

Construction and Immunogenicity Study of a 297-bp Humanized HIV V3 DNA of an Approximated Last Common Ancestor in Mice

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Attempts to develop human immunodeficiency virus (HIV) vaccines started with the confirmation of HIV as the cause of the acquired immunodeficiency syndrome (AIDS).¹⁻³ In spite of intensive international collaborative efforts, no effective HIV vaccine has been developed so far. One of the obstacles is the existence of several unknown factors in the development of the HIV vaccine, for examples, the antigenic epitopes to be used (*gag*, *pol* or *env*), the form of the immunogens (recombinant protein, live recombinant vector or DNA) or the nature of the protective immune correlates (neutralizing antibody or cytotoxic T lymphocytes, CTL). DNA immunization is one of the most attractive approaches since it can induce strong CTL responses⁴⁻⁶ and is relatively easy to produce. By optimizing codon usage to that favored by mammalian cells, humanized DNA vaccines have been found to be

SUMMARY DNA immunization represents one of the promising HIV-1 vaccine approaches. To overcome the obstacle of genetic variation, we used the last common ancestor (LCA) or "center-of-the-tree" approach to study a DNA fragment of the HIV-1 envelope surrounding the V3 region. A humanized codon of the 297-bp consensus ancestral sequence of the HIV-1 envelope (codons 291-391) was derived from the 80 most recent HIV-1 isolates from the 8 circulating HIV-1 subtypes worldwide. This 297-bp humanized "multi-clade" V3 DNA was amplified by a PCR-based technique. The PCR product was well expressed *in vitro* whereas the corresponding non-humanized V3 DNA (subtype A/E) could not be expressed. However, both V3 DNA constructs as well as the full-length HIV-1 envelope construct (A/E) were found to be immunogenic in mice by the footpad-swelling assay. Moreover, intracellular and extracellular interferon-gamma could be detected upon *in vitro* stimulation of spleen cells although the response was relatively weak. Further improvement of our humanized V3 DNA is needed.

superior to their non-humanized counterparts in enhancing both *in vitro* expression and *in vivo* immunogenicity.⁷⁻¹⁰ Here, we report the utilization of a PCR-based method to generate a 297-bp humanized DNA from selected immunodominant HIV envelope epitopes of an approximated last common ancestor (LCA) strain which is a hypothetical strain with consensus sequences derived from the 8 subtypes

worldwide. Its *in vitro* expression and immunogenicity were studied and compared to the non-humanized counterpart and to the full-length envelope DNA.

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MATERIALS AND METHODS

Epitope selection and DNA humanization

The potential immunodominant HIV envelope epitopes were selected by computer search of sequences reported in the Los Alamos National Laboratory's (LANL) HIV sequence database (<http://hiv-web.lanl.gov>). The selected region encompasses 297 basepairs (bp), i.e. codons 291-391 of the HIV envelope according to the CM240 reference strain (GenBank accession number U54771). It includes the V3 region, CTL epitopes, T helper epitopes, CXCR4 and CD4 binding sites and neutralizing epitopes.¹¹ A consensus sequence of the same HIV envelope region was calculated from an alignment of the most recently reported sequences from 80 independent HIV isolates across the 8 subtypes (10 sequences from each subtype which included subtypes A, B, C, D, CRF01_A/E, F, G, H) and translated to a predicted amino acid sequence using DNAMAN version 4.15 (Lynnon Biosoft, Seattle, WA, USA). This amino acid sequence was back translated to an estimated preferred human codon sequence using Backtranslate (Program Manual for the Wisconsin Package, Version 8, 1994, Genetics Computer Group, Madison, WI, USA). This approach, the last common ancestor (LCA) sequence, has the advantage of generating a sequence most similar to all currently circulating strains of interest throughout the world as compared to a representative sequence for one single subtype only.¹² It is anticipated that such a vaccine would cross-react with all subtypes and thus be more applicable worldwide.

Generation of the 297-bp humanized DNA (hu-V3 DNA)

Three primers overlapping one another with 25-mer regions were synthesized as 134 (hu-5'), 138 (AS-73-210) and 125-mer (AS-186-297), respectively (Proligo Singapore Pte Ltd, Singapore). All of the oligos were purified by crude desalting only. The primer sequences were as follows: **hu-5'** (134-mer): 5'*TATCGATAAGCTTGATATCGA ATTCGCCGCCACCATGAGCGT GGAGATCAACTGCACCAGGC CCAGCAACAACACCAGGACC AGCATCACCATCGGCCCCGGC CAGGTGTTCTACAGGACCGG CGACATCATCG* 3'; **AS-73-210** (138-mer): 5'*CTGGAAGATGATGGTCTTGTTCTTGAAGTGCT CCTTCAGCTTCTCGGTACCT GCTTCAGGGCCTCGTTCCTACT TGGTGCCGTTGATCTCGCAGT AGGCCTTCCTGATGTCGCCGA TGATGTCGCCGGTCTCTGTAG AA* 3'; **AS-186-297** (125-mer): 5'*CTAGTCTAGACTAGAACAGCT TGGTGGTGTTCAGTAGAAGA ACTCGCCCCTGCAGTTGAAGT GGTGCATGGTGTATCTCCAGGT CGCCGCCGCTGGGGGGCTGG AAGATGATGGTCTTGTTCTTG* 3' (The italic represents restriction sites, underline represents Kozak sequence, bold represents start or stop codons, bold and underline represent overlapping regions.)

The 134-mer included an *Eco* RI site, a Kozak sequence (to increase protein expression) and an ATG start codon. The 125-mer included a TAG stop codon and an *Xba* I site. The 3 primers were combined in a standard master mix for polymerase chain reaction (PCR) without adding any additional template. Five microliters each of 25 mM MgCl₂, 10x PCR buffer and 10

mM dNTPs were mixed with 20 pmol of each primer and 1.5 U of *Taq* polymerase (Promega, Madison, WI, USA) to a total volume of 50 µl. The PCR conditions were 94°C, 5 minutes, 32 cycles of 94°C, 30 seconds, 50°C, 30 seconds, 72°C, 1 minute, autoextension at 72°C for 7 minutes after which the PCR products were kept at 4°C. Nested PCR was performed with 2 µl of PCR product from the 1st PCR with 2 smaller primers (small hu-5' and small hu-3', **small hu-5'**: 5' *GATTTCGCCGCCACCATGAG* 3', **small hu-3'**: 5' *CTAGTCTAGACTAGAACAGCTTGGTG* 3') to amplify the whole 297-bp product. The conditions were the same as for the first round PCR but the number of cycles was increased to 40 cycles for the 3-temperature PCR. The first round PCR yielded 3 possible products (Fig. 1). Only the longest (full-length or PCR product 3) first round PCR product was nested by the 2 smaller primers (Fig. 1).

Generation of the 297-bp non-humanized subtype A/E DNA (non-hu-V3 DNA)

A DNA template for PCR amplification of the 297-bp HIV envelope region was extracted from peripheral blood mononuclear cells (PBMC)¹³ of an antiretroviral-naïve Thai patient infected with HIV-1 A/E of a non-syncytium inducing (NSI) phenotype using the 10-14 day co-cultivation technique as described in the ACTG Virology Manual for HIV Laboratories, version 5, 1997.¹⁴ The 297-bp non-hu-V3 PCR product was amplified by nested PCR using ss-envI and ss-envII as 5' and 3' outer primers (**ss-env I**: 5' *GCGCTAATCTTAGCAATAGTAGTGTGAAC* 3' [location

6013-6041 of HXB2], **ss-env II** : 5' GCTCCATGTTTATCTAGATC-TTGAGATAC 3' [location 8921-8892 of HXB2]). The PCR conditions were 94°C, 5 minutes, 40 cycles of 94°C, 1 minute; 55°C, 1 minute and 72°C, 5 minutes, then autoextension at 72°C for 7 minutes after which the PCR products were kept cool at 4°C. The inner primer sequences for the **env-6675** were: 5' TAAAGAATTTCGCCGCCACC-ATGTCTGTAGAAATCAATTG-TACC 3' (location 7091-7112 of HXB2), and for the **env-6971**: 5' TATTGTCGACCTAAAACAGTT-TTGTGTATTGC 3' (location 7397-7378 of HXB2). The PCR conditions were 94°C, 5 minutes, 35 cycles of 94°C, 30 seconds; 56°C, 30 seconds; 72°C, 1 minute, then auto-extension at 72°C for 7 minutes after which the PCR products were kept cool at 4°C.

Generation of the 2.5 kb full-length envelope DNA

The 2.5 kb envelope was amplified from HIV DNA using **ss-envI** and **ss-envII** as 5' and 3' outer primers and then nested with the primers **env-E-Xho I-5'** (5' ATCG-CTCGAGGCCGCCACCATGAGAGTGAAGG 3', location 6213-6225 of HXB2) and **env-E-EcoR I-3'** (5' ATCGGAATTCCTAGCAAA-GTCCTTTCTAA 3', location 8776-8761 of HXB2). The PCR conditions were 94°C, 5 minutes, 40 cycles of 94°C, 1 minute; 56°C, 1 minute; 72°C, 3 minutes, then auto-extension at 72°C for 7 minutes after which the PCR products were kept cool at 4°C.

DNA constructs

The amplified products were cloned into pCI mammalian expres-

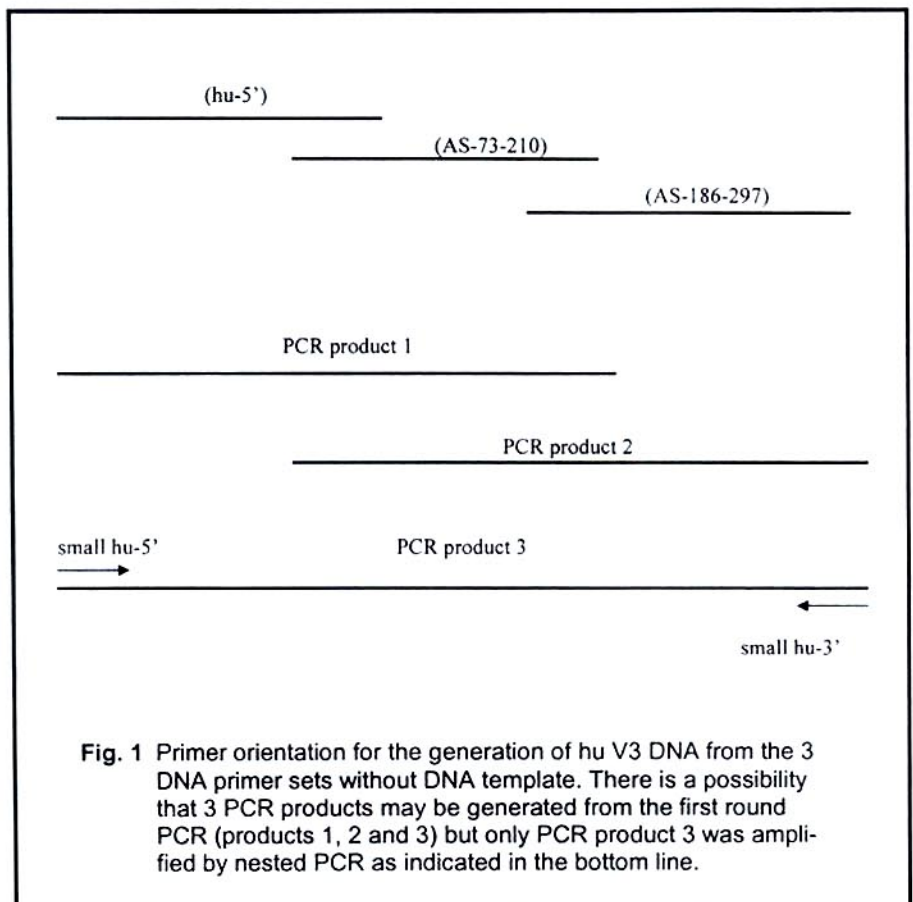


Fig. 1 Primer orientation for the generation of hu V3 DNA from the 3 DNA primer sets without DNA template. There is a possibility that 3 PCR products may be generated from the first round PCR (products 1, 2 and 3) but only PCR product 3 was amplified by nested PCR as indicated in the bottom line.

sion vectors (Promega, Madison, WI, USA) at *Eco* RI / *Xba* I and *Eco* RI / *Sal* I multiple cloning sites for hu-V3 DNA and non-hu-V3 DNA, respectively, by using T4 DNA ligase (Boehringer Mannheim, Germany) according to the manufacturer's protocol. The 2.5 kb full-length envelope was cloned into a pCI plasmid that had been modified by insertion of IRES (Internal Ribosome Entry Site) and *REV* (p1.1 cI IRES *REV*, a kind gift from Professor Kenji Okuda, Yokohama City University School of Medicine, Yokohama, Japan). The 3 ligated products were verified by restriction enzyme digestions and sequencing analyses.

In vitro protein expression

Both the hu-V3 DNA and

non-hu-V3 DNA plasmids were propagated in DH5 α and purified by using the QIAGEN endotoxin-free Giga kit (QIAGEN, Hilden, Germany). Two micrograms of each plasmid DNA construct were used to transfect HEK293 cells (Cat # 103, NIH AIDS Research and Reference Reagent Program, MD, USA) for 48 hours using Lipofectamine (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The cells were then washed once with cold PBS and lysed with NP-40 lysis buffer (50 mM Tris-Cl, pH 7.6 with 1 % Nonidet P-40). Eighteen microliters of the cell lysate were loaded into 4-12% gradient polyacrylamide gel (Invitrogen, Carlsbad, CA, USA) followed by transfer to a Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Buck-

inghamshire, England). The proteins were detected by using pooled anti-HIV positive plasma from HIV-1 CRF01_A/E-infected individuals. Peroxidase-conjugated rabbit anti-human IgG (Cat #309-035-003, Jackson ImmunoResearch Lab. Inc., PA, USA) was used as the second antibody. The β -actin band was used as a positive control.

Immunogenicity study in mice

Immunization protocol

Six to eight weeks old female BALB/c mice, weighing 20–25 g, were used in the experiments. Eight mice were immunized with pCI empty plasmids, which served as negative controls, 10 mice each with the hu-V3 DNA and non-hu-V3 DNA and 20 mice with the 2.5 kb full-length envelope. One hundred micrograms of individual construct in 100 μ l PBS were injected into the gastrocnemius muscles of the hind legs on days 0, 14 and 28. Spleen cells were collected on day 42 or 2 weeks after the third immunization. Half of the mice in each group were boosted with 25 μ g of recombinant gp120 of subtype A/E (a gift from MG Sarngadharan, Advanced Bioscience Laboratories, Inc., Kensington, MD, USA) on day 42. The spleen cells were collected from the boosted group on day 56 or 2 weeks after boosting.

In vivo immunogenicity assay

A delayed type hypersensitivity (DTH) skin test, a footpad-swelling assay, was performed 48 hours before sacrificing both the non-boosted (day 40) and the rgp120 boosted mice (day 54). The mice were injected with 8 μ g of a 15-residue peptide (V3 [E] peptide: GVH-

MGPGRVIFYRTGE for pCI control, non-hu-V3 and 2.5 kb full-length envelope groups and V3 [hu] peptide: SITIGPGQVFYRTGD for hu-V3 group) into both left and right hind footpads. The sequences of the 15-residue peptides were synthesized (Peptide Synthesizer, PSSM-8, Shimadzu Corporation, Kyoto, Japan) according to the amino acid sequences of the relevant V3 sequences. The thickness of both, the left and the right hind footpads was measured with a dial thickness gauge (Ozaki Seisakusho, Tokyo, Japan) both before and 24 hours after skin testing. The difference of the footpad thickness pre- and post-injection was considered as footpad swelling (DTH) and measured in millimeters (mm).

In vitro immunogenicity assays

Intracellular cytokine staining

Ten million splenocytes were stimulated over night with 20 μ g of specific V3 peptides, i.e. V3(hu) for hu-V3 group and V3(E) for pCI, non-hu-V3 and 2.5 kb full-length envelope groups. One microliter of Golgi Plug (the Cytofix/Cytoperm, Cat # 555028, BD Pharmingen, San Diego, CA, USA) was added to block intracellular protein transport for 2 hours prior to immunostaining with PE-labeled anti-mouse CD8a (Cat # 01045A, BD Bioscience Pharmingen), followed by FITC-labeled anti-mouse interferon-gamma (IFN- γ) (Cat # 11-7311-82, BD Bioscience Pharmingen) after permeating the cells with Cytofix/Cytoperm solution (Cat # 555028, BD Bioscience Pharmingen). CD8⁺ cells with intracellular IFN- γ were counted by flow cytometry. A sample was considered positive if the % positivity was more than or equal to the

mean % positivity plus 2 SD of the negative control (pCI) group.

ELISPOT assay

One million splenocytes were stimulated over night with 1 μ g of specific V3 peptides as described above. The secreted IFN- γ was assessed by anti-mouse IFN- γ biotin/streptavidin-alkaline phosphatase system. The spot-forming units (SFU) were counted by computer-assisted video image analysis¹⁵ and expressed as SFU/10⁶ cells. The results of the ELISPOT assay were determined by the difference in SFU between the stimulated and the non-stimulated cultures.

RESULTS

The 297-bp humanized DNA, 297-bp non-humanized DNA and 2.5 kb full-length envelope DNA were successfully amplified using nested PCR technique according to the procedures described in Materials and Methods. The amplified products were shown by agarose gel electrophoresis to have the correct size of DNA, i.e., 297-bp and 2.5 kb as shown in Fig. 2. The correct insertion products of 297-bp hu-V3 and non-hu-V3 DNA and 2.5 kb full-length envelope DNA were verified by restriction enzyme digestion (Fig. 3) and DNA sequencing analysis. The DNA sequence of the 297-bp hu-V3 and non-hu-V3 DNA (GenBank accession number AY366934) are shown in Fig. 4.

In vitro protein expression

The expression of hu-V3/pCI plasmid in HEK293 cells yielded a single protein of approximately 13 kDa, which is the predicted molecular size of the hu-V3 protein (Fig. 5-A).

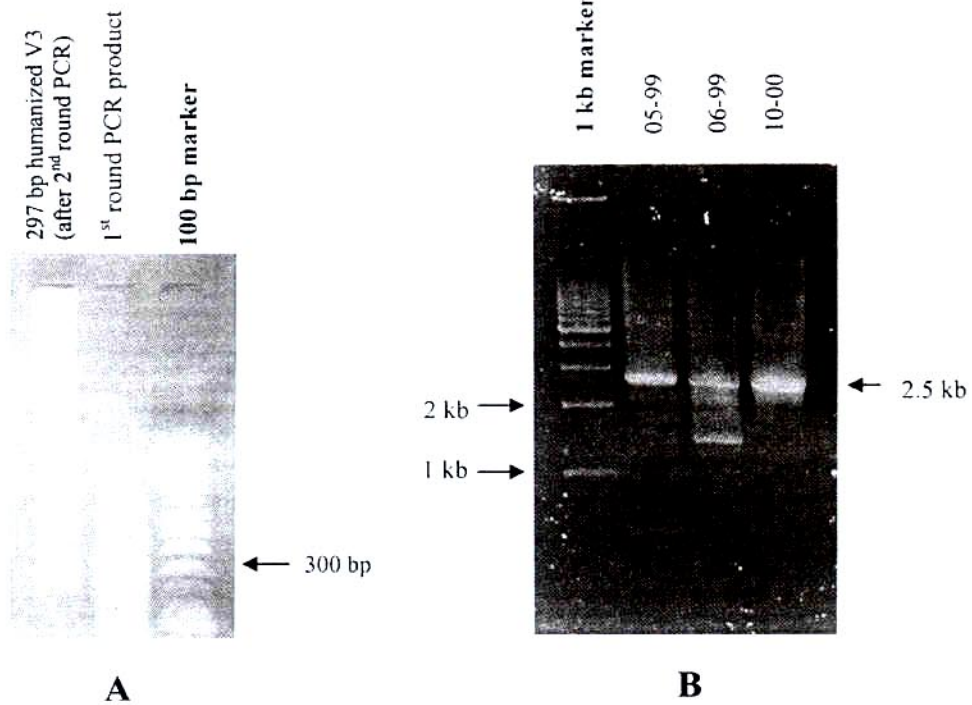


Fig. 2 Agarose gel electrophoresis depicting the PCR products of 297-bp humanized DNA (A) and of 2.5 kb full-length envelope DNA (B). Lanes 05-99, 06-99 and 10-00 in B indicate the 3 different HIV-1 isolates being amplified. The PCR product of the 297-bp non-humanized DNA is not shown since it has the same size as the 297-bp humanized DNA. The 100 bp marker is 100 bp EZ Load Molecular Ruler, and the 1 kb marker is 1 kb EZ Load Molecular Ruler, BIO-RAD, CA, USA.

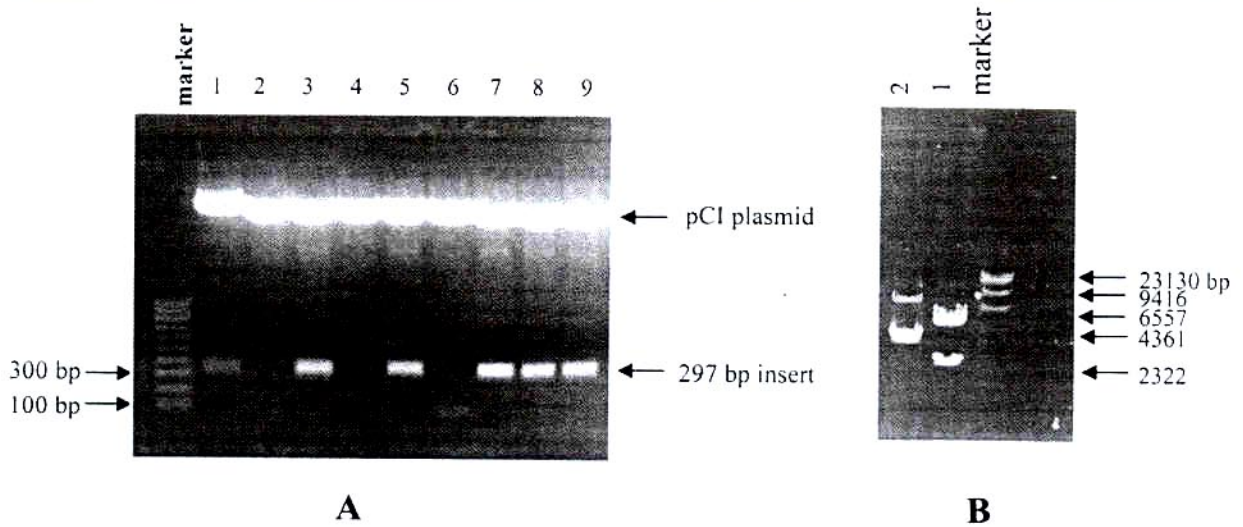


Fig. 3 Restriction enzyme digestions of cloning experiments of 297-bp humanized V3 DNA, 297-bp non-humanized V3 DNA (A) in a 1.5% agarose gel (lanes 1, 3, 5 indicate insertion of the 297-bp humanized V3 DNA, lanes 7-9 indicate insertion of the 297-bp non-humanized V3 DNA) and of the 2.5 kb full-length envelope DNA (B) in a 0.8% agarose gel (lane 1, digested with *Eco* RI yielded 2 bands of 4,932 and 2,571 bp; lane 2, digested with *Xho* I yielded 2 bands of 3,969, 3,534 bp).

Nucleotide sequences of 297-bp humanized DNA

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atgagcgtgg agatcaactg caccaggccc agcaacaaca ccaggaccag catcaccatc ggccccggcc
aggtgtctac aggaccggcg acatcatcgg cgacatcagg aaggcctact gcgagatcaa cggcaccaag
tgaacgagg cctgaagca ggtgaccgag aagctgaagg agcactcaa gaacaagacc atcatcttc
agccccccag cggcggcgac ctggagatca ccacacacca ctcaactgc aggggcgagt tcttctactg
caacaccacc aagctgttct ag
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Nucleotide sequence of 297-bp non-humanized DNA (AY366934)

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atgtctgtag aatcaattg taccagacc tcaacaaca caagaacagg tgtacatag ggaccaggac
gagtattcta tagaacagga gaaataatag gaaatataag aatagcatat tgtgagatta atggaacaca
atggaataaa actttaacac aagtagctga aaaattaaaa gagcacttta ataagacaat aatcttcaa
ccacaaccac cctcaggagg agatctagaa attacaatgc atcatttaa tttagaggg gaattttct
attgcaatac aacaaaactg ttttag
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Fig. 4 Nucleotide sequences of the 297-bp humanized DNA and 297-bp non-humanized DNA (GenBank accession number AY366934). It starts at position 7091 (after the ATG start codon) and ends at position 7397 (before the TAG stop codon) as compared to HXB2.

In contrast, we could not detect any protein expression from the non-hu-V3 DNA transfected HEK293 cells even when we increased the amount of DNA from 2 μ g to 4 μ g. The same results were also obtained when the hu-V3 DNA and the non-hu-V3 DNA were cloned into pcDNA 3.1 mammalian expression plasmids (Invitrogen, Carlsbad, CA, USA) (Fig. 5-A). The expression of 2.5 kb full-length envelope DNA (p1.1 cI envE IRESrev) yielded 2 bands at 160 and approximately 38 kDa (Fig. 5-B) which corresponded to gp160 and gp41 of HIV envelope structural proteins, respectively.

In vivo immunogenicity

The immunogenicity of the V3/envelope DNA constructs was studied by intramuscular injection into BALB/c mice with and without

recombinant gp120 (E) boosting as described in Materials and Methods. The delayed type hypersensitivity (DTH) response to the vaccine was measured by footpad swelling assay. The mean thickness of 76 hind footpads from 38 mice at baseline was 1.91 ± 0.05 mm. Twenty-four hours after the footpad antigen challenge, the footpad thickness of pCI-immunized mice (negative control group) remained unchanged (1.91 ± 0.04 mm, $N = 4$, $p > 0.05$). On the contrary, mice immunized with all the 3 DNA constructs, both boosted and non-boosted, had significant footpad swelling upon appropriate antigen challenge (Fig. 6), except those immunized with 2.5 kb full-length envelope DNA without rgp120 boosting. Recombinant gp120 (E) boosting in 297-bp humanized and non-humanized DNA primed mice further increased the footpad thick-

ness but not significantly different from the non-boosted mice. There was no significant difference in the DTH response induced by the 3 DNA constructs upon boosting. In addition, V3 (E) and V3 (hu) peptides were equally effective as skin test antigen in mice immunized with full-length envelope (E) DNA (Fig. 6).

In vitro immunogenicity

Intracellular cytokine staining

For the intracellular cytokine staining (ICS) assay, spleen cells from mice immunized with the 3 DNA constructs were stimulated with the corresponding V3 peptides (V3[hu] and V3[E]) for 18 hours. Stimulated CD8⁺ T cells producing interferon-gamma intracellularly were stained and counted by flow cytometry as described in

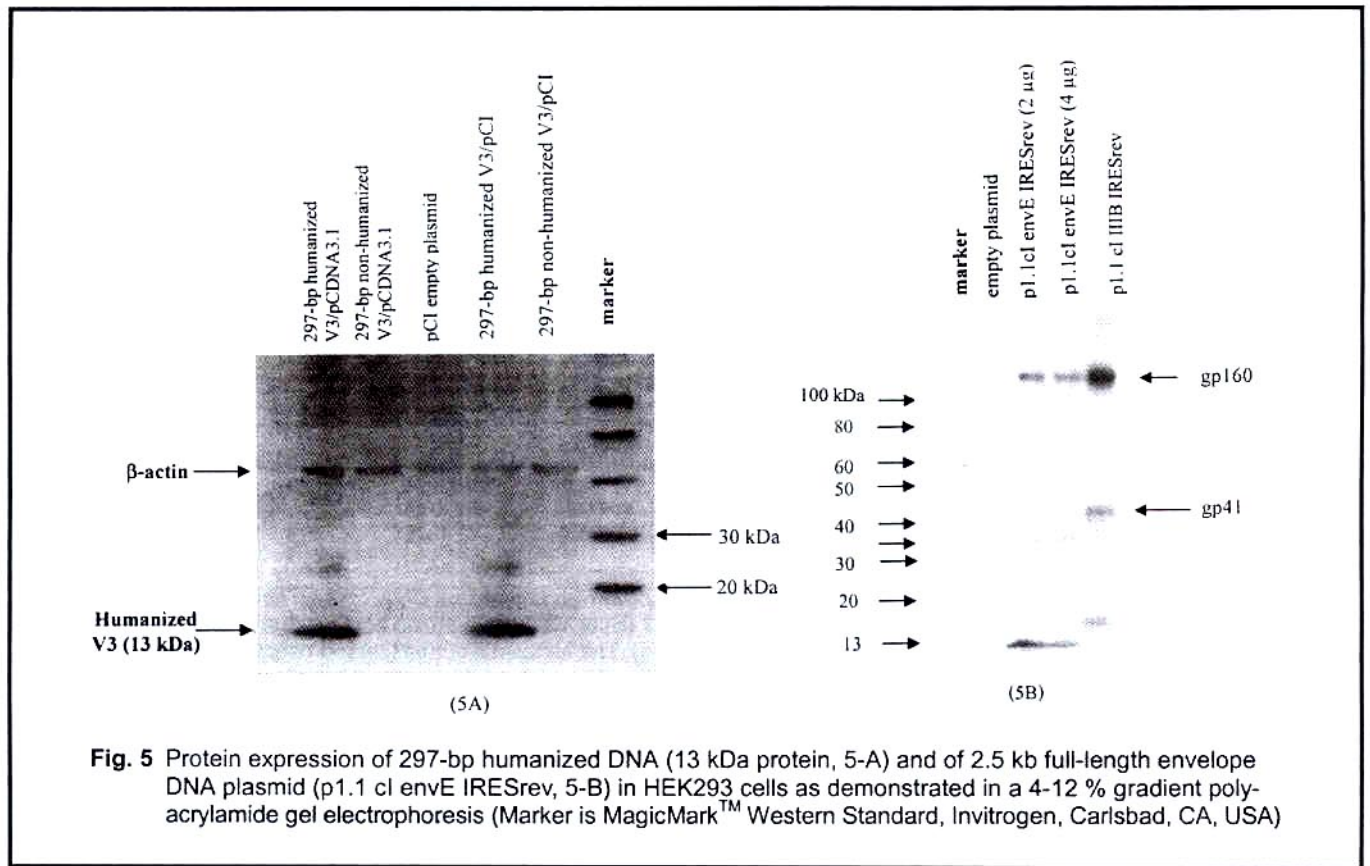


Fig. 5 Protein expression of 297-bp humanized DNA (13 kDa protein, 5-A) and of 2.5 kb full-length envelope DNA plasmid (p1.1 cl envE IRESrev, 5-B) in HEK293 cells as demonstrated in a 4-12 % gradient polyacrylamide gel electrophoresis (Marker is MagicMark™ Western Standard, Invitrogen, Carlsbad, CA, USA)

Materials and Methods. Representative results of ICS as shown in the flow cytometry are shown in Fig. 7 and the composite results of percent ICS of various groups are shown in Fig. 8.

For the 297-bp humanized and non-humanized DNA vaccinated groups, the percent ICS was quite low (Fig. 8-A). Using the standard criteria of percent ICS of more than or equal to the mean percent ICS of the negative controls (pCI immunized group) in that particular experiment plus 2 SD as the cut-off for positive ICS response, only 3/5 mice in the 297-bp humanized DNA immunized group had a positive response (% ICS = 0.08, 0.05, 0.08; cut-off = 0.05). Two out of 5 mice in the 297-bp non-humanized DNA group had a positive ICS response (% ICS = 0.06, 0.06; cut-off = 0.05).

Neither the % ICS nor the % positive ICS response increased after boosting with rgp120 (Fig. 8-B).

For the 2.5 kb full-length envelope DNA immunized group, the intracellular cytokine response seemed to be higher (Fig. 8-C). Unfortunately, only the results from those immunized with 3 doses of DNA plus rgp120 boosting could be analyzed. All the immunized mice responded to the stimulated antigens. The % ICS were 0.11, 0.06, 0.13, 0.09, 0.07, 0.07, 0.07, 0.06, 0.07; cut-off = 0.06. The results from the non-boosted group could not be analyzed due to cell death as seen in the flow cytometry.

Enzyme linked immunospot (ELISPOT)

The number of spleen cells

secreting interferon-gamma after an *in vitro* antigen challenge was measured by ELISPOT assay as described in Materials and Methods. Results of the ELISPOT assay are shown in Fig. 9.

For the non-boosted mice, the 297-bp humanized V3 DNA, 297-bp non-humanized V3 DNA and 2.5 kb full-length envelope DNA gave 118 ± 67 , 120 ± 45 and 123 ± 36 SFU/ 10^6 cells respectively, significantly higher than the negative control group (30 ± 36), p value < 0.05, < 0.025 and < 0.01 respectively (Fig. 10). The overall ELISPOT response was relatively weak as compared to mice immunized with rVVgp160(B) (positive control, vPE16, Cat # 362, NIH AIDS Research and Reference Reagent Program, MD, USA) which had 750 SFU/ 10^6 cells (Fig. 10). Unfortu-

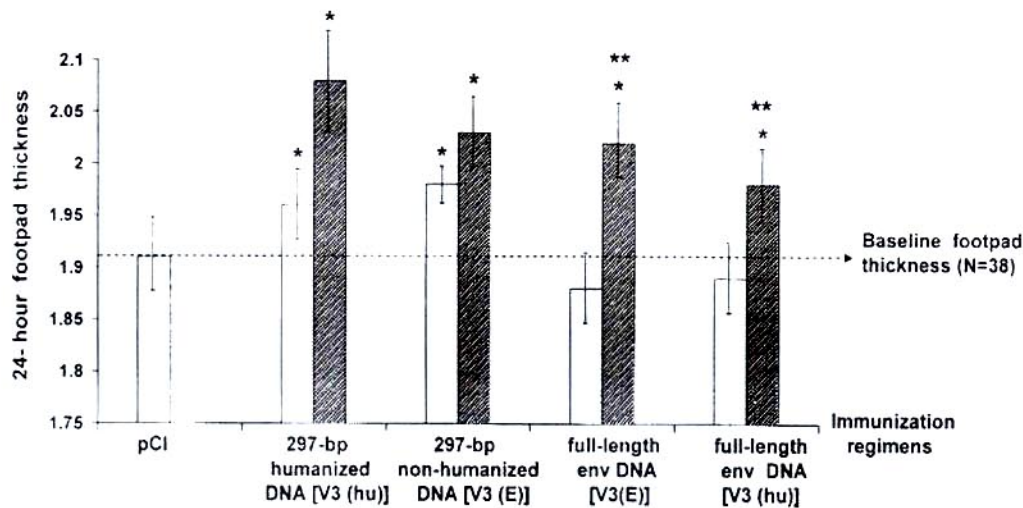


Fig. 6 Mean 24-hour footpad thickness of mice immunized with pCI, 297-bp humanized DNA, 297-bp non-humanized DNA and 2.5 kb full-length envelope DNA and skin tested with V3 (E) or V3 (hu) 15-amino acid residue peptides injected into the footpads (□ = without boosting, ■ = with boosting). The horizontal dotted line represents the mean pre-challenged footpad thickness of all 38 mice, serving as the baseline footpad thickness. * Indicates significant difference from negative (pCI) control. ** Indicates significant difference between boosted and non-boosted groups.

nately, the culture system in the boosting experiment was faced with a problem of cell survival. Very low ELISPOT was found including that of the positive control.

DISCUSSION

We reported here the production of three DNA constructs encoding the envelope region of HIV-1 to be studied as potential HIV-1 candidate vaccines. One of the constructs was the 2.5 kb full-length gp160 DNA and the second was a 297-bp DNA segment encoding the V3 region and the surrounding immunodominant epitopes. Both constructs were amplified from a Thai patient infected with HIV-1 subtype A/E and NSI phenotype. The third DNA construct was amplified from a hypothetical sequence encoding the same V3 region as the other 297-bp construct but the sequence was derived from the last common ancestor (LCA) of 8 HIV-1 subtypes (subtype A, B, C, D, CRF01_A/E,

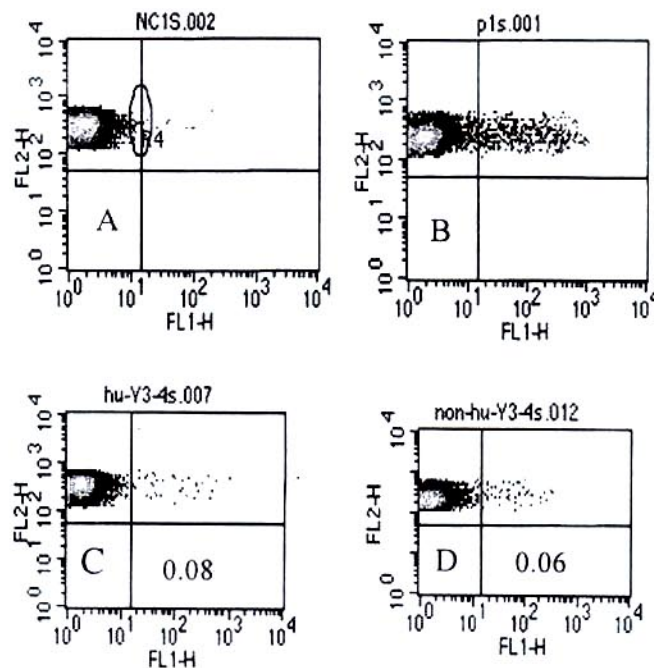


Fig. 7 Representative results of the intracellular cytokine assay of mice immunized with the DNA constructs as detected by flow cytometry. A: mouse immunized with pCI (negative control), stimulated with V3 (E); B: mouse immunized with rVVgp160 (B) (positive control), stimulated with V3 (B); C: mouse immunized with 297-bp humanized DNA, stimulated with V3 (hu); D: mouse immunized with 297-bp non-humanized DNA, stimulated with V3 (E).

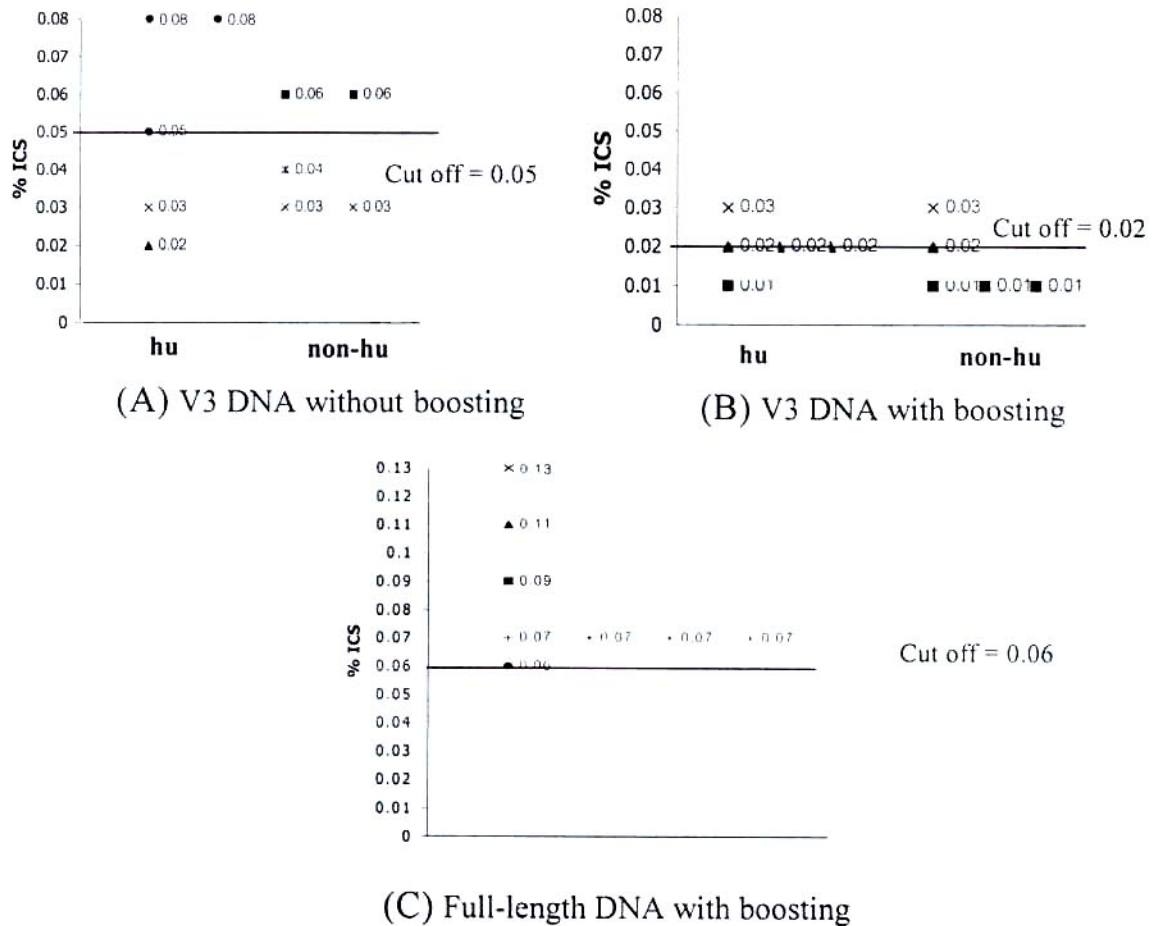


Fig. 8 Composite results of ICS in DNA-immunized mice. (A) mice immunized with 297-bp humanized and non-humanized DNA vaccine without *rgp120* (E) boosting (cut-off = 0.05), (B) mice immunized with 297-bp humanized and non-humanized DNA vaccine with *rgp120* (E) boosting (cut-off = 0.02), (C) mice immunized with 2.5 kb full-length subtype A/E envelope DNA vaccine with *rgp120* (E) boosting (cut-off = 0.06).

F, G, H), using 10 most recent isolates from each subtype. The DNA sequence of this hypothetical DNA construct was also optimized for human codon usage with the hope to get a better expression and to be more immunogenic. This hypothetical humanized V3 DNA construct was produced by PCR-based technique without the use of DNA templates. The technique is based on PCR amplification of overlapping DNA primers with nesting from the

2 far ends (Fig. 1). The technique has been used before to generate required DNA segments by other investigators¹⁰ and is much more economical, being at least 10 times cheaper than using a DNA synthesizer.

It is hoped that truncated envelope DNA such as our 297-bp non-humanized V3 DNA will be as immunogenic as the full-length envelope DNA. If this is the case, the

cost of DNA production and the side effects will be less since less DNA is administered. Our findings indicated that the 297-bp non-humanized V3 DNA was more immunogenic than the 2.5 kb full-length envelope DNA as it could induce DTH response without boosting (Fig. 6) although the higher immunogenicity may be due to the higher V3 DNA density in the 100 μ g DNA immunizing dose. This difference in immunogenicity may

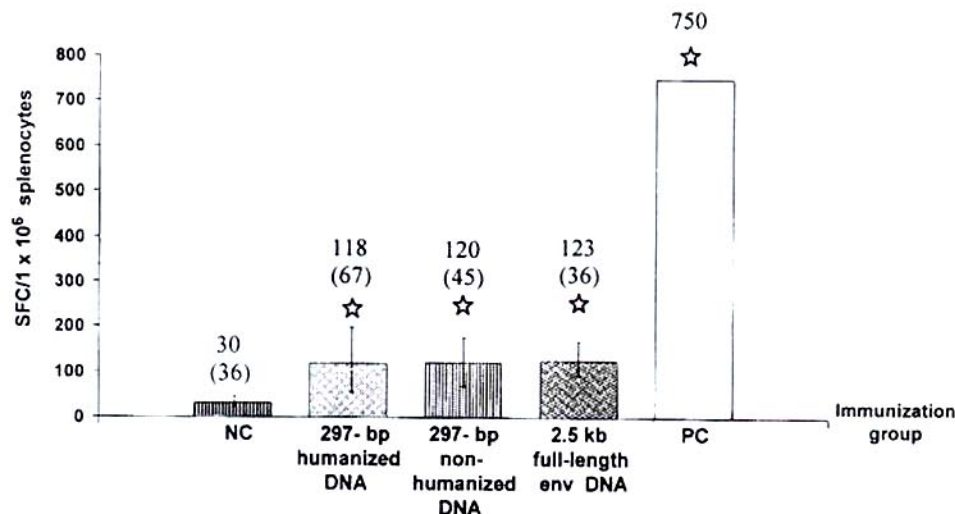


Fig. 9 Mean ELISPOT results of mice immunized with 297-bp humanized DNA, 297-bp non-humanized DNA and 2.5 kb full-length envelope DNA without rgp120 (E) boosting. NC = negative control, injected with pCI. PC = positive control, immunized with rVVgp160 (B). ☆ indicates a statistically significant difference as compared to the negative control. Numbers in parenthesis indicate standard deviations.

be due to the increased exposure of the immunogenic epitopes in the truncated envelope or due to the deletion of the immunosuppressive epitopes in the envelope as described previously.¹⁶⁻¹⁷

However, when the 297-bp non-humanized V3 DNA was compared to the humanized V3 DNA of the same region, the 297-bp humanized V3 DNA was much better expressed in the *in vitro* expression system (Fig. 5-A). The difference is most likely due to optimized codon usage as has been described by others^{8,10,18-19} although it is true that the 2 envelope peptides differ by 16/99 (16.2%) amino acid sequences. The difference in amino acid composition alone cannot explain the difference in protein expression *in vitro*. Despite the *in vitro* expression, the non-expressed 297-bp non-humanized V3 DNA could readily induce a DTH response upon immunization into mice (Fig. 6). The reasons that

a DNA construct which is not expressed *in vitro* can be immunogenic *in vivo* may be that the step of antigenic processing may render a weak immunogen more immunogenic or perhaps a more efficient *in vitro* expression system is needed for less immunogenic constructs.

Although recombinant gp120 (A/E) significantly enhanced the DTH response to the full-length envelope (A/E) DNA vaccine, it did not significantly enhance the response to 297-bp humanized V3 DNA (Fig. 6). The lack of such a prime-boost effect may indicate the non-cross-reaction between the boosting (subtype A/E) and the priming immunogens (last common ancestor of 8 subtypes). However, this could not explain the lack of boosting effect when rgp120 (A/E) was used to boost mice primed with 297-bp non-humanized V3 (A/E) DNA. It also could not explain the equal potency of 15 V3 (hu) and V3

(E) amino acid peptides when they were used as skin test antigens in mice primed-boosted with full-length envelope DNA and rgp120, all of subtype A/E (Fig. 6).

The cell-mediated immune response to DNA vaccination could be demonstrated *in vitro* by both the intracellular cytokine staining (ICS) and ELISPOT assays. It was seen more consistently with the ELISPOT than with the ICS assay (Figs. 8,9). The response was generally weak or of low level with all 3 DNA constructs. This may reflect the weak immunogenicity of our DNA constructs or the inappropriate selection of antigens used for *in vitro* stimulation. The immunogenicity of the DNA constructs, particularly the humanized DNA should be further enhanced by adding more of the immunostimulating CpG motifs²⁰⁻²¹ or the cytokine adjuvants.²²⁻²⁴ More potent routes of administration²⁵ (such as

intra-dermal or transdermal immunization) and more potent immunization schedules should be studied. In addition, appropriate peptide or peptide pools to be used as *in vitro* stimulating antigen have to be selected more carefully. Both CD4⁺ and CD8⁺ ICS and ELISPOT responses have to be studied in order to correlate with the *in vivo* DTH response, which is more a CD4⁺ or helper T cell response.²⁶

The concept of the last common ancestral (LCA) DNA sequence used in our study is relatively new and exciting. The objective of an "ancestral" and "center of the tree" DNA sequence is to derive at a consensus sequence which will cover all strains which diversified from that ancestor or center of the phylogenetic tree.^{12,27} Many groups of investigators have used this concept to study common ancestral clade B or C env and gag DNA vaccines and found that they could produce functional proteins with potential to be vaccine candidates.²⁸⁻²⁹ We used a similar concept but broadened the ancestral sequence to encompass the eight circulating subtypes (subtype A, B, C, D, CRF01_A/E, F, G, H) by using 10 most recent isolates from each of the 8 subtypes to generate the phylogenetic tree in order to derive at the LCA sequence used in our humanized hypothetical envelope DNA vaccine.

It will be essential to study further whether such a hypothetical multiclade DNA vaccine can indeed generate cross-clade immune responses. If this is the case, such a multiclade DNA vaccine would be applicable worldwide, i.e., one vaccine for the whole world despite diversified HIV-1 subtypes. In fact,

Weaver *et al.*³⁰ just recently described an artificial synthetic group M consensus envelope gene (CON6) which could induce cross-reactive CTL and neutralizing antibodies against primary isolates from multiple clades. These findings encourage further work on our humanized V3 DNA of last common ancestor origin.

ACKNOWLEDGMENTS

This work was supported by the Royal Golden Jubilee Program, the Thailand Research Fund. The authors would like to express their sincere thanks to Dr. K. Matsuo for his excellent technical advice. R. Oelrichs is the recipient of a Wellcome Trust Traveling Research Fellowship (055521/Z/98/Z).

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