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Award Number: DAMD17-98-1-8119

TITLE: Genetic Damage Caused by ALU Repeats in Breast Cancer

PRINCIPAL INVESTIGATOR: Prescott L. Deininger Ph.D.

CONTRACTING ORGANIZATION: Tulane University Medical Center
New Orleans, Louisiana 70112-2699

REPORT DATE: August 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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DTIC QUALITY INSPECTED 4

20010108 092

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE August 1999	3. REPORT TYPE AND DATES COVERED Annual (1 Aug 98 - 31 Jul 99)	
4. TITLE AND SUBTITLE Genetic Damage Caused by ALU Repeats in Breast Cancer			5. FUNDING NUMBERS DAMD17-98-1-8119	
6. AUTHOR(S) Prescott L. Deininger Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Tulane University Medical Center New Orleans, Louisiana 70112-2699 E-MAIL: pdeinin@tcs.tulane.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES This report contains colored photos				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) We hypothesize that some of the same factors that may contribute to genomic instability in breast tumors, will also increase levels of retrotransposition of Alu elements, resulting in further genetic damage. We have developed a PCR-based method that is capable of looking at some of the most recent Alu inserts and eliminate background from the 500,000 older Alus. We will use this assay to compare inserts between breast tumor and normal tissue from the same patient and determine the rate of this type of insertion for the first time. In order to make this assay capable of effectively measuring the retrotransposition rate, we are developing methods to display a higher percentage of new Alu inserts. We have developed several different methods to look at subsets of the 1000 recent Ya5 inserts. In the next year, we will make improvements to these technologies, exploring subtraction of recent inserts from normal and tumor tissues and start to apply these techniques to tumor samples.				
14. SUBJECT TERMS Breast Cancer				15. NUMBER OF PAGES 22
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

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X For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

9-1-97

PI - Signature Date

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(5) Introduction:

This project was based on the hypothesis that early cellular transformation events involved in breast cancer formation might influence the amplification of human Alu repeats. Any increases in Alu amplification, might contribute to further destabilization of the human genome and inactivation of tumor suppressors that could contribute to the progression of breast cancer. At least in sporadic cases, Alu insertions have been shown to contribute to a number of cancers, including at least one case of breast cancer due to inactivation of BRCA2 (1). We have previously shown that only a specific set of subfamilies of Alu elements are actively amplifying in the human genome(2,3). This project combines this information with an anchored PCR procedure we have developed to form displays of the most recently amplified Alu elements. We have demonstrated that this Allele-Specific Alu PCR (ASAP) will effectively display the members of the smallest of the recent Alu subfamilies as bands on an acrylamide gel. Our goal is to generalize these procedures to the larger subfamilies and explore various procedures to deal with the larger number of bands expected. We will then use these procedures to compare breast cancer and normal DNA from a number of individuals to determine whether there are new, tumor-specific Alu inserts. This will allow us to determine whether this form of genetic instability plays a role in human breast cancer.

(6) BODY

Goals of Year One:

First Six Months:

- Optimization of ASAP. Our primary goal will be to optimize the Allele-Specific PCR further. We will work to identify the very best PCR primers to allow the most effective allele-specific amplification of the Alu inserts and flanks. This will allow us to develop a procedure with both minimal steps and minimal background in the later experiments.
- No patient samples will be needed at this stage.

First Year:

- Optimization of Displays. We will utilize the ASAP procedure to generate test samples from all three relevant Alu subfamilies, which can then be utilized to improve the display procedures, in particular the subdivision with PCR into 16 subdivisions. We will begin to explore ways to utilize subtraction procedures on these samples.
- No patient samples will be needed at this stage

Previously Proposed Goals of Year 2

Second Year:

- Refinement of Subtraction Technology. Technical development will continue with refinement of the subtraction procedures and tests of the sensitivity of detection of bands and the ability to pool samples in the PCR reactions.
- Preliminary work on tumor samples. Work will begin with existing technology to carry out analysis on tumor samples. We expect to have carried out analysis of the first 10-20 samples in this year. We will use this experience to determine the best approach to generate data in a production mode. This will provide an initial feel for the level of diversity in the displays and a basic characterization of any diversity to determine

whether it is caused by insertions. Any evidence of other forms of genomic instability influencing the assay will be assessed at this point and procedures optimized to compensate.

Accomplishments of Year One:

We have accomplished most of the goals proposed for year one and explored several undescribed options as well. Although there are some limited technical difficulties with the developed procedures (discussed below), I believe that we are in an excellent position to proceed with the goals of year 2 (see above).

At the time this grant was funded, we had already obtained reasonable displays of the small, Ya8, subfamily of Alu repeats. Thus, the basic allele-specific PCR techniques were working and continue to work well. However, we needed to develop refined techniques that would allow us to display the Alu repeats from the larger subfamilies (Ya5 and Yb8) that are actively amplifying. We have particularly placed our emphasis on the Ya5 subfamily because the majority of new Alu inserts come from that subfamily and it may be the most technically challenging. We have cloned the amplified PCR products, however, and found that 10 out of 11 clones sequenced had the appropriate diagnostic sequence characteristics of the Ya5 subfamily. Therefore, we were successful at amplifying the Ya5 Alus. Our initial problem was that there are about 1000 bands in the Ya5 amplification, which provided an unresolved smear on a gel. Thus, although the PCR part of the procedure worked fine, we cannot use a simple gel system to display heterogeneity in the samples. Therefore, we needed to work we needed to develop methods to display only subsets of the bands. We have made several methods work. One method that is very effective is to cut the genomic DNA with a less frequent-cutting enzyme. In this case we tried *EcoR1* rather than the initially used *Mse1*. *Mse1* will cleave within 500 bases of almost all of the elements because it is an A+T preferring enzyme with four base recognition. *EcoR1* will cut on the average about 2-3 kb from the Alus. Thus, only a small fraction (e.g. 10-20%) will cleave within 500 bases and provide a readily PCR-amplifiable template. Figure 1 on the next page shows some examples of individual DNAs with linkers applied to the *EcoR1* sites and amplified for Yb8 and displayed on an agarose gel. We learned two things from this successful experiment. First, we got a lot less than the approximately 40 bands when we used *Mse1* and the average flanking sequence was much longer. Longer flanking sequences might be more effective in subtraction strategies and therefore are likely to be advantageous. However, an additional benefit of this procedure, particularly the use of the agarose gel to fractionate the slightly longer fragments, is that very minor length differences aren't picked up. In the PAGE-based methods, we sometimes saw bands of small difference in mobility that turned out to be one or two base length polymorphisms rather than new inserts. We isolated several fragments (marked on the figure) and generated new PCR primers from the Alu to the flanking sequence. This allowed us to demonstrate that two of these were polymorphic for presence/absence in different individuals (see panels on right, next page). This demonstrated the first direct proof that the method was detecting insertion polymorphisms and not other forms of polymorphisms between individuals.

Although the use of *EcoR1* was encouraging, it would still be likely to generate too many bands from the Ya5 subfamily. Accordingly, we also explored the use of third round primers that match the linker, but with 2 extra bases on the 3' end. These primers would only amplify about one sixteenth of the existing fragments. Figure 2 shows an amplification of Ya5 members

with a linker primer that has the dinucleotide CT added at the 3' end. This provides a reasonably clear banding pattern with a reasonable number of fragments and allows detection of modest levels of polymorphism. This is quite promising. Unfortunately, when we utilized a dinucleotide extension of TT, the banding pattern was more complex and less clear. This probably reflects the fact that the flanks of Alu elements are very A+T rich. Thus, there will be more TTs in that region than CCs. It could also reflect different effects with alternative base-pairing with Ts as well. We find that about half of the dinucleotide extensions work reasonably well, which should allow us to display about half of the potential new inserts.

I should mention that in the process of these studies we have carried out an exhaustive series of experiments on procedures that will increase the signal to noise ratio in these allele-specific amplifications. This includes studies to optimize all of the concentrations of DNA at each of the nested amplifications, modifications of the ends of the DNA fragments to suppress spurious background amplification, the use of biotin on some of the primers to select specific amplification products and a number of other variables in the PCR amplification reactions. We have also tried extensive variations of the linker sequences, as well as different allele-specific primers to find the most effective sequences.

We have just begun exploring an alternative strategy for displaying subsets of Ya5 and Yb8 Alu inserts. This new strategy, that was not described in the proposal, involves PCR amplification with an allele-specific primer and a ten base primer of arbitrary sequence. The arbitrary sequence will allow amplification of a small fraction of potential Alus. The one primer we have used amplified about ten quite robust bands. By adjusting conditions, changing primer length, etc., we should be able to adjust conditions to get more bands as well. By using a number of different arbitrary primers, we will be able to display a significant subset of the Alu inserts in this way as well. Thus, we have several methods that should work to display a large proportion of new Alu inserts in the tumor samples. However, we would still like to make improvements that would streamline the procedures.

Thus, our plan for the second year remains largely unchanged. If we can amplify the whole subset of Ya5 or Yb8 Alus at once and subtract the products from the tumor and the normal tissue, we could potentially characterize all Ya5 with a single display rather than requiring many displays. Thus, as planned, we will put our primary focus initially on implementing the subtraction strategies. We will also carry out tests of our ability to pool samples, either with the subtraction or the display procedures described above, so that we can potentially analyze more samples. We will then use either the displays described above, or the subtraction procedures to carry out an extensive analysis of a limited number of tumor samples. This will allow us to work out any specific difficulties with these comparisons and will also allow us to determine whether the rate is exceptionally high in the breast cancer samples.

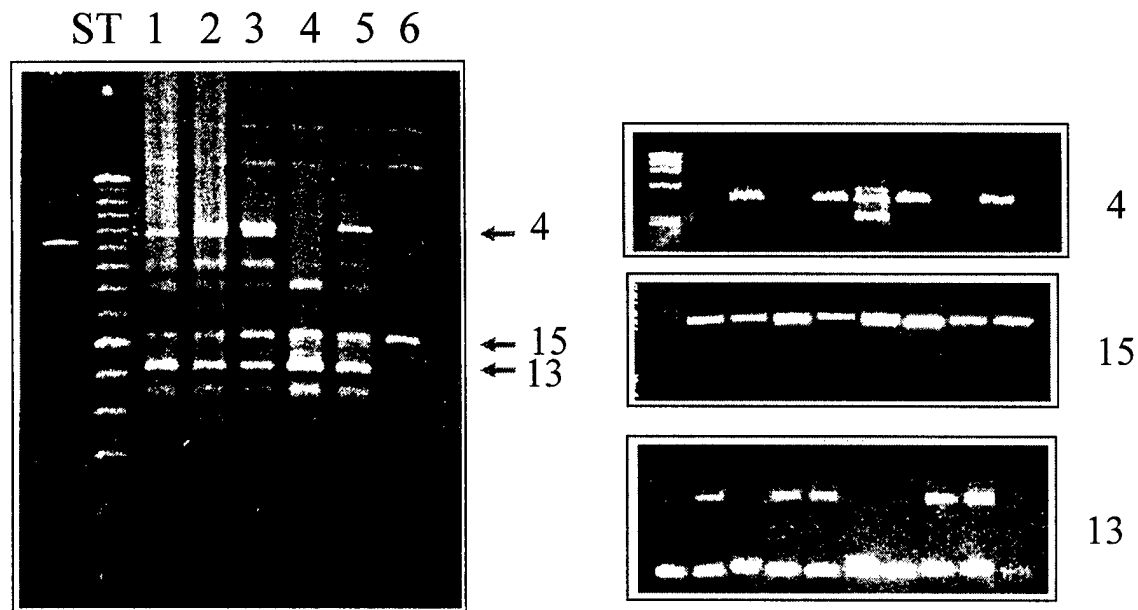
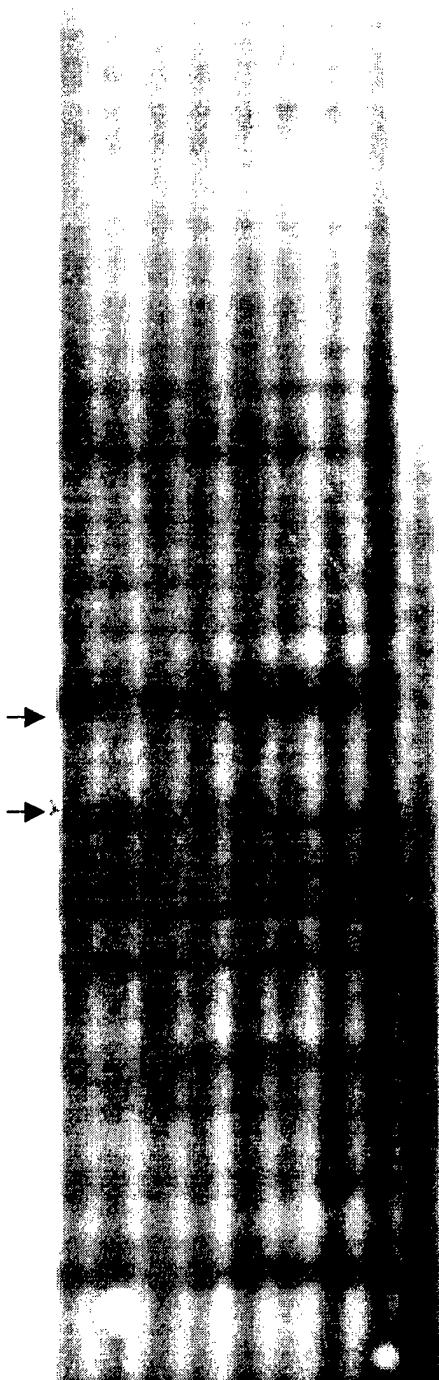


Figure 1. ASAP display of *EcoRI* cleaved human DNAs. The panel on the left represents a size marker (ST) and six individual human DNAs that have been cleaved with *EcoRI*, linked with the appropriate linker, and amplified with three nested rounds of amplification between Ya5-specific primers and the linker. The bands marked 4, 15 and 13 were excised from the gel and the sequences flanking the Alu determined. A primer was designed for each of these flanking sequences and used in conjunction with a Ya5-specific primer to amplify each of the specific loci in the panels on the right from a different group of individual humans. Bands 4 and 13, which show strong polymorphisms on the original gel on the left, show polymorphism when directly assayed through more individuals. This confirms the ability of this approach to detect differences between the DNA of different individuals.

Figure 2. Display of a CT subset of Ya5 Alus from multiple human DNA samples. The arrows mark several positions where polymorphisms are seen in the original autoradiogram. The scan could not fully reproduce the resolution of this sequencing gel. Although the complexity of the banding pattern might obscure some of the polymorphism, many potential polymorphisms can be detected with this display. Note also that the display is quite robust and worked similarly on all samples, with the exception of the last lane on the right which did not amplify larger products well. This may represent degraded DNA or a problem with inhibition of the PCR reaction



(7) Key Research Accomplishments

- Establishment of optimum conditions for amplification of the most recent subfamilies of Alu inserts
- Obtaining clear displays of the Ya8 subfamily on acrylamide and agarose gels which allow the isolation of insertion polymorphisms between different individuals.
- Demonstrating the use of modified primers that display subsets of the Ya5 elements that will allow at least a substantial portion of Ya5 inserts to be studied.

(8) Reportable Outcomes

At this point, we have written one review article that acknowledges this grant support. This review article helps demonstrate the potential importance of Alu insertions to human diseases, including cancer.

(9) Conclusions

This project requires the further development of existing techniques to answer the question of whether human mobile elements contribute significantly to genomic instability in breast cancer. The basic procedures have been developed that will allow a beginning to the measurements in actual tumors. However, further improvements are being worked on that will allow more of the potential insertions to be assayed more rapidly and with a more robust assay. This will increase the probability of detecting new Alu inserts and providing a reasonable measure of the insertion rate.

So What? It was previously shown that expression (not necessarily retroposition) of Alu elements increases in response to cellular transformation. One of my collaborators has recently shown that the expression of Alu elements also increases greatly in response to several genotoxic chemotherapy treatments that are commonly used with breast cancer patients. Determining whether this increased expression leads to a high level of insertion of Alu elements will allow an estimate of the contribution of Alus to genomic damage in tumors and as a secondary consequence following chemotherapy. This may then lead to approaches to repress the Alu expression or the use of chemotherapeutic regimens that we find has minimal stimulatory influences on Alus.

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APPENDIX

one reprint for:

Deininger, P. and Batzer, M. (1999) Mol. Gen and Metab 67, 183-193.

MINIREVIEW

Alu Repeats and Human Disease

Prescott L. Deininger^{*,†,1} and Mark A. Batzer[‡]

**Tulane Cancer Center, SL-66, and Department of Environmental Health Sciences, Tulane University Medical Center, 1430 Tulane Avenue, New Orleans, Louisiana 70112; †Laboratory of Molecular Genetics, Ochsner Medical Foundation, 1516 Jefferson Highway, New Orleans, Louisiana 70121; and ‡Departments of Pathology, Biochemistry and Molecular Biology Biometry and Genetics, Stanley S. Scott Cancer Center, Neuroscience Center of Excellence, Louisiana State University Medical Center, 1901 Perdido Street, New Orleans, Louisiana 70112*

Received March 25, 1999, and in revised form March 31, 1999

Alu elements have amplified in primate genomes through a RNA-dependent mechanism, termed retroposition, and have reached a copy number in excess of 500,000 copies per human genome. These elements have been proposed to have a number of functions in the human genome, and have certainly had a major impact on genomic architecture. Alu elements continue to amplify at a rate of about one insertion every 200 new births. We have found 16 examples of diseases caused by the insertion of Alu elements, suggesting that they may contribute to about 0.1% of human genetic disorders by this mechanism. The large number of Alu elements within primate genomes also provides abundant opportunities for unequal homologous recombination events. These events often occur intrachromosomally, resulting in deletion or duplication of exons in a gene, but they also can occur interchromosomally, causing more complex chromosomal abnormalities. We have found 33 cases of germline genetic diseases and 16 cases of cancer caused by unequal homologous recombination between Alu repeats. We estimate that this mode of mutagenesis accounts for another 0.3% of human genetic diseases. Between these different mechanisms, Alu elements have not only contributed a great deal to the evolution of the genome but also continue to contribute to a significant portion of human genetic diseases. © 1999

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Key Words: Alu repeats; recombination; insertion mutation; human disease; genetic diversity.

THE SPREAD OF Alu ELEMENTS IN THE HUMAN GENOME

Alu elements represent a sequence of approximately 300 nucleotides (nt) in length that are transcribed by RNA polymerase III. The RNA transcript is then reverse-transcribed and inserted into a new location in the genome. This RNA-mediated process for making new copies of the element is termed retroposition (1). Different Alu elements in the genome are not identical to one another. It appears that Alu elements that have integrated recently within the genome are quite homogeneous, and almost exact copies of one another (2). However, the older copies have accumulated random mutations, making them typically divergent by 20% or more from one another at the sequence level (3).

Alu elements began inserting early in primate evolution, approximately 65 mya (3). Although there are some related elements in mammals outside of the primate order, they do not have the specific structure of Alu elements. The rate of Alu amplification appears to have reached a maximum between 35 and 60 mya, and is currently amplifying at only 1% of the maximum rate. There are probably only about 2000 Alus specific to the human genome, and not found in chimpanzee and gorilla. Thus, about 99.8% of the 500,000 Alus in the human genome can

¹ To whom correspondence should be addressed at Tulane Cancer Center, SL-66, Tulane University Medical Center, 1430 Tulane Ave., New Orleans, LA 70112. Fax: (504) 588-5516. E-mail: pdeinin@tcs.tulane.edu.



TABLE 1
Alu Insertions and Disease

Locus	Distribution	Subfamily	Disease	Reference
CaR	Familial	Ya4	Hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism	(51)
Mlvi-2	<i>De novo</i> (somatic?)	Ya5	Associated with leukemia	(52)
NF1	<i>De novo</i>	Ya5	Neurofibromatosis	(53)
PROGINS	About 50%	Ya5	Linked with ovarian carcinoma	(54)
IL2RG	Familial	Ya5	XSCID	(55)
ACE	About 50%	Ya5	Linked with protection from heart disease	(35)
Factor IX	A grandparent	Ya5	Hemophilia	(56)
EYA1	<i>De novo</i>	Ya5	Branchio-oto-renal syndrome	(57)
2 × FGFR2	<i>De novo</i>	Ya5 & Yb8	Apert's syndrome	(41)
Cholinesterase	One Japanese family	Yb8	Cholinesterase deficiency	(58)
APC	Familial	Yb8	Hereditary desmoid disease	(59)
Btk	Familial	Y	X-linked agammaglobulinaemia	(55)
C1 inhibitor	<i>De novo</i>	Y	Complement deficiency	(60)
BRCA2	<i>De novo</i>	Y	Breast cancer	(61)
GK	?	Y	Glycerol kinase deficiency	(62)

be found at the same locus in all of the great apes, and 85% of the elements at specific loci can be found in all monkeys. Our best estimates of Alu amplification in the human genome are that there is one new insert in about every 200 new births (4). Although this is well below the peak rate, it is still high enough to represent a significant factor in human mutagenesis.

In addition to random mutations, which occur to Alu elements after their insertion in the genome, there are specific base changes that allow separation of Alu elements into different subfamilies (5–10). The different subfamilies were all inserted at different stages of primate evolution. Almost all of the insertions that have occurred specifically in the human genome come from four closely related subfamilies, Alu Y, Ya5, Ya8, and Yb8. Ya5 and Yb8 inserts represent the majority of the inserts and Alu Y inserts are relatively rare. All of the new inserts belong to a small group of the most recently created subfamilies (see Table 1). This demonstrates that only a small subset of Alus is capable of amplification (11).

Several explanations for the selective amplification of specific subfamilies have been proposed. One likely explanation is that a few specific loci are capable of active amplification, while almost all other loci are not, and that there are almost no such loci in the older subfamilies (11). Alternatively, one has to propose that loci from all subfamilies express, but that the RNAs expressed from the newer subfami-

lies interact with the retroposition apparatus much better than the older subfamily RNAs (12,13).

Alus AND L1 ELEMENTS

The other major mobile element in the human genome is the L1 element. Alu elements are RNA polymerase III-derived transcripts that have no coding capacity. Thus, they do not code for any proteins that might be involved in the retroposition process. L1 repeats, on the other hand, are much longer and have two open-reading frames (reviewed in (14)). One open-reading frame apparently codes for an RNA-binding protein whose exact function is unknown. The other open-reading frame codes for a protein that includes domains for reverse transcriptase, as well as for an endonuclease that apparently nicks the genome at the site of insertion (15–17). An assay that allows rapid L1 retroposition in cultured cells has been devised recently (18). This assay facilitates the dissection of the details of the L1 retroposition mechanism.

Alu elements must obtain the enzymes for their retroposition from somewhere. In addition, there are striking similarities between the mechanisms of Alu and L1 retroposition that make it very attractive to think that L1 elements may supply the necessary components for Alu retroposition (15,16,19,20). This idea is certainly very attractive, and thus the rate of Alu retroposition may be very dependent on the rate and evolution of L1 elements.

Alu ELEMENTS: FUNCTIONAL ROLE OR A PARASITE'S PARASITE

Alu repeats represent over 5% of the mass of the human genome. They are also spread throughout the entire genome, at varying densities. These observations, along with other specific properties of the Alu elements have led to a number of hypothetical functions for the Alu elements that might explain their ubiquitous presence in primate genomes. Some of the proposed roles involve an everyday function for the cell, while others are of a more sporadic nature.

The first role ever proposed for Alu elements was that they might be origins of DNA replication (21). This role is consistent with their high copy number and dispersed nature, but has not been substantiated by direct experimentation and seems like too important a function to be served by an element that is not found outside of primates.

More recently, evidence has been presented that Alu RNAs may stimulate protein translation by inhibiting a RNA-dependent protein kinase, PKR (22-24). Because Alu RNAs from many loci are stimulated by a number of cellular stresses, such as viral infection and heat shock, this would provide a mechanism by which dispersed sequences may contribute to a cellular process as a group. If this is a function of Alu elements, then it is likely to represent only a slightly modified regulation seen in nonprimate species that is filled by other RNAs or molecules in those species.

Evidence has been presented in yeast that retrotransposable elements may aid in healing chromosomal breaks (25,26). This suggests the possibility that Alu and L1 elements may provide the same role in the human genome.

There are several thoughts concerning the possible roles of Alu elements in the evolution of the human genome. As discussed below, Alu elements can lead to unequal recombination that results in deletion or duplication of sequences. These events could allow duplication of exons and therefore formation of new protein variants. They can also contribute to interchromosomal recombination that may lead to cytogenetic alterations that are involved in human speciation.

There are also several ways in which Alu repeats have been proposed to influence the evolution of gene expression. Because Alu elements are rich in CpG dinucleotides that represent the substrate for genomic methylation, Alu elements rep-

resent CpG-rich islands that make up about 30% of the methylation sites in the human genome (24). When an Alu element inserts in a new location in the genome, it introduces a CpG island at that new location. CpG islands have been associated with gene regulation, as well as imprinting of genes, and therefore Alu elements may contribute to the evolution of gene expression and imprinting in the human genome. In addition, Alu elements have been found to carry functional promoter elements for several of the steroid hormone receptors (27,28). Thus, insertion of a new Alu element in the vicinity of a gene may introduce new transcription factor-binding sites that could alter the regulation of gene expression. There are a number of cases where elements that influence gene expression have been mapped to within an Alu repeat (29), demonstrating that the introduction of these sequences can at least occasionally contribute to gene expression and regulation.

Although, there are numerous cases where individual Alu elements have had a positive impact on the human genome, it might be argued that none of them has been confirmed as a function. In this sense we would not define something that happens in a positive sense every few thousand years as being a function, because it would be occurring too sporadically to apply a positive selection for the presence of Alu elements. In addition, studies of individual Alu elements demonstrate that there is essentially no selective pressure on any given Alu repeat, although it is possible that selection does exist for a handful of master elements. Thus, it has been argued that Alu and L1 elements may both represent "selfish" DNA, or DNA that is only working to replicate itself. Selfish DNA may often have negative impacts on the host, but can be tolerated if it does not have too strong an adverse affect. Selfish DNA may also occasionally have positive benefits, but only by chance, and not by functional design. If L1 elements are essentially a parasite within the human genome, and if Alu relies on L1 elements for their amplification process, then one might describe Alu as a "parasite's parasite."

Alus AS MARKERS FOR HUMAN DIVERSITY

Although there is still a question as to whether there is a true functional role for Alu elements in the human genome, Alu elements have proved to be

useful in studies of human DNA. The presence of Alu repeats located ubiquitously throughout the human genome, but not in nonprimate species, has allowed detection of human DNA sequences that have been transfected into the cells of other organisms, such as mice. This has been useful in marker-rescue experiments in isolating a number of genes, including the first examples of oncogenes isolated by transforming rodent cell lines with human tumor DNAs (30). More recently, inter-Alu PCR (31,32) has found a broad range of uses in isolating specific human DNA regions from mouse/human hybrid cell lines and other complex sources containing large segments of human DNA.

Recent Alu insertions have also proven useful in a number of human population studies. In particular, there are over 1000 Alu insertions that occurred recently enough to be present only in a subset of human chromosomes. Because there does not seem to be any specific mechanism for removing Alu elements from the genome, once inserted they make a very stable genetic marker (33,34). This observation, along with the extremely low probability that any two recently integrated elements have inserted independently in the same chromosomal location, makes Alu insertions one of the best identical-by-descent (IBD) markers for human evolution studies. Any two individuals sharing an Alu insert almost certainly do so because they share a common ancestor in which the insertion occurred. Table 1 includes an example of an Alu insertion in the angiotensin-converting enzyme (ACE) locus that shows a useful association with protective advantages from heart disease (35). Many other Alu insertion polymorphisms have been identified either in random genomic loci or in specific genes, but without any known disease association. These Alu insertions are easy to assay for their presence or absence in a chromosomal location and have been found to be very powerful markers for human forensic and molecular anthropology studies (36,37).

RETROPOSITION OF Alu ELEMENTS AND DISEASE

Alu elements are located throughout the genome and in almost any location within a gene except those in which they would totally disrupt the function of that gene. Figure 1 illustrates some of the positions relative to a typical gene structure in which Alu may land. Alus landing far enough upstream of a gene may have no influence on that

gene's expression. However, Alus landing in or near the promoter/enhancer regions of a gene have been found to influence the expression of specific genes (reviewed in (29)), as well as to have the general potential to add transcription elements, like steroid hormone receptor elements (27,28), to the upstream gene region.

Very few Alu elements are found within the 5' noncoding or coding regions of exons, presumably because insertions in those locations are too disruptive to gene function. There are a number of instances where Alu elements have been found to be part of the region coding for the carboxy-terminus of a protein product (38,39). Presumably these Alus insert far enough downstream in the coding sequence to result in a new carboxy-terminus that does not disrupt the structure of the protein.

Insertions into the 3' noncoding regions of genes are found commonly and appear to have few negative effects. Similarly Alus are commonly found in introns, demonstrating that Alu insertions in much of the intronic region do not alter gene function significantly.

The vast majority of Alu insertions that have led to human disease insert into coding exons, or into introns relatively near an exon and presumably alter splicing. Table 1 is a list of the genetic defects that are thought to be caused by Alu insertion events. Not all of these cases have been demonstrated to be directly causative for the disease, but the rarity of Alu insertion events, coupled with the lack of other detectable mutations in these cases, strongly indicates that these are the causative events. The ACE insertion (35,40) is likely to be one example, however, that shows association with disease, but is highly unlikely to be the causative event.

The above examples demonstrate that Alu insertions are capable of causing genetic defects which lead to human disease. Examples of this type are being found at an increasing frequency as the tools for genetic analysis allow more mutations to be detected. Finding 16 Alu-based insertion mutations in the Human Genetic Mutation Database that contains 14374 characterized human mutations suggests that Alu elements contribute to approximately 0.1% of human genetic diseases. This number agrees well with a previous calculation based on a similar dataset of mutations where Alu and L1 insertions were estimated to each contribute approximately 0.075% of human mutations (16). In some cases, the insertional mutagenesis may make detection of mutations easier, biasing the results in favor of the

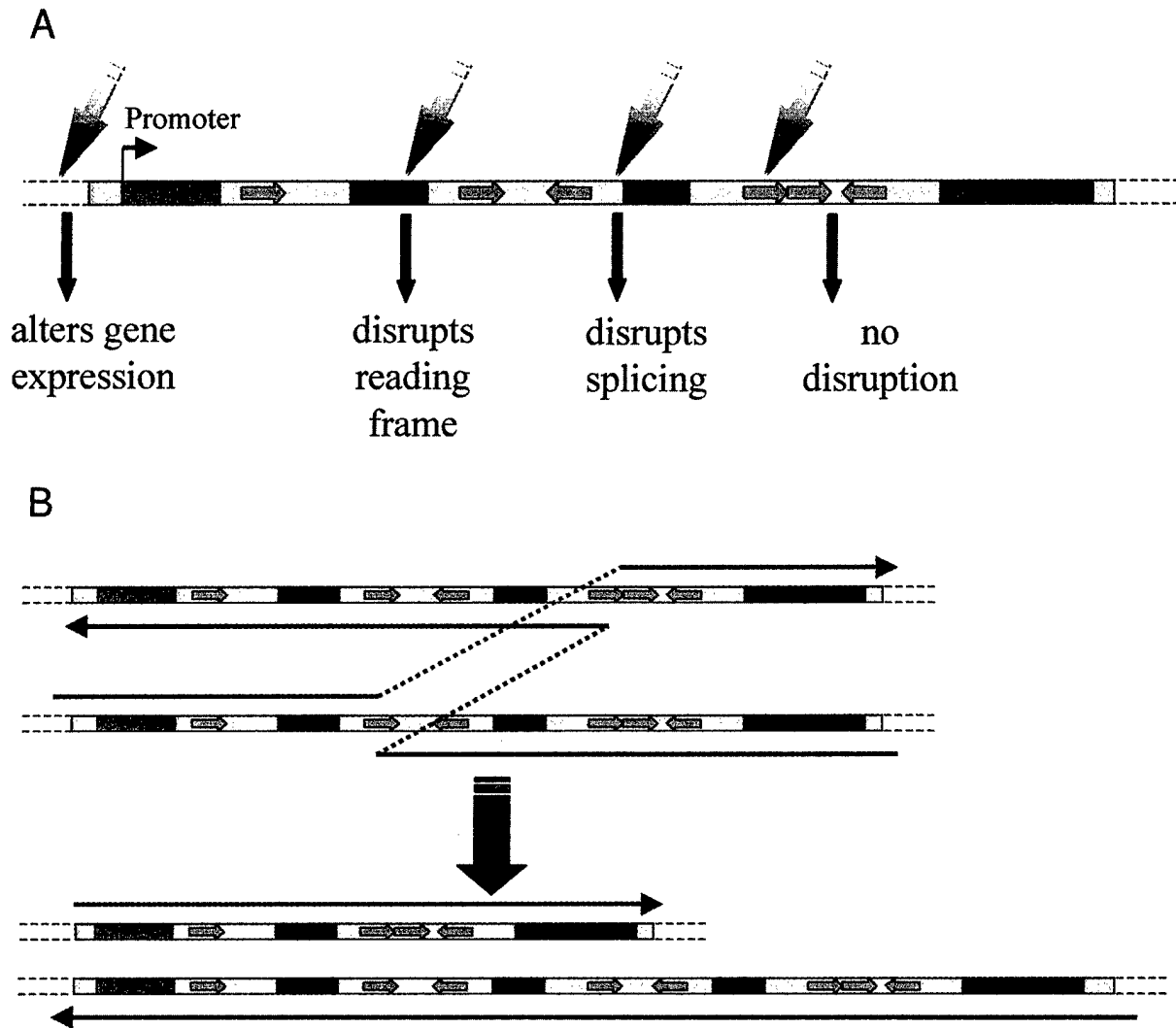


FIG. 1. Schematic of Alu-induced damage to the human genome. Panel A illustrates some of the potential consequences of insertion of a new element in the vicinity of a gene. The colored boxes represent various exons of the gene. The red arrows show existing Alu elements oriented in different directions in the introns of the gene. Depending on the site of insertion, the Alu element has varied probability of impact on the genome as shown. Panel B illustrates an unequal, homologous recombination occurring between two Alu elements in different introns of a gene. The arrows broken by dotted lines show the path of the recombination event. The genes below show that one copy will have a deletion while the other will duplicate gene sequences. Either is likely to be deleterious.

detection of Alu insertions. However, many mutation detection strategies are designed to identify point mutations, particularly in coding regions, and may overlook insertions, particularly if they occur in introns. In addition, many new mobile element insertions may be lethal during embryogenesis. Therefore, it is likely that these estimates of insertion frequencies are underestimates of the true contribution of new Alu insertions to human disease.

We expect that with increasing study of mutations, it will be found that some genetic diseases are

more likely than others to result from retroposon insertion. It has certainly been observed that some genes have a much higher Alu repeat content, making it reasonable that they will have a higher frequency of disabling Alu insertions. It has been observed that 2 out of 258 mutations in the *FGFR2* gene were caused by Alu insertions (41). This is the first case of multiple Alu insertion mutations being detected associated with a single disease, suggesting that this genetic locus may be more susceptible to retroposon insertions than other regions of the ge-

TABLE 2
Alu/Alu Recombination and Germ-Line Disease

Locus	Distribution	Disease	Reference
8 × LDLR	Kindreds	Hypercholesterolemia	(63-67)
5 × α-globin	Kindreds	α-thalassaemia	(68-71)
5 × C1 inhibitor	Kindred	Angioneurotic edema	(60,72)
Lys Hydrox.	Kindreds	Ehlers-Danlos syndrome	(73)
DMD	Kindred	Duchenne's muscular dystrophy	(74)
ADA	One patient	ADA deficiency-SCID	(75)
Apo B	One patient	Hypo-betalipoproteinemia	(76)
Ins. Rec. β	One patient	Insulin-independent diabetes	(77)
α-gal A	One patient	Fabry disease	(78)
HPRT	One patient	Lesch-Nyhan syndrome	(79)
Plat. Fibrinogen Receptor	Kindred	Glanzmann thrombasthenia	(80)
Phosphorylase kinase	One patient	Glycogen storage disease	(81)
GALNS	One patient	Mucopolysaccharidosis type IVA	(82)
Antithrombin	One patient	Thrombophilia	(83)
XY	One patient	XX male	(84)
β-HEXA	Classic form of disease	Tay Sachs	(85)
C3	Kindred	C3 deficiency	(86)
HEXB	27% of patients	Sandhoff's disease	(87)

nome. However, the number of insertions found so far is still fairly low making more definitive conclusions difficult.

RECOMBINATION BETWEEN Alu ELEMENTS ASSOCIATED WITH DISEASE

In addition to the potential impact of Alu element insertions in causing human disease, their dispersion throughout the genome provides ample opportunity for unequal homologous recombination which leads to a much higher level of mutations. Figure 1B illustrates how this unequal recombination can cause insertion or deletion mutations. When recombination occurs between Alu elements on the same chromosome, the result is that there is either duplication or deletion of the sequences between the Alus. Recombination may also occur between Alu elements on different chromosomes, resulting in chromosomal translocations or more complex chromosomal rearrangements.

Table 2 presents a compilation of Alu/Alu recombination events that have contributed to germ-line disease with Alu-based recombination events associated with cancer shown in Table 3. There are many more recombination than insertion events contributing to disease and the table of recombination events is not intended to be exhaustive in presenting all of the Alu/Alu recombinations that have contributed to human disease. In addition, there are many

recombination events that occurred between an Alu element and some other non-Alu-related sequence which may have been influenced by the presence of the Alu element (42). Although single Alu elements may contribute specifically to such recombination events, we have made no efforts to collect those data. The mutations resulting from Alu/Alu recombination include 33 mutations that are the result of germ-line recombination and 16 mutations that are the result of somatic events that led to cancer. Based on the calculations in the previous section, the germ-line recombination mutants would represent about 0.3% of mutants characterized. We expect that this number is an underestimate as mutation schemes aimed at detecting point mutants would often be expected to overlook large duplication and deletion events, and we have probably not reported all known Alu/Alu recombinations in the tables.

The data in Tables 2 and 3 show that Alu/Alu recombination events are highly biased towards specific genes. The first to show evidence for this was the LDLR gene, which has at least eight independent cases. It was also reported that these recombination events appeared to take place in a preferred location within the Alu element (42,43). These data suggested that Alu elements may represent hot spots for recombination by a mechanism that was more than simple homologous recombination. Multiple Alu/Alu recombination events have also occurred in the germ line involving two other genes.

TABLE 3
Alu/Alu Recombination and Cancer

Locus	Distribution	Disease	Reference
10 × ALL-1 (MLL)	Somatic	Acute myelogenous leukemia	(88-90)
2 × BRCA1	Somatic and kindreds	Breast cancer	(91,92)
MLH1	Two kindreds	HNPCC	(93)
TRE	Somatic	Ewing's sarcoma	(94)
RB	Common	Association with glioma	(95)
EWS	Subset of Africans	Protective against Ewing sarcoma?	(96)

Even more striking is the preferential recombination seen in somatic recombination. The All-1 gene which participates in a high proportion of acute leukemias is another hotspot for Alu/Alu recombination. This includes intragenic recombination which is the major cause of acute myelogenous leukemia in individuals without a cytogenetic defect, as well as a possible contribution to recombination between the All-1 gene and other chromosomal loci in causing more complex cytogenetic defects associated with leukemia (44-46).

The genes that show high levels of Alu/Alu recombination tend to have a large number of Alu sequences. Although Alu density may help contribute to this recombination, the correlation does not seem to hold up upon analysis of other Alu-rich genes. Therefore, it seems likely that some other factor contributes to the high recombination rates seen in these genes and that the Alu elements are likely to help in that process rather than to be the primary cause.

It has generally been found that longer stretches of sequence identity allow more efficient homologous recombination and that 300 bp of imperfect sequence identity would represent a relatively inefficient target (47). Therefore, as Alu elements accumulate random mutations after integration in the genome their recombination potential gradually decreases. Thus, early in primate evolution when a high proportion of Alu elements were closer matches to one another, Alu/Alu recombination may have contributed even more to the evolution and reshaping of primate genomes.

Based on the above considerations, one might expect the much longer L1 family of elements to contribute significantly to recombination, as well. Surprisingly, we are familiar with only two L1/L1 recombination events in the human genome (48). Therefore, it would appear that: (1) L1 elements are located in less recombinogenic regions of the human

genome; (2) the approximately 10-fold lower copy number of L1 elements is more than enough to offset their larger size in terms of probabilities of recombination; (3) some basic property of the Alu elements themselves makes them recombinogenic; or (4) the larger average spacing between L1 elements causes the vast majority of L1/L1 recombination events to be lethal. It is possible that all of these factors may contribute to this observed difference. Transient transfection experiments suggest that the third possibility may not be true since Alu sequences did not recombine more frequently than other control sequences (49). However, in their native chromatin environment, or in specific cell types or cell stimuli *in vivo*, Alus may still respond with higher recombination rates. We believe that the fourth possibility may be the dominant factor, however. The vast majority of Alu/Alu recombination events listed in the tables represent recombination between Alu elements within the same gene. This limits the effect of the recombination to a single gene defect. With their lower copy number and tendency to be located between genes rather than in genes, L1/L1 recombination events are likely either to involve only intergenic regions or to involve a much larger region that may cause defects in several genes simultaneously, resulting in loss of viability.

There is growing evidence that repetitive DNAs contribute to disease either through the mutations they cause during the retroposition process that forms them (16,50) or through recombination processes involving unequal cross-overs of repetitive elements. These recombination events may involve repetitive sequences of various repetition frequencies with the likelihood that longer and more perfect repeats that are near one another probably recombine well, while short, mismatched repeats (like Alu) recombine relatively poorly. However, the extremely high copy number of Alu elements makes them a

major factor in the molecular basis of human diseases.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health RO1 GM45668 to P.L.D., Department of the Army DAMD17-98-1-8119 to P.L.D. and M.A.B., and National Science Foundation SBR-9610147 to M.A.B.

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