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The Human T-Cell Leukemia Virus Type I Tax Protein Induces Apoptosis Which Is Blocked by the Bcl-2 Protein

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The Tax protein of human T-cell leukemia virus type I activates transcription of cellular and viral genes and can immortalize primary T lymphocytes. We have previously reported that the Tax protein transforms Rat-1 cells. Here we show that Tax-transformed Rat-1 cells detach from plates to undergo apoptotic cell death by serum deprivation. These cells exhibit DNA fragmentation into oligonucleosomal fragments and chromatin condensation. Constitutive expression of a proto-oncogene, *bcl-2*, effectively blocks Tax-mediated apoptosis caused by serum deprivation without affecting the levels of Tax expression and the transformed phenotype of the cells.

Human T-cell leukemia virus type I (HTLV-I) is an etiological agent of adult T-cell leukemia (9, 18). The HTLV-I genome has a unique pX region that encodes three proteins, Tax, Rex, and p21X, between the *env* gene and the 3′ long terminal repeat. Tax is a nuclear protein which transcriptionally *trans* activates not only its own enhancer in the long terminal repeat but also a number of cellular genes involved in cell proliferation control (23): the genes for interleukin 2 (IL-2) and the α subunit of IL-2 receptor, c-*fos*, c-*jun*, *egr-1*, and the gene for granulocyte-macrophage colony-stimulating factor. On the other hand, Tax represses the expression of human β-polymerase for DNA repair (27). Altered expression of these cellular genes may cause oncogenicity. Tax alone can immortalize rat embryo fibroblasts or primary T lymphocytes and transform them in cooperation with the oncoprotein Ras. Transgenic mice expressing Tax develop mesenchymal tumors and neurofibromas (30). We previously demonstrated transforming properties of Tax in Rat-1 cells (25). During an examination of serum dependency, we noticed that Tax-transformed Rat-1 cells detached and died from serum starvation.

Apoptosis is a physiological suicide mechanism including embryonic development, tissue homeostasis, tumorigenesis, and the lysis of virus-infected cells. Apoptotic cell death involves a characteristic morphology, detachment from surrounding cells, chromatin condensation, and fragmentation of chromosomal DNA to the size of oligonucleosomes, while the mitochondria and intracellular organelles are preserved (12, 34). There are several pieces of evidence that deregulated growth involves apoptotic cell death. Some *trans*-acting oncoproteins, such as E1A, c-Myc, and Fos, induce apoptosis that is prevented by expression of Bcl-2 (7, 19, 22, 28).

bcl-2 was discovered as a proto-oncogene from analysis of the specific chromosome translocation t(14;18) of follicular lymphoma cells. The Bcl-2 gene product is located in the inner membrane of mitochondria and has been shown to suppress many types of apoptosis (13, 33). In this report, we demonstrate that Tax induces apoptosis in serum-deprived fibroblasts and that Bcl-2 prevents this process.

All cells in this study were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 4 mM L-glutamine, 50 U of penicillin per ml, and 50 μg of streptomycin per ml and incubated at 37°C in a humidified 5% CO_2 atmosphere. W4 cells were generated by transfection of Rat-1 cells with a *tax* expression plasmid, pH2Otax, as described elsewhere (20). The *bcl-2* expression plasmid (pΔCj-bcl-2) (27) was transfected into Rat-1 cells or cotransfected with pSV2-bsr (10) into W4 cells (1:10) by electroporation using a Bio-Rad Gene Pulser at 400 V and 960 μF. After electroporation, the cells were allowed to recover in the complete medium and cultured for 2 days. Then the transfectants were selected in the presence of G418 at 600 μg/ml for Rat-1 cells or blasticidin S at 10 μg/ml for W4 cells. Cell clones were obtained by cylinder cloning. For colony-forming assays, 10^4 cells were suspended in complete medium containing 0.33% agarose (SeaPlaque; FMC) and cultured for 10 days. Colonies over 60 μm in diameter were counted, and colony-forming efficiencies were calculated.

The expression of Tax and Bcl-2 was analyzed by Western blotting (immunoblotting). Exponentially growing cells were harvested with a lysis buffer containing 50 mM Tris-HCl (pH 8), 150 mM NaCl, 2 mM phenylmethylsulfonyl fluoride, and 1% Nonidet P-40. Proteins from each soluble fraction were precipitated with 4 volumes of acetone, resolved by electrophoresis in sodium dodecyl sulfate (SDS)-polyacrylamide gels (10 or 12% polyacrylamide), and electrophoretically transferred onto nitrocellulose membranes (Schleicher and Schuell). The membranes were incubated with Bcl-2- or Tax-specific monoclonal antibodies (25, 27) and then reacted with peroxidase-conjugated anti-mouse immunoglobulin serum. Immunoreactivity was visualized by an enhanced chemiluminescence detection system (Amersham Corp.).

For analysis of DNA fragmentation, 2×10^6 cells were plated on 100-mm-diameter plates 20 h prior to serum starvation. The three plates of cells were washed twice with phosphate-buffered saline (PBS) and incubated in Dulbecco's modified Eagle's medium without serum. At the indicated time, floating cells were collected by centrifugation and attached cells were scraped in ice-cold PBS in the presence of 10 mM EDTA. Cells were put together into one tube, centrifuged at 1,500 × *g* for 5 min, washed with PBS, pelleted, and lysed in 500 μl of a lysis buffer containing 10 mM EDTA, 5 mM Tris-HCl (pH 7.6), and 0.5% Triton X-100. After 10 min on

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TABLE 1. Colony-forming efficiencies in 0.33% soft agar

Cell line	Tax	Bcl-2	Efficiency of colony formation (%)	Avg diam of colonies (μm)
Rat-1	−	−	0.0	ND[a]
Rat-1b-5	−	+	0.0	ND
W4	+	−	68.0	110.0
W4b-9	+	+	87.3	102.9
W4b-10	+	+	89.1	93.7

[a] ND, not determined.

ice, the lysates were centrifuged (13,000 × *g*) for 10 min at 4°C to separate the soluble fragmented DNA from insoluble intact chromatin. The supernatants were treated with proteinase K (200 μg/ml) in the presence of 1% SDS overnight at 37°C. Then the lysates were extracted first with phenol and then with phenol-chloroform–isoamyl alcohol (24:1), and the aqueous phase was made to 300 mM NaCl and precipitated with 2 volumes of ethanol. The pellet was rinsed with 70% ethanol, air dried, and dissolved in 15 μl of 10 mM Tris-HCl–1 mM EDTA (pH 7.5)–RNase A (0.6 mg/ml). After incubation at 37°C for 30 min, the samples were electrophoresed in a 2% agarose gel.

For electron microscopic examination, cells were stained with 2% glutaraldehyde in PBS at 4°C and then with 1% OsO_4 in the same buffer for 2 h at 4°C. The samples were dehydrated through an ethanol series and embedded in epoxy resin. The ultrathin sections were stained with uranyl acetate followed by lead citrate and were examined in the electron microscope.

For flow cytometric analysis, 5×10^5 to 10×10^5 cells were trypsinized and centrifuged (200 × *g*). After fixation with 70% ethanol in PBS, cells were pelleted and resuspended in 0.5 ml of PBS containing propidium iodide (40 μg/ml) and DNase-free RNase A (100 μg/ml). The cells were incubated at 37°C for 30 min and subjected to flow cytometric analysis.

The transforming activity of the Tax and Bcl-2 was determined by colony-forming assay in soft agar (Table 1). Parental Rat-1 cells and Bcl-2-expressing Rat-1 cells (Rat-1b-5) did not grow in soft agar. Regardless of the absence or presence of Bcl-2, Tax-expressing cells showed the transformed morphology and formed large colonies in soft agar with equivalent sizes and efficiencies (W4, W4b-9, and W4b-10). Thus, expression of Bcl-2 did not affect the transformed phenotype caused by Tax expression.

Expression of Tax and Bcl-2 proteins was determined by Western blotting (Fig. 1A and B). Figure 1A shows that only *bcl-2*-transfected cells (Rat-1b-5, W4b-9, and W4b-10) expressed Bcl-2 protein. W4, W4b-9, and W4b-10 cells expressed the same levels of Tax (Fig. 1B). Thus, the levels of expression of Tax were not affected by the presence of Bcl-2. The results indicate that the prevention of Tax-induced cell death by Bcl-2 is not due to the decrease of Tax expression. Microscopic analysis is shown in Fig. 1C. Exponentially growing Tax-expressing cells (W4, W4b-9, and W4b-10) showed the transformed phenotype, with a refractile appearance and smaller cells than the parental Rat-1 cells. Expression of *bcl-2* did not alter the apparent morphology of Rat-1 (Rat-1b-5) or W4 (W4b-9 and W4b-10) cells. When Rat-1 cells were serum starved, they were larger and flatter and most of the cells were still alive after 7 days of serum starvation. In contrast, W4 cells began to round up and detach after incubation in serum-free medium for 6 h, and after 24 h, the cell volume of the floating cells decreased. However, the cells expressing both Bcl-2 and Tax (W4b-9 and W4b-10) escaped detachment and cell death by serum starvation. By 7 days of incubation in serum-free medium, all W4 cells had died, while Rat-1, Rat-1b-5, W4b-9, and W4b-10 cells were still alive (data not shown). Tax-induced cell death was observed also in primary rat embryo fibroblasts and 3Y1 rat fibroblast cell lines expressing Tax, within 24 h of serum starvation (data not shown).

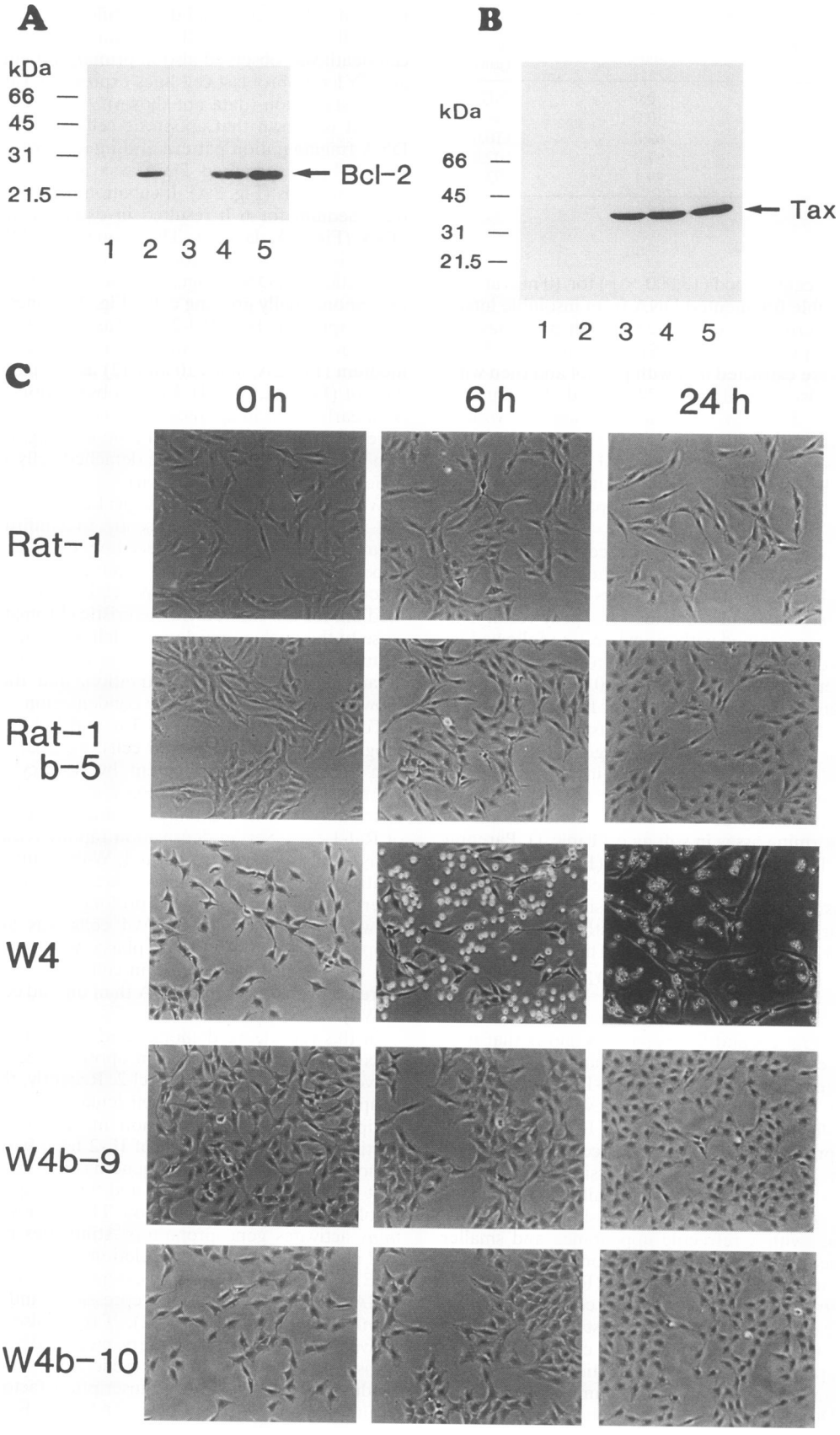
As it is known that apoptotic cells exhibit a characteristic DNA fragmentation pattern and integer-size multiples of 180 to 200 bp, fragmented DNA was analyzed by agarose gel electrophoresis (Fig. 2A). Incubation of W4 cells with serum-free medium for 6 h resulted in extensive fragmentation of DNA (Fig. 2A, lane 8). The fragmented DNA showed the distinctive pattern of oligonucleosomes commonly observed in apoptotic cells. DNA fragmentation was not observed in any of the exponentially growing cells (Fig. 2A, lanes 3, 7, 9, and 11). Cells expressing both Bcl-2 and Tax (W4b-9 and W4b-10) did not show fragmentation after 6 h of incubation in serum-free medium (Fig. 2A, lanes 10 and 12) as the parental Rat-1 cells did not (Fig. 2A, lane 4). In our observation, cell detachment is an early event in apoptosis because detached cells were still alive as determined by trypan blue staining. DNA fragmentation is observed mainly in the detached cells and rarely in the adherent cells (data not shown).

We examined the morphological changes of serum-starved W4 cells by electron microscopy to confirm the apoptotic nature suggested by the preceding findings (Fig. 2B). The upper panel of Fig. 2B shows exponentially growing W4 cells. About 45% of the detached W4 cells incubated in serum-free medium for 6 h showed characteristic chromatin condensation close to the nuclear envelope, which is a typical morphological feature of apoptosis (Fig. 2B, lower panel). Rat-1 cells did not detach by 6 h of serum starvation, and the attached cells showed no significant nuclear condensation.

To determine the effect of Tax and Bcl-2 on the cell cycle progression of serum-starved cells, we analyzed the distribution of cellular DNA content by flow cytometry (Fig. 3). Expression of both Tax and Bcl-2 did not affect the cell cycle distribution of exponentially growing cells compared with that of Rat-1 cells. Serum deprivation rapidly reduced the proportion of cells in S phase for Rat-1, W4b-9, and W4b-10 cells. In contrast, W4 cells showed no decrease in S-phase cells at 6 h after serum starvation. With prolonged serum starvation, the growth of cells except for W4 cells was arrested and the proportion of cells in G_0-G_1 phase, was increased. However, W4 cells showed a decrease in cells in G_0-G_1 phase, and an increase in cells with less DNA than diploid cells resulted (Fig. 3, W4).

In this report, we demonstrated that serum deprivation of cells expressing Tax results in apoptotic cell death, which is prevented by expression of Bcl-2. Recently, the occurrence of apoptosis in some adult T-cell leukemia cells obtained from peripheral blood by cultivation in serum-free medium and partial blocking by addition of IL-2 have been reported (26), although a responsible viral gene has not been reported.

It is known that oncogenes and tumor suppressor genes are involved in apoptotic regulation. The adenovirus E1A protein *trans* activates gene prompters, stimulates cell proliferation, and also causes apoptosis. Deletion of the conserved region 1 of E1A, which is known to be essential for induction of cellular DNA synthesis, enhancer repression, and transformation, eliminates apoptosis (19, 32). Tax is also known to *trans* activate cellular gene promoters, mediated by multiple cellular transcription factors such as cyclic AMP responsive element binding protein/activating transcription factor (CREB/ATF), serum response factor, Fos-Jun(AP-1), NF-κB, and Ets. Tax also increases the site-specific DNA binding activity of these


A
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0 h
6 h
24 h
Rat-1
Rat-1 b-5
W4
W4b-9
W4b-10

FIG. 1. Western blot analysis showing the levels of expression of Tax and Bcl-2 in parental Rat-1 cells and *tax*- and/or *bcl-2*-transfected Rat-1 cells. Exponentially growing cells were lysed with lysis buffer. Equal amounts of protein (50 μg per lane) were fractionated on an SDS–12% (A) or 10% (B) polyacrylamide gel, blotted onto a nitrocellulose filter, and probed with either anti-Bcl-2 (A) or anti-Tax (B) monoclonal antibodies. Lanes 1, Rat-1 cells; lanes 2, Rat-1b-5 cells; lanes 3, W4 cells; lanes 4, W4b-9 cells; lanes 5, W4b-10 cells. (C) Morphological changes of cells following serum starvation. Exponentially growing Rat-1, Rat-1b-5, W4, W4b-9, and W4b-10 cells were washed twice with PBS and placed in serum-free medium. Then the cells were incubated in 37°C and were examined by phase-contrast microscopy at the indicated time.

transcription factors (2). Smith and Greene reported that transcriptional activation through the CREB/ATF pathway plays an important role in Tax-mediated cellular transformation (24). Whether this CREB/ATF pathway is essential for induction of apoptosis remains to be determined.

Overexpression of the c-*myc* gene with or without serum starvation causes apoptosis (13). In an IL-3-dependent myeloid cell line, c-Myc has been overexpressed to cause apoptosis after IL-3 withdrawal (3). Addition of antisense oligonucleotides of c-*myc* in T-cell hybridomas suppressed apoptosis induced by anti-CD-3 treatment (21). There are contradicting reports on c-*myc* gene *trans* activation by Tax (6, 17). Whether the c-*myc* gene is involved in Tax-mediated apoptosis remains to be elucidated. Continuous expression of Fos induces apoptosis (22). In IL-2- and IL-6-dependent cell lines, mRNAs of c-*fos* and c-*jun* are induced soon after growth factor deprivation. Antisense oligonucleotides directed against c-*fos* and c-*jun* reduce the expression of these genes and prevent apoptosis induced by growth factor deprivation (5). In serum-deprived cells, negative regulation of c-*fos* transcription is mediated by the serum-responsive element (SRE) (14). HTLV-I Tax *trans* activates the c-*fos* promoter through four regulatory elements, including the SRE (1). There is a possibility that Tax blocks a resting signal through the SRE or continuously stimulates transcription of the c-*fos* gene even in the absence of serum, thereby promoting the apoptotic signal caused by serum starvation.

The p53 tumor suppressor gene also induces apoptosis. Overexpression of wild-type p53 can suppress cell growth and promote apoptotic cell death (35). Tumors frequently contain mutant or deleted p53 genes. The interactions of viral oncoproteins with p53 contribute to oncogenic transformation.

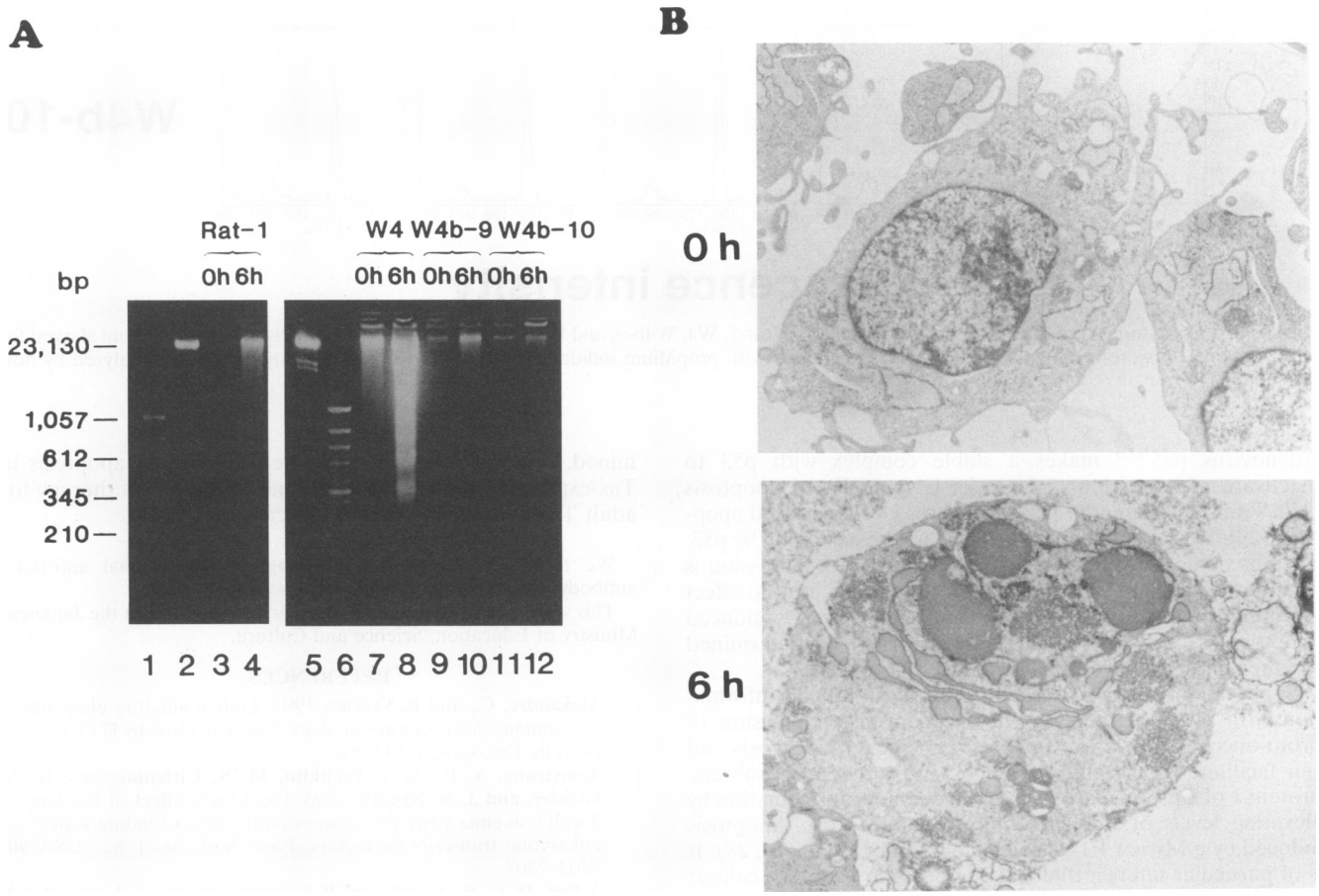

FIG. 2. (A) Agarose gel electrophoresis of fragmented DNA from serum-starved cells. Soluble DNA was collected from both attached and detached cells and fractionated by electrophoresis in a 2% agarose gel. Lanes 1 and 6, bacteriophage ϕX174 DNA digested with *Hae*II as a molecular marker lanes 2 and 5, bacteriophage λ DNA digested with *Hin*dIII as a molecular marker; other lanes contain DNA from the indicated cells after the indicated period of serum starvation. (B) Electron microscopic analysis of W4 cells. Upper panel, exponentially growing W4 cells; lower panel, W4 cells incubated in serum-free medium for 6 h.

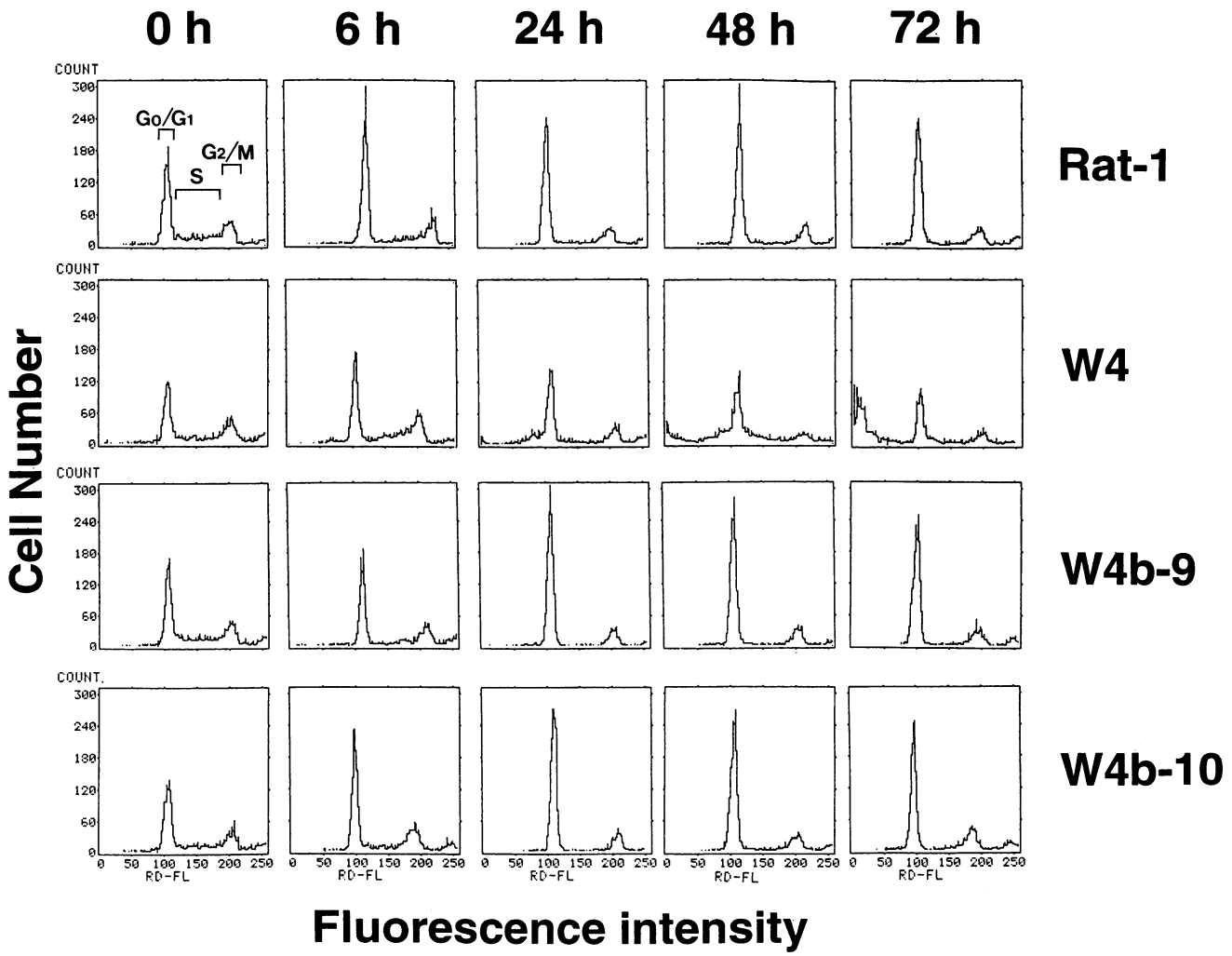

FIG. 3. Flow cytometric patterns of serum-starved cells. Rat-1, W4, W4b-9, and W4b-10 cells either exponentially growing or serum starved for 6, 24, 48, or 72 h were fixed with 70% ethanol and stained with propidium iodide. Then the DNA contents of the cells were analyzed by flow cytometry.

Adenovirus $p55^{E1B}$ makes a stable complex with p53 to inactivate p53 function and blocks E1A-mediated apoptosis (15). Transfected mutant p53 suppresses v-Myc-induced apoptosis (29). However, all apoptosis may not be explained by p53. Thymus tissue from transgenic mice lacking p53 expression is resistant to ionizing-radiation-induced apoptosis, but no effect on topoisomerase II inhibitor- and anti-CD3 antibody-induced apoptosis is seen (4, 16). It still remains to be determined whether p53 is involved in Tax-induced apoptosis.

Suppression of apoptosis may provide additional information with which to analyze the mechanisms. Expression of proto-oncogene *bcl-2* prevents several types of apoptosis and can facilitate tumor cell growth (13, 33). Latent membrane protein 1 of Epstein-Barr virus can facilitate transformation by elevating levels of Bcl-2 to inhibit apoptosis (8). Apoptosis induced by c-Myc or E1A is also inhibited by Bcl-2 (19, 28). It is of particular interest that Bcl-2 appears to keep W4 cells at G_0-G_1 phase under serum-starved conditions, inhibiting Tax-mediated cell cycle progression.

Apoptosis may represent a suicidal response against deregulated cell growth to reject malignant cells. Although the mechanisms of Tax-induced apoptosis still remain to be determined, we anticipate that selective induction of apoptosis in Tax-expressing cells could lead to new methods of therapy for adult T-cell leukemia in HTLV-I carriers.

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