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The Homeobox Protein MSX2 Interacts with Tax Oncoproteins and Represses Their Transactivation Activity*[S]

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Bovine leukemia virus (BLV) *tax* is an essential gene involved in the transcriptional activation of viral expression. Tax is also believed to be implicated in leukemogenesis because of its ability to immortalize primary cells *in vitro*. To gain insight into the molecular pathways mediating the activities of this important gene, we identified cellular proteins interacting with Tax. By means of a two-hybrid approach, we show that Tax specifically interacts with MSX2, a general repressor of gene expression. GST pull-down experiments and co-immunoprecipitation assays further confirmed binding specificity. Furthermore, the N-terminal residues 1–79 of MSX2 are required for binding, whereas the C-terminal residues 201–267 of MSX2 do not play a critical role. Whereas the oncogenic potential of Tax in primary cells was only slightly affected by overexpression of MSX2, the other function of Tax, namely LTR-dependent transcriptional activation, was inhibited by MSX2 in human HeLa and bovine B-lymphoblastoid (BL3) cell lines. This MSX2 repression function can be counteracted by overexpression of transcription factors CREB2 and RAP74. The Tax/MSX2 interplay thus results in repression of viral transcriptional activation possibly acting as a regulatory feedback loop. Importantly, this viral gene silencing is not strictly associated with a concomitant loss of Tax oncogenicity as measured by its ability to immortalize primary cells. And interestingly, MSX2 also interacts with and inhibits the transactivation function of the related Tax1 protein encoded by the Human T-cell leukemia virus type 1 (HTLV-1).

Human T-cell leukemia virus type 1 (HTLV-1)[1] and bovine leukemia virus (BLV) are members of the *Deltaretrovirus* genus in the Retroviridae family (1–4). In addition to the structural proteins Gag, Pol, and Env, these viruses also encode a series of regulatory proteins: Tax, Rex, p12/R3, and p13/G4. The Tax protein is a transcriptional activator, which increases the synthesis of viral proteins acting on a triplicate 21-bp element located in the 5′ long terminal repeat (LTR) (5–7). Tax does not interact directly with DNA but rather acts via cellular factors, such as members of the CREB/ATF family of basic leucine zipper proteins (8–11). The *tax* gene is believed to be essential because its presence is absolutely required for infectivity *in vivo* (12). Besides its role in the regulation of transcription, the Tax protein also exhibits an oncogenic potential (13). Tax behaves as an immortalizing oncogene because it is able to cooperate with the Ha-ras oncoprotein to fully transform primary rat embryo fibroblasts (14, 15). Both transactivation and immortalizing functions of Tax can be dissociated by mutations in specific regions of the protein. For example, mutation of the phosphorylation sites at serines 106 and 293 of BLV Tax abrogates immortalization potential *in vitro* but maintains transcriptional activity and viral oncogenicity *in vivo* (16, 17). Conversely, the transactivation of the LTR promoter is not required for Tax to transform primary cells *in vitro* (14). The Tax protein of HTLV-1 (Tax1) is known to activate several cellular genes including IL-2, IL-2Rα, IL-3, TNF-α, and GM-CSF (18–20). Tax1 is also involved in cell cycle regulation by direct activation of cyclin D3 and cyclin kinases cdk4 and cdk6 (21), or by inactivating the cyclin-dependent kinase inhibitor $p16^{INK4A}$ (22). In fact, protein-protein interactions with cellular factors are crucial for Tax1 to perturb the regulation of many cellular pathways (23). These HTLV-1 Tax binding factors include the human mitotic checkpoint protein HsMAD1 (24), MEKK1 (25), the IκB kinase (26), or the PCAF protein (27). In contrast, little is known about the cellular partners of the BLV Tax protein. In this study, we describe a functional interaction between the homeobox protein MSX2 and the BLV Tax oncoprotein.

EXPERIMENTAL PROCEDURES

Plasmids—Plasmids pSGMSX2 and pEGFPMSX2 were constructed by subcloning the human MSX2-cDNA (kindly offered by Dr. T. Iimura, Tokyo Medical and Dental University, Japan) into pSG5 (Stratagene) and pEGFP-C1 (Clontech), respectively. Plasmids pcDNAMSX2-c-Myc, pcDNAMSX2ΔN-c-Myc, and pcDNAMSX2ΔC-c-Myc, which express respectively, C-terminal c-Myc-tagged full-length, amino acids 80–267 and amino acids 1–200 of the human MSX-2 protein, were constructed by inserting the human MSX2 cDNA, or PCR-derived MSX2 truncation mutants into pcDNA3.1/myc-HisB (Invitrogen). Plasmid pcDNARAP74-flag was obtained by subcloning the human RAP74 cDNA (kindly offered by Dr. Z. Burton, Michigan State University) into pcDNA3.1Flag (Invitrogen).

The pLTRLuc, pLTR1Luc, and pCMVLuc reporter constructs contain, respectively, the BLV LTR, the HTLV-1 LTR, and the cytomegalovirus (CMV) promoter upstream of the firefly luciferase gene. Vectors

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[1] The abbreviations used are: HTLV-1, human T-cell leukemia virus type 1; LTR, long terminal repeat; CMV, cytomegalovirus; PBS, phosphate-buffered saline; PKA, cAMP-dependent protein kinase; GST, glutathione *S*-transferase; BLV, bovine leukemia virus; REF, rat embryo fibroblasts; FITC, fluorescein isothiocyanate; IL, interleukin; CREB, cAMP-response element binding protein.

pSGTax and pSGTax1 express the BLV and the HTLV-1 Tax proteins under the control of the SV40 promoter. Plasmid pCMVTax, provided by E. Wattel (Centre Léon Bérard, Lyon, France), is a mammalian expression vector for HTLV-1 Tax. Plasmids pSGCREB and pPKA express, respectively, the bovine CREB2 protein and the protein kinase A (28). Plasmids pBDTax, pBDTax(106 + 293), p53, and pLaminC express the DNA binding domain (BD) of the yeast transactivator Gal4 fused to the BLV Tax protein, the Tax mutant in which serine residues 106 and 293 were replaced by alanine sequences (16), the murine p53 (amino acids 72–390), and the human Lamin C (amino acids 67–230), respectively. Plasmids pADMSX2 and pSV40 code for the Gal4-activating domain (AD) fused to the human MSX2 protein or the amino acids 84–708 of the SV40 large T-antigen, respectively. Vector pGexTax codes for a fusion protein between Tax and the glutathione *S*-transferase (GST) (29).

β-Galactosidase Assay—Yeast cells were co-transformed with pADMSX2 and pBDTax plasmids and grown overnight in synthetic dropout medium (S.D.) lacking uracil, leucine, tryptophan, and histidine (Ura-Leu-Trp-His-) but supplemented with 2 mM 3-aminotriazole (3AT). Cells were then diluted 5-fold in YPAD-rich medium (1% bacto-yeast extract, 2% bacto-peptone, 2% glucose, and 0.6% adenine) and cultivated until the absorbance at 600 nm (A_{600}) reached 0.5–0.8. After a wash in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM Mg_2SO_4), yeast were lysed by three freeze-thaw cycles in liquid nitrogen. Cell lysates were then resuspended in 0.7 ml of Z buffer containing 0.27% β-mercaptoethanol and 160 μl of ortho-nitrophenyl-β-D-galactopyranoside (ONPG) 4 mg/ml in Z buffer, as a substrate of β-galactosidase. After incubation at 30 °C, the reactions were stopped by addition of 400 μl of 1 M Na_2CO_3, and the A_{420} was determined. β-Galactosidase activity was calculated using the following equation: β-galactosidase units = $1000 \times (A_{420}/t \times V \times A_{600})$ where t = time (min) of incubation and V = volume (ml) of culture.

GST Pull-down Assay—The HB101 strain of *Escherichia coli* was transformed with plasmid pGexTax or, as a control, pGex-2T. Overnight cultures were 6× diluted in fresh NZY medium (21 g/liter) (Invitrogen) containing 100 μg/ml ampicillin and incubated until the A_{600} reached 0.7. After induction with 1 mM of isopropyl-β-D-thiogalactoside (IPTG), bacteria were allowed to grow for an additional 3 h. After harvesting and washing with PBS (1 mM KH_2PO_4, 150 mM NaCl, 3 mM Na_2HPO_4 pH 7.4), bacteria were lysed by sonication and by incubation at 4 °C in the presence of 1% Triton X-100. After centrifugation at 10,000 × *g* for 15 min at 4 °C, the supernatant was mixed with glutathione-Sepharose beads (Amersham Biosciences) over 1 h at 4 °C. Finally, the beads were washed four times with PBS and stored at 4 °C as a 15% suspension in the presence of a mixture of protein inhibitors (Complete, Roche Applied Sciences). The GST-Tax, or GST polypeptides bound to the beads were quantified using the Bradford method (Bio-Rad assay). For the pull-down assay, equal amounts of fusion proteins were added to 5 μl of rabbit reticulocyte lysates (TnT *in vitro* transcription-translation, Promega) programmed with pcDNAMSX2-c-Myc, pcDNAMSX2ΔN-c-Myc, pcDNAMSX2ΔC-c-Myc, or pSGMSX1 plasmids in the presence of a mixture of [^{35}S]methionine and [^{35}S]cysteine (Promix, Amersham Biosciences). After gentle shaking for 3 h at 4 °C in NETN binding buffer (200 mM NaCl, 20 mM Tris-HCl pH 8, 1 mM EDTA, and 0.5% Nonidet P-40), the beads were washed four times in binding buffer. Bound proteins were eluted in SDS sample buffer, resolved by 12.5% SDS-PAGE, soaked in amplifying solution (Enlightning, PerkinElmer Life Sciences), and visualized by autoradiography.

Co-immunoprecipitation—HeLa cells were cultivated at 37 °C in 5% CO_2/air-humidified atmosphere in minimum essential medium (MEM) with L-glutamine (Invitrogen) supplemented with 10% fetal calf serum, 100 units of penicillin/ml, 100 μg/ml of streptomycin, and 1 mM sodium pyruvate. One day before transfection, the cells were divided and seeded in 6-well plates at a density of 3×10^5 cells per well. The cells were next transfected with 2 μg of plasmid DNA using the Lipofectamine reagent (Invitrogen) as described by the manufacturer. Thirty hours after transfection, the cells were washed in PBS buffer, scraped, and lysed in NET buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.5 mM EDTA, 0.5% Nonidet P-40, and 0.25% sodium deoxycholate) containing protease inhibitors (Complete).

The lysates were immunoprecipitated with an anti-GFP antibody (Molecular Probes), or a preimmune antiserum coupled with protein A-Sepharose beads (Amersham Biosciences). The immunoprecipitates were washed three times in NET buffer and once in TNE (10 mM Tris-HCl, pH 8.3, 150 mM NaCl, and 1 mM EDTA) and electrophoresed on a denaturing 12.5% polyacrylamide gel (SDS-PAGE). After transfer of the proteins onto polyvinylidene difluoride, the membranes were saturated in 1% Blocking Reagent (Roche Applied Science), incubated overnight with anti-Tax (5A5) antibody, washed in TBST (50 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.1% Tween 20), and revealed by chemiluminescence using horseradish peroxidase-conjugated anti-mouse immunoglobulin antibody.

Immunoprecipitations were also performed under similar experimental conditions using reticulocytes lysates programmed with pSG-Tax1 and pcDNAMSX2-c-Myc. Anti-Tax1 (αTax3, provided by F. Bex, Université Libre de Bruxelles) and anti-c-Myc (A-14, Santa Cruz Biotechnology) monoclonal antibodies were used for immunoprecipitation and Western blot experiments, respectively.

Confocal Microscopy—Two micrograms of plasmids (pSGTax, pcDNAMSX2-c-Myc, and pSGTax+pcDNAMSX2-c-Myc) were transfected into HeLa cells using Genejammer (Stratagene). Twenty-four hours post-transfection, cells were fixed in 3.7% formaldehyde (20 min at 4 °C), permeabilized with 0.1% Nonidet P40 (10 min), incubated with anti-c-Myc (A-14, Santa Cruz Biotechnology) and anti-Tax antibody (5A5), and then with fluorescein (FITC) or Alexa 546-coupled anti-rabbit or anti-mouse immunoglobulin conjugates (Molecular Probes). After nuclear staining with TOPRO-3 and fixation with mounting medium (Prolong Antifade kit, Molecular Probes), the cells were analyzed using a Zeiss fluorescence confocal microscope (Axiovert 200 with LSM 510).

Luciferase Assays—One microgram of reporter plasmids pLTRLuc, pLTR1Luc, or pCMVLuc and different amounts of effector vectors (pSGTax, pCMVTax, pSGCREB, pPKA, pcDNARAP74-flag, pcDNAMSX2-c-Myc, pcDNAMSX2ΔN-c-Myc, pcDNAMSX2ΔC-c-Myc, pSGMSX1, and pSGMSX2) were transfected into 3×10^5 HeLa cells using Genejammer, 8×10^5 BL3, or Jurkat cells using TransIT-Jurkat (Mirus Bio). Twenty-four hours post-transfection, cells were washed three times with PBS, lysed, and luciferase activities were determined using the Promega luciferase assay kit according to the manufacturer's instructions.

Primary Rat Embryo Fibroblasts (REFs) Transformation Assay—REFs were transfected with 2 μg of plasmids (pSGTax, pSGMSX2, pSV_2NeoEJ, pSV_2Myc, see Ref. 13) using Effectene reagent (Qiagen) as described by the manufacturer. Forty-eight hours post-transfection, cells were collected, washed with PBS, and either cultured in the presence of 400 μg of G418 per ml to score for foci formation, or injected subcutaneously into thymus-less nude mice. A total of six mice in three independent experiments were injected for each plasmid combination. The tumor volume was calculated by the ellipsoid formula: $4/3\Pi ab^2$ where a and b are the length and width of the tumor, respectively.

TABLE I
Tax interacts with MSX2 in yeast

The PJ696α yeast strain was transformed with indicated plasmids as described under "Experimental Procedures." Cell colony viability was evaluated: dead (−), efficiently growing (+), or forming very small colonies (*). The strengths of the interactions were next measured by titration of the β-galactosidase units (ONPG assay). Values indicated are the means and S.D. of three independent experiments.

Vectors	Growth	β-Gal units
pBDTax + pADMSX2	+	20 ± 5
pBDTax(106 + 293) + pADMSX2	+	18 ± 4
pBDTax	*	3 ± 2
p53 + pADMSX2	−	−
pLaminC + pADMSX2	−	−
p53 + pSV40	+	36 ± 8

RESULTS

Identification of MSX2 as a Ligand for Tax—Out of a yeast two-hybrid screen, we previously isolated several clones interacting with BLV Tax (29) among which three of them corresponded to the homeodomain protein MSX2. To confirm the interactions of this primary screen, expression vectors for both partners were extracted and retransformed into yeast. The different clones were then tested for their ability to specifically activate a β-galactosidase reporter construct that can be induced only in the presence of both partners. As shown in Table I, yeast transfected with pBDTax and pADMSX2 were able to form colonies (+) and expressed the β-galactosidase enzyme (20 units). In contrast, yeast containing MSX2+p53 or MSX2+Lamin C failed to grow (Table I, −) whereas those expressing only the Tax protein formed small colonies

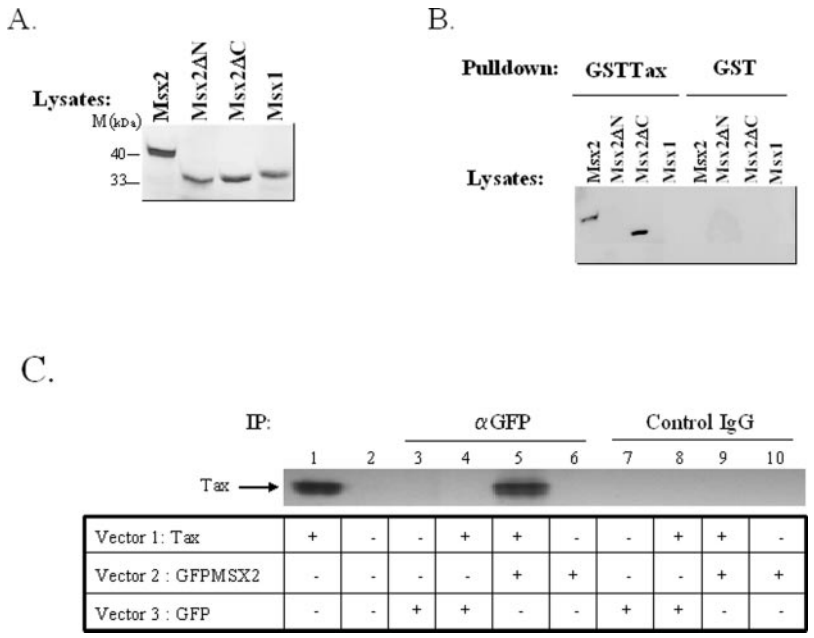

FIG. 1. **BLV Tax interacts with MSX2.** *A*, MSX2, MSX2ΔN, MSX2ΔC, and MSX1 proteins were synthesized using rabbit reticulocyte lysates in the presence of ^{35}S-labeled methionine and cysteine. Five microliters of the programmed lysates were then electrophoresed in a 12.5% SDS-PAGE gel, dried, and exposed to x-ray film. *B*, five microliters of lysates containing MSX2, MSX2ΔN, MSX2ΔC, or MSX1 were mixed with GST-Tax or GST protein bound to glutathione-Sepharose beads. The mixtures were incubated 3 h at 4 °C, washed extensively, and electrophoresed onto a 12.5% SDS-PAGE gel. [^{35}S]methionine-labeled MSX2 protein was then revealed by autoradiography. *C*, HeLa cells were co-transfected with plasmid pEGFPMSX2 expressing the GFPMSX2 protein and plasmids pSGTax for the BLV Tax protein. After 30 h, cells were lysed, and immunoprecipitation (*IP*) was then performed using rabbit anti-GFP antibody or, as control a rabbit antiserum (*Control IgG*). After extensive washes, the immunoprecipitates were used in Western blotting experiment using a mouse anti-Tax antibody (5A5). As negative controls, the same experiments were performed in parallel using lysates from HeLa cells transfected with pEGFP+pSGTax, pEGFP, and pEGFPMSX2. The amount of transfected DNA was maintained constant with plasmid pSG5.

(Table I, *) with a background enzymatic activity of 3 units. It thus appears that Tax, when bound to DNA, can generate weak but significant basal transcriptional activation of the reporter gene. As a positive control, yeast transformed by p53 and pSV40 expressed high levels of β-galactosidase (36 units). We conclude that BLV Tax specifically binds to MSX2 in yeast.

Tax and MSX2 Interact in Vitro and Coimmunoprecipitate in Cell Lysates—To assess the specificity of the interaction between BLV Tax and MSX2 *in vitro*, we performed GST pull-down experiments. To this end, BLV Tax protein was expressed as a fusion protein with GST using the pGexTax vector. On the other hand, MSX2, truncated mutants MSX2ΔN (lacking residues 1–79 of MSX2) and MSX2ΔC (lacking residues 201–267 of MSX2) and MSX1 (which has 51% identity with MSX2 in their N-terminal regions and 62% identity in their C-terminal regions) were synthesized in rabbit reticulocyte lysates in the presence of ^{35}S-labeled methionine and cysteine (Fig. 1*A*). The lysates containing the labeled MSX proteins were incubated with GST-Tax fusion protein bound to glutathione-Sepharose beads. The beads were then washed extensively in binding buffer, and the bound polypeptides subjected to SDS-PAGE analysis (Fig. 1*B*). MSX2, and its C-terminal-deleted mutant (MSX2ΔC) specifically bound to GST-Tax fusion protein but did not interact with GST alone. Under the same experimental conditions, MSX1 and MSX2ΔN mutant did not interact with GST-Tax (Fig. 1*B*). We conclude that BLV Tax binds to MSX2 *in vitro*, and that the N-terminal domain of MSX2 is required for the interaction.

To test whether BLV Tax and MSX2 interact in cell lysates, we first immunoprecipitated the MSX2 protein, and the presence of Tax in the complex was subsequently assessed by Western blot. For this purpose, two vectors pEGFPMSX2 (expressing a fusion protein between MSX2 and the green fluorescent protein) and pSGTax were transfected in HeLa cells and cultivated over 30 h. After cell lysis, proteins were immunoprecipitated using either rabbit anti-GFP or with a control rabbit antiserum (Fig. 1*C*). The immunoprecipitates were analyzed by SDS-PAGE, transferred onto a nylon membrane, and revealed with two Tax-specific monoclonal antibodies (5A5 and 6A7). A polypeptide of 34 kDa sharing antigenicity and co-migrating with the Tax protein (lane 1) was revealed in immunoprecipitates from lysates containing GFPMSX2 (lane 5) but not GFP alone (lane 4). As controls for specificity, Tax was not revealed when the cells were transfected with a control plasmid (lanes 2 and 3) or when the GFP antibody was omitted (lanes 7–10). It thus appears that Tax and MSX2 interact in HeLa cell lysates providing additional evidence for the specificity of their interaction.

Colocalization of Tax and MSX2—To assess the subcellular localization of Tax and MSX2 in mammalian cells we performed confocal microscopy. As demonstrated previously, MSX2 protein fused to GFP localizes mainly in the cell nucleus (29). To confirm and extend these observations, the MSX2 protein was tagged with the c-Myc epitope (in plasmid pcDNAMSX2-c-Myc). HeLa cells were transfected with this expression vector together with pSGTax coding for BLV Tax. Twenty-four hours post-transfection, cells were fixed, permeabilized, and incubated with antibodies against BLV Tax (5A5) and c-Myc (A-14). Finally, anti-Tax and anti-cMyc antibodies were revealed by Alexa-546- and FITC-coupled conjugates, respectively.

BLV Tax was localized in the nucleus (stained by TOPRO-3) as well as in the cytoplasm of the transfected cells, as revealed by the orange (Alexa 546) and red (TOPRO-3) fluorochrome profiles, two types of patterns were observed (Fig. 2, *Tax*). In contrast, MSX2-c-Myc protein was localized exclusively in the nucleus of HeLa cells (Fig. 2, *MSX2*). Tax and MSX2 co-localized in the nucleus, as revealed by the yellow color of the merged green and orange fluorochromes (Fig. 2, *Tax+MSX2*) and the perfect match of the profiles of the green and orange fluorochrome intensities (Supplemental Fig. 3). In addition, in Tax+MSX2 double positive cells, Tax was almost exclusively

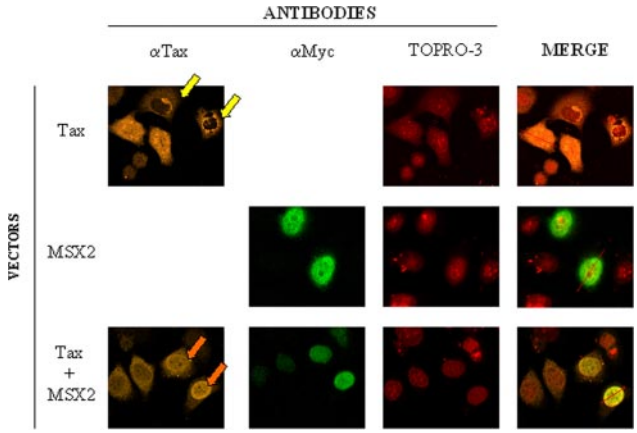

FIG. 2. **Co-localization between BLV Tax and MSX2 in HeLa cells.** HeLa cells were transfected with pSGTax (coding for BLV Tax), and pcDNAMSX2-c-Myc (expressing MSX2-tagged by c-Myc), as indicated. Twenty-four hours after transfection, cells were fixed, permeabilized, and labeled with Tax and c-Myc-specific antibodies and Alexa-546 or FITC-conjugated secondary antiserum. Finally, labeled cells were stained with TOPRO-3 and analyzed by confocal microscopy (Zeiss LSM510 coupled with an Axiovert 200 microscope). The *orange arrows* indicate differential localization of Tax in MSX2+Tax double positive cells whereas the *yellow arrows* depict Tax single positive cells.

concentrated in the nucleus (orange arrows). In contrast, in the absence of MSX2 (*e.g.* single Tax transfectants), Tax was also cytoplasmic (see yellow arrows). We conclude that the Tax subcellular distribution appears to be altered in the presence of MSX2.

MSX2 Does Not Interfere with the Tax-immortalizing Function in Vitro—The Tax protein exhibits an oncogenic potential in cell culture (13). Indeed, Tax cooperates with Ha-ras oncogene to transform primary REFs that are then capable of inducing tumors in nude mice (13, 15). Interestingly, a transdominant mutant of MSX2 has been shown to interfere with cell proliferation and transformation (30–32). To address the role of BLV Tax/MSX2 interaction in cell transformation, vectors expressing Tax and MSX2 (pSGTax and pSGMSX2) were co-transfected with the Ha-ras oncogene in REF cells. Three days post-transfection, cells were harvested and half of them cultured in the presence of G418 to select for stable transfectants. Cells co-expressing Tax and Ha-ras oncogenes form transformed colonies (foci) that can be counted from the culture media (Table II, Cell culture). The other collected cells were directly injected into nude mice, and the tumor volumes were determined 1 month post-injection (Table II). As a positive control for the REF transformation assay, co-expression of Myc and Ras induced formation of numerous foci (50 foci) and generated large tumors in nude mice (5000–7000 mm^3). As a negative control, REF cells co-transfected by Ras and an empty vector (pSG5) yielded only background levels. As expected, co-expression of Tax and Ras induced REF transformation (22 foci and tumors of 900–3000 mm^3), confirming our previous results (13). In contrast, MSX2 by itself is not tumorigenic in this system (0 foci and tumor volume $<$ 300 mm^3) further confirming and extending previous observations in NIH3T3 cells (32). Most importantly, co-transfection of MSX2 did not affect Tax transformation capacity (25 foci) although the tumor volume was slightly reduced (400–900 mm^3 *versus* 900–3000 mm^3). From these transformation assays, we conclude that MSX2 does not abrogate the ability of Tax to immortalize primary REF cells.

MSX2 Represses BLV Tax Transactivation—MSX2 is a general repressor of gene expression interacting with components of the basal transcriptional machinery, such as TFIIF (33). Indeed, MSX2 has been shown to inhibit the expression under

TABLE II

MSX2 does not interfere with the Tax immortalizing function in vitro

Primary REF cells were transfected with expressing vectors for indicated proteins and, as negative control, the empty vector pSG5. One-half of the cells were stably selected and transformed foci counted. The other half were injected into nude mice and tumor volumes calculated as described under "Experimental Procedures." The number of nude mice and the range of their tumor volumes are indicated.

DNA	Cell culture	Nude mice
Myc	50 foci (±7)	6/6 (5000–7000 mm^3)
pSG5	0	6/6 ($<$150 mm^3)
Tax	22 foci (±5)	6/6 (900–3000 mm^3)
MSX2	0	6/6 ($<$300 mm^3)
Tax + MSX2	25 foci (±4)	6/6 (400–900 mm^3)

the control of several promoters including the osteocalcin promoter (34, 35) and the SV40 promoter (36). Because MSX2 binds to Tax, we examined whether MSX2 could interfere with transcriptional activation of the viral LTR promoter. Therefore, we co-transfected HeLa cells with expression vectors for Tax (pSGTax) and MSX2 (pSGMSX2) together with the pLTRLuc reporter construct. This latter plasmid contains the BLV promoter cloned upstream of the firefly luciferase gene. Twenty-four hours post-transfection, the cells were harvested, and the luciferase activities were measured in the lysates. It appeared that, luciferase activity was gradually decreased by MSX2 in a dose-dependent manner (Fig. 3*A*). As illustrated for BLV Tax by a Western blot in *B*, inhibition of transactivation was not because of a decrease in the levels of Tax. We conclude that MSX2 very efficiently represses Tax transactivation of the LTR promoter. As expected, MSX2 also inhibited, but to a lesser extent, the cytomegalovirus promoter (Fig. 3*C*, *CMV-Luc*) further confirming the role of MSX2 as a general inhibitor of transcription. Because BLV infects B-lymphocytes, we performed similar transfection experiments using the bovine B-lymphoblastoid cell line (BL3). It appeared that MSX2 also inhibited Tax-dependent transactivation in BL3 cells (Fig. 3*D*). Together, these data demonstrate that Tax-dependent LTR transactivation activity is also inhibited by the homeodomain protein MSX2, similar to many cellular and viral promoters. Neither MSX1 nor the MSX2ΔN mutant unable to bind Tax (Fig. 1*B*) inhibited the Tax transactivation function, confirming the specificity of the MSX2 repression of Tax-dependent LTR transactivation (Fig. 3*E*).

Transcription Factors CREB2 and RAP74 Interfere with MSX2-dependent Repression of Tax Transactivation—To activate viral transcription, the Tax protein requires a 21-bp sequence (Tax-responsive element), which is repeated three times within the LTR (7, 37). These repeats share a motif resembling the cyclic AMP (cAMP)-responsive element (CRE) core sequence (TGACGTCA) (38). Tax does not bind directly to these motifs but interacts with CREB/ATF cellular transcription factors (8, 9). Among these, CREB2 induces LTR-dependent activation in the presence of PKA and activates Tax-directed transactivation (28).

To determine the role of MSX2 in this process, we co-transfected the pLTRLuc reporter vector together with expression vectors for Tax, CREB2, PKA, and MSX2 as indicated on Fig. 4. As expected, MSX2 represses Tax transactivation (Fig. 4*A*, compare *lanes 2* and *6*). Co-transfection of bovine CREB2 partially reverted MSX2 inhibition of Tax-transactivation (Fig. 4*A*, compare *lanes 2*, *3*, *6*, and 7). In the presence of bovine CREB2 and PKA, MSX2 completely lost its ability to inhibit Tax transactivation (Fig. 4*A*, compare *lanes 4* and *8*). Taken together, these data demonstrate that overexpression and activation of CREB2 by PKA reverts MSX2 repressor function.

It has been shown that MSX2 suppresses transcription through interaction with RAP74, the large subunit of TFIIF, and

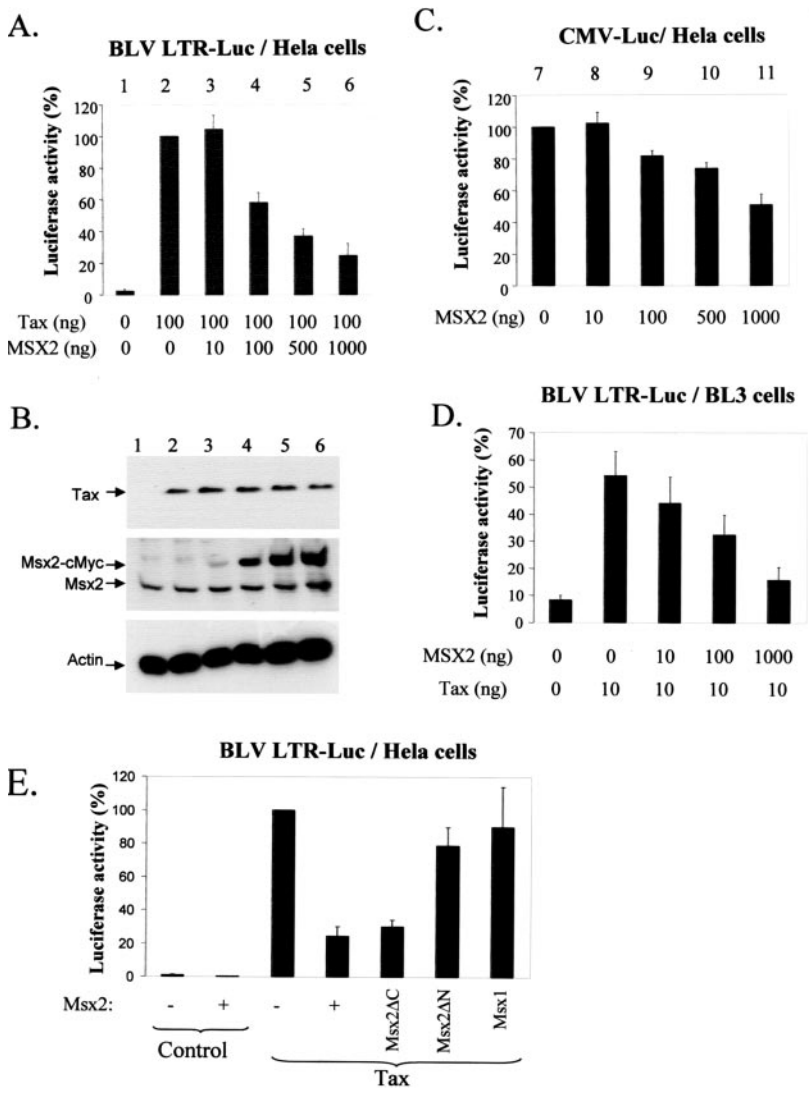

FIG. 3. **MSX2 inhibits Tax transactivation.** *A*, 1 μg of BLVLTR-Luc reporter plasmid, and, as indicated, different amounts of expressing vectors for MSX2 and Tax (pSGMSX2 and pSGTax) were transfected into HeLa cells. Luciferase activities were determined 24 h post-transfection. The data are the mean of relative luciferase activities of three independent experiments normalized to protein concentrations. *Error bars* represent S.D. *B*, Western blot analysis of transfected cells (30 μl equivalent to one-fifth of lysates corresponding to *lanes 1–6* of *A*) using anti-BLV Tax antibody, anti-Msx2 antibody, or anti-actin antiserum. *C*, 1 μg of CMV-Luc reporter plasmid and, as indicated, different amounts of expressing vector for MSX2 (pSGMSX2) were transfected into HeLa cells. Luciferase activities were determined 24 h post-transfection. The data are the mean of relative luciferase activities of three independent experiments normalized to protein concentrations. *Error bars* represent S.D. *D*, 1 μg of reporter plasmid BLVLTR-Luc, and, as indicated, different amounts of expressing vectors for MSX2, and Tax were transfected into BL3 cells by the *TransIT-Jurkat* procedure. Luciferase activities were determined 24 h post-transfection. The data are the means of relative luciferase activities of three independent experiments normalized to protein concentrations. *Error bars* represent S.D. *E*, 1 μg of BLVLTR-Luc reporter plasmid, and, as indicated, different expressing vectors for MSX2 (pcDNAMSX2-c-Myc, pcDNAMSX2ΔC-c-Myc, pcDNAMSX2ΔN-c-Myc), MSX1 (pSGMSX1), and Tax (pSGTax) were transfected into HeLa cells. Luciferase activities were determined 24 h post-transfection. The data are the mean of relative luciferase activities of three independent experiments normalized to protein concentrations. *Error bars* represent S.D.

that RAP74 is able to counteract the MSX2-repression of the osteocalcin promoter (33). To determine the role of RAP74 in MSX2 repression of the LTR promoter activation, we co-transfected pLTRLuc reporter vector together with expression vectors for Tax, MSX2, and increasing amounts of RAP74. As shown in Fig. 4*B*, RAP74 was able to reverse, in a dose-dependent manner, the MSX2 repression of LTR transactivation (Fig. 4*B*, compare *lanes 7–10*). To analyze the effects of RAP74 and Tax on MSX2 repression, we co-transfected the LTR reporter vector with expression vectors for RAP74, MSX2, and increasing amounts of Tax. We then calculated the percentage of MSX2 inhibition of LTR activity by comparing luciferase data from cell samples overexpressing MSX2 to those from cells lacking MSX2. As shown in Fig. 4*C*, MSX2 repression of LTR promoter activity gradually decreased in the presence of increasing amounts of Tax (Fig. 4*C*, compare *lanes 1–5*) and, as expected, the presence of RAP74 also interfered with MSX2 inhibition of the LTR activation (Fig. 4*C*, *lanes 6–10*). Taken together, these data demonstrate that overexpression of transcription factors CREB2 or RAP74 and the presence of high doses of Tax can independently interfere with MSX2 repressor function.

MSX2 Also Interacts with HTLV-1 Tax and Represses Its Transactivation Activity—To test whether MSX2 also interacts with HTLV-1 Tax, both proteins were synthesized separately in rabbit reticulocyte lysates using plasmids pSGTax1 and pcDNAMSX2-c-Myc in the presence of ^{35}S-labeled methionine and cysteine (Fig. 5*A*). Equal volumes (10 μl) of each lysate were incubated over 4 h, and the Tax1 protein immunoprecipitated using either the Tax1 antibody or a control antiserum. The immunoprecipitates were analyzed by SDS-PAGE and revealed by Western blotting with anti-c-Myc antibody. As shown in Fig. 5*B*, c-Myc-tagged MSX2 protein specifically co-immunoprecipitated with Tax1.

To examine whether MSX2 could also interfere with transcriptional activation of the HTLV-1 LTR promoter, we co-transfected HeLa or Jurkat cells with expression vectors for Tax1 (pCMVTax) and MSX2 (pSGMSX2) together with a HTLV-1 LTR luciferase reporter construct. Twenty-four hours post-transfection, the cells were harvested, and the luciferase activities were measured in the lysates. It appeared that MSX2 represses HTLV Tax1 transactivation in a dose-dependent manner (Fig. 5*C*).

Because BLV and HTLV-1 infect different cell types (*i.e.* respectively, B- and T-lymphocytes), we next compared the endogenous levels in a bovine B-lymphoblastoid cell line (BL3) and in Jurkat T-cells. In both cell lines, the endogenous MSX2 levels were similar (*lanes 1* and *3* of Fig. 6). In addition, the amount of MSX2-cMyc generated by plasmid transfection compared with the endogenous levels is about 2-fold higher in BL3 cells and 3-fold higher in Jurkat T cells (*lanes 2* and *4*, Fig. 6). We conclude that the homeobox protein MSX2 targets both BLV and HTLV-1 Tax oncoproteins and reduces their transactivation activities.

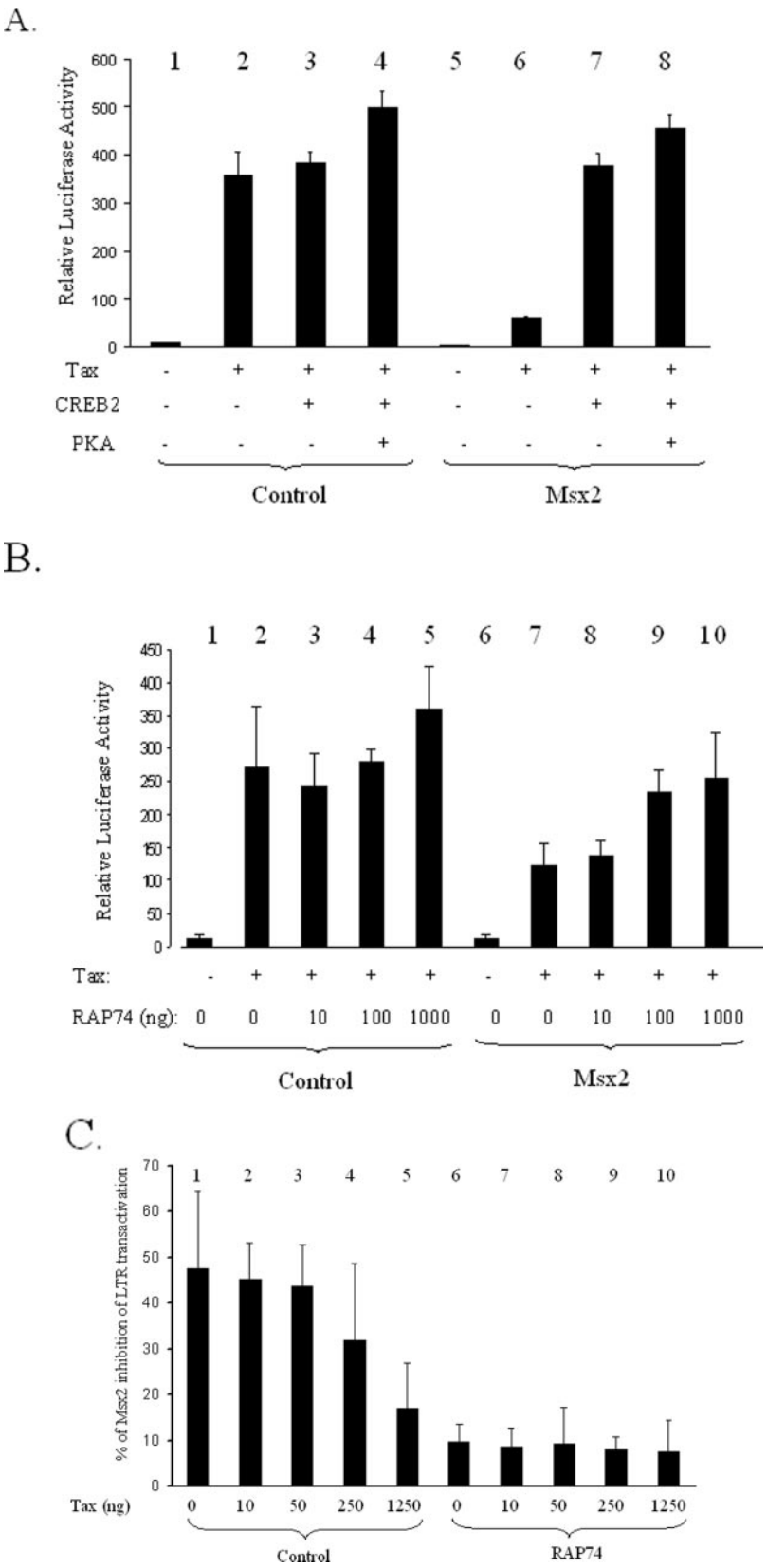

FIG. 4. **Transcription factors RAP74 and CREB2 interfere with the MSX2 repression of the LTR transactivation.** *A*, 1 μg of a reporter plasmid pLTR-Luc and different effector plasmids (pSGMSX2 for MSX2, pSGTax for BLV Tax, pSGCREB for bovine CREB2, and pPKA for protein kinase A) were transfected into HeLa cells. Luciferase activities were determined 24 h post-transfection. The data are the mean of relative Luciferase activities of three independent experiments normalized to protein concentrations. *Error bars* represent S.D. *B*, 1 μg of a reporter plasmid pLTR-Luc and, as indicated, increasing amounts of RAP74-expressing vector (pcDNARAP74-Flag) and two other effector plasmids (pcDNAMSX2-c-Myc for MSX2 and pSGTax for BLV Tax) were transfected into HeLa cells. Luciferase activities were determined 24 h post-transfection. The data are the mean of relative Luciferase activities of three independent experiments normalized to protein concentrations. *Error bars* represent S.D. *C*, 1 μg of a reporter plasmid pLTR-Luc and, as indicated, increasing amounts of Tax-expressing vector (pSGTax) and two other effector plasmids (pcDNAMSX2-c-Myc and pcDNARAP74-Flag) were transfected into HeLa cells. Luciferase activities were determined 24 h post-transfection. The luciferase activities data were normalized to protein concentrations and the percentage of MSX2-inhibition of the LTR transactivation was calculated by comparing samples in the presence or absence of MSX2. The data are the mean of the percentage of MSX2 inhibition of the LTR transactivation of three independent experiments. *Error bars* represent S.D.

DISCUSSION

BLV and HTLV-1 are closely related retroviruses sharing similar genomic organizations and affecting cells from the hematopoietic system. However, these two viruses infect different cell types (CD4 and CD8 T-lymphocytes for HTLV-1 and B cells for BLV) and are not subject to mutual cross-transmissions. The induced diseases are also different: ATL (adult T-cell leukemia) or TSP (tropical spastic paraparesis) for HTLV-1 and EBL (enzootic bovine leukemia) for BLV. However, for both viruses, the *tax* gene is thought to be a major actor in pathogenesis, and the aim of this report is to unravel the metabolic pathways involved in this process.

By means of the two-hybrid system, we identified MSX2 as a cellular partner specifically interacting with BLV Tax. Several

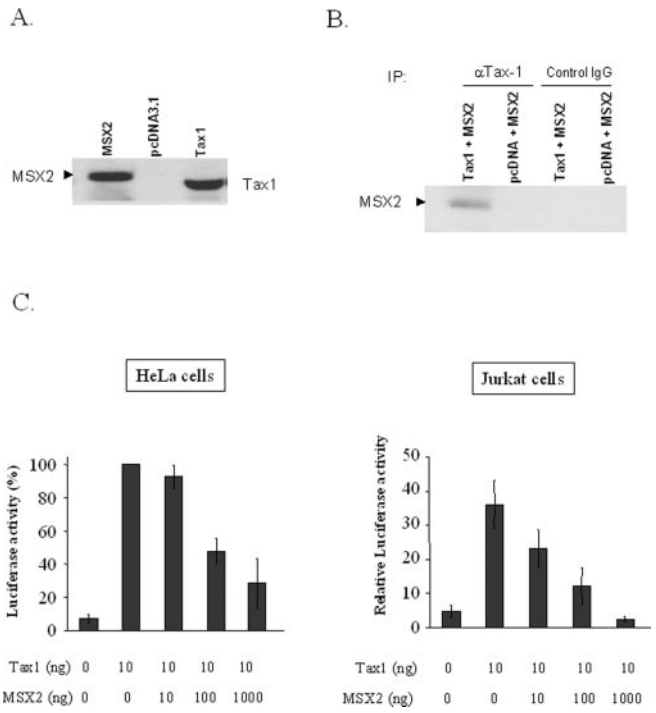

FIG. 5. **MSX2 interacts with HTLV-1 Tax and represses its transactivation activity.** *A*, MSX2 tagged by c-Myc and Tax1 proteins were separately synthesized using rabbit reticulocyte lysates in the presence of ^{35}S-labeled methionine and cysteine. Five microliters of the programmed lysates were then electrophoresed in a 12.5% SDS-PAGE gel, dried, and exposed to an x-ray film. The empty vector pcDNA3.1 was used as a negative control. *B*, 10 μl of each lysate containing *in vitro* translated Tax1 and MSX2-c-Myc proteins were mixed in 500 μl of NET buffer and incubated for 4 h at 4 °C. The Tax1 protein was then immunoprecipitated using specific antibody (αTax1) or a control antiserum (*Control IgG*). The immunoprecipitates were analyzed by SDS-PAGE, transferred onto a nylon membrane and revealed with anti-c-Myc antibody. *C*, 1 μg of reporter plasmid HTLV1LTR-Luc, and, as indicated, different amounts of expressing vectors for MSX2, and Tax1 were transfected into HeLa or Jurkat cells by the *GeneJammer* or *TransIT-Jurkat* procedures, respectively. Luciferase activities were determined 24 h post-transfection. The data are the mean of relative luciferase activities of three independent experiments normalized to protein concentrations. *Error bars* represent S.D.

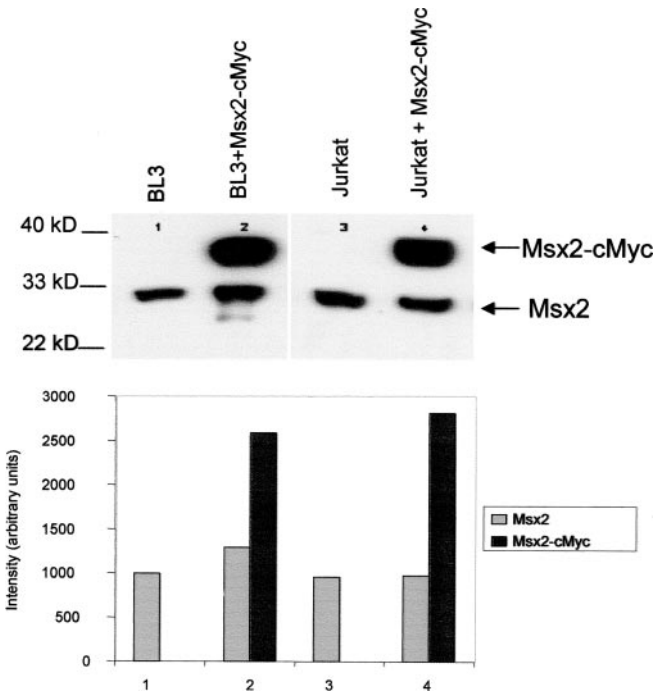

FIG. 6. **Expression of MSX2 in BL3 and Jurkat cell lines.** BL3 and Jurkat cell lysates were separated by SDS-PAGE and subjected to Western blot analysis using anti-Msx2 antibody (*lanes 1* and *3*, respectively). Lysates from BL3 and Jurkat cell lines transfected with 1 μg of pcDNAMSX2-c-Myc were analyzed in parallel on *lanes 2* and *4*, respectively. *Arrows* indicate transfected (Msx2-cMyc) and endogenous Msx2 proteins. The protein band intensities were measured using *Scion Image* tools (www.scioncorp.com).

lines of evidence support the specificity of interaction between Tax and MSX2. First, when the expression vectors for both partners were isolated and re-introduced into yeast, the resulting cells yielded high levels of β-galactosidase activity. It should be mentioned here that a weak signal was measured in yeast containing only the bait vector (Table I, pBDTax), further supporting the existence of an activation domain within Tax, as described previously (39). Other evidence for specificity for BLV Tax and MSX2 interaction have been obtained by two other independent techniques: GST pull-down and co-immunoprecipitations experiments (Fig. 1). Furthermore, we showed that the N-terminal residues 1–79 of MSX2 are required for binding, whereas the C-terminal residues 201–267 of MSX2 do not play a critical role in the interaction. The MSX gene family comprises three members (*MSX1*, *MSX2*, and *MSX3*). MSX1 and MSX2 are often co-expressed in tissues and share 98% of primary structure identity in their homeodomain, 62% identity in their C-terminal regions, and 51% identity in their N-terminal regions. We found that MSX1 did not bind Tax, further confirming the specificity of Tax/MSX2 interaction. Similar approaches extended these observations to HTLV Tax (Fig. 5). *In vivo*, BLV and HTLV infect different cell types (B and T lymphocytes, respectively). In this report, we showed that MSX2 affects both Tax proteins in the same way and that the MSX2 endogenous expression levels are comparable in B and T cell lines (Fig. 6). These data suggest that mechanisms involving similar cellular pathways are shared by the BLV and HTLV viruses.

MSX2 is a general negative regulator of gene expression known to interact with some components of the basal transcription machinery (33). Direct interaction with DNA is apparently not required for MSX2 suppressor function. MSX2 rather inhibits transcription via protein-protein interactions with components of the basal transcription machinery such as TFIIF (RAP74 and RAP30) (33). MSX2 belongs to a family of homeobox proteins, which have been implicated in epithelial-mesenchymal interactions during embryogenesis in various body tissues and their expression is often associated with high cellular growth potential (41–45). The MSX2 homeodomain protein has also been involved in the transcriptional mechanisms that regulate osteoblast proliferation, differentiation, and gene expression. MSX2 suppresses the osteocalcin promoter and induces craniosynostosis syndrome, characterized by precocious differentiation of calvarial osteoprogenitor (33, 46, 47). Most interestingly, overexpression of MSX2 was found in a large proportion of carcinoma cell lines and in several tumors (47). In addition, overexpression of antisense MSX2 cDNA interferes with cell transformation induced by the v-Ki-ras oncogene, suggesting its possible role in carcinogenesis (32).

Here, we have shown that the interaction between Tax and MSX2 results in an inhibition of LTR-directed gene expression in fibroblasts and lymphoid cell lines. Comparatively, transcription directed by the LTR was apparently more affected than that of other promoters like CMV (Fig. 3), SV40, and TRE (data not shown), suggesting a possible specificity of inhibition. Under these conditions, viral expression would thus be silenced without affecting the transcription of other cellular genes. We speculate about a possible mechanism allowing silencing of viral gene expression during activation and replication of the

host cell. Interestingly, MSX2 repression could be overcome by overexpression of CREB2 and the catalytic subunit of protein kinase A (Fig. 4*A*) In this context, several models are conceivable: (i) the CREB/PKA pathway activates a gene that inhibits MSX2, (ii) the CREB/Tax complex displaces MSX2 from the preinitiation complex, (iii) CREB and MSX2 have similar or overlapping recognition sites on Tax resulting in mutual exclusion of both proteins. In any case, we have shown that activation via the CREB/PKA pathway reverts MSX2 repression of Tax transactivation. It has been shown that RAP74 binds directly to the core suppressor domain of MSX2 and reverses inhibition of the osteocalcin promoter activity (33). Here, we extended these observations to the BLV LTR promoter activation by providing evidences that RAP74 can also interfere with MSX2-repression of BLV LTR-directed transactivation (Fig. 4, *B* and *C*). However, RAP74 and Tax require two different regions of MSX2 for binding (residues 132–148 and 1–79, respectively) and Tax does not influence RAP74/MSX2 interaction (Supplemental Fig. 1). Another major point of this report concerns the immortalizing potential of BLV Tax. We have indeed shown that MSX2 does not abolish the ability of BLV Tax to transform REF cells (Table II). It thus appears that MSX2 represses Tax-dependent transactivation but still permits cell transformation indicating that both functions of Tax are differently modulated by MSX2. It should be mentioned here that separable domains within the BLV Tax protein mediate transactivation and transformation (16). Indeed mutations in the zinc finger structure abrogate the capacity of Tax to activate viral expression without concomitant loss of immortalization (14). On the other hand, phosphoserines 106 and 293 of BLV Tax are required for *in vitro* transformation but not for transactivation (16). Dissociation of these two activities of Tax might be a major process which allows cell immortalization in the absence of viral structural gene expression. The Tax/MSX2 interplay may therefore play an important role in viral silencing and persistence as well as oncogenesis *in vivo*.

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