *et al.*, 1990; Ruben *et al.*, 1991) cloned into the Rc-CMV vector (In Vitrogen). ΔSP is a p50 DNA mutant encoding the dimerization but not the DNA binding domain (Logeat *et al.*, 1991).

In vitro mutagenesis

The κB motif located 37 bp upstream of the TATA box was deleted from the 0.38 kb promoter fragment (cloned into Bluescript-SK+), by using the Muta-gene kit (Bio-Rad), following the manufacturer's recommendations. The oligonucleotide used for mutagenesis consisted in 30 nucleotides flanking the target sites for deletion (15 nucleotides on each side: TGGTCTGACTGGCTTGAGCCTGACCCCGCC), synthesized in a 381A DNA synthesizer (Applied Biosystems) and purified in 12% denaturing polyacrylamide gels. The mutated clones were verified by DNA sequencing and the 0.37 kb promoter fragment containing the κB deletion was subcloned into the sites of the pGL2-basic plasmid, as above.

Cell cultures and transfection experiments

J.Jhan cells, derived from the Jurkat cell line, Daudi B cells, THP1 monocytes and K562 (an undifferentiated leukemia cell line), all of human origin, were cultured in RPMI 1640 medium containing glutamine, antibiotics and 5–10% fetal calf serum. Cells (5×10^5) were transfected with the indicated reporter plasmid by a modified DEAE-dextran method (Schwartz *et al.*, 1990). Twenty-four hours after transfection the cells were stimulated with 40 ng/ml PMA (Sigma Chemical, St Louis, MO) or 50 ng/ml human rTNF (a generous gift of W.Fiers, Gent, Belgium). Eight hours later the cells were lysed with 25 mM Tris-phosphate pH 7.8, 8 mM $MgCl_2$, 1 mM dithiothreitol, 1% Triton X-100 and 15% glycerol, and luciferase activity was measured in a luminometer (Berthold). The background obtained with the lysis buffer was subtracted from each experimental value. The experiments were performed in quadruplicate in 24-well plates, and the mean RLU (relative light units) value was calculated.

Gel mobility shift assays

Nuclear extracts were obtained by a previously described method (Dignam *et al.*, 1983). Binding reactions were performed by incubating 3 μg of nuclear extracts with [^{32}P]dCTP-labelled double-stranded oligonucleotides at 22°C for 15 min in a total volume of 10 μl, in the buffer described by Yano *et al.* (1987). The binding reaction was analyzed by electrophoresis on native 5% polyacrylamide gels. When indicated, 0.3 μl of rabbit anti-p50 (serum 2 in Kieran *et al.*, 1990) or anti-relA serum (raised against the 15 carboxy-terminal amino acids of human relA) or anti-v-rel (Ab 8542) antiserum (which also recognizes human c-rel; a kind gift of N.Rice, NCI, Frederick) was added to the standard reaction prior to the addition of the radiolabelled probe. The oligonucleotides used in the binding reactions were synthesized and purified as described for *in vitro* mutagenesis. KBF correponds to the κB sequence located in the promoter of the H-2 K^b gene (Kieran *et al.*, 1990), Igκ contains the κB sequence located in the Igκ intronic enhancer, and κB1, 2 and 3 correspond to the κB sites of the *MAD3* promoter.

Their sequences are as follows (with the κB motif in bold):

KBF: AGCTT**GGGGATTCCCC**AT
A**CCCCTAAGGGG**TATCGA

Igκ: GACAGA**GGGGACTTTCC**GAGAGG
GTCT**CCCCTGAAAGG**CTCTCCCT

MAD3/κB1: GATCTT**GGAAATTCCCC**GA
AA**CCTTTAAGGGG**CTCTAG

MAD3/κB2: GATCGT**GGGAAACCCC**AG
CA**CCCTTTGGGG**TCCTAG

MAD3/κB3: GATCGGA**AGGACTTTCC**AG
CCT**TCCTGAAAGG**TCCTAG

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