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Tax of the Human T-Lymphotropic Virus Type I Transactivates Promoter of the *MDR-1* Gene

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The *mdr-1* gene has been shown to confer resistance to chemotherapy of multiple drugs which share no obvious structural similarities. We and others have previously reported that some virus-associated malignant cells express high levels of MDR-1 (1,2), probably regulated by some viral proteins. In this study we have examined the role of Tax, the key protein of HTLV-1. An excellent correlation was found between the existence of HTLV-1 and the expression of MDR-1 among seven human T-cell lines. In the second part of the study, a 1.76-kb DNA fragment representing the upstream regulatory elements of human *mdr-1* gene was cloned into the CAT reporter plasmid. When the Tax expression plasmid was co-transfected with the MDR-1 reporter plasmid, a significant induction of CAT activity was observed. We conclude that Tax protein may up-regulate the expression of the *mdr-1* gene. © 1997 Academic Press

Viruses have been found to be associated with several human malignancies. In some endemic areas, such as the South China and Taiwan, a substantial portion (15-20%) of the malignancies are associated with viruses. For example, NPC, HCC, and UCC are closely linked to EBV, HBV/HCV, and HPV, respectively. Although a causative role of these viruses in carcinogenesis has been an issue of controversy, their existence in the tumor cells nevertheless may have an effect on the phenotypes of the tumors.

Recently, we and others have noticed that the existence of some viruses may have conferred drug resistance to their host tumor cells. For example, the recurrent EBV-associated peripheral T-cell lymphoma, as compared with its EBV-non-associated counterpart, was found to express a much higher level of the MDR-1/p-glycoprotein and resulted in drug resistance to the second-line chemotherapy (1). Furthermore, the recurrent HTLV-1-related ATL (2, 3) and HBV-related HCC (4) were all found to express a significant level of MDR-1. Together, these evidence suggest that expression of MDR-1 of the tumor cells may be regulated by some viral proteins. Although a biologic reason for this phenomenon remains elusive, it is important to clarify the possible link between drug-resistance and viruses, which may help shed light on the future treatment of the generally chemotherapy-refractory virus-associated malignancies. In our laboratory, a continual effort has been devoted to this aspect, and the pX protein of HBV has also been under intense investigation.

In this paper, Tax, one of the regulatory proteins encoded by HTLV-1, was chosen for detailed studies. We have provided evidence that, in addition to being able to transactivate a versatile array of genes as reported previously, Tax also transactivates, either directly or indirectly, the human *mdr-1* gene.

MATERIALS AND METHODS

Cell lines and cell culture. Three HTLV-1 negative human T-cell lines, H9, SR786, Hut78, and four HTLV-1 positive human T-cell lines, Hut102, MT-2, THAM-1 (5), and TATL-1 (established from a Taiwanese with ATL, unpublished data) were maintained, in suspension, in RPMI 1640 medium supplemented with 10% fetal calf serum (Gibco Co., New York, NY).

PCR Primers and their locations in respective genes. GAPDH-1 (5′-AGGTGAAGGTCGGAGTCAA), in 1st exon of *GAPDH.* GAPDH-2 (5′-GCAGAGGGGGCAGAGATGAT), in 5th exon of *GAPDH.*

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The abbreviations used are: ATL, adult T-cell leukemia/lymphoma; CAT, chloramphenicol acetyl transferase; EBV, Epstein-Barr virus; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HPV, human papilloma virus; HTLV-1, human T-lymphotropic virus type 1; MDR, multidrug-resistance; NPC, nasopharyngeal carcinoma; ONPG, o-nitrophenyl-β-D-galactopyranoside; RT-PCR, reverse transcription-polymerase chain reaction; UCC, uterine cervical carcinoma.

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MDR1-1 (5′-GGAAGCCAATGCCTATGACT), in 13th exon of *mdr-1*. MDR1-2 (5′-CGATGAGCTATCACAATGGT), in 15th exon of *mdr-1*. RPX3 (5′-ATCCCGTGGAGACTCCTCAA), in 2nd exon of *tax* gene. RPX4 (5′-AACACGTAGACTGGGTATCC), in 3rd exon of *tax* gene. LTR2 (5′-AGGTCAGGGCCCAGACTAA), in LTR of HTLV-1. LTR5 (5′-GGGCGCGTGAAGGAGAGA), in LTR of HTLV-1. MDRB5 (5′-TCCTGCACTGTTTAGGGAGGGTT), in promoter region of *mdr-1*. MDRP3 (5′-TTGAGCTTGGAAGAGCCGCTACT), in promoter region of *mdr-1*.

The expected amplification products are 145, 249, 372, and 297 bp for primer pairs of RPX3/RPX4 (6), MDR1-1/MDR1-2, GAPDH-1/GAPDH-2, and LTR2/LTR5, respectively. For the first three pairs, the upper and lower primers were separated by at least one intron so that RT-PCR would produce products easily distinguishable between properly spliced transcripts and their counterparts of genomic or unspliced copies.

PCR, reverse transcription, and RT-PCR. For detection of the presence of the HTLV-1 genome in total DNA of various cell lines by PCR (Fig. 1A), the HTLV-1 LTR specific primer pair of LTR2 and LTR5 was used. With 10 ng of total cellular DNA as the template, PCR was run at 94°C/30s denaturation, 58°C/60s annealing, and 72°C/30s elongation, for 35 cycles. For reverse transcription, total cDNA was synthesized by 200 units MMLV (Moloney murine leukemia virus) reverse transcriptase with random hexamer as primers and 2 μg of total RNA, which was extracted by the Chomczynsk method and was dissolved in formamide (7,8), as the template in a total volume of 50 μl. The volume of RNA added was less than 2 μl so that the formamide concentration was less than 4% in order not to interfere with reverse transcription. For detection of the tax-specific transcript (Fig. 1B), one μl of the reverse transcription mixture was directly used as templates in a standard PCR reaction with the primer pair of RPX3 and RPX4. PCR was run at 94°C/30s denaturation, 55°C/60s annealing, and 72°C/30s elongation, for 35 cycles. For semi-quantification of the MDR-1 transcript (Fig. 2), one μl of the reverse transcription mixture was directly used as the template in a standard PCR reaction with the primer pair of MDR1-1 and MDR1-2, the reaction was first run for 20 cycles at 94°C/30s denaturation, 54°C/60s annealing, and 72°C/30s elongation, then the primer pair of GAPDH-1 and GAPDH-2 was added, and was further run for another 20 cycles at the same setting as previously described (9). For electrophoretic analysis of each amplification, five μl of the total 50 μl PCR reaction products was resolved in a 2% agarose gel.

Plasmids for transfection. pMDRP1.7-CAT contains the CAT reporter gene under the control of the 1.76-kb regulatory region of the human *mdr-1* gene (−1730 to +29, relative to the major transcription initiation site) (10). This 1.76-kb DNA was generated from genomic DNA by PCR using the primer pair of MDRB5 and MDRP3. To construct pMDRP1.7-CAT, the amplified 1.76-kb fragment was blunt ended and ligated into HindIII-cut and blunt-ended pZE8-CAT vector. pCMVβ contains the *E. coli* β-galactosidase gene under the control of the human cytomegalovirus immediate early promoter/enhancer (11). pHISLES-XΔSph (tax^+/rex^-) and pHISLES-XΔAC (tax^-/rex^-) were kindly provided by Dr. Joseph Sodroski of the Dana-Farber Cancer Institute, Boston, USA. Both are driven by its own promoter in the LTR region. In pHISLES-XΔSph (tax^+/rex^-), the *rex* gene was inactivated by a CATG deletion which included its initiation codon. The *tax* gene remained intact. In pHISLES-XΔAC (tax^-/rex^-), both the *tax* and *rex* genes were inactivated by a 124-bp deletion which was in the coding sequences shared by both genes.

Transfection and harvesting of total cellular protein. The DEAE-dextran method was used for transfection. Briefly, 1×10^7 H9 cells in log phase were collected by centrifugation, washed once with TS buffer (137 mM NaCl, 5 mM KCl, 0.7 mM sodium phosphate, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 25 mM Tris, pH 7.4), re-suspended in 1 ml of TS/DNA/DEAE-dextran mixture, (containing 1 ml of TS buffer, 50 μl of 1% DEAE-dextran, and 5 μg of each plasmid DNA, pre-mixed for at least 15 min before use), incubated at room temperature for 15 min, briefly tapped once in the middle of incubation. Ten ml of RPMI medium containing 10% FCS and 100 μM chloroquine was then added, and incubated in a T25 flask at 37°C for 30 min. The transfected cells were then washed once with and re-suspended in 30 ml RPMI plus 10%FCS and cultured in a T75 flask at 37°C for two days before harvesting. For total protein extraction, the cells were washed once with PBS, once with 0.25 M Tris (pH7.8), and re-suspended in 200 μl of 0.25 M Tris (pH7.8), then lysed by three rounds of freezing and thawing. Cell debris was removed by centrifugation.

β-galactosidase assay. Standard procedure was followed (12). Briefly, in a total volume of 300 μl, cell extract equivalent to 100 μg total protein was incubated with ONPG (0.88 mg/ml) in 1 mM $MgCl_2$, 45 mM β-mercaptoethanol, 67 mM sodium phosphate, pH7.5, at 37°C for 30 min to 4 hr until an apparent yellow color developed. The reaction was stopped by adding 500 μl of 1M Na_2CO_3, reaction time recorded, and optical absorbance at 420 nm was measured.

CAT assay. CAT assays were performed as previously described (12). Briefly, in a total volume of 200 μl, extract equivalent to 100 μg of total protein was incubated with 0.4 mg/ml of acetyl-CoA and 3 μM of [^{14}C]-chloramphenical (60 Ci/mol) in 0.4 M Tris buffer, pH7.8, at 37°C for 2 hr. After separation of the reaction products by thin-layer chromatography, the amounts of acetylated [^{14}C]-chloramphenical were measured with a PhosphoImager (Molecular Dynamics, Inc., Sunnyvale, CA, USA).

RESULTS

Status of HTLV-1 in seven cell lines. The presence or absence of HTLV-1 genome in cell lines were confirmed by PCR amplification of the LTR region with the primer pair of LTR2 and LTR5. As shown in Fig.1A, a band of 297 bp was produced from the four HTLV-1-positive Hut102, MT-2, THAM-1, and TATL-1, but not from the HTLV-1-negative H9, SR786, and Hut78 cell lines. The expression of Tax-specific transcript was demonstrated by RT-PCR with the primer pair of RPX3 and RPX4. This primer pair should produce a DNA of 145 bp from a spliced transcript specific for the *tax* gene, but a DNA of 2262 bp from the full-length viral RNA and proviral DNA. Positive signals (the 145-bp band) were obtained only from the four HTLV-1 positive cell lines (Fig 1B). The results are consistent with the records of these cell lines.

Correlation of HTLV-1 (Tax) and MDR-1 expression. Expression of MDR-1 among these cell lines was examined by RT-PCR (Fig.2), using the GAPDH transcript as an internal control. The relative intensities of the MDR-1 band over the GAPDH band would reflect the level of the MDR-1 transcript. A positive correlation between HTLV-1 (Tax) and MDR-1 expression was observed. Three of the four HTLV-1 (Tax) positive cell lines (MT-2, THAM-1, and TATL-1) showed significantly more MDR-1 expression than the three HTLV-1 negative cell lines (H9, SR786, and Hut78), with Hut102 cell line as the only exception. To further examine the activities of the LTR promoter and the *mdr-1* promoter in various cell lines, cells were transfected

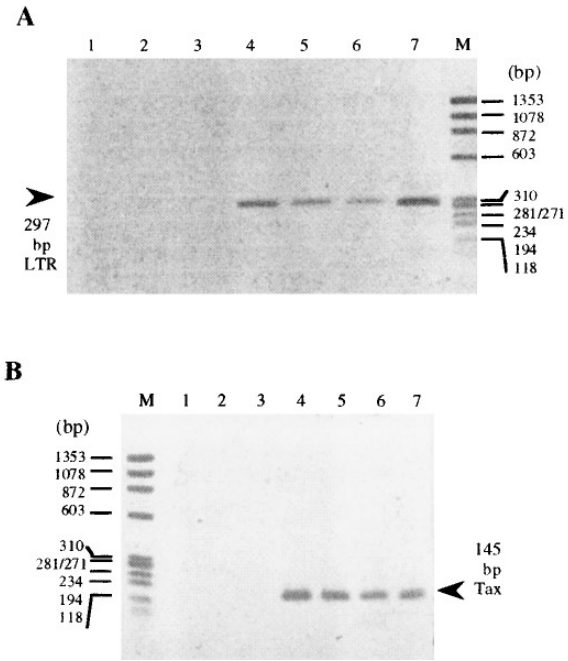

FIG. 1. HTLV-1 status in cell lines. **A,** LTR-specific PCR products (297 bp, arrow head) amplified with the LTR2/LTR5 primer pair using genomic DNA as the templates. **B,** Tax-specific RT-PCR products (145 bp, arrow head) amplified with the RPX3/RPX4 primer pair. M, DNA markers with lengths indicated aside (bp). Lanes 1-3, HTLV-1 negative cell lines, H9, SR786, and Hut78, respectively; Lanes 4-7, HTLV-1 positive cell lines, Hut102, MT-2, THAM-1, and TATL-1, respectively.

with the HTLV-1 LTR-CAT reporter plasmid pCHL4 or the MDR-CAT reporter plasmid pMDRP1.7-CAT. As expected, strong expression from the LTR promoter was observed in all three HTLV-1 positive cell lines (Fig. 3A, lanes 3-5) and none in the two HTLV-1 negative cell lines (Fig. 3A, lanes 1 and 2). Expression from the *mdr-1* promoter was observed in two of the three HTLV-1 positive cell lines (Fig. 3B, lanes 4 and 5) and none in the HTLV-1 negative cell lines (Fig. 3B, lanes 1 and 2). Hut102 was the only exception of being HTLV-1 positive and not expressing *mdr-1* promoter activity (Fig. 3B, lane 3). The transfection efficiencies of the five cell lines were similar, as monitored by a side-by-side transfection with the pCMVβ plasmid and followed by measuring their β-galactosidase activities present in the protein extracts of the transfected cells (23.0± 17.6 arbitrary units). The other two cell lines, SR786 and TATL-1, were not included in Figure 3 because of difficulties in introducing plasmid into these cell lines in our hands.

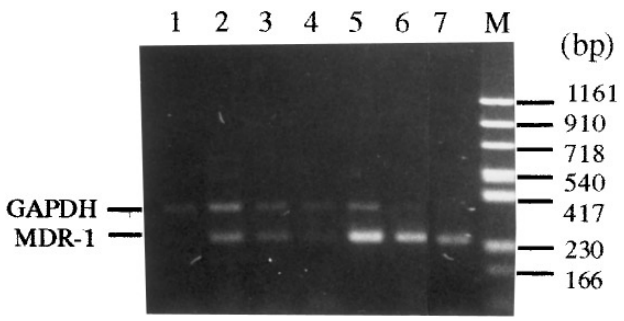

FIG. 2. MDR-1 transcript levels in cell lines measured by RT-PCR. M, DNA markers with length in bp indicated on the right. Lanes 1-7, cell lines as of those in figure 1. The amplification products for MDR-1 (249 bp) and the internal reference GAPDH (372 bp) are marked. Amplification was 40 cycles for MDR-1 and 20 cycles for the more abundant GAPDH. With the exception of Hut102, the levels of the MDR-1 transcript were positively correlated with the presence of HTLV-1.

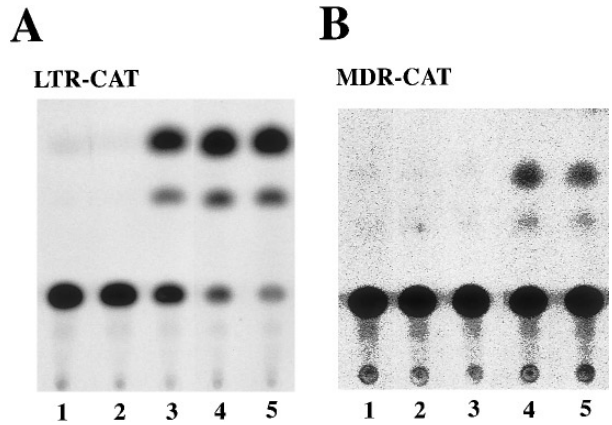

FIG. 3. HTLV-1 LTR and cellular *mdr* promoter activities in various cell lines (CAT assay). Either LTR-CAT or MDR-CAT reporter plasmid was separately transfected into each cell line, and CAT activity was measured 48 hr later. Lanes 1, 2, 3, 4, and 5 are H9, Hut78, Hut102, MT-2, and THAM-1, respectively.

HTLV-1 Tax transactivates mdr-1 promoter. To demonstrate that HTLV-1 Tax does transactivate the *mdr-1* promoter, co-transfection experiments were performed. Using H9 cells as a host, co-transfection of the pMDRP1.7-CAT reporter plasmid and the internal reference plasmid pCMVβ with the Tax-expressing plasmid pHISLES-XΔSph (tax$^+$) resulted in a small but clear CAT activity (Fig.4, lane 1). CAT activity was not detectable when co-transfected with the tax$^-$-control plasmid pHISLES-XΔAC (Fig.4, lane 2), indicating a transactivating effect of Tax on the *mdr-1* promoter. When the reference plasmid pCMVβ was omitted from co-transfection, a much more profound transactivation by Tax was observed (Fig.4, lane 3). Again, CAT activity was not detectable when co-transfection was with the tax$^-$-control plasmid pHISLES-XΔAC (Fig.4, lane 4), Therein, the transfection efficiency was monitored by using the reference plasmid pCMVβ alone, in parallel, in a separate transfection. The transfection efficiency differences were less than 15% between lanes 1

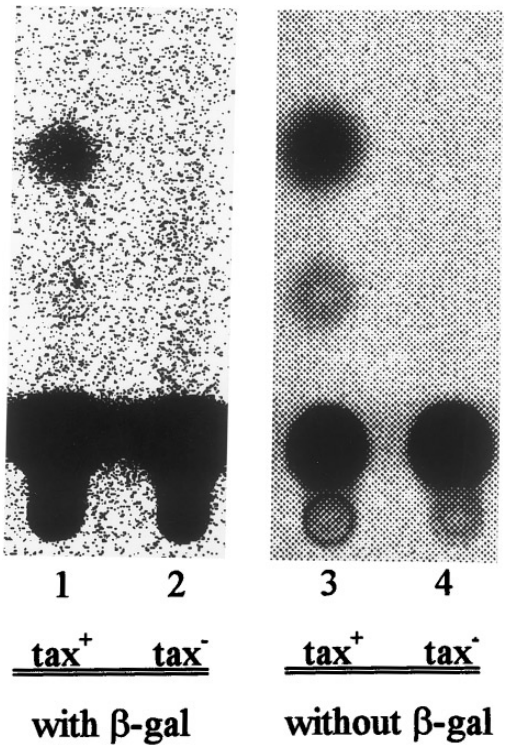

FIG. 4. Transactivation of the *mdr-1* promoter by Tax (CAT assay). Lanes 1 and 3, co-transfection of the pMDRP1.7-CAT reporter plasmid with pHISLES-XΔSph (tax^+). Lanes 2 and 4, co-transfection of pMDRP1.7-CAT with the control plasmid pHISLES- XΔAC (tax^-). Lanes 1 and 2, co-transfection with the internal reference plasmid pCMVβ. Lanes 3 and 4, co-transfection without pCMVβ.

and 2, and between lanes 3 and 4, as measured by the β-galactosidase activities following transfection. The low CAT activity was probably caused by interference from the strong CMV promoter/enhancer of the internal reference plasmid pCMVβ (compare lanes 1 and 3, Fig.4).

DISCUSSION

Some virus-associated malignant tumors have been reported to express a high level of MDR-1 (1-3). The *mdr-1* gene is therefore an attractive target for studying the viral effects on tumor phenotypes in respect to tumor's sensitivity to chemotherapeutic drugs. In this report, we have observed that the presence of HTLV-I virus is associated with the high expression level of MDR-1 in T-cell lines: while low MDR-1 transcript was found in all three HTLV-1-negative T-cell lines tested, three of the four HTLV-1-bearing T-cell lines expressed significantly higher levels of MDR-1 (Fig.2 and Fig.3). Furthermore, in CAT assays, co-transfection of the HTLV-1 Tax-expressing vector resulted in an increased CAT activity from the *mdr-1* promoter-driven CAT reporter plasmid (Fig.4). These results clearly demonstrated that Tax protein of the HTLV-1 virus transactivated the *mdr-1* gene. In an attempt to address the relationship between drug-resistance and MDR-1 expression level, we found that the correlation was only partial (data not shown). This was actually not a surprise to us, since drug resistance of cancer cells has been known to be a very complex phenomenon, and it is now becoming clearer that MDR-1 is just one of the players contributing to the final phenotype of drug resistance.

A few cis-elements have been identified in the promoter region of the *mdr-1* gene, including heat-shock responsive element, metal responsive element, phorbol ester-responsive element, GC box, and CAAT box (10). These elements, and probably more others, may mediate the induction of MDR-1 expression by various stimuli such as heat shock, sodium arsenite, dioxin, sodium butyrate, retinoic acid, DMSO, and the *ras* gene product, through their interaction with appropriate protein factors (10, and references therein).

Tax itself is not a DNA binding protein. It exerts its transactivation activities through interaction with various cellular transcription factors, such as CREB (13-17), ATF (17,18), NF-kB (19-23), AP-1 and AP-2 (24), SRF (23,25), Ets (26), and THP (27). Different types of cells may use different factors. Therefore, the cellular context is likely to affect how HTLV-1 Tax activates the MDR-1 expression. This may partly explain why Hut102 cells did not express MDR-1 transcript as high as other HTLV-1 containing cell lines (Fig. 2, lane 4 and Fig.3B, lane 3). It remains to be determined which cellular factor(s) in the H9 cells might have interacted with the HTLV-1 Tax protein in transactivating the *mdr-1* gene. Recently, we have demonstrated that pX of HBV also possesses similar transactivation activities on the *mdr-1* gene (Doong et al., unpublished results). The possibility that both Tax and pX can transactivate the *mdr-1* gene is supported by a report showing that pX of HBV and Tax of HTLV-1 share functional similarities (28).

In addition to being able to transactivate the HTLV-1's own promoter located in its LTR and the *mdr-1* promoter as reported here, Tax has also been shown to transactivate other cellular genes (29), including those for c-Myc (19), Fos (30,31), G-CSF and GM-CSF (20), IL-2 (32), IL-2Rα (6,30), IL-3 (33), IL-6 (9,21), IL-8 (34), INF-γ (35), Krox (25), proenkephalin (18), PTHrP (24,36), TGF-β1 (37), TNF-β (38), vimentin (22,39,40), ICAM-1 (41) and most early response genes (42). We postulate that the presence of viruses in general, is likely to influence the behavior of a tumor cell via their various transactivating proteins. Our observation is probably more than just a fortuitous phenomenon, and may have an important implication for chemotherapy of the virus-associated malignancies.

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