



An alternative pathway for *Alu* retrotransposition suggests a role in DNA double-strand break repair

Deepa Srikanta, Shurjo K. Sen, Charles T. Huang, Erin M. Conlin, Ryan M. Rhodes, Mark A. Batzer*

Department of Biological Sciences, Biological Computation and Visualization Center, Louisiana State University, 202 Life Sciences Building, Baton Rouge, LA 70803, USA

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ABSTRACT

The *Alu* family is a highly successful group of non-LTR retrotransposons ubiquitously found in primate genomes. Similar to the L1 retrotransposon family, *Alu* elements integrate primarily through an endonuclease-dependent mechanism termed target site-primed reverse transcription (TPRT). Recent studies have suggested that, in addition to TPRT, L1 elements occasionally utilize an alternative endonuclease-independent pathway for genomic integration. To determine whether an analogous mechanism exists for *Alu* elements, we have analyzed three publicly available primate genomes (human, chimpanzee and rhesus macaque) for endonuclease-independent recently integrated or lineage specific *Alu* insertions. We recovered twenty-three examples of such insertions and show that these insertions are recognizably different from classical TPRT-mediated *Alu* element integration. We suggest a role for this process in DNA double-strand break repair and present evidence to suggest its association with intra-chromosomal translocations, in-vitro RNA recombination (IVRR), and synthesis-dependent strand annealing (SDSA).

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Introduction

Alu elements are ubiquitous members of the Short Interspersed Element (SINE) family of mobile DNA elements, with copy numbers reaching ~1.2 million in the human genome and ~1 million in the rhesus macaque genome [1,2]. Full-length *Alu* elements are ~300 bp long, are comprised of two monomers joined by a 32 bp poly-A region and possess a variable-length poly-A tail [1]. *Alu* elements lack any protein-coding capacity and are therefore non-autonomous retrotransposons, that use the enzymatic machinery of another retrotransposon family, the L1 elements, for integration into the host genome [3]. Although the vast majority of genomic *Alu* integrations occur into non-coding sequence and have no phenotypic effect, occasionally new integrants disrupt gene expression and function, and have been implicated in a multitude of human diseases, including cancer, neurofibromatosis and hemophilia [4–6].

The majority of genomic *Alu* integration occurs through a process termed target site-primed reverse transcription (TPRT). During TPRT, the L1 endonuclease (EN) makes an initial single-strand nick at a specific site in the host genome (generally approaching the motif 5'-

T₂A₄-3') and the *Alu* mRNA anneals to the nick site using its 3' poly-A tail. Next, the L1 reverse transcriptase initiates reverse transcription using the *Alu* mRNA as a template. The second strand of DNA is nicked downstream of the initial cleavage site creating staggered breaks, which are later filled in by small (7–20 bp) direct repeats on either side of the element, termed target site duplications (TSDs) [7,8]. In the final two steps, the order of which is not yet clear, the integration of the newly synthesized *Alu* cDNA and synthesis of the second strand occur; the normal completion of TPRT results in creating unique structural hallmarks, i.e. intact TSDs and variable length poly-A tails [9,10]. Previous studies have established that the *Alu* family of retrotransposons acts as a “parasite's parasite” and hijacks L1 machinery during classical TPRT-mediated genomic integration [11].

Mobile DNA capture has been attributed to novel chimeric genes, genetic rearrangements and deletions within and around genes [12–17]. Recently, two analyses have documented an alternative model of mobile DNA capture, an endonuclease-independent L1 insertion mechanism [18,19] at DNA double-strand break repair sites. This pathway, initially observed in DNA repair-deficient rodent cell lines [19], has subsequently been shown to also occur in the human genome [18]. As *Alu* mobilization utilizes L1 machinery in *trans* [7,20–25], the possibility exists that the non-classical endonuclease-independent insertion mechanism seen in the L1 family may also occur with *Alu* elements [26]. To explore this hypothesis, we scanned the three primate genomes that were publicly available at the time of analysis (human, chimpanzee, and rhesus macaque). Through a combination of computational data mining and wet bench techniques, we recovered 23 *Alu* elements that have exploited this alternative pathway of

Abbreviations: NCAI, non-classical *Alu* insertion; CS, chimpanzee-specific; HS, human-specific; RS, rhesus-specific; DSBs, double-strand breaks; TPRT, target-site primed reverse transcription; SDSA, synthesis dependent strand annealing; IVRR, in vitro RNA recombination; PCR, polymerase chain reaction; NHEJ, non-homologous end-joining; EN, endonuclease; RT, reverse transcriptase; SINE, short interspersed element; OWM, Old World monkey; RM, RepeatMasker; TSD, target site duplication.

* Corresponding author. Fax: +1 225 578 7113.

E-mail address: mbatzer@lsu.edu (M.A. Batzer).

integration, which we term non-classical *Alu* insertion (NCAI). In each case, we verified the pre-insertion state of the locus by sequencing the orthologous position in an outgroup primate genome, and confirmed that the loci lack the characteristic hallmarks of TPRT-mediated insertions. We suggest that this mechanism may play a fortuitous role in genomic DSB repair. Overall, our results support the hypothesis that endonuclease-independent mobilization of non-LTR retrotransposons in primate genomes may have implications for the maintenance of genomic integrity.

Results and discussion

Genomic distribution of non-classical *Alu* insertions (NCAI)

Using a combination of computational data mining and wet-bench verification, we have analyzed three primate genomes (human, chimpanzee, and rhesus macaque) for evidence of an alternative, endonuclease-independent mode of *Alu* integration. We excluded all endonuclease-dependent TPRT-mediated insertions through a rigorous manual inspection of putative NCAI loci following a triple alignment of the three genomes and report a total of twenty-three atypical insertions using the hg18, panTro2 and rheMac2 assemblies. Of the Hominin-specific loci we recovered, four were specific to humans, four to chimpanzees, and one locus was shared between humans and chimpanzees; the other 8 loci were shared among all four Hominin genomes assayed in our PCR analyses (i.e., human,

chimpanzee, gorilla, and orangutan). Along with the truncated *Alu* elements, we found approximately 7.36 kb of non-*Alu* sequence inserted at experimentally confirmed NCAI loci.

Sequence architecture of NCAI loci and alignment to the ancestral full-length sequence

Alu elements at NCAI loci ranged in size from 34 bp to 276 bp in contrast to full-length *Alu* elements which are ~300 bp in length. We minimized the chance of erroneously selecting loci with post-insertion 3' truncations of preexisting TPRT-mediated *Alu* elements that mimic the typical structure of EN-independent insertions by rigorously comparing the orthologous flanking sequence in all three genomes. In theory, post-insertion random genomic deletions which remove the 3' segments of full-length *Alu* elements could mimic NCAI events. However, to pass our screening procedure, such random deletions would have had to arise in three separate primate genomes at exactly the same location [27]. The extremely low probability of this occurrence makes it unlikely that such loci are included in this study.

A multiple alignment of the *Alu* elements at NCAI loci reveals a tendency to cluster towards the 5' end of the consensus sequences of the respective full-length elements (Fig. 1). Indeed, only three insertions (NCAI 12, 13, & 14) align towards the 3' end of the consensus sequence. Eight of the Hominin NCAI loci were 5' intact and could not be traced back to a pre-existing *Alu* element when a triple alignment was performed. All other Hominin-specific NCAI loci

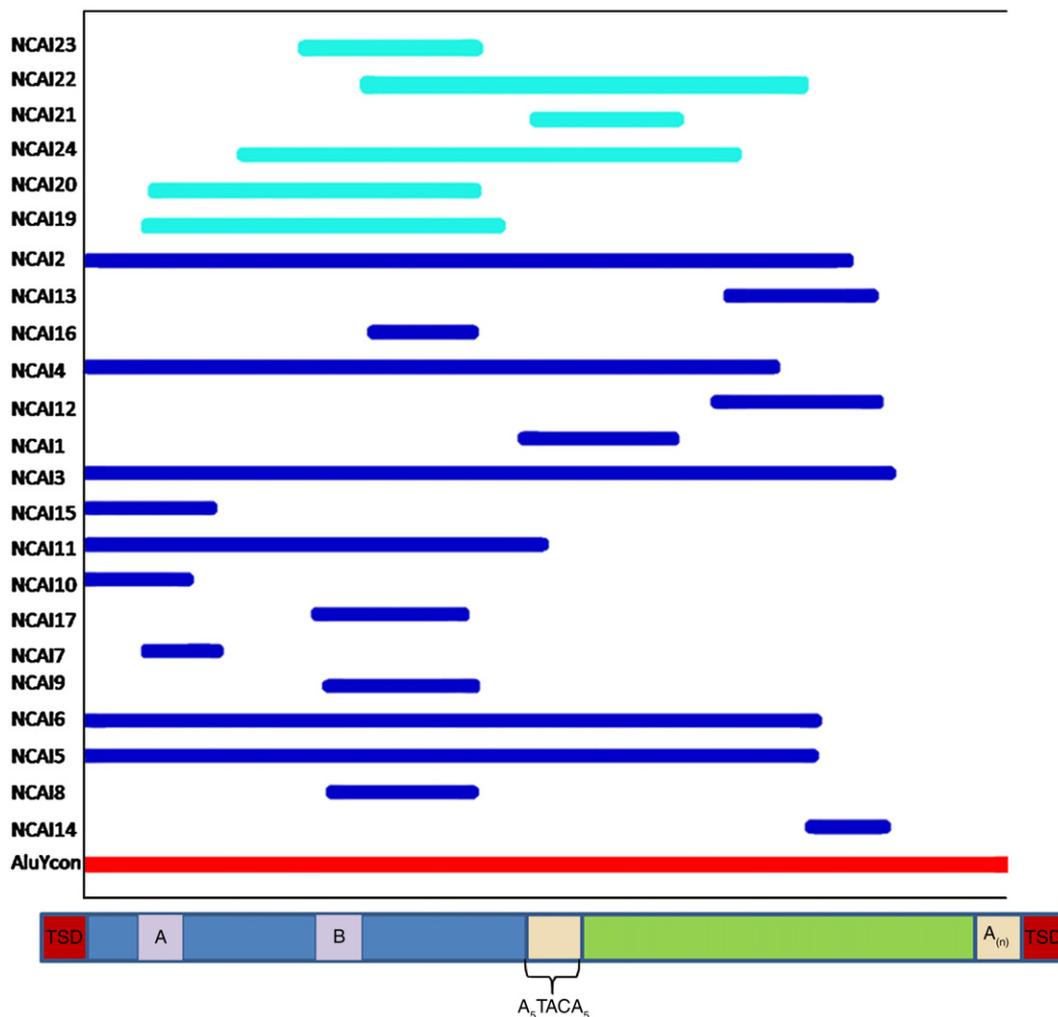


Fig. 1. NCAI fragments juxtaposed with a full-length *Alu* element consensus sequence from the RepeatMasker website. Hominin-specific loci are in dark blue and rhesus macaque-specific loci are in light blue. The consensus sequence is in red. A visualization of an *Alu* element is placed below the red consensus line.

showed 20 bp or more of 5' truncation. Ten Hominin-specific and five rhesus-specific NCAI loci had intact middle A-rich regions within the *Alu* element with four Hominin-specific and three rhesus-specific NCAI loci terminating in the middle A-rich region. One locus was retained based on the results from the computational output and sequencing of the out-groups. NCAI 17 is 761 bp long; it contains a 51 bp *Alu* fragment and is rich in simple repeats. Due to the simple repeats, PCR amplification and sequencing were not possible.

Based on the diversity of local sequence architecture features found adjacent to the NCAI loci we have recovered, we suggest that there is no one preferred model for endonuclease-independent *Alu* insertions and that this pathway is essentially an opportunistic mechanism for *Alu* integration. Over half of the 23 NCAI loci had non-*Alu* sequence inserted with them. One possibility is that these non-*Alu* sequences at NCAI loci represent “filler DNA”, small segments of which are often found at the junctions of genetic rearrangements [28,29] (Fig. 2). Previous studies have extensively documented the capture of mobile DNA at double-strand break sites in eukaryotic cells [19,30,31]. In the case of non-LTR retrotransposons in primate genomes, recent evidence supports the hypothesis that the L1 family may possess an endonuclease-independent mechanism that fills such genomic lesions both in cell culture analyses and in the publicly available human genome [18,19]. In view of the fact that the same enzymatic machinery is shared between the L1 and *Alu* families and that both are currently mobilizing in the human genome, we suggest that our results represent evidence for a similar endonuclease-independent insertion pathway operating for *Alu* elements to integrate into primate genomes. In this context, it is possible that similar to L1 elements, mature *Alu* mRNA molecules too can act as genomic Band-Aids® by opportunistically bridging DSBs in primate genomes [32]. Given that gene density and *Alu* density are strongly correlated across primate genomes, it is tempting to speculate that unrepaired DSBs in gene-rich regions of the genome, which would otherwise most likely be lethal, could be preferentially repaired by such *Alu* mRNA from actively transcribed elements located nearby.

Since RM cannot detect insertions under 30 bp in length, and half the loci we recovered were between 34 and 50 bp, it is likely that this

study represents a conservative estimate of NCAI activity, as any loci below 30 bp would remain undetected. The list was also narrowed by discarding all elements >2% diverged, rejecting those loci which had ambiguous sequence or putative TSDs >3 bp, and those in which the pre-insertion sequence could not be authenticated. There could potentially be more NCAI loci that have the hallmarks of endonuclease-independent insertion, but which have found homology with the 5' or 3' regions, thereby making it difficult to locate them computationally. These loci would appear as full-length elements using our search criteria, and would remain undetected.

Structural features of NCAI loci suggest a role in DNA double-strand break repair

In terms of their local sequence architecture, NCAI loci possess a distinct set of features that differentiate them from the larger set of “classical” TPRT-mediated insertions, which supports our hypothesis that two separate mechanisms operate for *Alu* integration. Below, we discuss some of these features:

Twenty of the twenty-three NCAI loci included target site deletions (i.e. deletions of the pre-insertion sequence) of varying size ranging from 1 bp to ~7 kb and adding up to approximately 16 kb of deleted sequence; this feature is thus common to both non-classical LINE and *Alu* insertions [18]. Among the deleted sequences, the largest deletion event was a little over 6 kb and associated with a Hominin-specific NCAI event. Three loci (NCAI 7, NCAI19, and NCAI20) were kept in the analysis even though they lacked target site deletions, because close inspection of the flanking sequence in the pre-insertion loci from the other 2 genomes and the NCAI revealed perfect matches. This suggests that these *Alu* insertions occurred with little to no loss of genomic material.

Very few, if any, TPRT-mediated *Alu* insertions include non-*Alu* DNA between the TSDs at either end [33]; in contrast, ~56% of NCAI loci (13 out of 23) in our study included non-*Alu* sequence along with the *Alu* fragment. The random segments of DNA range in size from 2 bp to ~2 kb. One possible explanation for this observation could be that the *Alu* mRNA may invade and attach to random DNA being used

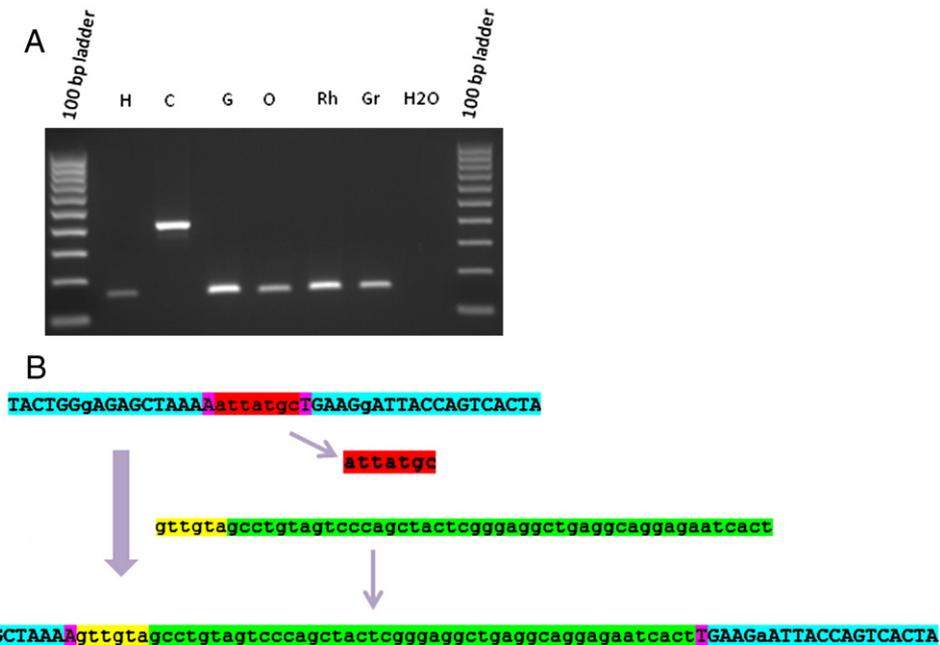


Fig. 2. Analysis of NCAI events. (A) Gel chromatograph of PCR products from a phylogenetic analysis of a chimpanzee-specific NCAI locus (NCAI 6). The DNA template used is indicated at the top of each lane (H, human; C, chimpanzee; G, gorilla; O, orangutan; Rh, rhesus macaque; and Gr, African green monkey). (B) Schematic diagram of an example NCAI locus (NCAI 6) showing *Alu* insertion (green box) associated with 7 bp deletion of target DNA (red box). Matching flanking sequences are shown as light blue boxes with pink sequence indicating exact sequence match at the ends of the indels. The yellow box indicates a small segment of non-*Alu* ‘filler’ DNA at the 3' end of this NCAI insertion.

process at NCAI loci, other cellular RNAs appear to have been transcribed along with the *Alu* fragment inserted at two loci (NCAI 9 and NCAI 17) (Fig. 2). There were also instances of capture of another retrotransposon RNA at a locus (NCAI 14, NCAI 11, NCAI 15, NCAI 9 and NCAI 13). L1 mRNA was captured most often, followed by other *Alu* mRNAs [24]. BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) [37] searches showed the extra nucleotides found at the 3' end of NCAI 9 were also found with almost a nearly perfect match at another location on the same chromosome, suggesting an intra-chromosomal translocation or *in vivo* RNA recombination. Enzymes associated with IVRR cause stopping or pausing of the DNA polymerase along the donor strand, which could lead to a truncated *Alu* if enzymatic activity was terminated [38]. Synthesis-dependent strand annealing (SDSA), an alternative model of DSB repair, could account for NCAI 14 wherein the invading strand initiates synthesis [39]. Of the 10 loci with extra sequence, at least three did not have a significant BLAST match when looking specifically at the non-*Alu* inserted sequence (NCAI 8 & NCAI 14) [30] and we did not find statistically significant matches in other cases.

NCAI microhomology and endonuclease cleavage site analyses

Multiple lines of evidence suggest the involvement of small stretches of complementary base pairing at the sites of mobile DNA capture at double strand break sites [40]. To examine whether a similar pattern was present at NCAI loci, we compared 6 bp stretches at both ends of the inserts with corresponding lengths in the pre-insertion flanking sequence following the procedure described in Sen et al. [18] (Fig. 3A). We excluded all loci where the 5' or 3' end of a locus included non-*Alu* inserted sequence along with the *Alu* fragment. *Alu* sequence was present at the 5' end of the NCAI locus in eleven cases and at the 3' end in thirteen cases. Our results indicate an increased level of microhomology at the 3' insertion junctions; however, at the 5' end we did not find a statistically significant increase in complementary bases [41] (Fig. 3B). This suggests that, though the NCAI mechanism supports opportunistic integration, microhomology at the attachment end of the fragment leads to higher rates of insertion as evidenced by higher levels of microhomology at positions 1 and 2 on the 3' end (10/13 and 9/13 loci analyzed, respectively).

Along with microhomology, all loci were inspected for the presence of deviation from the preferred L1 endonuclease cleavage site (5'TTTT/A). Analysis of the L1 EN cleavage sites is important in this regard because *Alu* elements use L1 machinery to insert into primate genomes and hence, characteristic TPRT-mediated insertion sites for *Alu* elements are similar to those for L1. Using a previously described point value system that accounts for the differential frequencies of transitions and transversions [42], NCAI loci were compared to a previous analysis of endonuclease-independent L1 insertions [18] and then to two recent analyses of TPRT-mediated L1 insertions (Fig. 4)

[19,42]. Comparison against the former suggests a similar trend towards more differences from the endonuclease cleavage site and comparison to TPRT-mediated insertions further strengthens this argument (Fig. 4). This provides further support to our hypothesis that *Alu* elements at NCAI loci are integrating without the activity of the L1 endonuclease. While atypical motifs for L1 EN cleavage sites do exist, a careful examination of NCAI loci revealed no insertions at such non-preferred TPRT cleavage sites, providing further evidence of EN-independent insertion [18,19,43,44].

Retrotransposition using a non-traditional route in primate genomes

In this analysis we have provided the first known evidence for the existence of an alternative *Alu* integration mechanism that appears to be independent of the L1 endonuclease activity. While TPRT-mediated insertions are much more abundant and without question form the preferred method of *Alu* mobilization, the structural features of loci discussed in this study leave little doubt that it has not been utilized in these cases. While previous research has shown that an endonuclease-independent pathway exists for L1 retrotransposition, both in cell culture and in the reference human genome [18,19], in our opinion the discovery of a similar mechanism for *Alu* elements is significant for a number of reasons.

In contrast to TPRT-mediated insertions, which are prone to causing genomic instability, the unique structural features of the NCAI mechanism discussed above lend credence to the hypothesis that they are associated with genomic DSB repair, and hence to the maintenance of genomic stability. The ubiquity of the *Alu* family in primate genomes implies that over evolutionary timescales, this endonuclease-independent pathway may have had an appreciable contribution to genome stability, and the relatively small numbers of insertions we have recovered in the three genomes probably represent a small fraction of the total number, for reasons we have discussed in the Materials and Methods section.

The human genome contains ~1.2 million, the chimpanzee ~1.1 million, and the Rhesus genome has ~1 million *Alu* elements [45]. Using the Tables utility from UCSC's BLAT website [46], and filtering for *Alu* elements showing divergence of 2% or less from the consensus sequence, we found 572 young inserts in the human genome, 160 in the chimpanzee genome, and 1075 in the Rhesus macaque genome. Based on the lineage-specific NCAI events recovered during the analysis of the human, chimpanzee, and Rhesus macaque genomes, including 4 human-specific, 4 chimpanzee-specific, and 6 Rhesus macaque-specific insertions, our data suggest the rate of insertion among young *Alu* elements by this endonuclease-independent pathway in each genome to be ~0.7%, 2.5%, and ~0.56%, respectively. This suggests that anywhere from as many as 27,500 to as few as 5581 NCAI events occurred within any of the three primate

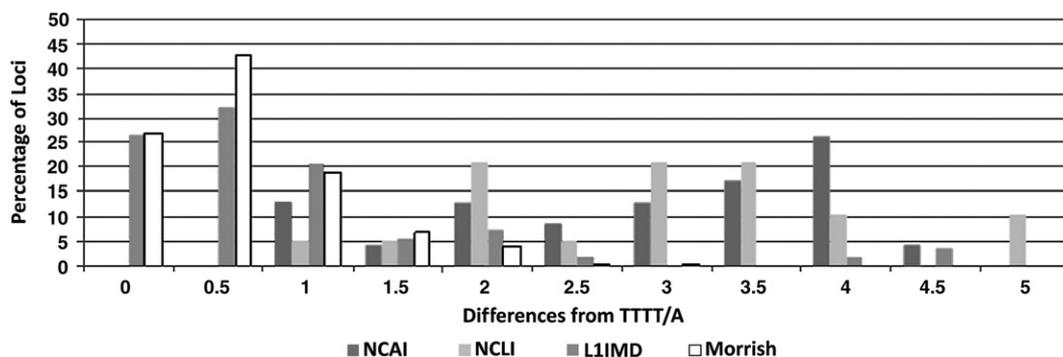


Fig. 4. Divergence from the L1 endonuclease cleavage site. The results indicate a large percentage of loci with a greater number of differences from the classical L1 endonuclease cleavage site seen in Target-site Primed Reverse Transcription. Atypical motifs of TPRT endonuclease cleavage sites exist, but a careful examination as compared to the cleavage sites found in NCAI showed that no insertions occurred at atypical TPRT cleavage sites, providing supplementary evidence of endonuclease-independent insertion.

lineages that were analyzed. Overall, there appears to have been a relatively homogeneous rate of NCAI throughout this portion of the primate lineage which includes humans, African apes and Old World monkeys. We believe the insertion rate in the chimpanzee genome may be inflated as a result of the calculations of percent divergence being based on the human *Alu* element consensus sequences [26]. These rates are much lower than those for TPRT-mediated insertions which encompass the vast majority of *Alu* insertions in primates [47].

DSB repair occurs using many pathways and a multitude of RNAs are recruited; we suggest *Alu* elements are preferentially caught at these breaks due to the large amount of free-floating retrotransposon RNA [18]. While the relative paucity of NCAI loci as compared to NCLI may be due to the greater length of the L1 mRNA providing a better chance of joining the separated ends of DSBs, in our opinion the fact that both of the most active non-LTR retrotransposon families in recent primate genome evolution (i.e. *Alu* and L1) are capable of participating in DSB repair is significant. In the sequence context of a recently created and unrepaired genomic DSB, the relative disadvantage of the shorter *Alu* mRNA as a repair tool compared to the longer L1 mRNA could potentially be offset by the fact that in contrast to L1 elements, the *Alu* family is concentrated in gene-dense areas, damage in which would likely be less tolerated hence giving NCAI a chance to be the genomic “first line of defense”. Indeed, it is possible to envision a scenario wherein the NCAI and NCLI mechanisms operate at two slightly different levels, with NCAI having access only to recent DSBs without much separation between the ends, while NCLI could act as a repair mechanism for breaks where the 300 bp *Alu* mRNA is unable to bridge the gap. Interestingly, this hypothesis is supported by the mean sizes of the deleted genomic sequences at NCAI and NCLI loci (712 bp vs. 1723 bp), which would provide an approximation of the mechanical separation between the two halves of the DSB at the breakpoint.

Conclusion

In conclusion, we have demonstrated an alternative *Alu* element integration method in primate genomes that may be utilized as a genomic damage repair pathway. By detailed inspection of the pre-insertion and post-insertion features of the sequence architecture, we have shown that this mechanism is distinct from the usual TPRT-mediated mode of integration and that TPRT and NCAI may have different consequences for primate genomes. On a global basis, TPRT-mediated *Alu* and L1 insertions are associated with disruption of gene function and are prone to post-insertion ectopic recombination. On the contrary, the endonuclease-independent NCAI we detected here, and the NCLI loci reported previously, and similar insertions in previous cell-culture analyses, show definite signs of being variants of DNA repair. In view of this evidence, it is now evident that both the L1 and *Alu* families contribute occasionally to the maintenance of genome stability, which provides additional insight into a hitherto neglected aspect of the biology of non-LTR retrotransposons, the most dynamic components of primate genomes.

Materials and methods

Computational screening and manual verification of putative NCAI loci

Classical TPRT-mediated *Alu* insertions are characterized by the presence of TSDs, L1 EN-cleavage sites falling within a limited spectrum of previously identified “preferred” motifs [19] and poly-A tails of varying length; the criteria used in the study identified *Alu* insertions that were truncated 3′ (lacking the poly-A tail), lacked TSDs, and did not have the structural hallmarks of an EN-cleavage site (typical or atypical) [9]. By looking for structural features similar to those described in Morrish et al [19] and Sen et al [18], the likelihood of finding false positives was reduced. To identify putative NCAI loci,

we modified the method outlined in Sen et al [18] for detecting similar insertions of L1 elements. Briefly, we downloaded whole-chromosome annotation files tabulating all mobile elements on each chromosome (available at <http://hgdownload.cse.ucsc.edu/downloads.html#human>) for the human (hg18) and chimpanzee (panTro2) genomes, and then using in-house Perl scripts, filtered out all non-*Alu* sequence, leaving only *Alu* elements [48]. Next, to scan for truncated *Alu* elements missing the poly-A tail that is used during classical TPRT-mediated integration, we wrote a set of programs to locate those elements which had 3′ truncations to positions numbering 276 or less, according to the 312 bp *AluY* consensus sequence used by the RepeatMasker (RM) software package at its default settings [49]. We chose this 3′ truncation limit to account for fluctuations in the poly-A tail length and maximize the number of putative loci while minimizing false hits. While the limit of 3′ truncation that we specified is arbitrary in terms of nucleotide position, we believe it is effective for the purpose of this analysis, as a manual inspection of putative loci attained by incrementally increasing the cutoff position from 276 towards the 3′ end of an intact element leads to an increase in false positives without returning any new loci fitting the criteria described above.

For the rhesus macaque genome (rheMac2), our strategy was slightly different due to the unavailability of whole-genome repeat annotations and the difference in *Alu* subfamily structure from the human and chimpanzee genomes. To locate putative NCAI loci in this genome, we first created a custom *Alu* element library and ran RM with varying 3′ truncation cutoff points to account for the different sizes of *Alu* subfamilies in the rhesus genome, which vary between 255 and 267 bp, not including the middle A-rich region or the poly-A tail [45,50].

Manual inspection of computationally detected loci involved extracting the putative truncated *Alu* along with 5000 bp of flanking sequence on both sides of each locus. Next, for any one primate genome (i.e., human, chimpanzee, or rhesus), we used this sequence to query the other two genomes using the BLAT software suite (<http://www.genome.ucsc.edu/cgi-bin/>) and created a triple alignment at the locus to analyze the local pre-insertion and post-insertion sequence architecture. In particular, we scanned for the presence of TSDs of any length and for any target site deletions present in the pre-insertion sequence but removed during the *Alu* insertion. By including the 5000 bp to either side of the locus, we were able to investigate the *Alu* element within the context of its flanking sequence and ascertain whether the element was truly young and truncated. To avoid including TPRT-mediated *Alu* elements partially masked by poly(N) stretches in the rhesus macaque genome, we only included *Alu* elements that were both 5′ (15–25 bp) and 3′ (35–50 bp) truncated and excluded all *Alu* elements flanked by unknown sequence. As we were only interested in relatively recent integrations for which we would be able to reconstruct the pre-insertion architecture from the other two primates, we discarded all elements >2% diverged from their respective consensus sequences according to the RM algorithm.

Loci matching all of the following five criteria were selected for experimental validation: 3′ truncation as specified above, absence of TSDs, absence of a poly-A tail, absence of typical or atypical EN cleavage site, and verifiable pre-insertion sequence structure in two other genomes. If the pre-insertion site in the orthologous genome contained any extraneous sequence between the starting points of the upstream and downstream matching flanking regions in the post-insertion genome, we cross-checked these against the putative NCAI to confirm that they were different (Table 1). Some putative chimpanzee and rhesus loci posed a problem as they were comprised of truncated *Alu* elements followed or preceded by a string of non-specific sequence (Ns). Wherever possible, we resequenced these loci to read through the poly-N stretches, and for the rhesus macaque loci we included African green monkey (*Chlorocebus aethiops*) DNA to

Table 1
NCAI loci and insertion site characteristics

Locus	Coordinates	Alu bp_ins	Non-Alu bp_ins	bp_del	Lineage	Intragenic	Non Alu seq	subfamily
NCAI1	chr11:130163514–130173562	45	6	7	H	–	–	Sc
NCAI2	chr22:41749851–41760112 ^a	258	0	21	H	–	–	Ya5
NCAI3	chr6:68781896–68792171	272	0	15	H	–	–	Ya5
NCAI4	chr14:88215334–88225566	233	0	2613	H	–	–	Ya5
NCAI5	chr4:123138133–123148385	246	0	28	C	–	–	Y
NCAI6	chr5:26686575–26696822	247	0	5	C	–	–	Y
NCAI7	chr16:13891929–13901955	25	186	0	C	–	5'	Y
NCAI8	chr3:79858862–79868909	48	341	33	C	–	3'	Yg
NCAI9	chr13:77357997–77368046	50	199	2	HC	MYC BP2	5'/3'	Yb8
NCAI10	chr2:131354400–131364436	35	3	258	HCG	–	–	Y
NCAI11	chr3:167550099–167560252	155	2075	2913	HCG	–	5'/3'	Yb9
NCAI12	chr13:25754437–25764491	55	67	47	HCG	–	5'/3'	Sg/x
NCAI13	chr16:70114181–70124229	49	134	136	HCG	–	5'/3'	Sg/x
NCAI14	chr2a:106680448–106690481	34	661	2436	HCG	–	3'	Yc3
NCAI15	chr5:64216227–64226270	43	2021	6670	HCG	–	5'	Y
NCAI16	chr16:63330452–63340488	34	0	966	HCG	–	–	Yc
NCAI17	chr19:51178572–51188621	50	639	18	HCG	OPA3	5'/3'	Sp
NCAI19	chr2:151660217–151670318	113	7	0	Rh	PPP2R3A	3'	YRa
NCAI20	chr3:42636756–42636861	107	53	0	Rh	–	5'/3'	YRd
NCAI21	chr4:103197391–103197423	41	7	49	Rh	–	–	YRa
NCAI22	chr12:81864628–81874777	148	2	51	Rh	–	–	YRb
NCAI23	chrX:65517270–65527328	58	17	1	Rh	–	3'	YRd
NCAI24	chr2:42702265–42712434	167	0	113	Rh	–	–	YR
Total (bp)	^a Callinan et al paper 2005	2513	6418	16382				

The letter in the column for 'Lineage' indicates the genome(s) to which the NCAI event is specific. In some cases the NCAI events were found in the Human, Chimpanzee, and Gorilla genomes, but were absent in the Rhesus macaque genome.

^a This locus was previously discussed in Callinan et al 2005.

accurately ascertain the pre-insertion sequence. To further confirm that loci fitting all the criteria described above were indeed atypical *Alu* insertions and not artifacts arising from sequence assembly errors, we PCR-amplified and resequenced all loci from a panel of primate genomes (Fig. 2).

PCR amplification and verification through resequencing

Primers surrounding each putative NCAI locus were designed using the Primer3 utility (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). PCR was performed in 25 µl reactions using 15–25 ng genomic DNA, 0.28 µM primer, 200 µM dNTPs in 50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-HCl (pH 8.4), and 2.5 units *Taq* DNA polymerase. Thermocycler programs were as follows: 95 °C for 2 min (1 cycle), [95 °C for 30 sec, optimal annealing temperature for 30 sec, 72 °C for 1 min] (35 cycles), 72 °C for 10 min (1 cycle). PCR products were visualized on 1–2% agarose gels stained with ethidium bromide. For PCR fragments larger than 1.5 kb, ExTaq™ (Takara) was used according to the manufacturer's specified protocol. All loci were amplified from the following genomes: *Homo sapiens* (HeLa; cell line ATCC CCL-2), *Pan troglodytes* (common chimpanzee; cell line from Coriell Cell Repositories AG06939B), *Gorilla gorilla* (Western lowland gorilla; cell line Coriell Cell Repositories AG05251), *Pongo pygmaeus* (orangutan; cell line GM04272A), *Macaca mulatta* (Rhesus macaque; cell line NG07109), and *Chlorocebus aethiops* (African green monkey; cell line ATCC CCL70). Primer sequences and annealing temperatures are available from the Publications section of the Batzer laboratory website (<http://batzerlab.lsu.edu>) under supplemental data.

Most loci were sequenced directly from the PCR amplicons after cleanup using Wizard® gel purification kits (Promega Corporation) or ExoSAP-IT® (USB Corporation). Samples that could not be sequenced directly from PCR products were cloned into vectors using the TOPO TA (fragments <1 kb) and TOPO XL (fragments >1 kb) cloning kits (Invitrogen). All sequencing was done using an ABI3130XL automated DNA sequencer. The resulting sequence files were analyzed using BioEdit and the SeqMan and EditSeq utilities from the DNASTar package® V.5. GC content in the flanking regions was calculated using GEECEE (available at: <http://mobylye.pasteur.fr/cgi-bin/MobylyePortal/>

portal.py?form=geecce). New DNA sequences generated during the course of this analysis have been submitted to GenBank under accession numbers EU263070-EU263102.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.ygeno.2008.09.016](https://doi.org/10.1016/j.ygeno.2008.09.016).

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