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Alternate RNA Splicing of Murine *nfkb1* Generates a Nuclear Isoform of the p50 Precursor NF-κB1 That Can Function as a Transactivator of NF-κB-Regulated Transcription

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The NF-κB1 subunit of the transcription factor NF-κB is derived by proteolytic cleavage from the N terminus of a 105-kDa precursor protein. The C terminus of p105NF-κB1, like those of IκB proteins, contains ankyrin-related repeats that inhibit DNA binding and nuclear localization of the precursor and confer IκB-like properties upon p105NF-κB1. Here we report the characterization of two novel NF-κB1 precursor isoforms, p84NF-κB1 and p98NF-κB1, that arise by alternate splicing within the C-terminal coding region of murine *nfkb1*. p98NF-κB1, which lacks the 111 C-terminal amino acids (aa) of p105NF-κB1, has a novel 35-aa C terminus encoded by an alternate reading frame of the gene. p84NF-κB1 lacks the C-terminal 190 aa of p105NF-κB1, including part of ankyrin repeat 7. RNA and protein analyses indicated that the expression of p84NF-κB1 and p98NF-κB1 is restricted to certain tissues and that the phorbol myristate acetate-mediated induction of p84NF-κB1 and p105NF-κB1 differs in a cell-type-specific manner. Both p84NF-κB1 and p98NF-κB1 are found in the nuclei of transfected cells. Transient transfection analysis revealed that p98NF-κB1, but not p105NF-κB1 or p84NF-κB1, acts as a transactivator of NF-κB-regulated gene expression and that this is dependent on sequences in the Rel homology domain required for DNA binding and on the novel 35 C-terminal aa of this isoform. In contrast to previous findings, which indicated that p105NF-κB1 does not bind DNA, all of the NF-κB1 precursors were found to specifically bind with low affinity to a highly restricted set of NF-κB sites in vitro, thereby raising the possibility that certain of the NF-κB1 precursor isoforms may directly modulate gene expression.

The NF-κB-like transcription factors comprise a group of homodimeric and heterodimeric proteins, all the subunits of which are encoded by a small multigene family related to the v-*rel* oncogene (1, 6, 17, 38). The best characterized of these factors, NF-κB, comprises a heterodimer of 50-kDa (p50) and 65-kDa (p65) proteins. NF-κB binds to decameric sequences conforming to the motif GGGARNNYYCC (κB site) found within the promoters and enhancers of viral genes and cellular genes (1, 26). In most cell types, NF-κB-like proteins are found in the cytoplasm in a non-DNA-binding form, associated with a family of regulatory proteins termed inhibitors (IκBs) (1, 6, 17). The cytoplasmic form of NF-κB is activated by a wide range of extracellular signals which lead to the phosphorylation of IκB (15, 27) and its dissociation from NF-κB and subsequent degradation (10, 21), thereby allowing NF-κB to be translocated to the nucleus.

In mammals, five different genes encoding proteins homologous to the v-*rel* oncoprotein have been identified. p65 (RelA), RelB, p52/p100(NF-κB2), p50/p105(NF-κB1), and the product of the c-*rel* proto-oncogene all share a highly conserved domain of approximately 300 amino acids termed the Rel homology domain that is located in the amino-terminal halves of these proteins. The Rel homology domain contains sequences important for DNA binding, protein dimerization, and nuclear localization (1, 6, 17, 38). Rel-related proteins can be divided into two groups. The c-*rel* protein, p65, and RelB each have divergent carboxyl-terminal halves to which transcriptional transactivating domains have been assigned (6, 11, 12, 38, 41, 43, 46).

By contrast, the p52 and p50 subunits, which consist largely of the Rel homology domain, are derived from the amino termini of larger precursor proteins, p100 and p105, respectively, by proteolytic cleavage (7, 13, 16, 25, 31, 36, 45). The carboxyl termini of both precursors, which are rapidly degraded after proteolytic cleavage (13, 31), contain seven repeated domains, each consisting of 34 to 40 amino acids, that are related to motifs found in the erythrocyte cytoskeletal protein ankyrin and a number of proteins involved in transcription, cell cycle control, and differentiation (6, 38).

The characterization of several different inhibitors has revealed that these proteins also contain six or seven ankyrin-like repeats related to those found in the C termini of p100 and p105, which in the case of IκBα and bcl-3 are essential for inhibiting DNA binding of NF-κB complexes (8, 24) and in the case of IκBα are essential for preventing nuclear translocation of NF-κB (3). The role of the ankyrin repeats in IκB proteins is consistent with the observation that the carboxyl-terminal halves of p100 and p105 both appear to inhibit the ability of the precursors to bind DNA in vitro and restrict the precursors to the cytoplasm (16, 22, 25). Furthermore, p100 and p105 can also act as cytoplasmic inhibitors of p50, p65, and c-*rel* (31, 40). Murine *nfkb1*, in apparent contrast to the human homolog, also encodes a 70-kDa inhibitor, termed IκBγ. This cytoplasmic protein, which corresponds to the carboxyl-terminal 607 amino acids of murine p105, is encoded by a 2.6-kb mRNA that is expressed in a wide range of tissues (14, 23, 28). We have recently identified isoforms of IκBγ generated by alternate RNA splicing of murine *nfkb1* (19). Initial characterization of this RNA splicing revealed that these splices are also found

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within the transcripts encoding the p50 precursor (19). Here we describe the functional properties of these p50 precursor isoforms and find that in contrast to p105, which is a cytoplasmic protein, certain isoforms are found in the nucleus. Moreover, one isoform is able to transactivate κB-regulated gene expression. The mechanism by which this transactivation occurs is examined, and the impact of these findings for gene regulation by NF-κB-like proteins is discussed.

MATERIALS AND METHODS

Cell culture. All cell lines with the exception of lymphoid cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) (Flow Labs). Murine B-cell lines and the murine T-cell line AT2.5 were grown in RPMI 1640–10% FCS–50 μM 2-mercaptoethanol (2-ME). The T-cell line p41.1 was grown in a solution containing RPMI 1640, 10% FCS, 50 μM 2-ME, and 10 U of interleukin 2 per ml. Confluent 10-cm-diameter dishes of short-term-passage NIH 3T3 fibroblasts were starved of serum for 24 h prior to stimulation with either 20% FCS or phorbol myristate acetate (PMA) (75 ng/ml). Concanavalin A (5 μg/ml) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (75 ng/ml) stimulation of the T-cell line p41.1 was done in RPMI 1640–1% FCS–50 μM 2-ME.

RNase protection mapping. Ten-microgram samples of total cytoplasmic RNA isolated from murine cell lines and the organs of BALB/c mice (spleen and thymus RNA) or mice deficient for the RAGI product (heart, lung, liver, brain, and testis RNA) (33) were hybridized to a 285-nucleotide [α-^{32}P] UTP-labeled antisense RNA probe encompassing nucleotides 2593 to 2878 of the murine p105 mRNA (16, 19). RNase A and RNase T_1 digestions were performed as described previously (4, 19), and the products were analyzed on 5% acrylamide–8 M urea gels.

cDNA cloning and nucleotide sequencing. λgt10 and λZA PII (Stratagene, San Diego, Calif.) cDNA libraries generated from various murine B-cell lines were screened with a ^{32}P-labeled 285-bp *Kpn*I fragment derived from the coding region of murine *nfkb1* (16, 19). All cDNA clones that demonstrated differences within the 285-bp *Kpn*I fragment were sequenced by using the dideoxynucleotide chain termination reaction.

PCR. One-microgram samples of total cytoplasmic RNA isolated from various murine B-cell lines and mouse organs were used as templates for cDNA synthesis and PCR amplification as described previously (14, 19). The 5′ oligonucleotide primers a and b distinguished the 4.0- and 2.6-kb mRNAs encoding the p50 precursor and IκBγ isoforms, respectively (14, 19), while 3′ primers specific for the spliced transcripts (primer c, 5′-CCCGGAGTTCATCTCATAGTTGTC-3′, for splice 1 and primer d, 5′-CCTTCATGTCCCCTGCCAGTT GGC-3′, for splice 2) spanned the splice junctions (19).

Plasmid constructs. cDNAs for p105, p98, and p84 were inserted into pBSKII (Stratagene) and the eukaryotic expression vector pCDNA-1 (Invitrogen). The coding regions of p105, p98, and p84 were also cloned into the eukaryotic expression vector PSTCO11 (47), such that the initiation codons of the precursor isoforms were in frame with a 9-amino-acid epitope tag (YPYDVPDYA) from influenza virus hemagglutinin HA1. The p98 precursor isoform lacking sequences necessary for DNA binding (amino acids 11 to 200 of p105 [29]) was cloned into pBSKII and pCDNA-1 and referred to as p98ΔN. A 2,590-bp *Fsp*I-*Bsa*I fragment encoding amino acids 1 to 861 of the p105 protein was also cloned into pCDNA-1 and PSTCO11, and it is referred to as p98ΔC. Eukaryotic expression vectors encoding murine p50, p65, $p75^{c\text{-}rel}$, and the p50-p65 chimeric protein have been described previously (14, 19, 20, 42).

Antisera and immunoprecipitations. Antibodies directed against the precursor isoforms were prepared by immunizing rabbits with keyhole limpet hemocyanin-conjugated synthetic peptides corresponding to amino acids 1 to 17 of murine p105 (MADDDPYGTGQMFHLNT [N1]), amino acids 445 to 471 of p105 (AKSDDEESLTLPEKETEGEGPSLPMAC [70.2]), the unique C-terminal 35 amino acids of both p98 and IκBγ-1 (MNSGIVTASVTVVWRHPSANSALQSLLLGTAH CYL [SP-1]), and amino acids 794 to 808 of p105 (TSDDILPQGD MKQLT [P3]). Peptide-specific antibodies were affinity purified on peptide-coupled Affi-Gel columns (Pharmacia). Immunoprecipitations from lysates of metabolically labeled cells were performed as described previously (19).

Pulse-chase labeling. Approximately 10^5 COS cells seeded on 30-mm-diameter tissue culture plates were transfected with expression vectors encoding the precursor isoforms containing the N-terminal hemagglutinin epitope tag (20). At 48 h posttransfection, cells were pulsed with 0.1 mCi of [^{35}S]methionine and processed for immunoprecipitation with the monoclonal antibody 12CA5 (49), which recognizes the hemagglutinin tag (Babco, Emeryville, Calif.), as described previously (13).

Transfections and chloramphenicol acetyltransferase assays. cDNAs encoding p105, p98, p84, and versions of p98 lacking the DNA binding domain or the unique C terminus were inserted into pcDNA1. The reporter construct consisted of four copies of κB3, the NF-κB binding site from the murine c-*rel* promoter (20), inserted upstream of the Tκ promoter in pBLCAT2 (30). Expression vectors for various NF-κB subunits (14, 19, 20) were transfected with a threefold molar excess of the precursor plasmids. Transient transfection of the Jurkat T-cell line was performed by a modification of the DEAE-dextran technique (48). At approximately 48 h after transfection, cells were harvested and extracts were prepared as described previously (20). Extracts from all transfections were standardized for protein content, and chloramphenicol acetyltransferase assays were performed with 15 μg of protein. Extracts were subjected to thin layer-chromatography and then exposed for autoradiography. The fraction of acetylated chloramphenicol was determined by PhosphorImager analysis (Molecular Dynamics) and expressed as a percentage of the total ^{14}C-labeled chloramphenicol. Each series of transfections was performed three to five times, with a maximum of approximately 15% variance observed among replicate experiments.

Immunofluorescence. Approximately 2×10^4 COS cells seeded onto chamber slides (Nunc) were transiently transfected (18) with expression vectors encoding versions of p105, p98, and p84 which had an N-terminal HA1 epitope tag. At 72 h posttransfection, cells were fixed and then incubated with either the monoclonal antibody 12CA5 or SP-1 antibodies (p98), and then they were stained with fluorescein isothiocyanate-conjugated affinity-purified sheep anti-mouse $F(ab)_2$ fragment (Silenus).

In vitro transcription and translation of the precursor isoforms. cDNAs for p105, p98, and p84 inserted into pBSKII were transcribed from linearized DNA templates by using T7 RNA polymerase (14), and equivalent amounts of RNA were translated in wheat germ extract (Promega).

EMSAs and UV cross-linking. The conditions used for electrophoretic mobility shift assays (EMSAs) were a modification of those described previously (20). Gel-purified ^{32}P-labeled oligonucleotides containing an NF-κB binding site from the mouse κ light-chain enhancer (κBE, 5′-GGGACT TCC-3′), the c-*rel* promoter (κB1, 5′-GGGATTTCTC-3′; κB3, 5′-GGGAAATCCC-3′; or κB3m, 5′-GGGAAATGAAT-3′, a

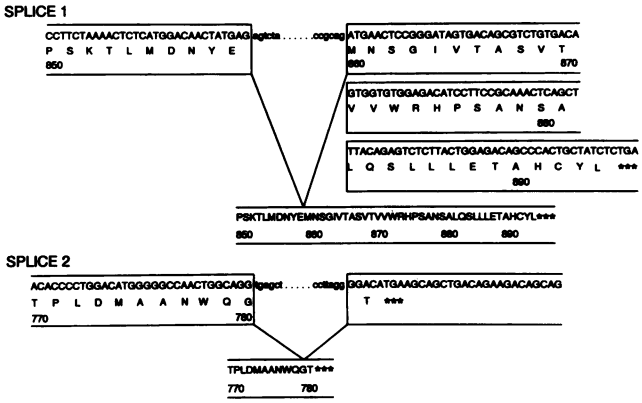

FIG. 1. Alternate RNA splicing within murine *nfkb1*. The nucleotide and predicted amino acid sequences of cDNA clones for splices 1 and 2 are compared with the corresponding genomic sequence of murine *nfkb1*. The genomic sequences corresponding to exons in splice 1 and splice 2 transcripts are represented by the boxed uppercase letters. Lowercase letters correspond to intronic sequences. The predicted amino acid sequences of the spliced products are indicated beneath the genomic sequences. The amino acid numbering is based on the sequence determined by Ghosh et al. (16). The single letter amino acid code is as follows: A, Ala; R, Arg; N, Asn; D, Asp; C, Cys; E, Glu; Q, Gln; G, Gly; H, His; I, Ile; L, Leu; K, Lys; M, Met; P, Pro; S, Ser; T, Thr; W, Trp; Y, Tyr; V, Val.

mutant version of κB3), the human immunodeficiency virus long terminal repeat (κBHIV, TGGGGACTTTCCA), the murine major histocompatibility complex enhancer (κBMHC, TGGGGATTCCCCA), or the human interleukin 2 promoter (κBIL2, 5′-GGGATTTCACC-3′) were used as probes in the DNA binding reactions. One- to two-microliter samples of the precursor translation mixtures were preincubated on ice for 30 min and then incubated for a further 20 min at room temperature upon the addition of probe. For competition experiments, nonradiolabeled DNA was incubated with extract for 10 to 15 min on ice before the addition of probe. When antibodies specific for Oct2 (50), the N terminus (N1) and C-terminal region (P3) of p105, or the unique C terminus of p98 (SP-1) were used in gel shift assays, translate and poly(dI-dC) were incubated on ice for 10 min, antibody was then added, and the incubation on ice continued for 1 h before the addition of probe. The incubation was continued for 20 min at room temperature, and then 2 μl of dye containing Ficoll was added, after which reaction mixtures were loaded on 5% nondenaturing polyacrylamide gels and run at 150 V for 40 min. After being dried, gels were exposed overnight to Kodak XAR5 films at −70°C with an intensifying screen.

The cross-linking method was adapted from that described by Ballard et al. (2). The palindromic oligonucleotide 5′-GTA AGCAGCGGGAAATCCCCCCCCCTC-3′, corresponding to the κB3 site in the c-*rel* promoter, was annealed to the complementary 10-mer (5′-GAGGGGGGGG-3′) and filled in with the Klenow fragment of DNA polymerase 1 in the presence of 10 mM Tris-Cl (pH 7.4), 6 mM $MgCl_2$, 50 mM NaCl, 6 mM 2-ME, 0.2 mg of gelatin per ml, 20 μM bromodeoxyuridine triphosphate, 20 μM (each) dATP and dGTP, and 100 μCi each of [α-^{32}P]dATP and [α-^{32}P]dCTP. The labeled probes were gel purified, and approximately 10^6 cpm was incubated with 4 to 6 μl of precursor isoform translate in a standard EMSA reaction (see above). Precursor translate-DNA complexes were electrophoresed as for a standard EMSA reaction, and gels covered with plastic wrap were irradiated directly at

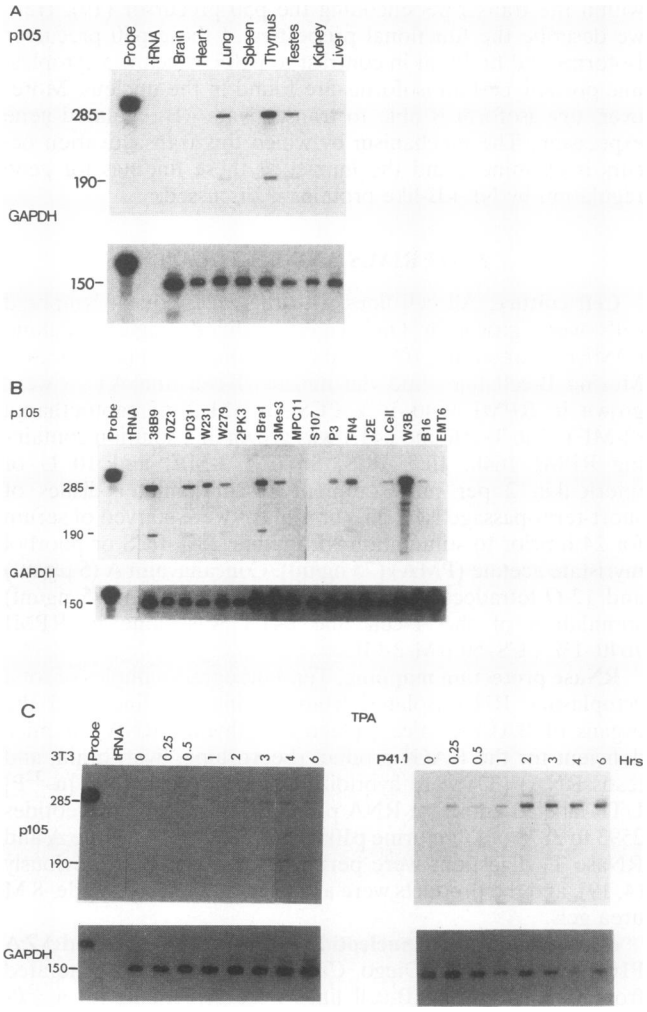

FIG. 2. Transcripts containing splice 2 are expressed in a wide variety of murine tissues and cell lines. (A) *nfkb1* expression in various murine organs. Ten-microgram samples of total cytoplasmic RNA were hybridized to the 285-nucleotide p105 probe and the 150-nucleotide rat GAPDH probe, digested with RNase A and T_1, and then fractionated on 5% acrylamide–urea gels. The gel was exposed for 24 h to autoradiography at −70°C. Spleen and thymus RNA was isolated from BALB/c mice, while brain, heart, lung, testis, kidney, and liver RNA was isolated from mice deficient for the *Rag1* gene (33). As the probe described here would yield a product only 4 nucleotides shorter than the predicted size of transcripts corresponding to splice 1, the product of splice 1 would not be identified in Fig. 2A. The p105 and GAPDH probes only and hybridization of these probes to yeast tRNA (negative control) are shown. The major RNase protection products (285 and 190 nucleotides for p105; 150 nucleotides for GAPDH) are indicated. The gel was subjected to 48 h of autoradiography. (B) *nfkb1* expression in murine cell lines. Ten-microgram samples of total cytoplasmic RNA were isolated from the following cell lines: 38B9, 70Z/3, and PD31, pre-B-cell lymphomas; W231, W279, 2PK3, 1Bra1, and 3Mes3, B-cell lymphomas; MPC11, S107, and P3, plasmacytomas; FN4 and J2E, erythroid leukemias; L cells and EMT6, fibroblasts; W3B, myeloid leukemia cells; and B16, a melanoma cell line; they were then subjected to RNase protection analysis with the 285-nucleotide p105 probe and the 150-nucleotide GAPDH probe. The gel was subjected to 48 h of autoradiography. (C) Transcripts encoding splice 2 are induced in PMA-stimulated 3T3 fibroblasts but not in T cells. Five-microgram samples of total cytoplasmic RNA isolated from serum-starved 3T3 fibroblasts or the T-cell line p41.1 at the indicated time points after the addition of TPA were subjected to RNase protection mapping. The gels were exposed for 48 h to autoradiography at −70°C.

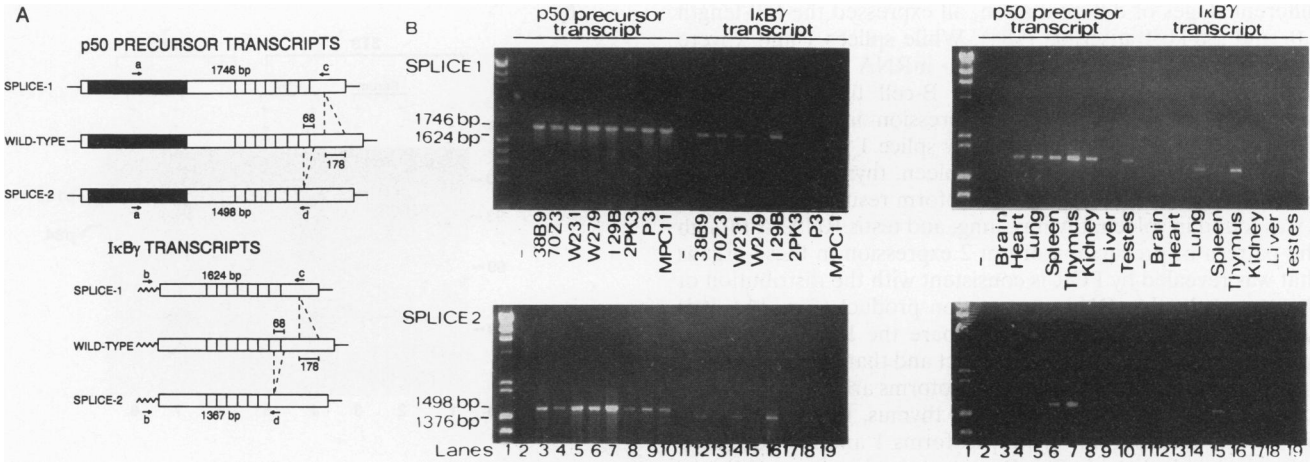

FIG. 3. Splices 1 and 2 are in both the 4.0- and 2.6-kb mRNA populations encoding the p50 precursors and IκBγ, respectively. (A) Strategy for detecting the 4.0- and 2.6-kb mRNAs containing splices 1 and 2. The closed box corresponds to the p50 precursor Rel homology domain, the open area represents the C-terminal half of the p105-p70IκBγ coding region, and the vertical bars correspond to the ankyrin repeats. The locations and sizes of the coding sequences spliced out of p105 or p70IκBγ are indicated by the broken lines. The 5′ primers a and b, specific for the 4.0- and 2.6-kb transcripts, respectively, have been described previously (14, 19). Primers c and d spanned the splice junctions of splices 1 and 2, respectively. The predicted sizes of PCR products for isoforms 1 and 2 derived from either the 4.0- or the 2.6-kb mRNA population are shown between the primers. (B) Transcripts (4.0 and 2.6 kb) containing splices 1 and 2 are differentially expressed in murine organs. One-microgram samples of total cytoplasmic RNA isolated from various B-cell lines (left panels) or from the organs of *Rag1*-deficient mice (with the exception of spleen and thymus RNA, which was from BALB/c mice) (right panels) were reverse transcribed and then subjected to PCR with 5′ primers specific for the 4.0- and 2.6-kb transcripts in combination with 3′ primers specific for splices 1 and 2. The sizes of the PCR products fractionated on 0.8% agarose gels were determined by comparison with *Hin*dIII-cleaved lambda DNA and *Hae*III-cleaved ϕX174 DNA (lanes 1). No-cDNA controls are shown in lanes 2 and 11. Lanes 3 through 10 show the PCR products for p50 precursor transcripts encoding splices 1 and 2, while the PCR products for the transcripts encoding the IκBγ isoforms are shown in lanes 12 through 19.

302 nm on a UV transilluminator for 1 h. The gels were then exposed to X-ray film, and the NF-κB complexes were localized. Upper complex a was eluted from gel slices into 0.5 ml of radioimmunoprecipitation assay buffer and incubated with N1 antiserum for 2 h on ice. Immunocomplexes were precipitated with protein A-Sepharose, and the DNA-protein adducts were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The gels were dried and exposed to X-ray film.

RESULTS

Alternate splicing of murine *nfkb1* generates p50 precursor and IκBγ isoforms. During the characterization of cDNA clones representing the 4.0- and 2.6-kb transcripts encoded by murine *nfkb1*, clones with deletions in the p105 C-terminal coding region (19) were isolated. In Fig. 1, the nucleotide sequences of two cDNA clones that encode putative proteins with changes C-terminal to ankyrin repeat 7 are compared with the corresponding genomic sequences of murine *nfkb1*. For one clone (splice 1), a deletion of 178 nucleotides corresponding to amino acids 861 to 918 of p105 introduced a reading frame shift (from amino acid 919 of p105), thereby generating a protein with a novel C-terminal domain of 35 amino acids. In the other clone (splice 2), the loss of 67 nucleotides corresponding to amino acids 780 to 802 created a frameshift that leads to the generation of a protein lacking the C-terminal 190 amino acids of p105.

RNase protection analysis was performed on RNA isolated from mouse tissues and cell lines by using a 285-nucleotide cDNA probe that spanned the region deleted in the splice 2 clone. This probe should yield a 190-nucleotide RNA product corresponding to splice 2. It should be noted that it was not possible to resolve the products of RNA splicing corresponding to splice 1 from the full-length 285-nucleotide product. A survey of mouse organs (Fig. 2A) revealed, in addition to the 285-nucleotide fragment, a smaller band of 190 nucleotides in thymus, testis, lung, and spleen tissue (very faint). The absence of RNase protection products from brain, heart, and kidney tissue was due to a low level of p105 expression in these tissues (18a). In murine cell lines (Fig. 2B), the 190-nucleotide product was seen in pre-B (38B9 and PD31), B (W231 and W279), and erythroid (FN4) cells but not in plasmacytomas (2PK3, S107, MPC11, and P3), L cells, or melanoma cell lines (B16 and EMT6). Other minor bands were also reproducibly observed in 1Bra1, a B-cell tumor, and W3B, a myelomonocytic leukemia. Both the 285- and 190-nucleotide RNase protection products were inducible in quiescent NIH 3T3 fibroblasts treated with the phorbol ester TPA (Fig. 2C). The 190-nucleotide RNase protection product was also induced in serum-stimulated fibroblasts (18a). By contrast, in T cells, TPA induced the 285- but not the 190-nucleotide product (Fig. 2C). The inability to induce the 190-nucleotide product in T cells was not due to limited sensitivity of the RNase protections, as it could not be detected by PCR (18a). Since only the 4.0-kb transcript is detected by Northern (RNA) blot analysis of mRNA from TPA-stimulated 3T3 fibroblasts (13a), the alternately spliced transcript arises from the 4.0-kb mRNA in these cells.

A PCR assay schematically outlined in Fig. 3A was used to survey expression of splices 1 and 2 in the 4.0- and 2.6-kb mRNA populations from various tissues and cell lines (Fig. 3B). The different transcripts were identified by using 5′ primers unique for the 4.0- and 2.6-kb mRNAs, in conjunction with 3′ primers that spanned the junctions of splices 1 and 2. Consistent with results of previous studies (14, 16, 19, 23) showing that transcripts encoding p105 and p70IκBγ are expressed in all murine tissues and in a majority of lymphoid cell lines, the B-cell lines analyzed (Fig. 3B), which represented

different stages of differentiation, all expressed the full-length 4.0- and 2.6-kb transcripts (18a). While splices 1 and 2 were present in both the 4.0- and 2.6-kb mRNA populations and were expressed in most of these B-cell lines, the spliced isoforms displayed differential expression in mouse tissues. The precursor isoform generated by splice 1 was expressed in a number of organs (heart, lung, spleen, thymus, kidney, and testis RNA), while the precursor isoform resulting from splice 2 was found in spleen, thymus, lung, and testis RNA. Although the pattern of precursor isoform 2 expression in these organs that was revealed by PCR is consistent with the distribution of the 190-nucleotide RNase protection product (Fig. 2A), it is not possible to quantitatively compare the level of the 190-nucleotide RNase protection product and that of the precursor isoform 2 PCR product. The IκBγ isoforms arising from splices 1 and 2 were also found in spleen, thymus, lung, and kidney tissue. The distribution of IκBγ isoforms 1 and 2 is restricted compared with that of p70IκBγ, which is ubiquitous (14).

Previously we have shown that the p50 precursor and IκBγ proteins encoded by splice 1 transcripts have molecular masses of 98 and 63 kDa, respectively, whereas the precursor and IκBγ isoforms encoded by splice 2 have molecular masses of 84 and 55 kDa, respectively (19). Figure 4 shows constitutive and induced precursor isoform expression in a variety of cell types. Both p105 and p84 were specifically immunoprecipitated in serum-stimulated (Fig. 4A, lanes 2 through 4) or PMA-stimulated (lanes 5 through 7) 3T3 fibroblasts, whereas only p105 was expressed in PMA-treated cells of the T-cell line AT2.5 (Fig. 4A, lanes 8 through 10). The absence of IκBγ isoforms in the 3T3 fibroblasts is consistent with previous findings (23). By using antibodies (SP-1) raised against the unique C-terminal epitope generated by splice 1, the p50 precursor (p98) and IκBγ (IκBγ-1 or p63) isoforms were detected in murine pre-B (70Z/3)-, B (129)-, and T (p41.1)-cell lines (Fig. 4B). The combined RNase protection, PCR, and immunoprecipitation analysis shows that alternate splicing of murine *nfkb1* generates p50 precursor and IκBγ isoforms. The rest of this paper will focus on the p50 precursor isoforms.

p50 precursor isoforms p98 and p84 are found in the nuclei of transfected cells. To determine the subcellular localization of p98 and p84, expression vectors encoding versions of p105 and precursor isoforms 1 and 2 tagged at the amino terminus with an epitope from influenza virus hemagglutinin (49) were transiently transfected into COS cells and then examined by immunofluorescence with antibodies specific for this epitope. While p105 localized to the cytoplasm (Fig. 5B), a finding consistent with those of previous studies (5, 22, 40), p98 and p84 were found in the cytoplasm and nucleus; p98 displayed equivalent staining in both compartments (Fig. 5C), whereas p84 was predominantly nuclear (Fig. 5D). To ensure that the nuclear p84 and p98 fluorescence was not due to the hemagglutinin tag or to processing of these precursors, generating a p50-like protein, the pattern of immunofluorescence was confirmed by using peptide-specific antibodies directed to epitopes in the C termini of the precursors. A representative example of these experiments is shown in Fig. 5E, in which p98 displays the cytoplasmic and nuclear staining pattern expected when antibodies (SP-1) specific for the unique C-terminal domain are used.

The p98 isoform is a transactivator of NF-κB-regulated reporters. As both p84 and p98 localize to the nuclei of transfected cells, we chose to determine if these p50 precursor isoforms can modulate transactivation of an NF-κB-regulated reporter in the Jurkat T-cell line. The results of these experiments are summarized in Fig. 6. While p105 and p84 did not transactivate (lanes 2 and 3), p98 alone promoted expression of

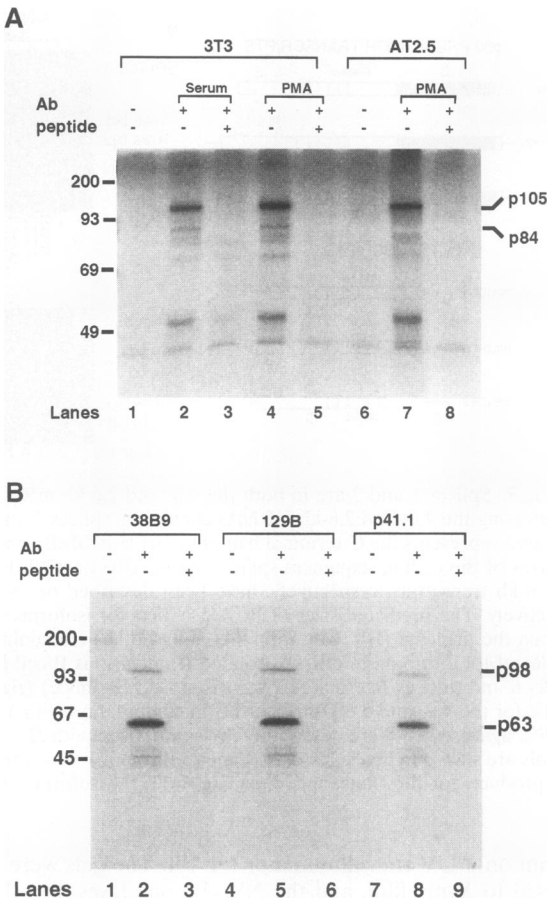

FIG. 4. Detection of the p50 precursor isoforms in metabolically labeled cells. (A) p105 and p84, the p50 precursors encoded by splice 2, are expressed in mitogenically stimulated fibroblasts. [^{35}S]methionine-labeled serum-stimulated (lanes 1 through 3) or PMA-stimulated (lanes 4 and 5) NIH 3T3 fibroblasts and PMA-stimulated AT2.5 T cells (lanes 6 through 8) were incubated with preimmune serum (lanes 1 and 6) or with 70.2 antiserum in the absence (−; lanes 2, 4, and 7) or presence (+; lanes 3, 5, and 8) of 70.2 peptide. The p105 and p84 precursors are indicated, while p98 could only be detected after prolonged autoradiography (data not shown). This exposure represents 24 h of autoradiography. The molecular masses (in kilodaltons) of protein standards are indicated on the left. (B) Detection of p50 precursor and IκBγ isoforms generated by splice 1 by using SP-1 antibodies specific for the unique C-terminal epitope. Metabolically labeled lysates from 38B9 (lanes 1 through 3), 129B (lanes 4 through 6), and p41.1 (lanes 7 through 9) cells were precipitated with preimmune serum (lanes 1, 4, and 7) or SP-1 antibodies in the absence (−; lanes 2, 5, and 8) or presence (+; lanes 3, 6, and 9) of SP-1 peptide. The p98 precursor and p63IκBγ isoform are indicated. Gels were exposed for 48 h to autoradiography at −70°C.

the reporter approximately eightfold above the background (lane 4). This transactivation was NF-κB site dependent (18a).

Since all of the p50 precursor isoforms can dimerize with p50, p65, and the c-*rel* protein (18a), we decided to assess the ability of the precursors to inhibit or enhance transcription mediated by different Rel or NF-κB subunits. This was tested by cotransfecting p105, p84, and p98 expression vectors and the reporter plasmid with vectors encoding various NF-κB subunits. p50 had no effect on the transactivating properties of p105 and p84 (Fig. 6, lanes 6 and 7, respectively), but it enhanced p98 transactivation approximately twofold (lane 8).

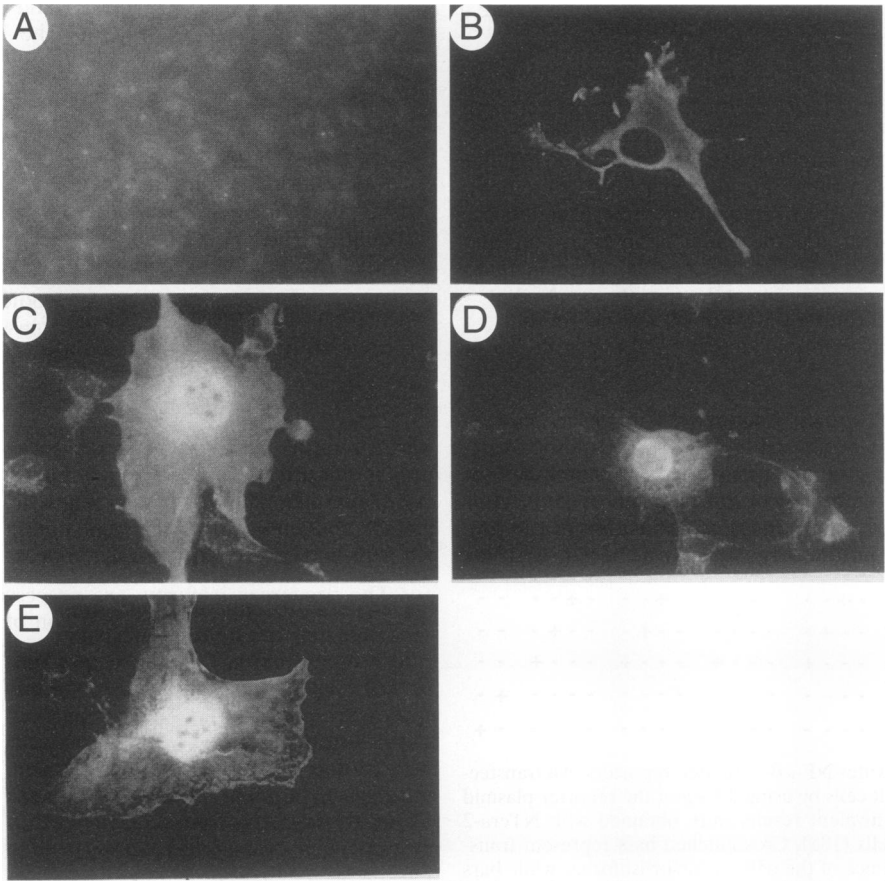

FIG. 5. p50 precursor isoforms display different subcellular localization. COS cells transiently transfected with expression vectors for p105 and the p50 precursor isoforms p98 and p84 were fixed and stained as described in Materials and Methods. The panels correspond to COS cells transfected with the expression vectors pSCT (A) (47), pSTCp105 (B), pSTCp98 (C), and pSTCp84 (D) and stained with 12CA5 antibodies and to COS cells transfected with pSTCp98 and stained with SP-1 antibodies (E).

While p84 reduced transactivation by the chimeric p50-p65 protein approximately threefold (compare lanes 5 and 7), a finding consistent with the p50 precursor behaving as an IκB, p105 was only weakly inhibitory (lane 6). By contrast, p98 and p50-p65 cotransactivation (lane 8) was significantly greater than the additive transactivation by p50-p65 and p98 alone. p65 transactivation, like p50-p65 transactivation, was not significantly affected by p105 (lane 10), and it was only weakly inhibited by p84 (lane 11). However, p65 also synergized with p98 to enhance NF-κB site-dependent transcription (lane 12). Finally, $p75^{c\text{-}rel}$-mediated transactivation, while not inhibited by p105 or p84 (lanes 14 and 15, respectively), was not enhanced by p98 (lane 16). The same results were obtained when these transfection experiments were repeated with NTera-2 cells, which have little or no endogenous NF-κB activity (10). To gain insight into the mechanism of p98-mediated transactivation, the properties of several p98 mutants were analyzed. While a mutation shown to affect p50 DNA binding (29) ablated p98 transactivation (lane 21), another mutant, p98ΔC, which is equivalent to p98 without the unique 35-amino-acid C terminus, is at best a weak transactivator (lane 22).

The p50 precursor isoforms bind DNA in vitro. Although previous studies indicate that the murine and human forms of p105 (16, 25) do not bind κB sites in vitro, the dependence of p98-mediated transactivation on sequences required for p50 DNA binding prompted a reinvestigation of the DNA binding properties of p105, p98, and p84 by using electrophoretic mobility gel shift assays.

Analysis by SDS-polyacrylamide gel electrophoresis of a typical set of precursor translates used for gel shifts showed that in addition to the full-length precursor isoforms, there were also proteins with lower molecular weights (Fig. 7A). This is a common observation for in vitro translates (16, 36, 39, 45), and it probably represents premature translation termination, translation initiation at internal methionine residues, or proteolytic cleavage.

To assess the DNA binding capacity of the p50 precursors, a panel of NF-κB binding sites were first tested for the ability to bind p98. These results, shown in Fig. 7B, indicate that an upper complex designated complex a and a lower, more diffuse complex, complex b, bound to κB3, an NF-κB binding site in the murine c-*rel* promoter (20). Complex a bound weakly to κB1, another NF-κB site in the c-*rel* promoter (20), and to the κB site in the human immunodeficiency virus long terminal repeat, but there was no detectable binding of complex a to the NF-κB sites in the immunoglobulin light-chain enhancer, the major histocompatibility complex class I enhancer, or the human interleukin 2 promoter. Because of the ability of κB3 to bind both complex a and complex b strongly, all subsequent binding experiments were done with this probe. Studies of the binding of p105, p84, and p98 translates to κB3 are shown in

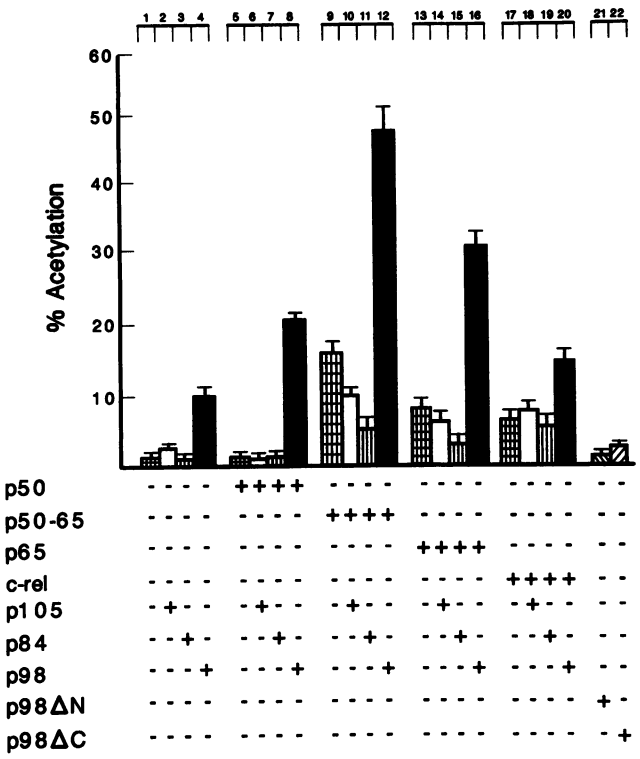

FIG. 6. p98 transactivates NF-κB-regulated reporters. All transfections were done in Jurkat cells by using 0.5 μg of the reporter plasmid κB3pBLCAT2 (20). Equivalent results were obtained with NTera-2 embryonal carcinoma cells (18a). Crosshatched bars represent transfections done in the absence of the p50 precursor isoforms, while bars that are open, bars with vertical lines, and bars that are closed correspond to transfections in which expression vectors encoding the Rel/NF-κB subunits were cotransfected with expression vectors for p105, p84, and p98, respectively. Lane 1, κB3pBLCAT2 alone; lanes 2 through 4, modulation of endogenous NF-κB activity by the p50 precursor isoforms; lanes 5 through 8, cotransfection of p50 isoforms with p50; lanes 9 through 12, cotransfection of p50 precursor isoforms with chimeric p50-p65; lanes 13 through 16, cotransfection of p50 precursor isoforms with p65; and lanes 17 through 20, cotransfection of p50 precursor isoforms with c-*rel*. Lanes 21 and 22 correspond to transfections of the p98 mutants p98ΔN and p98ΔC (4 μg), respectively. Each set of experiments was done six times with an observed maximum variance of 15%. The masses of the expression vector plasmids transfected into the cells were as follows: p50, p50-p65, and p65, 0.5 μg; $p75^{c\text{-}rel}$, 1 μg; and p105, p84, and p98, 4 μg.

Fig. 7C. DNA binding of complexes a and b from all translates (lanes 4, 7, and 10) is specific, as an excess of κB3 DNA (lanes 5, 8, and 11) but not mutant κB3 DNA (lanes 6, 9, and 12) competed for binding of both complexes. Binding of complexes a and b to κB3 is also dependent on sequences shown to be essential for DNA binding by p50 (18a).

To determine the composition of complexes a and b, antibodies specific for various epitopes in the precursor isoforms were used in the gel shifts. The results of these experiments are summarized in Fig. 7D. N1 antibodies, which recognize amino-terminal residues 1 to 17 of murine p105, ablated binding of both complexes (lanes 6 through 8). SP-1 antibodies, specific for the unique C-terminal 35 amino acids of p98, disrupted complex a, but not complex b, in translates of p98 (lane 12), and they had no effect on either complex in p105 or p84 translates (lanes 10 and 11). P3 antibodies, raised against amino acids 794 to 818 of murine p105, specifically eliminated binding of complex a from p105 and p98 but not from p84 translates. Neither the SP-1 nor the P3 antibodies affected complex b binding in any translate. Antibodies specific for Oct2 (50), as expected, did not affect complex a or b (lanes 18 through 20). The ability of certain antibodies, but not others, to differentially inhibit DNA binding of complexes a and b from different precursor translates is consistent with complex a containing the p50 precursor isoforms and complex b corresponding to a p50 homodimer-like complex. To determine if complex a comprised a homodimer of the precursor isoforms, this complex from p98 translates was cross-linked to κB3 and immunoprecipitated with N1 antibodies (Fig. 7E). The protein-DNA adducts with molecular masses of 110 and 60 kDa were of sizes consistent with the theory that the complex comprised p98 and p50-like subunits. The use of SP-1 antibodies confirmed that the 110-kDa adduct was p98, and immunoprecipitation of cross-linked complex a from p105 and p84 translates showed that these forms of this complex were also heterodimers of the precursor and p50-like subunits (18a). The presence of p50-like subunits in complexes a and b is most likely due to proteolytic cleavage of the precursor in the wheat germ extracts or premature translation termination.

The precursor isoforms are processed to p50 at different rates. While the nuclear localization of p84 and p98, coupled with the ability of p50 precursor isoforms to bind certain κB sites in vitro, supports a model in which the p98 subunit can directly activate transcription, other mechanisms could account for p98 transactivation. One alternative is that the p50 precursors which associate with endogenous NF-κB subunits such as p65 are processed by proteolysis to generate transactivating heterodimers. In this model, complexes that include p98 would be processed more readily than those containing p105 and p84. To test the processing of the precursor isoforms, vectors encoding forms of p105, p84, and p98 with the N-terminal hemagglutinin tag were transiently transfected into COS or Jurkat T cells, and the rate of proteolytic processing was monitored by pulse-chase analysis. Results of the experiments done with COS cells are shown in Fig. 8. Equivalent results were obtained with Jurkat cells (18a). p105 was relatively stable and showed no obvious processing over a 3-h period, which agrees with the findings of Mercurio et al. (31). While p84 displayed stability similar to that of p105 (Fig. 8B), a significant proportion of the p98 was processed to p50 within 60 min (Fig. 8C), even in the absence of TPA stimulation, which promotes p105 processing (31). In contrast, p98ΔC, the mutant lacking the unique C-terminal 35-amino-acid domain, exhibited stability similar to that of p105 and p84 over a 3-h period (Fig. 8D). These findings suggest that the unique C terminus of p98 influences proteolytic processing.

DISCUSSION

Previous studies have shown that the p50 precursor is a non-DNA-binding, cytoplasmic protein that is processed by proteolytic cleavage to yield the p50 subunit of NF-κB. Although these studies have focused on p105, the predominant form of the precursor, two-dimensional electrophoresis has revealed that the p50 precursor comprises a number of isoforms (37). While certain of these arise by posttranslational modifications such as phosphorylation (37), here we show that alternate splicing of transcripts encoded by *nfkb1* generates precursor isoforms with novel activities, which include differential subcellular localization, transactivating properties, and differences in proteolytic processing. The implications of these findings are discussed in the context of NF-κB regulation.

Both p84 expression and p98 expression appear to be

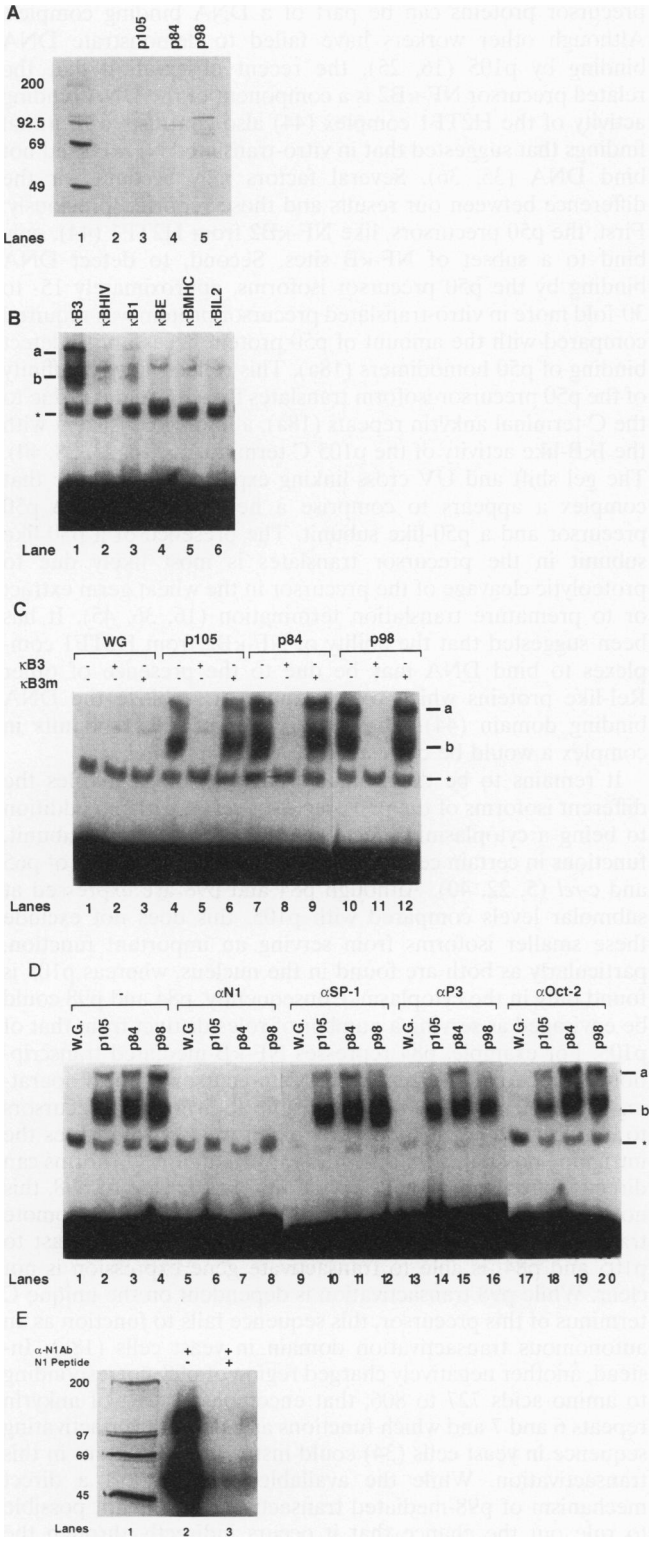

FIG. 7. Determining the DNA binding properties of the p50 precursor isoforms by gel shift analysis. (A) In vitro wheat germ translates of p105, p98, and p84. cDNA clones for p105, p98, and p84 were translated in wheat germ extracts in the presence of [^{35}S]methionine. Two-microliter samples of each translate were fractionated on an SDS–10% polyacrylamide gel, fixed, and prepared for autoradiography. Lanes: 1, ^{14}C-labeled molecular weight protein standards; 2, wheat germ translate alone; 3, p105; 4, p84; 5, p98. The minor products with lower molecular weights represent internal translation initiation or premature termination of translation. The molecular masses (in kilodaltons) of protein standards are indicated at the left of the panel. (B) Only a subset of NF-κB sites bind complexes from in vitro translates of the precursor isoform p98. EMSAs were performed on p98-programmed wheat germ translates as described in Materials and Methods by using several different NF-κB sites. Two specific complexes, designated a (upper complex) and b (lower complex), bound to the NF-κB site κB3 (lane 1). Lanes: 2, κBHIV; 3, κB1; 4, κBE; 5, κBMHC; 6, κBIL2. *, nonspecific complex seen in wheat germ extracts that binds to all κB sites used. (C) Two complexes from in vitro translates of p105, p98, and p84 specifically bind to the NF-κB site κB3. Gel mobility shift assays were performed with no competition (−) or competition with a 25-fold molar excess of unlabeled κB3 or the mutated κB3 probe κB3 m (+). Lanes: 1 through 3, wheat germ extract alone; 4 through 6, p105 translate; 7 through 9, p84 translate; 10 through 12, p98 translate. Competition analysis was done for the following gel shifts: lanes 2, 5, 8, and 11, competition with unlabeled κB3; and lanes 3, 6, 9, and 12, competition with unlabeled κB3m. (D) p50 precursor-specific antibodies block DNA binding of complex a. p105, p98, and p84 translates were prepared as described for panel A. The probe used for gel shifts was κB3. Lanes: 1, 5, 9, 13, and 17, wheat germ extract alone; 2, 6, 10, 14, and 18, p105 translate; 3, 7, 11, 15, and 19, p84 translate; 4, 8, 12, 16, and 20, p98 translate. Precursor-specific antibodies were added to the following gel shifts (regions to which antibodies are specific are in parentheses): lanes 5 through 8, N1 antibodies (amino acids 1 to 17 of p105); lanes 9 through 12, SP-1 antibodies (unique C-terminal amino acids of p98); lanes 13 through 16, P3 antibodies (p105 C-terminal amino acids 794 to 808); and lanes 17 through 20, Oct2-specific antibodies (50). (E) p98 is a component of the DNA binding activity in complex a. p98 translate equivalent to that shown in Fig. 7A was subjected to an EMSA using a bromodeoxyuridine triphosphate-substituted κB3 probe and electrophoresed on 5% nondenaturing gels as described in Materials and Methods. After direct UV irradiation, complex a was isolated, eluted, and immunoprecipitated with N1 antibodies, and the resultant proteins were analyzed on SDS–8% polyacrylamide gels. Lanes: 1, ^{14}C-labeled protein molecular weight standards; 2, N1 immunoprecipitates of cross-linked complex a; 3, complex a incubated with N1 antibodies in the presence of N1 peptide. The molecular masses (in kilodaltons) of protein standards are indicated at the left of the panel.

regulated in a complex manner. Transcripts encoding these isoforms are restricted to certain tissues and vary in abundance. Moreover, stimuli, such as serum or TPA, which activate NF-κB and promote transcription of *nfkb1* (9, 32) also induce expression of transcripts encoding p84 in a cell-type-specific fashion. The mechanism by which TPA stimulation differentially regulates the induction of splice isoform 2 remains to be determined. It is noteworthy that neither serum nor TPA induces expression of p98 (13a). Although the relative abundances of the transcripts encoding p105 and p84 appear to be equimolar in serum- or TPA-treated fibroblasts, the level of p84 in these cells is approximately 25% of that of p105, possibly indicating that the stability of p84 and that of p105 differ in these cells.

While published data and the findings presented here show that p105 is a cytoplasmic protein (5, 22, 40), mounting evidence suggests that under certain circumstances NF-κB precursor proteins may be found in the nucleus. In Jurkat T cells, the p50 precursor is composed of a heterogeneous group of cytoplasmic proteins, certain isoforms of which can be detected in the nuclei of phorbol ester-treated cells (37). It remains to be determined if the nuclear subset of p50 precursor isoforms detected in stimulated Jurkat cells, like p84 and p98, arise by alternate RNA splicing. Since p84 and p98 lack the C-terminal p105 epitope recognized by the antibodies used in the study conducted by Neumann et al. (37), the isoforms

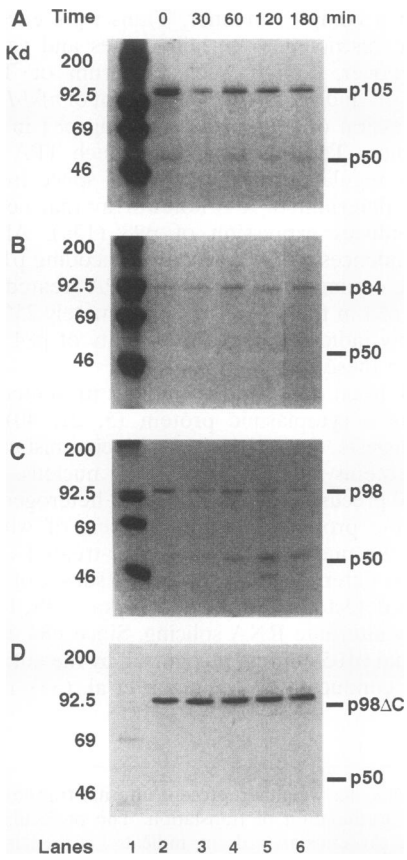

FIG. 8. Differential processing of the p50 precursor isoforms. Expression vectors for the epitopically hemagglutinin-tagged p50 precursors transiently transfected into COS cells were pulsed with [^{35}S]methionine for 20 min and then incubated in the absence of a radioisotope for the indicated times before the cells were lysed. Cell extracts were incubated with antihemagglutinin monoclonal antibodies (12CA5), and the immunoprecipitates were analyzed on SDS-polyacrylamide gels. Panel A, p105; panel B, p84; panel C, p98; and panel D, p98ΔC. The gels were exposed for 24 h to autoradiography.

described here must differ from those detected in the nuclei of stimulated T cells. The recent finding that p100NF-κB2 is a component of the DNA binding activity of the H2TF1 complex (44) lends support to the notion that NF-κB precursor proteins may under certain circumstances enter the nucleus. The localization of p84 and p98, but not p105, to the nuclei of transfected COS cells provides insight into the mechanism by which the p50 precursors are transported to the nucleus. It has been proposed that an acidic region within ankyrin repeat 6, required for the cytoplasmic maintenance of p105 (5, 22), normally masks the nuclear localization signal in the Rel homology domain of the precursor. While p84 lacks the C-terminal 190 amino acids of p105, including part of this acidic region, this sequence is retained in p98. This indicates that the nuclear transport of p50 precursors can be regulated by other mechanisms. For example, conformational changes in the isoforms created by the loss of sequences could make the nuclear localization signal in the Rel homology domain accessible to the nuclear transport machinery. Alternatively, a cryptic nuclear localization signal in the p50 precursor is unmasked by splice 1, or the C-terminal 35 amino acids of p98 act as a nuclear transport signal.

The findings presented here are the first to show that p50 precursor proteins can be part of a DNA binding complex. Although other workers have failed to demonstrate DNA binding by p105 (16, 25), the recent observation that the related precursor NF-κB2 is a component of the DNA binding activity of the H2TF1 complex (44) also contrasts with initial findings that suggested that in vitro-translated NF-κB2 did not bind DNA (35, 36). Several factors may account for the difference between our results and those reported previously. First, the p50 precursors, like NF-κB2 from H2TF1 (44), only bind to a subset of NF-κB sites. Second, to detect DNA binding by the p50 precursor isoforms, approximately 15- to 30-fold more in vitro-translated precursor protein was required compared with the amount of p50 protein necessary to detect binding of p50 homodimers (18a). This reflects a weak affinity of the p50 precursor isoform translates for κB3, and it is due to the C-terminal ankyrin repeats (18a), a finding consistent with the IκB-like activity of the p105 C terminus (5, 14, 23, 28, 40). The gel shift and UV cross-linking experiments indicate that complex a appears to comprise a heterodimer of the p50 precursor and a p50-like subunit. The presence of a p50-like subunit in the precursor translates is most likely due to proteolytic cleavage of the precursor in the wheat germ extract or to premature translation termination (16, 36, 45). It has been suggested that the ability of NF-κB2 from H2TF1 complexes to bind DNA may be due to the presence of other Rel-like proteins which could expose or stabilize the DNA binding domain (44). The presence of p50-like subunits in complex a would be consistent with such a model.

It remains to be determined what physiological roles the different isoforms of the p50 precursor serve. p105, in addition to being a cytoplasmic precursor of the NF-κB p50 subunit, functions in certain cell types as a cytoplasmic inhibitor of p65 and c-*rel* (5, 22, 40). Although p84 and p98 are expressed at submolar levels compared with p105, this does not exclude these smaller isoforms from serving an important function, particularly as both are found in the nucleus, whereas p105 is found only in the cytoplasm. Consequently, p84 and p98 could be envisaged as serving a number of roles distinct from that of p105. For example, p84 represses NF-κB-mediated transcription, a function consistent with this precursor isoform operating as a nuclear IκB. However, it is the ability of p50 precursors to bind certain κB sites in vitro, albeit weakly, that raises the intriguing possibility that the nuclear p84 and p98 isoforms can directly participate in gene regulation. In the case of p98, this notion is supported by the ability of this isoform to promote transactivation. The mechanism by which p98, in contrast to p105 and p84, is able to transactivate gene expression is not clear. While p98 transactivation is dependent on the unique C terminus of this precursor, this sequence fails to function as an autonomous transactivation domain in yeast cells (18a). Instead, another negatively charged region of p98, corresponding to amino acids 727 to 806, that encompasses part of ankyrin repeats 6 and 7 and which functions as a potent transactivating sequence in yeast cells (34) could instead be important in this transactivation. While the available data support a direct mechanism of p98-mediated transactivation, it is not possible to rule out the chance that it occurs indirectly through the processing of complexes in which exogenous p98 is associated with endogenous Rel/NF-κB subunits such as p65. Although the enhanced processing of p98 compared with the other precursors would fit this model, this mechanism seems unlikely, as p98 is able to transactivate reporter constructs just as effectively in NTera-2 cells, a human embryonal carcinoma cell line which has very low levels of endogenous NF-κB (10), as it does in Jurkat or COS cells. In vitro transcription assays

currently in progress should show whether p98 is able to act as a direct transactivator of NF-κB-dependent gene expression.

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