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Constitutive activation of NF-κB is essential for transformation of rat fibroblasts by the human T-cell leukemia virus type I Tax protein

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Human T-cell leukemia virus type I (HTLV-I) encodes a 40 kDa *trans*-acting protein, Tax, that regulates transcription of both the proviral and cellular genes, and can transform rat fibroblasts. To determine the functional importance of its *trans*-acting capacities in cell transformation, we have examined two representative pathways of transcriptional activation—HTLV-I long terminal repeat (LTR) mediated and NF-κB dependent—by mutational analysis of Tax. In contrast to a previous report, mutants lacking the ability to activate an NF-κB-dependent promoter failed to transform rat fibroblasts, whereas a mutation which abolishes the HTLV-I LTR-mediated *trans*-activation demonstrated a wild-type capacity for cell transformation. Stable expression of Tax competent for transformation caused enhanced DNA binding of NF-κB in rat fibroblasts. We also demonstrate that stable co-expression of the NFKB2 precursor, known as a member of the IκB proteins, with wild-type Tax blocked transformation as well as eliminated aberrant NF-κB activation by Tax without interference with the HTLV-I LTR-mediated *trans*-activation. Our results indicate that constitutive activation of NF-κB is essential for Tax-mediated transformation of rat fibroblasts.

Keywords: HTLV-I/NF-κB/Tax/transformation

Introduction

Elucidation of the mechanisms by which viruses transform cells has contributed greatly to our understanding of the principal mechanisms of normal growth control (reviewed in Nevins, 1992). Oncoproteins of DNA tumor viruses generally target cellular proteins which have central roles in normal growth regulation. For example, the adenovirus E1A protein, the SV40 large T antigen, the polyoma virus large T antigen and the E7 protein of human papilloma virus (HPV) target the retinoblastoma gene product for binding, thus releasing the E2F transcription factor (DeCaprio *et al.*, 1988; Whyte *et al.*, 1988; Dyson *et al.*, 1989; Münger *et al.*, 1989; Chellappan *et al.*, 1991). The adenovirus E1B protein, SV40 large T antigen and the E6 protein of HPV bind to p53, another important molecule for growth control (Lane and Crawford, 1979; Sarnow *et al.*, 1982; Werness *et al.*, 1990). Since the number of products encoded in a viral genome is quite limited, it is reasonable to assume that viral oncoproteins should have evolved to alter cellular behavior with maximum efficiency by targeting molecules critical for growth control of the infected cells. In this context, studies designed to understand how human T-cell leukemia virus type I (HTLV-I) transforms cells could allow us to discover another important molecular mechanism of normal growth control.

HTLV-I is a retrovirus etiologically associated with adult T-cell leukemia (ATL) as well as HTLV-I-associated myelopathy (Poiesz *et al.*, 1980; Yoshida *et al.*, 1982; Gessain *et al.*, 1985; Osame *et al.*, 1986). The HTLV-I genome contains no homologous sequence of a cellular proto-oncogene, and there is no preferred integration site among patients, ruling out insertional mutagenesis as the mechanism of leukemogenesis (Seiki *et al.*, 1983, 1984). Since there is a long latency period until the onset of disease and a relatively low incidence of developing ATL after HTLV-I infection, leukemogenesis by HTLV-I has been thought to represent a multistep process (Okamoto *et al.*, 1989). The viral-encoded *trans*-acting proteins, Tax and Rex, have been suggested to be involved in leukemogenesis, in which they could modulate the expression of cellular genes (Yoshida and Seiki, 1989).

Much attention has focused on the viral transcriptional modulator, Tax, which greatly stimulates transcription from the HTLV-I long terminal repeat (LTR) (Sodroski *et al.*, 1985; Seiki *et al.*, 1986). A number of cellular and viral genes are also upregulated by Tax. Among them are the c-*fos* gene, the interleukin-2 (IL-2) gene, the IL-2 receptor α-chain (IL-2Rα) gene, human immunodeficiency virus type 1 gene, the gene encoding granulocyte–macrophage colony-stimulating factor and several of the immediate early response genes (Maruyama *et al.*, 1987; Siekevitz *et al.*, 1987a,b; Fujii *et al.*, 1988; Miyatake *et al.*, 1988a,b; Alexandre *et al.*, 1991). The aberrant expression of these growth-related genes has been supposed to contribute to the establishment of the HTLV-I-associated proliferative pathogenesis. Indeed, several recent studies have demonstrated oncogenic properties of Tax. Expression of Tax in established rodent fibroblast cell lines results in their oncogenic transformation (Tanaka *et al.*, 1990). Tax cooperates with an activated RAS to transform rat embryo cells (Pozzatti *et al.*, 1990). Transgenic mice bearing the *tax* gene as a transgene develop multiple mesenchymal tumors and neurofibromas (Nerenberg *et al.*, 1987). Expression of the *pX* genes in human cord blood lymphocytes and thymus T cells by means of a herpesvirus saimiri vector results in T-cell clones that are immortalized, but whose proliferation is still dependent on IL-2 (Grassmann *et al.*, 1989). These findings strongly suggest that Tax may play cardinal roles in the process of leukemogenesis.

To investigate the mechanism of Tax-mediated transformation, we employed a cloned rodent fibroblast cell

line, since rodent fibroblasts have so far provided useful systems to study the oncogenic properties of various viral and cellular oncoproteins. Among these are Rat-1 cells susceptible to transformation by various nuclear and cytoplasmic oncoproteins, and having a low background of spontaneous foci (Topp, 1981). Moreover, the Rat-1 cell line allowed us to analyze accurately the biochemical pathways of transformation by Tax without considering the effects of an activated *ras* gene product.

Despite the accumulating knowledge on how Tax regulates transcription in cooperation with cellular transcription factors, including activating transcription factor/cyclic adenosine monophosphate response element binding protein (ATF/CREB), NF-κB-like factors, Ets-1 and serum response factor (SRF), it still remains unclear which of the diverse biological activities of Tax are actually essential for cell transformation (Fujisawa *et al.*, 1986; Leung and Nabel, 1988; Lowenthal *et al.*, 1988; Bosselut *et al.*, 1990; Fujii *et al.*, 1992).

Mutational analysis has shown that the diverse Tax *trans*-activations involve several cellular mechanisms which require distinct functional domains of the Tax polypeptide (Smith and Greene, 1990; Semmes and Jeang, 1992, 1995; Tsuchiya *et al.*, 1994; Adya and Giam, 1995; Yin *et al.*, 1995). The amino-terminal region of Tax seems to be important for interaction with CREB, while its carboxy-terminus is likely to be involved in communication with the basal transcriptional machinery. In the present study, we have analyzed which of the two representative *trans*-activating functions of Tax, namely the HTLV-I LTR-mediated or NF-κB-dependent *trans*-activation, is essential for its oncogenic activity.

Results

Construction of mutants

Three *tax* mutants were used to examine the relationship between Tax-dependent *trans*-activation and cell transformation (Figure 1). Mutant *m148* gene was initially cloned from a flat revertant cell line (designated EA4) which had been isolated in an attempt to obtain cellular variants by chemical mutagenesis of a Tax-transformed Rat-1 cell line. We found that EA4 cells were susceptible to re-transformation by wild-type Tax and that transient introduction of a chloramphenicol acetyltransferase (CAT) gene under the control of the HTLV-I LTR into EA4 cells resulted in strong activation of this CAT gene (data not shown). These observations suggested that EA4 cells may express a mutant Tax protein which is defective in cell transformation, but retains the ability to *trans*-activate the HTLV-I LTR. By means of the polymerase chain reaction (PCR) amplification of the genomic DNAs prepared either from EA4 cells or from Tax-transformed Rat-1 cells, 40MRatcl-1 cells (Tanaka *et al.*, 1990), the *tax* genes were cloned and sequenced. A 1.4 kb fragment encompassing the entire coding region of the *tax* gene from EA4 cells showed a single base alteration from GGA at codon 148 to GTA at the same position, resulting in an amino acid substitution of valine for glycine (Figure 1).

The other two mutants, *m319* and *m353vp*, were generated by PCR mutagenesis of the wild-type *tax* gene cloned from a 40MRatcl-1 cell as described in Materials and methods. *m319* directs two amino acid alterations at

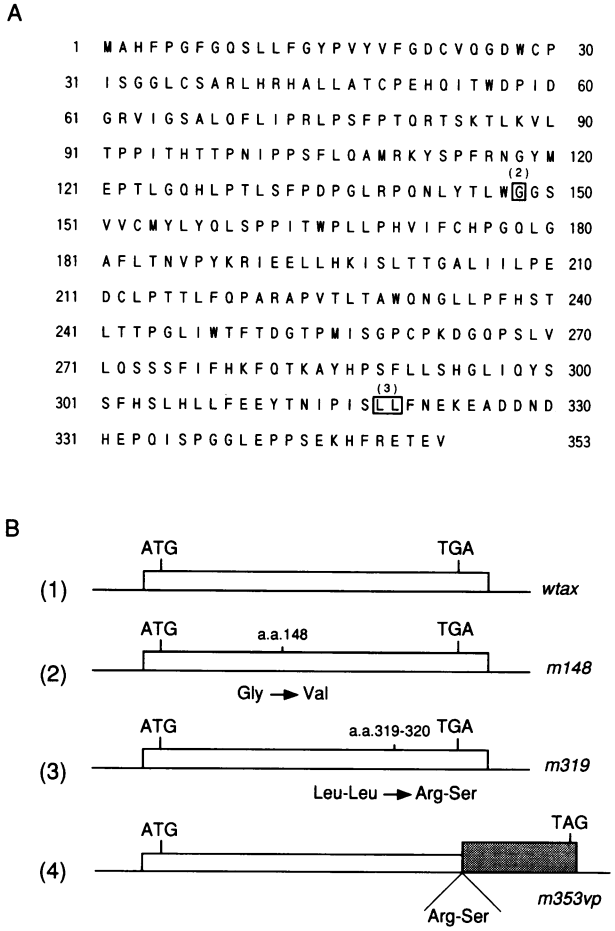

Fig. 1. Structures of *tax* mutants. (**A**) Amino acid sequence of the Tax protein deduced from the pUC*wtax* DNA sequence determined by dideoxynucleotide sequencing. The substituted amino acids are indicated by the boxed residues. (**B**) Schematic representation of each of the *tax* genes. (1) Wild-type *tax*; (2) *m148*; (3) *m319*; (4) *m353vp*. Symbols: open boxes, HTLV-I sequence; shaded box, sequence for the acidic activation domain of herpes simplex virus VP16; ATG, translation initiation codon for Tax; TGA or TAG, translation stop codon. Numbers above the open boxes refer to the amino acids substituted. The linker-encoded dipeptide, arginine–serine, located at the boundary between Tax and VP16 is indicated.

codons 319 and 320, from leucine–leucine to arginine–serine, which corresponds to the M47 Tax mutant generated by Smith and Greene (1990). *m353vp* was constructed by linking the entire coding region of the wild-type *tax* gene to a 0.24 kb DNA fragment encoding the acidic activation domain of the herpes simplex virus VP16 (amino acids 413–490) (Sadowski *et al.*, 1988). All the wild-type and mutant *tax* genes were transferred to a eukaryotic expression vector, pH2R*neo* (Tanaka *et al.*, 1990), in the sense orientation, and the resultant plasmids were designated pH2R*wtax*, pH2R*m148*, pH2R*m319* and pH2R*m353vp*. The encoded Tax proteins were designated wild-type Tax, M148, M319 and M353VP, respectively.

Mutants exhibit distinct trans-activation phenotypes

To test how these mutations affect Tax-mediated transcriptional activation of the two representative promoters, the HTLV-I LTR and an NF-κB-dependent promoter, Rat-1 cells were transiently co-transfected with each of the

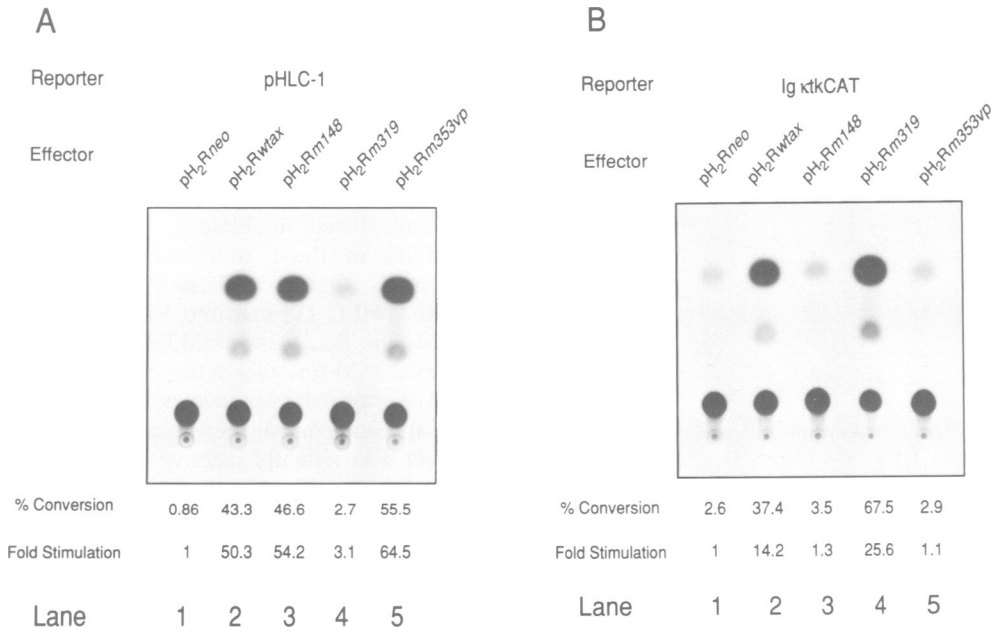

Fig. 2. The mutant Tax proteins selectively *trans*-activate differential promoter constructs. Rat-1 cells were transiently transfected with 1 μg of reporter plasmid [pHLC-1 in (**A**) or IgκtkCAT in (**B**), as shown], 1 μg of effector plasmid, 1 μg of pCMVβ DNA and 3 μg of sonicated salmon sperm carrier DNA (ss-DNA). Effector plasmids are indicated at the top: lanes A-1 and B-1, pH2R*neo* vector; lanes A-2 and B-2, pH2R*wtax*, lanes A-3 and B-3, pH2R*m148*; lanes A-4 and B-4, pH2R*m319*; lanes A-5 and B-5, pH2R*m353vp*. The amounts of radioactivity are shown below each lane as percent conversion. The fold stimulation indicates the degree of elevation of CAT activity relative to that obtained with pH2R*neo* vector [a value of 1 = 0.86% conversion in (A) and 2.6% in (B)]. The results are representative of independent transfections in triplicate.

Tax expression constructs and a CAT reporter plasmid containing either the HTLV-I LTR (pHLC-1) or seven copies of the NF-κB-responsive murine immunoglobulin κ light chain enhancer core sequence (IgκB) linked to the herpesvirus thymidine kinase (tk) gene promoter (IgκtkCAT) (Figure 2). Each transfection experiment included a plasmid which expresses the bacterial *lacZ* gene under the control of the cytomegalovirus promoter (pCMVβ) as an internal control. As expected, wild-type Tax efficiently stimulated transcription from both the HTLV-I LTR and the promoter containing the κB motifs (Figure 2, lanes 2). Mutants M148 and M353VP *trans*-activated the HTLV-I LTR as efficiently as wild-type Tax did, but failed to enhance the κB site-dependent transcription (Figure 2, lanes 3 and 5). Conversely, M319 barely activated the HTLV-I LTR, although it was fully active for the κB site-dependent promoter (Figure 2, lanes 4). Thus, under the transient assay condition, these three Tax mutants *trans*-activated either the HTLV-I LTR or the κB site-dependent promoter alone.

M319 induces stable transformation of Rat-1 cells, but M148 and M353VP do not

To determine the functional importance of the two independent *trans*-activating functions in cell transformation, we established Rat-1 cells stably expressing the mutant Tax proteins. Each of the Tax expression constructs, pH2R*wtax*, pH2R*m148*, pH2R*m319* and pH2R*m353vp*, was introduced into Rat-1 cells by the calcium phosphate precipitation technique. Selection of the transfected cells with the antibiotic G418 generated a large number of drug-resistant colonies ($>6\times10^2$ colonies/μg of each plasmid) 10–14 days after transfection. Twenty to thirty clones were isolated and expanded into cell lines for each construct, and were examined for Tax expression (Figure 3). The remainder of the cells were collected and expanded as pooled populations (Wmix, M148mix, M319mix and M353VPmix). NRRatcl-2 is one of the G418-resistant Rat-1 cell clones isolated from NRRatmix (Tanaka *et al.*, 1990) containing pH2R*neo* vector alone. For each form of Tax, two independent cell clones which express appropriate levels of Tax were selected and demonstrated in the present communication. Essentially similar biological phenotypes were observed among at least five independent cell clones for each form of Tax (data not shown). W4 cells expressed a relatively large amount of wild-type Tax from the pH2R*wtax* construct, whereas 40M-3 cells expressed a relatively small amount of wild-type Tax from another Tax expression construct, pH2R40M (Tanaka *et al.*, 1990). Both M148-5 cells and M148-8 cells expressed relatively high levels of M148 from the pH2R*m148* construct. M319-5 cells and M319-12 cells expressed relatively low levels of M319 from the pH2R*m319* construct. M353VP-11 or M353VP-19 cells expressed M353VP from the pH2R*m353vp* construct at levels comparable with those of 40M-3 or M319-5 cells.

Figure 4 shows the morphology of several transfectants. Consistent with our previous report (Tanaka *et al.*, 1990), wild-type Tax efficiently transformed Rat-1 cells; cells expressing wild-type Tax (Figure 4B and C) exhibited a marked increase in cell refractility and cell density compared with control cells transfected with pH2R*neo* vector alone (Figure 4A). Rat-1 cells expressing M319 (Figure 4E) were almost indistinguishable from those expressing wild-type Tax. Interestingly, cells expressing M148 (Figure 4D) or M353VP (Figure 4F) characteristically displayed reduced swirling of the cell monolayers and a moderate increase in cell density at confluence. The biological

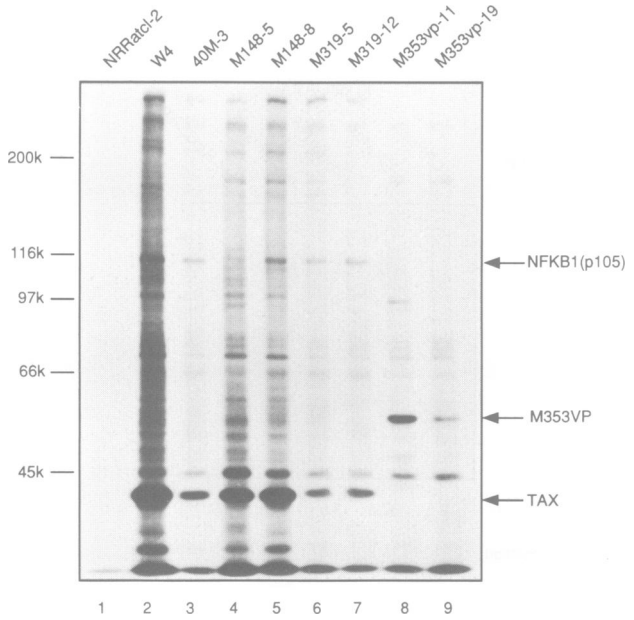

Fig. 3. Immunoprecipitations from ^{35}S-labeled Rat-1 transfectants. Lysates prepared from approximately equal numbers (1.5×10^6) of ^{35}S-labeled Rat-1 transfectants were immunoprecipitated with MI73 (anti-Tax monoclonal antibody). At the top of each lane is the name of the cell line tested. NRRatcl-2 cells were used as a negative control (lane 1). W4 cells and 40M-3 cells express wild-type Tax (lanes 2 and 3). Two independently arising cell lines were analyzed for each mutant. Lanes 4 and 5, M148 transfectants; lanes 6 and 7, M319 transfectants; lanes 8 and 9, M353VP transfectants. The proteins were resolved on a 7.5% SDS–polyacrylamide gel. The gel was processed by fluorography and visualized by autoradiography. The positions of the Tax proteins, the Tax–VP16 fusion protein and the NFKB1 precursor protein are indicated. Numbers on the left represent the molecular weights of standard size markers.

properties of the transfectants are summarized in Table I. Wild-type or M319 transfectants exhibited a similar transformed phenotype; they grew beyond a monolayer, produced colonies in soft agarose and gave rise to tumors in *nude* mice. In contrast, both M148 transfectants and M353VP transfectants underwent contact inhibition at confluence, could not grow in soft agarose and failed to elicit tumors in *nude* mice 6 weeks after inoculation. However, the pH2R*wtax* construct induced transformation of M148 or 353VP transfectants (data not shown), indicating that these mutants could not suppress transformation in a *trans*-dominant negative fashion. Virtually identical results were obtained in assays of the transformation of other established rat fibroblasts, normal rat kidney cells (NRK49F) (de-Larco and Todaro, 1978) and Rat-2 cells (Topp, 1981) (data not shown). We have concluded that M319 fully retains the transforming activity, while both M148 and M353VP are completely defective in the transformation of Rat-1 cells.

Mutants express differential phenotypes of trans-activation in the stable cell lines

Trans-activating properties of Tax observed in transient expression experiments (Figure 2) do not necessarily assure us of constitutive transcriptional activation of the target genes under long-term stable expression of Tax in Rat-1 transfectants. In fact, a previous report showed selective suppression of NF-κB activity following long-term expression of Tax in Jurkat T-lymphoid cells, implying that the short- and long-term effects of Tax expression could be quite different depending on the cellular environment (Ruben and Rosen, 1990). To investigate the functional contribution of Tax-dependent *trans*-activation to cell transformation, it is essential to know the actual condition of transcriptional activation in the Rat-1 transfectants listed in Table I. We therefore assayed CAT activity in Rat-1 transfectants with the two reporter plasmids (Figure 5). Transfection with the HTLV-I LTR CAT (pHLC-1) gene into Rat-1 transfectants expressing wild-type Tax, M148 or M353VP induced CAT activity to a level >20-fold that achieved by control cells. However, M319 transfectants gave no significant CAT activation. On the other hand, the κB-dependent CAT reporter construct was virtually inactive in both M148 and M353VP transfectants. This reporter construct demonstrated a >16-fold stimulation of CAT activity in wild-type Tax or M319 transfectants. Thus, stable expression of each mutant Tax protein causes constitutive transcriptional activation of the expected proper target gene in Rat-1 transfectants.

Wild-type Tax and M319 induce enhanced DNA binding of NF-κB in stable cell lines

Constitutive NF-κB DNA binding activity has been reported in HTLV-I-infected cells (Leung and Nabel, 1988; Lowenthal *et al.*, 1988; Li *et al.*, 1993; Lanoix *et al.*, 1994), Jurkat T-lymphoid cells stably or conditionally expressing the Tax protein (Ballard *et al.*, 1988; Böhnlein *et al.*, 1989; Arima *et al.*, 1991; Lacoste *et al.*, 1991; Li *et al.*, 1993; Kanno *et al.*, 1994a; Lanoix *et al.*, 1994) and Tax-transformed murine fibroblasts (Kitajima *et al.*, 1992). To understand the molecular basis of the differential responsiveness of the promoter containing the murine IgκB motifs to Tax mutants, proteins induced by Tax to bind to the κB sequence were analyzed by electrophoretic mobility shift assay (EMSA) (Figure 6). Nuclear extracts were prepared from control NRRatcl-2 cells or from cells stably expressing wild-type or mutant Tax, and were tested for binding to a double-stranded DNA probe containing a single copy of the murine IgκB motif. Consistent with the *trans*-activation assays, nuclear extracts from cells expressing either wild-type Tax or M319 showed high levels of NF-κB DNA binding activity, whereas those from control cells, M148 or M353VP transfectants showed equivalently low levels of NF-κB DNA binding activity. The band was competed with an excess of unlabeled IgκB oligonucleotide (Figure 6B, lane 2), but not with an excess of a mutated IgκB probe containing a single base substitution (Figure 6B, lane 3), demonstrating the specificity of binding. The mutant probe gave no specific retarded band (Figure 6B, lane 7).

To determine the polypeptide composition of the κB-specific complexes, we used NF-κB subunit-specific antibodies for the supershift assay. With the nuclear extract derived from W4 cells, addition of NFKB1-specific antibody completely supershifted the κB-specific band (Figure 6B, lane 5). RelA-specific antibody also supershifted the band, albeit not completely (Figure 6B, lane 6). Neither NFKB2-specific nor c-Rel-specific antibody affected formation of the κB-specific complexes (data not shown). These observations indicate that NFKB1 and RelA [presumably the NFKB1 (p50)/RelA heterodimer] represent

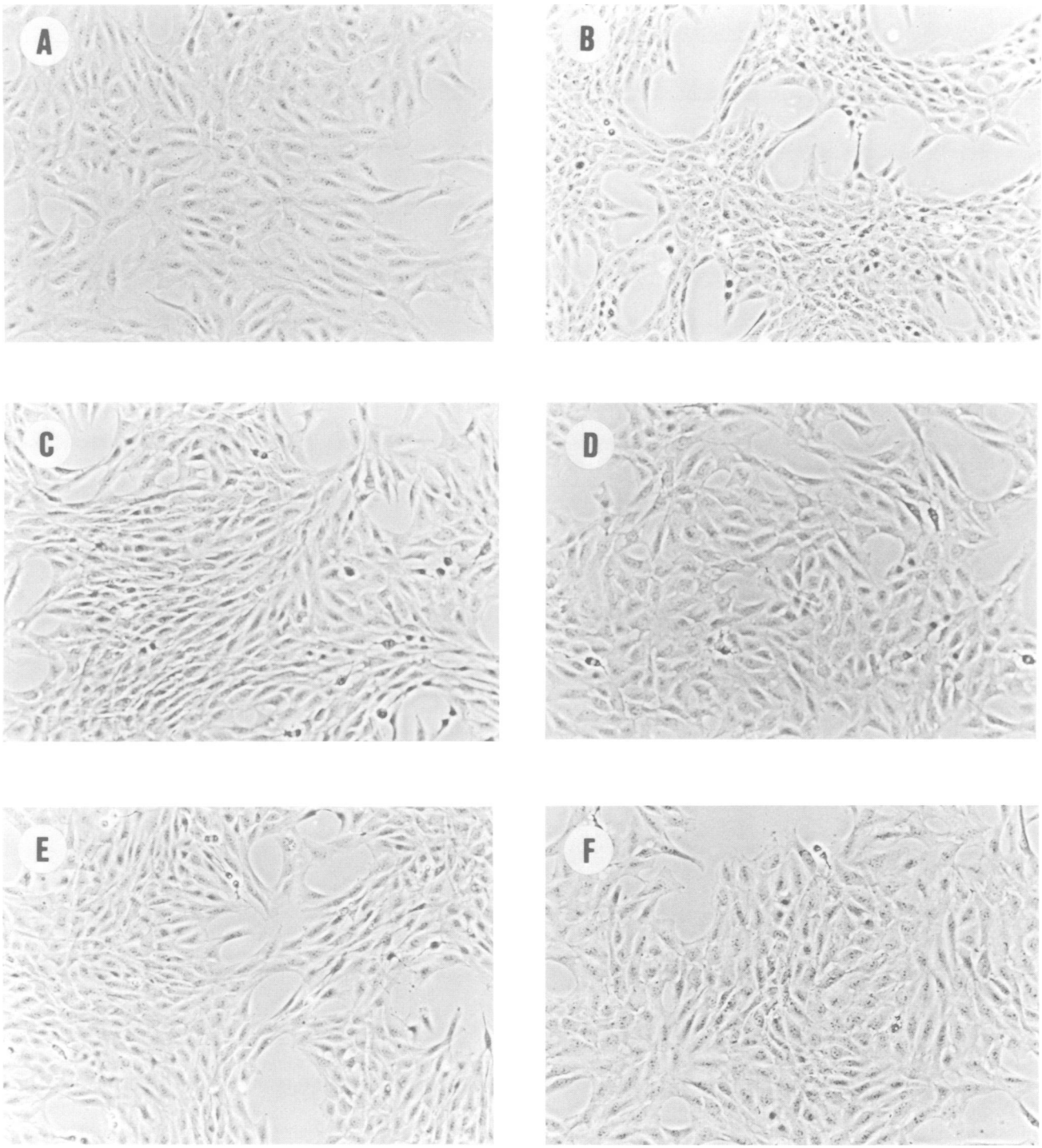

Fig. 4. Morphology of Rat-1 transfectants. Phase-contrast micrographs (original magnification ×100) of Rat-1 transfectants on monolayer cultures: (**A**) NRRatcl-2 cells; (**B**) W4 cells; (**C**) 40M-3 cells; (**D**) M148-8 cells; (**E**) M319-12 cells; (**F**) M353VP-11 cells.

the major NF-κB subunits activated in Tax-transformed Rat-1 cells.

Stable co-expression of NFKB2 blocked transformation by Tax

To further confirm the role of NF-κB in cell transformation, we attempted to eliminate selectively NF-κB activation in cells expressing wild-type Tax. As a candidate for selective suppressor, we chose NFKB2 (p100), which recently has been reported to be able to interact with Tax and efficiently interrupt Tax-mediated NF-κB activation in transient assay conditions (Béraud *et al.*, 1994; Kanno *et al.*, 1994b; Lanoix *et al.*, 1994; Watanabe *et al.*, 1994; Murakami *et al.*, 1995). Rat-1 cells were co-transfected with plasmid pKCR*b-wtax*, a Tax expression vector containing the blasticidin S-resistance gene, and plasmid pC*n*100, an NFKB2 (p100) expression vector containing the G418-resistance gene. Transfectants were selected with both

Table I. Biological properties of Rat-1 transfectants

Cell line	Phenotype of *trans*-activation	Contact inhibition at confluency[a]	Colony-forming efficiency in soft agar (%)[b]	Tumorigenicity in *nude* mice[c]	Tumor latency (weeks)[d]
Rat1	–	yes	<0.01	0/5	–
NRRatcl-2	–	yes	<0.01	0/5	–
40M-3	wild type	no	98.8	5/5	1.5–2
WTAXmix	wild type	no	42.6	5/5	1.5–2
W4	wild type	no	96.6	5/5	1–1.5
M148mix	HTLV-I LTR	yes	<0.01	0/5	–
M148-5	HTLV-I LTR	yes	<0.01	0/5	–
M148-8	HTLV-I LTR	yes	<0.01	0/5	–
M319mix	NF-κB	no	53.8	5/5	2–2.5
M319-5	NF-κB	no	80.7	5/5	1.5–2.5
M319-12	NF-κB	no	90.2	5/5	1.5–2
M353VPmix	HTLV-I LTR	yes	<0.01	0/5	–
M353VP-11	HTLV-I LTR	yes	<0.01	0/5	–
M353VP-19	HTLV-I LTR	yes	<0.01	0/5	–

[a]Cells (~1×10^5) were seeded onto 60 mm plastic dishes, re-fed every 3 days and inspected 14 days after seeding.
[b]Percentage of tested cells that gave rise to colonies >60 μm at 14 days after seeding in 0.33% agarose.
[c]Tumorigenicity in *nude* mice scored after injecting 5×10^6 cells for each cell line. The fraction indicates the number of mice with tumors at 6 weeks after inoculation/number injected.
[d]Latency indicates weeks to development of tumors 0.8 mm in diameter.

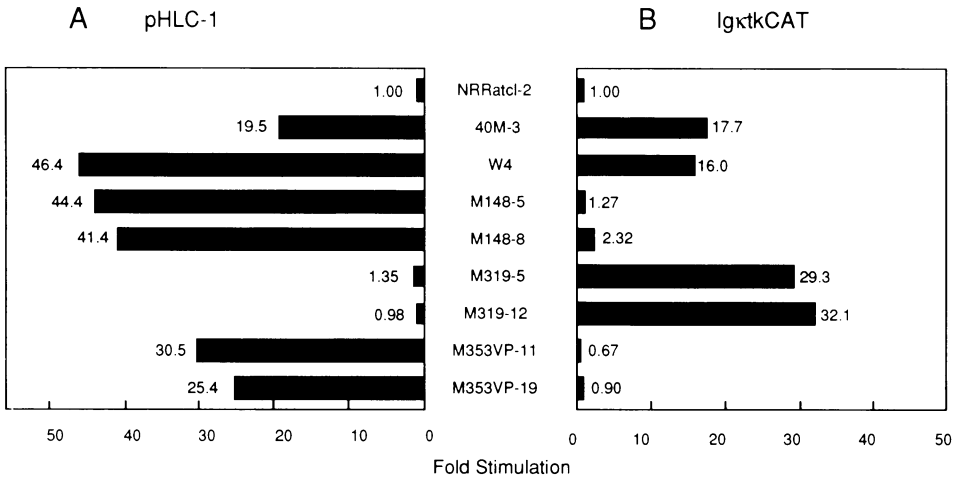

Fig. 5. Differential phenotypes of *trans*-activation by the mutant Tax proteins stably expressed in Rat-1 transfectants; a graphic representation of CAT activity produced by Rat-1 transfectants. The reporter plasmids are (**A**) pHLC-1 or (**B**) NF-κB-responsive IgκtkCAT. Each experiment contained 1 μg of reporter plasmid, 1 μg of pCMVβ DNA and 4 μg of ss-DNA so that CAT activity was determined within the linear range of the assay on cell lysates normalized for β-galactosidase activity. For each promoter construct, the CAT activity induced in Rat-1 transfectants is shown relative to the CAT activity produced in NRRatcl-2 cells, which was set to 1.0. The results represent an average from 4–5 independent experiments.

drugs and the resultant cell clones were analyzed for expression of both Tax and NFKB2. Although stable NFKB2 expression was relatively difficult to obtain, out of 50 clones examined, two cell clones designated TKB2-62 and TKB2-73, derived from independent transfections, were found to express significant amounts of Tax and NFKB2 proteins (Figure 7A and B). In addition, transfection of the wild-type Tax-expressing cell clones, W4 or 40M-3, with another NFKB2 expression vector, pC*b*100, containing the blasticidin S-resistance gene instead of the G418-resistance gene, generated cell lines, designated W4-20 and 40M-3-H4, in which NFKB2 is stably overexpressed with the expression of Tax maintained. Interestingly, biochemical fractionation experiments showed that stable co-expression of NFKB2 in these cells did not apparently alter the subcellular localization of Tax (Goh *et al.*, 1985; Kiyokawa *et al.*, 1985) (Figure 7B), although it was previously reported that Tax is redistributed predominantly in the cytoplasm when overexpressed transiently with NFKB2 in COS cells (Béraud *et al.*, 1994; Pepin *et al.*, 1994). Assays of transcription from either the HTLV-I LTR or the κB-dependent promoter in cells stably co-expressing Tax and NFKB2 demonstrated that the former promoter was fully activated, but that the latter promoter was almost inactive in these cells (Figure 8). Moreover, transfection of these cells with a RelA expression vector and the κB-dependent CAT construct led to a significant elevation of CAT activity, whereas co-transfection with a Tax expression vector and the κB-CAT construct could barely elevate CAT activity, suggesting that endogenous NF-κB could no longer be activated by additional Tax expression in NFKB2-expressing cells (data not shown). Figure 9 shows the morphology of cells co-expressing Tax and NFKB2 in monolayer cultures. Consistent with the results for cells expressing Tax mutants, these cells appeared to be phenotypically normal.

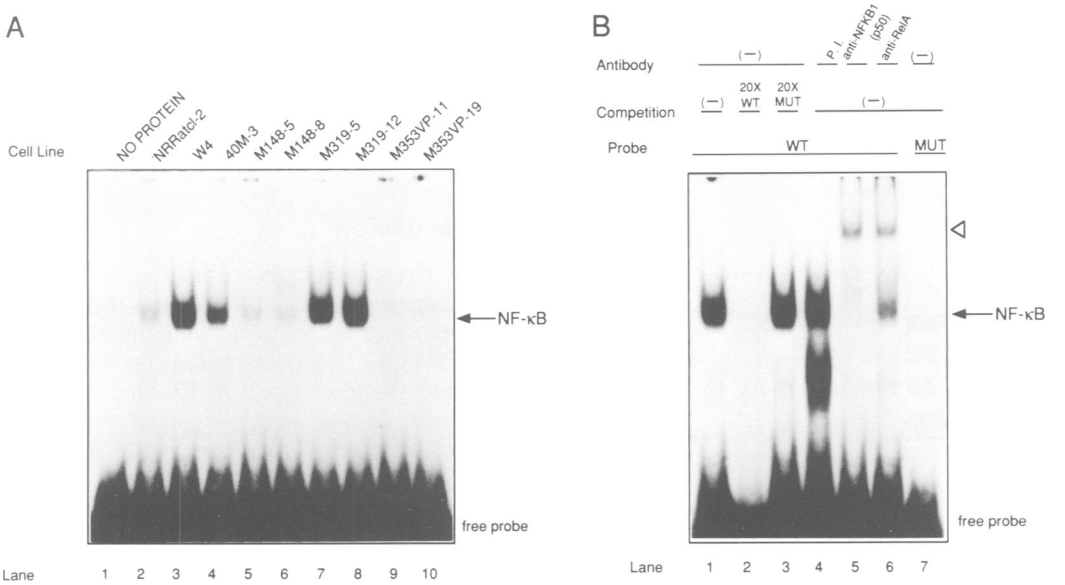

Fig. 6. Wild-type Tax and M319 induce nuclear κB DNA-binding proteins containing NFKB1 and RelA in stable transfectants. (**A**) EMSA containing the radiolabeled wild-type κB-oligonucleotide and nuclear extracts derived from the indicated cell lines. Lane 1, no protein added; lane 2, extract from control NRRatcl-2 cells; lanes 3 and 4, extracts from cells expressing wild-type Tax; lanes 5 and 6, extracts from cells expressing M148; lanes 7 and 8, extracts from cells expressing M319; lanes 9 and 10, extracts from cells expressing M353VP. The arrow indicates the position of the specific nuclear protein–DNA complex. (**B**) EMSA characterizing the nuclear protein–DNA complex. The ^{32}P-labeled DNA probes used are wild-type κB-oligonucleotide in lanes 1–6 and the mutated one in lane 7. W4 cell extract was loaded directly (lanes 1 and 7) or after pre-incubation as follows: lane 2, with 20 M excess of wild-type κB-oligonuleotide; lane 3, with 20 M excess of mutated κB-oligonucleotide; lane 4, with 2 μl of pre-immune rabbit serum; lane 5, with 1 μl of anti-NFKB1 antibody; lane 6, with 1 μl of anti-RelA antibody. The open arrowhead indicates the positions of supershifted bands.

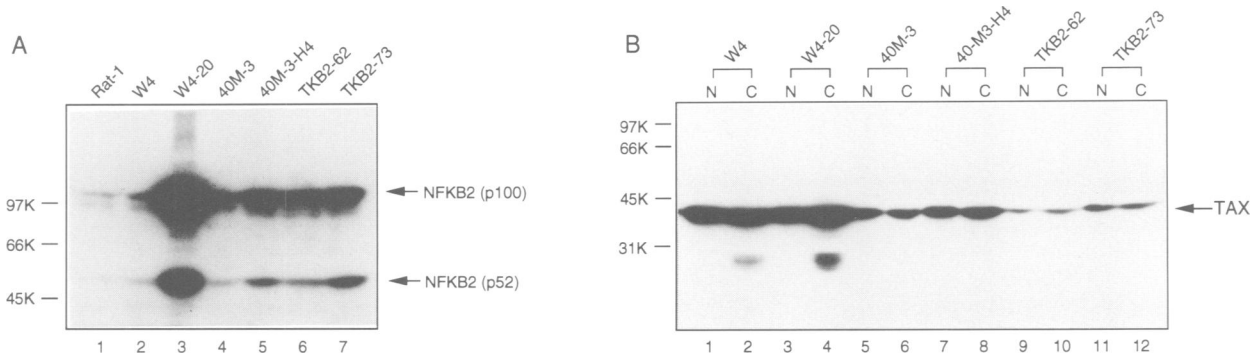

Fig. 7. Stable expression of the NFKB2 precursor does not significantly alter the subcellular distribution of Tax. (**A**) Immunoblot analysis of NFKB2 protein levels in Rat-1 transfectants. Whole-cell extracts (~100 μg of protein for each lane) were resolved on a 7.5% SDS–polyacrylamide gel, and the NFKB2 precursor (p100) as well as its processed form (p52) detected by an anti-NFKB2 antibody were visualized using [^{125}I]proteinA (Amersham). (**B**) Immunoblot analysis of nuclear and cytoplasmic fractions prepared from Rat-1 transfectants. Nuclear (odd-numbered lanes) and cytoplasmic (even-numbered lanes) fractions from W4 cells (lanes 1 and 2), W4-20 cells (lanes 3 and 4), 40M-3 cells (lanes 5 and 6), 40M-3-H4 cells (lanes 7 and 8), TKB2-62 cells (lanes 9 and 10) and TKB2-73 cells (lanes 11 and 12) were prepared, and equal cell equivalents of each fraction were subjected to immunoblot analysis using an anti-Tax monoclonal antibody (MI73) and [^{125}I]proteinA.

They could not grow in 0.33% soft agarose and did not elicit tumors in *nude* mice over a period of 6 weeks after injection of 5×10^6 cells. The above observations strongly indicate that constitutive activation of NF-κB is essential for Tax-mediated transformation of rat fibroblasts.

Mutation at codons 137 and 138 eliminates transformation

Finally, we tested another mutant form of Tax that has been previously reported by Smith and Greene (1991) as being unable to activate NF-κB, but remains competent to transform Rat-2 cells. This mutant, designated M137 in our experiments, has two amino acid substitutions at codons 137 and 138, from glycine–leucine to alanine–serine, which corresponds to the M22 Tax mutant generated by Smith and Greene (1990). M137 expression vector was constructed and introduced into Rat-1 cells. The individual M137-expressing cell clones, including M137-8 and M137-12, showed contact inhibition at confluency on monolayer cultures, could not grow in 0.33% soft agar and did not develop tumors in *nude* mice. Polyclonal pools of M137 transfectants also showed the same biological phenotype. Expression of comparable amounts of wild-type Tax or M137 in the individual cell clones was verified by Western blot analysis (Figure 10B). Transient transfection experiments in Rat-1 cells or stable M137

transfectants revealed that M137 cannot *trans*-activate the κB site-dependent promoter, as reported for the M22 mutant, thus further supporting our conclusion that NF-κB activation is critical for Tax-mediated transformation. Unexpectedly, M137 also failed to *trans*-activate the HTLV-I LTR (Figure 10C). As for *trans*-activation of the HTLV-I LTR, essentially identical results were obtained for COS7 cells, where M137 was abundantly expressed (data not shown). Thus, M137 is completely defective in cell transformation and functionally inactive in both of the *trans*-activation pathways.

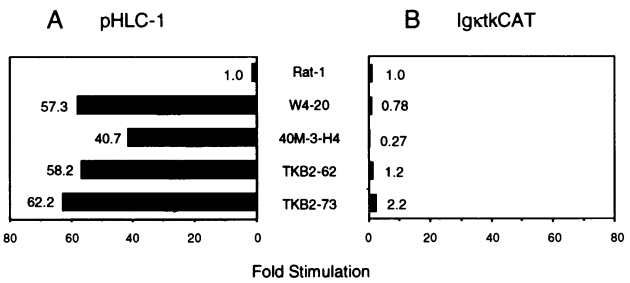

Fig. 8. Stable co-expression of Tax and the NFKB2 precursor eliminates aberrant NF-κB activation without affecting the HTLV-I LTR-mediated *trans*-activation; a graphic representation of CAT activity produced by Rat-1 cells stably expressing both Tax and NFKB2. The reporter plasmids are (**A**) pHLC1 or (**B**) NF-κ-responsive IgκtkCAT. Assays were carried out as described in the legend to Figure 5. CAT activity is shown relative to that for Rat-1 cells, which was set to 1.0. Transfection with each reporter plasmid into Rat-1 cells containing control vectors yielded CAT activity essentially equal to that for Rat-1 cells (data not shown). Mean values for at least three independent assays are presented.

Discussion

We have shown that mutations of Tax which abolish induction of active NF-κB in the nucleus completely eliminate transformation, while loss of *trans*-activation of the HTLV-I LTR does not affect transformation. Moreover, stable co-expression of NFKB2 led to a loss of constitutive NF-κB activation accompanied by a reversion of the transformed phenotype. Our results indicate that the ability of Tax to *trans*-activate the cognate HTLV-I LTR is dispensable for transformation; instead, persistent activation of NF-κB is essential for transformation. These results are in contrast to a previous observation by Smith and Greene (1991) in which the authors suggested that the induction of NF-κB is neither necessary nor sufficient to transform Rat-2 cells and that the ability to *trans*-activate the ATF/CREB-responsive HTLV-I LTR plays a central role in transformation of these cells. The difference in *trans*-activation of the HTLV-I LTR by our M137 or their M22 mutant could arise from the substitutions of eight

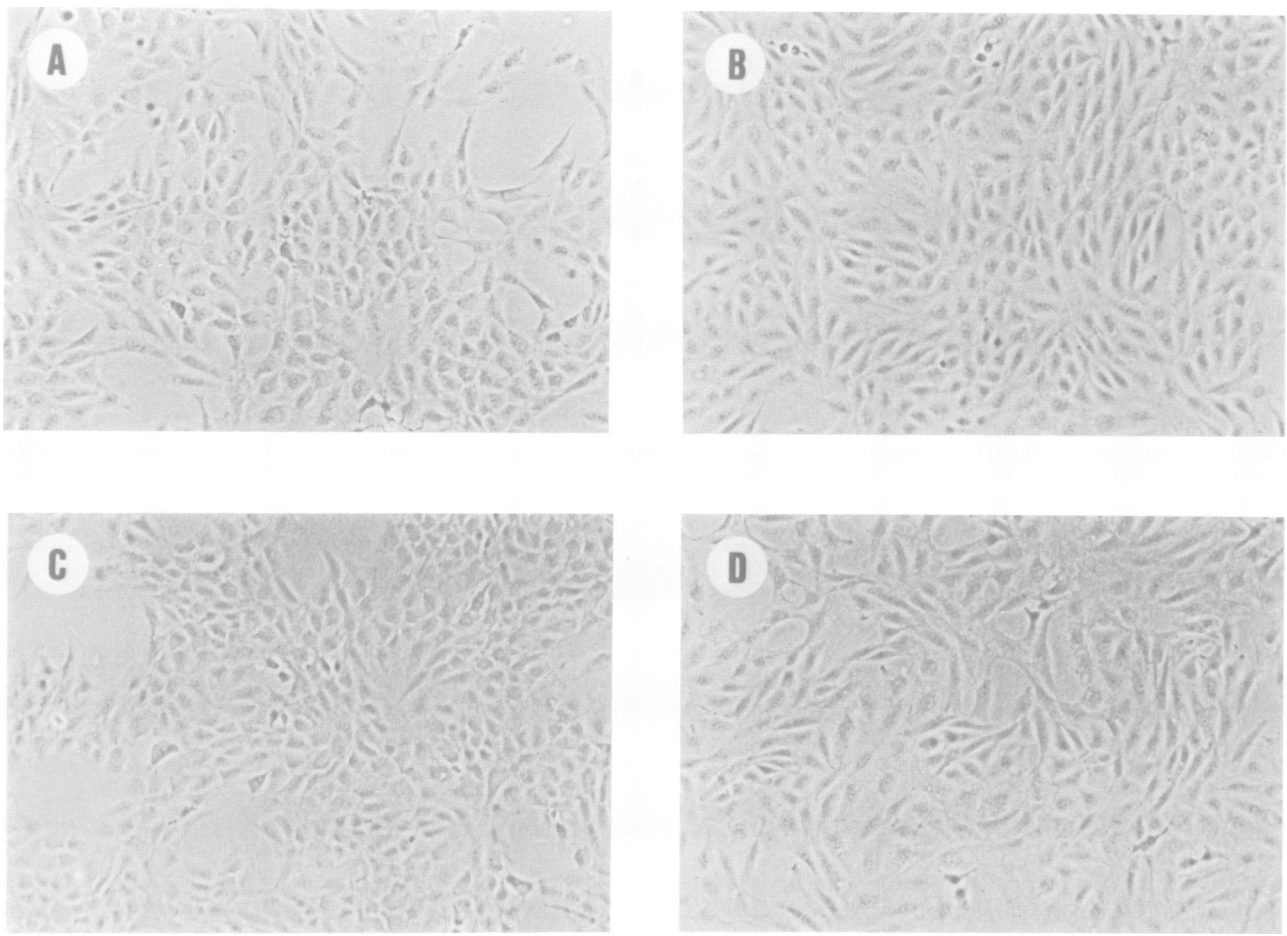

Fig. 9. Morphology of cells co-expressing Tax and NFKB2. Phase-contrast micrographs (original magnification ∞100) of NFKB2 transfectants on monolayer cultures: (**A**) W4-20 cells; (**B**) 40M-3-H4 cells; (**C**) TKB2-62 cells; (**D**) TKB2-73 cells.

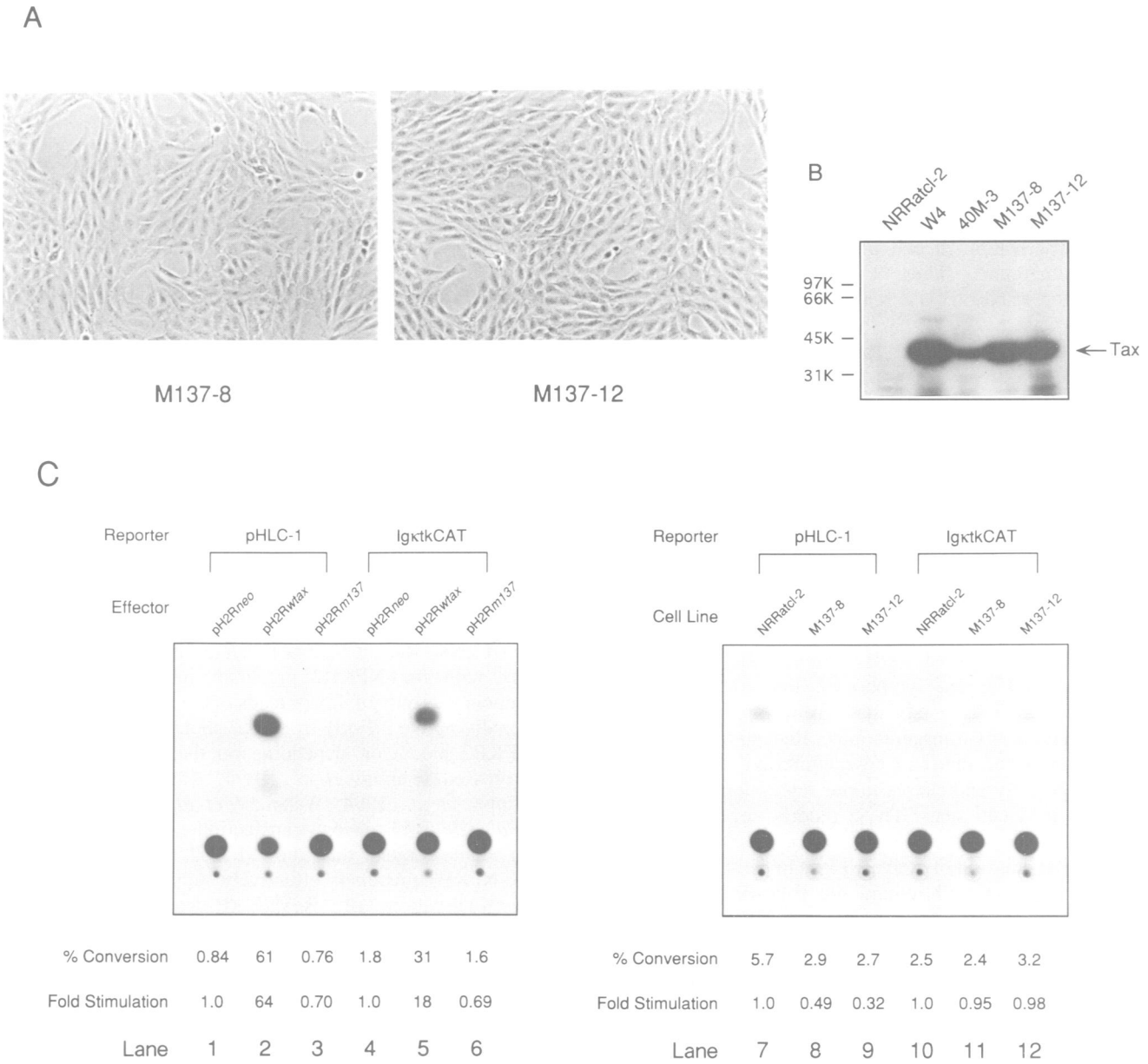

Fig. 10. M137 is defective in transformation and *trans*-activation. (**A**) Phase-contrast micrographs (original magnification ×100) of M137-expressing cell clones isolated from independent experiments on monolayer cultures (M137-8 cells on the left and M137-12 cells on the right). (**B**) Immunoblot analysis of Tax protein expression in M137 transfectants. Approximately 80 μg of total protein extracts from the indicated cell lines were resolved on a 10% SDS–polyacrylamide gel and subjected to immunoblot analysis with the MI73 antibody and [^{125}I]protein A. (**C**) Representative autoradiogram showing CAT activities by wild-type Tax or M137. Lanes 1–6, Rat-1 cells were transfected with 1 μg of the indicated reporter and effector plasmids; lanes 7–12, stable transfectants were transfected with 1 μg of the indicated reporter plasmid. Assays were carried out as described in the legends to Figures 2 and 5. The degree of CAT activation is presented as an average fold stimulation from three independent transfections.

amino acid residues originally present throughout each Tax polypeptide. In fact, the same mutation in another *tax* gene independently cloned from a leukemic cell also resulted in a complete loss of *trans*-activation of both the HTLV-I LTR and a κB-dependent promoter (J.Fujisawa, personal communication). The lack of transforming activity in M137 would be explained, at least in part, by the loss of NF-κB activation. Of note, while the HTLV-I LTR-dependent CAT activity achieved by the M22 mutant in a transient assay condition is reported to be 56% of that by wild-type Tax (Smith and Greene, 1990), our M148 and M353VP mutants can fully *trans*-activate the HTLV-I LTR (Figures 2A and 5A), allowing us to conclude that the ability to *trans*-activate the HTLV-I LTR is independent of transformation. Several lines of evidence indicate that the controversial results on cell transformation might be attributed to some differences in the background of cells used, but not to those in the expression systems employed. We obtained virtually identical results for the entire experiments when wild-type Tax or mutants were expressed under the control of the human cytomegalovirus immediate early promoter (data not shown). The failure of M148, M353VP and M137 to transform Rat-1 cells is not due to insufficient expression of the proteins because stable transfectants express equivalent or even greater amounts of Tax compared with 40M-3 cells (Figures 3

and 10B). The biological or biochemical characteristics of polyclonal pools of transfectants further verified the overall activities of each Tax mutant, while, in the previous report, those of several individual cell clones were described. We also demonstrated constitutive transcriptional activation of the target genes by Tax stably expressed in the transfectants (Figure 5). Moreover, wild-type Tax or M319 readily transformed Rat-2 and NRK cells, but M148 and M353VP failed, suggesting a crucial role of NF-κB activation in cell transformation under different cell backgrounds. Thus, it is obvious that constitutive activation of NF-κB is essential for fibroblast transformation by Tax.

Willems *et al.* (1992) previously demonstrated that mutations in the bovine leukemia virus (BLV) Tax protein which abrogate the cognate LTR-dependent *trans*-activation do not interfere with the ability of BLV Tax to transform rat embryo fibroblasts in cooperation with the H-*ras* oncogene. They concluded that the capacity of BLV Tax to *trans*-activate the BLVLTR is independent of cell transformation. Our idea is further supported by the experiments of Kitajima *et al.* (1992) showing that transcriptionally active NF-κB expression may be necessary for maintenance of the Tax-induced malignant phenotype. Inhibition of NF-κB expression by antisense oligonucleotides directed against RelA inhibited the growth of an HTLV-I-transformed lymphocyte line as well as Tax-transformed cells established from fibroblastic tumors developed in *tax*-transgenic mice. Recently, Higgins *et al.* (1993) reported that antisense inhibition of RelA blocks tumorigenicity and causes tumor regression using diverse transformed cell lines. These reports, together with our present observations, strongly support an essential role for nuclear expression of active NF-κB in cell transformation. However, there still remain the following possibilities. First, besides the ability to activate NF-κB, only wild-type Tax and M319 may share by chance some unknown biological activity of Tax essential for transformation. For instance, another cellular transcription factor involved in Tax-mediated *trans*-activation is SRF which binds to a CArG box (Fujii *et al.*, 1988; Norman *et al.*, 1988; Alexandre *et al.*, 1991). Tax is reported to interact with SRF *in vitro* and *in vivo*, and aberrantly activate its transcriptional activity (Fujii *et al.*, 1992). However, the SRF pathway of *trans*-activation appeared not to be essential for Tax transformation of rat fibroblasts because transfection with a SRF-responsive CAT reporter plasmid into Rat-1 transfectants listed in Figure 5 did not cause selective elevation of CAT activity in cells with the transformed phenotype (data not shown). Secondly, like oncoproteins of DNA tumor viruses, transformation may involve interaction or inactivation of certain cellular tumor suppressor gene products. Tax seems not to be physically associated with the retinoblastoma susceptibility gene product or p53 (data not shown). Likewise, wild-type Tax expression did not cause a significant increase in the binding activity of the E2F transcription factor (data not shown). It is not altogether unexpected because HTLV-I is a retrovirus which replicates in the integrated form, whereas DNA tumor viruses share a common requirement for maintenance of the cellular S phase activity for their efficient replication. However, it is still possible that Tax may interact with yet unknown tumor suppressor gene products.

In this regard, it is notable that Tax physically interacts with the NFKB1 precursor and the NFKB2 precursor (Hirai *et al.*, 1992, 1994; Béraud *et al.*, 1994; Lanoix *et al.*, 1994), processing of which may play an important role in NF-κB induction (Baeuerle, 1991). Recent experiments have demonstrated IκB-like functions of the NFKB1 and NFKB2 precursors, which potentially form stable complexes with other Rel/NF-κB family members *in vivo* and keep them in the cytoplasm, thus controlling nuclear translocation of the Rel/NF-κB members (Rice *et al.*, 1992; Mercurio *et al.*, 1993; Naumann *et al.*, 1993a,b). In our immunoprecipitation experiment, we detected a cellular polypeptide with an apparent mol. wt of 110 kDa co-immunoprecipitated with Tax from Rat-1 cells (Figure 3). Immunochemical analyses with specific antibodies further identified this polypeptide to be mostly the NFKB1 precursor, but not the NFKB2 precursor (data not shown). As previously reported by Hirai *et al.* (1992, 1994), the efficiency of co-immunoprecipitation of the NFKB1 precursor with each Tax variant appears to correlate with the degree of *trans*-activation of the κB-responsive promoter (Figures 3 and 5), suggesting that this interaction would be functionally important for NF-κB induction in Rat-1 cells. Recent reports indicate that the interaction of Tax with the NFKB2 precursor inhibits the NF-κB-inducing activity of Tax or leads to the release of transcriptionally active NF-κB from IκB molecules including the NFKB2 precursor, depending on their relative amounts expressed (Béraud *et al.*, 1994; Kanno *et al.*, 1994b; Muñoz *et al.*, 1994; Watanabe *et al.*, 1994; Murakami *et al.*, 1995). Transient transfection experiments have so far demonstrated that the NFKB2 precursor, but not the NFKB1 precursor, effectively suppresses Tax-induced *trans*-activation of Rel/NF-κB-dependent promoters (Béraud *et al.*, 1994; Watanabe *et al.*, 1994; Murakami *et al.*, 1995). This would partly be due to the different processing of these precursors in the presence of Tax. While processing of the NFKB1 precursor is remarkably enhanced by Tax, that of the NFKB2 precursor occurs much more slowly in the presence or absence of Tax (Mercurio *et al.*, 1993; Watanabe *et al.*, 1993, 1994; Lanoix *et al.*, 1994). This could enable the NFKB2 precursor, when overexpressed, to block Tax-induced translocation of free NF-κB released from other IκB molecules. These observations prompted us to render Tax-expressing cells stably overexpressing the NFKB2 precursor in order to abrogate Tax-induced NF-κB activation. Rat-1 cells stably expressing both Tax and the NFKB2 precursor lack constitutive NF-κB activity as well as the transformed phenotype, while the HTLV-I LTR can be fully *trans*-activated by Tax (Figures 7 and 8). Accordingly, the localization of Tax (nuclear versus cytoplasmic) in these cells is not significantly altered (Figure 7B). These results have two important implications. First, if NFKB2 is engaged exclusively in the regulation of the Rel/NF-κB transcription pathways, it can be concluded that constitutive NF-κB activation is essential for transformation of Rat-1 cells by Tax. Secondly, ablation of Tax-induced constitutive NF-κB activity does not necessarily require sequestration or inactivation of Tax itself by the NFKB2 precursor, but could be achieved by the strong

IκB activity of the NFKB2 precursor. Tax expression appears to induce increased expression of endogenous NFKB2 (Figure 7A), but it could not break Tax-mediated NF-κB activation until NFKB2 is ectopically overexpressed. Previous illustrations as to the functional consequences of Tax-NFKB2 interaction have come from transient transfection experiments. Our results from stable cell lines, which may represent more the physiological state of the cells, indicate that the NFKB2 precursor can abrogate Tax-induced NF-κB activation presumably through its IκB activity. Interestingly, stable expression of NFKB2 can also block tumor necrosis factor (TNF)-α-mediated NF-κB activation in Tax-expressing Rat-1 cells (our unpublished data), suggesting that Tax and TNF-α may share, at least in part, common pathways of NF-κB activation. Since this is the first demonstration that NF-κB activation can be abolished by stable expression of an IκB protein, it could be an excellent model to analyze the biological roles of NF-κB.

The fact that persistent activation of NF-κB has rarely been reported so far in other naturally occurring human neoplasms may reflect strict controls of Rel/NF-κB activity by multiple regulatory systems. Indeed, although transient activation of NF-κB can be observed in many cells by treatment with various stimuli (Baeuerle, 1991), nuclear expression of NFKB1 (p50) and RelA increases amounts of endogenous IκB proteins, which in turn sequester both NF-κB subunits in the cytoplasm until cells are specifically induced to translocate them to the nucleus (Brown *et al.*, 1993; Scott *et al.*, 1993; Sun *et al.*, 1993). Consistently, stable overexpression of RelA or its deletion mutant could not lead to transformation of rat fibroblasts (Grimm and Baeuerle, 1994) (data not shown). Therefore, it is noteworthy that stable expression of Tax alone achieves constitutive activation of NF-κB. Recent reports have demonstrated that Tax induces phosphorylation and proteolytic degradation of IκB-α (Kanno *et al.*, 1994a; Lacoste *et al.*, 1995; Sun *et al.*, 1994; Suzuki *et al.*, 1995). It would be quite reasonable that Tax somehow targets all the cytoplasmic IκB proteins so far examined to translocate NF-κB into the nucleus, resulting in constitutive transcriptional activity, although effects of Tax on IκB-β, another IκB isoform recently cloned (Thompson *et al.*, 1995), are currently unknown.

The evidence of an essential role for persistent NF-κB activation in Tax-mediated cell transformation of rat fibroblasts, whose growth is independent of the IL-2/IL-2Rα signaling pathway, raises the concept that NF-κB may govern an unknown important regulatory process involved in normal growth control. Two observations suggesting a role for the Rel/NF-κB family in fibroblast growth control are that NF-κB DNA-binding activity is induced during the G_0 to G_1 transition in mouse fibroblasts, resulting in transcriptional activation of a promoter containing the c-*myc* NF-κB binding site (Baldwin *et al.*, 1991), and that TNF-α initiates mitogenic events in mouse fibroblasts through phorbol 12-myristate 13-acetate-independent pathways (Cornelius *et al.*, 1990). Most recently, Mosialos *et al.* (1995) reported the interaction of latent membrane protein 1 (LMP-1) of Epstein–Barr virus with TNF receptor family-associated proteins, suggesting linkage of signal transduction from the TNF receptor family to LMP-1-mediated transformation, which seems to be closely correlated with NF-κB activation in Rat-1 cells (Huen *et al.*, 1995). Oncogenic potentials have been reported for several Rel/NF-κB family members, including Lyt10, v-Rel and Bcl-3 (Wilhelmsen *et al.*, 1984; Ohno *et al.*, 1990; Neri *et al.*, 1991). Thus, the Rel/NF-κB family of transcription factors may control the expression of a variety of genes not restricted to those for immune function, exerting a crucial influence on cell behavior. The identification of cellular target genes for NF-κB in Tax-mediated transformation would provide a clue to understanding how NF-κB is involved in the control of cell growth.

Materials and methods

Cell culture

All the cells derived from Rat-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 4 mM L-glutamine, penicillin (50 U/ml) and streptomycin (50 μg/ml) at 37°C in a humidified 5% CO_2 atmosphere.

Plasmid constructions and polymerase chain reactions

pHLC-1 and pCMVβ (Clontech) have been described previously (Mori *et al.*, 1987; MacGregor and Caskey, 1989). To construct IgκtkCAT, seven copies of a double-stranded oligonucleotide, used for a probe in EMSA and containing a single κB binding motif of the murine immunoglobulin κ light chain enhancer, were placed between the *Xba*I site of pBLCAT2 (Luckow and Schütz, 1987) and nucleotide −85 of the herpesvirus thymidine kinase (tk) gene promoter (McKnight and Kingsbury, 1982) within pBLCAT2. The coding sequences of wild-type *tax* and *m148* were amplified by PCR as follows. Genomic DNAs prepared either from 40MRatcl-1 cells (Tanaka *et al.*, 1990) or from EA4 cells were subjected to two-step PCR using Gene Amp Kit (Takara, Kyoto) with specific primers, MTax11, MTAX01, MTax1 and MTAX0 as described previously (Sakurai *et al.*, 1992). Each cycle of PCR included 20 s of denaturation at 94°C, 30 s of primer annealing at 52°C and 90 s of extension/synthesis at 72°C. PCR products were separated by agarose gel electrophoresis, purified, and subcloned into the *Hinc*II site of pUC119, generating pUC*wtax* and pUC*m148*. pH2R*wtax* and pH2R*m148* were constructed by transferring *Eco*RI–*Hin*dIII fragments of pUC*wtax* and pUC*m148* containing the PCR product to the unique *Hin*dIII site within the pH2R*neo* vector (Tanaka *et al.*, 1990) in the sense orientation. *m319* was obtained by PCR mutagenesis (Ho *et al.*, 1989) of pUC*wtax* which introduces a unique *Bgl*II site in the *tax* gene, resulting in substitution of the dipeptide arginine–serine for leucine–leucine at codons 319 and 320. *m353vp* was generated by fusing in-frame the entire *tax* coding sequence to a 0.24 kb DNA fragment encoding the acidic activation domain (amino acids 413–490) of the herpes simplex virus VP16 (Sadowski *et al.*, 1988). Two linker-encoded amino acids, arginine–serine, were inserted at the boundary of the fused polypeptides. *m137* was also generated by PCR mutagenesis of pUC*wtax* which introduced a *Nhe*I site in the *tax* gene, resulting in amino acid alteration at codons 137 and 138 from glycine–leucine to alanine–serine. Subsequently, pH2R*m319*, pH2R*m353vp* and pH2R*m137* were constructed in the same way as pH2R*wtax*. Mutations were confirmed by dideoxynucleotide sequencing with Sequenase (United States Biochemical). Another wild-type Tax expression vector, pKCR*b-wtax*, was constructed, as follows. Plasmid pSV2bsr (Izumi *et al.*, 1991) was digested with *Bam*HI, treated with Klenow enzyme and further digested with *Pvu*II. A resultant fragment containing the blasticidin S-resistance gene was cloned into the unique *Pvu*II site of plasmid pKCRH2 (Mishina *et al.*, 1984), generating pKCR*b* vector. To construct pKCR*b-wtax*, a 1.4 kb *Eco*RI fragment from plasmid pH2R*wtax* containing wild-type *tax* cDNA was cloned into an *Eco*RI site of pKCR*b* vector. The correct orientation was determined by restriction enzyme cleavage patterns. Two NFKB2 expression vectors, pC*n*100 or pC*b*100, containing either the G418-resistance gene or the blasticidin S-resistance gene, were constructed in the following way. The cDNA of NFKB2 (Schmid *et al.*, 1991), kindly provided by Dr Inder M.Verma, was subcloned into the *Bam*HI site of pCMV-Neo-Bam vector (Baker *et al.*, 1990), a generous gift of Dr Bert Vogelstein, generating pC*n*100, or subcloned into pCMV-Bsr-Bam vector which was created by replacing the G418-resistance

gene of pCMV-Neo-Bam vector with the blasticidin S-resistance gene from plasmid pSV2bsr, yielding pC*b*100.

Cell transfections, immunoblotting and CAT assays

All the transfections were performed by the calcium phosphate precipitation method (Graham and van der Eb, 1973). Rat-1 cells (1×10^6) were transfected with 20 μg of Tax or NFKB2 expression plasmid DNA and selected for stable expression in the presence of 600 μg of G418 (Sigma)/ml or 5 μg of blasticidin S (Funakoshi, Tokyo, Japan)/ml. Pooled populations of drug-resistant cells were established by collecting >90% of the colonies generated. Cell clones were screened for Tax expression by immunoblotting with an anti-Tax antibody (MI73) as described previously (Mori *et al.*, 1987; Yamaoka *et al.*, 1990), except that cells were lysed with a lysis buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 4 mM EDTA, 1% Nonidet P-40 (NP-40), 0.1% sodium dodecyl sulfate (SDS), 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride (PMSF). Expression of NFKB2 was determined by immunoblotting with an anti-NFKB2 antibody (sc-298) purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). For CAT assays, Rat-1 cells (4×10^5) were transfected with 1 μg of each of the Tax expression vectors, 1 μg of reporter CAT plasmid, 1 μg of pCMVβ DNA and 3 μg of sonicated salmon sperm carrier DNA (ss-DNA). Rat-1-derived stable transfectants (4×10^5) were transfected with 1 μg of reporter CAT plasmid, 1 μg of pCMVβ DNA and 4 μg of ss-DNA. Cells were harvested 48 h after transfection and CAT activity was determined within the linear range of the assay (Gorman *et al.*, 1982) on cell lysates normalized for β-galactosidase activity. CAT activity was quantitated by comparing the total amount of radioactivity in the acetylated form of chloramphenicol with the total amount of radioactivity in both the non-acetylated and acetylated forms of chloramphenicol using a radioanalytic imaging system (Fuji BAS 2000). Assays were performed with lysates obtained by 3–5 independent transfections.

Cell labeling and immunoprecipitation

Cells (~1.5×10^6 cells) were incubated (at 37°C) in medium without methionine and cysteine for 30 min, and labeled for 4 h with 250 μCi of Tran ^{35}S-label (ICN) per plate. The labeled cells were lysed on ice for 15 min in 1 ml of lysis buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 4 mM EDTA, 0.5 mM NaF, 0.5 mM sodium orthovanadate, 1 mM PMSF and 1 μg of leupeptin per ml]. Cellular debris was removed by centrifugation at 12 000 *g* for 5 min. The resulting lysate was pre-cleared with protein A–Sepharose (Pharmacia) coated with normal mouse IgG, and the supernatant was incubated sequentially with the anti-Tax monoclonal antibody (MI73) for 60 min at 4°C and with protein A–Sepharose for 60 min at 4°C. The precipitates were washed three times with lysis buffer, twice with high-salt washing buffer [50 mM Tris–HCl (pH 7.5), 500 mM NaCl and 1% NP-40] and once with low-salt washing buffer [50 mM Tris–HCl (pH 7.5), 50 mM NaCl and 1% NP-40]. After elution of protein in 1× Laemmli sample buffer [50 mM Tris–HCl (pH 6.8), 2% SDS, 0.1% bromophenol blue, 10% glycerol and 5% mercaptoethanol] with heating at 95°C for 5 min, samples were fractionated on a 7.5% SDS–polyacrylamide gel and the gel was processed by fluorography and visualized by autoradiography.

Extract preparation and electrophoretic mobility shift assay

Nuclear extracts were prepared from exponentially growing cells as described previously (Schreiber *et al.*, 1989). Protein concentrations were determined with the BCA protein assay (Pierce) so that each binding reaction was conducted with an equivalent amount (~10 μg) of protein. The wild-type κB probe used was a double-stranded oligonucleotide, 5′-AGCTTCAACAGAGGGGACTTTCCGAGAGGCTCGA-3′, containing a single κB motif derived from the murine immunoglobulin κ light chain enhancer (Kitajima *et al.*, 1992). The mutated κB probe was a double-stranded oligonucleotide, 5′-AGCTTCAACAGAGGCG-ACTTTCCGAGAGGCTCGA-3′, containing a single base substitution of C for G at the underlined site. The ^{32}P-radiolabeled probe was used at 2×10^4 c.p.m./reaction. Extracts were pre-incubated for 20 min on ice in buffer containing 10 mM Tris–HCl (pH 7.4), 50 mM NaCl, 0.5 mM $MgCl_2$, 1 mM EDTA, 0.1 mg bovine serum albumin/ml, 1 mM dithiothreitol, 4% glycerol and 2 μg of poly(dI-dC). Reactions were incubated with the ^{32}P-labeled probe for 30 min at 37°C and then loaded onto 5% non-denaturing polyacrylamide gels and electrophoresed in 0.5× Tris–borate–EDTA buffer. For supershift assays, extracts were pre-incubated with 1 μl of subunit-specific antibody on ice, 20 min prior to the addition of the radiolabeled probe. Anti-NFKB1 (p50) antibody (sc-114X), anti-RelA antibody (sc-109X), anti-NFKB2 (p52) antibody (sc-298X) and anti-c-Rel antibody (sc-70X) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Cell fractionation

Cell fractionation was performed as described previously (Schreiber *et al.*, 1989). Briefly, ~1×10^7 cells were suspended in 400 μl of buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol and 0.5 mM PMSF]. Cells were allowed to swell on ice for 15 min, after which 25 μl of a 10% solution of NP-40 were added. After vortex mixing for 15 s, the lysates were cleared of nuclei by centrifugation at 12 000 *g* for 90 s at 4°C and the supernatant was used as a cytoplasmic fraction. The pelleted nuclei were washed with buffer A, resuspended in 100 μl of buffer C [20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol and 1 mM PMSF], vortexed vigorously and agitated at 4°C for 30 min. Debris was removed by centrifugation at 12 000 *g* for 5 min and the supernatant was used as a nuclear fraction. Equal cell equivalents of nuclear and cytoplasmic fractions were subjected to immunoblot analysis.

Assays of colony formation in soft agar and tumorigenicity in nude mice

Analysis for anchorage-independent cell growth was performed as described previously (Tanaka *et al.*, 1990) by overlaying a single-cell suspension of ~1×10^4 cells in 1.5 ml of 0.33% low-melting-point agarose (Sea Plaque) onto a 60 mm tissue culture dish containing a 0.5% agarose base. All agarose media were made with DMEM supplemented with 10% FBS. Duplicate plates were prepared for each tested cell line and inspected for colony formation after incubation at 37°C for 14 days. Colonies >60 μm in size were scored positive. The tumorigenic potential was evaluated as described previously (Tanaka *et al.*, 1990) by injection of cells into 4- to 6-week-old Balb/c athymic *nude* mice. Cells (5×10^6) suspended in 0.1 ml of sterile phosphate-buffered saline were inoculated s.c. into mice. Animals were monitored at regular intervals for tumor formation over a period of 6 weeks and were scored positive when the tumor diameter exceeded 8 mm.

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After submitting this paper, Smith and Greene (1995) (*Genes Dev.*, **9**, 2324) corrected an error pertaining to the genotype of their Tax M22 mutant.