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Immunology

# Stable expression of the *tax* gene of type I human T-cell leukemia virus in human T cells activates specific cellular genes involved in growth

(interleukin 2/interleukin 2 receptor/granulocyte/macrophage colony-stimulating factor)

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**ABSTRACT** **Stable expression of the 40-kDa transactivator protein (Tax) from the type I human T-cell leukemia virus (HTLV-I) in Jurkat T cells leads to the activation and sustained expression of certain cellular genes that are transiently induced during normal T-cell growth. Cellular genes induced by Tax include those encoding the α subunit of the high-affinity interleukin 2 receptor (Tac), interleukin 2, and granulocyte/macrophage colony-stimulating factor. Tax induction of the interleukin 2 gene is synergistically amplified by mitogens that augment cytoplasmic levels of calcium. These changes in the pattern of cellular gene expression reflect a specific action of Tax, as they are undetectable in isogenically matched control cell lines expressing antisense *tax* cDNA. The spectrum of cellular genes regulated by Tax appears to be restricted: several other T-cell genes, either inducibly or constitutively expressed, are unaffected by this viral protein. These cell lines constitutively expressing Tax provide valuable reagents to explore the molecular basis for Tax action and to delineate the full spectrum of cellular genes regulated by this retroviral gene product.**

Human T-cell leukemia virus type I (HTLV-I) has been strongly implicated as the cause of adult T-cell leukemia (ATL) (1, 2). This aggressive, and often fatal, leukemia occurs in areas of the world where HTLV-I infection is endemic (3, 4). Furthermore, clonal patterns of HTLV-I provirus integration have been detected in the leukemic cells of most ATL patients studied (5). While this virus is able to immortalize human T cells *in vitro* (6, 7), it lacks a classical oncogene (8) and does not insert at specific sites within the host genome (9). HTLV-I-infected leukemic T-cell lines are phenotypically distinguished by their constitutive display of large numbers of interleukin 2 (IL-2) receptors (10–12) and the spontaneous secretion of many lymphokines characteristically produced by activated T cells (57).

Sequence analysis of HTLV-I (8) has revealed that its genome, like those of other retroviruses, is composed of tandem long terminal repeats (LTRs) and *gag*, *pol*, and *env* genes, but HTLV-I contains, in addition, a unique 3′ region, *pX*, that encodes at least three nonstructural proteins. A double splicing event involving one of these open reading frames (*tax*) generates a subgenomic mRNA encoding a 40-kDa protein, Tax (transactivator from the *pX* region, previously called tat-1, $p40^x$, and x-lor) (13–16). The Tax protein acts in trans through the 21-base-pair enhancer elements located in the HTLV-I LTR (17–19), producing a marked increase in viral gene transcription (20). Tax may also be capable of altering the expression of certain cellular genes perhaps centrally involved in HTLV-I-induced transformation (21–23). Particularly attractive cellular targets include the genes encoding the IL-2 receptor and IL-2, as the expression of these proteins regulates normal T-cell growth (24, 25). In this regard, Arima *et al.* (26) demonstrated autocrine proliferation in primary ATL tumor cells mediated by IL-2 and IL-2 receptors. Furthermore, transient cotransfections of *tax* expression vectors with reporter genes linked to IL-2 receptor α-chain (IL-2Rα) and IL-2 promoters in Jurkat T cells showed that Tax can markedly augment reporter gene activity (27–30). Transient expression of *tax* cDNA in Jurkat and HSB-2 T cells also induced small changes in the expression of the endogenous IL-2Rα and IL-2 genes (31). However, the low transfection efficiencies and transient nature of *tax* expression intrinsic to these experiments precluded firm conclusions regarding persistent effects of Tax on endogenous cellular gene expression. We now report the preparation of isogenically matched Jurkat T-cell lines stably transfected with either sense or antisense *tax* cDNA expression plasmids that are uniquely suited for such analyses. We have used these cell lines to demonstrate that the constitutive production of a functional *tax* gene product is associated with the induction and sustained expression of the cellular genes encoding IL-2Rα, IL-2, and granulocyte/macrophage colony-stimulating factor (GM-CSF).

## MATERIALS AND METHODS

**Plasmids.** The *tax* cDNA expression vector spXHF/82-C was used to prepare Jurkat T-cell lines constitutively producing the HTLV-I Tax protein. This plasmid contains a full-length *pX*-derived cDNA, encoding both Tax and the 27-kDa Rex protein, inserted downstream of the HTLV-I LTR. Thus, theoretically, high-level Tax production may be achieved by this vector as a result of "autotransactivation." Site-directed mutations within *tax*, but not *rex*, block activation of the IL-2Rα and IL-2 promoters in Jurkat T cells, indicating that Tax is the biologically active protein in this response. This plasmid also contains the neomycin-resistance gene promoted by the early region of simian virus 40 (SV40), thereby permitting G418 antibiotic selection of stable transfectants. The SV40-promoter–neomycin-resistance cassette was inserted in the same 5′ → 3′ orientation as the *tax* cDNA. This configuration appeared important, as multiple transfections with plasmids containing these elements in opposite polarity failed to yield stable *tax*-expressing transformants. This finding may reflect an antisense-mRNA arrest of the translation of the phosphotransferase mRNA respon-

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Abbreviations: ATL, adult T-cell leukemia; CAT, chloramphenicol acetyltransferase; GM-CSF, granulocyte/macrophage colony-stimulating factor; HTLV-I human T-cell leukemia virus type I; IL-*n*, interleukin *n*; IL-2Rα, IL-2 receptor α chain; LTR, long terminal repeat; PHA, phytohemagglutinin; PMA, phorbol 12-myristate 13-acetate; TCR, T-cell antigen receptor.

sible for drug resistance mediated by the *tax* mRNA (Y.W. and W.C.G., unpublished observations). To prepare isogenically matched control cell lines, Jurkat cells were also transfected with the spXHF/82-NC plasmid, which contains the same *tax* cDNA inserted in the opposite (noncoding) orientation.

**Preparation and Analysis of Jurkat T-Cell Lines Stably Producing Tax Protein.** The spXHF/82-C and spXHF/82-NC plasmids were introduced into Jurkat T cells by electroporation (32). In brief, $10^7$ cells were suspended in 300 μl of RPMI 1640 containing 10% fetal bovine serum (complete medium) at 4°C. Addition of 10 μg of linearized plasmid (*Bgl* II restriction) was followed by electroporation (250 mV, 960 μF). The cells were cultured for 2 days in complete medium and then seeded in individual microtiter wells ($10^4$ cells per well) and cultured for 21 days in the presence of complete medium supplemented with antibiotic G418 (800 μg/ml). Viable cells from multiple wells were recovered and cloned by limiting dilution. Multiple clones were then screened for *tax* mRNA by cytoplasmic RNA dot blotting (33). Four Jurkat *tax* clones (J-*tax*-2, -8, -9, and -19) and two anti-*tax* Jurkat cell lines (J-anti-*tax*-2 and -10) were selected for further study.

DNA and RNA isolated from these cell lines were analyzed by Southern and Northern blotting as described (34). DNA probes were $^{32}$P-labeled by the random priming method (35). Transient transfection of these cells with the HTLV-I LTR-CAT and RSV-CAT plasmids and measurement of chloramphenicol acetyltransferase (CAT) activity were performed as described (29). IL-2 production was measured by using IL-2-responsive murine CTLL cells (36).

## RESULTS

Southern blot analyses of *Bgl* II-digested DNA from each of the Jurkat *tax* and anti-*tax* cell lines confirmed the presence of expression-vector DNA apparently integrated at one or more unique sites (Fig. 1 *Left*). The less intense hybridization signals obtained with the J-*tax*-2 and J-*tax*-8 samples reflect the presence of less DNA in these lanes as assessed by ethidium bromide staining. Hybridization of *Eco*RI-digested genomic DNA from the *tax* and anti-*tax* cell lines and parental Jurkat cells with a T-cell antigen-receptor (TCR) β-chain probe (37) revealed identical patterns of TCR gene rearrangement in these cells (Fig. 1 *Right*). These results, coupled with equivalent reactivity of the cell lines with the clonotypic MX-6 monoclonal antibody specific for the Jurkat αβ TCR (data not shown), confirmed the identical genetic background of these experimental and control cell lines.

These isogenically matched *tax* and anti-*tax* cell lines were first evaluated for production of functional Tax by transfection of plasmids containing the HTLV-I LTR linked to the CAT reporter gene. The HTLV-I LTR was markedly transactivated in each of the four *tax* cell lines but not in either the parental Jurkat T cells or the two anti-*tax* cell lines (Fig. 2). With serial passage in culture over a period of 9 months, the J-*tax*-9 and -19 cell lines emerged as the highest Tax producers.

Potential alterations in cellular gene expression associated with the production of functional Tax were next studied by Northern blotting of poly(A)$^+$ mRNA isolated from the *tax* and anti-*tax* cell lines and parental Jurkat T cells (Fig. 3). For comparison, RNAs from phytohemagglutinin (PHA)-activated peripheral blood lymphocytes and two HTLV-I-infected cell lines (MT-1 and HUT 102B2) were analyzed in parallel. Constitutive expression of the 25S and 16S forms of IL-2Rα (Tac antigen) mRNA (20) was detected in each of the four *tax* cell lines, but not in either of the anti-*tax* cell lines. The most marked changes in IL-2Rα gene expression were present in the J-*tax*-9 and -19 cell lines, which, at the time of study, produced the greatest amounts of Tax. Considerably more IL-2Rα mRNA was present in the PHA-activated T cells, MT-1 cells, and HUT 102B2 cells. This quantitative difference may reflect intrinsic properties of the Jurkat T-cell line, as PHA and phorbol 12-myristate 13-acetate (PMA) stimulation of these cells consistently leads to at least a factor of 10 fewer IL-2 receptors than present on identically activated normal T lymphoblasts (38).

Potential Tax-induced changes in IL-2 gene expression were similarly analyzed. Small amounts of IL-2 mRNA were detected in the two highest producing *tax* cell lines, J-*tax*-9 and -19 (Fig. 3). In contrast, no IL-2 mRNA was detected in the other *tax* or anti-*tax* cell lines or the parental Jurkat cells. IL-2 mRNA was also present in the RNA isolated from PHA-activated T cells but not from the MT-1 or HUT 102B2 cell lines. These latter results are consistent with the observation that IL-2 gene expression is only rarely detected in long-term cultured ATL cell lines (39).

Tax-mediated changes in the expression of other inducible lymphokine genes were also evaluated. The Tax protein appeared to activate GM-CSF gene expression (40). GM-CSF mRNA was readily identified in three of the Tax-producing cell lines and HUT 102B2 cells but not in the anti-*tax* cell lines, parental Jurkat cells, or MT-1 cells. This Tax-inducible GM-CSF gene expression is intriguing in view of the previous finding that many HTLV-I- or HTLV-II-infected cell lines constitutively secrete this lymphokine (41, 42). In contrast to its effects on the IL-2 and GM-CSF genes, Tax did not appear

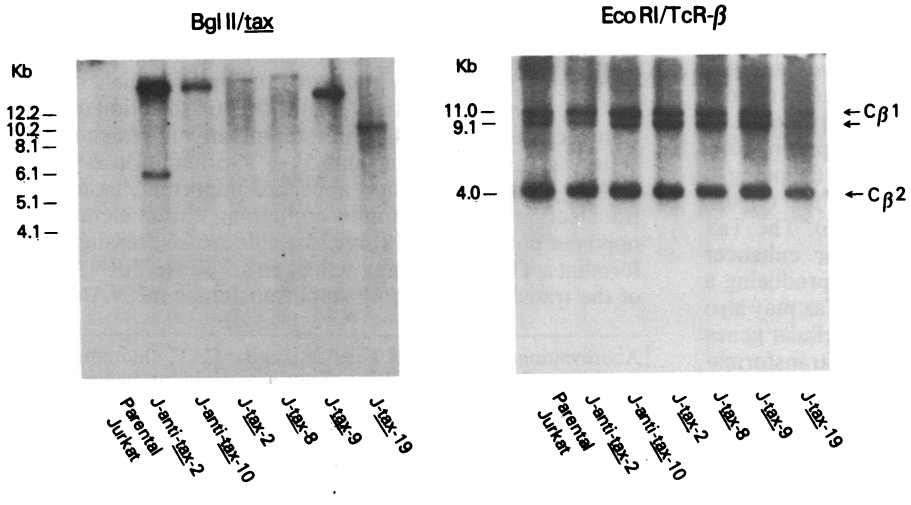


FIG. 1. (*Left*) Plasmid DNA integration in stably transfected *tax* and anti-*tax* Jurkat T-cell clones. Genomic DNA (10 μg) from each indicated cell line was digested with *Bgl* II, which cuts once within the transfected expression vector, electrophoresed in 0.8% agarose, transferred to nitrocellulose, and probed with a radiolabeled 2.0-kilobase (kb) *tax* cDNA from plasmid spXHF/82-C. Due to a loss during ethanol precipitation, only 2–4 μg of J-*tax*-2 and J-*tax*-8 DNA was analyzed. (*Right*) TCR β-chain gene rearrangement in *tax*, anti-*tax*, and parental Jurkat lines. Genomic DNA was digested with *Eco*RI and probed with a radiolabeled cDNA encoding human TCR β chain (37). The pattern of $C_\beta l$ constant-region rearrangement detected for each of the *tax* and anti-*tax* cell lines was identical to that detected for the parental Jurkat cells.

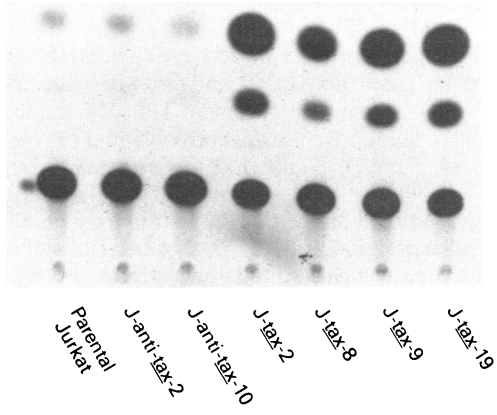


FIG. 2. Analysis of functional Tax production in the *tax* and anti-*tax* Jurkat T-cell lines. Plasmids containing the HTLV-I LTR linked to the CAT gene were transfected with DEAE-dextran (5 μg per $5 \times 10^6$ cells) into each of the indicated cell lines as described (29). After 48 hr of culture, cell extracts were assayed for CAT activity (29). Data reflect levels of conversion obtained by 30 min of incubation of the extracts with [$^{14}$C]chloramphenicol and acetyl-CoA. Unmodified [$^{14}$C]chloramphenicol and the mono- and diacetylated reaction products were separated by thin-layer chromatography. This experiment was performed three times with similar results. With time in culture, *tax* expression remained stable in the J-*tax*-9 and -19 cell lines but decreased slowly in the J-*tax*-2 and -8 cell lines.

to induce expression of the IL-1 (α and β), IL-3, IL-4, or granulocyte colony-stimulating factor (G-CSF) gene (data not shown). Further, Tax did not alter the constitutive expression of the gene encoding the δ-chain of the T3 complex (43) (Fig. 3). These findings not only confirm the presence of equivalent amounts of hybridizable mRNA in the various *tax* and anti-*tax* samples but also suggest that the progressive decline in T3 antigen expression observed in long-term cultures of HTLV-I-infected T cells (44) may not directly involve an action of Tax. Further, Tax did not alter expression of the *lck* gene, which encodes a T-cell-specific tyrosine kinase (45). Constitutive expression of *lck* is inhibited in many HTLV-I-transformed T cell lines (R. Perlmutter, personal communication); however, as with T3, our findings suggest that the *tax* gene product may not be responsible for this effect.

Previous studies (27–29) involving the transient coexpression of *tax* and IL-2 promoter–CAT plasmids in Jurkat T cells suggested that certain mitogens, which by themselves are ineffective signals for IL-2 promoter or gene induction, synergize markedly with Tax, leading to sharp increases in IL-2 promoter activity. To investigate whether the endogenous IL-2 gene was similarly regulated, the *tax*, anti-*tax*, and parental Jurkat T-cell lines were cultured in the presence of various mitogens (PHA, ionomycin, OKT3, PMA) and the secretion of biologically active IL-2 was measured 48 hr later (Table 1). The J-*tax*-9 and -19 cell lines constitutively produced low levels of IL-2 consistent with the RNA hybridization results in Fig. 3. In contrast, no IL-2 production was detected in the J-*tax*-2 and -8 cell lines or in the anti-*tax* and parental Jurkat T-cell lines. Stimulation of each of the J-*tax* lines with PHA, ionomycin, or the OKT3 monoclonal antibody, which reacts with the T3 component of the TCR complex, was associated with a pronounced increase in IL-2 production. However, these agents alone produced little or no stimulation of IL-2 production in the control J-anti-*tax* or parental Jurkat cells. Combinations of PHA and PMA did activate IL-2 gene transcription in these control cells, albeit at lower levels than that present in the *tax* cell lines. Stimulation of the *tax* cell lines with combinations of PHA and PMA consistently yielded levels of IL-2 lower than that

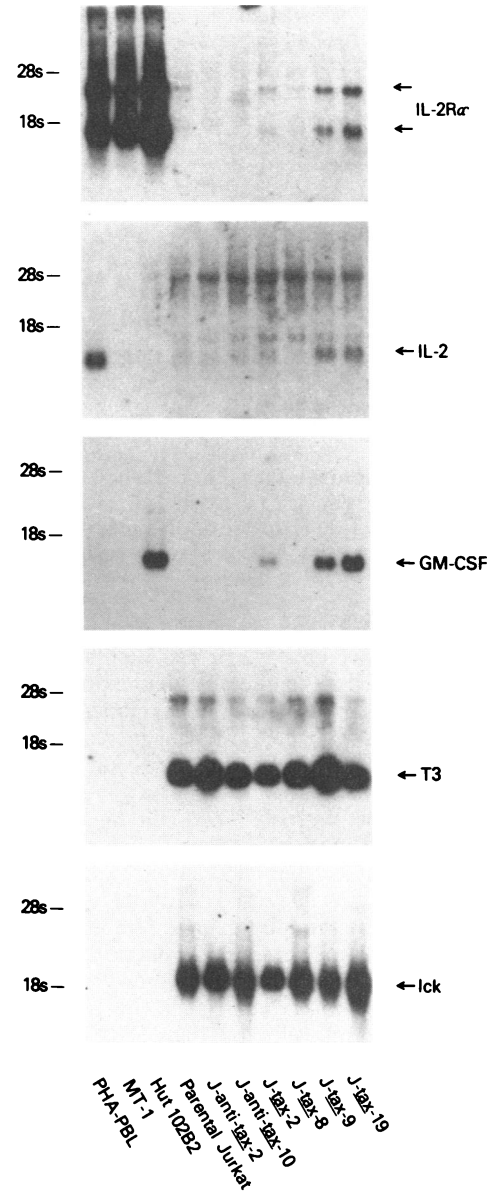


FIG. 3. Northern blotting analysis of cellular gene expression in the *tax* and anti-*tax* Jurkat T-cell lines. Poly(A)$^+$ RNA (10 μg) from the parental Jurkat T cells, J-anti-*tax*-2 and -10 cells, and J-*tax*-2, -8, -9, and -19 cells and poly(A)$^+$ RNA (1 μg) from PHA-activated peripheral blood lymphocytes (PHA-PBL) and the HTLV-I-infected cell lines MT-1 and HUT 102B2 were size-fractionated in 1% agarose/2.2 M formaldehyde gels and transferred to nitrocellulose filters. These filters were then serially hybridized with the indicated radiolabeled DNA probes. Migration positions of 28S and 18S rRNAs are indicated at left and the position(s) of the specific hybridizing mRNA species are indicated at right.

obtained with PHA alone. The most marked effects on IL-2 production in the *tax* cell lines were obtained with agents that induce increased intracellular calcium levels (e.g., PHA and the calcium ionophore ionomycin) (46–48). Synergistic activation of the *tax* cell lines by PMA, a protein kinase C agonist, was also evident.

We next studied whether it was possible to manipulate the levels of Tax production in the *tax* cell lines. The HTLV-I LTR contains functional sequences associated with inducibility by cAMP and phorbol ester (49). Since the HTLV-I LTR governed expression of the *tax* and anti-*tax* cDNAs in our vectors, the levels of *tax* mRNA present in these cell lines was compared before and after stimulation with PMA and forskolin (an adenylate cyclase agonist). These agents in-

Table 1. Synergy of various inducing agents with Tax in the activation of IL-2 production

| Cell line | IL-2 produced, units/ml | | | | | |
|---|---|---|---|---|---|---|
| | Medium | PHA | Iono-mycin | OKT3 | PMA | PHA + PMA |
| Jurkat | 0 | 3.3 | 0 | 0 | 0 | 8.1 |
| J-α-*tax*-2 | 0 | 0 | 0 | 0 | 0 | 0.03 |
| J-α-*tax*-10 | 0 | 0.7 | 0 | 0 | 0 | 3.4 |
| J-*tax*-2 | 0 | 94.0 | 17.0 | 0.3 | 3.5 | 29.7 |
| J-*tax*-8 | 0 | 66.0 | 14.0 | 0.1 | 0.3 | 16.2 |
| J-*tax*-9 | 0.04 | 145.0 | 146.0 | 24.0 | 8.4 | 38.6 |
| J-*tax*-19 | 0.10 | 132.0 | 190.0 | 30.0 | 4.4 | 57.0 |

The *tax* and anti-*tax* (α-*tax*) T-cell lines or parental Jurkat cells were incubated in medium alone (control) or with PHA (1 μg/ml), ionomycin (1 μg/ml), monoclonal antibody OKT3 (100 ng/ml), PMA (50 ng/ml), or PHA plus PMA. After 48 hr, IL-2 present in the culture supernatant was measured by the CTLL-2 bioassay (36). Units of IL-2 activity were determined by comparison of half-maximal responses with the IL-2 standard provided by the National Cancer Institute Biological Response Modifier Program. Three independent experiments yielded similar results.

duced a marked increase in *tax* mRNA expression in the *tax* but not the anti-*tax* cell lines (Fig. 4). Combinations of PMA and forskolin proved more effective than either agent alone. The sharp increases in *tax* mRNA levels induced by PMA and forskolin were accompanied by striking increases in IL-2Rα, GM-CSF, and IL-2 cellular gene expression (Fig. 4). Constitutive expression of each of these three genes was virtually undetectable in the untreated *tax* cell lines by dot blot analysis of cytoplasmic RNA isolated from $10^6$ cells. However, after PMA and forskolin induction, expression of each of these cellular genes increased ≥10-fold. Although PMA and forskolin may potentially exert direct effects on these cellular genes, a role for Tax in this induction seemed likely, as no changes in cellular gene expression were evident in the identically induced anti-*tax* cells (Fig. 4) or parental Jurkat cells (data not shown). In addition, these PMA- and forskolin-induced changes appeared specific, as the levels of c-*myb* and T3 δ-chain mRNA were not altered by these agents. Together, these results suggest that production of Tax in these *tax* cell lines can be enhanced by agents that modulate activity of the HTLV-I LTR.

## DISCUSSION

We have produced and characterized Jurkat T-cell lines stably expressing either sense or antisense *tax* cDNA derived from HTLV-I. The sense *tax* cell lines constitutively express biologically active Tax as assessed by transactivation of the HTLV-I LTR. Production of Tax in these Jurkat cells is associated with the induced and sustained expression of the endogenous T-cell genes encoding IL-2Rα, IL-2, and GM-CSF. These findings thus confirm and extend earlier transient-transfection studies describing stimulatory effects of Tax on the promoter sequences regulating IL-2Rα and IL-2 gene expression (27–30) and the respective endogenous genes (31). Of note, the IL-2 gene was activated in a synergistic manner in the Tax-producing cells by the addition of normally inactive agonists such as PHA, PMA, OKT3, and ionomycin. The inducing agents that mediated the greatest increases in the intracellular levels of calcium (PHA and ionomycin) (46–48) consistently produced the most pronounced stimulatory effects. Finally, the level of *tax* mRNA was sharply increased in each of the *tax* cell lines containing the integrated HTLV-I LTR–*tax* expression vector (spHXF/82-C) by the addition of agonists (PMA and forskolin) that activate the HTLV-I LTR through phorbol ester- and cAMP-responsive cis-acting elements (49). The increased Tax production induced by combinations of PMA and forskolin was accompanied by marked increases in expression of IL-2Rα, IL-2, and GM-CSF cellular genes.

These inductive effects of Tax on cellular genes directly involved in T-cell activation and growth have interesting implications for the mechanism of HTLV-I-induced transformation of human T cells. It is possible that the deregulated expression of the IL-2 and IL-2 receptor genes induced by Tax may produce a period of uncontrolled autocrine T-cell growth. Additional antigenic stimulation may be a prerequisite for full activation of the IL-2 gene, as Tax alone appears to produce only weak stimulatory effects on this cellular gene. This ensuing period of T-cell proliferation may in turn facilitate the occurrence of additional second-stage events required for the complete transformation of these virally infected cells and the emergence of growth-factor independence. Tax-induced activation of GM-CSF gene expression may similarly alter the growth or differentiation of these HTLV-I-infected T cells. In addition, Tax-induced production of GM-CSF may be responsible for the frequent clinical finding of neutrophilia in ATL patients (50).

These *tax*-expressing cell lines and matched anti-*tax* control cell lines provide important reagents to explore the mechanism of Tax action. In recent studies with these cells (51), it was found that Tax induces the expression of a series

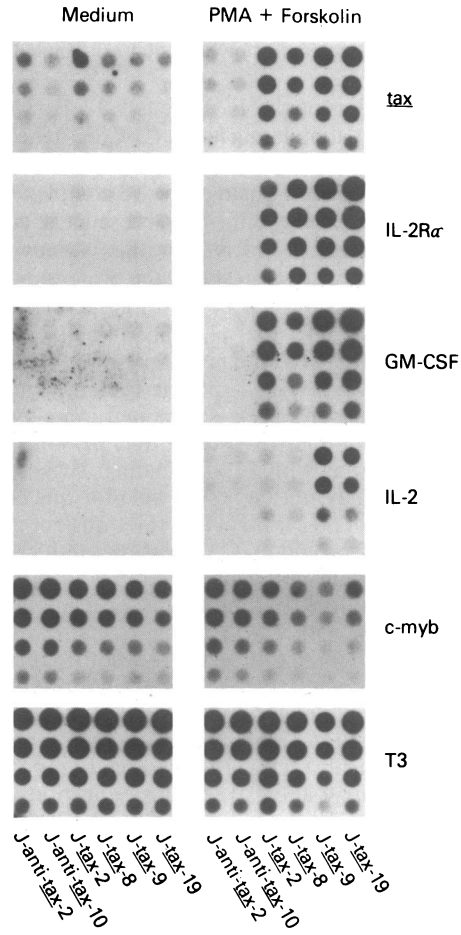


FIG. 4. Phorbol ester and forskolin activation of *tax* and cellular gene expression in the *tax*-expressing Jurkat T-cell lines. Cytoplasmic RNA from $10^6$ cells of each indicated cell line was isolated (33) before and after stimulation with PMA (50 ng/ml) and forskolin (10 μM) for 12 hr. Serial 1:2 dilutions of these RNA samples were blotted on nitrocellulose and hybridized with the indicated radiolabeled DNA probes (see text). Like the anti-*tax* cell lines, the parental Jurkat cells showed no alterations in the expression of the indicated cellular genes after induction with PMA and forskolin.

of host proteins, which in turn bind to and activate a κB-like sequence (resembling the immunoglobulin κ-chain enhancer element recognized by transcription factor NF-κB) present in the IL-2Rα promoter (53). Similarly, Leung and Nabel (54) and Ruben *et al.* (55) have assembled data supporting a role for a Tax-inducible host factor(s) in the altered expression of the IL-2Rα gene. A duplicated κB element is also present in the enhancer of the human immunodeficiency virus type 1 (HIV-1) (58), which interacts with at least some of the same proteins as the IL-2Rα κB element (52, 53) These latter results provide an attractive mechanism to explain the observation that the HTLV-I Tax protein augments transcriptional activity of the HIV-1 LTR (56). Together, these results suggest that Tax may function in an indirect manner serving to induce or modify the expression of normal cellular transcription factors, thereby leading to an activated T-cell phenotype. Effects of *tax* on more than one host-factor system seem likely, as Tax-induced transactivation of the HTLV-I LTR is apparently mediated by a separate set of cellular proteins utilizing a different DNA recognition sequence (17–19, 49).

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