Recovery of APOBEC3-edited human immunodeficiency virus G→A hypermutants by differential DNA denaturation PCR

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Virus genomes from the same family may exhibit a wide range in their DNA GC content, whereas viral hypermutants differ substantially in GC content from their parental genomes. As AT-rich DNA melts at lower temperatures than GC-rich DNA, use of a lower denaturation temperature during PCR should allow differential amplification of AT-rich genomes or variants within a quasispecies. The latter situation has been explored explicitly in a two-step process by using a series of well-defined viral sequences differing in their AT content. Firstly, the lowest denaturation temperature (T_D) that allowed amplification of the parental sequence was determined. Secondly, differential amplification of AT-rich viral variants was obtained by using a denaturation temperature 1–3 °C lower than T_D. Application of this sensitive method to two different viruses allowed us to identify human immunodeficiency virus type 1 G→A hypermutants in a situation where none were expected and to amplify AT-rich variants selectively within a spectrum of poliovirus mutants.

The human immunodeficiency virus (HIV) Vif protein intercepts the host-cell proteins APOBEC3F and APOBEC3G, preventing their incorporation into budding virions (Harris et al., 2003; Wiegand et al., 2004; Zheng et al., 2004). The resulting Vif/APOBEC3 complexes are shunted to the proteasome for degradation (Sheehy et al., 2003; Yu et al., 2003). Of the seven APOBEC3 genes on human chromosome 22, at least five are transcribed (Jarmuz et al., 1993). Although mRNA-editing functions have not yet been ascribed to any APOBEC3 molecule, APOBEC3C, -3F and -3G are able to extensively deaminate single-stranded DNA (Harris et al., 2003; Lecossier et al., 2003; Suspende et al., 2004; Wiegand et al., 2004; Yu et al., 2004).

In the singular context of a HIVΔvif virus, only APOBEC3F and -3G appear to be packaged into the virion (Harris et al., 2003; Bishop et al., 2004; Liddament et al., 2004; Wiegand et al., 2004; Zheng et al., 2004). It is of note that APOBEC3F and -3G are packaged during budding from the donor cell and do not enter the replication complex of an incoming virion. Consequently, as soon as minus-strand viral cDNA is synthesized in the next round of infection, the numerous multiple C residues are deaminated, yielding U. Following plus-strand DNA synthesis, the U residues are copied into A, giving rise to so-called G→A hypermutants, by reference to the viral plus strand (Pathak & Temin, 1990; Vartanian et al., 1991).

As G→A hypermutants are associated with a lethal phenotype, the absence of vif, their detection in a natural setting is, not surprisingly, highly variable and their frequency is often low. However, as G→A hypermutants frequently exhibit 20–60 % of G residues substituted by A, their base composition is shifted considerably from that of the parental sequence. As AT-rich DNA melts at a lower temperature than DNA with a higher GC content, this suggested a means to differentially amplify AT-rich hypermutants, simply by modulating the PCR denaturation temperature. The reverse is well-known: when working with GC-rich genes or genomes, PCR denaturation temperatures are usually increased to around 95 °C and occasionally higher (Smith et al., 1996). Hence, it should be possible to find a denaturation temperature that allows amplification of AT-rich variants and not the parental sequence.

Low denaturation temperatures have rarely been exploited to differentially amplify AT-rich DNA. One report described ligation-mediated PCR performed at low denaturation temperatures (notably 80–88 °C), to selectively amplify AT-rich segments within a bacterial genome (Masny & Plucienniczak, 2003).
From previous work on HIV G→A hypermutation, we had a large collection of molecular clones, corresponding to the V1V2 region of the HIV-1 envelope gene, that differed uniquely in the number of G→A transitions. A smaller region within this fragment was amplified by using Taq polymerase and degenerate primers that were derivatives of the standard SK122/SK123 pair (Goodenow et al., 1989), to result in better amplification of hypermutated genomes. Their sequences were: SK122intD, 5′-AAACCCTAAARCCATRTRTA; SK123intD, 5′-TAATGTATGGGAATTTGGYTAA. When the PCR denaturation temperature was lowered to 83 °C (the reaction profile was 5 min at 83 °C, 25 cycles of 1 min at 83 °C, 30 s at 45 °C and 30 s at 72 °C, followed by 10 min at 72 °C), it was possible to uniquely amplify clones harbouring at least three mutations, whilst not amplifying the parental sequence (no mutations; Fig. 1a, b).

To confirm that amplified material was indeed hypermutated and not a PCR artefact, products were electrophoresed in agarose gel containing HA-yellow (Hanse Analytik), a pegylated bisbenzamide that interacts preferentially with the minor groove of AT-rich DNA, thus retarding migration (Abu-Daya et al., 1995; Abu-Daya & Fox, 1997; Janini et al., 2001). As can be seen in Fig. 1(c), migration of PCR products in a gel containing 1 U HA-yellow (ml agarose)$^{-1}$ was retarded progressively when moving from 0 to 18 transitions per sequence, confirming the selective amplification of G→A-hypermutated DNA at 83 °C.

It is apparent from Fig. 1(b) that product recovery correlated with the extent of hypermutation. To explore more carefully the relationship between denaturation temperature and the number of G→A transitions per clone, another series of G→A-hypermutated reference clones spanning another locus within the V1V2 region was analysed by using Taq polymerase and a different pair of primers, RT3 and RT4 (Martinez et al., 1994). Lowering the denaturing temperature by 1 °C progressively amplified more extensively hypermutated sequences (Fig. 1d). Given the exquisite relationship between denaturation temperature and AT content of a sequence, the success of amplification may also depend on the calibration of the PCR machine and perhaps upkeep and make. Accordingly, all PCRs were performed on the same machine. These findings show that the selective amplification of G→A hypermutants is indeed generally related to the melting temperature of the target DNA. We refer to this method as differential DNA denaturation PCR, or 3D-PCR.

Fig. 1 also shows nested PCR product (293T/PBMC) corresponding to the same V1V2 region amplified from peripheral blood mononuclear cells (PBMCs) that had been infected with a Δvif derivative of HIV-1 pNL4.3 following transfection of 293T cells. The denaturation temperature was 83 °C. The fact that this material represented differentially amplified G→A hypermutants was indicated when the 3D-PCR products were electrophoresed in a gel containing HA-yellow (Fig. 1c, 293T/PBMC). When the 3D-PCR products were cloned and sequenced, the vast majority of sequences were extensively hypermutated, harbouring between three and 18 G→A transitions compared with the reference sequence (0). Of the 18 sites bearing G→A transitions, 15 were in the context GpA and the remaining 3 were in pA.
three were in the context GpG. Cloning and sequencing of PCR material amplified at 95 °C identified only wild-type DNA (not shown). The surprise here is that the HIV-1 Dvif virus stock was made by using the 293T cell line, which is widely used as not only can it be transfected easily, but also it is considered not to express APOBEC3 molecules. From what is known of the mechanism of GR A hypermutation, the simplest explanation is that the 293T cell line had become clonally heterogeneous, so that APOBEC3F [preference for 5'TpC dinucleotide, GpA on viral plus strand (Harris et al., 2003; Liddament et al., 2004; Wiegand et al., 2004; Zheng et al., 2004)] as opposed to APOBEC3G [5'CpC preference, or GpG on plus strand (Harris et al., 2003; Lecossier et al., 2003; Suspène et al., 2004)] was being expressed in a subset of cells. Presumably 3D-PCR was picking up DNA from viruses produced by this subset.

Clearly, 3D-PCR can be used to differentially amplify AT-enriched genomes compared with the parental genome. Although retroviral hypermutants are an obvious target for 3D-PCR, it could be applied to any sample in which there was a mutant spectrum. With this in mind, we sought a very different system. We settled on poliovirus VP1 PCR products from ten patients with post-vaccinal acute flaccid paralysis (Balanant et al., 1991). A smaller 480 bp nested segment was targeted and the denaturation conditions were investigated by using the primer pair UG1/UC1 (Guillot et al., 2000). Calibration using cloned DNA showed that the reference Sabin 1 sequence was amplified by using denaturation temperatures from 95 to 91 °C, but not from 90 to 80 °C. Sabin 2 and 3 targets were subtly different from Sabin 1 in that they could not be amplified below 92 °C. The higher denaturation temperature used here compared with the HIV-1 locus described earlier is explained by the higher GC content of the target (48 %, compared with 34 % for HIV-1).

Among the ten samples, only one yielded a strong signal by 3D-PCR, with the following reaction profile: 5 min at 90 °C, 25 cycles of 1 min at 90 °C, 30 s at 45 °C and 30 s at 72 °C, followed by 10 min at 72 °C. When cloned and sequenced, a series of AT-enriched sequences was obtained, with substitutions mapping particularly to VP1 residues 560–728 in the alignment of entoviral polyproteins.

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**Fig. 2.** A collection of G→A-hypermutated HIV-1 V1V2 region sequences derived from Δvif stock virus grown on 293T cells [293T/PBMC, Fig. 1(a–c)]. For clarity, only a 189 bp region of the 304 bp segment that was amplified is shown. Sequences are aligned with respect to the parental sequence. Only differences are shown. Hyphens denote gaps. Clone designation is shown to the left. Analysis of material from the 95 °C amplification failed to identify any hypermutated genomes.
The sequences carried between one and six substitutions per segment. Of the 34 distinct substitutions, 28 were non-synonymous (including two nonsense), which is typical of variation within a quasispecies that has not undergone purifying selection. All but one substitution yielded genomes that were enriched in A and T. Amplification, cloning and sequencing of PCR material obtained at 95°C revealed 17 clones that harboured only two substitutions in the locus shown in Fig. 3 (data not shown). Hence, it can be concluded that 3D-PCR was indeed amplifying the AT-rich end of the poliovirus mutant spectrum. As only one sample could be amplified differentially, the AT-rich variants presumably represent an unusually broad mutant spectrum and have nothing to do with the post-vaccination syndrome.

3D-PCR allows differential amplification of genomes that differ by just a few GC→AT transitions. As the degree of substitution directly affects the melting temperature of the DNA, the lower the denaturation temperature, the more substituted the genomes that are amplified. As different loci may have widely different base compositions, the conditions will have to be optimized for each segment. Although the method allows differential amplification, it is not quantitative per se. However, coupled to limiting dilution of input DNA, it should be possible to quantify the fraction of AT-rich genomes within a sample. 3D-PCR could be used to amplify AT-rich bacterial 16S rDNA sequences within a heterogeneous natural sample, neo-deaminated immunoglobulin V regions or promoter regions that have undergone extensive 5-MeC deamination following extensive methylation.

In the precise setting of HIV, 3D-PCR has shown that one cell line that is used widely to support the replication of Δvif genomes is probably clonally heterogeneous, meaning that there is a background G→A-hypermutated signal in any sample. The ability to discriminate AT-rich variants over background suggests that this technique might find a variety of applications to biological questions.

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References


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![Fig. 3. A collection of AT-rich poliovirus VP1 segments derived from a patient with post-vaccinal acute flaccid paralysis. For clarity, only a 109 bp region of the 480 bp segment that was amplified is shown. Sequences are aligned with respect to poliovirus Sabin 1. Only differences are shown. Clone designation is shown to the right. The 3D-PCR-amplified segments bore one to six GC→AT transitions compared with Sabin 1. Analysis of material from the 95°C amplification yielded two substitutions among 17 clones in the same sequence.](www.iah.bbsrc.ac.uk/virus/picornaviridae/SequenceDatabase/alignments/entero_pep.txt)


