UCSF
UC San Francisco Previously Published Works

Title

Susceptibility of Human Endogenous Retrovirus Type K to Reverse Transcriptase Inhibitors

Permalink

https://escholarship.org/uc/item/9vt2h3jj

Journal

Journal of Virology, 91(23)

ISSN

0022-538X

Authors

Contreras-Galindo, Rafael
Dube, Derek
Fujinaga, Koh
et al.

Publication Date

2017-12-01

DOI

10.1128/jvi.01309-17

Peer reviewed

Susceptibility of Human Endogenous Retrovirus Type K to Reverse Transcriptase Inhibitors

Rafael Contreras-Galindo,[a] Derek Dube,[a,e] Koh Fujinaga,[f] Mark H. Kaplan,[a] David M. Markovitz[a,b,c,d]

Department of Internal Medicine[a] and Programs in Immunology,[b] Cancer Biology,[c] and Cellular and Molecular Biology,[d] University of Michigan, Ann Arbor, Michigan, USA; University of Saint Joseph, West Hartford, Connecticut, USA[e]; University of California at San Francisco, San Francisco, California, USA[f]

ABSTRACT Human endogenous retroviruses (HERVs) make up 8% of the human genome. The HERV type K (HERV-K) HML-2 (HK2) family contains proviruses that are the most recent entrants into the human germ line and are transcriptionally active. In HIV-1 infection and cancer, HK2 genes produce retroviral particles that appear to be infectious, yet the replication capacity of these viruses and potential pathogenicity has been difficult to ascertain. In this report, we screened the efficacy of commercially available reverse transcriptase inhibitors (RTIs) at inhibiting the enzymatic activity of HK2 RT and HK2 genomic replication. Interestingly, only one provirus, K103, was found to encode a functional RT among those examined. Several nucleoside analogue RTIs (NRTIs) blocked K103 RT activity and consistently inhibited the replication of HK2 genomes. The NRTIs zidovudine (AZT), stavudine (d4T), didanosine (ddl), and lamivudine (3TC), and the nucleotide RTI inhibitor tenofovir (TDF), show efficacy in blocking K103 RT. HIV-1-specific nonnucleoside RTIs (NNRTIs), protease inhibitors (PIs), and integrase inhibitors (IIs) did not affect HK2, except for the NNRTI etravirine (ETV). The inhibition of HK2 infectivity by NRTIs appears to take place at either the reverse transcription step of the viral genome prior to HK2 viral particle formation and/or in the infected cells. Inhibition of HK2 by these drugs will be useful in suppressing HK2 infectivity if these viruses prove to be pathogenic in cancer, neurological disorders, or other diseases associated with HK2. The present studies also elucidate a key aspect of the life cycle of HK2, specifically addressing how they do, and/or did, replicate.

IMPORTANCE Endogenous retroviruses are relics of ancestral virus infections in the human genome. The most recent of these infections was caused by HK2. While HK2 often remains silent in the genome, this group of viruses is activated in HIV-1-infected and cancer cells. Recent evidence suggests that these viruses are infectious, and the potential exists for HK2 to contribute to disease. We show that HK2, and specifically the enzyme that mediates virus replication, can be inhibited by a panel of drugs that are commercially available. We show that several drugs block HK2 with different efficacies. The inhibition of HK2 replication by antiretroviral drugs appears to occur in the virus itself as well as after infection of cells. Therefore, these drugs might prove to be an effective treatment by suppressing HK2 infectivity in diseases where these viruses have been implicated, such as cancer and neurological syndromes.

KEYWORDS HERV-K, reverse transcriptase inhibitors, AZT, NRTIs, NtRTIs, NNRTIs

Human endogenous retroviruses (HERVs) are remnants of ancient infections of the germ line that make up approximately 8% of the modern human genome (1–3). Most of these proviruses have been inactivated by DNA mutations. However, the most

recent of those infections, attributed to the human endogenous retrovirus type K (HERV-K) HML-2 family, here referred to as HK2, have led to preserved provirus sequences that are partially intact and can code for all the viral proteins (2, 3). Nonetheless, a complete replication-competent HK2 virus has not yet been identified (4, 5). The genome of HK2 proviruses consists of four retroviral genes (gag, pro, pol, and env), flanked by two long terminal repeats (LTRs). Approximately 3,000 HK2 sequences are found in the modern human genome (6, 7). Of these, ~2,500 are solitary LTRs (solo LTRs), which originated by recombination between the flanking LTRs of full-length proviruses, leading to the removal of the internal genes (8). About 117 HK2 sequences exist as full-length proviruses (3, 9–12). We have additionally discovered over 100 or more HK2 in centromeric and pericentromeric regions (11–13).

As HK2 is one of the most recent retrovirus families to enter the genome, it is not surprising that HK2 is transcriptionally active (14–21). Under certain circumstances, viral RNA, proteins, and virus-like particles (VLPs) are produced, especially in breast cancer, leukemia, melanoma, and teratocarcinoma cell lines (16, 22–25). Increased expression of HK2 Env has been associated with breast and other cancers (16, 19, 26–28). HK2 produces two accessory proteins, Np9 and Rec, which also have been linked to malignancy. Np9 is highly expressed in transformed tissues and is associated with β-catenin, ERK, Akt, Notch1, and c-myc pathways implicated in carcinogenesis (29–33). Rec causes carcinoma in situ in mice and interacts with the testicular zinc finger protein and androgen receptor (34–36). HK2 sequences have been shown to produce not only mRNA and viral proteins but also VLPs in patients infected with HIV-1 and in patients with various malignancies, including lymphomas (11, 12, 37–40). HERV-K expression also has been linked to brain development and neurologic disorders (41, 42). While no single HK2 is capable of coding an infectious virus, the sum of these loci retain coding capacity necessary to produce each and every functional retroviral protein (4, 11, 38–40, 43, 44). Recombination or trans-complementation between these proviruses could lead to replication-competent viruses (9, 45, 46), as seen with endogenous retroviruses in mice (47, 48).

Recent reports have found evidence that modern HK2 particles may be infectious (11, 45, 49–51). Analysis of the HK2 env gene, which is necessary for infectivity, revealed that the envelope is under purifying selection, suggesting that replication-competent HK2 is still present in humans (6). Three groups have resurrected infectious viruses by deriving recombinant sequences from existing HK2 proviruses or by fixing mutations of the most recent HK2 forms (45, 49, 50). Our laboratory looked for replication of HK2 VLPs derived from cancer cell lines and from the blood of HIV-1 and cancer patients and found evidence of active viral env RNA recombination, suggesting that the viral genome replicated through reverse transcription. In addition, we found signs of purifying selection in the env gene, suggesting that the viruses were infectious, or at least that their envelopes were preserved for the sake of potential infectivity (11, 38). To test these observations, we devised a molecular beacon, termed Bogota, which can be packaged into HK2 VLPs to assess the infectivity and replication of viruses produced in cancer cell lines or peripheral blood cells of lymphoma patients. With these studies, we showed that present-day HK2 viruses can package and transmit viral genomic material from one cell to the next (51).

We have found features that make the replication cycle of HK2 different from the canonical replication cycle of retroviruses. The reverse transcription step of HK2 bears some resemblance to that of spumaviruses, a genus of the retroviridae family, which replicates through an infectious DNA form (52). Viral RNA genomes transcribed from HK2 proviruses can be reverse transcribed into cDNA within the cells, in already-released VLPs, and/or after particle infection (53). We previously noted that unlike conventional retroviruses, which after infection are integrated into the host genome, contemporary HK2 cDNAs autointegrate into 1-long terminal repeat (1-LTR) and 2-long terminal repeat (2-LTR) episomes (51). Although we cannot rule out the existence of genomic integration of contemporary HK2 viruses, we did not find any evidence of their existence despite an extensive search. In contrast, other investigators have found that

resurrected HK2 viruses, in addition to producing episomal forms, can integrate into the host genome (45, 49). Transmission of contemporary HK2 viruses requires bona fide reverse transcription that can be inhibited with reverse transcriptase inhibitors. Whether the viral RNA is reverse transcribed by an HK2 reverse transcriptase (RT) or another RT remains unknown. Previously, Berkhout et al. identified active HK2 RT enzymes encoded by at least three different loci in the genome of human bone marrow mononuclear cells (54). Two of the functional enzymes identified are encoded by the *pol* gene of HK2 7p22b (K108b) and 10p12.1 (K103), which are present polymorphically in humans (4, 5) and, therefore, may not exist in every HK2 VLP-producing cell line.

In the present report, we tested whether HK2s can code for functional RTs, as well as whether this enzyme, which of course is vital to viral replication, is susceptible to different reverse transcriptase inhibitors (RTIs) at different stages of the HK2 replication cycle. We show that a functional RT enzyme is encoded by at least one contemporary HK2, HERV-K 103, which is susceptible to a range of RTIs but not to HIV-1-specific RTIs and/or protease or integrase inhibitors. We provide evidence that RTIs inhibit the replication of the viral genome of viruses produced in cancer cell lines, preventing reverse transcription both in viral particles and in target cells. Thus, these drugs can be used to study the biology of HERVs and hold the potential to treat HK2-related pathogenesis.

RESULTS

Identification of RT-coding genes in the human genome. In order to determine which HK2 proviruses can code for functional reverse transcriptase (RT), we analyzed the reading frames of RT genes carried by all known HK2 proviruses thus far identified in the human genome (3, 10, 11, 54). We screened HK2 proviruses for complete ~1,788-bp *rt* genes with intact reading frames and the retroviral motifs LPQG and YIDD necessary for functional RT activity. Of all known proviruses analyzed, we identified 11 HK2 proviruses with coding potential for RT proteins. These HK2 proviruses have intact open reading frames (ORFs) for the complete *pol* gene. Six of these proviruses are found polymorphically inserted among humans (Fig. 1). We tested the expression capacity of RT-coding HK2 proviruses by reverse transcription-PCR (RT-PCR) and sequencing of the HK2 *env* gene in cell-free supernatants of four cell lines known to produce HK2 virus-like particles (NCCIT, Sk-Mel 103, T47D, and DT22; see Fig. S1 in the supplemental material). Interestingly, in the cell line NCCIT, which we have previously shown produces transmissible HK2 particles with the capacity for potential genomic replication (51), we found expression of six RT-coding HK2 proviruses, K102, K103, K107, K108b, K117, and KC19. Although all HK2-producing cells tested express different HK2 proviruses, the NCCIT cell line had the most RT-coding HK2 proviruses. We found HK2 *env* RNA expression of only K106 and K107 in the melanoma cell line Sk-Mel 103 and of only K106 in the breast cancer DT22 cell line. We did not detect expression of RT-intact HK2 proviruses in the particle-producing breast cancer cell line T47D.

We measured the RT activity in the supernatant of these cell lines. We found significantly increased RT activity in the cell lines Sk-Mel 103, Sk-Mel 19, and NCCIT (Fig. 2A). In contrast to these cells, DT22 cells showed a lower increase in RT activity. We did not observe apparent RT activity in T47D cells. We further tested the functional effects of HK2 reverse transcription in these cell lines by looking for recombinant *env* sequences that may have arisen by template switching during reverse transcription (Fig. S1). We identified several recombinant HK2 sequences in the supernatant of the cell line NCCIT and only one in the supernatant of the cell line Sk-Mel-103. This suggests that HK2 particles produced by the NCCIT cells, and perhaps Sk-Mel 103 cells, have the ability to reverse transcribe their viral genome. We did not find recombinant sequences in the supernatants of breast cancer cell lines. Similarly, in previous studies we did not find recombinant sequences in the blood of patients with breast cancer, suggesting that these HK2 particles do not contain a functional RT (38).

Inhibition of reverse transcription of HK2 in particle-producing cells. In order to further determine whether the genomes of HK2 particles produced by certain cell lines

```
                10        20        30        40        50        60        70        80        90       100       110
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
1q22/K102    KSRKRRNRLSFLGAATVEPPKPIPLTWKTEKPVWVNQWPLPKQKLEALHLLANEQLEKGHIEPSFSPWNSPVFVIQKKSGKWRMLTDLRAVN---AVIQPMGPLQPGLPS
10p12.1/K103 KSRKRRNRVSFLGAATVEPPKPIPLTWKTEKPVWVNQWPLPKQKLEALHLLANEQLEKGHIEPSFSPWNSPVFVIQKKSGKWRMLTDLRAVN---ALIQPMGPLQPGLPS
3q27.2/K117  KSRKRRNRVSFLGAATVEPPKPIPLTWKTEKPVWVNQWPLPKQKLEALHLLANEQLEKGHIEPSFSPWNSPVFVIQKKSGKWRMLTDLRAVN---AVIQPMGPLQPGLPS
3q13.2/K106  KSKKRRNRVSFLGAATVEPPKPIPLTWKTEKPWVNQCPLPKQKLEALHLLANEQLEKGHIEPSSSPWNSPVFVIQKKSGKWRMLTNLRAVN---AVIQPMGPLQPGLPS
5q33.3/K107  KSKKRRNRVSFLGAVTVEPPKPIPLTWKTEKPVWVNQWPLPKQKLEALHLLANEQLEKGHIEPSFSPWNSPVFVIQKKSGKWRMLTDLRAVN---AVIQPMGPLQPGLPS
7p22.1b/K108 KSRKRRNRESLLGAATVEPPKPIPLTWKTEKPVWVNQWPLPKQKLEALHLLANEQLEKGHIEPSFSPWNSPVFVIQKKSGKWRMLTDLRAVN---AVIQPMGPLQPGLPS
8p23.1a/K115 KSKKRRNRVSFLGVATIEPPKPIPLTWKTEKLVWVNQWPLPKQKLEALHLLANEQLEKGHIEPSFSPWNSPVFVIQKKSGKWRMLTDLRAVN---AVIQPMGPLQPGLPS
11q22.1/K118 KSKKRRNRVSFLGAATVEPPKPIPLTWKTEKPVWVNQWPLPKQKLEALHLLANEQLEKGHIEPSFSPWNSPVFVIQKKSGKWRMLTDLRAVN---AVIQPMGPLQPGLPS
19p12b/K113  KSKKRRNRVSFLGAATVEPPKPIPLTWKTEKPVWVNQWPLPKQKLEALHLLANEQLEKGHIEPSFSPWNSPVFVIQKKSGKWRMLTDLRAVN---AVIQPMGPLQPGLPS
19q11/KC19   KSKKRRNRVSFLGVATVEPPKPIPLTWKTEKPVWVNQWPLPKQKLEALHLLANEQLEKGHIEPSFSPWNSPVFVIQKKSGKWRMLTDLRAVNAVNAVIQPMGPLQPGLPS
Xq21.33      KSKKRRNRESFLGAATVEPPKPIPLTWKTEKPVWVNQWPPPKQKLGALHLLANEQLEKGHIEPSFSPWNSPVFVIQKKSGKWRMLTDLRAVN---AVIQPMGPLQPGLPS
```

                                                        LPQG   YIDD
```
               120       130       140       150       160       170       180       190       200       210       220
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
1q22/K102    PAMIPKDWPLIIIDLKDCFFTIPLAEQDCEKFAFTIPAINNKEPATRFQWKVLPQGMLNSPTICQTFVGRALQPVREKFSDCYIIHYIDDILCAAETRDKLIDCYTFLQA
10p12.1/K103 PAMIPKDWPLIIIDLKDCFFTIPLAEQDCEKFAFTIPAINNKEPATRFQWKVLPQGMLNSPTICQTFVGRALQPVREKFSDCYIIHYIDDILCAAETKDKLIDCYTFLQA
3q27.2/K117  PAMIPKDWPLIIIDLKDCFFTIPLAEQDCEKFAFTIPAINNKEPATRFQWKVLPQGMLNSPTICQTFVGRALQPVREKFSDCYIIHYIDDILCAAETKDKLIDCYTFLQA
3q13.2/K106  PAMIPKDWPLIIIDLKDCFFTIPLAEQDCEKFAFTIPAINNKEPATRFQWKVLPQGMLNSPTICQTFVGRALQPVREKFSDCYIIHYIDDILCAAETKDKLIDCYTFLQA
5q33.3/K107  PAMIPKDWPLIIIDLKDCFFTIPLAEQDCEKFAFTIPAINNKEPATRFQWKVLPQGMLNSPTICQTFVGRALQPVREKFSDCYIIHYIDDILCAAETKDKLIDCYTFLQA
7p22.1b/K108 PAMIPKDWPLIIIDLKDCFFTIPLAEQDCEKFAFTIPAINNKEPATRFQWKVLPQGMLNSPTICQTFVGRALQPVREKFSDCYIIHYIDDILCAAETKDKLIDCYTFLQA
8p23.1a/K115 PAMIPKDWPLIIIDLKDCFFTIPLAEQDCEKFAFTIPAINNKEPATRFQWKVLPQGMLNSPTICQTFVGRALQPVRKKFSDCYIIHYIDDILCAAETKDKLIDCYTFLQA
11q22.1/K118 PAMIPKDWPLIIIDLKDCFFTIPLAEQDCEKFAFTIPAINNKEPATRFQWKVLPQGMLNSPTICQTFVGRALQPVREKFSDCYIIHYIDDILCAAETKDKLIDCYTFLQA
19p12b/K113  PAMIPKDWPLIIIDLKDCFFTIPLAEQDCEKFAFTIPAINNKEPATRFQWKVLPQGMLNSPTICQTFVGRALQPVRDKFSDCYIIHYIDDILCAAETKDKLIDCYTFLQA
19q11/KC19   LAMIPKDWPLIIIDLKDCFFTIPLAEQDCEKFAFTIPAINNKEPATRFQWKVLPQGMLNSPTICQTFVGRALQPVREKFSDCYIIHYIDDILCAAETKDKLIDCYTFLQA
Xq21.33      PAMIPKDWPLIIIDLKDCFFTIPLAEQDCEKFAFTIPAINNKEPATRFQWKVLPQGMLNGPTICQTFVGRALQPVREKFSDCYIIHYIDDILCAAETKDKLIDCYTFLQA
```

```
               230       240       250       260       270       280       290       300       310       320       330
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
1q22/K102    EVANAGLAIASDKIQTSTPFHYLGMQIENRKIKQQKIEIRKDTLKTLNDFQKLLGDINWIRPTLGIPTYAMSNLFSILRGDSDLNSKRILTPEATKEIKLVEEKIQSAQI
10p12.1/K103 EVANAGLAIASDKIQTSTPFHYLGMQIENRKIKPQKIEIRKDTLKTLNDFQKLLGDINWIRPTLGIPTYAMSNLFSILRGDSDLNSKRILTPEATKEIKLVEEKIQSAQI
3q27.2/K117  EVANAGLAIASDKIQTSTPFHYLGMQIENRKIKPQKIEIRKDTLKTLNDFQKLLGDINWIRPTLGIPTYAMSNLFSILRGDSDLNSKRILTPEATKEIKLVEEKIQSAQI
3q13.2/K106  EVANAGLAIASDKIQTSTPFHYLGMQIENRKIKPQKIEIRKDTLKTLNDFQKLLGDINWIRPTLGIPTYAMSNLFSILRGDSDLNSKRMLTPEATKEIKLVEEKIQSAQI
5q33.3/K107  EVANAGLAIASDKIQTSTPFHYLGMQIENRKIKPQKIEIRKDTLKTLNDFQKLLGDINWIRPTLGIPTYAMSNLFSILRGDSDLNSQRILTPEATKEIKLVEEKIQSAQI
7p22.1b/K108 EVANAGLAIASDKIQTSTPFHYLGMQIENRKIKPQKIEIRKDTLKTLNDFQKLLGDINWIRPTLGIPTYAMSNLFSILRGDSDLNSKRMLTPEATKEIKLVEEKIQSAQI
8p23.1a/K115 EVASAGLAIASDKIQTSTPFHYLGMQIENRKIKPQKIEIRKDTLKTLNDFQKLLGDINWIQPTLGIPTYAMSNLFSILRGDSDLNSKRILTPEATKEIKLVEEKIQSAQI
11q22.1/K118 EVANAGLAIASDKIQTSTPFHYLGMQIENRKIKPQKIEIRKDTLKALNDFQKLLGDINWIRPTLGIPTYAMSNLFSILRGDSDLNSKRMLTPEATKEIKLVEEKIQSAQI
19p12b/K113  EVANAGLAIASDKIQTSTPFHYLGMQIENRKIKPQKIEIRKDTLKTLNDFQKLLGDINWIRPTLGIPTYVMSNLFSILRGDSDLNSKRMLTPETTKEIKLVEEKIQSAQI
19q11/KC19   EVANAGLAIASDKIQTSTPFHYLGMQIENRKIKPPKIEIRKDTLKTLNDFQKLLGDINWIRPTLGIPTYAMSNLFSILRGDSDLNSKRMLTPEATKEIKLVEEKIQSAQI
Xq21.33      EVANAGLAIASDKIQTSTPFHYLGMQIENRKIKPQKIEIRKDTLKTLNDFQKLLGDINWIRPTLGIPTYAMSNLFSILRGDSDLNSKRMLNPEATKEIKLVEEKIQSAQI
```

```
               340       350       360       370       380       390       400       410       420       430       440
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
1q22/K102    NRIDPLAPLQLLIFATAHSPTGIIIQNTDLVEWSFLPHSTVKTFTLYLDQIATLIGQTRLRIIKLCGNDPDKIVVPLTKEQVRQAFINSGAWQIGLANFVGIIDNHYPKT
10p12.1/K103 NRIGPLAPLQLLIFATAHSPTGIIIQNTDLVEWSFLPHSTVKTFTLYLDQIATLIGQTRLRITKLCGNDPDKIVVPLTKEQVRQAFINSGAWQIGLANFVGIIDNHYPKT
3q27.2/K117  NRIDPLAPLQLLIFATAHSPTGIIIQNTDLVEWSFLPHSTVKTFTLYLDQIATLIGQTRLRIIKLCGNDPDKIVVPLTKEQVRQAFINSGAWQIGLANFVGIIDNHYPKT
3q13.2/K106  NRIDPLAPLQLLIFATAHSPTGIIIQNTDLVEWSFLPHSTVKTFTLYLDQIATLIGQTRLRIIKLCGNDPDKIVVPLTKEQVRQAFINSGAWQIGLANFVGIIDNHYPKT
5q33.3/K107  NRIDPLAPLQLFIFATAHSPTGIIIQNTDLVEWSFLPHSTVKTFTLYLDQIATLIGQTRLRIIKLCGNDPDKIVVPLTKEQVRQAFINSGAWQIGLANFVGLIDNHYPKT
7p22.1b/K108 NRIDPLAPLQLLIFATAHSPTGIIIQNTDLVEWSFLPHSTVKTFTLYLDQIATLIGQTRLRIIKLCGNDPDKIVVPLTKEQVRQAFINSGAWKIGLANFVGIIDNHYPKT
8p23.1a/K115 NRIDPLAPLQLLIFATAHSPTGIIIQNTDLVEWSFLPHSTVKTFTLYLDQIATLIGQTRLRIIKLCGNDPDKIVVPLTKEQVRQAFINSGAWQIGLANFVGIIDNHYPKT
11q22.1/K118 NRIDPLAPLQLLIFATAHSPTGIIIQNTDLVEWSFLPHSTVKTFTLYLDQIATLIGQTRLRIIKLCGNDPDKIVVPLTKEQVRQAFINSGAWQIGLANFVGIIDNHYPKT
19p12b/K113  NRIDPLAPLRLLIFATAHSPIGIIIQNTDLVEWSFLPHSTVKTFTLYLDQIATLIGQTRLRIIKLCGNDPDKIVVPLTKEQVRQAFINSGAWQIGLANFVGIIDNHYPKT
19q11/KC19   NRIDPLATLQLLIFATAHSPTGIIIQNTDLVEWSFLPHSTVKTFTLYLDQMATLIGQTRLRIIKLCGNDPDKIVVPLTKEQVRQAFINSGAWQIGLANFVGIIDNHYPKT
Xq21.33      NRIDPLAPLQLLIFATAHSPTGIIIQNTDLVEWSFLPHSTVKTFTLYLDQMATLIGQTRLRIIKLCGNDPDKIVVPLTKEQVRQAFINSGAWQIGLANFVGIIDNHYPKT
```

```
               450       460       470       480       490       500       510       520       530       540       550
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
1q22/K102    KIFQFLKLTTWILPKITR-REPLENALTVFTDGSSNGKAAYTGPKERVIKTPYQSAQRAELVAVITVLQDFDQPINIISDSAYVVQATRDVETALIKYSMDDLNQLFNL
10p12.1/K103 KIFQFLKLTTWILPKITR-CEPLENALTVFTDGSSNGKAAYTGPKERVIKTPYQSAQRAELVAVITVLQDFDQPINIISDSAYVVQATRDVETALIKYSMDDLNQLFNL
3q27.2/K117  KIFQFLKMTTWILPKITR-REPLENALTVFTDGSSNGKAAYTGPKERVIKTPYQSAQRAELVAVITVLQDFDQPINIISDSAYVVQATRDVETALIKYSMDDLNQLFNL
3q13.2/K106  KIFQFLKLTTWILPKITR-REPLENALTVFTDGSSNGKAAYTGPKERVIKTPYQSAQRAELVAVITVLQDFDQPINIISDSAYVVQATRDVETALIKYSMDDLNQLFNL
5q33.3/K107  KIFQFLKLTTWILPKITR-REPLENALTVFTDGSSNGKAAYTGPKERVIKTPYQSAQRAELVAVITVLQDFDQPINIISDSAYVVQATRDVETALIKYSMDDLNQLFNL
7p22.1b/K108 KIFQFLKLTTWILPKITR-REPLENALTVFTDGSSNGKAAYTGPKERVIKTPYQSAQRAELVAVITVLQDFDQPINIISDSAYVVQATRDVETALIKYSMDDLNQLFNL
8p23.1a/K115 KIFQFLKLTTWILPKITR-REPLENALTVFTDGSSNGKAAYTGPKERVIKTPYQSAQRAELVAVITVLQDFDQPINIISDSAYVVQATRVVETALIKYSMDDLNQLFNL
11q22.1/K118 KIFQFLKLTTWILPKITR-REPLENALTVFTDGSSNGKAAYTGPKERVIKTPYQSAQRAELVAVITVLQDFD--INIISDSAYVVQATRDVETALIKYSMDDLNQLFNL
19p12b/K113  KIFQFLKLTTWILPKITR-REPLENALTVFTDGSSNGKAAYTGLKERVIKTPYQSAQRAELVAVITVLQDFDQPINIISDSAYVVQATRDVETALIKYSMDDLNQLFNL
19q11/KC19   KIFQFLKMTTWILPKITR-REPLENALTVFTDGSSNGKAAYTGPKERVIKTQYQSAQRAELVAVITVLQDFDQPINIISDSAYVVQATRDVETALIKYSMDDLNQLFNL
Xq21.33      KIFQFLKLTTWILPEITR-REPLENALTVFTDGSSNGKAAYTGPKERVIKTPYQSAQRAELVAVITVLQDFDQPINIISDSAYVVQATRDVETALIKYSMDDLNKLFNL
```

```
               560       570       580       590       600
           ....|....|....|....|....|....|....|....|....|....|.
1q22/K102    LQQTVRKR----NFPFYITHIRAHTNLPGPLTKANEQADLLVSSALIKAQELHA
10p12.1/K103 LQQTVRKR----NFPFYITHIRAHTNLPGPLTKANEQADLLVSSALIKAQELHA
3q27.2/K117  LQQTVRKR----NFPFYITHIRAHTNLPGPLTKANEEADLLVSSALIKAQELHA
3q13.2/K106  LQQTVRKR----NFPFYITHIRAHTNLPGPLTKANEEADLLVSSALIKAQELHA
5q33.3/K107  LQQTVRKR----NFPFYITHIRAHTNLPGPLTKANEQADLLVSSALIKAQELHA
7p22.1b/K108 LQQTVRKR----NFPFYITHIRAHTNLPGPLTKANEQADLLVSSALIKAQELHA
8p23.1a/K115 LQQTVRKR----NFPFYITHIRAHTNLPGPLTKANEQADLLVSSALIKAQELHA
11q22.1/K118 LQQTVRKR----NFPFYITHIRAHTNLPGPLTKANKQADLLVSSALIKAQELHA
19p12b/K113  LQQTVRKR----NFPFYITHIRAHTNLPGPLTKANEQADLLVSSALIKAQELHA
19q11/KC19   LQQTVRKRNFPRNFPFYITHIRAHTNLPGPLTKANEQADLLVSSALIKAQELHA
Xq21.33      LQQTVRKR----NFPFYITHIRAHTNLPGPLTKANEQADLLVSSALIKAQELHA
```
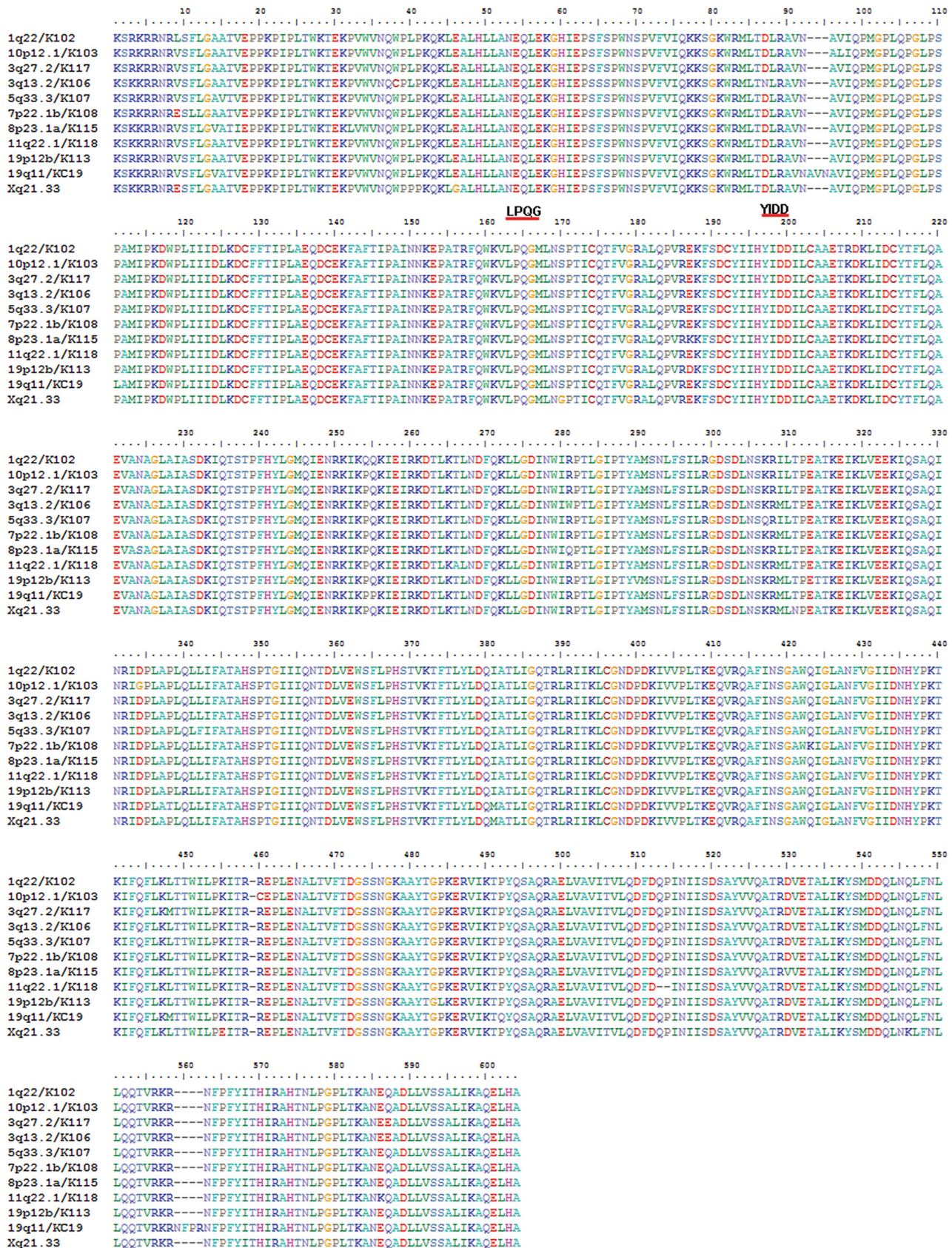
FIG 1 Intact HK2 reverse transcriptase genes in the human genome. An amino acid alignment of translated nucleotide reverse transcriptase (RT) genes carried by the HK2 family. The alignment shows 11 proviruses (left) that appear to code for an intact RT with the conserved functional motifs LPQG and
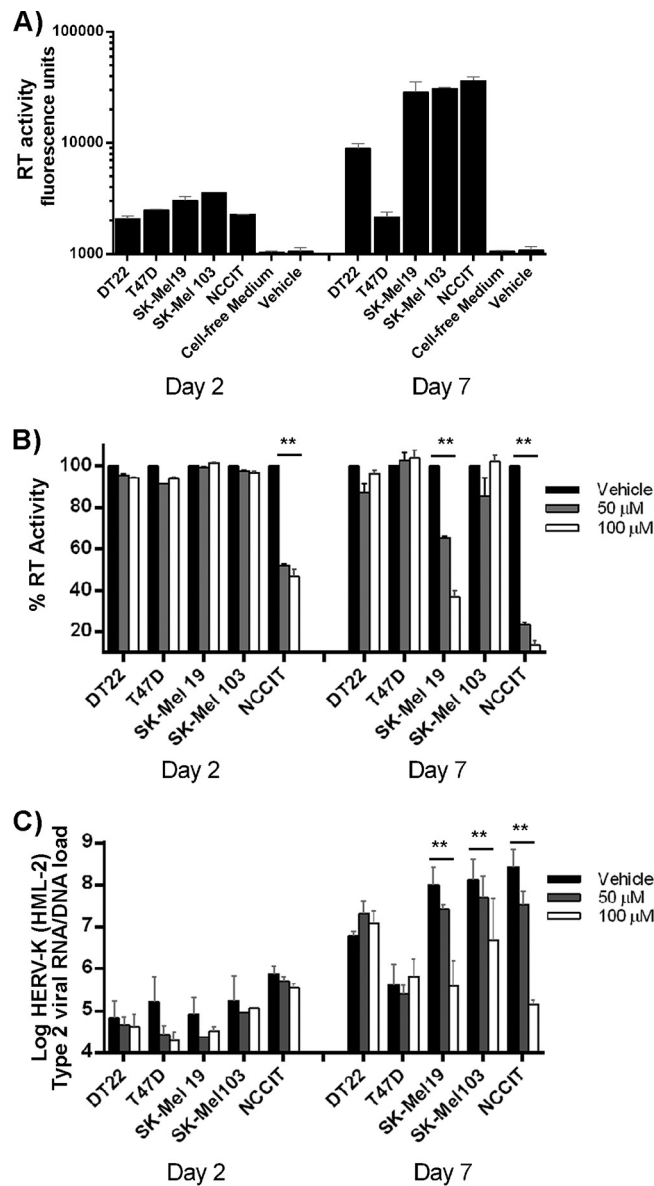
FIG 2 Inhibition of reverse transcription by AZT in HK2 particle-producing cells. Fifty thousand cells were seeded in 6-well plates, and the supernatants were collected to measure RT activity. Cells were treated with vehicle or with a 50 μM or 100 μM concentration of AZT for 2 or 7 days. After treatment, cell supernatants were collected and RT activity and HK2 viral genome expression were measured by RT assays and qRT-PCR, respectively. (A) RT activity of HK2 particle-producing cells DT22, T47D, Sk-Mel19, Sk-Mel103, and NCCIT at days 2 and 7 of culture. (B) Percent RT activity of cell line supernatants after treatment with AZT as measured by the EnzChek reverse transcription kit. (C) Abundance of HK2 type 2 *env* viral genomes in cell supernatants measured by qRT-PCR. Values shown represent the means $\pm$ standard deviations (SD) from at least three independent experiments. Stars indicate significant values ($P < 0.01$ by t test).

can be reverse transcribed, we measured the effect of the nucleoside reverse transcriptase inhibitor (NRTI) zidovudine (AZT), which has been shown to inhibit the reverse transcription of HK2 and other retroviruses (45, 49, 51). We treated HK2 particle-producing cells with 50 or 100 μM concentrations of AZT for 2 or 7 days and

FIG 1 Legend (Continued)
YIDD required for reverse transcription. HK2 proviruses K103, K106, K108b, K113, K115, and Xp21.33 are polymorphic among humans. All of these proviruses have intact ORFs in the *pol* gene. The accession numbers are the following: 1q22/K102, P63135.1; 10p12.1/K103, AAC63291.1 or AF164611; 3q27.2/K117, Q9UQG0.2; 3q13.2/K106, JN675022.1; 5q33.3/K107, P10266.2; 7p22.1b/K108, AAK11553.1; 8p23.1a/K115, P63133.1; 11q22.1/K118, P63136.1; 19p12b/K113, P63132.1; 19q11/KC19, Q9WJR5.2; and Xq21.33, KU054272.1.

determined the RT activity in cell-free supernatants. We and others have shown previously that these concentrations of AZT can block HK2 replication (49, 51). We observed that the levels of RT activity were significantly reduced in the supernatants of NCCIT cells 2 days after treatment with AZT, and these levels were much further reduced 7 days after treatment (Fig. 2A). Although we did not observe a significant reduction of RT activity in the supernatant of the cell line Sk-Mel-19 after 2 days of treatment, we saw such a reduction of RT activity following 7 days of treatment with AZT (Fig. 2B). We did not observe a significant decrease in RT activity in the breast cancer cell line T47D, but this was difficult to assess accurately, as we did not find an increase in RT activity in T47D cells over time in culture. Two cell lines, DT-22 and Sk-Mel-103, show RT activity that is resistant to AZT. It is possible these cell lines produce RT coded by retroelements in the genome other than HK2, or other cellular polymerases, which are not sensitive to AZT. Interestingly, we observed RT activity that was sensitive to AZT in the NCCIT cell line, which expresses polymorphic HK2 viruses with ORFs for RTs such as K103 but did not observe RT activity in T47D cells that express mostly proviruses with mutated RT genes (Fig. S1). Further experiments are required to determine the extent of expression of HK2 in the cell line Sk-Mel 19.

We next determined whether treatment with AZT was able to reduce the levels of HK2 viral genomes. We previously showed that the retroviral genome of HK2 virus particles is made up of RNA and recently reverse-transcribed cDNA (rt-cDNA) (53). Therefore, we measured the levels of viral RNA/cDNA in cell-free HK2 viral particles by RT-PCR, which can measure the levels of both viral RNA and rt-cDNA. Treatment of HK2-producing cells with AZT for 2 days showed no significant inhibition of the amount of HK2 viral genomes produced in Sk-Mel 19, Sk-Mel 103, and NCCIT cell lines. However, after 7 days of AZT treatment, we observed a significant reduction of virus particle-associated HK2 genomes produced by these cells (Fig. 2C). The inhibition of HK2 genomic replication was dose dependent. We did not observe significant changes in the levels of HK2 genomes in the breast cancer cells treated with AZT after 2 or 7 days of drug incubation, again consistent with our previous findings *in vivo*, suggesting activation but not replication of HK2 in breast cancer (38). Interestingly, although the RT activity of cell line Sk-Mel 103 is resistant to AZT, these cells produce HK2 RNA/DNA that is sensitive to AZT treatment. One possible explanation is that Sk-Mel 103 cells express high levels of RTs encoded by other retroelements and that these RTs are resistant to AZT. Taken together, these analyses demonstrate that AZT affects reverse transcription of HK2 particles in some, but not all, cell lines.

Purification and activity of HK2 RT enzymes. Having observed that the viral genomes of HK2 particles produced by certain cancer cell lines are inhibited by the nucleoside analog AZT, we studied whether expression and activity of the HK2 *rt* genes themselves in these cells could be responsible for the reverse transcription of HK2 viral genomes. We therefore amplified and cloned the *rt* genes in expression vectors in frame with a His tag coding gene. We cloned *rt* genes with intact coding frames for RT proteins as determined by our sequence analysis shown in Fig. S1. Three *rt* genes were chosen based on their potential to produce active RT enzymes, including the *rt* from proviruses K103, K108, and K113. We chose proviruses K108 and K113, as they are the most intact HK2 in the human genome and have the ability to produce virus particles. We selected K103, as Berkhout et al. previously showed that the K103 RT, named clone 10.9, had functional activity (54). The *rt* genes K108 and K113 were amplified from bacterial artificial chromosome (BAC) clones, whereas the K103 *rt* gene was amplified from DNA isolated from NCCIT cells. We expressed and purified these proteins in *Escherichia coli* and detected them by Western blot analysis using an anti-His antibody (Fig. 3A). We used the EnzChek RT kit to measure the RT activity of the purified proteins. We observed that, in contrast to the K108 and K113 RT proteins, the K103 RT enzyme had RT activity (Fig. 3B). Similar to findings reported by Beimforde et al. (50), we did not observe RT activity in the K113 RT or K108 RT. Therefore, we performed the subsequent experiments with K103 RT enzyme. We observed that, similar to the murine leukemia
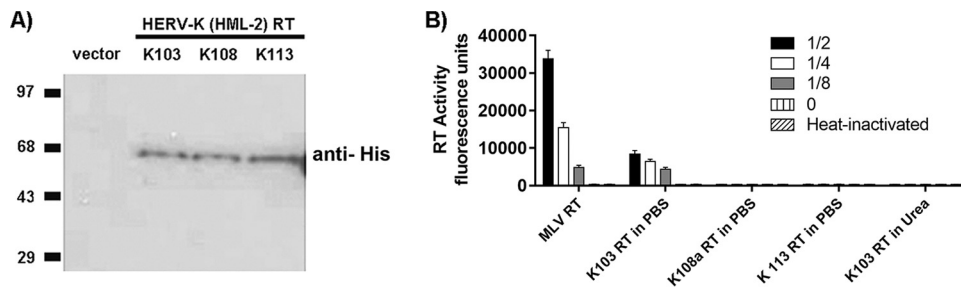
FIG 3 Purification and measurement of activity of HK2 reverse transcriptase. The *rt* gene of proviruses K103, K108, and K113 was cloned in frame with a His tag in the pET32-EK/LIC vector, and the recombinant RT protein was expressed in *E. coli* and purified using a Ni column. (A) Western blot analysis of the HK2 His-RT proteins. Purified proteins were electrophoresed in 12% PAGE, transferred onto a polyvinylidene difluoride (PVDF) membrane, and detected using an anti-His antibody. The control vector did not produce detectable bands. (B) RT activity of HK2 103, 108, and 113 RT. The RT activity of purified proteins was assessed using the EnzChek reverse transcriptase kit. As a positive control, we used MLV RT. The graph shows the RT activity produced by serially diluted RT enzymes in three independent experiments. No RT activity was detected from the K108b and K113 RT, in heat-inactivated proteins, or in K103 RT denatured in the presence of urea. Values represent the means ± SD from at least three independent experiments.

virus (MLV) RT control, the K103 RT enzyme possesses an RT activity that decreases when the enzyme is diluted in buffer. The RT activity is inactivated by heat or by treatment with urea (Fig. 3B).

We further assessed the capacity of the K103 RT to reverse transcribe HK2 RNA transcripts using real-time RT-PCRs. We observed that the K103 enzyme was able to reverse transcribe *gag* and *env* HK2 synthetic transcripts using RT-PCRs, similar to a commercial MLV RT enzyme (Promega) but with lower performance (Fig. 4A). This was done using RT-PCR conditions similar to those described in the QuantiTect RT-PCR kit (Qiagen), using either the MLV RT or the K103 RT. The PCR products were sequenced directly to identify nucleotide substitutions and calculate the misincorporation rate. K103 RT enzyme was able to reverse transcribe RNA templates on the order of 10^9 to 10^5 transcript copies per reaction efficiently, although the reaction generated primer dimer by-products (Fig. 4B and C). Preliminary analysis of the sequences of the amplification products also revealed that K103 RT misincorporated 1 or 2 bases per 100 bp of nucleotide sequence (Fig. 4D and E), in contrast to the MLV RT that did not misincorporate bases (Fig. 4F). The frequency of misincorporation by K103 RT is higher than that of other RTs, such as HIV-1 RT (error rate, 1 in 5,900 bp) and MLV RT (error rate, 1 in 37,000 bp) (55), suggesting that the K103 RT is more prone to make misincorporation errors and probably is not viable for a truly replicative retrovirus. A more precise method to calculate the misincorporation rate of the RT deserves further investigation in order to more accurately determine the error rate of HK2 RT and compare its error rate to that of different RTs. Taken together, these experiments show that K103 RT is functionally active and is able to reverse transcribe HK2 RNA transcripts, although with a lack of fidelity.

Effect of reverse transcriptase inhibitors on HK2 RT. Having identified a functional HK2 RT, we assessed whether reverse transcriptase inhibitors can impair the activity of K103 RT. We performed RT assays of K103 RT with increasing concentrations of triphosphate NRTIs, HIV nonnucleoside RTIs (NNRTIs), or an HIV protease inhibitor (PI). Nucleoside analogs need to be phosphorylated intracellularly to be functionally active (56). Therefore, in these *in vitro* experiments we used phosphorylated forms. We observed that K103 RT was inhibited by certain NRTIs (azidothymidine [or zidovudine] triphosphate [AZTTP], stavudine triphosphate [d4TTP], and lamivudine triphosphate [3TCTP]) and chain terminators (ddTTP, ddCTP, and ddGTP) in a dose-dependent manner but not by ddUTP, which did not affect K103 RT activity (Fig. 5A). We did observe a partial inhibition with the chain terminator ddATP (40%) using a concentration of 1,000 μM inhibitor in the RT reaction. As the EnzChek reverse transcriptase assay kit measures the RT activity by polymerizing a poly(A) ribonucleotide template with

FIG 4 Functional capacity and fidelity of K103 RT. Capacity of K103 RT to efficiently reverse transcribe HK2 K108 *env* transcripts produced *in vitro* in RT-PCRs. Serial dilutions of HK2 DNA-free RNA transcripts

(Continued on next page)

dTTP, it is likely that the chain terminator ddATP cannot be efficiently incorporated into a poly(A) template. Nonetheless, in this system, ddGTP and ddCTP were able to inhibit the activity of K103 RT and probably compete with dTTP in the RT reaction. K103 RT was not inhibited by HIV-specific NNRTIs (nevirapine, efavirenz, or etravirine) or by the HIV PI amprenavir (Fig. 5B). As expected, the MLV RT was inhibited by nucleoside analogues AZTTP, d4TTP, and 3TCTP and the chain terminator ddTTP but not by the HIV NNRTI nevirapine (Fig. 5C). These data suggest that K103 RT can be specifically inhibited by certain nucleoside analogues.

Effect of reverse transcriptase inhibitors in HK2 viral particles. Having determined that certain RTIs impair the function of HK2 RT, we assessed whether these inhibitors interfere with the replication capacity of the viral genome of HK2 virus particles released by NCCIT cells. We incubated NCCIT cells with increasing concentrations of NRTIs and HIV-specific NNRTIs and measured the RT activity in the supernatants after 7 days of drug treatment. As NCCIT cells were very sensitive to concentrations of AZT in the range of 50 to 100 μM, we used smaller concentrations of inhibitors that were in the range of 0.5 to 8 μM in order to obtain a linear dose-response. We observed that the RT activity in NCCIT supernatants was significantly reduced after treatment with the NRTIs AZT, emtracitabine (FTC), lamivudine (3TC), zalcitabine (ddC), stavudine (d4T), didanosine (ddI), abacavir (ABC), and tenofovir (TDF) in a dose-dependent manner (Fig. 6A). The NRTI ddI, which is metabolized to ddATP (57), did affect the RT activity of NCCIT cells. However, ddATP only partially blocked the K103 RT, perhaps as the K103 enzymatic assay was performed using a poly(A) template. Thus, it is likely that K103 enzyme will incorporate the complementary dTTP rather than ddATP. Surprisingly, we also observed inhibition of RT activity with the NNRTI etravirine (ETV) (Fig. 6B), although it did not block the enzymatic activity of the K103 RT (Fig. 3). It is possible that etravirine can block other HK2 RTs and thus interfere with HK2 replication. We did not observe any significant effect in cells treated with the HIV NNRTIs efavirenz (EFV) or nevirapine (NVP). As expected, treatment with the HIV-1 PIs atazanavir (ATV), amprenavir (APV), or indinavir (IDV) did not have an effect on the RT activity of NCCIT cells (Fig. 6C).

We next measured the effect of RTIs on the replication of HK2 viral genomes by RT-PCR, a method that can assess the effect of these drugs on HK2 RNA and DNA viral species. After drug treatment, HK2 RNA/rt-cDNA viral genomes were isolated from cell-free supernatants and quantitated by RT-PCR using primers that bind type 2 *env*. We observed a significant reduction in the amount of virus particle-associated HK2 *env* in cells treated with the NRTIs AZT, TDF, 3TC, ABC, and FTC in a dose-dependent manner (Fig. 7A). We did not observe any significant effect with treatments with the HIV NNRTIs ETV, EFV, and NVP. We further measured the effect of RTIs on the number of HK2 rt-cDNA copies in virus particles released by NCCIT. We measured the cDNA copy number of the retroviral genes *env* and *gag* by real-time PCR. We observed a reduction of both *env* and *gag* cDNA in virus particles released by NCCIT cells treated with the NRTIs AZT, 3TC, and FTC but not with the NNRTI EFV (Fig. 7B). These data suggest that NRTIs interfere with the genomic replication capacity of HK2 in viral particles produced by cancer cells.

FIG 4 Legend (Continued)
ranging from a concentration of 10^9 to 10^6 copies per microliter were subjected to RT-PCR using the MLV RT or the K103 RT using the master mix buffer provided by the Qiagen OneStep qRT-PCR kit. (A) An electrophoresis gel showing the amplification products generated by qRT-PCRs using serial dilutions of HK2 transcripts and the negative-control fetal bovine serum (FBS). The upper band represents HK2 amplicon, whereas the lower band represents primer dimer. Efficient amplification was observed when using a lower concentration of K103 RT. Amplification efficiency (B) and melting curve analysis (C) of reverse-transcribed RNAs generated by the MLV RT (left) and the K103 RT. The calibration curves display the squared correlation coefficient (R^2). The melting curve analysis shows the melting temperature peaks of the PCR amplicons and the primer dimers in each RT reaction using different enzymes. (D and E) Sequence of HK2 *env* type 2 (D) or *gag* (E) PCR amplicons produced by RT-PCRs using K103 RT on HK2 transcripts produced *in vitro*. Arrows indicate misincorporation of nucleotides, likely generated by the K103 RT. (F and G) Sequence chromatograms of *env* type 2 (F) and *gag* (G) on amplicons produced by RT-PCRs using MLV RT on HK2 transcripts. We did not find misincorporation of bases using the MLV RT.
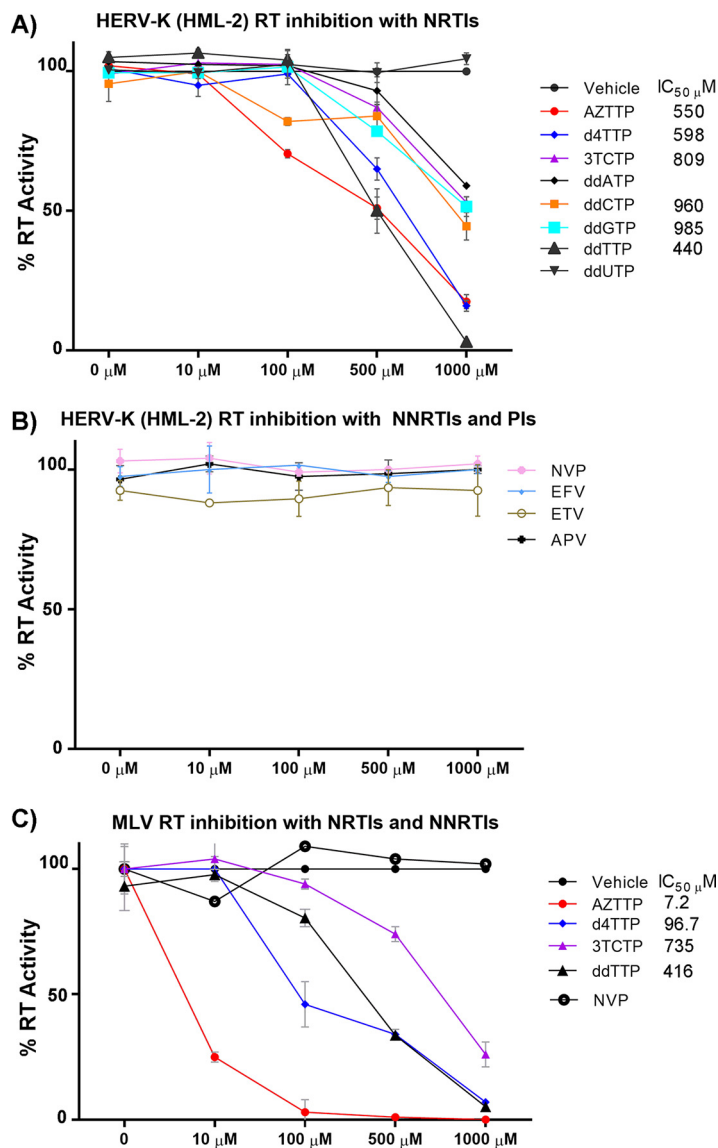
FIG 5 Activity of K103 RT in the presence of NRTIs (A) and NNRTIs and PIs (B). K103 RT activity was measured using the EnzChek reverse transcription kit in the presence of vehicle or different concentrations of triphosphate NRTIs (AZTTP, d4TTP, and 3TCTP); triphosphate chain terminators (ddATP, ddCTP, ddGTP, ddTTP, and ddUTP); NNRTIs (NVP, EFV, and ETV); and the PI APV. The graph shows the percent RT activity (y axis) of the K103 RT in the presence of increasing concentrations of NRTIs, NNRTIs, or PIs (x axis). (C) Activity of MLV RT in the presence of NRITs and NNRTIs. Shown is a plot indicating the RT activity of the enzyme MLV RT in the presence of the triphosphate NRTI AZTTP, d4TTP, ddTTP, or 3TCTP and the NNRTI nevirapine (NVP). RT activity was measured using the EnzChek reverse transcription kit. The graph shows the percent MLV RT activity (y axis) in the presence of increasing concentrations of NRTIs, an NNRTI, or the vehicle (x axis). Values represent the means ± SD from at least three independent experiments. Shown are the 50% inhibitory concentrations (IC$_{50}$s). No IC$_{50}$s are shown for drugs that did not inhibit RT activity.

Effect of reverse transcriptase inhibitors on intracellular HK2 DNA. We previously observed that HK2 RNA transcripts could be reverse transcribed intracellularly as well as in viral particles as noted above (53). We therefore measured the amount of HK2 *env* DNA in NCCIT cells after treatment with AZT. Measurement of cellular HK2 DNA assesses the levels of proviral DNA plus newly reverse-transcribed cDNA. We observed that treatment with 50 or 100 μM AZT decreases the total amount of DNA, likely cDNA, in NCCIT cells (Fig. 8). Taken together, these data suggest that NRTIs impair the replication capacity of HK2 genomes in NCCIT cells.
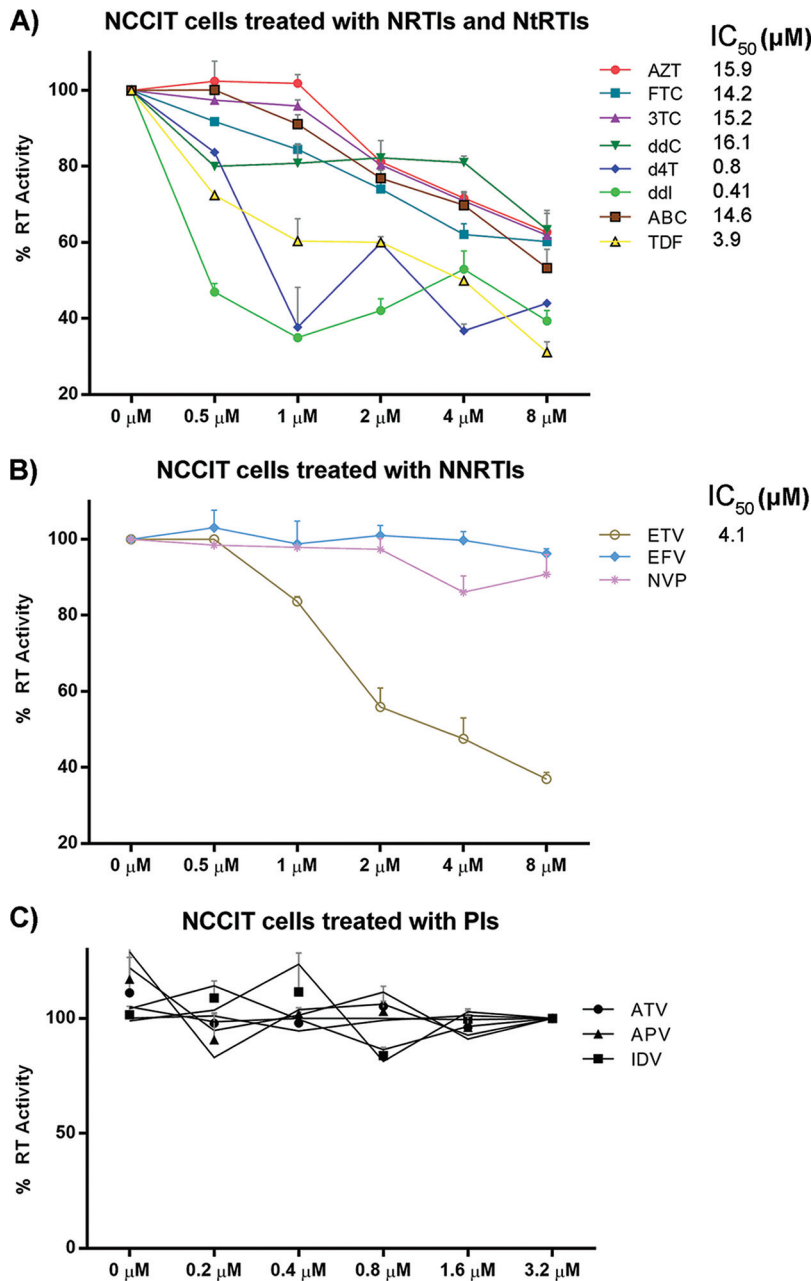
FIG 6 Effect of NRTIs (A) and NNRTIs (B) on the RT activity of NCCIT cells. NCCIT cells were treated with different concentrations (x axis) of NRTIs (AZT, FTC, 3TC, ddC, d4T, ddI, ABC, TDF, and ETV) (A) and NNRTIs (ETV, EFV, and NVP) (B) for 7 days. We measured the percent RT activity (y axis) in cell supernatants using the EnzChek reverse transcription kit. Values represent the means $\pm$ SD from at least three independent experiments. Shown are the IC_{50}s. (C) Treatment of NCCIT cells with viral protease inhibitors does not affect RT activity. NCCIT cells were treated with vehicle or increasing concentrations (x axis) of the protease inhibitors atazanavir (ATV), amprenavir (APV), or indinavir (IDV) for 7 days, and the RT activity was measured in the supernatants using the EnzChek reverse transcription kit. We used drug concentrations higher than the IC_{50} known to inhibit HIV particle assembly (63). The graph displays the percentage of RT activity in the supernatants of cells treated with increasing doses of PIs in three independent experiments. No IC_{50}s are shown for drugs that did not inhibit the RT activity.

Inhibition of the infectivity of HERV-K$_{CON}$ by RTIs. To directly address whether the RTIs that affect K103 RT and the reverse transcription of the viral genomes of HK2 particles produced by NCCIT can affect HK2 virus replication, we measured the effect of RTIs on the infectivity of HERV-K$_{CON}$. The HERV-K$_{CON}$ virus was created by correcting the mutations that occurred over time to the genes of the progenitor HK2 that infected
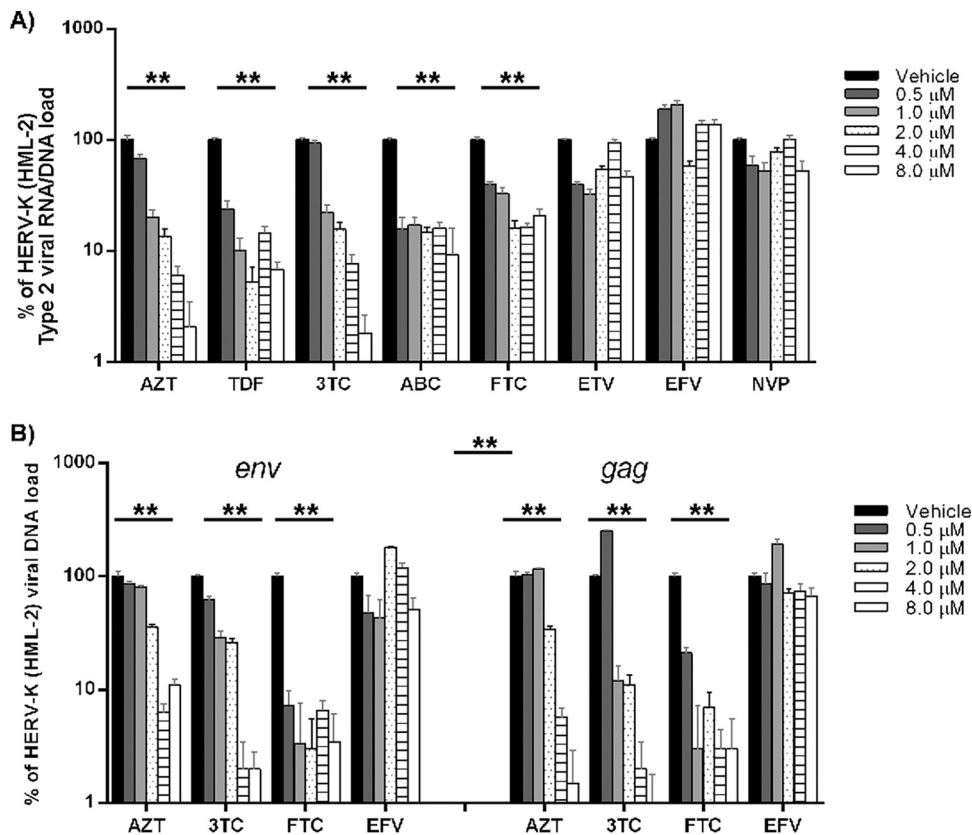
FIG 7 Effect of NRTIs and HIV-1 NNRTIs on HK2 viral RNA and DNA forms in the supernatants of NCCIT cells. NCCIT cells were treated with increasing concentrations of NRTIs (AZT, TDF, 3TC, ABC, and FTC) or HIV-1 NNRTIs (ETV, EFV, and NVP) for 7 days. Extracellular HK2 viral RNA and cDNA levels were measured by RT-PCR or PCR, respectively. HK2 type 2 *env* viral RNA/cDNA levels in supernatants (A) and HK2 *env* and *gag* viral cDNA levels in supernatants (B) from cells treated with increasing concentrations of NRTIs or HIV-1 NNRTIs. Values represent the means ± SD from at least three independent experiments. The IC$_{50}$s for *env* type 2 RNA/cDNA inhibition are the following: AZT, 0.69; TDF, 0.29; 3TC, 0.29; and FTC, 0.42. The NNRTIs did not inhibit HK2 RNA/cDNA. The IC$_{50}$s for *env* type 2 DNA inhibition are the following: AZT, 1.17; 3TC, 0.69; and FTC, 0.25. EFV did not inhibit HK2 DNA. The *y* axis is set to the log$_{10}$ scale. Stars indicate differences in the HK2 RNA/cDNA levels between vehicle-treated and drug-treated cells ($P < 0.01$ by *t* test).

human cells (49). Lee and Bieniasz and our laboratory have previously shown that HERV-K$_{CON}$ infection can be blocked by AZT (49, 51). We infected 293 cells with HERV-K$_{CON}$ virus and measured the number of HERV-K$_{CON}$ infectious units. Target cells were pretreated with 5 µM or 50 µM concentrations of NRTIs, HIV NNRTIs, HIV protease
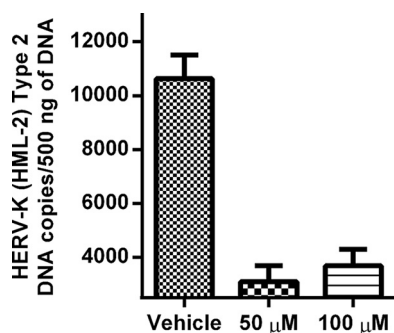
FIG 8 AZT treatment reduces the abundance of HK2 cDNA copies in NCCIT cells. NCCIT cells were treated with vehicle or 50 µM or 100 µM concentrations of AZT for 7 days. After treatment, DNA was isolated from the cells and the copy number of HK2 type 2 *env* DNA was measured by qRT-PCR on 500 ng of DNA. No differences were observed in the abundance of the cellular gene *gapdh* with or without AZT treatment. The graph displays the average number of type 2 *env* DNA copies as determined in each treatment category in three independent experiments.
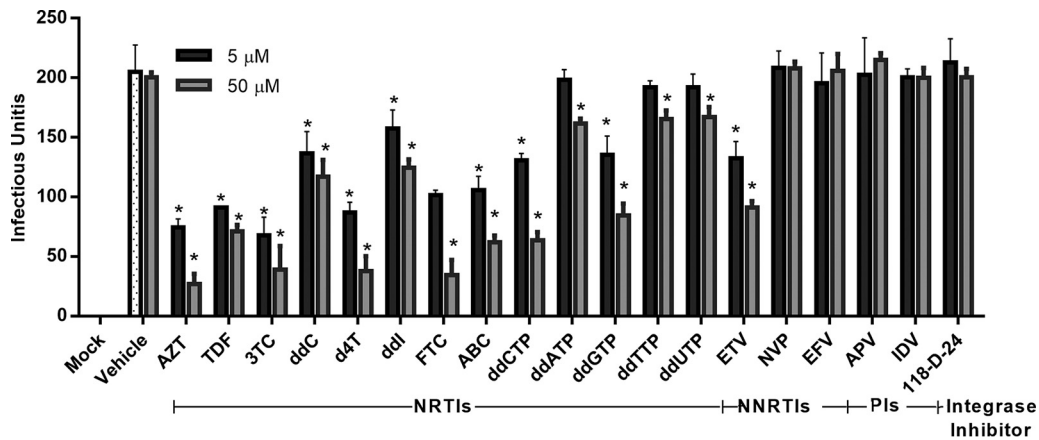
FIG 9 Effect of RTIs on infectious HERV-K$_{CON}$. Infectivity of HERV-K$_{CON}$ virus particles on target 293T cells treated with different concentrations of NRTIs (AZT, TDF, 3TC, ddC, d4T, ddI, FTC, and ABC), chain terminators (ddCTP, ddATP, ddGTP, ddTTP, and ddUTP), HIV-1 NNRTIs (ETV, NVP, and EFV), HIV-1 PIs (APV and IDV), or the HIV-1 integrase inhibitor 118-D-24. Infectious HK2 viral particles were produced in cells transfected with the plasmids HERV-K$_{CON}$ CHKCP and VSVG in the presence of HK2 Rec. 293T target cells pretreated for 24 h with NRTIs, HIV-1 NNRTIs, HIV-1 PIs, or an HIV-1 integrase inhibitor then were infected with HERV-K$_{CON}$. Target cell infection was measured by counting puromycin CFU. HERV-K$_{CON}$ infection results in stable and authentic integration into the target cell genome as well as episomal forms (49). Values shown represent the means ± SD from two independent experiments. Stars indicate significant differences in the number of infectious units between vehicle-treated and drug-treated cells ($P < 0.001$ by t test).

inhibitors, or the integrase inhibitor 118-D-24, which have been shown to inhibit the infectivity of HERV-K$_{CON}$. We observed that treatment with the NRTIs AZT, TDF, 3TC, ddC, d4T, ddI, FTC, ABC, ddCTP, and ddGTP inhibited the infectivity of HERV-K$_{CON}$ in a dose-dependent manner (Fig. 9). Treatment of cells with ddATP, ddTTP, or ddUTP moderately inhibited the infectivity of HERV-K$_{CON}$ at a concentration of 50 μM but not at a concentration of 5 μM. The infectivity of HERV-K$_{CON}$ was not affected by treatment of target cells with the HIV-1 NNRTIs ETV, NVP, or EFV or by treatment with HIV PIs or the HIV-1-specific integrase inhibitor 118-D-24. Taken together, these data suggest that certain NRTIs can inhibit (i) the infectious capacity of HERV-K$_{CON}$, (ii) the activity of K103 RT, and (iii) the RT activity and replication capacity of HK2 viral genomes produced by NCCIT cells.

DISCUSSION

HK2 is the most recent HERV entrant into the human genome. Members of this family are transcriptionally active and produce retrovirus-like particles that have been shown to be infectious to some extent. Modern HK2 viruses released by cancer cell lines appear to infect and replicate their genome, although to date no proof exists that these viruses can integrate into the cellular genome of a target cell (51). We have shown that HK2 virus particles produced from certain cancer cell lines can package viral RNA and passage it to a target cell (51). We further showed evidence that replication of the HK2 genome occurred in viral particles as well as in the infected cell (53), resembling the viral replication mechanism of spumaviruses (52). The infectious material of HK2 viruses can circularize and create episomal forms (51), similar to transforming viruses such as Epstein-Barr virus (EBV) and cytomegalovirus (CMV) (58, 59). It is possible, then, that despite no proof of HK2 integration, replication and episomal formation of HK2 viruses could lead to carcinogenesis or other disturbances in cellular replication in human cells.

In the present report, we show that HK2 can express functional RT enzymes that replicate HK2 genomes. We cloned and expressed RT proteins produced by the most recent HK2 proviruses K108a and K113, as well as the K103 RT enzyme. Consistent with a previous report, we detected RT activity produced by the K103 RT enzyme, called clone 10.9 by the group of Berkhout et al. (54). However, we did not find enzymatic activity for RT proteins coded by K108 and K113. Lack of RT activity in the K113 RT is consistent with the observations of the group of Beimforde et al., who identified six

inactivating mutations (50). The inactivation of the K108 RT protein can be explained by a mutation of Y to C in the RT catalytic motif YIDD of K108 that was verified by sequencing of the K108 expression vector. This mutation is present in one of the proviruses, K108a, that make up the tandem repeat K108 (4, 60). Three forms of K108 exist polymorphically inserted in humans: solo LTR, provirus K108a with the inactivating mutation, and the K108 tandem repeat that has the adjacent provirus K108b. K108b alleles with the YIDD catalytic motif and intact RT genes have been found in the population but at low frequency (60). Berkhout et al. showed previously that K108b RT, called clone 7.1, is functional (54).

We observed that zidovudine (AZT) blocked the RT activity of K103 RT in a dose-dependent manner *in vitro* in a manner similar to how it blocks RT activity produced by the cancer cell lines NCCIT and Sk-Mel 103, which express K103. Further, AZT was able to inhibit the reverse transcription of HK2 genomes within viral particles released by the NCCIT and Sk-Mel 103 cell lines. AZT did not affect the RT activity of cancer cell lines that do not express K103. These results suggest that expression of K103 contributes to reverse transcription of contemporary HK2 viruses in cancer cells, although other HK2 RTs might be involved. Although no solid evidence exists yet that infectious HK2 particles lead to carcinogenesis, our results indicate that HK2 RTs such as K103 RT are a therapeutic target to block HK2 and their potential pathogenic effects in cancer or the neurological syndromes in which HK2 has been implicated (22–42).

We tested the susceptibility of HK2 RT to several FDA-approved nucleoside reverse transcriptase inhibitors (NRTIs), HIV-1 specific nonnucleoside (NNRTIs), and protease inhibitors. Our studies indicate that K103 RT can be inhibited *in vitro* by certain nucleoside analogs (NRTIs) with different inhibitory concentrations. These same NRTIs also were able to inhibit the RT activity in NCCIT cancer cells. The NRTI didanosine (ddI) showed the most potent inhibitory concentration. The nucleotide reverse transcriptase inhibitor (NtRTI) tenofovir (TDF) was the second most potent drug at inhibiting the RT activity of NCCIT cells. The NRTIs emtracitabine (FTC) and abacavir (ABC) were also able to inhibit RT activity, although at higher doses. Treatment with AZT, TDF, 3TC, ABC, and FTC significantly lowered the levels of RNA and DNA genomic intermediates found in HK2 virus particles released from NCCIT in a dose-dependent manner. Treatment with AZT also inhibited the amount of HK2 cellular DNA. These results suggest that HK2 reverse transcription in NCCIT is dependent on HK2 K103 RT. All of the NRTIs and NtRTIs that inhibit the genomic replication of contemporary HK2 particles blocked the infectivity of HERV-K$_{CON}$ particles treated with these agents prior to exposure to target cells in a dose-dependent manner, especially the most potent drugs, AZT, 3TC, and d4T.

Interestingly, the HIV-1 specific NNRTI etravirine (ETV) inhibited the RT activity of NCCIT cells in addition to partially inhibiting the infectivity of HERV-K$_{CON}$ on target cells, but it did not inhibit the activity of K103 RT or affect the reverse transcription of contemporary HK2 particles. The other NNRTIs tested, efavirenz (EFV) and nevirapine (NVP), did not inhibit K103 RT activity, the production of HK2 particles released by NCCIT, or the infectivity of HERV-K$_{CON}$. ETV could inhibit the RT activity of NCCIT by a mechanism different from blocking HK2 RT, resulting in the partial inhibition of HERV-K$_{CON}$ infectivity.

A recent study by Tyagi et al. showed that NRTIs and HIV-1-specific NNRTIs and PIs blocked HK2 replication (61). While the present study confirms that NRTIs can inhibit HK2, our studies expand these observations and show a wide range of drugs that can inhibit HK2 with different potencies. Our studies also reveal how the RT inhibitors affect the enzymatic activity of HK2 RT. In contrast to Tyagi and colleagues, our studies showed that only ETV, but not other HIV-1 NNRTI or PIs, inhibited HK2 infectivity to any extent. However, ETV did not block the enzymatic activity of the K103 RT. Thus, it is possible that ETV can block other HK2 RTs and thereby interfere with HK2 replication. While our findings are consistent with the general understanding that NRTIs are more likely to have an effect on diverse retroviruses, whereas NNRTIs and PIs are thought to be specific to HIV, Tyagi and colleagues presented structural data to explain why drugs

from the latter two categories inhibit replication of HK2. Therefore, the discrepancy in the two sets of results remains to be explained.

Taken together, the findings in the present report cast further light on the genetics and enzymatic activity of diverse HK2s, especially those thought to be candidates for replication in modern humans. The work also provides a broad screen of the effect of NRTIs on the only functional HK2 reverse transcriptase found by us and by others: K103. The studies further demonstrate the effect of specific agents, especially NRTIs, on HK2 reverse transcription both within the virus itself and in cells. These results could prove to be helpful in determining which HK2 inhibitors can be useful for treating diseases related to HK2 pathology. Likewise, these studies will be essential to ongoing efforts to discern whether and how specific modern and ancient HK2 can/could be replication competent.

MATERIALS AND METHODS

Plasmid constructs. The plasmid CHKCP, coding for the infectious HERV-K$_{CON}$, and the plasmid pCR3.1/K-Rec were kindly provided by Paul Bieniasz. HERV-K$_{CON}$ contains a puromycin reporter cassette inserted into the *env* gene, and its expression is driven by the CMV promoter (49). pCR3.1/K-Rec encodes the HK2108 Rec protein. The VSVG plasmid codes for the vesicular stomatitis virus (VSV) envelope.

Chemical agents. The chemicals AZT, APV, ABC, TDF, ETV, IDV, NVP, EFV, FTC, 3TC, d4T, ddC, ddI, and 118-D-24 were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS. The chemicals ddATP, ddCTP, ddGTP, ddTTP, and ddUTP were purchased from TriLink Biotechnologies.

Culture of cell lines. The cell lines 293, NCCIT, PA-1, DT22, T47D, Sk-Mel19, and Sk-Mel103 were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin. Cells were grown in 6- or 24-well plate dishes.

Cloning and expression of the HK2 *rt* gene. The *rt* gene of provirus K103 was amplified from DNA isolated from NCCIT cells. PCR products from *rt* genes derived from NCCIT cells were cloned into TOPO TA vector. The clones were screened by sequencing to identify clones with the K103 *rt* gene. The *rt* gene of proviruses K108 and K113 was amplified by PCR from BAC clones RP11-33P21 (K108) and RP11-398B1 (K113). The RT amplicons were cloned and verified by sequencing. The *rt* genes of K103, K108, and K113 were reamplified and cloned in frame with a His tag into the vector pET32-EK/LIC (Novagen). The RT gene was amplified using the primers RT-F-EK/LIC (<u>GAC GAC GAC AAG AT</u>G AAA TCA AGA AAG AGA AGG AAT AG) and RT-R-EK/LIC (<u>GAG GAG GAG CCC GGT</u> TAA GCA TGA AGT TCT TGT GCT T). The sense and antisense primers contain 5′ 5-ligation independent cloning (LIC) extensions for cloning into the pET-32 EK/LIC vector with LIC overhangs (underlined sequences). Expression vectors were verified by sequencing. The recombinant RT protein was expressed in *E. coli* and purified using nickel-nitrilotriacetic acid (Ni-NTA) agarose in a column (no. 30210; Qiagen). The RT proteins were analyzed by Western blotting and the RT activity validated by RT assays.

Western blot analysis. To prepare Western blot samples, purified RT-His tag proteins were resuspended in 2× Laemmli sample buffer with 50 mM dithiothreitol (no. 161-0737; Bio-Rad). Samples were boiled for 10 min and loaded into SDS-PAGE. Recombinant proteins were detected using a mouse anti-His tag antibody (no. 34660; Qiagen) and a rabbit anti-mouse IgG antibody conjugated to horse-radish peroxidase (no. AP160P; Sigma). SuperSignal West Pico chemiluminescent substrate (Thermo Scientific) was added and protein bands detected by X-ray film.

Reverse transcriptase assays. The RT activity was measured in 5 μl of cell supernatant or purified protein using the EnzChek RT assay kit (Invitrogen) as described by the manufacturer. Serial dilutions of murine leukemia virus RT (Promega) were used for calibration. The purified His tag RT proteins were tested for RT activity in the presence of RT inhibitors at different concentrations for 2 h. We used concentrations of inhibitors at 0.1× to 10× the concentration of the competitor dTTP (100 μM), included in the reaction buffer. Reactions were stopped with 200 mM EDTA, and the RT activity was determined by PicoGreen fluorescence (excitation, ~480 nm; emission, ~520 nm) using a plate reader.

Viral RNA/DNA and cellular DNA extraction. Supernatants were cleared of cellular debris by centrifugation at 2,000 rpm for 10 min and treated with 200 U RNase-free DNase for 1 h at 37°C. Viral RNA and DNA were extracted from cell-free supernatants using the QIAamp viral RNA minikit (Qiagen) by following the manufacturer's protocol as previously described (51). This kit contains reagents that preserve the integrity of the RNA during extraction but uses a silica column that purifies both RNA and DNA. Viral RNA and DNA copies were calculated in 60 μl of extracted nucleic acid material. Viral nucleic acids were eluted from the QIAamp columns in equal volumes and stored at −80°C. Cellular DNA was extracted with the QIAmp DNA minikit by following the manufacturer's instructions (Qiagen).

Quantitative RT-PCR. Viral RNA/DNA and cellular DNA were analyzed for expression of the endogenous retrovirus HK2 *gag* and type 2 *env*, as well as the housekeeping gene *gapdh*, by real-time RT-PCR using the primers described below. Quantitative RT-PCR was done using the Brilliant III ultrafast SYBR green quantitative RT-PCR (qRT-PCR) kit (Agilent Technologies). Quantitative PCR was performed using the SYBR green master mix. The quantitative reactions were run in the StepOne Plus real-time PCR system (Applied Biosystems). *In vitro* RNA transcripts for the *gag* and *env* genes were used as calibrators to generate standard curves that were used to determine viral RNA quantity as described previously (11). Levels of *gag* and *env* DNA were measured using the $\Delta\Delta C_T$ method, normalizing to the threshold cycle (C_T) values of the *gapdh* gene.

Virus infection. Production of infectious HERV-K$_{CON}$ viruses was performed as described previously (49, 51). Briefly, plasmid CHKCP encoding HERV-K$_{CON}$ was transfected into 293 cells with plasmids expressing HK2 Rec and VSVG. Virus-containing supernatant was cleared of cellular debris with two centrifugations at 1,000 × g at 4°C, followed by filtration through a 0.45-μm Whatman filter. Infection was performed by exposing the purified virus to 60 to 70% confluent target 293 cells seeded in 6- or 24-well plates in the presence of 5 μg/ml of Polybrene. Target cells were preincubated in the presence or absence of nucleoside and nonnucleoside reverse transcriptase inhibitors (RTIs), protease inhibitors (PIs), or the HIV-1 integrase inhibitor 118-D-24 for 24 h. We used concentrations of inhibitors in the range of 5 μM to 100 μM, which have been used previously to inhibit HERV-K$_{CON}$ (49). Cells transduced with HERV-K$_{CON}$ were selected with 2 μg/ml puromycin for approximately 10 to 20 days. Selected cells were stained with Coomassie blue for visualization, the number of resistant colonies was scored, and the percentage of infectious virus was determined by calculating the percentage of resistant CFU in treated cells versus that in untreated infected cells.

Sequence analysis. The full-length *env* sequences existing in HK2 viruses were amplified from DNase-treated cell-free supernatants from particle-producing cell lines and then cloned and sequenced as previously described (11). The *env* gene was amplified using the OneStep RT-PCR kit (Qiagen, Valencia, CA) with primers ES1 and ES2 that expand the full-length *env* SU region and generate PCR products of 1,159 bp for type 1 HK2 elements and 1,451 bp for type 2 HK2 elements. The sequences were aligned in BioEdit and exported to the MEGA 6 matrix. Sequences were aligned to known HK2 proviruses (11). Phylogenetic trees were constructed and corroborated by the neighbor-joining method, using the statistical bootstrap test (10,000 replicates) of inferred phylogeny and the Kimura 2-parameter model (62). Intact ORFs of RT sequences from known HK2 proviruses were determined using translated-BLAST in the NCBI database and aligned in the BioEdit platform.

Test for recombination. We evaluated sequences for potential recombination events using several methods, as previously described (11). First, we inspected the neighbor-joining tree for each data set. Potential recombinant sequences were verified and the parent sequences identified using RIP 3.0 (http://www.hiv.lanl.gov). We verified the sequence similarity between the putative parent and query sequence on each side of the recombination spot. In 8 of 10 recombination plot analyses, recombinants were more than 99% similar to each parental sequence.

List of primers. The following primers were used:

KgagRTF, 5'-AGC AGG TCA GGT GCC TGT AAC ATT-3';

KgagRTR, 5'-TGG TGC CGT AGG ATT AAG TCT CCT-3';

Kenvtype2F, 5'-AGA CAC CGC AAT CGA GCA CCG TTG A-3';

Kenvtype2R, 5'-ATC AAG GCT GCA AGC AGC ATA CTC-3'; GAPDHF, 5'-TGC ACC ACC AAC TGC TTA GCA CCC-3'; GAPDHR, 5'-CTT GAT GAC ATC ATA TTT GGC AGG-3'; ES1, 5'-AGA AAA GGG CCT CCA CGG AGA TG-3'; and ES2, 5'-ACT GCA ATT AAA GTA AAA ATG AA-3'.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JVI.01309-17.

SUPPLEMENTAL FILE 1, PDF file, 14.1 MB.

ACKNOWLEDGMENTS

REFERENCES

1. Jern P, Coffin JM. 2008. Effects of retroviruses on host genome function. Annu Rev Genet 42:709–732. https://doi.org/10.1146/annurev.genet.42.110807.091501.
2. Nelson PN, Carnegie PR, Martin J, Davari Ejtehadi H, Hooley P, Roden D, Rowland-Jones S, Warren P, Astley J, Murray PG. 2003. Demystified. Human endogenous retroviruses. Mol Pathol 56:11–18. https://doi.org/10.1136/mp.56.1.11.
3. Subramanian RP, Wildschutte JH, Russo C, Coffin JM. 2011. Identification, characterization, and comparative genomic distribution of the HK2 group of human endogenous retroviruses. Retrovirology 8:90. https://doi.org/10.1186/1742-4690-8-90.
4. Barbulescu M, Turner G, Seaman MI, Deinard AS, Kidd KK, Lenz J. 1999. Many human endogenous retrovirus K (HERV-K) proviruses are unique to humans. Curr Biol 9:861–868. https://doi.org/10.1016/S0960-9822(99)80390-X.
5. Lenz J. 2016. HK2 diversity among humans. Proc Natl Acad Sci U S A 2016 113:4240–4242. https://doi.org/10.1073/pnas.1603569113.

6. Belshaw R, Pereira V, Katzourakis A, Talbot G, Paces J, Burt A, Tristem M. 2004. Long-term reinfection of the human genome by endogenous retroviruses. Proc Natl Acad Sci USA 101:4894–4899. https://doi.org/10.1073/pnas.0307800101.

7. Paces J, Pavlícek A, Zika R, Kapitonov VV, Jurka J, Paces V. 2004. HERVd: the Human Endogenous RetroViruses Database: update. Nucleic Acids Res 32:D50. https://doi.org/10.1093/nar/gkh075.

8. Hughes JF, Coffin JM. 2004. Human endogenous retrovirus K solo-LTR formation and insertional polymorphisms: implications for human and viral evolution. Proc Natl Acad Sci U S A 101:1668–1672. https://doi.org/10.1073/pnas.0307885100.

9. Turner G, Barbulescu M, Su M, Jensen-Seaman MI, Kidd KK, Lenz J. 2001. Insertional polymorphisms of full-length endogenous retroviruses in humans. Curr Biol 11:1531–1535. https://doi.org/10.1016/S0960-9822(01)00455-9.

10. Wildschutte JH, Williams ZH, Montesion M, Subramanian RP, Kidd JM, Coffin JM. 2016. Discovery of unfixed endogenous retrovirus insertions in diverse human populations. Proc Natl Acad Sci U S A 113:E2326–E2334. https://doi.org/10.1073/pnas.1602336113.

11. Contreras-Galindo R, Kaplan MH, Contreras-Galindo AC, Gonzalez-Hernandez MJ, Ferlenghi I, Giusti F, Lorenzo E, Gitlin SD, Dosik MH, Yamamura Y, Markovitz DM. 2012. Characterization of human endogenous retroviral elements in the blood of HIV-1-infected individuals. J Virol 86:262–276. https://doi.org/10.1128/JVI.00602-11.

12. Contreras-Galindo R, Kaplan MH, He S, Contreras-Galindo AC, Gonzalez-Hernandez MJ, Kappes F, Dube D, Chan SM, Robinson D, Meng F, Dai M, Gitlin SD, Chinnaiyan AM, Omenn GS, Markovitz DM. 2013. HIV infection reveals widespread expansion of novel centromeric human endogenous retroviruses. Genome Res 23:1505–1513. https://doi.org/10.1101/gr.144303.112.

13. Zahn J, Kaplan MH, Fischer S, Dai M, Meng F, Saha AK, Cervantes P, Chan SM, Dube D, Omenn GS, Markovitz DM, Contreras-Galindo R. 2015. Expansion of a novel endogenous retrovirus throughout the pericentromeres of modern humans. Genome Biol 16:74. https://doi.org/10.1186/s13059-015-0641-1.

14. Johnston JB, Silva C, Holden J, Warren KG, Clark AW, Power C. 2001. Monocyte activation and differentiation augment human endogenous retrovirus expression: implications for inflammatory brain diseases. Ann Neurol 50:434–442. https://doi.org/10.1002/ana.1131.

15. Ruda VM, Akopov SB, Trubetskoy DO, Manuylov NL, Vetchinova AS, Zavalova LL, Nikolaev LG, Sverdlov ED. 2004. Tissue specificity of enhancer and promoter activities of a HERV-K(HML-2) LTR. Virus Res 104:11–16. https://doi.org/10.1016/j.virusres.2004.02.036.

16. Seifarth W, Baust C, Murr A, Skladny H, Krieg-Schneider F, Blusch J, Werner T, Hehlmann R, Leib-Mösch C. 1998. Proviral structure, chromosomal location, and expression of HERV-K-T47D, a novel human endogenous retrovirus derived from T47D particles. J Virol 72:8384–8391.

17. Sugimoto J, Matsuura N, Kinjo Y, Takasu N, Oda T, Jinno Y. 2001. Transcriptionally active HERV-K genes: identification, isolation, and chromosomal mapping. Genomics 72:137–144. https://doi.org/10.1006/geno.2001.6473.

18. Tönjes RR, Löwer R, Boller K, Denner J, Hasenmaier B, Kirsch H, König H, Korbmacher C, Limbach C, Lugert R, Phelps RC, Scherer J, Thelen K, Löwer J, Kurth R. 1996. HERV-K: the biologically most active human endogenous retrovirus family. J Acquir Immune Defic Syndr Hum Retrovirol 1:S261–S267. https://doi.org/10.1097/00042560-199600001-00039.

19. Wang-Johanning F, Frost AR, Johanning GL, Khazaeli MB, LoBuglio AF, Shaw DR, Strong TV. 2001. Expression of human endogenous retrovirus k envelope transcripts in human breast cancer. Clin Cancer Res 7:1553–1560.

20. Yi JM, Kim HM, Kim HS. 2001. Molecular cloning and phylogenetic analysis of the human endogenous retrovirus HERV-K long terminal repeat elements in various cancer cells. Mol Cells 12:137–141.

21. Gonzalez-Hernandez MJ, Cavalcoli JD, Sartor MA, Contreras-Galindo R, Meng F, Dai M, Dube D, Saha AK, Gitlin SD, Omenn GS, Kaplan MH, Markovitz DM. 2014. Regulation of the human endogenous retrovirus K (HK2) transcriptome by the HIV-1 Tat protein. J Virol 88:8924–8935. https://doi.org/10.1128/JVI.00556-14.

22. Bieda K, Hoffmann A, Boller K. 2001. Phenotypic heterogeneity of human endogenous retrovirus particles produced by teratocarcinoma cell lines. J Gen Virol 82:591–596. https://doi.org/10.1099/0022-1317-82-3-591.

23. Büscher K, Trefzer U, Hofmann M, Sterry W, Kurth R, Denner J. 2005. Expression of human endogenous retrovirus K in melanomas and melanoma cell lines. Cancer Res 65:4172–4180. https://doi.org/10.1158/0008-5472.CAN-04-2983.

24. Löwer R, Boller K, Hasenmaier B, Korbmacher C, Müller-Lantzsch N, Löwer J, Kurth R. 1993. Identification of human endogenous retroviruses with complex mRNA expression and particle formation. Proc Natl Acad Sci U S A 90:4480–4484. https://doi.org/10.1073/pnas.90.10.4480.

25. Muster T, Waltenberger A, Grassauer A, Hirschl S, Caucig P, Romirer I, Födinger D, Seppele H, Schanab O, Magin-Lachmann C, Löwer R, Jansen B, Pehamberger H, Wolff K. 2003. An endogenous retrovirus derived from human melanoma cells. Cancer Res 63:8735–8741.

26. Golan M, Hizi A, Resau JH, Yaal-Hahoshen N, Reichman H, Keydar I, Tsarfaty I. 2008. Human endogenous retrovirus (HERV-K) reverse transcriptase as a breast cancer prognostic marker. Neoplasia 10:521–533. https://doi.org/10.1593/neo.07986.

27. Ejthadi HD, Martin JH, Junying J, Roden DA, Lahiri M, Warren P, Murray PG, Nelson PN. 2005. A novel multiplex RT-PCR system detects human endogenous retrovirus-K in breast cancer. Arch Virol 150:177–184. https://doi.org/10.1007/s00705-004-0378-8.

28. Johanning GL, Malouf GG, Zheng X, Esteva FJ, Weinstein JN, Wang-Johanning F, Su X. 2017. Expression of human endogenous retrovirus-K is strongly associated with the basal-like breast cancer phenotype. Sci Rep 7:41960. https://doi.org/10.1038/srep41960.

29. Chen T, Meng Z, Gan Y, Wang X, Xu F, Gu Y, Xu X, Tang J, Zhou H, Zhang X, Gan X, Van Ness C, Xu G, Huang L, Zhang X, Fang Y, Wu J, Zheng S, Jin J, Huang W, Xu R. 2013. The viral oncogene Np9 acts as a critical molecular switch for co-activating β-catenin, ERK, Akt and Notch1 and promoting the growth of human leukemia stem/progenitor cells. Leukemia 27:1469–1478. https://doi.org/10.1038/leu.2013.8.

30. Denne M, Sauter M, Armbruester V, Licht JD, Roemer K, Mueller-Lantzsch N. 2007. Physical and functional interactions of human endogenous retrovirus proteins Np9 and rec with the promyelocytic leukemia zinc finger protein. J Virol 81:5607–5616. https://doi.org/10.1128/JVI.02771-06.

31. Büscher K, Hahn S, Hofmann M, Trefzer U, Ozel M, Sterry W, Löwer J, Löwer R, Kurth R, Denner J. 2006. Expression of the human endogenous retrovirus-K transmembrane envelope, Rec and Np9 proteins in melanomas and melanoma cell lines. Melanoma Res 16:223–234. https://doi.org/10.1097/01.cmr.0000215031.07941.ca.

32. Armbruester V, Sauter M, Roemer K, Best B, Hahn S, Nty A, Schmid A, Philipp S, Mueller A, Mueller-Lantzsch N. 2004. Np9 protein of human endogenous retrovirus K interacts with ligand of numb protein X. J Virol 78:10310–10309. https://doi.org/10.1128/JVI.78.19.10310-10319.2004.

33. Armbruester V, Sauter M, Krautkraemer E, Meese E, Kleiman A, Best B, Roemer K, Mueller-Lantzsch N. 2002. A novel gene from the human endogenous retrovirus K expressed in transformed cells. Clin Cancer Res 8:1800–1807.

34. Hanke K, Chudak C, Kurth R, Bannert N. 2013. The Rec protein of HERV-K(HK2) upregulates androgen receptor activity by binding to the human small glutamine-rich tetratricopeptide repeat protein (hSGT). Int J Cancer 132:556–567. https://doi.org/10.1002/ijc.27693.

35. Kaufmann S, Sauter M, Schmitt M, Baumert B, Best B, Boese A, Roemer K, Mueller-Lantzsch N. 2010. Human endogenous retrovirus protein Rec interacts with the testicular zinc-finger protein and androgen receptor. J Gen Virol 91:1494–1502. https://doi.org/10.1099/vir.0.014241-0.

36. Galli UM, Sauter M, Lecher B, Maurer S, Herbst H, Roemer K, Mueller-Lantzsch N. 2005. Human endogenous retrovirus rec interferes with germ cell development in mice and may cause carcinoma in situ, the predecessor lesion of germ cell tumors. Oncogene 24:3223–3228. https://doi.org/10.1038/sj.onc.1208543.

37. Contreras-Galindo R, Kaplan MH, Markovitz DM, Lorenzo E, Yamamura Y. 2006. Detection of HERV-K(HK2) viral RNA in plasma of HIV type 1-infected individuals. AIDS Res Hum Retrovir 22:979–984. https://doi.org/10.1089/aid.2006.22.979.

38. Contreras-Galindo R, Kaplan MH, Leissner P, Verjat T, Ferlenghi I, Bagnoli F, Giusti F, Dosik MH, Hayes DF, Gitlin SD, Markovitz DM. 2008. Human endogenous retrovirus K (HK2) elements in the plasma of people with lymphoma and breast cancer. J Virol 82:9329–9336. https://doi.org/10.1128/JVI.00646-08.

39. Contreras-Galindo R, Almodóvar-Camacho S, González-Ramírez S, Lorenzo E, Yamamura Y. 2007. Comparative longitudinal studies of HERV-K and HIV-1 RNA titers in HIV-1-infected patients receiving successful versus unsuccessful highly active antiretroviral therapy. AIDS Res Hum Retrovir 23:1083–1086. https://doi.org/10.1089/aid.2007.0054.

40. Contreras-Galindo R, López P, Vélez R, Yamamura Y. 2007 HIV-1 infection increases the expression of human endogenous retroviruses type K

(HERV-K) in vitro. AIDS Res Hum Retrovir 23:116–122. https://doi.org/10.1089/aid.2006.0117.

41. Mortelmans K, Wang-Johanning F, Johanning GL. 2016. The role of human endogenous retroviruses in brain development and function. APMIS 124:105–115. https://doi.org/10.1111/apm.12495.

42. Li W, Lee MH, Henderson L, Tyagi R, Bachani M, Steiner J, Campanac E, Hoffman DA, von Geldern G, Johnson K, Maric D, Morris HD, Lentz M, Pak K, Mammen A, Ostrow L, Rothstein J, Nath A. 2015. Human endogenous retrovirus-K contributes to motor neuron disease. Sci Transl Med 7:307ra153. https://doi.org/10.1126/scitranslmed.aac8201.

43. Mayer J, Sauter M, Rácz A, Scherer D, Mueller-Lantzsch N, Meese E. 1999. An almost-intact human endogenous retrovirus K on human chromosome 7. Nat Genet 21:257–258. https://doi.org/10.1038/6766.

44. Mayer J, Meese E, Mueller-Lantzsch N. 1997. Multiple human endogenous retrovirus (HERV-K) loci with gag open reading frames in the human genome. Cytogenet Cell Genet 78:1–5.

45. Dewannieux M, Harper F, Richaud A, Letzelter C, Ribet D, Pierron G, Heidmann T. 2006. Identification of an infectious progenitor for the multiple-copy HERV-K human endogenous retroelements. Genome Res 16:1548–1556. https://doi.org/10.1101/gr.5565706.

46. Hanke K, Hohn O, Bannert N. 2016. HERV-K(HK2), a seemingly silent subtenant–but still waters run deep. APMIS 124:67–87. https://doi.org/10.1111/apm.12475.

47. Delviks-Frankenberry K, Paprotka T, Cingöz O, Wildt S, Hu WS, Coffin JM, Pathak VK. 2013. Generation of multiple replication-competent retroviruses through recombination between PreXMRV-1 and PreXMRV-2. J Virol 87:11525–11537. https://doi.org/10.1128/JVI.01787-13.

48. Young GR, Eksmond U, Salcedo R, Alexopoulou L, Stoye JP, Kassiotis G. 2012. Resurrection of endogenous retroviruses in antibody-deficient mice. Nature 491:774–778. https://doi.org/10.1038/nature11599.

49. Lee YN, Bieniasz PD. 2007. Reconstitution of an infectious human endogenous retrovirus. PLoS Pathog 3:e10. https://doi.org/10.1371/journal.ppat.0030010.

50. Beimforde N, Hanke K, Ammar I, Kurth R, Bannert N. 2008. Molecular cloning and functional characterization of the human endogenous retrovirus K113. Virology 371:216–225.

51. Contreras-Galindo R, Kaplan MH, Dube D, Gonzalez-Hernandez MJ, Chan S, Meng F, Dai M, Omenn GS, Gitlin SD, Markovitz DM. 2015. Human endogenous retrovirus type K (HERV-K) particles package and transmit HERV-K-related sequences. J Virol 89:7187–7201. https://doi.org/10.1128/JVI.00544-15.

52. Rethwilm A. 2010. Molecular biology of foamy viruses. Med Microbiol Immunol 199:197–207. https://doi.org/10.1007/s00430-010-0158-x.

53. Dube D, Contreras-Galindo R, He S, King SR, Gonzalez-Hernandez MJ, Gitlin SD, Kaplan MH, Markovitz DM. 2014. Genomic flexibility of human endogenous retrovirus type K. J Virol 88:9673–9682. https://doi.org/10.1128/JVI.01147-14.

54. Berkhout B, Jebbink M, Zsíros J. 1999. Identification of an active reverse transcriptase enzyme encoded by a human endogenous HERV-K retrovirus. J Virol 73:2365–2375.

55. Ji JP, Loeb LA. 1992. Fidelity of HIV-1 reverse transcriptase copying RNA in vitro. Biochemistry 31:954–958.

56. Varga A, Lionne C, Roy B. 2016. Intracellular metabolism of nucleoside/nucleotide analogues: a bottleneck to reach active drugs on HIV reverse transcriptase. Curr Drug Metab 17:237–252. https://doi.org/10.2174/1389200217666151210141903.

57. Kewn S, Hoggard PG, Henry-Mowatt JS, Veal GJ, Sales SD, Barry MG, Back DJ. 1999. Intracellular activation of 2',3'-dideoxyinosine and drug interactions in vitro. AIDS Res Hum Retrovir 15:793–802.

58. Richardson AK, Currie MJ, Robinson BA, Morrin H, Phung Y, Pearson JF, Anderson TP, Potter JD, Walker LC. 2015. Cytomegalovirus and Epstein-Barr virus in breast cancer. PLoS One 10:e0118989. https://doi.org/10.1371/journal.pone.0118989.

59. Giller RH, Grose C. 1989. Epstein-Barr virus: the hematologic and oncologic consequences of virus-host interaction. Crit Rev Oncol Hematol 9:149–195.

60. Reus K, Mayer J, Sauter M, Scherer D, Müller-Lantzsch N, Meese E. 2001. Genomic organization of the human endogenous retrovirus HERV-K(HK2.HOM) (ERVK6) on chromosome 7. Genomics 72:314–320.

61. Tyagi R, Li W, Parades D, Bianchet MA, Nath A. 2017. Inhibition of human endogenous retrovirus-K by antiretroviral drugs. Retrovirology 14:21. https://doi.org/10.1186/s12977-017-0347-4.

62. Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791.

63. Lv Z, Chu Y, Wang Y. 2015. HIV protease inhibitors: a review of molecular selectivity and toxicity. HIV/AIDS (Auckland, NZ) 7:95–104. https://doi.org/10.2147/HIV.