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Human T-Cell Leukemia Virus Type 1 Tax Protein Transforms Rat Fibroblasts via Two Distinct Pathways

KAYOKO MATSUMOTO,[1]* HIROTOSHI SHIBATA,[1] JUN-ICHI FUJISAWA,[2] HIROKAZU INOUE,[3] AKIRA HAKURA,[3] TOMONORI TSUKAHARA,[4] AND MASAHIRO FUJII[4]

Osaka Red Cross Blood Center, Morinomiya 2-4-43, Johtoh-ku, Osaka 536,[1] Department of Microbiology, Kansai Medical University, Moriguchi, Osaka 570,[2] Department of Tumor Virology, Research Institute for Microbial Diseases, Osaka University, Yamadaoka 3-1, Suita, Osaka 565,[3] and Department of Immunotherapeutics, Medical Research Division, Tokyo Medical and Dental University, Yushima, Bunkyo-ku, Tokyo 113,[4] Japan

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The human T-cell leukemia virus type 1 (HTLV-1) Tax protein activates the transcription of several cellular genes. This function is thought to play a critical role in the Tax-dependent transformation step in HTLV-1 leukemogenesis. Tax activates transcription via three enhancers: the cyclic AMP response element (CRE)-like sequence, the κB element, and the CArG box. Their involvement in the transformation of rat fibroblasts by Tax was examined by colony formation of Rat-1 cells in soft agar and Ras cooperative focus formation of rat embryo fibroblasts (REF). Among Tax mutants, those retaining activity for the CArG box transformed REF like wild-type Tax, while those inactive for the CArG box did not. Thus, the activation of the CArG box pathway is essential for the transformation of REF by Tax. In contrast, activation of the κB element correlated with the transformation of Rat-1 by Tax. These results show that Tax transforms rat fibroblasts via two distinct pathways.

Human T-cell leukemia virus type 1 (HTLV-1) is the etiological agent of adult T-cell leukemia (19, 35, 55). The viral protein Tax has been proposed to play a critical role in leukemogenesis because of its transforming activity in various experimental systems. Established rodent fibroblast cell lines transfected with *tax* form colonies in soft agar and induce tumors in nude mice (41, 49). Tax also transforms primary fibroblasts (rat embryo fibroblasts [REF]) together with activated Ras protein (36). Transgenic mice carrying the *tax* gene develop fibrosarcoma (34). In addition, Tax immortalizes primary T cells in the presence of interleukin 2 (IL-2) (1, 17).

Tax was originally identified as a factor which activates transcription from the HTLV-1 long terminal repeat (15, 43). Tax also activates a number of cellular genes, including proto-oncogenes (c-*myc*, c-*jun*, c-*fos*), immediate early genes (*egr-1*, *egr-2*), and genes encoding growth factors (IL-2, IL-6, transforming growth factor β, gp34 [OX ligand]) and their receptors (α chain of IL-2 receptor, OX40) (8, 10–12, 21, 25, 28, 30, 33, 39, 53).

Analysis of the promoter region of Tax-inducible genes identified at least three different enhancers activated by Tax: the cyclic AMP response element (CRE)-like sequence, the κB element, and the CArG box. Tax activation of IL-2 receptor α chain and IL-6 is mediated by the κB element (4, 26, 53), while Tax activation of c-*fos*, *egr-1*, and *egr-2* is mediated by the CArG box (2, 3, 14). CRE mediates the Tax-dependent activation of the HTLV-1 long terminal repeat, as well as that of some cellular genes, such as *fra-1* (6, 16, 38, 50).

Tax does not directly bind to any of these three enhancer sequences but, rather, interacts with cellular transcription factors. CREB/CREM is bound to CRE, p65/c-Rel on the κB element, and SRF on the CArG box. Tax activates transcription via binding with these transcription factors (14, 44, 46, 56). The κB element is also activated in another manner by Tax. By interacting with its inhibitors (p100, p105, IκBα, or IκBγ), Tax releases NF-κB/rel, which then activates transcription via the κB element (5, 20, 22, 23, 47, 51). Recent evidence showed that Tax facilitates phosphorylation and degradation of IκBα and IκBβ (7, 24).

Activation of cellular genes is thought to be essential for Tax-mediated cell transformation. However, the respective roles of the three Tax-inducible pathways in cell transformation remain to be clarified. Here, we show that two of the three Tax-inducible pathways mediate the respective transformation of two different rat fibroblasts.

MATERIALS AND METHODS

Cell culture. Primary REF were prepared from 16-day-old embryos and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). The established rat cell lines Rat-1 and Rat-2 were cultured in DMEM supplemented with 5% FCS.

Plasmids. Wild-type *tax* and *tax* mutant genes were cloned into pHβPr.1-neo, which has a β-actin promoter for protein expression as well as neomycin resistance for G418 selection (19). The Tax mutants TaxM22, TaxSH-1, TaxSH-2, Tax703, and TaxΔ3 have been previously characterized (29, 40, 45). Tax410 has the same amino acid substitution as Tax mutant M44 (40). Substituted amino acids are as follows (positions in parentheses): Thr(130)Leu-AlaSer for TaxM22, Pro(58)-Ser for TaxSH-1, Leu(205)-Ala for TaxSH-2, Leu(310)Leu-AlaSer for Tax703, and Glu(319)Glu-AlaSer for Tax410. TaxΔ3 has three N-terminal amino acid deletions. p21x6-CAT, pκBx4-CAT, and pCArG-CAT are chloramphenicol acetyltransferase (CAT) expression plasmids regulated by a hexamer of the 21-bp viral enhancer (CRE), a tetramer of the κB element, and a monomer of the CArG box from the c-*fos* gene, respectively.

Transactivation assay. Cells were seeded at 5×10^5/60-mm-diameter plate in DMEM supplemented with FCS (10% FCS for REF and 5% FCS for cell lines) and incubated overnight. They were then transfected with the Tax expression plasmids and the CAT reporter plasmids by the calcium phosphate method. After 7 h of incubation with the precipitates, the incubation medium was changed. Each culture was harvested 48 h later. A CAT assay was carried out by diffusion of reaction products into scintillation fluid (37). The averages of three independent experiments are presented.

Gel mobility shift assay. Rat-1 cells (10^7) were washed with phosphate-buffered saline and were then treated with 0.2% Nonidet P-40 in lysis buffer (20 mM

* Corresponding author. Mailing address: Osaka Red Cross Blood Center, Morinomiya 2-4-43, Johtoh-ku, Osaka 536, Japan. Phone: (6)962-8649. Fax: (6)962-7652.

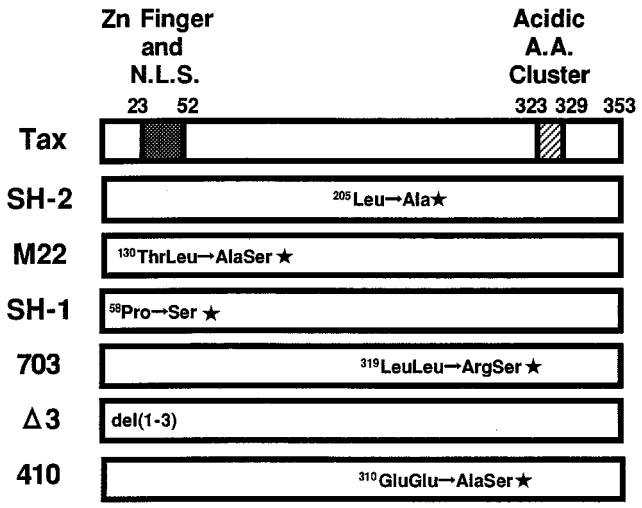


FIG. 1. Schematic illustration of wild-type Tax and its mutants. The three known domains of the Tax protein are shown: Zn finger, nuclear localization signal (NLS) (42), and acidic amino acid cluster (13).

HEPES [pH 7.9], 20 mM NaF, 1 mM Na_3VO_4, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg of leupeptin per ml, 1 μg of aprotinin per ml). After centrifugation, the pellets were further treated with lysis buffer supplemented with 420 mM NaCl and 20% glycerol for 4°C for 30 min, and centrifugation was repeated. The resulting supernatant was used as the nuclear extract in a gel mobility shift assay. A 10-μg amount of the nuclear extract was preincubated with 2 μg of poly(dI-dC) in 20 μl of binding buffer consisting of 13 mM HEPES (pH 7.9), 65 mM NaCl, 0.15 mM EDTA, 8% glycerol, and 1 mM dithiothreitol for 15 min on ice. Approximately 1 ng of labeled oligonucleotide was added to the reaction mixture, and the mixture was further incubated for 30 min at 25°C. Complexes formed were separated from the unbound probe by electrophoresis in a 4% polyacrylamide gel containing 0.5× Tris-borate-EDTA and 2.5% glycerol and dried. The gel was then exposed to X-ray film. A double-stranded synthetic oligonucleotide corresponding to the NF-κB binding site (top strand: AGCTTTGGGAAATTCCTCGGGTGGTAC) from the gene coding for interferon was labeled with [γ-^{32}P]ATP by polynucleotide kinase and employed as the κB site probe.

Colony formation in soft agar (CFSA) assay. Rat-1 or Rat-2 cells were seeded at 5×10^5/60-mm-diameter plate in DMEM supplemented with 5% FCS and incubated overnight. Cells were then transfected with each Tax expression plasmid (5 μg) as described above. After 48 h of incubation, the cells were transferred to two 100-mm-diameter plates and incubated in G418 selection medium for 2 weeks. Drug-resistant colonies (more than 2,000) were pooled, and medium containing 0.4% Noble agar was inoculated with 10^4 cells. Data are expressed as percentages of colonies more than 0.25 mm in diameter 3 weeks after plating.

Ras cooperative focus formation (RCFF) assay. REF were seeded at 5×10^5/60-mm-diameter plate in DMEM supplemented with 10% FCS and incubated overnight. They were then transfected with 7.5 μg of the indicated Tax expression plasmid and 7.5 μg of pEJRas (the expression plasmid of an activated Ras oncoprotein) by the calcium phosphate method. After 7 h, the cells were washed, and 48 h later, each culture was transferred to two 100-mm-diameter plates and incubated in DMEM supplemented with 2.5% FCS. The cell medium was changed every 7 days, and focus formation was scored 3 weeks after transfection.

RESULTS

Transactivation activities of Tax mutants. To examine the transactivation activities of Tax mutants (Fig. 1), REF were transfected with CAT reporter plasmids with an enhancer (CRE, the κB element, or the CArG box) together with Tax expression plasmids. As shown in Fig. 2, a Tax mutant, TaxSH-2, stimulated the CAT activities of all three reporters. This activity was equivalent to that of wild-type Tax. TaxM22 effectively activated CRE and the CArG box but not the κB element. In contrast, TaxΔ3 activated only the κB element. TaxSH-1 and Tax703 activated CRE and the κB element but did not affect the CArG box. Tax410 retained activity only for CRE. The level of this activity was one-third that of the wild-type Tax.

The transactivation phenotypes of Tax mutants in Rat-1 cells were similar to those in REF, but some differences were observed. The activity of the Tax mutants for the κB element in Rat-1 cells were reproducible, and their phenotypes were the same as those in REF. However, the extent of their activities in Rat-1 cells was less than in REF. TaxSH-1, inactive for the CArG box in REF, weakly activated this enhancer in Rat-1 cells. Tax703 activated CRE to a lesser extent in Rat-1 cells

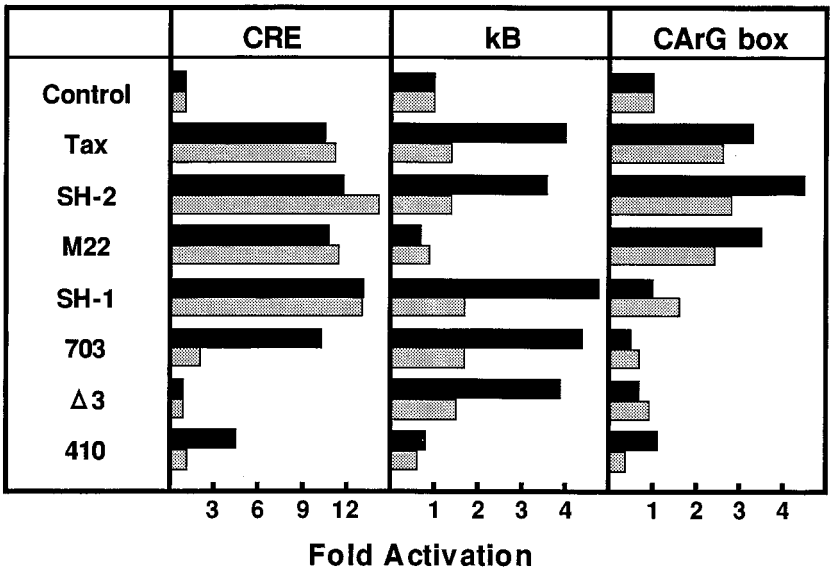


FIG. 2. Transactivation phenotype of Tax mutants. REF (solid bars) and Rat-1 cells (dotted bars) were transfected with reporter CAT plasmids with the CRE (5 μg), the κB element (5 μg), or the CArG box (11 μg) together with expression plasmids encoding Tax (1 μg) or its indicated mutants (1 μg). The CAT activity in the harvested cells was measured as described in Materials and Methods. Fold activation shows the ratio of CAT activity in the cells cotransfected with the expression plasmid encoding Tax or its mutant with respect to that of cells cotransfected with the vector plasmid.

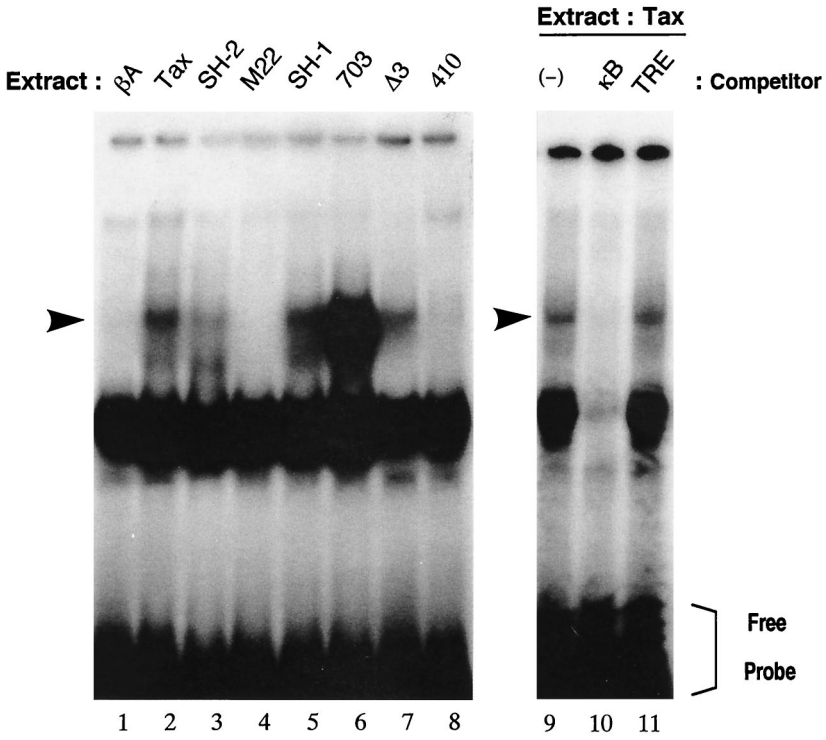


FIG. 3. The NF-κB DNA-binding activity in Rat-1 cells transfected with *tax* and *tax* mutant plasmids. Nuclear extract was prepared from Rat-1 cells transfected with the indicated *tax* mutant plasmids and incubated with a ^{32}P-labeled κB site probe in the absence (lanes 1 to 9) or presence (lanes 10 and 11) of homologous oligonucleotides or an unrelated one (TRE of the *collagenase* gene). The formed complex was separated on a 4% acrylamide gel. The specific complex is indicated (arrowhead).

than in REF, while Tax410 displayed little activity in Rat-1 cells (Fig. 2). The findings obtained with Rat-2 or 3Y1 cells were almost identical to those obtained with Rat-1 cells (data not shown).

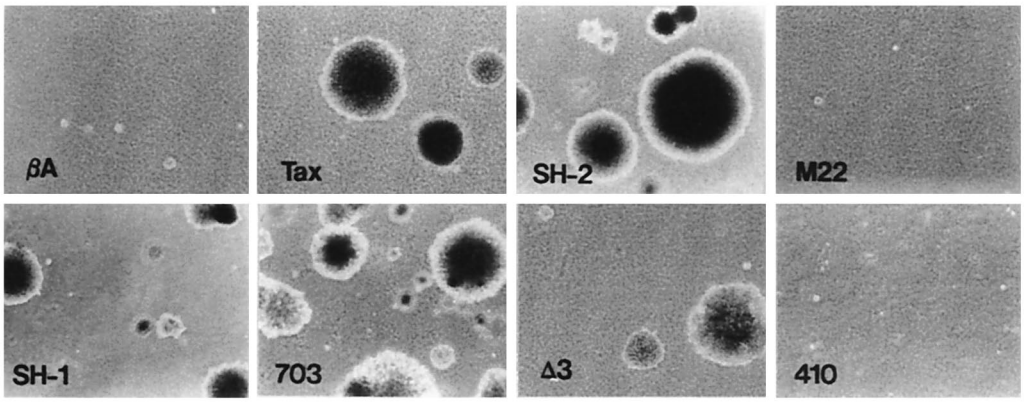
NF-κB DNA-binding activity. Tax induces NF-κB DNA-binding activity (activity of binding of cellular factors to the κB element) in T-cell lines. To examine the effect of Tax on NF-κB DNA-binding activity in Rat-1 cells, Rat-1 cells were transfected with each Tax expression plasmid characterized above and selected with G418. The drug-resistant colonies were pooled and examined for NF-κB activity. A gel mobility shift assay using the nuclear extract prepared from the Rat-1 transfectants indicated that all the Tax mutants active for the κB element in the CAT assay (Tax, TaxSH-2, TaxSH-1, Tax703, and TaxΔ3) showed nuclear NF-κB activity in Rat-1 cells. In contrast, Rat-1 cells transfected with two mutants inactive for the κB element (TaxM22 and Tax410) slightly expressed the NF-κB activity, like Rat-1 cells with the control plasmid. Tax703 induced the NF-κB activity in Rat-1 cells more than the other mutants active for the κB element. This may be due to the greater expression of the Tax703 protein in Rat-1 cells (data not shown). The binding specificity was confirmed by the selective inhibition of the complex by the homologous κB oligonucleotide but not by an unrelated one (tetradecanoyl phorbol acetate-responsive element [TRE] of the gene coding for collagenase) (Fig. 3). Thus, the NF-κB DNA-binding activity in Rat-1 cells transfected with the Tax mutant plasmids correlated with those obtained by a transactivation assay (Fig. 2).

CFSA in rat fibroblast cell lines. The transforming activities of Tax mutants were examined in immortalized cell lines. Rat-1 cells were transfected with each Tax expression plasmid characterized above, and colonies were selected with G418. The drug-resistant colonies were pooled, and cells were inoculated into soft agar for anchorage-independent growth assay. Rat-1 cells transfected with a vector plasmid produced small colonies (less than 0.4 mm in diameter) with very low frequency. Like wild-type Tax, TaxSH-2, which activated all three enhancers in Rat-1 cells, formed large colonies with high frequency (Fig. 4A and B). TaxΔ3, which activated only the κB element, also formed large colonies with high frequency. In contrast, TaxM22, which activated CRE and the CArG box but not the κB element, did not produce any large colonies. Two other mutants, TaxSH-1 and Tax703, both of which activated the κB element, showed transformation activity equivalent to that of wild-type Tax. These findings indicate that activation via the κB pathway is essential for Rat-1 transformation by Tax.

To further confirm the involvement of the κB pathway in the transformation of immortalized cells by Tax, the CFSA activity of the Tax mutants used above was analyzed in another rat fibroblast cell line, Rat-2. TaxΔ3, which activated only the κB element, showed high CFSA activity, whereas TaxM22, inactive only for the κB element, showed none (Fig. 4C). Thus, the indispensable role of the κB pathway in the transformation by Tax was confirmed in two rat cell lines.

RCFF activities of Tax mutants in REF. Next, we examined the transforming activities of Tax mutants in primary cells. Expression of activated H-*ras* alone or *tax* alone in REF produced fewer than three foci (data not shown), whereas their coexpression produced more than 100. Thus, coexpression of *tax* with *ras* transforms REF. As shown in Table 1, RCFF ability was observed in two Tax mutants, TaxSH-2 and TaxM22. The former activated all three pathways, while the latter activated the CRE and the CArG box but not the κB

A



B

	Colony Formation in Soft Agar [%]
βA	0.1
Tax	14.6
SH-2	12.2
M22	0
SH-1	10.6
703	16.1
Δ3	15.5
410	0

C

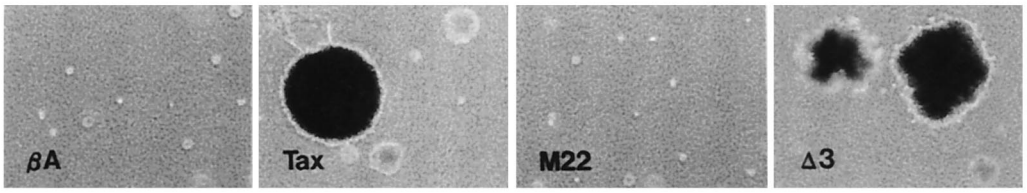


FIG. 4. CFSA activity of Tax or its mutant proteins. Rat-1 (A and B) and Rat-2 (C) cells were transfected with expression plasmids encoding Tax or the indicated Tax mutants and selected in medium containing G418. Pools of resistant cells (10^4) were inoculated in medium containing 0.4% Noble agar for 3 weeks. Values indicate percentages of colonies more than 0.25 mm in diameter. βA indicates a Rat-1 transformant with a vector plasmid without the Tax coding region.

element. Therefore, activation of the κB pathway by Tax is not essential to RCFF activity. In contrast to these two mutants, the other four mutants inactive for the CArG box did not show RCFF activity. Thus, the activation of the CArG box pathway by Tax is essential for RCFF activity. Activation of the CRE pathway alone was not sufficient to induce RCFF activity, since TaxSH-1 and Tax703, which activate CRE, did not show RCFF activity. The present findings do not exclude the possibility that both the CRE and the CArG box pathways are required for the RCFF, since all the Tax mutants active for the CArG box retained activity for the CRE. The inability of some Tax mutants to transform Rat-1 and REF was not due to inefficient protein expression in cells, since the levels of expression of mutant and active genes were almost equal in the two cell types (Fig. 5).

DISCUSSION

Using Tax mutants to segregate the three Tax-inducible pathways, we showed that activation of the κB element and the

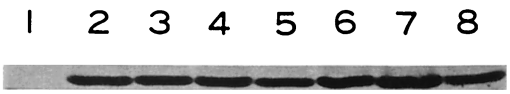


FIG. 5. Expression of Tax and Tax mutant proteins in REF. Cell lysates were prepared from REF transfected with vectors encoding vector plasmid (βA) (lane 1) or with expression vectors encoding wild-type Tax (lane 2) or its mutants: TaxSH-2 (lane 3), TaxM22 (lane 4), TaxSH-1 (lane 5), Tax703 (lane 6), TaxΔ3 (lane 7), and Tax410 (lane 8). These lysates were resolved by electrophoresis on sodium dodecyl sulfate–12% polyacrylamide gels and transferred to a nitrocellulose sheet (Bio-Rad). Tax proteins in the sheet were detected by enzyme-linked immunosorbent assay with anti-Tax antibody (a high-titer anti-Tax human serum).

TABLE 1. RCFF activity of Tax mutants in REF

Tax mutant	RCFF (no. of foci)
βA	2
Tax	113
SH-2	119
M22	70
SH-1	3
703	0
Δ3	3
410	0

CArG box by Tax correlated with transformation of Rat-1 cells and REF, respectively (Fig. 6). Thus, there are at least two distinct pathways by which Tax mediates transformation in rat fibroblasts.

The mechanism underlying the different requirements for transformation by Tax of Rat-1 cells and REF remains unclear. Rat-1 is an immortalized cell line, while REF are primary cells. Thus, the dispensability of CArG box activation to the transformation of Rat-1 may be caused by a cellular change(s) accompanying immortalization. On the other hand, the dispensability of the NF-κB pathway to the transformation of REF may be due to coexpression of activated Ras protein, since Ras can activate the NF-κB pathway.

In contrast to our findings, Smith and Greene (41) previously reported that transcriptional activation through the CRE, but not the κB, pathway plays an essential role in Tax-mediated transformation of Rat-2. In addition to Rat-1 and Rat-2 cells, we observed that transcriptional activation through the κB pathway plays an important role in the abnormal cell growth (focuslike phenotype) of another rat fibroblast cell line, 3Y1 (data not shown). Yamaoka et al. (52) also demonstrated that the κB, but not the CRE, pathway is essential and sufficient for the transformation of Rat-1 cells. Thus, we believe that activation of the κB pathway is generally essential for the transformation of rat cell lines by Tax.

Although the cellular genes responsible for the transformation of REF by Tax remain to be determined, they are activated by Tax via the CArG box (Fig. 3). A number of immediate early genes are induced by mitogenic signals via the CArG box, including *egr-1*, *egr-2*, and the proto-oncogene c-*fos* (2, 3, 14). Thus, their products may mediate the transformation of REF by Tax.

The present findings suggest that the cellular genes responsible for the transformation of Rat-1 by Tax are different from those of REF and are regulated by the κB pathway. Tax reportedly activates the transcription of a number of cellular genes via this pathway, including a c-*myc* proto-oncogene and cytokine genes (10, 32, 53). Moreover, Tax can interact with $p16^{INK}$, an inhibitor of cyclin-dependent kinases (CDKs) (CDK4 and CDK6), by using the domain overlapping with that involved in the NF-κB activation (48). Thus, Tax may stimulate cell growth via these CDKs without transcriptional activation. Further analysis is required to determine the respective roles of the activation of NF-κB and of CDKs in cell transformation by Tax.

Tax immortalizes T cells in the presence of IL-2 (1, 17), and these immortalized cells constitutively express the IL-2 receptor. Thus, the κB pathway regulating IL-2 receptor is likely necessary for this transformation event (55). The roles of the other two pathways in T-cell immortalization by Tax, however, have yet to be determined. The activation of the proto-oncogene c-*fos* via the CArG box by IL-2 is suggested to play an essential role in the growth-stimulatory function of IL-2 (31). Therefore, the CArG box pathway may enhance or even bypass the IL-2-dependent signaling in the Tax-induced immortalization process in T cells. Thus, these two pathways activated by Tax may play two distinct roles in the transformation not only of rat fibroblasts, but also of T cells.

Many transforming viruses have two transforming proteins,

	Rat-1				REF			
	CFSA	CRE	kB	CArG	RCFF	CRE	kB	CArG
Tax	+	+	+	+	+	+	+	+
SH-2	+	+	+	+	+	+	+	+
M22	-	+	-	+	+	+	-	+
SH-1	+	+	+	+*2	-	+	+	-
703	+	-*1	+	-	-	+	+	-
Δ3	+	-	+	-	-	-	+	-
410	-	-	-	-	-	+*3	-	-

FIG. 6. Summary of transactivation and transformation activities of Tax mutants examined in Fig. 2 and 3 and Table 1. *1, 17% activity relative to that of wild-type Tax in Rat-1 cells; *2, 60% activity in Rat-1 cells; *3, 43% activity in REF.

such as E1A and E1B of adenovirus and E6 and E7 of papillomavirus. In addition, large T antigens of simian virus 40 can interact with two tumor suppressor proteins, p53 and the retinoblastoma protein, and the interaction plays a role in the transformation of cells (9, 27). Thus, the activation of multiple pathways associated with transformation may be a common strategy of these viruses for replication in vivo. Therefore, analysis of the role of each of the Tax-inducible pathways in transformation may facilitate understanding of the replicative strategy of the transforming viruses in vivo.

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