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Differential *Alu* Mobilization and Polymorphism Among the Human and Chimpanzee Lineages

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Alu elements are primate-specific members of the SINE (short interspersed element) retroposon family, which comprise ~10% of the human genome. Here we report the first chromosomal-level comparison examining the *Alu* retroposition dynamics following the divergence of humans and chimpanzees. We find a twofold increase in *Alu* insertions in humans in comparison to the common chimpanzee (*Pan troglodytes*). The genomic diversity (polymorphism for presence or absence of the *Alu* insertion) associated with these inserts indicates that, analogous to recent nucleotide diversity studies, the level of chimpanzee *Alu* diversity is ~1.7 times higher than that of humans. Evolutionarily recent *Alu* subfamily structure differs markedly between the human and chimpanzee lineages, with the major human subfamilies remaining largely inactive in the chimpanzee lineage. We propose a population-based model to account for the observed fluctuation in *Alu* retroposition rates across primate taxa.

[The sequence data from this study have been submitted to GenBank under accession nos. AY569161–AY569170.]

Alu elements are primate-specific members of the SINE (short interspersed element) family of retroposons. They have enjoyed enormous success over the course of primate evolution and, by conservative estimates, comprise some 10% of the human genome (Schmid 1996; Lander et al. 2001). Largely as a result of the human genome project, a wealth of knowledge has been accumulated concerning the underlying biology, retroposition activity, and associated population genetics of *Alu* repeats (Schmid 1998; Batzer and Deininger 2002). The ubiquitous presence of *Alu* sequences within primate genomes has been the cumulative result of a “copy and paste” mechanism, in which an RNA polymerase III-generated transcript is reverse-transcribed and integrated into the genome (Burke et al. 1999). In addition to being wholly dependent upon host cellular processes for their transmission through the germline, *Alu* elements also lack the ability to generate the endonuclease and reverse transcriptase necessary for their own retroposition. Instead, they must appropriate the necessary enzymatic machinery from L1, a member of the LINE (long interspersed element) retroposon family (Jurka 1997; Kajikawa and Okada 2002). As a result of this obligatory relationship with their genomic host and other transposable elements, the *Alu* family has been characterized as a “parasite’s parasite” (Schmid 2003). Despite the family’s various designations as “junk,” “parasites,” and “selfish DNA,” researchers have been reluctant to dismiss them as entirely self-serving genomic entities. A number of investigators have suggested a potential role for *Alu* elements within their host genomes, and recent implications of *Alu* element involvement in alternative splicing, segmental duplications, and DNA repair serve to further fuel these arguments (Morrish et al. 2002; Bailey et al. 2003; Lev-Maor et al. 2003; Salem et al. 2003a). Whether these observations constitute adaptations, exaptations (i.e., they have been commandeered for their current roles, despite not having been evolved for

them; Brosius 1999), or are simply coincidental by-products of their presence in the genome remains a subject of debate. To address these and other questions will require a better understanding of the manner in which *Alu* elements have propagated and adapted themselves within nonhuman primate lineages. As the fate of the *Alu* retroposon is necessarily linked to that of its genomic host, major events in primate evolutionary history will likely have left their mark within the *Alu* “fossil record” that is present in the genomes of all living primates.

Given the relatively recent divergence time (5 to 6 Mya) of the human and chimpanzee lineages (Wildman et al. 2003), it would be reasonable to expect *Alu* transpositional activity and the underlying molecular biology associated with retrotransposition in the chimpanzee might closely parallel that of humans. However, initial examination of ~10.6 Mb of sequence from multiple primate genomes by Liu et al. (2003) revealed a significant deficit in chimpanzee *Alu* insertions compared with humans and baboons. Their results suggest that substantial variation in transposition and/or fixation rates may exist among primate lineages. Whether these differences are attributable to underlying differences in biology, stochastic fluctuations in *Alu* proliferation, and/or broader population-genetic processes remains to be determined.

Here we present the first chromosomal-level comparison of *Alu* retroposition dynamics and associated polymorphism between chimpanzees and humans. We have surveyed common chimpanzee chromosome 22, and its human homolog, chromosome 21, for lineage-specific *Alu* sequences and determined the insertion polymorphism associated with each of these insertions. We also examined the nucleotide composition of the observed inserts to better understand evolutionarily recent *Alu* activity. Finally, we propose a population-based model to account for fluctuations in *Alu* activity within and between primate lineages. In contrast to prior studies of *Alu* diversity, which have largely relied upon inferred “young” *Alu* sequence characteristics to identify loci for investigation, the present comparative approach allows for a more unfiltered appraisal of *Alu* retroposition activity since we last parted ways with our chimpanzee relatives.

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RESULTS

Alu Insertion Levels

For the purpose of our comparison, all available sequence from human chromosome 21 and chimpanzee chromosome 22 was first aligned by using a local installation of BLAT (Kent 2002), resulting in ~32 Mb of aligned sequence that was subsequently screened for evidence of lineage specific *Alu* insertions (see Methods). To reduce the likelihood of misidentifying deletion events in one lineage as insertions in the other, the identification of *Alu* insertions was restricted to loci exhibiting distinct, individually inserted *Alu* elements (see Methods). As a consequence, several questionable insertion/deletions from both the human and chimpanzee were excluded as probable lineage-specific deletion events. Of the remaining putative insertions, the possibility of deletion events masquerading as *Alu* insertion events was further excluded by using the gorilla as an outgroup to determine the ancestral state of the locus. In all, 46 lineage-specific *Alu* insertions were identified in chimpanzee chromosome 22, whereas 101 lineage-specific elements were identified in human chromosome 21, demonstrating a 2.2 \times increase in the number of detectable human insertions (Table 1). These results are in excellent agreement with those of Liu et al. (2003), who found 11 chimpanzee and 23 human insertions (2.1 \times) in their ~10.6-Mb human–chimp comparison; as their sequence data was derived from multiple genomic locations, this correspondence suggests that our data are reflective of the genome as a whole and not endemic to the particular chromosomes surveyed.

Although the cross-species comparison allowed us to classify loci as putatively specific to either the human or chimpanzee lineage, there remained the possibility that (1) some of the insertions were shared polymorphisms in which only one lineage's sequenced individual possessed the insertion, and (2) there were "fixed present" insertions in one species that remained polymorphic in the other. Extensive surveys of hundreds of human *AluYa5*, *AluYb8*, and *AluYc1* insertions in which representative common chimpanzee and bonobo (*Pan paniscus*) samples were analyzed in nonhuman primate controls have demonstrated that the sharing of *Alu* polymorphism between species for these young *Alu* subfamilies would be negligible (Carroll et al. 2001; Roy-Engel et al. 2001, 2002a). In addition, theoretical estimates of the rate of decay of shared polymorphism (Clark 1997), as well as empirical nucleotide data from human, chimpanzee, and gorilla sequences (Hacia et al. 1999), indicate that the number of

shared polymorphisms expected given the number of loci involved in our study would be at most one, and therefore, this effect would not appreciably alter our results. However, to address the possibility that some unknown property of *Alu* insertions might cause them to deviate substantially from these expectations, we evaluated all non-*Ya5/Yb8/Yc1* human insertions (most likely to be shared) and 25 chimpanzee-specific insertions in population panels (80 humans and 12 common chimpanzees) from the opposite species and found no instances of shared *Alu* polymorphism. In addition, these results also give no indication that an appreciable number of elements fixed in human populations remain polymorphic in the chimpanzee. This is further evidenced by the fact that surveys of human *Alu* elements found that shared insertion in chimpanzee was extremely rare (Carroll et al. 2001; Roy-Engel et al. 2001). Were there a significant number of fixed human elements remaining polymorphic in the chimpanzee, insertion status of the chimpanzee reference samples in these large surveys would have occurred with higher frequency.

To aid in distinguishing whether the observed *Alu* insertion disparity represents a decrease in the chimpanzee *Alu* retroposition rate or an increase in the human retroposition rate within a local phylogenetic context (human, chimpanzee, gorilla), we examined a 1.5-Mb segment of homologous 7q31 sequence available in all three species for *Alu* insertions specific to a given species. The results of this comparison indicate a gorilla *Alu* transposition/fixation level that is near that of *P. troglodytes*, with four *Alu* inserts in *Gorilla gorilla* compared with three in *P. troglodytes* and eight in humans. The small amount of gorilla sequence available for comparison resulted in too few *Alu* insertions to yield significant results ($P \sim 0.25$). However, the trend exhibited between humans and chimpanzees in this region (8:3) echoes that of our larger chromosome 21 survey, leading us to believe that the gorilla insertion numbers are also representative of its genome. Although more extensive sequence comparisons using gorillas and orangutans will be required before definitive conclusions can be drawn, our data favor a human-specific increase in *Alu* retroposition activity within the local phylogenetic context. Examination of the subfamily composition of human and chimpanzee elements (see below) lends further support to this interpretation.

Distribution of Insertions

Qualitatively, the evolutionarily recent *Alu* insertions were found distributed relatively evenly throughout the chimpanzee and human chromosomes, with expected lower densities near telomeric and centromeric regions primarily due to unsequenced heterochromatic regions. *Alu* density has previously been established to be strongly correlated with both GC-content and gene density (Schmid 1996; Lander et al. 2001). Chromosome 21 exhibits a 42% GC content, compared with 48% on chromosome 22 and 49% on chromosome 19, which contains both the highest GC content and highest gene density (Lander et al. 2001). Correspondingly, overall *Alu* density is highest on chromosome 19, followed by chromosome 22 (Chen et al. 2002). Chromosome 21 is relatively gene poor, with an average density of approximately seven genes per megabase compared with the 11.1 per megabase genomic average (Hattori et al. 2000). However, recent genomic surveys of young *AluYb8* and *AluYa5* subfamilies demonstrate no significant deficit of young subfamily insertions on chromosome 21 (Carter et al. 2004; data not shown). This may partially be attributable to the fact that the *Alu* GC and genic distribution bias appears to be more pronounced for evolutionarily older insertions (Lander et al. 2001; Jurka et al. 2004). As a result of the relatively small numbers of recently inserted *Alu* elements in our survey, larger genome-wide comparisons of young *Alu* inserts

Table 1. Lineage-specific *Alu* Insertions

	Human	Human/ Chimp ratio	Chimpanzee
Observed inserted total	101	2.20	46
PCR tested	78	—	43
Fixed present	63	—	26
Observed polymorphic	16	—	18
Observed polymorphic fraction	0.21	0.50	0.41
Adjusted polymorphic ^a	31–33	—	35–37
Adjusted polymorphic fraction	0.33–0.34	0.56–0.60	0.57–0.59
Adjusted inserted total	116–118	1.84–1.93	61–63

^aAdjusted polymorphic fraction was calculated based upon simulation of the frequency of polymorphic *Alu* elements observed in a given genome by sampling alleles from a uniform frequency distribution (see Methods). Ranges indicated were generated based on 95% confidence intervals derived by simulation.

will be necessary for adequately detecting any changes in distribution between species. However, we do note here that, in agreement with previous studies of total *Alu* content (Lander et al. 2001; Chen et al. 2002), human- and chimpanzee-specific insertions on chromosomes 21/22 had a tendency to insert in GC-rich genic regions, with >20% of the insertions in our survey being located within the introns of known genes, and an even higher frequency (>50%) when predicted genes are considered. Based on estimates of known and predicted gene number and average chromosome 21 gene sizes, we estimate that these gene categories span ~20% and 8% of the sequenced region of the chromosome, respectively. In addition, *DSCAM*, an alternatively spliced gene involved in neural development (Yamakawa et al. 1998), demonstrated a total of five human-specific insertions. This may not in itself be remarkable, as *DSCAM* spans 840 kb, making it a rather large target for insertion. However, all five inserts are in the antisense orientation relative to gene transcription, a feature that has been linked to alternative splicing (Lev-Maor et al. 2003). Given intronic *Alu* orientation frequencies of 0.47 (sense) and 0.53 (antisense) calculated from a survey of 179 *AluYb8* and *AluYa5* gene insertions, this configuration of antisense *Alu* elements deviates significantly from expectation ($P < 0.05$).

Anomalous Loci

In addition to the lineage-specific insertions found in our study, one element, designated *CS12*, was determined to be exclusive to gorilla and chimpanzee genomes and not present in human, implying a relationship contrary to the orthodox phylogeny of ([HC],[G]). Such discrepancies have been reported elsewhere (Salem et al. 2003b) and most likely represent lineage sorting of an ancestral polymorphism present in the common ancestor of humans, chimpanzee, and gorilla. The existence of such sorting events serves to highlight the relatively short period of time, evolutionarily speaking, during which these three lineages emerged. For the purposes of this study, however, putative lineage sorting events were excluded from further analysis, as they could not be classified as lineage specific for either humans or chimpanzee.

Another locus, *HS6*, exhibited phylogenetic inconsistencies that were less readily explained. PCR analysis of the locus showed insertions in orangutan, gorilla, and human to the exclusion of chimpanzee. The maintenance of a polymorphism over this period of time—approximately 6 Myr from the branching of orangutan to the divergence of humans and chimpanzees—would be unlikely, prompting us to consider the possibility of an *Alu* excision at the chimpanzee locus. For further examination, we sequenced the orthologous loci in *G. gorilla*, *P. paniscus*, and *Pongo pygmaeus* (Fig. 1). The *HS6* insertions in human, gorilla, and orangutan contained direct repeats that were identical in both sequence and length, strongly indicating identical by descent insertions. Unexpectedly, the chimpanzee locus was a perfect pre-integration site, consisting of only one copy of the direct repeat (Fig. 1). In the only previously reported instance in which an *Alu* element appeared to be excised from a genome, remnants of the *Alu* insertion remained in the sequence (Edwards and Gibbs 1992). As the precise excision of an *Alu* insertion appeared to be a remote possibility, we began to explore other potential explanations

for our observations. One such possibility is that a segmental duplication in a great ape common ancestor produced a pair of paralogous loci, only one of which received an *Alu* insertion. This paralogous locus, which would itself be polymorphic and subject to lineage sorting, could have resolved itself into the observed phylogenetic situation. Our inability to detect evidence through PCR for more than one uninserted locus among the tested species indicates that this long-term maintenance of a duplication polymorphism is no more probable than that of a long-lived *Alu* insertion polymorphism. However, when considered together, these alternative pathways to the same observed state makes the observed insertion states somewhat more likely. On further examination of the *HS6* locus, we discovered two immune-related genes, *CXADR* and *CHODL*, within 1 Mb of *HS6*. It is conceivable that balancing selection acting at these nearby loci served to maintain the *HS6* polymorphism, ultimately resulting in the unusual phylogenetic distribution of this *Alu* insertion. Additional investigation of the genes at this locus will be required to verify this hypothesis.

Subfamily Composition

Human *Alu* elements inserted on chromosome 21 were classified according to subfamily structure as previously reported (Fig. 2; Batzer et al. 1996). All human-specific insertions were members of the *AluY* subfamily or one of its derivatives. Of these, the *AluYa5* and *AluYb8* subfamily constituted the largest percentage, comprising 25% and 38% of the loci, respectively. For those elements categorized as members of *AluY*, their sequences were screened against the human genome database to determine if they belonged to previously uncharacterized subfamilies. Several of these elements appeared to be members of small (10- to 100-member) *Alu* subfamilies that had previously remained unidentified. Comparative analysis of additional chromosomes will likely reveal additional small subfamily structure that remained undetected by previous molecular and computational methods.

At present, very little is known about the subfamily structure of *Alu* elements within the chimpanzee genome. Multiple alignments of all observed *P. troglodytes* chromosome 22 lineage-specific inserts uncovered two candidates for active subfamilies. The first group, consisting of 27 elements, has a consensus sequence identical to that of *AluYc1* in humans. Whether this subfamily is identical by descent or state to its human counterpart is unclear, as *AluYc1* differs from the canonical *AluY* sequence by a single G→A nucleotide substitution. Human *AluYc1* insertions exhibit a relatively young (1 to 3 Myr) average age (Garber et al. 2004). Our estimates of the chimpanzee *AluYc1* family place it between 1.2 and 2.6 Myr old. Although this is suggestive of an independent parallel mutation, the human *AluYc1* elements may have remained relatively dormant in the human genome until some time subsequent to *Pan-Homo* split. To better localize the chimpanzee *AluYc1* activity in time, we examined the insertion status of 18 *P. troglodytes*-specific *AluYc1*-like elements in a representative bonobo (*P. paniscus*), estimated to have diverged from *Pan troglodytes* ~1.8 Mya (Yu et al. 2003). Eleven elements were present in the *P. troglodytes* population but absent from our *P. paniscus* individual and seven elements were present in both species, indicating that the chimpanzee *AluYc1*-like subfamily had

Human	TGCCAATAGAGATAGAAAAGAAATGGATGGAAACAGACATGCATTTAAGAAGGTTCA<ALU>AAGAAGGTTTCAGCAGAGTGTGGTGAAGACTGGGC
Gorilla	TGCCAATAGAGATAGAAAAGAAATGGATGGAAATAGACATGCATTTAAGAAGGTTCA<ALU>AAGAAGGTTTCAGCAGAGTGTGGTGAAGACTGGGC
Orangutan	TGCCAATAGAGATAGAAAAGAAATGGATGGAAATAGACATGCATTTAAGAAGGTTCA<ALU>AAGAAGGTTTCAGCAGAGTGTGGTGAAGACTGGGC
Chimpanzee	TGCCAATAGAGATAGAAAAGAAATGGATGGAAATAGACATGCATTTAAGAAGGTTCA-----GCAGAGTGTGGTGAAGACTGGGC
Owl Monkey	TGCCAATAGAGAGAAAAGAAATGGATGGAAATAGAGATGGATTTAACAAAGGTTAA-----GCAGAGTGTGGTGAAGACTGGGC

Figure 1 Reconstructed *Alu HS6* insertion sites in human and nonhuman primates. Shaded area indicates direct repeat region. Chimpanzee site demonstrates no evidence for an extracted insertion.

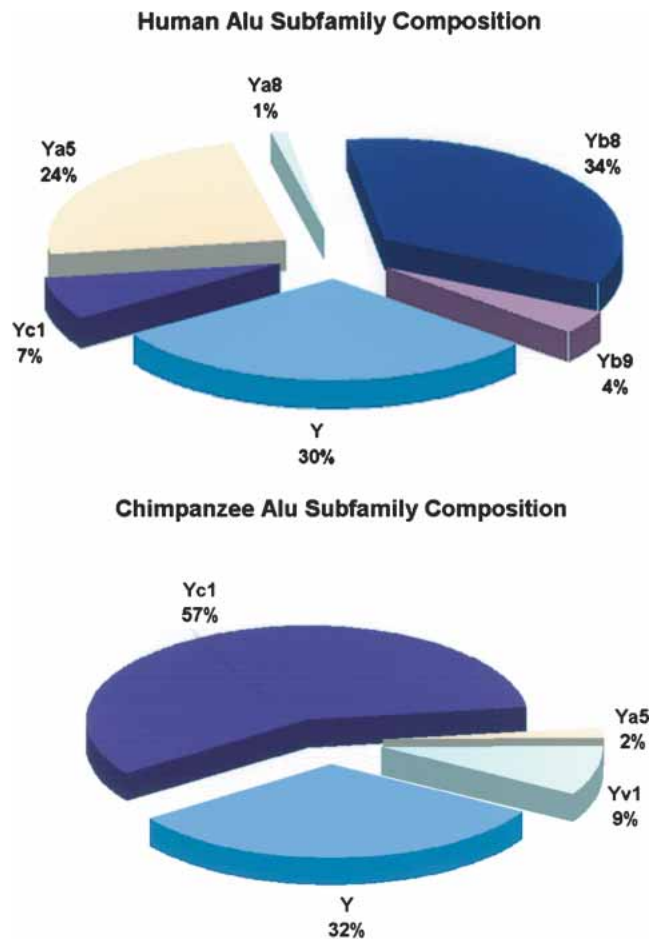


Figure 2 Subfamily composition of lineage-specific *Alu* insertions in humans and common chimpanzee.

began amplifying prior to the *P. troglodytes*–*P. paniscus* divergence. This places a lower bound on the chimpanzee *AluYc1* family age of ~2 Mya, not ruling out the possibility that these subfamilies are of common descent.

The second group of four elements (designated YV1) were distinguished by five diagnostic mutations from the *AluY* consensus. Screening of the human genome database revealed several matches within humans, indicating that this subfamily was not restricted to the chimpanzee lineage and has been amplifying, albeit slowly, since before the human–chimpanzee split. Here, there is little possibility of a parallel forward mutation event, as YV1 is distinguished by five mutations.

Alu Insertion Polymorphism

To assess the diversity of individual lineage-specific *Alu* insertions on human chromosome 21, 78 *Alu* elements that were amenable to PCR were amplified on a panel of 80 human individuals from four geographically diverse populations (African American, Asian, German Caucasian, and South American). Among the four represented populations, 16 of 78 (20.51%) elements demonstrated polymorphism in our panel. Allele frequencies of all polymorphisms, as well as primers used in this study, are available at our Web site (<http://batzerlab.lsu.edu>). Forty-three chimpanzee-specific insertions were evaluated on our chimpanzee panel of 12 unrelated *P. troglodytes*. Because of the small size of our *P. troglodytes* sample, we assessed its adequacy in evaluating loci for polymorphism (see *Methods*). Assuming a uniform distribution of

Alu allele frequencies, we estimated that our 12 individual (24-chromosome) sample would capture ~88% to 93% of the polymorphism present at the examined loci. In all, 18 of 43 (41.86%) elements exhibited polymorphism in our chimpanzee panel. The 2.0 ratio of human-to-chimpanzee polymorphism fraction is somewhat higher than the 1.5 ratio of a recent nucleotide heterozygosity study (Yu et al. 2003). If adjustments for unequal polymorphism levels are made, however, the values become closer (see Discussion).

DISCUSSION

Alu Transposition Levels and Subfamily Structure

Our results suggest that an elevation in human *Alu* retroposition activity, largely mediated by two human *Alu* subfamilies (*AluYa5* and *AluYb8*), occurred some time subsequent to the divergence of the human and chimpanzee lineages. The most current estimates for the ages of these subfamilies place them amplifying between 2.5 and 3.5 Mya (Carroll et al. 2001). A survey of a 4-Mb X-Y translocation event (Schwartz et al. 1998), which has previously been dated to ~3.5 to 4 Mya (Sargent et al. 2001), suggests no appreciable retroposition activity of *AluYa5* and *AluYb8* families prior to that time period. This is indicated by the absence of *AluYb8* and *AluYa5* elements duplicated at the time of the translocation event. These observations place the onset of significant *AluYa5* and *AluYb8* mobilization subsequent to the divergence of the human and chimpanzee lineages, indicating that a contraction in population size during or immediately following speciation does not account for the chimpanzee–human *Alu* disparity.

The question arises as to whether or not the *AluYa5* and *AluYb8* subfamily expansions were simultaneous or distinct events. Although current age estimates date them to roughly the same period, polymorphism levels of *AluYb8* (20%) and *AluYa5* (25%) suggest a somewhat younger overall age for the *AluYa5* subfamily, as more of its members remain unfixed in the population (Carroll et al. 2001). However, the polymorphism fraction may only serve to indicate that the bulk of *AluYa5* insertions are distributed closer to the present than that of *AluYb8*, and is not necessarily reflective of the initial appearance date of the subfamily.

An additional factor with the potential to influence the estimated ratio of *Alu* insertion numbers in species is the existence of unequal diversity levels within humans and chimpanzees for *Alu* insertions. By using the observed *Alu* diversity in chimpanzee and human, we estimated the extent to which this effect may have skewed our results (see *Methods*). Our estimates suggest that in 95% of cases, 42% to 58% of the polymorphic *Alu* insertion loci would be missed by sequencing a single representative human genome or chimpanzee genome. When we adjust insertion numbers within both lineages for these missed *Alu* loci, our estimate of the human/chimpanzee insertion ratio is 1.84 to 1.93 (Table 1).

The paucity of evolutionarily recent *Alu* insertions observed on the *P. troglodytes* chromosome 22 restricts our ability to completely capture the chimpanzee *Alu* substructure. However, assuming that young *Alu* subfamily dispersal in humans is distributed proportional to chromosome size, the chance of missing a major young *Alu* family (>300 elements) in our chimpanzee chromosome 22 survey would be remote (<5%). Our data indicate that the major lineages that constitute the bulk of recent human activity, *AluYa5* and *AluYb8*, are only present at negligible levels in *P. troglodytes*. A solitary *AluYa5* element was found on chimpanzee chromosome 22, and although GenBank database queries indicate that a small number of authentic *AluYb8* chimpanzee insertions are present in the *P. troglodytes* genome, quantitative PCR results suggest that their copy number is negli-

gible compared with that of humans (Walker et al. 2003). The *AluYc1*-type subfamily appears to dominate the *Pan* lineage (Fig. 2), but we can not conclusively say if it is identical by descent to the subfamily that is found in humans. If it is indeed the same family, it would be curious that, given their estimated ages (1 to 3 Myr), the source sequence would have remained relatively dormant in both lineages only to become active, independently, at a later time. Alternatively, the independent parallel success of these source mutations may suggest a selective advantage for the G→A consensus substitution, or it could simply be a base position where such change is tolerated in the *Alu* source or “master” genes.

Although several of the *Alu* polymorphic loci in chimpanzee contained sequence characteristics that were present in only a single copy on chromosome 22, these insertions will serve as excellent starting points to search for further chimpanzee *Alu* family substructure, as they likely represent chromosome 22 representatives of smaller, active *Alu* subfamilies analogous to those recovered in the human sequence.

The presence of *AluYb8* and *AluYa5* members in small copy numbers within the chimpanzee and gorilla genomes (Leeflang et al. 1993) demonstrates that the sequence evolution of successful subfamilies begins well before their peak activity. These subfamilies appear to undergo a lengthy period during which low baseline mobilization occurs. A chance insertion within a suitable genomic context, however, could initiate a burst of activity from the locus within a given host lineage. In conjunction with L1 enzyme availability and population genetic factors (see below), such fortuitous insertions would initiate the expansion phase of the *Alu* subfamily.

Alu Insertion Polymorphism

Our *Alu* insertion diversity data demonstrate two times higher *Alu* polymorphism in chimpanzee compared with humans. If we adjust the estimates of polymorphic *Alu* loci by accounting for the insertion polymorphisms that were predicted to be missed in chimpanzee and human sequences (see Methods), our ratio of chimpanzee-to-human *Alu* polymorphism decreases to 1.67 to 1.78. A number of previous studies, making use of multiple genetic systems, have attempted to assess the level of genetic diversity of chimpanzees relative to that of humans. Mitochondrial and nuclear genome surveys have generated seemingly conflicting depictions of chimpanzee diversity. Mitochondrial diversity has been estimated to be as much as 10 times higher among chimpanzees than humans (Rogers and Jorde 1995). Nuclear nucleotide diversity estimates, in contrast, have yielded chimpanzee heterozygosities that are lower than human levels for protein-coding loci (King and Wilson 1975; Satta 2001). Surveys of additional coding and noncoding loci have reported nucleotide heterozygosity estimates three to four times higher in chimpanzee than humans (Deinard and Kidd 1999; Kaessmann et al. 1999). Our range of 1.67 to 1.78 times higher common chimpanzee diversity best corresponds to that of Yu et al. (2003), who estimated nucleotide diversity in common chimpanzee at 1.5 times higher than that of human, with a lower value for bonobo.

The previously reported disparity of heterozygosity values exhibited by different genetic systems (mitochondrial, microsatellite, nuclear SNPs) can potentially be explained by a population bottleneck in humans that had a more severe effect on mitochondrial diversity due to its smaller (1/4 autosomal) effective population size (Yu et al. 2003). The existence of a bottleneck in human evolutionary history has been suggested by many studies (Harpending et al. 1998; Chen and Li 2001; Lonjou et al. 2003). Although our chromosome 21/22 data are consistent with this

scenario, we can not exclude other possibilities, such as selective sweeps reducing mitochondrial diversity.

If the correspondence between *Alu* insertion polymorphism ratios and the nucleotide diversity ratios between humans and chimpanzees is not simply coincidental, it would appear that the effective population size is the dominant influence determining the fraction of *Alu* insertion polymorphisms in these genomes. That is, despite markedly different subfamily composition and retroposition histories between the two lineages, *Alu* insertion polymorphism generally parallels nucleotide polymorphism in behavior. This is a somewhat surprising result, given that fluctuations in *Alu* activity over time could result in one lineage having an excess or deficit of younger polymorphic *Alu* insertions relative to the other lineage, largely independent of effective population size. However, this situation could conceivably be explained if the more dramatic changes in *Alu* insertion rates occurred in more distant evolutionary history and have had little influence on current polymorphism levels. In this scenario, relatively uniform insertion rates within individual lineages over recent evolution history have resulted in effective population size being the dominant determinant of polymorphism levels. Further resolution of the insertion dates of human and chimpanzee *Alu* elements will be necessary to clarify this issue.

A Population-Based Model for Fluctuations in *Alu* Mobilization

Under standard neutral or “nearly neutral” population genetics theory, three scenarios could conceivably account for the relative increase in fixed *Alu* insertions within humans compared with chimpanzees. First, a smaller long-term effective population size in the human lineage could have resulted in the fixation of otherwise slightly deleterious *Alu* insertions at a higher rate in humans. Under this scenario, the roughly twofold increase in observed human insertions would need to be accounted for by deleterious elements. Although this possibility can not presently be excluded, the fixation of hundreds of deleterious *Alu* insertion loci would no doubt represent a considerable burden to a population. An explanation that avoided such a genetic calamity would appear to be more parsimonious. A second scenario would be that the existing *Alu* polymorphism that was present at the time of human–chimpanzee speciation was funneled through a *Homo* lineage bottleneck, resulting in an increased fixation of *Alu* elements within humans. In this situation, the differences in *Alu* insertion number would be attributable to many more of these ancestral polymorphisms fixing in the human lineage than the chimpanzee. This scenario is unlikely as well, however, as the sequence structure of *Alu* insertions of humans, comprised largely of two young subfamilies, differs considerably from that of chimpanzee (Fig. 2). This suggests that they were not derived from a common pool of *Alu* insertions that were polymorphic at the time of speciation. In addition, the major retroposition activity within the *AluYa5* and *AluYb8* subfamilies can be reasonably dated by independent lines of evidence to a period subsequent to the human–chimpanzee speciation (see Results). The third possibility, which we favor, is an increase in the *Alu* retroposition rate itself. This would be analogous to an increased nucleotide mutation rate within a given lineage. However, in the case of retroposition, there is an added layer of complexity in the interaction among insertion rates, fixation rates, and population size that must be addressed.

The population dynamics of *Alu* elements within their hosts can account for much of the insertion variance observed within and between primate lineages. The basic components of our model are as follows: (1) variation in source *Alu*-producing loci exist in the population, (2) stochastic sampling of these source

variants either at speciation or during bottleneck events alters the population-level *Alu* transposition activity (*insertions per birth*), and (3) although the previous two conditions are sufficient to produce variation within and between lineages, smaller effective population sizes will both increase the sampling variance of *Alu* sources and reduce a given population's ability to select against deleterious source loci. This may result in a substantially increased population-level *Alu* activity (*insertions per birth*) brought about by environmental insults, speciation events, etc.

Aside from their observed GC-rich distribution bias, there has been no evidence indicating that *Alu* insertions behave appreciably different than nucleotide polymorphisms as genetic markers once inserted in the genome (Perna et al. 1992; Stoneking et al. 1997; Watkins et al. 2001, 2003; Bamshad et al. 2003). As such, the behavior of *Alu* elements should be consistent with other neutral or "nearly neutral" characters. The probability of a given *Alu* insertion reaching fixation in a population is therefore contingent upon its initial frequency in the population, $1/2N$, where N is the population size (Kimura 1983). In the context of *Alu* retrotransposition, however, not all of the further assumptions of neutral theory hold. Although the number of novel nucleotide mutations arising each generation in a population is dictated by the size of the population (i.e., total number of mutable sites) and the frequency of mutations arising each generation, the number of novel *Alu* insertions has a more complex relationship with population size. As the majority of new *Alu* copies are known to arise from a select number of "master" or source loci, these loci themselves will be subject to allelic variation in both transpositional competency and/or insertion status. Evidence for such allelic variation in retrotransposition capability has been observed in members of the L1 subfamily (Lutz et al. 2003) and within *Alu* may be attributable to variation at PolIII promoter efficiency, variation in target-primed reverse transcription, oligo dA tail instability (Roy-Engel et al. 2002b), and insertion status polymorphism for the source locus itself. Additional evidence from L1 sequence transduction events demonstrate that retroposon source sequences can produce "offspring" that proceed to fixation, whereas the parent sequences are ultimately lost (Boissinot et al. 2001). As a consequence of this source allele variation, a reduction in overall human population size may occur, whereas the number of novel *Alu* insertions *per individual birth* actually *increases* due to the stochastic effects of sampling the active source variants (Fig. 3). In effect, unlike nucleotide substitution rates, the equivalent of the *Alu* substitution rate will itself fluctuate along with population size. The intensity of these

fluctuations will increase as the population size becomes smaller. Simultaneously, a reduced effective population size is less capable of selecting against detrimental source variants as the population size grows smaller. This effect is exacerbated because the *Alu* source is effectively "screened" by its indirect relationship to the deleterious insertion loci it generates. As a consequence, transposition may run rampant when the population size is no longer large enough to effectively select against *Alu* "hyperactivity." Within a window of selective pressure, deleterious insertions would still be effectively removed from the genome, but the source or sources generating the deleterious insertions become(s) essentially neutral (i.e., having a selective coefficient $\ll 1/2N$).

An attractive feature of this explanation is that it does not necessitate the presence of a large number of fixed deleterious loci to account for differential lineage *Alu* insertion counts. Furthermore, it does not require the invocation of any novel biology to account for changes in the relative number of insertions per generation. One prediction of the model is that the onset of increased *Alu* transposition activity would tend to be coincident with population size decreases, and as a consequence, *Alu* transposition rates may change *rapidly* within and between lineages. By developing better analytical tools to estimate the ages of individual *Alu* insertions, it may be possible to localize transposition events in time and estimate the rate at which *Alu* transposition activity fluctuates. A further prediction is that isolated inbred populations would be at an increased risk for *Alu* "hyperactivity," as they would experience a decreased capacity to select against active source loci. Genomic display, ATLAS, and similar methodologies that have the potential to exhaustively examine retroposon insertions within individual genomes will allow testing in extant populations for evidence of this effect.

METHODS

DNA Samples

Cell lines used to isolate DNA samples were as follows: A chimpanzee diversity panel of 12 *P. troglodytes* of unknown geographic origin was obtained from the SouthWest foundation for Biomedical Research, gorilla (*G. gorilla*), lowland gorilla *Coriell AG05253A*, owl monkey (*A. trivirgatus*), ATCC CRL1556, and pygmy chimpanzee (*P. paniscus*), *Coriell AG05253A*. Human DNA from South American populations was purchased as part of the Human Variation Panel available from the Coriell Institute for Medical Research. DNA samples from the European, African

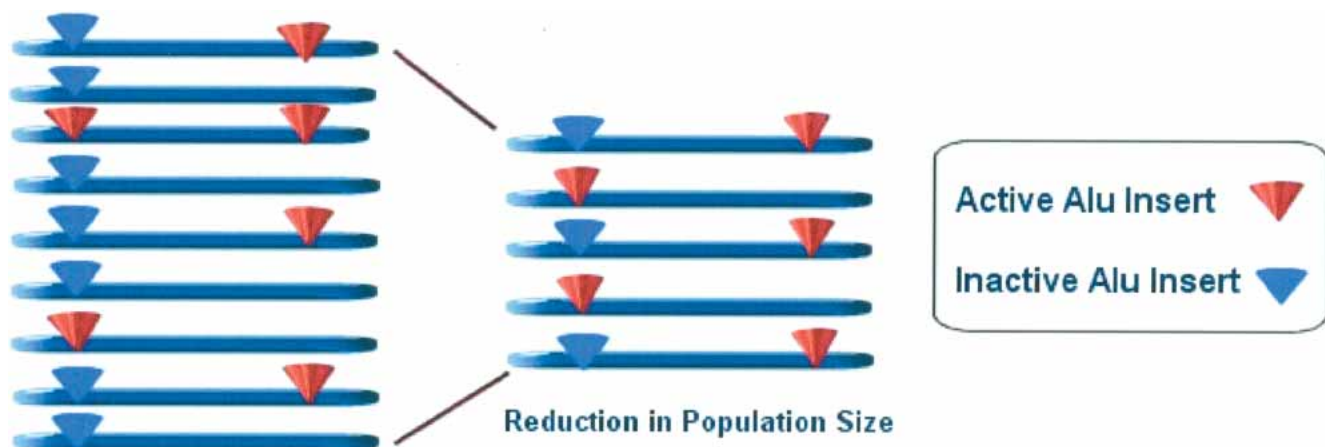


Figure 3 Variation in the insertion status and retroposition capability of *Alu* elements at two loci. Reduction in population size leads to variation in the number of active elements.

American, and Asian population groups were isolated from peripheral blood lymphocytes available from previous studies.

Human–Chimpanzee Comparison

DNA sequences for chromosome 22 (~43 Mb, including overlapping sequence) were obtained from The Chimpanzee Chromosome 22 Sequencing Consortium (<http://chimp22pub.gsc.riken.go.jp>). Sequence for human chromosome 21 was obtained from the University of California, Santa Cruz (UCSC) June 2003 assembly data. Human chromosome 21 and chimpanzee chromosome 22 alignments were generated by using a local installation of BLAT (Blast-like Alignment Search Tool; Kent 2002), resulting in ~32 Mb of aligned sequence out of an estimated 33.8 Mb total chromosome 21 sequence (Hattori et al. 2000). BLAT results were subsequently screened by using a Perl script for all insertions/deletions of sizes 100 to 1000 bp. These sequences, along with 200 bp of flanking sequence, were extracted for further examination. In addition, a separate manual BLAT screen of the human genome database (using UCSC Web interface) using the chimpanzee chromosome 22 sequence was conducted to assess the accuracy of our script-generated results. Indel sequences were screened by using a local installation of RepeatMasker (<http://repeatmasker.genome.washington.edu/cgi-bin/RepeatMasker>) to determine their repetitive element content. Subsequent sequence alignments were done with MEGALIGN program, part of the DNASTAR package. Redundant repeat insertions resulting from overlapping BLAT fragments were excluded by verifying unique flanking sequence. An additional ~1.5 Mb of human, chimpanzee, and gorilla homologous sequence from chromosome 7 was obtained from the National Institutes of Health (NIH) Intramural Sequencing Center (www.nisc.nih.gov). Sequences were aligned with BLAT and/or MEGALIGN to identify species-specific indels, and RepeatMasker was used to determine their repetitive element content.

All putative *Alu* insertions were manually verified as authentic by determining if the insertions met established criteria for evolutionarily recent *Alu* insertions. Authentic *Alu* insertions were required to have only 5' truncations, as 3' truncations have not been observed to occur upon insertion. Any "partial" *Alu* indels in which a fragment of the *Alu* is already present at the locus prior to the indel event were excluded, as these are more characteristic of partial deletions of elements. *Alus* that were contained within larger insertion/deletion events were also excluded, as these did not represent authentic *Alu* transposition events. To further resolve ambiguities, all putative insertions were amplified from the gorilla genome to determine the ancestral state of the insertion.

Statistical Methods

Estimating the Number of Detected Polymorphic *Alu* Insertions

Estimations of the number of polymorphic insertions that would be detected in a single sequenced genome were conducted by generating 1000 samples of a genome (set of detectable alleles) from a uniform distribution of *Alu* insertion frequencies. This choice of distribution was based on observations of the allele frequencies of human *Alu* inserts (Carroll et al. 2001; Roy-Engel et al. 2001), and reasoning that the higher long-term effective population size of chimpanzee would result in an even more uniform (flat) distribution of *Alu* insertion frequencies due to the lack of recent bottlenecks and/or expansions (Harpending et al. 1998). In our simulation, the probability of discovering a given allele was proportional to its frequency in the population. The mean fraction of detections was 0.5, with a variance inversely proportional to the number of actual polymorphic loci. Our 1000 replicates using 100 loci yielded a standard deviation of 4%, which was used to calculate a 95% confidence interval for unsampled polymorphisms of 42% to 58%.

Detection of Polymorphism

The probability of detecting an *Alu* insertion polymorphism at a given locus is contingent upon its minor allele frequency

$1 - [(1 - q)^N]$, where q is the minor allele frequency and N is the number of sampled chromosomes. Consequently, the number of detectable *Alu* variants will be subject to the distribution of allele frequencies in the population. If we assume this is roughly uniform, then summing over i minor allele frequencies $\sum [1 - [(1 - q_i)^N]]$ yields the fraction of polymorphic sequences detected. By simulating 1000 trial detections of uniformly distributed minor alleles, we estimate that 95% of the time our human panel of 80 individuals (160 chromosomes) would detect 97.3% to 99.7%, and our chimpanzee panel of 12 individuals (24 chromosomes) would detect 89% to 93% of the polymorphism at PCR evaluated loci. Within the observed polymorphism, there should be a skew toward higher frequency alleles, as these are more likely to appear in a given sequenced genome. Because we restricted our analysis to polymorphic/fixed status, this bias should not affect our conclusions.

PCR Analysis

Oligonucleotide primers for the PCR amplification of each *Alu* element were designed using the 700- to 1200-bp flanking unique sequence fragments and Primer3 software (Whitehead Institute for Biomedical Research; http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The sequences of the oligonucleotide primers, annealing temperatures, PCR product sizes and chromosomal locations for all *Alu* elements in this study can be found on our Web site (<http://batzerlab.lsu.edu>). PCR amplification was performed in 25 μ L reactions using 10 to 50 ng target DNA, 200 nM each oligonucleotide primer, 200 μ M dNTPs in 50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-HCl (pH 8.4), and 1 U *Taq* DNA polymerase. Each sample was subjected to an initial denaturation step of 150 sec at 94°C, followed by 32 cycles of PCR at 1 min of denaturation at 94°C, 1 min at the annealing temperature, 1 min of extension at 72°C, followed by a final extension step of 10 min at 72°C. The resulting products were then evaluated for polymorphism on Ethidium Bromide (EtBr)-stained 2% agarose gels and visualized with UV lighting.

DNA Sequencing

DNA sequencing was performed on gel-purified PCR products that had been cloned by using the TOPO TA cloning vector (Invitrogen) using chain termination sequencing on an Applied Biosystems 3100 automated DNA sequencer. All sequences generated in this study are available in the GenBank database (accession nos. AY569161–AY569170).

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- http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi; Primer3 software, Whitehead Institute for Biomedical Research.

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Differential *Alu* Mobilization and Polymorphism Among the Human and Chimpanzee Lineages

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