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# Infectivity of chimeric human T-cell leukemia virus type I molecular clones assessed by naked DNA inoculation

**(human T-cell leukemia virus type I *rex* gene/cloned retrovirus/quantitative competitive-PCR)**

T. M. ZHAO, M. A. ROBINSON, F. S. BOWERS, AND T. J. KINDT*

Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, Twinbrook II Facility, National Institutes of Health, Rockville, MD 20852

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**ABSTRACT** **Two human T-cell leukemia virus type I (HTLV-I) molecular clones, K30p and K34p were derived from HTLV-I-infected rabbit cell lines. K30p and K34p differ by 18 bp with changes in the long terminal repeats (LTRs) as well as in the *gag*, *pol*, and *rex* but not *tax* or *env* gene products. Cells transfected with clone K30p were infectious *in vitro* and injection of the K30p transfectants or naked K30p DNA into rabbits leads to chronic infection. In contrast, K34p did not mediate infection *in vitro* or *in vivo*, although the cell line from which it was derived is fully infectious and K34p transfectants produce intact virus particles. To localize differences involved in the ability of the clones to cause infection, six chimeric HTLV-I clones were constructed by shuffling corresponding fragments containing the substitutions in the LTRs, the *gag/pol* region and the *rex* region between K30p and K34p. Cells transfected with any of the six chimeras produced virus, but higher levels of virus were produced by cells transfected with those constructs containing the K30p *rex* region. Virus production was transient except in cells transfected with K30p or with a chimera consisting of the entire protein coding region of K30p flanked by K34p LTRs; only the transfectants showing persistent virus production mediated *in vitro* infection. *In vivo* infection in rabbits following intramuscular DNA injection was mediated by K30p as well as by a chimera of K30p containing the K34p *rex* gene. Comparisons revealed that virus production was greater and appeared earlier in rabbits injected with K30p. These data suggest that several defects in the K34p clone preclude infectivity and furthermore, provide systems to explore functions of HTLV-I genes.**

Human T-cell leukemia virus type I (HTLV-I) infection is associated with adult T-cell leukemia (1–3), a progressive myelopathy termed HAM/TSP (4–6) and is implicated in a number of chronic disease conditions (7–10). The majority of HTLV-I infected individuals remain asymptomatic, raising questions concerning the relationship between virus variation and outcome of infection. Surveys of viral sequence from different patient groups have failed to identify mutations leading to disease. Experimental manipulations of HTLV-I have been limited by difficulty in achieving infection using molecular clones of this human retrovirus. Recently, infectious molecular clones of HTLV-I were reported by several groups (11–13). A clone (K30p) developed in our laboratory caused infection *in vitro* and cell lines transfected with K30p caused infection in rabbits (11).

The present study examines a second molecular clone, K34p, derived from an HTLV-I-infected rabbit cell line that was shown to produce acute leukemia-like disease or a chronic cutaneous lymphoma upon injection into rabbits (14–16). Although the sequences of K30p and K34p vary by only 18 nucleotides, the K34p molecular clone did not mediate infection as did K30p. Extensive comparisons of the two clones, including intramuscular injection as naked DNA, revealed that although K34p transfectants produced virus, no *in vitro* or *in vivo* infectivity was detected. Chimeric clones incorporating combinations of genes that differ between the two viruses were assayed allowing assignment of genetic regions important to *in vivo* or *in vitro* virus production.

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## MATERIALS AND METHODS

**Cell Lines.** The cell line RH/K30 was derived from rabbit peripheral blood mononuclear cells (PBMC) by coculture with irradiated MT-2 cells, a human T-cell line chronically infected with HTLV-I (17). RH/K34 was derived in a similar manner except for using the *in vivo* transformed rabbit cell line, RHT-16, as the source of virus. RHT-16 was derived from the PBMC of a rabbit injected with irradiated MT-2 cells. The rabbit T-cell line RL-5 is a lymphoid line derived from a B/J rabbit strain inoculated with the virus *Herpesvirus ateles* (18). All cell lines were grown in complete RPMI 1640 medium (GIBCO/BRL) supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum.

**Construction of Chimeric HTLV-I Molecular Clone.** DNA from RH/K30 and RH/K34 cells was digested with *Eco*RI and cloned into the bacteriophage λEMBL4 (Stratagene), then subcloned into a plasmid vector pSV2-gpt (19). K30 plasmid clone (K30p) contained a full-length HTLV-I provirus (9 kb) flanked by rabbit genomic DNA (2.5 kb at the 5′ end and 2.6 kb at the 3′ end). K34 plasmid clone (K34p) contained a full-length HTLV-I provirus (9 kb) flanked by rabbit genomic DNA (5.5 kb at the 5′ end and 0.5 kb at the 3′ end). Six chimeric HTLV-I clones (designated K30.1p, K30.2p, K30.3p, K34.1p, K34.2p, and K34.3p) were constructed by digestion with combinations of restriction enzymes (*Not*I, *Sal*I, and *Mlu*I) then shuffling segments between K30p and K34p. Structures were verified by sequence analysis (Fig. 1). The HTLV-I clones K30p and K34p are available for research purposes from the AIDS Research and Reference Reagent Program (Ogden Bioservices, Rockville, MD); the sequences are available from GenBank under numbers L03561 and L03562.

**Transfection.** Transfection of one microgram of cloned DNA into RL-5 cells using DEAE–dextran was performed as described (11). Once a week, 50% of culture was replaced by fresh complete medium. Viral pellets were prepared by ultracentrifugation of the culture supernatant (40,000 × *g* for 30 min). The amounts of HTLV-I $p24^{gag}$ and $p19^{gag}$ proteins in the samples were determined utilizing commercial antigen capture assay (Coulter and Cellular Products). Transfected cell lines

Abbreviations: HTLV-I, human T-cell leukemia virus type I; LTR, long terminal repeat; PBMC, peripheral blood mononuclear cells; QC-PCR, quantitative competitive PCR; RT, reverse transcriptase.

*To whom reprint requests should be addressed at: National Institute of Allergy and Infectious Diseases, Twinbrook II Facility, 12441 Parklawn Drive, Rockville, MD 20852.

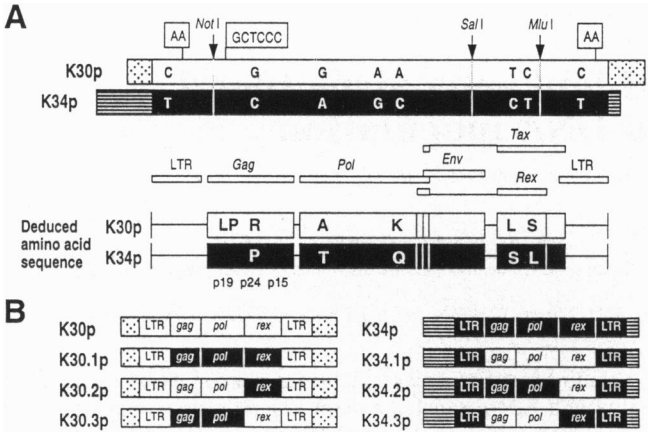


FIG. 1. HTLV-I clones and chimeras. Comparison of K30p and K34p nucleotide and deduced amino acid sequences showing only differences. There is one nucleotide substitution and one insertion (AA) in the long terminal repeat (LTR). In the *gag* region, RH/K30 contains a 6 base insertion (GCTCCC) which results in a two amino acid insertion in $p19^{gag}$; there is a replacement substitution in $p24^{gag}$. Other differences leading to amino acid substitutions were found in the *pol* region and in the gene encoding the regulatory protein Rex. Arrowheads indicate restriction endonuclease sites. The nucleotide positions (numbered according to ref. 14) of *Not*I, *Sal*I, and *Mlu*I are 842, 5681, and 7489 in K30p; 840, 5673, and 7481 in K34p, respectively. Chimeric HTLV-I molecular clones constructed by shuffling corresponding K30p and K34p fragments obtained by *Not*I, *Sal*I, and *Mlu*I digestion. The sequences flanking the 5′ and 3′ LTRs are rabbit genomic DNA.

are designated by cell line and the clone used for transfection; for example, RL-5/K30p is RL-5 transfected with K30p.

**Preparation of DNA-Free Virus Stocks.** Viral particles were prepared from cell cultures by low speed centrifugation (3,000 × *g* for 15 min) followed by ultracentrifugation of the supernatant (40,000 × *g* for 60 min). Pellets were treated with DNase (80 units/ml) (Promega) for 1 h at 37°C and filtered through a 0.45 μm pore-size filter. Before use, the virus preparations were washed by resuspension in Tris·$MgCl_2$ buffer (10 mM Tris·HCl, pH 8.3/1.5 mM $MgCl_2$) and pelleted by centrifugation at 40,000 × *g* for 30 min. The density of the virions was measured by ultracentrifugation on a 20–60% sucrose gradient at 27,000 rpm in a Beckman SW28 rotor for 20 h; HTLV-I $p24^{gag}$ concentration was determined for each fraction.

**Oligonucleotide Primers and Probe.** The following is a list of sequences (5′-3′) of the primers used for PCR and quantitative competitive PCR (QC-PCR). The numbers correspond to the nucleotide positions on HTLV-I clone K30p (14). env: 5363F GCGGATCAGGCCCTACAG (nt 5363–5380), 5689R AGCGTCGACTAGAAGGGAGAA (nt 5689–5669); tax: 7488F GACGCGTTATCGGCTCAGCT (nt 7488–7507), 7721R GGAGTCCGGGGTCTGGAA (nt 7721–7704). HTLV-I env probe (1497 bp, nt 5198–6685) was used for Southern blot analysis.

**PCR and QC-PCR.** High molecular weight DNA was prepared by using the Wizard Genomic DNA purification kit (Promega). The PCR mixture contained a total volume of 20 μl [10 mM Tris·HCl, pH 8.3/50 mM KCl/1.5 mM $MgCl_2$/0.001% (wt/vol) gelatin/0.2 mM dATP/0.2 mM dCTP/0.2mM dGTP/0.2 mMdTTP/1 μM of each oligonucleotide forward and reverse primer/0.5 unit of *Taq* DNA polymerase (Perkin–Elmer)/2 μl template DNA]. After denaturation for 5 min at 95°C, samples were subjected to 30 cycles of PCR in a DNA Thermal Cycler (Perkin–Elmer). Each cycle included 95°C for 30 sec, 58°C for 30 sec, and 72°C for 1.5 min followed by a final extension at 72°C for 5 min. PCR products were analyzed by agarose gel electrophoresis. A region (nt 5099–5689) in the HTLV-I *env* gene was selected as QC-PCR target sequence. A fragment, designated K30envΔ, containing a 91 bp internal deletion was generated by PCR and cloned into pUC18 (Life Technologies, Gaithersburg, MD). QC-PCR reactions were performed using primer pairs 5363F, 5689R (shown above), and 10-fold dilutions of quantified K30envΔ as a competitive template.

**Detection of Reverse Transcriptase (RT) Activity and Viral RNA.** A PCR-based technique, in which endogenous viral RT was used to make cDNA copies of viral templates, was developed to detect RT and viral RNA simultaneously (11). Briefly, the pelleted DNA-free virus samples were lysed in 5–10 μl of lysis buffer [10 mM Tris·HCl, pH 8.3/1.5 mM $MgCl_2$/0.25% Tween-20 (vol/vol)]. cDNA synthesis was carried out in a total volume of 25 μl consisting of 10 μl of viral lysate and 15 μl of RT cocktail [50 mM Tris·HCl, pH 8.3/75 mM KCl/3 mM $MgCl_2$/2 mM DTT/1 unit of RNase inhibitor (Inhibit-Ace, 5 Prime → 3 Prime, Inc.)/1 mM dATP/1 mM dCTP/1 mM dGTP/1 mM dTTP/2 μM oligo-$d(T)_{18}$]. This reaction mixture was incubated at 37°C for 1 h, then at 95°C for 5 min to inactivate endogenous RT activity and cooled quickly on ice. The synthesized cDNA was purified and dissolved in TE solution for PCR analysis. PCR was performed using 2 μl of synthesized cDNA in a 20 μl reaction mixture. Negative and positive controls, in which either water or cloned HTLV-I plasmid DNA was substituted for viral lysate, were included with each assay. PCR products were analyzed as described above.

**Assay for *in Vitro* Infectivity.** Assays for *in vitro* infectivity were carried out as described (20). Briefly, rabbit PBMC were stimulated by phytohemagglutinin at 37°C for 72 h in complete medium containing 100 units of recombinant human interleukin 2 (IL-2) per ml (BioSource International, Camarilla, CA). Activated cells ($10^7$) and an equal number of irradiated (10,000 rads) RL-5 cells transfected with the HTLV-I constructs were maintained in 10 ml of IL-2 supplemented complete medium. Once per week, 5 ml of media were removed and the culture replenished with an equal volume of fresh media. The levels of HTLV-I $p24^{gag}$ protein in the supernatants were determined.

**Assays for *in Vivo* Infectivity.** The ability of HTLV-I transfected cell lines to transmit infection *in vivo* was evaluated by intravenous injection of $10^7$ irradiated (10,000 rad) cells into adult rabbits. Controls included age and sex matched rabbits injected with an equal number of nontransfected RL-5 cells or RL-5 cells transfected with plasmid vector pSV2-gpt. Infectivity of cloned DNA was evaluated by naked DNA inoculation (21). Rabbits were injected in the quadriceps with 100 μl of 0.5% bupivacaine hydrochloride, followed in 24 h by 100 μg of DNA (in 1 ml saline) in the same area. This process was repeated biweekly for a total of four inoculations. HTLV-I infection was monitored by testing DNA samples from PBMC for the presence of HTLV-I sequences by PCR using primers and conditions described above. Serum anti-HTLV-I antibodies were assayed using a commercial ELISA kit (Cellular Products). PBMC were isolated from the inoculated rabbits and cultured in complete medium containing 100 units of IL-2 per ml; supernatants were tested for HTLV-I $p24^{gag}$ protein at weekly intervals.

## RESULTS

**Characterization of K30p and K34p.** The HTLV-I molecular clone, K30p, mediates both *in vitro* and *in vivo* infectivity (11). A second clone, K34p, obtained from the rabbit cell line RH/K34, was tested using methods by which the infectivity of K30p was established. Despite the minor differences in sequence between the cloned viruses, cells transfected with K34p did not mediate *in vitro* or *in vivo* infection. Comparisons of the properties and the levels of virus produced by the original cell lines and by RL-5 cells transfected with K30p and K34p give

little insight into differences in ability to mediate infection (Table 1). Both RL-5/K30p and RL-5/K34p cells produced viral particles that had appropriate densities (1.15–1.17 g/ml), contained HTLV-I RNA sequences, and had comparable levels of RT activity. The K34p transfectant did not produce as many virus particles as did the K30p transfectant. However, this difference reflects levels of virus produced by the native cell lines and does not explain adequately the differences in infectivity considering that the parent RH/K34 line is infectious.

To further examine viral factors involved in this differential ability to cause infection, six chimeric HTLV-I clones were constructed by shuffling the regions of K30p and K34p that show variation (Fig. 1*A*). Construction of the chimeras utilized restriction sites that separate the LTRs, the *gag/pol* region and the *tax/rex* region (Fig. 1*B*). Differences between K30p and K34p clones include insertion/deletion and substitutions in the LTR, replacement substitutions, and an insertion/deletion in $p19^{gag}$, replacements substitutions in $p24^{gag}$ and in *pol* and *rex* region. Identical amino acid sequences are predicted for the env and tax proteins encoded by the two clones (Fig. 1*A*). RL-5 cells were transfected with the parental clones or with the chimeras; all transfectants were tested initially for production of virus and for *in vitro* infectivity using activated rabbit PBMC as targets.

**Virus Production by RL-5 Cells Transfected with HTLV-I Clones.** Major differences in virus production and *in vitro* infectivity were observed among transfected RL-5 cells when examined over extended periods of time (Fig. 2). Virus isolated from cells transfected with the parental clones or with any of the chimeras contained all of the major HTLV-I structural proteins, RT activity, and genomic viral RNA (Table 2). Although Southern blots of cellular genomic DNA revealed provirus in all transfectants, only certain of these retained provirus for extended periods. RL-5/K30p cells produced virus and retained the HTLV-I genome for up to 36 months posttransfection, whereas RL-5/K34p cells produced virus transiently and HTLV-I provirus could be detected only up to about 3 months. Of cells transfected with the six chimeric clones, only RL-5/K34.1 maintained viral production and retained the proviral genome for the entire 10-month period of observation. K30p and K34.1p differ only in their LTR and flanking regions. Cells transfected with the other clones varied in level of virus protein and time of optimal production, but none persisted as did the K30p or the K34.1p transfectants. Cells receiving the other clones produced $p24^{gag}$ protein for several weeks before levels began to decrease; no $p24^{gag}$ was detected in these cultures by day 75 (Fig. 2). The only two transfectants that transferred infectivity *in vitro* were RL-5/K30p and RL-5/K34.1p, the two lines that maintained persistent virus production.

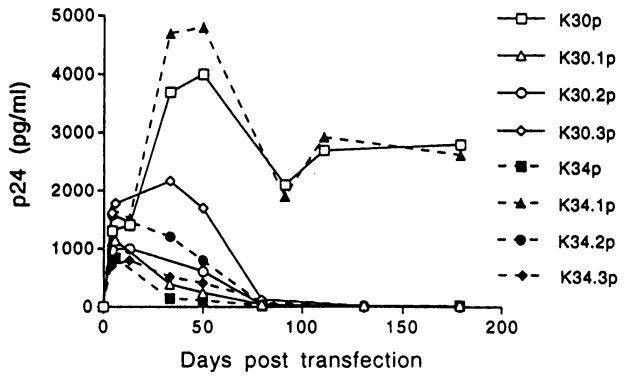


FIG. 2. HTLV-I $p24^{gag}$ protein production by the rabbit T-cell line RL-5 following transfection with HTLV-I molecular clones.

Significant differences in the amounts of viral $p24^{gag}$ protein produced by the cells transfected with the different clones were observed. To determine the relationship between levels of virus production and viral structure, the eight clones were transfected into RL-5 cells under identical conditions and amounts of viral $p24^{gag}$ protein that accumulated in the culture supernatants were compared in replicate experiments. The data are reported with the clones divided into four paired groups based upon difference in the *rex* region (Fig. 3*A*). Cells transfected with constructs containing the K30p *rex* region sequence produced significantly more supernatant $p24^{gag}$ than those with that of K34p. To assure that the supernatant $p24^{gag}$ reflects virus production, levels of viral $p24^{gag}$ and $p19^{gag}$ in pelleted virus were compared for each of the transfectants (Fig. 3*B*). Results obtained for both gag proteins in virus pellets concur with those obtained for supernatant $p24^{gag}$, and confirm that the *rex* gene substitutions influence the amount of virus produced in the transfectants.

***In Vivo* Infection with HTLV-I Clones.** Rabbits were injected with irradiated RL-5 cells transfected with parent and selected chimeric clones (Table 3). RL-5/K34.1p was chosen because it retained virus production for an extended period of observation (see Fig. 2). All transfected lines produced infection except RL-5/K34p even though it was injected when it was producing optimum levels of virus.

Injection of the HTLV-I clones into rabbits as naked DNA provided a further test of infectivity. A total of 10 rabbits, including 1 control, were given intramuscular inoculations of the 2 parents and 3 selected chimeric DNA clones (Table 3). K30p was injected into three rabbits and all became infected as indicated by seroconversion, by presence of viral DNA in

Table 1. Comparison of RL-5 cells transfected with K30p and K34p and their parent cells

| | RL-5/K30p | RL-5/K34p | RH/K30 | RH/K34 |
|---|---|---|---|---|
| Virus produced by the cells* | | | | |
| vp × $10^6$/ml culture | 12 | 2.2 | 10.2 | 4.3 |
| Density (g/ml) | 1.16-1.17 | 1.15 | 1.16 | 1.17 |
| RT enzymatic activity | + | + | ++ | ++ |
| HTLV-I RNA | + | + | + | + |
| HTLV-I provirus† | + | + | + | + |
| Infectivity of the cells | | | | |
| Infectivity *in vitro*‡ | + | − | + | + |
| Infectivity *in vivo*§ | + | − | + | + |

*Viral particle count was performed by Advanced Biotechnologies (Columbia, MD). The RT enzymatic activity is measured by the level of incorporation of radiolabeled deoxyribonucleoside triphosphates into high molecular weight products (1). HTLV-I RNA *tax*, *env* sequences were detected by RT-PCR.
†HTLV-I provirus was detected by Southern blot analysis of cell genomic DNA using an HTLV-I env probe.
‡Determined by coculture of irradiated cells with rabbit PBMC.
§Determined by intravenous injection of irradiated cells into rabbits.

Table 2. Comparison of RL-5 cells transfected with HTLV-I clones

| Cell | Virus particle* | | | | Persistence of virus production | Provirus in genomic DNA† | *In vitro* infectivity‡ |
|---|---|---|---|---|---|---|---|
| | RNA | RT | $p24^{gag}$ | $gp46^{env}$ | | | |
| RL-5/K30p | + | + | + | + | >36 mo | + | + |
| RL-5/K30.1p | + | + | + | + | 2–3 wk | + | − |
| RL-5/K30.2p | + | + | + | + | 2–3 wk | + | − |
| RL-5/K30.3p | + | + | + | + | 2–3 wk | + | − |
| RL-5/K34p | + | + | + | + | 2–3 wk | + | − |
| RL-5/K34.1p | + | + | + | + | >10 mo | + | + |
| RL-5/K34.2p | + | + | + | + | 2–3 wk | + | − |
| RL-5/K34.3p | + | + | + | + | 2–3 wk | + | − |

*Viral RNA and RT activity were simultaneously determined by RT-PCR analysis. Viral protein $p24^{gag}$ was detected utilizing commercial antigen capture assay. The $gp46^{env}$ glycoprotein was detected by Western blot analysis.
†Provirus was detected by Southern blot analysis of cell genomic DNA using an HTLV-I env probe.
‡*In vitro* infectivity was determined by coculture of irradiated cells with rabbit PBMC.

PBMC, and by the production of virus by cultured blood. K30p inoculated rabbits seroconverted by 2 months after inoculation and remained seropositive for the period of observation (Fig. 4). Immunoblot analysis of rabbit serum samples revealed antibodies to all major virus proteins (data not shown). Rabbits injected with K34p ($n$ = 2) or with plasmid DNA ($n$ = 1) showed no signs of infection as measured by the same criteria. In addition, DNA from the chimeric clones, K34.1p or K30.3p, did not mediate infection in inoculated rabbits. The negative result for K34.1p was unexpected because, as shown above, it gave rise to persistent infection of RL-5 cells *in vitro* and the transfectant mediated *in vivo* infection. *In vitro* analyses of the injected K34.1p DNA sample ruled out introduction of further substitutions or deletions during expansion of the clone as cause for the lack of *in vivo* infectivity by direct DNA injection.

The clone, K30.2p, which contained all K30p genes except those in the *rex* region, also gave rise to infection despite the lack of infectivity or long-term persistence of the clone *in vitro*. Both seroconversion and detection of virus DNA in PBMC were delayed in the rabbit given K30.2p compared with those inoculated with K30p. Antibodies appeared at 3 months postinjection in the rabbit injected with K30.2p, and at 2 months in those given K30p. Virus was detected in PBMC cultures of K30p inoculated rabbits at day 43 after injection, whereas no virus was detected in cultures from the K30.2p inoculated rabbit until day 90 postinjection. The mean concentrations of $p24^{gag}$ in K30p derived cultures were 640 ± 338 (SEM) pg/ml compared with 64 ± 42 (SEM) pg/ml for those from the K30.2p injected animal. In addition, when the levels of HTLV-I provirus were compared by QC-PCR, about 50,000 copies of HTLV-I DNA per μg of PBMC DNA were observed for two K30p injected rabbits (X178 and X190) and 10-fold less (≈5,000 HTLV-I copies per μg of PBMC DNA) was observed in samples from the K30.2p injected rabbit X191 (Fig. 4). Sequence analysis of virus from PBMC of the K30.2p inoculated rabbit indicated that the *rex* gene retained the substitutions characteristic of K34p.

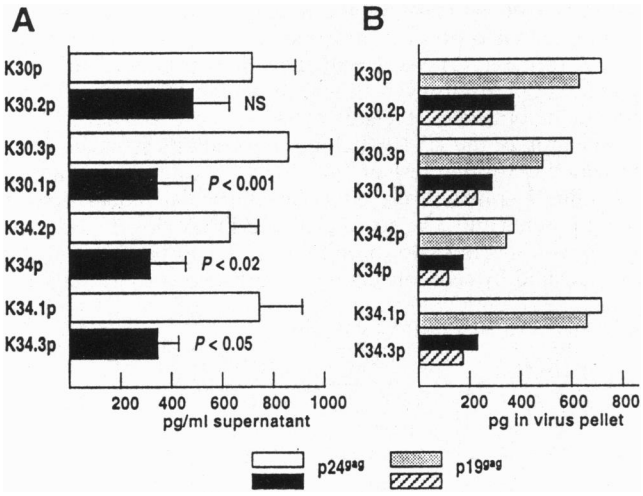


FIG. 3. Comparison of HTLV-I protein production following transfection with HTLV-I molecular clones by rabbit T-cell line RL-5. The two clones in each pair compared differ only in that one contains K30p *rex* region (open and shaded bars) and the other contains the K34p *rex* region (solid and hatched bars). The mean amount of $p24^{gag}$ protein accumulated in 1 ml of culture medium sampled at intervals of 3–4 days within 12 days posttransfection. Data (mean and SEM) are from three independent experiments; $P$ values were calculated for each pair compared. The difference between K30p and K30.2p was not significant (NS). $p19^{gag}$ and $p24^{gag}$ protein in virus pellets were prepared from 1 ml of culture supernatant from the transfected RL-5 cells.

## DISCUSSION

HTLV-I causes serious disease in only a small percentage of infected patients and usually only after a lag period of many years suggesting a complex interaction between the virus and the host in HTLV-I *in vivo* infection. Surveys of virus structure from different patient populations have not identified virus sequences influencing the outcome of HTLV-I infection. While animal models for HTLV-I have mimicked certain features of human infection and disease (22–25), limitations in ability to manipulate the virus have hampered detailed studies of viral gene activity. The combined use of infectious HTLV-I molecular clones and the biologic assays reported here allow a direct study of factors related to infectivity of HTLV-I. Comparison of the K30p and K34p clones can provide infor-

Table 3. *In vivo* infectivity of HTLV-I clones

| Clone | Clone injected into rabbits | |
|---|---|---|
| | RL-5 transfectant | Naked DNA |
| Parent clones | | |
| K30p | 2/2* | 3/3 |
| K34p | 0/2 | 0/2 |
| Chimeras | | |
| K34.1 | 2/2 | 0/2 |
| K30.2p | 1/1 | 1/1 |
| K30.3p | 1/1 | 0/1 |
| Control | | |
| pSV2-gpt | 0/1 | 0/1 |

HTLV-I clones were administered by either intravenous inoculation of irradiated transfected cells or by intramuscular injection of naked plasmid DNA.
*Numbers show rabbits positive for *in vivo* infection/number inoculated. Infection was monitored by HTLV-I antibodies in the serum detected by ELISA and by virus isolation from rabbit PBMC.

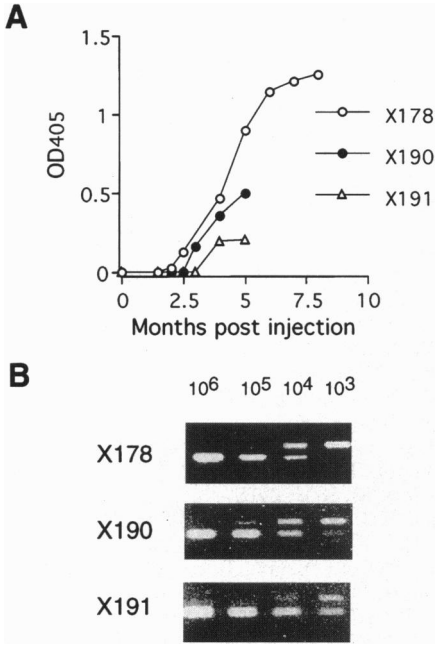


FIG. 4. Analysis of samples from rabbits inoculated with HTLV-I naked DNA. (*A*) Detection of antibodies to HTLV-I proteins in serum from rabbits injected with K30p (X178 and X190) or K30.2p (X191). Antibodies were detected with a commercial ELISA kit; numbers shown are optical densities at 405 nm ($OD_{405}$) for serum samples diluted 1:100. Provirus copy number in rabbit PBMC DNA estimated by amplification of an HTLV-I env fragment by QC-PCR using the standard competitor K30envΔ, a env fragment containing a 91 bp internal deletion. Shown is the ethidium bromide stained gel with the copy number of the competitor at top. Upper bands are the wild-type HTLV-I env fragment (327 bp); lower bands are standard K30envΔ fragment (236 bp). Rabbits X190 and X191 DNA samples were taken at 5 months postinjection; X178 at 15 months.

mation not only about the action of different viral genes, but also about some basic features of the HTLV-I infection processes.

Because the molecular clone K30p is infectious in all assay systems used, the K34p clone reflects the more commonly reported HTLV-I clones that do not mediate infectivity. This lack of infectivity is not easily explained for K34p, which was derived from a clonal T-cell line containing a single copy of the integrated proviral genome. Trivial explanations for the inability of K34p to cause infection, such as small deletions or mutations in the cloned gene, are largely ruled out by sequence analysis of the clone compared with direct sequencing of RH/K34 viral DNA. Furthermore, pelleted virus from the K34p transfectants display all viral structural proteins, have RT activity, and contain the RNA genome; integrity of K34p genes is further shown by the fact that infectivity is restored in certain chimeric constructs with K30p. The data obtained with the six chimeras reported herein establish at least preliminary relationships between virus structure and different aspects of infectivity.

One surprising result was that all of the clones mediated production of virus that appeared intact by all criteria tested. The particles had the appropriate density and morphology and included all structural proteins. Despite differences between K30p and K34p *gag* genes, the quality and quantity of p19 and p24 gag proteins were not influenced. No obvious differences in distribution of viral proteins were observed by immunoblot analyses of pelleted virus. Detection of the major RNA transcripts and immunologic detection of gp46 envelope glycoprotein in virus produced by all transfectants eliminate defective splicing of virus message or posttranslational processing of protein as causes for differences in infectivity. Detection of RT activity using the virus RNA as a template argues for intact replication machinery in the virus. Another factor considered in the comparisons was the presence of the proviral DNA integrated into host genomic DNA. Integrated provirus was detected in all transfectants and persisted beyond the time of detectable production of virus protein. In the case of RL-5/K34p, provirus remained detectable for 3 months following transfection, whereas no viral protein was detected after two months.

Several easily measurable functional differences between K30p and K34p were observed and genetic regions influencing these could be assigned by analyses of chimeric constructs. One major difference between K30p and K34p was the ability to cause persistent infection in the rabbit RL-5 cell line. Persistent infection was only observed in cells transfected with K30p or with one of the chimeras, K34.1p, which contains all K30p genes except the 5′ and 3′ LTR and flanking rabbit genomic DNA. Cells transfected with this chimera were infectious for PBMC *in vitro* and gave rise to chronic infection *in vivo* when irradiated and inoculated into rabbits. This observation indicates that the K34p LTR substitutions do not preclude persistence of cellular infection or the passage of infection to primary cells in culture or *in vivo*. The clones are flanked by rabbit genomic sequences that have minimal identity to one another (T.M.Z., unpublished data); the role of these sequences has not been examined, although the present comparison of K30p and K34.1p suggest that the differences have no major effect on *in vitro* infectivity.

A second clear cut difference involved the level of virus expression mediated by K30p and K34p. Analyses of the chimeras implicated sequence differences in the K34p *rex* region in the lowered levels of virus production. For example, chimera K34.2p, which contains all K34 genes except those of the *rex* region, produced as much virus as K30p, suggesting that this phenotype is conferred by the substitutions in the *rex* region. Although *rex* region products determined levels of virus produced by transfectants, the relationship between these quantitative effects and infectivity remain obscure.

The *rex* gene product induces cytoplasmic expression of unspliced and single spliced mRNAs that encode the structural proteins gag and env required in the assembly of infectious virions. The action of *rex* is dependent upon a cis-regulatory sequence termed the Rex response element (26, 27). K30p and K34p clones have identical Rex response element sequences, but, there are nucleotide substitutions leading to two amino acid changes in predicted protein sequence of rex (Fig. 1*A*). It may now be possible to determine whether quantitative differences in viral level are associated directly with the *rex* gene product or with proteins that may be encoded by other open reading frames within the *pX* region (28). The strong association between levels of HTLV-I production and substitutions in this region provides impetus for a more detailed analysis of K30p and K34p *pX* regions and their gene products.

A third measurable difference between K30p and K34p is in their ability to mediate *in vivo* infection upon injection of naked DNA. The infectivity of naked K30p DNA was demonstrated by detection of HTLV-I antibodies and integrated proviral DNA as well as isolation of virus in PBMC cultures. No infection was detected in rabbits inoculated with K34p DNA. Inoculation of DNA from chimeric clones revealed differences in factors required for *in vivo* and *in vitro* infectivity. Although K34.1p was infectious *in vitro*, and the transfectant RL-5/K34.1p mediated infection in rabbits, no signs of infection were detected in two rabbits injected with naked K34.1p DNA. Surprisingly, a chimeric clone, K30.2p, which did not mediate persistent infection *in vitro*, caused infection of rabbits upon injection of naked DNA or transfected cells. K30.2p contains all genes of K30p except the *rex* region that derives from K34p. The delayed onset of infection and the lowered level of virus in PBMC of rabbit inoculated with

K30.2p compared with those given K30p support conclusions drawn from *in vitro* data that the substitutions in *rex* region genes play a quantitative role in virus production. The precise role that variations in K34p *rex* region genes play in infectivity is not clear from our findings, but the fact that K30.2p mediates infection as naked DNA suggests that these differences are not absolute determinants of the ability of the clones to cause infection.

These results indicate that there is not a clear parallel relationship between infectivity and *in vitro* expression of HTLV-I particles in cells transfected with different molecular clones. These studies further suggest that HTLV-I *in vivo* infection may occur by different mechanisms depending on whether infection is mediated by infected cells or by naked DNA. Characteristics of the host cells infected by HTLV-I are likely to also play an important role in the infectivity of virus constructs as well as on the pathogenesis produced by the cell once infected. These cellular characteristics have not been examined but may now be investigated using the clones and methods developed in these analyses.

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