A 10-Amino Acid Domain within Human T-cell Leukemia Virus Type 1 and Type 2 Tax Protein Sequences Is Responsible for Their Divergent Subcellular Distribution*§

Received for publication, January 16, 2004, and in revised form, July 1, 2004
Published, JBC Papers in Press, July 21, 2004, DOI 10.1074/jbc.M40497200

Laurent Meertens‡¶, Sébastien Chevalier‡¶, Robert Weil, Antoine Gessain‡,
and Renaud Mahieux‡**

From the §Unite´ d’Epide´ miologie et Physiopathologie des Virus Oncoge` nes, Institut Pasteur and Unite´ de Biologie Mole´ culaire de l’Expression G´ enique, FRE 2364 CNRS, Institut Pasteur, 75724 Paris Cedex 15, France

Human T-cell leukemia virus type 1 and type 2 (HTLV-1/2) are related retroviruses that infect T lymphocytes. Whereas HTLV-1 infection can cause leukemia, HTLV-2 has not been demonstrated to be the agent of a hematological malignant disease. Nevertheless, the virally encoded Tax-1 and Tax-2 transactivators display a high percentage of similarity. Tax-1 is a shuttling protein that contains a noncanonical nuclear localization signal as well as a nuclear export signal. The presence of the nuclear localization signal and the nuclear export signal domains in the Tax-2 sequence has not been determined. The distribution of Tax-2 in infected cells is not known but has been assumed to be similar to that of Tax-1. By using a Tax-2-specific antibody, we report here that Tax-2 is located predominantly in the cytoplasm of the HTLV-2 immortalized or transformed infected T-cells. These results were confirmed after transient transfection of untagged Tax-1 and Tax-2 constructs, histidine tag Tax1/Tax2, GFP-Tax, and Tax-GFP fusion constructs in several cell lines. We show that this unanticipated localization is not due to a default in the Tax-2 nuclear localization signal functions nor to major differences in Tax-2 versus Tax-1 binding to the IKKα/NEMO protein. In addition, we demonstrate that inhibiting the proteasome results in a relocalization of Tax-1 in the cytoplasm, similar to that of Tax-2. By using a series of Tax-1/Tax-2 chimeras, we determined that the minimal domain that is necessary for Tax-2 peculiar distribution encompasses amino acids 90–100. Finally, we show a high correlation between intracellular localization of Tax and their NF-xB or CREB transactivating ability.

HTLV-1 and HTLV-2 are closely related retroviruses that infect lymphoid T-cells with a preferential tropism for CD4+ and CD8+ cells, respectively (1–4). HTLV-1 is the etiological agent of the adult T-cell leukemia/lymphoma (5) and of the tropical spastic paraparesis/HTLV-1-associated myelopathy (TSP/HAM) (6). Even if originally isolated from a case of atypical hairy T-cell leukemia (1, 7) and capable of inducing the survival and proliferation of CD34+ cells (8, 9), of immortalizing human lymphocytes, or of transforming rat cells (10–13), HTLV-2 has not been epidemiologically demonstrated to be the agent of a malignant hematological disease (14). There is in fact only one cutaneous T cell lymphoma case report where HTLV-2 was likely to be involved in the pathogenesis of the disease (15). Nevertheless, an overall increase of the CD8+ T-cell population is often detected in HTLV-2-infected individuals (16). HTLV-2 infection has also been linked occasionally to some TSP/HAM “like” cases (17, 18).

Both HTLV-1 and HTLV-2 encode a viral transactivator named Tax (19–22). Tax-1 possesses an oncogenic potential and is responsible for cell transformation in vitro and in vivo. Tax-1 and Tax-2 share roughly 75% nucleotide sequence homology (21). So far, four subtypes of HTLV-2 (A to D) have been described. HTLV-2B, -2C, and -2D Tax proteins retain similar but not identical length (356, 356, and 344 amino acids, respectively), whereas HTLV-2A Tax is shorter (331 amino acids) (23). Strikingly, however, several reports have now demonstrated that although the functional regions of the proteins are well conserved (i.e. NF-xB and CREB/ATF activation domains) (24), Tax-1 and Tax-2 possess some major phenotypical differences: Tax-1 but not Tax-2 is capable of inducing transcription from the ICAM-1 gene in T-cells (25); Tax-1 but not Tax-2 induces micronuclei (26); and Tax-1 but not Tax-2 suppresses the maturation and development of human CD34+ cells in vitro (27). As compared with Tax-1, Tax-2A is also largely impaired for the inhibition of p53 functions (28, 29). Both proteins transactivate the human EGR-1 promoter but through different cis-acting sequences (30). In vitro, HTLV-2 transform T-cells independently of Janus tyrosine kinase/signal transducers and activators of transcription activation (31), and most importantly, Tax-2 transforms rat fibroblasts less efficiently than Tax-1 (11).
Differences between Tax-1 and -2 Subcellular Localization

Soon after the discovery of HTLV-1, several laboratories investigated the localization of the Tax-1 protein in infected T-cells, using cellular fractionation, immunoelectron microscopy, immunocytochemistry, or immunofluorescence (32-41). Tax-1 was found to be present both in the nucleus and cytoplasmic fractions in the infected T-cells, in proportions that depend on the cell line and on the method of quantification used. In SLB-1, ~80% of the Tax-1 signal was in the nucleus (33), whereas this nuclear signal represented only 20-30% in HUT 102 and less in MT2 (38). However, it was also reported that between 50 and 60% of Tax-1 was present in the nuclear fraction of both HUT 102 and C8166 cell lines (39). These discrepancies might be linked to rough calculation methods. Within the nucleus, Tax-1 is almost equally distributed between the nucleoplasm and the nuclear matrix (34). The situation is nearly equivalent in Tax-1-transfected cells (32), where the protein colocalizes in discrete nuclear bodies (also called Tax Speckled Structures (26)), components of the spliceosome (42), with transcription factors and specific transcripts of genes that are activated by Tax (43, 44). The amount of Tax-1 present in the cytoplasm depends on the cell line used for the experiment (45). In this cellular compartment, Tax-1 was reported to associate with a molecular chaperone complex containing hTid-1 and HSP70 (46).

Proteins enter and exit the nucleus by using lysine-rich signals that are referred to as nuclear localization signal (NLS) and nuclear export signal (NES) (47). With respect to NLSs, the best characterized transport sequences consist of one or two short stretches of basic amino acids (aa). Recently, NLSs that are not particularly rich in lysine residues have been identified in various viral and cellular proteins of diverse function. Some of these NLSs have been characterized as nonconventional importin-interacting motifs (47). Several laboratories have mapped the different domains of Tax. A first report (36) suggested that the minimal region of Tax-1 necessary for a nuclear distribution of the protein encompasses residues 17–48. The same group further reported that the 48 N-terminal residues of Tax-1 contained a noncanonical functional NLS sequence (35). This was confirmed using either a Tax-1 mutant that was deleted for amino acids 2–59 (Δ58 Tax-1) (48) or the Δ24–60 mutant (49). Both mutants failed to accumulate in the nucleus. Most interestingly, Tax-1 has a cysteine- and histidine-rich region (zinc finger domain) between amino acids 22 and 53, which therefore overlaps the putative NLS (49). Whether the Tax-1 zinc finger domain is required for the nuclear localization of the protein is controversial, however (36, 49). Altogether, these results lead to the basic paradigm that Tax-1, as transcription factors, is a nuclear protein. Nevertheless, Tax-1 also activates the NF-κB transduction pathway (50), which implies that it also has a cytoplasmic function (51). It was indeed demonstrated recently that Tax-1 was a shuttling protein (52). Based on sequence scan, the same authors made also the suggestion that Tax-1 may contain a Rev-like nuclear export signal at amino acids 190–203 (52). This hypothesis was later confirmed when another group demonstrated that this region functions as an NES that is insensitive to leptomycin B (45).

Because of the lack of an available Tax-2-specific antibody, the intracellular localization of the protein in HTLV-2-infected cells has not been investigated but was assumed to be similar to that of Tax-1. We have engineered a rabbit Tax-2-specific antibody, which allowed us to investigate the subcellular distribution of Tax-2. Unexpectedly, the protein appears to locate predominantly in the cytoplasm of the HTLV-2-infected cells, regardless of the Tax subtype tested (i.e., 2A or 2B). These results were confirmed in several cell lines after transient transfection of either untagged Tax-1 and Tax-2 constructs, histidine tag Tax1/Tax2, GFP-Tax, or Tax-2 fusion constructs. This unexpected localization is not due to a fault in the Tax-2 putative NLS signal since fusing the first 60 N-terminal amino acids of Tax-2 to the GFP protein results in a massive distribution of the protein into the nucleus. In order to map the Tax-2 domain that is responsible for such a difference, we constructed a series of Tax-1 and Tax-2 chimeric plasmids. This allowed us to deduce that the minimal domain of Tax-2 that is responsible for this peculiar subcellular distribution encompasses amino acids 90–100. Most interestingly, we also show a correlation between the localization of the proteins and their transactivating activity. Our results therefore suggest that the predominant cytoplasmic localization of Tax-2 in the cell is not only linked to the presence of an NLS sequence but that at least another region (aa 90–100), which is also different from the NES, is responsible in part for its peculiar intracellular distribution.

EXPERIMENTAL PROCEDURES

Cell Culture and Drug Treatments—HeLa, COS-7, HEK, and 293T cell lines were grown in Dulbecco’s modified Eagle’s medium supplemented with fetal bovine serum (10%) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). HTLV-1-transformed cell lines C8166, C91/PL, HUT 102, HTLV-2A-transformed cell lines C19 and MO or HTLV-2B immortalized (IL-2 dependent) GabMD2 and Pgy590101, as well as uninfected CEM cells were grown in RPMI 1640 supplemented with 20% fetal bovine serum, antibiotics, and 1-glutamine. Interleukin-2 was added to Pgy590101 and GabMD2 cells. All cell lines were maintained at 37 °C in 5% CO2. When indicated, cells were incubated with leptomycin B (20 nM for 6 h) (Sigma) as previously described (53), lactacystin (Sigma) (20 μM for 6 h), and epoxomycin (Affiniti) (1 μM for 7 h).

GFP-Tax, Tax-GFP, Histidine-Tax, and pSG5M-Tax Protein Construction—The cDNA sequences coding Tax-1, Tax-2B, and Tax-2A sequences were amplified from pTax, pCG-Tax2B, and pCG104-Tax2A, respectively (24, 36, 54). They were inserted downstream or upstream from the GFP sequence after SacI/EcoRI or Xhol/PstI digestion and cloned into pGFP-C3 and pEGFP-N1 vectors (Clontech). This resulted in constructs in which Tax-1 or Tax-2 were either fused to the C or to the N terminus of the green fusion protein (GFP). Chimeric sequences were also obtained by the same method. pSG5M-Tax-1 and pDNAHismax-Tax2/1 were constructed by inserting the Tax-1 or Tax-2 sequences into the EcoRI/BamHI or NotI/XbaI restriction endonuclease sites within the pSG5M or pcDNAHismax plasmids, respectively. The nucleotide sequence of all constructs was determined using the DYEnamic ET Terminator Cycle Sequencing kit (Amersham Biosciences) on an Applied Biosystems 373A DNA sequencer. Except where indicated, Tax-1 implies Tax-2 throughout the text.

Tax-2-specific Antibody—Based on the comparison sequences of Tax-2A and Tax-2B, two synthetic oligopeptides representing separate domains (central and C-terminal) of the protein were synthesized (Eurogentec). The peptides were selected from regions in the sequence found to be hydrophilic. Oligopeptide A consists of amino acids 74–88, and oligopeptide B consists of amino acids 320–331 of the Tax-2 sequence. They were coupled to keyhole limpet hemocyanin and injected to specific pathogen-free New Zealand White rabbits. After several booster injections, the serum (GP3738) was tested for the presence of anti-Tax-2 antibodies. Pre-immunization serum was also tested for the presence of non-specific reactivity.

Western Immunoblot Analyses—Twenty hours after transfection, cells were washed twice with PBS, lysed (50 mM Tris-HCl, pH 7.4, 120 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 0.2 mM NaV3O5, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) in the presence of protease inhibitors (Complete, Roche Applied Sciences) on an Applied Biosystems 373A DNA sequencer. Except where indicated, Tax-1 implies Tax-2B throughout the text.

Because of the lack of an available Tax-2-specific antibody, the intracellular localization of the protein in HTLV-2-infected cells has not been investigated but was assumed to be similar to that of Tax-1. We have engineered a rabbit Tax-2-specific antibody, which allowed us to investigate the subcellular distribution of Tax-2. Unexpectedly, the protein appears to locate predominantly in the cytoplasm of the HTLV-2-infected cells, regardless of the Tax subtype tested (i.e., 2A or 2B). These results were confirmed in several cell lines after transient transfection of either untagged Tax-1 and Tax-2 constructs, histidine tag Tax1/Tax2, GFP-Tax, or Tax-2 fusion constructs. This unexpected localization is not due to a fault in the Tax-2 putative NLS signal since fusing the first 60 N-terminal amino acids of Tax-2 to the GFP protein results in a massive distribution of the protein into the nucleus. In order to map the Tax-2 domain that is responsible for such a difference, we constructed a series of Tax-1 and Tax-2 chimeric plasmids. This allowed us to deduce that the minimal domain of Tax-2 that is responsible for this peculiar subcellular distribution encompasses amino acids 90–100. Most interestingly, we also show a correlation between the localization of the proteins and their transactivating activity. Our results therefore suggest that the predominant cytoplasmic localization of Tax-2 in the cell is not only linked to the presence of an NLS sequence but that at least another region (aa 90–100), which is also different from the NES, is responsible in part for its peculiar intracellular distribution.
with anti-rabbit or with anti-mouse horseshoe peroxidase-conjugated secondary antibodies (Amersham Biosciences) and developed using either the SuperSignal West Pico or SuperSignal West Fenta Chemiluminescent substrate kit (Pierce). To control for the amount of protein loaded per well, membranes were stripped with the Re-blot Plus Kit (Chemicon International) and reprobed with a specific anti-β-tubulin antibody (sc9104 Santa Cruz Biotechnology 1:1000).

Tax/IKK/NEMO Coimmunoprecipitation—HEK cells were transfected using CaCl2 with GFP (5 µg), GFP-Tax-1 (5 µg), GFP-Tax-2 (5 µg), and GFP-Tax-2–ha22 (5 µg). In some cases, 5 µg of an IKK/NEMO expressing vector were also transfected (55). Twenty four hours later, cells were lysed in Chris buffer (50 mM Tris, pH 8.0, 0.5% Nonidet P-40, 200 mM NaCl, and 0.1 mM EDTA supplemented with 10 µg/ml each of the protease inhibitors leupeptin, aprotinin, N-tosyl-l-phenylalanyl-l-chloromethyl ketone, N-tosyl-l-lysine chloromethyl ketone, and phenylmethylsulfonyl fluoride, as well as the phosphatase inhibitors sodium fluoride (100 mM) and sodium orthovanadate (2 mM)). Whole cell extracts (800 µg) were incubated with anti-IKK/NEMO polyclonal antibody (55) at 4 °C for 1 h. Protein A- or protein G-Sepharose beads were then added, and the reaction was incubated for an additional 30 min. After extensive washing with lysis buffer, the complexes were run on 8% polyacrylamide gels. Inputs were run on 10% Tris-glycine (Invitrogen) gels. Western blot analysis was performed to detect GFP fusion proteins by using a monoclonal anti-GFP antibody (Clontech) or to detect IKK/NEMO with the IKK/NEMO polyclonal antibody (55) as described previously (56).

Transient Transfections—For microscopic analyses, HeLa, HEK, 293T, or COS-7 cells were seeded on an eight-well chamber glass slide at a concentration of 3 × 104 cells/well and transfected the next day with 0.2 µg of DNA using Effectene or Polyfect reagents (Qiagen). For immunoblot analyses or luciferase assays, HEK and 293 T-cells were seeded on a 6-well plate at 6 × 105 cells/well and transfected the next day with 2 µg of DNA using the Polyfect reagent (Qiagen) following the manufacturer’s instructions.

Green Fluorescent Protein Analyses—Twenty four hours after transfection, the cells were washed with PBS, fixed with 4% paraformaldehyde (Sigma), and washed with PBS. Nuclei acids were stained with DAPI, a fluorescent phalnendilimine dihydrochloride (DAP)-containing mounting medium ( Vectashield, Vector Laboratories). Cells were visualized with a Zeiss Axioplan 2 imaging microscope, ×40, with a CoolSnapHq digital camera and the SimplePCI software (Hamamatsu).

Indirect Immunofluorescence—Twenty four hours post-transfection, cells were washed with PBS and fixed with 4% paraformaldehyde (Sigma). Following 0.5% Triton X-100 treatment, cells were preincubated with a 5% PBS/milk solution and incubated with anti-Tax1 (168A-5142), anti-Tax 2 (GP3738), or anti-histidine (sc-804, Santa Cruz Biotechnology) or anti-p53 (Ab-6, Oncogene Research) antibodies at a dilution 1:100, 1:40, 1:100, and 1:1000, respectively, in a 5% PBS/milk solution for 1 h at 37 °C. Samples were stained with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Vector Laboratories), CY3-conjugated goat anti-IgG mouse (Amersham Biosciences), or CY3-conjugated goat anti-IgM mouse (Amersham Biosciences) at dilutions 1:200, 1:1000, and 1:1000, respectively, in a 5% PBS/milk solution for 1 h at 37 °C. The coverglass was finally washed, mounted with DAPI-containing Vectashield, and examined for the protein localization by two different investigators in a blinded manner. A similar procedure was performed for HTLV-1- or HTLV-2-infected cells. In some cases (Fig. 1, m–r), HTLV-2-infected cells were mixed on the slide with uninfected CEM cells at a ratio of 1 to 3 in order to control for the specificity of the signal.

Luciferase assay—HEK cells were transiently transfected with HTLV-1-LTR-luc, HTLV-2-LTR-luc, NF-κ B-luc, or IL-8–luc (57) plasmids together with pSG5M-Tax plasmids as described previously (28, 58). The amount of DNA transfected was equalized by addition of a control vector. All the transfections were carried out in the presence of a pBRG-TK vector in order to normalize the results for the transfection efficiencies. Reporter activities were assayed 24 h post-transfection using the dual-luciferase reporter assay system (Promega). Luciferase assays were performed with a Berthold LB9500 luminometer as described elsewhere (59).

RESULTS

Tax-1 and Tax-2 Intracellular Distribution Is Different in Infected Cells—None of the available Tax-1 monoclonal or polyclonal antibodies recognizes Tax-2A and Tax-2B (28). An anti-GST-Tax-2B antibody is available but does not always reliably detect Tax-2A or Tax-1 in Western blot analyses (11, 60). Therefore, we prepared two synthetic peptides based on the amino acid sequences of Tax-2A and Tax-2B and injected them into rabbits. The Tax-2 antiserum was then tested by Western blot for its ability to recognize the Tax-1 and/or Tax-2 proteins that were present in cell extracts from HTLV-1- or HTLV-2-infected cell lines (Fig. 1A). Most interestingly, the Tax-2 antiserum (GP3738) recognized the Tax-2A protein that was present in C19 and MO HTLV-2 cell extracts and the Tax-2B protein that is present in GabMD2 and Pyg9590101 HTLV-2 immortalized cells (Fig. 1, 1st, 2nd, 9th, and 10th lanes) but not the Tax-1 protein (Fig. 1A, 4th to 6th lanes). As a positive Tax-2B control, extracts from Tax-2B-transfected 293T were used (Fig. 1A, 8th lane). The Tax-1 monoclonal antibody (Tab172) reacted specifically against the Tax-1 protein that was present in C8166, C91/PL, and HTU 102 HTLV-1-infected cells (Fig. 1A, 4th to 6th lanes) but not against Tax-2A or Tax-2B.

By using the Tax-2 antibody, we then determined the protein distribution in HTLV-2-infected cells for the first time. We undertook a series of immunofluorescence experiments on HTLV-1- (C8166), HTLV-2A- (C19), or Pyg9590101- and GabMD2 (HTLV-2B-infected) cells. The Tax-1 (clone 168A-5142) monoclonal antibody was also used. As reported previously, this antibody stained nuclear structures in the C8166 HTLV-1-transformed T lymphocytes (43) (Fig. 1B, c) but not in C19 cells (data not shown). As expected, we could not detect any signal when the cells were incubated only with the CY3-conjugated goat anti-IgG mouse (Fig. 1B, b). The same experiment was then performed with the Tax-2-specific serum. Most strikingly, in C19 (HTLV-2 subtype A) cells, but also in Pyg9590101- and GabMD2-infected cell lines (HTLV-2 subtype B), the Tax-2-specific signal was predominantly detected in the cytoplasm (Fig. 1B, k, n, and q). As controls, we could not detect any signal in HTLV-uninfected CEM cells or in HTLV-1-infected MT2 cells (data not shown) with the Tax-2 antibody, neither in the wells where cells have been incubated with the secondary antibody only (Fig. 1B, h) or with pre-immunization serum (data not shown). Altogether, these results suggested that Tax-2 does have the same cellular localization as Tax-1 in the infected cells.

Tax-1 and Tax-2 Do Not Have a Similar Localization in Transfected Cells—In order to determine whether the different localization profiles between Tax-1 and Tax-2 would also be observed when expressed in the same cell line and in the absence of the other viral proteins, we transfected pSG5M-Tax-1 and pSG5M-Tax-2B plasmids in HeLa cells that have been extensively used for Tax localization studies (42, 45, 51, 52, 61, 62). As we observed for HTLV-1-infected cells, Tax-1 was mainly present in nuclear structures in transfected cells (Fig. 2A, b and c). On the contrary, Tax-2 signal was predominant in the cytoplasm of Tax-2B (or Tax-2A, data not shown)-transfected HeLa cells (Fig. 2A, e and f). Similar results were obtained in HEK, COS-7, and Jurkat cells (data not shown). The expression levels of Tax-1 and Tax-2 proteins in the transfected HeLa cells are shown in Fig. 2B. In order to confirm those differences by using the same antibody, we constructed a series of histidine tag Tax-1 and Tax-2 plasmids. Immunofluorescence experiments using a rabbit anti-histidine antibody led to the same conclusions, i.e. that Tax-1 signal was predominant in the nucleus, whereas Tax-2 signal was predominant in the cytoplasm (Fig. 2A, h for Tax-1 and q for Tax-2). Similar results were obtained when the experiment was performed with a mouse anti-histidine antibody (data not shown). As a control experiment, histidine-tagged Tax-1 and Tax-2 proteins were also detected with anti-Tax-1 and anti-Tax-2 antibodies, respectively, and displayed the same distribution (Fig. 2A, h for Tax-1 and i for Tax-2).
and n). Finally, histidine tag Tax protein expression was monitored by Western blot (Fig. 2C).

**GFP-Tax and Tax-GFP Proteins Display a Similar Cellular Distribution as Untagged Constructs in All Cell Lines Tested**—A previous report (46) demonstrated that GFP-Tax-1 has the same cellular distribution as nontagged Tax-1. We hypothesized that GFP-Tax constructs could therefore be used for mapping the Tax-1 and -2 domains that are responsible for their different cellular localization. In order to exclude any positional effect of the GFP protein, we cloned it either upstream or downstream from the Tax-1 or Tax-2 sequence. These plasmids were then transfected into HeLa cells, and the distribution of the protein was determined. In the absence of Tax, the GFP protein was found to be located both in the nucleus and in the cytoplasm of the cells (Fig. 3A, b). On the contrary, GFP-Tax-1 or Tax-1-GFP was found to be primarily expressed in the nucleus of the cells (Fig. 3A, e, and data not shown). We therefore conclude that the position of the GFP vis à vis Tax did not interfere with Tax-1 localization. As with untagged Tax-2 or histidine-tagged Tax-2, we found that the GFP-Tax-2B as well as the Tax-2B-GFP fusion proteins were predominantly restricted to the cytoplasm in HeLa cells (Fig. 3A, h, and data not shown) as well as in HEK, COS-7, and Jurkat cells (data not shown). Comparable results were obtained with a GFP-Tax-2A construct (data not shown) implying that the 25 C-terminal amino acid sequence of Tax-2 does not play a critical role in the nuclear localization of the protein. Finally, the expression of the constructs was found to be similar by Western blot (Fig. 3B). Of note, the cellular detection of GFP-Tax gave similar results at 48 and 72 h post-transfection (data not shown).

**Mapping the Tax Domain That Is Responsible for the Differ-**

ent Subcellular Localizations—Because the NLS domain of Tax-1 has been reported to be consist of the first 60 amino acids (aa) of the proteins, we aligned Tax-1 and -2 sequences. This revealed five changes between Tax-1 and Tax-2 (aa 13, 26, 31, 38, and 54) in this region. Point mutations were then
introduced in the Tax-1 sequence to replace sequentially Tax-1 aa 13, 26, 31, 38, and 54 with those of Tax-2. The resultant Tax-1 and GFP-Tax-1 mutants exhibited, however, the same localization as wild-type Tax-1 (data not shown). This suggested that more than one modification was requested for altering the Tax-1 localization. Consequently, chimera plasmids that would contain the 60 N-terminal aa of Tax-1 combined to the remaining sequence of Tax-2, or vice versa, were made (Fig. 4A). Unexpectedly, switching the putative NLS sequences did not modify the localization of the chimeric GFP-Tax protein. As an example, GFP-Tax1-(1–60)-Tax2 localizes like Tax-1, whereas GFP-Tax-2wt (see Fig. 4A, a and c). These results imply that the 1–113-amino acid sequence includes a domain that is critical for Tax localization. Protein expression was determined either with a GFP antibody or with a Tax-1 monoclonal antibody raised against a C-terminal epitope. As expected this antibody allowed the detection of the chimeras that contain the C-terminal part of Tax-1 only (Fig. 4B, Tab172 panel see 3rd, 5th, and 7th lanes). To confirm these results, we then constructed another series of chimera in which only the Tax-1-(60–113) domain was replaced with the corresponding Tax-2 sequence. Similarly, we replaced Tax-2-(60–113) sequence with Tax-1 sequence (Fig. 4C). Consistent with the previous observations, the GFP-Tax-2-Tax-1-(60–113)-Tax2 had a localization that was similar to that of Tax-1, whereas GFP-Tax-1-Tax-2-(60–113)-Tax-1 localization was similar to that of Tax-2 (Fig. 4C). Protein expression was determined (Fig. 4D). Most interestingly, Smith and Greene (36) also observed that mutating some aa in the 60–110 domain of Tax-1, i.e. outside the putative NLS/zinc finger domain, resulted in an altered localization (cytoplasm > nucleus).

The 90–100 Domain of Tax-2 Is Necessary for the Protein Localization—As see above, the construct that contains the 113 N-terminal sequence of Tax-2 or the 60–113 region of Tax-2 inserted in the Tax-1 sequence has the same localization

Fig. 2. Tax-1 and Tax-2 distribution is different in HeLa-transfected cells. A, pSG5M-Tax-1 and -2B (a–f) or histidine-Tax-1 and -2B plasmids (g–r) were transiently transfected into HeLa cells. Twenty four hours post-transfection, cells were fixed, stained as described under “Experimental Procedures” with a Tax-1 monoclonal antibody (168A-5142), a polyclonal rabbit Tax-2 serum (GP3738) or a rabbit anti-histidine antibody (sc-803 Santa Cruz Biotechnology) and mounted with DAPI-containing mounting medium (Vectorshied, Vector Laboratories). Images of cells that are representative of the entire population are shown. Cells were visualized with a Zeiss Axioplan 2 imaging microscope x40 using a CoolSnapHQ digital camera and the SimplePCI software (Hamamatsu). B and C, Western-blot analysis of Tax-1 and Tax-2. Cell lysates (70 μg) were subjected to electrophoresis on a 10% TG gel and probed with Tax-1 (Tab172), Tax-2 (GP3738), histidine (sc-803 Santa Cruz Biotechnology) or β-tubulin (sc9104 Santa Cruz Biotechnology) antibodies.
as Tax-2wt, whereas GFP-Tax2-(1–80)-Tax1 localizes like Tax-1. We therefore concluded that the region that is necessary for Tax-2 localization includes aa 80–113. In order to confirm these results, we built a series of untagged chimeras that contain a minimal sequence of Tax-2 that exchanges either 20, 23, or 33 aa in the 80–113 Tax-1 sequence (Fig. 5A). This allowed us to determine the localization of the chimeric proteins with a Tax-1 antibody. As a control, the Tax-1, Tax-2, and Tax2-(1–113)-Tax1 were used and displayed the same localization as their GFP-Tax counterparts (Fig. 5A, e, h). Most interestingly, the four constructs that contain the Tax-2 amino acid sequences, 80–100, 80–113, 90–100, and 90–113, had a cellular distribution that was roughly similar, but not identical, to Tax-2, i.e. a strong cytoplasmic signal, with a faint punctate nuclear staining that is usually the hallmark of Tax-1 (Fig. 5A, see k–u). These results therefore suggest that the 90–100 domain of Tax-2 is critical for determining Tax-2 localization. This region is different from the previously described NLS and NES sequences (35, 42, 45) and strikingly overlaps the domain of Tax-1 (aa 89–110) that has been shown to contact DNA (63). Part of this region is also involved in the Tax-1-IKK/H9253 binding domain (64, 65). Most strikingly, however, affixing the Tax-1-(90–100) or the Tax-2-(90–100) domain to the GFP had no effect on the GFP localization (Fig. 5B) suggesting that this sequence per se is not sufficient when not placed within the context of the whole protein. As a control, protein expression was also monitored in transfected cells (Fig. 5C).

We did a comparative amino acid sequence alignment of Tax-1 and Tax-2B sequence in the 80–113 region that revealed several changes, most of them being located in the 90–113 region (Fig. 6A). We then tested the ability of the plasmids encoding the chimeras described in Fig. 5 to activate transcription from different promoters for which Tax localization is critical, i.e. HTLV-2-LTR (nuclear function) and NF-κB (cytoplasmic activity) (Fig. 6B and C). Most interestingly, we were able to demonstrate that the NF-κB activation is increased when Tax is in the cytosol (Fig. 6C). On the opposite, the Tax1-(1–113)-Tax2, which localizes like Tax-1, i.e. preponderantly in the nucleus, was as active as Tax-1 to induce the transactivation of the HTLV-2-LTR reporter construct (Fig. 6B). Altogether, these results showed a strong correlation between the localization of the proteins and their respective activity on the HTLV-LTR and on an NF-κB-luc construct. Of note, we cannot also formally exclude that these chimeric proteins are somehow impaired for CREB activation because they do not have a proper folding, although this is unlikely because they can activate the NF-κB pathway.

Finally, we wanted to determine whether the differences between Tax-1 and Tax-2 transcriptional activity would also be measurable on a cellular promoter. To this end, we used an IL-8 reporter construct that was shown previously to be acti-
FIG. 4. The 60–113 domain of Tax is critical for the protein localization. A (panels a–l) and C (panels a–f), HeLa cells were transiently transfected with GFP and GFP-Tax chimera plasmids. Twenty four hours later cells were fixed and stained with DAPI-containing mounting medium (Vectashield, Vector Laboratories). Cells were visualized with a Zeiss Axioplan 2 imaging microscope ×40 using a CoolSnapHQ digital camera and the SimplePCI software (Hamamatsu). Images of cells that are representative of the entire population are shown. B and D, Western blot analysis of GFP and GFP-Tax-chimeras. Cell lysates (70 μg) were subjected to electrophoresis on a 10% TG gel and probed with a GFP antibody (B and D) (Invitrogen), a Tax-1 monoclonal antibody (Tab 172) raised against the C terminus part of the protein (B), or a β-tubulin antibody (B and D) (sc9104 Santa Cruz Biotechnology).
vated by Tax-1 through NF-κB-binding sites (57). The experiment was performed with Tax-2wt, Tax-2-M22, and Tax-2wt constructs in the presence of the IκB/H9260B-binding sites (57). The experiment was performed with Tax-1 monoclonal antibody (168A-5142) (A), and mounted with DAPI-containing mounting medium (A and B) (Vectashield, Vector Laboratories). Cells were visualized with a Zeiss Axioskop 2 imaging microscope ×40 using a CoolSnapHQ digital camera and the SimplePCI software (Hamamatsu). Images of cells that are representative of the entire population are shown. C, cell lysates (70 µg) from B were subjected to electrophoresis on a 10% TG gel and probed with a GFP antibody (Invitrogen) or a β-tubulin antibody (sc9104 Santa Cruz Biotechnology).

Western blot experiments showing in parallel the expression of the different chimeras were performed as controls (Fig. 6E). As expected, the Tax-1 monoclonal antibody allowed the detection of the proteins that contain the C-terminal part of the protein (Tax-1wt, Tax2-(1–113)-Tax1, Tax1–2-(80–100)-Tax1, Tax1–2(90–100)-Tax1, Tax1–2-(80–113)-Tax1, and Tax1–2-(90–113)-Tax1), whereas Tax-2-specific antiserum only allowed the detection of the proteins that contain either the 70–80-aa N-terminal region of Tax-2 or the C-terminal domain of Tax-2 (Tax-2wt, Tax2-(1–113)-Tax1, and Tax1-(1–113)-Tax2).

Tax-2 Localization Is Not CRM-1-dependent, however, the Proteasome Is Involved in Tax-1 Distribution—In order to define whether the differential distribution was due to a strong export of Tax-2 from the nucleus or to a degradation of the protein in the nucleus, we treated the transfected cells with different chemicals (Figs. 7 and 8). First, leptomycin B (LMB), a CRM-1-mediated nuclear export inhibitor was used. However, as compared with control treatment, incubation of Tax-2B-transfected cells with LMB did not result in a significant change in its cellular distribution (Fig. 7A, h versus k). This result was not due to an inefficient LMB treatment, since endogenous p53, whose export is CRM-1-dependent (66, 67), accumulated in the nucleus upon treatment (Fig. 7A, n versus q). As described previously (45), Tax-1 localization was not modified by LMB (Fig. 7A, b versus e). This suggested that as with Tax-1, Tax-2 is not exported from the nucleus through the CRM-1-dependent pathway.

A previous report (68) has demonstrated that Tax-1 associates with but is not degraded through the proteasome. Never-
theless, we determined whether Tax-2 was actively processed in the cells through this pathway (Fig. 8). Lactacystin or epoxomycin treatments had no measurable effect on Tax-2 localization. Addition of cycloheximide to the cells in the presence of proteasome inhibitors did not result in an increase of the Tax-2 nuclear signal (data not shown). Most strikingly however, treatment of Tax-1-expressing cells with epoxomycin or lactacystin resulted in a massive relocalization of the protein in the cytoplasm of the cell (Fig. 8A, e and h versus b). These results also suggest that under normal conditions Tax-1 may be transiently sequestrated in the cytoplasm by its physical interactions with another protein that is then degraded by the proteasome. Inhibition of the proteasome could therefore prevent Tax-1 from translocating in the nucleus. Alternatively, Tax-1 ubiquitination may have a role in its nuclear-cytoplasmic transport, as reported for other proteins (69). As a control for proteasome inhibition, cellular extracts obtained from untreated and treated cells were run and probed for the presence of poly-ubiquitinated proteins that are the hallmark of proteasome inhibition, and then for GFP to detect the Tax fusion proteins (Fig. 8B).

**DISCUSSION**

The transport of macromolecules into and out of the nucleus is essential for regulating most aspects of cell growth and is mediated by gated channels that permeate the nuclear envelope, commonly referred to as nuclear pore complexes (70). Although most small molecules can passively diffuse through the central channel of the nuclear pore, many proteins (larger than 40 kDa) comprise signals that facilitate more rapid and energy-dependent entry into the nucleus (70). There are several types of nuclear localization signals that mediate nuclear import by directly binding to one or more import receptors of the importin-α or importin-β families. The ensuing discovery of protein sequences that signal rapid export from the nucleus is more recent. Cellular or viral proteins containing both nuclear import and export signals have the capacity to “shuttle” between the nucleus and cytoplasm, and by regulating this dynamic movement, the cell is able to control the localization and activity of individual proteins and protein complexes (70).

Tax-1 is one of these proteins that shuttles from the nucleus to the cytoplasm (52), using NLS and NES sequences (35, 45, 49). We have hypothesized that one reason for the Tax-1 and Tax-2 phenotypical differences that have been observed by many could be their subcellular distribution. We report here that while Tax-1 is mainly located in the nucleus, both in infected lymphocytes and in transfected cells (32, 34, 35, 37–40, 42, 43, 61, 62), Tax-2A or Tax-2B is mainly found in the cytoplasm of the infected or transfected T- and non-T-cells. Two previous articles (24, 26) mentioned that the Tax-2A protein is restricted to the nucleus of COS or 293T-transfected cells. Of note, the anti-Tax-1 sera that have been used for these Tax-2 studies (and which are no longer available) were specifically raised against Tax-1 but not Tax-2 sequences and were neither tested by immunofluorescence nor by straight Western blot on HTLV-2-infected cells (21, 26). In addition, by using the Tax-1 antibody, Slamon et al. (21) made the observation of a difference in Tax-1 versus Tax-2 protein expression by immunoprecipitation and Western blotting. They suggested that their results probably reflected a lower affinity of their Tax-1 antibody for Tax-2. Whether this lower affinity had an impact on the previously published Tax-2 immunofluorescence result specificity remains to be determined.
FIG. 6. Correlation between localization of the Tax proteins and their ability to activate the NF-κB or the CREB/ATF pathways. A, comparative amino acid sequence alignment of the 80–113 domains of Tax-1 (top) and Tax-2 (bottom). Changes are marked with arrows. B–D, HEK cells were transiently transfected as described under “Experimental Procedures” with Polyfect reagent (Qiagen) with 125 ng of HTLV-2 LTR-luc (B) or 100 ng of NF-κB-luc together with the different Tax constructs (C). D, 200 ng of the IL-8-luc plasmid (57) were used. For all experiments, 10 ng of phRG-TK (Promega) were used, and DNA concentrations were adjusted with vector control so that equivalent amount of DNA was transfected. Transfection results were normalized to renilla activity. The results presented in B–D are the average values of 2–4 independent experiments. E, Western blots. 70 μg of protein extracts from the lysates obtained after transfection were run on 10% TG gels and probed for Tax-1, Tax-2, or β-tubulin antibodies.
Previous studies on Tax-1 intracellular localization using several cell types and methods of Tax-1 detection have shown that the amount of cytoplasmic Tax-1 can differ from one cell line to another (45). However, we have used the same cell lines as those used by Ross et al. (24) and by Semmes et al. (26) for their Tax-2 localization reports, and our Tax-2-specific antibody has been raised against almost the same peptidic domain as the Tax-1 antibody used by Ross et al. (24). In any cell lines transfected with a Tax-2 construct, the Tax-2 signal that was detected either with anti-Tax-2 antibody, with an anti-histidine (mouse or rabbit) antibody, or by direct GFP visualization was predominantly cytoplasmic. These data were reproducible in all three HTLV-2A- or -2B-infected cell lines tested. Furthermore, our Tax-2A construct (which has the same localization as Tax-2B) was derived from that of Ross et al. (24), which excludes any bias that could have occurred in the arbitrary choice of a Tax-2 sequence in the data base. An obvious reason that would explain why Tax-1 and Tax-2 have different distributions would have been the absence or the presence of a non-functional NLS sequence in Tax-2. However, our results clearly demonstrate that the Tax-2 NLS is able to direct nuclear importation of GFP. We show that the 60–113-aa sequence (which is different from the published Tax-1 NLS and NES sequences) contains a domain that is responsible for the Tax-1 versus Tax-2 differential localization. A computer-based analysis using the PSIPRED software (72) revealed that this region includes two secondary \(\alpha\)-helix structures (aa 65–73 and 103–111) separated by a linker sequence. Both of these domains are present in Tax-1 and Tax-2. However, the first \(\alpha\)-helix is shorter in Tax-1 (aa 67–72), whereas the second \(\alpha\)-helix is shorter in Tax-2 (aa 103–109). The remaining Tax sequence (aa 80–100) is predicted not to form a secondary structure. Replacing part of this sequence (i.e. aa 90–100) in Tax-1 with that of Tax-2 redirects most of the chimeric protein into the cytoplasm.
Most interestingly, this stretch of amino acids corresponds almost perfectly to a previously published protease-sensitive linker sequence joining two protein structural domains which is involved in the Tax-1 DNA binding (63). The 94–114 domain of Tax-1 has also been reported to be critical for its binding to IKKγ/H9253/NEMO (64). However, our results do not demonstrate major differences between Tax-1 and Tax-2 as far as IKKγ/H9253/NEMO binding (see supplemental Fig. 9). Nevertheless, it remains to be determined how this sequence could also be implicated in the intracellular distribution of Tax. It would also be interesting to examine the rate at which Tax-2 is exported from the nucleus relative to Tax-1. The ability of the noncanonical NLS of Tax-1 to bind importins is not known. Such studies are now also in progress in our laboratory.

Tax has the unique ability to activate both the CREB/ATF and the NF-κB pathways (36). One of these signals (NF-κB) is initiated in the cytoplasm (51), whereas the other (CREB/ATF) takes place in the nucleus (22, 32, 48, 49). Both Tax-1 and Tax-2 can activate these two pathways, regardless of the cell line tested (73), suggesting that Tax-2 may contain an NLS and an NES sequence. However, such studies have not been conducted. A number of reports have also shown that although the Tax-1 and Tax-2 functional domains are similar, they are not identical (24). Overall, these results are paradoxical. If the domains are comparable, why do the two proteins present so many biological differences (11, 25–31, 74, 75)?

As an example, Jeang and co-workers (26) compared the HTLV-1 and -2 Tax proteins in two selected aspects, micronuclei induction and transcriptional activation of LTRs. Their results indicate that Tax1 is a potent inducer of micronuclei formation in cells, whereas Tax-2 lacks micronuclei inductive ability. Fujii and co-workers (11) also recently showed that Tax-1 is more transformant than Tax-2. Finally, Feuer and co-workers (27) also demonstrated that conversely to Tax-2, Tax-1 expression perturbs development and maturation of pluripotent hematopoietic progenitor cells. In addition, they have demonstrated that Tax-1 inhibited the cellular replication to a higher degree in comparison to Tax-2 (75). Altogether, these results, in addition to numerous epidemiological studies (18), clearly prove that there are phenotypic differences between Tax-1 and Tax-2. One reason that could explain the disparities between Tax-1 and Tax-2 may be the difference in the localization of the two proteins. We believe that Tax-2, which is predominantly expressed in the cytoplasm, should act pref-
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especially on the activation of the IkappaB-kinase complex; conversely, Tax-1 should act mostly at the LTR transcriptional level. Such studies are now in progress; nevertheless, our transient transfection results are already in accordance with this hypothesis.

Tax-1 has been shown to form homodimers (48, 76, 77), and its dimerization is known to be critical of a phosphorylation-independent CREB activation (78, 79). It is also very likely to be necessary for NF-kB activation (51, 80). The Tax-1 domains that are needed for the protein dimerization are still a matter of debate. It has been suggested that Tax-1 dimer formation deserves our attention.

Linked to its less pathogenic properties is a challenge that has been revealed in the transfection experiments (see Fig. 6) were deleted in the dimerization domains. In addition, they were also capable of activating NF-kB. This suggests that the folding or the dimerization of these constructs is not impaired. In conclusion, whereas HTLV-1 infection leads to leukemia and TSP/HAM, HTLV-2 can cause, at best, myelopathy. Determining whether Tax-2 intracellular localization is directly linked to its less pathogenic properties is a challenge that deserves our attention.

Acknowledgments—We thank Emmanuel Perret for help with the imaging; Dr. Warner Greene for the gift of the pcTax plasmid; Dr. William Hall for the gift of the NF-kB-luc and the pcGTA2B6 plasmids; Dr. Patrick Greene for the pG104-TaxA2A plasmid; Dr. Claudine Pique for the pSG5M backbone vector; Dr. Roberto Accolla for the HTLV-2 LTR-luc plasmid; and Dr. Mukaida for the gift of the IL-2lac construct. We also thank Dr. Nathalie Arhel and Dr. Timothy Stinear for their critical comments and Dr. Gerold Feuer for sharing unpublished data.

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