Genotypic and Phenotypic Heterogeneity in the U3R Region of HIV Type 1 Subtype C

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Abstract

Approximately 20% of all HIV-1 mother-to-child transmission (MTCT) occurs *in utero* (IU). In a chronic HIV infection, HIV-1 exists as a complex swarm of genetic variants, and following IU MTCT, viral genomic diversity is restricted through a mechanism that remains to be described. The 5' U3R region of the HIV-1 long terminal repeat (LTR) contains multiple transcription factor (TF) binding sites and regulates viral transcription. In this study, we tested the hypothesis that sequence polymorphisms in the U3R region of LTR are associated with IU MTCT. To this end, we used single template amplification to isolate 517 U3R sequences from maternal, placental, and infant plasma derived from 17 HIV-infected Malawian women: eight whose infants remained HIV uninfected (NT) and nine whose infants became HIV infected IU. U3R sequences show pairwise diversities ranging from 0.2% to 2.3%. U3R sequences from one participant contained two, three, or four putative NF- κ B binding sites. Phylogenetic reconstructions indicated that U3R sequences from eight of nine IU participants were consistent with placental compartmentalization of HIV-1 while only one of eight NT cases was consistent with such compartmentalization. Specific TF sequence polymorphisms were not significantly associated with IU MTCT. To determine if replication efficiency of the U3R sequences was associated with IU MTCT, we cloned 90 U3R sequences and assayed promoter activity in multiple cell lines. Although we observed significant, yet highly variable promoter activity and TAT induction of promoter activity in the cell lines tested, there was no association between measured promoter activity and MTCT status. Thus, we were unable to detect a promoter genotype or phenotype associated with IU MTCT.

Introduction

WOMEN ACCOUNT FOR 69% OF THE estimated 23.5 million people living with HIV-1 in sub-Saharan Africa.¹ In the absence of interventions, such as antiretroviral therapy, replacement feeding, and elective cesarean sections, approximately one-third of the children born to HIV-infected women will become HIV infected.² HIV-1 mother-to-child transmission (MTCT) accounts for 90% of new pediatric HIV infections.^{3,4} MTCT can occur *in utero*, intrapartum (during delivery), or postpartum (through breastfeeding); *in utero* (IU)

transmission comprises approximately 20% of MTCT.⁵ Viral genetic diversity is severely restricted during IU MTCT,⁵ and previous work from our group suggests an association between Env polymorphisms and IU MTCT. In addition to an Env-mediated phenotype, it is plausible that IU-transmitted virions also have a replication phenotype, manifest through sequence polymorphisms in the HIV promoter.

HIV-1 provirus contains identical long terminal repeats (LTRs) on the 5' and 3' ends of the viral genome that are formed during reverse transcription, when the HIV-1 genome is converted from a single-stranded RNA to double-stranded

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DNA.⁶ Although the LTRs are identical, the 5' LTR functions as the promoter for the viral genome and the 3' LTR regulates polyadenylation of viral RNA transcripts as well as other functions.⁶ Each LTR contains approximately 640 nucleotides and is divided into three regions: U3, R, and U5. The U3 and R regions are subdivided into a modulatory domain, which contains ETS1, NFAT, C/EBP, and TCF1a binding sites,⁷⁻⁹ an enhancer domain, which contains NF- κ B binding sites,¹⁰ and a core domain, which contains three SP1 binding sites and the TATA box.¹¹ Thus, the 5' LTR serves as the point at which host transcription factors, basal transcriptional machinery, and viral proteins converge to modulate viral transcription.

Conservation of specific transcription factor binding sites within U3R/LTR is important for transcriptional regulation; for example, the TATA box and ETS1 binding elements are essential for both basal and TAT-induced LTR transcription.^{12,13} Sequence variations within U3R could alter the interactions with host transcription factors and consequently alter viral replication. For example, C/EBP binding site polymorphisms have been correlated with promoter activity in monocytic and brain-derived populations.^{7,14} In addition, several studies have indicated that polymorphisms within the κ B enhancer sites, such as noncanonical consensus binding sequences or alterations in the number of κ B sites, can alter gene expression up to 5-fold.¹⁵⁻¹⁹ Overall, LTR sequence heterogeneity, including nucleotide insertions, deletions, and polymorphisms, has been characterized within other HIV-specific contexts, including longitudinal studies of HIV infection, transmission via injection drug use, and heterosexual transmission.²⁰⁻³²

In this study, we characterized genetic heterogeneity within HIV-1 subtype C U3R sequences obtained from nine *in utero* transmission and eight nontransmission cases and experimentally characterized promoter activity associated with the U3R sequences. The working hypothesis postulates that sequence polymorphisms in the U3R region of LTR are associated with IU MTCT.

Materials and Methods

Study participants

Clinical isolates were obtained from participants enrolled in the Malaria, HIV, and Pregnancy cohort (MHP) from 2001 to 2004.³³⁻³⁶ Institutional Review Board (IRB) approval for the parent study was obtained at the University of North Carolina at Chapel Hill and the Malawi College of Medicine Research Ethics Committee; additional approval for this substudy was received from The Ohio State University IRB. All participants provided informed, written consent. Women were ineligible for the MHP cohort if they were in labor, were under 15 years of age, or had hypertension, multiple gestations, altered consciousness, or were participating in other research studies. Participants were considered HIV positive if two independent HIV-1 serological tests were positive.³⁴ HIV-positive mothers, on the onset of delivery, and their infants, at birth, were given nevirapine according to the HIVNET012 protocol.³⁷ Pediatric HIV infection was characterized as IU transmission if infants were HIV-1 DNA positive within 48 h after delivery and nontransmission (NT) if initial and 6-week postdelivery tests were HIV-1 DNA negative.³⁸ Maternal CD4⁺ T cell levels were quantified by FACSscan (Becton Dickinson, San Jose, CA) and HIV-1 RNA concentration was quantified using the Amplicor HIV-1 Monitor v1.5 (Roche Diagnostics, Branchburg, NJ).

Clinical sample selection

Samples were obtained from maternal peripheral blood, placental biopsies, placental plasma, umbilical cord blood, and infant heel stick plasma. Peripheral blood was collected through venipuncture and plasma was isolated with standard protocols. Placental blood was collected as described by Kumar *et al.*³⁶ Placental plasma and placental biopsy samples were combined into a single category termed "placenta." Infant blood collected from heel-pricks or the umbilical cord vein were combined into a single category termed "infant."⁵ Selection of the nine IU cases was based on the ability to polymerase chain reaction (PCR) amplify *vpu-rev-env-u3r* cassettes, as described in Kumar *et al.*³⁶ For this study, isolated NT(s) were matched to IU cases based on CD4⁺ T cell counts and viral load. Participant 2400 delivered fraternal twins; the placental and infant sequences from the two infants were combined for this analysis. A minimum of seven U3R amplicons were sequenced from each tissue compartment.

Viral extraction, cDNA synthesis, and single template amplification

Viral RNA was extracted from plasma using the QIAamp viral RNA mini kit, according to the manufacturer's protocol. RNA was extracted from 100 mg of placental biopsies, according to Kumar *et al.*³⁶ RNA was [reverse] transcribed to cDNA with Superscript III (Invitrogen, Carlsbad, CA) using 5.0 μ l extracted viral RNA, 0.25 μ M HIV-1 subtype C [OFM19] primer 5'-GCACTCAAGGCAAGCTTTATTGAGGCTTA-3' (nt 9604 to 9632 of HXB2 sequence),³⁹ and 0.5 mM deoxynucleotide triphosphates (dNTPs) (Invitrogen, Carlsbad, CA) according to the methods of Salazar-Gonzalez *et al.*^{36,39,40} Single template amplification of clinical samples was performed according to documented protocols.^{36,39,41,42} All reactions used High Fidelity Platinum Taq (Invitrogen, Carlsbad, CA) with subtype C-specific outer primers: [VIF] 5'-GGGTTTATTA CAGGGACAGCAGAG-3' (nt 4900 to 4923 of HXB2 sequence) and [OFM19] 5'-GCACTCAAGGCAAGCTTTATTGAGGCTTA-3' (nt 9604 to 9632 of the HXB2 sequence).³⁹ A U3R-specific nested PCR used the following inner primers: [JKLTR4] 5'-CTAGCTAGCGGCTTAAGCAGTGGGT-3' (nt 9564 to 9612 of the HXB2 sequence) and [JKU3R] 5'-CGGGGTACCGGGG-GACTGGAWGGGGTTA-3' (nt 9077 to 9097 of the HXB2 sequence).

DNA sequencing

Prior to sequencing, U3R amplicons were incubated with alkaline phosphatase (Roche) and exonuclease I (New England Biolabs) at 37°C for 30 min, followed by enzyme inactivation at 95°C for 5 min.⁴³ U3R amplicons were directly sequenced with JKLTR4 (forward primer) and JKU3R (reverse primer), providing a 2-fold coverage of the U3R region, from start site of forward primer through the TAR region [nt 9077 to 9612 of the HXB2 reference sequence] (Genewiz, Inc., South Plainfield, NJ). Sequence ends were trimmed to remove ambiguous nucleotides, and chromatograms were manually examined for multiple peaks. A total of 568 U3R sequences were collected from nine IU transmission and eight nontransmission participants, 51 (~9%) of which were discarded from further analysis owing to double peaks or low-quality sequencing results. In all, 517 sequences were

assembled using Geneious software (www.geneious.com) and examined using the Los Alamos National Laboratory HIV database quality control tools (www.hiv.lanl.gov). No laboratory isolate contamination was detected and subtype C was inferred for all sequences (data not shown). U3R sequences were examined for APOBEC hypermutation using the hypermut tool (<http://hiv.lanl.gov>). The U3R sequences have been deposited into GenBank (<http://ncbi.nlm.nih.gov>) under accession numbers JQ778319–JQ778843.

Sequence alignment, diversity, and phylogenetic tree construction

Cleaned U3R sequences were aligned using Multiple Sequence Comparison Using Log-Expectation (MUSCLE).⁴⁴ To infer epidemiological linkage, the alignment was subjected to a RAXML tree search⁴⁵ with a general time-reversible substitution model with an estimate of the proportion of invariable sites (GAMMAI). The best scoring tree after 100 replicates is shown in Fig. 1. Alignments and phylogenetic trees for the U3R sequences from each mother–infant pair were generated in the same way and are shown in Supplementary Fig. S1 (Supplementary Data are available online at www.liebertpub.com/aid). The phylogenetic reconstruction depicts maternal sequences as blue squares, placental sequences as red circles, and infant sequences as green diamonds. All trees used IU transmission participant 2400 maternal sample #42 as an outgroup to root the tree, except for participant 2400, which is rooted using IU transmission participant 1468 placental sample #2 as an outgroup. Sequences on the phylogenetic tree marked by an asterisk were selected for cloning (see below). Polymorphisms, insertions, and gaps were denoted with the default Highlighter scheme, as follows: A = green, T = red, G = orange, C = light blue, gaps = gray. Swarm diversities of mother–placental pairs were determined using DIVEIN,⁴⁶ which compares each variant within each population in a pairwise fashion. Subtype C sequences from heterosexual transmission cases (accession numbers: FJ496185–FJ496214)⁴⁷ as well as subtype B sequences from vertical and MSM transmission cases (accession numbers: DQ848353–DQ848563, FJ495818–FJ496184, JN024126–JN024491, and JN944911–JN944934)^{47–50} were downloaded from GenBank for comparison purposes. Diversity levels were tested for correlations with maternal CD4⁺ T cell counts and peripheral viral loads using Spearman's correlation analysis implemented with Graphpad Prism. Shannon Entropy values were calculated with the entropy tool available from the Los Alamos HIV Database (<http://hiv.lanl.gov>). Variations within the ETS1, TATA box, TCF1a, NFAT, C/EBP, and NF- κ B binding sites were established with the following consensus sequences indicated by the HXB2 sequence within the HIV sequence compendium (www.hiv.lanl.gov): ETS1 (5'-CATCCG-3'), TATA box (5'-TATAA-3'), TCF1a (5'-GTA CTTCAGAACTGCTG-3'), NFAT (5'-GGAAA-3'), C/EBP (5'-AGCTTTCTACAA-3'), and NF- κ B (5'-GGGACTTTCC-3'). Evidence of intrapatient sequence recombination was tested using GARD with default/suggested parameters (www.datamonkey.org).^{51,52}

Compartmentalization testing

Phylogenetic compartments were detected using a Slatkin–Maddison⁵³ test. Each nontransmission and IU transmission

FIG. 1. Phylogenetic reconstruction indicating epidemiological linkage of 517 U3R sequences amplified from 17 Malawian mother-placental pairs. Participant IDs are displayed to the right of U3R sequences amplified from each corresponding mother-placental pair. Parameters of alignment and phylogenetic reconstruction are described in the Materials and Methods section.

mother–placenta–infant pair was tested for compartmentalization independently. The Hudson nearest-neighbor test,⁵⁴ a robust measurement when unequal numbers of sequences are compared,⁵⁵ was also used to test for compartmentalization. All compartmentalization tests included duplicate sequences. Both tests were performed using the standard implementation in the HYPHY analysis package⁵² with 1,000 null simulation replicates and default/suggested options otherwise.

U3R cloning

To determine replication activity of the U3R promoter sequences, 5–10 U3R sequences were selected from the phylogenetic trees of each mother–placenta–infant pair, as denoted with an asterisk in Supplementary Fig. S1 (totaling 90 U3R-*luc2* constructs). U3R promoter sequences were selected for cloning based on position within the phylogenetic reconstruction and tissue microenvironment. Selected U3R amplicons were directionally cloned into a promoterless *luc2* vector (pGL4.10, Promega, Madison, WI) using *NheI* and *KpnI* restriction sites engineered into the inner PCR primers (JKLTR4 and JKU3R). Owing to the presence of an *NheI* restriction site within selected U3R sequences, samples from Participants 1639 and 3321 used primer JKLTR4-XHOI (5'-CTACTC GAGGGCTTAAGCAGTGGGT-3') instead of JKLTR4. After double digestion with *KpnI* and *NheI* or *KpnI* and *XhoI* (New England Biolabs), U3R sequences were ligated into pGL4.10 using T4 ligase (New England Biolabs). Constructs were transformed into MAX Efficiency DH5 α Chemically Competent Cells (Invitrogen, Carlsbad, CA) per the manufacturer's instructions. Plasmids were purified using the Qiagen Plasmid Miniprep Kit (Qiagen, Valencia, CA). Correct insertion of the U3R promoter was verified by sequencing with pGLRVprimer3 (5'-CTAGCAAATAGGCTGTCCC-3'), which is located approximately 50 nucleotides upstream of the U3R insert. All sequences were inserted in the proper orientation (data not shown).

Cell lines

293T cells (ATCC CRL-11268) were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and penicillin-streptomycin (Gibco, Life Technologies, Grand Island, NY). THP-1 cells were obtained through the AIDS research and reference reagent program, division of AIDS, NIAID, NIH: THP-1ATCC from Drs. Li Wu and Vineet N. KewalRamani.⁵⁶ Jurkat cells were obtained through the AIDS research and reference reagent program, division of AIDS, NIAID, NIH: Jurkat Clone E6-1 from Dr. Arthur Weiss.⁵⁷ THP-1 and Jurkat cells were maintained in Roswell Park Memorial Institute Medium (RPMI) with 10% fetal bovine serum and penicillin-streptomycin. BeWo cells (ATCC CCL-98) were maintained in DMEM/Ham's F-12 medium with 10% FBS and penicillin-streptomycin (Gibco, Life Technologies, Grand Island, NY).

U3R-*luc* activity assay

U3R-pGL4.10 constructs were transfected into 293T, Jurkat, THP-1, or BeWo cells using the Expressfect Transfection Reagent (Denville Scientific, Inc.) or TransIT-Jurkat Transfection Reagent (Mirus Bio, Madison, WI) per the manufacturer's instructions (totaling two biological replicates per cell line). Cells were seeded in 96-well dishes, in triplicate, at 3×10^4 cells/well in 100 μ l DMEM or RPMI containing 10% FBS and without penicillin-streptomycin. Nonadherent cells (THP-1 and Jurkat) were seeded into v-bottom 96-well plates. Cells were incubated at 37°C, and 24 h later transfected with 150 ng of each U3R-pGL4.10 plasmid $\pm$ 150 ng of pC-Tat.BL43.CC plasmid; pC-Tat.BL43.CC, a mammalian expression vector containing HIV-1 subtype C *tat*, was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID,

NIH from Dr. Udaykumar Ranga. pBlue3'LTR-luc-C plasmid, which contains the LTR from a subtype C isolate, served as a positive control (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr. Reink Jeeninga and Dr. Ben Berkhout). pGL4.10 backbone, which does not contain a promoter, was used as a negative control. To control for differential transfection efficiency, all U3R-luc constructs were cotransfected with 15 ng pGL4.74, which contains a CMV-driven hRLuc [*Renilla*] gene.

Transfected cells were incubated at 37°C for 48 h, after which time the medium was aspirated; nonadherent cells were centrifuged at $295 \times g$ for 5 min prior to media aspiration. Cells were washed with 100 μ l phosphate-buffered saline (PBS) and lysed with 50 μ l $1 \times$ passive lysis buffer (25 mM Tris-phosphate pH 7.8, 2 mM DTT, 2 mM 1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid, 10% glycerol, 1% Triton X-100); plates were freeze-thawed three times. Twenty microliters of each lysate was placed into an opaque 96-well plate, and luciferase activity was quantified on a dual injection luminometer, Glomax-96 Microplate Luminometer (Promega), using 100 μ l of luciferase buffer (previously described by Dyer *et al.*⁵⁸) with a 2-s integration time and a 5-s read time. *Renilla* activity was quantified with 100 μ l Stop and Glo reagent (Promega, Madison, WI). U3R-luc constructs with activity less than five times the negative control were considered nonfunctional and were discarded (three cloned promoters). The data are presented as normalized activity, which were obtained by dividing firefly luciferase activity by its corresponding *Renilla* activity (*Renilla* light units were divided by 1,000 beforehand). TAT induction was determined by dividing the luciferase activity with TAT addition by the luciferase activity without TAT addition. Data were plotted using Graphpad Prism, and statistical differences between the groups were determined using either a paired Wilcoxon matched pairs test or unpaired Mann-Whitney test, as appropriate (Graphpad Prism).

nef open reading frame sequence analysis

nef sequences were aligned using HIVAlign (<http://hiv.lanl.gov>). The alignments were adjusted for codon alignment and translated using Geneious (www.geneious.com). Codons under positive and negative selection were identified as follows. Three different approaches were used to identify codons under negative selection: single likelihood ancestor counting (SLAC), fixed-effects likelihood (FEL), and internal fixed-effects likelihood (IFEL). SLAC and FEL detect sites under selection at the external branches of the phylogenetic tree, while IFEL identifies sites along the internal branches.^{59–61} Sites were classified as negatively selected with $p < 0.05$ by at least two methods. Codons under positive selection were identified using a mixed-effects model of evolution (MEME) ($p < 0.05$). MEME uses an algorithm comparable to FEL, with increased sensitivity in identifying evolution due to positive selection in a small proportion of branches.

Results

Interpatient and inpatient heterogeneity within U3R sequences

We used single-template amplification to isolate HIV-1 U3R regions from maternal (peripheral plasma), placental (placental plasma and placental tissue biopsies), and infant (umbilical

TABLE 1. SUMMARY OF CLINICAL SAMPLES FROM 17 MOTHER-PLACENTAL PAIRS

Participant ID	Transmission status	CD4 ⁺ T cell count	Peripheral viral load (copies/ml)	No. U3R sequences amplified		
				Maternal plasma	Placenta	Infant plasma
1639	NT	375	160,259	7	19	NA
1669	NT	485	81,178	8	13	NA
1702	NT	273	NA	12	7	NA
2437	NT	445	13,529	14	9	NA
2502	NT	38	154,972	13	17	NA
2512	NT	222	24,301	7	12	NA
2544	NT	NA	587,702	18	15	NA
3274	NT	103	NA	16	13	NA
1468	IU	274	36,453	20	8	NA
1472	IU	397	51,551	14	13	15
1485	IU	329	125,813	11	22	11
1646	IU	95	902,778	11	11	4
1851	IU	761	NA	16	19	NA
2080	IU	91	165,000	11	9	NA
2400	IU	439	98,793	13	36	23
2797	IU	399	NA	16	12	9
3321	IU	220	NA	9	10	2

NT, nontransmission; IU, *in utero* transmission; NA, samples not available.

cord blood and heel-prick plasma) microenvironments. Table 1 describes the clinical characteristics of the participants including transmission status, peripheral CD4⁺ T cell count, and peripheral viral load. Of the 517 sequences examined, none contained evidence of APOBEC-induced hypermutation and three mother-placenta-infant pairs demonstrated signs of one possible recombination breakpoint (data not shown). All U3R sequences were aligned and a maximum-likelihood tree with general time-reversible substitution was constructed (Fig. 1). The percent pairwise diversity of the U3R sequences obtained from the participants ranged from 0.2% to 2.3% (Supplementary Fig. S2) with an overall 8% diversity among all 517 U3R sequences from the 17 participants; the IU transmission cases were marginally more diverse than the NT transmission cases (IU=1.5% versus NT=1.3%, $p < 0.0001$, Mann-Whitney test). No correlation between pairwise diversity and either peripheral viral load ($r = -0.17$, $p = 0.6$) or peripheral CD4⁺ T cell count ($r = -0.05$, $p = 0.9$) was detected.

Next, we analyzed the correlation between U3R diversity and tissue of origin. In seven of the eight NT participants, median U3R diversity in placental isolates is equal to or lower than the U3R diversity in maternal peripheral isolates (Fig. 2). Among all IU cases, the median U3R sequence diversity in placental isolates was lower than the median diversity in maternal peripheral isolates. Three of the five infant populations were less diverse than the corresponding maternal and placental populations. Among IU participants, overall median U3R sequence diversity was highest in maternal peripheral blood (1.1% on average for each participant) with subsequently decreasing values in the placenta (0.6% on average for each participant) and infant (0.2% on average for the five participants for which infant sequences were available), $p < 0.0001$, Mann-Whitney. In summary, U3R sequence diversity in the placenta was less than in peripheral blood for both nontransmission and IU isolates.

Sequence diversity within the nef-overlapping and non-coding regions of the 517 U3Rs is shown in Fig. 3; Shannon

entropy values indicate that there is a wide range of variation throughout the U3R. A number of canonical binding sites are conserved within the population, for example, the TATAA box (99% of sequences) and ETS1 (97% of sequences), while binding sites, such as the TCF1a, C/EBP, NFAT, NF- κ B, and SP1 sites, contain sequence variation within as much as 55% of the sequences analyzed. More specifically, a 30-64 nucleotide insertion overlapping the TCF1a binding site, when compared to the HXB2 reference sequence, is observed within the 517 U3Rs from this study. Fifteen sequences contained a 35-nucleotide insertion containing a fourth canonical NF-(B binding site and eight sequences contained only two canonical NF-(B binding sites. Overall, a wide range of sequence variation throughout the U3R sequences is observed with nucleotide insertions found within and/or following the TCF1a as well as within the NF- κ B binding site region.

Evidence of U3R compartmentalization for IU transmission cases

Sequence alignment and phylogenetic reconstruction show that each participant's viral population formed a distinct clade, confirming epidemiological linkage of matched mother-placenta-infants sequences (Fig. 1 and Supplementary Fig. S2). Rakes of identity, indicators of active viral replication⁶² containing a minimum of four HIV-1 variants, were noted in four of the eight nontransmission cases and three of the nine IU cases. In seven of the eight NT participants, the U3R sequences from different tissues were intermingled, while U3R sequences from eight of the nine IU participants showed statistically significant segregation into tissue-specific populations (e.g., maternal peripheral blood; placental blood). Tree-based (Slatkin-Maddison) and distance-based (Hudson nearest-neighbor) analyses confirmed placental compartmentalization in one out of the eight NT and eight out of the nine IU participants (Fisher's exact probability test, $p = 0.003$, Table 2). Thus, HIV-1 variants from cases of IU transmission form a distinct population compartment associated with the placenta.

FIG. 2. U3R sequence diversity decreases in the placenta. Pairwise sequence diversity, stratified by tissue origin, of sequences isolated from (A) nontransmission cases, $n=8$, and (B) *in utero* transmission cases, $n=9$. Dots represent median values per participant. Gray lines connect matched samples extracted from individual subjects; black lines represent median value of all samples. Statistical significance between overall median values was tested with the Mann-Whitney test.

Basal and TAT-induced transcription are highest in placental cytotrophoblast-like cells

TAT activates viral transcription at U3R through the recruitment of the PTEF-b complex and other cellular host factors.^{63,64} To assess transcriptional activity in several cellular contexts, we determined basal and TAT-induced activity of the 90 U3R-luciferase constructs in 293T, Jurkat, THP-1, and BeWo cells. Median U3R-mediated basal transcription was lowest in 293T [4,959 normalized relative luciferase light units (RLU)] and THP-1 (4,347 RLU) cells, followed by Jurkat (77,032 RLU) and BeWo cells (255,260 RLU). Tat-induced transcription was lowest in HEK-293T cells (median induction = 1.8-fold), followed by Jurkat (6.6-fold), THP (14.8-fold), and BeWo cells (23.8-fold) (Fig. 4).

Transcriptional activities of variants from nontransmission and IU transmission cases are comparable

Basal and TAT-induced transcription levels of the selected U3R-luciferase constructs within the aforementioned cell lines were reanalyzed based on transmission status and tissue

source (e.g., maternal plasma, placenta). Within BeWo cells, *luc*-based transcriptional activity of NT constructs is 1.4-fold higher than IU constructs ($p=0.007$); the biological significance of this small difference is unknown (Fig. 5). Overall, the U3R-luc constructs had a relatively wide distribution of luciferase activity, covering three- $\log_{10}$ normalized units. Stratification of the U3R-luc constructs by transmission status or tissue origin did not identify any meaningful differences in activity between the groups, regardless of cell type (Supplementary Figs. S3 and S4).

Sequence polymorphisms within the *nef* open reading frame (ORF) do not significantly alter amino acid sequence within 3' functional domains

To determine whether U3R polymorphisms might affect *nef* function, we analyzed *nef* sequences in the region overlapping U3R (positions 9,094–9,418, HIV-1 HXB2 reference sequence). The overlapping region begins with *nef* codon 99, and thus does not include defined functional regions including the well-defined (PxxP)₃ polypurine tract domain, as well as domains required for myristoylation and interaction with cellular factors such as PACS1. Within the 517 U3R nucleotide sequences, *in silico* translation of *nef* indicated that both synonymous and nonsynonymous polymorphisms occurred (data not shown). However, overall conservation of an amino acid sequence in comparison to a consensus sequence was high, with frequency of conservation of consensus amino acids across the Nef C-terminal 108 codons averaging 90% (EntropyOne, LANL). The averaged conservation of amino acid sequences located in functional domains was higher (94%) than positions located outside reported functional domains (82%).⁵⁹

To determine whether selection pressures contribute to decreased Nef diversity despite increased nucleotide polymorphisms, synonymous (dS) and nonsynonymous (dN) substitution rates were analyzed for each codon using models of molecular evolution. A mixed effects model of evolution (MEME) capable of measuring both episodic and pervasive positive selection identified eight codons predicted to be under positive (diversifying) selection ($p < 0.05$).⁶⁵ None of these positions was located in defined functional domains, including residues required for maintaining Nef structure and interacting with cellular adapters such as PAK.⁵⁹ Similar site-by-site analysis, using three additional programs (see Materials and Methods), identified 37 codons reported under negative (purifying) selection.⁶¹ These positions were interspersed throughout the ORF with many localized in Nef functional domains, including those necessary for binding to CD4 and β -COP.⁵⁹

These results show that conservation of amino acid sequences in the portion of the *nef* ORF overlapping U3R is high, with most substitutions resulting in purifying selection ($p=0.0005$, Fisher's exact test). Therefore, the majority of the polymorphisms in the *nef* ORF result in silent mutations.

Discussion

The objective of this study was to test the hypothesis that there is an association between U3R genotypic variance and HIV-1 IU mother-to-child transmission. We observed (1) a decrease in viral U3R diversity from maternal to placental/infant environments, (2) HIV-1 placental compartmentalization only

FIG. 3. Sequence diversity varies throughout the U3R. Shannon entropy values are represented along the *y*-axis for each nucleotide position within the U3R (start through TATA box) of the aligned 517 sequences; the *x*-axis indicates the nucleotide position within U3R. Select transcription factor binding sites are indicated below the *x*-axis; the *nef* open reading frame is illustrated by gray shading.

among IU transmission cases, (3) TAT induction that was highest in BeWo cells and lowest in HEK-293T cells, and (4) no difference in transcriptional activity among U3R sequences stratified by MTCT status or tissue microenvironment.

In a similar study, *Mehta et al.*⁴⁸ showed that HIV-1 subtype B sequences obtained from six vertical transmission cases had low viral heterogeneity, based on DNA evolution rates and conserved κ B binding sites. We reanalyzed the *Mehta* sequences using the methods outlined in this article and our results indicate that the 218 U3R sequences were shown to have pairwise sequence diversities from 1.4% to 5.3% with a population average of 7.7% diversity,⁴⁸ which is similar to the 8% pairwise diversity observed in this study. Although the pairwise diversity was similar, Shannon entropy plots revealed that the *Mehta et al.* sequences contained a frequent 30-nucleotide insertion between the TCF1a and NF- κ B binding sites and single polymorphisms throughout the U3R, but no other sequence alterations. The 517 U3Rs within this study contained the insertions and single polymorphisms mentioned previously; however, they contain additional unique alterations within the NF- κ B binding site region (Fig. 3). Overall, sequence diversities were similar, although sequence analyses reveal divergent sequence polymorphisms among the two study populations.

To put the observed sequence diversity from this study in perspective, we compared our results to the results of studies

from men who have sex with men (MSM) or heterosexual transmission that also used single genome amplification. The sequences from MSM and heterosexual transmission shared a multitude of single polymorphisms as well as a 30-64 nucleotide insertion either adjacent or bifurcating the TCF1a binding site, a frequent insertion site within this study's 517 sequences. Of the 517 U3Rs obtained in this study, 15 sequences contained an approximately 35-nucleotide insertion conferring a fourth NF- κ B binding site and eight sequences contained a deletion creating a two NF- κ B binding site region, which is divergent from the conservation of the NF- κ B binding site region found within the MSM and heterosexual transmission studies.^{47,49,50} Overall, this study's U3R sequences contain common sequence alterations within the TCF1a and Sp1 binding sites, but in contrast contain unique alterations within the NF- κ B binding site region.

Although we were unable to ascribe a promoter-based phenotype to the observed genotypic heterogeneity, U3R substantially overlaps the *nef* ORF. The *nef* gene encodes a 27-kDa N-terminal myristoylated protein that contributes to HIV pathogenesis through modulation of cellular signaling pathways.⁶⁶ While the Nef amino acid sequence across loop regions has been shown to be polymorphic within subtypes,⁶⁷ this polymorphism has not been associated with HIV disease progression^{59,67} and previous studies have shown that *nef* is conserved during MTCT.⁶⁰ Our analyses demonstrated that

TABLE 2. EVIDENCE OF U3R COMPARTMENTALIZATION WITHIN *IN UTERO* TRANSMISSION CASES

Participant	Transmission status	Maternal vs. placental	
		Maddison-Slatkin	Hudson nearest-neighbor
1639	NT	0.5	0.3
1669	NT	0.8	0.8
1702	NT	0.3	0.07
2437	NT	0.05	0.02
2502	NT	0.06	0.5
2512	NT	0.3	0.8
2544	NT	1.0	0.7
3274	NT	0.7	0.6
1468	IU	<0.001	<0.001
1472	IU	<0.001	0.4
1485	IU	<0.001	<0.001
1646	IU	0.5	0.05
1851	IU	<0.001	0.001
2080	IU	0.1	0.003
2400	IU	0.07	0.05
2797	IU	0.006	0.005
3321	IU	0.2	0.4

Results of tree-based (Slatkin-Maddison) and distance-based (Hudson nearest-neighbor) tests. Statistically significant compartmentalization is indicated by italics ($p \leq 0.05$).

NT, nontransmission; IU, *in utero* transmission.

Nef amino acid sequences were highly conserved across functional domains, averaging 94% conservation in comparison to a consensus sequence across defined functional domains. Overall, the majority of the polymorphisms in the *nef* ORF result in silent mutations that likely do not affect Nef function. In addition to serving as a promoter and its overlap with *nef*, U3R polymorphisms could also regulate

FIG. 4. TAT induction of subtype C U3R-luciferase constructs varies across cell lines. Each gray dot represents an individual U3R construct; median values are demonstrated by the black horizontal line. HEK-293T (epithelial), THP (monocytic), Jurkat (CD4⁺ T cell), and BeWo (choriocarcinoma) cells are displayed. Statistical significance was determined with the Wilcoxon matched-pairs signed rank test.

FIG. 5. Transcriptional activity of U3R-luciferase constructs ranges three-log₁₀ in BeWo cells. All normalized luciferase light units were categorized as nontransmission promoter or *in utero* (IU) transmission promoter with and without TAT. Each gray dot represents an individual U3R construct; median values are demonstrated by the black vertical line. Statistical significance [nontransmission (NT) and IU comparison] was determined with the nonparametric Mann-Whitney test.

mRNA stability or the recently identified antisense RNA transcription.⁶⁸

The placenta is a short-lived microenvironment composed of tree-like villi surrounded by maternal blood; the tree-like villi are multilayered, a continuous syncytiotrophoblast layer covering discontinuous cytotrophoblast cells⁶⁹; this unique subset of cells is a potential target for HIV replication. U3R sequences isolated from IU transmission cases showed strong evidence of placental compartmentalization (Table 2). HIV-1 compartmentalization has been shown in the brain,^{70,71} kidney,⁷² semen,⁷³ placenta,^{36,74} metastatic tissue,⁷⁵ and breast milk.^{76,77} This finding is consistent with a previous study from our group, which identified placental compartmentalization of *env* sequences from an overlapping set of clinical isolates.³⁷ We used a placental cytotrophoblastic cell line (BeWo) to model the placental environment. We observed that TAT induction of the U3R variants was highest in BeWo and Jurkat cells, when compared to the epithelial or monocytic cell lines. The differences in TAT induction do indicate that the promoters have higher transcription in the placental cell line when induced with TAT (Figs. 4 and 5); this could be due to levels of TAT-recruited cellular components that regulate transcript elongation by the RNA polymerase II, Cyclin T1, and CDK9⁷⁸ complex or other host cellular components. The component(s) responsible for this increased transcriptional activity in CD4⁺ T cells and cytotrophoblastic cells is unknown.

In summary, U3R sequences extracted from 17 mother-infant pairs contained a high level of sequence diversity, most notably within the NF- κ B binding site region, in contrast to heterosexual and MSM transmission populations. Within the cell lines tested, specific polymorphisms and U3R transcriptional activity did not correlate with transmission status or tissue of origin, although a three-log₁₀ transcriptional difference is observed for the *U3R-luc2* constructs.

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Author Disclosure Statement

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