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A human T lymphotropic virus type I (HTLV-I) long terminal repeat-directed antisense c-*myc* construct with an Epstein–Barr virus replicon vector inhibits cell growth in a HTLV-I-transformed human T cell line

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A panel of EB virus replicon-based vectors was constructed to examine the relative utility of four distinct eukaryotic promoters for high-level gene expression in a HTLV-I-transformed human T cell line, HUT102. We found that HTLV-I LTR, which is trans-activated by the viral *tax* protein, was most suited for EBV vector-based stable gene expression in it. We prepared a HTLV-I LTR-directed antisense c-*myc* construct with an EBV vector. This antisense plasmid suppressed c-*myc* expression and inhibited growth of HUT102 cells in vitro with unaltered expression of *tax*. Non-specific plasmid toxicity was excluded by showing that the antisense construct had little effect on growth and c-*myc* expression of HTLV-I-negative Jurkat T cells, in which the viral LTR is expected to be less active. Our results indicate that c-*myc* may play an important role in the deregulated growth of HTLV-I-transformed T cells.

HTLV-I; EBV replicon vector; Gene transfection; Antisense RNA; c-*myc*

1. INTRODUCTION

HTLV-I is etiologically associated with ATL [1–3], and can immortalize primary human T cells in vitro [4,5]. HTLV-I does not carry a typical *onc* gene in its genome, but has a unique 3′ region designated as pX besides three common retroviral genes [6,7]. The pX region encodes the viral trans-activator, *tax* protein, which can transcriptionally activate its own LTR [8–10] as well as several cellular gene promoters [11–15]. Albeit many previous experiments have suggested that the *tax* protein contributes to the virus-associated immortalization and malignant transformation of T cells [11–19], the long latency of disease onset implies that the viral infection represents a necessary but an insufficient step by itself in leukemogenesis.

To analyze the roles of specific genes, including known oncogenes and suppressor genes, in the genesis of ATL and HTLV-I-mediated immortalization of human T cells, gene transfection strategies in HTLV-I-infected cells may offer powerful approaches. To date, however, there has been no systemic investigation about gene transfection and expression in such cells. We have therefore explored the use of EBV replicon as expression vector for achieving high-level sense and antisense RNA transcription in a HTLV-I-transformed human T cell line, HUT102. We describe a comparative functional analysis of a selected panel of eukaryotic promoters in it and utility of HTLV-I LTR for such purposes. We have also constructed HTLV-I LTR-directed antisense c-*myc* vector to dissect a biological role of c-*myc* in HTLV-I-transformed cells since it has never been directly examined whether c-*myc* is indispensable for the deregulated growth of HTLV-I-transformed, *tax*-expressing human T cells. This antisense plasmid has suppressed c-*myc* expression and inhibited growth of HUT102 cells in vitro. These results indicate that c-*myc* may play an important role in the growth regulation of HTLV-I-transformed T cells.

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Abbreviations: HTLV-I, human T lymphotropic virus type I; ATL, adult T cell leukemia; EBV, Epstein–Barr virus; RSV, Rous sarcoma virus; CMV, cytomegalovirus; LTR, long terminal repeat; CAT, chloramphenicol acetyltransferase; FCS, fetal calf serum; PBS, phosphate-buffered saline; mAb, monoclonal antibody; ECL, enhanced chemiluminescence.

2. MATERIALS AND METHODS

2.1. *Plasmid construction*

The selected promoters and CAT gene were assembled in a multicloning site of the EBV replicon-based vector p220.2 [20] as illustrated in Fig. 1; pE2/RSVCAT was constructed with RSV LTR derived from RSVCATα/220.2 (provided by M.L. Tykocinski; [20]); pE2/CMVCAT with the CMV immediate early promoter [21] from CDM8; pE2/HβAPrCAT with the human β-actin 5′ promoter region from pHβAPr-1-neo [22]; and pE2/HTLV-ICAT with the HTLV-I LTR from pCHL4 (provided by K. Sugamura; [10]); pE2/OCAT (pro-

moter-less CAT) was generated from pE2/RSVCAT by excising the promoter region.

An antisense human c-*myc* RNA expression vector was prepared as follows (Fig. 1). A 1.4-kb *Acc*II–*Ava*III fragment containing exons 2 and 3 of human c-*myc* cDNA was excised from pBRmyc (provided by T. Naoe). After attaching the *Hin*dIII linker to the blunted *Ava*III site, the fragment was ligated into the backbone fragment of *Hin*dIII–*Bgl*II-digested pMTV*dhfr* to generate pAMYC. A 2.2-kb *Hin*dIII–*Bam*HI CAT gene fragment in pE2/HTLV-ICAT was exchanged for a 2.3-kb *Hin*dIII–*Bam*HI fragment, containing the antisense c-*myc* and SV40 early splice and polyadenylation signals, of pAMYC to generate pE2/LAMYC.

2.2. *Cell culture and transfection*

HUT102, a HTLV-I-transformed human $CD4^+$ T cell line [1,23], and Jurkat, a HTLV-I-negative human T cell line, were cultured in RPMI 1640 with 10% FCS. Five million cells were transfected with 50 μg of vector DNA by electroporation (Gene Pulser; Bio-Rad, Richmond, CA) at 350 V in PBS. The cells were then seeded on 48-well culture plates at 1×10^5/well. Two days later, selection was begun with 250 μg/ml of hygromycin B (Calbiochem, La Jolla, CA).

2.3. *CAT assay*

CAT assay was performed as previously described [24]. Briefly, cells (5×10^6) were lysed in 100 μl of 250 mM Tris-HCl (pH 7.8) and cellular extracts (30 μl) were mixed with 0.1 μCi of [^{3}H]acetyl coenzyme A (DuPont-NEN, Boston, MA) and chloramphenicol. The mixtures were then overlaid with water-immiscible scintillation fluid (Econofluor; DuPont-NEN) in glass scintillation vials. After the enzymatic reaction proceeded at 37°C for 1 h, radioactive acetylated chloramphenicol diffusing into the scintillation fluid was detected by a liquid scintillation counter.

2.4. *Southern and Northern blots*

Total DNA was isolated by SDS-proteinase K digestion with extensive dialysis [24]. *Bam*HI and *Eco*RI digested samples (10 μg) were separated by electrophoresis on 0.8% agarose, transferred to a nylon membrane (Gene Screen plus; DuPont-NEN) and detected using the *Acc*II–*Ava*III fragment of c-*myc* cDNA labeled by random-primer extension (Amersham, Bucks, UK) with [α-^{32}P]dCTP. Hybridization and washing were performed as instructed by DuPont-NEN.

RNA was prepared using guanidinium thiocyanate-CsCl centrifugation [24]. Total RNA (15 μg) was electrophoresed on a 1.2% agarose-formaldehyde gel and transferred to a nylon membrane. To detect sense or antisense c-*myc* transcripts, a 0.36-kb *Cla*I–*Msp*I fragment of c-*myc* cDNA exon 3 was subcloned into the plasmid pBluescript SK(+) (Stratagene, La Jolla, CA), then [α-^{32}P]UTP-labeled sense or antisense RNA probes were prepared from the linearized plasmids using T7/T3 RNA polymerases. Membranes were hybridized with RNA probes and washed at 70°C twice in 2 × SSC, 1% SDS for 20 min each and twice in 0.1 × SSC, 1% SDS for 20 min each.

2.5. *Western blot*

Cells were lysed in SDS sample buffer. Samples were size-fractionated by SDS-PAGE (9%) and electroblotted onto PVDF membranes (Immobilon; Millipore, Bedford, MA). The protein blots were blocked in PBS with 5% non-fat dry milk. The membranes were reacted with anti-c-*myc* mAb CT14 (obtained from ATCC) or anti-p40*tax* mAb Lt4 (provided by Y. Tanaka; [25]) followed by incubation with peroxidase-labeled goat anti-mouse IgG antibodies (Cappel, West Chester, PA). The probed proteins were visualized using the ECL system (Amersham).

2.6. *Cell growth studies*

Logarithmically growing cells were pelleted, washed with medium and resuspended at a concentration of 1×10^5/ml in fresh medium containing 10 or 2% FCS. The cell suspension was portioned at 4×10^4/well into 48-well plates and cultured. At daily intervals thereafter, the viable cell number in each well was determined by Trypan blue exclusion in a hemocytometer.

3. RESULTS

3.1. *HTLV-I LTR provides high-level promoter activity in stably transfected HUT102 cells*

To develop high-efficiency transfection and an expression system in HTLV-I-infected T cells, we comparatively monitored the activity of selected eukaryotic promoters, the RSV LTR, the CMV immediate early promoter, the human β-actin promoter and the HTLV-I LTR with an EBV replicon-based vector in a HTLV-I-transformed human T cell line, HUT102 by stable CAT expression [20]. These constructs were transfected into HUT102 cells by electroporation. After 3 weeks in hygromycin B selection, the transfection efficiency was determined (Table I). The vectors carrying the CMV promoter or the HTLV-I LTR revealed high transfection efficiency, whereas others were similar to that of the promoterless construct.

CAT assays were performed by transfectants with various promoter-CAT/220.2 constructs (Fig. 2). Among 4 promoters, the HTLV-I LTR yielded the

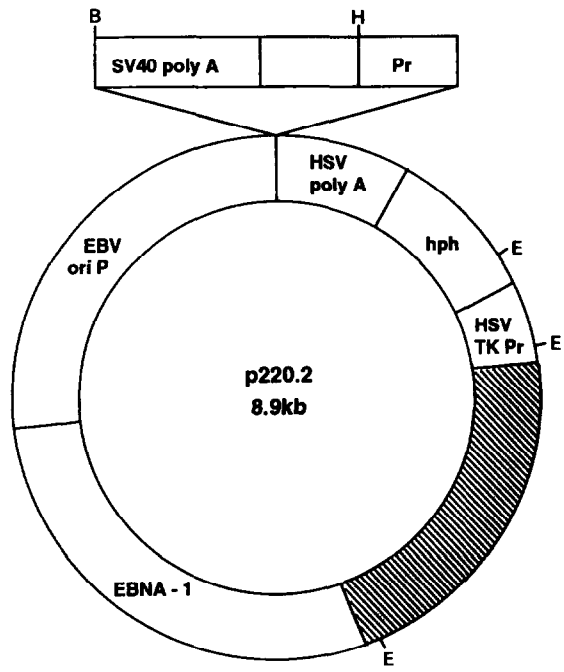

Fig. 1. Structure of the EBV replicon-based expression plasmid. Upper boxes represent an expression cassette consisting of various promoters (Pr) as detailed in the text, CAT or antisense c-*myc* gene (stippled box) and SV40 early splice and polyadenylatin signals (SV40 poly A). The expression cassette was inserted into the EBV replicon-based vector p220.2 containing the EBV origin of replication (EBV oriP), the EBV nuclear antigen 1 (EBNA-1), bacterial *hph* gene (*hph*), herpes simplex virus thymidine kinase gene promoter and polyadenylation signal (HSV TK Pr and HSV poly A), as well as pBR322-derived sequences for bacterial propagation (hatched box). E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII.

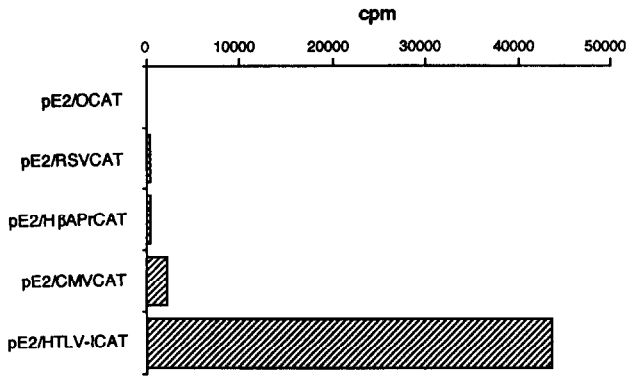

Fig. 2. CAT enzymatic activities in HUT102 cells stably transfected with p220.2 carrying various promoter-CAT constructs, HUT102 cells were transfected with 50 μg of each plasmid by electroporation. Pooled transfectants corresponding to each plasmid were used for CAT assay as described in section 2.

highest promoter activity. This was 100-fold more active than the RSV LTR, which is reportedly an efficient promoter in human cytotoxic T cell clones and therefore used in antisense RNA-mediated gene inhibition studies [20]. The CMV promoter, one of the most efficient eukaryotic promoters, was also 5-fold more active than the RSV LTR, but was much less active than HTLV-I LTR. The human β-actin promoter demonstrated low acetylation values. The strong promoter activity of the HTLV-I LTR was also supported at the transcriptional level by Northern analysis in other HUT102 transfectants harboring an antisense HTLV-I *env* construct similarly driven by each promoter (data not shown).

3.2. *The HTLV-I LTR-directed antisense* c-myc *construct reduces* c-myc *expression in HUT102 cells*

We used the HTLV-I LTR to express antisense c-*myc* RNA in HUT102 cells. The p220.2-based antisense c-*myc* RNA expression plasmid termed pE2/LAMYC with the HTLV-I LTR, was constructed as described in section 2. We also prepared a control plasmid, pE2/LACD4, which contained antisense CD4 (provided by H. Nakauchi) in place of c-*myc* in pE2/LAMYC. Plasmids pE2/LAMYC and pE2/LACD4 were stably introduced into HUT102 cells like pE2/HTLV-ICAT. The efficiency of transfection was equal for all these plasmids.

Table I

Transfection efficiency of the EBV-based vector carrying various promoter-CAT constructs in HUT102 cells*

	% of wells containing hygromycin B resistant colonies				
	OCAT**	RSVCAT	HβAPrCAT	CMVCAT	HTLV-ICAT
Exp.1	12	ND***	42	94	ND
2	2	8	10	96	90
3	4	25	10	ND	100

* HUT102 cells (5×10^6) were transfected with 50 μg of EBV replicon- based vector p220.2 containing each promoter-CAT expression cassette by electroporation and seeded into 48-well culture plates at 1×10^5/well. After three weeks in hygromycin B selection (250 μg/ml), the number of wells containing antibiotic resistant colonies was counted using an inverted microscope.

** Each promoter-CAT construct was inserted into p220.2.

***ND, not determined.

We established three antisense c-*myc* lines carrying a complete and unrearranged antisense c-*myc* expression cassette. Southern blot hybridization of total DNA with a human c-*myc* cDNA probe revealed only endogenous c-*myc* sequences in HACD4, the control bulk pE2/LACD4 cells (Fig. 3a). In LAMP2, LAMP5 and LAMC2 antisense c-*myc* lines, an additional 4.3-kb band, the expected size for antisense c-*myc* construct, was detected (Fig. 3a). The copy number of the antisense c-*myc* was estimated at 1–2 copies per cell.

To examine the expression of antisense c-*myc* RNA, Northern blotting was performed. Total RNA was hybridized with strand-specific RNA probes to identify sense or antisense transcripts. In addition to backgrounds due to ribosomal RNA, all three lines expressed 2.4-kb antisense RNA but expression levels were more prominent in LAMP5 and LAMC2 than in LAMP2 (Fig. 3b). No obvious reduction in the steady-state level of 2.4-kb endogenous c-*myc* mRNA was observed in antisense lines when compared with control HACD4 line (data not shown).

We then investigated expression levels of c-*myc* protein by Western blotting (Fig. 4a). It was conceivable that suppression of c-*myc* leads to alteration in total amount and distribution of cellular proteins. Thus, we

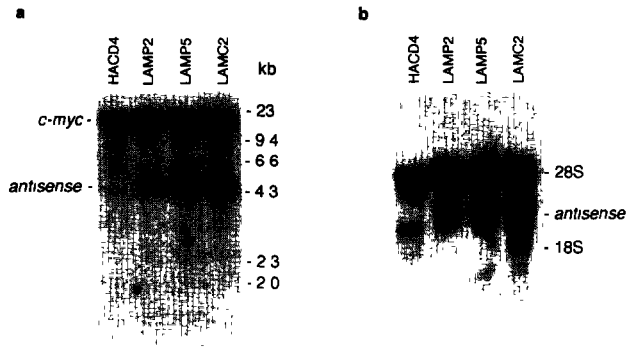

Fig. 3. (a) Southern blot of introduced antisense c-*myc* plasmid in HUT102 transfectants. Total DNA prepared from control antisense CD4 expression plasmid transfectant (HACD4) and antisense c-*myc* expression plasmid transfectants (LAMP2, LAMP5 and LAMC2) was digested with *Eco*RI and *Bam*HI and Southern blotted with a ^{32}P-labeled human c-*myc* cDNA probe. Upper bands represent an endogenous c-*myc* gene, whereas the lower bands of 4.3-kb indicate the presence of a complete and unrearranged antisense c-*myc* RNA expression cassette (see Fig. 1). (b) Expression of antisense c-*myc* transcripts in HUT102 transfectants. Total RNA prepared from control or antisense lines was analyzed by Northern blot hybridization with a ^{32}P-labeled RNA probe complementary to antisense c-*myc*.

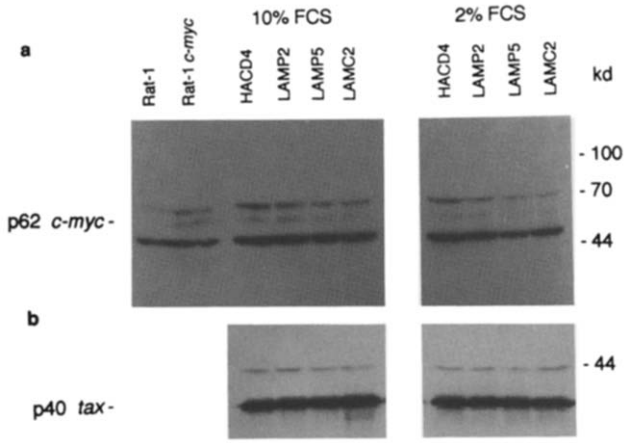

Fig. 4. Western blot of the c-*myc* (a) and *tax* (b) proteins in HUT102 transfectants. Control HACD4 and antisense c-*myc* lines LAMP2, LAMP5 and LAMC2 were cultured in medium with 10% or 2% FCS for 48 h, harvested and lysed in SDS sample buffer. Lysates equivalent to 5×10^5 cells were analyzed with mAb for c-*myc* or *tax*. Rat-1 and human c-*myc*-transfected Rat-1 cells were also included in Western blot of c-*myc* as negative and positive controls.

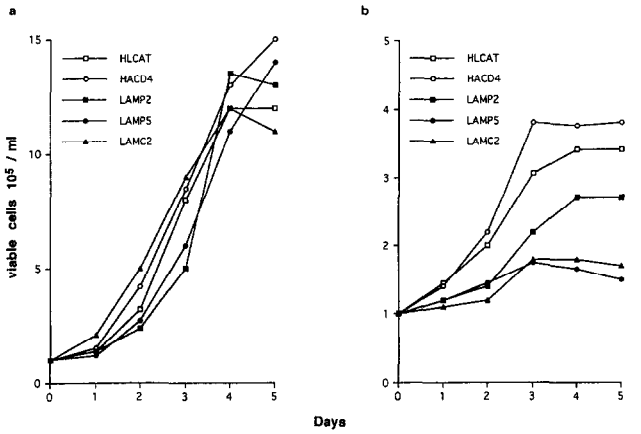

Fig. 5. Cell growth of control HUT102 lines HACD4 or HLCAT (pooled pE2/HTLV-ICAT transfectants) and antisense c-*myc* lines LAMP2, LAMP5 or LAMC2 in the presence of 10% (a) or 2% (b) FCS. Logarithmically growing cells in medium with 10% FCS were pelleted and resuspended at a concentration of 10^5/ml in fresh medium containing the indicated concentration of FCS. Cells were daily stained with trypan blue and counted in a hemocytometer. Similar results were obtained in three separate experiments.

adjusted loading dose of lysates for each lane by cell number rather than by total protein amount. When transfectants were cultured under 10% FCS condition, two antisense lines, LAMP5 and LAMC2, showed somewhat reduced c-*myc* protein levels compared to HACD4. A notable reduction in the steady-state level of c-*myc* protein was observed in these two lines when cultured in medium with 2% FCS (Fig. 4a). LAMP2 and the control HACD4 exhibited comparable c-*myc* protein levels under a similar 2% FCS condition. It is not clear whether two additional bands (70 kDa and 50 kDa) detected by anti-c-*myc* mAb CT14 are antigenically related to c-*myc*. Densitometric analysis showed that in LAMP5 and LAMC2, the intensity of c-*myc* bands decreased to 25% and 29% of that in the control HACD4 cells under 2% FCS condition whereas 50 kDa bands did to 62% and 84%, respectively. Although we do not know exact reasons for reduction in the levels of these additional bands, we believe that c-*myc* protein amount per cell specifically decreased in LAMP5 and LAMC2 antisense lines. Inconsistent loading of samples was excluded by reproducibility of results and also by unaltered *tax* protein levels in same samples. The levels of antisense RNA seemed to correlate with c-*myc* suppression (Figs. 3b and 4a). As described above, no difference of *tax* expression was observed between HUT102 transfectants with reduced and non-reduced c-*myc* expression, whether they were cultured in medium with 10% FCS or with 2% FCS (Fig. 4b).

3.3. *Inhibited cell growth of* c-myc-*suppressed HUT102 cells cultured under lowered serum conditions*

The growth characteristics of the transfectants were then investigated. In the presence of 10% FCS, the antisense lines LAMP2, LAMP5 and LAMC2 grew as well as the control lines HACD4 and HLCAT (pooled pE2/HTLV-ICAT transfectants) (Fig. 5a). The c-*myc*-suppressed lines, LAMP5 and LAMC2, however, showed apparently reduced-growth rates in medium containing 2% FCS. At day 5, viable cells in c-*myc*-reduced lines remained at about half that of the control lines (Fig. 5b). Reduced thymidine incorporation into the HUT102 cells with suppressed c-*myc* was also observed during the culture with lowered serum condition (data not shown). LAMP2 having normal c-*myc* protein with a moderate antisense RNA expression showed slightly reduced growth property (Fig. 5b).

3.4. *HTLV-I LTR-directed antisense* c-myc *construct does not influence growth of HTLV-I-negative Jurkat T cells*

It is known that HTLV-I LTR is strongly trans-activated by the viral *tax* protein. Thus, we monitored the effect of the viral LTR-directed antisense c-*myc* plasmid in HTLV-I-negative cells. We introduced pE2/LAMYC and control pE2/LACD4 into a HTLV-I-negative human T cell line, Jurkat, and obtained a control line and two antisense lines carrying a complete and unrearranged antisense c-*myc* expression cassette (Fig. 6a). These two lines showed a marginal change, if any, in c-*myc* expression and proliferation rate under 2% FCS condition when compared with control pE2/LACD4 line (Fig. 6b and c). These results indicate that HTLV-I LTR-directed antisense c-*myc* plasmid was less active in HTLV-I-negative cells and also was not non-specifically toxic.

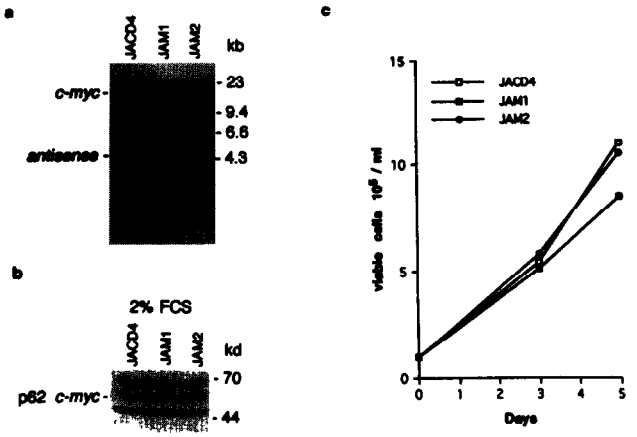

Fig. 6. (a) Southern blot of introduced antisense c-*myc* plasmid in Jurkat transfectants. Total DNA (10 μg) prepared from control line JACD4 (pE2/LACD4 transfectant) or antisense c-*myc* lines JAM1 and JAM2 (pE2/LAMYC transfectants) was Southern blotted with a human c-*myc* probe. (b) Western blot of c-*myc* protein in Jurkat antisense c-*myc* lines cultured in 2% FCS. Lysates equivalent to 1×10^6 cells were analyzed with mAb for c-*myc*. (c) Cell growth of Jurkat antisense c-*myc* lines in the presence of 2% FCS.

4. DISCUSSION

4.1. c-myc *may play an essential role in deregulated growth of HTLV-I-transformed T cells*

c-*myc*, one of proto-oncogenes, encodes a nuclear oncoprotein which contains both DNA binding and protein dimerization motifs and may also act as a transcription factor [26]. It has been observed that c-*myc* is important for cell growth, cell transformation, cell differentiation and also programmed cell death [27–33]. Several experiments using an antisense strategy have provided direct evidence confirming such a role for c-*myc* [28–31,33]. We decreased the c-*myc* expression of a HTLV-I-transformed human T cell line HUT102 with a HTLV-I LTR-directed antisense construct. The suppressive effect of antisense c-*myc* transcripts became more apparent when the cells were cultured at a lowered serum concentration (Fig. 4a). One explanation for this may be the fact that in HUT102 cells, reduced FCS concentration appeared to induce repressed expression of c-*myc* (Fig. 4a). On the other hand, expression of *tax*, which is transcribed from the viral LTR, was not affected by the reduced serum concentration (Fig. 4b), suggesting that LTR-directed antisense activity was essentially unchanged under similar conditions.

Previous reports showed that decreased c-*myc* expression by antisense RNA leads to increased serum dependency for growth of murine erythroleukemia cells [31] and *ras*-transformed NIH3T3 cells in vitro [33]. In our study, the c-*myc*-suppressed HUT102 lines (LAMP5 and LAMC2) displayed increased growth factor requirements for cell growth, while their growth in 10% FCS medium did not deteriorate too much (Fig. 5). Although growth suppression of antisense cells seemed to correlate with the c-*myc* protein levels, LAMP2 having comparable c-*myc* protein with a moderate antisense RNA expression showed slightly reduced growth property (Figs. 3b, 4a and 5b). It may be possible that antisense RNA partially influenced growth of this line, for reasons not clear at present. Non-specific toxicity of the plasmid was excluded by showing that the antisense construct had little effect on growth of HTLV-I-negative Jurkat T cells (Fig. 6), in which the viral LTR is expected to be less active.

The immortalizing and oncogenic potential of *tax* has been suggested by several investigators [16–19]. Similar to other viral nuclear proteins such as SV40 T and adenovirus E1a, cotransfection of *tax* (in place of c-*myc*) into rat embryo fibroblasts with the *ras* oncogene induced neoplastic transformation [18,27,34]. This finding suggests functional compatibility between c-*myc* and *tax*. Recently, M.P. Duyao et al. reported that *tax* activates murine c-*myc* promoter via NFκB sites [35]. They also described that *tax* can activate the human c-*myc* promoter [35]. On the other hand, stably introduced *tax* had no effect on endogenous c-*myc* expression in Jurkat T cells [36]. In our analysis, c-*myc*-suppressed and growth-inhibited HUT102 transfectants showed unchanged *tax* protein levels (Figs. 4 and 5). While this constitutive expression of the *tax* protein ensured antisense transcription from the viral LTR, these results proved the inability of *tax* to functionally replace c-*myc* in growing HUT102 cells. Expression of c-*myc* may therefore stay critical for growth of these cells. Further investigation about the role of c-*myc* in terms of *tax*-mediated immortalization and tumor development is ongoing in our laboratory.

4.2. *The utility of the HTLV-I LTR-directed stable expression vector with the EBV replicon in HTLV-I-infected cells*

The development of stable transfection strategies for directing high-level gene expression in HTLV-I-infected cells may offer a useful clue for analyzing the cellular physiology of virus-infected cells as well as for designing an effective gene therapy to eliminate HTLV-I-infected cells. The HTLV-I LTR is appropriate since it gave high acetylation values in stable CAT experiments (Fig. 2) and also showed sufficient level of antisense expression in HUT102 cells (Fig. 3b). Since the promoter activity of HTLV-I LTR increases to 50- to 100-fold in the presence of *tax* [8–10], the LTR may be suitable for achieving high levels of selective antiviral or antiproliferative gene expression in *tax*-expressing cells. In our investigation, HTLV-I LTR-directed antisense c-*myc* construct seemed to be less active in virus-negative Jurkat cells than virus-infected HUT102 cells, indicating a possible therapeutic application of our antisense strategy. Recently, similar in vitro approaches for the human immunodeficiency virus have been attempted using the HIV LTR-directed expression systems [37,38]. To-

gether, these results indicate the usefulness of the HTLV-I LTR-directed vector for attaining sense and antisense expression in HTLV-I-infected cells.

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