(mean ± SD of three experiments) (Fig. 4). The inability of rCD4-dgA to kill Daudi cells at concentrations $>5 \times 10^{-8} M$ may be due to a lower binding affinity of rCD4-dgA to class II molecules or to the inability of class II antigens to be internalized after binding rCD4-dgA.

If infected cells from HIV-positive individuals can also be killed by rCD4-dgA, it might be possible to prevent or delay the onset of clinical disease. The effective application of such a therapeutic strategy, however, must take into account the mode of latent HIV infection and the factors contributing to viral activation.

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Fidelity of HIV-1 Reverse Transcriptase

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The human immunodeficiency virus type 1 (HIV-1) shows extensive genetic variation and undergoes rapid evolution. The fidelity of purified HIV-1 reverse transcriptase was measured during DNA polymerization in vitro by means of three different assays. Reverse transcriptase from HIV-1 introduced base-substitution errors in DNA from the bacteriophage ϕX174 *amber*3 at estimated frequencies of 1/2000 to 1/4000. Analyses of misincorporation rates opposite a single template adenine residue showed that HIV-1 reverse transcriptase catalyzed nucleotide mismatches with a specificity of A:C >> A:G > A:A. The high error rate of HIV-1 reverse transcriptase in vitro translates to approximately five to ten errors per HIV-1 genome per round of replication in vivo. This high error rate suggests that misincorporation by HIV-1 reverse transcriptase is, at least in part, responsible for the hypermutability of the AIDS virus. The specificity of misincorporation may provide a basis for the systematic construction of antiviral nucleosides.

GENOMIC HETEROGENEITY IS A hallmark of HIV-1 (*1–3*). Studies on sequential HIV-1 isolates from persistently infected individuals suggest that HIV-1 evolves at a rate approximately a million times as great as that of eukaryotic DNA genomes (*2*). This hypermutability could be central to the pathogenesis of HIV-1 and could thwart efforts to develop effective vaccines.

The high rate of HIV-1 mutagenesis is shared by other retroviruses (*4*) and presumably originates in mechanisms unique to the retroviral life cycle. Replication of retroviral genomes proceeds by a series of enzymatic reactions involving virus-encoded reverse transcriptase (RT) and integrase, as well as host cell–encoded DNA polymerases and RNA polymerase II (*5*). The viral RT polymerizes deoxyribonucleotides by using viral RNA as a template and also acts as a DNA polymerase in converting the resulting minus strand DNA into double-stranded DNA (*6*). The high error rate of avian myeloblastosis virus RT in vitro, with both deoxyribonucleotide and ribonucleotide templates (*7*), implicates RTs as major contributors to retroviral mutagenesis. In contrast, provirus replication is not very error-prone (*8*), presumably because replication is achieved by the more accurate host cell DNA polymerase α (*9*).

We used three different methods to evaluate the fidelity of DNA synthesis by HIV-1 RT. First, as a qualitative assessment of misincorporations opposite multiple template sites, we used the "minus" sequencing gel assay (*10, 11*). In the presence of only three 2′-deoxyribonucleoside 5′-triphos-

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Table 1. Kinetic parameters for site-specific misincorporation. Apparent K_m and V_{max} values ± standard errors were determined by the method of Wilkinson (*22*) from the data in Fig. 2. The relative insertion frequencies were calculated from the V_{max}/K_m ratios and are expressed relative to dTTP.

RT	dNTP	K_m(app) (μ*M*)	V_{max}(app) (%/min)	V_{max}/K_m [%/(min *M*)]	Relative insertion frequency
HIV-1 (virion)	T	3.7 ± 1.0	80 ± 10	2.1×10^7	1
	C	290 ± 56	4.2 ± 0.2	1.4×10^4	1/1,500
	G	1200 ± 240	0.4 ± 0.05	3.2×10^2	1/66,000*
	A	ND†	ND	$<1.3 \times 10^2$	<1/170,000‡
HIV-1 (clone)	T	4.0 ± 1.7	133 ± 27	3.4×10^7	1
	C	370 ± 72	2.9 ± 0.27	7.8×10^3	1/4,300
	G	ND	ND	1.5×10^2	1/200,000‡
	A	ND	ND	$<1.3 \times 10^2$	<1/270,000‡
AMV (virion)	T	0.7 ± 0.2	32 ± 3.0	4.6×10^7	1
	C	1500 ± 370	2.7 ± 0.4	1.8×10^3	1/26,000
	G	ND	ND	$<1.3 \times 10^2$	<1/370,000‡
	A	ND	ND	$<1.3 \times 10^2$	<1/370,000‡

*Calculated from incubations containing excess RT (0.04 unit/μl). †Could not be accurately determined because of the low levels of misincorporation. ‡Estimated from the slope of the initial velocity versus [dNTP] plot.

phates (dNTP), DNA polymerases or viral RTs readily extend an oligonucleotide primer up to, but not opposite, the template positions that normally pair with the absent dNTP. Extension opposite or beyond these positions arises by nucleotide misincorporation. A comparison of oligonucleotides extended by cloned HIV-1 RT with those extended by RT from avian myeloblastosis virus (AMV) and Moloney murine leukemia virus (M-MuLV), as well as the mammalian DNA polymerase α–primase complex, shows that each of these enzymes pauses at template sites for which the complementary nucleotide is lacking, but HIV-1 RT copies past these sites at the highest frequency (Fig. 1). The frequency of misinsertion at a given template site can be estimated from the yield of oligonucleotides extended opposite and beyond the target site. When averaged over all detected sites in all minus reactions, the relative frequencies of misinsertion were HIV-1 RT > DNA polymerase α > AMV RT > M-MuLV RT. Moreover, the relative abilities to extend nascent mismatches, as determined from the yields of oligonucleotides terminating immediately opposite target sites, were HIV-1 RT > AMV RT > DNA polymerase α. (Note: mismatch extension by M-MuLV RT could not be determined because of the low yields of oligonucleotides with single mispairs.) Thus, under the conditions of this assay, HIV-1 RT is highly error-prone in its ability to both insert and extend nucleotide mispairs.

To characterize the fidelity of misinsertion by HIV-1 RT, we used a second assay that measures site-specific nucleotide misincorporation (*12*). Rates of nucleotide insertion were examined opposite the template A residue at position 587 of φX174 *am3* DNA primed with a 15-nucleotide oligomer that was labeled with ^{32}P at the 5′ end (Fig. 2A). To reach the target site, the polymerase must first incorporate two complementary C's. Rates of primer extension from the resultant 17-nucleotide oligomer to products ≥18 nucleotides were measured by polyacrylamide gel electrophoresis (*12*). With only 10 μ*M* dCTP, the RTs rapidly extended the 15-nucleotide oligomer primers up to, but not beyond, 17 nucleotides. On addition of the correct nucleotide, dTTP, or high concentrations of the incorrect nucleotides, dATP, dCTP, or dGTP, the production of oligomers ≥18 nucleotides increased linearly with time to about 4 min (*13*). Plots of the rates of primer elongation past the template A (v_i) versus dNTP concentration showed saturation kinetics for

Table 2. Fidelity of RTs in the φX174 mutagenesis assay. φX174 *am3* DNA was primed (*23*) with a twofold molar excess of the pentadecamer 5′-^{32}P-labeled d(GGAAAGCGAGGGTAT)-3′ that hybridizes to nucleotide positions 590 to 604 of the φX174 genome (*24*); this yields a 15-nucleotide oligomer–φX174 *am3* partial-duplex molecule with the primer 3′-terminus positioned three nucleotides from the A residue at position 587 of the *am3* codon. Each reaction (50 μl) contained: 20 m*M* tris-HCl, *p*H 8.0; 24 m*M* KCl; 5 m*M* $MgCl_2$; 2 m*M* dithiothreitol; 0.1 mg of BSA per milliliter; 50 μ*M* each of dATP, dCTP, dGTP, and dTTP; 0.0023 pmol of the template-primer per microliter; and either 0.4 units of AMV RT per microliter or 0.2 units of cloned HIV-1 RT per microliter. Incubation was for 30 min at 30°C. Both the HIV-1 and AMV RTs extended >90% of the oligonucleotide primers yielding nascent strands with an average length of approximately 700 nucleotides (as determined by 15% polyacrylamide sequencing gels; data not shown). The polymerized molecules were transfected into spheroplasts prepared from KT-1 *Escherichia coli*, and the resultant progeny phage were titered on HF4714 (Sup$^+$) and HF4704 (Sup$^-$) *E. coli* to detect *am3* and wild-type revertant phage, respectively (*18*).

Polymerase	Phage titer		Average reversion frequency* ($\times 10^{-6}$)	Estimated error rate†
	am3 ($\times 10^8$)	Wild type ($\times 10^2$)		
None (control)	120; 120‡	340; 400	3	
HIV-1 RT	16; 56	650; 2500	43	1/4000
AMV RT	37; 86	810; 2100	23	1/9000§

*Calculated as the ratio of wild-type/*am3* titers. †Calculated from the following formula with the use of 17% for the biologic expression of the nascent minus strand (*23*): error rate = $1/[(RF_{pol} - RF_{control})/0.17]$, where RF_{pol} is the reversion frequency in the presence of polymerase and $RF_{control}$ is the reversion frequency in the absence of polymerase. ‡Values separated by semicolons represent the results of duplicate determinations. §Average error rate ± standard deviation of five independent experiments (7) = 1/10,000 ± 1/6,000.

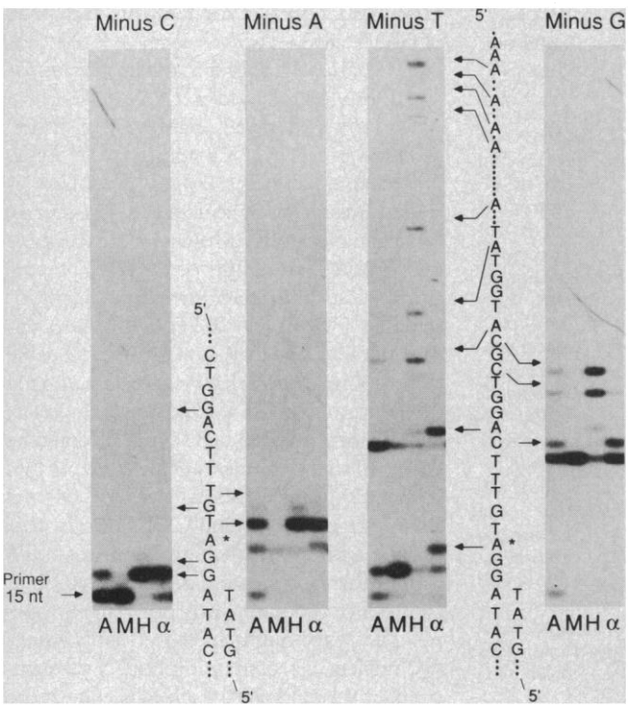

Fig. 1. "Minus" sequencing gel assay for nucleotide misincorporation. A twofold molar excess of the oligodeoxyribonucleotide ^{32}P-labeled at the 5′ end d(GGAAAGCGAGGGTAT)-3′ was hybridized to nucleotide positions 590-604 of φX174 *am3* DNA (*23, 24*). The resultant 5′-^{32}P-labeled 15-nucleotide oligomer–φX174 *am3* duplex DNA (0.0023 pmol/μl) was incubated with the indicated RT or DNA polymerase at 30°C for 30 min in a total volume of 10 μl containing: 20 m*M* tris-HCl (*p*H 8), 24 m*M* KCl, 6 m*M* $MgCl_2$, 2 m*M* dithiothreitol, 0.1 mg of BSA per milliliter, and only three dNTPs at 50 μ*M* each [minus C = 2′-deoxyadenosine 5′-triphosphate (dATP), 2′-deoxyguanosine 5′-triphosphate (dGTP), and 2′-deoxythymidine 5′-triphosphate (dTTP); minus A = 2′-deoxycytidine 5′-triphosphate (dCTP), dGTP, and dTTP, and so on]. High-performance liquid chromatography (HPLC) analysis of the dNTPs [Pharmacia (FPLC-pure) or Sigma] immediately before use (*11*) showed no detectable cross-contamination or deamination products (<0.06%). All incubations contained 0.4 unit of polymerase or RT per microliter (1 unit catalyzes the incorporation of 1 pmol of [α-^{32}P]dTTP into 0.023 pmol of 15-nucleotide oligomer–φX174 duplex DNA in 30 min at 30°C in the presence of all four dNTPs). Product oligonucleotides were isolated by Sephadex G-100 chromatography and analyzed by electrophoresis through 15% polyacrylamide sequencing gels (*23*). The primer-template sequences are shown with arrows indicating the relative nucleotide positions on each autoradiograph. Lane A, AMV RT; lane M, M-MuLV RT; lane H, cloned HIV-1 RT; and lane α, calf thymus DNA polymerase α. AMV RT and M-MuLV RT were obtained from Pharmacia and Bethesda Research Laboratories, respectively. Cloned HIV-1 RT was purified to apparent homogeneity and contained approximately equimolar amounts of p66 and p51. Calf thymus DNA polymerase α–primase complex was purified by antibody affinity chromatography (*25*). The position of nucleotide 587 is shown by the asterisk.

the incorporation of dTTP or dCTP by both HIV-1 and AMV RTs (Fig. 2, B and C). Plots of [dNTP]/v_i versus [dNTP] and $1/v_i$ versus 1/[dNTP] were also linear for these nucleotides (*14*). With both enzymes there was very low misincorporation of dGTP and virtually no detectable misincorporation of dATP even at 4000 μ*M*. We calculated the apparent Michaelis constant (K_m) and maximum velocity (V_{max}) values and the relative insertion frequencies for each dNTP (Table 1). If we assume steady-state conditions and assume that the rate-limiting step for the insertion of correct and incorrect nucleotides is the same, then the relative efficiency of nucleotide insertion (that is, V_{max}/K_m) is a measure of the accuracy of the RT (*12, 15*). Cloned HIV-1 RT, virion HIV-1 RT (*16*), and AMV RT all formed base pairs opposite the template A residue with similar relative frequencies, that is, A:T >> A:C >> A:G > A:A. However, with this method HIV-1 RT was about ten times as error-prone as AMV RT. Cloned and virion HIV RTs exhibited very similar K_m and V_{max} values for each of the dNTP substrates. The K_m values for dTTP incorporation opposite the A residue in this DNA (3.7 to 4.0 μ*M*, Table 1) are similar to those reported for virion HIV-1 RT with a poly(rA) template [2.1 to 3.8 μ*M* (*17*)]. Thus, in addition to the similar biochemical properties of the cloned and virion RT (*17*), their fidelity and mispairing specificities are also similar.

The relative misinsertion frequencies deduced from V_{max}/K_m ratios are based on measurements carried out with single dNTPs in the absence of the correct nucleotide. To determine the fidelity of HIV-1 RT during polymerization in the presence of all four dNTPs, we used a third assay that measures reversion of a single base mutation in ϕX174 *am*3 DNA. In this assay, all possible base substitutions at position 587 can be detected by reversion of the *amber* codon to wild type (*18*). In vitro polymerization by AMV RT or the cloned HIV-1 RT yielded revertant phage at frequencies about 8 or 14 times, respectively, as high as the uncopied controls (Table 2). This corresponds to estimated error rates of approximately 1/4000 for HIV-1 RT and 1/9000 for AMV RT. When the ratio of noncomplementary to complementary dNTPs was increased to 1000:1, the reversion frequency of AMV RT–copied ϕX174 *am*3 increased about 50-fold while that of HIV-1 RT-copied DNA increased only 3-fold. This insensitivity of HIV-1 RT to dNTP pool bias suggests that other, presumably lethal, mutations also occur at high frequency during polymerization of the ϕX174 genome in vitro. The ϕX174 fidelity assay scores only base substitutions at one site on the DNA template. Mutations at other sites could be detected in a forward mutation assay (*19*).

The data from all three assays indicate that HIV-1 RT introduces base-substitution errors at frequencies ranging from 1/2000 to 1/4000 during polymerization of a DNA template. If these same error rates occur in vivo during both minus strand and plus strand DNA synthesis, HIV-1 RT would catalyze five to ten mutations per HIV-1 genome per round of replication. This is similar to estimates of the rates of mutagenesis of RNA viruses in vivo (*4*).

The specificities of nucleotide misincorporation and subsequent extension of mispaired primers distinguish HIV-1 RT from mammalian DNA polymerase α. Although calf thymus DNA polymerase α inserts noncomplementary nucleotides, the resultant mispaired primer termini are not efficiently extended (Fig. 1) (*20*). In contrast, HIV-1 RT both incorporates and extends nucleotide mispairs with relatively high efficiency. This difference between HIV-1 RT and DNA polymerase α is especially observed opposite template A and C residues (Fig. 1). The specificity of mismatch formation by HIV-1 RT opposite the template A residue of ϕX174 *am*3 (A:C >> A:G > A:A) (Table 1) is also different from that of mammalian DNA polymerase α (A:A ≥ A:C >> A:G) (*20*). A detailed analysis of misincorporation by HIV-1 RT and host cell DNA polymerases is necessary to determine if unique mismatch specificities also occur at other template sites. Moreover, this analysis could guide the design of antiviral nucleoside analogs that are preferentially incorporated by HIV-1 RT. Three approaches appear feasible: (i) analogs with unextendable 3′ positions could selectively terminate viral DNA synthesis; (ii) analogs that are preferentially incorporated by HIV-1 RT could be designed to function as suicide reagents that covalently link the RT to the nascent viral DNA strand (*21*); and (iii) analogs that mispair with unusually high frequency could further increase the viral mutation rate so as to produce predominantly nonviable progeny. The latter might include doubly modified nucleosides containing both a substituent that mispairs with high efficiency and a

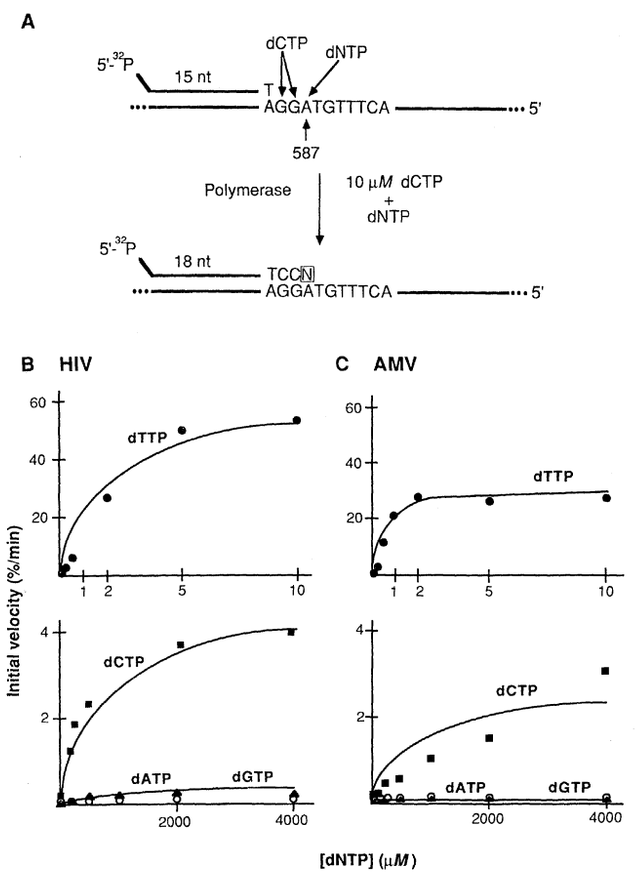

Fig. 2. Kinetic assay for site-specific nucleotide misincorporation. (**A**) Schematic diagram of procedure used to measure rates of dNTP incorporation opposite the A residue at position 587 of ϕX174 *am*3 DNA. 5′-^{32}P-labeled 15-nucleotide oligomer–primed ϕX174 *am*3 DNA (0.0023 pmol/μl; primed at a 1:1 molar ratio) was incubated at 30°C (total volume = 20 μl) with limiting polymerase (0.005 unit of cloned HIV RT per microliter, 0.01 unit of virion HIV RT per microliter, or 0.01 unit of virion AMV RT per microliter) in the presence of 10 μ*M* dCTP and increasing concentrations of single dNTPs. All other reaction components were as in Fig. 1. Aliquots (5 μl) were removed after incubation of 0, 2, 4, or 8 min and added immediately to 1 μl of 0.5*M* EDTA on ice. Product oligonucleotides were isolated by Sephadex G-100 chromatography and analyzed by electrophoresis through 15% polyacrylamide gels and quantified by densitometric scanning of gel autoradiographs (*12*). The relative rates of site-specific nucleotide incorporation are shown as functions of dNTP concentration during catalysis by virion HIV RT (**B**) and virion AMV RT (**C**). Initial velocities (v_i) of nucleotide incorporation opposite the A residue at position 587 were calculated from the relative band intensities with the formula (*12*): $v_i = (I_3/I_2)(I_2 + I_3)/t$, where I_2 and I_3 are the integrated intensities (expressed as percent of total primer) of 17-nucleotide oligomers and oligomers of ≥18 nucleotides, respectively, and t is the incubation time (2 min).

large substituent that precludes incorporation by the host cell DNA polymerase, but allows incorporation by HIV-1 RT.

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The Accuracy of Reverse Transcriptase from HIV-1

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A study was conducted to determine the fidelity of DNA synthesis catalyzed in vitro by the reverse transcriptase from a human immunodeficiency virus type 1 (HIV-1). Like other retroviral reverse transcriptases, the HIV-1 enzyme does not correct errors by exonucleolytic proofreading. Measurements with M13mp2-based fidelity assays indicated that the HIV-1 enzyme, isolated either from virus particles or from *Escherichia coli* cells infected with a plasmid expressing the cloned gene, was exceptionally inaccurate, having an average error rate per detectable nucleotide incorporated of 1/1700. It was, in fact, the least accurate reverse transcriptase described to date, one-tenth as accurate as the polymerases isolated from avian myeloblastosis or murine leukemia viruses, which have average error rates of ~1/17,000 and ~1/30,000, respectively. DNA sequence analyses of mutations generated by HIV-1 polymerase showed that base substitution, addition, and deletion errors were all produced. Certain template positions were mutational hotspots where the error rate could be as high as 1 per 70 polymerized nucleotides. The data are consistent with the notion that the exceptional diversity of the HIV-1 genome results from error-prone reverse transcription.

THE ABILITY OF HIV-1 TO ESCAPE the host's immune response (*1*), in part the result of extensive genetic variation, is one of the key features of this virus that complicates development of an effective vaccine for the prevention of AIDS. However, no information is available on the rate of mutation per HIV-1 growth cycle. In fact, the only calculation of a mutation rate for any retrovirus comes from a recent study (*2*) of an avian retroviral expression vector. Nevertheless, comparisons of HIV-1 isolates from patients in different geographic locations allow estimates of the frequency of change in terms of nucleotide substitutions per site per year, and values of ≥0.002 have been obtained (*3, 4*). Mutation frequencies have also been estimated from the nucleotide sequences of HIV-1 isolates taken from a single patient at different times after infection (*5*). Minimum estimates for the variation rate in the *env* gene range from 0.001 to 0.01 nucleotide substitutions per site per year, whereas mutation frequencies for the *gag* gene are about one-tenth as high. The most remarkable feature of HIV-1 variability is the presence of hypervariable regions within specific portions of the *env* and *nef* genes (*4*). The diversity in these regions is characterized by a wide variety of changes, including transitions, transversions, insertions, and deletions (*5*).

Genetic variability in HIV-1 may involve one or all of several steps in the viral life cycle (*6*), including reverse transcription, integration into the host chromosome to establish the proviral state, provirus replication by the host replication machinery, and transcription by RNA polymerase II into RNAs to be packaged into virions. In this study we focused on the HIV-1 reverse transcriptase (RT) as a potential source of diversity. We have used the RT as purified from virus particles as well as the purified enzyme that had been overproduced in *E. coli* carrying the cloned gene for HIV-1 RT. This recombinant enzyme appears to have the same subunit structure and kinetic properties as the natural enzyme (*7*).

We first examined HIV-1 RT for associated 3′ → 5′ proofreading exonuclease activity using two separate assays for excision of a mismatched base from a primer terminus. In the first assay (*8*), a mismatched substrate was constructed by hybridizing a 15-base oligonucleotide labeled with ^{32}P at the 5′ end to M13mp2 single-strand DNA; this created an A (template):G mispair at the 3′ primer terminus. The products of excision

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