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Tax Induces Nuclear Translocation of NF-κB through Dissociation of Cytoplasmic Complexes Containing p105 or p100 but Does Not Induce Degradation of IκBα/MAD3

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The activity of the NF-κB transcription factor is controlled through cytoplasmic retention by either of two types of molecules: the inhibitor IκBα/MAD3 or the p105 and p100 precursors of the p50 and p52 DNA-binding subunits. Treatment of cells with classical NF-κB inducers such as tumor necrosis factor, interleukin-1, phorbol myristate acetate, and lipopolysaccharide results in MAD3 degradation followed by nuclear translocation of NF-κB. On the other hand, the mechanisms involved in the dissociation of the cytoplasmic p105/p100-containing complexes are largely unknown. The Tax protein encoded by human T-cell leukemia virus type 1 is a potent activator of viral and cellular gene transcription. It does not bind DNA directly but seems to activate transcription indirectly either by enhancing the activities of the transcription factors that recognize responsive elements located in the promoters of the Tax-responsive genes or by forming ternary complexes with these factors and DNA. It has been previously shown that Tax is able to induce nuclear translocation of NF-κB. We demonstrate here that Tax can induce translocation of members of the NF-κB family retained in the cytoplasm through their interaction with either p105 or p100. On the other hand, Tax induces no apparent degradation of MAD3, although experiments using cycloheximide indicate that it decreases the half-life of MAD3. However, this activity is shared by a mutant of Tax which is unable to activate NF-κB. These results suggest that Tax activates NF-κB essentially through the p105/p100 retention pathway.

The Tax protein, encoded by human T-cell leukemia virus type 1 (HTLV-1), is responsible for transcriptional activation of the viral genome through conserved 21-bp repeats located in its promoter (18, 56). Tax also activates the transcription of cellular genes such as those for interleukin-2 (IL-2), IL-2 receptor, granulocyte-macrophage colony-stimulating factor, vimentin, c-Fos, and c-Jun, as well as the major histocompatibility complex class I genes (1, 2, 20, 21, 27, 30, 32, 33, 41, 44, 46, 49). The transcriptional activation of cellular genes is likely to be responsible for the pathogenesis induced by HTLV-1 infection. At least four Tax-responsive elements have been identified: the 21-bp repeats of the HTLV-1 long terminal repeat, the NF-κB binding motif (κB site), the serum-response element, and the AP1 site, which binds members of the Fos/Jun family. Tax does not directly bind to these DNA sequences but seems to activate transcription indirectly either by enhancing the activity of the transcription factors that recognize these responsive elements or by forming ternary complexes with these factors and DNA (3, 4, 16, 17, 31, 58, 63, 65). Nuclear factor κB (NF-κB), one of the transcription factors which is involved in transactivation by Tax, is implicated in the regulation of numerous cellular genes as well as some viruses (reviewed in references 10 and 22 and references therein). It is made of hetero- or homodimeric combinations of several proteins belonging to the same family. The most common species are heterodimers consisting of a 50-kDa subunit (p50) and either a 65-kDa protein (RelA [formerly p65]) or the product of the c-*rel* proto-oncogene. These complexes are constitutively expressed as an active nuclear form in mature B cells, monocytes, and some T-cell lines. In most other cell types, NF-κB is found in an inactive cytoplasmic form, bound to the inhibitor IκB.

Several IκB proteins have been purified (reviewed in references 5, 45, and 51) and shown to be inhibitors of the DNA binding activity of NF-κB, through their interaction with either RelA or c-Rel (7, 28, 61). A cDNA clone called MAD3 has been isolated recently and demonstrated to code for IκBα, the main species responsible for NF-κB retention in the cytoplasm (15, 23, 62). Upon cell activation by phorbol esters, mitogens, or cytokines, IκB undergoes specific modifications (most likely involving phosphorylation) and is subsequently degraded (6, 11–13, 19, 24, 28, 36, 39, 47, 52, 57), resulting in rapid translocation of the active NF-κB complex to the nucleus.

Tax has been demonstrated to induce nuclear translocation of NF-κB in various cell types (3, 31, 32, 34, 35), although the mechanism responsible for this activation is not yet understood. Recently we and others have demonstrated that an alternative way of retaining active members of the NF-κB family in the cytoplasm is through interaction with the p105 or p100 precursors of the p50 and p52 DNA-binding subunits of NF-κB (40, 42, 43, 48, 50). It is possible that such cytoplasmic inactive complexes might be released in response to stimuli distinct from the ones classically associated with release from IκB. Recently it has been shown that Tax interacts with the p105 precursor of the p50 subunit of NF-κB, and also with the p50 subunit itself, both in the presence and in the absence of a κB site (25, 26, 59, 64). These data led us to investigate the nature of the cytoplasmic complexes which are the targets of Tax. We report here that Tax-induced NF-κB activation is not

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correlated with MAD3 degradation and that Tax can induce translocation of members of the NF-κB family which are retained in the cytoplasm through interaction with p105 or p100.

MATERIALS AND METHODS

Cells. Monkey COS-7 cells were grown in Dulbecco modified Eagle medium supplemented with 10% newborn calf serum. 70Z/3, a murine pre-B cell line, was maintained in RPMI medium supplemented with 10% fetal calf serum.

Antisera. The antisera used to detect p105, c-Rel, and RelA have been described previously (48): 1140 (105C) was raised against the C-terminal 15 amino acids of p105, 1157 (105I) was raised against amino acids 339 to 357 of p105, 1207 (65N) was raised against amino acids 2 to 17 of human RelA, 1226 (65C) was raised against amino acids 537 to 550 of human RelA, and 265 (hrelC) was raised against amino acids 573 to 587 of human c-Rel. Sera 265 and 1267 were a kind gift of N. R. Rice (Frederick, Md.). The S5 antiserum, a gift of R. T. Hay (St. Andrews, United Kingdom) has been raised against recombinant MAD3 (39). The Tax antiserum has been raised against the 14 C-terminal amino acids of the protein and was a gift from S. Oroszlan (Frederick, Md.).

Plasmids. Expression vectors for transfection into COS-7 cells were obtained by subcloning cDNAs encoding p105, c-Rel, RelA, or MAD3 into the Rc-CMV vector (Invitrogen). pSG-Tax was generated by excising the Tax-coding region from the plasmid pSGT and recloning into pSG5 (14). The M9, M22, M44, and M47 Tax mutants were obtained from W. C. Greene (55). The (Igκ)3-conaluc plasmid was constructed by inserting three copies of the immunoglobulin κ chain enhancer κB site upstream of the conalbumin promoter followed by the luciferase gene.

Transfections. COS-7 cells were grown to 40% confluency in complete Dulbecco modified Eagle medium in 100-mm-diameter petri dishes and transfected with 1.5 μg of expression vector coding for p105 or p100 per ml or with 0.25 μg of expression vector coding for c-Rel or RelA per ml. The concentration of the Tax-expressing vector (wild type [wt], M22, M44, or M47) was between 0.25 and 0.5 μg/ml except when otherwise stated. Transfections were performed by the calcium phosphate coprecipitation procedure. Eighteen hours later, the cells were washed with phosphate-buffered saline and supplied with fresh medium. Forty hours after transfection, cytoplasmic and nuclear fractions were prepared as described below.

70Z/3 cells were cotransfected by a modified DEAE-dextran method (60), using 500 instead of 250 μg of DEAE-dextran per ml. Twenty-four hours after transfection, the cells were stimulated for 6 to 8 h and then lysed in 25 mM Tris-phosphate (pH 7.8)–8 mM $MgCl_2$–1 mM dithiothreitol–1% Triton X-100–15% glycerol, and luciferase activity was measured in a luminometer (Berthold). The background value obtained with the lysis buffer was subtracted from each experimental value. The experiments were performed in triplicate, and the mean relative luciferase units (see legend to Fig. 1) were calculated.

Immunofluorescence. COS-7 cells were grown on coverslips in 24-well plates and transfected by the calcium phosphate coprecipitation method. Immunofluorescence was carried out as described previously (9).

Cell extracts and mobility shift assays. Cytoplasmic and nuclear extracts from transfected COS-7 or 70Z/3 cells were prepared as described previously (9). Binding reactions were performed by incubating 3 μg of nuclear extract with an end-labelled oligonucleotide probe corresponding to the κB-binding site located in the enhancer region of the H-2 K^b promoter, KBF (29). When indicated, a specific antiserum (0.4 μl) was included in the standard reaction mixture prior to the addition of the radiolabelled probe.

Immunoblots. Immunoblots were carried out as described previously (48). Detection was by the ECL system (Amersham).

Purification of Tax and M9 Tax mutants. The Tax and M9 cDNAs were cloned into the pGEX-2T vector, and the glutathione *S*-transferase–Tax or M9 fusion proteins expressed in *Escherichia coli* were purified as previously described (54). Thrombin digestion was carried out on glutathione beads, and Tax or M9 mutants were recovered from the supernatant following centrifugation.

RESULTS

Extracellular Tax protein induces NF-κB binding and transactivation but not degradation of IκBα/MAD3. It has been demonstrated that purified extracellular Tax protein can be taken up by normal peripheral blood lymphocytes (37) as well as by lymphoid cell lines (34, 35, 38). In murine 70Z/3 pre-B cells, expression of nuclear NF-κB can be detected as soon as 30 min after exposure to extracellular Tax. To compare the action of Tax with that of other NF-κB inducers in 70Z/3 cells, we stimulated the cells with either recombinant purified Tax, IL-1, phorbol myristate acetate (PMA), or lipopolysaccharide (LPS). We observed by immunoblotting that IL-1, LPS, and PMA induced rapid degradation of MAD3 (Fig. 1A and data not shown) (6, 24, 47) and subsequently an increase in NF-κB binding activity (Fig. 1B [for PMA] and data not shown). In contrast, extracellular Tax, although unable to induce disappearance of MAD3 (Fig. 1A), mediated a clear increase in NF-κB binding activity (Fig. 1B). In addition, Tax was able to transactivate a luciferase reporter construct driven by three κB sites, demonstrating that our preparation of Tax protein is functional in this cell line (Fig. 1C). These results suggest that Tax can induce NF-κB activity independently of the MAD3 degradation pathway, which seems to be required for stimulation by IL-1, LPS, and PMA. However, it is possible that Tax increases the turnover of MAD3 but that constant resynthesis would not allow its detection in an experiment which assays steady-state levels as in Fig. 1. This would possibly allow a continuous nuclear translocation of small amounts of NF-κB. We therefore carried out experiments using cycloheximide to measure the half-life of MAD3 in the presence or absence of Tax. The half-life of this protein in 70Z/3 cells is approximately 2 to 3 h, and Fig. 2A shows that Tax does indeed decrease the half-life of MAD3 but that a mutant of Tax (M9 [55]) which is unable to induce NF-κB DNA binding activity (Fig. 2B) or to transactivate a κB-luciferase construct (data not shown) has the same effect on the stability of the MAD3 protein. Therefore, Tax increases the turnover of MAD3, but this seems to be unrelated to its ability to activate NF-κB (see Discussion).

Tax is able to override cytoplasmic retention of c-Rel or RelA by p105. We and others have recently demonstrated that the p105 and p100 precursors of the p50 and p52 subunits of NF-κB exhibit IκB activity and are able to retain RelA, c-Rel, or p50 in the cytoplasm of various cell lines (40, 42, 43, 48, 50). However, the stimuli inducing the nuclear translocation of the NF-κB/Rel factors by modifying these inactive cytoplasmic complexes remain unknown. It has been shown recently that Tax interacts with p105 (25, 64) and as a consequence enhances the nuclear translocation of p50 and RelA to the nucleus (64). We therefore extended these results and assayed the possible involvement of the Tax protein in such a mecha-

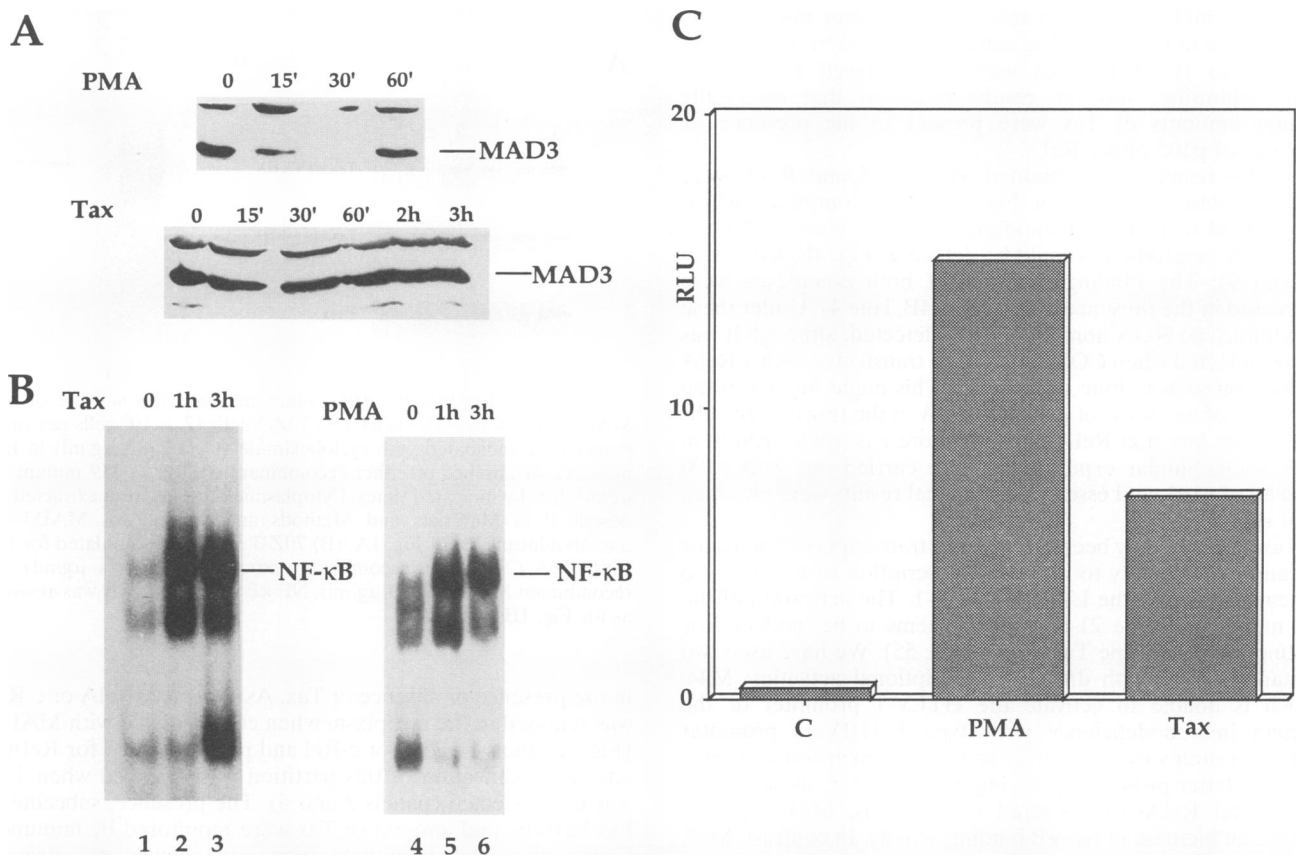

FIG. 1. Extracellular Tax induces NF-κB activation in mouse 70Z/3 pre-B cells but no apparent MAD3 degradation. Cells (10^6/ml) were stimulated with PMA (20 ng/ml) or purified recombinant Tax (1 μg/ml) for the indicated times. Cytoplasmic and nuclear extracts were prepared as described in Materials and Methods. (A) Immunobloting analysis of cytosolic fraction of 70Z/3 cells. Twenty micrograms of cytoplasmic proteins was applied to each lane. Immunoblots were probed with the S5 antiserum raised against recombinant MAD3. The specificity of the band labelled MAD3 was checked by competition with an excess of recombinant MAD3 (not shown). (B) NF-κB binding activity in 70Z/3 cells stimulated with PMA or Tax. Three micrograms of nuclear proteins from stimulated cells collected at the times indicated was subjected to electrophoretic mobility shift assay with the KBF probe. The NF-κB complex (p50/RelA) is indicated. (C) Activation of a κB-driven luciferase construct by extracellular Tax in 70Z/3 cells. One microgram of the (Igκ)3-conaluc plasmid per 10^6 cells was transfected into 70Z/3 cells as described in Materials and Methods, and 24 h later the culture medium was supplemented with PMA (20 ng/ml) or recombinant Tax (1 μg/ml) for 6 h and the luciferase activity was measured in triplicate as previously described. Relative luciferase units (RLU) represent the ratio of the observed activity to the value obtained with nonstimulated cells. Results shown are representative of three independent experiments. C, control.

nism by transfection of COS-7 cells followed by immunofluorescence analysis (Fig. 3). As previously shown (48), transfection of COS-7 cells with a p105 expression vector resulted in a strictly cytoplasmic localization of this protein (Fig. 3, panel 4), while c-Rel and RelA were nuclear when transfected alone (panels 2 and 3). When p105 was cotransfected with either RelA or c-Rel at a molar ratio of 6:1, the c-Rel and RelA molecules were almost completely excluded from the nucleus (Fig. 3, panels 5 and 6). Under these conditions, we found that Tax was able to partially reverse this cytoplasmic retention: approximately 30 to 40% of the cells cotransfected with p105, c-Rel or RelA, and Tax exhibited a nuclear staining of c-Rel or RelA (Fig. 3, panels 8 and 9). The expression of Tax was monitored by immunofluorescence, and the percentage of Tax-positive cells was identical to that of cells expressing c-Rel or RelA (not shown). Also, as previously described, we observed that the Tax protein was present both in the cytoplasm and in the nucleus when transfected alone (Fig. 3, panel 7) and that this subcellular repartition was not altered by the presence of p105 plus c-Rel (panel 10) or p105 plus RelA (panel 11).

We confirmed these results by using a bandshift assay to analyze the κB binding activities present in nuclear fractions prepared from transfected cells. Figure 4A shows that Tax alone induced a weak NF-κB activity in COS-7 cells (lane 10). Transfection of p105 alone resulted in the detection of small amounts of p50 homodimers (Fig. 4A, lane 2). This binding activity was clearly increased in the presence of Tax (Fig. 4A, lane 3) and was not due to the transactivation by Tax of the cytomegalovirus (CMV) promoter driving p105, since cotransfection of Tax with a CMV-p50 expression vector did not result in an increased p50 homodimer DNA binding activity (lanes 4 and 5). Cotransfection of c-Rel with p105 did not modify the binding activity detected when p105 was transfected alone (Fig. 4A, lanes 7 and 8). In contrast, when Tax was included in the experiment, two strong complexes could be detected (Fig. 4A, lane 9). Addition of specific antisera in the binding reaction mixture demonstrated that these two complexes correspond to p50/p50 homodimers and p50/c-Rel heterodimers (Fig. 4A, lanes 11 to 13). Under our experimental conditions, we were not able to detect c-Rel/c-Rel homodimers even in COS-7 cells transfected with c-Rel alone; this may result either from the inability of c-Rel/c-Rel homodimers to bind to the KBF/κB site

or from a high dissociation rate of the complex impairing its observation in this assay. The extracts analyzed by bandshift in lanes 6 to 10 of Fig. 4A were also assayed for Tax by immunoblotting, and the results indicated that essentially similar amounts of Tax were present in the presence or absence of p105 plus c-Rel.

Similar results were obtained when p105 and RelA were cotransfected, as shown in Fig. 4B. Two complexes which correspond to p50/p50 homodimers and the classical NF-κB p50/RelA heterodimers could be detected (Fig. 4B, lanes 3, 7, 8, and 9). The binding activities of both complexes were increased in the presence of Tax (Fig. 4B, lane 4). Under these conditions, no RelA homodimer was detected, although it was easily detected when COS-7 cells were transfected with a RelA expression vector alone (see Fig. 7). This might be due to the presence of an excess of p50 over RelA in the transfected cells and to the fact that RelA interacts more easily with p50 than with itself. Similar experiments were carried out with p100 instead of p105, and essentially identical results were obtained (not shown).

Tax had originally been defined as a transcriptional activator because of its ability to increase transcription from the 21-bp repeats located in the LTR of HTLV-1. The activation of the κB motifs or of the 21-bp repeats seems to be mediated by distinct regions of the Tax protein (53, 55). We have used two mutants of Tax with distinct transcriptional activities: M44, which is unable to activate the HTLV-1 promoter or the human immunodeficiency virus type 1 (HIV-1) promoter (which contains two κB sites), and M47, which transactivates only the latter promoter (55). Figure 4B, lane 5, shows that in p105- and RelA-cotransfected COS-7 cells, M44 failed to induce an increase in NF-κB binding activity. In contrast, M47, as expected, behaves like wt Tax (Fig. 4B, lanes 4 to 6). These results have been confirmed by immunofluorescence analysis (data not shown). The amount of Tax was assayed by immunoblotting, and the results showed that identical amounts of wt, M44, and M47 proteins were present in various transfected cells (Fig. 4B, lanes 1 to 6, lower panel). Since the M44 mutant is unable to activate either the HTLV-1 or the HIV-1 LTR, one possible explanation for its lack of activity is that the mutations introduced strongly interfere with its normal conformation. Therefore, to confirm our hypothesis with a more relevant mutant, we used the M22 mutant, which does not activate the HIV-1 LTR (i.e., does not induce nuclear translocation of NF-κB) but is still able to activate the HTLV-1 LTR, suggesting that its structure is not grossly distorted (55). This mutant behaved like M44 and was unable to relieve cytoplasmic retention by p105 (not shown). Together, these data indicate a good correlation between the ability of Tax to activate transcription through κB motifs and its capacity to override the inhibitory activity of p105.

The results of the bandshift assay were confirmed by immunoblot analysis of cytoplasmic and nuclear fractions of transfected COS-7 cells. We observed that c-Rel is indeed excluded from the nucleus when cotransfected with p105 (Fig. 5, lanes 3 and 4) and that cotransfection of Tax induces nuclear translocation of these c-Rel molecules (lane 5). In addition, as expected from the results shown above, an increase in the amount of nuclear p50 was also observed in the presence of Tax in p105/c-Rel-cotransfected cells (Fig. 5, lane 5, lower panel) or in p105/RelA-cotransfected cells (data not shown).

Tax does not prevent cytoplasmic retention of c-Rel or RelA by MAD3. In a parallel set of experiments we assayed the effect of Tax on the retention of NF-κB by the IκB/MAD3 molecule. We first performed indirect immunofluorescence experiments with COS-7 cells cotransfected with MAD3 and c-Rel or RelA

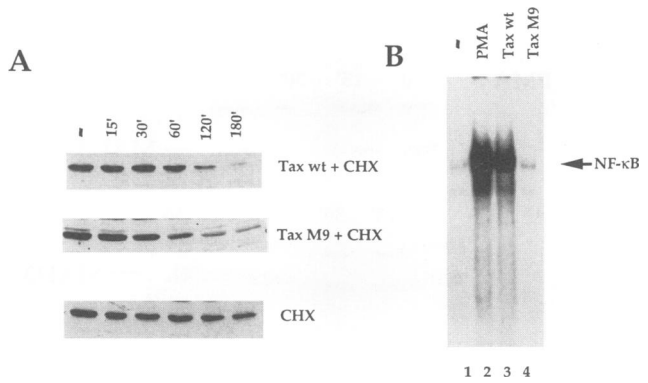

FIG. 2. wt Tax and the M9 mutant decrease the half-life of the MAD3 protein in 70Z/3 cells. (A) 70Z/3 cells (2×10^6 cells per time point) were incubated with cycloheximide (CHX) (10 μg/ml) in the presence or absence of either recombinant wt Tax or M9 mutant (1 μg/ml) for the indicated times. Cytoplasmic proteins were extracted as described in Materials and Methods and assayed for MAD3 by immunoblotting as for Fig. 1A. (B) 70Z/3 cells were stimulated for 1 h with PMA (20 ng/ml), recombinant extracellular Tax (1 μg/ml), or recombinant M9 mutant (1 μg/ml). NF-κB binding activity was assayed as for Fig. 1B.

in the presence or absence of Tax. As expected, RelA or c-Rel was retained in the cytoplasm when cotransfected with MAD3 (Fig. 6, panels 1 and 2 for c-Rel and panels 4 and 5 for RelA), and no modification of this partition was observed when Tax was cotransfected (panels 3 and 6). The presence, subcellular localization, and amount of Tax were monitored by immunofluorescence and immunoblotting and showed no obvious variation related to the various coexpressed proteins (not shown). To confirm these results we prepared nuclear extracts of transfected COS-7 cells. Figure 7 shows that, as discussed above, transfection of RelA results in the appearance of RelA/RelA, p52/RelA, and p50/RelA complexes (lane 4). Inclusion of MAD3 induced disappearance of these complexes (Fig. 7, lane 2) and Tax could not reverse this inhibitory effect (lane 3).

DISCUSSION

The HTLV-1-encoded Tax protein activates viral or cellular genes through precise DNA sequences but is not a sequence-specific DNA-binding protein (1, 2, 20, 21, 27, 30, 32, 33, 41, 44, 46, 49). Four sequences so far have been demonstrated to mediate the effect of Tax. The 21-bp repeats located in the HTLV-1 LTR represent one class of Tax-responsive motifs and contain sequences that serve as recognition elements for the CREB/ATF family of transcription factors. It has been demonstrated recently that Tax is able to enhance the binding of CREB to the 21-bp repeat through direct protein-protein interaction, probably as a result of increased dimerization (4, 58, 63, 65). The sequence recognized by the serum response factor, which is located in the promoters of several genes, including c-*fos*, *Krox-20*, and *Krox-24*, as well as the binding sites for the AP1 family of transcription factors represent other targets for Tax. Indirect association between Tax and serum response factor-binding sequences (CArG boxes) can be mediated through direct interaction between Tax and serum response factor (17, 59). In addition, this direct interaction has been shown to result in an increased transcriptional activity of the target promoter (17). More generally, a recent report suggests that Tax acts by increasing the DNA binding activities

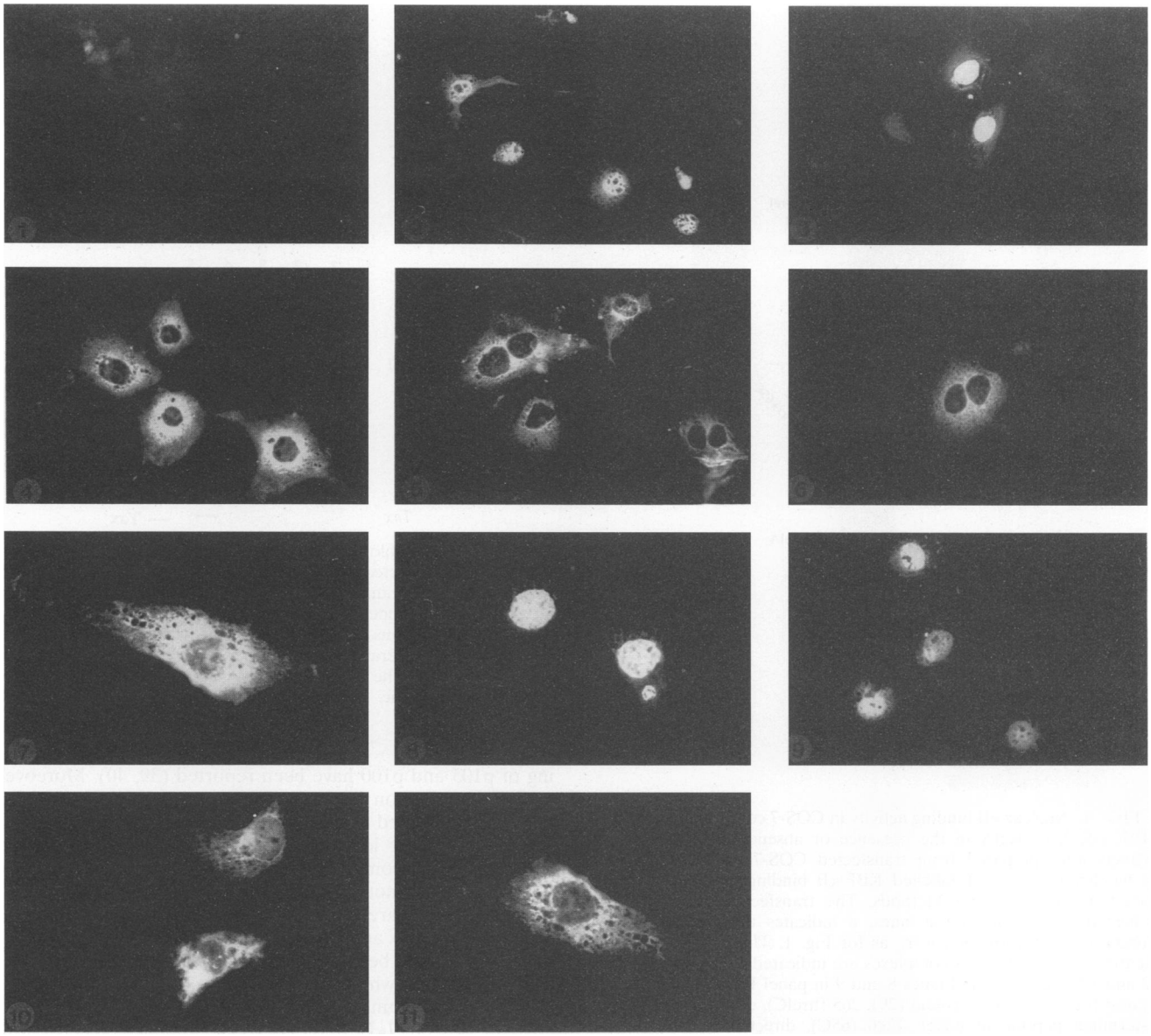

FIG. 3. Tax is able to override cytoplasmic retention of c-Rel or RelA by p105 in transfected COS-7 cells. COS-7 cells were transfected with expression vectors encoding c-Rel (panel 2), c-Rel plus p105 (panel 5), c-Rel plus p105 plus Tax (panels 8 and 10), RelA (panel 3), RelA plus p105 (panel 6), RelA plus p105 plus Tax (panels 9 and 11), p105 alone (panel 4), or Tax alone (panel 7). The amount of plasmid DNA transfected was 1.5 μg/ml for CMV-p105 and 0.25 μg/ml for CMV–c-Rel, CMV-RelA, and pSG-Tax. Forty hours after transfection, the cells were prepared for indirect immunofluorescence as described in Materials and Methods. The antisera used have been described previously (48): p105 was detected with antiserum 1140 (105C) in panel 4; c-Rel was detected with antiserum 265 (hrelC) in panels 2, 5, and 8; RelA was detected with antiserum 1207 (65N) in panels 3, 6, and 9; and Tax was detected with anti-Tax antiserum in panels 7, 10, and 11. Panel 1 shows untransfected COS-7 cells stained with the second antibody only.

of a number of sequence-specific transcription factors (4). Another family of Tax-responsive elements is represented by κB sequences, which are binding sites for the NF-κB family of transcription factors. The mode of action of Tax in this case is not clearly understood. The DNA binding activity of NF-κB (p50/RelA heterodimer) is only marginally induced by Tax (4). On the other hand, a direct interaction between Tax and the p50 subunit has been demonstrated recently, although only in vitro or under conditions of overexpression in transfected cells (59). Since the main mechanism of regulation of NF-κB activity is by exclusion from the nuclear compartment through cytoplasmic retention, another possible effect of Tax is the induction of nuclear translocation of NF-κB. The classical way by which NF-κB complexes can be retained in the cytoplasm is through the IκB family of inhibitors. Recent results indicate that several signals classically known to activate NF-κB induce degradation of the IκB/MAD3 inhibitor. This degradation probably follows phosphorylation of the IκB molecule, resulting in either dissociation of the NF-κB/IκB complex or targeting of IκB for degradation, or both (6, 11–13, 19, 24, 28, 36, 39, 47, 52, 57). Therefore, one possibility is that Tax directly or indirectly induces dissociation of the NF-κB/IκB complex or

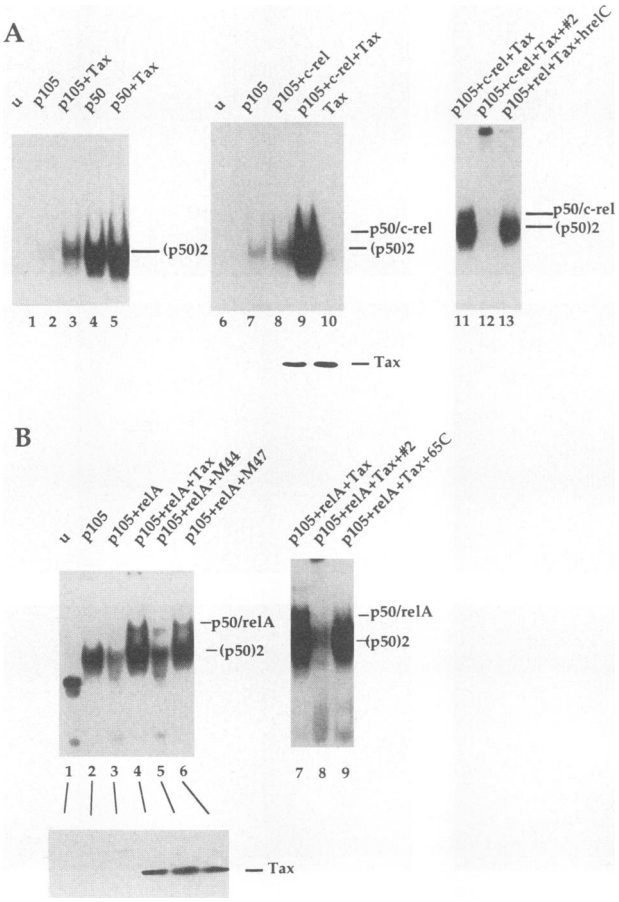

FIG. 4. Nuclear κB binding activity in COS-7 cells transfected with p105, c-Rel, or RelA in the presence or absence of Tax. Nuclear extracts were prepared from transfected COS-7 cells and assayed by bandshift with a ^{32}P-labelled KBF/κB binding sequence as described in Materials and Methods. The transfected constructs are indicated at the top of the lanes; u indicates untransfected cells. Conditions of transfection were as for Fig. 1. The antisera used to identify the protein/DNA complexes are indicated at the top of lanes 12 and 13 in panel A and lanes 8 and 9 in panel B: sera 2, produced against the entire p105 protein (29); 265 (hrelC), directed against the C-terminal peptide of c-Rel; 1226 (65C), directed against the C-terminal peptide of human RelA; and 1267, directed against the N-terminal peptide of p100. Autoradiograms were developed after 2 and 12 h of exposure for panels A and B, respectively. The labelled probe was in excess in all cases. (A) p105, c-Rel, and Tax; (B) p105, RelA, and Tax. The M44 and M47 mutants of Tax are described in the text. The amounts of Tax proteins (wt and mutants) were assayed by immunoblotting with extracts corresponding to lanes 6 to 10 in panel A and lanes 1 to 6 in panel B.

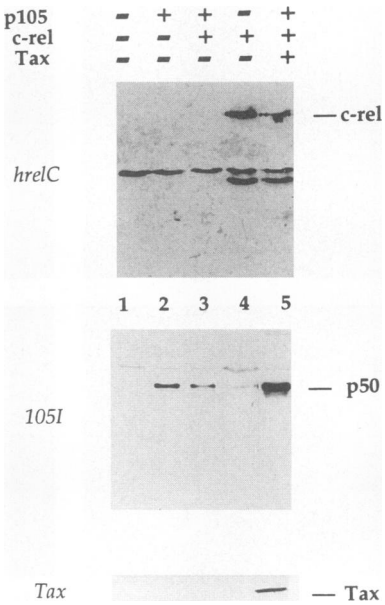

FIG. 5. Immunoblot analysis of nuclear and cytosolic fractions of COS-7 cells transfected with p105, c-Rel, and Tax expression vectors. COS-7 cells were transfected with combinations of p105, c-Rel, and Tax, as indicated, under the conditions described in the legend to Fig. 1. Twenty micrograms of nuclear proteins was subjected to immunoblot analysis using sera 265 (hrelC) for c-Rel, 1157 (105I) for p50, and anti-Tax for Tax. The bands migrating faster than c-Rel in the top panel are nonspecific.

degradation of IκB. Another mechanism of cytoplasmic retention of NF-κB is through interaction of p50, RelA, or c-Rel with the p105 or p100 precursors of the p50 and p52 subunits (40, 42, 43, 48, 50). In this case either the release would result from processing of the precursors or, alternatively, dissociation of the complex could be induced without processing, following a modification of either subunit. These two mechanisms are not necessarily exclusive. The stimuli responsible for dissociation of this type of complex have not been clearly identified. However, some data which suggest that tumor necrosis factor might induce an increase in the processing of p105 and p100 have been reported (39, 40). Moreover, a direct interaction between Tax and cytoplasmic p105 has been demonstrated recently (25), raising the possibility that Tax might act at least in part by inducing dissociation of p105-containing complexes (64). Some data suggest that Tax apparently does not induce nuclear translocation of NF-κB complexes which are retained in the cytoplasm by IκB/MAD3. First, unlike p105 and p100 (8, 25; our unpublished results), MAD3 could not be demonstrated to interact with Tax when cotransfected or when cotranslated in vitro (not shown). In addition, Tax is unable to relieve cytoplasmic retention of c-Rel or RelA by IκB/MAD3 in COS-7 transfected cells, while it can relieve retention by p105 or p100 (see below). In order to study the effect of Tax in a more physiological system, we analyzed the effect of extracellular Tax on the endogenous MAD3 molecules in murine pre-B 70Z/3 cells. It has been reported that extracellular Tax can be taken up by 70Z/3 cells and induce nuclear translocation of NF-κB by a protein synthesis-independent mechanism (34, 35). We show here that nuclear translocation of NF-κB following addition of recombinant Tax in the culture medium of 70Z/3 cells is not associated with any decrease in the steady-state amount of MAD3, while treatment of these cells with IL-1, PMA, or LPS results in degradation of MAD3 that correlates with the appearance of NF-κB DNA binding activity in the nucleus. Although experiments with cycloheximide indicate that Tax decreases the half-life of MAD3, this property is also exhibited by a mutant of Tax that can no longer activate NF-κB, indicating that NF-κB activation and increase in MAD3 turnover are independent properties of the Tax protein. In conclusion, the evidence we present points to a lack of a specific effect of Tax on MAD3 that would result in NF-κB activation. However, we cannot exclude the possibility that Tax induces

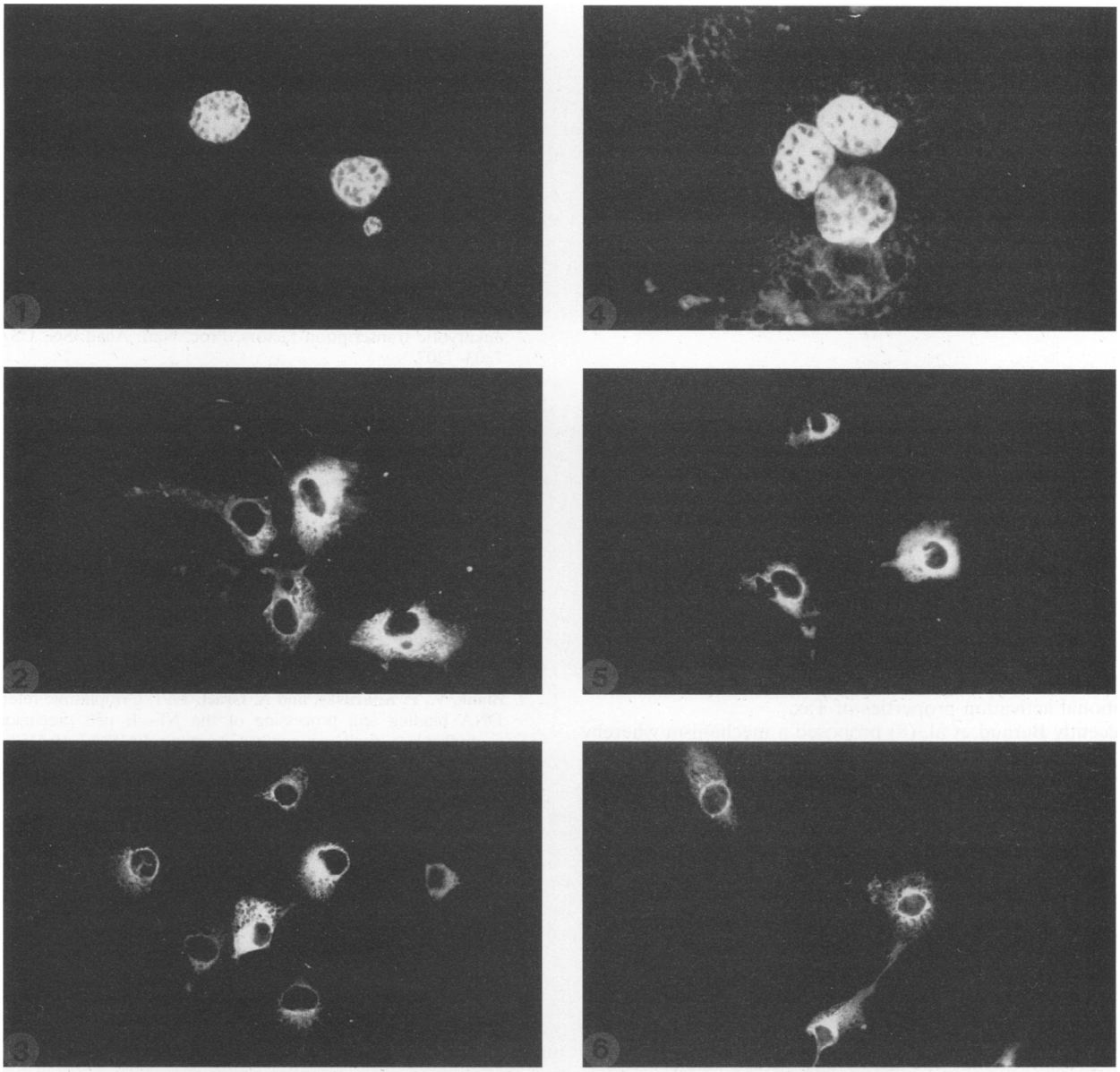

FIG. 6. Tax is unable to override cytoplasmic retention of c-Rel or RelA by MAD3 in transfected COS-7 cells. COS-7 cells were transfected with CMV-MAD3 (1.5 μg/ml) in panels 2, 3, 5, and 6, with CMV–c-Rel (0.25 μg/ml) in panels 1, 2, and 3, with CMV-RelA (0.25 μg/ml) in panels 4, 5, and 6, and with pSG-Tax (0.25 μg/ml) in panels 3 and 6. Indirect immunofluorescence was performed as for Fig. 1 with anti-c-Rel antiserum 265 (hrelC) (panels 1 to 3) or with anti-RelA antiserum 1207 (65N) (panels 4 to 6).

dissociation of MAD3-containing complexes without inducing degradation, although a previous report indicates that purified recombinant Tax is unable to dissociate an NF-κB/IκB complex in vitro (34). The situation might of course be different in vivo. In addition, the increased MAD3 turnover that we observe might only be the first in a series of events leading to the release of some NF-κB heterodimers. We are currently testing these hypotheses.

Since these results suggest that Tax does not activate NF-κB through the classical IκB/MAD3 pathway, we turned to the alternative mechanism of cytoplasmic retention involving the p105 and p100 precursors. As previously demonstrated by cotransfection into COS-7 cells (48), p105 can retain cotransfected c-Rel or RelA in the cytoplasm. We confirmed and extended these results by showing that Tax can overcome retention by p105 or p100 and induce nuclear translocation of c-Rel or RelA. Under the same conditions, neither tumor necrosis factor nor PMA seems to be able to induce such a release (our unpublished observations). The significance of these observations was further validated by the use of three Tax mutants, M22, M44, and M47 (55). M44 was originally characterized by its inability to activate the HIV-1 and HTLV-1 LTRs (namely, it can activate transcription neither through κB nor through CREB/ATF sites), while M22 activates only the HTLV-1 LTR and M47 activates only the HIV-1 LTR. We found that M47 is able to overcome cytoplasmic retention of c-Rel by p105, while M22 and M44 are not. This indicates a correlation between this anchorage-releasing activ-

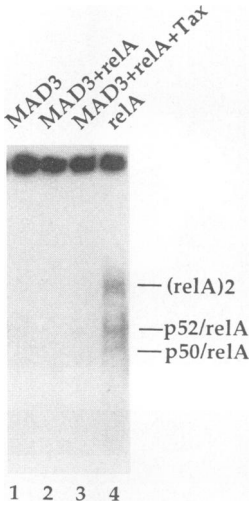

FIG. 7. Confirmation of results shown in Fig. 6. Electrophoretic mobility shift assay was performed as for Fig. 5 with nuclear proteins (3 μg per lane) derived from COS-7 cells transfected with the expression vectors indicated at the top of the lanes, under the conditions described for Fig. 6. The identities of the proteins contained in the complexes in lane 4 were ascertained by the use of specific antisera (not shown).

ity directed towards p105-containing complexes and the transcriptional activation properties of Tax.

Recently Beraud et al. (8) proposed a mechanism whereby the p100 precursor of the p52 subunit of NF-κB blocks the activity of Tax through association with and cytoplasmic retention of this molecule. The data we present here do not contradict these results, since depending on which protein is in excess, the anchoring activity of p100 or the NF-κB-releasing activity of Tax will be observed.

In summary, the results presented here demonstrate that Tax activates NF-κB through a mechanism which differs from the one postulated in the case of induction by tumor necrosis factor, PMA, IL-1, or LPS, i.e., degradation of IκB/MAD3 and nuclear translocation of complexes originally associated with this molecule in the cytoplasm. However, at this time we cannot totally exclude the possibility that Tax could induce some conformational change in the MAD3 molecule that would release some NF-κB complexes. Experiments are in progress to characterize in more detail the mechanism by which Tax induces dissociation of p105- or p100-containing complexes and in particular to determine whether this effect is direct or whether it involves additional cellular components.

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