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HIV Inter-subtype Recombination - Consequences for the Epidemic

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Abstract

Until just a few years ago, HIV-1 was believed to evolve primarily by accumulating point mutations, leading to gradual rather than abrupt changes in virological properties. More extensive sampling and development of new laboratory and data-analysis methods have revealed that this picture was incomplete. HIV-1 has in recent years been shown to use recombination as an additional mechanism for the generation of variation and diversity. As data continues to accumulate, the role of recombination for viral evolution, and indeed the evolution of the global epidemic, has become increasingly evident. Factoring in recombination between divergent HIV strains and especially between different subtypes has brought about changes, not only in the genetic HIV-1 classification system, but also in the ways we must think about developing vaccine and prevention strategies. The continuous dissemination of different HIV-1 subtypes throughout the world will lead to multiple opportunities for intersubtype recombination, of which the consequences will be discussed below.

Key words

HIV. Recombination. Subtypes. Classification

Mechanisms of HIV variability

Mutation

HIV is one of the most variable human pathogens known today. As for all organisms that use RNA as their genetic material in some stages of their genome replication, the inability of the replicative RNA polymerases (and reverse transcriptases) to correct misincorporations leads to a high primary replication error rate[1-5]. For the majority of RNApolymerases, this basic misincorporation rate is in the range of 10^{-5} to 10^{-4}.

The high mutation rate sometimes also leads to a high evolutionary rate. This is certainly the case with HIV, where evolutionary rates of approximately 0.5-1%/year have been observed for the V3 loop region of the genome[6-9]. However, evolution of the genome seems to take place in all regions of the genome, although the specific rate in different regions varies. For HIV there seems to be less bias between silent and non-silent sites than in many other viruses such as the enteroviruses which vary mainly in the silent positions of the coding sequence.

Most RNA viruses deal with the problems associated with the polymerase error rate with multiple means: first, RNA genomes are relatively small, minimising the proportion of truly defective genomes generated per replication cycle. Secondly, some viruses, among them HIV, seem to have flexible protein structure requirements, so that many mutations can be tolerated without loss of function. Thirdly, in many cases evolutionary mechanisms that counteract the effects of random-point mutations have evolved. One such mechanism is the segmented genome structure of many virus groups. Use of a segmented

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genome allows the virus to compensate defective genome segments with non-defective ones, if the multiplicity of infection is high. For retroviruses, the analogous mechanism is recombination during reverse transcription. This is possible, since retroviruses are diploid, carrying two RNA molecules within the particle.

Superinfection and recombination

Retroviral recombination occurs during the reverse transcription step, before integration and is dependent on co-packaging of two different viral genomes. This, in turn, requires simultaneous infection of the same cell by two different strains and subsequent integration of two different parental generation proviruses in the same nucleus. Simultaneous expression and packaging of viral RNA effectively generates a population of heterozygous first-generation virus particles. The actual recombination event only takes place upon reinfection of these first generation heterozygote particles into new host cells, after entry into the cytoplasm, as the different RNA molecules in the particles are reverse transcribed. In this reaction, the growing DNA strand can jump from one RNA template to the other by a copy-choice mechanism, creating chimeric second-generation proviruses. The viral particles produced by the second-generation proviruses will now contain recombinant RNA forms[10-12].

HIV groups and subtypes

The high evolutionary rate of HIV results in a continuous and gradual change of the virus over time. However, in contrast to some other viruses such as the Influenza A type, there is no replacement of one dominant global lineage over time, but multiple evolutionary lineages co-exist simultaneously[13]. This is evident from phylogenetic analyses using HIV sequences, which result in phylogenetic trees of a star-like radiation, in which groups of strains branch from a central node and form distinct clusters of strains[14-16] (Fig. 1).

The main HIV-1 clusters were initially recognised using relatively short subgenomic sequences of the *gag* and *env* genes[14]. As the clustering patterns of the major groups seemed to be mostly consistent between the two genes (see next subsection for a discussion on the exceptions from this rule), they were named "genetic subtypes". The "genetic subtype" term has mainly been used as a classification unit below the "type" level (HIV-1 and HIV-2 are the known "types" of immunodeficiency viruses infecting humans). Subtypes have been named alphabetically, primarily in the order of discovery, and currently 10 subtypes, designated by the letters A-K, are recognised[17]. Recent advances in PCR- and sequencing methods have made determination of sequences for complete HIV-1 genomes much easier than before[18-20]. This has resulted in rapid growth in the number of complete HIV-1 sequences available for study. Several research groups (among them our own group) have systematically tried to even the bias towards subtype B sequences which earlier existed in the International HIV sequence database[21]. During the last few years, this has resulted in the generation of the first complete genome sequences for all HIV-1 subtypes, which can now be used as references for a variety of purposes[17,19,22-28]. Analysis of complete genome sequences has verified the subtype grouping[17], and has shown that the subtype clustering is a property repeated over the entire genome (Fig. 1). This comes as no big surprise, since the subtypes are evolutionary lineages, and evolution affects the entire genome.

In a recent development of the classification system, a proposal has been put forward suggesting the introduction of the concept of the "sub-subtype", which are strains forming subclusters within the main subtype clusters in phylogenetic trees[17]. Currently, subtype F is divided into sub-subtypes F1 and F2 (Fig. 1). Subtypes B and D also form a sub-subtype relation, but their names have not been altered since they are so well established in the literature.

In addition to the above described HIV-1 subtypes, outlier strains that clearly associate with HIV-1 in phylogenetic analyses have been described[21]. These strains form two other clusters within the HIV-1 type cluster, but branch off separately outside the subtypes described above. Upon discovery of these outlier strains, an intermediate classification unit was introduced. This is the "group" unit. Therefore, all the above described A-K subtypes belong to the M (main) group of HIV-1, and the outlier strains are called the O (outlier) and N (new or non-M, non-O) groups. Both the O and N groups have a very low prevalence and are almost exclusively found in central Africa. Much less is known about their genetic variability than for the M group, which constitutes the bulk of the global HIV-1 epidemic[29-33]. Even less information is available for the N group[34].

While less data is available for HIV-2 than for HIV-1, it appears that there is a roughly similar structure of subtypes within HIV-2 as for HIV–1, although most strains belong to subtypes A and B[35]. However, the evolution of HIV-2 is complicated by its clear close-relatedness to SIVsm from sooty mangabey monkeys, and relatively recent cross-species transmission events. A detailed description of HIV-2 evolution is beyond the scope of this review, but has been recently addressed in earlier publications[36-39].

In summary, the current HIV classification and nomenclature system follows the hierarchy of "type" (1 and 2) -> "group" (M, N and O) -> "subtype" (A-K) -> "sub-subtype" (F1 and F2) -> "strain" -> "isolate" -> "clone". The last three levels follow the conventions in the literature, although the strain/isolate name is sometimes used synonymously. The current nomenclature and classification system, as well as requirements for assigning novel subtypes for HIV, is described in detail in a recent review[17].

Inter-subtype recombination and Circulating Recombinant Forms (CRFs)

A practical consequence of the small bias in mutation distribution and simultaneous relatively high

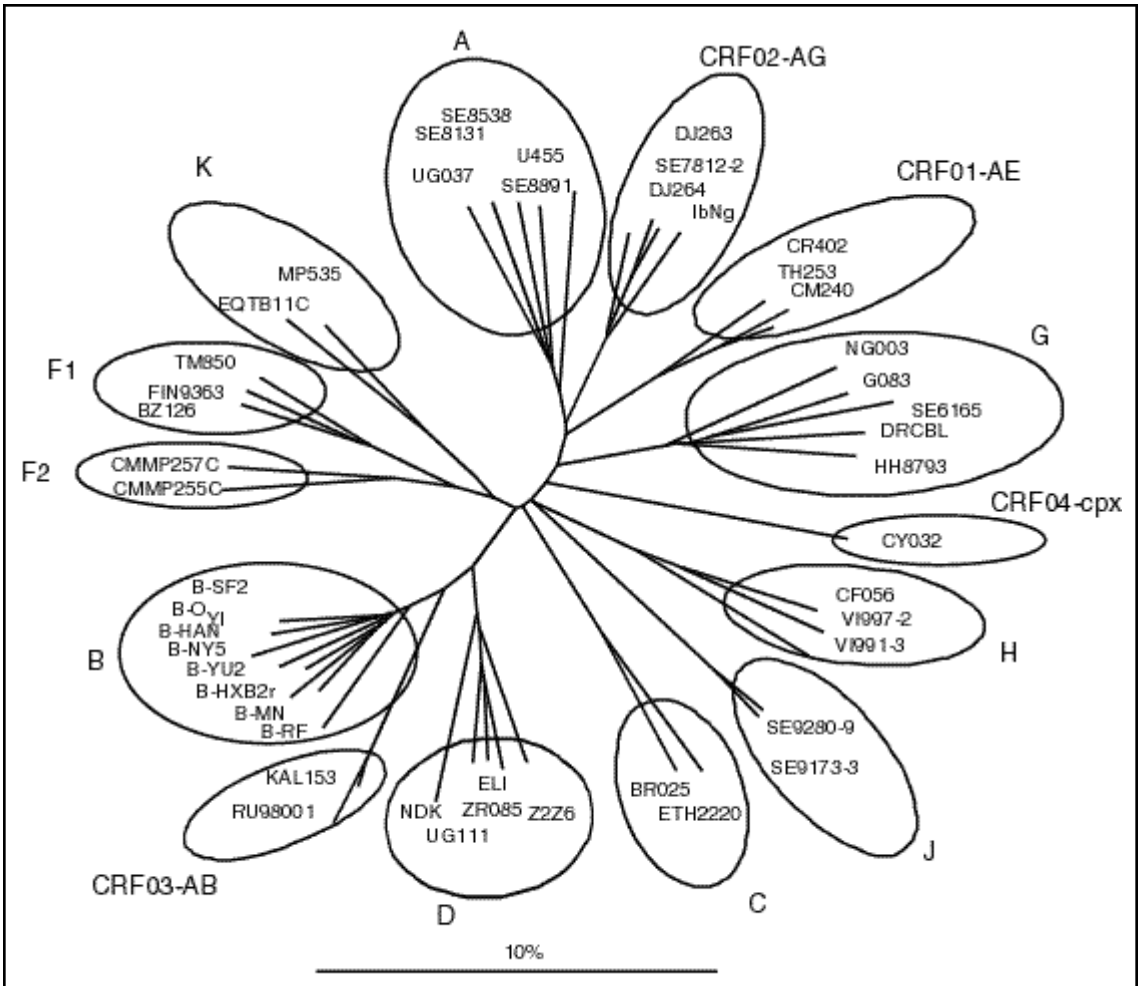

Fig. 1. *Phylogenetic tree of current HIV-1 subtypes and Circulating Recombinant Forms (CRF). The tree was constructed using a reference set of virtually complete genome sequences available in the Los Alamos HIV sequence database[20].*

fixation rate along the HIV genome has been that detecting recombination between different HIV-1 evolutionary lineages is relatively easy and straightforward. This is especially true for detecting recombination between different subtypes of the virus. As already described above, the HIV subtypes are defined as clusters of sequences in a phylogenetic tree, with additional describing parameters of intra- and intercluster variability or genetic distance. In plain text, this means that the genetically defined HIV-1 subtypes are almost equidistant from each other in all genome regions. Therefore, recombination can be detected by observing the similarity (or dissimilarity, which is the inverse; sometimes estimates of genetic distance are also used) of a genome segment to reference sequences representing the different genetic subtypes. Using this method in an average overwindow fashion (dubbed distance- or similarity plotting), recombination breakpoints in intersubtype recombinant strains can be mapped with relatively high accuracy, i.e. approximately within 100 bp[27,40]. The alignment is broken into consecutive overlapping windows and the similarity between the unknown and the reference sequences is calculated. Values for each segment are then plotted at the midpoint of each window along the genome. The genetic subtype of each region is revealed by the most similar reference sequence. Another methodology, which we originally developed, employs a similarly sliding window analysis method, but uses the bootstrap values of clustering the unknown with type clades or sequences to map recombination bases[41,42]. This method has the advantage of being visually more easy to interpret and allows for studies of robustness of clustering groups in addition to individual sequences, but is computationally much more demanding than similarity plotting. Neither of these methods is foolproof, but they perform reasonably well when sequences have not diverged too much since the recombination event took place. Both methods are highly dependent on the reference sequence data set.

The recent increase in complete genome sequencing of a large number of non-B type viruses also has revealed a surprisingly large number of strains that appear to be inter-subtype recombinants. Earlier studies had already shown that for some HIV-1 isolates, subtyping using different subgenomic regions indicated discordant results (such as subtype "E", which maps with subtype A in all regions but

the envelope and LTR regions)[14,27]. When such results were first discovered, they were often viewed as either results of limitations in the analysis techniques (phylogenetic analyses), laboratory errors (sample mix-ups or contamination), PCR generated artefacts (PCR-induced recombination in a mixed isolate/sample), or as the consequence of evolutionary forces driving certain strains into different clusters in different parts of the genome. Therefore, the early results were frequently dismissed as artefacts. Earlier laboratory studies seemed to indicate that super- or co-infection of different HIV strains would be unlikely, since interference based superinfection inhibition in cell culture takes place, and is caused by viral entry receptors being down-regulated by the virus[43-46]. This phenomenon would prevent HIV recombination, since co-infection of the same cell is required for retroviral recombination. However, due to the frequent detection of intersubtype recombinant forms, it has become evident that co-infection *in vivo* must be relatively common. Nevertheless, it is still unknown whether there are any differences in susceptibility to superinfection/co-infection depending on disease stage. Several studies have also addressed the laboratory error or artefact issues, and have provided convincing evidence to indicate that these are not significant confounding factors[18,41].

While many of the intersubtype recombinant forms seem to be unique, and have only been found as single isolates, there are some examples of the opposite. Some recombinant strains have reached high prevalence and significant geographical spread. Thus, they form epidemiologically equally important entities as the subtypes themselves, and, reflecting this fact, they have been taken into account in the current HIV subtype classification and nomenclature system.

Recombinant forms that: 1) share a common chimeric structure; 2) cluster together in monophyletic groups upon phylogenetic analysis regardless of genome region and 3) have reached significant prevalence and/or geographical spread, can currently be given the taxonomic classification of circulating recombinant forms, or CRFs (Fig. 1). However, to avoid future confusion, it has been agreed that similar to new subtypes, at least three complete genome sequences from epidemiologically unlinked sources verifying the structure must be completed before formal CRF status is granted. Exception may be granted if there are two sequences plus additional shorter sequence data from other samples[17,47]. Taxonomically, the CRFs are at the same level as the subtypes. Each CRF is given a consecutive number in order of discovery, followed by a letter series describing the parental subtypes. Currently, four CRFs have been given a taxonomic name, CRF01-AE, CRF02-AG, CRF03-AB and CRF04-cpx (Fig. 2). The last of these consists of more than two subtypes (A, G, H, K and unclassified regions) and has therefore been given the cpx-denomination (short for "complex"). It is also the lineage which was formerly thought to have contained regions of a putative "subtype I" for which no isolates classified as "I" throughout the genome have been found[48]. Recent re-analysis of originally unclassifiable regions of this isolate have shown them to have different phylogenetic origins, and therefore "subtype I" was removed from the subtype classification list. Recently, two other recombinant lineages that may constitute additional CRFs have been described and provisionally named CRF05-DF and CRF06-cpx[23,49,50]. However, these two cases do not fulfil the formal criteria established above, so they have only been provisionally named.

Recombinant and CRF Epidemiology

It has now become more and more evident that HIV recombination is a very common phenomenon, and that, in areas where multiple subtypes circulate and prevalence is sufficiently high, recombinant viruses may even dominate the epidemic. This can clearly be seen in many countries of sub-Saharan Africa, where systematic studies have been conducted[26,51-57]. In these countries, more than 50% of the strains sampled may be recombinant. Also, the previous view of subtype A as the dominant variety of HIV in central and western Africa has been shown to be too simplistic[26]. The differences in the distribution and prevalence of the varieties of HIV between subregions are large.

One clear distinction or divider is evident in a division between, on the one hand the western-central sub-Saharan African axis, and, on the other hand the eastern-southern axis. Beginning with the latter, there is a dominance of subtypes A, C and D, with subtype C being the absolutely dominant subtype, probably accounting for more than 90% of the epidemic in Ethiopia and South Africa. At least in countries such as Uganda, Tanzania, and Kenya there are more subtype A and D infections in a variable mix. Recombinant forms are quite common, but generally only isolated strains are found, so that no circulating recombinant forms unique to this region have yet been described. Systematic data on the proportion of recombinant forms compared to "pure" strains is unavailable, but small-scale studies from Tanzania and some other countries suggest that >50% strains in the region may be recombinant[58].

In the western-central sub-Saharan African direction, on the other hand, the picture is quite different. In this region, all subtypes are found at some frequencies, but the dominant variety of HIV is a CRF. CRF02-AG (prototype strain IbNG[24]) causes a large proportion of HIV-1 infections in the region, specifically in countries like the Ivory Cost, Nigeria, Gabon, Senegal, Cameroon, the Congo, the Central African Republic and the DRC. Original studies suggested that, in these regions, subtype A was the dominating HIV variant. As more sequence data has become available, it has been revealed that most of these strains belong to the CRF02-AG recombinant strain[26,52,53,59]. Many other unique recombinant strains have also been reported in this region[50,54,59,60].

Another epidemiological pattern of a dominant CRF is the classical example of the CRF01-AE (pro-

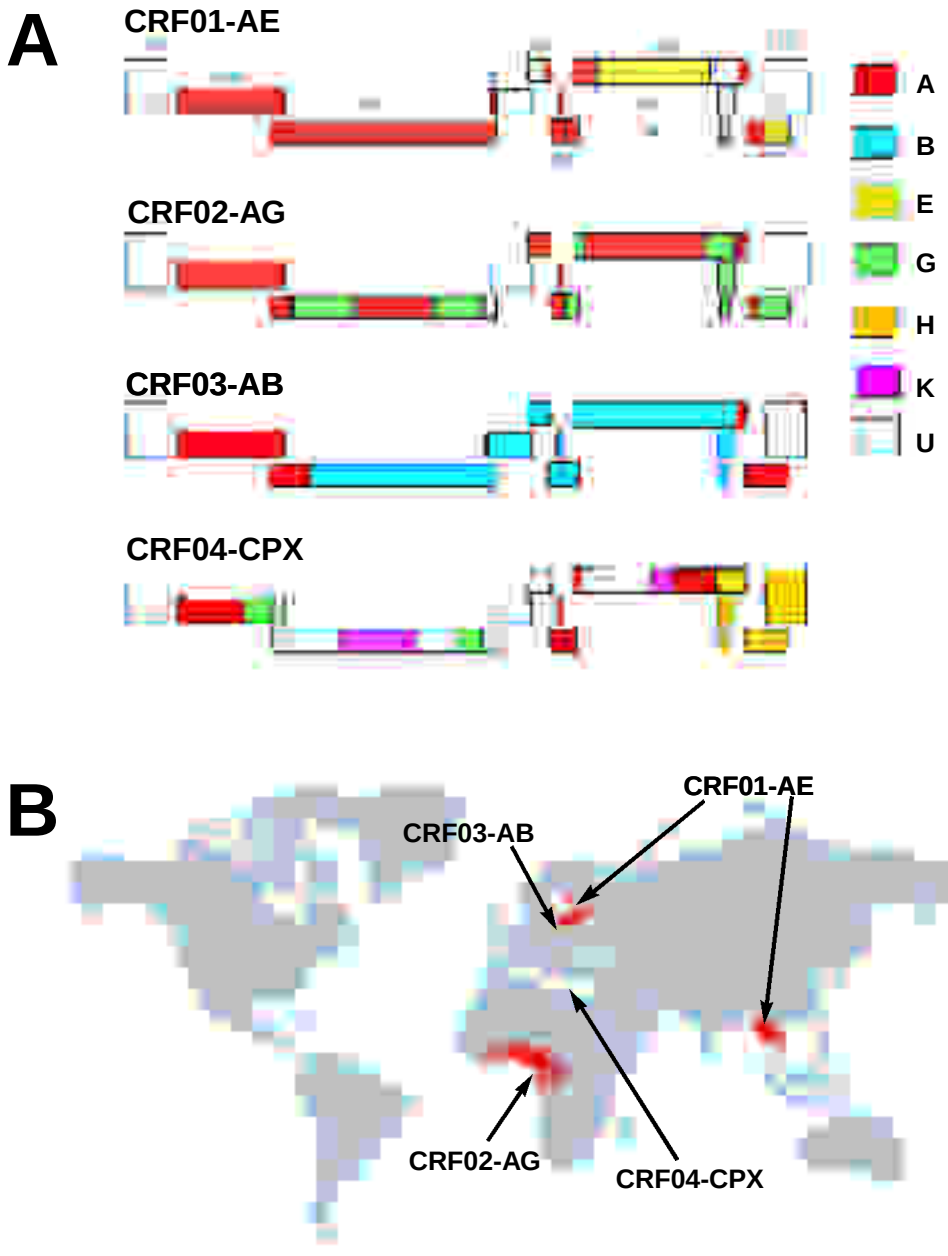

Fig. 2. *Currently recognized CRFs. A) Genetic structure and B) distribution of the currently recognized CRFs (red). "U" denotes unclassified regions.*

totype strain CM240[27]) which is highly prevalent in southeast Asia. This CRF first started a sexual transmission associated epidemic in Thailand in the early nineties, but has since spread to many other countries in the region, including Vietnam and Cambodia and, to some extent, also the Philippines, India, China, Taiwan and Japan[61-66]. While CRF01-AE originally was described as spreading mainly in association with sexual transmission and commercial sex work (CSW), it has, according to later studies, also become more and more prevalent among users of intravenous drugs[65,67-71]. CRF01-AE was first described as a new subtype, subtype E, but later studies by ourselves and others showed that the virus is actually a recombinant of subtypes A and independent "E" regions[27,72]. Using current nomenclature requirements, the "E" subtype would not qualify for subtype status, since no full-length "E" strain has been described. For reasons of consistency in the literature and the epidemiological significance of this variant, the "E" subtype designation has been retained in the name of CRF01-AE[17].

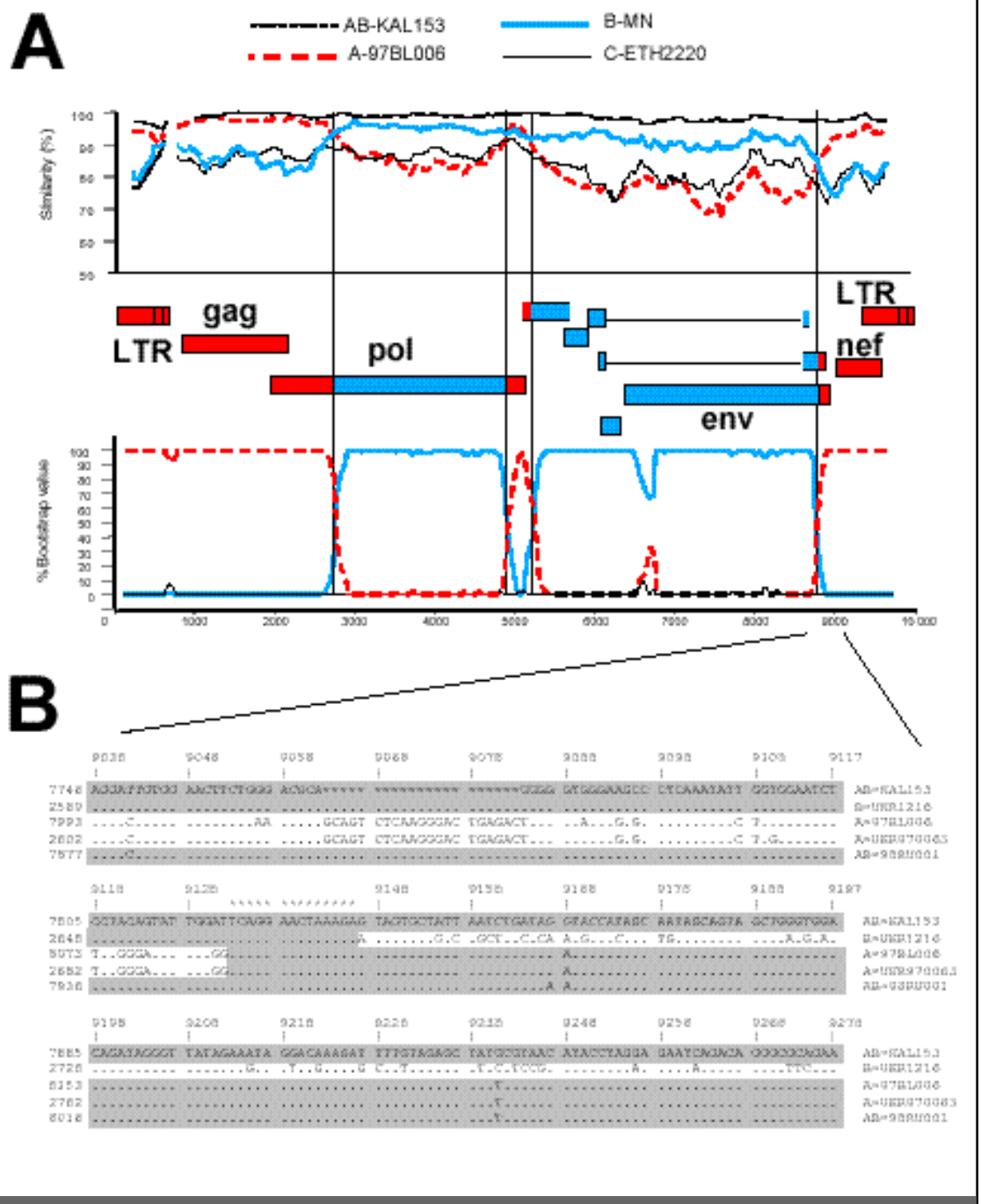

Fig. 3. *Subtype structure of CRF03-AB. A) Similarity plot and Bootscan using A- and B-reference sequences and outgroup (subtype C). B) Magnification of alignment of representative CRF03-AB (Kal153 and 98RU001), Parental B (UKR1216) and parental A (UKR970063 and 97BL006) strains. The regions of highest similarity are shaded, and the putative 14 bp recombination point is indicated by the stars. Numbering follows alignment and clone positions[93].*

CRF01-AE has also shown some tendency of at least spurious global dissemination. Occasional infections with this CRF have been found in practically all molecular epidemiology studies conducted in the western hemisphere and in these cases often linked to sex-tourism[51,73-79]. Despite such low-level importation, there has been no evidence for significant spread associated with sexual-transmission in the west[79].

However, in at least one region of north-western Europe, CRF01-AE has recently become epidemiologically a very significant factor. In Finland, injecting drug use (IDU) associated HIV/AIDS was rarely reported prior to 1998, despite a quite comprehensive national surveillance system (HIV is a notifiable disease and cases are reported by both laboratories and primary physicians to the National Public Health Institute with identification). The few IDU-transmission associated HIV cases that were found between 1981-1996 were also exclusively imported, and were all unrelated subtype B strains when analysed. No evidence for domestic spread associated

with these early cases was evident. The potential for an IDU associated HIV epidemic was nevertheless there, since HCV incidence in Finland especially among young people is on a quite high level[80]. In mid-1998 this threat became reality and an HIV epidemic among the IDUs, especially in the capital region around Helsinki, commenced.

The epidemic has since spread to other parts of the country, too, and in 1999, 55% of all newly reported HIV cases were associated with IDU. Surprisingly, upon typing, the variant of HIV causing the epidemic was not a member of either subtypes A or B (prevalent among IDUs in eastern and western Europe, respectively), but CRF01-AE[81]. It is quite clear that this event will have a profound effect on the epidemiology of HIV in Finland, and may even have implications on future vaccine choices as discussed in more detail below. How the CRF01-AE became the variant of HIV to establish itself in the Finnish IDU population will probably remain forever elusive, but is a perfect example of the great unpredictability of the HIV epidemic.

CRFs are also apparently generated due to IDU: in Russia and in China, subtypes A and B *versus* C and B, respectively, seem to have recombined and generated epidemics caused by the recombinant variants[65,82-85]. The IDU epidemic of the Russian Kaliningrad region, located between Lithuania and Poland, is caused by CRF03-AB. This CRF is the product of a subtype A strain which is very prevalent among IDU in the Ukraine and has also been described in the IDU context from Byelorussia, many parts of Russia, Kazakhstan, Moldova and even Latvia[83,86-94]. The B parent has also been described from southern Russia and recently in Georgia (Jean Carr, personal communication).

In China, the IDU-associated HIV epidemic was first described to have been caused by a triplet of strains. Subtypes B, C and the CRF01-AE were all found among IDUs, but further detailed characterisation and extensive molecular epidemiological studies have shown that there is a significant contribution of a BC recombinant strain which is evidently also originally derived by recombination between the original pure B and C strains[85,95]. Preliminary evidence suggests that a single form of the recombinant has been spread to a large part of the country. Therefore, this strain is a clear candidate for a novel CRF, but formal classification will have to await more thorough molecular characterisation.

Availability of both parental strains in the case of the Russian recombinant strains has also allowed, for the first time, very precise mapping of the breakpoints in an intersubtype recombinant strain (Fig. 3). First, the breakpoints in the strain were mapped by the above-described techniques (bootscanning and similarity plotting) and then the alignment of the mapped regions was visually examined to pinpoint the probable breakpoints. Assuming that the breakpoint is located where high sequence similarity of one parent and the recombinant switches to high similarity between the other parent and the recombinant (as measured by aligned nucleotide position identity), we were able to map a recombination point in the envelope gene to within a region of 14 bases. This region was perfectly conserved between all strains and bounded by the switch in similarity between parental strains. Further assuming that recombination has required exact identity or has fixed the sequence of the actual hybridisation point during the strand transfer, we concluded that, at least in this case, 14 nucleotides of identical sequence has been sufficient for intersubtype recombination[86].

Although the estimate of required sequence homology derived from the above case is very simplistic, and other types of less perfectly matched base-pairing in the region may have been important, stretches of 14 bases of exact identity between subtypes are quite common in most regions of the HIV genome. Therefore, it is hardly surprising that so much recombination can seen between strains. In addition, several experimental studies have recently shown that recombination is frequent even in viral cell culture systems[96,97].

Recombination evidently also will affect the epidemic in South America. In most South American countries that have been surveyed, subtype B dominates the epidemic[98,99]. However, subtypes F and C also seem to have gained a stronghold. Recombinants of subtypes B and F have been described from multiple countries in the region. Based on the data available from South America, it is still unclear whether the epidemic is characterised by contiguous generation of multiple, individual recombinant forms or whether there is a sub-epidemic(s) of a circulating recombinant form(s). Nevertheless, several studies have suggested that multiple recombinant lineages may be concurrently generated[98,100]. It remains to be seen whether any of these recombinant forms will gain similar dominance as has happened in the cases of CRF01-AE (south-east Asia and Finnish IDUs), CRF03-AB (Russian Federation and some neighbouring countries) or the potential BC CRF in China.

Implications of recombination for HIV diversity and evolution

Generation and selection in vivo – why do we see the recombinant forms?

Looking at the mechanisms generating HIV recombinant forms *in vivo* and the dynamics of HIV replication from the point of view of the viral dynamics[101-104], some potentially important hypotheses are immediately evident. First of all, if we make the simplistic, but reasonable (for the purpose of the argumentation), assumption that the biochemical recombination reaction occurs with a similar frequency all over the length of the genome, then a huge number of different recombinant proviral forms are most likely produced in every co-infected individual[97]. These recombinant forms must then compete with not only other recombinant forms, but also with all other non-recombinants for contribution to the *quasispecies* swarm[103,105]. From this it follows that,

if the amount of effectively replicating viruses is high, then the likelihood of any of the single newly-generated unique recombinant to reach a dominating role in the *quasispecies* becomes very low. In other words, it should be very difficult for any single, novel recombinant form to overgrow the huge existing *quasispecies*. Certainly, if it would be left to chance alone, overgrowth should be very unlikely under these conditions[102].

Evidently, some recombinant forms nevertheless achieve overgrowth, since we can detect them both *in vivo* and spreading as CRFs in populations. There are some possible explanations that could explain why some recombinants are successful enough to gain a dominant role. The first could be that the actual replicating number of virus clones producing the large amount of progeny *in vivo* is actually small, i.e. only few of the produced virions actually result in proviruses that then produce all the next generation virions. In such a case, chance shifts in *quasi-species* main variants are much more likely to take place due to a bottleneck effect. Some studies seem to suggest that this could be the case[106]. Another explanation, however, could be selection. If some intersubtype recombinant forms would be more fit than others *in vivo* (by whatever mechanism, including neutralisation escape, wider target cell range, *etc.*), then their offspring would be much more likely to take over the dominant *quasispecies* role[102].

This is an interesting hypothesis, since recombination as a mechanism is generally accepted as having evolved because of its potential to generate novel combinations of existing inter-breadable lineages. Theoretically, combination of separately evolved lineages is favoured because it allows the combination of independently acquired properties in a rapid way. Therefore, it is at least possible that recombinants emerge as dominating forms in a *quasispecies* because a few of them have combined favourable features of their parental lineages and have become more fit than the rest of the *quasispecies*. It may even be argued that HIV (and retroviruses in general) has evolved diploidy due to its favourability in generation of recombinant forms. However, there are no experimental studies that have addressed the question of why recombinants emerge as dominant *in vivo* forms, so the final answer to the question is still not available.

Population level dynamics – founder effects and spuriousness

The apparent success of some recombinant forms in establishing globally or locally significant epidemics combined with the theoretical evolutionary competition advantages, has also led to suggestions that inter-subtype recombinants would be more fit than the non-recombinant HIV strains on a population level[107]. The very rapid spread of some of these intersubtype recombinants, evidenced by both sero-epidemiological and molecular sequence data, has led some authors to suggest that some property of these strains would favour their transmission over others. Examples of properties that have been suggested, and even studied, are higher infectivity in sexual transmission and/or preferential infectivity towards certain cell types[108,109].

It is, however, very difficult to address the question of the relative fitness of different HIV varieties on a population level. Fitness, in it's classical definition, is the ability of a variant to produce viable offspring, i.e. the more viable offspring a variant produces, the more fit it is. Viewed strictly by this definition, all the globally prevalent varieties of HIV-1 are the offspring of successful clones. This also applies to those CRFs that have rapidly gained high prevalence. However, many confounding factors complicate the picture. The above definition of fitness assumes that the environmental factors between the strains to be compared are equal, which is clearly not true. The environments in which different HIV-epidemics occur differ in many ways, including not only mode of transmission, but also in social and behavioural patterns and society's response to an epidemic.

Viewing some of the CRF-HIV epidemics (such as those caused by CRF01-AE and CRF03-AB) from this point of view emphasises the probable contribution of environmental factors that may lead to rapid amplification of a single strain. In both cases, a particular recombinant form that probably pre-existed at low prevalence within the framework of a larger mature epidemic, set off an explosive epidemic within a vulnerable, but previously largely unaffected population. CRF01-AE has even managed to do this multiple times, going from a relatively rare strain in Africa through multiple amplification cycles primarily in south-east Asia, but as the example from Finland shows, with the full potential for the same anywhere, given the opportunity. For CRF03-AB, the strain was probably generated in the Ukraine or neighbouring areas, but only caused a significant epidemic when it entered the unaffected IDU population in Kaliningrad[83,86,87].

The above epidemics, where a pathogen has rapidly been amplified in a population that has not seen it before, have lead to founder effects reflected in the genetic variability of sampled strains[93,110]. Since a single strain effectively established the epidemics, genetic variation is initially low, and viruses cluster very closely together in phylogenetic analyses. In early studies, when less was known about the parameters of HIV variation, the low interstrain variability was even mistaken as a special property of these strains. We now know that, as time passes and the epidemic matures, viral interstrain diversity will increase, and will reach levels seen in the classical intra-subtype comparisons[8].

Critical examination of the known HIV epidemics caused by CRFs has failed to identify factors of increased viral dissemination associated with recombinant genome structure *per se*. Rather, the evidence points towards random introductions of these particular strains into populations where conditions favourable for HIV transmission already exist. Also, the case of CRF01-AE seems to counter-indicate any preference towards mode of transmission, since this variety of HIV started as a mostly heterosexual-

ly transmitted strain, but has since shown its full potential for IDU-associated spread, too.

In conclusion, current evidence seems to point towards the direction that the governing factor of whether a particular recombinant becomes a successful CRF or remains a spurious singly-occurring strain, is random chance and environment rather than the theoretically superior fitness of some recombinant strains. However, the caveat of the difficulty of separating these factors on a population level with a strict human pathogen goes both ways, so we will probably not have the final answer to this question for a long time.

Mixing and merging – will the subtypes vanish?

As we have seen, the effect of recombination is to combine separately evolving lineages. If recombination would occur freely in the HIV population, this would implicate that over time the current subtypes would probably erode and perhaps even vanish as entities, due to multiple inter-subtype recombination cycles. There are evidence of such processes taking place, since multiple subtype recombinant strains have been described and viral heterogeneity is highest and subtyping most difficult in regions where multiple subtypes coexist. Examples of subtype mixing through recombination have been described from central Africa for multiple subtypes, but also South America for subtypes B and F[98,100]. Therefore, the real possibility exists that the classical single-subtype strains in these regions will indeed be replaced by a swarm of related, but differing, recombinant lineages, which will not be classifiable by the current methods of phylogenetic analysis. This is especially likely in sub-Saharan Africa, where HIV prevalence is high and multiple subtypes coexist. It may well be that sequential sampling of viruses from this region will show a continuous gradual blurring of the phylogenetic tree of HIV-1, resulting in a bushlike picture, with no clear clusters evident. Some studies already point towards such a future[56,111,112].

Speciation - could the genetic subtypes be ancient CRF founder-effects?

There is, however, a caveat to the picture above: we do have the clearly defined subtypes right now, so how were they generated in the first place? Recombination is certainly not a newly acquired property of the virus, since it employs the basic replication mechanisms of the entire retrovirus group. Therefore, if recombination is eroding the subtypes now, why it did not do it earlier, too? On top of this, we have the problem of how the subtypes evolved in the first place. HIV accumulates mutations in a consistent, even clocklike manner[8], and, therefore, it is actually surprising to find clusters of separated groups of sequences like the subtypes. It would be more likely to see a continuum of different strains branching off all over the tree. Where did all these intermediate strains disappear?

An argument has been put forward that the subtypes themselves represent foundereffects of the past. This is a valid hypothesis, since foundereffects in Africa must have occurred in the past when the prevalence of HIV in the human population was much lower and the virus was spreading only slowly. In such circumstances foundereffects can easily occur if individual strains are brought into a new population. However, this still does not necessarily provide a complete answer. In foundereffects like the one above, the origin of a founding strain can usually still be determined. This is possible, because the foundereffect cluster will branch off from earlier related strains and not from the centre of a phylogenetic tree. It is difficult to generate a reasonable model where multiple founder-effects would occur, with the simultaneous extinction of all earlier related strains.

The above, however, only applies if one disregards recombination as a co-factor in the founder-effect mechanisms. Here I would like to propose that recombination might at least sometimes offer the additional mechanism for a “fresh start” of a novel lineage branching off from the centre of the HIV-1 tree. If recombinant strains carry approximately equal proportions of the different parental strains, they will branch off from the centre of a phylogenetic tree. If the recombinant, simultaneously, is sufficiently amplified, the net effect will be a cluster of strains originating from the centre of the tree, i.e. similar to a subtype. Depending on the original prevalence of the parental strains, they either can be recognised as parental forms, or never be seen due to sampling limitations. The hypothesis put forward here is that HIV-1 intersubtype recombination, coupled to founder-effects, in effect is a mechanism somewhat analogous to speciation, generating novel genetic lineages. While there are numerous problems with this hypothesis, it may provide at least one possible explanation to the puzzle of how the HIV-1 subtypes have originated.

Vaccine implications

Superinfection and vaccine strain selection

The recognition that HIV-1 inter-subtype recombination occurs and even seems to be common, if given the opportunity, has clear negative implications for vaccine design. Earlier, when superinfection was considered unlikely, the goal of a vaccine was to establish a vaccination regimen that would mimic the immune response of the natural infection as closely as possible. It was believed that if it would be possible to generate a similar immune response as in natural infection, but without the pathogenesis, this would probably also protect from infection with wild-virus. In addition, a common view was that natural-kind immunity generated with a single strain HIV-1 vaccine could potentially protect from other strains, since many cell-mediated immunity governing antigenic epitopes are conserved among HIV strains.

The evidence for superinfection indirectly evident from recombination seems to suggest that a natural

infection-like immune response will be not be enough for an effective vaccine. This also probably means that live, attenuated viral vaccines will not be a sufficiently safe alternative, since they could regain infectivity by recombining with challenge strains. There is even some indirect evidence for this taking place in the chimpanzee animal model[113-115] as well as direct evidence from a SIVmac model[116]. Therefore, we may need HIV vaccines that generate immune responses that are clearly better than those occurring during natural infection.

Another concern of the consequences of recombination may be that it will further complicate strain selection for vaccine development. Some vaccine development strategies try to incorporate the HIV antigenic variability by taking a multivalent approach, where the genetic subtypes of HIV are used as surrogates of antigenic variation[117,118]. While the subtypes are clearly not antigenic types *per se,* they have been taken as the best approximation available for including antigenic variability. From this starting point, several projects that aim to collect representative vaccine candidate strains are currently in place[117,118]. If this strategy is to be consistently implemented, the most prevalent CRFs should be included in the collection of candidate vaccine strains. Certainly at least CRF02-AG will be very high on this priority list due to it's high prevalence in Africa.

As a final summary, we can see from the above that currently, and in the future, HIV evolution and epidemiology will be heavily influenced by intersubtype recombination. Therefore, it is a biological property of the virus which must be taken into account in designing both prevention and treatment measures. While the implications of recombination for the evolution of drug resistance were beyond the direct scope of this review, it has clear effects on this aspect of HIV treatment, too[119,120].

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