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Retrotransposons Revisited: The Restraint and Rehabilitation of Parasites

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Retrotransposons, mainly LINEs, SINEs, and endogenous retroviruses, make up roughly 40% of the mammalian genome and have played an important role in genome evolution. Their prevalence in genomes reflects a delicate balance between their further expansion and the restraint imposed by the host. In any human genome only a small number of LINE1s (L1s) are active, moving their own and SINE sequences into new genomic locations and occasionally causing disease. Recent insights and new technologies promise answers to fundamental questions about the biology of transposable elements.

The seminal discovery that genomes contain pieces of DNA capable of moving to new locations challenged prevailing notions of genes as static "beads on a string" passed essentially unchanged from one generation to the next. Studying mosaic coloration in maize, Barbara McClintock described Dissociator and Activator "mutable loci" and with prescience called them "controlling elements" that regulate genes. The notion was at first poorly received.

Following the discovery of transposons in plants and bacteria, the presence of mobile DNA in eukaryotic species gained widespread acceptance. However, the concept of "controlling elements" gave way to disparaging terms such as selfish DNA and "junk DNA." Nevertheless, the notion of transposable elements as merely molecular parasites, benign at best and powerful mutagens at worst, that hijack cellular mechanisms for their own selfish propagation, seemed incomplete to some biologists. Given that evolution tends to dispose of that which is useless and harmful for a species, it was curious that the genome should be cluttered with so much "junk." Now we understand that genomes have coevolved with their transposable elements, devising strategies to prevent them from running amok while coopting function from their presence. Repetitive DNA, and retrotransposons in particular, can drive genome evolution and alter gene expression. Evolution has been adept at turning some "junk" into treasure.

There are two major groups of so-called "jumping genes" (Figure 1). Class II elements or DNA transposons comprise about 3% of the human genome and most move by a "cut and paste" mechanism. No currently active DNA transposons have been identified in mammals. Class I elements comprise three groups, all moving in a "copy and paste" manner involving reverse transcription of an RNA intermediate and insertion of its cDNA copy at a new site in the genome. Penelope-like elements form a diverse group, are apparently absent in mammals, and are in the very early stages of characterization (Gladyshev and Arkhipova, 2007). Retroviral-like or long terminal repeat (LTR) retrotransposons include endogenous retroviruses, relics of past rounds of germline infection by viruses that

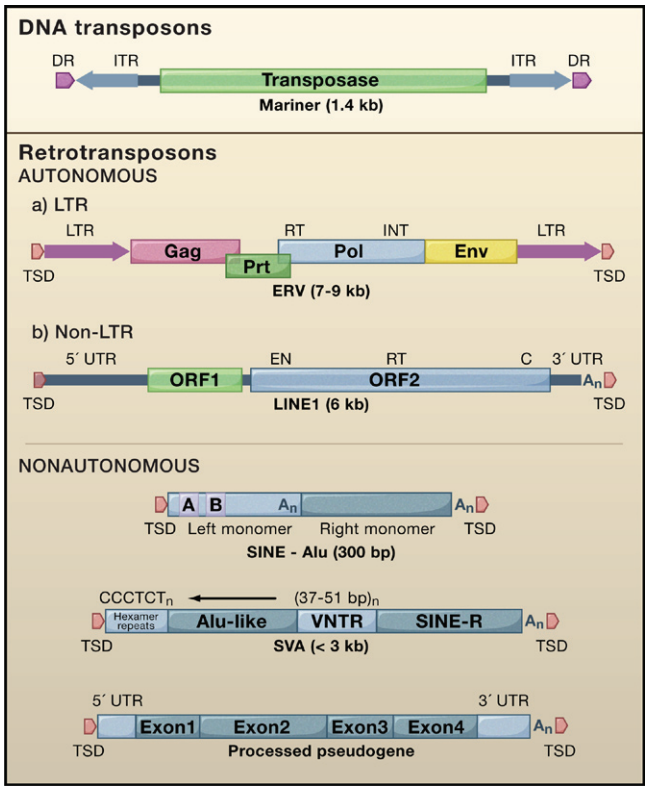

Figure 1. Types of Transposable Elements in Mammals
Mariner-like DNA transposons are inactive relics in mammalian genomes. Retrotransposons that contain many, but not all, of the activities necessary for their mobility are called autonomous. They are endogenous retroviruses (ERVs) and LINEs. The L1 is the only LINE known to be actively mobile in eutherian species. Nonautonomous elements, such as Alus and SVAs, are dependent on L1 for their mobility. Processed pseudogenes are spliced mRNAs copied and inserted in the genome by the L1s. Abbreviations: DR, direct repeat; ITR, inverted terminal repeat; Gag, group-specific antigen (capsid proteins); Prt, protease; Pol, polymerase; Env, envelope; RT, reverse transcriptase domain; INT, integrase domain; TSD, target site duplication; LTR, long terminal repeat; EN, endonuclease domain; C, zinc knuckle domain; A_n, poly(A); A/B, A- and B-box Pol III promoter; SVA, (SINE-R, *V*NTR, *A*lu); VNTR, variable number tandem repeats.

lost their ability to reinfect and became trapped in the genome. These elements undergo reverse transcription in virus-like particles by a complex multistep process. The transposition process for non-LTR retrotransposons is fundamentally different. RNA copies of these elements are likely carried back into the nucleus where their reverse transcription and integration occur in a single step on the DNA itself.

We focus here on recent discoveries in the biology of the two major groups of mammalian non-LTR retrotransposons, LINEs (long interspersed nucleotide elements) and SINEs (short interspersed nucleotide elements). SINEs are nonautonomous elements that do not encode protein and as a consequence require LINEs for their propagation. We also consider aspects of their biology that remain unclear but that involve important questions: How do retrotransposons jump? Where do they jump? When do they jump? Why don't they jump more? And what have been the consequences of all that jumping?

A Heavy Genomic Load

Non-LTR retrotransposons are as old as the earliest multicellular organisms, and their 15 clades have origins in the Precambrian Era of 600 million years ago (Eickbush and Jamburuthugoda, 2008). In mammals, members of four clades are known. The RTE (*retro*transposable *e*lement) clade, absent in humans and rodents, includes Bov-B elements from ruminant and afrotherians (a diverse clade that includes species such as elephants and aardvarks) as well as families prevalent in the opossum. LINE2 (L2) elements form 3% of marsupial genomes and almost 20% of monotreme (platypus) genomes. Although ancient and extinct, L2s occupy greater than 2% of human DNA and their impact has probably been significant. For example, Donnelly et al. (1999) showed that L2s are capable of acting as T cell-specific gene silencers. Low-copy number and degenerate CR1/L3 clade members form only 0.05%, 0.3%, and 0.5% of mouse, human, and platypus genomes, respectively, but have expanded to 2.3% of opossum DNA (Jurka et al., 2005; Gentles et al., 2007; Mikkelsen et al., 2007; Warren et al., 2008). Comparative analyses reveal significant differences between vertebrate lineages in their histories of expansion, contraction, and rates of activity for retrotransposon families (reviewed in Böhne et al., 2008; see SnapShot by P.K. Mandal and H.H. Kazazian in this issue). It seems that genomes differ in their abilities to control and coexist with these elements. Little is known of the biology of the non-L1 LINEs.

L1s, the only currently active autonomous transposons in humans, have been evolving during at least 160 million years (Myr) of mammalian radiation. Multiple active lineages of L1s coexisted in ancestral primates, but for the past 40 Myr, a single unbroken lineage of subfamilies has evolved (Boissinot and Furano, 2001; Khan et al., 2006). Expansion of L1s was massive, and roughly 500,000 copies occupy about 18% of the human genome. From 25 Myr ago the expansion slowed, and most insertions are (fortunately) molecular fossils—truncated, rearranged, or mutated and incapable of further retrotransposition. L1 has also been responsible for genomic insertion of 8000 human processed pseudogenes (many of which are transcribed, often in testes) and over a million SINEs (Zhang et al., 2003; Vinckenbosch et al., 2006). Most SINE 5′ ends are derived from Pol III-transcribed small cellular RNAs. B1s and Alus, the predominant SINEs of mice and men, originate from a portion of the 7SL RNA component of the protein signal recognition particle. Alus are about 300 bp in length and have a dimeric structure; B1s are monomeric. Other mammalian SINEs, such as mouse B2s, have tRNA sequence homology at their 5′ ends, and AmnSINEs of chickens and mammals contain both tRNA and 5S rRNA-like regions (Nishihara et al., 2006; see Review by Kramerov and Vassetzky, 2005). About 40,000 snoRNA/RTE LINE-derived chimeric retrotransposons have recently been found in platypus (Schmitz et al., 2008).

Many SINEs derived from tRNAs, notably in fish and reptiles, share 3′-end homology with a LINE family member from the same genome. In mammals these include Ther-1, Ther-2, Mon1, Bov-tA, and Bov-A2 SINEs and their related L2, L3, and Bov-B LINEs (reviewed in Ohshima and Okada, 2005). Presumably homologous SINE sequence binds LINE-encoded protein to foster retrotransposition, a hypothesis supported by cell culture assays with an eel SINE and its corresponding LINE (Kajikawa and Okada, 2002). Yet, except for its poly(A) tail, L1s lack sequence homology with any SINE.

Hominid genomes also contain SVAs (*S*INE-R, *V*NTR, *A*lu], which are composite elements apparently mobilized by L1s. Despite their small copy number (3000 in humans), SVAs are probably quite active, being highly polymorphic and the cause of five known cases of human disease (Ostertag et al., 2003; Wang et al., 2005). The coming million years or so will tell if SVAs are the "next big thing" in human mobile DNA.

On LINE Chat

The 6.0 kb full-length human L1 has a 900 nucleotide (nt) 5′ untranslated region (UTR) that functions as an internal promoter, two open reading frames (ORF1 and ORF2), and a short 3′ UTR that ends in the poly(A) signal and tail. The mouse L1 5′ UTR is distinguished by having tandem repeats. An unconventional termination/reinitiation mechanism translates ORF2, which encodes a 150 kDa protein (ORF2p) with endonuclease and reverse transcriptase activities (Alisch et al., 2006). More attention has focused on the 40 kDa ORF1 protein (ORF1p), mostly because it is expressed at much higher levels than ORF2p and is easier to study. While mutational analysis has shown ORF1p to be essential for retrotransposition, its precise role remains unclear, although it forms trimeric complexes and possesses nucleic acid chaperone activity in vitro. ORF1p has been detected in the cytoplasm and to a lesser degree in nuclei of carcinoma and germ cells and has been isolated in ribonucleoprotein (RNP) particles together with L1 RNA and ORF2p activity (Martin, 2006; Kulpa and Moran, 2006). Although several other proteins coimmunoprecipitate in ORF1p RNPs (Goodier et al., 2007), with the exception of a few transcription factors, little is known of the non-L1 proteins directly participating in the complex process of retrotransposition. Information on L1 RNP assembly and transport into the nucleus is fragmentary. Kubo et al. (2006) found no retrotransposition in quiescent G_0 cells but significant levels in nondividing G_1/S phase-arrested Gli36 tumor cells, suggesting active RNP transport across the nuclear membrane. (L1 biology is reviewed in Moran and Gilbert, 2002 and Babushok and Kazazian, 2007.)

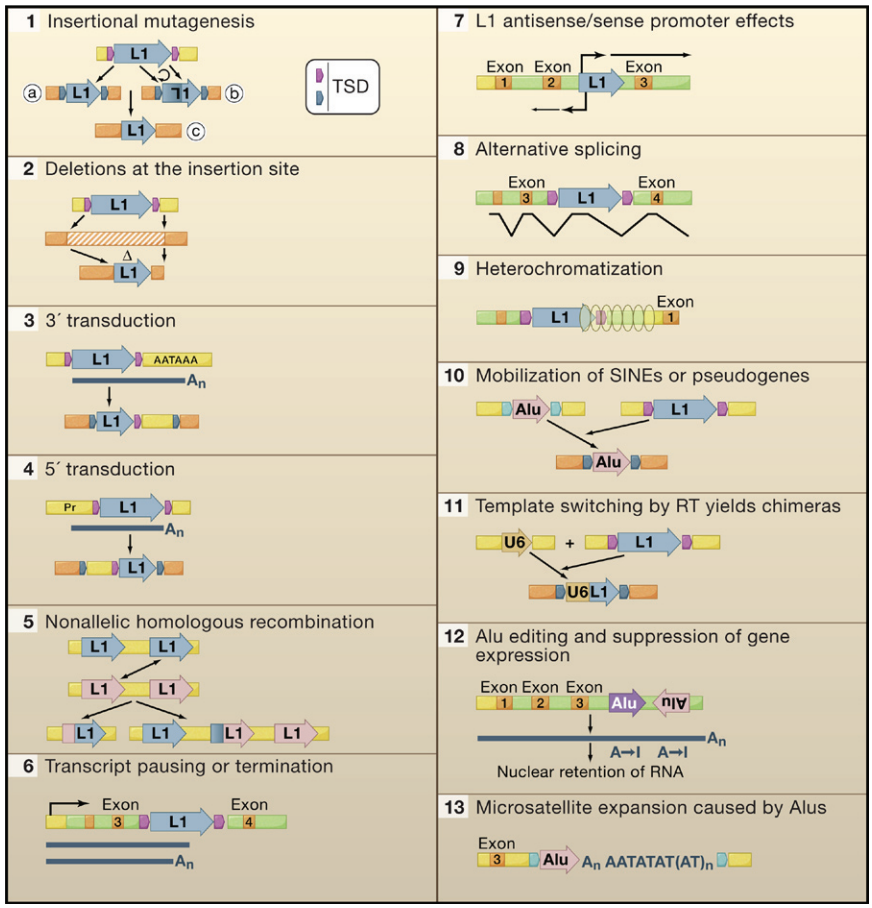

Figure 2. How Retrotransposons Affect the Cell

Insertions into new locations may be full length or 5′ truncated (1a) or contain inversions and deletions (1b). Endonuclease-independent insertions can also occur at low frequency (1c). Insertions may be accompanied by deletion at the insertion site (2). Flanking sequence, either 3′ or 5′ to L1, may be carried along with the element upon retrotransposition (3 and 4). Mispairing and crossing over between LINE or SINE elements can lead to deletions or duplications (5). Transcriptional pausing can occur within retrotransposon sequence, and poly(A) signals within an L1 can lead to premature termination of transcription (6). The antisense promoter in the L1 5′ UTR can produce new transcription start sites for genes upstream of the L1 on the opposite strand (7). Splice sites within L1s residing in introns can lead to new exons within genes (8). L1s can alter the chromatin state, thereby altering gene expression (9). L1 reverse transcriptase can mobilize Alu, SVA, mRNA, and small noncoding RNAs, leading to further genome expansion (10). Template switching of L1 reverse transcriptase from L1 RNA to other sequences, such as U6 RNA or Alu RNA, can produce chimeric insertions in the genome (11) (Buzdin et al., 2007; Garcia-Perez et al., 2007a). Editing by ADAR of inverted Alus can suppress gene expression by nuclear retention of the mRNA (12). Alu elements seed formation and expansion of microsatellites that have been occasionally associated with disease (13) (Arcot et al., 1995).

It is believed, but not confirmed in vivo, that L1s retrotranspose by target primed reverse transcription (TPRT), a process characterized for insect non-LTR retrotransposons. According to this model, L1 ORF2-encoded endonuclease nicks the bottom strand of target DNA to expose a 3′-hydoxyl that primes reverse transcription of L1 RNA. Second-strand DNA synthesis follows, possibly initiated by a second ORF2 molecule, and the integrant is resolved in a manner still poorly understood (Eickbush and Jamburuthugoda, 2008). Consistent with the TPRT model, short target site duplications, but occasionally deletions, are generated at the L1 insertion site. Evidence from endogenous human insertions and from cell culture assays indicates that L1 proteins have *cis*-preference, tending to bind their own encoding RNA. Interestingly, Alus and B1s co-opt some, but not all, of the L1 retrotransposition machinery given that ORF1p is not required for insertion (Dewannieux et al., 2003). Clearly a subset of non-L1 RNAs, including Pol III transcripts and RNAs associated with the nucleolus, are preferred targets for retrotransposition (Buzdin et al., 2007). Detailed "ribonomic" studies of the RNA components of the L1 RNP are required.

Knowledge of the mechanisms of L1 retrotransposition has lagged behind the exquisitely detailed studies of insect R1 and R2 elements and yeast and bacterial group II introns. Cell culture assays available for both L1 and Alu retrotransposition (Moran et al., 1996; Ostertag et al., 2000; Dewannieux et al., 2003) and improving in vitro assays for L1 endonucleolytic cleavage, TPRT, and reverse transcription should move the field forward more rapidly (Cost et al., 2002; Kulpa and Moran, 2006).

On Good and Evil

There are 65 known human disease-causing insertions of L1s, Alus, and SVAs. However, simple insertion mutation is but one of a startling number of ways that retrotransposons reshuffle the genome and alter gene expression, and this list is sure to grow (Figure 2; reviewed in Han and Boeke, 2005; Medstrand et al., 2005; Belancio et al., 2008).

Retrotransposition occasionally generates target site deletions, some quite large, as illustrated by an L1 insertion that obliterated 46 kb of the gene encoding pyruvate dehydrogenase complex, component X *(PDHX)* and caused pyruvate dehydrogenase complex deficiency. Another example is the deletion of an entire HLA-A gene likely caused by an SVA and resulting in leukemia (Gilbert et al., 2002; Symer et al., 2002; Mine et al., 2007; Takasu et al., 2007). Nonretrotransposon DNA can also be added to the genome by a phenomenon termed 3′ transduction (Moran et al., 1999). About 10% to 20% of the time, 3′ end processing ignores the weak L1 poly(A) signal and utilizes instead a downstream signal, causing flanking sequence to be carried along with the retrotransposon to the new site of insertion. Less frequently, transcription initiates

from a chance upstream promoter and the L1 mobilizes 5′ flanking sequence. In these ways, retrotransposons may serve as vectors for exon shuffling and the creation of new genes. One definitive instance involved an SVA transduction of the entire AMAC1 (acyl-malonyl condensing enzyme 1) gene to generate multiple transcriptionally active copies in the human genome. The SINE-R component of the SVA appears to drive transcription of the SVA-AMAC chimeras (Xing et al., 2006).

Recombination between retrotransposons causes deletions, duplications, or rearrangements of gene sequence. This is especially true for Alus, which have been implicated in almost 50 disease-causing recombination events. Moreover, Alus are significantly enriched at boundaries of human segmental duplications. Mouse segmental duplications are enriched in LTR and recent LINE1 retrotransposons but (unlike humans) not SINEs (Bailey and Eichler, 2006; She et al., 2008). Comparisons of the human and chimp genomes identified more than 10,000 species-specific transposable element insertions that have occurred since these species diverged 6 million years ago. About 95% of these inserts were L1s, Alus, and SVAs, along with some endogenous retroviruses (summarized in Mills et al., 2007). By examining orthologous loci, the Batzer lab has estimated the extent of retrotransposon-mediated give and take: approximately 900 kb and 7.5 Mb of primate sequence lost, respectively, at the sites of Alu and L1 insertions, 400 kb of human DNA removed by Alu-Alu recombination, and 53 kb inserted by human SVA-mediated DNA transductions (Xing et al., 2007). With recent publication of the finished mouse genome, draft assemblies for 20 other mammalian species, and sequencing of 26 more mammals in progress, a wealth of data from comparative analyses is coming soon.

The greatest impact of retrotransposon insertions may be on the expression of nearby genes. Ongoing retrotransposition peppers genomes with new splice sites, adenylation signals, promoters, and transcription factor-binding sites that can reorganize gene expression and build new transcription modules, as hypothesized by Britten and Davidson (1971) over 35 years ago. For instance, a mouse B1 subfamily (B1-X35S) is distinguished by three mutations that recruit transcription factors Slug and dioxin receptor Ahr, causing repression of physiologically important genes (Roman et al., 2008).

The recent discovery that thousands of DNA fragments are highly conserved in sequence and sometimes synteny among evolutionarily distant vertebrate genomes, and that many of these fragments originate from transposable elements, especially SINEs and LINEs, has sparked interest in the field. Such conservation by strong purifying selection predicts function. Several superfamilies of these preserved sequence bits have been identified, and with them intriguing tales of exaptation (the adoption of a feature that had a different function in its ancestral form). For example, some SINEs contain a conserved central 65 bp "core." These were first described as mammalian-wide interspersed repeats (MIRs) and are now considered members of the CORE-SINE superfamily. The pro-opiomelancortin gene neuronal enhancer nPE2 is a CORE-SINE (Santangelo et al., 2007). Bejerano et al. (2006) discovered that members of the ancient LF (lobed-fin) SINE family, conserved in sequence in coelacanth fish and land tetrapods, have been exapted as exon fragments, and apparently in one instance as the distal enhancer of the neuro-developmental gene ISL1. This same group also found that >10,000 conserved mammalian transposon sequences are preferentially retained near genes involved in the regulation of transcription and development (Lowe et al., 2007). Most recently, AmnSINEs, members of the Deu-SINE superfamily, have been shown to exert enhancer activity on Fgf8 and Satb2 gene expression in the developing mammalian forebrain (Sasaki et al., 2008).

The L1 is especially adept at disrupting transcription of its host genes. L1 sequences are found in the noncoding regions of about 80% of human genes, and L1 density inversely correlates with mRNA expression of those genes. Resident L1s cause pausing in transcriptional elongation and premature transcript termination due to cryptic polyadenylation signals (Perepelitsa-Belancio and Deininger, 2003, Han et al., 2004). Possessing functional promoters on the sense and antisense strands of its 5′ UTR, an L1 can also initiate both upstream and downstream transcription. Many transcripts in the expressed sequence tag (EST) databases originate from the antisense promoter, which may even regulate tissue-specific expression of some genes (Mätlik et al., 2006). Promoter co-option is not limited to L1s. Analyzing a cluster of microRNA genes on human chromosome 19, Borchert et al. (2006) found some transcribed by upstream Alu Pol III promoters, as well as 50 others overlapping Alus and other repeats. Indeed, high-throughput analyses of cDNA ends reveal that tens of thousands of antisense transcripts originate from both class I and class II transposable elements sitting within genes (Conley et al., 2008).

Sequence from transposable elements may be captured and incorporated into gene mRNAs, a process called "exonization." It has been estimated that 5% of human alternatively spliced internal exons originate in Alus, a phenomenon fostered by "pseudosplice sites" and occasionally RNA editing that alters the nucleotide sequence of transcripts (for reviews see Häsler et al., 2007 and Sorek, 2007). A recent comprehensive study by Zhang and Chasin (2008) confirms that high copy number repeats are the most important sources of new genes in humans and mice, and by implication new proteins. Nekrutenko and Li (2001) claimed that 4% of human protein coding regions contained transposable element sequence (mostly L1s and Alus), although Gotea and Makalowski (2006) believed 0.1% a more reasonable estimate, arguing that most alternative transcripts containing transposable elements do not generate actual protein. Others make different assumptions and place the number significantly higher (reviewed in Piriyapongsa et al., 2007). Retrotransposons may also be exapted as noncoding genes, as in the case of the primate *BC200* small RNA gene that originated from an Alu and the analogous rodent *BC1* gene that derived from a retrotransposed tRNA (and itself subsequently spawned a subclass of ID SINEs). Both *BC1* and *BC200* small RNAs function as translational repressors in neurons by targeting the catalytic activity of factor eIF4A (Volff and Brosius, 2007; Lin et al., 2008).

Retrotransposon-mediated pseudogene insertions have also exonized to create functional fusion genes. Retrotransposition of mRNA from an X-linked gene into the intron of an autosomal gene generated a bicistronic and new retrogene

product, *Utpl46*, important in mouse spermatogenesis. Intriguingly, X-to-autosome retrotransposition is not uncommon, and it has been proposed as a mechanism that modulates gene dosage effects of X chromosome inactivation (Bradley et al., 2004). The *POTE* group 3 fusion gene subfamily formed after an actin pseudogene inserted immediately downstream of a *POTE* gene precursor prior to the Old World monkey/ape divergence (Lee et al., 2006). Retrotransposition of the cyclophilin gene into an intron of *TRIM5* created a fusion gene that explains resistance of the owl monkey to HIV-1 infection (Sayah et al., 2004). Also, Babushok et al. (2007) described how intergenic splicing of two RNAs transcribed from neighboring genes (which encode phosphatidylinositol-4-phosphate 5-kinase, PIP5K1A, and the 26S proteasome subunit, PSMD4) generated a single RNA template that was reverse transcribed and inserted into the genome of an early hominoid as a fusion retrogene.

Overexpressing L1 ORF2 protein may be bad for the cell. Gasior et al. (2006) transiently transfected an L1 construct into tumor cells and discovered double-strand chromosome breaks in numbers significantly greater than expected for target-primed reverse transcription alone. Damage depended upon an intact endonuclease domain and the DNA damage-response kinase ATM (although the effect disappeared by 48 hr). The results are consistent with the observation that L1 overexpression may induce apoptosis and senescence in some cell lines (Belgnaoui et al., 2006; Wallace et al., 2008). Although these observations require validation in vivo, if endogenous L1 expression induces chromosome breaks independent of retrotransposition, this implies a greater role for L1s than previously thought in fostering recombination and translocation. Such genetic instability would be aggravated in germ cells, stem cells, and cancers where L1 expression is highest (see below).

The Search for Meaning

The increasing number of documented instances of gene expression modified by transposable elements support McClintock's original hypothesis but still do not explain why the cell has allowed so many mobile elements to accumulate while evolving multiple strategies to control their activity (as discussed below). The hunt is on for a more overarching explanation of why half our genome is not just half junkyard. Several studies report that expression of retrotransposon RNA and proteins has marked effects on cell function and metabolism.

Endogenous reverse transcriptase activity has been detected in some tumors and patients with certain pathologies. Following upon studies identifying elevated reverse transcriptase activity in spermatozoa, epididymis, oocytes, and early-stage embryos (Evsikov et al., 2004; Crowell and Kiessling, 2007), the Spadafora lab investigated effects of inhibiting this activity with antireverse transcriptase antibodies, the nonnucleoside inhibitor nevirapine, and siRNA against L1s and human endogenous retroviruses (HERVs) and posited intriguing roles for endogenous reverse transcriptase in early normal embryonic development, cell differentiation, and tumor progression (Spadafora, 2008). A caveat is the difficulty of controlling off-target effects in broad-based inhibition studies, especially when so many transposable elements reside in genes. Also, a recent study reported inhibition of L1 retrotransposition in cell culture by nucleoside analog reverse transcriptase inhibitors but not by nevirapine (Jones et al., 2008).

The relationship between retrotransposons, DNA damage, and cell stress is poorly understood. Genotoxic poisons, radiation, heat shock, viral infection, and heavy metals all induce SINE or LINE expression (Li et al., 1999; Kale et al., 2006; reviewed in Farkash and Prak, 2006). This raises the question of why a cell under duress would permit upregulation of mobile elements whose mutagenicity might compound damage inflicted by the original stress. Perhaps they play some role in coping with stress (see Capy et al., 2000).

Upon heat shock, transcription of many housekeeping genes is repressed while temperature response genes, such as the chaperone *HSP70*, are activated. Recent investigations suggest that coincident increase in SINE transcription may be part of this adaptive response. The Kugel and Goodrich groups showed in vitro that mouse *B2* RNA and human dimeric *Alu* RNA occupied promoters of heat shock-repressed genes, bound Pol II at high affinity, and caused its inactivation at these promoters. Repression was partially relieved by an antisense oligonucleotide against the Alu RNA. Alu RNAs were not found at the promoters of activated *HSP70* or *U2* genes (Mariner et al., 2008). Translation inhibition likely requires Alu RNA to be bound in an RNP with signal recognition particle (SRP) 9/14 proteins (Häsler and Strub, 2006).

Retrotransposons have also been linked to DNA-damage repair. Morrish et al. (2002) demonstrated an endonuclease-deficient pathway of L1 integration in Chinese hamster ovary (CHO) cells defective in nonhomologous end-joining (NHEJ) double-strand break repair. Furthermore, in CHO cells impaired for both NHEJ and telomere maintenance due to DNA-PKcs deficiency, L1s integrated at dysfunctional telomeres, leading the authors to propose that endonuclease-independent retrotransposition is an ancestral remnant of an RNA-mediated DNA-repair mechanism extant before the acquisition of endonuclease by non-LTR retrotransposons (Morrish et al., 2007). Endonuclease-independent insertions are indeed seen in the human genome, although only one de novo human L1 insertion at a breakpoint has been reported (Sen et al., 2007, Liu et al., 1997). In yeast, double-strand break repair by Ty1 LTR retrotransposons is well known. Drawing a connection between telomere targeting and retrotransposons across a broad swath of eukaryota, a subset of Penelope-like retrotransposons that lack endonuclease but share common ancestry with TERT (telomerase reverse transcriptase) have been found at telomeres in rotifers, fungi, stramenopiles, and plants. HeT-A, TART, and Tahre non-LTR retrotransposons play a critical role in telomere maintenance in the fruit fly *Drosophila*, a species without active telomerase (Pardue et al., 2005; Gladyshev and Arkhipova, 2007). Indeed, TERT, which maintains the ends of chromosomes in most species, itself is thought to derive from an ancient non-LTR retrotransposon (Nakamura and Cech, 1998).

In placental mammals, L1 sequences may play a role in X-inactivation, the process of silencing many genes on one X chromosome early in female embryogenesis. Inactivation

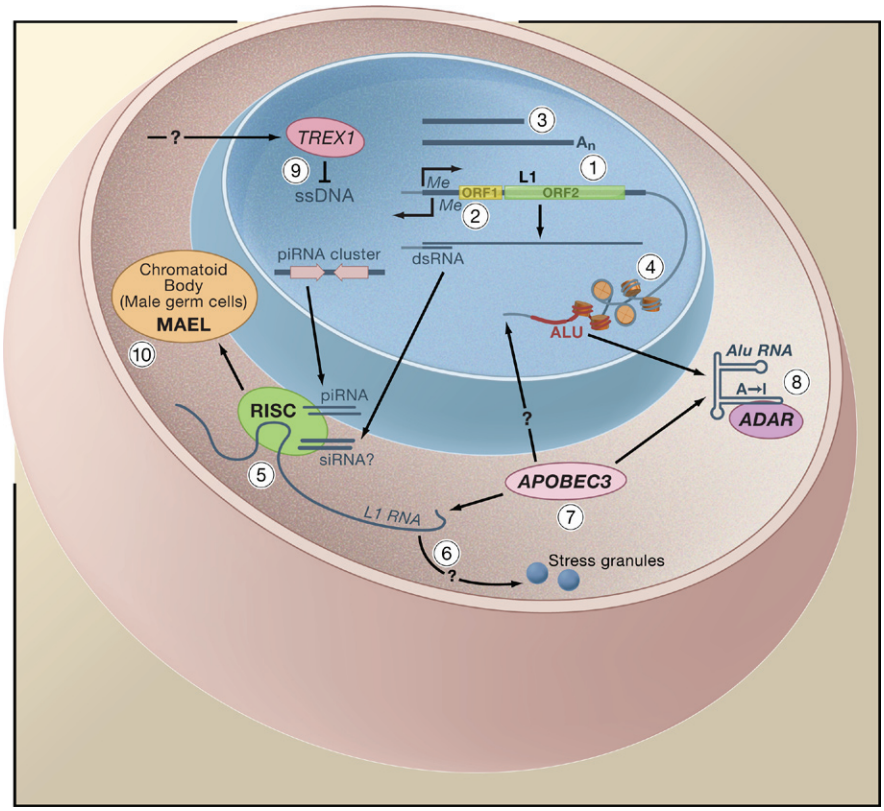

Figure 3. How the Cell Affects Retrotransposons

Truncation, mutation, and rearrangement inactivate the L1 (1). Methylation of the L1 5′ UTR inhibits its expression in cells (2). Pausing and premature termination of transcription inhibits L1 expression (3). Heterochromatization of L1s and Alus suppresses expression (4). Double-strand RNA produced by transcription from both the sense and antisense promoters may inhibit L1 retrotransposition by an RNA interference mechanism (5). Small piRNAs inhibit L1 expression in germ cells (5). ORF1 protein and L1 RNA may be sequestered in stress granules in the cytoplasm (6). Various APOBEC3s reduce L1 retrotransposition in cell culture by an unknown mechanism unrelated to cytosine deamination. It is unclear whether the effect occurs in the nucleus or cytoplasm (7). Alus are altered by ADAR RNA editing (8). TREX1, a DNA nuclease, prevents accumulation of retroelement reverse-transcribed single-strand DNA. Although predominantly cytoplasmic, TREX1 can translocate to the nucleus, its presumed site of action on L1 single-stranded DNA (ssDNA) (9). MAEL, present in the chromatoid body in male germ cells, induces silencing of retrotransposons in mice (10).

involves the activity of XIST, a small noncoding RNA that coats the inactive X. Mary Lyon hypothesized that L1 sequences function as "booster" elements, *cis*-acting sequences fostering the spread of inactivation. Indeed, L1s are enriched on the X chromosome and their density correlates with the degree of inactivation, although interaction of L1s and XIST RNPs remains to be shown. Marsupial species lack an XIST homolog, and L1 density is not enriched on the opossum X (Mikkelsen et al., 2007; reviewed in Lyon, 2006).

With so many ways that insertions of transposable elements can influence genes, a modest increase in transposition could drive evolutionary change at a rate not possible by random nucleotide mutation. This could advantage a species faced with a deteriorating environment and the need to adapt or die. Slotkin and Martienssen (2007) noted, "stress-reactivated TEs (transposable elements) might generate the raw diversity that a species requires over evolutionary time to survive the specific stress," and harkened back to Barbara McClintock who proposed that transposons were activated in response to genomic challenge. Interestingly, Ohshima et al. (2003) discovered simultaneous bursts of Alu and processed pseudogene amplifications 40 to 50 Myr ago, coincident with the radiation of the higher primates and possibly linked with the activity of several L1 subfamilies. Using a smaller data set, Devor and Moffatt-Wilson (2003) identified additional pseudogene peaks at 38 Myr ago, following the Catarrhini divergence, and 23 Myr ago, a time when hominoid species began to appear. Investigation of more genomes should show if transposition bursts accelerate speciation.

Preemptive Strikes

Given that retroelements affect the genome in so many ways, it is not surprising that the cell has marshaled various forces to regulate their activity (Figure 3). Foremost is transcript malformation due to splicing and premature polyadenylation (as described above) and DNA methylation. A majority of methylated cytosines in human genomic DNA occur in repetitive sequences, and indeed it has been proposed that DNA methylation evolved primarily as a defense mechanism against transposable elements (Yoder et al., 1997). Maintenance DNA methyltransferase Dnmt1, and de novo methyltransferases Dnmt3a and Dnmt3b, are all involved in methylation of intracisternal A particle (IAP) retrotransposons in mouse embryos and/or germ cells (Walsh et al., 1998; Kato et al., 2007). Dnmt3L, a Dnmt3a/b homolog without a catalytic domain, is expressed in premeiotic spermatagonia and when absent causes complete loss of post-zygotene spermatocytes. Male mice lacking Dnmt3L display hypomethylation at the promoters of both IAPs and L1s and a dramatic increase in their transcription in spermatocytes and spermatagonia (Bourc'his and Bestor, 2004). In an analogous manner, germ cells of female mice lacking lymphoid-specific helicase (Lsh) show defective chromosome synapsis and hypomethylation with loss of IAP silencing. Lsh is a homolog of *Arabidopsis* Ddm1 (decrease in DNA methylation I), a SNF2 ATPase-type gene that promotes chromatin condensation and transposon silencing (De La Fuente et al., 2006).

Chromatin condensation may suppress the activity of transposable elements. Conversely, structural change initiating within transposable elements may nucleate local DNA structure. Methylation spreads from plant SINEs into flanking

regions. Mouse B1 SINEs can also act as "methylation centers," and methylation spreading from human Alus has been implicated in silencing some tumor suppressor genes (Graff et al., 1997; Arnaud et al., 2000). At the chromatin level, Kondo and Issa (2003) found Alus to be a target for H3K9 histone methylation. On the other hand, Martens et al. (2005) detected enriched lysine methylation at mouse satellite repeats and IAP retrotransposons but not at L1s and only weakly at B1s. Such studies assay average histone modifications across a genome; most informative would be comparisons of local chromatin structure at individual loci polymorphic for an insertion, or in cell culture lines in which a retrotransposon is present or absent. Although promiscuous spread of altered chromatin can engulf and repress proximal genes, one may also speculate that the numerous repetitive elements scattered about the genome play a role in organizing higher-order chromatin structure.

It has been proposed that RNA-induced silencing is an ancient form of immunity that evolved to quell viruses and transposable elements. Control of transposable elements by RNA intereference (RNAi) was identified first in plants and subsequently in other eukaryotes. Micro-RNAs (miRNAs) and small-interfering RNAs (siRNAs) are cleaved from short hairpin and double-stranded RNA precursors by Dicer endonucleases and bound by Argonaute proteins in a large multiprotein complex called RISC. RISC is targeted to homologous sequence in cellular RNAs, forcing their degradation or suppressing translation. Transcription of murine L1 and IAP elements is elevated in embryonic stem cells of Dicer knockout mice (Kanellopoulou et al., 2005). Interestingly, about 12% of miRNAs in the mirBASE database have significant homology with all classes of transposable elements—L2, MIR, and DNA transposon sequences being most common. Also, almost 30 human miRNAs share 5′ seed target complementarity to a conserved site within Alus (Smalheiser and Torvik, 2005, 2006). The presence of transposons within mRNAs means that many genes may also be regulated by miRNAs related to transposable element sequence.

Forward and reversed retrotransposons sitting within transcribed genes, along with both sense and antisense activity from the promoter of the L1 5′ UTR, predict a pool of double-stranded RNAs available for siRNA production. Recent deep sequencing of small RNA libraries from mouse oocytes found a preponderance of L1, LTR retrotransposon, and pseudogene fragments (Watanabe et al., 2008; Tam et al., 2008). Cotransfecting L1 siRNAs diced in vitro with an L1 active in cell culture reduced its retrotransposition (Soifer et al., 2005). Depletion of Dicer in cultured cells doubles L1 retrotransposition, although transcription is modestly increased (Yang and Kazazian, 2006). Double-stranded small RNAs hybridizing with probes from the L1 5′UTR were detected but not confirmed as bona fide siRNAs. Whether or not siRNAs suppress L1s remains an open question.

Evidence is stronger that a second class of small RNA silences mammalian retrotransposons. In *Drosophila*, the Piwi group of Argonaute proteins bind 26 to 31 nt piRNAs that are enriched in sequences of retrotransposons and other repetitive elements. piRNAs are apparently not derived from double-strand RNA precursors but from single-strand RNA transcribed from piRNA gene clusters. Disruption of piRNA pathways causes marked derepression of retrotransposons in the *Drosophila* male germline (reviewed in Klattenhoff and Theurkauf, 2008). Deletion of two RNAs, *Nct1/2* within a piRNA cluster on mouse chromosome 2, increased L1 RNA and ORF1p expression many fold in spermatocytes (Xu et al., 2008). Mouse Piwi proteins, Mili, Miwi, and Miwi2, are important for spermatogenesis. Although initial studies reported that their bound piRNAs are depleted in repeat-derived sequences in pachytene-stage male germ cells, more recent experiments have identified prepachytene and fetal germ cell piRNAs enriched in repeats. Loss of Mili and Miwi2 impairs de novo IAP and L1 promoter methylation, together with a reduction in repeat-associated piRNAs and derepression of retrotransposon transcription in male germ cells of newborn mice (Kuramochi-Miyagawa et al., 2008; reviewed in O'Donnell and Boeke, 2007, Aravin et al., 2007; Girard and Hannon, 2008). The mechanism linking piRNAs with DNA methylation is not understood.

Both L1 RNA and ORF1 protein accumulate in stress granules, which are cytoplasmic aggregates that form in stressed cells to store inactive RNAs and occasionally target them for degradation (Goodier et al., 2007; J.L.G., unpublished data). One interpretation is that cells sequester L1 RNPs so they do no harm. Stress granules are associated with another somatic RNA granule, the processing body (P body), and share components including members of RISC. In male germ cells of mice, the perinuclear chromatoid body is analogous to P bodies and is a reservoir for miRNAs and Argonaute/Dicer proteins (Kotaja et al., 2006). It is also the abode of MAEL protein (homolog of the *Drosophila* nuage protein Maelstrom), recently shown to be important for suppression of retroelement transcription in testes and control of IAP and L1 retrotransposition in cell culture (Soper et al., 2008). Loss of MAEL also results in impaired DNA methylation and spermatogenesis, drawing an interesting parallel with the phenotypes of mice lacking Mili or Miwi2.

Another component of the cell's arsenal for retrotransposon control is nucleic acid editing. The single cytosine deaminase gene *Apobec3* in rodents has expanded to seven variants in the primate lineage. APOBEC3G was first identified through its ability to inhibit HIV strains with defective *vif* (viral infectivity factor) genes by causing C to U hypermutation of minus strand DNA. Human APOBEC3A, 3B, 3C, and 3F are inhibitors of LINE-1 retrotransposition in cell culture. The effect of APOBEC3G on L1s is uncertain due to contradictory evidence, although it strongly downregulates Alu retrotransposition. Intriguingly, suppression of Alu, L1, and mouse IAP and musD LTR retrotransposons by APOBEC3 proteins is not accompanied by hypermutation and in the case of Alu inhibition does not even require an active cytosine deaminase domain. This alternative path of APOBEC activity, which may involve sequestering retrotransposon RNAs in high-molecular-weight cytoplasmic complexes, requires more detailed investigation (summarized in Schumann, 2007; Chiu and Greene, 2008).

In contrast to the APOBEC family, the ADAR (adenosine deaminase acting on dsRNA) family of RNA-editing enzymes catalyzes adenosine to inosine changes in double-stranded RNA. In the human genome extensive editing of the noncoding region of genes occurs largely within Alus, as well as some LINEs. Mouse B1 SINEs are edited but to a lesser degree. ADARs bind nonspecifically to any dsRNA, and Alus may be favored

targets due to their dimeric structure and occasional head-to-tail orientation (reviewed in Nishikura, 2007). An examination of two genes, *Nicolin1* and *Lin28*, found that inverted Alus in their 3′ UTRs were hyperedited and suppressed expression of the genes in a manner correlated with nuclear retention by binding protein $p54^{nrb}$. This phenomenon might be extrapolated to some of the 333 other genes that also contain inverted Alus in their 3′ UTRs (Chen et al., 2008). Although a role for ADAR editing in the control of transposable elements remains unclear, intriguing connections with chromatin remodeling and repression of interfering RNA production have been proposed (Nishikura, 2007).

Viral nucleic acids trigger interferon production, a critical component of the immune response to infection. It is desirable, however, that RNA and cDNA from endogenous retroelements do not, as overproduction of interferons may initiate autoimmune disease. A new study by Stetson et al. (2008) revealed that the 3′-5′ exonuclease TREX1 targets reverse-transcribed single-strand DNA and prevents its accumulation in mice. Overexpression of TREX1 dramatically reduced retrotransposition rates of L1 and IAP elements in cell culture. This raises interesting questions, such as how TREX1 complexes recognize their nucleic acid targets, and what part reverse transcriptases may play in autoimmunity.

Losing Control

Suppression of retrotransposition is relaxed in some cell types. Early studies detected L1 ORF1p and full-length RNA transcripts in mouse embryonal carcinoma cells and testicular germline tumors, and in several human tumorigenic cell lines, but rarely in differentiated somatic cells. Cell culture retrotransposition assays also detect robust retrotranspositon in many tumor lines but not in most primary nontransformed cells. The reasons for repression in normal cells are unclear, but it is possible that mutations that predispose a cell to become cancerous also activate retrotransposition. Failure of transcriptional control might occur for a number of reasons. Altered miRNA profiles and reduced ADAR editing characterize some tumor phenotypes (Paz et al., 2008). Dramatic changes in global methylation, with consequent repatterning of the transcriptome, frequently involve hypomethylation of Alus, LINEs, and HERVs, although it remains untested if this demethylation leads to increased endogenous retrotransposition. Elevated retrotransposition within a developing cancer could alter the tumor phenotype or hasten tumor progression (discussed in Schulz, 2006; Hauptmann and Schmitt, 2006). Furthermore, an unlucky hit into a tumor suppressor gene of a normal cell could set it on the path to cancer, as apparently occurred in a case of colon cancer (Miki et al., 1992). Synergy between retrotransposition and cancer remains an unexplored question.

If we think of retrotransposons as genetic parasites, it makes sense that they should evolve to be active in the germ line, and so pass on to future generations, but remain inactive in somatic cells and not risk harming the host. This notion is challenged by recent evidence for activity in certain normal somatic cells. Elevated levels of L1 protein have been detected in human testicular vascular endothelial and Leydig cells (Ergun et al., 2004). Employing a chimeric L1-adenoviral delivery system to improve transfection, Kubo et al. (2006) detected retrotransposition (>2%–3%) in primary fibroblasts and hepatocytes. Cultured human embryonic stem cells support some retrotransposition (Garcia-Perez et al., 2007b). Muotri et al. (2005) reported a propensity for retrotransposition in neuronal progenitor cells in cell culture, and in a mouse transgenic for a human L1, and speculated that derepressed retrotransposition could contribute to neuronal plasticity and "uniqueness of individuals within a population." Equally likely, however, are insertion-induced neural pathologies, including cancer.

Expression of some retrotransposons is high in late oocytes and early-stage embryos, diminishing in later stages. Peaston et al. (2004) found that 13% of transcripts from mouse EST libraries of late-stage oocytes contained sequence from transposable elements, most often beginning within LTRs of MaLR and endogenous retrovirus-L class retrotransposons and extending into flanking sequences. Erasure of methylation during preimplantation development may open windows of opportunity for retrotransposition. Of course, retrotransposons can only become established in the genome if insertion occurs in germ cells or their progenitors, or in early-stage embryonic cells destined to become germ cells. Male germline insertions have been observed in two mouse models of human L1 retrotransposition, and early somatic insertion in a third, although in each case the transgene included a heterologous promoter with unknown effects on cell-specific activity (Ostertag et al., 2002; Prak et al., 2003; An et al., 2006). More recent transgenic mouse and rat studies in our lab also suggest that retrotransposition is elevated in early embryogenesis (H.K. and H. Kano et al., unpublished data). On the clinical side, an insertion into the *CYBB* gene of one patient caused chronic granulomatous disease and revealed that retrotransposition may occur either during meiosis I in female germ cells or in the early embryo (Brouha et al., 2002). A second patient suffering from X-linked choroideremia caused by a full-length L1 insertion led to the discovery that the mother was both a germline and somatic mosaic, evidence that retrotransposition can occur early in human embryonic development (van den Hurk et al., 2007). These observations lead us to speculate that retrotransposons, along with other known factors such as toxins and chromosome abnormalities, may contribute to the high rate of spontaneous abortions (estimated at 50% of pregnancies) that occur in humans.

Valuable information concerning mobile element regulation and cell development will be gained by profiling de novo insertion events in tumor versus normal cells, in the developing embryo, in differentiating stem cells, and so on. Techniques for "fingerprinting" and isolating new L1 insertions have been described and are being refined (Sheen et al., 2000; Badge et al., 2003). Deep sequencing techniques, such as 454/Roche and Solexa/Illumina, together with tiling array and other microarray technologies, are the new tools available for rapidly cataloguing novel retrotransposition events across entire genomes.

Private LINEs, Private SINEs

The Human Genome Project and subsequent sequencing efforts revealed only 0.1% variation among individual genomes. Pairs of individuals from different populations are often more similar than pairs of individuals from the same

population, supporting a recent evolutionary origin for modern humans. It is comforting that we are all essentially the same under the skin.

Much of the individual genome variation that does exist is due to chromosome rearrangements, copy number variants, single-nucleotide polymorphisms (SNPs), and repetitive elements. Although the Human Genome Project indicated about 2000 L1s and 7000 Alus specific to humans, in reality the number is far greater due to individual polymorphisms and ongoing retrotransposition. Up to 5% of newborn children are estimated to have a new retrotransposon insertion (Cordaux et al., 2006). Bennett et al. (2004) examined DNA sequencing trace data derived from 36 humans from diverse populations to identify 25% to 35% of the more than 2000 estimated transposon-related insertion/deletion (indel) polymorphisms. Akagi et al. (2008) found inbred mouse strains to have a significantly greater number of transposable element indels, most due to L1s, and consistent with many more potentially active mouse L1s than human L1s (Goodier et al., 2001; Zemojtel et al., 2007). Interestingly, L1 activity appears to have been extinguished in some South American rodents and megabats (Cantrell et al., 2008).

Ongoing activity of retrotransposons, their random and stable integration, and their essentially homoplasy-free nature have created a pool of polymorphic and fixed alleles that are increasingly valuable for evolution, population, and forensic analyses (reviewed in Xing et al., 2007). How many polymorphic elements are active in a human genome? Brouha et al. (2003) determined that 44% of young intact L1s were polymorphic for presence in the genome and that about half of these were capable of retrotransposition in cell culture, predicting 80 to 100 potentially retrotransposition-competent L1s in the average diploid genome. Interestingly, only a small number are very active, or "hot," for retrotransposition, and these have accounted for most de novo insertions. However, when several of these "hot" Ta-1 L1s were examined across diverse human populations, considerable individual allelic variation affected their ability to retrotranspose (Seleme et al., 2006). Epigenetic variation in methylation and chromatin structure may partially account for variation in the regulation of some homologous retrotransposons between different individuals. Mapping a person's unique complement of mobile DNA, their "transposome," and its activity is a hot topic being pursued in several labs. It would not be unreasonable to expect that when various human populations are analyzed, the total extant number of active L1s may be in the hundreds of thousands.

Gene expression levels, termed "the expression phenotype," are both highly variable and heritable in humans and other species. Morley et al. (2004) assayed CEPH cell lines from 14 pedigrees and found 3554 genes that varied in expression levels. It is unclear to what degree polymorphic transposable elements exert an effect, but it could be significant. Variegating repression of allelic transposable elements can cause tissue or individual differences in the silencing of proximal genes. The phenomenon of "transcriptional interference" was dramatically illustrated when a mouse IAP inserted upstream of the yellow agouti gene and, due to differential methylation of a cryptic promoter in the IAP, caused an extreme range of coat colors in the offspring of an isogenic strain. The maternal epigenetic state was partially inherited, whereas methylation patterns were erased in the male germline (Morgan et al., 1999). Similarly, phenotypic variation in Axin-fused ($Axin^{Fu}$) allelic mice, manifested as kinked tails, correlated with differential methylation of an adjacent IAP LTR and in this case was passed through both the male and female germlines (Rakyan et al., 2003). Examples such as these support the notion that transposable elements in mammalian genomes cause considerable cellular phenotypic variation, making each individual a "compound epigenetic mosaic" (Whitelaw and Martin, 2001).

Epilogue

Mobile DNA has been described as the genome's "dark matter": a significant part of its mass, difficult to understand, and often ignored. Transposable elements may also be seen as "dark energy," a dynamic force that not only accelerates expansion but also helps set the warp and weft of genomes, for better and for worse. Transposable elements arose as intracellular parasites that became domesticated. A new insertion is most likely benign, very occasionally harmful, and with extreme rarity beneficial. However, over the course of eukaryote evolution some beneficial inserts have been retained, those harmful have been lost, and as a group transposable elements have contributed to chromosome architecture to the degree that we cannot really understand our own genome without understanding their biology.

Twenty years ago an unfortunate insertion into the *factor VIII* blood clotting gene caused hemophilia A in a boy and demonstrated that L1s can be actively mobilized in human beings (Kazazian et al., 1988). Since then many researchers have been revealing the biology of retrotransposons in mammals. Progress has been due in large part to knowledge of mammalian genome sequences producing evolutionary insights, an assay for retrotransposition in cultured cells, and transgenic rodent models that allow analysis of retrotransposition in vivo. Although considerable new information has been obtained, progress on a number of key questions has been disappointing. We still have a rudimentary understanding of the biochemistry of insertion. We know little of the host factors important for retrotransposition. Our knowledge concerning when and in what cells most retrotransposition occurs is lacking. However, it is likely that these deficiencies will be eliminated with the emerging use of tiling arrays, massive cDNA transfection arrays, and high-throughput DNA sequencing, among others. We foresee an explosion of progress on the key questions and more complete understanding of the role of retrotransposons in the etiology of disease and the regulation of genes. Now it begins to get truly interesting.

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