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Cloning of the DNA-binding subunit of human nuclear factor κB: The level of its mRNA is strongly regulated by phorbol ester or tumor necrosis factor α

(transcription factor/protein purification/DNA sequencing/regulation of nuclear factor κB expression/multigene family)

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ABSTRACT **The DNA binding subunit of nuclear factor κB (NF-κB), a B-cell protein that interacts with the immunoglobulin κ light-chain gene enhancer, has been purified from nuclei of human HL-60 cells stimulated with tumor necrosis factor α (TNFα), and internal peptide sequences were obtained. Overlapping cDNA clones were isolated and sequenced. The encoded open reading frame of about 105 kDa contained at its N-terminal half all six tryptic peptide sequences, suggesting that the 51-kDa NF-κB protein is processed from a 105-kDa precursor. An *in vitro* synthesized protein containing most of the N-terminal half of the open reading frame bound specifically to an NF-κB binding site. This region also showed high homology to a domain shared by the *Drosophila* dorsal gene and the avian and mammalian *rel* (proto)oncogene products. The level of the 3.8-kilobase mRNA was strongly increased after stimulation with TNFα or phorbol ester. Thus, both factors not only activate NF-κB protein, as described previously, but also induce expression of the gene encoding the DNA-binding subunit of NF-κB.**

Nuclear factor κB (NF-κB) (for review, see ref. 1) is a pleiotropic transcription factor that is involved in the expression of various viral and cellular genes. It was first discovered as one of the B-cell proteins interacting with the immunoglobulin κ light-chain gene enhancer (2), and its binding site was found to be critical for the B-cell-specific transcriptional activity of the κ light-chain enhancer (3). NF-κB is either constitutively active, as in mature B-lymphocytes (2), monocytes, and macrophages (4), or present in an inactive cytosolic complex together with an inhibitory protein called IκB (5). Inactive NF-κB could be activated by stimulation of cells with a variety of agents, such as phorbol esters [phorbol 12-myristate 13-acetate (PMA)], bacterial lipopolysaccharides, cAMP, the cytokines tumor necrosis factors α and β (TNFα and TNFβ) and interleukin 1 (IL-1), the human T-cell lymphotrophic virus type I tax protein, and double-stranded RNA (refs. 6–9 and references therein). *In vitro* activation was obtained by treatment of the inhibited cytosolic complex with sodium desoxycholate or formamide (5), involving dissociation of IκB from its cytosolic complex with NF-κB. It is presumed that phosphorylation of IκB leads to its dissociation and release of NF-κB as shown recently *in vitro* (10).

NF-κB has been purified from human B lymphocytes and has been shown to contain 51-kDa and 68-kDa proteins (p51 and p68), of which p51 was binding to DNA (11). This purified fraction could stimulate transcription from the human immunodeficiency virus type 1 promoter *in vitro* (11). Similar sizes of 50 and 65 kDa were obtained for NF-κB purified from HeLa cell cytosol (p50 and p65) (12). Whereas p50 was found to be the DNA-binding component, p65 was required for inactivation of NF-κB by IκB (12).

Because of the importance of NF-κB in the expression of genes involved in immune responses, we decided to clone and sequence the gene encoding the DNA-binding protein of NF-κB.§

After completion of our work, two reports appeared (13, 14) describing cloning of murine NF-κB p50 and human KBF1. According to the one report (14), KBF1, a protein involved in the constitutive expression of the *H-2* and β_2-microglobulin gene enhancers (15), is identical to NF-κB p50. The cDNA we cloned differs in having one amino acid less than the other reported human cDNA (ref. 14; also see below). We demonstrate that the encoding mRNA is strongly inducible by phorbol ester or TNFα and discuss a mechanism involving regulation of the gene encoding NF-κB.

MATERIALS AND METHODS

Cell Culture and Preparation of Nuclear Extracts. HL-60 cells were grown as described (16). At a density of 4.7×10^6 cells per ml, 1.2 nM TNFα (20 ng/ml) was added. After 1 hr nuclear extracts were prepared essentially as described (17).

Protein Sequencing of NF-κB. NF-κB was purified from 500 ml of nuclear extract as described (11). Approximately 50 μg of protein from the purest fraction was transferred to a polyvinylidene difluoride membrane after CCl_3COOH precipitation and SDS/PAGE. The 51-kDa band was excised and the fixed protein was blocked with polyvinylpyrrolidone (0.5% in 100 mM acetic acid) and then digested with trypsin (1 μg in 100 mM sodium bicarbonate). Peptides were separated by HPLC using a 2.1 × 100 mm Brownlee Aquapore reversed phase 300 column and sequenced with an Applied Biosystems 470A sequencer.

Renaturation After SDS/PAGE. Proteins were renatured essentially as described (18). Pellets of eluted proteins were dissolved in the presence of 7 M urea for 20 min at room temperature and diluted 40-fold at 0°C. For corenaturation, different protein samples were mixed immediately after dilution. After 20 hr of renaturation at 4°C, binding reactions were performed.

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Abbreviations: NF-κB, nuclear factor κB; PCR, polymerase chain reaction; PMA, phorbol 12-myristate 13-acetate; ORF, open reading frame; TNFα and TNFβ, tumor necrosis factors α and β; IL-1, interleukin 1.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M58603).

Construction of the cDNA Bank and Isolation of cDNA Clones. Total RNA from the human lymphoblastoid cell line BJA-B was isolated, and poly(A)$^+$ RNA was purified by oligo(dT) chromatography. Oligo(dT)-primed cDNA was ligated into phage λ Zap II (Stratagene) with *Eco*RI linkers. Plaques (1.2 million) from the nonamplified cDNA library were screened with "best guess" oligonucleotides (19) based on two tryptic sequences (amino acids 165–180 and 231–243 in Fig. 2*B*). The oligonucleotides were: 5′-GCCTGIAGGTAGGCIAGGTCAGGGTGCACIAGIAGGCCAGGGTTGTA and 5′-GTCATAGATGGCATCIGAGACCACAGGCTCIAG, in which I is inosine. The filters were washed in 0.90 M NaCl/0.09 M sodium citrate, pH 7/0.05% sodium pyrophosphate at 50°C. The isolated cDNAs were sequenced on both strands (20).

Expression of Protein Fragments by Using *in Vitro* Transcription–Translation. Polymerase chain reactions (PCRs) were performed essentially according to the Perkin–Elmer/Cetus *Thermus aquaticus* (*Taq*) polymerase protocol. The 5′ PCR primer contained the phage T7 promoter sequence 5′-TAATACGACTCACTATAGGGAGA, followed by a translation-initiation sequence, 5′-CCACCATG (21), and in-frame sequences from nucleotides 506 to 523 (see Fig. 2*B*), the 3′ PCR primer being complementary to nucleotides 1606–1622 (Fig. 2*B*). The PCR products were expressed in coupled transcription–translation reactions (22), and products were tested for DNA binding by using electrophoretic mobility shift assays (11). Endogenous NF-κB-like activity in the lysate was depleted by pretreatment with the same amount (vol/vol) of NF-κB affinity resin for 1 hr at 0°C where indicated.

RNA (Northern) Blot Analysis. HL-60, HeLa, and 70Z/3 cells were stimulated by incubation at 37°C for 3 hr with either 40 nM PMA or 10 nM TNFα. Total RNA was prepared from treated and nontreated cells by the guanidine thiocyanate procedure (22). Poly(A)$^+$ RNA was isolated with oligo(dT)-cellulose, resolved on 1% agarose gels containing formaldehyde (21), and transferred to a Zeta-Probe membrane. The filter was hybridized overnight at 65°C in 0.5 M sodium phosphate, pH 7.2/1 mM EDTA/7% SDS/1% bovine serum albumin. The filter was washed at high stringency (0.015 M NaCl/0.0015 M sodium citrate, pH 7/0.1% SDS at 65°C).

RESULTS

Purification of NF-κB from HL-60 Cells. NF-κB was purified from HL-60 cells stimulated with TNFα (7) essentially as described (11). As observed in the purification from human B cells (11), the purest fraction obtained from stimulated HL-60 cells also consisted of 51- and 65-kDa species (Fig. 1*A*). The purified NF-κB bound to the κ-chain gene enhancer binding site (Fig. 1*B*, lane 2), giving rise to two complexes C1 and C2. In methylation interference experiments (data not shown), both complexes showed the same typical pattern, as observed earlier (11). However, the exact nature of both complexes is not known.

Proteins were isolated from several molecular mass regions of the SDS gel (Fig. 1*A*), renatured (18), and assayed for DNA binding. Only p51 bound directly to DNA (Fig. 1*C*, lane 2). The resulting complex, C3, had an intermediate mobility compared to the C1 and C2 complexes obtained with native NF-κB. Corenaturation of p51 and p65 yielded the C1 and C2 complexes (Fig. 1*C*, lane 5) as observed with native NF-κB. p65, which does not bind itself (Fig. 1*C*, lane 4), had to be renatured together with p51 to interact with p51, whereas mixing of separately renatured p65 and p51 yielded only the DNA–protein complexes obtained with p51 alone (Fig. 1*C*, compare lanes 5 and 7). An interaction similar to that of our highly purified protein components was also observed for the cytosolic NF-κB subunits (12), indicating further that we have purified *bona fide* NF-κB.

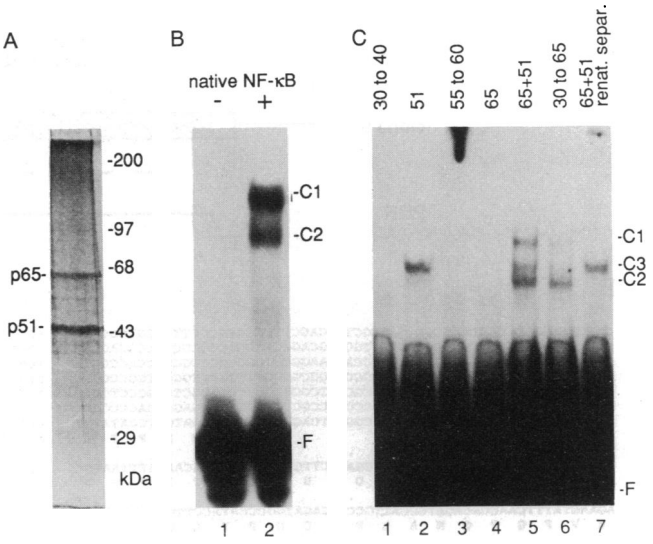
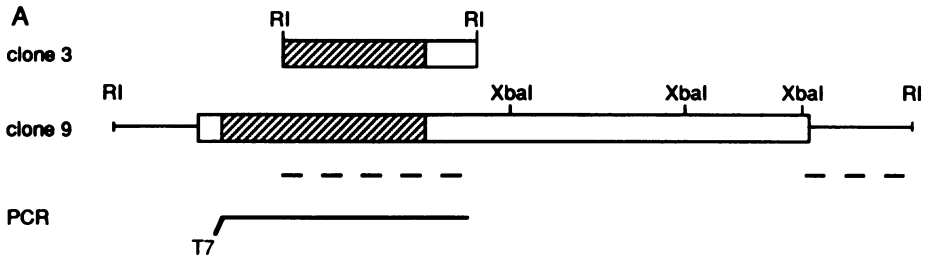


FIG. 1. Purification of NF-κB. (*A*) Fifteen microliters of the purest fraction obtained was analyzed by SDS/PAGE and silver staining. The positions of the p51 and p65 proteins and the molecular mass standards are indicated. (*B*) A radiolabeled oligonucleotide containing the κ-chain gene enhancer binding site (11) was incubated without (lane 1) or with (lane 2) purified NF-κB. Protein–DNA complexes were resolved on 4% native polyacrylamide gels. (*C*) Proteins were eluted from different regions of the SDS/PAGE gels shown in *A*, renatured, and tested for binding to the NF-κB binding site. For lane 7, p51 and p65 were renatured separately and then mixed for DNA binding incubation. The positions of the protein–DNA complexes C1–C3 are indicated as well as the position of the unbound oligonucleotide (F). Numbers at the top of the lanes indicate molecular mass regions in kDa.

Protein Sequencing and Isolation of the Gene Encoding the p51 Subunit of NF-κB. The purified NF-κB fraction (Fig. 1*A*) was blotted onto a polyvinylidene difluoride membrane after SDS/PAGE, and the 51-kDa band was isolated and used for sequencing. Whereas the N terminus was blocked, six tryptic peptides were sequenced after HPLC separation (Fig. 2*B*). Best-guess oligonucleotides were synthesized by using two of the peptide sequences and were used to screen a human cDNA library. Two overlapping clones were obtained (Fig. 2*A*) that hybridized to both probes, and the entire sequence of the 3.85-kilobase (kb) insert of clone 9 was determined (Fig. 2*B*). An open reading frame (ORF) was found that potentially encodes a protein of 105 kDa. All six peptide sequences were located in the N-terminal half of the ORF (Fig. 2*B*), suggesting that the 51-kDa protein is posttranslationally processed from the N-terminal half of the predicted 105-kDa precursor protein (p105). The methionine at position 398 (Fig. 2*B*) is preceded by a stop codon and could represent the N terminus of p51. A 51-kDa protein species could then be encoded by the first 490 amino acids of the ORF in the absence of posttranslational modifications. Sequence comparison with the cDNA sequence of human KBF1/NF-κB p50 (14) revealed the lack of one whole codon for Ala-515 (Fig. 2*B*) in our sequence. This may account for an allelic difference.

Strong homology was found between the region containing amino acids 42–366 of the ORF and the *Drosophila* dorsal and the vertebrate c-*rel* gene products (Fig. 3). The homology was highest with the rel protein showing 47% identity and 63% similarity, whereas rel and dorsal shared a similarity of nearly 80% (23).

The N-Terminal Part of the ORF Encodes a Polypeptide Binding to NF-κB Sites. We used PCR (25) to generate DNA



B

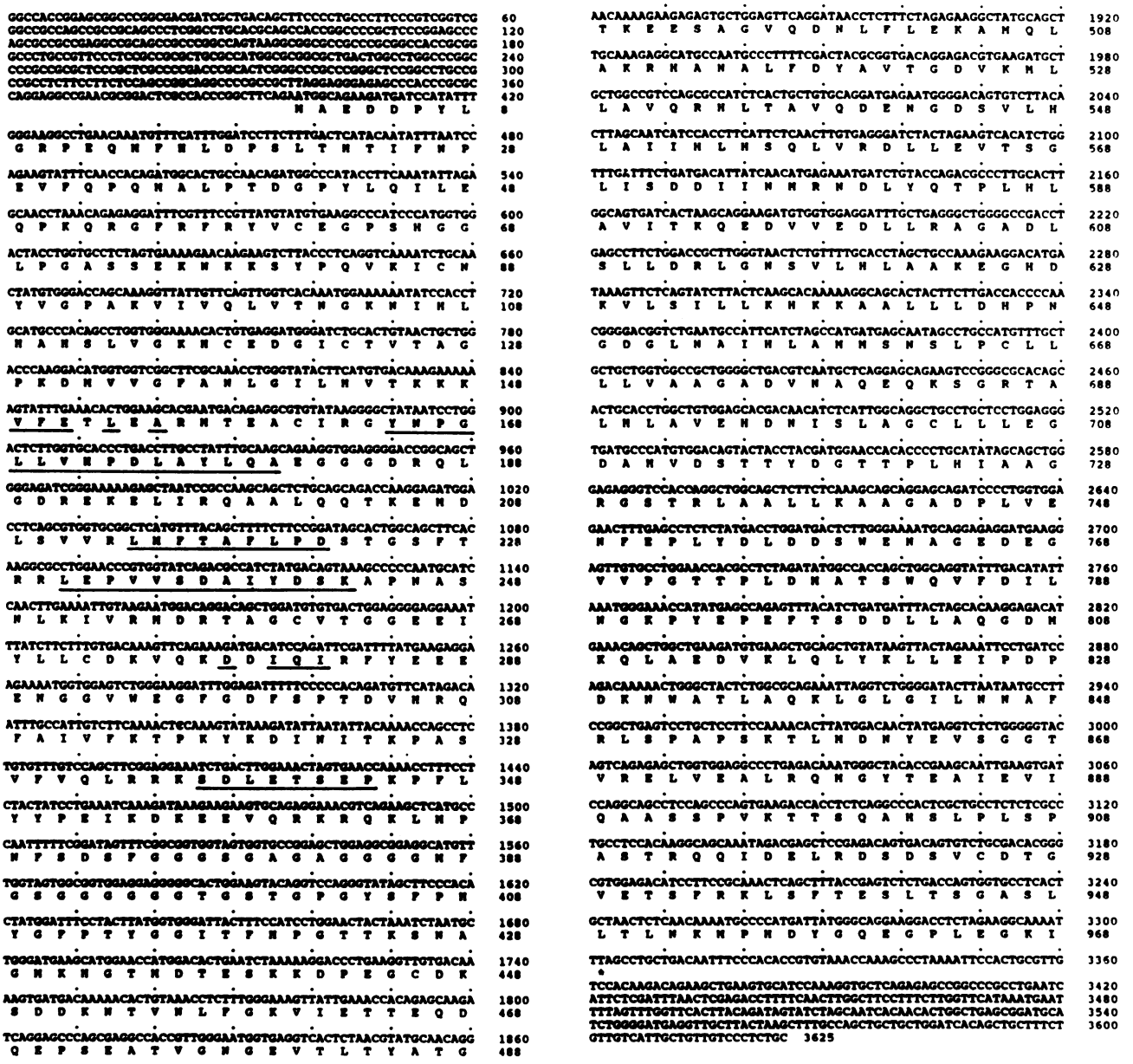


FIG. 2. (*A*) Diagram of clones 3 and 9. ORFs are shown by bars; nontranslated regions, by solid lines; the conserved region, by filled bars; and the Northern blot probes, by broken lines. The position of the PCR product used for *in vitro* transcription–translation is indicated with "T7" followed by a line. (*B*) DNA sequence of the cDNA and the predicted and determined protein sequences. The tryptic peptide sequences are underlined.

fragments suitable for coupled transcription–translation of clone 9. Translation products of the amplified and transcribed DNA fragments were used in gel shift assays with the NF-κB binding site probe. The reticulocyte lysate contained an endogenous NF-κB-like activity (Fig. 4*A*, lanes 1–3, endogenous binding factor). Whereas translation products of the full-length ORF were refractory to DNA binding (data not shown), a DNA–protein complex was obtained with a translation product containing amino acids 37–408 (Fig. 2*A*). The DNA binding of this protein was specific (Fig. 4*A*, lanes 4–6) and was also assayed in reticulocyte lysates that were affinity-depleted of most of the endogenous NF-κB-like activity (Fig. 4*B*). Thus, specific DNA binding is obtained when the homology region (Fig. 3) is present in the protein and does not

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P51      39 TDG....PYLQILEQPKQRGFRFRYVCEGPSHGGLPGASSEKNKKSYPQV
C-REL     1 ISE....PYIEIFEQPRQRGMRFRYKCEGRSAGSIPGEHSTDNNKTFPSI
DORSAL   40 TKNVRKKPYVKITTEQPAGKALRFRYECEGRSAGSIPGVNSTPENKTYPTI

P51      85 KICNYVGPAKVIVQLVTNGKNIHLHAHSLVGKH.CEDGICTVTAGPKDMV
C-REL    47 QILNYFGKVKIRTTLVTKNEPYKPLPHDLVGKD.CRDGYYEAEFGPERRV
DORSAL   90 EIVGYKGRAVVVVSCVTKDTPYRPHPHNLVGKEGCKKGVCTLEINSETMR

P51     134 VGFANLGILHVTKKKVFETLEARMTEACIRGYNPGLLVHPDLAYLQAEGG
C-REL    96 LSFQNLGIQCVKKKDLKESISLRISKK.INPFN.................
DORSAL  140 AVFSNLGIQCVKKKDIEAALKAR.EEIRVDPFKTGF..............

P51     184 GDRQLGDREKELIRQAALQQTKEMDLSVVRLMFTAFL.PDSTGSFTRRLE
C-REL   123 ............VPEEQLHNIDEYDLNVVRLCFQAFL.PDEHGNYTLALP
DORSAL  175 .SHRF..............QPSSIDLNSVRLCFQVFMESEQKGRFTSPLP

P51     233 PVVSDAIYDSKAPNASNLKIVRMDRTAGCVTGGEEIYLLCDKVQKDDIQI
C-REL   165 PLISNPIYDNRAPNTAELRICRVNKNCGSVKGGDEIFLLCDKVQKDDIEV
DORSAL  210 PVVSEPIFDKKA..MSDLVTCRLCSCSATVFGNTQIILLCEKVAKEDISV

P51     283 RFYEEEENGGVWEGFGDFSPTDVHRQFAIVFKTPKYKDINITKPASVFVQ
C-REL   215 RFVLDN.....WEAKGSFSQADVHRQVAIVFRTPPFLR.DITEPITVKMQ
DORSAL  258 RFFEEKNGQSVWEAFGDFQHTDVHKQTAITFKTPRYHTLDITEPAKVFIQ

P51     333 LRRKSDLETSEPKPFLYYPEIKDKEEVQRKRQK.........LMPNFSDS
C-REL   259 LRRPSDQEVSEPMDFRYLPDEKDPYGNKAKRQR.........STLAWQKL
DORSAL  308 LRRPSDGVTSEALPFEYVPMDSDPAHLRRKRQKTGGDPMHLLLQQQQKQQ
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FIG. 3. Comparison of the conserved domain in the DNA binding protein of NF-κB (p51) and the *Drosophila* dorsal and human c-*rel* gene products. Conserved amino acids are shaded, and identical amino acids are printed in boldface letters. Only parts of the protein sequences of the *Drosophila* dorsal (23) and chicken c-rel (24) proteins are shown. No significant homologies were seen outside of these regions.

require sequences C-terminal to amino acid 408. A translation product containing the protein sequence from position 145 to position 438 in Fig. 2*B* and lacking the first third of the conserved domain did not bind to DNA (data not shown), implying that the conserved domain contains the DNA binding domain of NF-κB.

Levels of mRNA Encoding Human NF-κB Are Increased Upon Treatment with TNFα and Phorbol Ester. Northern blot analysis was performed with different fragments of the cloned cDNAs as probes. With a probe from the 5′ region of clone 9 (Fig. 2*A*), a 3.8-kb transcript was detected under stringent hybridization conditions in the unstimulated human cells (Fig. 5*A*, lanes 1 and 3). The transcript levels were strongly increased when HL-60 and HeLa cells were stimulated with TNFα or the phorbol ester PMA (Fig. 5*A*, lanes 2, 4, and 5), while the mouse pre-B-cell line 70/Z3 did not show any signal even after stimulation (lanes 6–8). This was presumably due to species-dependent sequence differences and the high stringency used. In fact, in unstimulated 70/Z3 cells, only a weak signal was observed even with the homologous murine probe (13). The same pattern was obtained when the blot was hybridized with a 3′ probe of clone 9 (Figs. 2*A* and 5*C*). The complete removal of the 5′ probe before hybridization with the 3′ probe is shown in Fig. 5*B* and control hybridization with an actin probe is shown in Fig. 5*D*. Thus, expression of the NF-κB gene seems to be strongly regulated by TNFα and phorbol esters at the level of mRNA abundance.

DISCUSSION

In an attempt to dissect the components involved in gene regulations by NF-κB, we molecularly cloned the DNA-binding subunit of NF-κB, p51. A precursor was isolated

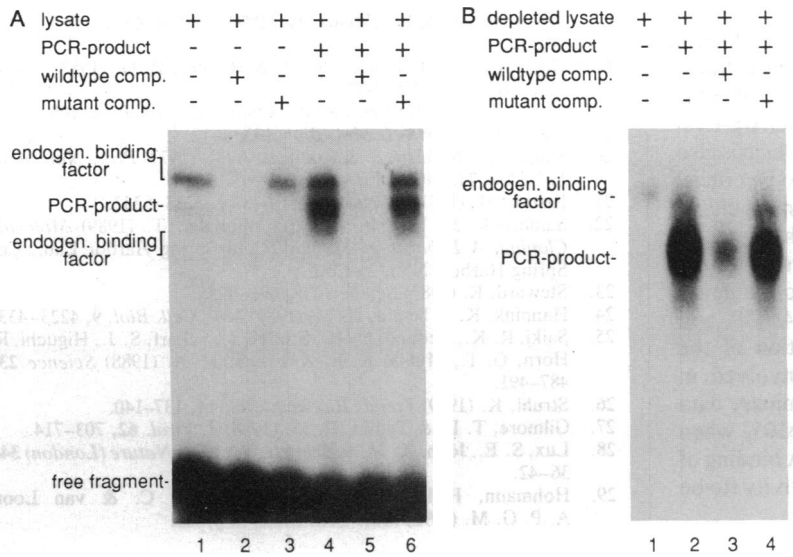

FIG. 4. (*A*) A protein fragment containing amino acids 37–408 was produced by coupled transcription–translation of PCR products in reticulocyte lysates. Electrophoretic mobility-shift assays were performed with either reticulocyte lysate alone (lanes 1–3) or lysate programmed with the PCR product (lanes 4–6). Either the wild-type κ-chain gene enhancer binding site oligonucleotide (11) (lanes 2 and 5) or the mutant oligonucleotide (11) (lanes 3 and 6) was added as competitor in 60-fold molar excess. (*B*) The reticulocyte lysate was depleted of (most of) the endogenous NF-κB-like activity prior to use in *in vitro* protein synthesis. Either wild-type (lane 3) or mutant competitor DNA (lane 4) was added. The unbound DNA left the gel.

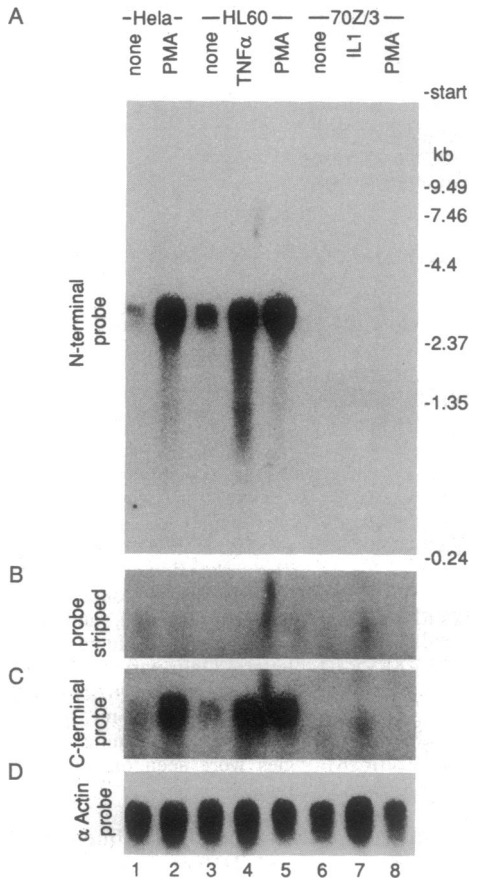

FIG. 5. Northern blot. Poly(A)$^+$ RNA was prepared from HeLa, HL-60, and 70Z/3 cells, which were stimulated with PMA, TNFα, or IL-1 where indicated. (*A*) The blot was hybridized with the 1-kb *Eco*RI fragment of clone 3 (Fig. 2*A*, N-terminal probe). The filter was rehybridized with a human α-actin probe under identical hybridization conditions after removal of the first probe by heating (*D*). After complete removal of this probe (*B*), the filter was rehybridized with an *Xba* I probe of clone 9 (*C* and Fig. 2*A*, C-terminal probe). Molecular masses of RNA markers are indicated.

whose N-terminal half encoded all tryptic peptide sequences and a region of homology to the *Drosophila* dorsal and vertebrate *rel* gene products. This region also encoded specific DNA-binding activity and, having no homology to other human DNA-binding motifs, such as leucine zipper, zinc finger, and helix–loop–helix (26), seems to contain a different type of conserved DNA-binding domain.

Since the homology is shared by proteins whose biological function involves, at least in part, cytoplasmic–nuclear translocation, still other functions might be contained in it. One obvious motif is a conserved nuclear transfer signal (Arg-Lys-Arg-Gln-Lys, positions 361–365 in Figs. 2*B* and 3), which is also present in rel and dorsal (23) and has been shown to be crucial for nuclear localization of v-rel (27). Other functions could be the interaction with other protein components such as p65.

Interestingly, the C-terminal half of the precursor protein contains seven ankyrin-related repeats (28), each of 31 amino acids, starting at positions 543, 582, 615, 651, 684, 719, and 772 in the protein sequence (Fig. 2*B*). A function of the repeats remains obscure, but they might be involved in attachment to cytosolic structures (28). Our preliminary data indicate that regions of the C-terminal half of p105, when expressed as a separate molecule, can inhibit DNA binding of native NF-κB, suggesting an encoded inhibitor activity (to be published elsewhere).

Whatever the exact nature of one or more C-terminal processing product(s) of the precursor is, the synthesis in one transcriptional unit provides tightly coordinated expression, which might be required for stoichiometric components of multiprotein complexes. Because of the strong inducibility of NF-κB p51 precursor mRNA (see below), there should be also a similar inducibility of the p65 subunit, given its stoichiometric appearance with p51 in native NF-κB and the apparent absence of free cytosolic p65 (12).

It has been reported that TNFα activates within minutes the appearance of nuclear NF-κB (7), which originates from the inactive cytosolic complex of NF-κB with the IκB protein (5). As found in cycloheximide studies, active NF-κB is highly unstable (29), and the existing cytosolic pool of inactive NF-κB is rapidly exhausted, thus requiring *de novo* synthesis for maintenance of NF-κB activity. Here we directly show that the levels of the mRNA encoding NF-κB p51 are strongly increased upon incubation of cells with TNFα or PMA. These data indicate that for fast responses to external signals preformed cytosolic NF-κB is activated but that longer lasting responses need the direct induction of NF-κB mRNA, where NF-κB itself may be its own second messenger.

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