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Full Length Research Paper

Frequency and site mapping of HIV-1/SIVcpz, HIV-2/SIVsmm and other SIV gene sequence cleavage by various bacteria restriction enzymes: Precursors for a novel HIV inhibitory product

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Resistance, toxicity and virologic failure have underlined the need to develop new HIV inhibitory products. Base on the natural bacteria "restriction modification system" antiviral immune model, we set out to analyze the effects of various restriction enzymes on the HIV genome. A computer simulated model using Web cutter Version 2.0, and cytogenetic analysis. 339 restriction enzymes from Promega database, 10 HIV-1/SIVcpz genes, 10 HIV-2/SIVsmm genes and 10 other SIV genes. Gene sequences were fed into Web cutter 2.0 set to search enzymes with at least 6 recognition base pairs (palindromes). A background *in vitro* cytogenetic control analysis using HIV-1/SIVcpz GAG, POL and ENV genes was done. Of the 339 enzymes used, 238 (70.2%) cleaved the HIV-1/SIVcpz A1.BY.97.97BL006_AF193275 genome with 9037 bp compared to 225 (66.4%) and 219 (64.6%) for the HIV-2/SIVsmm genome (9713 bp) and other SIV B.FR.83.HXB2_LAI_IIIB_BRU_K03455 genome (9719 bp), respectively. Individual genes had differing but potent susceptibility to the enzymes, with a 98.9% Web cutter PPV (95%CI, 97.2%-99.6%) for *in vitro* cytogenetics. The natural bacteria RMS antiviral immune model offers precursors for developing novel HIV and other viral therapeutic molecules.

Key words: HIV/AIDS, Novel Microbicides Strategies, Restriction Modification Systems, HIV genomes, Combination-Microbicides

INTRODUCTION

Over two decades since the first case of Acquired Immunodeficiency Syndrome (AIDS) was clinically described (CDC. Pneumocystis pneumonia- Los Angeles MMWR, 1981), the syndrome still has no cure, and global infection rates have soared from epidemic to pandemic levels (Fanci, 1999; Joint UNAIDS/WHO, 1997; WHO. Removing obstacles to health development. Geneva, 1999; UNAIDS. Report on the global HIV-AIDS epidemic, 2002). Human Immunodeficiency Virus (HIV), the virus that causes AIDS, survives and reproduces *in vivo* by incorporating its genome into that of susceptible human cells (Cains and Souza 1998; Levy, 1996; Rosenberg et al., 1998; Barre-Sioussi, 1996), and many lines of therapeutic interventions have been developed using the principle of inhibiting this replication cycle (Challand et al., 1997; Mansky and Temin, 1995). The international AIDS Society, USA current treatment options include three drug classes: two Reverse Transcriptase inhibitor classes (the Nucleoside RTI; NRTIs and the Non-nucleoside RTIs; NNRTI classes), and the Protease Inhibitors; PIs, with the Integrase (fusion) Inhibitor MK-0508 (DHHS. Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents, 2006; Hammer et al., 2006; Bartlett et al., In press; Bartlett et al., 2001). However, resistance, toxicity and virologic failure have underlined the need to develop newer HIV inhibitory products (Mansky et al., 1995), and newer products such as chemokine receptor inhibitors and monoclonal antibody entry inhibitors are in final clinical trials (Mayer et al., 2006; Gulick et al., 2006; Sansone et al., 2006; Pugach et al., 2006;

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Anastasopoulou et al., 2006; Norris et al., 2005; Norris et al., 2006; Davison et al., 2006; Delmedico et al., 2006; Markowitz et al., 2006).

Gene therapy offers another line of therapeutic intervention, and as of to day, the use of U1snRNAs to inhibit gene expression (Sajic et al., 2006), RNA interference pathway to stop early and late HIV replication through post transcriptional gene slicing (Barichievy et al., 2006; Meshcheryakova et al., 2006), autologous T cell anti-HIV anti-sense Ribonucleic acid (RNA) delivered by the VRX496 lentiviral vector (Rebello et al., 2006), and ribozyme enzymes cleaving HIV RNA(Liu et al., 2006) are being developed. Naturally, bacteria have a remarkable immunity to viral infection. Through the operation of a special enzyme system called the Restriction Modification System (RMS), whose main biological role is to protect the bacteria cell from incoming foreign deoxyribonucleic acid (DNA), many bacteria are rendered immune to viral tropism. Four types of RMS exist, with over 2,000 restrictions enzymes isolated and tested. Most of the enzymes found in nature are however attributed to the type I and II RMS, which systems comprise of two enzymes: a specific restriction endonuclease and a specific DNA methyltransferase. Both enzymes of this system recognize the same 4-8 base pair nucleotide sequence in the invading DNA with the former catalyzing cleavage of foreign double standard DNA and the later functioning to protect the host genomic DNA from cleavage by the cognate restriction enzyme (Murray, 2000; Nelson et al., 1972; Roberts and Macelis, 1991; Janulaitis et al., 1992; Kessler and Manta, 1990; Nelson and McClelland, 1991; Radasci and Bickle, 1996; Barcus and Murray, 1995)

We designed computer simulated assays with a background cytogenetic analysis control aimed at identifying which enzymes are active against the HIV genome as a means of identifying precursors for a restriction enzyme based HIV inhibitory product (Misaki, www.aegis.org/news/nv/2005/NV050301.html), and present here the results.

METHODS

Design

Computer simulated analysis employing web cutter Version 2.0 preset to analyze linear nucleotide sequences for frequency of restriction enzyme cuts and site mapping of recognition sequences greater than 6 base pairs (bp) (Makerere Develops a Model for an AIDS Cure, Web cutter Version 2.0. http://rna.lundberg.gu.se/cutter2/), followed by a control cytogenetic analysis employing HIV-1/ Simian Immunodeficiency Virus (SIV) cpz POL, GAG and ENV genes

Enzymes and genes

339 restriction enzymes accessed from the Promega (Promega Corp. http://www.promega.com/default.asp) data base, HIV genes from the HIV sequence database (HIV gene sequence Database. www.hiv.lanl.gov/content/hiv-db/ALIGN_CURRENT/ALIGN-INDEX.html): 3 HIV whole genomes (HIV-1/SIVcpz A1.BY.97.97BL006_AF193275 genome with 9037 bp, HIV-2/SIVsmm A.GM.x.MCN13_AY509259 genome with 9713 bp, and other SIV B.FR.83.HXB2_LAI_IIIB_BRU_K03455 genome with 9719 bp), 10 HIV-1/SIVcpz genes [LTR; A1.CD.97.97ZR49_AF196750 (632 bp), GAG; A.KE.97.M104gag_AY772953 (1506 bp), POL; A1.TZ.01.A341_AY253314 (3012 bp), ENV; A.CD.97.KCC2_AJ401034 (2589 bp), VIF; A.CD.97.KCC2_AJ401034 (579 bp), TAT; A1.BY.97.97BL006_AF193275 (308 bp), REV; A1.TZ.01.A341_AY253314 (372 bp), VPU/VPX; A1.BY.97.97BL006_AF193275(246 bp), VPR; A.UA.x.98UA0116_AF413973 (291 bp), and NEF; A1.BY.97.97BL006_AF193275(624 bp)], 10 HIV-2/SIVsmm genes [LTR; A.PT.x.1139D3_AY622982(380 bp), GAG; G.GH.00.GH31_AY396866
(358 bp), ENV; MAC.US.x.239_M33262 (2640 bp), VIF; A.GM.x.MCN13_AY509259 (648 bp), TAT; A.GM.x.MCN13_AY509259 (393 bp), REV; A.GM.x.MCN13_AY509259 (324 bp), VPU/VPX; A.GM.x.MCN13_AY509259 (339 bp), VPR; A.GM.x.MCN13_AY509259 (318 bp) and NEF; MAC.US.x.239_M33262 (792 bp)] and 10 other SIV genes [LTR; B.FR.83.HXB2_LAI_IIIB_BRU_K03455 (634 bp), GAG; B.FR.83.HXB2_LAI_IIIB_BRU_K03455 (1503 bp), POL; B.FR.83.HXB2_LAI_IIIB_BRU_K03455 (3009 bp), ENV; B.FR.83.HXB2_LAI_IIIB_BRU_K03455 (2571 bp), VIF; B.FR.83.HXB2_LAI_IIIB_BRU_K03455 (579 bp), TAT; B.FR.83.HXB2_LAI_IIIB_BRU_K03455 (306) REV; B.FR.83.HXB2_LAI_IIIB_BRU_K03455 (351 bp), VPU/VPX; DRL.x.x.FAO_AY159321, VPR; A1.UG.85.U455_M62320 (291 bp) and NEF;
B.FR.83.HXB2_LA1_IIIB_KO3455 (621 BP)]

Measured variables

Number of cutting enzymes, frequency of cuts, and site mapping of the recognition sequences. Positive Predictive value (PPV) of Computer assay for actual *in vivo* cleavage.

RESULTS

Of the 339 enzymes analyzed, 238 (70.2%) enzymes cleaved the HIV-1/SIVcpz genome as compared to 225 (66.4%) and 219 (64.6%) for HIV-2/SIVsmm and other SIV whole genomes. For the individual genes, HIV-1/SIVcpz: LTR 62 (18.3%), GAG 70 (20.7%), POL 118 (34.8%), ENV 138 (40.7%), VIF 33 (9.7%), TAT 15 (4.4%), REV 25 (7.4%), VPU/VPX 83 (24.5%), VPR 36 (10.6%) and NEF 61 (18%);HIV-2/SIVsmm: LTR 24 (7.1%), GAG 29 (8.6%), POL 129 (38.1%), ENV 150 (44.3 %), VIF 54 (15.9%), TAT 67 (19.8%), REV 27 (8.0%), VPU/VPX 39 (11.5%), VPR 52 (15.3%) and NEF 61 (18.0%); and other SIV LTR 53 (15.6%), GAG 91 (26.8%), POL 97 (28.6)%), ENV 102 (30.1 %), VIF 37 (10.9%), TAT 38 (11.2%), REV 41 (12.1%), VPU/VPX 33 (9.7%), VPR 41 (12.1%) and NEF 76 (22.4%); and other SIV. Details of number of cuts and enzymes cutting is 4 or > times are shown in Tables 1, 2 and 3. Site Mapping for gene cleavage is demonstrated in Figure 1. For the *in vitro* cytogenetic analysis of actual HIV-1 GAG, ENV and POL exposed to enzyme activity, 69 (20.4%), 137

Table 1. Table of enzyme activity on HIV-1/SIVcpz[+].

# Gene (size by base pairs)	No. cutting enzymes; n (%of N*)	Freq. of cuts;number of enzyme	Enzymes cutting 4 or > times
1. LTR A1.CD.97.97ZR49_AF196750 (632 bp)	62 (18.3)	1;55 2;7	0
2. GAG A.KE.97.M104gag_AY772953 (1506 bp)	70 (20.7)	1;42 2;18 3;3 4;7	7 [all 7BssT1I, DraII, Eco130I, EcoO109I, EcoT14I, ErhI and StyI) cleaving four times]
3. POL A1.TZ.01.A341_AY253314 (3012 bp)	118 (34.8)	1;66 2;30 3;7 4;11 5;2 11;2	15 [11 (AccI, BbsI, Bbv16II, BpiI, BpuAI , BstX2I , BstXI , BstYI , DraI , MfiI and XhoII) cleaving four times, 2 (SfcI and BstSFI) cleaving five times, and 2 (AcsI and ApoI) cleaving 11 times]
4. ENV A.CD.97.KCC2_AJ401034 (2589 bp)	138 (40.7)	1;90 2;34 3;7 4;1 5;4 7;2	7 [1 (MslI) cleaving four times, 4 (BssT1I, Eco130I, ErhI and StyI) Cleaving five times, and 2 (BstSFI and SfcI) cleaving seven times]
5. VIF A.CD.97.KCC2_AJ401034 (579 bp)	33 (9.7)	1;31 2;2	0
6. TAT A1.BY.97.97BL006_AF193275 (308 bp)	15 (4.4)	1;9 2;6	
7. REV A1.TZ.01.A341_AY253314 (372 bp)	25 (7.4)	1;24 2;1	0
8. VPU/VPX A1.BY.97.97BL006_AF193275 (246 bp)	83 (24.5)	1;71 2;9 2;3	0
9. VPR A.UA.x.98UA0116_AF413973 (291bp)	36 (10.6)	1;25 2;7 3;4	0
10. NEF A1.BY.97.97BL006_AF193275 (624 bp)	61 (18)	1;43 2;12 3;6	0

*N = Total enzymes analyzed (339).
[+]For the entire HIV-1/SIVcpz A1.BY.97.97BL006_AF193275 genome with 9037 bp, 238 (70.2%) enzymes exhibited cutting properties.

(40.4%) and 116 (34.2%) enzymes exhibited cleavage respectively, yielding a Web cutter positive predictive value (PPV) of 98.6% (95%CI, 97.2%-99.6%) for actual *in vivo* enzyme activity.

DISCUSSION

Our results generally indicate that all human immunodefiency virus (HIV) genome are still highly susceptible to restriction enzyme activity, with HIV-1/SIVcpz (70.2%) and HIV-2/SIVsmm (66.4%) being more susceptible than other simian immunodefiency virus (SIV) genomes (64.6%). Considering that HIV1 and 2 evolved from SIV, this could imply that the original native SIV had acquired minimal resistance to restriction enzyme activity, and the evolutionary crossover to humans came with a loss of some of this protective property. This means that precursors from restriction enzymes have a high potential to

Table 2. Table of enzyme activity on HIV-2/SIVsmm genes+.

# Gene (size by base pairs)	No. cutting enzymes; n (%of N*)	Freq. of cuts;number of enzyme	Enzymes cutting 4 or > times
1. LTR A.PT.x.1139D3_AY622982 (380 bp)	24 (7.1)	1;23 2;1	0
2. GAG G.GH.00.GH31_AY396866 (358 bp)	29 (8.6)	1;29	0
3. POL A.GM.x.MCN13_AY509259 (3096 bp)	129 (38.1)	1;61 2;33 3;13 4;14 4;6 9;2	22 [14 (BalI, BsrDI, BssT1I, BstXI, CfrI, EaeI, Eco130I, EcoT14I, ErhI, MluNI, MscI, PpuMI, Psp5II and StyI) cleaving four times, 6(BstX2I, BstYI, DraII, EcoO109I, MflI and XhoII) cleaving five times, and 2 (AcsI and ApoI) cleaving nine times].
4. ENV MAC.US.x.239_M33262 (2640 bp)	150 (44.3)	1;102 2;28 3;13 4;4 5;3	7 [4 (BstX2I, BstYI, MflI and XhoII) cleaving four times, and 3 (BpmI, GsuI and MslI) cleaving five times].
5. VIF A.GM.x.MCN13_AY509259 (648 bp)	54 (15.9)	1;41 2;6 3;7	0
6. TAT A.GM.x.MCN13_AY509259 (393 bp)	67 (19.8)	1;62 2;2 3;3	0
7. REV A.GM.x.MCN13_AY509259 (324 bp)	27 (8.0)	1;21 2;6	0
8. VPU/VPX A.GM.x.MCN13_AY509259 (339 bp)	39 (11.5)	1;30 2;9	0
9. VPR A.GM.x.MCN13_AY509259 (318 bp)	52 (15.3)	1;47 2;5	0
10. NEF MAC.US.x.239_M33262 (792 bp)	61 (18.0)	1;48 2;10 3;3	0

*N=total enzymes analyzed (339).
+For the entire HIV-2/SIVsmm A.GM.x.MCN13_AY509259 genome with 9713 bp, 225 (66.4%) enzymes exhibited cutting properties.

suppress or kill HIV with less resistance or virologic failure arising.

Enzyme activity among individual enzyme genes demonstrates that it is not the size of gene, but rather the presence of specificity palindromes that determines the number of cuts a particular enzyme will produce on any given HIV gene. This is especially an important fact, when we project these results to a future human gene therapy model for HIV, as it explains that although the genetic code is universal, as long as targeted cells do not contain the specificity palindrome, the human genome will be protected against the enzyme activity. This reduces the risk of toxicity, without of course eliminating other shortcomings in the developing of effective gene therapy. If we consider the HIV-1/SIVcpz GAG, POL and ENV genes for instance, the largest genes in the entire genome, by only looking at enzymes cleaving 4 or > times, we may be made to draw the wrong conclusion that the larger the gene, the more susceptible it is to enzyme activity (1503 bp/7, 3012 bp/15 and 2889 bp/7cuts > or =4). However, on further analysis, we see that the ENV gene (2889 bp) which is 423 bp less then POL (3012 bp) was susceptible to 20 more enzymes than the POL (138/118). To support this further, the GAG gene with

Table 3. Table of enzyme activity on other SIV genes[+].

# Gene name(size by base pairs)	No. cutting enzymes; n (%of N*)	Freq. of cuts; number of enzyme	Enzymes cutting 4 or > times
1. LTR B.FR.83.HXB2_LAI_IIIB_BRU_K03455 (634 bp)	53 (15.6)	1;43 2;6 4;4	4 [all4enzyme(BstX2I, BstYI, MflI and XhoII) cleaving four times]
2. GAG B.FR.83.HXB2_LAI_IIIB_BRU_K03455 (1503 bp)	91 (26.8)	1;64 2;12 3;12 4;2 5;1	3 [2 (AcsI and ApoI) cleaving four times, and 1(Eco57I) cleaving five times].
3. POL B.FR.83.HXB2_LAI_IIIB_BRU_K03455 (3009 bp)	97 (28.6)	1;50 2;23 3;17 4;4 5;1 10;2	7 [4 (AccI, BstSFI, BstXI and SfcI) cleaving four time, 1 (DraI) cleaving five times, and 2 (AcsI and ApoI) cleaving ten times]
4. ENV B.FR.83.HXB2_LAI_IIIB_BRU_K03455 (2571 bp)	102 (30.1)	1;56 2;22 3;13 4;5 5;6	11 [5 (BstX2I, BstYI, Eco57I, MflI and XhoII) cleaving four times, and 6 (BssT1I, BstSFI, Eco130I ,EcoT14I, ErhI and StyI) cleaving five times].
5. VIF B.FR.83.HXB2_LAI_IIIB_BRU_K03455 (579 bp)	37 (10.9)	1;31 2;7	0
6. TAT B.FR.83.HXB2_LAI_IIIB_BRU_K03455 (306) (351 bp)	38 (11.2)	1;31 2;7	0
7. REV B.FR.83.HXB2_LAI_IIIB_BRU_K03455	41 (12.1)	1;34 2;7	0
8. VPU/VPX DRL.x.x.FAO_AY159321 (? bp)	33 (9.7)	1;22 2;9 3;1 4;1	1 [BseRI]
9. VPR A1.UG.85.U455_M62320 (291 bp)	41 (12.1)	1;33 28	0
10. NEF B.FR.83.HXB2_LA1_IIIB_KO3455 (621 bp)	76 (22.4)	1;64 3;12	0

*N=total enzymes analyzed (339).
[+]For the entire other SIV B.FR.83.HXB2_LAI_IIIB_BRU_K03455 genome with 9719 bp, 219 [64.6%] enzymes exhibited cutting properties.

1083 bp less than the ENV, had a similar susceptibility to enzymes cleaving 4 or > times. Moreover GAG had 7 enzymes cutting 4 times, as compared to only 1 of ENV. More evidence for site specificity rather than size determining enzyme activity is demonstrated by the REV (372bp) and VPU/VPX (246) genes, with the larger REV gene having only 25 cutting enzymes in general compared to 83 for the smaller VPU/VPX gene. Other issues of safety are dependant upon choice of enzyme gene delivery, and although various *ex vivo and in vivo* gene transfer techniques exist, we believe that a recom-binant HIV viral vector whose pathogenesis gene, NEF has been removed to render it impotent, could offer a more targeted delivery. The pharmacokinetics of gene therapy

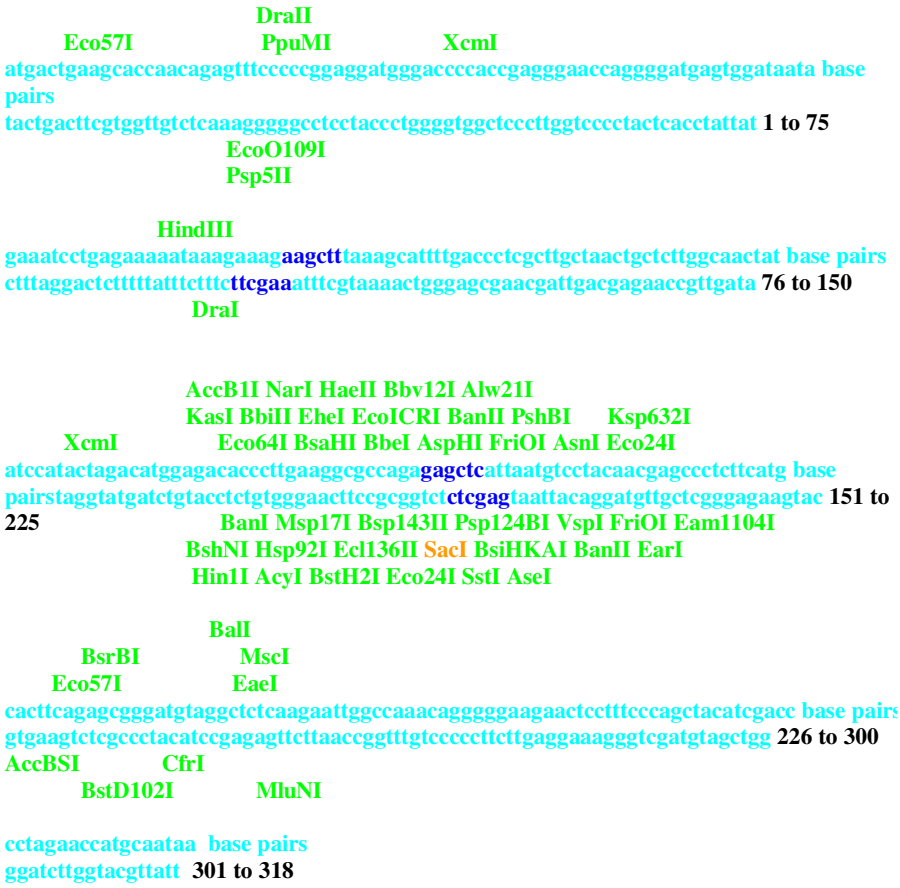

Figure 1. Graphic map of 6 bps Palindromic recognition nucleotide sequences in the HIV-1/SIVcpz VPR gene. **Color key:** *cac* = unrecoginsed VPR gene sequence, *aagctt* = recognized specificity 6 bps sequences *BanI* = enzyme cutting.

is also dependant on the choice of delivery (Perelson et al., 1996; Preslson et al., 1997; Miller et al., 1993; Jolly, 1994; Weissman et al., 1995; Ledley and Ledley, 1994).

One property that adds value to the RMS model is that specificity genes from enzymes in the same class, say type I can recombine to yield specificity proteins with different specificity properties from the parent enzymes. This is a major tool for developing newer restriction enzymes with new specificities, and thus fighting HIV resistance (Murray, 2000; Nelson et al., 1972; Roberts and Macelis, 1991; Janulaitis et al., 1992; Kessler and Manta, 1990; Nelson and McClelland, 1991; Radasci and Bickle, 1996; Barcus and Murray, 1995; Misaki, Makerere Develops a Model for an AIDS Cure. www.aegis.org/news/nv/2005/NV050301.html).

The options for developing an HIV drug or micro biocide using restriction enzyme precursors is not to be overlooked.

Conclusion

Although HIV is addressed here as the primary target, the RMS model offers prospects for developing other antiviral and anti-virus induced tumor therapy. It is this concept, the use of the bacterial RMS model in developing antiviral therapies that we have termed bacteriovirogenics.

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