The p65 subunit of NF-κB regulates IκB by two distinct mechanisms

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Transcription factor NF-κB (p50/p65) is generally localized to the cytoplasm by its inhibitor IκB. Overproduced IκB, free from NF-κB, is rapidly degraded. Overexpression of p65 increases endogenous IκB protein in both carcinoma and lymphoid cells by two mechanisms: protein stabilization and increased transcription of IκB mRNA. In contrast, p65Δ, a naturally occurring splice variant, fails to markedly augment IκB protein levels. Both overexpressed p65 and coexpressed p50 are cytoplasmic, whereas p65Δ is partly nuclear, indicating that the IκB induced by p65 can maintain NF-κB in the cytoplasm. Thus, p65 and IκB are linked in an autoregulatory loop, ensuring that NF-κB is held in the cytoplasm until cells are specifically induced to translocate it to the nucleus.

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NF-κB is a widely expressed factor important for transcription of the immunoglobulin κ gene in mature B-lymphoid cells and for rapid response to external stimuli in other cells (Blank et al. 1992; Nolan and Baltimore 1992). NF-κB is composed of two subunits, p50 and p65, that associate with a third protein, IκB, in many cell types (Baeuerle and Baltimore 1989). p50 and p65 exhibit amino-terminal homology to the proto-oncogene c-*rel*, heterodimerize through interaction sites in their Rel homology domains, and bind to κB sites in a variety of genes to stimulate transcription (Ghosh et al. 1990; Kieran et al. 1990; Zabel et al. 1991). In addition to its Rel homology region, p65 contains an acidic carboxyl terminus that functions in transcriptional activation (Nolan et al. 1991; Ruben et al. 1991; Fujita et al. 1992). A naturally occurring splice variant of p65, p65Δ, which lacks 10 *rel*-homologous amino acids, has been suggested to transform Rat-1 fibroblasts and to exhibit differential expression during hemopoietic cell development (Narayanan et al. 1992). The 38-kD protein, IκB-α, that copurifies with NF-κB and inhibits its binding to DNA, can be dissociated from p50/p65 by deoxycholate (Baeuerle and Baltimore 1988; Zabel and Baeuerle 1991). A cDNA clone, MAD-3, originally isolated from adherent macrophages, encodes a protein that exhibits the properties of and contains peptide sequences from IκB-α (Davis et al. 1991; Haskill et al. 1991; Link et al. 1992). Notable in its sequence are repeats of a motif present in *cdc10*, SWI4, SWI6 and the erythrocyte structural protein ankyrin (ankyrin repeat; Andrews and Herskowitz 1989; Lux et al. 1990).

The mechanisms controlling the levels of NF-κB constituents are not yet established. Transient activation of NF-κB can be achieved in many cells by treatment with stimuli like active phorbol esters (PMA) or lipopolysaccharide (LPS; Sen and Baltimore 1986). PMA stimulates protein kinase C, which can phosphorylate IκB in vitro, eliminating its ability to inhibit DNA binding by p50/p65 (Ghosh and Baltimore 1990). Thus, the control of NF-κB may involve, at least partially, the controlled phosphorylation of IκB.

We have found that p65 overexpression, directed either by transfection or by retroviral infection, causes a striking increase in endogenous IκB-α protein. The induced IκB-α is quite stable. It differs markedly in this sense from IκB-α overproduced by plasmid-directed expression, which is short-lived. Combination of IκB-α with p65 then appears to stabilize the inhibitor protein, explaining part of the increased IκB-α produced by p65 expression. Overexpression of p65 also induces increased IκB-α mRNA; run-on experiments show that the increase is the result of a highly specific stimulation of transcription. In contrast to p65, retrovirus-directed expression of the 10-amino-acid natural deletion mutant p65Δ fails to increase intracellular levels of IκB-α. p65Δ binds with lower affinity to IκB and does not activate transcription from κB-dependent promoters (Ruben et al. 1992). The localization of overproduced p65 and p65Δ reflects their ability to induce IκB: p65 is cytoplasmic, whereas p65Δ is partly nuclear. In addition, coexpressed p50 and p65 are maintained in the cytoplasm owing to the induced IκB, whereas p50 alone is nuclear. These findings are explained most simply by the idea that IκB-α induced by p65 maintains NF-κB in the cytoplasm. Therefore, it appears that this system of proteins is autoregulated through the ability of p65 to determine the

level of IκB-α protein. Similar conclusions were reached in two articles published while this paper was being reviewed (Brown et al. 1993; Sun et al. 1993).

Results

IκB levels parallel those of p65, not p65Δ

cDNAs coding for IκB-α, p65, and p65Δ were each inserted into a retroviral vector and then transfected by the calcium phosphate technique into 293 cells (human embryonic kidney cells transformed by the adenovirus E1A protein). Using Western blotting to detect the protein, untransfected cells exhibited nearly undetectable levels of p65, whereas lysates prepared 48 hr after transfection with each of three separately prepared constructs contained high levels of p65 (Fig. 1A, lanes 5–7). The untransfected cells also had virtually undetectable levels of IκB-α, but the cells that made p65 contained a large quantity of IκB-α (Fig. 1B, lanes 5–7). This inductive effect was specific to wild-type p65, because control cells transfected with the activated tyrosine kinase v-*abl*, the IκB-like molecule *bcl*-3, or empty vector (pGD) alone showed no increase in IκB-α (lanes 1–4,8). The levels of IκB-α induced by p65 were surprisingly high. By Western blotting, the amount of p65-dependent IκB-α was similar to that achieved through retroviral expression of the IκB-α gene product alone (lane 9). In contrast, cells transfected with the p65Δ construct produced p65Δ but exhibited little, if any, induction of IκB-α expression (lane 11) compared with cells transfected with the parent retroviral vector (lane 8); at most, a twofold increase might occur compared with the very marked increase induced by p65.

Time-course studies also suggested that the observed increases in IκB-α expression depended on p65 expression. Lysates were prepared from separate dishes of cells that were transfected simultaneously with a p65 construct and harvested at the times indicated in Figure 2. Western blotting and detection with antibodies to p65 revealed low levels of the proteins until 24 hr after transfection. IκB-α levels in the same lysates remained low at early time points and rose only when p65 was seen (Fig. 2B, lanes 7,8).

The striking parallels between p65 and IκB-α expression were not limited to transfected or adherent cells. To introduce the p65 retroviral expression construct into pre-B and B cells by retroviral transduction, we first cotransfected 293 cells with both the replication-defective p65 retrovirus construct and a clone encoding replication-competent Moloney murine leukemia virus (MuLV) to generate virus stocks that could infect murine 70Z/3 and WEHI 231 cells. Two days after transfection, lymphoid cells were added to the transfected (adherent) producer cells and cocultured for 24 hr. Medium containing the infected nonadherent lymphoid cells was then aspirated from the coculture. Lysates from the nonadherent cells were analyzed by Western blotting. Like the transfected 293 cells, both murine B-lymphoid cell types expressed elevated amounts of IκB-α after introduction of the p65 gene (p65 levels in Fig. 3A; IκB-α levels in Fig. 3B). The p65 and IκB-α that we detected were unlikely to result from contamination of the nonadherent cell population by retrovirus-producing 293 cells for two reasons: (1) Free virus infection of 70Z/3 and WEHI 231 cells has been carried out with identical, but quantitatively less, impressive results (data not shown); (2) human nonadherent lymphoid (Namalwa) cells that are not infectable by ecotropic murine virus were cocultivated

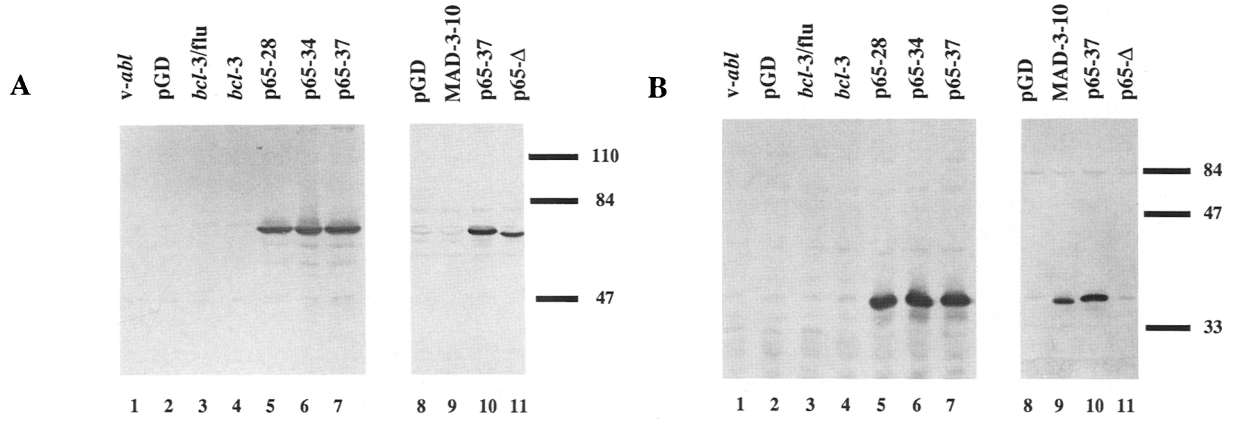

Figure 1. p65 and IκB-α proteins in transfected cells. Human renal carcinoma (293) cells were transfected with plasmids directing expression of the genes indicated at the *top* of *A* and *B*. pGD (lanes *2*, *8*) is the parent retroviral vector without insert. v-*abl* Refers to a construct containing the Abelson murine leukemia virus tyrosine kinase gene (lane *1*). *bcl*-3 and *bcl*-3/flu (lanes *3*,*4*) refer to constructs containing a murine cDNA encoding the *bcl*-3 proto-oncogene with or without a carboxy-terminal influenza virus HA epitope. p65-28, p65-34, and p65-37 (lanes *5*–*7*) are independent clones of a p65 cDNA in the pGD vector. MAD-3-10 (lane *9*) refers to a pGD plasmid containing the IκB-α (MAD-3) cDNA. p65Δ (lane *11*) was derived from pGD–p65-34. Whole-cell protein lysates were boiled in SDS/mercaptoethanol, electrophoresed through denaturing gels, and transferred to nitrocellulose. One filter was incubated with anti-p65 serum (*A*); the second filter was incubated with antibody to IκB-α (*B*). Molecular masses (in kilodaltons) are indicated by the numbers at *right*. Lanes *1*–*7* and *8*–*11* are from separate transfections.

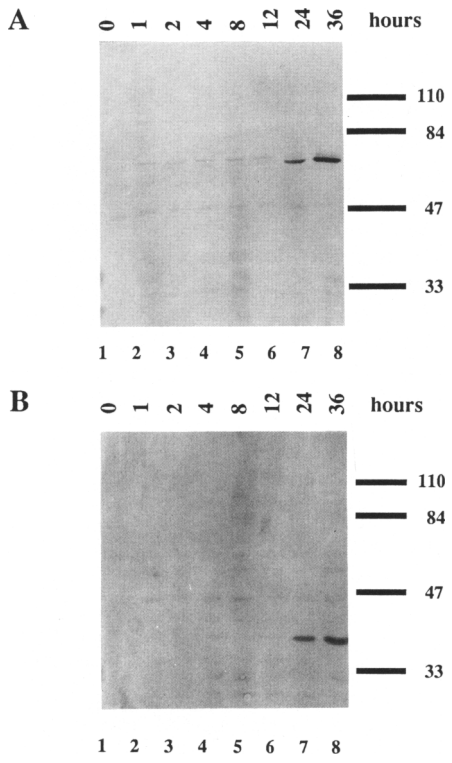

Figure 2. Time course of p65 and IκB-α expression in transfected cells. 293 cells grown in separate 60-mm dishes were transfected simultaneously with pGD–p65-34 DNA as in Fig. 1. At times indicated (in hours) after addition of DNA as a calcium phosphate precipitate, cells from each dish were lysed and boiled in SDS/mercaptoethanol sample buffer. Duplicate polyacrylamide gels of the samples were transferred to nitrocellulose. One filter was incubated with p65 antiserum (*A*); the second filter was incubated with affinity-purified antibody to IκB-α (*B*). Molecular masses (in kilodaltons) are indicated by the numbers at *right*. Bands demonstrating retrovirally expressed p65 and endogenous IκB-α are visualized in the 24- and 36-hr time points (lanes 7,8).

with retrovirus-producing 293 cells by the same protocol and displayed no detectable level of p65 or IκB-α (data not shown). We therefore conclude that IκB-α levels in a variety of cell types may be determined by the levels of p65 expression in those cells.

The mechanism of increased IκB expression is multifactorial

To determine the mechanism of increased IκB-α expression in p65-transfected cells, we first carried out protein-labeling experiments. 293 cells were transfected with either a retroviral p65 construct or an IκB-α (MAD-3) retroviral plasmid and labeled for 30 min with [^{35}S]methionine. Immunoprecipitations at the times indicated after labeling showed that the half-life of p65 was long, on the order of 12–24 hr (Fig. 4A; despite strongly denaturing lysis and wash conditions, a smaller protein that migrates at a size identical to overexpressed IκB-α was also observed in the immunoprecipitations of p65). Overexpressed IκB-α was, in the absence of p65, a very short-lived protein with a $t_{1/2}$ of ~30 min (Fig. 4B). Immunoprecipitation of IκB-α from p65-transfected cells (Fig. 4C,D) showed that protein stabilization was a significant factor in increasing the levels of the inhibitor. In the presence of p65, the half-life of IκB-α was extended at least 25-fold to >12 hr (Fig. 4D). The upper limit of the half-life is not clearly established by the above experiment, which was carried out three times with no reproducible drop in the amount of protein detected 24 hr after labeling.

To investigate the importance of transcription or mRNA stabilization to the observed increase in IκB-α protein levels, poly(A)-containing RNA from 293 cells transfected with retroviral p65, p65Δ, or pGD vectors was isolated and hybridized with p65- and IκB-α-specific probes. Specifically bound probe was quantitated using a PhosphorImager. Cells transfected with the p65 or p65Δ constructs exhibited the expected 20- to 50-fold increases of p65 mRNA (Fig. 5A). Importantly, the p65-transfected cells contained approximately seven times as much IκB-α mRNA as did cells transfected with p65Δ or the pGD vector (Fig. 5B). Thus, p65 can both stabilize IκB-α protein and increase the steady-state concentration of its mRNA.

To determine whether the increased IκB-α mRNA was the result of increased transcription of the gene, we carried out nuclear run-on studies. We used retroviruses to introduce *rel*- or IκB-related genes into NIH-3T3 cells and obtain run-on RNA free of contaminating plasmid sequences. Viruses transducing p50, an influenza hem-

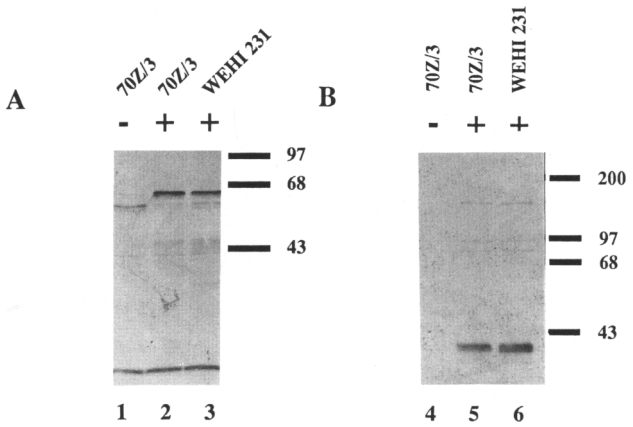

Figure 3. Retrovirus-induced p65 and IκB-α proteins in lymphoid cells. 70Z/3 pre-B cells were infected with Moloney MuLV alone (−, lanes *1,4*) or with pGD–p65 and MuLV helper virus (+, lanes *2,5*); WEHI 231 cells were infected with pGD–p65 and MuLV helper virus (+, lanes *3,6*). Twenty-four hours after cocultivation with adherent cells producing the indicated viruses, lymphoid cells were aspirated from the culture, pelleted by centrifugation, and lysed in RIPA buffer. Lysates were then subjected to gel electrophoresis and Western blotting as in Figs. 1 and 2. (*A*) Proteins detected by p65-specific antiserum; (*B*) proteins detected by IκB-α-specific antibodies.

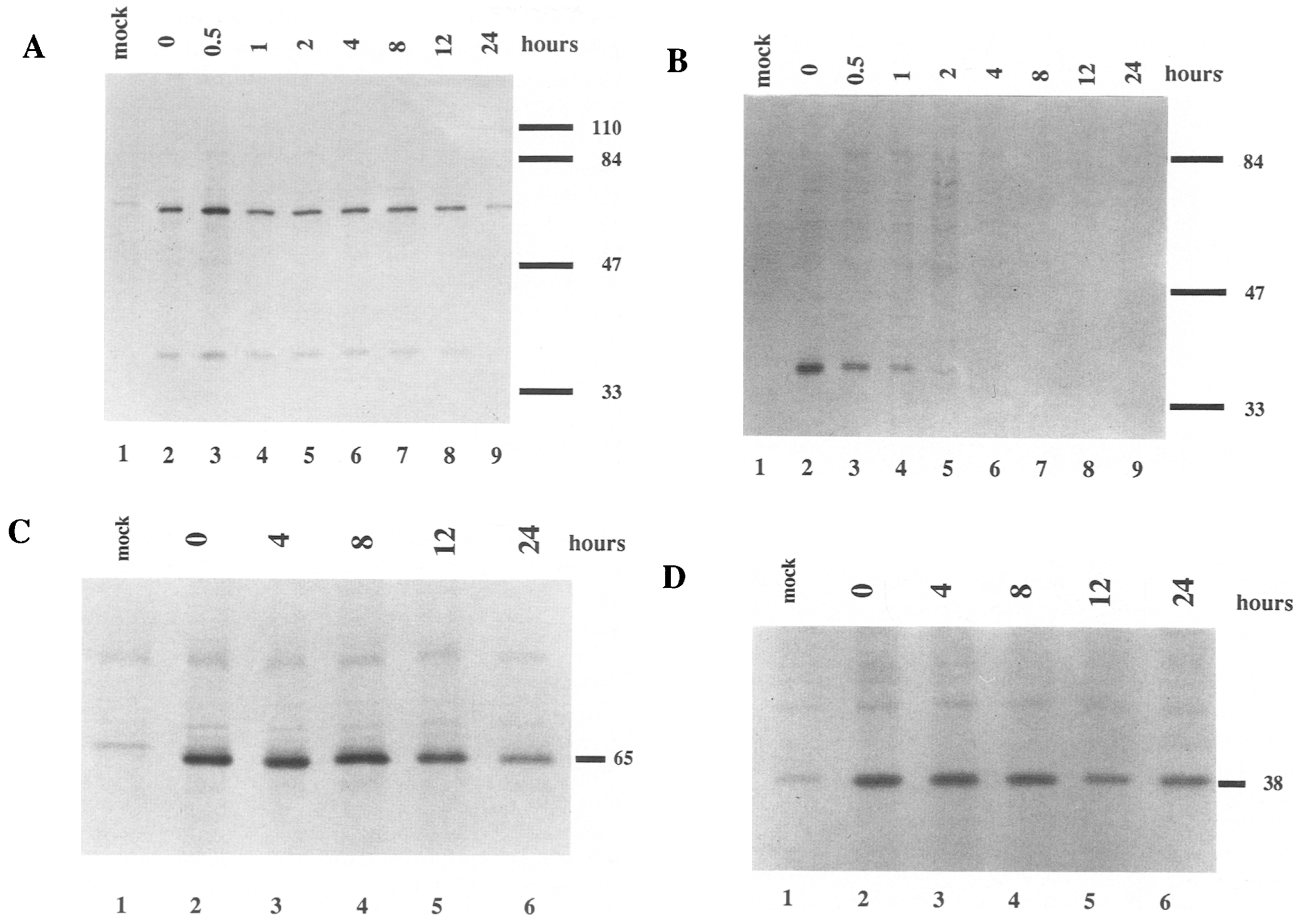

Figure 4. Pulse-chase labeling of p65 and IκB-α proteins in transfected cells. (*A*) Separate plates of 293 cells were transfected with pGD–p65-34 DNA or pGD vector DNA (mock, lane *1*). Thirty-six hours after addition of the DNA, all plates were starved in methionine-free medium for 30 min, and pulse labeled with [^{35}S]methionine for 30 min. All cells were then refed with normal medium. Just after feeding, lysates were prepared from mock-transfected cells and pGD–p65-transfected cells (time 0, lane *2*). Lysates from separate plates were then prepared at the times indicated (in hours) and stored at −70°C. All samples were then immunoprecipitated using anti-p65 serum, resolved by electrophoresis on SDS–polyacrylamide gels, and autoradiographed. (*B*) Separate plates of 293 cells were transfected with retroviral DNAs encoding IκB-α (pGD–MAD-3-10) or pGD vector alone (mock) and processed as described in *A*. These IκB-α-transfected cell lysates were then immunoprecipitated using affinity-purified anti-IκB-α antibody, analyzed on SDS–polyacrylamide gels, and autoradiographed. (*C,D*) Separate plates of 293 cells were transfected with pGD–p65-34 DNA or pGD vector DNA (mock, lane *1*). Thirty-six hours after the addition of the DNA, all plates were labeled with [^{35}S]methionine, refed, and lysed at the indicated times (in hours) after labeling as before. Lysates from each plate were then divided. Half of the lysate from a given time point was immunoprecipitated using anti-p65 serum (*C*); the other half was precipitated with anti-IκB-α antibody (*D*). The precipitates were analyzed on separate SDS–polyacrylamide gels and autoradiographed as shown.

agglutinin (HA) epitope-tagged variant of p50, p65, p65Δ, bcl-3, and IκB-α were tested. RNAs from the nuclei of infected cells were in vitro labeled and hybridized to immobilized plasmid DNAs coding for various *rel*- and IκB-related genes, as well as others (Fig. 6). In each case, the increased transcription of the retrovirally transduced gene was detected. The only endogenous gene that responded to any retroviral infection was the IκB-α gene; its transcription rate increased 5- to 10-fold after infection by the p65-encoding retrovirus (lane 3) but not after infection with the other viruses. Although strong binding between p50 and both bcl-3 and IκB-γ have been demonstrated (Franzoso et al. 1992; Wulczyn et al. 1992), no transcriptional response to p50 overproduction was evident among any of the genes tested. Thus, p65 specifically increases the rate of IκB-α transcription, accounting for the increased steady-state amount of IκB-α mRNA.

Localization of p65

A central tenet of previous thinking about IκB/NF-κB regulation has been the idea that functional IκB tethers NF-κB in the cytoplasm of cells where it is dormant. Therefore, if the IκB-α protein induced by p65 is functional, overexpressed p65 should be confined to the cytoplasm. To determine the subcellular localization of p65, we used helper-containing retroviral stocks to transmit the p65 gene to NIH-3T3 cells for immunofluores-

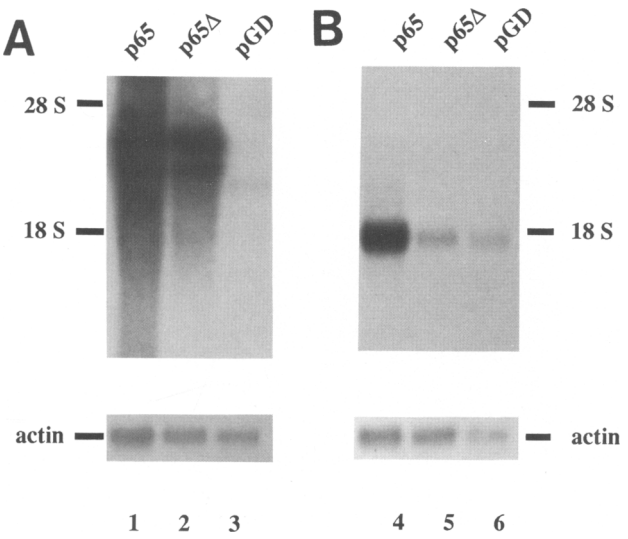

Figure 5. p65 and IκB-α mRNAs in transfected cells. 293 cells were transfected with DNA constructs encoding p65 or p65Δ expressed from a retroviral promoter, or with the empty retroviral vector (pGD), as described in Materials and methods. Poly(A)-containing RNA (2.5 μg/lane) from each population of cells was fractionated by electrophoresis through a formaldehyde–agarose gel, transferred to nitrocellulose, and hybridized with ^{32}P-labeled probe. The transfected expression construct is indicated at the *top* of each lane. Bars at the *right* indicate the positions of 18S and 28S RNAs, determined by ethidium bromide staining of a separate lane from the gel. (*A*) Filter 1 was hybridized to a p65-specific probe. Intensely hybridizing bands represent the RNA transcribed from the p65 (lane *1*) and p65Δ (lane *2*) expression constructs. The weak band in lane *3* represents endogenous p65 mRNA. Hybridization of the same filter to an actin probe is shown below. (*B*) Filter 2 was hybridized to an IκB-α-specific probe. The band visualized in lanes *4–6* represents endogenous IκB-α mRNA. Hybridization of the same filter to an actin probe is shown below.

cence studies. Under the staining conditions used, endogenous p65 was essentially undetectable (Fig. 7A,B). As predicted, retrovirally transduced wild-type p65 was almost completely limited to the cytoplasm (Fig. 7C). In contrast, the transcriptionally inactive p65Δ was found in both the nucleus and the cytoplasm of infected cells (Fig. 7D).

If the p65 regulatory effect is the central mode of NF-κB regulation, then the induced IκB should maintain the whole NF-κB complex in the cytoplasm. To test this possibility, we simultaneously infected NIH-3T3 cells with retroviral stocks that could transmit NF-κB p50 (tagged at its amino terminus with a 9-amino-acid influenza virus HA epitope and detectable by a mouse monoclonal antibody; Field et al. 1988) or p65 (detectable by specific rabbit antiserum). Immunofluorescence staining 2 days after infection (Fig. 8) revealed populations of cells singly infected by either p50- or p65-transducing viruses, cells simultaneously infected by viruses transducing both genes, and uninfected cells. Figure 8A is the phase photomicrograph of a field in which one cell overexpressing p65 is stained with a fluorescein-conjugated secondary antibody (Fig. 8B); neighboring cells lack expression. Examination using a rhodaminated anti-mouse antibody (Fig. 8C) shows that the p65-expressing cell is also expressing p50 and that both molecules are wholly cytoplasmic. As a control, another cell in the field expresses p50 but not p65. Its fluorescence is entirely nuclear. Thus, the IκB-α induced by p65 maintains the p50/p65 NF-κB complex in the cytoplasm, even at very high levels of expression. In contrast, p50 alone induces only a small amount of IκB (data not shown), and virtually all of the protein is found in the nucleus.

Discussion

NF-κB p50/p65 heterodimers in most cells are held in the cytoplasm by an inhibitor molecule, IκB. In many cell types, specific signals such as LPS, PMA, or TNF-α can release p50/p65 from IκB, causing transport of transcriptionally active heterodimers to the nucleus. This scheme requires that inhibitor levels in unstimulated cells always equal or exceed transcription factor levels. Our studies suggest that the importance of controlling transcription from κB-dependent genes is such that higher organisms have evolved at least two ways to assure parity of NF-κB and IκB levels. First, p65-dependent stabilization of IκB-α protein can increase inhibitor half-life at least 25-fold. Because p65 is stable, but free IκB-α is unstable, cells must make a large molar excess of IκB-α that is available to be stabilized by any increased synthesis of p65. Second, IκB-α mRNA levels are at least sevenfold inducible by p65 as a consequence of increased transcription of the IκB-α gene. Therefore, even larger increases of p65 can trigger increased synthesis of IκB-α.

Stabilization of IκB-α

We found that the half-life of retrovirally expressed IκB-α, free from p65, is short (~30 min). Similar to findings by Sun et al. (1993) and Brown et al. (1993), published while this paper was being reviewed, we have observed the rapid disappearance of endogenous IκB after PMA or LPS treatment of pre-B cells, presumably reflecting the difference in half-life between the unstable free and longer-lived bound forms (H.-C. Liou, M.L. Scott, and D. Baltimore, unpubl.). Two structural features of IκB-α predict a short half-life for the free protein. The two amino acids just following the initiating methionine (F and Q) are by the amino-end rule "destabilizing" residues (Bachmair and Varshavsky 1989); interestingly, two lysine residues, 18 amino-acids carboxy-terminal to the Q, are at a distance such that one or both could serve as acceptors for ubiquitin and target the protein for degradation (Hershko 1991). Also, the presence of PEST sequences associated with rapid protein turnover has been noted previously (Rogers et al. 1986; Haskill et al. 1991). Thus, binding of IκB-α to p65 must prevent the inhibitor molecule from interacting with one or more degradative pathways

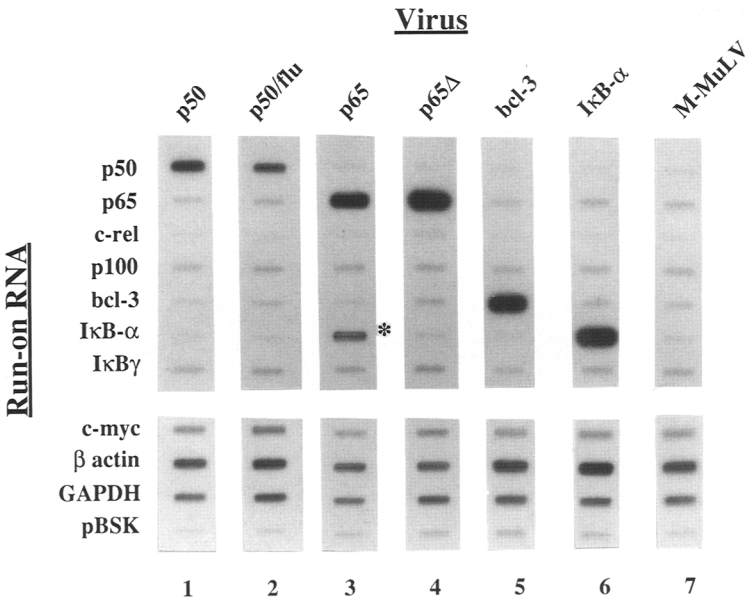

Figure 6. Nuclear run-on RNAs from Rel- and IκB-expressing cells. Retrovirus stocks transducing the genes indicated at the *top* (Virus) were used to infect NIH-3T3 cells. Thirty-six hours after infection, ^{32}P-labeled nuclear run-on RNA was prepared from each population of infected cells and hybridized to slot-blotted plasmids containing the cDNAs indicated at the *left* (see Materials and methods). The asterisk (*) indicates the increased IκB-α transcription in p65-expressing cells.

in the cell. In unstimulated cells, IκB-α is probably made in excess, with the unbound fraction being continually degraded, such that fluctuations in the level of p65 will not lead to the unprogrammed nuclear appearance of NF-κB (p50/p65) or $(p65)_2$. This suggests a mechanism by which cells that have cytoplasmic NF-κB (p50/65) can also have nuclear $(p50)_2$ (KBF-1; Kieran et al. 1990). If p50 were made in excess over p65, its transport to the nucleus would not be inhibited by IκB-α. Consistent with this idea, we have observed that overexpression of p50 induces a small amount of IκB-α but has no effect on *bcl-3* or IκB-γ, the two regulators that interact most strongly with p50 itself (Franzoso et al. 1992; Inoue et al. 1992; Liou et al. 1992; Wulczyn et al. 1992).

IκB-α protein levels are also regulated by p65-dependent changes in IκB mRNA. We have shown here that retrovirus-directed expression of murine p65 can cause at least a sevenfold increase in IκB-α mRNA in transfected 293 cells. Nuclear run-on studies of RNA from NIH-3T3 cells demonstrated that most of the effect arises from increased transcription (Fig. 6). Although we cannot rule out a small contribution from post-transcriptional events, the simplest explanation for our findings involves the presence of κB sequences in the IκB-α promoter. These sequences could bind $(p65)_2$, p50/p65, or p65 complexed with another *rel*-related protein. Two recent findings are consistent with this hypothesis. First, we have demonstrated that $(p65)_2$ can function as a transcriptional activator in vitro (Fujita et al. 1992). Second, R. de Martin and F. Bach (pers. comm.) have sequenced the promoter region of a porcine genomic locus that encodes a structural and functional homolog of IκB-α; this promoter contains two κB sites that are functional in EMSA assays. Increased transcription of IκB-α is probably a physiologic response that can control increases in p65 or NF-κB beyond those that can be accommodated by protein stabilization.

Localization

The fluorescence data on p65 subcellular localization that we have presented here differ from the results of Beg et al. (1992) and Zabel et al. (1993), who found that overexpressed p65 localized to the nucleus. At least three experimental details distinguish our work from theirs. First, we used wild-type p65 and IκB-α detected with rabbit sera specific to them, not proteins amino-terminally modified with an influenza virus HA epitope detected with an epitope-specific monoclonal antibody. Although the epitope-tagged proteins are largely similar in in vitro function to their normal counterparts, it is sometimes difficult to be certain that their in vivo behavior is identical. To rule out this possibility, we have also carried out coinfection experiments similar to those shown in Figure 8 using viruses that encode wild-type p50 and p65. Detection with p50-specific antiserum revealed a population of cells expressing cytoplasmic p50, which is not seen in cells transduced by p50 virus alone (data not shown). Second, the other studies were carried out using vectors containing a cytomegalovirus (CMV) promoter that, in our hands, yields expression levels at least 10-fold higher than the retrovirus-based constructs that we have employed. This partly results from the presence of κB sites in the CMV promoter itself. Expressed proteins may therefore up-regulate their own synthesis. A third potential explanation involves the cell types studied. Some studies (Beg et al. 1992) were carried out in COS cells that can amplify the transfected constructs, whereas our investigation employed renal carcinoma, pre-B, and B-lymphoid cells without the ability to amplify. It is our belief that the last two differences are probably the source of the discrepancy. We suspect that in our experiments, the amount of p65 does not exceed the capacity of a cell to respond by synthesis of an equimolar amount of IκB-α. In the other studies there is prob-

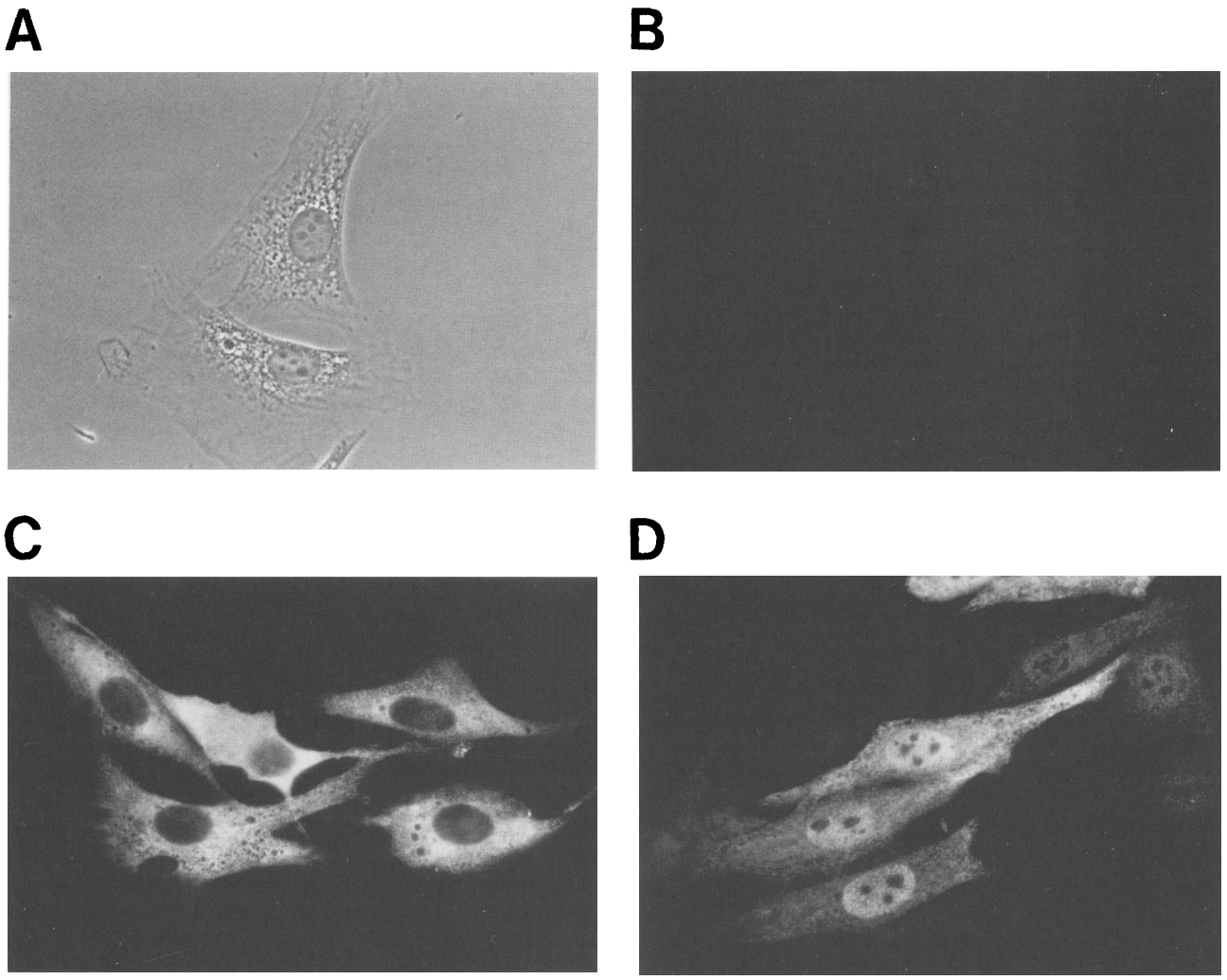

Figure 7. Immunolocalization of p65 and p65Δ in NIH-3T3 cells. (*A*) A phase photomicrograph of cells infected with Moloney MuLV (helper) virus alone (60× objective). (*B*) A fluorescence micrograph of the cells seen in *A*. This sample was stained with p65-specific primary antibody (1 : 300 dilution) and secondary rhodaminated donkey antirabbit serum (1 : 200 dilution), and was then photographed under the conditions used to identify the cells shown in *C* and *D*. (*C*) NIH-3T3 cells infected with the p65-transducing retroviral construct pGD–p65-34 were stained with p65-specific serum and photographed (60× objective) under conditions identical to those employed in *B*. Greater than 50% of the cells in this sample expressed detectable p65 (all cytoplasmic). (*D*) NIH-3T3 cells infected with the p65-transducing retroviral construct pGD–p65-Δ4 were stained with p65-specific serum and photographed (60× objective) under conditions identical to those employed in *B*. Greater than 30% of the cells in this sample expressed detectable p65Δ, all with a mixture of cytoplasmic and nuclear staining.

ably such an excess of p65 that some or most of it is free from IκB-α and is therefore transported to the nucleus. It seems unlikely that cells can make such an excess of p65 from their endogenous gene that it would overwhelm the IκB response system. In this regard it is probably significant that transfection of a plasmid containing the genomic p65 promoter and natural exons causes increases in p65 and IκB-α similar to those with retroviral constructs (W. Sha, M. Scott, and D. Baltimore, unpubl.).

The p65Δ splice variant both fails to stabilize IκB-α and fails to induce elevated transcription of IκB-α mRNA, properties that likely cause its localization to the nucleus. It has recently been reported that p65Δ interacts only weakly with IκB-α, possibly because the splice variant has little ability to dimerize with itself or other Rel variants (Beg et al. 1992). Thus, it may be the affinity of IκB-α for its full-length p65 target that stabilizes the inhibitor protein (Ganchi et al. 1992). Such high-affinity binding could mask a site of interaction with a proteolytic system or could be the signal for a modification of IκB-α that makes it insensitive to a proteolytic system. How these properties relate to the transforming potential of p65Δ (Narayanan et al. 1992) remains to be determined.

Materials and methods

Viruses and cells

p65 virus inserts were prepared by PCR using as template the murine p65-5 cDNA clone (Nolan et al. 1991). One microgram

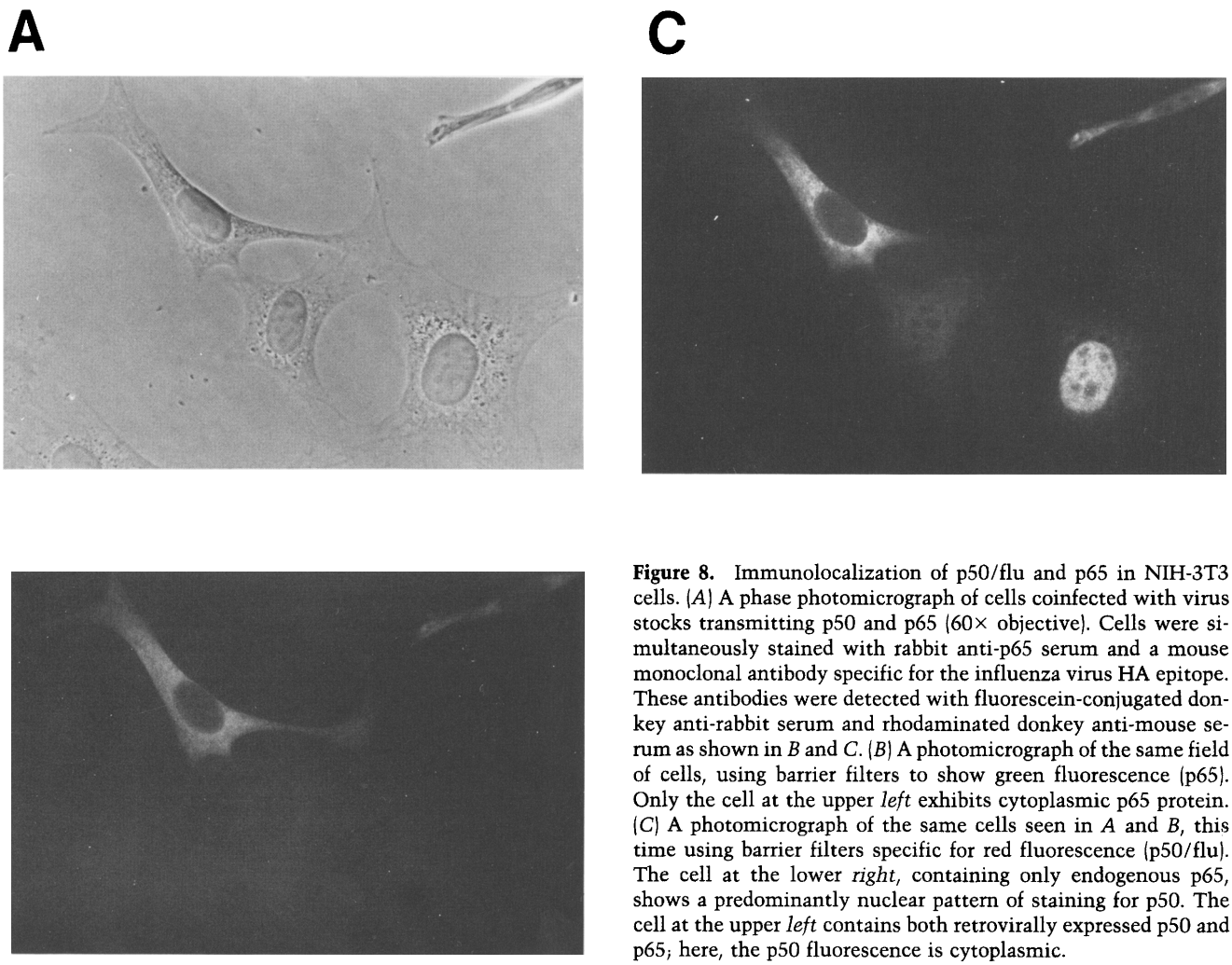

Figure 8. Immunolocalization of p50/flu and p65 in NIH-3T3 cells. (*A*) A phase photomicrograph of cells coinfected with virus stocks transmitting p50 and p65 (60× objective). Cells were simultaneously stained with rabbit anti-p65 serum and a mouse monoclonal antibody specific for the influenza virus HA epitope. These antibodies were detected with fluorescein-conjugated donkey anti-rabbit serum and rhodaminated donkey anti-mouse serum as shown in *B* and *C*. (*B*) A photomicrograph of the same field of cells, using barrier filters to show green fluorescence (p65). Only the cell at the upper *left* exhibits cytoplasmic p65 protein. (*C*) A photomicrograph of the same cells seen in *A* and *B*, this time using barrier filters specific for red fluorescence (p50/flu). The cell at the lower *right*, containing only endogenous p65, shows a predominantly nuclear pattern of staining for p50. The cell at the upper *left* contains both retrovirally expressed p50 and p65; here, the p50 fluorescence is cytoplasmic.

of template DNA was amplified for 10 cycles under conditions including 10% DMSO and 4 mM $MgCl_2$ described by the manufacturer of *Taq* polymerase (Stratagene) to yield a 1.6-kb product with the following structure: *Sal*I–*Eco*RI–Kozak–p65–*Eco*RI–*Cla*I. After gel isolation, this product was inserted into the pGD retroviral vector (Daley et al. 1990) at its *Xho*I and *Cla*I sites, removing the neomycin-resistance cassette from the virus. Three independent clones (pGD–p65-28, pGD–p65-34, and pGD–p65-37) were identified; all yielded the same increase in IκB-α levels described in the text. A p65Δ mutation, deleting amino acids EDIEVYFTGP from the murine cDNA, was introduced using a 30-base oligonucleotide to prime second-strand synthesis of uracil-substituted pBSK-p65-5 phagemid DNA (Ausubel et al. 1989). A clone containing the p65Δ mutation was identified by DNA sequencing. The *Sph*I–*Bsi*WI fragment carrying this deletion was inserted into the pGD–p65-34 retroviral construct. Clones pGD–p65Δ-4 and pGD–p65Δ-11 were selected on the basis of *Sph*I, *Bsi*WI, and *Sac*I–*Eco*RI digestion (the last yielding a 228-bp fragment 30 bp shorter than wild type) for further characterization. The human IκB-α (MAD-3) cDNA was amplified under the conditions described above, using 51-mer and 48-mer primers to generate a *Bam*HI–*Eco*RI–IκB-α–*Eco*RI–*Sal*I product. This gel-isolated product was ligated to a modified pGD vector generated by *Xho*I digestion of the parent plasmid, followed by addition of a *Bcl*I linker to the blunted site. The *Bcl*I cloning vector (pGDB) so generated was digested with *Bcl*I and *Sal*I to allow insertion of the corresponding MAD-3 cDNA. Retroviral constructs encoding murine *bcl*-3 and influenza HA epitope-tagged or wild-type murine p50 were also prepared and are described elsewhere (Nolan et al. 1993).

Virus stocks and virus-producing cells were prepared by the method of W. Pear, G. Nolan, M. Scott, and D. Baltimore (in prep.) using pGD–p65, p65Δ, or IκB-α constructs and a replication-competent helper virus clone pZAP (Goff et al. 1982). In cocultivation experiments, 70Z/3 or WEHI 231 cells (10^6/ml in RPMI) were added to the adherent producer cell culture. Twenty-four hours thereafter, the medium containing nonadherent cells was aspirated and lysates for Western blotting were prepared as described below.

Protein immunoblotting

Lysates of transfected 293 cells or infected lymphoid cells were prepared at either 48 hr or at the times indicated after the addition of the p65 or IκB-α DNAs as calcium phosphate precipitates. Cells were washed once with cold calcium/magnesium-free PBS and scraped from the plates in RIPA buffer [25 mM Tris-Cl at pH 7.4, 0.5% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 0.5 mM EDTA, 150 mM NaCl, 1 mM PMSF, 10 μg/ml aprotinin (Sigma)], 1.0 ml/100-mm plate of cells. After clarifi-

cation in an Eppendorf centrifuge (14000*g* for 10 min), 5–10 μl of each boiled lysate was applied to 10% polyacrylamide–SDS gels, electrophoresed, and transferred to supported nitrocellulose.

Rabbit sera specific to murine p65 were obtained after immunization with full-length protein derived from a baculovirus expression construct (Fujita et al. 1992). The serum was used at a dilution of 1 : 1000 on immunoblots. Affinity-purified antibodies specific to IκB-α were prepared by immunizing rabbits with a glutathione *S*-transferase/IκB-α fusion protein (Liou et al. 1992) and passing the immune sera over an Affigel 15 matrix (Bio-Rad) to which IκB-α fusion protein had been coupled as per the manufacturer's instructions. Antibody bound to the column was eluted as specified (Harlow and Lane 1988) and used at a concentration of 0.5–1.0 μg/ml. An alkaline phosphatase-coupled donkey anti-rabbit preparation (Jackson Immunoresearch) was used to detect bound primary antibody in transfected cells.

In vivo protein labeling

293 cells seeded in 60 mm plates at 2.5×10^5 cells per plate were transfected with 5 μg of retroviral DNA per plate and incubated 48 hr before labeling. After a 30-min period of starvation in methionine-free Dulbecco's minimal essential medium (DMEM) supplemented with 5% dialyzed fetal calf serum, fresh labeling medium with [^{35}S]methionine (Amersham, 800 Ci/mmole, 200 μCi/ml) was added for 30 min. At the end of labeling, radioactive medium was aspirated and replaced with standard DMEM/10% FCS (time 0). Lysates from the subsequent time points were prepared as described above and frozen at −70°C before immunoprecipitation. Bicinchoninate assays (Pierce) revealed less than twofold differences among the protein concentrations of all lysates. Each lysate was precleared by the addition of 5 μl of preimmune rabbit serum for 30 min on ice; this serum was then collected on a 20-μl bed volume of protein A–Sepharose 4B (Pharmacia) for 30 min.

To precipitate p65 from the precleared lysates of cells transfected with pGD–p65, 5 μl of specific serum was added. After incubation on ice for 60 min, another 20-μl bed volume of protein A–Sepharose (precoated with a cold lysate from untransfected 293 cells) was added for 30 min. The protein A–Sepharose beads were washed twice in RIPA buffer, twice in high salt buffer (1% Triton X-100, 0.5 M NaCl, 20 mM Tris-Cl at pH 7.6), and twice in low salt buffer (1% Triton X-100, 10 mM NaCl, 20 mM Tris-Cl at pH 7.6). After the last wash, each pellet was resuspended in 30 μl of protein sample buffer and boiled for 5 min. Five microliters of each sample was loaded onto SDS–polyacrylamide gels and visualized by autoradiography.

A similar protocol was used to precipitate IκB-α protein from cells transfected with either the p65 or IκB-α expression constructs. Instead of whole sera, affinity-purified antibody (5 μg per lysate) was used. As an additional demonstration of specificity, 5 μg of affinity-purified antibody to IκB-α was preincubated with 50 μg of fusion protein for 30 min on ice and then added to precleared lysates and processed as described above. This preincubation eliminated the 38-kD-labeled band precipitated from either p65 or (IκB-α)-transfected cells (not shown).

Immunofluorescence

Virus stocks transducing the p50, p65, and p65Δ genomes were prepared by transient transfection of 293 cells as described above and used to infect NIH-3T3 cells. 3T3 cells were seeded onto glass coverslips at 10^4 cells/well in 24-well plates. The cells were exposed, 24–36 hr later, to the indicated viruses in 4 μg/ml of Polybrene for 3 hr. For mixed infections, equal volumes of viral supernatants from 293 cells singly transfected with p50 or p65 constructs were added to the plate of 3T3 cells. Cells were then refed with DMEM/10% calf serum and incubated for 36–48 hr. (For some experiments, cells were infected at the indicated density in 35-mm plastic dishes, incubated for 24–48 hr, and seeded onto coverslips for staining 1 day thereafter.) After washing in PBS, coverslips were fixed in PBS/4% paraformaldehyde for 10 min, quenched with 50 mM glycine (pH 8.0) in PBS for 10 min, and permeabilized with 1% Triton X-100/PBS for 10 min. Cells remaining on the coverslips were then blocked in a solution of PBS, 5% normal donkey serum, and 1 mg/ml of BSA for 60 min. Primary rabbit antiserum specific to p65 was diluted 1 : 300 in blocking solution and added to the coverslips for 45 min. The coverslips were subsequently washed for 4–12 hr in three or four changes of PBS and reblocked for 30 min. Secondary antibody (rhodaminated donkey antirabbit Fab_2; Jackson Immunoresearch) diluted 1 : 200, as described above, was added for 30–45 min and washed away in PBS (two or three changes) for 1–3 hr before inversion and mounting on glass slides. Cells were visualized and photographed using a Nikon fluorescence microscope with a 60× objective.

RNA isolations and hybridizations

293 cells (10^7 cells/150-mm plate) were transfected with p65, p65Δ, or empty vector (pGD) retroviral DNAs and incubated at 37°C for 48 hr. After lysis in an NP-40-containing solution as per standard procedures (Ausubel et al. 1989), total cytoplasmic RNAs obtained from each population of transfected cells were applied to an oligo(dT)–cellulose column. Samples (2.5 μg) of each poly(A)-containing RNA were electrophoresed through formaldehyde–agarose gels, transferred to nitrocellulose, and hybridized to ^{32}P-labeled probes derived from gel-isolated p65 and MAD-3 cDNAs. Probe bound to each filter was detected by exposure to XAR film and quantitated by using a PhosphorImager (Molecular Dynamics). The filters were stripped and rehybridized to an actin probe, and the process was repeated to demonstrate equal loading of RNA in each lane.

Nuclear run-on assays were performed as described (Greenberg and Ziff 1984) using nuclei isolated from three 150-mm plates of NIH-3T3 cells, 36 hr after infection, with the indicated virus stocks prepared as described above. Plasmids (7.5 μg) encoding p50 (Ghosh et al. 1990), p65Δ (above), c-*rel* (Bull et al. 1990), human p100 (Schmid et al. 1991), *bcl*-3 (Nolan et al. 1993), human IκB-α, murine IκB-γ (Liou et al. 1992), c-*myc* (Saksela et al. 1991), glyceraldehyde phosphate dehydrogenase, and β-actin were linearized, phenol extracted, and slot blotted to supported nitrocellulose before hybridization with labeled RNA.

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