HTLV or induced by HTLV infection, substantially augment gene expression directed by HTLV LTR sequences. The phenomenon of *trans*-activation distinguishes HTLV from other retroviruses. The unusual structure of the 3′ terminus of HTLV also distinguishes these from most other retroviruses. For this reason, we suggest that the protein encoded by the LOR region may mediate transcriptional changes observed in HTLV-infected cells. In this regard, we note that transcription directed by the HTLV-I LTR is activated to high levels in a cell line, C81-66, that expresses the 42,000-dalton HTLV-I–associated protein but not HTLV *gag*, *pol*, or *env* products (*8*). We further suggest that the HTLV LOR product mediates both the *trans*-activating and transforming effects of HTLV infection. We note that *trans*-acting transcriptional activities have been associated with the transforming genes of other tumor viruses, notably adenovirus and SV40 (*10, 11*). The existence of a potential transforming function within the HTLV genome may explain the ability of the virus to transform cells in vitro, as well as the absence of specific integration sites in tumor cells and the absence of chronic viremia in target tissues (*12–14*). Such a transforming function would differ from that of other retroviruses because, unlike the oncogenes, the sequence that encodes the putative transforming gene will not anneal to the highly conserved cellular sequences (*4*).

Comparison with the bovine leukemia virus genome. We noticed that the 3′ genome of another retrovirus, bovine leukemia virus (BLV), also contains an LOR frame located 3′ to the envelope glycoprotein gene that could encode a protein of a size similar to that of HTLV (*15, 16*) (Fig. 2). There is evidence for the existence of a subgenomic spliced mRNA species that contains the 3′ open reading frame but not the *gag*, *pol*, and *env* gene sequences in BLV-producing cell lines (*17*).

Although the similarity in structure of the HTLV and BLV proteins is insufficient to indicate that they have a common functional role, the overall similarity in genomic structure, including the location of a 5′ NCR and 3′ LOR frame, and the previously described similarity in protein antigenicity of the two viruses (*1, 14*) suggests that they are functionally similar. Moreover, there is a similarity in the distribution of hydrophobic and hydrophilic regions of the HTLV and BLV polypeptides. We note that the disease induced by BLV has characteristics similar to those associated with HTLV-I, namely, a long latent period sometimes preceded by persistent lymphocytosis, an absence of chronic viremia in target organs preceding disease, and an absence of preferred integration sites in tumor cells (*18*). These features could be expected of viruses that contain an LOR product mediating transformation.

The biology, structure, and pathology of HTLV and BLV differ from other transforming retroviruses such that we propose that they be considered a new subgroup of retroviruses distinct from both the nonacute transforming viruses that contain only the *gag*, *pol*, and *env* genes and the acute transforming viruses that encode oncogenes.

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Sequence of the Envelope Glycoprotein Gene of Type II Human T Lymphotropic Virus

Abstract. *The sequence of the envelope glycoprotein gene of type II human T lymphotropic virus (HTLV) is presented. The predicted amino acid sequence is similar to that of the corresponding protein of HTLV type I, in that the proteins share the same amino acids at 336 of 488 residues, and 68 of the 152 differences are of a conservative nature. The overall structural similarity of these proteins provides an explanation for the antigenic cross-reactivity observed among diverse members of the HTLV retrovirus family by procedures that assay for the viral envelope glycoprotein, for example, membrane immunofluorescence.*

Human T-cell leukemia viruses have been implicated as the etiological agents of several human diseases. The most prevalent type, HTLV-I, is associated with a high incidence of an aggressive form of adult T-cell leukemia (ATLL) and several unusual forms of mycosis fungoides and Sezary syndrome (*1*). A second member of the family, HTLV-II, has been isolated from a patient with benign hairy cell leukemia of T-cell origin (*2*). Recently, a new group of viruses, HTLV-III, was isolated from patients with acquired immune deficiency syndrome (AIDS) (*3*).

The envelope glycoprotein is the major antigen recognized by the serum of persons infected with HTLV (*4*). In this respect HTLV resembles several other retroviruses for which the envelope glycoprotein is typically the most antigenic viral polypeptide (*5*). Moreover, most

neutralizing antibodies are directed toward the envelope glycoproteins of retroviruses (*5, 6*).

The envelope glycoproteins of HTLV-I, HTLV-II, and HTLV-III have common antigenic determinants. Serum samples from patients infected with HTLV-II recognize the envelope glycoproteins of HTLV-I (*8*). Samples from AIDS patients, some of whom are known to be infected with HTLV-III, also frequently immunoprecipitate the envelope glycoproteins of HTLV-I and -II (*4, 7, 8*). These antigenic cross-reactions motivated us to a study of the HTLV-II envelope glycoprotein gene. Here we present the complete nucleotide sequence of this gene (Fig. 1).

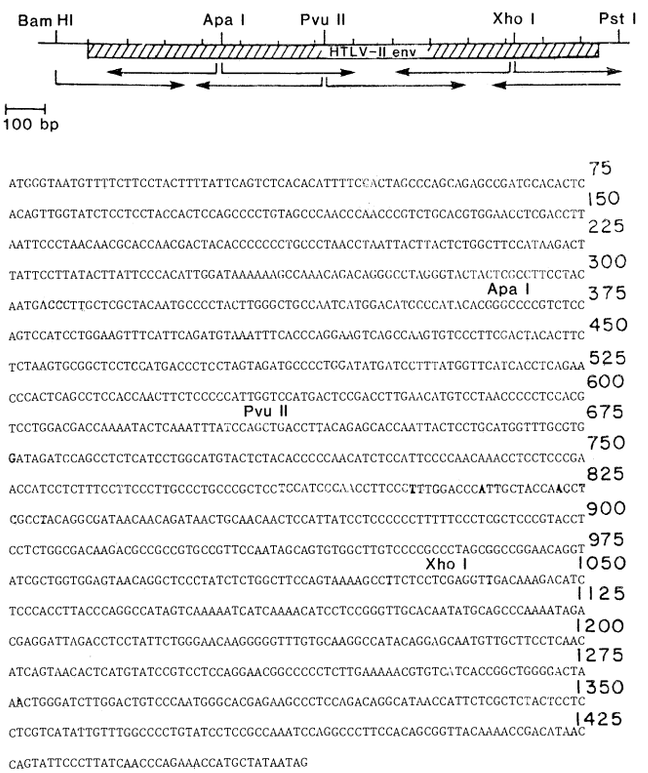

Fig. 1. Nucleotide sequence of the HTLV-II *env* gene. The strategy for sequencing the MO15A (*10*) *env* gene is shown in the top figure. Sites depicted were 5′ and 3′ end-labeled and sequenced according to the procedure of Maxam and Gilbert (*11*). The lower figure depicts the nucleotide sequence of the entire envelope gene with restriction endonuclease sites denoted above the sequence line.

The *env* gene sequence contains an open reading frame 484 amino acids long. The predicted amino acid sequence of the *env* glycoprotein precursor protein is shown in Fig. 2. The most direct evidence that the predicted sequence is the *env* gene is derived from the partial amino acid sequence of the amino terminal regions of the 67,000, 52,000, and 21,000 dalton glycoproteins (gp67, gp52, and gp21) seen in immunoprecipitates of HTLV-II–infected cells (*8*). These proteins represent the fully glycosylated envelope precursor, the processed exterior glycoprotein, and the transmembrane glycoprotein, respectively (*4, 8, 9*). The location of the cysteine and serine residues of the gp67 and gp52, and of the valine residues of gp21 relative to the amino termini, has been determined (*8*). There is an exact correspondence of the residues with the amino acids if one assumes that the amino terminus of the mature exterior glycoprotein is located at amino acid 21 in the precursor protein, and that the amino terminus of the transmembrane protein is located at amino acid 309. The molecular weight of the unglycosylated precursor protein, 53,000, is close to that predicted by this sequence (53,856) (*9*).

The exterior glycoprotein. A number of structural features of the *env* gene products can be deduced from the predicted primary amino acid sequence. Useful for such analysis is computation of the hydrophobic and hydrophilic properties of subdomains of the protein. Such a display is presented in Fig. 3. Potential glycosylation sites and location of cysteine residues are also indicated.

The first 20 amino acids of the postulated *env* precursor of HTLV II are hydrophobic. This region probably constitutes the leader sequence. The partial amino acid sequence of gp67 and gp52 indicates that the first 20 amino acids are cleaved to generate the mature protein.

Amino acids 21 to 309 probably constitute the exterior glycoprotein. This is sufficient to encode a protein of molecular weight 33,000, the size of the unglycosylated version of gp52 (*9*). This region contains four potential glycosylation sites. Overall the protein is hydrophobic. The hydrophilic regions of this protein are small, one located near the amino terminus of the protein (residues 85 to 93) and one located at the extreme car-

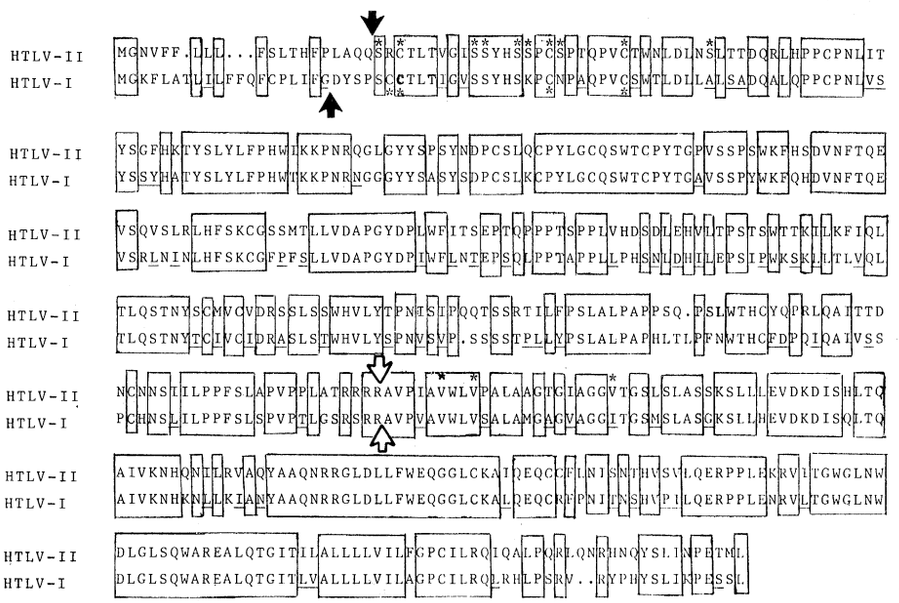

Fig. 2. Amino acid sequence of the HTLV-II and HTLV-I envelope proteins as deduced from the nucleic acid sequence. The HTLV-I sequence is derived from Seiki *et al.* (*12*). Identical amino acids between the two envelope proteins are boxed. Conservative amino acid changes are underlined. Dark arrows indicate the site where the leader sequence is cleaved from the mature envelope glycoprotein. The position of this site has been verified by protein sequencing of envelope proteins labeled with cysteine and serine (for HTLV-II) and with cysteine (for HTLV-I) (*4, 8*). The positions of these labeled residues are indicated by asterisks. The open arrows indicate the point of cleavage of the exterior glycoprotein from the transmembrane protein. The position of this site has also been confirmed by protein sequencing the transmembrane protein after radioactive labeling with valine (shown by asterisks) (*8*). Dots indicate gaps in the sequence placed to maximally align the type I and type II envelope sequences.

boxyl terminus of the protein. Two other short hydrophilic regions are located in the center of the protein. The solubility of the exterior glycoprotein in aqueous medium must depend in large measure on glycosylation.

The transmembrane protein. The transmembrane protein begins at residue 309 and is 178 amino acids long, sufficient to encode a polypeptide of molecular weight 19,500. The sequence contains one potential glycosylation site.

The transmembrane protein contains two long hydrophobic regions, one located near the amino terminus, 26 amino acids long, and a second located at the carboxyl terminus, 27 amino acids long. There are three cysteine residues located near the potential glycosylation site. The extreme carboxyl terminal domain of the protein is hydrophilic.

Comparison of the sequence of HTLV-I and -II. The structure of the envelope glycoproteins of HTLV-I and -II deduced from the predicted amino acid sequence is very similar (Figs. 2 and 3). The envelope precursor proteins are 488 and 486 amino acids long, respectively. The hydrophobic leader sequences are both 20 amino acids long; the exterior glycoproteins are 292 and 288 amino acids long; and the transmembrane proteins are 176 and 178 amino acids long. The proteins share the same amino acids at 336 of the 488 positions, and 68 of the 152 differences represent conservative changes in protein sequence. The greatest divergence in protein sequence occurs at the extreme amino terminal and carboxyl terminal regions in the *env* gene precursor, both regions that may be absent from the mature protein. The transmembrane protein is more highly conserved—85 percent similarity—than is the exterior glycoprotein—65 percent similarity. The overall similarity in protein sequences is also reflected at the DNA level, with 50 percent of the nucleotides being identical, although the regions encoding the extreme amino and carboxyl termini show little or no similarity in DNA sequence.

The distribution of hydrophobic and hydrophilic regions is strikingly similar for HTLV-I and HTLV-II *env* gene products (Fig. 3). Sites of three of the four potential glycosylations of the exterior glycoprotein are shared. The location of the fourth potential glycosylation site near the carboxyl terminus differs in HTLV-I and -II. The location of the single glycosylation site in the transmembrane protein is the same for HTLV-I and -II. The location of the cysteine residues is also conserved throughout the length of the envelope precursors of HTLV-I and -II.

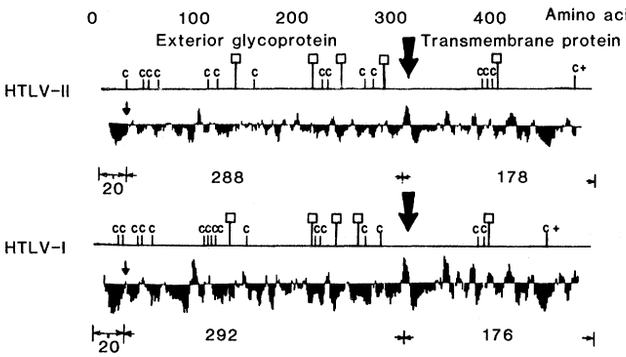

Fig. 3. Schematic representation of the HTLV-II and HTLV-I envelope proteins based on the predicted amino acid sequence. Numbers on top represent amino acid residues from the amino terminus of the complete envelope precursor. The positions of the cysteine residues (c) and potential glycosylation sites (□) are shown. Large arrows above the line drawings depict the site of cleavage of the exterior glycoprotein from the transmembrane protein. The + notes the position of a positively charged amino acid that is positioned on the inner side of the host cell membrane. The small arrowheads underneath the line drawings depict the site of cleavage of the hydrophobic leader sequence from the exterior glycoprotein. The hydrophilic (up) and hydrophobic (down) character of the envelope proteins as determined by the method of Hopp and Woods (*13*) is shown underneath the line drawings. Note the hydrophobic nature of both the leader sequence at the amino terminus and the transmembrane region near the carboxyl terminus of the protein. Also note the alternating hydrophobic and hydrophilic character of the region surrounding the cleavage site between exterior and transmembrane proteins. Numbers beneath each figure represent the lengths of the leader sequence, the mature exterior glycoprotein, and the transmembrane protein, respectively.

The location of several cysteine residues is well conserved among this family of proteins. Especially well conserved are cysteine residues spaced eight amino acids apart in the central domain of the transmembrane protein of all the retroviruses, and cysteine residues spaced four amino acids apart that are located near one of the three conserved potential glycosylation sites (the conserved glycosylation site located nearest the carboxyl terminus of the exterior glycoprotein). Because these cysteine residues are invariant among the glycoproteins, we suspect that they play a key role in the structural properties of the *env* genes, and are probably the sites at which disulfide bonds between the transmembrane protein and the exterior glycoprotein are formed.

The conserved spacing of cysteine residues suggests that the shape and rigidity of the exterior glycoprotein is determined by specific disulfide bonds. The similarity in placement of the potential glycosylation sites also suggests that these proteins assume a similar tertiary configuration. The likelihood of overall structural similarity, as well as the identity of much of the primary amino acid sequence, provides a natural explanation for the antigenic cross-reactivity between the envelope glycoproteins of HTLV-I and -II. On the basis of these considerations, the two proteins ought to have common sequence-specific and configuration-specific epitopes. Antigenic differences between the HTLV-I and HTLV-II envelope proteins detected by neutralization of vesicular stomatitis virus pseudotypes (*14*) are probably due to differences in primary amino acid sequence in a limited region of the exterior glycoprotein. The cross-reactivity of the serum of AIDS patients, who are presumably infected with HTLV-III, with the envelope glycoproteins of HTLV-I and -II (*4, 7, 8*) may be explained by similar structural conservation of the envelope glycoprotein of all known members of this retrovirus family.

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Sindbis Virus Mutants Selected for Rapid Growth in Cell Culture Display Attenuated Virulence in Animals

Abstract. *Mutants of Sindbis virus were selected for rapid growth in baby hamster kidney (BHK) cell cultures and screened for attenuation of virulence in suckling mice. Comparisons among independently isolated virulent and attenuated strains, as well as a classical reversion analysis, showed that accelerated penetration of BHK cells was correlated with attenuation in vivo. Both phenotypic changes resulted from a reorganization of virion structure as detected by monoclonal antibodies. These results suggest that mutants selected for rapid growth in cell culture may be useful as attenuated vaccines and for studies of the molecular basis of virus pathogenesis.*

It has long been recognized that blind serial passage of viruses in cell culture results in the selection of strains that have decreased pathogenicity in animals and are useful as live virus vaccines. However, the molecular mechanism or mechanisms responsible for attenuation by serial passage are not well understood. One possible basis for this enrichment is that serial passage in cell culture exerts a selective pressure for mutants that efficiently utilize the synthetic processes or structural elements of the tissue culture host. Such mutants presumably would be less efficient in their utilization of the analogous processes in target cells of the natural, animal host, thus restricting their ability to cause overt disease in vivo. Our objective was to test this hypothesis by applying a stringent selective pressure for efficient replication of Sindbis virus (SB) in baby hamster kidney (BHK) cells and by screening the resulting mutants for attenuation in suckling mice. We found that selection for efficient replication in cell culture enriched for mutations affecting one of the SB glycoproteins and that these mutations governed both penetration in vitro and attenuation in animals.

Sindbis virus is a member of the alphavirus genus, a group of viruses carried by arthropods that includes the agents of Eastern, Western, and Venezuelan equine encephalitis (*1*). SB virions consist of a single-stranded RNA of positive polarity (*2*) that is complexed with capsid protein (30,000 molecular weight) to form an icosahedral nucleocapsid (*3*). The nucleocapsid is enclosed within a lipoprotein envelope composed of cellular lipids (*4*) and two virus-specified glycoproteins, E1 and E2 (50,000 and 45,000 molecular weight, respectively) (*3, 5*). Upon subcutaneous inoculation of suckling mice, a single plaque-forming unit (PFU) is sufficient to cause death within 5 days (*6, 7*). The virus replicates locally at the site of injection and gives rise to a viremia that peaks 24 hours after inoculation (*7*). Invasion of the central nervous system occurs by infection of endothelial cells lining the small capillaries of the brain. By 2 days after inoculation, viral antigen is found throughout the brains of infected animals, and high virus titers are sustained until death. Mortality is invariably 100 percent.

Since viruses are obligate intracellular parasites, their replication depends heavily on the interaction of virus-specified products with structural and enzymatic elements of the host. We reasoned (i) that the efficiency of these types of interactions between virus and cell could be affected by mutation, (ii) that a significant increase in efficiency would lead to a decrease in the time required for the completion of the entire replicative cycle, and (iii) that mutants exhibiting improved efficiency would be among those progeny virions released from infected cells soon after the end of the latent period. Accordingly, SB was grown in BHK cells, and virus was harvested 5.5 hours after infection, which is 1 to 2 hours after the end of the normal latent period (*8*). Undiluted virions were then used as inoculum for a second passage, and the rapid-growth selection was continued in this manner for 12 serial passages (*9*). Thirty putative mutants were cloned by plaque purification from each of passages 4 to 12, and over 100 of these were tested for virulence in suckling mice. The isolates fell into three virulence categories: those which resembled wild-type, infected mice having a mean survival time of 5 to 6 days and 100 percent mortality; a marginally attenuated group, infected mice having a mean survival time of 8 to 9 days and an occasional survivor; and an attenuated group characterized by mortality rates ranging from 90 to 0 percent with mean survival times from 10 to 14 days (Fig. 1).

Attenuated isolates were found in passage 4, and the proportion of attenuated strains increased at each round of rapid growth selection from 7 percent in passage 4 to 70 percent in passage 12. In contrast to mutants selected for rapid growth, our wild-type SB stock had been maintained by passage with an 18-hour growth cycle. Of 30 independent plaque-purified isolates from wild-type stock, all showed 100 percent mortality and a mean survival time of 4 to 5 days. Selection for rapid growth therefore increased the number of mutants that displayed reduced virulence.

A prototype attenuated mutant from passage 11 was characterized further. This mutant had a reduced latent period in BHK cells compared to SB and was designated SB-RL. This mutant was indistinguishable from its wild-type parent on the basis of the following criteria: temperature sensitivity; *p*H optima for hemagglutination or cell-to-cell fusion from within or without; attachment to BHK cells; ability to induce homologous interference; induction of interferon in vitro or sensitivity to interferon; ability to produce defective-interfering particles after passage at high multiplicities of infection; buoyant density in potassium tartrate gradients; sedimentation velocity in sucrose; mobility in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of the virion proteins; and two-dimensional analysis of ribonuclease T1 oligonucleotides derived from the genomic RNA's (*10*). However, the reduction in the SB-RL latent period resulted from two aspects of SB-RL rep-