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Constitutive NF-κB Activity in Neurons

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NF-κB is an inducible transcription factor present in many cell types in a latent cytoplasmic form. So far, only immune cells including mature B cells, thymocytes, and adherent macrophages have been reported to contain constitutively active forms of NF-κB in the nucleus. A recent study showed that the human immunodeficiency virus type 1 (HIV-1) promoter is highly active in several brain regions of transgenic mice (J. R. Corboy, J. M. Buzy, M. C. Zink, and J. E. Clements, Science 258:1804–1807, 1992). Since the activity of this viral enhancer is governed mainly by two binding sites for NF-κB, we were prompted to investigate the state of NF-κB activity in neurons. Primary neuronal cultures derived from rat hippocampus and cerebral cortex showed a high constitutive expression of an HIV-1 long terminal repeat-driven luciferase reporter gene, which was primarily dependent on intact NF-κB binding sites and was abolished upon coexpression of the NF-κB-specific inhibitor IκB-α. Indirect immunofluorescence and confocal laser microscopy showed that the activity of NF-κB correlated with the presence of the NF-κB subunits p50 and RelA (p65) in nuclei of cultured neurons. NF-κB was also constitutively active in neurons in vivo. As investigated by electrophoretic mobility shift assays, constitutive NF-κB DNA-binding activity was highly enriched in fractions containing neuronal nuclei prepared from rat cerebral cortex. Nuclear NF-κB-specific immunostaining was also seen in cryosections from mouse cerebral cortex and hippocampus. Only a subset of neurons was stained. Activated NF-κB in the brain is likely to participate in normal brain function and to reflect a distinct state of neuronal activity or differentiation. Furthermore, it may explain the high level of activity of the HIV-1 enhancer in neurons, an observation potentially relevant for the etiology of the AIDS dementia complex caused by HIV infection of the central nervous system.

In eukaryotic cells, regulation of transcription factor activity is a widely used mechanism to control inducible gene expression. In many cases, such gene-regulatory proteins preexist in the cell and become active only when their DNA-binding or -transactivating potential is unleashed upon ligand binding, covalent modification, and/or association or release of regulatory protein subunits.

NF-κB was originally described as a constitutive transcription factor in mature B-cell lines (43). Subsequently, it was found that NF-κB was present in an inducible form in many other cell types (21, 35, 37, 43). The inducible form can be activated by treatment of cells with an extreme variety of conditions, including viral infection, bacterial lipopolysaccharide, inflammatory cytokines, and oxidants (for a review, see reference 2). Extensive studies over the past 3 years revealed that the DNA-binding subunits of NF-κB belong to a multigene family currently comprising five distinct proteins in higher vertebrates (for a review, see reference 8). The five DNA-binding subunits share an ~300-amino-acid-long region of sequence homology, encompassing domains required for dimerization, DNA binding, nuclear translocation, and association with regulatory subunits. Two of them, p50 and p52, can transactivate only when dimerized with one of the three transactivating DNA-binding subunits RelA, c-Rel, and RelB. A very frequent dimer combination is composed of p50 and RelA.

The DNA binding and nuclear uptake of NF-κB dimers is tightly controlled by association with a second type of protein subunit, called IκB. When IκB is released in response to extracellular stimuli, the DNA-binding dimer is translocated to the nucleus. Induction of nuclear transport of NF-κB involves unmasking of nuclear location signal sequences in p50 and RelA subunits (7, 18, 50). Distribution of NF-κB between the nucleus and cytoplasm is thus controlled by IκB and directly correlates with the activation state of the transcription factor.

The reactions leading to the disruption of interactions between IκB and RelA (or c-Rel) are complex. Coincident with the appearance of active NF-κB in cells, the α form of IκB is proteolytically degraded (6, 11, 26, 44). Several protease inhibitors can prevent the activation of NF-κB in intact cells, showing that the activity of an as yet unidentified protease is required for activation (26). Hydrogen peroxide appears to act as a common upstream messenger for many inducing conditions, as is evident from the strong inhibitory effect of many structurally unrelated antioxidants and the induction of NF-κB upon H_2O_2 treatment in some cells (40, 41). Protein phosphorylation events are also involved in controlling the activation of NF-κB (6, 19, 30).

IκB proteins belong to a multigene family as well (reviewed in references 8 and 21). In higher vertebrates, five members, which display distinct binding specificity and function, are currently known. Two are produced in *cis* with the DNA-binding subunits p50 and p52, giving rise to the inactive precursor molecules p105 and p100, respectively. These precursors have IκB-like function toward RelA and c-Rel subunits and require proteolytic processing to release their DNA-binding portions. The α and β forms of IκB seem to be involved in forming the inducible complexes. The nuclear Bcl-3 protein is unique in that it is an inhibitor toward p50 dimers but associates with p52 dimers on DNA to form a transactivating complex (9, 17, 24, 36, 38, 48). A common structural

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characteristic of IκB-like proteins is five to seven closely adjacent ~30-amino-acid repeats constituting the minimal requirement for protein-protein interaction and inhibitory activity.

Only a limited number of lymphoid cells have so far been found to contain constitutively active, nuclear NF-κB. These include mature B cells (41), thymocytes (32), a few T lymphomas (25), and some monocytic cell lines (20). In these cells, the constitutive activation of NF-κB is only partial; the cytoplasm still contains some of the factor in an inactive IκB-complexed form (4). The molecular mechanism leading to a permanent shift from IκB-bound to uncomplexed NF-κB is not understood in detail. There is evidence to suggest that constitutively active NF-κB in B and T cells serves to maintain an activated state of the immune cells by continuously upregulating the expression of cytokines and their receptors (for a review, see reference 21).

NF-κB is also abundant in brain tissues, as has been shown by electrophoretic mobility shift assays (EMSAs) (29). Most of the brain NF-κB was found in an inducible form. The predominant forms of NF-κB in the brain apparently consist of p50-RelA and RelA–c-Rel dimers. Immunofluorescence labeling of retina slices, subcellular brain fractionation, and colocalization with synatophysin suggested that a fraction of the inducible NF-κB as well as the p105 precursor in the brain are contained in synapses.

Inducible expression of human immunodeficiency virus type 1 (HIV-1) is almost entirely dependent on two high-affinity binding sites for NF-κB in the long terminal repeat (LTR) which synergize with three binding sites for the constitutive factor Sp1 (35). Transgenic mice which expressed an HIV-1 LTR-driven reporter gene in various regions of the mouse brain (13) have been described. This observation prompted us to investigate the state of NF-κB activity in primary neurons from cerebral cortex and hippocampus, using transiently transfected κB-controlled luciferase reporter constructs. In addition, we used immunofluorescence microscopy to examine whether NF-κB subunits were present in nuclei seen in cryosections from cerebral cortex and hippocampus. Strong evidence that NF-κB is constitutively active and present in the nuclei of cultured neurons as well as in subsets of neurons in vivo was obtained. We discuss possible roles for the differential activation state of NF-κB in neurons and the implication of our findings for HIV-1 infection of the brain.

MATERIALS AND METHODS

Primary cultures of hippocampal and cortical neurons. Primary cultures were obtained as described previously (5). Briefly, brains of Wistar rat embryos 17 days postcoitus (for hippocampi) or 12 days postcoitus (for cortices) were dissected and freed of meninges. After dissection of the corresponding regions, the tissue was homogenized by pipetting with a fire-polished Pasteur pipette. For transfection, 10^5 living cells were seeded on 6-cm-diameter plastic dishes coated with poly-L-lysine. For antibody staining, neurons were cultured on Lab-Tek chamber slides (Nunc). To suppress the growth of nonneuronal cells, culture was performed in serum-free medium.

Plasmids, transfection, and luciferase assay. The wild-type HIV-1 LTR-driven luciferase reporter and the corresponding construct with mutated κB sites were constructed (28) and kindly provided by N. Israël (Institut Pasteur). The tk(NF-κB)$_6$ luciferase reporter construct contains six reiterated copies of the HIV-1 κB site in front of a truncated herpes simplex thymidine kinase gene (*tk*) promoter spanning positions −105 to +51 (1). HeLa cells were cultured as described previously (26) and transfected by a modified calcium phosphate protocol (47). Primary cultures were transfected by using Lipofectin as instructed by the manufacturer (Bethesda Research Laboratories). Twenty hours after transfection, cells were lysed and assayed for luciferase activity (14). At least three independent transfection experiments were performed with different preparations of neurons. All experiments gave the same qualitative results. One representative experiment is shown.

Cryosectioning and antibody staining of mouse brains. Brains from BALB/c mice were dissected. Tissues were embedded in Tissue-Tek (Miles-Bayer, Leverkusen, Germany) and quick-frozen in liquid nitrogen. Eight-micrometer sections were cut on a Leitz cryotome (Leitz Instruments, Wetzlar, Germany) and collected on gelatin-coated slides. After fixation in methanol (−20°C, 5 min), tissue was blocked with 5% goat serum. For immunohistochemistry, the first antibody was diluted 1:100. Bound antibody was detected by a second antibody (biotin-conjugated anti-rabbit immunoglobulin G [IgG]) complexed to streptavidin peroxidase, and complexes were visualized by a Vectastain Elite kit (Serva, Heidelberg, Germany). For indirect immunofluorescence, blocking was performed with 5% goat serum, and the first antibody was diluted 1:20. Its binding was detected with a 1:100 dilution of a second antibody (anti-rabbit IgG with biotin spacer; Dianova, Hamburg, Germany) complexed to 1:300-diluted streptavidin-conjugated Cy3 (Dianova). For control sections, antibodies were preabsorbed with an equal amount of the corresponding peptide and incubated for 0.5 h at 37°C. Microphotographs were taken on a Zeiss photomicroscope equipped with epifluorescence. Confocal equipment was from Zeiss Instruments, Oberkochen, Germany.

Isolation of neuronal nuclei and EMSA. Nuclei from brain tissue were isolated essentially as described previously (46). Briefly, cerebral cortices were isolated from two rats and homogenized in 4 volumes of 2.0 M sucrose–1 mM $MgCl_2$ with a Potter homogenizer. The homogenate was mixed with an equal volume of 2.0 M sucrose solution and centrifuged at 64,000 × g_{av} (Beckman SW.28 rotor). The pellet was resuspended in 2.4 M sucrose–1 mM $MgCl_2$, overlaid with 1.8 M sucrose–1 mM $MgCl_2$, and then centrifuged at 85,000 × g_{av}. The neuronal nuclei concentrating at the interface of sucrose layers (N^1) and the nuclear population on the bottom of the tube (N^2) were collected and pelleted. Proteins were extracted with buffer C as described by Dignam et al. (16). EMSAs were performed as described by Henkel et al. (26), using 3.5 μg of nuclear proteins from N^1 and N^2 fractions and a ^{32}P-labeled oligonucleotide encompassing the NF-κB site from the murine κ light-chain enhancer (Promega). The specificity of protein-DNA complexes was tested by addition of 1 μl of two distinct p65-specific antisera (kind gifts of R. Bravo and L. Schmitz) to the DNA-binding mix.

RESULTS

The constitutive transcriptional activity of the HIV-1 LTR in primary neurons is dependent on NF-κB. Primary neuronal cultures were prepared from rat hippocampus and cerebral cortex and transfected with HIV-1 LTR-driven luciferase reporter plasmids. Luciferase was strongly expressed in primary hippocampal neurons, as assayed in cell extracts by light emission in the presence of luciferin and ATP (Fig. 1A, column 1). A reporter construct in which the two NF-κB sites in the enhancer were rendered inactive by mutations showed a strongly diminished activity (column 3), indicating that cultured neurons contained a constitutive transcriptional activator

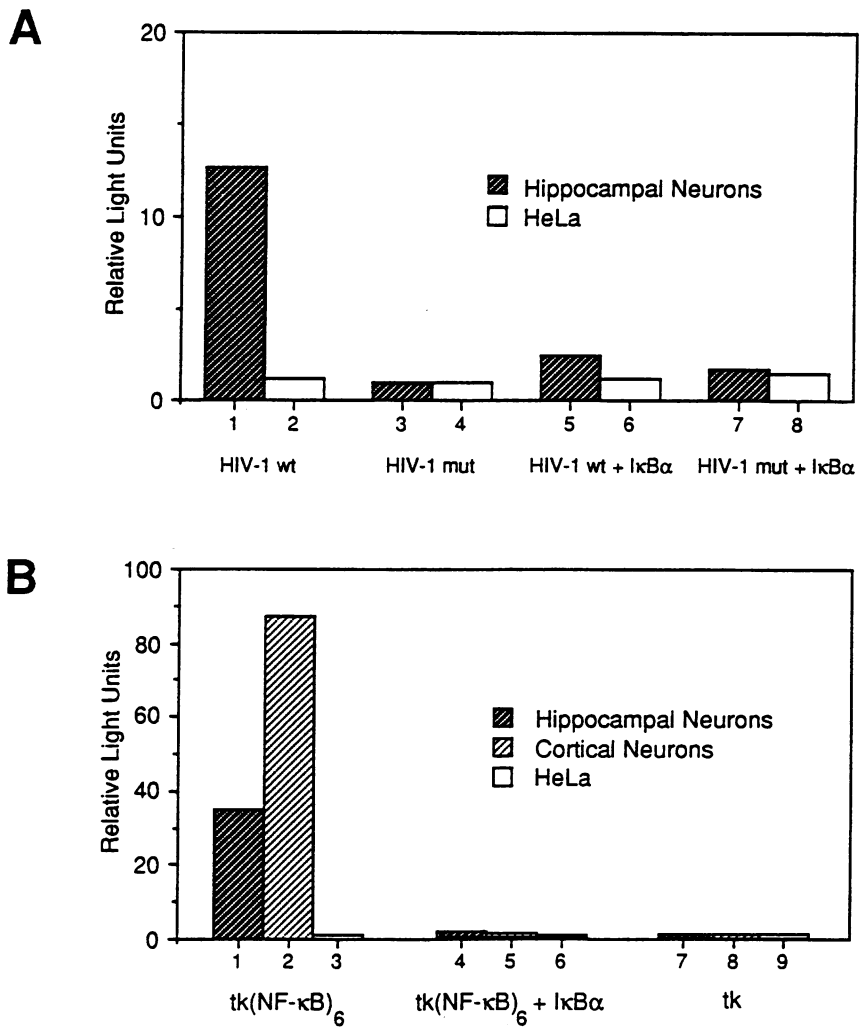
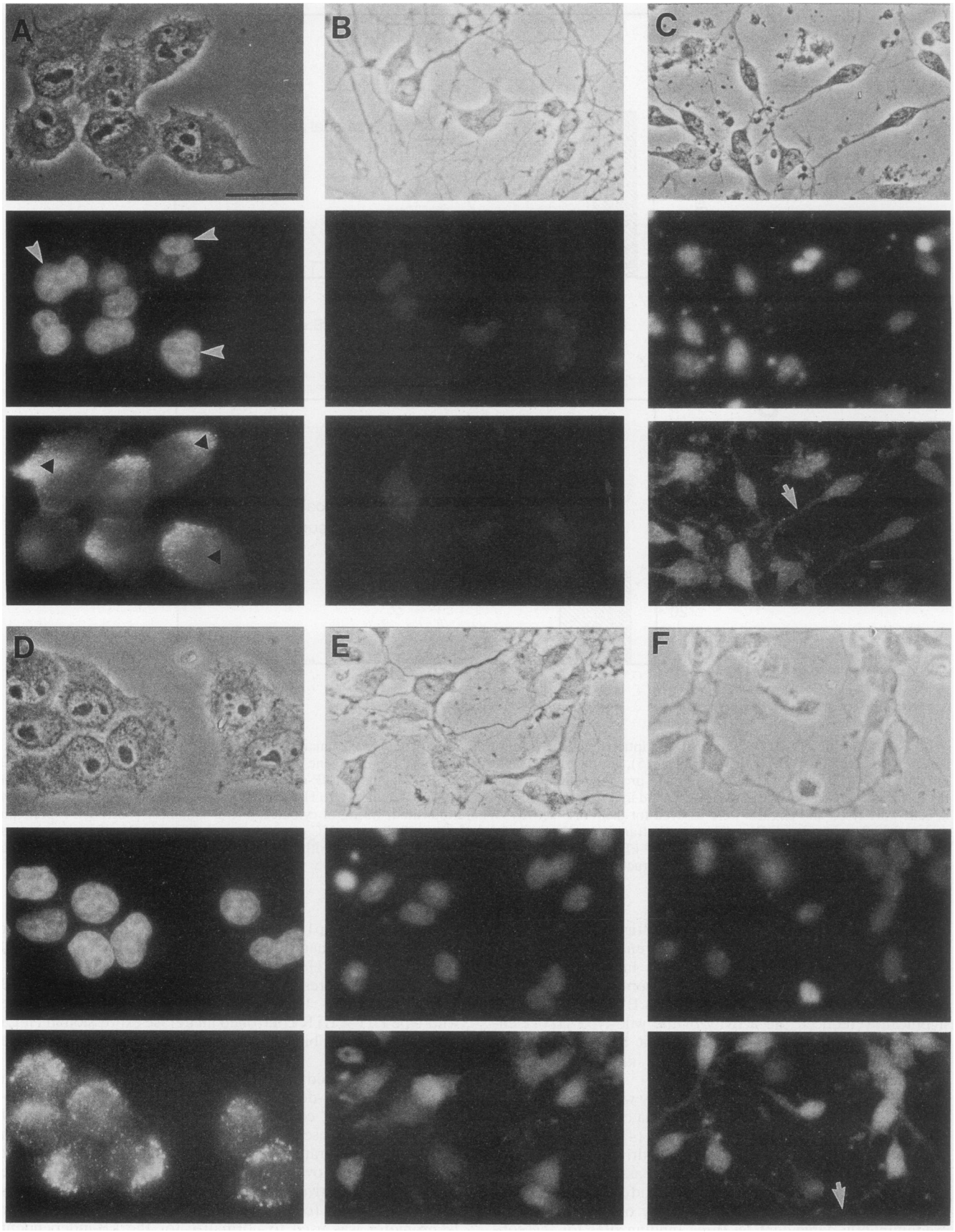


FIG. 1. Constitutive, κB-dependent transcriptional activity in cultured primary neurons. Primary neurons from rat hippocampus and cerebral cortex were prepared as described previously (5), and the expression of a luciferase reporter gene under control of wild-type (HIV-1 wt) and κB mutant HIV-1 (HIV-1 mut) LTRs (A) or a *tk* promoter under control of six κB elements [tk(NF-κB)$_6$] (B) was determined. Two micrograms of the luciferase reporter plasmids was transfected into primary neurons by using Lipofectin or into HeLa cells by using calcium phosphate. After 24 h, cells were lysed and extracts were assayed for luciferase activity. Where indicated, 1.5 μg of cytomegalovirus enhancer-controlled expression vector encoding a human IκB-α cDNA was cotransfected. In control transfections, 1.5 μg of pUC plasmid was used instead. Luciferase activity is shown in relative light units. To compare the κB-dependent luciferase activities of neurons with each other and with that of HeLa cells, the activities measured with the HIV-1 mut construct and the *tk* promoter construct were arbitrarily set to 1.0 for all cell types.

requiring two intact NF-κB sites. To investigate the identity of this activator, a cDNA expression vector encoding the human IκB-α protein was cotransfected with a luciferase reporter. IκB-α is a specific inhibitor of NF-κB complexes containing transactivating Rel subunits (23, 49). IκB-α overexpression significantly reduced the activity of the wild-type HIV-1 enhancer (compare columns 1 and 5) but had no inhibitory influence on the basal activity of the κB mutant HIV-1 enhancer (compare columns 3 and 7).

The level of constitutive NF-κB activity was compared with that of HeLa cells, an epithelial carcinoma cell line containing predominantly inducible forms of NF-κB (4). The constitutive activity of the mutant κB HIV-1 LTR-driven luciferase reporter construct was used to normalize assays for the basal transcriptional activity in HeLa cells and cultured neurons (Fig. 1A, columns 3 and 4). The activity of this construct is predominantly dependent on three binding sites for the constitutive factor Sp1 (35). The wild-type HIV-1 enhancer showed a more than 10-fold-higher activity in hippocampal neurons than in HeLa cells (compare columns 1 and 2), indicative of the presence of high levels of constitutive NF-κB activity in neurons. In contrast to neurons, the activity of the wild-type HIV-1 LTR-controlled luciferase expression in HeLa cells was not inhibited by IκB-α overexpression (compare columns 2 and 6).

Neurons prepared from the cerebral cortex showed an even higher level of κB-dependent activity than hippocampal neurons did (Fig. 1B; compare lanes 1 and 2). In this series of experiments, we used a luciferase reporter construct which contained six reiterated κB motifs upstream from a minimal *tk* promoter. Upon overexpression of IκB-α, the constitutive transcriptional activity of the κB-dependent construct was virtually abolished (columns 4 and 5). The basal activity of the *tk* promoter was used to normalize for the κB-independent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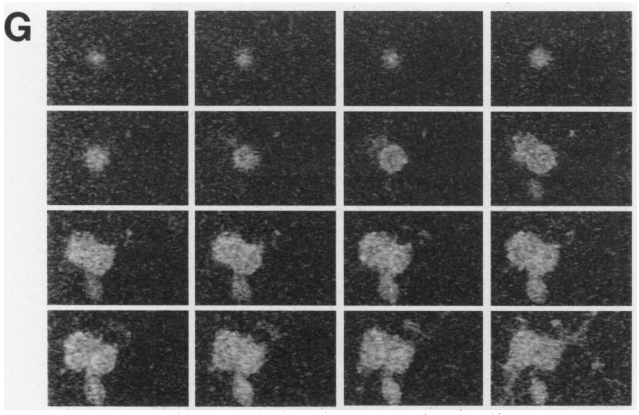

FIG. 2. Indirect immunofluorescence localization of p50 and RelA NF-κB subunits in HeLa cells and primary neuronal cultures. (A and D) HeLa cells; (B, E, and G) primary hippocampal neurons; (C and F) primary cerebral cortex neurons. HeLa cells and 2-day-old cultures of primary cells were fixed and stained with affinity-purified p50-specific (α-p50N [26]; A to C) or RelA-specific (α-p65M [29]; D to G) antipeptide rabbit IgG. Bound IgG was visualized by a biotin-streptavidin-based amplification system using an anti-rabbit secondary antibody (bottom panels). Cultures are also shown by phase-contrast microscopy (top panels) and stained for DNA with DAPI (middle panels). The bar in panel A represents 40 μm. Photographs of primary cells and HeLa cells were taken at the same magnification. (G) Confocal microscopy of a group of three primary hippocampal neurons stained with α-p65M. Two-hundred-nanometer optical sections are shown from the top (upper left panel) to the bottom (lower right panel) of the culture dish.

luciferase activity from cultures of hippocampal neurons, cortical neurons, and HeLa cells (columns 7 to 9). As seen with the HIV-1 enhancer-controlled construct (Fig. 1A), the luciferase activity in HeLa cells was very low compared with that of the neurons and was barely affected by IκB-α overexpression (Fig. 1B, columns 3 and 6).

The high constitutive activity of two unrelated reporter constructs, the dependence on κB-binding motifs, and the inhibitory effect of IκB-α overexpression strongly suggest that cultured neurons contain constitutively activated NF-κB.

Primary neurons contain p50 and RelA in nuclei as well as in cytoplasm. Cultures of HeLa cells (Fig. 2A and D) and cultures highly enriched by serum-free culture for primary neurons from the hippocampus (Fig. 2B and E) and cerebral cortex (Fig. 2C and F) were assayed by indirect immunofluorescence labeling for the presence and subcellular distribution of the two NF-κB subunits p50 and RelA. Phase-contrast microscopy shows neurons with outgrowing neurites (Fig. 2B, C, E, and F, top panels). The positions of cell nuclei were visualized by staining DNA with 4′,6-diamidino-2-phenylindole (DAPI) (Fig. 2, middle panels). In HeLa cells, the immunostaining of p50 and RelA did not overlap with that of nuclei (Fig. 2A and D; compare middle and bottom panels). As indicated by arrowheads (Fig. 2A) and as seen upon confocal laser microscopy (data not shown), nuclei were largely excluded from immunostaining. The cytoplasmic immunofluorescence staining was seen with peptide affinity-purified antibodies against p50 (Fig. 2A) and RelA (Fig. 2D).

In contrast to HeLa cells, the nuclei of both hippocampal and cortical neurons were stained with rabbit antipeptide antibodies specific for the p50 (Fig. 2B and C) and RelA (Fig. 2E and F) NF-κB subunits, respectively. In addition, the cytoplasm of neurons, including neurites (marked by arrows), was stained. The specificity of the antisera was demonstrated in several earlier studies (27, 29, 50) and is also shown in Fig. 4 and 5.

The presence of the transactivating RelA subunit in nuclei of hippocampal neurons was verified by confocal laser microscopy. A group of three neurons stained with anti-RelA (Fig. 2G, lower right panel) was analyzed in various focal planes. When the focal plane was moved in small increments upward from the surface of the cell culture dish, spherical nuclear structures were seen (middle and top panels). In none of the sections was a ring-like cytoplasmic staining excluding the nuclei evident. Taken together, these findings suggest that in cultured neurons, a significant portion of the cellular NF-κB is present in the nucleus, which would explain the constitutive expression of κB-controlled reporter genes in these cells.

Neuronal nuclei from rat cerebral cortex contain constitutive NF-κB activity. It cannot be excluded that NF-κB was artifactually activated in primary neurons as a result of cell culture. For instance, we have recently observed that serum-free conditions induce NF-κB in 293 cells (21a). To obtain biochemical evidence for active forms of NF-κB in neurons, nuclei were isolated from cerebral cortices of rats and neuronal nuclei enriched by sucrose gradient centrifugation, using a published procedure (46). High-salt nuclear extracts were then analyzed by EMSA using a ^{32}P-labeled oligonucleotide encompassing the NF-κB-binding site from the murine κ light-chain enhancer. The N^1 fraction, which is enriched for neuronal nuclei, formed a strong complex with the κB probe (Fig. 3, lane 1), whereas an equal amount of protein from the N^2 fraction formed four times less complex (lane 2), as quantified by β imaging. The protein-DNA complex detected in the N^1 fraction contained the transactivating subunit p65 (RelA), since it reacted with two distinct sera raised against p65 (lanes 3 and 4)

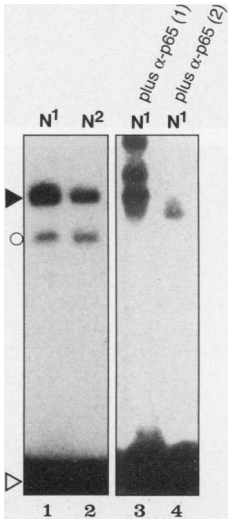

FIG. 3. Constitutive NF-κB activity in nuclei from rat cerebral cortex. Neuronal nuclei were highly enriched by sucrose gradient centrifugation, and nuclear extracts from the N^1 (lane 1) and N^2 (lane 2) fractions were analyzed for κB-specific DNA-binding activities, using EMSA with a ^{32}P-labeled κ enhancer oligonucleotide. The specificity of protein-DNA complexes was tested by adding two distinct p65-specific antisera to the binding reaction (lanes 3 and 4). The filled arrowhead indicates the position of an immunoreactive complex comigrating with a RelA-p50 complex (data not shown), the open arrowhead indicates the position of uncomplexed DNA probe, and the circle indicates the position of an unidentified complex.

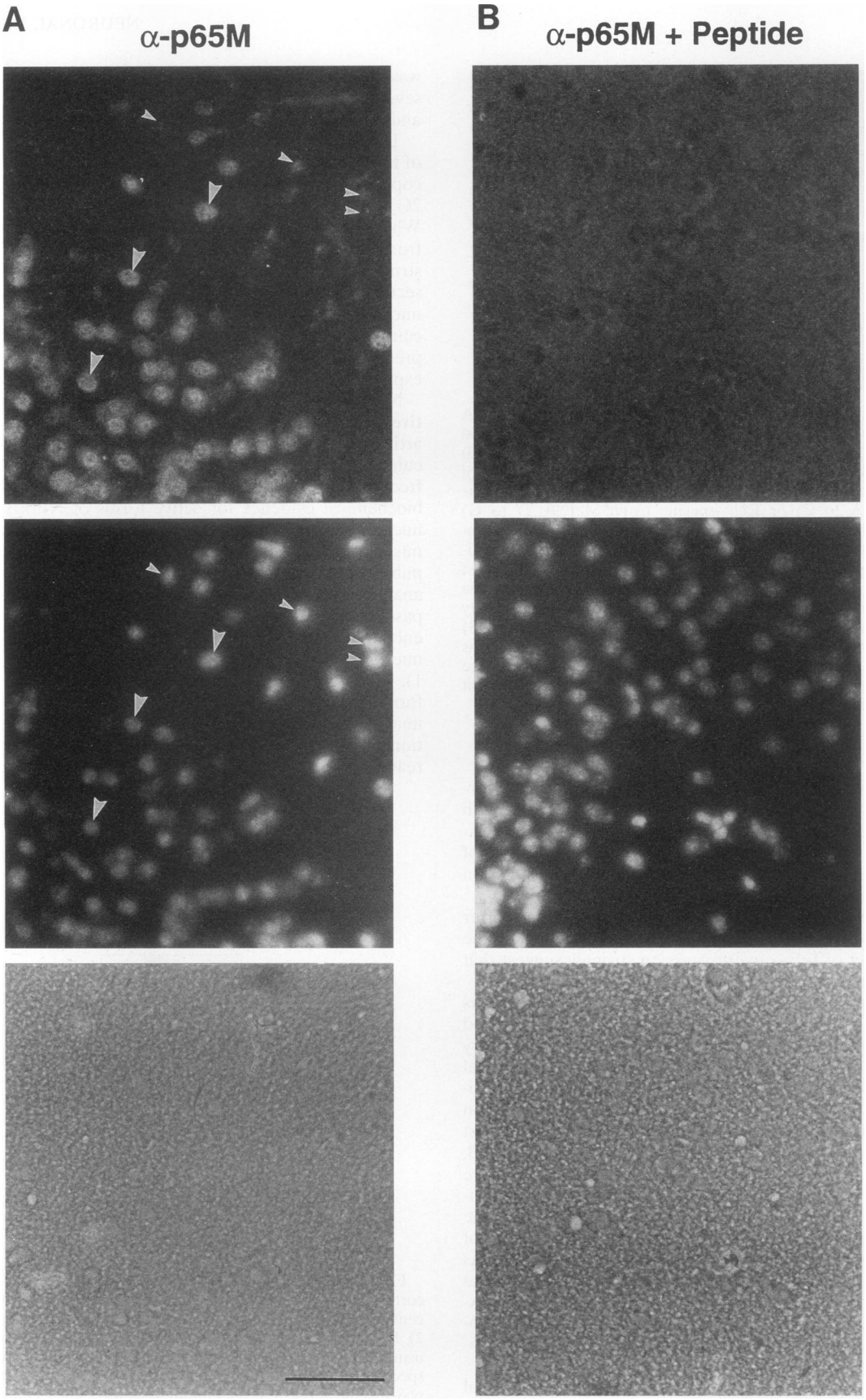

FIG. 4. Immunolocalization of RelA and p50 in cryosections from mouse cerebral cortex. Sagittal cryosections were stained with α-p65M (designated α-p65NLS in reference 50; A, top) and peptide-preabsorbed α-p65M (B, top). Bound IgG was visualized by a biotin-streptavidin-based amplification system. Middle panels show nuclear staining with DAPI, and bottom panels show phase-contrast micrographs of the sections used. Large arrowheads depict strong nuclear immunoreactivity; small arrowheads indicate weak or no NF-κB immunostaining. The bar represents 50 μm.

but not with control sera (data not shown). One antiserum caused immune complexes with decreased mobility (lane 3); the other abolished the DNA binding of the complex (lane 4). These data show that nuclei from cortical neurons contain constitutively active NF-κB.

Detection of nuclear NF-κB by immunofluorescence analysis of cryosections from the cerebral cortex. The particular mechanism of NF-κB activation predicts that the occurrence of RelA and p50 in the cell nucleus correlates with an activated state of the factor. To investigate the subcellular distribution of NF-κB subunits, cryosections from mouse cerebral cortex were immunostained with two distinct anti-NF-κB antisera. One, called α-p65M, was raised against a peptide epitope of RelA which is masked by IκB in the inducible cytoplasmic complex but is exposed in the active nuclear form (designated α-p65NLS in reference 50). The other, α-p50N, recognizes the N terminus of p50 and of its cytoplasmic precursor, p105. Because the gene encoding p105 is induced by NF-κB (45), an enhanced immunoreactivity with α-p50N is also indicative of enhanced κB-dependent transcription. In addition, nuclear immunostaining with α-p50N indicates the presence of p50 in the nucleus either in complex with RelA or as a p50 homodimer. However, no evidence for the existence of constitutive p50 dimers in neuronal tissue has been obtained (Fig. 3, lane 1) (29, 39).

Nuclei in cryosections were stained with DAPI, and the primary antipeptide antibodies were visualized by indirect immunofluorescence staining using a biotin-streptavidin-based amplification system. In the section shown in Fig. 4A, certain neuronal nuclei clearly show a strong nuclear staining with α-p65M (top panel, large arrowheads). No specific immunostaining was obtained if the antibody was preincubated with a peptide corresponding to the nuclear localization signal of RelA (Fig. 4B). This sequence is fully conserved between rodent and human RelA. Interestingly, not all nuclei in the section were stained with α-p65M, showing that NF-κB is activated only in a subset of cells (nonimmunoreactive nuclei are marked with small arrowheads). A similar picture emerged with α-p50N. Only a subset of cells were strongly stained with the p50-specific antibody both in nuclei and in the cytoplasm (Fig. 5A, top panel, large arrowheads). Also with α-p50N, only a subset of nuclei is immunoreactive (large versus small arrowheads). The cytoplasmic staining might be partially due to p105. No specific immunostaining was evident if the α-p50N serum was preabsorbed with a peptide corresponding to the N terminus of p50/p105 (Fig. 5B, top panel). These data strongly support the view that NF-κB is constitutively active in a subset of neurons in the intact brain as well.

Detection of nuclear NF-κB in neurons of the hippocampus. Cryosections from mouse hippocampus were also analyzed for the cytoplasmic or nuclear distribution of NF-κB subunits. The primary antipeptide antibodies α-p65M and α-p50N were visualized by peroxidase-conjugated second antibodies, and sections were developed with nickel-enhanced diaminobenzidine staining. Both p50- and RelA-specific antibodies stained neurons within the hippocampus (Fig. 6A and B, respectively). Staining was present in all fields of the Ammon's horn (CA1 and CA3 in Fig. 6) and in the dentate gyrus. Cells within the habenular nucleus (Fig. 6) were also stained. The immunostaining was not observed if the antibodies were preincubated with the respective peptides (data not shown). To investigate the subcellular distribution of NF-κB immunoreactivity in more detail, sections of the CA3 region and dentate gyrus were analyzed at high resolution by nuclear staining with DAPI and indirect immunofluorescence staining for the p50 NF-κB subunit, using a biotin-streptavidin-based amplification system. The immunostaining obtained with the anti-p50 antibody showed a significant overlap with the nuclear DAPI staining (arrows in Fig. 7), indicating the occurrence of p50 in nuclei of hippocampal neurons in vivo. Clearly, not all nuclei were stained by anti-p50. Some staining patterns did not overlap with a nuclear staining, suggesting that they were cytoplasmic. The nuclear and cytoplasmic staining patterns of p50 were overlaid with a punctate staining. In a recent study, we provided evidence that the punctate staining may be due to p50 and its precursor p105 in synapses (29). As is evident from the low-resolution analysis (Fig. 6), α-p65M produces a staining very similar to that seen with α-p50N. These data suggest that neurons in the hippocampus also contain active NF-κB in their nuclei. In a previous study, the same areas of the hippocampus were shown to highly express an HIV-1 LTR-controlled reporter gene in transgenic mice (13).

DISCUSSION

This study reports that subsets of neurons in the hippocampus and cerebral cortex, like activated B and T cells, contain constitutively active NF-κB in their nuclei. On one hand, the evidence for constitutive NF-κB in neurons is based on experiments with primary neurons, which showed a high-level, κB-dependent expression of luciferase reporter genes and nuclear presence of the DNA-binding NF-κB subunits p50 and RelA. HeLa cells, which predominantly contain inducible forms of NF-κB (4, 37), neither exhibited significant κB-dependent expression of the reporter gene nor showed strong NF-κB immunoreactivity in their nuclei. The data on cultured neurons are consistent with a recently published report by Rattner et al. (39).

Because it cannot be ruled out that cell culture conditions activated NF-κB in primary neurons, we tried to obtain evidence for the presence of constitutively active NF-κB in neurons in the intact brain. Both the enrichment of NF-κB-binding activity in neuronal nuclei and the nuclear immunostaining for RelA and p50 subunits in neurons of cryosections from the cerebral cortex and hippocampus strongly suggest that NF-κB was also present in brain tissue in an IκB-released, activated form. Hence, the constitutive activation of NF-κB observed in cultured neurons might not have been an artifact of cell culture. According to Rattner et al. (39), it is unlikely that the constitutive brain factor BETA is responsible for the κB-specific activity in cultured neurons, since this factor poorly binds the motifs from the HIV-1 LTR.

The constitutive NF-κB activity in neurons appears to be in contrast to studies reporting low to undetectable levels of NF-κB DNA-binding activity in total brain extracts (12, 29, 31, 39). Most of the NF-κB in cell extracts from total brain required prior deoxycholate treatment in order to bind to DNA (29), apparently because most of the NF-κB was associated with IκB. It must, however, be considered that most brain cells are glial cells. According to a recent report (34), those cells contain inducible NF-κB. Moreover, not every neuron may contain constitutive NF-κB (compare Fig. 4 and 5). Hence, the small percentage of brain cells containing constitutive NF-κB might not give a significant signal in biochemical assays. Our data, in fact, showed that isolation of neuronal nuclei by sucrose gradient fraction centrifugation allowed an enrichment for constitutive NF-κB-binding activity.

The transcriptional activity of the HIV-1 LTR is governed by two NF-κB and three Sp1 sites (35). Corboy et al. (13) fused two distinct HIV-1 LTRs derived from central nervous system-isolated viruses, called HIV-$1_{JR\text{-}CSF}$ and HIV-$1_{JR\text{-}FL}$, to a β-galactosidase reporter construct in order to investigate the

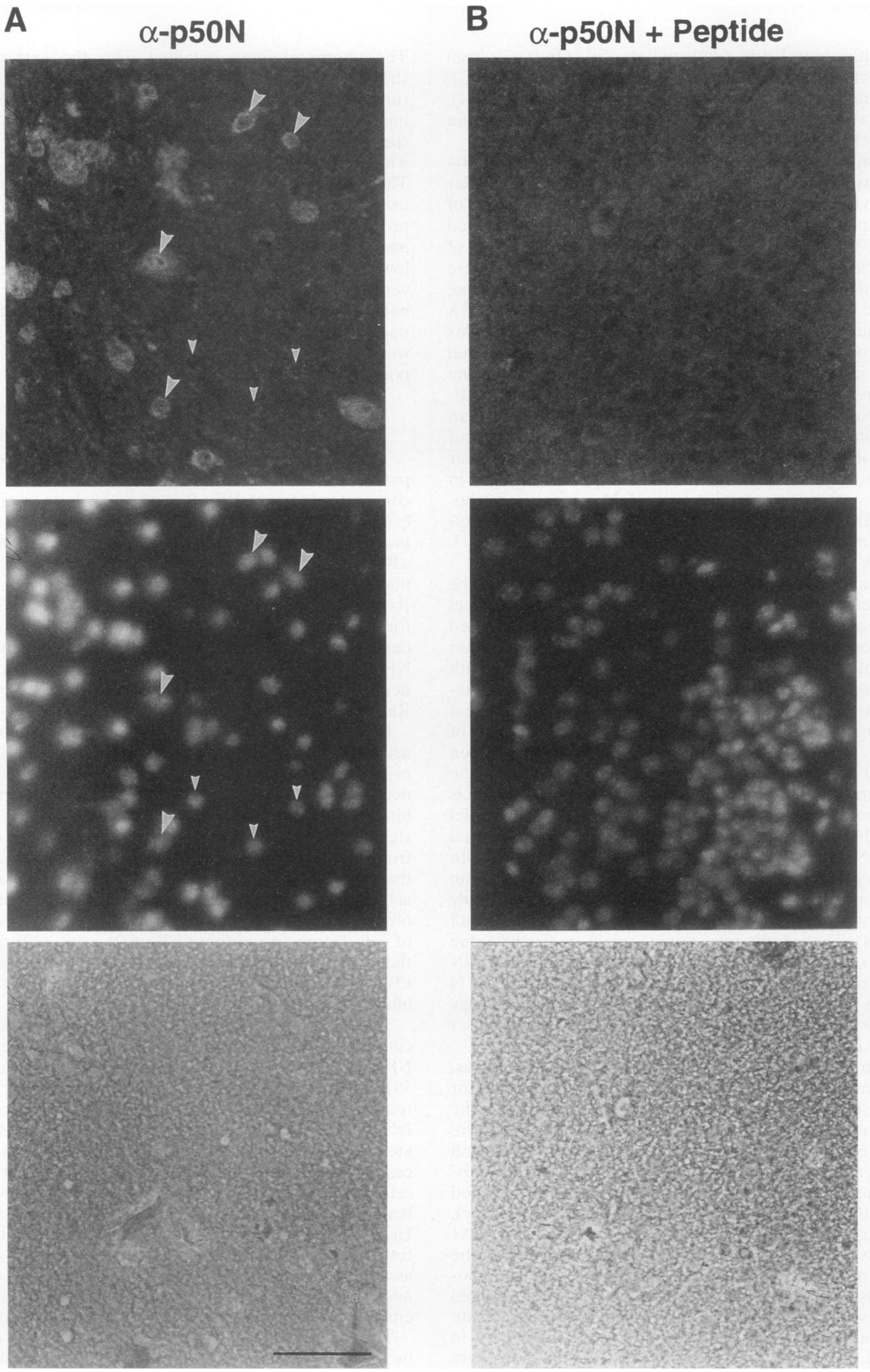

FIG. 5. Sections stained with α-p50N (27) (A, top) and peptide-preabsorbed α-p50N (B, top). Staining was done as described for Fig. 4. Middle panels show nuclear DAPI staining, and bottom panels show phase-contrast micrographs. Some nuclei with strong p50 immunostaining are indicated with large arrowheads. Nuclei with weak or no NF-κB immunostaining are indicated by small arrowheads. The bar represents 50 μm.

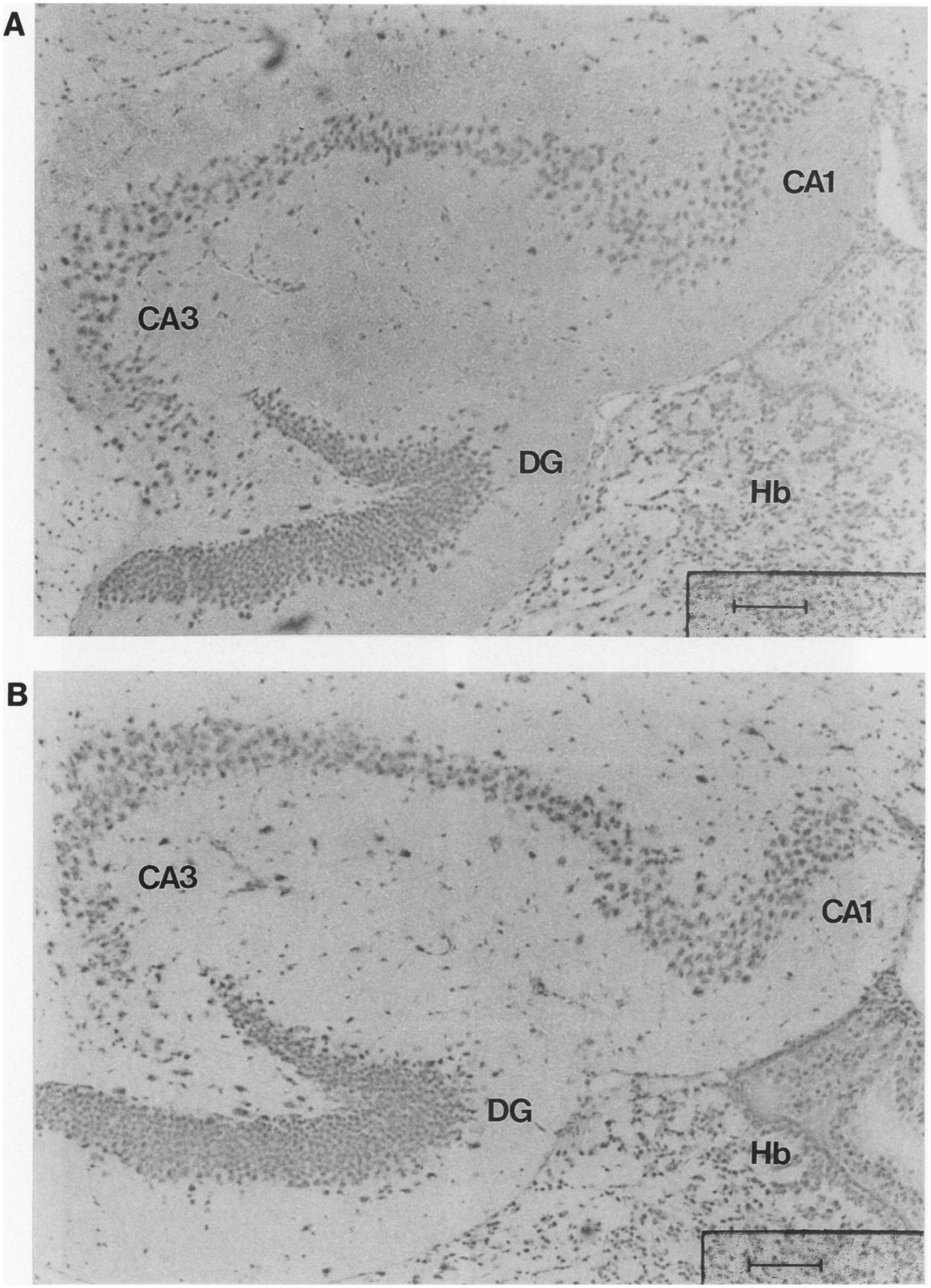

FIG. 6. Immunolocalizations of NF-κB subunits in mouse hippocampus cryosections. Sagittal cryosections were incubated with α-p50N (A) or α-p65M (B) peptide affinity-purified antibody. Bound IgG was decorated with anti-rabbit secondary antibodies conjugated to peroxidase and developed by nickel-enhanced diaminobenzidine staining. Hippocampal fields CA1 and CA3 and dentate gyrus (DG) are labeled. Hb, habenula nucleus. The bar represents 160 μm.

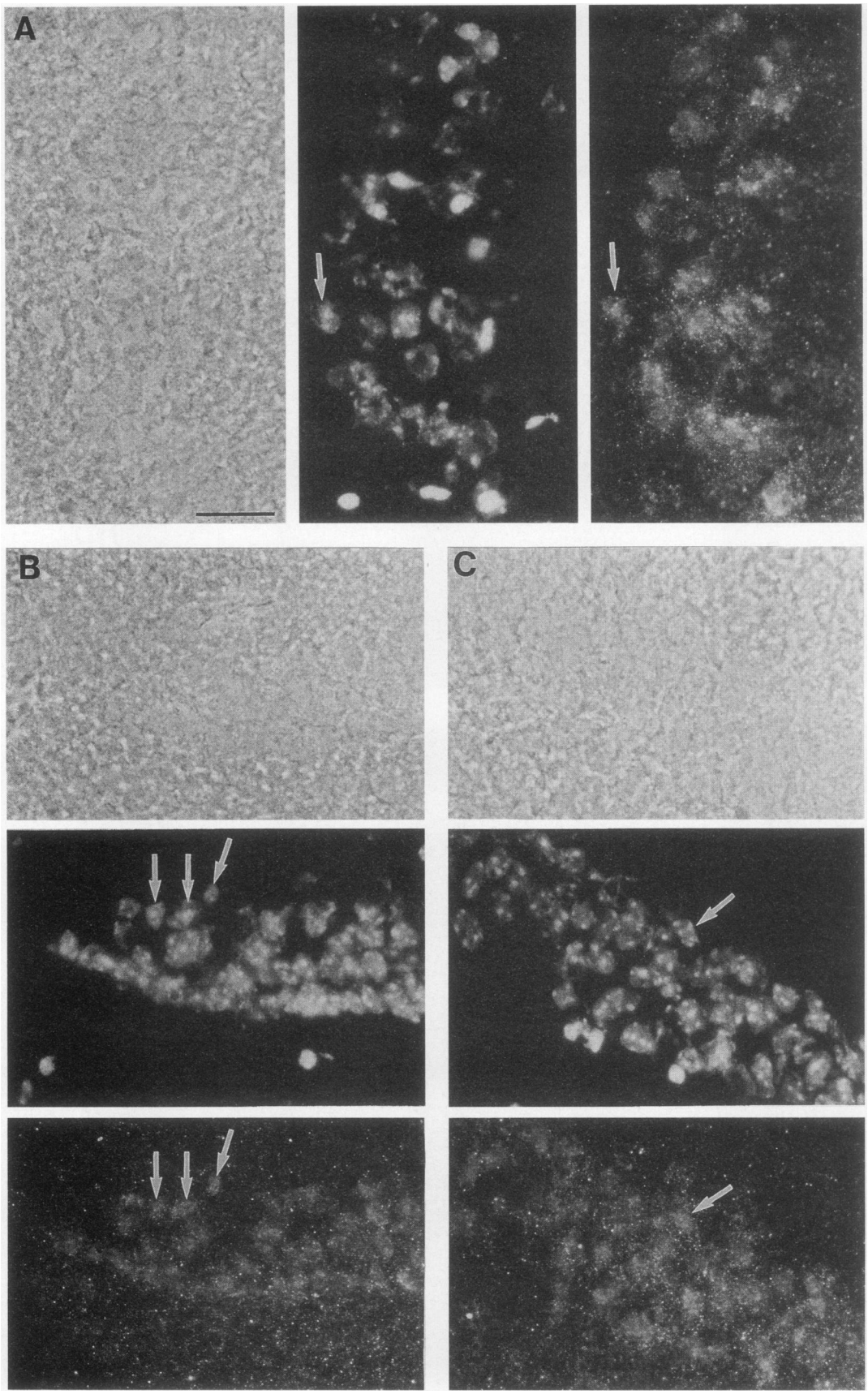

FIG. 7. Nuclear immunofluorescence localization of p50 in hippocampal neurons. Cryosections of three distinct hippocampal fields were incubated with affinity-purified α-p50N, and bound IgG was visualized by a biotin-streptavidin-based amplification system (third panels). (A) CA3; (B) tip of dentate gyrus; (C) CA1. Phase-contrast microscopy (first panels) and nuclear DNA staining with DAPI (middle panels) are shown. The bar represents 40 μm. Arrows indicate examples of cells in which DAPI and p50 immunostaining overlap.

activity of the viral enhancers in brains of transgenic mice. Although the LTRs had multiple nucleotide differences, their binding sites for NF-κB and Sp1 were conserved. Our data suggest that the high-level expression of the HIV-1 LTR-controlled β-galactosidase reporter genes in neurons of transgenic mice is mainly due to a constitutively activated form of NF-κB rather than to changes in the level of the constitutive factor Sp1. This is evident from the observation that the activity level of a wild-type HIV-1 LTR in cultured neurons was much higher than that of a κB mutant LTR, whose activity is mainly dependent on the remaining three Sp1 sites. The nuclear occurrence of NF-κB in selected brain regions is in good agreement with the expression pattern found for HIV-$1_{JR\text{-}CSF}$ LTR-controlled β-galactosidase in brains of transgenic mice (13). This LTR directed β-galactosidase expression to all fields of the hippocampus as seen with p50 and RelA (Fig. 6). The LTR from isolate JR-FL directed expression only to CA3 and the dentate gyrus. Both HIV-1 LTRs directed expression of the reporter gene to the habenula nucleus and to various layers of the cerebral cortex, consistent with a nuclear NF-κB staining in these brain regions. Hence, it is likely that the expression of the HIV-1 LTR-controlled β-galactosidase mainly reflects the state of NF-κB activity in brains of transgenic mice. It is currently unknown what caused the differential tissue activity of LTRs from isolates JR-CSF and JR-FL. One possibility is that nucleotide differences between the LTRs gave rise to additional binding sites for *trans*-acting factors. In the LTR from HIV-$1_{JR\text{-}FL}$, the presence or absence of an additional *cis*-acting element could have restricted the activity of the viral enhancer to fewer brain regions than that of HIV-$1_{JR\text{-}CSF}$.

The constitutive activity of NF-κB in neurons might be relevant for understanding the AIDS dementia complex (ADC). ADC is characterized by mental disorder and progressive loss of memory (for a recent review, see reference 10) which may be caused by neuronal cell death as a consequence of inflammatory reactions. HIV-1 replication within the central nervous system is frequently found in the CD4-positive microglial cells (33). Microglial cell reactions involving cytokines are discussed as a trigger for ADC (15). Neurons lack the CD4 receptor, but an infectious pathway involving galactosyl ceramide as an alternative cell surface receptor was recently described for neuronal cells (22). We report here that neurons contain the transcription apparatus permissive for HIV gene expression due to the presence of constitutive NF-κB activity. Future studies should investigate whether NF-κB-dependent expression of HIV in neurons causes aberrant neuronal function or programmed cell death.

An intriguing observation was that the activity of NF-κB, as reflected by its subcellular distribution, showed topic differences in cells of the cerebral cortex. Apparently, some cells had nuclear NF-κB, while other cells had it in the cytoplasm but not in the nucleus. Further studies are required to explore this finding in more detail by correlating the activation state of NF-κB with cortical and hippocampal architecture and neuronal activity.

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