ANTISENSE AND VIRUS *TRANS*-ACTIVATOR DECOY APPROACHES OF INHIBITING REPLICATION OF HUMAN T-CELL LYMPHOTROPIC VIRUS TYPE 1 (HTLV-1)

BY

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V. Bratslavska, O., Ivanova, K., Kozireva, S., Kalnberza, G., Tomsone, V., Murovska, M. (2000) Malignant transformation of human non-lymphoid cell line, infected by HTLV-I. *Experimental Oncology*, **22**,110-117.

ABBREVIATIONS

A	Adenine
ACs	Asymptomatic carriers
AP-1	Activator protein-1
as	Antisense
ATF	Activating transcription factor
ATLL	Adult T-cell leukemia-lymphoma
BLV	Bovine leukemia virus
bp	Base-pair
C	Cytosine
cAMP	Cyclic adenosine monophosphate
CBP	CREB binding protein
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
CNS	Central nervous system
CRE	cAMP-response element
CREB	cAMP response element binding protein
CREM	cAMP response element binding protein modulator
CSF	Cerebrospinal fluid
CTL	Cytotoxic T lymphocyte
DCs	Dendritic cells
ds	Double stranded
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-lynked immunosorbent assay
env	envelope gene
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
G	Guanine
gag	Group antigen gene
GFP	Green fluorescent protein
GLUT-1	Glucose transporter 1
HAM/TSP	HTLV-1-associated myelopathy/tropical spastic paraparesis

HBV	Hepatitis B virus
HBZ	HTLV-1 basic region zipper factor
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
hnRNP	Heterogeneous nuclear ribonuclear protein
HOS	Human osteosarcoma cell line
HPV	Human papillomavirus
HSV	Herpes simplex virus
HTLV-1	Human T-cell lymphotropic virus type 1
IFA	Indirect immunofluorescence assay
IFN	Interferon
IgG	Immunoglobuline G
IL	Interleukine
IL2R	Interleukine 2 receptor
IκB	Inhibitor of NF-κB
kb	Kilobase
kDa	Kilodalton
LTR	Long terminal repeat
MAD	Mitotic arrest deficiency protein
MHC	Major histocompatibility complex
M-MuLV	Moloney Murine Leukemia Virus
MPSV	Myeloproliferative sarcoma virus
mRNA	Messenger RNA
MRP	Multidrug resistance protein
neo	Gene for the neomycin phosphotransferase
NF-AT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor κB
ODN	Oligodeoxynucleotide
ORF	Open reading frame
PBLs	Peripheral blood lymphocytes
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen

PCR	Polymerase chain reaction
PEI	Polyethylenimine
pol	Polymerase gene
Poly (A)	Polyadenosine tail
pRb	Retinoblastoma protein
R	Repeated
rex	Gene for regulation of expression
RNAi	RNA interference
RT	Reverse transcription
RT-PCR	Reverse transcriptase-polymerase chain reaction
RxRE	Rex-responsive element
SA	Syncytia assay
siRNA	Small interfering RNA
SRE	Serum responsive element
SRF	Serum response factor
STAT5	Signal transducer and activator of transcription 5
SV40	Simian virus 40
T	Thymine
TAR	*Trans*-activation response element
tax	gene for *trans*-activator
TCR	T-cell receptor
TGF-β	Transforming growth factor β
TK	Thymidine kinase
TNF	Tumour necrosis factor
TRE	Tax-responsive element
TREB	Tax-responding element binding protein
tRNA	Transfer RNA
$tRNA^{pro}$	Transfer RNA for proline amino acid
TXBP	Tax binding protein
U3	Unique 3'
U5	Unique 5'
VSV	Vesicular stomatitis virus

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INTRODUCTION

Despite vaccination, which has reduced the incidence of several viral infections (e. g. polio, mumps, measles), the treatment of diseases caused by many viruses, as retroviruses (e. g. HTLV/BLV group, human immunodeficiency virus - HIV), hepatitis B virus (HBV), hepatitis C virus (HCV), human papillomaviruses (HPV) and others, remains problematic. Molecular therapy is emerging as a potential strategy for the treatment of various diseases for which few known effective therapies exist. Specific inactivation of gene expression is an attractive approach for development of successful antiviral therapy. A number of strategies for inhibition of gene expression have been developed. Some, such as a triple helix forming or decoy transcription factor binding oligodeoxynucleotides seems to disrupt gene expression at the level of transcription. Others, such as antisense oligodeoxynucleotides (ODNs), small interfering RNA molecules and the use of ribozymes, attempt to disrupt expression at the level of mRNA translation (Kalota *et al.*, 2004). Ribozymes are known to catalitically cleave specific target RNA, leading to its degradation, whereas antisense molecules inhibit translation by binding to mRNA sequences on a stoichiometric basis. The more recently small interfering RNA (siRNA) has been shown to inhibit target gene expression. Over the past few years tremendous progress has been made toward using nucleic acids as therapeutic agents. Antisense (as) nucleic acids, complementary to certain chosen virus genome sequences have been proved to be efficient antiviral agents, possessing high specificity and low toxicity (Whitton, 1994; Varga *et al.*, 1997). Traditional approaches allow targeting of protein functions, whereas antisense therapy can be directed toward not only the protein-coding regions, but also against a different level of viral function, such as nucleic acid sequences that control replication, transcription, and translation. Nevertheless, the evaluation and use of nucleic acid drugs in control of diseases remains at an early stage of development. Clinical trials with antisense oligonucleotides have been already started. Gene therapy of viral infections, first introduced as intracellular immunization, may offer hopes for new treatments to be used alone, or in conjunction with, conventional drugs. Inhibition of virus replication by means of molecular therapy (RNA based technologies, viral transactivator decoy, suicide genes) has now been reported for numerous viruses, including such important human pathogens as HIV-1 (Michienzy *et al.*, 2002; Xing *et al.*, 2004; Lu *et al.*, 2004), HCV (Zhang *et al.*,

2004; Kronke *et al.*, 2004)), HBV (Xu *et al.*, 2003; Guha *et al.*, 2003), HPV (Alvarez-Salas *et al.*, 2003; Butz *et al.*, 2003; Clawson *et al.*, 2004), poliovirus, Coxsackievirus B3 (Yuan *et al.*, 2005) and influenza virus A. Presently much effort is devoted to development of anti-HIV therapy. Many different approaches of molecular therapy have been applied for HIV-1. This include the use of antisense RNA, siRNA, ribozymes and RNA decoy of HIV-1 *trans*-activator protein Tat as well as their combinations. Some of them have been used also in clinical trials. At 8th Conference on Retroviruses and Opportunistic Infections in Chicago (February 4-8, 2001) was reported about clinical trials, where asRNAs have been used for anti-HIV therapy. The continued expression of the anti HIV-1 antisense genes in HIV-1-infected subjects was detected in peripheral blood mononuclear, $CD4^+$ cells, and bone marrow aspirate $CD34^+$cell populations isolated post-infusion of the tranduced bone marrow $CD34^+$ stem cells (Liu *et al.*, 2001).

Successful treatment of HTLV-1 associated diseases requires inhibition of the viral transcription as well as pathology specific therapy. It might be possible to design gene therapy strategy that allow altogether avoid viruses and their drawbacks. Although some progress in the treatment and prophylaxis of HTLV-1 infection has been made, neither vaccine, nor satisfactory treatment of HTLV-1 associated diseases is currently available. Thus, the development of suitable theraupeutic means against HTLV-1 infection remains of great importance. Different molecular strategies depending on the nature of the pathological condition could be applied to inhibit certain virus. These include using of different types of nucleic acid compounds, administration routes applied and ways of delivery into a cell, therapy target choice, as well as combination of multiple targets for therapy simultaneously. To accumulate a needed experience for the practical application of modern nucleic acid technologies for the suppression of certain viruses (e. g. HTLV-1) more trials *in vitro* and especially *in vivo* should be performed.

The aim and objectives of the present study

The aim of the present study was to investigate antiviral effect of asRNA and viral *trans*-activator decoy sequences on HTLV-1 replication. In accordance with the aim, the main objectives were:

1) to establish convenient monolayer cell culture model for investigation of HTLV-1 infection;

2) to construct the plasmids carrying HTLV-1 sequences, suitable to study their effect on viral replication;

3) to study antiviral activity of asRNA genes targeted at LTR U3 and pX regions of HTLV-1 as well as virus *trans*-activator decoy sequences in cells persistently infected with the virus: a) in the established monolayer cell line infected with HTLV-1; b) in well described HTLV-1 producing lymphoid cell cultures: the lymphoid rabbit cell line Ra-1 and human T-cell line MT-2.

LITERATURE REVIEW

BIOLOGY OF HTLV-1

Human T-cell lymphotropic virus type 1 is an exogenous oncogenic retrovirus, which belongs to the *Deltaretrovirus* genus of the *Orthoretrovirinae* subfamily in the *Retroviridae* family as defined by the International Committee on the Taxonomy of Viruses (ICTV).

Structure of the virion

The mature virions are seen as spherical and enveloped C-type particles (Fig. 1) with a diameter of 110 to 140 nm (Poiesz *et al.*, 1980; Yoshida *et al.*, 1982). The host cell-derived membrane contains the glycoprotein spikes encoded by the viral *env* gene which encodes two protein components: a 21 kDa transmembrane protein and a receptor binding 46 kDa membrane surface glycoprotein (Ha *et al.*, 2002). The center of the HTLV-1 virion consists of a highly dense, spherical nucleocapsid containing two copies of the 9 kb genomic RNA (which bears all of the characteristics of eucariotic mRNA), the virus encoded enzymes - reverse transcriptase (Trentin *et al.*, 1998) and integrase (Bertola *et al.*, 2001), $tRNA^{pro}$ which is required as a primer for the initiation of reverse transcription, and the viral protease (Kobayashi *et al.*, 1991), which is responsible for the cleavage of HTLV-1 structural proteins.

Chronically infected cell lines could contain also defective proviruses and release different type particles. It was reported that in HTLV-1 transformed MT-2 cell line, which contains one complete provirus and seven defective HTLV-1 genomes, two distinct types of virions are released: the major "classic" type as described above and "light", containing chimeric Gag-pX protein p28, RT activity and the 3.4 kb RNA transcript (Morozov and Weiss, 1999).

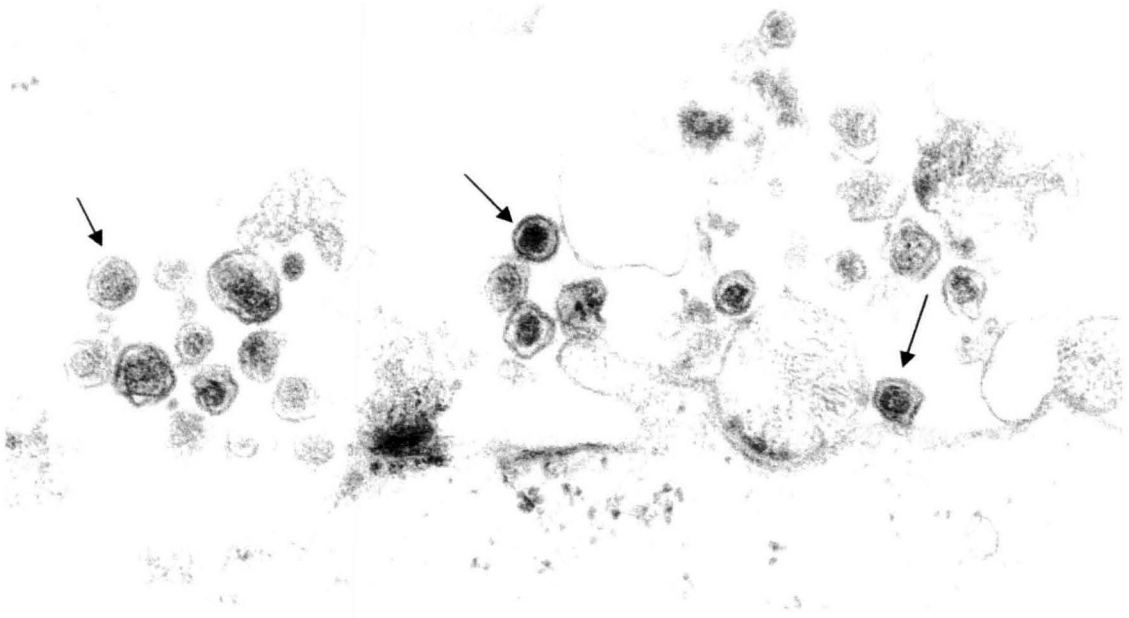


Fig. 1. Electron microscopic photograph of HTLV-1 particles in the HUT102 cell line (courtesy of M. Murovska). C-type retrovirus particles are indicated by arrows.

HTLV-1 life cycle

Major events in the viral replication cycle include adsorption and entry, reverse transcription, nuclear transport and integration, viral gene expression, and viral protein synthesis, processing, and assembly.

Efficient HTLV-1 entry into the host cell usually requires direct cell to cell interaction, although successful *in vitro* infections with cell-free virus particles have been documented in several cell lines (Clapham *et al.*, 1983; Ho *et al.,* 1984). Although the cell to cell spread of viruses has been documented for many years, this mode of viral propagation is less well understood than cell-free virus infection. In the last year publications new term – "virological synapse" is invented. First detailed description of such specialized virus induced cell to cell contact was published recently for the HTLV-1 (Igakura *et al.,* 2003; reviewed in Piguet and Sattentau, 2004).

After entry, reverse transcriptase within the viral capsid initiates the synthesis of the viral DNA by utilizing the single-stranded viral RNA as a template (Trentin *et al.*, 1998). It is important to note that the mutation rate of HTLV-1 during reverse transcription is about fourfold lower than that of another retrovirus HIV-1 (Mansky, 2000). The synthesized double-stranded proviral DNA is then transported into the nucleus where integration of the proviral DNA into the host genome proceeds with the assistance of viral integrase carried within the HTLV-1 virion (Bertola *et al.*,

2001). No specific HTLV-1 provirus insertion sites have been identified. HTLV-1 integration appears to take place randomly, preferentially in AT-rich transcriptionally active DNA regions (Leclercq *et al.*, 2000; Ozawa *et al.*, 2004).

After integration, the viral life cycle proceeds into the second stage which includes transcription of viral genes, translation of viral proteins, virion assembly, and virion release. All of these processes require participation of cellular transcription, translation, and transport machinery, as well as the assistance of a number of viral proteins. The integrated provirus can be passively spread to daughter cells following host cell division and can remain latent for prolonged period of time. Following cellular stimulation, the nature of which remains to be defined (Franchini *et al.*, 2003; Dumais *et al.*, 2003; Wycuff *et al.*, 2004), the provirus enters an active replication cycle which results in production of progeny virions. From the integrated provirus the cell transcribes viral mRNAs. Single–spliced (4.2 kb) and double-spliced (2.1 kb) subgenomic mRNAs and also the full-length (8.5 kb) viral genomic mRNA are transported to the cytoplasm where in the early phase the spliced mRNAs and in the late phase genomic RNAs are translated into viral proteins. The assembly of virions is still not clearly understood. As seen from electron microscopic images, the assembly of capsids with genomic RNA and budding are simultaneous. By budding through the cell plasma membrane the immature virus particles acquire a lipid bilayer membrane containing envelope glycoproteins. HTLV-1 Gag protein has a special PPPY and a PTAP motif, which play important role in viral particle assembly and release (Le Blanc *et al.*, 2002; Heidecker *et al.*, 2004). Typically, mature intracellular virions are not observed; rather, the crescent-shaped patches on the cell membrane where budding starts are the first visible forms. Final maturation steps in the newly released immature viral particles include processing of assembled polyproteins by the viral protease to yield fully infectious virions (Heidecker *et al.*, 2002). *In vitro* HTLV-1 virions are not efficiently released into the cell culture media from the infected cells. Also the low diversity found in HTLV-1 isolates could not be explained only by low mutation rate during reverse transcription. These facts together with other data are supporting the hypothesis that HTLV-1 replicates primarily as a provirus during cellular division, rather than via reverse transcription. Until recently, it was believed that HTLV-1 was largely latent also *in vivo*, as it is difficult to detect HTLV-1 mRNA, proteins or virions in a fresh blood. But the strong and chronically activated T-cell response to the virus indicates that HTLV-1 proteins are expressed persistently.

Continuous expression of the viral antigens would not be possible since such cells would be rejected by the host immune response. Therefore, it is reasonable to predict that a certain level of viral antigens (particularly Tax) is transiently expressed in a limited population of infected cells at one time and in another cell population at another time (Yoshida, 2001).

Genome structure and encoded proteins

The HTLV-1 genome contains elements common to many retroviruses, as well as genes unique to HTLV-1 (Fig. 2). The structural proteins, the virion-associated enzymes, and envelope proteins are encoded by the *gag* (group-specific antigens), *pol* (polymerase), and *env* (envelope) genes respectively, which are common to all known retroviruses. After translation into polyprotein, Gag is eventually cleaved into the 19 kDa matrix, 24 kDa capsid, and 15 kDa nucleocapsid proteins (Ha *et al.*, 2002). HTLV-1 protease (responsible for generating mature Gag products) is encoded by an open reading frame (ORF) that spans 3'end of *gag* to the 5'end of *pol*. HTLV-1 *pol* encodes enzymes that perform three distinct functions: reverse transcription, proviral DNA integration, and RNaseH digestion of RNA-DNA duplexes (Trentin *et al.*, 1998). The *env* gene encodes the 61-69 kDa viral membrane protein which after the series of posttranslational modifications forms a 21 kDa transmembrane protein and a 46 kDa membrane surface glycoprotein (Delamarre *et al.*, 1997).

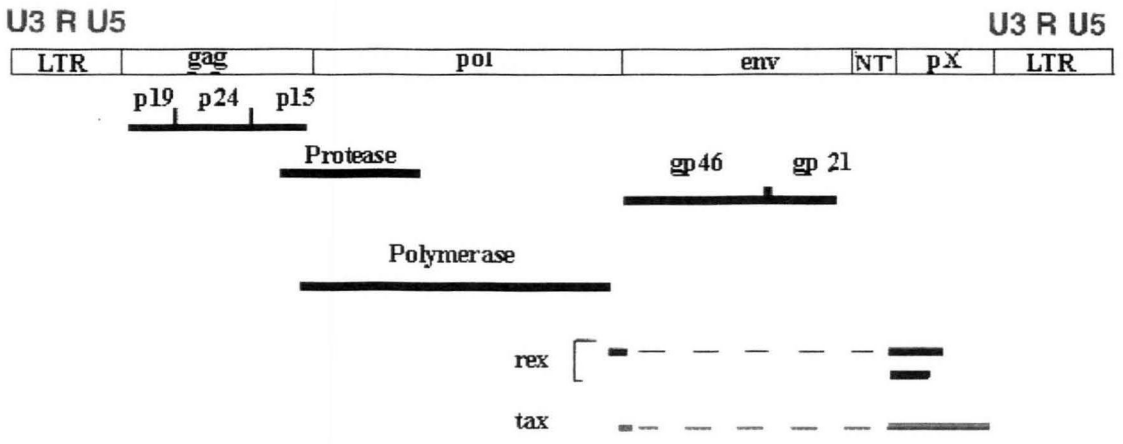


Fig. 2. Genomic structure of HTLV-1 proviral DNA (modified from Yao and Wigdahl, 2001). The viral mRNAs and the corresponding viral proteins are also shown. Dashed lines represent introns in the viral mRNAs.
The structural proteins, the virion-associated enzymes, and envelope proteins, are encoded by the *gag* (group-specific antigens), *pol* (polymerase), and *env* (envelope) genes respectively, which are common to all known retroviruses. The HTLV-1

genome is flanked at each end by long terminal repeat, LTR. Each LTR is composed of U3 (unique 3'), R (repeated) and U5 (unique 5') regions. LTR is important in regulating proviral gene expression as well as mRNA termination and polyadenilation. The U3 region contains three 21 basepair repeats which are responsible for Tax-mediated trans-activation of viral transcription. NT is a non-translated region. Two important viral regulatory proteins, Tax and Rex, are encoded by the pX region. Both are translated from doubly-spliced subgenomic mRNAs and are essential for the viral life cycle. The functions of other regulatory proteins ($p12^{I}$, $p13^{II}$, $p30^{II}$, not shown here), encoded by pX region are not clearly understood yet.

In addition to the structural *gag*, *pol* and *env* genes, HTLV genome has the unique regulatory region pX between the *env* gene and the 3' long terminal repeat (LTR) region. The pX region comprises at least four ORFs: X-I, X-II, X-III and X-IV (Princler *et al.*, 2003). Two important viral regulatory proteins, Tax and Rex, are encoded in the distal portion of this region. Both are translated from doubly-spliced subgenomic mRNAs and are essential for the viral life cycle (Seiki *et al.*, 1985). While the 27 kDa protein Rex is primarily encoded by the X-III ORF, the 40 kDa protein Tax is mainly encoded by the X-IV ORF. Rex (a nuclear phosphoprotein), modulates viral gene expression at the posttranscriptional level (Hidaka *et al.*, 1988). It increases the expression of viral structural genes *gag*, *pol* and *env* and inhibits the synthesis of Tax and Rex by promoting the nuclear export of nonspliced or singly spliced viral mRNAs (Inoue *et al.*, 1991). Tax is a viral transcriptional activator (also a nuclear phosphoprotein) and can dramatically increase viral gene transcription through interaction with the 5'LTR of the proviral genome. Tax can also interact with multiple cellular transcription factors and signal molecules to exert pleiotropic functions. In addition, a 21 kDa $p21^{Rex}$ protein is encoded by ORF X-III and X-IV (Berneman *et al.*, 1992). Although function of $p21^{Rex}$ is not clear yet, limited studies have suggested that it may act antagonistically with Rex (Kubota *et al.*, 1996).

ORF X-I and X-II can be transcribed into four different mRNAs by alternative splicing. ORF X-I transcripts can be either singly or doubly spliced, however both encode only one 12kDa protein - $p12^{I}$. $p12^{I}$ is a weak oncogenic protein that localizes to the endoplasmic reticulum and Golgi apparatus, it interacts with the interleukin 2 receptor (IL-2R) β and γ_c chains and activates signal transducer and activator of transcription 5 (STAT5) mediated transcriptional activity, and subsequently T-cell proliferation (Mulloy *et al.*, 1996; Bindhu *et al.*, 2004). It also affects calcium release

from the cells which in turn may activate nuclear factor of activated T-cells (NF-AT)-dependent transcription (Ding *et al.*, 2001).

Two protein species are derived from ORF X-II by two different mRNA splicing events. While the singly spliced mRNA yields 13 kDa protein $p13^{II}$, the doubly spliced mRNA encodes 30 kDa protein $p30^{II}$. A protein $p13^{II}$ has been shown to localize in the nucleus and mitochondria and affects mitochondrian membrane potential. Recent evidence demonstrated that $p30^{II}$ can regulate HTLV-1 replication through binding to Tax and Rex mRNA and reducing protein expression (Nicot *et al.*, 2004; Younis *et al.*, 2004).

Last, a protein of 31 kDa, HTLV-1 basic region zipper factor (HBZ), is encoded by 3'end antisense RNA (Gaudray *et al.*, 2002). HBZ forms heterodimers with cyclic adenosine monophosphate responding element (CRE) binding protein 2 (CREB2) and suppresses Tax- and CREB2-mediated transcription from the viral LTR (Gaudray *et al.*, 2002).

The sequences required for dimerisation of the 5' leader of the HTLV-1 RNA genome are located between the primer binding site (upstream) and the splice donor site (Monie *et al.*, 2004). The primary dimer initiation site of HTLV-1 has been located to a 14 nucleotide palindrome containing sequence, and dimerisation is shown to be dependent on specific sense-sense RNA interactions (Monie *et al.*, 2004)

LTRs. The HTLV-1 genome is flanked at each end by LTR. LTR is retroviral regulatory system essential to virus reverse transcription, integration and transcription. The two LTRs located at the 5' and the 3' ends of the provirus are identical in nucleotide sequence but different in function. They function as transcriptional initiator and terminator, respectively. Each LTR is composed of a U3 (unique 3'), R (repeated), and U5 (unique 5') region. The U3 region is important in regulating proviral gene expression as well as mRNA termination and polyadenilation. A particular feature of the HTLV U3 region is that TATA box, which determines the transcriptional initiation site, is located downstream of the polyadenosine tail - poly (A) signal. This arrangement prevents premature termination of the transcript in the 5' LTR. The functions of the R and U5 regions are not well known. They may play a role in posttranscriptional control of gene expression. A Rex-responsive element (RxRE) has been identified for HTLV-1 in the U3-R region of 3'LTR. The RxRE can form a highly stable complex stem-loop RNA secondary structure which is required for Rex responsiveness (Ballaun *et al.*, 1991). Independent

of its importance for Rex functioning, the RxRE also plays a role in the 3' processing of all viral transcripts (Bar-Shira *et al.*, 1991).

LTR U3 region harbour three imperfect tandem 21–bp repeats which are responsible for Tax–mediated trans-activation, collectively termed the Tax Responsive Element 1 (TRE-1) – and a sequence located between the central 21-bp repeat and the proximal repeat called Tax Responsive Element 2 (TRE-2) (Datta *et al.*, 2000). The three 21-bp repeat transcriptional enhancers act in a position and direction independent manner. Each 21-bp repeat contains three completely conserved domains designated A, B, and C from distal end to proximal end (Yao and Wigdahl, 2001) (Fig. 3.). These three domains comprise 13 nucleotides of the 21 bp repeat. Domain B contains the first five of eight bp (TGACGTCA) of the CRE [cyclic adenosine monophosphate (cAMP) response element] sequence, and is sufficient for the Tax-mediated trans-activation in combination with either domain A or domain C. At least two copies of 21-bp repeat sequences are required for significant transactivation by Tax (Yao and Wigdahl, 2001). Electrophoretic mobility shift analysis have shown that the DNA-protein complexes common to each 21-bp repeat consist of activating transcription factor/CRE binding proteins (ATF/CREB) family members - CREB, CREB modulator (CREM) protein, Tax responding element binding protein TREB5, ATF-1, ATF-2, ATF-4 or CREB2, whereas DNA–protein complexes unique to the promoter proximal repeat involved Sp1 and Sp3 transcription factors (Lundblad *et al.*, 1998; Gachon *et al.*, 1998; Datta *et al.*, 2000). In addition, AP-1 transcription factor components c-Fos and c-Jun derived from either the U-373 MG glioblastoma cell line or the monocytic cell line specifically bind the promoter central repeat (Wessner and Wigdahl, 1997). Barnhart and co-authors (Barnhart *et al.*, 1997) have clearly demonstrated competitive binding of Sp1 and CREB to the promoter proximal repeat. TREB5 (hXBP-1) protein is a transcription factor that also recognizes the CRE–like element in enhancers of HTLV-1 and MHC II gene and activates their transcription (Matsuzaki *et al.*, 1995). However, assembly of 21-bp-repeat –CREB-Tax complex itself may not be sufficient to initiate transcription. Additional cellular transcriptional factors which interact with the C-terminal trans-activation domain of Tax are required for transcriptional activation. Youn and coauthors showed that a cellular factor TAXREB803 (serum response-related protein) enhanced both - the Tax dependent transcription and the CREB binding to TRE in cooperation with Tax (Youn *et al.* 2003).

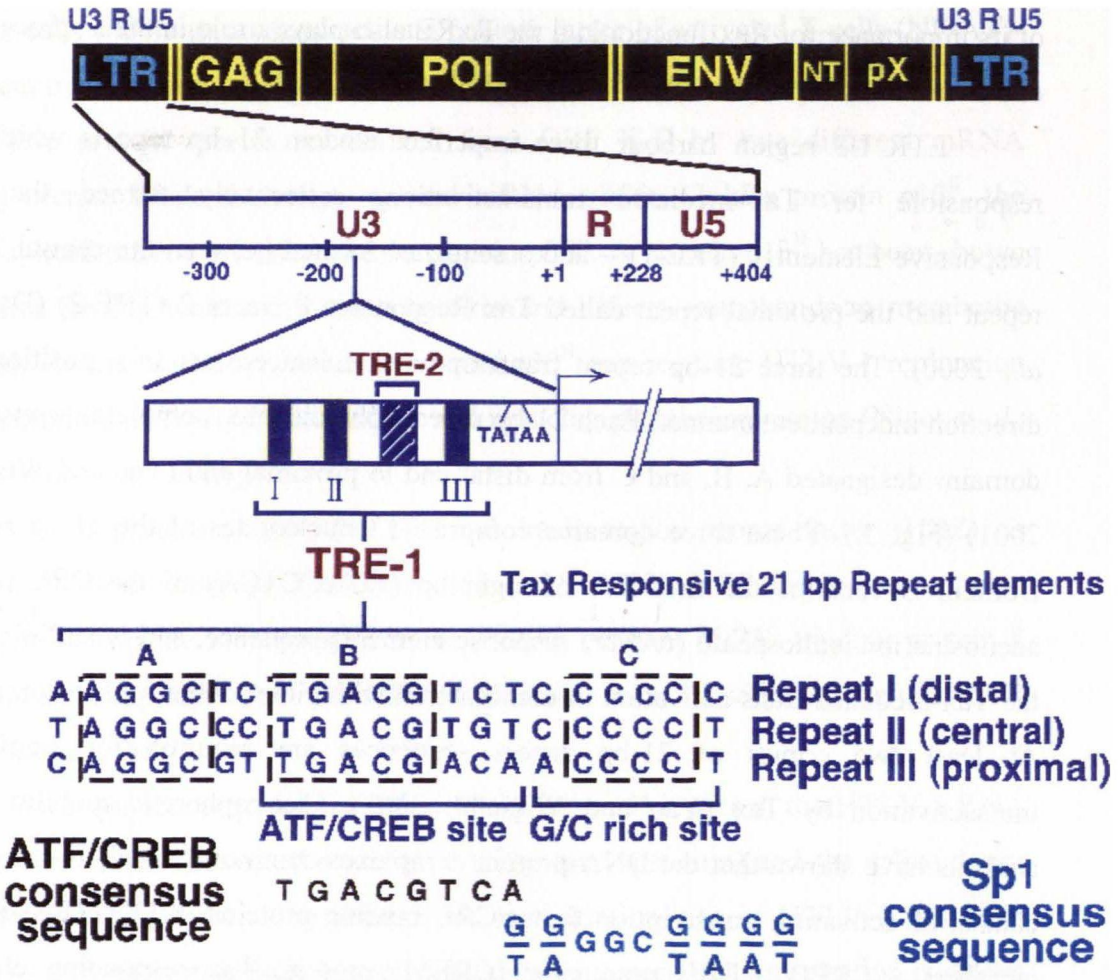


Fig. 3. HTLV-I LTR structure. The viral LTRs are located at the both ends of the viral genome. Viral transcription is regulated by the sequence within the U3 region of the 5' LTR. Three 21-bp Tax-responsive elements, which are collectively referred to as Tax-responsive element 1 (TRE-1), are positioned within U3 region of the LTR at positions -251 to -231, -203 to -183, and -103 to -83 relative to the start of transcription. In addition, a second Tax-responsive element 2 (TRE-2) is located between the promoter proximal repeat and the promoter central repeat. The nucleotide sequences of the three 21-bp repeats as well as ATF/CREB and potential Sp1 binding sites are also illustrated (Yao and Wigdahl, 2001).

The viral oncoprotein - Tax

The virally encoded oncoprotein Tax has been implicated in HTLV-1-mediated cellular transformation. The exact mechanism by which this protein contributes to the oncogenic process still remains to be clarified. Generally, viral oncogenes are not required for their replication; however Tax is essential for gene expression and replication of HTLV-1. Also in case of some other oncogenic viruses viral encoded proteins, such as T antigen of SV40 virus and poliomavirus, E1A protein of adenoviruses, and E6/E7 proteins of papillomaviruses, are required for viral replication and modify cellular regulation through multiple mechanisms (Helt and Galloway, 2003; Duensing and Münger, 2003; Al Moustafa *et al.*, 2004).

Tax is 40 kDa phosphoprotein mainly localized in the nucleus and originally identified as a transactivator of viral gene expression responding to the 21-bp enhancer in the LTR (Suzuki *et al.*, 1993). To activate the viral LTR, Tax requires at least two copies of the 21-bp enhancer containing an imperfect CRE to which binds CREB/ATF family of transcription factors (Shimotohno *et al.*, 1986). The interaction of Tax with CREB and the CRE in the LTR results in a CRE-CREB-Tax ternary complex (Tie *et al.*, 1996). The mechanisms by which the HTLV-1 LTR is transactivated by Tax are not yet fully appreciated. It has been a long held position that Tax does not bind to the viral DNA directly but, rather it exerts its effect by specific interactions with cellular intermediaries. However, recent evidence suggests that Tax does directly interact with the enhancer GC-rich DNA motifs (Lundblad *et al.*, 1998). Tax and cellular transcription factors compete for the co-activators - CREB binding protein and p300 (CBP/p300), and their expression levels and affinity to CBP/p300 affect the efficiency of transcription (Georges *et al.*, 2003).

The related proteins CBP and p300 are transcriptional co-activators that act with other factors to regulate gene expression and play roles in many cell-differentiation and signal transduction pathways (Kawasaki *et al.*, 1998). Both proteins have intrinsic histoneacetyltransferase activity and may act directly on chromatin, of which histones are a component, to facilitate transcription (Kawasaki *et al.*, 1998).

Tax exerts: a) trans-activation and –repression of transcription of different sets of cellular genes through binding to groups of transcription factors and co-activators (Ballard *et al.*, 1988; Fujii *et al.*, 1994; Armstrong *et al.*, 1993), b) dysregulation of cell cycle through binding to inhibitors of cyclin-dependent kinases 4 and 6

(CDK4/6): $p16^{INK4a}$, $p15^{INK4b}$, and interaction with the human orthologue of mitotic arrest deficiency protein 1 (MAD-1), designated also as Tax binding protein 181 (TXBP181) (Jin *et al.*, 1998; Schmitt *et al.*, 1998; Suzuki *et al.*, 1996) and c) inhibition of some tumor suppressor proteins (p53) (Pise-Masison *et al.*, 2000). The target molecules are CREB, CREM, NF-κB (nuclear factor κB), IκB (inhibitor of NF-κB), SRF (serum response factor) and CBP/p300 (activation of genes); E47, p53 and CBP/p300 (repression), $p16^{INK4a}$ $p15^{INK4b}$, $p18^{INK4c}$, MAD-1 (cell cycle promotion) (Jeang *et al.*, 2004; reviewed in Yoshida, 2001 and in Marriott *et al.*, 2002).

Transcriptional activation is basically achieved by two independent mechanisms: the first is through direct binding of Tax to transcription factors such as CREB, NF-κB and SRF in the nucleus and, the second is specific to activation of NF-κB in the cytoplasm. The first is a general mechanism for three different enhancers and explains well how Tax is able to respond to structurally unrelated enhancers. The second is the inhibition or destabilization of inhibitors of transcription factors such as IκB in cytoplasm, which results in promoting of NF-κB transport to the nucleus. Tax directly interacts with NF-κB family members (NF-κB1 p50, NF-κB2 p52, NF-κB p65) and acts cooperatively with NF-κB p65 or c-Rel to augment the expression of the promoters containing NF-κB binding sites (Bex *et al.*, 1997; Robek and Ratner, 1999). Therefore, Tax can modulate NF-κB through a number of distinct processes leading to activation of gene expression.

Tax enhances gene expression also via direct interaction with SRF and promoters containing a serum responsive element (SRE). When Tax is present the transcription of the promoters containing a SRE site [e. g. the *c-fos*, the *fos related antigen 1* (*fra-1*), the *early growth response gene 1* and *2* (*egr1* and *egr2*) oncogenes] can be activated without mitogenic signals (Fujii *et al*, 1994).

Another less studied aspect of Tax is its stimulation of AP-1 transcriptional activity, which is very important for T-cells because AP-1 is one of several transcription factors involved in activation of IL-2 promoter (Mori *et al.*, 2000).

NF-κB pathway is a central regulatory pathway for the growth and survival of T-cells. Tax has been shown to transcriptionally activate the promoters of IL-2 and IL2Rα chain through NF-κB pathway (Maruyama *et al.*, 1987). Similarly, transcription of IL-15 and the IL-15Rα chain is increased by Tax through NF-κB (Azimi *et al.*, 1998; Mariner *et al.*, 2001). Tax also activates IL-12, IL-4, IL-6, IL-8

and IL-10 through NF-κB pathway (Li-Weber *et al.*, 2001; Mori *et al.*, 1998_a; Mori *et al.*, 1998_b); Yamashita *et al.*, 1994). The number of cytokines and receptors transcriptionally activated by Tax is likely to increase over time.

Through repression of DNA polymerase β Tax influence cellular base excision repair and through activation of expression of proliferating cell nuclear antigen PCNA – Tax affect nucleotide excision repair (Jeang *et al.*, 1990; Kao *et al.*, 1999; Lemoine *et al.*, 2000). The hypothesized mechanism of transrepression is direct competition by Tax for E-box binding protein E47 recruitment of CBP-p300 (Suzuki *et al.*, 1999). The E-box motif is present in the promoter region of β-polymerase gene, thyrosine kinase Lck, p53 and $p18^{INK4c}$. Recent data suggest that Tax represses the expression of human telomerase (hTert) (Gabet *et al.*, 2003). Tax has also recently been shown to repress expression of transforming growth factor β 1 (TGF-β1) (Arnulf *et al.*, 2002). This finding may have important implications in HTLV-1 pathogenesis since TGF-β is a potent inhibitor of T-cell proliferation and cytotoxicity.

Tax may enhance, through the NF-κB pathway, the activity of a kinase that phosphorylates p53 on serine residues 15 and 392, which in turn may impair p53 interaction with the transcription factor IID (TFIID). Another possibility for p53 inactivation is direct competition between Tax and p53 recruitment of coactivators CBP/p300, perhaps in cells where CBP/p300 is limiting in amount (Pise-Masison *et al.*, 2000). Stabilization of p53 is associated with failure of HTLV-1-infected cells to arrest in G1 phase of cell division after DNA damage. Promotion of G1/S transition may prevent cells from pausing and repairing DNA.

The combined effects of Tax on base excision repair, nucleotide excision repair, DNA end stability, telomerase, and cell cycle progression create a setting in which repair of mistakes is compromised. These combined dysregulation might explain the observed 2.8 fold increase in genomic mutation frequency in HTLV-1 infected cells (Miyake *et al.*, 1999).

Tax now appears to interact with many cellular proteins, and certainly, more will be found. For instance, 32 proteins associated with Tax were identified by Wu and coworkers (Wu *et al.*, 2004). Many of these proteins belong to the signal transduction and cytoskeleton pathways and transcription/chromatin remodeling (Wu *et al.*, 2004).

A surprising aspect is that a single protein, Tax, is able to affect so many targets, mostly directed to cell proliferation. It could be speculated that Tax may mimic a few possible molecules that coordinate divergent and redundant regulatory machinery for cell proliferation and differentiation. In summary, most of the pleiotropic functions of Tax protein cooperate in promoting cell proliferation, accumulation of DNA damage, and avoiding apoptosis of abnormal cells infected with HTLV-1.

Target cell and receptor

HTLV-1 preferentially targets and transforms $CD4^+$ T-lymphocytes (Collins *et al.*, 1996). Also B cells and cells of monocytic/macrophage lineage was found to be infected *in vivo*, nevertheless $CD4^+$ appear to be the most permissive cells for both viral replication and transformation (Koyanagi *et al.*, 1993). HTLV-1-infected monocytes/macrophages may contribute to the persistent infection in tissues, including central nervous system (CNS) and joints. CNS contains other cells of monocyte/macrophage lineage, e. g. microglial cells, which are not derived from circulating monocytes and could be also infected by HTLV-1. The role of HTLV-1 positive B cells is unknown. Studies with vesicular stomatitis virus (VSV) and defective HIV pseudotypes bearing the envelope glycoproteins of HTLV-1 showed that there is a broad range of cells susceptible to pseudotype infection (Hoshino *et al.*, 1985; Sutton and Littman, 1996). Thus, the expression of HTLV receptors is not restricted to lymphoid cells, because many cell types derived from diverse mammalian species are permissive for HTLV-1 adsorption and penetration (Tatcno *et al.*, 1984; Yamamoto and Hinuma, 1985). HTLV-1 can infect a wide variety of cells *in vitro*, also some non-lymphoid cell lines as human osteosarcoma cell line (Clapham *et al.*, 1983). Co-cultivation of HTLV-1 producing cells with a variety of human and animal non-lymphoid cell types induces cell fusion, leading to the formation of large, multinucleated cells - syncytia as a result of HTLV-1 expression and transmission (Sagara *et al.*, 1997). These data about broad cellular tropism of HTLV-1 are in contrast with its preferential infection of CD4+ cells *in vivo*. Although HTLV-1 was the first human retrovirus to be isolated and characterized, its study has been hampered by poor viral infectivity as manifested by low cell-free and cell associated virus titers. Several different approaches have been utilized to identify the receptor for HTLV-1. Sommerfelt and co-workers generated a series of human-mouse somatic cell hybrids and correlated susceptibility of these hybrids to VSV HTLV-1 pseudotype

infection with the presence of particular human chromosome (Sommerfelt *et al.*, 1988). In this experiments, all hybrids which were susceptible to VSV HTLV-1 pseudotype infection contained human chromosome 17. Additional studies have localized the gene which encodes the receptor to the long arm of chromosome 17, and the gene product of approximately 30-31 kDa was identified as a cell surface receptor for HTLV-1 (Gavalchin *et al.*, 1995). Several other candidate antigens have previously been suggested to be involved in HTLV-1 surface adhesion and syncytium formation. They include HLA A2 (Clarke *et al.*, 1983), IL-2R (Kohtz *et al.*, 1988), CD2 (Duc Dodon *et al.*, 1989), membrane glycoprotein C33 (Fukudome *et al.*, 1992), an 80 kDa membrane glycoprotein (Agadjanyan *et al.*, 1994), 71 kDa heat shock cognate protein (Sagara *et al.*, 1998). Recently Manel and co-workers reported that the receptor binding domain of HTLV-1 envelope glycoproteins inhibit glucose transport by interacting with GLUT-1, the ubiquitous vertebrate glucose transporter encoded by short arm of human chromosome 1 and proposed it as the true receptor for HTLV-1 (Manel *et al.*, 2003).

HTLV-1 associated diseases

HTLV-1 is associated with two distinct types of disease: the malignancy, known as adult T-cell leukemia lymphoma (ATLL) and a range of chronic inflammatory conditions including the CNS disease - HTLV-1 associated myelopathy/tropical spastic paraparesis. HTLV-1 was the first human retrovirus isolated and was shown to be the causative agent of ATLL (Poiesz *et al.*, 1980; Yoshida *et al.*, 1984). Approximately 5 years after its discovery, epidemiological data linked HTLV-1 infection with a chronic progressive disease of CNS termed HTLV-1 associated myelopathy in Japan (Osame *et al.*, 1986) and tropical spastic paraparesis in the Caribbean (Gessain *et al.*, 1985). The two syndromes were determined to be the same disease and were termed HAM/TSP (Gessain and Gout, 1992). HTLV-1 is endemic in the southern region of Japan, the Caribbean, and the equatorial regions of Africa and in South America (Osame *et al.*, 1986; Gessain *et al.*, 1986). It is estimated that one to two million people are infected by HTLV-1 in Japan alone, where the virus is endemic and, approximately 10 to 20 million people are HTLV-1 carriers worldwide (Franchini, 1995). In Latvia seropositivity for the HTLV-1 among blood donors is 0.3%, which is about ten times higher comparing to Western Europe (Kukaine *et al.*, 1993). HTLV-1 does not efficiently replicate *in vivo* and is transmitted through infected T cells in breast milk (from mother to child), in semen

(from male to female), and in blood (transfusion, intravenous drug users). The adult HTLV-1 seroprevalence rate in some of endemic areas can be as high as 30%, however only 1-5% develops HAM/TSP or ATLL, the remainder are asymptomatic carriers (ACs) of the virus (Tajima *et al.*, 1985). Why one group of HTLV-1 seropositive individuals develop a neurological disease, another - leukemia and the majority remain clinically well, is unknown and an area of intense investigation. Possible differences between the diseased and asymptomatic states currently under study include analysis of virus strain (Daenke *et al.*, 1990; Niewiesk *et al.*, 1994; Furukawa *et al.*, 2000), human histocompatibility leukocyte antigen (HLA) (Jeffery *et al.*, 1999; Vine *et al.*, 2002), viral load and immune function (reviewed in Bangham 2003). There are case reports of HAM/TSP developing within a few months of transfusion with HTLV-1-infected blood, but cases of ATLL have not been reported so soon after infection (Gout *et al.*, 1990). Possibly, the route of infection determines the provirus load and the risk of different HTLV-1-associated diseases. It has also been suggested that infection by the oral route might lead to a degree of immunological tolerance of HTLV-1. HTLV-1 is also associated with arthritis, uveitis, infective dermatitis, polymiositis and other pathologies (Yodoi and Uchiyama, 1992). The list of diseases associated with the HTLV-1 has been more extended in the past several years (for the review see Yao and Wigdahl, 2001).

HAM/TSP. HAM/TSP is a chronic neurodegenerative disorder whose symptoms are primarily localized to functions associated with the lower spinal cord, including spastic paraplegia of the lower extremities, loss of bladder control, and sexual dysfunction. Pathology is mainly limited to the lower and middle thoracic cords, where marked degeneration of the corticospinal tracts and demyelination are evident, accompanied by perivascular mononuclear infiltrates consisting primarily of $CD4^+$ T lymphocytes in early lesions, followed by the appearance of $CD8^+$ T lymphocytes in older lesions (Levin and Jacobson, 1997). The incubation from time of infection to onset of disease is typically from years to decades, but can be as short as 18 weeks following blood transfusion with HTLV-1 contaminated blood (Gout *et al.*, 1990). The age of onset is usually 35 to 45 years, but can be as early as 12 years of age. The disease is three times more prevalent in women than men (Levin and Jacobson, 1997).

HAM/TSP is thought to be an autoimmune disease induced by activated T-cells. Also the possibility that HTLV-1 specific antibodies can recognize a cellular

antigen expressed by CNS cells is suggested and this molecular mimicry may play a role in pathogenesis of HAM/TSP. Specifically, it is reported that HAM/TSP patients developed antibodies that cross-react with heterogeneous nuclear ribonuclear protein (hnRNP) A1 highly expressed in neurons. Discovered cross-reaction of antibodies to HTLV-1 Tax protein with hnRNP A1 suggests molecular mimicry between the two proteins (Kalume *et al.*, 2004). A number of studies have described both cellular and humoral immune responses in patients with HAM/TSP and compared these to HTLV-1 asymptomatic individuals and normal HTLV-1 seronegative controls. HAM/TSP patients have elevated antibodies titers to HTLV-1 in sera and cerebrospinal fluid (CSF), they may have up to 50 times more HTLV-1 proviral DNA in PBL compared to ACs (Bangham, 2003). Direct damage of HTLV-1 infected cells is unlikely to contribute disease pathogenesis, because few (Lehky *et al.*, 1995) if any (Hara *et al.*, 1994) resident CNS cells become infected with HTLV-1. Contrary, it is shown that HTLV-1 specific T-cells themselves are more frequently infected with HTLV-1 than are T cells specific to other antigens. This preferential infection is evident in both CD4+ T-cells and CD8+ T-cells (Goon *et al.*, 2004). It is proposed that tissue damage is caused by the release of cytokines from highly activated T-cells that infiltrate the CNS. HTLV-1 infected activated CD4+ T cells spontaneously secrete proinflammatory, neurotoxic cytokines such as interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) and high levels of these cytokines have been demonstrated in the sera, CSF and spinal cord lesions of patients with HAM/TSP (Umehara *et al.*, 1994). It is found that the median frequency of HTLV-1-specific IFN-γ positive CD4+ T-cells is 25-fold greater in patients with HAM/TSP than in ACs with a similar proviral load (Goon *et al.*, 2002). Patients with HAM/TSP showed significantly higher frequencies of activated Th1 type HTLV-1 Env specific CD4+ lymphocytes compared to ACs (Goon *et al.*, 2002).

Patients with HAM/TSP develop a $CD8^+$ HLA 1 restricted cytotoxic T lymphocyte (CTL) response specific for immunodominant HTLV-1 peptides. The Tax protein usually is immunodominant in the CTL response to HTLV-1, although in some individuals vigorous responses can also be detected with the other HTLV-1 proteins, especially Pol (Parker *et al.*, 1992; Ozden *et al.*, 2004). One of the largest single factors that accounts for the variation between individuals in the equilibrium provirus load in healthy carriers of HTLV-1 is individual variation in the efficiency of

the CTL response to the virus. An efficient CTL response, associated with a low provirus load, is characterized by strong mRNA expression of granzymes and other CTL lysis related genes (Bangham 2003, Vine *et al.*, 2004), and rapid killing of HTLV-1 infected lymphocytes. The molecular basis for this high CTL-responsiveness to HTLV-1 is unknown, although it is associated with certain class 1 HLA alleles (A*02, Cw*08) in southern Japan. It is shown that in southern Japan a higher risk of HAM/TSP is associated with following host genotype polymorphisms for HLA antigen, TNF-α promoter and the SDF-1 chemokine gene: HLA-A2-, HLA-Cw8-, HLA-DR1+, TNF-α-863A+, SDF-1 801A- and with infection with HTLV-1 subgroup A (Furukawa *et al.*, 2000; Vine *et al.*, 2002). Thus, although the frequency of HTLV-1 specific CD8+ cells is lower in subjects with a low provirus load (the median frequency of specific CD8+ cells is 2- to 4-fold greater in patients with HAM/TSP than in carriers with an equivalent provirus load), such individuals express higher total levels of mRNAs of lysis-related genes in their circulatory CD8+ cells than do individuals with a high provirus load. These observations therefore strongly support the idea that variation in CTL efficiency accounts for variation in provirus load. Abundant, chronically activated $CD8^+$ T-cell response would be expected to exert significant selection pressure on the virus. It was found that naturally occurring sequence variants of Tax escape recognition by fresh autologous CTLs (Niewiesk *et al.*, 1995). However, recombinant Tax proteins that contained these putative CTL escape mutations were highly defective in their transactivating activity (Niewiesk *et al.*, 1995).

Dendritic cells (DCs) are the most potent antigen presenting cells and so can stimulate not only naive CD4+ T-cells but also CD8+ T-cells. It is suggested that HTLV-1 infected DCs could also be implicated in the disease pathogenesis and could play a role in the production of autoreactive T-cells in HAM/TSP patients (Makino *et al.*, 1999).

ATLL. Adult T-cell leukaemia-lymphoma is defined as a peripheral CD4+/ CD25+ T-cell neoplasm caused by HTLV-1, which is believed to be a neoplasm of lymph-node cell origin (Yamada and Tomonaga, 2003). After infection with HTLV-1, which is mostly transmitted by breast feeding, there is a long latent period as HTLV-1 carrier until overt ATLL develops. The median age of patients is 57-60 in Japan, and the proportion of patients younger than 40 is less than 10%; patients in the Caribbean,

one of the other endemic areas, are much younger than Japanese patients (Yamada and Tomonaga, 2003). The grade of aggressiveness differs greatly among patients; some survive for more than 10 years without chemotherapy but others die within several months even though intensive chemotherapy has been applied. Most patients with aggressive ATLL do not have a prodromal phase and develop ATLL suddenly, but some patients have a history of indolent ATLL, and it gradually turns to a more aggressive form. ATLL was subclassified into four subtypes: acute type and lymphoma type as an aggressive form, and chronic type and smoldering type as an indolent form (Kawano *et al.*, 1985; Shimoyama 1991). The clinical symptoms are heterogenous. It is often associated with lymphadenopathy, infiltrative skin lesions, hepatosplenomegaly, hypercalcaemia, bone marrow infiltration, and lytic bone lesions (Yamada *et al.*, 1997). Patients are usually in a severely immune-suppressed condition and susceptible to opportunistic infections. Characteristic, the so-called flower cells with multilobulated nuclei are observed in blood smears of ATLL patients (Uchiyama *et al.*, 1977).

Initially, the transformed cells show a polyclonal pattern of HTLV-1 integration. During intermediate states toward ATLL, the integration pattern of the provirus changes to oligoclonal and finally monoclonal by a predominant growth of some infected cells and subsequently followed by an uncontrolled clonal proliferation of these T cells carrying a single copy of integrated virus (Takemoto *et al.*, 1994). The monoclonal integration of the provirus is a typical character of the transformed leukaemic cells contrary to oligoclonal pattern of HTLV-1 integration observed in HAM/TSP (Yoshida *et al.*, 1994). Clonal expansion of cells carrying the provirus occurs likely after antigenic stimulation.

It has been proposed that the HTLV-1 regulatory Tax protein may have an important role in the transformation process. However, only low levels of *tax* mRNA could be detected *in vivo* from ATLL patients by polymerase chain reaction (PCR), suggesting that *tax* gene expression is critical for the initiation of transformation but not essential for maintaining the growth of tumour cells (Takeda *et al.*, 2004). Tax expression during repeated T-cell division and clonal expansion by shortening G1/S cell cycle progression interferes with DNA repair and may favor the genetic instability and accumulation of genetic defects in clonally expanded T–cells. These cells may not express viral proteins and may have acquired genetic lesions that recapitulate, for example, the effect of Tax on NF-κB transcriptional pathway, cell

cycle and apoptosis. In the late stage of infection, acquisition of stable somatic mutations may favor selection of deleted/mutated provirus which decreases risk of elimination by the immune system. One of these clones will outgrow the others and cause ATLL in the host.

Both NF-κB and AP-1 transcription factors are constitutively active in ATLL cells (Mori *et al.*, 2000). $p16^{INK4A}$ and $p15^{INK4B}$ often are deleted or methylated in ATLL cells, and this circumstance is associated with progression of ATLL (Trovato *et al.*, 2000). Similarly, alteration of the retinoblastoma *Rb* gene has been reported in ATLL (Hatta *et al.*, 1997). The *p53* gene is mutated in approximately 30% of ATLL cases and is stabilized and functionally impaired in ATLL cells (Sakashita *et al.*, 1992; Takemoto *et al.*, 2000). Genes that regulates survival of cells (*e. g.* Bcl-xL, survivin, an inhibitor of apoptosis protein family member, $p21^{WAF1/CIP1}$) are elevated in *ex vivo* samples from ATLL patients, even in the absence of demonstrable Tax expression (Kawata *et al.*, 2003; Nicot *et al.*, 2000). Constitutive activation of one of the STAT5 proteins has been associated with active DNA synthesis (S phase) and G2/M transition in ATLL cells (Takemoto *et al.*, 1997). Noteworthy, that ATLL cells are resistant to chemotherapeutic agents. Such chemo-resistance of ATLL cells may be partly explained by the fact that ATLL cells express products of the multidrug-resistance protein gene *MRP* (Su *et al.*, 1991).

Current treatments of ATLL fail to induce long-term remission, and even the clinically less aggressive forms of ATLL are fatal. Also no effective means to prevent the development of ATLL has been found.

THERAPEUTIC NUCLEIC ACIDS AND THE MECHANISMS OF THEIR ACTION

Antisense nucleic acids

The discovery of gene silencing by antisense RNA in bacterium *Escherichia coli* (Light and Molin, 1983; Mizuno *et al.*, 1983), found also in vertebrates (Robb *et al.*, 2004), has improved our knowledge and understanding of regulation of gene expression. This natural phenomenon offered a unique opportunity to artificial manipulation of gene expression and became a powerful genetic tool used by researchers.

Two strands of nucleic acids can form a non-covalently bound duplex as a result of Watson-Crick base pairing, where adenine can form a hydrogen bond with thymine/uracyl, while cytosine can bond with guanine. In the case of DNA, one strand serves to store the genetic code and is called the sense strand, while the other provides the complementary supporting strand and is known as the antisense strand. RNA is generally copied from the antisense strand and has the same sequence as the DNA sense strand.

The following main mechanisms of action have been reported for the antisense nucleic acids: 1) oligonucleotides (ODNs), designed in antisense orientation, hybridize to their target mRNA in a strict basepair specific manner (Watson – Crick base pairing) and thus block the translation; 2) in case of antisense RNA, the effects of double stranded (ds) RNA could be involved in protection mechanisms additionally. Short 21-23 nt dsRNA produced by cellular RNase III like enzyme – Dicer from longer RNA duplexes, known as small interfering RNAs, employed cellular mechanisms of RNA silencing (see RNA interference). Longer dsRNAs, frequently expressed in cells infected by viruses, activates mechanisms that efficiently kill the infected cells, thereby preventing spread of the virus (induction of interferon) (Friedrich *et al.*, 2004); 3) ODNs can bind to the genomic DNA in the nucleus and thus block the transcription (Hoogsteen-type base triplets). Another, unspecific mechanism of the action is the binding of the oligonucleotide to a target protein that has been referred to as antisense aptamer-binding (Lavrovsky *et al.*, 1997). So, inhibition can take place on different levels (transcription, translation).

Mainly two types of as-nucleic acids are used: ODNs and asRNAs. As-ODNs are short (15-25 basepairs) single stranded DNA molecules complementary to the target mRNA or DNA sequence, which are administered exogenously into a cell. As-

RNAs usually are produced intracellularly from an expression vector, which could be introduced into a cell by different approaches (Mahato *et. al.*,1997).

A major concern with the use of as-ODNs has been their stability in tissue culture medium and, ultimately, in the living host. Such short nucleic acids (due to constraints in permeability of a cell membrane for big molecules) are susceptible to nuclease degradation, when are administered into an organism. Modifications in the base, sugar, and phosphate moieties of oligonucleotides have been reported to stabilize the molecules (e. g. phosphorothioates, morpholino) (Summerton *et al.*, 1997).

RNA interference

The term RNA interference (RNAi) describes the use of dsRNA to target specific mRNAs for degradation, thereby silencing their expression. RNAi is one manifestation of a broad class of RNA silencing phenomena that are found in plants, animals and fungi. The discovery of RNAi has changed understanding how cells guard their genomes, led to the development of new strategies for blocking gene function, and may yet yield RNA-based drugs to treat human disease. RNAi-like phenomena are found throughout eukaryotes, suggesting that the RNAi machinery is quite ancient, having evolved prior to the divergence of animals, plants, and fungi. In both plants and animals, one key function of the RNAi pathway is to maintain integrity of the genome by suppressing the mobilization of transposons and the accumulation of repetitive DNA in the germline. A growing body of data suggests that the RNAi, in whole or part, serves to regulate expression of endogenous genes (reviewed in Zamore, 2001). In higher plants RNAi plays also the antiviral function and forms the basis of a highly elaborate immune system (Lecellier and Voinnet, 2004). But it remains unclear if RNA silencing plays a defensive role against viral infection in higher vertebrates. Understanding the interactions of the RNAi machinery with viruses in animals will be essential in the quest to develop small interfering RNA (siRNA)-based therapies for human disease (Joost Haasnoot *et al.*, 2003).

RNAi is initiated by the RNase III-like nuclease Dicer, which promotes cleavage of long dsRNAs into 21-23-nt short siRNAs with 2-nt 3' overhangs. Subsequently, the siRNAs are incorporated into an RNA-induced silencing complex and the protein-RNA effector nuclease complex recognizes and destroys the target mRNAs. In worms and flies, only a few molecules of dsRNA per cell are required to silence thousands of target mRNA molecules (Martinez *et al.*, 2002). The fact that

synthetic siRNAs or purified siRNAs cleaved from long dsRNA can efficiently mediate RNAi *in vitro* suggests that long dsRNA are more effective because they are more efficiently processed into siRNAs, perhaps because Dicer binding or cleavage is highly cooperative. However, the use of dsRNAs longer than 30 bp in mammalian cells appeared to be of limited utility, because of triggering interferon responses through the activation of dsRNA-dependent protein kinase and 2' ,5' –oligoadenilate synthetase (Park *et al.*, 2002).

Ribozymes

Behind mentioned antisense approaches, catalytic RNA molecules, called ribozymes are being assessed as potential antiviral agents. The leading study was conducted by Thomas Cech on ciliated protozoan *Tetrahymena Thermophila* group I introns in 1980s, which showed that RNA can participate in the intramolecular catalysis of the self-splicing and acts as protein enzyme (Cech *et al.,* 1983). Five classes of ribozymes have been described based on their unique characters in the sequences as well as three-dimensional structures. They are denoted as the Tetrahymena group I intron, RNase P, the hammerhead ribozyme, the hairpin ribozyme and the hepatitis delta virus ribozyme (Lavrovsky *et al.,* 1997; James and Gibson, 1998). They may catalyse self-cleavage (intramolecular or "in-*cis*" catalysis) as well as the cleavage of external substrates (intermolecular or "in-*trans*" catalysis). Ribozymes can cleave other RNAs and after destroying one target molecule, can move to the next, thus offering the potential benefit of cycling (Whittton, 1994). The antisense sections of ribozyme RNA allow specific targeting and a catalytic domain cleavage of the desired target mRNA. Thus, ribozymes combine enzymatic processes with the specificity of antisense base pairing (James and Gibson, 1998). For gene therapy the hammerhead, the hairpine and RNase P type ribozymes are used. Ribozymes can be delivered to cells as preformed ribozymes (exogenous delivery) or as ribozyme genes, a method of endogenous delivery.

Transcription factor decoy

A successful nucleic acid based approach has been the use of nucleic acid molecules with high affinities to a target transcription factor which could be introduced into cells as decoy *cis*-elements to bind these factors and alter gene expression. The competition for gene enhancer sequences recognized by cellular transcription factors: the decoy oligonucleotide technology, offers great promise as a tool for defining cellular regulatory processes and for treating cancer, viral diseases

and other pathological conditions. Different decoy nucleotides targeting such transcription factors as CREB/ATF family, NF-κB and E2F were designed and successfully applied by researchers to inhibit growth of cancer cells (Cho *et al.*, 2002; Cho-Chung, 2003, Morishita *et al.*, 2004, Ahn *et al.,* 2003). The decoy approach has been used also for the inhibition of human retrovirus HIV-1. A number of reports describe the use of RNA molecules containing TAR sequence recognized by HIV-1 transactivator protein Tat in control of the viral replication (Michienzy *et al.*, 2002, Ding *et al*, 2002).

CONSTRUCTION OF THERAPEUTIC NUCLEIC ACIDS' EXPRESSION VECTORS

Choice of a promoter

A long-termed approach is to clone as-sequence into a mammalian expression vector, with the intent to control expression of the as-sequence or ribozyme. A large excess of asRNA molecules over target RNAs is important for effective inhibition of virus replication. To achieve this goal, the as-gene should be inserted into an expression vector behind an appropriate promoter sequence. A promoter is a regulatory sequence of DNA that is located upstream of a gene and to which proteins (transcription factors and RNA polymerase) bind to initiate the synthesis of mRNA and subsequently protein. Appropriate promoter systems allow ensuring high levels of as-gene expression in target tissue, required for effective antiviral action of asRNAs. Therefore, the promoter choice is one of the crucial points in the construction of the asRNA expression vectors.

Usually strong promoters, mostly derived from pathogenic viruses, are used to drive the expression of asRNA genes (Shayakhmetov *et al.,* 1997, James and Gibson, 1998). Two strategies are commonly used. First, the as-gene is inserted behind a strong promoter for RNA polymerase II, which may be of viral origin or a strong endogenous promoter (e. g. the actin gene promoter). One of the main advantages of the RNA polymerase II promoter is the availability of a tissue-specific and inducible promoter. Second, an alternative to RNA polymerase II is use of RNA polymerase III promoter. The RNA polymerase III transcribes a variety of small nuclear and cytoplasmic RNAs that are abundant in all cell types (e. g. tRNA). However, in their present form, these type promoters can not be regulated, and its use in certain applications is limited (Lavrovsky *et al.,* 1997; James and Gibson, 1998).

Strong viral promoters have been successfully used in mammalian cell cultures *in vitro* where the inflammatory cytokines are not present. However, it has been reported that the expression of sequences cloned behind some widely used strong viral promoters (e. g. cytomegalovirus promoter) can be affected by cytokines produced by immune system cells (INFγ, TNF-α*) in vivo* (Gribaudo *et al.*, 1993).

The ideal asRNA constructs must provide expression of antiviral asRNA genes only in those cells of the organism which could be infected by the virus, and only when they are infected. The study of Shayakhmetov and co-authors demonstrated that these criteria can be achieved, at least partially, by using the own promoter of the virus to drive the asRNA gene (Shayakhmetov *et al.*, 1997). Authors showed a 75% inhibition of bovine leukemia virus (BLV) replication by asRNA targeted to the BLV LTR RU5 region driven by the BLV U3 promoter. This strategy was also applied successfully for HTLV-1. It was demonstrated that HTLV-1 LTR driven antisense *c-myc* construct suppressed *c-myc* expression and inhibited the growth of HTLV-1-infected and transformed cells of the human T-cell line HUT102 (Fujita and Shiku, 1993).

Choice of the target

An important issue in creation of an antisense therapeutic molecule is to address the as-nucleic acid to a proper virus target gene. As it is known, certain virus sequences are highly conserved. Such "immutable" sites would be difficult to target as protein, but may be more accessible to antisense inhibition. In this case, the emergence of antisense-resistant mutants is unlikely. In the case of animal virus BLV which is closely related to HTLV-1, effective inhibition was demonstrated by using as-RNAs targeted to virus pX and LTR RU5 regions. A number of constructs were obtained under a control of various promoters (HSV TK, SV40). The most effective suppression of BLV replication was observed with asRNA against the RU5 region of BLV LTR. A significant but less marked suppression of BLV was observed with asRNA targeted to the BLV pX region (Murovska *et al.*, 1992; Shayakhmetov *et al.*, 1997). Peng and co-authors also showed that intracellular expression of HIV-1 sense or antisense U3RU5 sequences conferred long-term inhibition of HIV-1 replication, despite continuous presence of viral challenge in the transduced Jurkat T-cells (Peng *et al*, 1997).

A promising candidate for HTLV-1 suppressive gene therapy is the Tax protein, as it is an early transactivator of the expression of all HTLV-1 genes. Therefore, the as-nucleic acids targeted to the *tax* gene and to the HTLV-1 LTR RU5 region are prospective targets for HTLV-1 suppressive therapy.

As LTR U3 region contains the Tax-responsive enhancer sequence (three 21 bp repeats), it would be of interest to study the antiviral activity of constructs, targeted to the HTLV-1 *tax* gene and LTR U3 region sequences simultaneously. Also, in the case of HTLV-1, one could expect that a mechanism other than antisense binding could be involved additionally. Briefly, when a plasmid vector expressing asRNA to the LTR U3 region is introduced into a cell, also the sense LTR U3 DNA sequence of the introduced vector can compete with viral and cellular DNA sequences for the virus transactivator Tax (Tax protein decoy). Such a possibility was assumed in the work of Shayakhmetov and co-authors with BLV, when the inhibition of the virus has been observed using a plasmid harbouring only the virus promoter LTR U3 sequence as a control (without as-gene) (Shayakhmetov *et al.*, 1997). Theoretically, this strategy could be promising for more efficient inhibition of virus transcription and Tax-mediated oncogenic and immunogenic effects.

MOLECULAR THERAPY OF HTLV-1 INFECTION

The use of the nucleic acid approach to inhibit HTLV-1 replication has been described mostly *in vitro,* in cell cultures. There are only a few publications where it was demonstrated *in vivo*.

Studies *in vitro*

Antisense oligodeoxynucleotides. Maeda and co-authors showed a 59% inhibition of syncytium formation between HTLV-1 producing human T-cell line C91/PL cells, and HTLV-1-uninfected human glioma cell line U251-MG cells, by antisense oligonucleotides complementary to the region of initiation codon of *tax* gene (Maeda *et al.*, 1997). Also, the effects of ODNs, complementary to the first splice junction, Rex-responsive site, *gag*, *env*, *tax*, *rex*, and *p21,* on syncytium formation, have been evaluated. Syncytium formation was significantly inhibited by as-ODNs to *env, tax, gag, p21*, and *rex*, with as-ODNs to *env* being the most inhibitory. Antisense ODNs to *env* and *tax* also inhibited reverse transcriptase activity (Maeda *et al.*, 1998).

It is important to note that the action of exogenuously introduced ODNs is short-term. Therefore, ODNs must be introduced repeatedly many times. Such a

strategy does not seem optimal for therapy of integrative viruses. Also, it is not known how specifically these short nucleic acids will act *in vivo*. Nevertheless, this system is valuable for primary screening of potentially active antisense sequences.

Antisense RNAs. Von Ruden and Gilboa in 1989 demonstrated that primary human T-lymphoid cells could be made partially resistant to HTLV-1 via asRNA-mediated inhibition. In that study, two segments of HTLV-1 were chosen as the targets: (1) the sequence spanning 5'end of mRNA, harbouring *cis*-elements, essential for viral gene expression (5'splice site) and virus replication (tRNA primer-binding site), and (2) the pX region corresponding to the first kilobase of the *tax* gene. It was shown that asRNA expression leads to significant, although not complete, inhibition of HTLV-1 replication (Von Ruden and Gilboa, 1989).

Ribozymes. Antiviral activity of ribozymes, targeted to the HTLV-1 *tax* and *rex* genes has also been reported. Hammerhead ribozyme targeted against HTLV-1 *tax/rex* mRNA was introduced into synovial cells obtained from patients with HTLV-1-associated arthropathy and from patients with HTLV-1-negative rheumatoid arthritis. The ablation of Tax expression as well as the ability of the cells to stop proliferating and to undergo apoptosis were examined. Both transcription of *tax* mRNA and Tax protein synthesis were inhibited significantly, resulting in inhibition of synovial cell growth and induction of apoptosis (Kitajima *et al.,* 1997a) Intracellular activities of the ribozymes targeted to *tax/rex* mRNA were studied also in HTLV-1 *tax* cDNA-transfected rat embryonic fibroblasts (Rat/Tax cells), which expressed the Tax. Tax protein levels were decreased by about 95%, while Tax as-ODNs reduced Tax expression by about 20% (Kitajima *et al.,* 1997b).

siRNAs. Nomura and co-authors used siRNA against Tax in a rat HTLV-1-infected T-cell line. The expression of siRNA targeting Tax successfully downregulated Tax expression. Repression of Tax expression was associated with resistance of the HTLV-1-infected T-cells to Tax-specific CTL killing. Furthermore, T-cells with Tax downregulation appeared to lose the ability to develop tumors in T-cell-deficient nude rats, in which the parental HTLV-1-infected cells induce ATLL-like lymphoproliferative disease (Nomura *et al.,* 2004).

CRE decoy. As HTLV-1 contains CRE consensus sequence in their LTR and it is implicated in the viral pathogenesis it would be of interest to learn existing information relevant to CRE element decoy. The CRE transcription factor complex plays a critical role in response to hormonal signals for cell proliferation,

differentiation and apoptosis. It functions in glucose homeostasis, growth-factor-dependent cell survival and has been implicated in learning and memory (Mayr *et al.*, 2001). Many transcription factors bind to this element and regulate expression of a wide variety of cellular and viral genes. CRE-binding factors have a definite role in T-cell apoptosis: they are involved in cAMP protection of TCR-induced apoptosis (Muller Igaz *et al.*, 2002). It has been shown that CRE-transcription factor decoy oligonucleotides inhibit CRE- and AP-1-directed gene transcription and promote growth inhibition *in vitro* and *in vivo* in a broad spectrum of cancer cells, without adversely affecting normal cell growth (Park *et al.*, 1999; Cho-Chung, 2003). The growth inhibition was accompanied by changes in cell morphology and apoptosis. It was reported that CRE-decoy oligonucleotide treatment results in an increase in the p53 protein level in MCF-7 human breast cancer cells that express wild-type p53. The stabilization and activation of p53 may have contributed to the growth inhibition induced by CRE-transcription factor decoy oligonucleotide in MCF-7 cells. The $p21^{WAF1/Cip1}$ protein levels were also increased accompanying a reduction in CDK2- and cyclin E–dependent kinase activity and pRb phosphorylation (Lee *et al.*, 2000). A marked reduction in the expression of the regulatory and catalytic subunits of protein kinase A by CRE –transcription factor decoy in ovarian cancer cells was also reported (Alper *et al*, 2001).

Experiments *in vivo*

Very little has been research has been conducted so far with respect to investigation of anti-HTLV-1 activity of asRNAs and ribozymes in animal model systems. The existing animal models of HTLV-1 infection display different patterns of infection and resulting pathologies; thus choice of the animal model depends on the pathology studied. In 1992 Kitajima and co-authors reported about suppression of fibroblastic tumors, developed in HTLV-1 Tax transgenic mice by as-ODNs to NF-kB transcription factor (Kitajima *et al.*, 1992). Treatment with ODNs to Tax showed virtually complete suppression of Tax expression, but not regression of the tumors.

Another strategy for inhibition of HTLV-1-associated disease has been applied by Murata and co-authors in 1997 on a rat tumor model (Murata *et al.*, 1997). Cells of HTLV-1 transformed Tax expressing rat T-cell line TARS-1 were transduced by a retroviral vector carrying the herpes simplex virus thymidine kinase (HSV TK) gene under the control of the LTR of HTLV-1 (LNLTK virus). The HSV TK gene is the most commonly used suicide gene. Cells transduced with the HSV TK gene become

sensitive to nucleoside analogs such as ganciclovir. Newborn rats were inoculated subcutaneously with TARS-1 cells, which gave growth of tumor in rats. Dramatic repression of tumor growth upon ganciclovir treatment was observed in the case of TARS-1 cells transduced with LNLTK virus construct.

Due to many technical difficulties associated with the production of transgenic animals, there are few reports of the anti-viral effects of asRNAs genes in transgenic mammals. However, the results of these few studies indicate that asRNA-mediated inhibition can effectively prevent viral infections *in vivo*. Kozireva and co-authors in 1996 have studied the sensitivity of rabbits to BLV infection, both in wild type and transgenic animals, in the latter with the asRNA gene targeted at LTR RU5 region of BLV. The obtained results indicated that the anti-BLV asRNA gene confers enhanced resistance to BLV infection in transgenic rabbits compared to wild type animals (Kozireva *et al.*, 1996). Continuing this investigation, the authors found that the expression level of asRNA in transgenic rabbits was not sufficient to abort infection in rabbits (Murovska et al., 2001). Therefore, the investigation should be continued to elucidate optimal targets and as-gene-expressing constructs that can inhibit effectively the virus replication and prevent pathologies.

CONCLUSIONS AND FUTURE PROSPECTS

Data on inhibition of HTLV-1 and other retrovirus infections by using antisense technology suggest that antisense nucleic acids and ribozymes can effectively inhibit HTLV-1 replication *in vitro*. In previos studies with ODNs, the most preferential target sequences for effective inhibition of the virus have been determined. The next logical step is the design of vectors expressing as- and si-RNAs and ribozymes, to create more specific and flexible nucleic acid therapeutic systems. The use of specific vectors for direct delivery of genetic materials into certain cells and tissues, and application of strong or inducible promoters to regulate the expression of as-sequences, allow the development of new as-constructs for antiviral protection. As the pathogenesis of HTLV-1 associated diseases is very complex, a panel of antisense drugs targeted not only to the virus, but also to some virus-activated cellular transcription factors and genes (e. g. NF-kB, *c-myc,* others) needs to be established. With improvements in stability, delivery, and design therapeutic nucleic acids, the nucleic acid approaches are certain to become one of the most important tools in gene therapy.

MATERIALS AND METHODS

Cell cultures

The following cell lines were used in the study: Ra-1 – HTLV-1 Japanese strain producing continuous lymphoid rabbit cell line; MT-2 - HTLV-1 Japanese strain producing continuous human T-cell line; HOS TE85 – human caucasian osteogenic sarcoma, monolayer epithelioid cell line. HeLa – human cervical carcinoma continuous monolayer epithelioid cell line; Vero – African green monkey kidney continuous epithelioid cell line.

HTLV-1–producing MT-2 human T-cell line (Miyoshi *et al.*, 1979) and Ra-1 (Miyoshi *et al.*, 1983) rabbit lymphoid cells were maintained in RPMI-1640 medium (Gibco BRL, UK) supplemented with 10 % foetal bovine serum (FBS) (Gibco BRL, UK), 2 mM L-glutamine and antibiotics and incubated at 37 ^{0}C in 5% CO_2.

HOS, HeLa and Vero monolayer cell lines were maintained in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% heat inactivated FBS (Gibco BRL, UK), 300 μg/ml L-glutamine and 50 μg/ml gentamycin. The cells were reseeded 2-3 times a week at $2x10^5$/ml cells, using a mixture of trypsine-EDTA.

HTLV-1 infection of HOS cells. HTLV-1 infected cell line HOS was established by co-cultivating HOS cells with HTLV-1 producing Ra-1 cells, which were treated by mitomycin C (5 μg/ml) for 1 hour at 37 ^{0}C. Co-cultivation was carried out using suspension of Ra-1 lymphocytes at concentration $1.5x10^5$cells/ml. The virus infected HOS subline was designated as RaHOS.

Detection of HTLV-1 provirus integration

Polymerase chain reaction (PCR) analysis. Total DNA was isolated from up to $3x10^6$ cells by proteinase K digestion followed by standard phenol/chloroform extraction (Sambrook *et al.*, 1989). Quality of DNA was assessed by PCR using primers for human β-globin gene (Vandamme *et al.*, 1995) and only samples positive in this assay were further processed.

1 μg of DNA was analyzed for the presence of HTLV-1 provirus by nested PCR using primers targeted to HTLV-1 *gag, env, tax* genes and 5' LTR region. The primer sequences and PCR conditions were described previously (Vandamme *et al.*, 1997; Miyada *et al.*, 1995; Liu *et al.*, 1994; Liu *et al.*, 1996).

DNA from the HTLV-I positive cell line Ra-1 was used as the positive control and DNA from the HTLV-I free cell line HOS as the negative control. The products

of DNA amplification were analyzed by electrophoresis on 1.9-% agarose gel followed by ethidium bromide staining and visualization in UV light for the presence of DNA bands of appropriate sizes. In further documentation and analysis of amplified fragments Kodak Electrophoresis Documentation and Analysis System 290 (USA) was used.

Dot-hybridization. Total cellular DNA and RNA were isolated from the transfected RaHOS cell culture clones by proteinase K digestion and by extraction with guanidinethiocyanate, accordingly, followed by standard phenol/chloroform extraction (Sambrook *et al.*, 1989) and 5 μg of each nucleic acid sample was immobilized on positively charged nylon membrane (Hybond-N+, Amersham Pharmacia Biotech, Sweeden). Hybridization was carried out using Rapid-hyb buffer (Amerscham Pharmacia Biotech, Sweden) and an [α-^{32}P] ATP labeled oligonucleotide probe according to the manufacturer's protocol and was followed by autoradiography. 2ng of HTLV-1 proviral DNA excised from the plasmid pMT2 was used as the positive control. The intensity of autoradiography sygnal comparing to the sygnal of the positive control was analysed by the "LabWorks" computer program (Bio-Rad Laboratories). The radiolabeled probes were synthesized by PCR from the plasmids pMHTs (to the U3 region) and pMP1100as (to the pX region) with the addition of [α-^{32}P] ATP into reaction mix.

Detection of the plasmid sequences in the transfected cells by PCR

The following primers were used:

to the MPSV promoter -	MP01 - 5'CCC AAG GAC CTG AAA TGA-CC 3'
	MM2 - 5' TCT ATC GGA GGA CTG GCG CG 3'
to the HTLV-1 LTR U3 -	Ba2 - 5'GCTTAGAGCCTCTCAGTGAA 3'
	MM1 - 5'AGG ACG GCT TGA CAA ACA TG 3'
to the HTLV-1 pX region -	PX02 - 5'AAA CAG TCC TCG GGT AGA AT 3'
to the P5neo and P3neo -	P5s - 5' CCG CGG CTA GAC CCG GGG 3'
	P5as - 5'CCC ACT CGT GCA CCC 3'.

The MPSV promoter sequence was detected using primers MP01 and MM2, the length of the amplified fragment was 140 bp. To detect P4neo and P2 neo plasmid sequences, the following combinations of the primers to MPSV promoter and viral LTR U3 sequence were used: MP01 and MM1 (P4neo) and MP01 and BA2 (P2neo), the length of amplified fragment was 370 bp. To detect P3neo and P5neo plasmid

sequences the primers P5s and P5as were used. The length of expected fragments was 312 bp (P3neo) and 538 bp (P5neo). To detect pGHT sequences, commercially available primers complementary to the RNA polymerases promoters of bacteriophages T7 and SP6 - Forvard and Reverse were used (Promega, USA).

The reaction was performed in 50 μl of 50 mmol/L Tris-HCl, pH 9.0; 20 mmol/L NH_4SO_4; 1.5 mmol/L $MgCl_2$; 50mmol/L of each deoxyribonucleotide triphosphate; 1 unit of Taq polymerase. PCR was carried out for 35 cycles (94 ^{0}C 30 s, 57 ^{0}C 30 s, and 72 ^{0}C 45 s). All primers and PCR reagents were purchased from MBI Fermentas, Lithuania.

Detection of HTLV-1 expression and HTLV-1 LTR U3 asRNA expression

Isolation of total cellular RNA and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Total RNA was extracted from up to $3x10^6$ cells and from 1,000 fold concentrated by ultracentrifugation virus containing culture media of RaHOS cells by using "RNeasy Total RNA Kit" (Qiagen, Germany). It was then treated with DNase I (Sigma, USA) and subjected to RT-PCR. For the reverse transcription MBI Fermentas, Lithuania reagents was used and it was performed in 20 μl volume according to the manufacturer's protocol. Reaction mixture contained RT buffer (50 mM Tris•HCl pH 8.3 at 25 ^{0}C, 50mM KCl, 4mM DTT), 200 ng of RNA, random hexamer primers (1μM), ribonuclease inhibitor (1U/μl) and M-MuLV (Moloney Murine Leukemia Virus) reverse transcriptase (1U/μl). After reverse transcription at 37 ^{0}C for 1 hour, followed by 5 min at 99 ^{0}C and 5 min at 5 ^{0}C, samples were subjected to amplification with the primers to the HTLV-1 *gag* and *tax* genes.

For the semi-quantitative RT-PCR analysis of viral RNA in control virus producing RaHOS, MT-2 and Ra-1 cells and in their transfected sublines, total cellular RNA was extracted using TRIzol reagent (Invitrogen, UK) according to manufacturer's protocol. After treatment with DNase I (Sigma, USA), 500 ng of the RNA sample was subjected to reverse transcription using MBI Fermentas RT-PCR reagents or, alternatively, RNA PCR Kit (AMV) Ver. 2.1 (Takara, Japan) in a 20 μl reaction mixture volume according to the manufacturer's protocol.

After reverse transcription, amplification of 2μl RT aliquote in 50 μl of 50 mmol/L Tris-HCl, pH 9.0; 20 mmol/L NH_4SO_4; 1.5 mmol/L $MgCl_2$; 50mmol/L of each deoxyribonucleotide triphosphate; 1 unit of Taq polymerase, was performed. Primers specific to HTLV-1 LTR U3 region BA2 (sense):

5'GCTTAGAGCCTCTCAGTGAA 3' (position 36-55) and MM1 (antisense): 5'AGGACGGCTTGACAAACATG 3' (position 249-231) were used at a final concentration of 200 nmol/L. As the RT-PCR control, the primers for the human β-actin gene with the same amount of cDNA aliquote were used in parallel amplification (Noppen *et al.*, 1997). 25 cycles of amplification at 94^0C for 30 seconds, 58^0C for 30 seconds, and 72^0C for 45 seconds, were performed. Amplification products were separated in 1.5 % agarose and visualised with ethidium bromide staining. For the detection of the HTLV-1 LTR U3 antisense sequence expression the sense primer BA2 was used in the reverse transcription step. Dot-hybridization of the total cellular RNA isolated from the transfected RaHOS cell clones was performed as described earlier for the proviral integration analysis. Northern blot hybridization analysis. Northern blot hybridization was performed using DIG Northern Starter Kit (Roche, Germany). DIG labeled RNA probes were synthesized using plasmid pGHT from its T7 and SP6 phages promoters by the manufacturer's protocol. Then total cellular RNA was isolated from MT-2 cells and its transfected sublines with TRIzol reagent (Invitrogen, UK) and 1μg of each RNA sample was subjected to electrophoresis in 1% formaldehyde denaturation gel followed with overnight transfer to a positively charged nylon membrane (Hybond-N+, Amersham Pharmacia Biotech, Sweden) as described in Sambrook *et al.*, 1989. Overnight hybridization with HTLV-1 LTR U3 specific probe and β-actin specific probe at 68 ^{0}C followed by autoradiography was performed accordingly manufacturer's instructions.

Detection of viral particles in cell culture supernatant

Concentration of virions from cell culture supernatant. RaHOS cells and its transfected sublines were seeded at concentration $2x10^5$ per ml, MT-2 and Ra-1 cells, as well as their transfected sublines were seeded in concentration $1x10^6$ cells per ml. After 72 hours of cultivation cell culture supernatant was collected. After centrifugation at 10,000 g for 20 minutes, 30 ml of cell-free supernatant was used for ultracentrifugation. Ultracentrifugation was performed through sacharose cushion (5ml of 20% sacharose in Hanks' buffer) at 100,000 g for 4 hours. The sedimented virus particles were resuspended in 15 μl of phosphate buffered saline (PBS) and 5 μl aliquote was used in RT-PCR as it is described above.

Quantitative HTLV-I/II p19 antigen ELISA. To assess the amount of HTLV-1 particles in cell culture supernatant, enzyme linked immunosorbent assay (ELISA) was performed by using commercial HTLV-I/II p19 antigen ELISA kit (ZeptoMetrix Corporation, USA) according to manufacturer's protocol. RaHOS cells and obtained transfected RaHOS cell culture clones were seeded on 24 well plate $2x10^5$ cells per well and cell culture media was collected after 48 hours of cultivation for the p19 antigen ELISA analysis. After centrifugation at 12,000 g for 20 minutes, 20 times diluted supernatant was used in ELISA assay. Ra-1 cells were seeded at concentration $3x10^5$ per ml; MT-2 cells were seeded $1.5x10^5$ cells per ml and then placed at +4 ^{0}C for 8 hours for synchronization. After 72 hours of cultivation at 37 ^{0}C, the cell culture media were collected and centrifuged at 12,000 g for 20 minutes and 50 times diluted supernatant was used in ELISA analysis.

Indirect immunofluorescence assay (IFA)

Cells, growing on glass coverslips, were fixed with methanol at –20 ^{0}C for 20 min, air-dried and then incubated at 37 ^{0}C for 40 min with the primary antibodies diluted in PBS. As the primary antibodies two sera of Japanese ATLL patients were used. Two HTLV-1 negative sera from normal persons were used as negative control. After incubation the cells were washed with cold PBS four times for 15 min and incubated with the secondary fluorescein isothiocyanate-conjugated antibody diluted in PBS. The secondary antibody was then removed by washing as described above. As the secondary antibody a goat anti-human FITC labeled IgG were used.

Syncytia assay (SA)

The ability of the RaHOS cells to mediate the formation of multinuclear giant cells – syncytia, was assessed by co-cultivation them with HeLa indicator cells. The HeLa cells were seeded at a density $5x10^4$ per ml into 35 mm Petri dish in DMEM with 5% FCS. After over-night incubation at 37 ^{0}C in 5% CO_2 the HeLa cells were co-cultivated with $5x10^3$ RaHOS cells which were pretreated by mitomycin C (5 μg/ml) in DMEM + 10% FCS for 2 days. Then the cells were washed twice with PBS, fixed in methanol for 15 min and stained by the Romanowsky-Giemsa technique. Syncytia were counted per 5,000 cells under the light microscope at x20 magnification. Only multinuclear cells, containing more than five nuclei were scored. Inhibition of syncytia formation by anti-HTLV-I serum was done using sera from two

Japanese patients with ATLL. Two sera from healthy blood donors were used as a negative control.

Proliferative activity assay

Proliferative activity of HOS and RaHOS cells was estimated by incorporation of ^{3}H-thymidine in DNA. Cells were seeded at concentration $1x10^{5}$ per well in 24 well plates. After 24, 48, 72 and 96 hours of cultivation ^{3}H-thymidine (1.0 mCi/ml, 25 Ci/mmol -Amersham) was added (2 μCi/well) and cells were incubated for 1 hour at 37 ^{0}C, 5% CO_2 atmosphere. Then cells were collected by using trypsine-EDTA solution and transferred to the Millipore filter (diameter of pores – 1.5 μm). The filters were washed 2 times with PBS and 3 times with 5 ml of 5% trichloroacetic acid to precipitate DNA. The DNA was fixed by 1 ml of 96% ethanol and air dried at 37 ^{0}C. The incorporation of ^{3}H-thymidine was measured in a Packard liquid scintillation counter.

Soft agar assay

1.000 and 10.000 viable single cells were suspended in 1 ml DMEM medium containing 0.3% Noble agar (Difco) and 15% FBS and were placed in six well plates over a base layer of DMEM medium, containing 0.6% Noble agar and 15% FCS in double. Cells were incubated in 5% CO_2 atmosphere at 37 ^{0}C in humid box for two weeks. Cultures were fed every 3 days, colonies larger than 0.1 mm was counted (Freschney, 1994).

Cytogenetic analysis

After 48h of cultivation 1 μg/ml of colchicine (Sigma) was added to culture liquid of RaHOS and HOS cells. The cells were incubated with colchicine at 37 ^{0}C in 5% CO2 incubator for 30 min. Then the cells were treated with trypsin-EDTA solution, suspended in DMEM with 5% FBS, sedimented by centrifugation and hypotonized with 1% sodium citrate for 30 min at 37 ^{0}C. The cells were fixed with a mixture of methanol and glacial acetic acid (3:1), dripped onto slides and stained with Giemsa (azure-eosin methylene blue in PBS) (Seabright, 1971).

Plasmids

pMPSVEH with cloned MPSV promoter (Artelt P. *et al.*, 1988), pMHTs, pMHTas with 226 bp fragment of HTLV-1 LTR U3 cloned into pMPSVEH vector under MPSV promoter in sense and antisense orientations as well as pGHT (pGEM5Zf+ with cloned 226 bp HTLV-1 LTR U3 fragment), pMP1100as with

HTLV-1 *tax* gene fragment cloned into pMPSVEH vector under MPSV promoter in antisense orientation and pCMV-β-Gal-SPORT and pEQ176 carrying β-galactosidase gene under the control of immediate early CMV promoter, were kindly provided by Alexei Borisenko (Moscow Research Institute for Viral Preparations, Academy of Medical Sciences, Russia). Plasmids phrGFP-1 with *gfp* reporter gene cassette and pKO Scrambler NTKV-1907 with *neo* selection gene cassette were obtained from Stratagene (USA). Plasmids pGEM5Zf+ and pBluescript KS$^-$ were obtained from Promega, USA. pHTLV-1 and pMT-2 with cloned complete HTLV-1 provirus was used to obtain HTLV-1 LTR U3 fragment for cloning.

Plasmid isolation and construction

The DH-5α and XL-1 blue *E. coli* strains were used. Isolation and further purification of plasmid DNA in CsCl gradient were performed as described in Sambrook *et al.*, 1989; and alternatively by using Qiafilter Midi kit (Qiagene, Germany) according to the manufacturer's protocol. The A_{260}/A_{280} ratio of the isolated plasmid DNA was in the 1.8-2.0 range. All enzymes were purchased from MBI Fermentas (Lithuania) and used according to manufacturer's instructions. The isolation of DNA fragments from the low melting temperature agarose (Sigma, USA) was performed as described by Sambrook *et al.* (1989).

Transfection

Transfection of RaHOS cells by the calcium phosphate co-precipitation method was performed using standard protocol, as described in Graham and Van der Eb, 1973.

Transfection with the ExGen 500 transfection reagent - 22 kDa linear polyethylenimine (PEI) (MBI Fermentas, Lithuania) was performed according to manufacturer's protocol. Briefly, cells grown in 24-well plate were transfected with 2 μg plasmid DNA mix with 6 equivalents of ExGen500 in 100 μl of 150 mM NaCl solution per well. For electroporation $2x10^7$ MT-2 or Ra-1 cells were suspended in 400 μl of RPMI-1640 with 50% FBS, mixed with 20 μg plasmid, placed in 4 mm gap cuvette model 640 (BTX, USA) and electroporated with 100 V electric pulse for 50 msec followed by 200 msec pause for five cycles.

Nucleofection of MT-2 cells was performed with Cell Line Optimization Nucleofector^{tm} kit according to manufacturer's instructions using the apparatus provided by Amaxa biosystems (Germany). 10^6 cells were suspended in 100 μl of

nucleofector solution V, mixed with 5 μg of the plasmid and subjected to nucleofection using protocol A-23.

Estimation of transfection efficiency and selection of transfected cells

Transfection efficiency was assessed by microscopic examination of GFP fluorescence in non-fixed cell preparations 24 hours after transfection with phr-GFP-1 or by transfection with plasmid pCMV-β-Gal-SPORT carrying β-galactosidase gene and subsequent staining of transfected cells with X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) –MBI Fermentas, Lithuania (Okimoto *et al.*, 1999).

Selection of G418 resistant MT-2 and Ra-1 cells was carried out at 1200 μg/ml of antibiotic for 4 weeks in 24 well plates. Selection of G418 resistant RaHOS cells was carried out at 400 μg/ml for 4 weeks in 6 well plates. The G418 resistant cell colonies were isolated as described in Freschney, 1994. Then transfected cells were maintained in the growth medium supplemented with 400 or 200 (for the RaHOS cells) μg/ml of the antibiotic G418.

RESULTS

The establishment of experimental model of HTLV-1 infection in monolayer cell line HOS

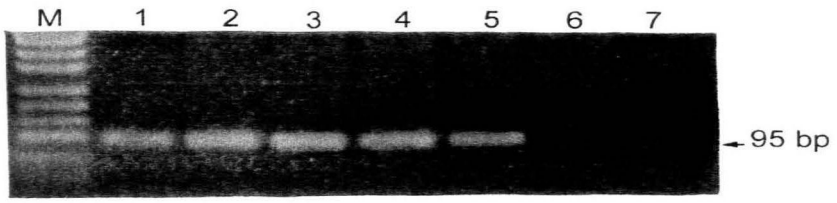
(Papers IV and V)

It is known that different lines of cultured cells vary by several orders of magnitude in their ability to take up and express exogenously added DNA (Sambrook *et al.*, 1989). The efficiency of establishing stably transfected cell lines is dependent on the efficiency of gene transfer into a given cell line, as well as on the survival of successfully transfected cells when a selective pressure is applied. Lymphocyte cell cultures mostly are transfected using electroporation techniques. The disadvantage of this method is the high incidence level of cell damage which makes it difficult to obtain cell clones harbouring the introduced nucleic acid. Known HTLV-1 producing cell lines are of lymphocyte origin and therefore are susceptible to cell damage induced by transfection procedures. Monolayer cell culture would allow usage of more effective and convenient transfection techniques such as calcium co-precipitation or liposome- or polycation-mediated gene delivery. Many non-lymphoid cell types derived from human and varieties of animal species have been shown to be permissive for HTLV-1 adsorption and penetration *in vitro* (Clapham *et al.*, 1983). However, HTLV-1 was incapable continuously replicate in these cells. Only non-lymphoid human osteosarcoma (HOS) cell culture and human endothelial cells were permissive for HTLV-1 replication (Clapham *et al.*, 1983; Hoxie *et al.*, 1984). To obtain a convenient monolayer cell culture model to study effect of asRNA on HTLV-1 replication, a monolayer HOS cell line was infected with HTLV-1.

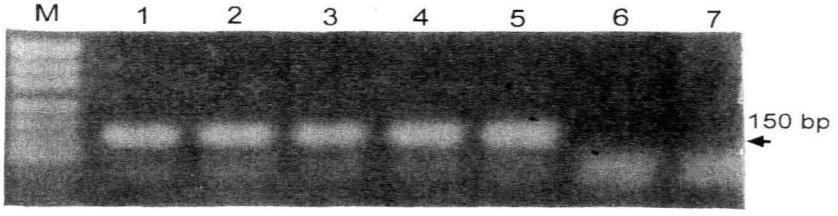
Infection of HOS cells by HTLV-1. Efficient HTLV-1 entry into the host cell requires direct cell to cell interaction and usually for the experimental infection of susceptible cells the method of co-cultivation with irradiated or mitomycin C treated (to prevent cell division) HTLV-1 producing cells is used (Miyoshi *et al.*, 1989). HOS cells were infected by co-cultivation with the mitomycin C treated virus-producing lymphoid cells of rabbit cell line Ra-1. Co-culture was carried out using suspension of Ra-1 cells at concentration 1.5×10^5 cells per ml. After co-cultivation of initial HOS cell culture with Ra-1 cells the polynuclear cells – syncytia containing from 5-12 nuclei have been registered. Their amount was increased for the next 6 passages of infected HOS cells with following decrease. By passage 14 only single syncytia

remained. Sometimes the single multinuclear cells were observed during the rest of cultivation of infected HOS cells. The formation of syncytia in HOS cells after co-culture with HTLV-1-producing Ra-1 cells indicated transmission and productive HTLV-1 infection in the HOS cells. The obtained cell culture was designated as RaHOS. Detection of HTLV-1 proviral DNA in RaHOS cells. PCR analysis for the virus regulatory (*tax*, 5' LTR) and structural (*gag, env*) genes showed the integration of the provirus DNA in RaHOS cells. Using a set of HTLV/STLV generic outer and HTLV-1 specific inner primers for the *tax* gene, DNA samples extracted from RaHOS cells were found to be positive for HTLV-1 sequence in dynamics, as shown for the passages 45, 57, 77 and 130 (Fig. 4A). HTLV-1 infection was further confirmed by the presence of the *gag* and *env* genes, and the 5'LTR region sequences as shown for the passages 45, 57, 77 and 130 (Fig. 4B, 4C, 4D). The results of PCR had proved that integration of the virus remained stable during the observation period, for more than 150 passages of cultivation.

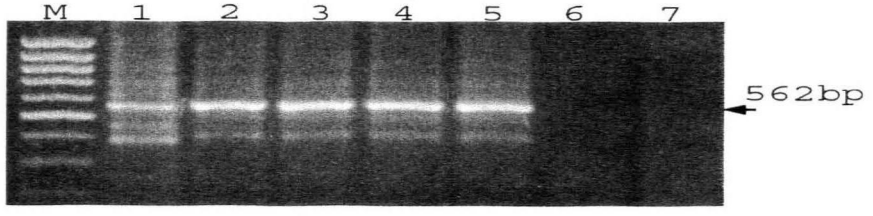
A.



B.



C.



D.

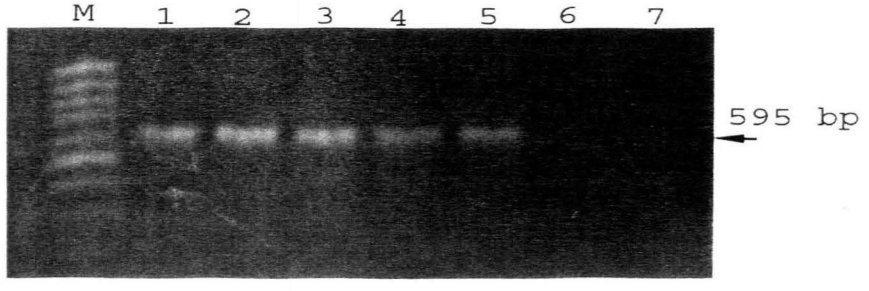
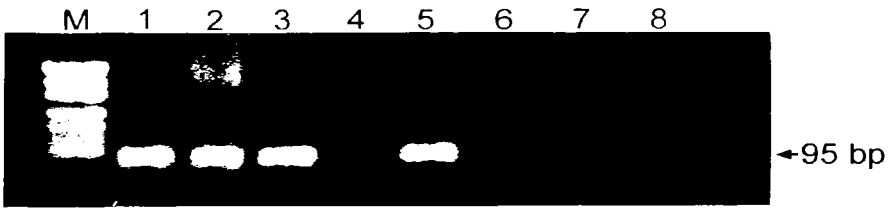


Fig. 4. Detection of HTLV-1 proviral sequences in DNA of RaHOS cells. DNA was amplified with primers complementary to the regions of the *tax* gene (A); *gag* gene (B); *env* gene (C) and 5'LTR (D). M-markers: pUC19 DNA/MspI (A, B), MassRuler DNA Ladder, Low range (C, D); 1 – DNA extracted from HTLV-1-producing Ra-1 cells (positive control); 2-5 – DNA extracted from RaHOS cells after 45, 57, 77, 130 passages, respectively; 6 – DNA extracted from HOS cells (negative control); 7 – control without DNA. The size of the amplification product (bp) is indicated on the right side.

Detection of HTLV-1 mRNA expression. To determine whether the cell line is persistently producing the virus or is latently infected, the RT-PCR analysis with HTLV-1 specific primers complementary to the *gag* and *tax* genes was performed.. The expression of the *gag* and *tax* genes was detected in RaHOS cells throughout all the observation period (20-150 passages). The detection of viral mRNA for the *tax* and *gag* genes in the cells at the passage 106 using the RT-PCR method is shown at the Fig. 5. To confirm the specificity of the amplification product and to exclude contamination with genomic DNA, simultaneus reactions were performed in the ansence of RT (Fig. 5, line 4). The results of RT-PCR indicated that viral mRNA transcripts in the obtained RaHOS cell culture were continuously expressed.

A



B

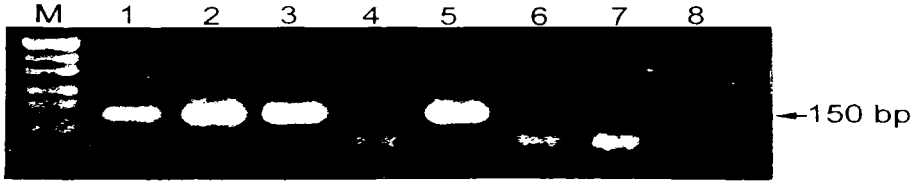
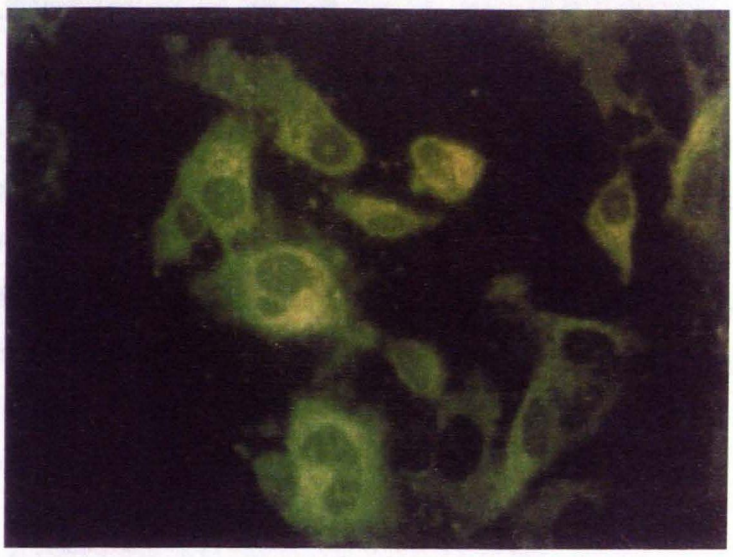


Fig. 5. Expression of HTLV-1 A. *tax* and B. *gag* sequences in RaHOS cells (passage 110) and RaHOS cell culture supernatant (passages 106-110). M – markers: pUC19 DNA/MspI; 1 – DNA extracted from HTLV-1-producing Ra-1 cells; 2 – RT-PCR: RNA from HTLV-1-producing Ra-1 cells; 3 – RT-PCR: RNA isolated from RaHOS cells after 110 passages; 4 – RNA from RaHOS cells, PCR without RT; 5 – RT-PCR: RNA extracted from RaHOS cell culture supernatant; 6 – RT-PCR: RNA extracted from HOS cells (negative control); 7 – RNA extracted from HOS cells, PCR without RT; 8 – control without RNA. The size of the amplified product (bp) is indicated on the right side.

Detection of HTLV-1 antigens. The presence of HTLV-1 antigens was estimated by indirect immunofluorescence analysis (IFA) and syncytia assay (SA). Bright fluorescent regions in cytoplasm and diffuse fluorescence around the nucleus

were detected in 75-80 % of RaHOS cells at 65 passage, when IFA was carried out with HTLV-1 positive sera (Fig. 6A). At the same time, fluorescence was not observed in HOS cells incubated with HTLV-1 positive sera, or in RaHOS cells incubated with HTLV-1 negative sera (Fig. 6B). The specific fluorescence in cytoplasm of RaHOS cells was observed during the whole observation period up to the 150^{th} passage. However, the number of fluorescence positive cells as well as fluorescence intensity decreased with increasing the passage number and at passage 150 the specific fluorescence was observed only in 5% of cells.

A



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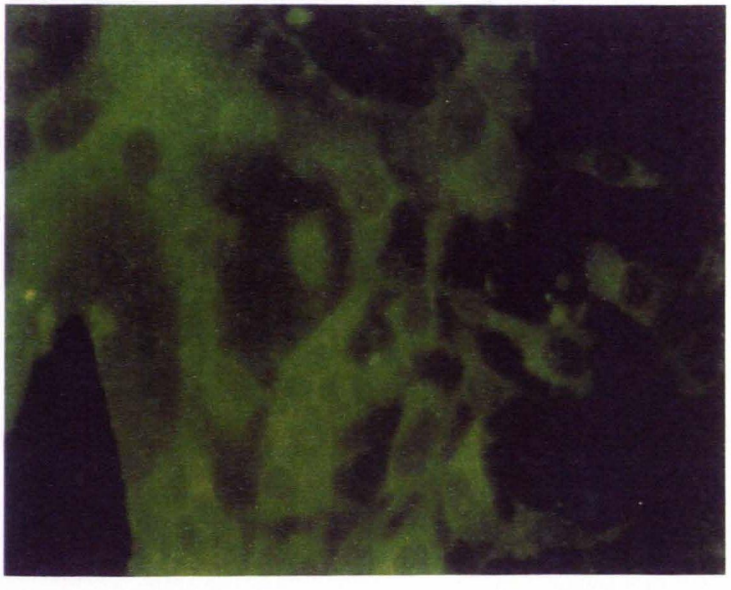
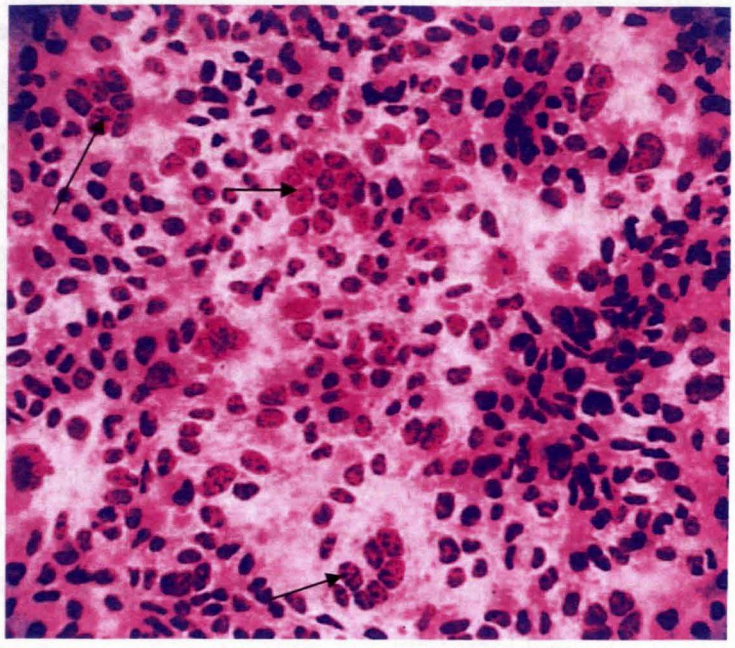


Fig. 6. Detection of HTLV-1 antigens in RaHOS cells by IFA. RaHOS cells at passage 65 were incubated with serum of ATLL patient (A) or with HTLV-1 negative serum (B). Fixation and staining were performed as described in Materials and Methods, magnification x 600

It is known that the free viral particles of HTLV-BLV group viruses are poorly infectious and viral transmission occurs almost exclusively via cell-to-cell contacts. The capacity of the virus to mediate cell-to-cell transmission is known to correlate with the syncytia-forming ability of the infected cells. The capacity of RaHOS cells to induce syncytia in HeLa cells was studied. The proportion of multinuclear cells in control HeLa cell culture did not exceed 0.6% and the number of nuclei in these cells was up to 5. When RaHOS cells were mixed with HeLa cells, syncytia containing 5-15 nuclei appeared in the indicator HeLa cells, and the total amount of syncytia increased by up to 1.5-4.0 % (Fig. 7A). The replication of HTLV-1 in the RaHOS cell line was also confirmed by inhibition of syncytia formation in the presence of anti-HTLV-1 antibodies from HTLV-1 positive patient sera (Fig. 7B), while in the presence of HTLV-1 negative sera, the syncytia inhibition was not observed. As the cell fusion is mediated by HTLV-1 envelope glycoproteins (Delamarre *et al.*, 1997; Sagara *et al.*, 1997), the induction of syncytia in indicator HeLa cell culture by co-culture with RaHOS cells indicated the expression of viral envelope proteins in RaHOS cells.

A.



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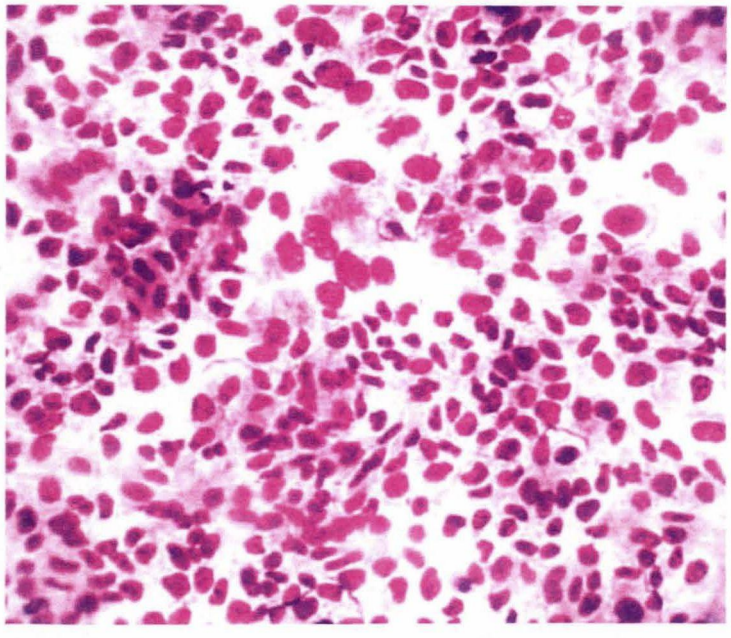


Fig. 7. Syncytia formation in HeLa cells co-cultivated with RaHOS cells (A) and inhibition of syncytia formation in the presence of serum of ATLL patient (B). The multinuclear cells are marked by arrows. SA was performed as indicated in Materials and Methods

Productive infection of HTLV-1 in RaHOS cell line. The production of HTLV-1 virions in RaHOS cell culture was showed by the presence of the HTLV-1 genomic RNA in cell-free RaHOS culture supernatant. For this purpose, the virions were sedimented by ultracentrifugation and isolated RNA was subjected to RT-PCR with the HTLV-1 *gag* and *tax*-specific primers. Both the HTLV-1 *gag* and *tax*-specific amplification products were detected, as it is shown for the 106-110 passages of cultivation (Fig. 5). These results were reproducible throughout the all observation period (20-110 passages). Thus, the HTLV-1 replication was demonstrated by PCR, RT-PCR, IFA and SA methods (Fig. 8). IFA and SA confirmed the expression of HTLV-1 antigens in the cell cytoplasm and on the cell surface. The presence of viral RNA in cell-free culture medium gave evidence of viral particle production by RaHOS cells. Thus, RT-PCR, IFA and SA indicated that HTLV-1 infection in RaHOS cells is productive, stable and long-term.

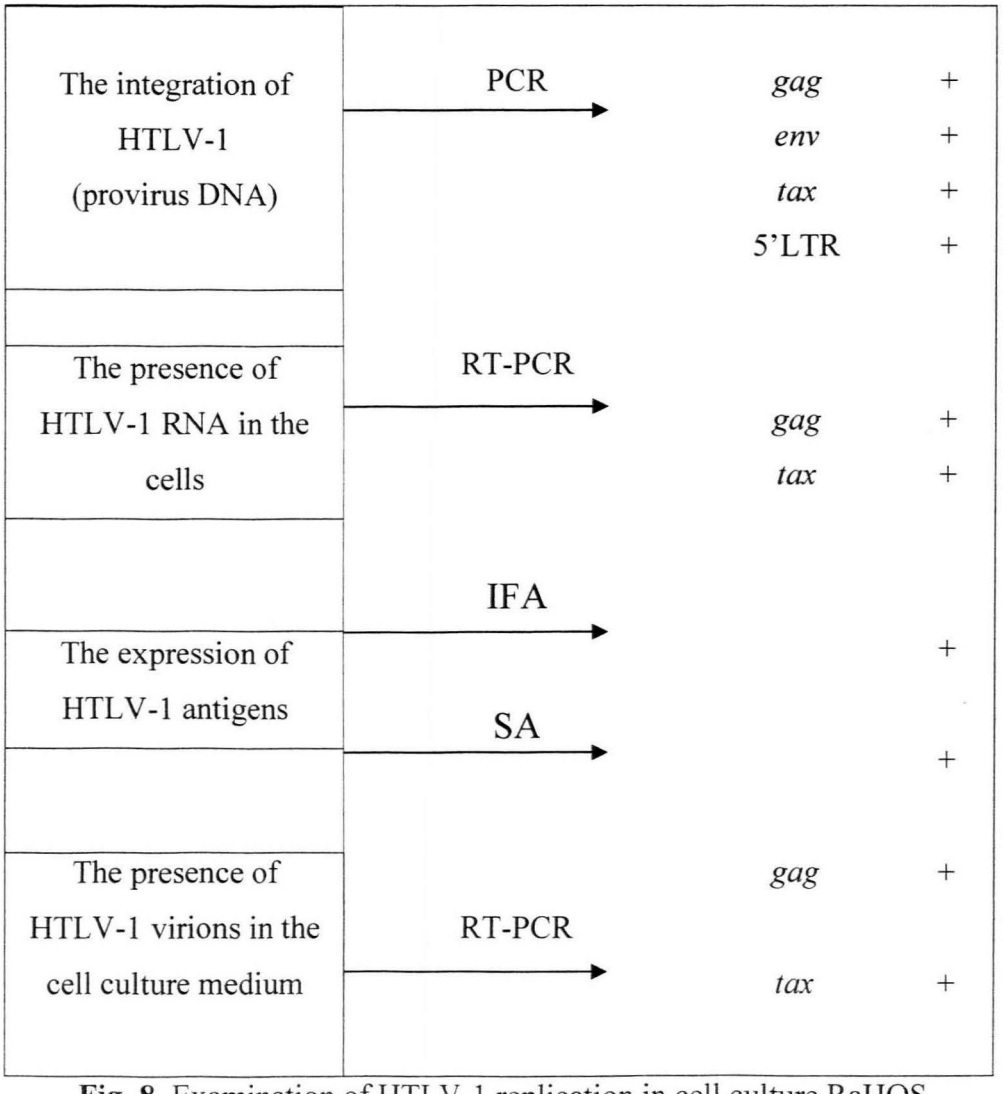


Fig. 8. Examination of HTLV-1 replication in cell culture RaHOS.

Cytogenetic analysis of RaHOS cells. Cytogenetic analysis of RaHOS cells of the 60^{th} and the 125^{th} passages showed human karyotype with modal number of chromosomes identical to those in HOS cells – 48 (Fig 9). However, at passage 125, the polyploidy percentage in the RaHOS cell culture was slightly elevated in comparison with HOS cell culture (5.4 % and 4.6 % respectively). Morphological features of RaHOS cell culture. Morphological studies of RaHOS cell culture (using Romanowsky-Giemsa staining) revealed some signs of transformation, compared to the initial HOS cell culture. Although both cell cultures were cultivated simultaneously in equal conditions, after 60 passages, the focuses of multilayer growth had appeared in the RaHOS cell culture (Fig. 10A), while the initial HOS cell culture lacked such features (Fig. 10B). After 30 passages, the RaHOS cells had got the ability to form colonies in soft agar. 0.1 % of the cells gave growth in colonies of about 0.1 mm in diameter. The colonie- forming capacity increased during further passages, and after 60 and 150 passages, already 0.4 % and 1.3 % of cells, respectively, had formed clumps of increased size (0.15-0.20 mm in diameter). The initial cell culture HOS TE85, used in this study as the control, did not form colonies in soft agar.

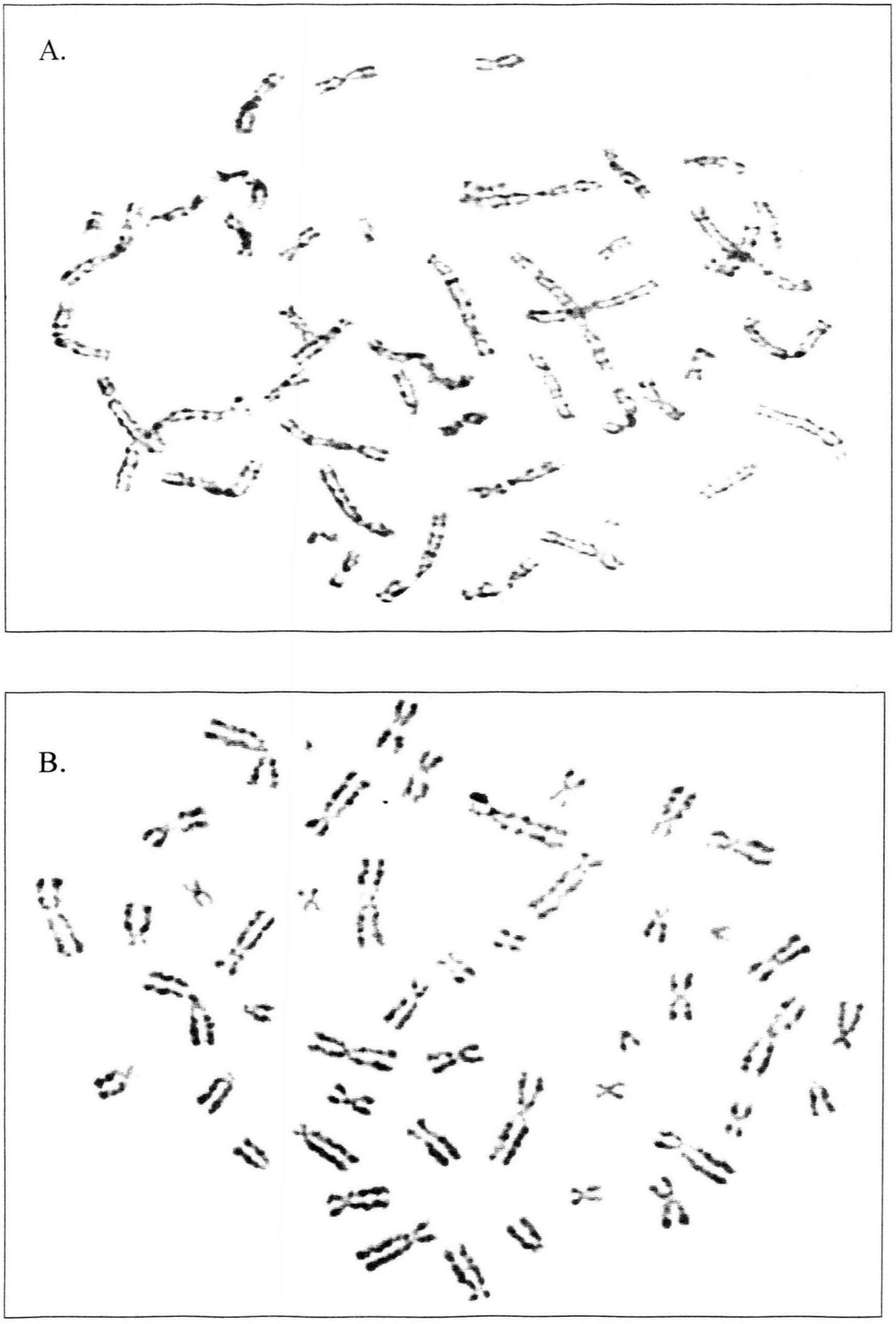
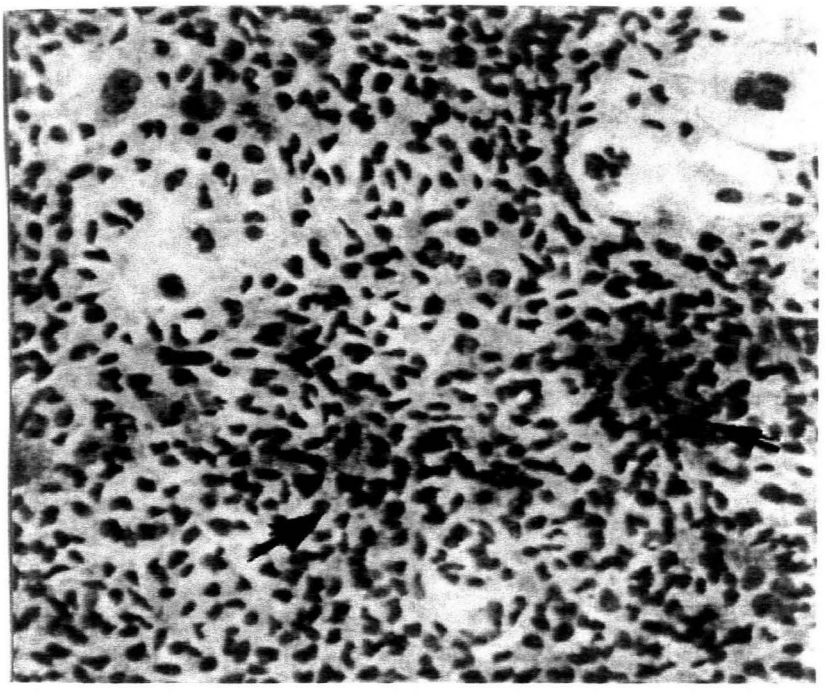


Fig. 9. Cytogenetic analysis of HOS and RaHOS cells.
A. Metaphase chromosomes of HOS cells.
B. Metaphase chromosomes of RaHOS cells
Cytogenetic analysis was performed as described in Materials and Methods.

A



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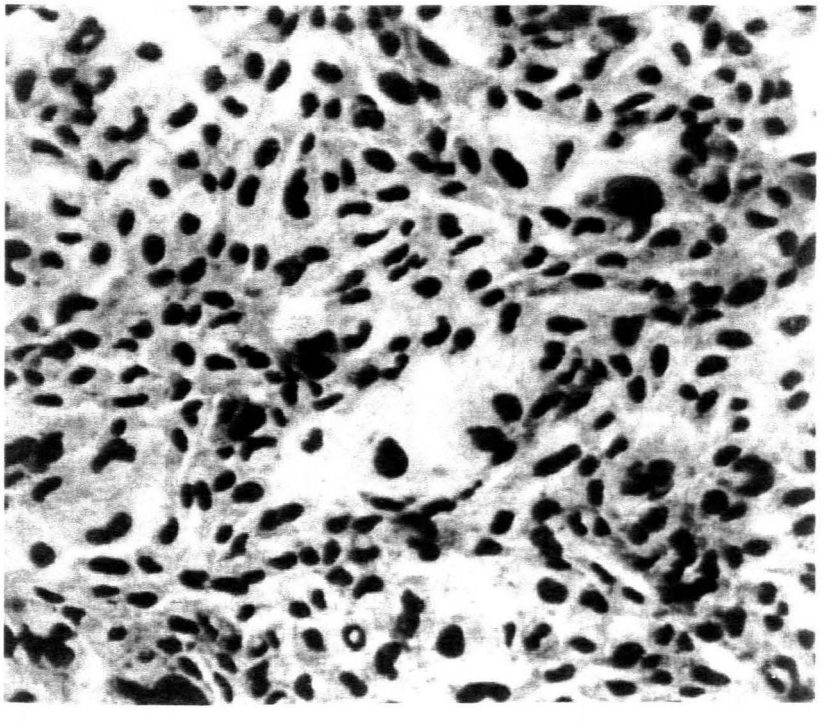

Fig. 10. Formation of foci of multilayer growth in RaHOS cells (passage 105) upon 72 h of culture (A). The control HOS cells upon 72 h of culture (B). The foci of multilayer growth are marked by arrows.

Also, the proliferative activity of RaHOS cells differed from the initial HOS cell culture. After 18 passages, the incorporation of ^{3}H-thymidine in RaHOS cells was 1.4 times higher, and at the 135 passage 2 times higher, in comparison with HOS cells. At 96 hours, the incorporation of ^{3}H-thymidine in RaHOS cells remained high, while in HOS cells its level was decreased (Fig. 11).

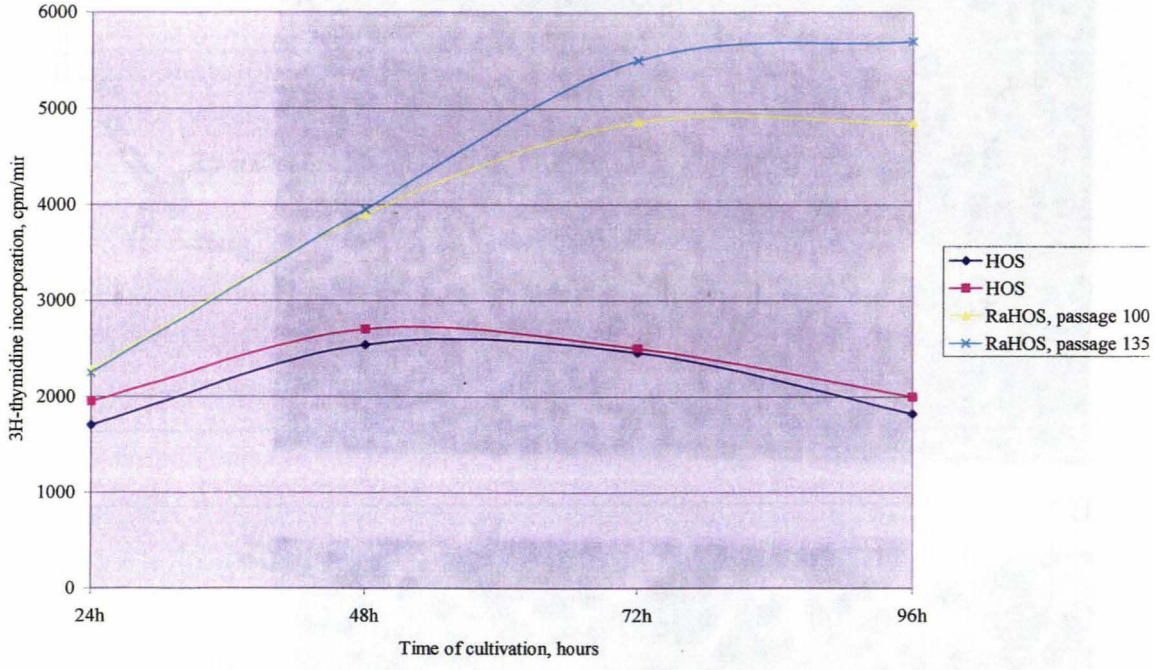

Fig. 11. Proliferative activity of RaHOS and HOS cells. Proliferative activity assay was performed as described in Materials and Methods.

Characteristics of the transformed cells *in vitro* included a loss of contact inhibition as demonstrated by focus formation in monolayer cell culture and acquisition of anchorage-independent growth as demonstrated by the ability to form colonies in soft agar. Therefore, RaHOS had a higher saturation density of the monolayer, higher proliferative activity, enhanced formation of multilayer growth focuses and the ability to form colonies in soft agar. The features of malignancy of RaHOS cells increased during continuous cultivation. The observed morphological changes apparently were the result of long-term HTLV-1 replication in these cells. The acquired phenotypic changes of the obtained cell culture indicated a change of the phenotype towards malignant transformation of the cells, previously not shown for HTLV-1 infected non-lymphoid cells. This cell culture could be a useful tool for investigation of the changes in cell genetic regulation which occur upon HTLV-1 infection. Cloning of RaHOS cell line. To improve and standartizy the established cell

culture model, the RaHOS cell line was cloned by dilution and four cell clones were obtained. Two of them contained integrated provirus, as detected by PCR analysis of extracted DNA. These two clones were designated as D1 and H9. Further analysis of the clones by RT-PCR confirmed the expression of viral mRNA (Fig. 12) and the IFA, as well as SA showed the expression of HTLV-1 antigens in the cells of these clones. Moreover, IFA and SA analyses showed more intensive viral expression in the clone H9. The specific fluorescence of cells was observed in 68% of H9 cells comparing to 27.9 % in D1 clone cells. The ability to grow in semisolid medium showed enhanced capacity of clone H9 to form colonies in soft agar in comparison with the D1 clone. After two week cultivation in soft agar about 0.6 % of clone H9 cells had formed clumps 0.06-0.17 mm in diameter comparing to 0.02% of D1 clone cells wich formed clumps 0.04 - 0.08 mm in diameter. Also in RT-PCR analysis of viral RNA expression in the clone H9 the amplification product of first PCR round was already visible comparing to the clone D1, where the virus specific amplification product was detectable only in the second PCR round (Fig. 12). Therefore, the clone H9 of RaHOS cell culture was used in further work in investigation of anti-viral effect of asRNA and viral trans-activator decoy constructs.

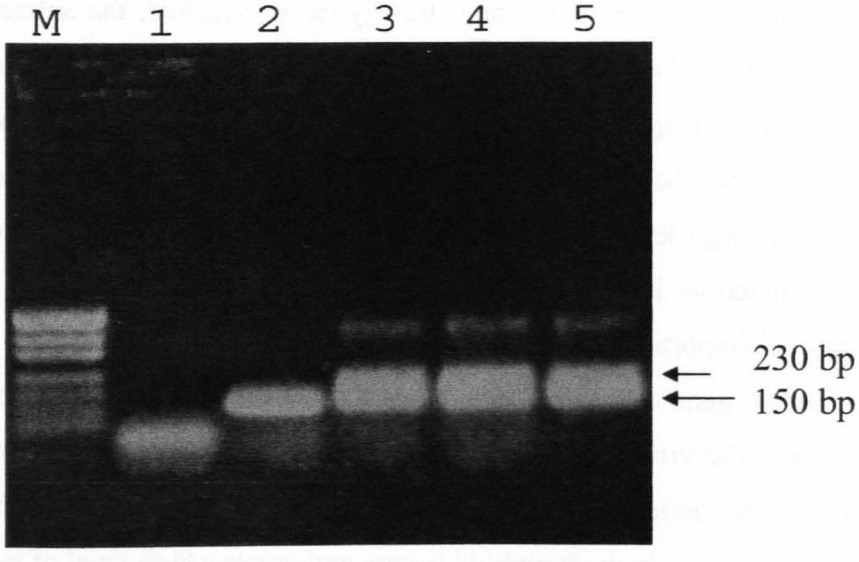

Fig. 12. Detection of viral RNA expression in RaHOS cell culture clones D1 and H9. RT-PCR with primers complementary to the HTLV-1 *gag* gene sequence. M – marker: pUC19 DNA/MspI; 1 – RNA isolated from HOS cells (negative control); 2 – RNA isolated from the RaHOS clone D1; 3 – RNA isolated from the RaHOS clone H9; 4 – RNA isolated from HTLV-1-producing cell line C91/PL; 5 – DNA of HTLV-1 positive person (positive control). By arrows on the right side are indicated 150 bp specific amplified product of second PCR round and also visible 230 bp fragment of the first PCR round.

Construction of the plasmids carrying asRNA genes targeted at HTLV-1 LTR U3 and pX region and Tax decoy HTLV-1 LTR U3 sequence

(Papers I and II)

There are as yet no general rules for choosing neither the optimal target site within a given viral genome nor the optimal length of the asRNA transcript. Therefore, these parameters still has to be established experimentally for the certain virus. Based on the investigations with the BLV, which is closely related to HTLV, as well as HIV, which uses similar strategy in its genome regulation and on the experiments with asODNs and ribozymes reported for the HTLV-1, the pX region and LTR U3 region of HTLV-1 genome were chosen as a promising targets for the nucleic acid based therapy of the virus.

Stable intracellular expression of as-sequences is currently the most efficient method by which as-nucleic acid technology can be used to inhibit gene expression. A major limitation to the use of stable expression of as-sequences as a therapy for viral infection is that long-term high levels of asRNA expression are required for effective inhibition of viral replication. The stoichiometry of as-sequences must be at a minimum 1:1, but ratios 5:1 or 10:1 lead to more effective inhibition of viral replication. Thus, for an as-gene therapy strategy to be effective, the antisense gene expression must be higher than the levels of HTLV-1 expression. Standard gene therapy vectors containing polymerase II promoters often do not produce sufficient levels of as-sequence to inhibit viral replication. Subsequently, polymerase III promoters ensures high level of gene expression, but are not cell/tissue specific and can not be regulated as it is possible in case of polymerase II promoters. Thus, a promoter choice is important in construction of asRNA gene carrying vector.

The asRNA gene carrying plasmids were constructed based on the principles mentioned above. The viral LTR U3 region and pX region sequences were cloned in reversed orientation under the strong MPSV (Myeloproliferative sarcoma virus) promoter, which is active in the lymphoid tissues and ensures high level of expression comparing to SV40 immediate early promoter and HIV transactivated promoter (Artelt P. *et al.*, 1988; Lin *et al.*, 1994; Engels *et al.*, 2003).

Construction of the plasmid carrying asRNA gene targeted at HTLV-1 LTR U3 (Paper I). The plasmid pMT2, containing full HTLV-1 genome was used as the source of HTLV-1 LTR U3 sequence fragment for construction of HTLV-1 LTR U3 asRNA gene carrying plasmid. For this purpose HTLV-1 LTR U3 226 bp fragment

excised from the plasmid pMT-2 with *Sma*I and *Hinc*II restriction enzymes (Fig.13) was inserted into *Sma*I site of the pMPSVEH vector (Fig 14) under MPSV promoter. The orientation of HTLV-1 LTR U3 fragment in obtained recombinant plasmids was examined by restriction analysis and antisense pMHTas plasmid construct was selected (Fig. 15B). As a control the plasmid with the same HTLV-1 LTR U3 fragment in sense orientation (pMHTs) also was used (Fig. 15A).

1 GGAAAAACTT GGAGTGTAGT TCTGACAATG ACCATGAGCC CCAAATATCC CCCGGGGGCT

61 TAGAGCCTCC CAGTGAAAAA CATTTCCGAG AAACAGAAGT CTGAAAAGGT CAGGGCCCAG

121 ACTAAGGCTC TGACGTCTCC CCCCGGAGGG CAGCTCAGCA CCGGCTCGGG CTAGGCCCTG

181 ACGTGTCCCC CTGAAGACAA ATCATAAGCT CAGACCTCCG GGAAGCCACC AAGAACCACC

241 CATTTCCTCC CCATGTTTGT CAAGCCGTCC TCAGGCGTTG ACGACAACCC CTCACCTCAA
301 AAAACTTTTC ATGGCACGCA TATGGCTCAA TAAACTAGCA GGAGTCTATA AAAGCGTGGA
361 GACAGTTCAG GAGGG

Fig. 13. HTLV-1 LTR U3 sequence, starting from nucleotide 23 (Seiki *et al.,* 1983).
Bright green – *Sma*I restriction endonuclease recognition site; violet – position of the BA2 primer; red – 21 bp repeats; blue – position of the MM1 primer; green – *Hinc*II restriction endonuclease recognition site; yellow – polyadenilation signal; underlined – TATA box

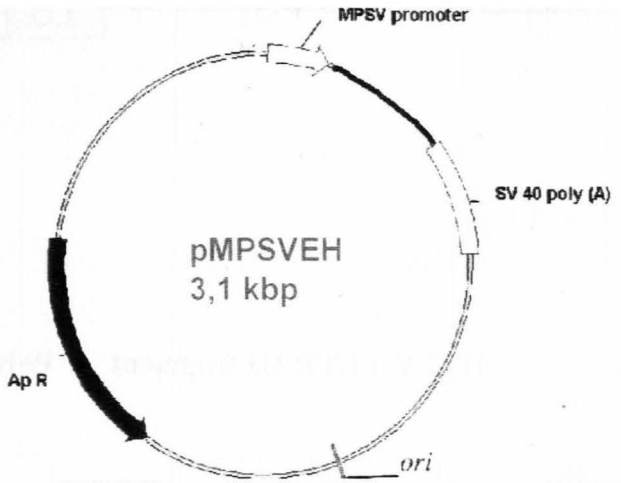

Fig. 14. Schema of the pMPSVEH vector

Abbreviations: MPSV - Myeloproliferative sarcoma virus; SV 40 poly (A) - Simian Virus 40 polyadenilation signal; ApR - ampicillin resistance gene; *ori* – pBR322 replication origin of the plasmid.

Construction of the plasmid carrying asRNA gene targeted at HTLV-1 pX region (Paper I). To construct a plasmid carrying asRNA targeted to HTLV-1 pX region, 1100 bp fragment of HTLV-1 pX region was cutted out from the plasmid pCO–12-20 with *EcoR*I and *Pst*I restriction enzymes and subcloned into pBluescript KS (pB1100). Then the fragment was excised from pB1100 with *BamH*I and *Hind*III restriction enzymes and inserted by *Hind*III and *BamH*I restriction sites into pMPSVEH vector under the MPSV promoter to obtain the antisense orientation required (Fig. 16).

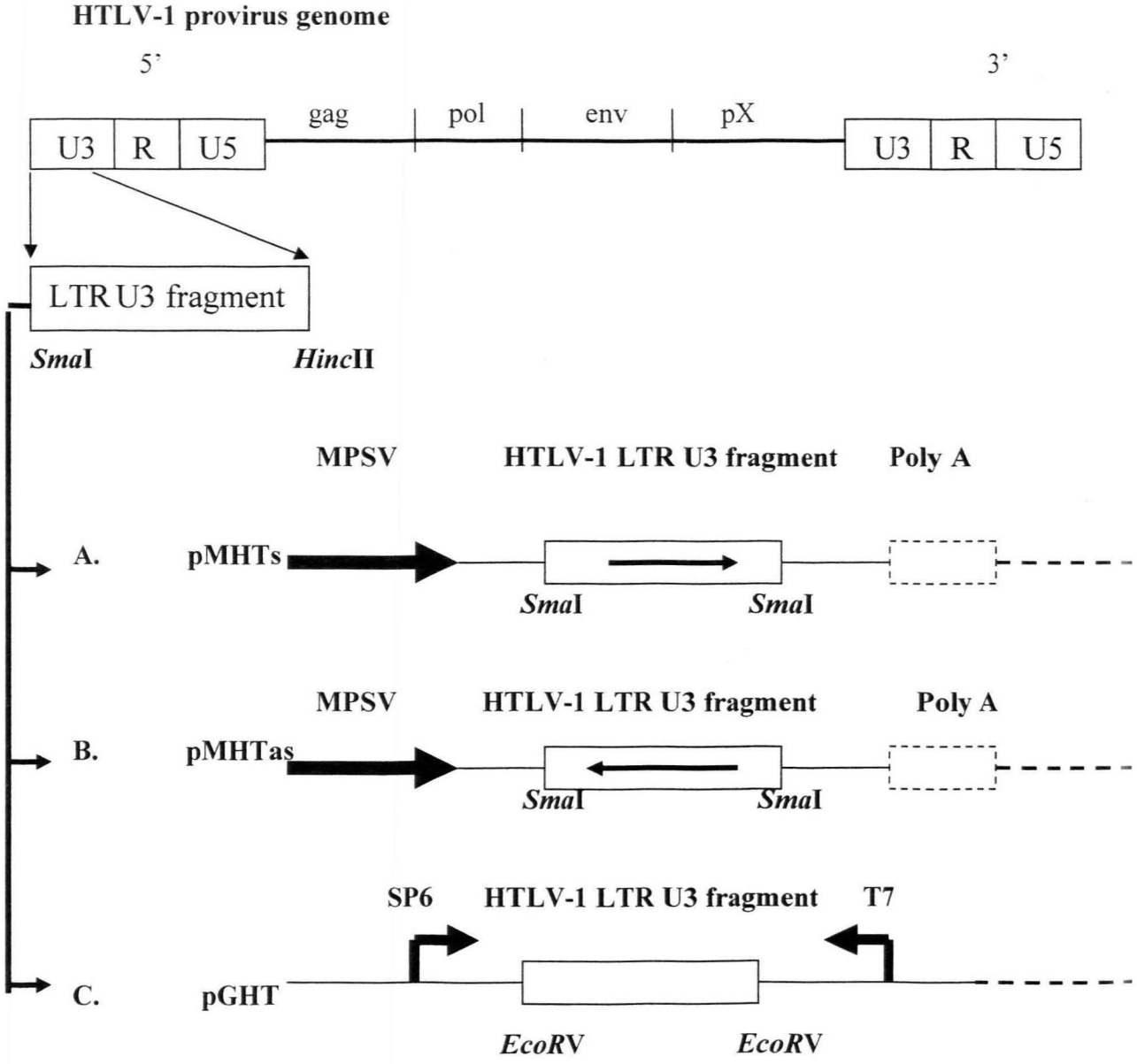

Fig. 15. Construction of pMHTs, pMHTas and pGHT plasmids.
HTLV-1 LTR U3 226 bp fragment was excised from the plasmid pMT-2 with *Sma*I and *Hinc*II restriction enzymes. The exicised fragment was inserted into *Sma*I site of the pMPSVEH vector under MPSV promoter in sense and antisense orientation obtaining pMHTs (A) and pMHTas (B) constructs, correspondingly. The same HTLV-1 LTR U3 fragment was inserted also into *EcoR*V restriction site of pGEM5Z+ plasmid without eukariotic promoter and is not expressed. The obtained Tax decoy construct was designated as pGHT (C).
Abbreviations: MPSV - Myeloproliferative sarcoma virus promoter; poly (A) - Simian Virus 40 polyadenilation signal; SP6 and T7 – promoters of the phages SP6 and T7, respectively

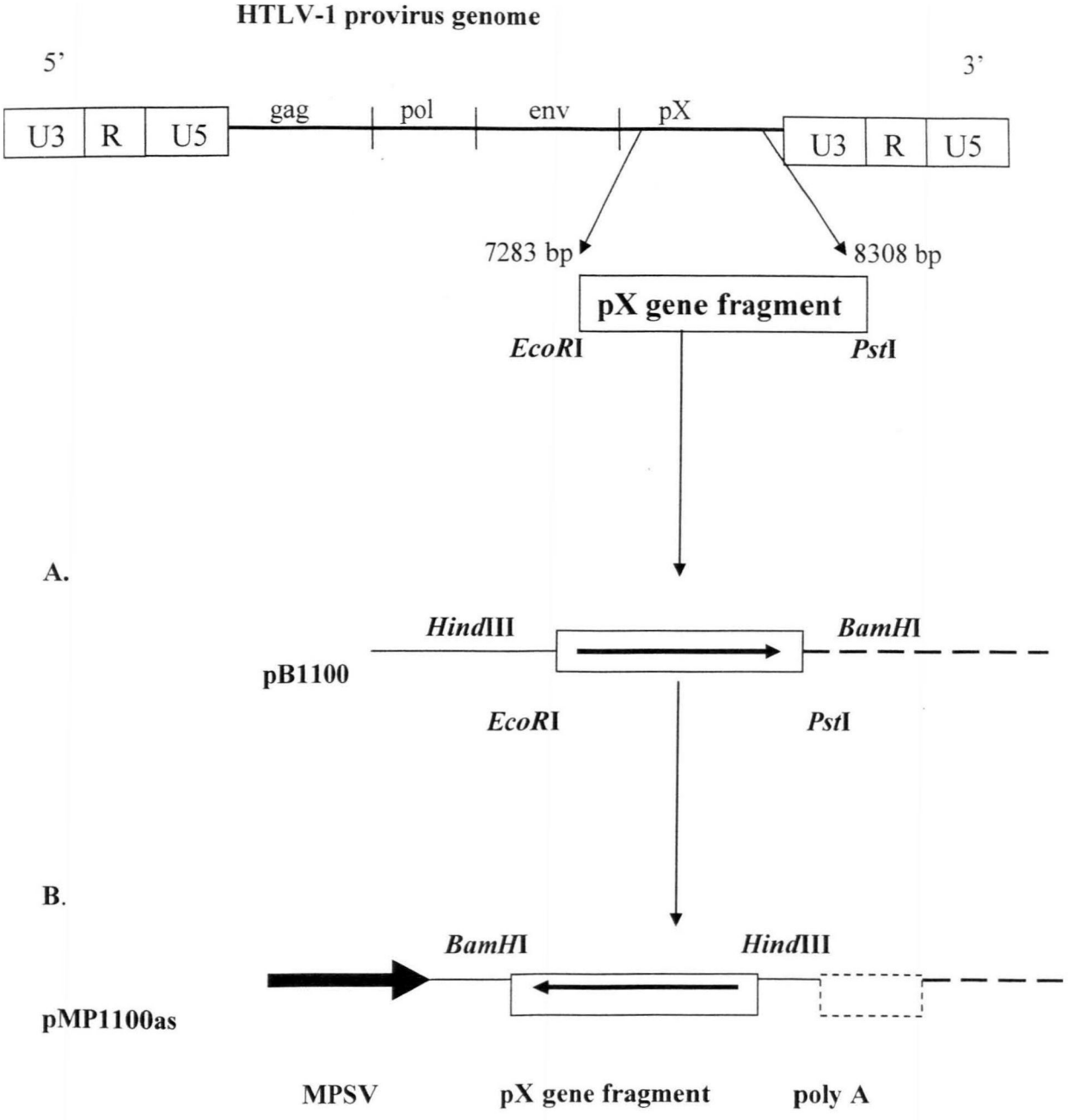

Fig. 16. Construction of the plasmid pMP1100as
A. HTLV-1 pX region fragment (1100 bp) was excised from pCO-12-20 with *EcoR*I and *Pst*I and inserted between *EcoR*I and *Pst*I sites of pBluescript KS$^-$ (pB1100).
B. Insertion of HTLV-1 pX region fragment into pMPSVEH vector in antisense orientation. pX gene fragment was excised with *BamH*I *and Hind*III from intermediate construct pB1100 and inserted into *BamH*I *and Hind*III restriction site of the pMPSVEH vector (pMP1100as).
Abbreviations: MPSV - Myeloproliferative sarcoma virus promoter; poly (A) - Simian Virus 40 polyadenilation signal

Construction of the Tax decoy plasmid, carrying HTLV-1 U3 fragment. To construct the plasmid carrying virus transactivator decoy sequences the same 226 bp HTLV-1 LTR U3 fragment excised from pMT-2 plasmid was used (Fig13). This fragment is containing two full 21 bp repeats, which are recognised by viral transactivator Tax (shown in the Fig. 13 by red). The fragment was inserted into pGEM5Z+ plasmid into *EcoRV* restriction site without eukariotic promoter and is not expressed. The obtained Tax decoy construct was designated as pGHT (Fig 15C).

The plasmids pMHTs, pMHTas, pMP1100as as well as the plasmid pGHT used in this study were constructed in Laboratory for Viral Infections Gene Therapy of the Moscow Research Institute for Viral Preparations, Academy of Medical Sciences, Russia in the frame of collaboration project „Design and testing of recombinant genes programming informational immunity against human T-cell leukemia virus". It was expected that the plasmid pMHTas could employ both: asRNA and Tax decoy mechanisms of action, as the Tax decoy sequences are transcribed from the MPSV promoter to asRNA. Subsequently, plasmid pMHTs could act as Tax decoy construct with simultaneous expression of viral LTR U3 RNA fragment and plasmid pGHT –exclusively as Tax decoy construct.

The optimization of HTLV-1 antisense and Tax decoy constructs (Paper II). As the original vector pMPSVEH with cloned MPSV promoter do not contain any selection/reporter gene, the cells have to be co-transfected with plasmid, which harbour marker gene. Taking in account low transfection efficiency, highly traumatic electroporation technique, laborious and time-consuming cloning procedure the probability to obtain cell clones harbouring therapeutic gene decreases strongly in case of co-transfection. To improve the efficiency of transfection the created as-constructs were modified by the insertion of the *neo* (neomycin phosphotransferase) selection gene into the plasmids pMHTs and pMHTas with 226 bp fragment of HTLV-1 LTR U3, cloned under MPSV promoter in sense and antisense orientations, respectively. Also the construct harbouring *neo* gene and non-expressed HTLV-1 LTR U3 sequence, which contains 21 bp repeats recognized by HTLV-1 trans-activator protein Tax, was made. Insertion of the *neo* gene into pMHTs and pMHTas. The *neo* gene was excised with *Hind*III and *BamH*I restrictases from the pKO Scrambler NTKV-1907 plasmid and inserted between *Hind*III and *BamH*I sites of the

plasmids pMHTs and pMHTas, respectively (Fig.17A). The new constructs were designated as P4neo and P2neo, accordingly. Construction of Tax decoy plasmid, harbouring *neo* selection gene. To construct the plasmid harbouring only HTLV-1 LTR U3 sequence, which contains 21 bp repeats, recognised by HTLV-1 *trans*-activator protein Tax (analogue of pGHT with *neo* gene), the pKO Scrambler NTKV-1907 plasmid was used as a backbone. First, the thymidine kinase gene was excised with *Rsr*II. Then the 226 bp fragment of the HTLV-1 LTR U3 was cutted out from pHTLV-1 by *Sma*I and *Hinc*II digestion and inserted into *SmaI* site of the intermediate construct (Fig. 17B). The new construct was named as P5neo.

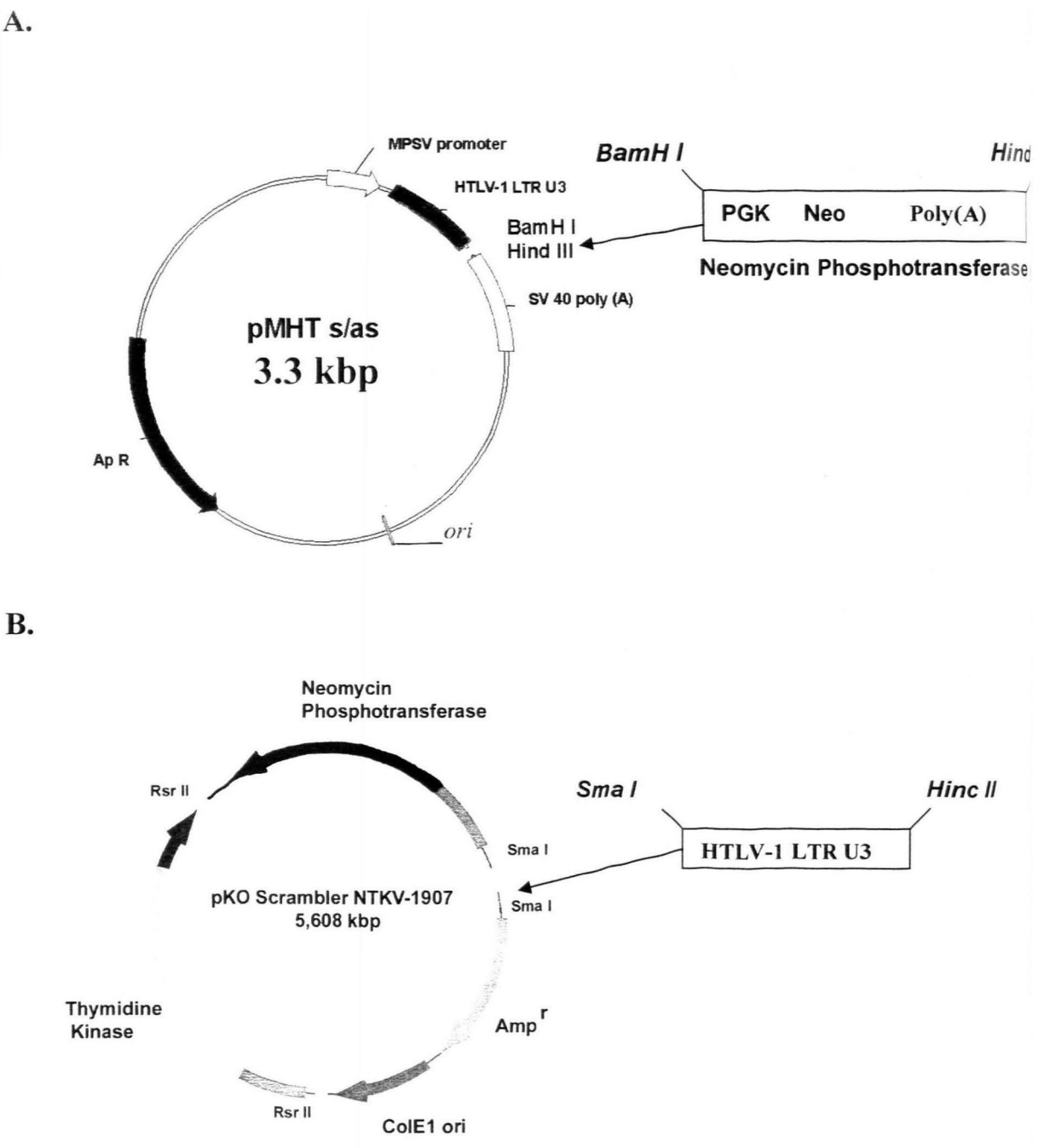

Fig. 17. Construction of the plasmids P2neo, P4neo and P5neo
A. Neomycin Phosphotransferase gene (1647 bp) was excised from pKO Scrambler NTKV-1907 with *BamH I* and *Hind III* and inserted between *BamH I* and *Hind III* sites of pMHTas and pMHTs, respectively.
B. Insertion of HTLV-1 LTR U3 fragment into pKO Scrambler NTKV-1907. First, the thymidine kinase gene (2019 bp) was excised with *Rsr II*. Then HTLV-1 LTR U3 fragment (226 bp) was cloned into *Sma I* restriction site of the construct.
Abbreviations: MPSV - Myeloproliferative sarcoma virus; SV 40 poly (A) - Simian Virus 40 polyadenilation signal; *Ap R* - ampicillin resistance gene; *ori* - replication origin of the plasmid; PGK – mouse phosphoglycerol kinase promoter; *Neo* – Neomycin Phosphotransferase; Poly (A) – polyadenilation signal; ColE1 ori – ColE1 replication origin of the plasmid; *Amp*^{r} – ampicillin resistance gene

Examination of asRNA expression in modified constructs. To assess, if the introduced *neo* gene did not affect the expression of asRNA from the new-made constructs, Vero cells were transfected with the P2neo and P4neo plasmids using ExGen 500 transfection reagent (22 kDa linear PEI). Vero cells were used as easy transfectable and HTLV-1 negative cell line to avoid crossreaction with viral mRNA when examining by RT-PCR its expression as one could expect in HTLV-1 producing cells. Total cellular RNA from transfected Vero cells was isolated and checked for HTLV-1 LTR U3 RNA expression by RT-PCR. In all tested samples expression of HTLV-1 LTR U3 fragment was clearly observed (Fig. 18) suggesting that the introduced marker genes did not affect asRNA synthesis. Thus, the new constructs will allow applying better and faster selection in further experiments on the efficiency of asRNA approach in antiviral therapy.

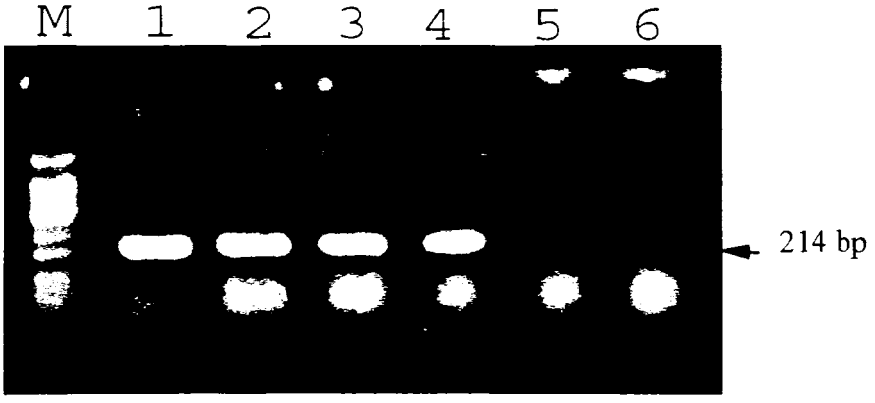

Fig. 18. Expression of HTLV-1 LTR U3 RNA in Vero cells transfected with P2neo, P4neo (RT-PCR)

M – 100 bp DNA Ladder (Promega, USA); 1 – Ra-1 RNA, positive control; 2 – RNA of Vero cells transfected with P2neo; 3- RNA of Vero cells transfected with P4neo; 4 – RNA of Vero cells transfected with P2GFP plasmid; 5 – RNA of intact Vero cells (negative control); 6 – control without RNA. Specific amplified fragment of 214 bp is indicated on the right side.

The investigation of the effect of HTLV-1 asRNA and Tax decoy plasmids on HTLV-1 replication in RaHOS cells

(Paper I)

Transfection of RaHOS cell line by HTLV-1 antisense and Tax decoy constructs and establishment of transfected cell clones. The plasmid constructs pMHTs, pMHTas, pMP1100as and pGHT were co-transfected with plasmid pSV2*neo* coding the *neo* selection gene into RaHOS cells by calcium co-precipitation method. The transfection efficiency was assessed by transfection with plasmid pCMV-β-Gal-SPORT carrying β-galactosidase gene under control of immediate early CMV promoter with subsequent staining of the cells with X-Gal (staines blue). The transfection efficiency of RaHOS cells was about 2-3% as assessed by amount of stained cells. This transfection efficiency is relatively low, comparing with other monolayer cell lines, for example HeLa or Vero cells, where it could be more than 30% (Chen and Okayama, 1987; Nikcevic *et al.,* 2003). Therefore, RaHOS cell line is relatively hard to transfect, nevertheless it allows usage of such convenient transfection techniques as calcium co-precipitation and others in contrast to lymphocyte cell cultures where these methods are very inefficient. After one month selection with 400 μg/ml of geneticin (G418), more than 40 of G418 resistant cell colonies were obtained. The established G418 resistant stable cell clones were analysed by PCR for MPSV promoter sequence (Fig. 19) and for the pGHT vector sequence (Fig. 20) to ensure the presence of the introduced plasmids in selected cells. Among the obtained antibiotic resistant clones, only 9 were positive for the introduced plasmids. As it is shown in the Fig. 19. the clones Nr. 12 and Nr.14 (pMHTs), Nr. 33 and Nr. 38 (pMHTas), Nr. 31 and Nr. 35 (pMP1100) were positive for MPSV promoter sequence and in the Fig. 20. the clones Nr. 25, Nr. 27 and Nr. 32 (pGHT) were positive for the pGHT sequence. The long lasting resistance of established cell cultures to G418 and repeated detection of the introduced plasmid sequences after passage of the cells allow to suggest integration of introduced sequences into genomic DNA of obtained RaHOS clones. The clones Nr. 14 (pMHTs), Nr. 38 (pMHTas), Nr. 35 (pMP1100as) and Nr. 25 (pGHT) were chosen for further analysis of the effect of introduced sequences on viral mRNA synthesis.

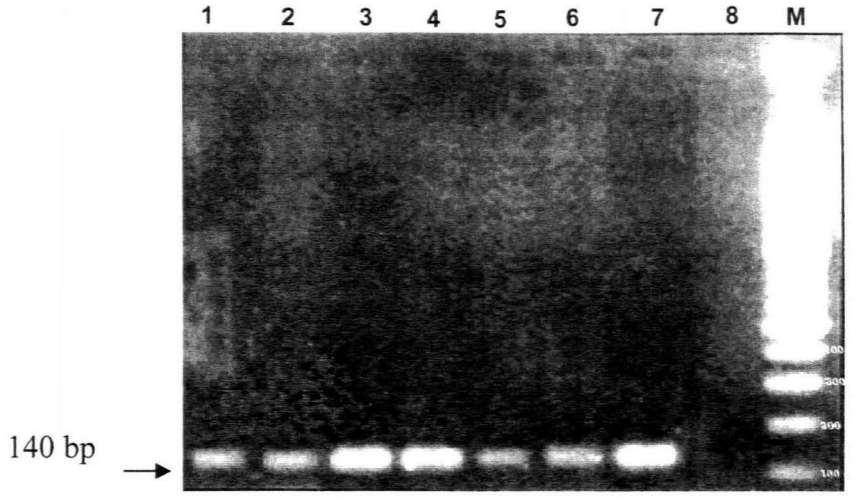

Fig. 19. Detection of MPSV promoter sequence in the G418 resistant cell clones of RaHOS cell culture transfected with plasmids pMHTs, pMHTas and pMP1100as. 1, 2 – DNA extracted from cell clones Nr. 12 and Nr.14, respectively, transfected with pMHTs; 3, 4 – DNA extracted from cell clones Nr. 33 and Nr. 38, respectively, transfected with pMHTas; 5,6 – DNA extracted from cell clones Nr. 31 and Nr. 35, respectively, transfected with pMP1100as; 7 – pMHTs plasmid DNA (positive control); 8 – DNA extracted from RaHOS cells (negative control); M- DNA size marker: DNA Step Ladder (Promega). Specific 140 bp amplified fragment is indicated on the left side.

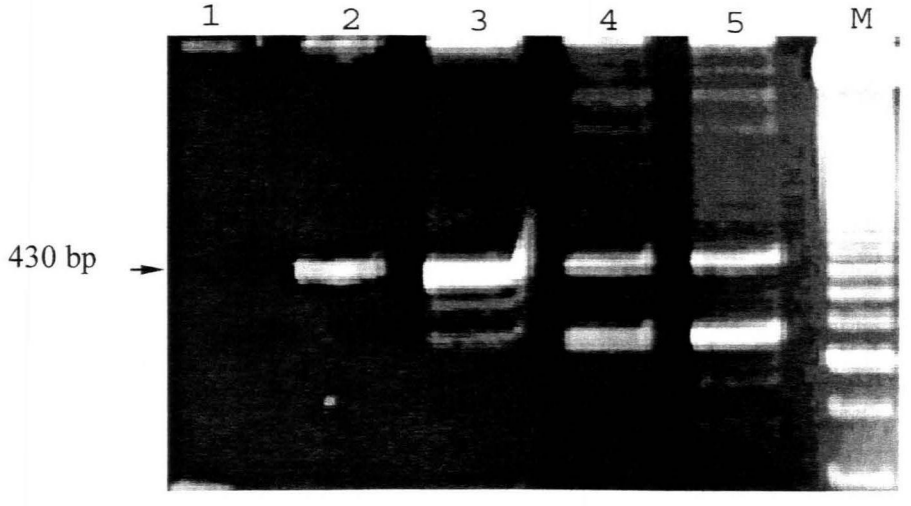

Fig. 20. Detection of pGHT sequence in the G418 resistant cell clones of transfected RaHOS cell culture. 1 – DNA extracted from RaHOS cells (negative control); 2 – pGHT plasmid DNA (positive control); 3, 4, 5 – DNA extracted from cell clones Nr. 25 , Nr. 27 and Nr. 32, respectively; M – DNA size marker (Applied Biosystems). Specific 430 bp amplified fragment is indicated on the left side.

Investigation of the effect of HTLV-1 antisense and Tax decoy constructs on HTLV-1 mRNA synthesis. To analyze the possible effect of the introduced antisense and decoy sequences on HTLV-1 replication, sensitive technique allowing quantitative analysis of samples should be applied. Dot-hybridization analysis of nucleic acids bound on nitrocelluloze membrane is sensitive enough to detect specific viral DNA or RNA in the sample and allows to compare the amount of analysed specific sequences in the samples by intensity of radioactive signal given by specifically bound probe with incorporated radioactive ^{32}P isotope. The HTLV-1 specific probes for the dot-hybridization analysis used in this study were made by PCR amplification of HTLV-1 LTR U3 and pX sequences from the pMHTs and pMP1100as plasmids, correspondingly, with the addition of $\alpha^{32}P$ dATP into reaction mix. Examination of integrated provirus by dot-hybridization in obtained RaHOS cell culture clones with the introduced sequences. First, genomic DNA of all clones was examined for the integrated proviral DNA. To differentiate between viral sequences introduced with the constructs and own proviral sequences of HTLV-1 infected cells, the clones Nr. 14, 38, 35 containing plasmids with HTLV-1 LTR U3 sequence were hybridized with probe to pX region and subsequently the clone Nr. 25, harbouring antisense sequence to pX region was hybridized with LTR U3 specific probe. All examined clones were positive for the proviral sequences. The results of proviral DNA analysis of clones with pX specific probe are showed in Fig. 21. The RaHOS cell culture and its clone H9 were used as the positive controls, the HOS cell culture as the negative control and 2ng of HTLV-1 genome excised from the pMT2 plasmid served as the quantitative control for hybridization. As it is shown in the Fig. 21. the signal of hybridization with proviral sequences is strong enough, comparing with the signal in RaHOS and its clone H9, exept the clone Nr. 38 with pMHTas sequence. The faint hybridization signal in the clone Nr. 38 allow to suggest that the amount of integrated provirus in these cells was lower.

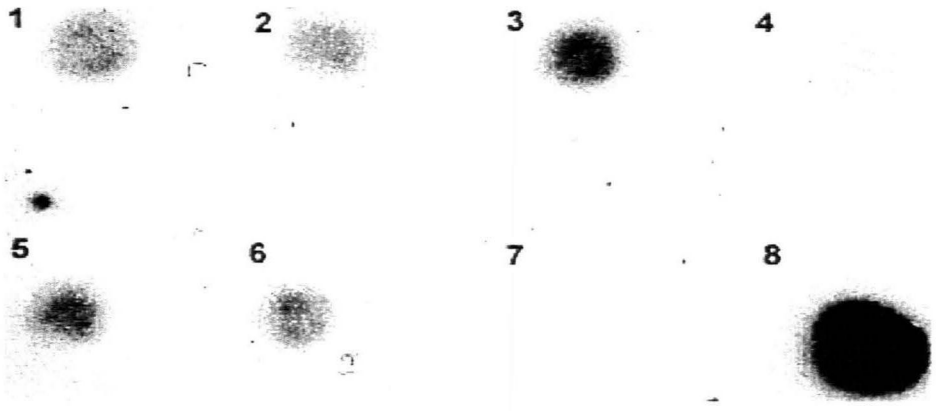

Fig. 21. Dot-hybridization analysis of genomic DNA extracted from RaHOS cell culture clones containing pMHTs, pMHTas, pMP1100as and pGHT constructs. The DNA is hybridized with the probe complementary to pX region of HTLV-1. 1 – DNA extracted from the RaHOS clone Nr. 14., containing pMHTs; 2 – DNA extracted from the RaHOS clone Nr. 25, containing pGHT; 3 – DNA extracted from the RaHOS cell clone Nr. 35, containing pMP1100as; 4 – DNA extracted from the RaHOS cell clone Nr. 38, containing pMHTas; 5 – DNA extracted from the RaHOS cell clone H9; 6 – DNA extracted from RaHOS cell culture; 7 – DNA extracted from HOS cells (negative control); 8 – 2 ng of pMT2 plasmid DNA (positive control).

The dot-hybridization analysis of viral RNA in the transfected clones. To analyze the possible effect of the introduced as- and decoy sequences on HTLV-1 replication the dot-hybridization analysis of viral RNA was performed. For the detection of viral RNA in the transfected cell clones, the probe corresponding to pX region was used to analyse cells containing HTLV-1 LTR U3 constructs (clones Nr. 14, 38, 35) (Fig. 22) and probe to HTLV-1 LTR U3 region to analyse the clone Nr. 25, harbouring antisense gene to HTLV-1 pX region (data not shown). The analysis of RNA dot-hybridization results using computer program „Lab Works" (Bio-Rad) clearly showed the strong, 90 % inhibition of viral RNA synthesis in clone Nr. 25, harbouring pGHT - HTLV-1 Tax decoy construct. Also in the clone Nr. 14, containing pMHTs plasmid and clone Nr. 35, harbouring pMP1100as plasmid, the inhibition of viral RNA synthesis was approximately 50%. No significant inhibition in the clone Nr. 38, harbouring antisense construct pMHTas, was observed.

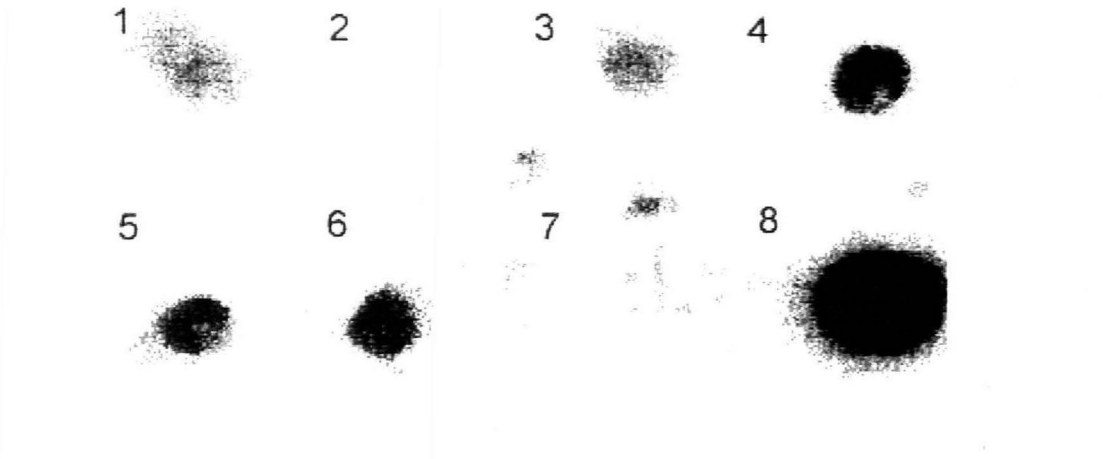

Fig. 22. Dot-hybridization analysis of RNA extracted from RaHOS cell culture clones containing pMHTs, pMHTas, pMP1100as and pGHT constructs. The RNA was hybridized with the probe complementary to pX region of HTLV-1. 1 – RNA extracted from the RaHOS clone Nr. 14., containing pMHTs; 2 – RNA extracted from the RaHOS clone Nr. 25, containing pGHT; 3 – RNA extracted from the RaHOS cell clone Nr. 35, containing pMP1100as; 4 – RNA extracted from the RaHOS cell clone Nr. 38, containing pMHTas; 5 – RNA extracted from the RaHOS cell clone H9; 6 – RNA extracted from RaHOS cell culture; 7 – RNA extracted from HOS cells (negative control); 8 – 2ng of pMT2 plasmid DNA (positive control).

It was anticipated that the plasmid pMHTas, harbouring HTLV-1 LTR U3 enhancer sequence under control of MPSV promoter, may employ both – antisense and viral *trans*-activator decoy mechanisms of action. The obtained results did not confirm this preposition. One of possible explanations for it could be following: binding of cellular transcription proteins to the DNA sequence of MPSV promoter and subsequent transcription of asRNA gene could be affected by viral protein Tax bound to its enhancer sequences in the asRNA gene and controversily, binding of Tax to its enhancer sequences could be hampered by transcription of these sequences from MPSV promoter. As a result of such disturbances caused by close position of two regulatory DNA sequences, the expression of asRNA from such construct may be hampered, as well as the binding of Tax protein to its enhancer. Therefore, one of the probable explanations of the inhibition of viral mRNA synthesis found in the cell clone harbouring control pMHTs construct could be its Tax decoy action. As it was shown by Shayakhmetov and co-authors, the efficient inhibition of BLV was achieved with a plasmid containing non-expressing BLV U3 promoter sequence starting from 20 molar excess of the plasmid over the BLV proviral DNA (Shayakhmetov *et al.*, 1997). In contrast, in the case of asRNA gene to BLV LTR R U5 region, the efficient

inhibition of BLV was achieved at 1:5 ratio of proviral to asRNA-encoding DNA (Shayakhmetov *et al.*, 1997). Another possible explanation of viral mRNA synthesis inhibition in cell clone harbouring sense pMHTs construct could be the phenomenon of co-suppression discovered first in plants and later also in animals when co-expression of introduced sequence together with the same natural sequence cause not the expected higher expression of the product but, opposite, inhibition of such sequence expression in the organism. (Napoli *et al.*, 1990; Pickford and Cogoni, 2003). Subsequently, the absence of significant effect found in pMHTas harbouring cell clone could be explained by possible disturbances in asRNA synthesis due to close position of strong MPSV promoter and HTLV-1 enhancer sequences by one hand and insufficient copy number of integrated pMHTas plasmid in the cells to act as Tax decoy by the other hand. At the same time the strong inhibition of viral RNA synthesis found in the RaHOS clone Nr. 25, could be due to high number of copies of Tax decoy construct in these cells. It could be judged undirectly from the PCR amplification results (Fig. 20), where DNA of clone Nr. 25 gave much stronger signal, comparing to others two clones. Transfection of RaHOS clone H9 with antisense P2neo and sense P4neo constructs. Thus, the results obtained with RaHOS cell culture clones showed efficient inhibition of viral mRNA synthesis in the cell clone harbouring pMHTs plasmid – HTLV-1 LTR U3 sense gene and no effect in the clone with plasmid pMHTas – HTLV-1 LTR U3 asRNA gene. It was reasonable to assume that the individual difference between clones in copy number/integration pattern of introduced plasmids may influence the assessment of their antiviral action. In order to obtain and analyse more clones with the HTLV-1 LTR U3 sense and asRNA genes, the constructs P2neo and P4neo (pMHTs and pMHTas with inserted *neo* gene) were transfected into RaHOS clone H9 cells using linear 22 kDa PEI. The transfection efficiency, assessed by transfection with plasmid carrying β-galactosidase reporter gene pEQ176, was about 2-4%. After three week selection with 400 μg/ml of G418, the cell cultures resistant to G418 antibiotic designated as RaHOS-P2neo and RaHOS-P4neo were obtained. The genomic DNA was extracted from these cell cultures and analysed for the introduced plasmid sequences by PCR. Both RaHOS-P2neo and RaHOS–P4neo cell cultures were positive for the corresponding plasmid sequences (Fig. 23).

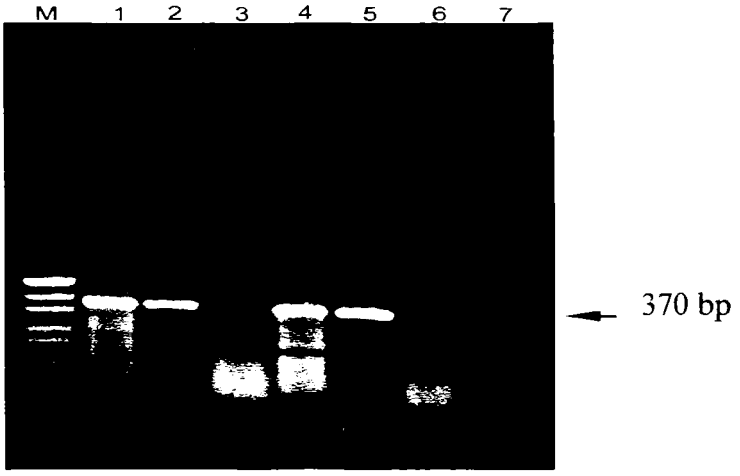

Fig. 23. Detection of P2neo and P4neo sequences in transfected G418 resistant RaHOS cells. M – marker: pUC19/MspI; 1 P2neo plasmid DNA (positive control); 2 – DNA isolated from G418 resistant RaHOS cells transfected with P2neo; 3 – DNA isolated from RaHOS cells (negative control); 4 – P4neo plasmid DNA (positive control); 5 – DNA isolated from G418 resistant RaHOS cells, transfected with P4neo; 6 – DNA isolated from RaHOS cells (negative control); 7 – control without DNA. Specific 370 bp amplified fragment is indicated on the right side.

The analysis of asRNA expression in cell culture RaHOS-P2neo. The results of analysis of viral expression in RaHOS cell clone harbouring antisense construct pMHTas showed no effect on virus RNA synthesis. Therefore at first it was important to analyse the expression of asRNA from the pMHTas plasmid in RaHOS cell culture. Previously, in the HTLV-1 negative Vero cells transfected with P2neo plasmid and using the same transfection reagent – 22 kDa linear PEI, it was found that the P2neo plasmid is functioning and expressing HTLV-1 asRNA (Fig. 18). The expression of asRNA in the RaHOS-P2neo cells, containing plasmid P2neo was analysed by RT-PCR, using sense primer in RT reaction for cDNA synthesis from asRNA strand as described in Materials and Methods. The expression of asRNA in RaHOS-P2neo cell culture was not detected (Fig. 24).

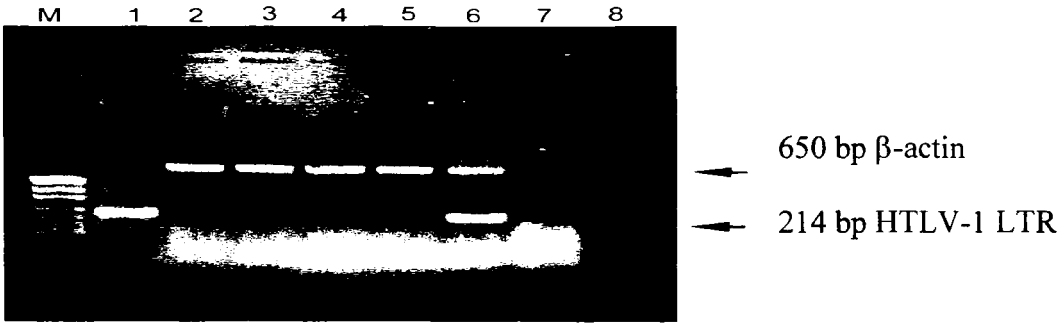

Fig. 24. Examination of HTLV-1 LTR U3 asRNA expression in RaHOS-P2neo cells (RT-PCR). M – marker: pUC19/MspI; 1 – P2neo plasmid DNA (positive control); 2 – RNA isolated from RaHOS cells; 3 – RNA isolated from RaHOS-P2neo cells; 4 – RNA isolated from RaHOS –P4neo cells; 5- RNA isolated from HOS cells (negative control) 6 – RNA isolated from Vero cells transfected with P2neo (positive control); 7 – DNA isolated from HOS cells (negative control); 8 – control without DNA. HTLV-1 specific 214 bp fragment and β-actin specific 650 bp fragment (RT-PCR control) are indicated on the right side.

By the same time, resistance to G418 and presence of plasmid P2neo sequence in the obtained RaHOS–P2neo cell culture, confirm integration of *neo* gene and plasmid sequence into cellular genome. These results suggest that the expression of asRNA from the P2neo plasmid probably is hampered in HTLV-1 infected cells through binding of HTLV-1 transactivator Tax to its enhancer sequences in HTLV-1 LTR U3 asRNA gene. There could be also other explanations of our fail to detect asRNA expression in the cells. For example, the effects of dsRNA, which could be formed by asRNA with its complementary cellular counterpart and subsequently could be degraded in the cell or processed to small fragments by the RNase III-like nuclease Dicer, which promotes cleavage of long dsRNAs into 21-23-nt short siRNAs with 2-nt 3' overhangs. However, in analogical work with BLV the detection of expressed asRNA clearly correlated with the antiviral action of the as-sequences (Tomsons *et al.*, 1993). Nevertheless, the detection of asRNA expression could not be always directly relevant to its antiviral effect. Analysis of viral RNA expression in RaHOS-P2neo and RaHOS-P4neo cell cultures. Thus, the expression of viral mRNA transcripts in RaHOS-P2neo and RaHOS-P4neo cell cultures was analysed by RT-PCR method for the LTR U3 sequences, as it is described in Materials and Methods. The results of RT-PCR are showed at Fig.25. No significant difference in fluorescence intensity of corresponding RT-PCR fragments was observed. Probably, the sensitivity of RT-PCR method is too high to detect smaller differences in specific

RNA amount, also by this method all viral RNA transcripts was detected, some of which may be truncated and not functional.

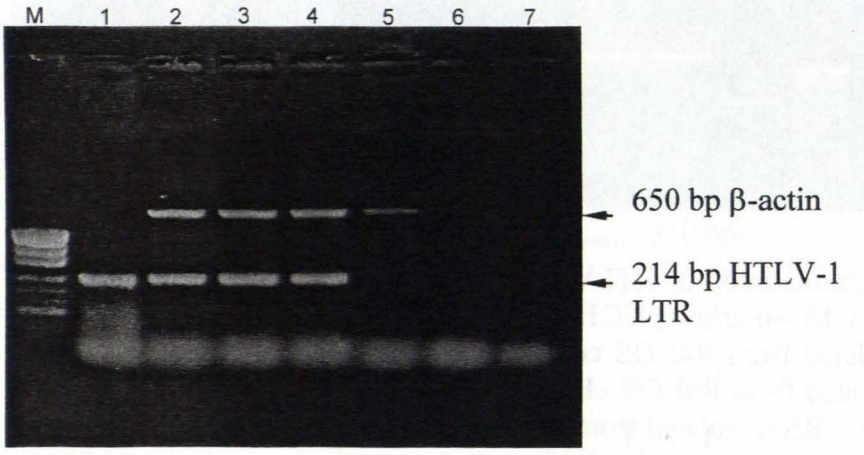

Fig. 25. Analysis of viral RNA expression in RaHOS-P2neo and RaHOS-P4neo cell cultures (RT-PCR). M – marker: pUC19 DNA/MspI; 1 – DNA extracted from RaHOS cells (positive control); 2 – RNA extracted from RaHOS-P2neo cells; 3 – RNA extracted from RaHOS-P4neo cells; 4 - RNA extracted from RaHOS cells; 5 – RNA extracted from HOS cells; 6 – DNA extracted from HOS cells (negative control); 7 – control without DNA. The amplified HTLV-1 specific 214 bp and β-actin gene specific 650 bp fragments (RT-PCR control) are indicated on the right side.

Analysis of RaHOS-P2neo and RaHOS-P4neo cell culture media for the viral particles. To examine viral particle release cell-free supernatant of transfected RaHOS-P2neo and RaHOS-P4neo cell cultures was analysed by quantitative ELISA for the amount of HTLV-1 p19 matrix protein coded by *gag* gene. The results of ELISA are shown in Fig. 26. The significant decrease of viral p19 protein in cell culture media was found for RaHOS–P2neo cells – 9460 pg per ml comparing to 26400 pg/ml for RaHOS H9 cells (64% inhibition) and 15620 pg/ml for RaHOS-P4neo cells (41 % inhibition). The results of ELISA also were in concordance with RT-PCR analysis of RNA isolated from viral particles concentrated by ultracentrifugation of cell culture media through sacharose cushion (Fig. 27).

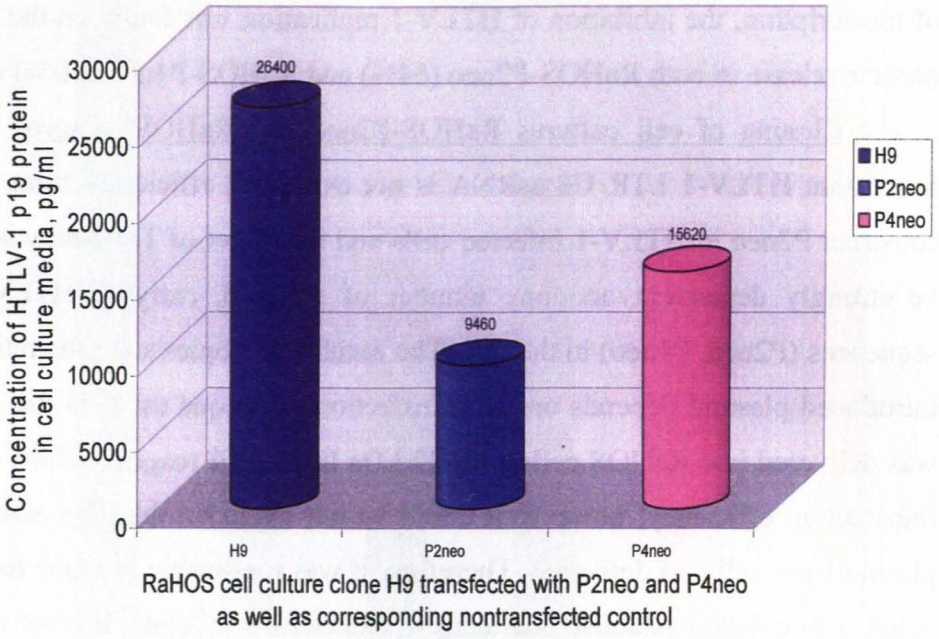

Fig. 26. HTLV-1 p19 antigen ELISA analysis of RaHOS cell cultures transfected with P2neo and P4neo

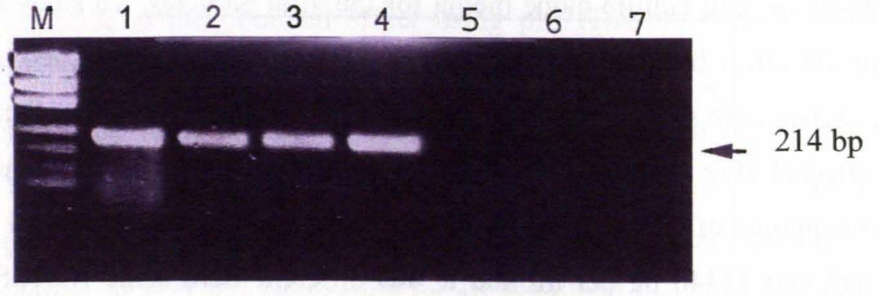

Fig. 27. Analysis of RaHOS-P2neo and RaHOS-P4neo cell culture media for the viral particles (RT-PCR). M – marker: pUC19 DNA/MspI; 1 – P2neo plasmid DNA (positive control); 2 – RNA isolated from RaHOS-P2neo cell culture supernatant; 3 – RNA isolated from RaHOS-P4neo cell culture supernatant; 4 – RNA isolated from RaHOS cell culture supernatant; 5 – RNA isolated from HOS cells; 6 – DNA extracted from HOS cells (negative control); control without DNA. Amplified 214 bp HTLV-1 LTR specific fragment is indicated on the right side.

Thus, despite of the fail in detection of asRNA expression in RaHOS-P2neo cells and no difference in viral RNA synthesis found by RT-PCR method on the level of transcription, the inhibition of HTLV-1 replication was found on the level of viral particle release in both RaHOS-P2neo (64%) and RaHOS-P4neo (41 %) cell cultures.

Cloning of cell cultures RaHOS-P2neo and RaHOS –P4neo. Thus, it was found that HTLV-1 LTR U3 asRNA is not expressed efficiently from the antisense construct P2neo in HTLV-1 infected cells and the effect of Tax decoy is supposed to be strongly dependent on copy number of plasmid, carrying HTLV-1 enhancer sequences (P2neo, P4neo) in the cell. The number of copies and integration pattern of introduced plasmid depends on the transfection technique used; in this case plasmid was delivered into RaHOS cells with 22 kDa linear PEI reagent, which showed good transfection efficiency, however it could be not excluded that less amount of intact plasmids per cell was delivered. Therefore, it was reasonable to clone transfected cell cultures in order to establish and analyse clones in which the effect of the constructs on HTLV-1 replication could be expressed more clearly. The transfected cell cultures were cloned using method of isolation of colonies formed by individual cells resistant to G418 antibiotic. Three subclones - 2D, 2H, 2Z, of RaHOS cell culture clone H9 containing plasmid P2neo and three subclones – 4B, 4C, 4B, containing plasmid P4neo were obtained. All subclones were examined for the presence of corresponding construct by PCR analysis as described in Materials and Methods and all were positive for the corresponding sequences. ELISA examination of RaHOS-P2neo and RaHOS-P4neo cell culture clone media for the viral particles. To study the variation of antiviral effect between clones the cells of obtained RaHOS-P2neo and RaHOS-P4neo clones were seeded on 24 well plate $2x10^5$ cells per well and cell culture media was collected after 48 hours of cultivation for the p19 antigen ELISA analysis (Fig. 28). The amount of HTLV-1 p19 protein in cell culture media in control RaHOS cell clone H9 was 11440 pg per ml and it was different from some RaHOS-P2neo and RaHOS P4neo clones, where HTLV-1 p19 protein amount in culture media was significantly lower in the clones 2D (2640 pg/ml – 77% inhibition), 4C (2805 pg/ml – 75% inhibition) and 4E (3520 pg/ml – 69%).

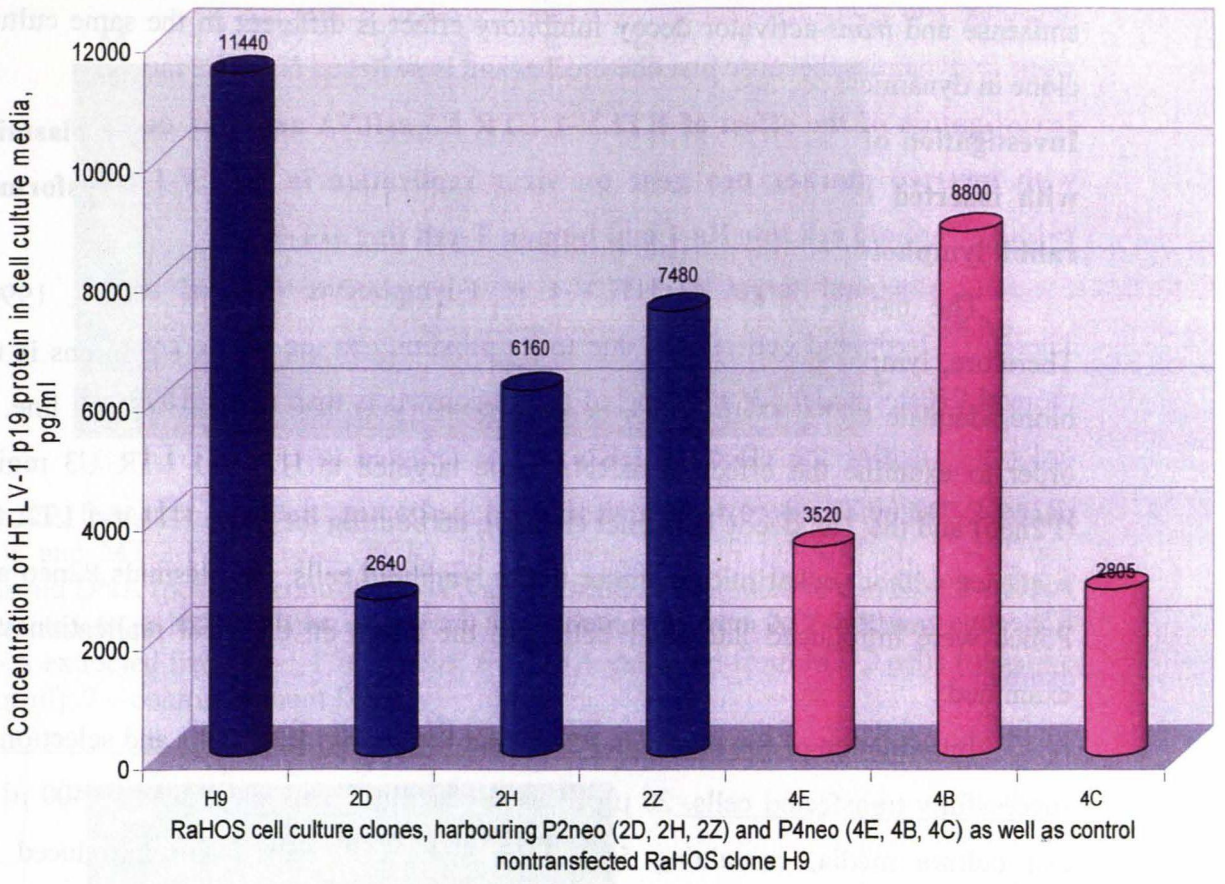

Fig. 28. HTLV-1 p19 antigen ELISA analysis of cell free culture media of RaHOS cell clones harbouring P2neo and P4neo plasmids

Thus, significant inhibition of HTLV-1 replication was found in both RaHOS subclones, containing antisense P2neo and sense P4neo constructs. Therefore, it is difficult to conclude if the obtained effect using pMHTas construct is due to the antisense mechanism and this investigation should be continued.

Together, the investigation of the antiviral effect of as-genes targeted at HTLV-1 LTR U3 and pX region and the virus transactivator Tax decoy sequences on HTLV-1 replication in virus producing monolayer cell culture RaHOS showed effective inhibition of HTLV-1 replication by all examined constructs. Nevertheless, the anticipated stronger effect of pMHTas construct as well as its analogue P2neo was not detected. Possibly, the effects of antisense and Tax decoy are not combined and may work separatedly; it could be dependent on the intracellular concentrations of viral *trans*-activator Tax and transcription factors involved in transcription from MPSV promoter and competition between these factors in the cell. Probably,

antisense and *trans*-activator decoy inhibitory effect is different in the same culture clone in dynamics, because just one mechanism is switched on at the moment.

Investigation of the effect of HTLV-1 LTR U3 asRNA and Tax decoy plasmids with inserted marker neo gene on virus replication in HTLV-1 transformed rabbit lymphoid cell line Ra-1 and human T-cell line MT-2

The natural target of HTLV-1 is T-lymphocyte (Collins *et. al.*, 1996). Therefore, lymphoid cell culture due to its proximity to the native conditions is the more adequate model for a testing of the as-constructs than the RaHOS cell line. In order to examine the effect of asRNA gene targeted to HTLV-1 LTR U3 region (P2neo) and the Tax decoy construct (P5neo), harbouring the same HTLV-1 LTR U3 sequence without eukariotic promoter in the lymphoid cells, the plasmids P2neo and P5neo were introduced into Ra-1 cells and the effect on the viral replication was examined.

Introduction of the plasmids P2neo and P5neo into Ra-1 cells and selection of successfully transfected cells. 20 μg of each plasmid P2neo and P5neo in 400 μl of cell culture media, containing 50% FBS and $2x10^7$ cells were introduced by electroporation into HTLV-1 transformed rabbit lymphoid cell line Ra-1. Transfection efficiency was estimated by microscopic examination of GFP (green fluorescent protein) fluorescence and was in the range of 0.5%- 3%, cell damage was between 60-80%. Thus, lymphoid cells are much more difficult to transfect comparing to monolayer cell cultures as well as electroporation technique is highly traumatic to cells resulting in low density of survived cells and difficulties with their further culturing and selection. Further, the survived cells were selected with 1.2 mg/ml G418 during four weeks and then maintained in the media containing supporting 400 μg/ml concentration of G418. The genomic DNA was isolated from the established G418 resistant transfected Ra-1 sublines and analysed by PCR for the presence of the introduced P2neo and P5neo plasmid sequences. The selected cells were positive for the introduced plasmid sequences (Fig. 29 and Fig. 30) and were designated as Ra-1-P2neo and Ra-1-P5neo, correspondingly.

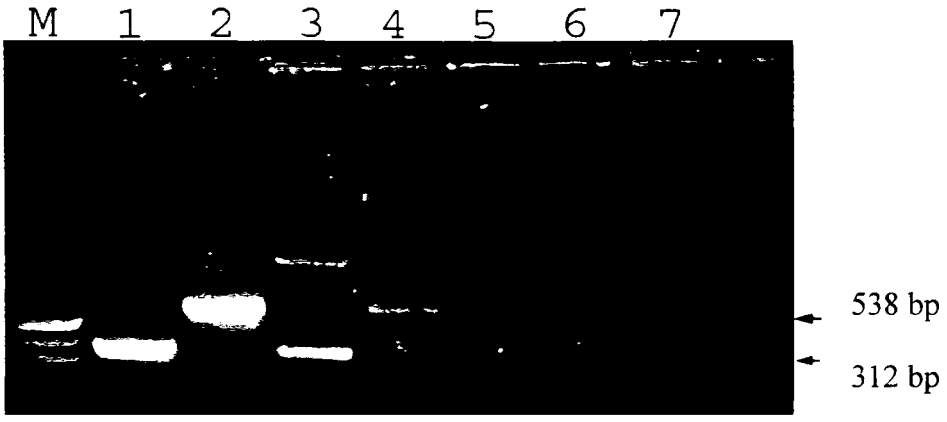

Fig. 29. Detection of P3neo and P5neo sequences in Ra-1-P5neo cells, MT-2-P5neo cells and MT-2-P3neo cells (PCR). M – marker: pUC19 DNA/MspI; 1 – P3neo plasmid DNA (positive control); 2 – P5neo plasmid DNA (positive control); 3 – DNA extracted from MT-2-P3neo cells; 4 – DNA extracted from MT-2-P5neo cells; 5 – DNA extracted from Ra-1-P5neo cells; 6 – DNA extracted from MT-2 cells (negative control); 7 – control without DNA.

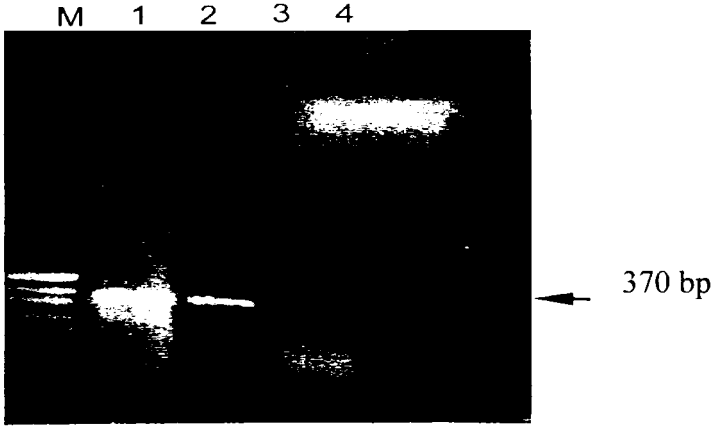

Fig. 30. Detection of P2neo sequence in Ra-1-P2neo cells (PCR). M –marker: pUC19 DNA/MspI; 1 – P2neo plasmid DNA (positive control); 2 – DNA isolated from Ra-1-P2neo cells; 3 – DNA isolated from Ra-1 cells (negative control); 4 – control without DNA. 370 bp specific amplified fragment is indicated on the right side.

RT-PCR analysis of asRNA expression in Ra-1 cells. In RaHOS cells transfected with antisense construct P2neo the expression of antisense RNA was not found, probably due to competition between MPSV promoter and HTLV-1 enhancer sequences of the construct, resulting in abortion of RNA synthesis. It was interesting to examine if it is true for lymphoid cells, where activities of MPSV promoter and Tax protein could be different. For this purpose the RNA of Ra-1 cells, containing P2neo construct was subjected to RT-PCR analysis using sense HTLV-1 LTR specific

primer in RT reaction. The expression of asRNA was not detected also in lymphoid Ra-1 cells (Fig. 31).

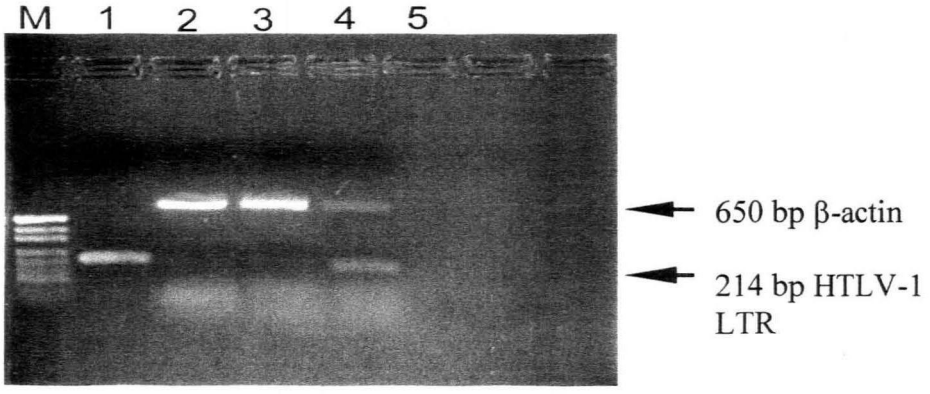

Fig. 31. RT-PCR analysis of asRNA expression in Ra-1 cells. M – marker: pUC19 DNA/MspI; 1- P2neo plasmid DNA (positive control); 2 – RNA isolated from Ra-1 cells (negative control); 3 – RNA isolated from Ra-1-P2neo cells; 4 – RNA isolated from Vero cells transfected with P2neo (positive control); 5 – control without DNA. The amplified HTLV-1 specific 214 bp and RT-PCR control β-actin gene specific 650 bp fragments are indicated on the right side.

Taking in account strong viral promoter, showed to ensure high expression in lymphoid cells and very sensitive PCR technique, the fail of asRNA detection in both monolayer RaHOS and lymphoid Ra-1 cells could not be explained by sensitivity of the method or improper promoter and probably have the same reasons in both cell cultures. Therefore, the comparison of the antiviral effect of P2neo and P5neo constructs with inserted HTLV-1 LTR U3 fragment carrying HTLV-1 enhancer sequences, could gave indirect evidence if the asRNA construct is really working by antisense mechanism. Analysis of HTLV-1 RNA synthesis and release of viral particles in transfected Ra-1 cells. With the purpose to compare viral mRNA expression and virion production in Ra-1 and its transfectants Ra-1-P2neo and Ra-1-P5neo, $3x10^5$ cells per ml were seeded and placed at +4 ^{0}C 8 hours for synchronization. After 72 hours of cultivation the cells and the cell culture media were collected for HTLV-1 RNA and HTLV-1 p19 antigen ELISA analyses. The results of RT-PCR analysis of HTLV-1 LTR U3 sequence expression are shown at Fig. 32.

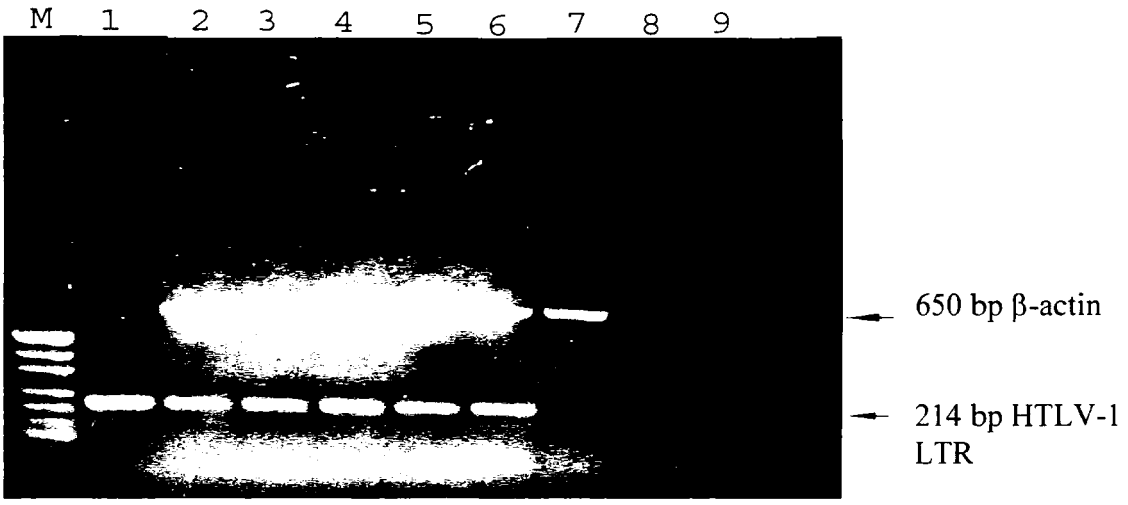

Fig. 32. RT-PCR analysis of HTLV-1 RNA synthesis in MT-2 –P3neo, MT-2-P5neo, Ra-1 P2neo and Ra-1-P5neo cells. M – DNA size marker: pUC19/MspI; 2 – DNA isolated from MT-2 cells (positive control); 2 – RNA isolated from MT-2-P3neo cells, 3 – RNA isolated from MT-2-P5neo cells; 4 – RNA isolated from Ra-1 cells; 5 – RNA isolated from Ra-1-P2neo cells; 6 – RNA isolated from Ra-1-P5neo cells; 7 – RNA isolated from HOS cells; 8 – DNA isolated from HOS cells; 9 – control without DNA.

No significant difference in the fluorescence intensity of obtained amplified fragments was detected. The results of quantitative HTLV-1 p19 antigen ELISA analysis of cell culture supernatant are shown in Fig. 33.

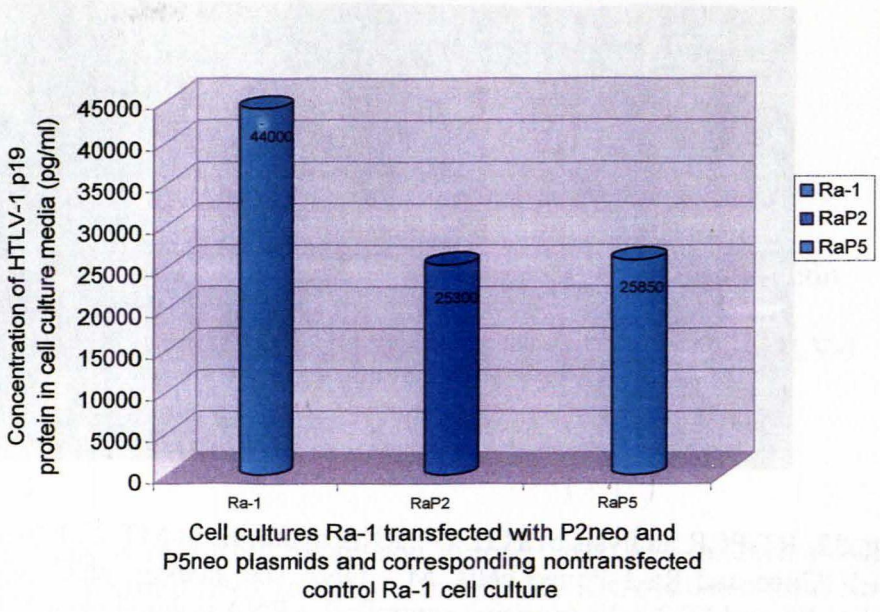

Fig. 33. HTLV-1 p19 antigen ELISA analysis of Ra-1 cell culture transfected with P2neo and P5neo plasmids.

These results show similar level - approximately 41% inhibition in Ra-1 P5neo cell culture (44000 pg/ml in Ra-1 comparing to 2585 pg/ml in Ra-1-P5neo) and approximately 43% inhibition in Ra-1-P2neo (44000pg/ml in Ra-1 comparing to 25300 pg/ml in Ra-1-P2neo subline) and confirm suggestion about the action of constructs preferentially as Tax decoy without additional stronger effect in cells harbouring construct P2neo with asRNA gene, which sequence could act also as Tax enhancer.

The investigation of the effect of Tax decoy plasmid with inserted marker *neo* gene P5neo on virus replication in HTLV-1 transformed human T-lymphocyte cell line MT-2.

The data obtained in experiments with RaHOS cell culture and Ra-1 cell culture allowed to suggest that Tax decoy could be prospective approach in attempt to inhibit HTLV-1 replication and prevent its oncogenic action. Thus, it could be valuable to study effects of Tax decoy in human T-cells infected with HTLV-1. Human T-cell line MT-2 is one of the first obtained and best studied HTLV-1 transformed cell lines. Therefore, study of Tax decoy effect was continued using MT-

2 cell culture model. Introduction of the plasmids P5neo and P3neo into MT-2 cells and selection of successfully transfected cells. The constructs P5neo and P3neo (vector control) were introduced into MT-2 cells by electroporation. The efficiency of transfection was estimated by microscopic examination of cells electroporated with phr-GFP-1 plasmid by counting GFP fluorescent cells 24 hours after transfection. The efficiency of transfection was in the range from 0.5% to 4% and cell damage was in the range of 50-80%. The survived cells were selected with 1.2 mg/ml G418 for four weeks and G418 resistant transfected MT-2 sublines were obtained. The presence of introduced plasmids P5neo and P3neo in obtained G-418 resistant MT-2 sublines was confirmed by PCR analysis of cellular DNA for the corresponding sequences (Fig. 29). Further, the obtained MT-2-P3neo and MT-2-P5neo cell cultures were maintained in media containing supporting 400 μg/ml of G418 to ensure retention of the introduced sequences in the cells. Analysis of HTLV-1 RNA synthesis and release of viral particles in transfected MT-2 cells. The effect of the introduced Tax decoy sequence on the viral RNA synthesis in MT-2 cells was estimated by Northern hybridization analysis, which allows direct analysis of cellular RNA comparing with RT-PCR, where the amount of amplified cDNA is estimated. For the RNA analysis of MT-2-P3neo and MT-2-P5neo cells by Northern blot hybridization the RNA probe to HTLV-1 LTR U3 region was synthesized from the pGHT plasmid. For the estimation of RNA amount used in hybridization analysis and its recovery, the probe for the β-actin RNA was used simultaneusly with HTLV-1 specific probe. Northern blot hybridization showed 70% inhibition of viral RNA synthesis in MT2-P5neo cells comparing to MT-2-P3neo cells (Fig. 34.). No any signal exept β-actin mRNA was detected in HTLV-1 negative Vero cells.

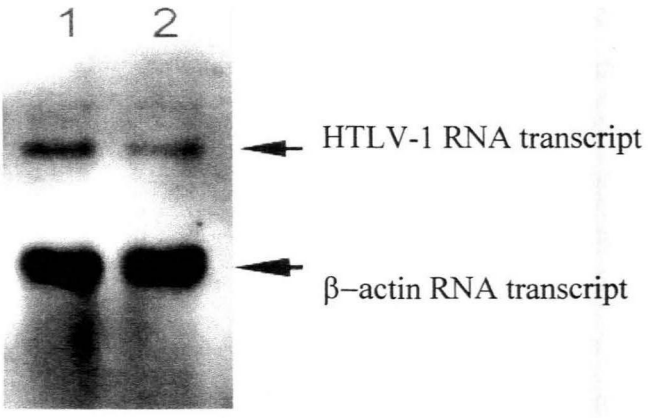

Fig. 34. Northern blot analysis of HTLV-1 RNA synthesis in MT-2-P3neo and MT-2-P5neo cells. 1 – RNA isolated from MT-2-P3neo cells; 2 – RNA isolated from MT-2-P5neo cells. HTLV-1 specific RNA and β-actin RNA transcripts are indicated on the right side.

The effect on viral RNA synthesis was seen also in RT-PCR analysis of cellular RNA, but not as clearly as it was detected by Northern hybridization (Fig. 33). Thus, in MT-2 cells the inhibition of viral mRNA synthesis was found on the level of viral mRNA transcription. In order to determine, if the viral particle release also is inhibited, the RT-PCR analysis of RNA isolated from viral particles concentrated by ultracentrifugation of MT2-P3neo and MT2-P5neo cell-free culture media was performed. RT-PCR results showed decrease in viral particle release in MT-2-P5neo cell culture comparing to MT-2-P3neo subline (Fig. 35).

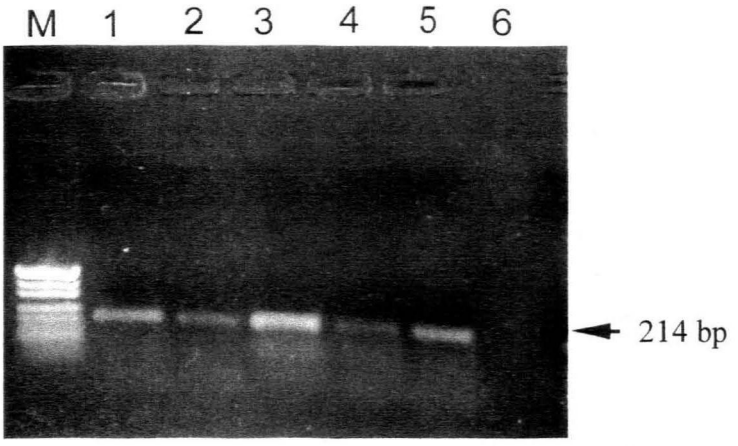

Fig. 35. RT-PCR analysis of RNA isolated from viral particles concentrated by ultracentrifugation of MT2-P3neo and MT2-P5neo cell free culture media. M – marker: pUC19 DNA/MspI; 1 – RNA isolated from MT-2-P3neo cell culture supernatant; 2 – RNA isolated from MT-2-P5neo cell culture supernatant; 3 – RNA isolated from Ra-1 cell culture supernatant; 4 – RNA isolated from Ra-1-P2neo cell culture supernatant; 5 – RNA isolated from Ra-1-P5neo cell culture supernatant; 6 – RNA isolated from HOS cells (negative control). HTLV-1 specific 214 bp amplified fragment is indicated on the right side.

The effect on viral particle release was examined also by quantitative p19 protein ELISA of synchronized MT-2, MT-2-P3neo and MT-2-P5neo cell cultures media, collected after 72 hours of cultivation. The concentration of HTLV-1 p19 protein in MT-2 cell culture supernatant was 20350 pg/ml, in control MT-2-P3neo cell culture supernatant - 25740 pg/ml (26% higher than in original MT-2 cells) and for the MT-2-P5neo cells - 8250 pg/ml (60% inhibition comparing with MT-2 cells and 68% comparing with vector control MT-2-P3neo cells) (Fig. 36).

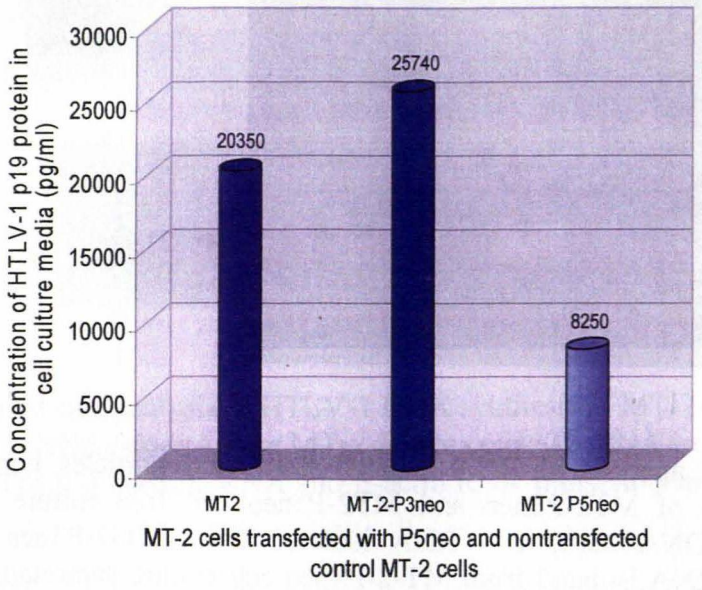

Fig. 36. HTLV-1 p19 antigen ELISA analysis of MT-2 cells transfected with P3neo and P5neo plasmids.

The inhibition of viral particle release found in MT-2 cells was about 20% higher than in Ra-1 cells transfected with the same P5neo plasmid by the same electroporation procedure. By comparison of the level of HTLV-1 expression found in Ra-1 cell culture (concentration of p19 protein for Ra-1 cells was 44000 pg/ml) and in MT-2 cells (20350 pg/ml) it become evident, that in case of lower HTLV-1 expression, as it was in MT-2 cells, the inhibitory effect could be detected more clearly. Taking in account low HTLV-1 expression level *in vivo*, the effective antiviral action of the Tax decoy construct could be achieved by a lower cellular concentration of Tax decoy sequences *in vivo* comparing with *in vitro* experiments.

DISCUSSION

The transfection procedure of HTLV-1 producing cell cultures of lymphocytic origin is difficult because of high level cell damage induced by electroporation technique. It makes difficult to obtain cells expressing the introduced gene and transfection efficiency is low, therefore selection of cells with the introduced gene is required. Monolayer cell cultures are privileged because they allows usage of more effective and convenient transfection techniques such as calcium co-precipitation and liposome- or polycation-mediated gene delivery.

It is shown that HTLV-1 *trans*-activator protein Tax could transform both lymphoid cells and the cells of non-lymphoid origin (Matsumoto *et al.*, 1997). At the same time in transgenic mice expressing the *tax* gene mesenchymal tumours and leukemia develop (Grossmann *et al.*, 1995; Nerenberg *et al.*, 1987). In HTLV-1 carriers and patients with HTLV-1-associated diseases the virus replicates preferentially in $CD4^+$ T-cells; HTLV-1 could also infect and transform $CD4^+$ cells *in vitro* (Collins *et al.*, 1996; Yodoi and Uchiyama, 1992; Del Mistro *et al.*, 1986). Up to the present non-lymphoid cells were not known as the possible targets for HTLV-1 transformation. It could be explained by an inability of HTLV-1 to replicate continuously in such cell cultures.

HOS cells are known to be permissive for HTLV-1 replication (Clapham *et al.*, 1983). On the other hand, HOS cells could be transformed by nitrosoguanidine or Kirsten virus (Rhim *et al.*, 1975; Rhim *et al.*, 1977). Therefore, we could expect that HTLV-1 would be capable to transform them.

It is known that the free viral particles of HTLV-BLV group viruses are poorly infectious and viral transmission occurs almost exclusively via cell-to-cell contacts. The capacity of the virus to mediate such a cell-to-cell transmission correlate with the syncytia-forming ability of the virus (Delamarre *et al.*, 1997; Sagara *et al.*, 1997). Therefore, we infected HOS TE85 cells co-cultivating them with HTLV-1-producing rabbit Ra-1 lymphoid cells. HTLV-1 infected HOS cell culture designated as RaHOS maintained human karyotype. The numerous syncytia in RaHOS cells were observed through the first six passages after co-cultivation. As the cell fusion is mediated by HTLV-I envelope glycoproteins (Delamarre *et al.*, 1997; Sagara *et al.*, 1997) this could be indicative of HTLV-1 expression in the RaHOS cells. The integration of HTLV-1 provirus and expression of viral antigēns as well as HTLV-1 particle release

were confirmed by PCR, IFA, RT-PCR and SA. PCR analysis for the virus regulatory (*tax*, 5' LTR) and structural (*gag, env*) genes in dynamics showed the integration of the provirus DNA in RaHOS cells. The results of PCR have proved that integration of the virus remained stable through the all observation period within 150 passages of cultivation. The detection of viral mRNA in the cells at different passages using RT-PCR method indicated continuous replication of the virus in this cell culture. IFA and SA confirmed the expression of HTLV-1 antigens in the cell cytoplasm and on the cell surface. The presence of viral RNA in cell free culture medium gave evidence of viral particle production by RaHOS cells. Thus, RT-PCR, IFA and SA indicated that HTLV-1 infection in RaHOS cells is productive, stable and long-term.

Newbound and co-wokers (Newbound *et al.*, 1996) demonstrated that cell tropism of HTLV-1 is determined by the expression levels or activation states of Tax-responsive cellular transcription factors binding to HTLV-1 LTR, the rate of viral transcription and protein production in HTLV-1 infected primary $CD4^+$ cells being higher than in primary $CD8^+$ cells. This increase was most notably observed in the presence of the viral *trans*-activating Tax protein. These data suggested that unique or activated transcription factors, particularly Tax-responsive factors in $CD4^+$ T cells, recognised regulatory sequences within HTLV-1 LTR, mediating the enhanced viral transcription in these cells (Newbound *et al.*, 1996). It could be supposed that the differences in levels or activation states of transcription factors between various cell lines and various types of cells could determine the permissiveness to HTLV-1 infection. Long-term replication of HTLV-1 in RaHOS cells suggest that initial HOS cells possessed transcription factors required for the activation of HTLV-1 transcription from its LTR. At the same time long-term Tax action could result in the shortage of cell transcriptional factors interacting with Tax or even to negative selection of Tax-expressing cells (Los *et al.*, 1998; Ruben *et al.*, 1990), which seemed to be a probable explanation of the decrease in the number of cells expressing HTLV-1 antigens upon long-term passage history of RaHOS cells.

After a long period of cultivation (more than 60 passages), the morphology of the obtained RaHOS cell culture had changed, comparing to the initial cell culture HOS. RaHOS had a higher saturation density of the monolayer, higher proliferative activity, enhanced formation of multilayer growth focuses and the ability to form colonies in soft agar. The cytogenetic analysis of RaHOS cell culture confirmed that the karyotype was identical to that of initial HOS cells. Therefore, the observed

morphological changes apparently were the result of long-term HTLV-1 replication in these cells. The acquired phenotypic changes of the obtained cell culture indicate a change of the phenotype towards malignant transformation of the cells, previously not shown for HTLV-1 infected non-lymphoid cells. Thus, this cell culture can be a useful tool for investigation of the changes in cell genetic regulation which occur upon HTLV-1 infection. The obtained RaHOS cell culture allows also the possibility of a comparative analysis with the analogous non-infected HOS cell line, which is not possible in case of existing HTLV-1 infected T-cell lines due to the absence of analogous non-infected T-cell clones.

Selective blockage of the virus on the gene expression level offers the possibility to develop highly specific alternatives to traditional pharmacological antagonists providing a promising new therapeutic strategy. Traditional approaches allow targeting of protein functions, whereas asRNA therapy can be directed toward not only the protein-coding regions, but also against nucleic acid sequences that control replication, transcription, and translation of the virus. These regulatory sequences mostly are highly conserved, therefore targeting them helps to avoid drug resistance problem, actual also for viral chemotherapy.

The gene regulation functions of as-nucleic acids strongly depend on the target sequences selected in the viral genome. Transcript of HTLV-1 pX region was chosen as target for asRNA, since spliced mRNA of the pX region includes ORFs for Tax and Rex proteins which are transcriptional and post-transcriptional activators and play important roles in viral replication (Seiki *et al.*, 1985). HTLV-1 protein Tax is the promising candidate for gene therapy of HTLV-1-associated diseases, as it is the early transactivator of the expression of all HTLV-1 genes and is able to transactivate numerous cellular genes, leading to the cell transformation. Extracellular Tax protein also plays an important role in pathogenesis of HTLV-1 associated neurodegenerative diseases (Cowan *et al.*, 1997). The LTR U3 region is one of the most promising targets in the HTLV-1 genome also, since this region contains transcriptional enhancer recognized by Tax protein and sequences, recognized by a number of cellular transcription factors.

In experiments on BLV, which is closely related to HTLV, was shown that asRNA targeted at the LTR and pX regions of BLV efficiently inhibited replication of the virus (Murovska *et al.*, 1992; Kozireva *et al.*, 1996). Moreover, in spite of more efficient inhibition of BLV replication in the cells expressing asRNA targeted to BLV

LTR RU5 region, the cells expressing asRNA targeted to BLV pX region showed diminished tumorigenic potential in nude mice comparing to the control cells. In studies using transient and stable transfection assays *in vitro*, it was shown that a retroviral vector expressing antisense HIV-1 TAR, the region to which viral *trans*-activator Tat binds, inhibited Tat-mediated *trans*-activation of a reporter plasmid with the HIV-1 LTR genes (VandenDriessche *et al.*, 1995). In another study, retroviral vectors expressing HIV-1 tat or rev asRNA can protect the cells after challenge with HIV-1 (Peng *et al.*, 1996). Efficient inhibition of HTLV-1 was achieved by asRNA targeted to pX region corresponding to the first kilobase of the *tax* gene (Von Ruden and Gilboa, 1989). Hammerhead ribozyme targeted against HTLV-I tax/rex mRNA and introduced into infected synovial cells; significantly inhibited both tax mRNA expression and Tax protein synthesis, resulting in inhibition of synovial cell growth and induction of apoptosis (Kitajima *et al.*, 1997). Therefore, the efficient inhibition of HTLV-1 replication by our as-construct pMP1100as carrying asRNA gene targeting first 1100 nucleotides of HTLV-1 pX region was in concordance with the results of other researchers.

RaHOS cell culture was transfected with the antisense and Tax decoy plasmid constructs together with the *neo* selection gene carrying plasmid. Strong, 90% inhibition of viral RNA transcription was found in cell clone, harbouring Tax decoy plasmid pGHT (HTLV-1 LTR U3 without eukariotic promoter). Also the effective inhibition (50%) of viral RNA transcription was found in control cell clone with LTR U3 sense RNA construct pMHTs.

The asRNA targeted at the LTR U3 region inhibited HTLV-1 replication to a various degree. The most effective inhibition of HTLV replication was observed in RaHOS cells comparing to Ra-1 cells. It could be explained by lower expression level of HTLV-1 in monolayer cell line than in lymphoid cells, wich are natural targets for HTLV-1. Observed various degree of inhibition by the plasmids carrying HTLV-1 LTR U3 asRNA gene are consistent with the observation of Shayakhmetov and co-authors in work with BLV, where authors found that the effects of plasmid carrying asRNA gene under control of own viral promoter and control construct with empty BLV promoter are variable, depending on the amount and relationship between the introduced asRNA gene and the virus. In our experiments less inhibition of HTLV-1 replication was observed in cell culture with higher viral load (by RNA dot-hybridization, RT-PCR and Northern hybridization) and productive infection (viral

particles release, detected by p19 antigen ELISA and RT-PCR after ultracentrifugation of cell culture medium).

In co-transfection experiments of RaHOS cell culture, only 1 of 5 obtained G418 resistant cell clones contained the introduced HTLV-1-specific sequence. To improve the efficiency of transfection the as-constructs were modified by the insertion of the *neo* gene into the original plasmids, carrying HTLV-1 LTR U3 sequence driven by MPSV promoter. The construct harbouring HTLV-1 LTR U3 sequence, without eukaryotic promoter and carrying the *neo* selection gene, also was made (Tax decoy). The introduced marker gene did not affect the expression of HTLV-1 LTR U3 fragment as it was shown in transfected HTLV-1 negative Vero cells. Thus, the obtained constructs allow applying better and faster selection in further experiments on the efficiency of asRNA approach in antiviral therapy.

A major advantage of the use of asRNA is the long-term expression of the as-compound inside the cell after a single administration. To conclude that an asRNA expression vector is acting through a true antisense mechanism several conditions should be followed: a) the antisense vector enters the cells and persists for a sufficient period of time; b) it generates asRNA inside the cells; c) it reduces the level of the target protein; d) it produces appropriate and specific biological effect. In the present study stable integration of asRNA expression vector within long-term culture of the transfected cells was detected advocating to its integration into cellular genome. In Vero cells transfected with P2neo plasmid carrying asRNA gene targeted at HTLV LTR U3 region the expression of asRNA was clearly observed. In one of RaHOS cell culture clones without integrated HTLV-1 provirus, after transfection with pMHTas plasmid stable integration and expression of the introduced pMHTas plasmid was found. However, in the virus infected RaHOS cell clones with the introduced asRNA vector we failed to detect asRNA transcript. The same was observed also in rabbit cell line Ra-1. This finding leads us to the conclusion that viral transcription could interfer with transcription of asRNA in the cells persistently infected by the virus. Many investigations showed correlation between detection of asRNA transcripts in the cells and their antiviral effect, the same was also observed with BLV (Tomsons *et al.*, 1993). The absence of asRNA transcripts in the transfected RaHOS and Ra-1 cells could be explained by possible influence of Tax protein binding to its enhancer sequences located within the asRNA gene of our pMHTas and P2neo constructs.

The investigation of antiviral effect of HTLV-1 LTR U3 fragment harbouring plasmids in Ra-1 and MT-2 cell cultures confirmed that Tax decoy could be prospective approach in attempt to inhibit HTLV-1 replication and prevent its oncogenic potential. In work of Shayakhmetov and co-authors efficient inhibition of BLV was achieved with a plasmid containing non-expressing BLV U3 promoter sequence starting from 20 molar excess of the plasmid over the BLV proviral DNA. In contrast, in the case of asRNA gene to BLV LTR RU5 region, the efficient inhibition of BLV was achieved at ratio 1:5 of proviral to asRNA-encoding DNA (Shayakhmetov *et al.*, 1997). The authors concluded that a low molar excesses (1:1 to 5:1) expression of the asRNA gene causes highly efficient inhibition of BLV replication and the decoy effect does not play a significant role. In its turn, at high molar excesses (10:1 and greater) the *trans*-activator decoy mechanism becomes the prevailing factor in virus inhibition. In the intermediate range both mechanisms play an equal role. In the mentioned study BLV U3 promoter sequence was used by to inhibit viral infection *de novo* in BLV negative cell culture subsequently infected with BLV. The results provided in the present study indicate that introduction of HTLV-1 *trans*-activator Tax decoy sequences into chronically infected cells may also efficiently inhibit HTLV-1 replication.

Marked inhibition of virus replication by plasmid containing an empty promoter suggests that trapping of transactivator proteins *in vivo* can significantly decrease the concentration of transcriptional components which are necessary for productive viral infection. This approach provides attractive possibility of regulated action of the construct, dependent on expression of viral transactivator in the cell. Thus, such type of sequences will work only in the cells expressing the virus. In the presence of viral Tax protein they, probably, could work as CRE decoy elements exhibiting antiproliferative effect. Studies on HTLV-1 have shown that the concentration of Tax *in vivo* is very low. Therefore, it should be feasible to achieve competitive inhibition of virus by *trans*-activator trapping. Since many types of viruses encode special *trans*-activators for enhanced transcription from viral promoters, the idea of use *trans*-activator decoy could be applied also for other viruses.

CONCLUSIONS

- The convenient monolayer cell culture RaHOS for studying of the anti-retroviral activity of antisense nucleic acids is established.
- The ability of HTLV-1 to transform not only the lymphoid cells, but also cells of non-lymphoid origin is demonstrated for the first time.
- Transfection efficiency of RaHOS monolayer cell line was not as high as Vero or HeLa cell lines. However, it allows the use of convenient lipofection and other poly-cation delivery techniques, which are inefficient in lymphoid cells.
- The insertion of *neo* selection gene into original constructs allowed fast and convenient evaluation of the antiviral effect also in lymphoid cells which are natural target for HTLV-1.
- asRNA genes targeted to HTLV-1 LTR U3 and pX regions are able to inhibit the expression of the virus in stable cell culture model.
- It is shown that Tax decoy is efficient and promising approach for the inhibition of HTLV-1 replication.
- The HTLV-1 LTR U3 asRNA gene construct has background of two inhibition mechanisms - antisense and Tax decoy. In the cells with high Tax expression level Tax decoy mechanism could become prevailing in virus replication control.

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ВЛИЯНИЕ ГЕНОВ acРНК И ПОСЛЕДОВАТЕЛЬНОСТЕЙ «ЛОВУШКИ» ВИРУСНОГО ТРАНСАКТИВАТОРА НА СИНТЕЗ РНК HTLV-I

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РЕЗЮМЕ

Значительное количество научных публикаций посвященных ингибированию вирусной репликации с помощью генов acРНК показывает, что этот подход является по-прежнему актуальным для генной терапии вирусных инфекций. Для изучения возможности подавления репродукции вируса HTLV-I acРНК нами был создан ряд рекомбинантных плазмид, содержащих гены acРНК направленные к U3LTR и pX областям HTLV-I как под контролем промотора вируса миелопролиферативной саркомы (MPSV), так и без него. Методом стабильной кальций-фосфатной трансфекции с последующей селекцией в присутствии G-418 были получены клеточные клоны на основе линии RaHOS, несущие не только гены acРНК, но и последовательности, способные связывать собственный трансактиваторный белок HTLV-I, т.е. «ловушку» вирусного трансактиватора (ЛВТ). Судя по данным дот-гибридизационного анализа вирусной РНК, выделенной из клеточных клонов RaHOS, последовательности ЛВТ способны подавлять синтез вирусных РНК на 90%, в то время как acРНК направленные к области pX на 50%.

EFFECT OF ANTISENSE RNA AND "TRAP" FOR VIRAL TRANSACTIVATOR ON HTLV-I RNA SYNTHESIS

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SUMMARY

A number of scientific publications devoted to the inhibition of viral replication by complementary addressed polynucleotides (antisense RNA and DNA, ribozymes, etc.) have clearly showed that this approach still remains one of preferred ones in the gene therapy of viral infections. To investigate an inhibition possibility of HTLV-I replication by antisense RNA (asRNA) we have constructed recombinant plasmids containing asRNA genes targeted to U3 LTR and pX regions under control of myeloproliferative sarcoma virus (MPSV) promoter and enhancer sequence recognized by viral transactivator Tax – " trap" of viral transactivator (TVT). The constructs were transfected into HTLV-I-infected monolayer cell line RaHOS and cell clones, carrying the introduced constructs were established. According to dot-hybridization analysis of viral RNA, TVT sequence decreases the level of HTLV-I RNA synthesis about 90% and asRNA gene targeted to pX region about 50%.

ВВЕДЕНИЕ

Т-лимфотропный вирус человека I типа (HTLV-I) является онкогенным ретровирусом, способным вызывать лейкозы/лимфомы у инфицированных людей после длительного латентного периода. Т-клеточный лейкоз взрослых (ATL), вызываемый этим вирусом, характеризуется злокачественным течением и рядом других клинических особенностей (1, 2). Помимо онкологических заболеваний, этот вирус способен вызывать HTLV-I-ассоциированную миелопатию, известную также как Тропический спастический парапарез (TSP) (3,4), и другие неврологические расстройства (5,6), а также целый ряд воспалительно-дегенеративных заболеваний (миозиты, увеиты, дерматиты, бронхопневмонии и т.д.) (7).

Инфицирование HTLV-I не приводит к относительно быстрому развитию какого-либо заболевания, как в случае ВИЧ-инфекции. От момента инфицирования до возникновения, например, Т-клеточного лейкоза взрослых проходит от 10 до 50 лет. При этом только 1-2 человека на 1000 инфицированных "доживают" в Японии, эндемичном для HTLV-I районе, до развития у них Т-клеточного лейкоза/лимфомы.

В лечении заболеваний, связанных с HTLV-I, широко применяют интенсивную полихимиотерапию, комбинацию производных антиретровирусного агента азидотимидина (AZT) с α-интерфероном, трансретиноевую кислоту. Но, несмотря на это, добиться стойкого результата в лечении не удается; развивается резистентность к химиотерапии, иммунодепрессия и прогноз для больных чаще всего неблагоприятен. Продолжительность жизни больного в острой стадии заболевания при интенсивной химиотерапии не превышает 10 месяцев.

Поэтому остается проблема поиска принципиально новых препаратов для лечения заболеваний, связанных с HTLV-I-инфекцией.

Одним из широко исследуемых за последние 20 лет направлений в лечении вирусных заболеваний, является генная терапия с помощью комплементарно-адресованных полинуклеотидов (КАП). КАП представляют собой антисмысловые РНК (асРНК), рибозимы, синтезированные химически антисмысловые олигодезоксинуклеотиды и т.д. Особую ценность комплиментарным полинуклеотидам как потенциальным терапевтическим агентам придает их практически абсолютная специфичность и, видимо, низкая токсичность (4). Кроме того, возможно создание рекомбинантных плазмид с аддитивным эффектом и тканеспецифичностью. асРНК были предложены в качестве потенциальных терапевтических агентов для профилактики и лечения ряда заболеваний. Несколькими авторами показана способность асРНК ингибироватьрепродукцию следующих вирусов: RSV (9,10), Ad5 (11), BLV (12), HIV (13), SIV (14), FIV (15), HBV (16).

Еще одним агентом, направленным на подавление вирусной репродукции, являются ДНК-последовательности «ловушек» вирусных трансактиваторов (ЛВТ). Эффект их действия был рассмотрен нами ранее на примере подавления репликации вируса лейкоза крупного рогатого скота (BLV) (17). Таким образом, изучение влияния асРНК и последовательностей ЛВТ на синтез РНК HTLV-I является актуальной задачей в разработке новых подходов к лечению HTLV-I-ассоциированных заболеваний.

Цель настоящей работы - изучение ингибирующего действия асРНК, направленных на различные области генома HTLV-I и ДНК-последовательностей ЛВТ на синтез вирусной РНК, а также их аддитивного эффекта в культуре клеток RaHOS, не только содержащей интегрированный провирус HTLV-I, но и экспрессирующей вирусные антигены.

МАТЕРИАЛЫ И МЕТОДЫ.

Материалы. В работе были использованы лабораторные штаммы E. coli DH5α, JM109, XL1-blue. Для выращивания бактерий были использованы среды LB, SOB и SOC. Клонирование фрагментов ретровирусного генома проводили с помощью следующих плазмидных векторов: pGEM5Zf+ (Promega, USA), pBluescript KS$^-$ ("Stratagene"), рекомбинантной плазмиды pMPSVEH, любезно предоставленной д-р Artelt и плазмиды pCO21, любезно предоставленной О.А. Павлиш (Институт канцерогенеза РАМН, Москва).

При проведении экспериментов были использованы клеточные линии HOS и RaHOS, любезно предоставленные д-р М.Муровска. Линия HOS представляет собой клетки остеосаркомы человека и является пермиссивной для репликации HTLV-I. Линия RaHOS получена в результате сокультивирования линии HOS с культурой лейкоцитов кролика Ra-1, продуцирующей HTLV-I. Для культивирования клеток применяли следующие питательные среды: DMEM ("HyClone", США) и DMEM/F12

(производства МПБП, Москва), содержавшие 10% эмбриональную сыворотку крупного рогатого скота (FBS) (Белорусский институт микробиологии, Минск).

Методы.

Все генно-инженерные манипуляции при получении рекомбинантных плазмид проводили по стандартным протоколам (18).

Электронная микроскопия

Клеточный монослой фиксировали 2,5% забуференным глутаровым альдегидом 4 часа с последующей дофиксацией 1% OsO_4 в течение 2 часов, заливали в смесь эпон-аралдит. Ультратонкие срезы контрастировали уранилацетатом и цитратом свинца, наблюдали и фотографировали в электронном микроскопе Хитачи Н-500.

Трансфекция, селекция и клонирование клеток

Клеточные линии HOS и RaHOS выращивались на среде МЕМ модифицированной Дальбеко (ДМЕМ) с добавлением 10% сыворотки эмбрионов коров (FBS), гентамицина и амфотерицина до конечной концентрации 50 мкг/мл и 2,5 мкг/мл соответственно при 37^0С и при 5% CO_2. Субкультивирование проводили путем обработки клеточного монослоя смесью 0,0025% раствора трипсина и раствора Версена в равном соотношении при 37^0С в течении 10-15 мин. с предварительной 3-х кратной отмывкой и с последующим ресуспендированием в 5-1,0 мл ростовой среды. Затем количество клеток определяли подсчетом в камере Горяева и высевали при более низких концентрациях.

Перенос чужеродных генов в клетки млекопитающих осуществлялся методом Са-фосфатной преципитации по Graham [19].

Селекция клеток COS-I на среде с неомицином проводили следующим образом.

Через 24 часа после трансфекции клетки переносили с 6 см чашек на две 10 см чашки Петри, содержащие в среде G-418. Селективная концентрация генетицина была подобрана эмпирически ранее и составляла 400 мкг/мл. Смену ростовой селективной среды производили через каждые 2-3 дня. Через 10-14 дней полученные клеточные линии субпассировали трижды на селективной среде и переводили на обычную ростовую среду.

Выделение ДНК и РНК из клеток.

Монослой клеток с двух культуральных флаконов размером 25 $см^2$ снимали обработкой смесью 0,25% трипсина, 1мМ ЭДТА и PBS, дважды отмывали PBS и лизировали в 350 мкл буферного раствора, содержавшего 50 мМ Трис-HCl (pH 8.0), 100 мМ ЭДТА, 100 мМ NaCl, 0,01% SDS и 100 мкг/мл

протеиназы К. Далее к лизату добавляли равный объем фенола, насыщенного Трис-HCl (pH 8.0). Пробы центрифугировали при 15000 об/мин 5 минут. Надосадочную жидкость отбирали в чистые пробирки и проводили экстракцию центрифугированием с равным количеством смеси фенол/хлороформ на 15000 об/мин в течение 5 минут. Вновь надосадочную жидкость отбирали в чистые пробирки, добавляли равный объем хлороформа и центрифугировали при ранее описанных условиях. Осаждение ДНК из надосадочной жидкости проводили холодным этанолом 96° в присутствии ацетата натрия. Осадок ДНК растворяли в буферном растворе ТЕ, добавляли 1 мкл РНКазы А в концентрации 1мкг/мл и инкубировали в течение 30 минут при температуре 37°С.

Для выделения РНК клеточный лизат растворяли в равном объеме гуанидинтиоционата. Затем проводили экстракцию смесью фенол/хлороформ и дважды хлороформом при ранее описанных условиях. Осаждение РНК проводили инкубацией надосадочной жидкости с 8 М LiCl в количестве трети от исходного объема в течение 12-14 часов при температуре 4°С и последующем центрифугировании на 15000 об/мин. Затем осадок промывали раствором 2М LiCl.

Дот- гибридизационный анализ ДНК-РНК.

Для проведения дот-гибридизации готовили радиоактивно меченый зонд с помощью ПЦР при использовании специфичных для HTLV-I праймеров (MP01, MM2, PX) с добавлением в реакционную смесь α^{32}РдАТФ. При проведении ПЦР в качестве матрицы использовали плазмиды со встроенными в них фрагментами ДНК HTLV-I: pMHTs и pMP1100as. В качестве положительного контроля прохождения реакции использовали полноразмерный вирусный геном HTLV-I, вырезанный из плазмиды pMT-2. Наряду с анализируемыми клеточными линиями использовали исходные линии HOS и Ra-HOS в качестве контролей. Измерение сигнала проводили радиоафтографией. Полученные данные переводили в количественный эквивалент ДНК или РНК с помощью программы "LabWorks" (Bio-Rad Laboratories).

Условия проведения полимеразной цепной реакции.

В реакциях использовали 10X буфер для ПЦР: 500 мМ KCL, 100 мМ Трис-HCL, pH 8,4, 25 мМ $MgCL_2$; 10X dNTP: по 2 mM dATP, dCTP, dGTP, dTTP; праймеры по 20-100 пмоль каждого на реакцию; термостабильную *Tag*-полимеразу из расчета 1 ед. фермента на 1 мкг ДНК, исследуемые образцы ДНК и минеральное масло.

Длина праймеров составляла 20 нуклеотидов. В ходе проведения работы были использованы следующие праймеры: MP01 ccc-aag-gac-ctg-aad-tga-cc; PX02 aaa-cag-tcc-tcg-ggt-aga-at; MM1 agg-acg-gct-tga-caa-aca-tg; MM2 tct-atc-gga-gga-ctg-gcg-cg; MM3 aaa-cat-ttc-cgt-gaa-aca-ga; MM4 ggg-agg-aaa-tgg-gtg-

gtt-cc, коммерческие праймеры Forvard и Revers для T7 и Sp6 промоторов фага M13 (Promega).

Расчет температуры отжига проводился по стандартной методике: $4^{o}C*(G+C)+2^{o}C*(A+T)-5$. Денатурация двухцепочечной ДНК проходила при $94^{o}C$ в течение 30 секунд. Длительность циклов отжига составляла 30-60 секунд. Длительность цикла элонгации определяли в зависимости от длины ожидаемого амплификата из расчета 1 минута на 1000 нуклеотидов и проводили при $72^{o}C$.

Объем реакционной смеси составлял 25-50 мкл, из них 1-2 мкл приходилось на ДНК. Реакция проходила в автоматическом устройстве "Терцик" (Диа-М, Россия) для проведения цепной реакции полимеризации с участием Taq-полимеразы.

РЕЗУЛЬТАТЫ И ОБСУЖДЕНИЕ.

Конструирование рекомбинантных плазмид с генами асРНК основывается на двух принципах. Первый - выбор в вирусном геноме оптимальной мишени для действия асРНК. Как правило, мишенями служат гены регуляторных белков, области, ответственные за транскрипцию вирусной РНК, за экспрессию поверхностного антигена и т.д.. Второй - выбор сильного конститутивного промотора для экспрессии этих генов. Вышеизложенные принципы были положены в основу получения рекомбинантных плазмид.

Конструирование рекомбинантных плазмид, содержащих гены асРНК.

Судя по имеющимся данным о HTLV-I и некоторых других ретровирусах, удобной мишенью для асРНК является U_3 область LTR HTLV-I. Во-первых, она играет важную роль в процессе трансактивации провирусной активности, во-вторых, участвует в репликации генома HTLV-I. Другой интересной мишенью для действия асРНК является область pX, кодирующая регуляторные белки Tax и Rex. В литературе описаны эксперименты по влиянию асРНК на трансформирующую способность HTLV-I в первичных Т-лимфоцитах человека. В этих экспериментах асРНК были направлены к области pX или к cis-действующему элементу на 5' конце вирусной РНК (20). В других работах было показано действие рибозимов и олигодезоксинуклеотидов на репродукцию вируса HTLV-I в культуре клеток. Было выяснено, что наиболее оптимальной мишенью для действия антисмысловых олигодезоксинуклеотидов была область гена *env* (21). Для BLV оптимальной мишенью для действия антисмысловых РНК оказался участок R области вирусного LTR, перекрывающий донорный сайт сплайсинга для всех субгеномных вирусных мРНК (22). Перед нами стояла задача получить рекомбинантные плазмиды с генами асРНК, направленными к областям U_3 и pX и проверить их действие на синтез РНК HTLV-I в условиях клеточно-культуральной модели инфекции.

В качестве источника фрагментов для генов асРНК были взяты плазмиды pMT-2, содержащая полноразмерный геном HTLV-I, и pCO–12-20, содержащая область pX. В качестве вектора была использована плазмида pMPSVEH, содержащая промотор вируса миелопролиферативной саркомы человека (MPSV). Выбор MPSV промотора обусловлен тем, что он, судя по литературным данным, способен обеспечивать значительный уровень генной экспрессии в фибробластах, клетках миелоидного и лимфоидного ряда по сравнению с другими промоторами, такими как ранний промотор SV40 и транс-активируемый промотор HIV (23).

В ходе проделанной работы были получены следующие генно-

инженерные конструкции. Первая конструкция содержит большую часть области pX (7283 п.о. - 8308 п.о.), встроенную в плазмиду pMPSVEH в обратной ориентации по отношению к MPSV промотору. Для этого плазмида pCO–12-20, содержащая область pX, была обработана рестрикционными эндонуклеазами *EcoR*I и *Pst*I. Полученный фрагмент, размером 1100 п.о., был вставлен в плазмиду pBluescript KS$^-$ по сайтам рестрикции *EcoR*I и *Pst*I (конструкция pB1100). Отбор рекомбинантных плазмид на этом этапе и далее проводился с помощью рестрикционного анализа плазмидной ДНК клонов, полученных после трансформации *E-coli*. Также было проведено определение нуклеотидной последовательности по концам встроенного фрагмента и полученные данные подтвердили результаты рестрикционного анализа. Затем полученная плазмида pB1100 была обработана рестрикционными эндонуклеазами *BamH*I и *Hind*III и полученный фрагмент был встроен в плазмиду pMPSVEH по тем же сайтам рестрикции в обратной ориентации по отношению к MPSV промотору. Полученная конструкция содержит ген асРНК к области pX и называется pMP1100as. Схема получения конструкций представлена на **рис. 1.**

Вторая конструкция содержит фрагмент U_3 области вирусного LTR размером 226 п.о. (29 п.о. - 255 п.о.). Данный фрагмент был вырезан по сайтам рестрикции *Sma*I, *Hinc*II из плазмиды pMT-2 и вставлен по сайту *Sma*I в вектор pMPSVEH. Из проанализированных клонов были отобраны те, что содержали вставку в обратной ориентации по отношению к MPSV промотору. Полученную конструкцию обозначили pMHTas. Таким образом, данная конструкция содержит ген асРНК к U_3 региону HTLV-I. Схема получения конструкции представлена на **рис. 2.**

Конструирование рекомбинантных плазмид, содержащих последовательности ЛВТ. Впервые действие ДНК-последовательностей, называемых «ловушками» вирусных трансактиваторов было показано для BLV в культуре клеток CC81. Эффект подавления транскрипционной активности промотора U_3 области LTR, обусловленный конкуренцией за связывание вирусного трансактиваторного белка p38tax, наблюдали в присутствие 20-ти – 30-ти кратных молярных избытков плазмиды, несущей энхансерные последовательности вирусного промотора (12). Нам представлялось интересным проверить эффект конкурентного связывания конструкциями с ЛВТ белка p40tax HTLV-I. Фрагмент U_3 области LTR размером 226 п.о. (29 п.о. - 255 п.о.), содержащий участки связывания белка p40tax, был встроен в вектор pMPSVEH по сайту рестрикции *Sma*I в прямой ориентации относительно промотора. Эта конструкция была названа pMHTs.

Следующая конструкция - pGHT содержала вышеуказанный фрагмент U_3 области, встроенный в вектор pGEM5Z по сайту рестрикции *EcoR*V и являлась транскрипционно неактивной.

К этой же группе следует отнести ранее описанную плазмиду pMHTas,

где одна и та же встроенная последовательность содержит не только ЛВТ, но и способна транскрибировать асРНК. Схемы получения конструкций представлены на **рис. 2.**

Предполагалось, что все три конструкции - pMHTs, pMHTas и pGHT - будут проявлять эффект ловушки вирусных трансактиваторов, так как содержат специфические последовательности связывания трансактиваторного белка p40tax; при этом ориентация фрагмента не должна влиять на связывание белка. Конструкция pMHTas предположительно могла дать двойной эффект, поскольку помимо последовательностей ЛВТ содержит гены асРНК.

Получение клеточных линий, стабильно трансфицированных плазмидами, содержащими гены асРНК или последовательности ЛВТ

В качестве клеточной модели вирусной инфекции, вызываемой HTLV-I, могут быть использованы лимфоциты человека, обезьяны, кролика или мышей. Экспериментально доказано, что Т клетки человека могут быть инфицированы, а также иммортализованы и трансформированы при сокультивации с HTLV-I-продуцирующими лимфоцитами, полученными от больных ATL (24, 25). Также было показано, что HTLV-I может инфицировать и трансформировать клетки нелимфоидной природы, такие как клетки остеосаркомы и эндотелиальные клетки (26, 27, 28, 29). При этом процессинг структурных белков вируса, являющихся продуктами генов *gag* и *env*, идет по пути, аналогичному для клеток лимфоидной природы. Было отмечено, что транс-активирующая функция белков была выражена в этих линиях сильнее, чем в лимфоидной линии МТ-2, продуцирующей HTLV-I и полученной от больного ATL. Методом непрямой иммунофлуоресценции показали, что в трансфицированной линии HeLa белок p40 (трансактиватор) большей частью локализован в ядре, в то время как другие структурные белки в цитоплазме и/или мембране. Мы в своей работе с HTLV-I использовали клеточные линии остеосаркомы человека HOS и RaHOS. Для получения линии RaHOS монослойная клеточная линия HOS была инфицирована сокультивированием с HTLV-I-продуцирующими лимфоидными клетками кролика линии Ra-1. Интеграция провируса в культуре RaHOS была выявлена с помощью полимеразной цепной реакции для последовательностей *gag*, *env*, *tax* и LTR. Экспрессия вирусных антигенов и размножение HTLV-I были установлены методами непрямой иммунофлуоресценции, реакции синцитиеобразования и с помощью обратно-транскриптазной полимеразной цепной реакции (30). После длительного пассирования клетки RaHOS приобрели признаки трансформированных: после 18-го пассажа повысилась пролиферативная активность, после 30-го пассажа клетки приобрели способность образовывать колонии в мягком агаре, после 60-го в культуре появились

очаги многослойного роста (30,31). Проведенное нами электронно-микроскопическое исследование линии RaHOS не подтвердило продукцию этими клетками вирионов, типичных для ретровирусов, что можно объяснить низким титром вируса и недостаточной чувствительностью данного метода электронной микроскопии.

Далее проводились эксперименты по трансфекции линии RaHOS рекомбинантными плазмидами, содержащими гены асРНК и/или ЛВТ. Модель для проведения трансфекции была предварительно отработана сначала на линии HOS, а затем на линии RaHOS на основе маркера эффективности транзиентной трансфекции с использованием плазмиды pCMV -β–Gal-SPORT, несущей ген β-галактозидазы под контролем предраннего CMV-промотора. Экспрессию гена β-галактозидазы выявляли окрашиванием фиксированных клеток раствором, содержащим хромогенный субстрат X-gal. С помощью микроскопического исследования оценивали количество ярко окрашенных синих клеток на фоне бесцветного монослоя через 48 часов после трансфекции. Эффективность трансфекции определяли делением числа окрашенных клеток на общее количество трансфицированных клеток и выражали в процентах. Эффективность как временной, так и стабильной трансфекций в наших экспериментах составляла 2%-3%.

В перевиваемую клеточную линию RaHOS методом Са-фосфатной котрансфекции вводили ДНК рекомбинантных плазмид, содержавших гены асРНК и/или последовательности ЛВТ, а также плазмиду pSV2neo, несущую ген устойчивости к неомицину (G-418). Далее для получения клеточных клонов проводили селекцию клеток в присутствии неомицина, который добавляли на третий день после трансфекции. Трансфицированные клетки выращивали в присутствии неомицина в концентрации 350 мкг/мл в течение 4 недель до получения отдельных клеточных клонов. Долговременная устойчивость клеток к неомицину, полученных в результате стабильной трансфекции, являлась свидетельством интеграции гена неомицинфосфотрансферазы в геном клетки. Вместе с плазмидой pSV2neo в геном клетки могли встроиться в конкатомерных формах рекомбинантные плазмиды с последовательностями генов антисмысловых РНК и/или ЛВТ.

Изолированные клоны клеток наращивали и далее анализировали на наличие интегрированных рекомбинантных плазмид с генами асРНК или ЛВТ методом полимеразной цепной реакции.

Для этого из полученных клеточных линий выделяли суммарную ДНК и с помощью специфических праймеров MP01 и MM2 определяли присутствие MPSV промотора для конструкций на основе плазмиды pMPSVEH (**рис. 3**).

Такой выбор праймеров и мишени обусловлен тем, что исследование проводилось на инфицированной HTLV-I культуре клеток, и мы не могли использовать праймеры, специфичные для встроенного в плазмиду фрагмента. В этом случае они одновременно отжигались бы как на вирусном геноме, так и на встроенном фрагменте, являющемся частью вирусного генома. Также не удалось подобрать праймеры, один из которых нацелен на MPSV промотор, а другой на фрагмент вирусного генома. В этом случае образование неспецифичных продуктов ПЦР затруднило анализ трансфицированных клонов. В качестве положительного контроля в ПЦР использовали плазмиду pMHTs, а отрицательного – ДНК клеток линии RaHOS. Электрофорез продуктов ПЦР проводили в 1,5%-ом агарозном геле. Специфический фрагмент размером 120 п.о., свидетельствующий о наличии MPSV промотора, показан на рисунке 3 стрелкой слева.

Для выявления плазмиды pGHT в клеточных клонах использовали коммерческие праймеры Forvard и Revers (Promega), фланкирующие полилинкер плазмиды pGEM5Zf+ (**рис. 4**). Как и в предыдущем случае мы не могли использовать праймеры, специфичные встроенному фрагменту и, соответственно, вирусному геному. Положительным контролем служила плазмида pGHT, а отрицательным – ДНК из клеток линии RaHOS. Электрофоретический анализ проводили в 8%-ом полиакриламидном геле. Стрелкой слева обозначен специфический продукт ПЦР размером 430 п.о. Как видно на рисунке, 27 и 32 клоны содержат помимо ожидаемого фрагмента еще и неспецифический продукт меньшего размера, поэтому для дальнейших исследований был выбран 25 клон, содержащий наименьшее количество неспецифических продуктов.

Анализ влияния генов антисмысловых РНК и последовательностей ловушки вирусного трансактиватора на синтез РНК HTLV-I в клеточных клонах линии RaHOS. Для оценки противовирусного эффекта асРНК и ЛВТ необходимо было использование высокочувствительного метода количественного определения уровня синтеза РНК HTLV-I. С этой целью использовали метод дот-гибридизации вирусной ДНК и РНК. Метод гибридизации нуклеиновых кислот несколько уступает по чувствительности ПЦР, но в то же время дает возможность учета количества связанного с нуклеиновой кислотой зонда. Так, при использовании в качестве зонда радиоактивно меченных специфических

нуклеиновых кислот (ПЦР-фрагментов) интенсивность радиоактивного сигнала и соответствующее ей количество нуклеиновой кислоты, специфичной зонду, можно определить с помощью компьютерного анализа при использовании специальных программ.

В нашем случае выбор праймеров для получения зонда осложнялся тем фактом, что анализ проводился на инфицированной вирусом культуре. Зонд, специфичный фрагменту вирусного генома, встроенному в рекомбинантную плазмиду, будет также специфичен и последовательности ДНК или РНК вируса. Таким образом, использование специфичного для U3 области зонда возможно было лишь на ДНК клонов RaHOS, полученных в результате трансфекции конструкцией с последовательностью pX (плазмида pMP1100as). В этом случае зонд гибридизуется только с вирусной нуклеиновой кислотой и мы можем достаточно точно оценить количество вирусной ДНК в данных клонах. Аналогично мы поступали и при выборе зонда для клонов RaHOS, содержавших фрагмент U3 области LTR HTLV-I. Зонд был специфичен области pX и использовался при изучении клонов RaHOS, содержавших рекомбинантные плазмиды pMHTs, pMHTas, pGHT (содержат фрагмент U3 области). Такая перекрестная система позволила избежать дополнительного специфичного связывания зондов помимо вирусной ДНК или РНК с конструкциями. Для получения зонда к последовательности pX использовали плазмиду pMP1100as и праймеры MP01 и PX01, один из которых был направлен к MPSV промотору, а второй к pX региону, ближе к его 3' концу. Для получения зонда к U3 региону использовали плазмиду pMHTs и праймеры MP01 и MM2, где первый праймер так же направлен к MPSV промотору, а второй к U3 области. При проведении ПЦР в реакционную смесь добавляли α^{32}РдАТФ.

В качестве положительного контроля прохождения реакции использовали вырезанный из плазмиды pMT-2 полноразмерный вирусный геном HTLV-I в концентрации, равной 2 нг ДНК на точку. Наряду с анализируемыми клеточными линиями, полученными в результате трансфекции рекомбинантными плазмидами, использовали исходные линии HOS и RaHOS в качестве контролей. Измерение сигнала проводили с помощью радиоафтографии. Полученные данные переводили в количественный эквивалент с помощью программы "LabWorks" (Bio-Rad). Результаты дот-гибридизационного анализа представлены на **рис. 5** и **рис. 6**.

Провести анализ экспрессии асРНК в условиях данной системы оказалось сложно, т.к. экспрессируемые молекулы асРНК идентичны участкам вирусных мРНК. В этом случае радиоактивный зонд может гибридизоваться как с асРНК, так и с вирусной РНК и сигнал радиоавтографа будет содержать в себе их суммарное значение.

Анализ количества РНК HTLV-I проводили методом дот-гибридизации РНК. Для этого выделяли суммарную РНК клонов клеточной линии RaHOS, полученных в результате трансфекции данной линии рекомбинантными

плазмидами. Выбор последовательности зондов определяли, руководствуясь рассуждениями, как и при выборе для анализа количества вирусной ДНК, т.е. один был направлен к области pX и использовался на клонах с конструкциями, содержащими U3 область. Второй зонд направлен к региону U3 и использовался на клонах с конструкциями, содержащими последовательность pX. В реакционную смесь для ПЦР добавляли 32РдАТФ. Результаты анализа представлены на **рис. 7.**

Как видно из **рис. 7,** на радиоавтографе слабый сигнал во второй точке, что свидетельствует о небольшом количестве вирусной РНК в 25 клоне линии RaHOS и, соответственно, ингибировании вирусной репродукции последовательностями ЛВТ. В третьей точке гибридизация зонда шла не только с вирусной РНК, но и с асРНК к гену pX.

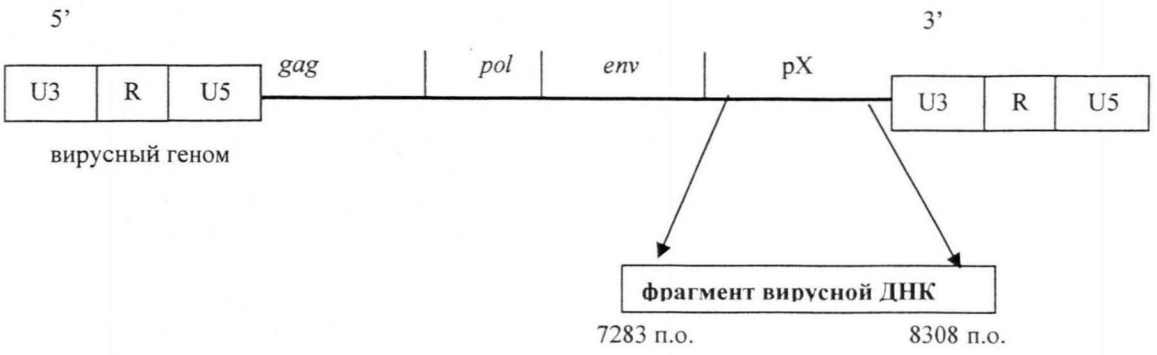
Оценивая результаты по снижению уровня РНК HTLV-I (**рис. 8**), можно заключить, что ингибирующее действие на HTLV-I оказали ЛВТ-последовательности и асРНК к области pX. Эффект ЛВТ выражен для транскрипционно неактивной плазмиды pGHT и составляет около 90% ингибирования экспрессии вирусной РНК. Антивирусный эффект в случае транскрипционно активной плазмиды pMHTs составляет около 50%. В отношении асРНК для региона U3 мы не наблюдали антивирусного эффекта. Конструкция pMHTas содержит ЛВТ и одновременно экспрессирует асРНК. Возможно, при считывании транскрибируемой области ЛВТ, удерживающей белки p40tax, между РНК-полимеразой и комплексом p40 taxCREB возникает конкуренция за ДНК-матрицу. Такое взаимодействие может снижать как образование асРНК, так и препятствовать связыванию белка p40tax. В отношении асРНК для области pX уровень подавления вирусной репродукции составил около 50%.

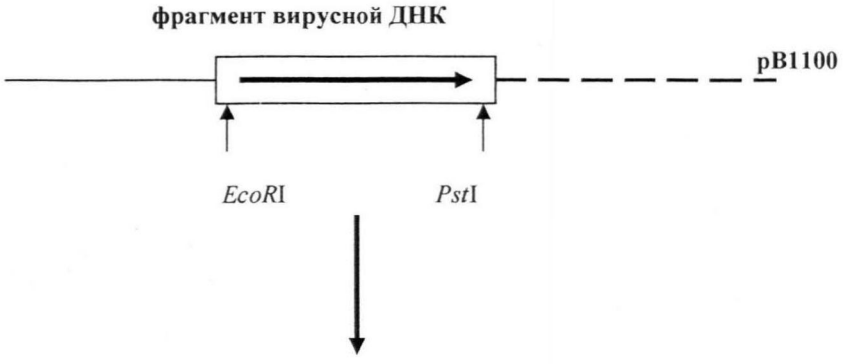
Таким образом, проведенные нами исследования показывают, что в условиях хронической вирусной инфекции можно добиться снижения уровня синтеза РНК HTLV-I с использованием асРНК и ДНК-последовательностей ЛВТ. Для усиления их эффекта представляется интересным исследование совместного действия ЛВТ и генов асРНК направленных к области pX. Поскольку последовательности ЛВТ подавляли репродукцию HTLV-I почти на 90%, а асРНК направленная к области pX почти на 50%, то в дальнейшем их следует изучить в системе *in vivo*. В качестве животной модели инфекции для HTLV-I подходят обезьяны, кролики (32, 33), мыши (34) и крысы (35, 36). Такие исследования позволят определить эффективность использования асРНК и ЛВТ в живых системах, а так же помогут выявить возможные негативные стороны данных терапевтических средств, такие как токсичность, тератогенность, генетическая нестабильность генома хозяина и т.д.

ИЛЛЮСТРАЦИИ

2 РИСУНКА, 1 ГИСТОГРАММА, 5 ФОТОГРАФИЙ

Рис. 1.


5'
3'
U3
R
U5
gag
pol
env
pX
U3
R
U5
вирусный геном
фрагмент вирусной ДНК
7283 п.о.
8308 п.о.


фрагмент вирусной ДНК
pB1100
EcoRI
PstI

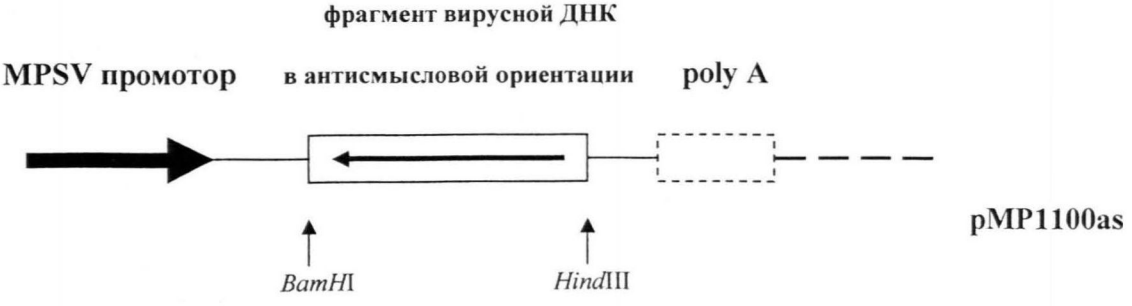

фрагмент вирусной ДНК
MPSV промотор
в антисмысловой ориентации
poly A
pMP1100as
BamHI
HindIII

Рис. 1. Схема получения конструкции pMP1100as.

Обозначения: MPSV – промотор вируса миелопролиферативной саркомы человека, обозначен стрелкой, poly A – сигнал полиаденилирования, обозначен штриховым прямоугольником. Клонируемый фрагмент обозначен прямоугольником, в котором стрелкой указана ориентация фрагмента при клонировании.

Рис. 2.

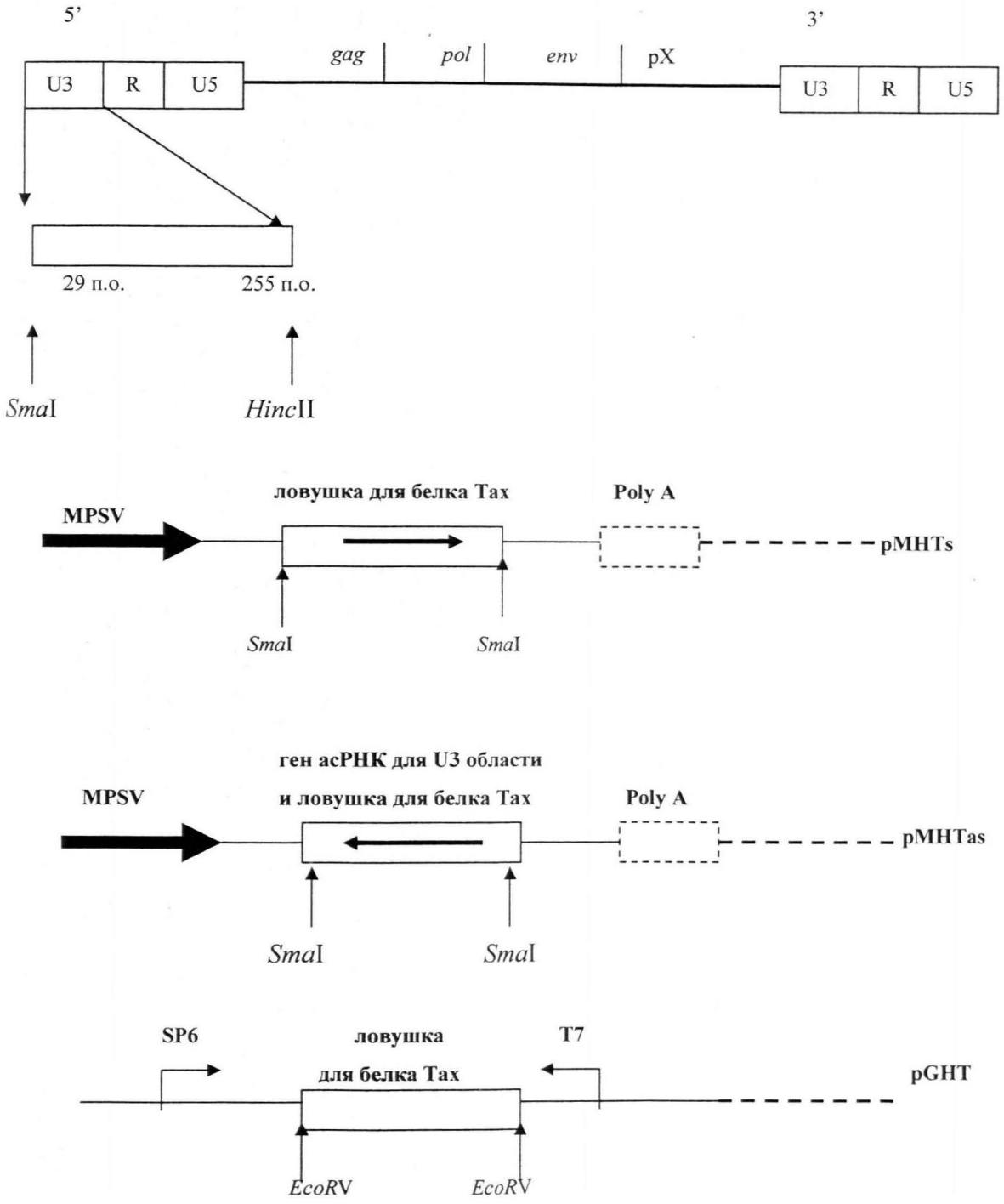

5'
3'
gag
pol
env
pX
U3
R
U5
U3
R
U5
29 п.о.
255 п.о.
SmaI
HincII
MPSV
ловушка для белка Tax
Poly A
pMHTs
SmaI
SmaI
ген асРНК для U3 области
и ловушка для белка Tax
MPSV
Poly A
pMHTas
SmaI
SmaI
SP6
ловушка
для белка Tax
T7
pGHT
EcoRV
EcoRV

Рис. 2. Схема получения плазмид, содержащих последовательности "ловушки" вирусного трансактиватора. Обозначения: MPSV – промотор вируса миелопролиферативной саркомы человека, показан стрелкой, poly A – сигнал полиаденилирования, обозначен штриховым прямоугольником, T7 - промотор для РНК-полимеразы фага T7, SP6 - промотор для РНК-полимеразы фага SP6. Клонируемый фрагмент обозначен прямоугольником, стрелками показана ориентация фрагмента при клонировании.

Рис. 3.

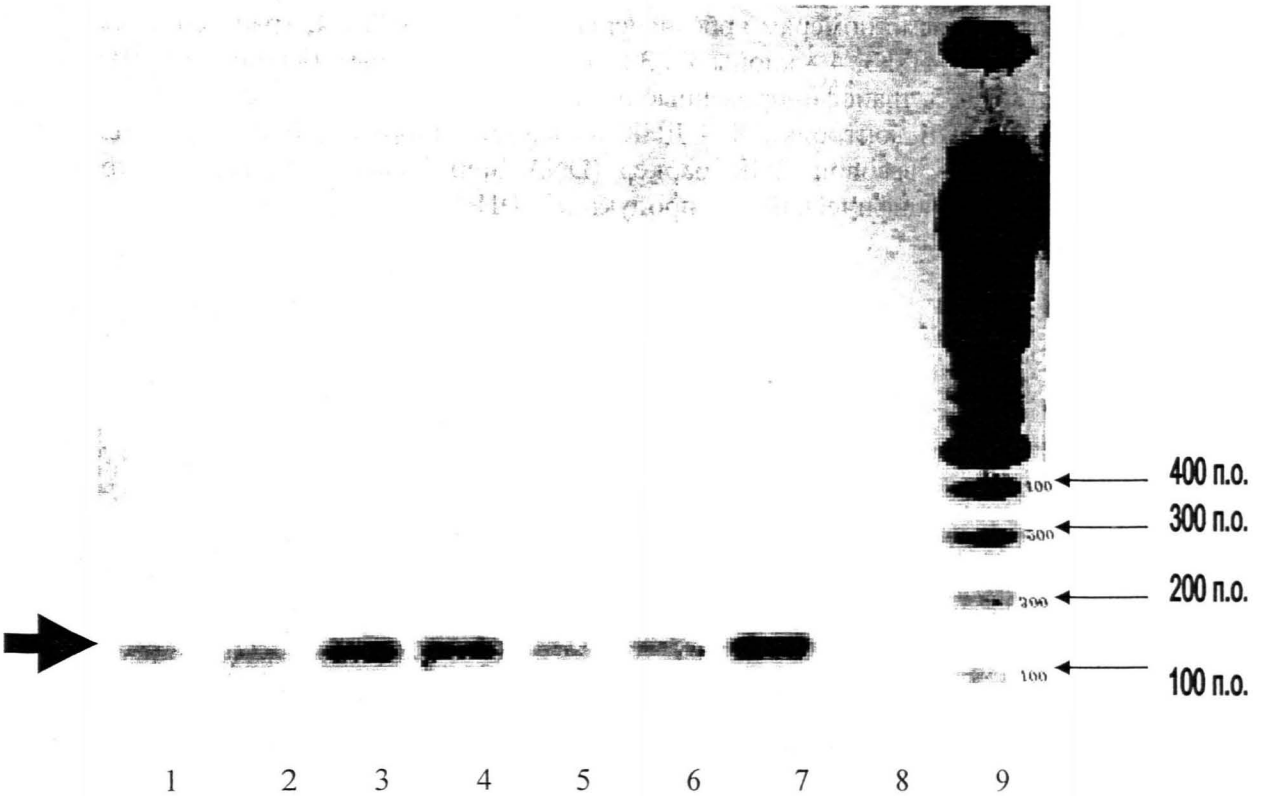


Рис. 3. Анализ наличия MPSV промотора в клонах клеточной линии RaHOS.

Порядковыми номерами обозначены: 1, 2 - клоны 12, 14, трансфицированные плазмидой pMHTs; 3, 4 – клоны 33, 38, трансфицированные плазмидой pMHTas; 5, 6 - клоны 31, 35, трансфицированные плазмидой pMP1100as; 7 – плазмида pMHTs (положительный контроль); 8 – ДНК из клеток линии Ra-HOS (отрицательный контроль), 9 – весовой ДНК маркер (DNA Step Ladder, “Promega”); стрелкой показан специфический продукт ПЦР, размером 120 п.о.

Рис. 4.

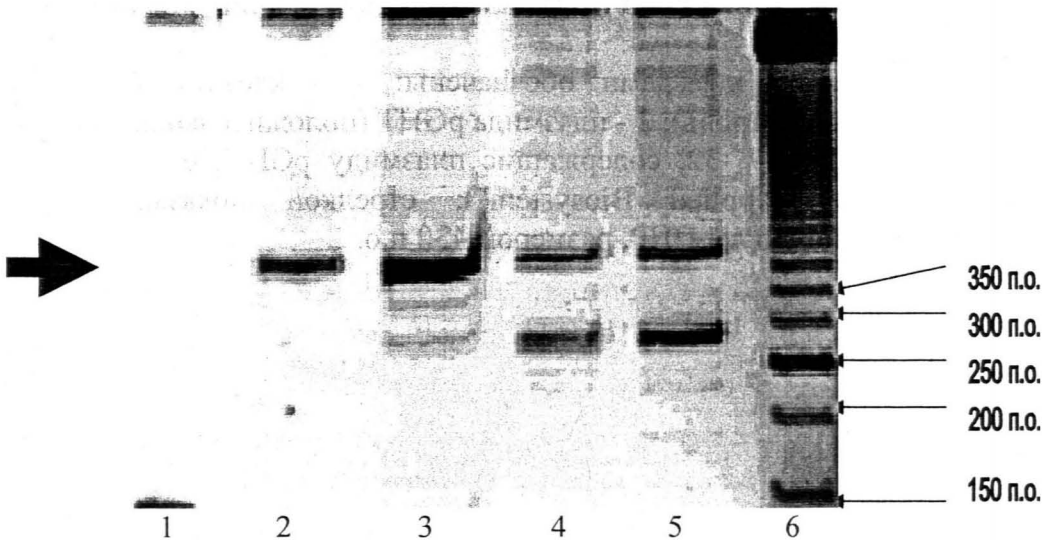
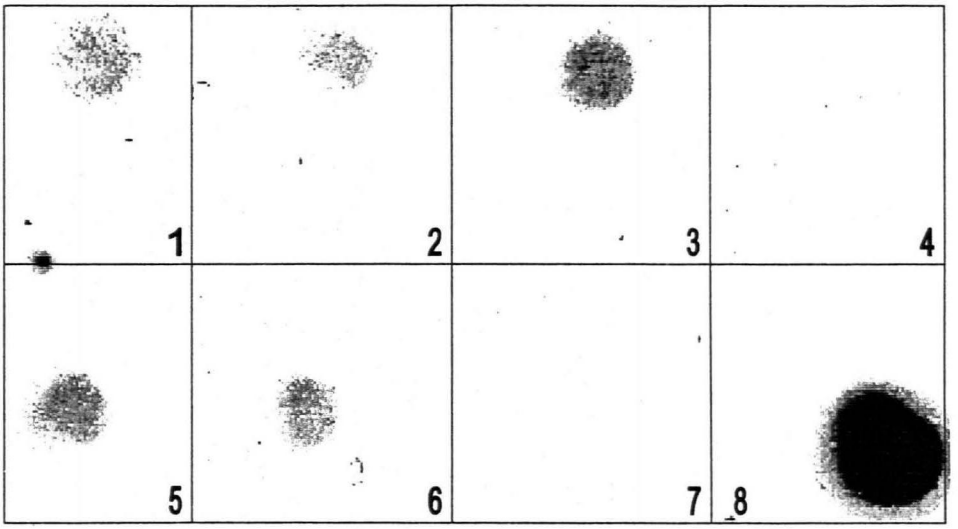


Рис. 4. Анализ наличия плазмиды pGHT в клонах клеточной линии RaHOS.

Порядковыми номерами обозначены: 1 - клетки линии Ra-HOS (отрицательный контроль); 2 - плазмида pGHT (положительный контроль); 3, 4, 5 – клоны 25, 27, 32, содержащие плазмиду pGHT; 6 – весовой ДНК маркер (DNA "Applied Biosystem"); стрелкой показан ожидаемый специфический продукт ПЦР, размером 430 п.о.

Рис. 5.

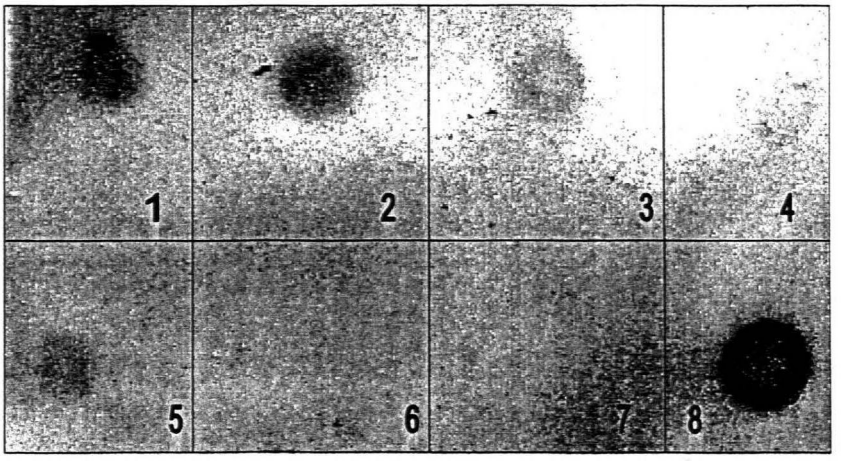


Точка	1	2	3	4	5	6	7	8
Количество ДНК в нг	1,18	0,94	1,58	0,34	0,8	1,17	0	2

Рис. 5. Результаты выявления с помощью дот-гибридизации вирусной ДНК и интегрированной плазмидной ДНК, содержащей участок региона pX, выделенных из клонов клеток RaHOS.

Используемый зонд направлен к области pX. Порядковыми номерами обозначены: 1 - 14 клон, содержащий плазмиду pMHTs; 2 – 25 клон, содержащий плазмиду pGHT; 3 – 35 клон, содержащий плазмиду pMP1100as; 4 – 38 клон, содержащий плазмиду pMHTas; 5 – клон, полученный из линии RaHOS, содержащей интегрированный провирус HTLV-I (положительный контроль); 6 – ДНК из клеток линии RaHOS (положительный контроль); 7 – ДНК из клеток линии HOS (отрицательный контроль); 8 – ДНК HTLV-I.

Рис. 6.

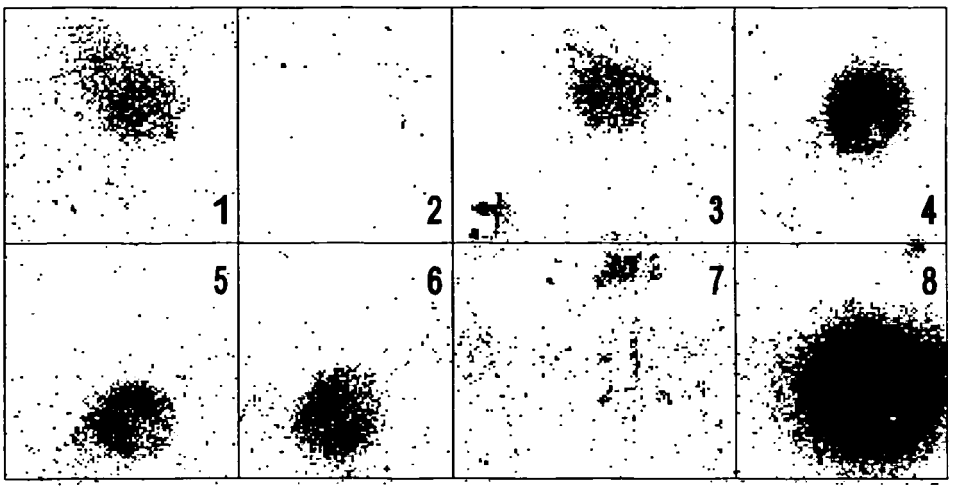


Точка	1	2	3	4	5	6	7	8
Количество ДНК в нг	1,79	1,77	0,6	0,2	1,13	-	0	2

Рис. 6. Результаты выявления с помощью дот-гибридизации вирусной ДНК и интегрированной плазмидной ДНК, содержащей участок U3 региона, выделенных из клонов клеток RaHOS.

Используемый зонд направлен к U3 региону. Порядковыми номерами обозначены: 1 - 14 клон, содержащий плазмиду pMHTs; 2 – 25 клон, содержащий плазмиду pGHT; 3 – 35 клон, содержащий плазмиду pMP1100as; 4 – 38 клон, содержащий плазмиду pMHTas; 5 – клон, полученный из линии RaHOS, содержащей интегрированный провирус HTLV-I (положительный контроль); 6 – ДНК из клеток линии RaHOS (положительный контроль); 7 – ДНК из клеток линии HOS (отрицательный контроль); 8 – ДНК HTLV-I.

Рис. 7.



точка	1	2	3	4	5	6	7	8
Количество РНК в нг	0,6	0,06	0,62	1,23	1,2	0,98	0	2

Рис. 7. Результаты выявления с помощью дот-гибридизации вирусной РНК, выделенной из клонов клеток RaHOS.

Используемый зонд направлен к области pX. Порядковыми номерами обозначены: 1 - 14 клон, содержащий плазмиду pMHTs; 2 – 25 клон, содержащий плазмиду pGHT; 3 – 35 клон, содержащий плазмиду pMP1100as; 4 – 38 клон, содержащий плазмиду pMHTas; 5 – клон, полученный из линии RaHOS, содержащей интегрированный провирус HTLV-I (положительный контроль); 6 – ДНК из клеток линии RaHOS (положительный контроль); 7 – ДНК из клеток линии HOS (отрицательный контроль); 8 – ДНК HTLV-I.

Рис. 8.

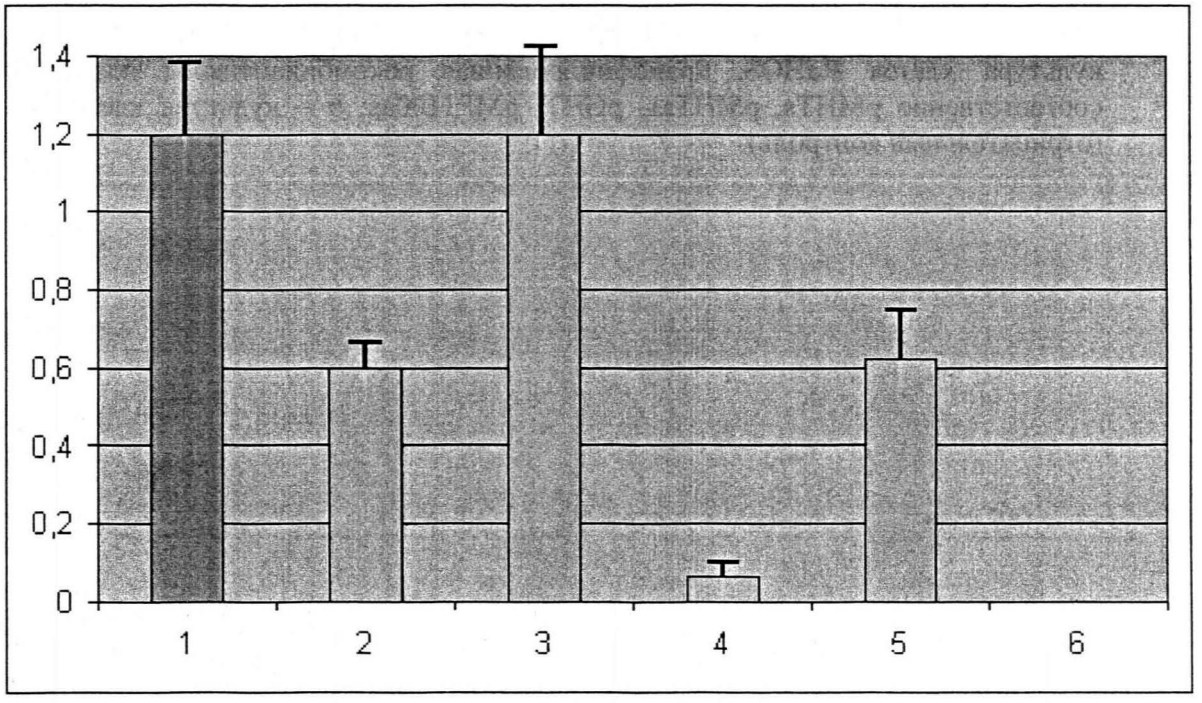


Рис. 8. Подавление синтеза РНК HTLV-I антисмысловыми РНК и ЛВТ в клетках RaHOS.

Обозначения: 1 - нетрансфицированная рекомбинантными плазмидами культура клеток Ra-HOS, содержащая интегрированный провирус HTLV-I; 2, 3, 4, 5, - культура клеток RaHOS, трансфицированная рекомбинантными плазмидами соответственно pMHTs, pMHTas, pGHT, pMP1100as; 6 – культура клеток HOS (отрицательный контроль).

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Construction of the Plasmids Carrying HTLV-1 Sequences Suitable for Study of Their Effect on Viral Replication

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Plazmīdu konstruēšana, kuras satur HTLV-1 provīrusa secības un kas ir pielietojamas vīrusa replikācijas inhibīcijas pētīšanai

Viena no daudzsološākajām metodēm efektīvas pretvīrusu terapijas izstrādē ir specifiska gēnu ekspresijas kavēšana ar antiinformācijas nukleīnskābēm. Darbā ir aprakstītas plazmīdas, kas satur HTLV-1 LTR U3 rajonu, klonētu informācijas un antiinformācijas virzienā attiecībā pret promoteru, kā arī plazmīda, kas satur HTLV-1 LTR U3 secību bez promotera. Šāda veida plazmīdas bija konstruētas ar divu veidu selekcijas gēniem: *neo* un *gfp*, kas vienkāršo ievadītās vīrusspecifiskās informācijas detekciju šūnās. Konstruēto plazmīdu ievadīšanai šūnās bija pielietotas dažādas transfekcijas metodes un pārbaudīta ievadītā marķiera gēna ekspresija transfecētajās šūnās. Labākie rezultāti bija iegūti ar jauno, uz elektroporācijas metodi bāzēto nukleofekcijas metodi. Salīdzinājumā ar standarta elektroporācijas metodi tā uzrādīja augstāku transfekcijas efektivitāti ar vismazāko šūnu bojājumu. Parādīts, ka visas iegūtās konstrukcijas, ievadītas MT-2 un Ra-1 šūnās, bija funkcionējošas, tādējādi atvieglojot transfecēto šūnu selekciju pēc ievadītā marķiera gēna. Lietojot Vero šūnas, parādīts, ka visās ievadītajās konstrukcijās selekcijas gēni netraucē ne informācijas, ne antiinformācijas HTLV-1 LTR U3 secību ekspresiju. Iegūtie rezultāti liecina, ka visas selekcijas gēnus nesošās konstrukcijas ir pielietojamas ātrākai un efektīvākai mērķa ģenētisko materiālu nesošo šūnu atlasei turpmākajos HTLV-1 antiinformācijas RNS vīrusinhibējošās darbības pētījumos.

Raksturvārdi: HTLV-1 vīruss, replikācijas nomākšana, plazmīdu konstruēšana, pretvīrusu terapija.

Introduction

Human T-cell lymphotropic virus type 1 (HTLV-1) is an oncogenic retrovirus, the causative agent of adult T-cell leukaemia, HTLV-1 associated myelopathy/tropical spastic paraparesis and other severe diseases. HTLV-1 preferentially targets and transforms CD4+ T-lymphocytes (Collins *et al.*, 1996); it lacks a typical oncogene (Weiss, 1984) and integrates randomly into the cell genome (Leclercq *et al.*, 2000).

The viral regulatory protein Tax has been shown to be responsible for the oncogenic potential of the virus. The Tax protein enhances the transcription of the virus through its interaction with a specific sequence (three 21 base pair repeats) in the virus LTR U3 region (Suzuki *et al.*, 1993). Through the interaction with the groups of cellular transcription factors and coactivators (e. g. NF-kB transcription factor family), Tax exerts transactivation of transcription of the number of cellular genes, including cellular oncogenes (*c-fos, c-jun, c-myc, c-ras*) and growth factors (IL-2, IL-6, TNF-a, TNF-b, GM-CSF) (Matsumoto *et al.*, 1997.). It deregulates the normal cell cycle through binding to inhibitors of cyclin dependent kinases 4 and 6, and can also inhibit some tumour repressor genes (Wu *et al.*, 2003). Altogether, Tax is able to bind and regulate many cellular proteins that regulate transcription and cytosceletal related pathways. These effects on a wide variety of cellular targets seem to cooperate in promoting cell proliferation, which is an effective viral strategy to amplify its proviral genome through replication of infected cells (Yoshida, 2001). Nevertheless, it is suggested that HTLV-1 in infected patients is not silent and is transcriptionally active (Asquith *et al.*, 2000). Thus, successful treatment of HTLV-1 associated diseases requires inhibition of the viral transcription as well as pathology specific therapy. Since neither effective chemotherapy nor vaccines are currently available, it is important to find a suitable therapy against HTLV-1 associated diseases.

Selective blockage of the virus on the gene expression level offers the possibility of developing highly specific alternatives to traditional pharmacological antagonists providing a promising new therapeutic strategy. Although there was originally scepticism toward the possibility of inhibiting gene expression using antisense (as) RNAs, in the past several years numerous studies have proved their potential utility as therapeutic drugs in neurology, psychiatry, cardiology, infectious diseases and oncology (Weiss *et al.*, 1999; Park *et al.*, 2002; Hilleman, 2003; Sun *et al.*, 2003; Vassalli *et al.*, 2003). Traditional approaches allow targeting of protein functions, whereas as-RNA therapy can be directed toward not only the protein-coding regions, but also against nucleic acid sequences that control replication, transcription, and translation of the virus. These regulatory sequences mostly are highly conserved, thus the possibility to target them helps to avoid drug resistance problems, also relevant for viral chemotherapy. To use as-RNAs as realistic therapeutic agents, several tasks should be fulfilled. These tasks are the efficient delivery of genetic material to a high percent of the cell population, efficient long-term expression from a vector, colocalization with the target, and specific action with the desired mRNA.

The use of specific vectors for direct delivery of genetic materials into certain cells and tissues, and application of strong and inducible promoters for effective and controlled expression of as-sequences, allows the development of optimal constructs for antiviral protection. At present there are intense studies searching for suitable promoter systems for controlled gene expression (Chang *et al.*, 2002).

An important issue in the creation of an antisense therapeutic molecule is to address the as-nucleic acid to a proper virus target gene. It has been shown previously that as-RNA targeted at the LTR and pX regions of Bovine leukaemia virus (BLV) efficiently inhibited replication of the virus (Murovska *et al.*, 1992; Kozireva *et al.*, 1996). Furthermore, efficient inhibition of BLV was achieved with a plasmid

containing only the BLV U3 promoter sequence devoid of any as-RNA genes. It was concluded that the viral promoter sequence could trap the viral transcription co-activator protein and decrease its intracellular concentration, thus inhibiting replication of the virus (Shayakhmetov *et al.*, 1997). As BLV is closely related to HTLV, it was expected that the sequences targeted to corresponding genes in HTLV-1 genome could also be active suppressors of the virus replication. Based on this assumption, plasmid harbouring HTLV-1 LTR U3 226 base pair (bp) sequence in antisense orientation was constructed (pMHTas). As controls the plasmids with HTLV-1 LTR U3 sequence in sense orientation (pMHTs), as well as the original cloning vector pMPSVEH (Artelt P. *et al.*, 1988) were used. The as-sequence was cloned under the strong MPSV (Myeloproliferative sarcoma virus) promoter, which is active in the lymphoid tissues (Artelt P. *et al.*, 1988).

HTLV-1 producing cell cultures of lymphocytic origin are very hard to transfect. The high level of damage to cells induced by electroporation technique makes it difficult to obtain cells expressing the introduced gene. When transfection efficiency is low, the selection of the cells with the introduced gene is required. For this purpose resistance to such antibiotics as geneticin (G418, neomycin antibiotic group) and hygromycin is usually used in mammalian cells (Stanley *et al.*, 1989, Sambrook *et al.*, 1989). Introduction of neomycin resistance gene is most common. It is also possible to select cells by fluorescent reporter gene, *gfp* (*g*reen *f*luorescent *p*rotein) derivatives, which are very popular and widely used in recent years. The insertion of such a gene into the structure allows not only selection using flow cytometry, but also convenient visualisation and non-invasive *in vivo* detection of the cells of interest (Sturm *et al.*, 2003).

As the original vector pMPSVEH with cloned MPSV promoter do not contain any selection/reporter gene, the cells have to be cotransfected with plasmid, which harbour the marker gene. Taking into account low transfection efficiency, highly traumatic electroporation technique, and laborious and time-consuming cloning procedure, the probability to obtain cell clones harbouring the therapeutic gene decreases strongly in case of cotransfection. To improve the efficiency of the transformation system the created as-constructs were modified by the insertion of the *neo* and *gfp* reporter genes into the plasmids mentioned above. Also the constructs harbouring only HTLV-1 LTR U3 sequence, which contains 21 bp repeats recognized by HTLV-1 transactivator protein Tax, were made.

Materials and methods

Plasmids

pMPSVEH with cloned MPSV promoter (Artelt P. *et al.*, 1988), pMHTs, pMHTas with 226 bp fragment of HTLV-1 LTR U3 cloned under MPSV promoter in sense and antisense orientations were kindly provided by Alexei Borisenko (Moscow Research Institute for Viral Preparations, Academy of Medical Sciences, Russia). Plasmids phrGFP-1 with *gfp* reporter gene cassette and pKO Scrambler NTKV-1907 with *neo* selection gene cassette were obtained from Stratagene (USA). pHTLV-1 with cloned complete HTLV-1 provirus was used to obtain HTLV-1 LTR U3 fragment for cloning.

Plasmid Isolation and Construction

Isolation and further purification of plasmid DNA was performed using Qiafilter Midi kit (Qiagene, Germany) according to the manufacturer's protocol. The A_{260}/A_{280} ratio of the isolated plasmid DNA was in the 1.8-2.0 range. All enzymes were purchased from MBI Fermentas (Lithuania) and used according to manufacturer's instructions. The isolation of DNA fragments from the low melting temperature agarose (Sigma, USA) was performed as described by Sambrook *et al.* (1989).

Cell Cultures

HTLV-1–producing MT-2 human T-cell line (Miyoshi *et al.*, 1979) and Ra-1 (Miyoshi *et al.*, 1983) rabbit lymphoid cells were maintained in RPMI-1640 medium supplemented with 10 % foetal bovine serum (FBS) (Gibco BRL, UK), 2 mM L-glutamine and antibiotics. Vero cells were grown in DMEM medium supplemented with 10 % (FBS), 2 mM L-glutamine and antibiotics.

Transfection

Transfection with the ExGen 500 transfection reagent - 22 kDa linear polyethylenimine (MBI Fermentas, Lithuania) was performed according to the manufacturer's protocol. Briefly, cells grown in 24-well plate were transfected with 2 mg plasmid DNA mix with 6 equivalents of ExGen500 in 100 ml of 150 mM NaCl solution per well. For electroporation $2x10^7$ MT-2 cells were suspended in 400 ml of RPMI-1640 with 50% FBS, mixed with 20 mg plasmid, placed in 4 mm gap cuvette model 640 (BTX, USA) and electroporated with 100 V electric pulse for 50 msec followed by 200 msec pause for five cycles.

Nucleofection of MT-2 cells was performed with Cell Line Optimization Nucleofector™ kit according to manufacturer's instructions using the apparatus provided by Amaxa biosystems (Germany). 10^6 cells were suspended in 100 ml of nucleofector solution V, mixed with 5 mg of the plasmid and subjected to nucleofection using protocol A-23.

Estimation of Transfection Efficiency and Selection of Transfected Cells

Transfection efficiency was assessed by microscopic examination of GFP fluorescence in non-fixed cell preparations 24 hours after transfection. Selection of G418 resistant MT-2 cells was carried out at 1200 mg/ml of antibiotic for 4 weeks. Then transfected cells were maintained in the growth medium supplemented with 400 mg/ml of the antibiotic G418.

Isolation of Total Cellular RNA and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total cellular RNA was extracted using TRIzol reagent (Invitrogen, UK) according to the manufacturer's protocol. After treatment with DNase I (Sigma, USA), 100 ng of the RNA sample was subjected to reverse transcription using RNA PCR Kit (AMV) Ver. 2.1 (Takara, Japan) in a 20 ml reaction mixture volume according to the manufacturer's protocol. After reverse transcription, amplification in 50 ml of 50 mmol/L Tris-HCl, pH 9.0; 20 mmol/L NH_4SO_4; 1.5 mmol/L $MgCl_2$; 50mmol/L of each deoxyribonucleotide triphosphate; 1 unit of Taq polymerase, was performed. Primers specific to HTLV-1 LTR U3

region BA2 (sense): 5'GCTTAGAGCCTCTCAGTGAA 3' (position 36-55) and MM1 (antisense): 5'AGGACGGCTTGACAAACATG 3' (position 249-231) were used at a final concentration of 200 nmol/L. 35 cycles of amplification at 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 45 seconds, was performed. Amplification products were separated in 1.5 % agarose and visualised with ethidium bromide staining.

Results and Discussion

Insertion of the *neo* Gene into pMPSVEH, pMHTs, pMHTas

The *neo* selection gene was excised with *Hind III* and *BamH I* restrictases from the pKO Scrambler NTKV-1907 plasmid and inserted between *Hind III* and *BamH I* sites of plasmids pMPSVEH, pMHTs, pMHTas, respectively (Fig.1A). The new constructs were designated as P1neo, P4neo and P2neo, accordingly. To construct the plasmid harbouring only HTLV-1 LTR U3 sequence, which contains 21 bp repeats, recognised by HTLV-1 transactivator protein Tax, the pKO Scrambler NTKV-1907 plasmid was used as a backbone. First, the thymidine kinase gene was excised with *Rsr II*. Then the 226 bp fragment of the HTLV-1 LTR U3 was cut out from pHTLV-1 by *Sma I* and *Hinc II* digestion and inserted into *Sma I* site of the intermediate construct (Fig. 1B). The new construct was named as P5neo.

Insertion of *gfp* Gene

The MPSV promoter with the HTLV-1 LTR U3 sequence was excised with *Pst I* from the plasmids pMHTs and pMHTas and inserted into *Nsi I* restriction site of phrGFP-1 (Fig. 2A). The constructs were designated as P4GFP and P2GFP, respectively. To construct the plasmid harbouring only HTLV-1 LTR U3 sequence with *gfp* reporter gene, the 226 bp fragment excised with *Sma I* and *Hinc II* from pHTLV-1 was inserted into phrGFP-1. For this purpose the phrGFP-1 plasmid was cut with *Nsi I* and protruding termini were filled in by T4 DNA polymerase. The new construct was named as P5GFP (Fig.2B).

Transfection of MT-2 and Ra-1 Cells and Selection of Transfectants

In order to test the expression of the inserted marker genes, MT-2 cells were transfected by different methods. Results of transfection using the *gfp* gene as a marker are summarised in Table 1. No significant differences in transfection rates were observed with different plasmids. All constructs harbouring *gfp* caused bright green fluorescence in the transfected cells during at least one week of observation. Transfection efficiency by electroporation in this set of experiments was in accordance with literature data (Sambrook *et al*, 1989). This is the first report to our knowledge which decscribes transfection of lymphocyte cell lines, like MT-2 and Ra-1, using ExGen 500 transfection reagent. The transfection rates achieved by this method are very low (Table 1) and although they may be probably improved it is obvious that application of new methods like nucleofection is preferable. The high efficiency of the nucleofection in comparison with other non-viral transfection methods is noteworthy. Probably, it can be explained by the ability of this method to deliver DNA directly into cell nucleus and initiate expression of transgene in few hours (Lai *et al*

2003; Trompeter *et al.*, 2003). The efficiency depends strongly on the cell line used, and transfection conditions have to be found selectively for each type of cells. Similar results were obtained when Ra-1 cell line was transfected by electroporation.

To test efficiency of the *neo* gene carrying constructs P1neo, P2neo, P4neo and P5neo, ca. 10^6 MT-2 cells were transfected with 5mg DNA of each plasmid using most efficient novel nucleofection technique. After 24 hours cells were transferred on growth medium containing 1200 mg/ml of G418, and after two more weeks viable cells were counted. The efficiency was calculated as a number of viable cells per 1ģg of DNA used for transfection (Table 2). Obvious are differences in the transfection rates when a control plasmid P1neo (not harbouring any virus sequence), or constructs containing HTLV-1 sequences in various orientations are used. It is tempting to speculate that this may be due to the expression of the cloned virus sequences in the transfected cells, but this assumption should be tested more carefully. It should also be mentioned that no differences were observed with constructs harbouring the *gfp* gene (Table 1).

Table 1

Transfection efficiency with the constructs harbouring the *gfp* gene (average results of 3-4 experiments)

	ExGen500 reagent		**Electroporation**		**Nucleofection**	
	gfp positive cells, %	Dead cells, %	*gfp* positive cells, %	Dead cells, %	*gfp* positive cells, %	Dead cells, %
MT2	0.05-0.1	2	3-4	50-80	15-17	10-15
Ra-1	ND	ND	3-4	60-80	ND	ND
Vero	65 -70	1	ND	ND	ND	ND

ND - not done

Table 2

Transfection efficiency of the MT-2 cells after two weeks of cultivation on medium containing G418 (calculated as a number of viable cells per 1mg DNA used)

Plasmid	Transfection efficiency
P1neo	1.2×10^5
P2neo	3.7×10^3
P4neo	8×10^4
P5neo	8×10^4

Examination of as-RNA Expression in Modified Constructs

To assess whether the introduced genes (*neo, gfp)* did not affect the expression of as-RNA from the new-made constructs, Vero cells were transfected with the P2neo, P4neo and P2GFP constructs using ExGen 500 transfection reagent. Vero cells were used as easy transfectable and HTLV-1 negative cell line to avoid crossreaction with viral mRNA as one could expect in HTLV-1 producing MT-2 and Ra-1 cells. Total cellular RNA from transfected Vero cells was isolated and checked for HTLV-1 LTR U3 RNA expression by RT-PCR. In all tested samples expression of HTLV-1 LTR U3 fragment was clearly observed (Fig. 3) and the introduced marker genes did not affect it. Thus, the new constructs will allow applying better and faster selection in further experiments on the efficiency of as-RNA approach in antiviral therapy.

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Summary

The use of antisense RNA and other nucleic acid agents to suppress viral replication is a promising alternative to traditional therapy. To investigate the possibility of HTLV-1 inhibition the plasmids carrying HTLV-1 LTR U3 sequence in sense and antisense orientation driven by MPSV promoter as well as non-expressing plasmid with cloned HTLV-1 LTR U3 fragment were used. As lymphocytic HTLV-1 producing cell lines are very hard to transfect, the selection of successfully transfected cells is required. To enhance efficiency of the selection procedure and to avoid laborious and time-consuming cloning, the analogical plasmids harbouring marker *neo* and *gfp* genes were constructed. In order to test the expression of the inserted marker genes, different transfection methods were applied. The best results were obtained with novel nucleofection technique based on electroporation, which showed higher transfection efficiency and lowest cell damage comparing to conventional electroporation. It was shown that all obtained constructs are functioning and allow convenient selection by introduced genes in MT-2 and Ra-1 cells. Also inserted *neo* and *gfp* genes did not affect the expression of HTLV-1 RNA from MPSV promoter in HTLV-1 negative Vero cells, transfected with the new-made plasmids. Thus, the new constructs will allow applying better and faster selection in further experiments on the efficiency of the antisense RNA approach in antiviral therapy.

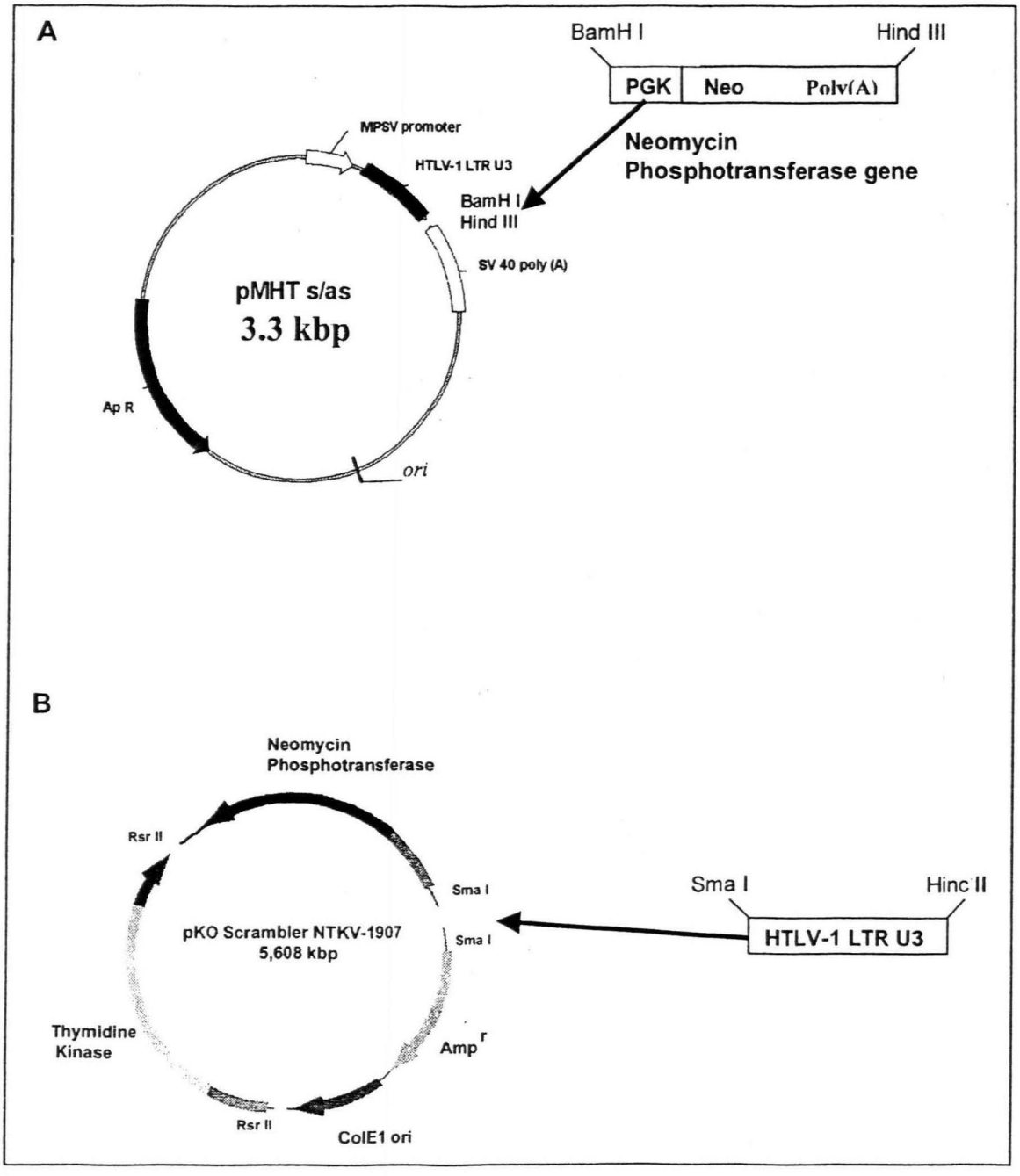

Figure 1. Construction of the plasmids P2neo, P4neo and P5neo.

A. Neomycin Phosphotransferase gene (1647 bp) was excised from pKO Scrambler NTKV-1907 with *BamH I* and *Hind III* and inserted between *BamH I* and *Hind III* sites of pMHTas and pMHTs, respectively.

B. Insertion of HTLV-1 LTR U3 fragment into pKO Scrambler NTKV-1907. First, the thymidine kinase gene (2019 bp) was excised with *Rsr II*. Then HTLV-1 LTR U3 fragment (226 bp) was cloned into *Sma I* restriction site of the construct.

Abbreviations:

MPSV - Myeloproliferative sarcoma virus; SV 40 poly (A) - Simian Virus 40 polyadenilation signal; *Ap R* - ampicillin resistance gene; *ori* - replication origin of the plasmid; PGK – mouse phosphoglycerol kinase promoter; *Neo* – Neomycin Phosphotransferase; Poly (A) – polyadenilation signal; ColE1 ori – ColE1 replication origin of the plasmid; *Amp*^{r} – ampicillin resistance gene.

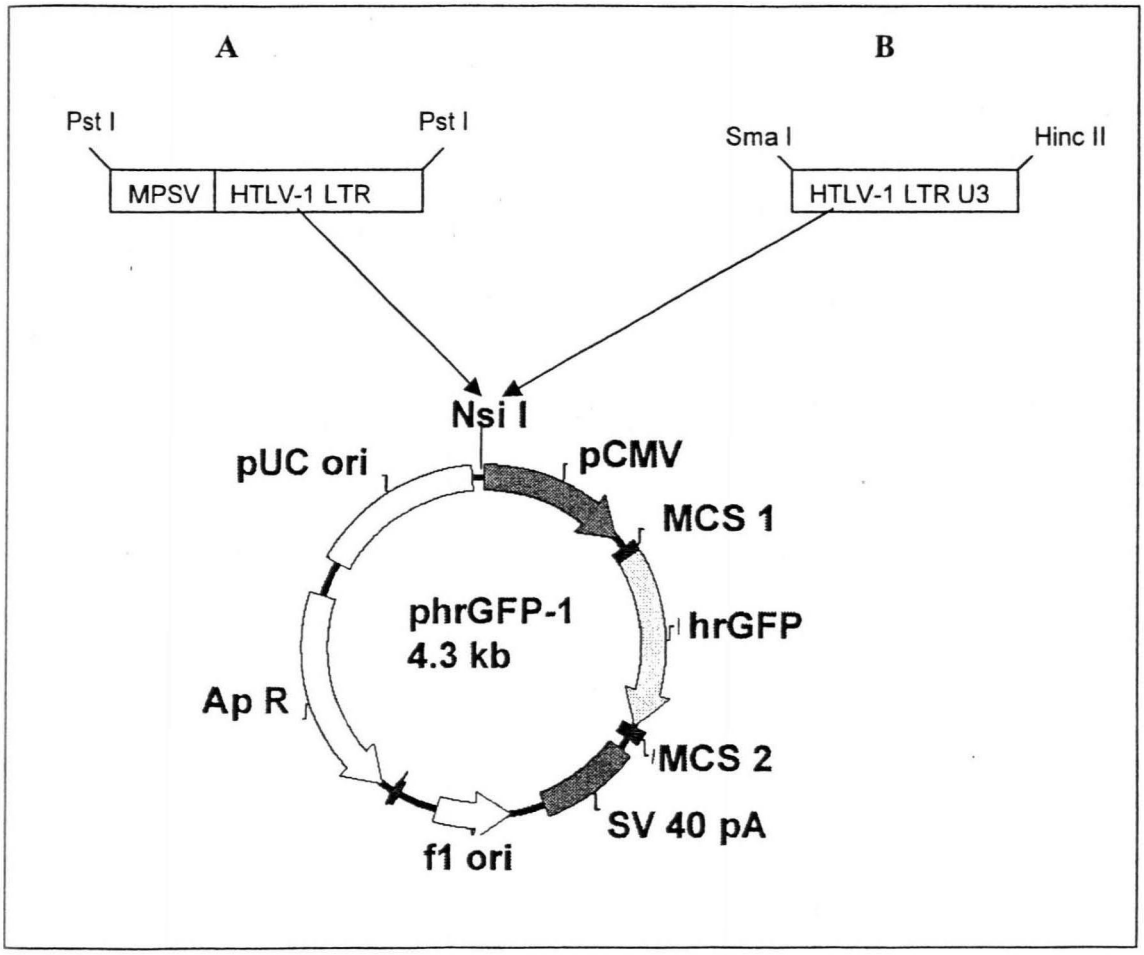

Figure 2. Construction of the plasmids P2GFP, P4GFP and P5GFP

A. MPSV HTLV-1 LTR U3 fragments (about 900 bp) were excised from pMHTas and pMHTs with *Pst I* and inserted into *Nsi I* restriction site of the phrGFP-1.

B. HTLV-1 LTR U3 fragment (226 bp) was excised from pHTLV-1 with *Sma I* and *Hinc II* and inserted into *Nsi I* restriction site of phrGFP-1.

Abbreviations:

pCMV – cytomegalovirus promoter; MCS 1 - multiple cloning site 1; *hrGFP* – *g*reen *f*luorescent *p*rotein; MCS 2 - multiple cloning site 2; SV 40 pA – Simian Virus 40 polyadenilation signal; f1 *ori* – f1 origin of single stranded DNA replication; *Ap R* – ampicillin resistance gene; pUC *ori* – pUC origin of replication of the plasmid

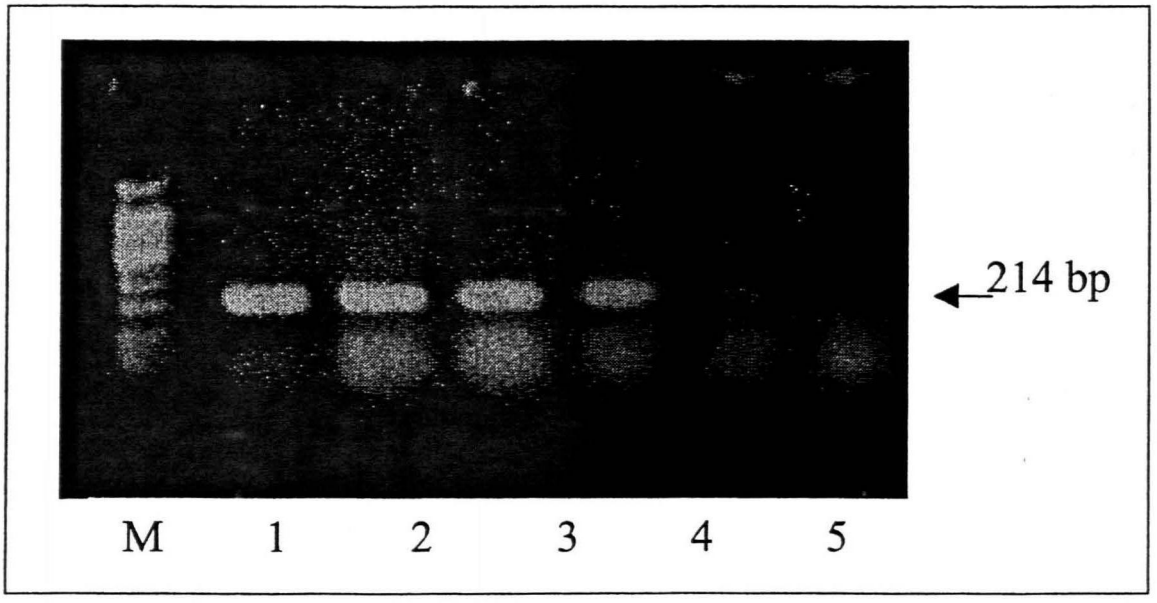

Figure 3. Expression of HTLV-1 LTR U3 RNA in Vero cells transfected with P2neo, P4neo and P2GFP (RT-PCR).

M – 100 bp DNA Ladder (Promega, USA); line 1 – Ra-1 RNA, positive control; line 2 – RNA of Vero cells transfected with P2neo; line 3- RNA of Vero cells transfected with P4neo; line 4 – RNA of Vero cells transfected with P2GFP; line 5 – RNA of intact Vero cells, negative control; line 6 – water.

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Review

ANTISENSE APPROACH OF INHIBITING REPLICATION OF HUMAN T-CELL LYMPHOTROPIC VIRUS TYPE 1 (HTLV-1)

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Specific inactivation of gene expression is an attractive approach for development of successful antiviral therapy. Antisense nucleic acids have great promise in the treatment of retroviral infection. The potential application of the antisense approach for suppression of human retrovirus HTLV-1 is discussed. A brief overview of the biology and pathogenicity of HTLV-1, the antisense approach, the action of different classes of antisense compounds, and the delivery methods of antisense drugs into organisms, are provided. Also, the choice of the target gene for antisense inhibition of HTLV-1 and possible therapy strategies are dicussed. In vitro *studies of antisense nucleic acids effect on HTLV-1 replication are reviewed.*

Key words: *HTLV-1, antisense oligodeoxynucleotides, antisense RNA, ribozyme.*

INTRODUCTION

Over the past few years, tremendous progress has been made towards using antisense (as) nucleic acids as therapeutic agents. As-nucleic acids, complementary to specific chosen virus genome sequences, have proved to be efficient antiviral agents with high specificity and low toxicity (Whitton, 1994; Varga *et al.*, 1999). Nevertheless, the evaluation and use of antisense drugs in control of viral infection remains at an early stage of development. Researchers still have to demonstrate that this technique works in human cells and in human bodies. Clinical trials with antisense nucleic acids have been already started, and currently more than a dozen successful biotechnology companies are focused on antisense technology. The goal of this review is to provide an overview of the current status in the development of antisense drugs against HTLV-1, the first human retrovirus to be described, and to assess the future potential of as-nucleic acid-based drugs as therapeutic agents against HTLV-1 infection.

BIOLOGY AND PATHOGENICITY OF HTLV-1

Human T-cell lymphotropic virus type 1 is an exogenous oncogenic retrovirus which is associated with a variety of severe human diseases. It is estimated that one to two million people are infected by HTLV-1 in Japan alone, where the virus is endemic, and approximately 10 to 20 million people are HTLV-1 carriers worldwide (Franchini, 1995). The main transmission routes are from mother to child, sexual transmission, blood transfusion, and shared contaminated needles among drug addicts (Tajima *et al.*, 1994).

HTLV-1 is the first characterised human retrovirus belonging to the HTLV/BLV (bovine leukaemia virus) group. HTLV-1 preferentially targets and transforms CD4+ T-lymphocytes (Collins *et al.*, 1996). The virus infects cells mostly via cell-to-cell contact which results in viral envelope-mediated membrane fusion between HTLV-1-infected cells and adjacent uninfected cells, while infection by free virus particles is less possible (Sagara *et al.*, 1997). Such cell-to-cell fusion leads to the production of a multinucleate cell – syncytium. A cellular receptor(s) for HTLV-1 still remains undetermined. Therefore, the pfenomenon of syncytium formation is used extensively to study virus entry into cells, as well as for quantitative assesment of the virus (Delamarre *et al.*, 1997; Niyogi and Hildreth, 2001). HTLV-1 lacks a typical oncogene (Weiss, 1984) and integrates randomly into the cell genome (Leclercq *et al*, 2000). In addition to the structural *gag*, *pol*, and *env* genes, the HTLV-1 genome has the unique pX region located between the *env* gene and the 3'LTR (long terminal repeat) region, which encodes the Tax and Rex proteins (Figure 1). The viral regulatory protein Tax has been shown to be responsible for the oncogenic potential of the virus. The Tax protein enhances the transcription of the virus through its interaction with a specific sequence (three 21 basepair repeats) in the virus LTR U3 region (Suzuki *et al*, 1993). Through interaction with the groups of cellular transcription factors and coactivators (e. g. NF-κB transcription factor family), Tax induces trans-activation of transcription of a number of cellular genes, including cellular oncogenes (e. g. *c-fos, c-jun, c-myc, c-ras*) and growth factors (e. g. IL-2, IL-6, TNF-a, TNF-b, GM-CSF) (Matsumoto *et al.*, 1997; Yao and Wigdahl, 2001). It deregulates the normal cell cycle through

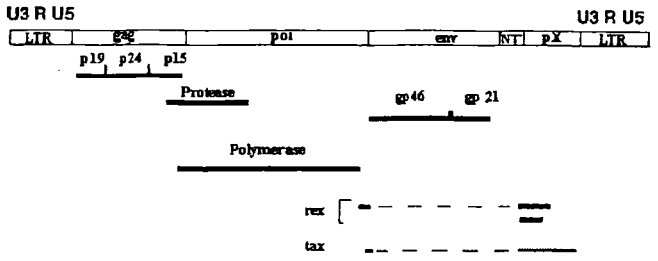

Fig. 1. Genomic structure of HTLV-1 proviral DNA (modified from Yao and Wigdahl, 2001). The viral mRNAs and the corresponding viral proteins are also shown. Dashed lines represent introns in the viral mRNAs.

The structural proteins, the virion-associated enzymes, and envelope proteins, are encoded by the *gag* (group-specific antigens), *pol* (polymerase), and *env* (envelope) genes respectively, which are common to all known retroviruses. The HTLV-1 genome is flanked at each end by a long terminal repeat, LTR. Each LTR is composed of U3 (unique 3'), R (repeated) and U5 (unique 5') regions. LTR is important in regulating proviral gene expression as well as mRNA termination and polyadenylation. The U3 region contains three 21 basepair repeats wich are responsible for Tax-mediated trans-activation of viral transcription. NT is a non-translated region. Two important viral regulatory proteins, Tax and Rex, are encoded by the pX region. Both are translated from doubly-spliced subgenomic mRNAs and are essential for the viral life cycle. The functions of other regulatory proteins ($p12^{I}$, $p13^{II}$, $p30^{II}$, not shown here), encoded by pX region are not clearly understood yet.

binding to inhibitors of cyclin dependent kinases 4/6, and can it inhibit also some tumour repressor genes (Cereseto *et al.*, 1996; Low *et al.*, 1997). These effects on a wide variety of cellular targets seem to co-operate in promoting cell proliferation. This is an effective viral strategy to amplify its proviral genome through replication of infected cells (Yoshida, 2001). Nevertheless, it is suggested that HTLV-1 in infected patients is not silent and is transcriptionally active (Asquith *et al.*, 2000). Thus, successful treatment of HTLV-1-associated diseases requires inhibition of the viral transcription as well as pathology-specific therapy.

Persistent infection by HTLV-1 can cause an agressive and lethal disease, adult T-cell leukemia (ATL), which is characterised by clonal expansion of HTLV-1-transformed $CD4^{+}$ lymphocytes and a long latency period (20–30 years) (Kawano *et al.*, 1985). HTLV-1 is the etiologic agent of the neurodegenerative disease HTLV-1-associated myelopathy/ tropical spastic paraparesis (HAM/TSP). A characteristic feature of this peripheral nervous system disorder is a strong, destroying response of the immune system to HTLV-1 antigens (particularly Tax) (Bangham, 2000). HTLV-1 is also associated with arthritis, uveitis, infective dermatitis, polymyositis, and other pathologies (Yodoi and Uchiyama, 1992). The list of diseases associated with the HTLV-1 has been ever more extended in the past several years (for a review, see Yao and Wigdahl, 2001). Despite vaccination, which has reduced the incidence of several viral infections (e. g. polio, mumps, rubella, measles), the treatment of diseases caused by retroviruses (e. g., the HTLV/BLV group, the human immunodefficiency virus HIV) remains problematic. Although some progress in the treatment and prophylaxis of HTLV-1 infection has been made, neither vaccines nor satisfactory treatment of HTLV-1 associated diseases is currently available. Th the development of suitable theraupeutic means agai HTLV-1 infection is still of great importance.

ANTISENSE TREATMENT STRATEGIES

A promising new therapeutic strategy is selective block of the gene involved into pathogenesis of a disease. It mi be possible to design gene therapy strategies that altoget avoid viruses and their drawbacks. Traditional approac allow targeting of protein functions, whereas antisense th apy can be directed toward not only the protein-coding gions but also against nucleic acid sequences that cont replication, transcription, and translation of the virus. D ferent antisense strategies depending on the nature of pathological condition can potentially be applied to inhi certain viruses. These include using different types of compounds, administration routes applied and ways of livery into a cell, therapy target choice, as well as combi tion of multiple targets for therapy simultaneously. To antisense nucleic acids as realistic therapeutic agents, s eral tasks should be first fulfilled. These tasks are the e cient delivery to a high percent of the cell populatio... e cient long-term expression from a vector, co-localisat with the target, and specific action with the desired mRN

Antisense nucleic acids and the mechanism of their tion. Two strands of nucleic acids can form a n covalently bound duplex as a result of Watson–Crick b pairing, where adenine can form a hydrogen bond w thymine/uracyl, and cytosine can bind with guanine. In case of DNA, one strand serves to store the genetic co and is called the sense strand, while the other provides complementary supporting strand and is known as the tisense strand. RNA is generally copied from the antise strand and has the same sequence as the DNA sense stran

Three main mechanisms of action have been reported the antisense nucleic acids: 1) oligonucleotides, designe antisense orientation, hybridise to their target mRNA i strict base-pair specific manner (Watson–Crick base p ing) and thus block the translation; 2) they can bind to genomic DNA in the nucleus and thus block the transcr tion (Hoogsteen-type base triplets), 3) the third, unspec mechanism of the action is the binding of the as-nucl acid to a target protein that has been referred to as antise aptamer-binding (Lavrovsky *et al.*, 1997). Thus, inhibit can take place on different levels of gene expressi (mRNA transcription, processing, and translation) (Fig 2).

Mainly two types of as-nucleic acids are used: as-oli deoxynucleotides (ODNs) and as-RNAs. As-oligodeo nucleotides are short (15-25 base-pairs), single-stran DNA molecules complementary to the target mRNA DNA sequence, which are administered exogenously int cell. As-RNAs are produced intracellularly from an exp sion vector which can be introduced into a cell by differ approaches (Mahato *et al.*, 1997).

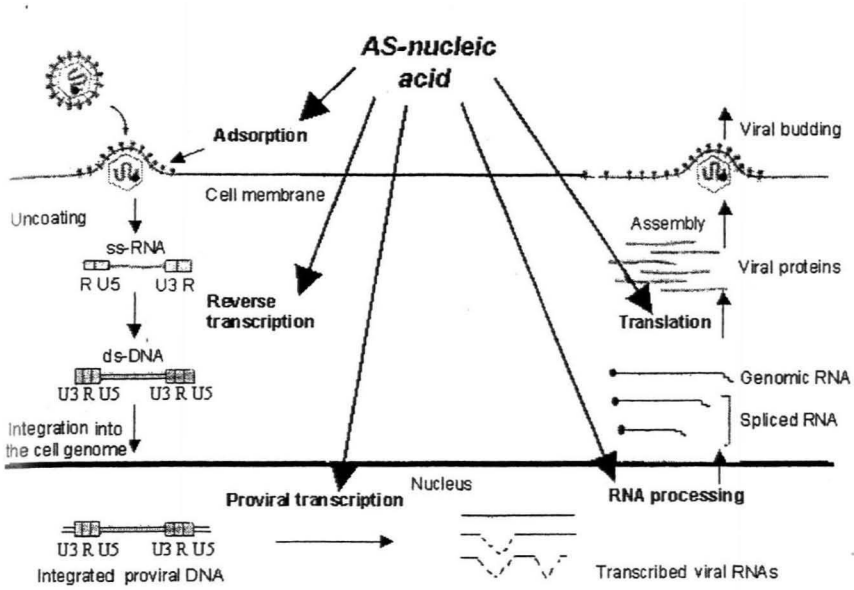

Fig. 2. The stages of retrovirus replication which could be affected by as-nucleic acids; ss-RNA, single-stranded RNA; ds-DNA, double-stranded DNA.

During the retrovirus life cycle, as-nucleic acids can affect the virus at different levels of its replication: reverse transcription, viral mRNA transcription, processing and protein synthesis (translation). Adsorption of the virions on the cell membrane also could be affected by exogenously added nucleic acids, due to unspecific interaction with the cell membrane (Miyano-Kurosaki *et al.*, 1996). At the stage of reverse transcription, effective inhibition could be achieved with as-RNAs complementary to the viral reverse transcription intermediate, a plus-strand strong-stop DNA (LTR region) (Peng *et al.*, 1997). Complementary binding of as-DNA or as-RNA to viral mRNA transcripts or proviral DNA physically obstructs their translation or transcription, correspondingly. An additional effect of as-nucleic acid binding to the targeted DNA or RNA sequence is the cleavage of DNA-RNA and RNA-RNA duplexes by cellular enzymes RNase H and RNase L, respectively.

A major concern with the use of as-ODNs has been their low stability in tissue culture medium and, ultimately, in the living host. Such short nucleic acids (due to constraints in the permeability of cell membranes for big molecules) are susceptible to nuclease degradation when they are administered into an organism. Modifications in the base, sugar, and phosphate moieties of oligonucleotides have been reported to stabilise the molecules (e. g. phosphorothioates, morpholino) (Summerton and Weller, 1997).

Antisense RNAs hold great promise for therapeutic use in the future. Contrary to as-oligonucleotides, as-RNAs are produced in the cell continuously and, in comparison with short oligonucleotides, they can bind longer sequences and block their expression. Therefore, the as-RNA action is expected to be much more specific and more long-term (Weiss *et al.*, 1999).

These "classic" antisense approaches, catalytic RNA molecules called ribozymes, are being assessed as potential antiviral agents. Ribozymes can cleave other RNAs and, after destroying one target molecule, they can move to the next, thus offering the potential benefit of cycling (Whitton, 1994). The ribozyme RNA consists of antisense sections (which allow to target it specifically) and a catalytic domain. Two classes of ribozymes, defined by the secondary structure of the ribozyme RNA catalytic core, are being used as antiviral agents: hammerhead and hairpin ribozymes. Ribozymes combine enzymatic processes with the specificity of antisense base pairing (James and Gibson, 1998). They can be delivered to cells as preformed ribozymes (exogenous delivery) or as ribozyme genes, a method of endogenous delivery.

Introduction of therapeutic nucleic acids into an organism. Methods for introduction of an alien gene into an organism are not completely developed so far. There is no "good universal" vehicle to transfer the gene of interest. All of the vectors that are currently available have both advantages and disadvantages. Traditionally, viral vectors have been used as an effective means of gene therapy. To use virus as a vector for gene therapy, specific genes involved in viral replication of the intact wild-type virus are modified or deleted, and a transgene is inserted into the viral genome using molecular biological techniques. Viral gene delivery offers many intrinsic advantages: 1) specific cell-binding and entry properties; 2) efficient targeting of the transgene to the nucleus of the cell and; 3) the ability to avoid intracellular degradation (Robbins and Ghivizzani, 1998). However, the use of viral vectors requires expensive cell culture methods, because the cell population must be expanded significantly to produce a sufficient quantity of the virus. Also, it can cause an inflammatory response of the organism leading to undesirable side effects. Therefore, in the past few years, intensive research has been focused on the development of non-viral gene delivery vectors that employ plasmid DNA encoding a target gene (for a review, see Mahato *et al.*, 1997). Non-viral transfection techniques allow to reduce the risk of introduction of a potentially replicating virus and the respective inflammatory response of the organism. A major problem in development of non-viral delivery systems is the low transfection efficiency which limits their application. Nevertheless, this is an attractive *in vivo* gene delivery strategy which is simpler, less expensive, and lacks some of the risks inherent in the viral systems. It has been demonstrated that lyposomes, receptor-mediated polycation systems, and that virus-like particles are promising carriers for delivery and expression of plasmid DNA encoding genes into the target cells (Boussif *et al.*, 1995; Abe *et al.*, 1998; Pumpens and Grens, 1999).

The *in vivo* approach involves the transfection of tissue cells by introducing an as-drug through systemic administration or *in situ* through direct injection into target organs. Gene therapy by systemic administration of an as-drug with a non-viral carrier does not seem to be practical because of the rapid elimination of the plasmid DNA from circulation,

poor target specificity, and possible toxicity to non-target tissues (Mahato *et al.*, 1997). Local administration methods, such as direct injection into the target site (liver, thymus, bone marrow, etc.) are being attempted in *in vivo* gene therapy (Wood and Prior, 2001). The importance of bone marrow cells, particularly the sub-population of hematopoietic cells, has never been underestimated in the context of gene therapy. Their potential for self renewal and differentiation into all hematopoietic lineages makes them very attractive as targets for gene transfer, especially when long-term transgene expression is required. These techniques could be of potential value in the antisense therapy of human leukemias. Clinical trials of anti-HIV-1 therapy, where as-RNAs were transduced with a retrovirus-based vector into hematopoietic cells, have been reported (Liu *et al.*, 2001). Autologous $CD34^+$ hematopoietic stem cells from HIV-1-infected patients were transduced with three antisense sequences targeted either to the TAR or to two separate sites of the TAT region in the HIV-1 genome. The continued expression of the anti-HIV-1 antisense genes in HIV-1-infected subjects was detected in peripheral blood mononuclear, $CD4^+$ (98% pure), and bone marrow $CD34^+$ cell populations isolated from the patients infused with the transduced bone marrow $CD34^+$ stem cells. Such a strategy could be prospective also for the treatment of adult T-cell leukemia caused by HTLV-1.

Construction of as-nucleic acids expression vectors. Choice of a promoter. A long-term approach is to clone an as-sequence into a mammalian expression vector, with the intent to control expression of the as-sequence or ribozyme. A large excess of as-RNA molecules over target RNAs is important for effective inhibition of virus replication (Shayakhmetov *et al.*, 1997). To achieve this goal, the as-gene should be inserted into an expression vector behind an appropriate promoter sequence. A promoter is a regulatory sequence of DNA that is located upstream of a gene, and to which proteins (transcription factors and RNA polymerase) bind to initiate the synthesis of mRNA and subsequently protein. Appropriate promoter systems allow to ensure high levels of as-gene expression in a target tissue, required for effective antiviral action of as-RNAs. Therefore, the promoter choice is one of the crucial points in the construction of the as-RNA expression vectors.

Usually, strong promoters, mostly derived from pathogenic viruses, are used to drive the expression of as-RNA genes (Shayakhmetov *et al.*, 1997; James and Gibson, 1998). Two strategies are commonly used. First, the as-gene is inserted behind a strong promoter for RNA polymerase II, which may be of viral origin or a strong endogenous promoter (e. g. the actin gene promoter). One of the main advantages of the RNA polymerase II promoter is the availability of a tissue-specific and regulable promoter. Second, an alternative to RNA polymerase II is use of the RNA polymerase III promoter. The RNA polymerase III transcribes a variety of small nuclear and cytoplasmic RNAs that are abundant in all cell types (e. g. tRNA). However, in their present form, this type of promoter cannot be regulated, and its use in certain applications is limited (Lavrovsky *et al.*, 1997; Ja and Gibson, 1998).

Strong viral promoters have been successfully used *in v* in mammalian cell cultures where the inflammatory c kines are not present. However, it has been reported that expression of sequences cloned behind some widely u strong viral promoters (e. g. cytomegalovirus promoter) be affected by cytokines produced by immune system c (INFγ, TNF-α) *in vivo* (Gribaudo *et al.*, 1993).

The ideal as-RNA constructs must provide expressior antiviral as-RNA genes only in those cells of the organ which can be infected by the virus, and only when they infected. Shayakhmetov *et al.* (1997) demonstrated these criteria can be achieved, at least partially, by using own promoter of the virus to drive the as-RNA gene. authors showed a 75 % inhibition of BLV replication as-RNA targeted to the BLV LTR RU5 region driven by BLV U3 promoter. This strategy was also applied succ fully for HTLV-1. It was demonstrated that the HTL LTR driven antisense *c-myc* construct suppressed *c-myc* pression and inhibited the growth of HTLV-1-infected transformed cells of the human T-cell line HUT102 (Fu and Shiku, 1993).

Choice of the target. An important issue in creation of antisense therapeutic molecule is to address the as-nuc acid to a proper virus target gene. As it is known, certain rus sequences are highly conserved. Such "immutable" s would be difficult to target as protein, but they may be m accessible to antisense assault. In this case, the emerge of antisense-resistant mutants is unlikely. In the case of animal virus BLV which is closely related to HTLV-1, fective inhibition was demonstrated by using as-RNAs geted to the virus LTR RU5 and pX regions. A number constructs were obtained under the control of various p moters (HSV TK, SV40). The most effective suppressior BLV replication was observed with as-RNA against RU5 region of BLV LTR. A significant but less mark suppression was observed with as-RNA targeted to the B pX region (Murovska *et al.*, 1992; Shayakhmetov *et* 1997). Peng *et al.* (1997) also showed that intracellular pression of HIV-1 sense or antisense U3RU5 sequen conferred long-term inhibition of HIV-1 replication, desp the continuous presence of viral challenge in the transduc Jurkat cell line.

A promising candidate for HTLV-1 suppressive gene th apy is the Tax protein, as it is an early transactivator of expression of all HTLV-1 genes. Therefore, the as-nucl acids targeted to the *tax* gene and to the HTLV-1 LTR R region are prospective targets for HTLV-1 suppressive th apy.

As the LTR U3 region contains the Tax-responsive enh cer sequence (three 21 base-pair repeats), it would be of terest to study the antiviral activity of constructs targeted the HTLV-1 *tax* gene and LTR U3 region sequences sim taneously. Also, in the case of HTLV-1, one could exp

that a mechanism other than antisense binding could be involved additionally. Briefly, when a plasmid vector expressing as-RNA to the LTR U3 region is introduced into a cell, also the sense LTR U3 DNA sequence of the introduced recombinant DNA can compete with viral and cellular DNA sequences for the virus transactivator Tax (trap for the Tax protein). Hence, the effects of aptamer binding and blockage of Tax translation through an antisense mechanism (by as-RNA produced from the plasmid vector) may be combined. Such a possibility was assumed by Shayakhmetov *et al.* (1997) in work with BLV, where the inhibition of the virus was observed using a plasmid harbouring only the virus promoter LTR U3 sequence as a control (without the as-sequence). Theoretically, this strategy could be promising for more efficient inhibition of the virus transcription and Tax-mediated oncogenic and immunogenic effects.

INHIBITION OF REPLICATION OF HTLV-1 BY ANTISENSE NUCLEIC ACIDS

The use of the antisense approach to inhibit HTLV-1 replication has been described mostly *in vitro,* in cell cultures. There are only a few publications where it was demonstrated *in vivo.*

Studies *in vitro.* Antisense oligodeoxynucleotides. Maeda *et al.* in 1997 showed a 59 % inhibition of syncytium formation between HTLV-1-producing human T-cell line C91/PL cells, and HTLV-1-uninfected human glioma cell line U251-MG cells, by antisense oligonucleotides complementary to the region of initiation codon of *tax* gene. Also, the effects of ODNs complementary to the first splice junction, the Rex-responsive site, *gag, env, tax, rex,* and *p21,* on syncytium formation, have been evaluated. Syncytium formation was significantly inhibited by as-ODNs to *env, tax, gag, p21,* and *rex,* with as-ODNs to *env* being the most inhibitory. Antisense ODNs to *env* and *tax* also inhibited reverse transcriptase activity (Maeda *et al.*, 1998).

It is important to note that the action of exogenuously introduced ODNs is short-term. Therefore, ODNs must be introduced repeatedly many times. Such a strategy does not seem optimal for therapy of integrative viruses. Also, it is not known how specifically these short nucleic acids will act. Nevertheless, this system is valuable for primary screening of potentially active antisense sequences.

Antisense RNAs. Von Ruden and Gilboa in 1989 demonstrated that primary human T-lymphoid cells could be made partially resistant to HTLV-1 via as-RNA-mediated inhibition. In that study, two segments of HTLV-1 were chosen as the targets: (1) the sequence spanning 5'end of mRNA, harbouring *cis*-elements, essential for viral gene expression (5'splice site) and virus replication (tRNA primer-binding site), and (2) the pX region, corresponding to the first kilobase of the *tax* gene. It was shown that as-RNA expression leads to significant, although not complete, inhibition of HTLV-1 replication (Von Ruden and Gilboa, 1989).

In our laboratory, recombinant DNA harbouring the HTLV-1 LTR U3 antisense sequence under the strong MPSV (Myeloproliferative sarcoma virus) promoter, which is active in the lymphoid tissues (Artelt *et al.*, 1988), is being tested. This antisense construct was transfected into a HTLV-1-infected monolayer cell culture, originally obtained in our laboratory (Bratslavska *et al.*, 2000; Ivanova *et al.*, 2001). Four cell clones containing the as-RNA gene were obtained. It was found that the as-RNA gene is integrated into cell genome and inherited during passaging of the cells. The study showed also stable expression of the introduced sequence in the cells during more then 40 passages. Preliminary results indicate that the expression of HTLV-1 in these clones is inhibited. Previous reports and our results demonstrate that as-RNAs genes targeted to regulatory sequences of the HTLV-1 genome inhibit the expression of HTLV-1 in cultured cells, and that they can be proposed as potential therapeutic agents (Von Ruden and Gilboa, 1989; Maeda *et al.*, 1998).

Ribozymes. Antiviral activity of ribozymes targeted to the HTLV-1 *tax* and *rex* genes has also been reported. Hammerhead ribozyme targeted against HTLV-I *tax/rex* mRNA was introduced into synovial cells obtained from patients with HTLV-1-associated arthropathy and from patients with HTLV-I-negative rheumatoid arthritis. The ablation of Tax expression as well as the ability of the cells to stop proliferating and to undergo apoptosis were examined. Both transcription of tax mRNA and Tax protein synthesis were inhibited significantly, resulting in inhibition of synovial cell growth and induction of apoptosis (Kitajima *et al.*, 1997a). Intracellular activities of the ribozymes targeted to HTLV-I *tax/rex* mRNA were studied also in HTLV-1 tax cDNA-transfected rat embryonic fibroblasts (Rat/Tax cells) which expressed the Tax. Tax protein levels were decreased by about 95 %, while Tax antisense oligodeoxynucleotides reduced Tax expression by about 20 % (Kitajima *et al.*, 1997b).

Experiments *in vivo.* Very little research has been conducted so far with respect to investigation of anti-HTLV-I activity of as-RNA and ribozymes in animal model systems. The existing animal models of HTLV-I infection display different patterns of infection and resulting pathologies; thus, choice of the animal model depends on the pathology studied. Kitajima *et al.* (1992) reported the occurrence of suppression of fibroblastic tumours developed in HTLV-I Tax transgenic mice by as-ODNs to the NF-kB transcription factor. Treatment with ODNs to Tax showed virtually complete suppression of Tax expression, but not regression of the tumours (Kitajima *et al.*, 1992).

Due to many technical difficulties associated with the production of transgenic animals, there are few reports of the anti-viral effects of as-RNAs genes in transgenic mammals. However, the results of these few studies indicate that as-RNA-mediated inhibition can effectively prevent viral infections *in vivo.* Kozireva et al. (1996) studied the sensitivity of rabbits to BLV infection, both in wild type and transgenic animals, in the latter with the as-RNA gene targeted at

the LTR RU5 region of BLV. The obtained results indicated that the anti-BLV as-RNA gene confers enhanced resistance to BLV infection in transgenic rabbits compared to wild type animals. Continuing this investigation, the authors found that the expression level of as-RNA in transgenic rabbits was not sufficient to block completely BLV reproduction, although it was sufficient to abort infection in rabbits (Murovska *et al.*, 2001). Therefore, the investigation should be continued to elucidate optimal targets and as-gene-expressing constructs that can inhibit effectively the virus replication and prevent pathologies.

CONCLUSIONS AND FUTURE PROSPECTS

Data on inhibition of HTLV-I and other retrovirus infections by using antisense technology suggest that antisense nucleic acids and ribozymes can effectively inhibit HTLV-I replication *in vitro*. In previous studies with ODNs, the most preferential target sequences for effective inhibition of the virus have been determined. The next logical step is the design of vectors expressing antisense RNAs and ribozymes, to create more specific and flexible antisense therapeutic systems. The use of specific vectors for direct delivery of genetic materials into certain cells and tissues, and application of strong or inducible promoters to regulate the expression of as-sequences, allow the development of new as-constructs for antiviral protection. As the pathogenesis of HTLV-I associated diseases is very complex, a panel of antisense drugs targeted not only to the virus, but also to some virus-activated cellular genes (e. g. NF-kB and *c-myc*), needs to be established. With improvements in stability, delivery, and design of as-nucleic acids, the antisense approach is certain to become one of the most important tools in gene therapy. Despite the present drawbacks, the potential power of antisense therapy remains undisputable, and soon these techniques will make an important contribution to viral infection therapy. To accumulate needed experience for the practical application of modern antisense technologies for the supression of the certain viruses (e. g. HTLV-I), more trials *in vitro* and especially *in vivo* should be performed.

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HTLV-I INHIBĪCIJAS IESPĒJAS, IZMANTOJOT ANTIINFORMĀCIJAS PIEEJU

Viena no daudzsološākajām metodēm efektīvas pretvīrusu terapijas izstrādē ir specifiska gēnu ekspresijas kavēšana ar antiinformācijas nukleīnskābēm. Īpaša nozīme šīs metodes izmantošanai var būt retrovīrusu infekciju profilaksē un ārstēšanā. Apskata rakstā ir analizētas cilvēka retrovīrusa HTLV-I inhibīcijas iespējas, lietojot antiinformācijas tehnoloģiju. Dots īss pārskats par HTLV-I bioloģiju un lomu patoloģiskos procesos, par antiinformācijas nukleīnskābju pamattipiem, to iedarbības principiem un ievadīšanas paņēmieniem šūnā un organismā. Apspriesta terapijas mērķgēna izvēle HTLV-I genomā un aprakstīti *in vitro* pētījumi, lai noskaidrotu uz HTLV-I genoma dažādiem rajoniem adresētu antiinformācijas nukleīnskābju pretvīrusa iedarbību.

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A CELL CULTURE MODEL FOR HUMAN T-CELL LYMPHOTROPIC VIRUS TYPE 1 ANTIVIRAL DRUG SEARCHING

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The aim of this work was to obtain a convenient monolayer cell culture model to study antisense RNA inhibitory effect on HTLV-1 replication and transforming ability. For this purpose, a monolayer human osteosarcoma (HOS) cell line was infected with HTLV-1 by co-cultivation with the virus-producing lymphoid rabbit cell line Ra-1. It was demonstrated that HTLV-1 infection of HOS cells was productive, stable and long-term. We also found that the obtained cell culture acquired features of transformation, compared to the initial cell line HOS. This study describes a useful cell culture system for further examination of new antiviral agents such as antisense polynucleotides.

Key words: *HTLV-1, monolayer cell culture, antisense nucleic acids, cell transformation.*

IV

INTRODUCTION

The human T-cell lymphotropic virus type 1 (HTLV-1) is a transforming human retrovirus which can cause adult T-cell leukemia (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis. HTLV-1 infection is also associated with various inflammatory disorders such as arthritis, uveitis, and infective dermatitis (Sarma and Gruber, 1990; Yodoi and Uchiyama, 1992).

Since no effective chemotherapy is currently available, it is important to find suitable therapeutic treatments against HTLV-1-associated diseases. Antisense RNAs have proved in many cases to be efficient antiviral agents in cell cultures (Veres *et al.*, 1998). Up to now, the known HTLV-1-producing lymphocyte cell lines are susceptible to cell damage induced by transfection procedures: the cell density decreases after selection with antibiotics which usually leads to apoptosis (Brielmeier *et al.*, 1998). Therefore, it is difficult to obtain cell clones which would contain antisense nucleic acid and could be used in the study of antisense construction inhibitory effect and mechanisms of action.

The aim of this work was to obtain a convenient monolayer cell culture model to study the antiviral activity of antisense constructions against HTLV-1. It has been shown previously that HTLV-1 can productively infect the non-lymphoid human osteosarcoma (HOS) cell line (Clapham *et al.*, 1983). By infecting cell culture HOS with HTLV-1, it was found that HTLV-1 infection in HOS cells is stable and long-term. Moreover, after a long passage, the infected cell line obtained features of transformation.

MATERIALS AND METHODS

Cell cultures. HOS line TE85, human cervical carcinoma (HeLa) monolayer continuous cell lines, and the HTLV-1-producing rabbit lymphoid cell line (Ra-1) were used. HOS and HeLa cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % foeta bovine serum (FBS; Gibco BRL, UK), 2 mM L-glutamine and antibiotics. Ra-1 cells were grown in RPMI-1640 medium supplemented with 15 % FBS, 2 mM L-glutamine and antibiotics.

HTLV-1 infection of HOS cells. The HOS cells were infected with a HTLV-1 Japanese strain, by co-cultivation with virus-producing Ra-1 cells which were previously treated with mitomycin C (5 μg per ml, for 1 hour at 37 ^{0}C). Co-cultivation was carried out using a suspension of Ra-lymphocytes at a concentration of $1.5 \cdot 10^5$ cells per ml. The virus-infected HOS subline was designated as RaHOS.

Cytogenetic analysis. Cytogenetic analysis of 48-h monolayers of the RaHOS and HOS cell lines in the phase of exponential growth was performed. The cells were incubated with colchicine (1 μg per ml; Sigma, USA) for 30 min, hypotonised with 1 % sodium citrate for 30 min, then fixed with mixture of methanol and glacial acetic acid (3:1) and stained

with Giemsa (azure-eosin-methylene blue in phosphate buffered saline) according to Seabright (1971). Metaphase chromosomes were analysed under a light microscope.

Cell proliferative activity. The proliferative activity of HOS and RaHOS cells was estimated by incorporation of ^{3}H-thymidine (Bratslavska *et al.*, 2000).

Soft agar assay. The ability of RaHOS cells to grow in semi-solid media was tested according to the protocol of Freshney (1994, pp. 166–167). One thousand viable single cells were suspended in 1 ml DMEM medium containing 0.3 % Noble agar (Difco, USA) and 15 % FBS. The cells were incubated for three weeks by regular addition of fresh medium.

Detection of HTLV-1 proviral DNA. Polymerase chain reaction (PCR) analysis was used. Genomic DNA was isolated from up to 3×10^6 HOS, RaHOS, Ra-1 cells by proteinase K digestion followed by standard phenol/chloroform extraction. DNA quality was confirmed by PCR for human β-globin gene according to Vandamme *et al.* (1995).

One μg of DNA was analysed for the presence of HTLV-1 provirus by nested PCR using primers targeted to HTLV-1 *gag, env,* and *tax* genes and the 5' long terminal repeat (5' LTR) region. The sequences of primers and PCR conditions have been previously described (Bratslavska *et al.*, 2000). The DNA from Ra-1 cells was used as a positive control, and sterile water and the DNA from HTLV-1 free HOS cells were used as negative controls. The amplified samples were analysed further by electrophoresis on 1.7 % agarose gel, followed by ethidium bromide staining and visualisation in UV light.

Detection of HTLV-1 expression. Reverse transcriptase–polymerase chain reaction (RT–PCR) analysis. Total RNA was extracted using an "RNeasy Total RNA Kit" (Qiagen, Germany), from 3×10^6 HOS, RaHOS and Ra-1 cells as well as from 1000 fold concentrated (by ultracentrifugation) virus-containing medium of RaHOS cell culture. After treatment with DNase I (Sigma, USA), 200 ng of the RNA sample was subjected to reverse transcription using M-MuLV reverse transcriptase (MBI Fermentas, Lithuania), in a 20 μl reaction mixture volume according to the manufacturer's protocol. After reverse transcription, amplification with primers to the HTLV-1 *tax* and *gag* genes was performed.

Indirect immunofluorescence assay (IFA). Cells grown on cover slips were fixed for 20 min with methanol at –20 ^{0}C and then air-dried. IFA was performed by a common procedure. Sera from ATL patients were used as the primary antibodies for HTLV-1 antigen detection. As secondary antibodies, a goat anti-human immunoglobulin G labeled with fluorescein isothiocyanate (FITC) was used.

Syncytia assay (SA). The ability of the RaHOS cells to induce multinuclear giant cells (syncytia) in HeLa indicator cells was assessed by co-cultivation at a ratio of 1:10 respectively (5×10^3: 5×10^4 cell per ml) (Hayami *et al.*, 1984).

RESULTS

Morphology and cytogenetic features of the RaHOS cell line. Morphological studies of RaHOS cell cultures (using Romanowsky-Giemsa staining) revealed some signs of transformation, compared to the initial HOS cell culture. Although both cell cultures were cultivated simultaneously in equal conditions, after 60 passages of cultivation, focuses of multilayer growth in the RaHOS cell culture appeared while the initial HOS cell culture lacked such features.

Also, the proliferative activity of RaHOS cells differed from the initial HOS cell culture. After 18 passages, the incorporation of ^{3}H-thymidine in RaHOS cells was 1.4-times higher, and at the 135th passage 2-times higher, in comparison with HOS culture. At 96 hours, the incorporation of ^{3}H-thymidine in RaHOS cells remained high, while in HOS cells, its level was decreased.

After 30 passages, the RaHOS cells possessed the ability to form colonies in soft agar. About 0.1 % of the cells formed colonies of ca. 0.1 mm in diameter. The capacity to form colonies increased during further passaging, and after 60 and 150 passages, already 0.4 % and 1.3 % of cells, respectively, had formed clumps of increased size (0.15–0.20 mm in diameter). The initial cell culture HOS TE85, used in our study as the control, did not form colonies in soft agar.

Cytogenetic analysis of RaHOS cells showed that this cell culture had the human karyotype. The modal number of chromosomes in RaHOS (60 and 125 passages) and HOS cells was identical – 48. However, in the RaHOS cell culture (125th passage), the percentage of polyploid cells was slightly elevated in comparison with HOS cells (5.4 % and 4.6 %, respectively).

Presence of HTLV-1 proviral DNA in RaHOS cells. Using a set of HTLV/STLV generic outer and HTLV-1 specific inner primers for the *tax* gene, DNA samples extracted from RaHOS cells were positive for HTLV-1 sequences in repeated experiments. HTLV-1 infection was further confirmed by the presence of *gag* and *env* genes, and the 5'LTR region of the virus. During the entire observation period (up to 150 passages), the integrated proviral DNA remained.

HTLV-1 mRNA expression. To determine the virus mRNA transcripts in infected cells, RT-PCR analysis with HTLV-1 specific primers complementary to the *gag* and *tax* genes was applied. The expression of *gag* and *tax* genes was detected in RaHOS cells at different passages of cultivation during the observation period from 20 to 150 passages. The RNA samples were free from contaminating DNA, which was confirmed by the absence of any amplification products after PCR with omission of RT.

Presence of HTLV-1 antigens. The presence of HTLV-1 antigens was estimated by IFA. Bright fluorescent regions in cytoplasm and diffuse fluorescence around the nucleus were detected in 75–80 % of RaHOS cells at the 65th passage, when IFA was carried out with HTLV-1 positive sera.

At the same time, fluorescence was not observed in HOS cells incubated with HTLV-1 positive sera, nor in RaHOS cells incubated with HTLV-1 negative sera. The specific fluorescence in cytoplasm of RaHOS cells was observed during the whole observation period up to the 150th passage.

The capacity of RaHOS cells to induce syncytia in HeLa cells was studied. The proportion of multinuclear cells in control HeLa cell culture did not exceed 0.6 % and the number of nuclei in these cells was up to 5. When RaHOS cells were mixed with HeLa cells, syncytia containing 5–15 nuclei appeared in the indicator HeLa cells, and the total amount of syncytia increased by up to 1.5–4.0 %. The replication of HTLV-1 in the RaHOS cell line was also confirmed by inhibition of syncytia formation in the presence of anti-HTLV-1 antibodies, while in the presence of HTLV-1 negative sera, the syncytia inhibition was not observed.

The productive infection of HTLV-1 in RaHOS cell line. The production of HTLV-1 virions in RaHOS cell culture was shown by the presence of the HTLV-1 genomic RNA in RaHOS culture liquid. For this purpose, the virions were sedimented by ultracentrifugation and viral RNA was subjected to RT-PCR with HTLV-1 *gag*- and *tax*-specific primers. By using RNA from the cell-free RaHOS supernatant, HTLV-1 *gag*- and *tax*-specific amplification products in RT-PCR were detected. These results were reproducible in dynamics during the observation period from 20 to 110 passages.

Thus, the HTLV-1 replication was demonstrated by the PCR, RT-PCR, IFA, and SA methods (Figure 1).

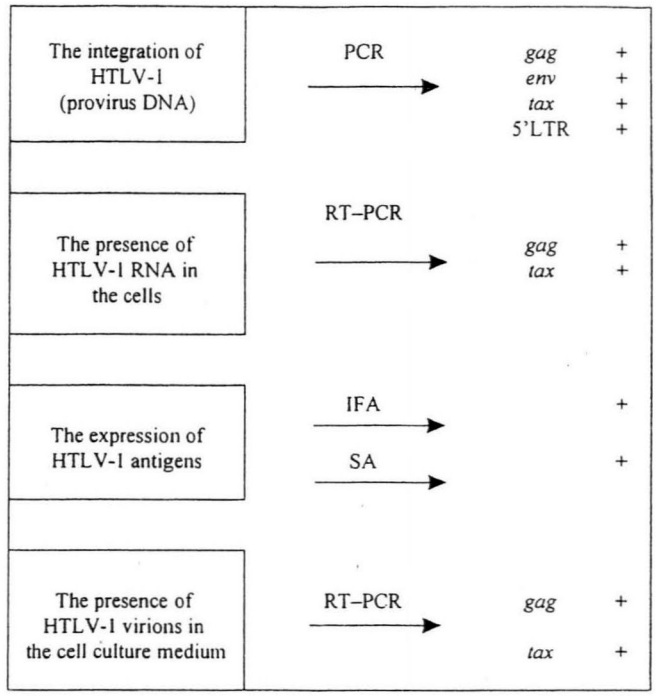

Fig. 1. Detection of human T-cell lymphotropic virus type I (HTLV-I) in RaHOS cell culture.

RaHOS, HTLV-1 infected human osteosarcoma cell line; PCR, polymerase chain reaction; RT–PCR, reverse transcriptase–polymerase chain reaction; IFA, indirect immuno fluorescence assay; SA, syncytia assay; *gag*, *env*, and *tax*, HTLV-1 genes; LTR, long terminal repeat.

DISCUSSION

HTLV-1 preferentially targeted and transformed CD4+ cells. However, the expression of HTLV-1 receptors is not restricted to CD4+ cells only (Sagara *et al.*, 1998). Co-cultivation of HTLV-1-producing cells with a variety of human and animal non-lymphoid cell types induces cell fusion leading to the formation of large multinuclear syncytia as a result of the expression of HTLV-1 envelope antigens (Delamarre *et al.*, 1997). Only some non-lymphoid cell types have been shown to be permissive for the replication of the virus (Clapham *et al.*, 1983; Hoxie *et al.*, 1984). Cell culture HOS was infected with HTLV-1 using co-cultivation with HTLV-1-producing rabbit lymphocytes. PCR analysis for virus regulatory (*tax*, 5' LTR) and structural (*gag, env*) genes in dynamics showed integration of the viral DNA in RaHOS cells. The results of PCR have proved that integration of the virus remained stable during the observation period, for more than 150 passages of cultivation. Also, the detection of viral mRNA in the cells at different passages using the RT-PCR method indicated continuous replication of the virus in this cell culture. IFA and SA confirmed the expression of HTLV-1 antigens in the cell cytoplasm and on the cell surface. The presence of viral RNA in cell free culture liquid gave evidence of viral particle production by RaHOS cells. Thus, RT-PCR, IFA, and SA indicate that HTLV-1 infection in RaHOS cells is productive, stable, and long-term. The continuous HTLV-1 replication in non-lymphoid cells can be explained by the presence in these cells of unique or activated cellular transcription factors required for the transactivation of HTLV-1 transcription from its long terminal repeat (Newbound *et al.*, 1996).

It was also found that, after a long period of cultivation (more than 60 passages), the morphology of the obtained RaHOS cell culture had changed, comparing to the initial cell culture HOS. RaHOS had a higher saturation density of the monolayer, higher proliferative activity, enhanced formation of multilayer growth focuses and the ability to form colonies in soft agar. The cytogenetic analysis of RaHOS cell culture confirmed that the karyotype was identical to that of initial HOS cells. Therefore, the observed morphological changes apparently were the result of long-term HTLV-1 replication in these cells. The acquired phenotypic changes of the obtained cell culture indicate a change of the phenotype towards malignant transformation of the cells, previously not shown for HTLV-1 infected non-lymphoid cells. Thus, this cell culture can be a useful tool for investigation of the changes in cell genetic regulation which occur upon HTLV-1 infection. In the case of the existing HTLV-1 infected T-cell lines, the analogous non-infected clones are absent, preventing the possibility of a comparative analysis.

It is known that different lines of cultured cells vary by several orders of magnitude in their ability to take up and express exogenously added DNA (Sambrook *et al.*, 1989, pp. 16.30–16.31). The efficiency of establishing stable transfected cell lines is dependent on the efficiency of gene transfer into a given cell line, as well as on the survival of successfully transfected cells when a selective pressure is applied. Lymphocyte cell cultures are mostly transfected by using electroporation techniques. The disadvantage of this method is the high incidence level of damage to cells, which makes it difficult to obtain cell clones expressing the introduced gene. Monolayer cell culture allows using more effective and convenient transfection techniques such as Ca^{++} co-precipitation or liposome- or polycation-mediated gene delivery.

A non-lymphoid monolayer cell line, which continuously produces HTLV-1, was obtained. This cell line possesses signs of transformation and can serve as a useful system for further investigation of the HTLV-1 biology and malignant potential, as well as for examination of new antiviral agents such as antisense RNAs.

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ŠŪNU KULTŪRA KĀ MODELIS HTLV-I ANTIVIRUSĀLO ĀRSTNIECĪBAS LĪDZEKĻU PĒTĪŠANAI

Darba mērķis bija pētīt HTLV-I spēju ilgstoši replicēties cilvēka izcelsmes nelimfoīdās šūnās un iegūt ērtu monoslāņa šūnu kultūras modeli, lai pētītu antiinformācijas RNS inhibējošo ietekmi uz HTLV-I replikāciju un transformējošajām īpašībām. Cilvēka osteosarkomas (HOS) šūnu līnija tika inficēta ar HTLV-I, kokultivējot to ar truša limfocitāro šūnu līniju Ra-1. Bija parādīts, ka HTLV-I HOS šūnās izsauc stabilu un ilgstošu produktīvu infekciju. Salīdzinot ar sākotnējo HOS šūnu līniju, HTLV-I inficētajā RaHOS šūnu līnijā parādījās transformācijas pazīmes. Aprakstīto šūnu kultūru kā ērtu modeli var izmantot tālākiem antivīrusu savienojumu pētījumiem, ieskaitot antiinformācijas nukleīnskābes.

ORIGINAL PAPERS

MALIGNANT TRANSFORMATION OF HUMAN NON-LYMPHOID CELL LINE INFECTED BY HTLV-I

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ЗЛОКАЧЕСТВЕННАЯ ТРАНСФОРМАЦИЯ НЕЛИМФОИДНОЙ ЛИНИИ КЛЕТОК ЧЕЛОВЕКА, ИНФИЦИРОВАННЫХ HTLV-I

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Human T-cell lymphotropic virus type I (HTLV-I) is an etiological agent of T-cell leukemia and HTLV-I-associated myelopathy/tropical spastic paraparesis. The virus is known to target and transform preferentially $CD4^+$ cells. The aim of this work was to investigate a transforming activity of HTLV-I in non-lymphoid cell line permissive for its replication as well as to establish a convenient model for studying the antiretroviral substances. For this purpose monolayer human osteosarcoma HOS cells were infected with HTLV-I by co-cultivation with HTLV-I-producing rabbit lymphoid Ra-1 cells. Cytogenetic analysis of HTLV-I-infected HOS cell culture (RaHOS) confirmed the human karyotype identical to that of the initial HOS cells. Integration of HTLV-I provirus was detected by polymerase chain reaction (PCR) for HTLV-I *gag, env, tax* and LTR sequences. Expression of viral antigens and HTLV-I replication in RaHOS cells were confirmed by immunofluorescence assay, RT-PCR and syncytia inhibition assay. The features characteristic of malignant transformation of RaHOS cells developed in the following order: increasing proliferative activity (after 18 passages), colony-forming ability (after 30 passages), appearance of the focuses of multilayer cell growth (after 60 passages). All these features increased progressively throughout passage history of the cells. At the same time the initial HOS cells did not form colonies in soft agar and focuses of multilayer cell growth. Thus, RaHOS cells is the first characterized monolayer cell culture expressing HTLV-I in which HTLV-I transforming activity is observed. This cell line could be a suitable model for studying the changes in expression of different cell genes upon HTLV-I infection as well as the effects of various anti-retroviral compounds.
Key Words: HTLV-I, transformation, HOS cells.

Т-лимфотропный вирус I типа (HTLV-I) – этиологический агент Т-клеточного лейкоза – инфицирует и трансформирует преимущественно $CD4^+$-лимфоциты. Целью работы явилось исследование возможного трансформирующего действия HTLV-I в нелимфоидной клеточной культуре, пермиссивной для его репликации. Монослойная клеточная линия остеосаркомы человека (HOS) была инфицирована кокультивированием с HTLV-I-продуцирующими кроличьими лимфоидными клетками линии Ra-1. Цитогенетический анализ инфицированной HTLV-I культуры (RaHOS) показал, что она имеет кариотип человека, идентичный таковому в исходной культуре. Интеграция провируса HTLV-I в культуре RaHOS была выявлена с помощью полимеразной цепной реакции для последовательностей *gag, env, tax* и LTR. Экспрессия вирусных антигенов и репликация были установлены методами непрямой иммунофлуоресценции, ингибиции синцитиеобразования и с помощью обратно-транскриптазной полимеразной цепной реакции. После длительного пассирования клетки RaHOS приобрели признаки трансформированных, которые проявлялись в следующей последовательности: после 18-го пассажа повысилась их пролиферативная активность, после 30-го пассажа клетки RaHOS приобрели способность образовывать колонии в мягком агаре, после 60-го – в культуре RaHOS появились очаги многослойного роста. Все эти особенности прогрессивно нарастали в процессе дальнейшего культивирования клеток. Полученная нами культура клеток RaHOS – первая охарактеризованная монослойная культура клеток, в которой выявлена не только экспрессия HTLV-I, но и его трансформирующая активность. Клетки RaHOS имеют неинфицированный аналог и поэтому могут служить удобной моделью при исследовании изменений в экспрессии клеточных генов, обусловленных инфекцией HTLV-I, и при изучении ингибирующего эффекта соединений, обладающих противоретровирусной активностью.
Ключевые слова: HTLV-I, трансформация, клетки HOS.

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Abbreviations used: AMP — adenosine monophosphate; ATL — adult T-cell leukemia; BLV —bovine leukemia virus; Cdk — cyclin-dependent kinase; CREB/ATF — cyclic AMP response element binding protein/activating transcription factor; FCS — fetal calf serum; FITC — fluorescein isothiocyanate; GM-CSF — granulocyte-macrophage colony-stimulating factor; HAM/TSP — HTLV-I-associated myelopathy/tropical spastic paraparesis; HOS — human osteosarcoma cell line; HTLV-I — human T-lymphotropic virus type I; IFA — indirect immunofluorescence assay; PCR — polymerase chain reaction; Ra-1 — HTLV-I infected rabbit lymphocyte cell line; RaHOS — HOS cell line infected by HTLV-I; RT-PCR — reverse transcriptase-polymerase chain reaction; SIA — syncytia inhibition assay; SRF — serum response factor.

Human T-cell lymphotropic virus type I (HTLV-I) is the first characterized human exogenous retrovirus, a member of the Oncovirinae subfamily. HTLV-I is an etiologic agent of an aggressive and lethal disease — adult T-cell leukemia (ATL) and the neurodegenerative disease, HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP). HTLV-I is also associated with arthritis, uveitis, infective dermatitis, polymyositis and mild immunosupression [48, 62].

In addition to the structural *gag, pol* and *env* genes HTLV genome has the unique pX region between *env* and the 3' LTR encoding Tax, Rex, p12^{I}, p13^{II} and p30^{II} proteins [1]. Tax is a potent transcriptional activator of the HTLV LTR [51, 52]. While Tax activates the transcription of HTLV-I itself, this protein is also known as *trans*-activator of a number of cell genes such as *c-fos, c-jun, fra-1, c-myc* proto-oncogenes [11, 37, 53], immediate early serum responsive genes *egr1* and *egr2* [12], genes encoding growth factors (IL-2, IL-6, TNFα, TNFβ, GM-CSF, TGF-β1) [2, 7, 16, 23, 57, 60] as well as the gene of α-chain of IL-2 receptor [33]. Tax exerts pleiotropic effects on virus-infected cells by interacting directly with key transcription factors including the cyclic AMP response element binding protein (CREB/ATF), serum response factor (SRF), and the components of the NF-κB/rel signalling pathway [27, 40, 46, 61].

Tax deregulates the normal cell cycle control through protein-protein interaction, for example Tax binds specifically with cyclin-dependent kinase (cdk) inhibitor p16$^{INK\,4A}$ and *trans*-activates the promoter of another cdk inhibitor, p21$^{waf1/cip1}$, which is overexpressed in HTLV-I infected T cells [3, 32]. Apoptosis is also known to be affected by Tax [31, 36]. This protein is thought to be responsible for the transforming features of HTLV-I. Tax has been shown to immortalize T lymphocytes and to transform rodent fibroblasts *in vitro* [13, 22, 59]. Transgenic mice expressing Tax develop leukemia and mesenchymal tumors [14, 38].

HTLV-I preferentially infects and transforms CD4$^+$ cells [6, 8, 62]. However, expression of HTLV-I receptors is not restricted to CD4$^+$ T cells. HTLV-I can also infect other blood mononuclear cells including CD8$^+$ cells, B cells and monocytes [10, 17, 25, 30]. Many non-lymphoid cell types derived from human and various animal species also turned out to be permissive for HTLV-I adsorption and penetration *in vitro* [15, 26, 49]. However, HTLV-I was incapable to replicate continuously in these cells. Only non-lymphoid human osteosarcoma HOS cells and human endothelial cells were permissive for HTLV-I replication [5, 18].

Although HTLV-I Tax protein involvement in cell transformation is well known, precise mechanisms of HTLV-I-related leukemogenesis as well as the role HTLV-I in the pathogenesis of HAM/TSP is far from being elucidated. It is not yet known whether HTLV-I could readily transform the cells of non-lymphoid origin. Therefore, the analysis of HTLV-I transforming activity in the different types of cells is important to fully understand the mechanisms of the malignant transformation. With this aim in view HTLV-I-producing non-lymphoid cell line RaHOS has been initiated by co-cultivation of HOS cells with HTLV-I-producing culture of rabbit lymphocytes (Ra-1 cells). Upon long-term passage history HTLV-I-infected RaHOS cells became transformed. The model obtained seems to be useful for studying the mechanisms of HTLV-I-related transformation as well as for *in vitro* screening of anti-HTLV-I substances, in particular, complementary addressed polynucleotides. At the same time as far as RaHOS has its non-infected counterpart — HOS, it allows us to follow up the changes in expression of cell genes in the HTLV-I-infected culture.

MATERIALS AND METHODS

Cell cultures. The following cell lines were used in the study: Ra-1 — HTLV-I Japanese strain producing lymphoid rabbit cell line; HOS TE85 — human Caucasian osteogenic sarcoma, monolayer epithelioid cell line; HeLa — human cervical carcinoma monolayer epithelioid cell line.

Ra-1 cells were cultured in suspension in 35 mm Petri dishes (3 ml per dish, 1 · 10^6 cells/ml) and reseeded twice a week. Cells were grown in RPMI-1640 medium (Gibco BRL, USA) supplemented with 15% heat inactivated fetal calf serum (FCS), 300 μg/ml L-glutamine and 50 μg/ml gentamycin and incubated at 37°C in 5% CO_2. HOS and HeLa cells were maintained in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% heat inactivated FCS, 300 μg/ml L-glutamine and 50 μg/ml gentamycin. The cells were reseeded 2–3 times a week at 2 · 10^5 cell/ml using trypsine-EDTA mixture.

HTLV-I infection of HOS cells. HTLV-I-infected HOS cell line was established by co-cultivating HOS cells with HTLV-I-producing Ra-1 cells (1.5 · 10^{5}cells/ml), which were treated by mitomycin C (5 μg/ml) for 1 h at 37°C. The virus-infected HOS subline was designated as RaHOS.

Detection of HTLV-I provirus integration by PCR. Total DNA was isolated from up to 3 · 10^6 cells by proteinase K digestion followed by standard phenol/chloroform extraction [47]. Quality of DNA was assessed by PCR using primers for human β-globin gene [54] and only samples positive in this assay were further processed.

1 μg of DNA was analyzed for the presence of HTLV-I provirus by nested PCR using primers targeted to HTLV-I *gag, env, tax* genes and 5' LTR region. The primer sequences and PCR conditions were described previously [28, 29, 35, 55] and are shown in Table. DNA from the HTLV-I positive cell line Ra-1 was used as the positive control and DNA from the HTLV-I free cell line HOS as the negative control. The products of DNA amplification were analyzed by electrophoresis on 1.9% agarose gel followed by ethidium bromide staining and visualization in UV light for the presence of DNA bands of appropriate sizes.

Detection of HTLV-I expression by RT-PCR. Total RNA was extracted using "RNeasy Total RNA Kit" (Qiagen) from 3 · 10^6 cells and from virus containing culture media of RaHOS cells 1,000 fold concentrated by ultracentrifugation. The samples were then treated with DNase I (Sigma, USA) and subjected to RT-PCR. Reverse transcription was performed in 20 μl volume according to the manufacturer protocol (MBI Fermentas, Lithuania). Reaction mixture contained RT buffer (50 mM Tris-HCl pH 8.3 at 25°C, 50 mM KCl, 4 mM DTT), 200 ng of RNA, random hexamer primers (1 μM),

ribonuclease inhibitor (1U/μl) and M-MuLV (Moloney murine leukemia virus) reverse transcriptase (1U/μl). After reverse transcription at 37°C for 1 h followed by 5 min at 99°C and 5 min at 5°C the samples were subjected to amplification with the primers for HTLV-I *gag* and *tax* genes. All PCR and RT-PCR reagents were from MBI Fermentas (Lithuania). The primer sequences and PCR conditions used are shown in Table.

Indirect immunofluorescence assay (IFA). Cells growing on glass coverslips were fixed with methanol at — 20°C for 20 min, air-dried and then incubated at 37°C for 40 min with the primary antibodies (two sera of Japanese ATL patients) diluted in phosphate buffered saline (PBS). Two HTLV-I negative sera from normal persons were used as the negative control. After incubation the cells were washed with cold PBS four times for 15 min and incubated with the secondary antibody (goat anti-human FITC labeled IgG) diluted in PBS. The secondary antibody was then removed by washing as described above.

Syncytia inhibition assay (SIA). The ability of the RaHOS cells to mediate the formation of multinuclear giant cells — syncytia, was assessed by co-cultivation of RaHOS cells with HeLa indicator cells. HeLa cells were seeded at a density $5 \cdot 10^4$ cell/ml into 35 mm Petri dish in DMEM with 5% FCS. After over-night incubation at 37°C in 5% CO_2 HeLa cells were co-cultivated with $5 \cdot 10^3$ RaHOS cells which were pretreated by mitomycin C (5 μg/ml) in DMEM + 10% FCS for 2 days. Then the cells were washed twice with PBS, fixed in methanol for 15 min and stained by the Romanowsky — Giemsa technique. Syncytia were counted per 5,000 cells under the light microscope at x 20 magnification. Only multinuclear cells containing more than 5 nuclei were scored. Inhibition of syncytia formation by anti-HTLV-I serum was done using sera from two Japanese patients with ATL. Two sera from healthy blood donors were used as a negative control.

Proliferative activity assay. Proliferative activity of HOS and RaHOS cells was estimated by incorporation of ^{3}H-thymidine in DNA. Cells were seeded in 24-well plates at a concentration $1 \cdot 10^5$ cells per well. After 24, 48, 72 and 96 h of cultivation ^{3}H-thymidine (25 Ci/mmol, Amersham, England) was added (2 μCi/well) and cells were incubated for 1 h at 37°C, 5% CO_2. Then cells were collected using trypsine-EDTA and transferred to the Millipore filters (diameter of pores — 1.5 μm). The filters were washed twice with PBS and 3 times with 5 ml of 5% trichloroacetic acid to precipitate DNA. The DNA was fixed by 1 ml of 96% ethanol and air dried at 37°C. The incorporation of ^{3}H-thymidine was measured in Packard liquid scintillation counter.

Soft agar assay. 1,000 viable single cells were suspended in 1 ml DMEM medium containing 0.3% Noble agar (Difco, USA) and 15% FCS and were placed in 6-well plates over a base layer of DMEM medium, containing 0.6% Noble agar and 15% FCS. Cells were incubated at 37°C in the humid box with 5% CO_2 for two weeks. Cultures were fed every 3 days, and colonies larger than 0.1 mm were counted.

Cytogenetic analysis. After 48h of cultivation 1 μg/ml of colchicine (Sigma, USA) was added to culture liquid of RaHOS and HOS cells. The cells were incubated with colchicine at 37°C in 5% CO_2 incubator for 30 min. Then the cells were treated with trypsin-EDTA, suspended in DMEM with 5% FCS, sedimented by centrifugation and hypotonized with 1% sodium citrate for 30 min at 37°C. The cells were fixed with a mixture of methanol and glacial acetic acid (3 : 1), dripped onto slides and stained with Giemsa (azure-eosin-methylene blue in PBS).

Table. Primer sequences and PCR conditions used for HTLV-I detection

Target region	Code	Position	Sequence (5'-3')	Conditions	Product length (bp)	Reference
tax	AV45	7501-7517	GGACGCGTT(A/G)TC(A/G)GCTC	94°C- 30s 50°C- 15s 72°C- 45s } x 45		[55]
	AV46	7803-7783	(G/T)GG(A/G)GAIAG(C/T)TGGTA(G/T)AGGTA		100	
	AV49	7626-7647	CCCTCCTTCCTCCAGGCCAT	95°C- 30s 55°C- 30s 72°C- 45s } x 40		
	AV80	7725-7702	GGTCTGGAAAAGACAGGGTTG			
gag	gagM2	1313-1337	TCCGGCTTGCGGTGCAGCAGTTTGA	94°C- 1min 60°C- 1min 72°C- 2min } x 30		[35]
	gagM3	1543-1519	GCCAGAGTTGCTGGTATTCTCGCCT		150	
	gagM4	1351-1375	GACCTCCAAGACCTCCTGCAGTACC	94°C- 1min 60°C- 1min 72°C- 1min } x 35		
	gagM5	1500-1476	ATTGTTGGCTTGGACACGGAGGGGA			
5'LTR	HFL9	124-144	AAGGCTCTGACGTCTCCCCCC	95°C- 30s 55°C- 30s 72°C- 45s } x 30		[29]
	HFL6	901-881	GTTAAGCCAGTGATGAGCGGC		595	
	HFL5	202-220	TCATAAGCTCAGACCTCCG	95°C- 30s 55°C- 30s 72°C- 45s } x 30		
	HFL10	796-779	TCCCGGACGAGCCCCCAA			
env	env3	5142-5160	CTGCATGCCCAAGACCCGT	95°C- 30s 55°C- 30s 72°C- 45s } x 30		[28]
	env4	6707-6689	GGCTGGAGGCGATGTGGTT		562	
	env7	6048-6067	GCTCCGTCAGCTACGACACC	95°C- 30s 55°C- 30s 72°C- 45s } x 30		
	env8	6609-6590	CCTCCGTCAGCTACGACACC			

Experimental animals. 5 to 6 weeks–old female nude mice of BALB/c background were obtained from animal facility of August Kirchenstein Institute of Microbiology and Virology. The mice were kept at 24 ± 2°C in filtered laminar air, and supplied with sterile food, water and bedding.

Tumorigenicity of RaHOS cells in nude mice. Tumorigenicity of HOS and RaHOS cells was tested by inoculating $1 \cdot 10^6$–10^7 viable cells in 0.2 ml PBS to each mouse subcutaneously into a subscapular area. Cell viability was determined by trypan blue exclusion and phase–contrast microscopy. The animals were observed for 2 months. Each group included 3 mice. All experiments were performed according to the approval of the Ethics Committee of the Latvian Council of Science on investigations using laboratory animals [24].

RESULTS

The presence of HTLV–I proviral DNA in RaHOS cells. The presence of HTLV–I proviral DNA sequence in the RaHOS cell line was analyzed by PCR. Using a set of HTLV/STLV generic outer and HTLV–I specific inner primers for *tax* gene, DNA samples extracted from RaHOS cells were clearly positive for HTLV–I sequence (Fig. 1, *b*). The HTLV–I infection was further confirmed by the presence of *gag*, *env* genes and 5' LTR region of the viral genome (Fig. 1, *a*, *c*, *d*). The results were reproducible in at least three independent experiments.

Productive infection of HTLV–I in RaHOS cells. We employed RT–PCR analysis with HTLV–I specific primers complementary to the *gag* and *tax* genes to determine whether the cell line is persistently producing the virus or is latently infected. To confirm the specificity of the amplification product and to exclude contamination with genomic DNA, simultaneous reactions were performed in the absence of RT (Fig. 2). The expression of *gag* and *tax* genes in RaHOS cells was detected throughout all the observation period (20–110 passages) (Fig. 2). The results were reproducible in RT–PCR analysis using another primer set derived from pX region (data not shown).

The replication of HTLV–I in RaHOS cells was monitored by the presence of the HTLV–I virions in RaHOS culture medium. HTLV–I *gag* and *tax* specific amplification products in RT–PCR were detected using RNA from the cell–free RaHOS supernatant (Fig. 2). These results were reproducible 20 through 110 passages.

Presence of HTLV–I antigens. HTLV–I antigens were estimated by IFA. Bright fluorescent regions in cytoplasm and diffuse fluorescence around the nucleus were detected in 75–80% of RaHOS cells at passage 65, with HTLV–I positive sera (Fig. 3, *a*). At the same time fluorescence was observed neither in HOS cells incubated with HTLV–I positive sera nor in RaHOS cells incubated with HTLV–I negative sera (Fig. 3, *b*). The specific fluorescence in the cytoplasm of RaHOS cells was observed throughout all the period of observation (150 passages). However, the number of fluorescence positive RaHOS cells decreased with increasing number of passages, and at passage 150 the fluorescence was observed only in 5% of cells.

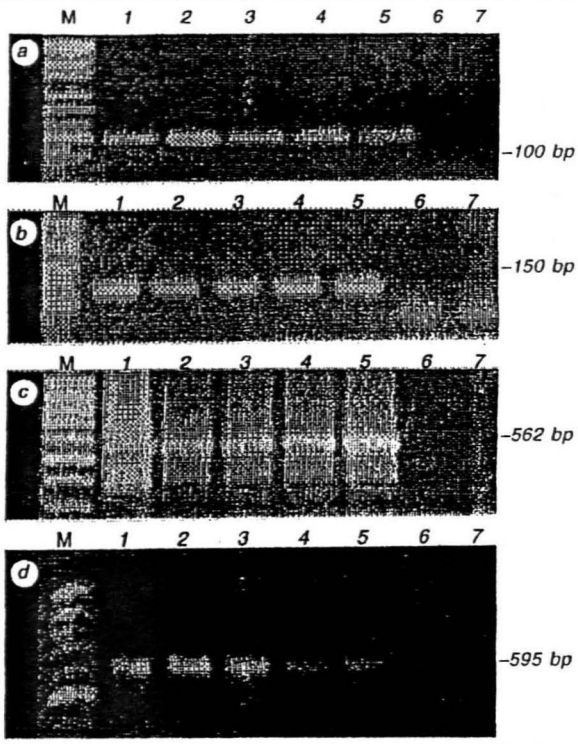

Fig. 1. Detection of HTLV–I proviral sequences in DNA of RaHOS cells. DNA was amplified with primers complementary to the regions of *tax* gene (*a*); *gag* gene (*b*); *env* gene (*c*); 5'LTR (*d*). M — markers: pUC19 DNA/MspI (*a*, *b*), MassRuler DNA Ladder, Low range (*c*, *d*); *1* — DNA extracted from HTLV–I producing Ra–1 cells line (positive control); *2–5* — DNA extracted from RaHOS cells after 45, 57, 77, 130 passages, respectively; *6* — DNA extracted from HOS cells (negative control); *7* — control without DNA. The size of the amplification product (bp) is indicated on the right side

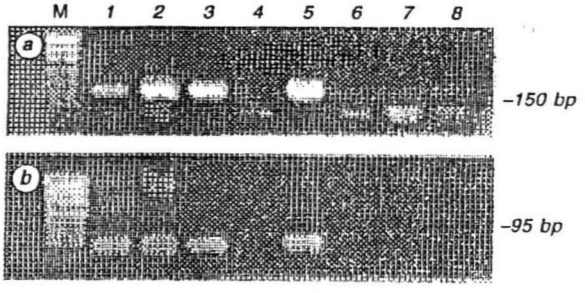

Fig. 2. Expression of HTLV–I *gag* (*a*) and *tax* (*b*) sequences in RaHOS cells (passage 110) and RaHOS cell culture supernatant (passages 106–110). M — markers: pUC19 DNA/MspI; *1* — DNA extracted from HTLV–I–producing Ra–1 cells (positive control); *2* — RT–PCR: RNA from HTLV–I–producing Ra–1 cells; *3* — RT–PCR: RNA isolated from RaHOS cells after 110 passages; *4* — RNA from RaHOS cells, PCR without RT; *5* — RT–PCR: RNA extracted from RaHOS cell culture supernatant; *6* — RT–PCR: RNA extracted from HOS cells (negative control); *7* — RNA extracted from HOS cells, PCR without RT; *8* — control without RNA. The size of the amplified product (bp) is indicated on the right side

Syncytia inhibition assay (SIA). The replication of HTLV–I in RaHOS cells was confirmed also by SIA. While the amount of multinuclear cells in control HeLa cells did not exceed 0.6% with no more than 5 nuclei, upon co–cultivation of RaHOS cells with HeLa cells syncytia

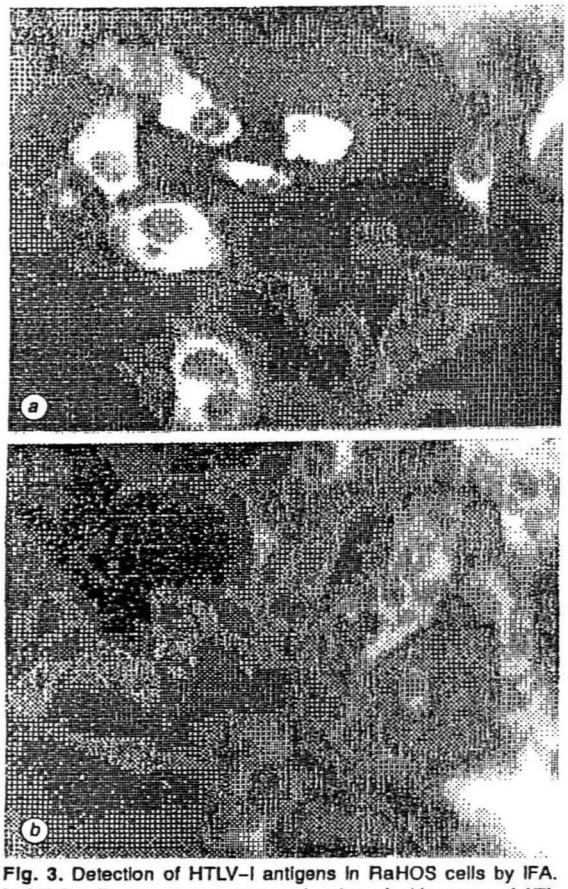

Fig. 3. Detection of HTLV-I antigens in RaHOS cells by IFA. RaHOS cells at passage 65 were incubated with serum of ATL patient (*a*) or with HTLV-I negative serum (*b*). Fixation and staining were performed as described in Materials and Methods, magnification x 600

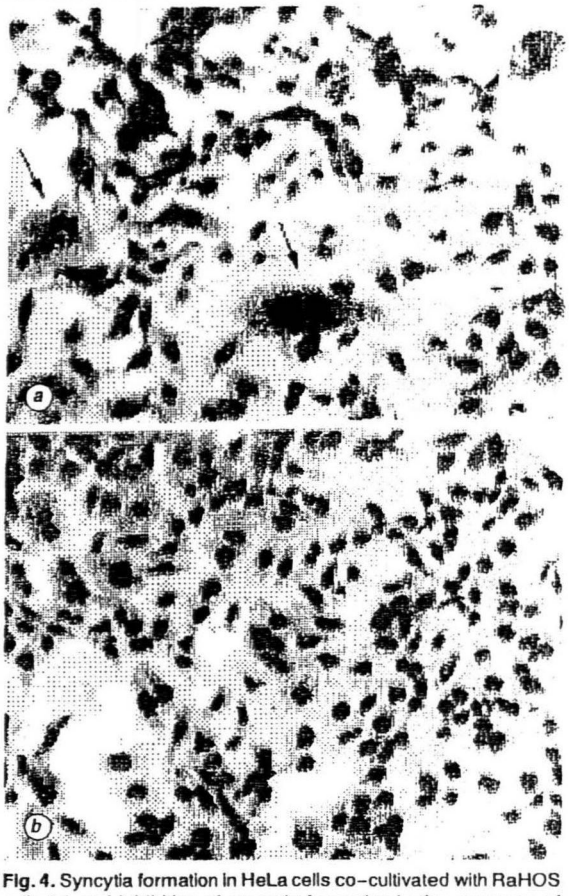

Fig. 4. Syncytia formation in HeLa cells co-cultivated with RaHOS cells (*a*) and inhibition of syncytia formation in the presence of serum of ATL patient (*b*). The multinuclear cells are marked by arrows. SIA was performed as indicated in Materials and Methods

containing 5–15 nuclei appeared (Fig. 4, *a*) and the total amount of syncytia increased up to 1.5–4%. The formation of syncytia was inhibited in the presence of anti-HTLV-I antibodies (Fig. 4, *b*) while in the presence of HTLV-I negative sera the syncytia inhibition was not observed.

Morphology, cytogenetic and transforming features of RaHOS cell line. After co-cultivation of initial HOS cell culture with Ra-1 cells the syncytia containing 5–12 nuclei have been registered. Their amount increased for the next 6 passages of RaHOS cells with following decrease. By passage 14 only single syncytia remained. Sometimes the single multinuclear cells were observed during the rest of cultivation of RaHOS cells. After 60 passages the focuses of multilayer growth had appeared in RaHOS cells (Fig. 5).

Cytogenetic analysis of RaHOS cells showed human karyotype with the modal number of chromosomes identical to those in HOS cells — 48. However, at passage 125 the polyploidy percentage in RaHOS cells was slightly elevated in comparison with HOS cells (5.4 and 4.6% respectively).

The proliferative activity of RaHOS cells differed from that in the initial HOS cells. After 18 passages the incorporation of ^{3}H-thymidine in RaHOS cells was 1.4 times higher and after 135 passages — twice higher

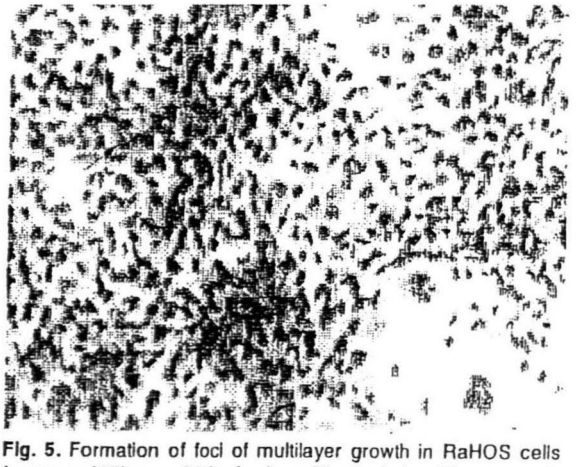

Fig. 5. Formation of foci of multilayer growth in RaHOS cells (passage 105) upon 72 h of culture. The foci of multilayer growth are marked by arrows

than in HOS cells (Fig. 6). Moreover, it is interesting to note that the maximal ^{3}H-thymidine incorporation into the HOS cells was observed at 48–72 h decreasing by 96 h. The incorporation of ^{3}H-thymidine in RaHOS cells at 96 h remained high.

After 30 passages RaHOS cells had got the ability to form colonies in soft agar. 0.1% of the cells gave

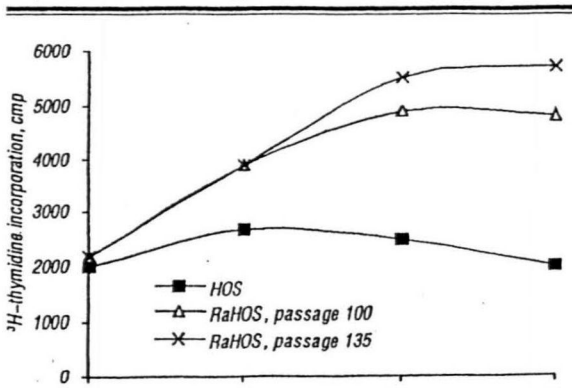

Fig. 6. Proliferative activity of RaHOS and HOS cells. Proliferative activity assay was performed as described in Materials and Methods

growth in colonies of about 0.1 mm in diameter. The colony-forming capacity increased during further passages (0.4 and 1.3% of the cells respectively, after 60 and 150 passages, with 0.15–0.20 mm colony size in diameter). The initial cell culture HOS TE85 used in the study as the control neither formed the colonies in soft agar nor developed tumors in nude mice. Meanwhile, RaHOS cells also were not tumorigenic in nude mice.

DISCUSSION

It is clearly demonstrated that HTLV-I Tax protein could transform both lymphoid cells and the cells of non-lymphoid origin [34]. At the same time in transgenic mice expressing the *tax* gene mesenchymal tumors and leukemia develop [14, 38]. In patients with HTLV-I associated diseases virus replicates preferentially in CD4$^+$ T cells; HTLV-I could also infect and transform CD4$^+$ cells *in vitro* [6, 8, 62]. Up to the present non-lymphoid cells were not known as the possible targets for HTLV-I transformation. It could be explained by an inability of HTLV-I to replicate continuously in such cell cultures.

HOS cells are known to be permissive for HTLV-I replication [5] . On the other hand, HOS cells could be transformed by nitrosoguanidine or Kirsten virus [42, 43]. Therefore, we could expect that HTLV-I would be capable to transform them.

It is known that the free viral particles of HTLV-BLV viruses are poorly infectious and viral transmission occurs almost exclusively via cell-to-cell contacts. The capacity of the virus to mediate such a cell-to-cell transmission is known to correlate with the syncytia-forming ability of the virus [9, 45]. So we infected HOS TE85 cells co-cultivating them with HTLV-I producing rabbit Ra-1 lymphoid cells. HTLV-I-infected HOS cell culture designated as RaHOS maintained human karyotype. The numerous syncytia in RaHOS cells were observed through the first six passages after co-cultivation. As the cell fusion is mediated by HTLV-I envelope glycoproteins [9, 45] this could be indicative of HTLV-I expression in RaHOS cells. The integration of HTLV-I provirus and expression of viral antigens as well as HTLV-I replication were confirmed by PCR, IFA, RT-PCR and SIA. In contrast to HOS TE85 cells RaHOS cells were changing their morphology throughout their passage history.

The features characteristic of malignant transformation of RaHOS cells developed in the following order: increasing proliferative activity (after 18 passages), colony-forming ability (after 30 passages), appearance of the focuses of multilayer cell growth (after 60 passages). Meanwhile, RaHOS cells were incapable to form tumors in nude mice. We do not know whether this could be attributable to insufficient tumorigenic potential of RaHOS cells or whether the transplanted cells were rejected by NK cells of the host. Such a rejection holds true in case of HTLV-I-transformed human T-cell line MT-2 [21, 41]. Imada *et al.* have shown that only leukemic non-producing HTLV-I-infected cell clones and fresh cells from ATL patients were capable to proliferate in SCID mice (CB17 scid/scid), while HTLV-I-infected lymphoid cell lines of non-leukemic origin could not be engrafted in SCID mice [19, 20]. However, HTLV-I-transformed human T-cell line (MT-2) was successfully grafted in SCID mice treated with anti-asialo GM-1 antibody [21], while according to the data of Inodi *et al.* [19] MT-2 cells without such a preliminary treatment could not be engrafted. Thus, it is possible that the ability of HTLV-I-transformed cells to produce virus switches on the defense mechanisms remaining in immunodeficient mice sufficient to reject the foreign cells.

In 1983 Chen *et al.* and Sodroski *et al.* [4, 50] had made the construct of HTLV-I and HTLV-II LTR with reporter gene and found that reporter gene expression was restricted to lymphoid cells. They suggested that *trans*-acting regulatory protein expressed only in lymphoid cells may act directly on LTR to enhance the expression. Authors also suggested that a product of the HTLV X gene itself acts as the *trans*-regulatory protein on LTR function [50, 58]. Later it had been shown that activation of HTLV-I gene expression involves direct interaction of Tax with the CREB/ATF family of cellular transcription factors leading to the enhanced dimerization and subsequent binding of these factors to 21-bp repeats in the HTLV-I LTR [56, 63]. Newbound *et al.* [39] demonstrated that cell tropism of HTLV-I is determined by the expression levels or activation states of Tax-responsive cellular transcription factors binding with HTLV-I LTR, the rate of viral transcription and protein production in HTLV-I-infected primary CD4$^+$ cells being higher than in primary CD8$^+$ cells. This increase was most notably observed in the presence of the viral *trans*-activating Tax protein. These data suggested that unique or activated transcription factors, particularly Tax-responsive factors in CD4$^+$ T cells, recognised regulatory sequences within HTLV-I LTR mediating the enhanced viral transcription in these cells [39]. It could be supposed that the differences in levels or activation states of transcription factors between various cell lines and various types of cells could determine the permissiveness to HTLV-I infection. Long-term replication of HTLV-I in RaHOS cells suggest that initial HOS cells possessed transcriptional factors required for HTLV-I LTR activation. At the same time long-term Tax action could result in the shortage of cell transcriptional factors interacting with Tax or even to the negative selection of Tax-expressing cells [31, 44] which seemed to be a

probable explanation of the decrease in the number of cells expressing HTLV-I antigens upon long-term passage history of RaHOS cells.

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