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Inhibition of transcription factors belonging to the rel/NF-κB family by a transdominant negative mutant

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The KBF1 factor, which binds to the enhancer A located in the promoter of the mouse MHC class I gene H-2K^b, is indistinguishable from the p50 DNA binding subunit of the transcription factor NF-κB, which regulates a series of genes involved in immune and inflammatory responses. The KBF1/p50 factor binds as a homodimer but can also form heterodimers with the products of other members of the same family, like the *c-rel* and *v-rel* (proto)oncogenes. The dimerization domain of KBF1/p50 is contained between amino acids 201 and 367. A mutant of KBF1/p50 (ΔSP), unable to bind to DNA but able to form homo- or heterodimers, has been constructed. This protein reduces or abolishes *in vitro* the DNA binding activity of wild-type proteins of the same family (KBF1/p50, c- and v-rel). This mutant also functions *in vivo* as a *trans*-acting dominant negative regulator: the transcriptional inducibility of the HIV long terminal repeat (which contains two potential NF-κB binding sites) by phorbol ester (PMA) is inhibited when it is co-transfected into CD4$^+$ T cells with the ΔSP mutant. Similarly the basal as well as TNF or IL1-induced activity of the MHC class I H-2K^b promoter can be inhibited by this mutant in two different cell lines. These results constitute the first formal demonstration that these genes are regulated by members of the *rel*/NF-κB family.

Key words: heterodimer/KBF1/rel/NF-κB/transdominant negative mutant

Introduction

Nuclear factors that bind to the promoter of the H-2K^b mouse major histocompatibility complex (MHC) have been identified (Kimura *et al.*, 1986; Baldwin and Sharp, 1987; Israël *et al.*, 1987; Burke *et al.*, 1989). A factor called KBF1 which binds to the enhancer A sequence (Kimura *et al.*, 1986) has been suggested to be responsible for basal expression of MHC class I genes (Israël *et al.*, 1989a,b). This protein was purified (Yano *et al.*, 1987) and the cDNA coding for KBF1 has been cloned (Kieran *et al.*, 1990). Recently, we have demonstrated that the DNA binding subunit (p50) of NF-κB (Baeuerle and Baltimore, 1989) and KBF1 are indistinguishable and that they belong to a family of structurally related factors that also contains the *c-* and *v-rel* (proto)oncogenes and the *Drosophila dorsal* gene products (Kieran *et al.*, 1990; see also Ghosh *et al.*, 1990; Gilmore, 1990). The factor NF-κB which binds to the κB site located in the enhancer of the immunoglobulin κ light chain gene (Sen and Baltimore, 1986) and to the enhancer A sequence of H-2K^b (Baldwin and Sharp, 1988) is composed of two different subunits (Baeuerle and Baltimore, 1989): a 50 kd DNA binding subunit (KBF1/p50) and a non-DNA binding 65 kd subunit (p65). The cytoplasmic inactive form of NF-κB is composed of p50, p65 and IκB (Baeuerle and Baltimore, 1988). IκB inhibits the DNA binding activity of NF-κB and prevents its translocation into the nucleus. In addition, the DNA binding activity of KBF1/p50 is also controlled by a proteolytic event which releases p50 from a 105 kd precursor (Kieran *et al.*, 1990; Ghosh *et al.*, 1990).

Many transcription factors bind DNA as dimers and they can form heterodimers with other members of the same family (Jun/Fos: Turner and Tjian, 1989; MyoD/Id: Benezra *et al.*, 1990; retinoic acid/thyroid hormone receptor: Glass *et al.*, 1989). The combination of different monomers may have different transcriptional effects (Jones, 1990). Our previous results (Kieran *et al.*, 1990) demonstrate that KBF1/p50 binds to its site as a dimer and that KBF1/p50 and v-rel can form heterodimers *in vitro*. Structure-function studies of different eukaryotic transcription factors have allowed the defining of a certain number of specific motifs responsible for DNA binding and dimerization (Mitchell and Tjian, 1989; Murre *et al.*, 1989). KBF1/p50 contains no obvious homology with DNA binding and dimerization domains characterized so far, but preliminary experiments have demonstrated that the DNA binding and dimerization domain are contained within amino acids 19-367 (Ghosh *et al.*, 1990; Kieran *et al.*, 1990). We have assayed various deletion mutants for either DNA binding or dimerization ability and report here the functional characterization of a mutant which allows dimerization but not DNA binding.

Results

A series of internal or C-terminal deletion mutants have been assayed for DNA binding activity by bandshift (Ghosh *et al.*, 1990; Kieran *et al.*, 1990). Lack of DNA binding activity could be due to disruption or deletion of either the dimerization or DNA binding domains (or both). We therefore decided to localize the dimerization region of KBF1/p50. We made the assumption that the same region is necessary for the formation of p50-p50 homodimers and p50-rel heterodimers, as suggested by the strong sequence conservation between p50 and rel products, and assayed various deletion mutants of p50 for their ability to associate with full-length v- or c-rel. Association was first evaluated by co-immunoprecipitation of v- or c-rel, using an anti-KBF1/p50 antiserum (Kieran *et al.*, 1990) or anti-v-rel (Rice *et al.*, 1986) or anti-c-rel (Brownell *et al.*, 1989) antisera. The critical DNA constructs are shown in Figure 1. After *in vitro* transcription and translation, dimerization was tested as described in Materials and methods, and the results are

shown in Figure 2. As a positive control we used the Xba construct which codes for a protein (amino acids 1−502) slightly larger than p50. This protein can be immunoprecipitated by anti-p50 (Kieran *et al.*, 1990). As shown in panel A, lane 6 compared with lanes 4 and 5, this construct can associate with c-rel. The Taq construct (amino acids 287−502) does not co-immunoprecipitate with c-rel using anti-c-rel antiserum (lane 1), and conversely c-rel was not co-precipitated with Taq using anti-p50 antiserum (lane 2). Results shown in panel B similarly demonstrate that the Spe construct (amino acids 1−341, lanes 1 and 2) cannot associate with c-rel. Similarly the NH construct (amino acids 253−361) cannot associate with v-rel (panel C). To confirm the results obtained by co-immunoprecipitation we assayed homodimer formation by glutaraldehyde crosslinking of *in vitro* translated products followed by immunoprecipitation. The results shown in Figure 3 indicate that, as expected, the Xba construct is able to dimerize (lanes 1 and 2). Conversely, the NH construct which is unable to form heterodimers with v- or c-rel (not shown) is also unable to homodimerize (lanes 5 and 6). Identical results have been obtained with the Taq (lanes 3 and 4) and Spe (lanes 9 and 10) constructs. These results support our initial hypothesis that the same domain is used for p50−p50 homodimer formation and p50−rel heterodimerization.

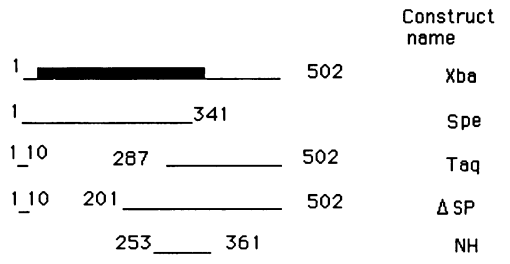

Fig. 1. Schematic diagram of the constructs used in this study. Relevant amino acid numbers are indicated. Shaded box: rel homology region.

We then assayed by co-immunoprecipitation the ΔSP construct which corresponds to a deletion of amino acids 11−200 in the Xba construct; this deletion mutant can associate with v-rel (Figure 4, lane 3) although it is not able to bind DNA (Kieran *et al.*, 1990). Lanes 1 and 2 show that ΔSP is correctly recognized by the anti-p50 antiserum, and lane 5 shows that v-rel is not recognized by this antiserum.

Oligomeric proteins which contain independent functional domains can be inhibited *in vitro* and *in vivo* by a non-DNA binding mutant which has retained its dimerization domain (Herskowitz, 1987). We asked first if the ΔSP mutant could act as a transdominant inhibitor of the wild-type *in vitro*. Various concentrations of ΔSP RNA were co-translated with a constant amount of the Xba construct RNA (wild-type form: Figure 5A, lanes 1−4) or of the v-rel construct (lanes 5−8) or of the c-rel construct (not shown). As shown in panel B, ΔSP is able to inhibit binding of the Xba product to the H-2K^b site when assayed in a gel-shift assay (lanes 1−4). Similar results can be obtained with v-rel (lanes 5−7) and with c-rel (lanes 9 and 10).

We then assayed the *in vivo* effect of the ΔSP mutant by asking whether its product could inhibit *in vivo* the binding of KBF1/p50 or NF-κB to their consensus sequence, as measured by a detectable inhibition of transcriptional activity. We decided to test first whether the introduction of an expression vector coding for ΔSP in T cells could prevent the activation of the NF-κB dependent enhancer of HIV-1. With this aim, we transfected J.Jhan T cells, either with the plasmid LTR-luc (where the reporter gene luciferase is under the control of the HIV-1 LTR) or with 3-Enh-TK-luc (where three copies of HIV-1 enhancer are cloned upstream of the herpes virus-1 thymidine kinase promoter in the TK-luc vector; see Materials and methods) (Schwartz *et al.*, 1990). As expected (Nabel and Baltimore, 1987), addition of phorbol ester (PMA) in the culture medium resulted in a reproducible enhancement of luciferase activity with either plasmid. The amplifications obtained were respectively 7- and 12-fold when LTR-luc and 3-Enh-TK-luc were used (not shown). This stimulation effect was decreased when the

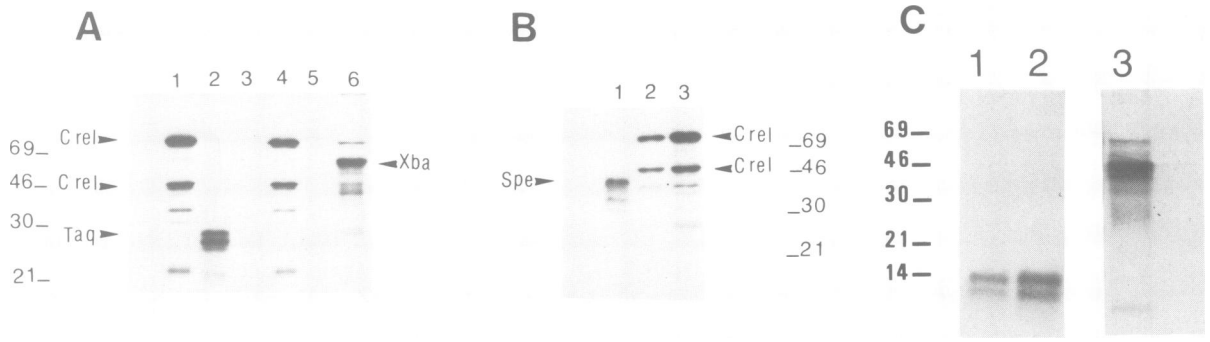

Fig. 2. Co-immunoprecipitation of c-rel with KBF1/p50 constructs. The cDNAs encoded by the constructs shown in Figure 1 were co-translated either alone or together with c-rel cDNA in wheat germ extracts, and immunoprecipitated by anti-KBF1/p50 polyclonal antiserum No. 2 (Kieran *et al.*, 1990) or with anti-c-rel antiserum (Rice *et al.*, 1986; Brownell *et al.*, 1989). The immune complexes were analyzed on a 10% SDS−polyacrylamide gel (panels A and B) or on 12% Tricine−SDS−polyacrylamide gels (panel C). Mol. wt markers (kd) are shown on the side. **A.** Lane 1, immunoprecipitation of co-translated Taq and c-rel constructs with the anti-c-rel antiserum; lane 2, same products immunoprecipitated with the anti-p50 antiserum No. 2 (Kieran *et al.*, 1990); lane 3, immunoprecipitation of the Taq construct with anti-c-rel antiserum; lane 4, immunoprecipitation of the c-rel construct with the anti-c-rel antiserum; lane 5, immunoprecipitation of the c-rel construct with the anti-p50 antiserum; lane 6, immunoprecipitation of co-translated Xba and c-rel constructs with the anti-p50 antiserum. **B.** Lane 1, immunoprecipitation of co-translated Spe and c-rel with the anti-p50 antiserum; lane 2, same products immunoprecipitated with the anti-c-rel antiserum; lane 3, immunoprecipitation of the c-rel product with the anti-c-rel antiserum. The two bands labeled c-rel correspond to the full-length protein (upper band) and to a truncated form probably caused by premature termination (lower band). **C.** Lane 1, immunoprecipitation of co-translated NH and v-rel constructs with the anti-p50 antiserum; lane 2, immunoprecipitation of the NH construct with the anti-p50 antiserum; lane 3, immunoprecipitation of co-translated NH and v-rel constructs with the anti-v-rel antiserum.

CMV-ΔSP construct (where the ΔSP cDNA has been cloned downstream of the cytomegalovirus promoter/enhancer) was co-transfected (Figure 6, lower panels). The level of inhibition of the induced activity of both constructs was dependent on the concentration of CMV-ΔSP DNA used in the co-transfection assays. With both luciferase expression vectors, the inhibition reached ~80% with the highest concentration of CMV-ΔSP DNA used (corresponding to a ratio of 6.7 between CMV-ΔSP and the luciferase reporter plasmid). As a control we replaced CMV-ΔSP by the vector Rc-CMV, which contains no cDNA sequence. We did not observe significant variations in luciferase activity, regardless of the plasmid concentration (upper panels).

We performed similar competition experiments using the enhancer or promoter of the mouse MHC class I H-2K^b gene in HeLa and Cos-7 cells. The results are shown in Table I. It can be seen that both a promoter (containing potential NF-κB binding sites; Israël *et al.*, 1989b) and an enhancer construct derived from the H-2K^b promoter were efficiently inhibited by a five times molar excess of the CMV-ΔSP construct. The inhibition observed with the promoter construct was not as strong as with the enhancer construct, probably due to the fact that there are other elements involved in the basal level of expression of the H-2K^b promoter in the 393 bp of the (393) H2-CAT construct (Kimura *et al.*, 1986). Interestingly both basal and induced levels of the K^b promoter were inhibited by the ΔSP construct. Induction of K^b expression by TNF (Tumor Necrosis Factor) in HeLa cells has been shown to be due to binding of NF-κB to the enhancer sequence while KBF1/p50 (and possibly AP2) are probably responsible for basal level of expression (Israël *et al.*, 1989b). For the Cos-7 cells induction experiments we have used interleukin 1 (IL1) instead of TNF because the stimulation observed was much stronger; bandshift assays have demonstrated that IL-1 treatment resulted in NF-κB induction (not shown). The observed inhibition is more

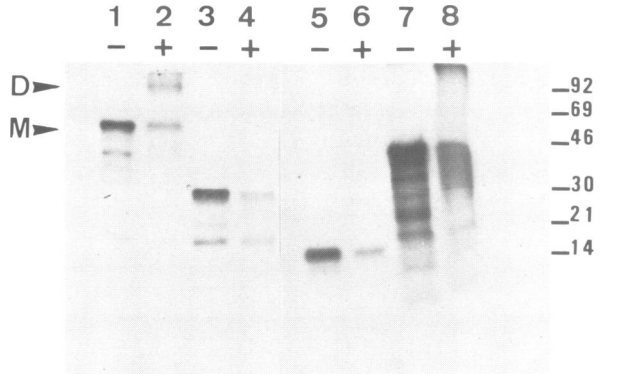

Fig. 3. Glutaraldehyde crosslinking of KBF1/p50 products. *In vitro* translated proteins were either untreated (−) or treated with glutaraldehyde (+) as described in Materials and methods, followed by analysis on Tricine−SDS−polyacrylamide gels: lanes 1 and 2, Xba construct; lanes 3 and 4, Taq construct; lanes 5 and 6, NH construct; lanes 7 and 8, Spe construct. M: monomer. D: dimer.

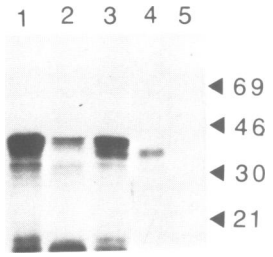

Fig. 4. Co-immunoprecipitation of the v-rel and ΔSP products. The ΔSP construct shown in Figure 1 was translated either alone or together with the v-rel cDNA in wheat germ extracts and immunoprecipitated with anti-p50 or anti-v-rel antiserum (Brownell *et al.*, 1989). The immune complexes were analyzed on a 10% SDS−polyacrylamide gel. Lane 1, immunoprecipitation of ΔSP by the anti-p50 antiserum; lane 2, one-fifth the amount of protein as in lane 1; lane 3, immunoprecipitation of co-translated ΔSP and v-rel by the anti-p50 antiserum; lane 4, immunoprecipitation of v-rel by anti-v-rel antiserum; lane 5, immunoprecipitation of v-rel by anti-p50 antiserum.

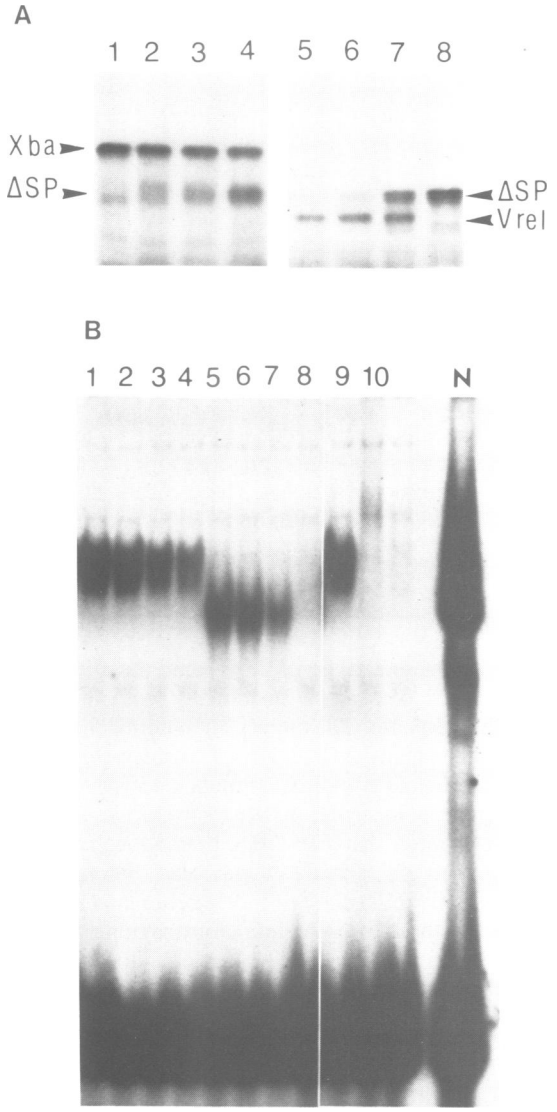

Fig. 5. Transdominant suppression of DNA binding. The following RNAs were translated in wheat germ extracts and the products were either analyzed by PAGE on 10% gels (panel A) or assayed by retardation using the H-2K^b palindrome KBF1 binding site as described in Kieran *et al.* (1990) (panel B). Panels **A** and **B**: lane 1, 1 μl of Xba RNA; lane 2, 1 μl of Xba RNA + 0.5 μl of ΔSP RNA; lane 3, 1 μl of Xba RNA + 1 μl of ΔSP RNA; lane 4, 1 μl of Xba RNA + 3 μl of ΔSP RNA; lane 5, 0.6 μl of v-rel RNA; lane 6, 0.6 μl of v-rel RNA + 0.5 μl of ΔSP RNA; lane 7, 0.6 μl of v-rel RNA + 1 μl of ΔSP RNA; lane 8, 1 μl of ΔSP RNA. Panel **B** only: lane 9, 1 μ l of c-rel RNA; lane 10, 1 μl of c-rel RNA + 2 μl of ΔSP RNA; lane N, 1 μl of HeLa nuclear extract.

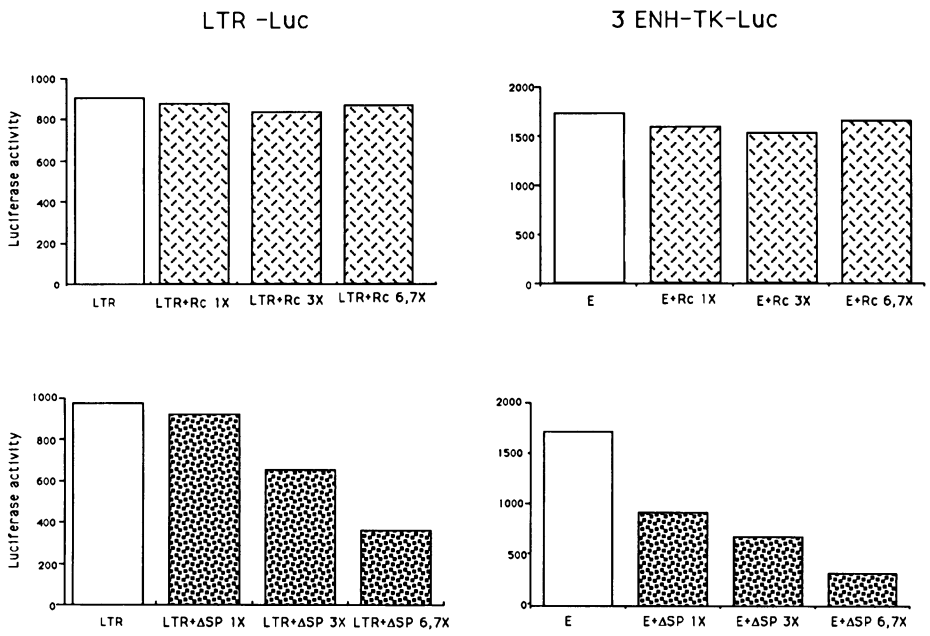

Fig. 6. Inhibition of HIV-1 enhancer or promoter activation by the CMV-ΔSP construct. J.Jhan cells (4×10^5) were co-transfected either with Rc-CMV (upper panels) or with CMV-ΔSP (lower panels) in various amounts, together with a fixed amount of LTR-luc (left panels) of 3-Enh-TK-luc (right panels; E refers to the 3-Enh-TK-luc plasmid). Transfected cells were exposed to PMA from 24 to 40 h after transfection, after which the cells were processed for luciferase assay. The ratio between the competitor (Rc refers to the Rc-CMV vector, ΔSP to the CMV-ΔSP expression vector coding for the ΔSP mutant) and the test plasmid is indicated under the histogram bars. Each bar represents the mean of luciferase activities obtained in four independent experiments. The standard deviation never exceeded 10%.

efficient when cells are induced, suggesting that under these conditions the enhancer region plays a more prominent role in the overall promoter activity. These results demonstrate that the ΔSP mutant can inhibit both KBF1/p50 and NF-κB activities *in vivo* (see Israël *et al.*, 1989b, and Discussion).

Discussion

Experiments presented here show that the dimerization domain of KBF1/p50 can be functionally separated from the DNA binding domain. The dimerization domain is localized between amino acids 201 and 367. Further studies using *Bal*31 digestion and site-directed mutagenesis should allow its more precise localization. Recently, Ip *et al.* (1991) have demonstrated that the DNA binding activity of the *Drosophila* protein dorsal, which also belongs to the rel/NF-κB family (Ghosh *et al.*, 1990; Kieran *et al.*, 1990), is mediated by the NF-κB-rel homology region. An internal deletion between amino acids 245 and 325 does not abolish DNA binding activity. Although dorsal recognizes sites that are slightly different from those recognized by NF-κB, if we assume that the two proteins use the same type of dimerization domain, these results suggest that this domain might be split into two parts and that the region between amino acids 245 and 325 in dorsal (which corresponds to amino acids 270–350 of the KBF1/p50 protein) could be dispensable for dimerization. However, it must be kept in mind that we have not been able to demonstrate association between dorsal and KBF1/p50 by co-immunoprecipitation of co-translated proteins using an anti-p50 antiserum (Kieran *et al.*, 1990). Further studies will be required to answer those questions.

We found no obvious homology with previously characterized motifs in this region, suggesting that KBF1/p50, v-rel and c-rel, which all can form heterodimers, use a novel type of dimerization domain. The internally deleted construct ΔSP, which cannot bind DNA but can form homo- or heterodimers (with KBF1/p50 or c- or v-rel) can inhibit the DNA binding activity of the wild-type construct *in vitro* by forming inactive heterodimers. The same construct acts *in vivo* by inhibiting the transcriptional activity of promoter or promoter elements which have been demonstrated to bind KBF1/p50 or NF-κB, most likely by the same mechanism. These results show that the DNA binding domain of each monomer is required for binding to DNA. However, this does not necessarily imply that the NF-κB complex uses the same surfaces to contact DNA.

Table I. Inhibitory effect of the CMV-ΔSP construct on MHC class I H-2K^b gene promoter or enhancer expression in HeLa and Cos-7 cells

	(ab)4-CAT		(393) H2-CAT			
	HeLa	Cos-7	HeLa		Cos-7	
			−TNF	+TNF	−IL-1	+IL-1
pUC	1[a]	1[b]	1[c]	3.5	1[d]	5.0
Rc-CMV	0.95	0.80	1	3.0	0.9	3.8
CMV-ΔSP	0.24	0.41	0.6	1.1	0.5	0.5

2 μg of each CAT construct was co-translated with 10 μg of either pUC (negative control), Rc-CMV (to assay possible promoter competition) or CMV-ΔSP. 2 μg of RSV-βgal was co-transfected in each case. Cells were either untreated (−) or stimulated with TNF (500 U/ml: HeLa cells) or IL-1 (100 U/ml: Cos-7 cells) during the last 16 h before collection. Cytoplasmic extracts were prepared and CAT and β-galactosidase activities were measured. CAT activity was normalized according to the β-gal activity. The table presents the results of two independent experiments; variation between the observed inhibition values was <20%.

[a,b,c,d]These values were arbitrarily taken as 1, the actual values were: [a]3 CAT units/mg protein (one unit corresponds to 1 nmol of acetylated chloramphenicol/h); [b]8 units/mg protein; [c]0.8 units/mg protein; [d]1.8 units/mg protein.

The recent observation that the p65 protein is also related to the rel family (Ghosh *et al.*, 1990) as well as the clear-cut differences observed between KBF1/p50 and NF-κB in their respective DNA binding properties (Kieran *et al.*, 1990) might suggest a situation similar to the jun-fos system (Turner and Tjian, 1989) where p50 and p65 would bind DNA together, although p65 does not seem to be able to bind by itself (Baeuerle and Baltimore, 1989).

The fact that the ΔSP construct can inhibit both basal and induced expression of MHC class I genes in two cell lines as well as induced HIV expression in a $CD4^+$ T cell line constitutes the first formal proof that these cellular or viral sequences are regulated by a member (or several) of the NF-κB-rel family. It has recently been suggested that the product of the protooncogene *c-rel* might be involved in the overall 'NF-κB' activity observed in activated T cells (Ballard *et al.*, 1990; Molitor *et al.*, 1990). Experiments shown in Figure 5 suggest that ΔSP could inhibit *c-rel* activity *in vivo* as well. Conversely it suggests that the previously cloned factors MBP-1 (Baldwin *et al.*, 1990) or PRDIIBF1 (Fan and Maniatis, 1990) are probably not involved in the expression of these genes at least in the cell lines studied (which include for HIV-1 a $CD4^+$ T cell line which is its physiological host), because their sequences contain no motif homologous to rel or KBF1/p50 and most likely could not form heterodimers with ΔSP. The recent observation that the C-terminal region of the *c-rel* proto-oncogene contains a transcriptional activation domain (Bull *et al.*, 1990) and that it can be translocated into the nucleus of activated T cells (Ballard *et al.*, 1990) suggests that its role in the regulation of a series of genes described as responsive to NF-κB alone has probably been overlooked.

Constitutive over-expression of the ΔSP protein (or similar deletion mutants) is expected to down-regulate all genes expressed under the control of a member of the rel/$NF\kappa_{13}$ family, which include among others MHC class I, immunoglobulin κ-chain, the IL-2 receptor and the β-interferon genes. It could thus interfere with B and T cell activation. In this respect it will be interesting to analyze the phenotype of transgenic mice over-expressing ΔSP. Another intriguing issue is whether there exist 'natural' negative transdominant mutants of NF-κB, similar to the Id member of the helix−loop−helix family of transcription factors (Benezra *et al.*, 1990), that could act to finely tune the expression and activity of this critical factor. The characterization of other members of the rel/NF-κB family should help to answer this question.

Materials and methods

Plasmids

The v-rel cDNA in Bluescript was kindly provided by M.Hannink and H.Temin (Hannink and Temin, 1989). It was cut at the *Hin*cII site for *in vitro* transcription and translation, giving rise to a full-length protein. The *c-rel* cDNA was kindly provided by N.Rice (Brownell *et al.*, 1989) and was cut at the *Xba*I site in the polylinker after the end of the coding region. Xba, Spe and ΔSP plasmids have been described in Kieran *et al.* (1990). The Taq construct (see Figure 1) was derived from the Xba construct by restriction with *Taq*I, filling-up with the Klenow enzyme and digestion with *Xba*I. The resulting fragment (amino acids 287−502) was ligated between the *Stu*I (amino acid 10) and *Xba*I site of the Xba construct. This construct was linearized with *Xba*I for *in vitro* transcription. The NH construct was derived from the cDNA coding for the mouse precursor of p50 (Ghosh *et al.*, 1990; kindly provided by S.Ghosh and D.Baltimore) by digestion with *Nsi*I and *Hin*dIII (this fragment codes for amino acids 244−361, but uses as a start codon an ATG located at position 253; data not shown). This fragment was then cloned between the *Pst*I and *Hin*dIII sites of pBluescript KS+, and linearized with *Hin*dIII for *in vitro* transcription.

The expression vectors used for transient transfections into mammalian cells were constructed as follows: the ΔSp cDNA was excised from the Bluescript construct by digestion with *Hin*dIII and *Xba*I and subcloned in the same sites of the Rc-CMV expression vector (Invitrogen), to give CMV-ΔSP. The Ltr-luc plasmid carries the reporter luciferase gene under the control of the U3R (*Bgl*II−*Hin*dIII fragment) of HIV-1 (LAV1 Bru strain) LTR. The 3-Enh-TK-luc construct contains three copies of the synthetic oligonucleotide:

a g c t t ACAAGGGACTTTCCGCTGGGGACTTTCCAGGGa
a TGTTCCCTGAAAGGCGACCCCTGAAAGGTCCCt t c g a

corresponding to the two 10 bp repeats of the HIV-1 enhancer (−107 to −76 in the HIV-1 LTR) cloned upstream of a truncated HSV TK promoter (−105 to +51) in a TK-luc expression vector (Schwartz *et al.*, 1990). Lower case letters correspond to *Hin*dIII compatible linker ends that do not belong to the LTR sequence; the two putative NF-κB binding sites are in bold.

The (ab)4-CAT construct has been described in Israël *et al.* (1989b) and contains the enhancer element of the H-$2K^b$ promoter (coordinates −171 to −158: TGGGGATTCCCCAT) cloned in the *Bam*HI site located upstream of the conalbumin transcription start site in the conaCAT expression vector (Kimura *et al.*, 1986). The (393) H2-CAT construct contains 393 bp of the MHC class I H-$2K^b$ promoter [including the enhancer element of the (ab)4-CAT construct] cloned upstream of the CAT gene and has been described in Kimura *et al.* (1986).

Cell cultures and reagents

The $CD4^+$ human lymphoblastoid T cell line J.Jhan derived from the Jurkat cell line (a kind gift from J.D.Fox, London) was grown in RPMI 1640 (GIBCO) medium supplemented with 10% FCS (Boehringer) and antibiotics. PMA was from Sigma (St Louis). Human HeLa and monkey Cos-7 cells were grown in DMEM supplemented with 10% new-born calf serum.

Transfection

J.Jhan cells were transfected by DEAE−dextran using a microtransfection technique (Schwartz *et al.*, 1990). Briefly, cells (4×10^5) were transfected with a fixed amount (30 ng) of either LTR-luc or 3-Enh-TK-luc. CMV-ΔSP was co-transfected with either plasmid at various concentrations (30−200 ng). As a control, Rc-CMV was used at the same concentrations as CMV-ΔSP. Transfected cells were exposed to PMA (20 ng/ml) for 16 h, from 24 to 40 h after transfection. Luciferase activity was measured according to Schwartz *et al.* (1990).

HeLa and Cos-7 cells were transfected using the calcium phosphate co-precipitation technique as described in Kimura *et al.* (1986). The RSV-βgal construct was co-transfected along with the various CAT constructs, and the β-galactosidase activity was used to correct for variations due to variable transfection efficiencies (Kimura *et al.*, 1986).

In vitro transcription and translation

4 μg of various derivatives of the p50 cDNA cloned in pBluescript were linearized with appropriate restriction enzymes and *in vitro* transcribed using T7 or T3 polymerase according to the manufacturer's recommendation (Stratagene). RNA was extracted, ethanol precipitated and redissolved in 20 μl of water. RNA (2 μl) was translated in wheat germ extracts (Promega) adjusted to 60 mM potassium acetate.

Immunoprecipitations

These were carried out as described in Murre *et al.* (1989) in a 100 μl volume containing 2 μl of *in vitro* translated products, 1 μl of anti-p50 antiserum No. 2 (Kieran *et al.*, 1990), anti-v-rel (Rice *et al.*, 1986) or anti-c-rel (Brownell *et al.*, 1989) and 20 μl of protein A−Sepharose CL4B (Pharmacia). The immune complexes were analyzed by SDS−polyacrylamide (Laemmli, 1970) using 10 or 12% Tricine−SDS gels (Schägger and von Jagow, 1987).

Gel-shift assays

These were performed as described in Israël *et al.* (1987).

Glutaraldehyde crosslinking

Equal amounts of *in vitro* translated products were diluted 15 times and treated or not with 0.002% glutaraldehyde for 1 h at room temperature. Samples were then immunoprecipitated with anti-p50 antiserum in a volume of 500 μl and analyzed on 12% polyacrylamide−SDS−Tricine gels.

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