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RNA interference: The molecular immune system

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Summary

Introduction of double-stranded RNA (dsRNA) into cells expressing a homologous gene triggers RNA interference (RNAi), or RNA-based gene silencing (RBGS). The dsRNA degrades corresponding host mRNA into small interfering RNAs (siRNAs) by a protein complex containing Dicer. siRNAs in turn are incorporated into the RNA-induced silencing complex (RISC) that includes helicase, RecA, and exo- and endo-nucleases as well as other proteins. Following its assembly, the RISC guides the RNA degradation machinery to the target RNAs and cleaves the cognate target RNA in a sequence-specific, siRNA-dependent manner. RNAi has now been documented in a wide variety of organisms, including plants, fungi, flies, worms, and more recently, higher mammals. In eukaryotes, dsRNA directed against a range of viruses (i.e., HIV-1, RSV, HPV, poliovirus and others) and endogenous genes can induce sequence-specific inhibition of gene expression. In invertebrates, RNAi can be efficiently triggered by either long dsRNAs or 21- to 23-nt-long siRNAs. However, in jawed vertebrates, dsRNA longer than 30 bp can induce interferon and thus trigger undesirable side effects instead of initiating RNAi. siRNAs have been shown to act as potent inducers of RNAi in cultured mammalian cells. Many investigators have suggested that siRNAs may have evolved as a normal defense against endogenous and exogenous transposons and retroelements. Through a combination of genetic and biochemical approaches, some of the mechanisms underlying RNAi have been described. Recent data in *C. elegans* shows that two homologs of siRNAs, microRNAs (miRNAs) and tiny noncoding RNAs (tncRNAs) are endogenously expressed. However, many aspects of RNAi-induced gene silencing, including its origins and the selective pressures which maintain it, remain undefined. Its evolutionary history may pass through the more primitive immune functions of prokaryotes involving restriction enzymes that degrade plasmid DNA molecules that enter bacterial cells. RNAi has evolved further among eukaryotes, in which its wide distribution suggests early origins. RNAi seems to be involved in a variety of regulatory and immune functions that may differ among various kingdoms and phyla. We present here proposed mechanisms by which RBGS protects the host against endogenous and exogenous transposons and retroelements. The potential for therapeutic application of RBGS technology in treating viral infections such as HIV is also discussed.

Introduction

Life on the green planet most likely found its roots in RNA, which essentially plays a passive role as a storage database and an active one as a catalyst. As evolution progressed and continually larger amounts of data required management, these roles were assumed by the comparatively more stable DNA, and additional proteins, complex carbohydrates, and lipid molecules took over the tasks of enzymes and structural building blocks. Meanwhile, RNA appeared to take on an intermediate role as translator of the massive amounts of information comprising the DNA database. However, we have recently begun to realize that, throughout evolution, the role of RNA in a discreet yet significant innate anti-viral defense system has been conserved; as such, RNA potentially functions as the major vanguard in preserving the integrity of the host organism's genomic structure via RNA interference (RNAi), or RNA-based gene silencing (RBGS) (reviewed in Fire 1999, Hamilton & Baulcombe 1999, Sharp 1999, Tuschl 1999, Bass 2000, Grishok & Mello 2002, Hannon 2002, Hutvager & Zamore 2002, Lindenbach & Rice 2002).

The concept of "molecular immunity" results from a fusion of concepts from diverse fields including plant biology, molecular genetics, immunology, and biochemistry; they have contributed to describing a mechanism by which eukaryotes are able to regulate gene expression, fight double-stranded RNA (dsRNA) viral infections, and protect their genomes from genetic parasites such as transposons (the DNA mobile elements that move from one location to another), retrotransposons (the DNA mobile element that use RNA intermediate), and retroviruses (Fire 1999, Hamilton & Baulcombe 1999, Ketting *et al.* 1999, Sharp 1999,

Tabara *et al.* 1999, Tuschl 1999, Bass 2000, Zamore *et al.* 2000, Grishok & Mello 2002, Hannon 2002, Hutvagner & Zamore 2002, Lindenbach & Rice 2002, Salo *et al.* 2002, Urwin *et al.* 2002, Seitz *et al.* 2003). In the last several years, numerous examples of RNA-based intracellular molecular defense mechanisms have been described. All of these are epigenetic and are based upon recognition of nucleic acid sequence homology at the mRNA level, the degradation of a homologous RNA by dsRNA, or triplex RNA (Bagasra & Amjad 1997, Bagasra & Amjad, 2000). RBGS has been shown to inhibit production of retroviruses (the human immunodeficiency virus, HIV-1 (Bagasra & Amjad 2000, Jacque *et al.* 2002), Rous sarcoma virus (reviewed by Hu *et al.* 2002, Joost *et al.* 2003), RNA viruses (respiratory syncytial virus and poliovirus: Joost *et al.* 2003), and a DNA virus (human papillomavirus (reviewed in Bagasra 1999, Gitlin & Andino 2003, Joost *et al.* 2003).

The phenomenon of RNAi was originally detailed in 1998 by Fire *et al.* (1998) of the Carnegie Institution of Washington (at Johns Hopkins University), and further explored by several leading groups including Sharp and Zamora and their team at MIT (Sharp 1999, Zamore *et al.* 2000), and Baulcombe and his group at the Sainsbury Laboratory in UK (Hamilton & Baulcombe 1999). To date, the RNAi effect has been documented in a variety of organisms including plants, fungi, flies, worms, and also complex mammals (Fire 1999, Hamilton & Baulcombe 1999, Ketting *et al.* 1999, Sharp 1999, Tabara *et al.* 1999, Tuschl 1999, Bass 2000, Zamore *et al.* 2000, Grishok & Mello 2002, Hannon 2002, Hutvagner & Zamore 2002, Lindenbach & Rice 2002, Salo *et al.* 2002, Urwin *et al.* 2002). From an immunological perspective, this RNA-associated function may represent the most primal form of innate immunity, allowing individual cells to intracellularly control (at a post-transcriptional but pre-translational level) the expression of 'undesirable' mRNAs of either exogenous or endogenous origins.

Here we will review some of the recent work concerning RBGS and present a novel hypothesis that includes possible alternate mechanisms which may function *in vivo* to mediate immunity associated with the control of retroelements and transposons (Bagasra 1999, Ketting *et al.* 1999, Tabara *et al.* 1999). We shall also offer some thoughts on the significance of these mechanisms from an evolutionary perspective. Indeed, RBGS and related processes may help to clarify some of the genetic processes of evolution, particularly in regard to the 'punctuated' evolution (a mechanism by which a new species appears suddenly) so often observed among eukaryotes of the paleontologic past. These same evolutionary processes may explain why the genomes of virtually all eukaryotes are packed with so-called "junk DNA" (non-coding genes) and why they are being maintained as essential parts of non-coding gene sequences.

The gene silencing concept

Among eukaryotes, RBGS mechanisms may be the primary molecular defense against retroviruses, pararetroviruses, transposons, retrotransposons, and dsRNA viruses. These agents are fundamentally different from all other infectious particles; retroelements are able to manipulate genomic DNA and insert themselves into a host's genome, occasionally even into the germ line DNA. Over time, retroviral DNA can spread throughout the genome of an entire species. Humans and the majority of eukaryotes carry within their genomes a large repertoire of endogenous retroviruses from their evolutionary past (reviewed in Seitz *et al.* 2003). Some of these endogenous retroviruses remain surprisingly active, particularly during embryogenesis and oncogenesis (Fire 1999, Sharp 1999; Bass 2000, Seitz *et al.* 2003).

Besides RNAi, RBGS involves molecular immunity (Bagasra & Amjad 1997, Bagasra 1999, Bagasra & Amjad 2000), microRNA (miRNA) (Grishok & Mello 2002), tncRNA (Ambros 2001), homology-dependent gene silencing (Salo & Baguna 2002), quelling (Hamilton & Baulcombe 1999), co-suppression (in Fire 1999, Hamilton & Baulcombe 1999, Sharp 1999, Tuschl 1999, Bass 2000, Zamore *et al.* 2000, Grishok & Mello 2002, Hannon 2002, Hutvagner & Zamore 2002, Lindenbach & Rice 2002), RNA dependent DNA methylation (Fire 1999, Hamilton & Baulcombe 1999, Sharp 1999, Tuschl 1999, Bass 2000, Zamore *et al.* 2000, Grishok & Mello 2002, Hannon 2002, Hutvagner & Zamore 2002, Lindenbach & Rice 2002), and apparently all host defenses that rely on host-parasite gene sequence homology (Ambros 2001, Ambros *et al.* 2003). The RBGS mechanism functions to prevent new retroelements, dsRNA viruses, and transposons from entering the host genome. Of note, over 60% of human gene sequences appear to be retroviral in origin, and over 98% of the human genome is composed of non-coding RNAs (Bagasra, 1999, Ambros 2001, Ambros *et al.* 2003). The extended maintenance of retroviral sequences suggests a beneficial function of these sequences through evolution as well as the ability of some of these agents to pass through the molecular defenses, (i.e., the emergence of AIDS for example) (Bagasra 1999, Hamilton & Baulcombe 1999, Tuschl 1999, Hul *et al.* 2000). Therefore, this massive accumulation of genomic retroelements may account for its defensive function against newly-arriving retroviruses. By utilizing gene sequences accommodated in the past, the host may preserve its genome from further encroachment by potentially hostile new retroviruses *via* RBGS. However, these systems appear to exhibit

flexibility. If a host encounters a retroelement in which the precise homologous siRNAs are not expressed by the endogenous host genome, then the host can accommodate new and evolving retroelements and viruses, thus arming itself with new gene sequences and protecting against subsequent invasion using these new siRNAs. New viruses, transposons, and retroelements periodically emerge *via* recombination between two or more kinds of retroviruses, transmitted by zoonosis (infection of one species by a microorganism from different species) (reviewed in Bagasra 1999, Ambros 2001, Ambros *et al.* 2003, Seitz *et al.* 2003). These siRNAs can be utilized to defend against genetically homologous viruses, and at times the remainder of the uninfected host cell as well (in Bagasra 1999, Fire 1999, Hamilton & Baulcombe 1999, Sharp 1999, Tuschl 1999, Bass 2000, Zamore *et al.* 2000, Ambros 2001, Grishok & Mello 2002, Hannon 2002, Hutvagner & Zamore 2002, Lindenbach & Rice 2002, Ambros *et al.* 2003). Periodically, the acquired gene sequences can be passed on, arming the next generation with these protective genes sequences (Jakowitsch *et al.* 1999, Hul *et al.* 2000, Jensen *et al.* 2002).

The actions of endogenous retroviruses may allow for the reshuffling of genetic sequences within genomes. McClintock's (1950) "jumping genes" are excellent examples of this. It is becoming clear that small hairpin RNAs or microRNAs (miRNAs) participate in the regulation of endogenous retroviruses, as the activity is generally confined to the nucleus. It is interesting how a major portion of non-coding genes, significant numbers of which contain active promoters and fully coding retroelements and mobile genes, are regulated by a finely tuned RBGS system. Control of these reshuffling processes is crucial; while a certain amount of genetic change is desirable, too much is deleterious (Fire 1999, Hamilton & Baulcombe 1999, Sharp 1999, Tuschl 1999, Bass 2000, Zamore *et al.* 2000, Grishok & Mello 2002, Hannon 2002, Hutvagner & Zamore 2002, Lindenbach & Rice 2002). Nonetheless, the reshuffling of genetic sequences in large chunks is a critical mechanism in eukaryotic evolution as large changes occur very quickly during adaptation to changing environments. Such a mechanism helps to explain the evolution of novel genetic sequences for which no intermediate forms are evident (Adams *et al.* 2002).

Transposons and retroelements in the human genome

The human genome is comparatively more populated with transposable elements than those of other species whose genomes have been analyzed. These "jumping genes" can cause disease and alter the genome in a number of different ways (reviewed in Ostertag & Kazazian 2001). There are two main classes of transposable elements: DNA transposons and retrotransposons. Retrotransposons are copied into RNA, which is then reverse-transcribed into DNA and inserted into the genome at a new location. These elements therefore expand in number by a duplicative "copy-and-paste" mechanism. The human genome is littered with remnants of the most prominent non-long-terminal repeat (non-LTR) retrotransposons and human long repetitive elements (LINES), roughly a half million of which are 5′ truncated, inverted, or mutagenically inactivated. However, approximately 5000 are full-length elements of at least 6 kb, 60–100 of which are still capable of retrotransposition. Full-length elements contain a 5′ untranslated region with an internal promoter, 1 kb open reading frame (ORF), one ORF1 that encodes a protein with an RNA binding capability, a 4 kb ORF2 that encodes a protein with endonuclease and reverse transcriptase activities, a short 3′ untransted region, and a poly (A) tail. It is hypothesized that their insertion into chromosomal DNA occurs by the process of target-primed reverse transcription (TPRT), whereby the endonuclease of ORF2 nicks a single strand of DNA and leaves a 3′-OH that serves as the primer for reverse transcription with the L1 RNA as a template. In summary, L1s account directly or indirectly for about one-third of the human genome (Ostertag & Kazazian 2001).

It has been shown that apparently endogenous retroviruses can "pop out" of a genome and again become infectious particles carrying active packets of genetic information. These particles bear the potential to cross species barriers, as ongoing studies of the historically recent HIV-1 epidemic clearly demonstrate (reviewed in Bagasra 1999).

Amplification, communication, and epigenetic persistence of RNAi

It is clear that either endogenous or exogenous small (~22 bp) dsRNAs initiate a cascade of events that allow enzymes such as Dicer (or RNaseIII or its equivalent, Bernstein *et al.* 2003) to cleave the target mRNA into ~22 bp fragments (Hul *et al.* 2000, Ambros 2001, Seitz *et al.* 2003). The annealing reaction between double stranded siRNAs and single stranded target mRNA forms a triplex complex. RNA-dependent RNA polymerase amplifies these "silencing complexes" that are cast off in the helicase reaction. This step involves the formation of the ATP-helicase-triplex complex that culminates in the unwinding and release of complex siRNAs (Fire 1999, Hamilton & Baulcombe 1999, Sharp 1999, Tuschl 1999, Bass 2000, Zamore *et al.* 2000, Grishok & Mello 2002, Hannon 2002, Hutvagner & Zamore 2002, Lindenbach & Rice 2002). These siRNAs bind mRNA and generate more siRNAs while degrading target mRNA. The resulting

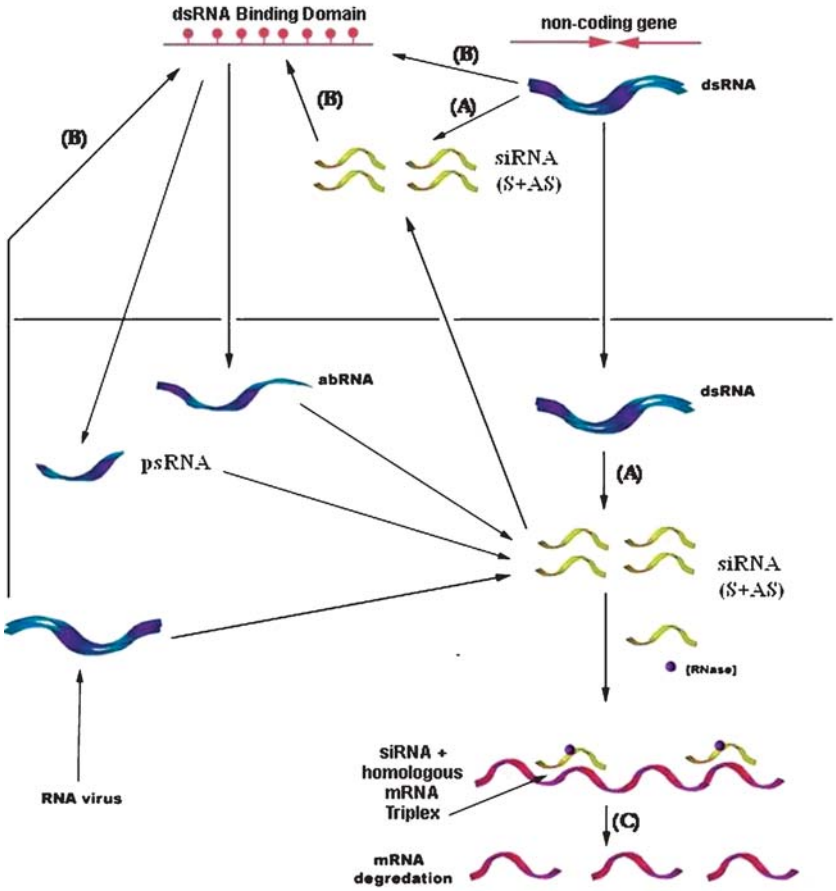

Figure 1. A model for the mechanism of mRNA degradation by RNAi. Small interfering RNAs (siRNAs) are the result of non-coding RNAs, part of introns, and other endogenous retroelements, accumulated throughout evolution. These pre-siRNAs are expressed as dsRNA a part of the large transcripts (blue ribbons) and broken down to small fragments of ~22 bp as siRNAs (yellow ribbons: step A). These siRNAs remain as dsRNA: sense (S) and antisense (AS). In the initial step (A), the dsRNA that initiates RNAi is bound by the RNAi nuclease (not shown), and then degraded to small dsRNAs (siRNAs) that remain stably bound to the RNAi nuclease. The enzymes coat the dsRNA in a precise register to generate specific fragments directing the cleavage of the mRNA at specific sites. When a retrovirus or other genetic element enters the cells, the siRNAs bind to the homologous gene sequences of the invading virus. This homologous binding forms a triplex and initiates a cascade of events resulting in the generation and amplification of siRNAs. The reaction is catalyzed by several enzymes including a Dicer (or RNA-dependent RNA Polymerase III or equivalent) that contains a dsRNA binding domain (red knob) on one or more ribonuclease domains (B), as well as an RNA helicase domain. In the subsequent step (C), these small template sequence-specific pieces cleave the mRNA. The helicase domain of the protein catalyzes an ATP-dependent strand exchange, replacing the sense stand(s) of the small dsRNA template with the mRNA. The mRNA is then cleaved to regenerate the RNAi nuclease with its small dsRNA. These siRNAs recycle and are amplified, transmitted to other cells, and silence the gene of interest in those cells. In addition, these RNA-based gene-silencing mechanisms also degrade aberrant RNA transcripts (abRNA) as well as pseudogenes (psRNA), and RNA viruses.

fragments, which are specific to the gene being silenced, may be distributed widely throughout the organism. The experimental data clearly show that the RBGS function is accompanied by the development and accumulation of significant quantities of siRNA fragments. Such has been demonstrated *via* Northern blotting techniques (Fire 1999, Hamilton & Baulcombe 1999, Sharp 1999, Tuschl 1999, Bass 2000, Zamore *et al*. 2000, Grishok & Mello 2002, Hannon 2002, Hutvagner & Zamore 2002, Lindenbach & Rice 2002). Furthermore, in both *C. elegans* and *Neurospora*, the memory of specific RNA sequences that have been silenced persists epigenetically through several generations. The signal then seems to fade if the progeny are not ''challenged'' by the mRNA in question (Fire 1999, Hamilton & Baulcombe 1999, Sharp 1999, Tuschl 1999, Bass 2000, Zamore *et al*. 2000, Grishok & Mello 2002, Hannon 2002, Hutvagner & Zamore 2002, Lindenbach & Rice 2002). In these species siRNA signals are readily transmitted from cell to cell, although the mechanisms of heritability and intracellular communication underlying these observations are not yet explained (Figure 1. Fire 1999, Hamilton & Baulcombe 1999, Ketting *et al*. 1999, Sharp 1999, Tabara *et al*. 1999, Tuschl 1999, Bass 2000, Zamore *et al*. 2000, Grishok & Mello 2002, Hannon 2002, Hutvagner & Zamore 2002, Lindenbach & Rice 2002, Salo *et al*. 2002, Urwin *et al*. 2002).

A unified hypothesis of RBGS mechanisms

RNAi, miRNA, tncRNA, molecular immunity, homology-dependent gene silencing, and DNA dependent methylation are host defenses that rely on homologous host-retroelement gene sequences (Bagasra 1999, Fire 1999, Hamilton & Baulcombe 1999, Ketting *et al*. 1999, Sharp 1999, Tabara *et al*. 1999, Tuschl 1999, Bass 2000, Zamore *et al*. 2000, Grishok & Mello 2002, Hannon 2002, Hutvagner & Zamore 2002, Lindenbach & Rice 2002, Salo *et al*. 2002, Urwin *et al*. 2002). These defenses have evolved to accommodate both the host and the parasite (Bagasra & Amjad 1997, Bagasra 1999, Fire 1999, Hamilton & Baulcombe 1999, Ketting *et al*. 1999, Sharp 1999, Tabara *et al*. 1999, Tuschl 1999, Bagasra & Amjad 2000, Bass 2000, Zamore *et al*. 2000, Grishok & Mello 2002, Hannon 2002, Hutvagner & Zamore 2002, Lindenbach & Rice 2002, Salo *et al*. 2002, Urwin *et al*. 2002). The RBGS mechanism functions to prevent new retroelements and transposons from entering the host genome. The extensive retention of retroviral sequences in eukaryotic life forms suggests a beneficial function for these sequences in the course of evolution (Ambros 2001, Ambros *et al*. 2003, Seitz *et al*. 2003). Therefore, this massive accumulation of retroelements in eukaryote genomes may account for its defensive function against newly-arriving retroviruses. We have hypothesized that non-coding sequences are expressed in the form of siRNAs (Bagasra *et al*. 1993, Bagasra & Amjad 1997, Bagasra 1999, Bagasra & Amjad 2000). Recently, Ambros and colleaguese (Carrington & Ambros, 2003) have analyzed miRNAs and tncRNAs in *C. elegans* and have discovered that among the short cDNA sequences that correspond to protein-coding sequences, only 49 were from the sense strand of an mRNA, whereas 746 distinct cDNA sequences were antisense to the coding strand of mRNAs. They reported that the siRNAs that accumulate in *C. elegans* in response to exogenous dsRNA triggers are also primarily antisense to the targeted mRNA (Ambros 2001, Ambros *et al*. 2003, Carrington & Ambros 2003), suggesting that the antisense sequences they identified represent endogenous siRNAs. A similar result was obtained for the plant *Arabidopsis thaliana*, where a significant fraction of ~22 nt cDNAs that were analyzed corresponded to protein coding sequences (reviewed in Carrington & Ambros 2003). In the *Arabidopsis* case, cDNA sequences were obtained for either the sense or antisense orientation relative to the open reading frame, which is consistent with the duplex nature of plant siRNAs (Carrington & Ambros 2003).

It appears that in using gene sequences accommodated in the past, the host protects its genome against encroachment by potentially hostile new retroviruses *via* the RBGS system. However, these systems appear to be flexible. If a host encounters a parasite in which the precise homologous siRNAs are not expressed by the host genome, then the host has the ability to accommodate new and evolving retroelements and viruses, arming the host with new gene sequences and thus protecting against the next invasion *via* these new siRNAs. New viruses, transposons, and retroelements are known to emerge periodically by recombination between two or more kinds of retroviruses and by zoonosis (reviewed in Gitlin & Andino 2003). These non-coding siRNAs can be utilized to defend against invasions by viruses that carry homologous gene sequences, and at times the remainder of the uninfected host cell as well (Bagasra 1999, Bagasra *et al*. 1993, Carrington *et al*. 2003, Seitz *et al*. 2003). In some cases, the newly acquired gene sequences can be passed on to future generations (Carrington & Ambros, 2003).

Role of siRNAs in development of HIV-1 vaccine

The ultimate and most dramatic use of RBGS would be to silence HIV-1 genes. Why are naturally occuring siRNAs not effective against HIV-1, yet are likely able to protect chimpanzees against SIVcpz? The solution most likely derives from understanding how chimpanzees, which are genetically close to humans, are able to remain healthy after SIVcpz (which bears >90% homology to HIV-1) infection, whereas humans are susceptible to HIV-1. There are a few possible explanations to this puzzle. For example, chimps in the wild carry SIVcpz and show a low to moderate viral load yet appear to be immune to this lentivirus against the development of AIDS (Ondoa *et al*. 2001). Similarly, several African non-human primates carry various versions of SIVs but remain unaffected by them. How do they become resistant to SIVs? One possibility may be that all the African primates acquired lentiviruses by slow exposure to various pre-lentiviruses over millions of years. They could have accumulated and amplified protective siRNAs without significant population impact. The accumulation of new siRNAs most likely took place when they invaded other primate colonies. These newly acquired viral sequences could then incorporated into their somatic cells as well as being partially or completely silenced (depending upon the repertoire of siRNAs that their respective genomes were expressing at the time of exposure); after many generations these new sequences were then incorporated into their germ lines, endowing their progeny with resistance to newer lentiviral sequences. That chimps and other African primates incompletely block their natural SIVs suggests that these viruses may be serving an evolutionary protective role by replicating at low levels to keep the RBGS mechanism activated until homologous genes are incorporated into the germ line.

The chief reason to entertain the notion that SIV gene fragments accumulated in the African primates slowly and did not result in high mortality is the observation that, in captivity, many African primates are exposed to different kinds of lentiviruses but without any major illness (reviewed in Bagasra 1999, Muchmore 2001). Humans also follow a similar course of events upon exposure to a single strain of SIV. For example, there are cases of accidental monkey bites in Africa and elsewhere in the world, primarily among animal care workers. However, they have not led to an HIV-1 like illness or epidemic (Bagasra 1999). Similarly, many African tribes rely upon primates, including chimpanzees, as their main diet. There is no evidence, however, that these tribes carry SIVs or have even seroconverted to a significant degree during the process, suggesting that natural exposure to a small dose of a single SIV strain in human or non-human primates (through bites) causes no significant illness.

HIV-1 may have arisen from massive recombination events between various SIVs (SIVcpz, SIVsm and SIVagm: reviewed in Salemi *et al.* 2003, Apetrei *et al.* 2004) that resulted in numerous new forms of lentiviral sequences *via* zoonosis (Salemi *et al.* 2003). Several of these new lentiviruses became infectious because the human host lacked matching endogenous siRNAs with which to neutralize them (Bagasra 1999). We hypothesize that, after the accidental introduction into human populations, these new lentiviral sequences went through a selection process, and those least homologous to the human RBGS (siRNAs) gave rise to the most successful versions of HIV-1 (this is similar to recombinant BSV described by Jakowitsch *et al.* 1999, Hul *et al.* 2000, Jensen *et al.* 2002). Since there is a great deal of variation between human populations with regard to endogenous siRNAs, they may be expressing different sequences of siRNAs and therefore the success rate of various HIV-1 clades differ significantly from one geographic locale to another (reviewed in Otting *et al.* 2002). This selection is constantly taking place, and new recombinants are constantly adapting to evade the pre-existing molecular defenses (Robertson *et al.* 1995, Sharp *et al.* 2000). Since germline integration into the human genome is rare, the natural integration of the new protective sequences will most likely occur very slowly.

Interestingly, one would expect that a few groups or individuals who are exposed to low doses of multiple strains of HIV-1 (or SIVcpz) would have a better opportunity to activate and amplify specific siRNAs against HIV-1 quasispecies, and hence would be more resistant to HIV-1 than an individual who was exposed to a single strain of a large dose of HIV-1. In recent years, several reports have described HIV-1 resistant individuals among African sex workers (Kaul *et al.* 2001, Messele *et al.* 2001). These individuals are regularly exposed to various HIV-1 strains due to unprotected sex (reviewed by Kaul *et al.* 2002). Numerous additional reports indicate that certain individuals or groups are immune to HIV-1 altogether (Baur *et al.* 1989, Bryson *et al.* 1995, Deacon *et al.* 1995, Roques *et al.* 1995, Paxton *et al.* 1996, Kaul *et al.* 2000, Kulkarni *et al.* 2003), while others remain AIDS free for extensive periods of time (Otting *et al.* 2002). In order to develop sufficient human protection against HIV-1 variants, a number of homologous sequences will be required in the endogenous repertoire (Hiroshika *et al.* 2000, Donze & Picard 2002, Hohjoh 2002, Jeong *et al.* 2002, Leirdal & Sioud 2002, Miyagishi & Taira 2002, Paddison *et al.* 2002, Paul *et al.* 2002, Sui *et al.* 2002, Yu *et al.* 2002). Through genetic technology, however, a rapid HIV-1 inhibitory siRNAs specific repertoire can perhaps be designed. These siRNAs could be encoded and delivered by appropriate vectors and then utilized in preventative or therapeutic vaccines (Hiroshika *et al.* 2000, Donze & Picard 2002, Hohjoh 2002, Jeong *et al.* 2002, Leirdal & Sioud 2002, Miyagishi & Taira 2002, Paddison *et al.* 2002, Paul *et al.* 2002, Sui *et al.* 2002, Yu *et al.* 2002). In conclusion, we are optimistic that the RBGS system could be utilized therapeutically to silence HIV-1.

Issues with current models of RNAi

Virtually all of the data published to date have been generated using experimental models in which the RBGS mechanism is examined with artificial dsRNA strands that are generated either *in vitro* or by vectors producing dsRNA upon transfection/transduction (reviewed in Hamilton & Baulcombe 1999, Fire 1999, Sharp 1999, Tuschl 1999, Bass 2000, Zamore *et al.* 2000, Grishok & Mello 2002, Hannon 2002, Hutvagner & Zamore 2002, Lindenbach & Rice 2002). Meaningful translation of *in vitro* data must, however, be approached with caution: to date, there has been no clear description of an *in vivo* model analogous to the siRNA effect. This is somewhat unusual, given the considerable years of experimentation as well as the extraordinary ubiquity of the observed mechanism in a wide variety of life forms (Hamilton & Baulcombe 1999, Fire 1999, Ketting *et al.* 1999, Sharp 1999, Tabara *et al.* 1999, Tuschl 1999, Bass 2000, Zamore *et al.* 2000, Grishok & Mello 2002, Hannon 2002, Hutvagner & Zamore 2002, Lindenbach & Rice 2002, Salo *et al.* 2002, Urwin *et al.* 2002). Therefore, the extent of the role played by RBGS as an antiviral system remains to be fully characterized. It is unclear whether RNA silencing can be elicited naturally after exposure to retroelements, transposons or dsRNA viruses. Some of the critical questions that must be addressed include:

(i) *Are siRNAs generated during natural infection with retroelements and transposons?* Reactivation of

transposons after the siRNA generating system supports this notion (Ketting *et al.* 1999, Tabara *et al.* 1999), but more work is needed before this issue is resolved.

(ii) *Are RBGS components upregulated during viral infection?* Several critical enzymes have been identified which participate in the RBGS system. These include Dicer, RNA-dependent RNA polymerase (RdRP), RNA degradation enzymes, and several other enzymes (reviewed in Grishok & Mello 2002). The upregulation of siRNA-generating components during viral infection would support the role of this system in antiviral defense.

(iii) *Have viruses evolved mechanisms to suppress or escape a RBGS system?* Several viral proteins have been identified which can quench the RBGS system (reviewed in Gitlin & Andino 2003), but more evidence is needed.

(iv) *Can RBGS in one infected cell trigger a systemic antiviral response?* In *C. elegans,* injection of dsRNA into one region of the worm triggers its spread to many different tissues, including the gonad (Urwin *et al.* 2002). Similar observations were made for plants (Hamilton & Baulcombe 1999, Salo & Baguna 2002) and mammalian cells, including human (Donze & Picard 2002, Hohjoh 2002, Leirdal & Sioud 2002, Miyagishi & Taira 2002, Yu *et al.* 2002). It is not known how the dsRNAs exit the cell in which they are produced, how they are systemically disseminated, or how they are taken up by distant target cells. It will thus be important to address whether RNA silencing in mammals also induces a systemic response. Few reports have yet suggested this to be the case (Desset *et al.* 2003); additional studies are needed.

(v) *What is the origin of RBGS?* One way to answer this question is to evaluate what happens to an organism when certain genes, presumed to be involved in RBGS, are altered. Mutations in several genes render an organism incapable of generating siRNAs; in some cases, these mutations reactivate transposons and retroelements (Ketting *et al.* 1999, Tabara *et al.* 1999, Hirochika *et al.* 2000, Nakayashiki *et al.* 2001, Jeong *et al.* 2002, Paddison *et al.* 2002, Paul *et al.* 2002, Sui *et al.* 2002). Proteins essential for the homologous RNA degradation pathway are highly conserved in fungi, plants, vertebrates, and mammals (reviewed in Grishok & Mello 2002). These proteins have been shown to play essential roles in organismal development, germline fate, and host defenses against both transposable elements and dsRNA viruses. However, the presence of these proteins and genes may be a small piece of a large puzzle. The real siRNA sequences involved in RBGS may be hidden from our eyes. How they are expressed, and how is the expression process coordinated? We will need deeper probing to solve this mystery.

(vi) *Does an adoptive immune system, in which genetic recombination occurs among a limited number of genes (i.e., VDJ), similarly lead to diversity within the siRNA generating mechanism?* If this is true, then one ought to be able to recognize the homologous enzymes and proteins involved, such as: RAG1/RAG2-recombination enzymes, ligase IV, helicase, and the dsDNA restriction and splicing elements (reviewed in Martin 2002).

(vii) *In what form are siRNAs transported? Are ssRNA, dsRNA or triplex siRNA or nucleic acid-complexs transported from cell-to-cell?* Our current understanding of molecular biology would suggest that the safest and most stable mode of transmission of molecular immunity would be in the form of triplex complexes (Bagasra 1999), since ss and ds nucleic acids can be readily digested by numerous nucleases active intracellularly or extracellularly. However, currently there is no nuclease known that digests triplex nucleic acid complex. This important issue still remains essentially uninvestigated.

We are unaware of conclusive evidence thus far of persistent double-stranded RNAs that exist naturally, other than in certain transient or rare circumstances involving viral activity, or in an infection with a relatively rare class of viruses that utilize dsRNA (such as *Reoviridae*, *Orbivirus*, *Cyoviruses*–or the once obscure West Nile virus). Furthermore, in these viral examples, the dsRNA is always encapsulated in a viral protein structure while in the host cell cytoplasm. The dsRNA is therefore not readily available for interaction with the normal enzymes or siRNA of the cell (Gitlin & Andino, 2003). For example, the bluetongue virus produces capped mRNA internally within an enclosed core particle, releasing only ssRNA into the cytoplasm of the host cell. Even more intriguing is that some of the members of the *Orthoreovirus* carry a protein designed to quench any dsRNA that might have been released accidentally into the cellular cytoplasm, thereby avoiding detection by an RNAi defense system (Mallory *et al.* 2001, Gitlin & Andino 2003).

Nonetheless, double-stranded RNA is always required in the various experiments cited in order to stimulate the RBGS response. All of the RNAi data reviewed thus far should therefore be viewed in light of this *in vitro* component, in which a type of ''molecular mimicry'' may actually be occurring. Instead, we hypothesize that these experimental protocols are actually simulating a natural system of a slightly different function that evolved for specific purposes. It appears that in eukaryotes, this system is primarily a molecular

immune mechanism specializing in fighting retroviruses, pararetroviruses, transposons, retrotransposons, aberrant transcripts, and protecting unnecessary gene arrangements (Hamilton & Baulcombe 1999, Fire 1999, Ketting *et al*. 1999, Sharp 1999, Tabara *et al*. 1999, Tuschl 1999, Bass 2000, Zamore *et al*. 2000, Grishok & Mello 2002, Hannon 2002, Hutvagner & Zamore 2002, Lindenbach & Rice 2002, Salo *et al*. 2002, Urwin *et al*. 2002, Seitz *et al*. 2003).

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