Human T-cell leukemia virus type 1 (HTLV-1) is the etiologic agent of adult T-cell leukemia/lymphoma and HTLV-1-associated myelopathy/tropical spastic paraparesis (17, 20, 30, 32). In addition to the gag, pol, and env genes common to most retroviruses, the HTLV-1 genome contains a region between env and the 3′ long terminal repeat (LTR) known as the pX region. The pX region consists of four open reading frames (ORFs), termed pX-I, pX-II, pX-III, and pX-IV, which are alternatively spliced to encode a number of proteins. ORFs pX-IV and pX-III encode the Tax and Rex proteins, which are transcriptional and posttranscriptional regulators of viral gene expression, respectively (3, 13, 21, 22, 34). ORFs pX-I and pX-II are each found in alternatively singly and doubly spliced transcripts which encode a total of three proteins (6, 24, 26). The single- and double-spliced transcripts of pX-I both utilize the same splice acceptor site, and both encode a protein of 12 kDa known as p12I (6, 24, 26). In addition, the double-spliced form of pX-I contains the coding potential for a 27-kDa protein (termed Rof), although expression of this protein in infections in rabbits (10), has been previously described. Mutations in the ACH clone that were predicted to result in the loss of expression of the p12I, p30II, and p13II proteins were constructed (Fig. 1). The ACH.p12I mutant contained a deletion of 4 bp of a PstI site in the 3′ flanking sequence (26). However, despite these initial characterizations of the pX-I- and pX-II-encoded proteins, little is known about the actual role of p12I, p30II, and p13II in viral replication or in the immortalization of primary lymphocytes.

A molecular clone of HTLV-1 known as ACH, which produces infectious virus particles capable of immortalizing primary human lymphocytes (23) and establishing productive infections in rabbits (10), has been previously described. Mutations in the ACH clone that were predicted to result in the loss of expression of the p12I, p30II, and p13II proteins were constructed (Fig. 1). The ACH.p12I mutant contained a deletion of 4 bp of a PstI site as a result of treatment with T4 DNA polymerase, which deletes the adenine in the conserved AG splice acceptor site utilized by both the single- and double-spliced pX-I transcripts. The ACH.p12I/p30II mutant contained a 2-bp deletion resulting in a frameshift mutation at a SacII site located 291 bp into the 725-bp p30II ORF. In addition to frameshifting the p30II ORF, this mutation also replaced the last 5 aa of p12I with 55 aa derived from a third reading frame. As p12I is predicted to contain two membrane-spanning domains, it is likely that this mutation disrupted the stability and/or processing of p12I. The ACH.p30II mutant contained a linker inserted at the same SacII site, which restored the p12I ORF and introduced a termination codon into the p30II ORF. The ACH.p30II/p13II mutant, constructed by site-specific PCR mutagenesis, contained a deletion of the initiator methionine codon of p13II (changing it to a GgII site), as well as the p30II mutation previously described. The structures of the proviral clones were confirmed by restriction enzyme and nucleotide sequence analyses. These mutations did not affect the ability of the virus to express a functional Tax protein capable of transactivating an HTLV-LTR-luciferase reporter vector when cotransfected with the mutant ACH clones in COS7 or 293T cells (data not shown). In addition, these clones produced comparable amounts of virus particles when transfected in 293T cells with similar Gag and Env protein compositions as determined by Western blotting of concentrated cell culture supernatants with anti-p19MA monoclonal antibody (MAb) 12/1.2 (provided by M. Robert-Guroff).
and anti-gp46 SU envelope MAb 0.5α (provided by Y. Matsushita) (data not shown).

These mutants also retained the ability to immortalize primary human peripheral blood mononuclear cells (PBMC). Human PBMC were isolated from normal donors by Ficoll-Paque (Pharmacia, Piscataway, N.J.) centrifugation and activated for human peripheral blood mononuclear cells (PBMC). Huhusushita) (data not shown).

There cell lacked expression of CD8, consistent with an activated T-helper phenotype (data not shown).

In addition, the cell lines derived from these cultures expressed similar amounts of p12lg and ACH.p30II mutants immortalized the transfected cells on three of four, two of four, four of four, and two of two attempts, respectively. The immortalized cell lines to confirm the absence of the p12lg double-spliced fragments (Fig. 2). The PCR products were separated on a 1% agarose gel, transferred to a nylon membrane, and probed with a digoxigenin (Boehringer Mannheim, Indianapolis, Ind.)-labelled probe derived from the pX-I ORF. Whereas the 2,041-bp fragment was present in both the ACH.wt and ACH.p12lg clones, the 490-bp pX-I fragment was only detected in the ACH.wt-immortalized cells. This analysis also confirmed the lack of additional cryptic p12lg mRNA splice sites, which could be functioning in the absence of the preferred p12lg splice acceptor site, as additional spliced transcripts would have been detected by this probe. This analysis does not, however, exclude the unlikely possibility that the p12lg protein could be expressed from a polycistronic message. To assess this possibility, we introduced a polynucleotide linker coding for the influenza hemagglutinin epitope at the 3′ end of the p12lg ORF (data not shown).

To further confirm the presence of the ACH.p12lg and ACH.p30II mutations in the proviruses of the immortalized cell lines, two separate strategies were employed. As the ACH.p12lg mutant contains a splice acceptor site deletion, reverse transcription-PCR (RT-PCR) was performed on total RNA isolated from the ACH.wt- and ACH.p12lg-immortalized cell lines to confirm the absence of the p12lg double-spliced fragment (Fig. 3A). The primer pair utilized amplifies both a 2,041-bp fragment corresponding to the gag-pol and env transcripts and a 490-bp fragment corresponding to the double-spliced pX-I transcript. The PCR products were separated on a 1% agarose gel, transferred to a nylon membrane, and probed with a digoxigenin (Boehringer Mannheim, Indianapolis, Ind.)-labelled probe derived from the pX-I ORF. Whereas the 2,041-bp fragment was present in both the ACH.wt and ACH.p12lg clones, the 490-bp pX-I fragment was only detected in the ACH.wt-immortalized cells. This analysis also confirmed the lack of additional cryptic p12lg mRNA splice sites, which could be functioning in the absence of the preferred p12lg splice acceptor site, as additional spliced transcripts would have been detected by this probe. This analysis does not, however, exclude the unlikely possibility that the p12lg protein could be expressed from a polycistronic message. To assess this possibility, we introduced a polynucleotide linker coding for the influenza hemagglutinin epitope at the 3′ end of the p12lg ORF in the ACH.wt and ACH.p12lg clones. However, we were un-
able to detect the expression of the tagged p12I protein with either clone in transiently transfected 293T cells by Western blotting or radioimmunoprecipitation (data not shown). This is most likely due to the low level of expression of the p12I transcript and is consistent with the fact that the translated p12I protein has not yet been detected in HTLV-1-infected cells (6, 24).

To confirm the ACH.p12I mutation in the immortalized cell lines, the p30II ORF was amplified by PCR from both the original plasmid and from the genomic DNA from the ACH.wt- and ACH.p12I/p30II-immortalized cells, cloned into the pTM3 expression plasmid (which places the p30II ORF under the control of the T7 promoter) (16), and expressed in vitro with the TNT-coupled reticulocyte lysate system (Promega, Madison, Wis.). Only the p30II ORF derived from the ACH.wt plasmid or genomic DNA expressed a 30-kDa product, while the product was absent from the ACH.p12I/p30II-immortalized cell DNA-derived expression vectors (Fig. 3B).

As the p12I protein has been demonstrated to bind to the IL-2 receptor β and γ chains (29) and may therefore influence the IL-2 requirements for cellular proliferation, we examined the IL-2 dependency of the ACH.p12I- and ACH.p12I/p30II-immortalized cell lines. The WT and mutant ACH-immortalized cell lines were all dependent on the addition of exogenous IL-2 for cellular viability and proliferation. Furthermore, over the course of 3 days in culture, the ACH.wt-, ACH.p12I-, and ACH.p12I/p30II-immortalized cell lines all manifested a similar IL-2 dose-response growth relationship (Fig. 4). Finally, long term cultures in gradually decreasing concentrations of IL-2 also revealed no difference in IL-2 dependency, with all cell lines remaining dependent on 3 to 6 U of IL-2 per ml after 6 months in culture (data not shown).

To confirm that the immortalized cell lines were producing virus which was infectious and capable of immortalizing uninfected PBMC, 5 × 10^5 ACH.wt-, ACH.p12I-, ACH.p12I/p30II-, and ACH.p30II-immortalized cells were exposed to lethal gamma radiation (6,000 rads) and cocultured in PBMC growth medium with 4 × 10^6 uninfected, activated PBMC and cell viability was monitored by a MTT conversion assay (19). The PBMC alone proliferated for a short period of time, and the irradiated cells alone did not proliferate. In contrast, the cocultured cells continued to proliferate indefinitely, indicating the productive infection and immortalization of the uninfected cells (Fig. 5).

To further quantify the infectivity and immortalizing ability of these mutants, we employed a microtiter infectivity/immortalization assay as described by Persaud et al. (31). In this assay, 10^5 uninfected activated PBMC were cocultured in PBMC growth medium with 10-fold dilutions (10^4, 10^3, 10^2, and 10) of lethally gamma-irradiated (6,000 rads) ACH mutant-immortalized cells in replicates of 10 to 20 in 96-well microtiter plates. The numbers of cultures which became immortalized were then determined at 9 to 16 weeks after the coculture by microscopic examination of the individual wells. The results of this analysis indicated that the viruses from the ACH.p12I, ACH.p12I/p30II, ACH.p30II, and ACH.p30II/p13II mutant-immortalized cell lines were all capable of infecting and immortalizing the uninfected PBMC with relatively similar efficiencies at the different dilutions of infected cells (Table 1). Furthermore, additional microtiter assays with the ACH.p12I mutant in decreasing concentrations of IL-2 (50, 10, and 2 U/ml) again revealed no difference in immortalizing activity compared to the ACH.wt clone, with 50 U of IL-2 per ml being required for the immortalization of uninfected PBMC for both the WT and p12I mutant (data not shown).
In conclusion, the p12I, p30II, and p13III putative accessory proteins of HTLV-1 appear to be dispensable for viral replication and the immortalization of T-lymphocytes in vitro. The role of these proteins in the viral life cycle has remained elusive in part due to their low level of expression in infected cells. Although the pX-I and pX-II transcripts have been detected by RT-PCR in HTLV-1-infected T-cell lines (24), HeLa cells transfected with an HTLV-1 proviral clone (6), freshly isolated infected macrophages (25), the translated proteins have not yet been detected. However, it has been reported that a small fraction of HTLV-1-positive individuals may seroconvert to HTLV-2 (12). Our studies extend these results, though, by demonstrating that virus derived from the ACH.p12I mutant is incapable of establishing a productive infection in rabbit models (18). The HTLV-2 pX-II ORF encodes a protein known as p28xII which is 56% similar and 37% identical to the p12I protein (7, 18). The HTLV-2 pX-II ORF is dispensable for viral replication in quiescent macrophages but not in dividing cells (29, 38). Preliminary results with a rabbit model of HTLV-2 infection indicate that virus derived from the ACH.p12I mutant is incapable of establishing a productive infection in rabbits, implying a role for p12I in viral replication in vivo (9). Therefore, it remains possible that the HTLV-1 p30II and p13III proteins are also playing a role in viral infectivity or replication in vivo.

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