Detailed methods

Cell culture and transfections

HeLa cells were cultured in RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 µl/ml streptomycin and 10% (v/v) newborn calf serum (Invitrogen Corporation, Carlsbad, California, U.S.A.) and incubated at 37°C under a humidified atmosphere of 5% CO₂. Transfections were performed using Lipofectamine 2000 transfection reagent (Invitrogen Corporation) according to the manufacturer’s instructions.

Plasmid constructs

Near full-length proviral cDNA were PCR-amplified from the individuals’ peripheral blood mononuclear cells (PBMCs) at each time point as previously described [1]. First-exon tat sequences were amplified from the cDNA via a nested PCR, firstly using the external primers 5’-AGGAGTGGAAGCCATAATAA-3’ and 5’-ATTGCCACTGTCTTCTGCTGT-3’, and then the internal primers 5’-ACAAGTTTGTACAAAAAAGCAGGCTATGGAGCCAGTAGAT-3’ and 5’-ACCACCTTTGTACAAAAAAGCAGGCTATGGAGCCAGTAGAT-3’. Amplicons were cloned into vector pDONR201 (via attB sites, underlined in the internal primer sequences) and then into the expression vector pcDNA-DEST40 using Gateway cloning technology (Invitrogen Corporation) in a manner that places a stop codon immediately after the first-exon tat sequence. Up to five clones per time point were isolated, totalling 89 clones. Clones were sequenced using dye terminators version 3.1, translated and aligned against HIV-1 clone SF2 [2] Tat using the European Molecular Biology Open Source Software Suite programs TRANSEQ and SHOWALIGN [3]. Unique clones from each individual were then chosen for further study. The sequences of these unique clones are available from the GenBank database with accession numbers EU184659 – EU184684.

The one-exon tat sequence from SF2 was similarly cloned into pcDNA-DEST40 and used as a control for the transactivation study. The study was performed using a luciferase assay system,
which included a luciferase reporter plasmid constructed by cloning the HindIII to BamHI fragment of the SF2 long terminal repeat (LTR) into vector pGL3-basic (Promega Corporation, Madison, Wisconsin, U.S.A.) to form pGL3-LTR. A CMV immediate-early promoter-driven β-galactosidase expression plasmid (pCMV-lacZ) was also included as a control for transfection efficiencies.

**Phylogeny analysis**

The alignment of tat gene sequences of HIV-1 strains with reference subtypes was done using CLUSTAL-W from the GCG package, followed by minor manual adjustments considering protein sequences. Gaps (insertions/deletions) were stripped prior to phylogenetic and bootscan analyses, using the BioEdit Sequence Alignment Editor (version 5.9.0, T. Hall, North Carolina State University, Raleigh, NC). Phylogenetic analysis was done using the PHYLIP package (version 3.5c; J. Felsenstein, University of Washington, Seattle, WA) based on default parameters. Pairwise nucleotide evolutionary distances were calculated using the Kimura two-parameter model with a transition/transversion ratio of 2.0. Phylogenetic trees were then constructed using the neighbour-joining method. The reliability of the nodes was confirmed by bootstrap analysis with 100 replicates using Seqboot and Consense. Bootstrap values of ≥70% were considered to be significant for assignment of parenthood [4].

**Transactivation assay**

HeLa cells were co-transfected in 24-well plates with Lipofectamine 2000 and 300 ng each of individual’s tat expression plasmids, pGL3-LTR and pCMV-lacZ. The SF2 tat expression plasmid was similarly transfected as a control. Cells were harvested and lysed 24 h post-transfection by incubating in Glo Lysis Buffer (Promega Corporation) and manually aspirating with a pipette. Lysates were assayed for luciferase activity using the Steady-Glo luciferase assay kit (Promega Corporation) as per the manufacturer’s instructions. Light output was quantitated on a Wallac TriLux MicroBeta counter (PerkinElmer, Inc., Wellesley, Massachusetts, USA). Lysates were
assayed for β-galactosidase activity using the chlorophenol red-β-D-galactopyranoside (CPRG)-based assay [5].

**Statistical analyses**

The null hypothesis for the transactivation study was that the relative luciferase output of cells transfected with each individual’s tat clone was equal to the relative luciferase output of clone A1-1. The hypothesis was tested using Student’s t-test against a two-tailed distribution assuming homoscedastic variances.

**References**


