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RNA Interference and mRNA Silencing, 2004: How Far Will They Reach?

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Submitted October 9, 2003; Revised October 30, 2003; Accepted November 3, 2003
Monitoring Editor: Thomas Pollard

The discoveries of RNA interference and RNA-mediated posttranscriptional gene silencing have opened an unanticipated new window on the regulation of gene expression as well as a facile and highly effective tool for knocking down gene expression in many organisms and cells. In addition, RNA interference and RNA silencing may conceivably be exploited for human therapeutics sometime in the future, possibly bringing greater clinical impact than have the so far disappointing antisense endeavors. This essay summarizes recent developments and offers some personalized perspectives, with emphasis on what we do not yet know.

INTRODUCTION

The discovery of RNA interference (Guo and Kemphues, 1995; Fire et al., 1998) was unusual in the postmodern era of molecular biology in that, like immunoglobulin gene rearrangement, for example, it was almost entirely unanticipated. Beyond the action of RNA interference and RNA silencing at the translational level of gene expression, RNA-mediated phenomena are now known to direct the transcription-level silencing of some genes, and even the "editing down" of an organism’s genome by selective excision events in certain cases (Selker, 2003; Yao et al., 2003).

In addition to possessing the element of surprise, RNA interference and RNA silencing also explained certain previous findings that had been puzzling, and thus the discoveries genuinely warranted the term breakthrough. In retrospect, were there any clues that were missed?

Hints of RNAs That Base Pair with mRNA

In the early 1970s, intramolecular double-stranded regions of large nuclear RNA molecules were described in both mammalian cells and sea urchin embryos (Jelinek and Darnell, 1972; Kronenberg and Humphreys, 1972; Ryskov et al., 1972). These studies used extracted, deproteinized nuclear RNA, but the in vivo authenticity of these double-stranded RNA (dsRNA) regions of nuclear RNA was subsequently verified in intact, living cells (Calvet and Pederson, 1979). An intriguing finding was that although cytoplasmic mRNA did not contain these intramolecular dsRNA regions, the dsRNA elements of nuclear RNA could nevertheless hybridize with cytoplasmic mRNA (Stamper et al., 1972; Naora and Whitelam, 1975; Ryskov et al., 1976; Jelinek et al., 1978). The implication was that a portion of a given nuclear dsRNA region is conserved in mRNA with the remainder of the dsRNA region being eliminated, i.e., the nuclear dsRNA-containing molecules are mRNA precursors. But alternatively, if a dsRNA-containing nuclear RNA molecule produced a small RNA that ended up hydrogen-bonded to a complementary stretch in a (separately encoded and generated) cytoplasmic mRNA, as in the RNA silencing of mRNA that has now been discovered, the observations would have been the same as the ones made.

The idea that intermolecular RNA–RNA interactions were involved in gene regulation was also advanced on the basis of other studies (Britten and Davidson, 1969), and later experiments revealed that duplex regions form between HeLa cell heterogeneous nuclear RNA molecules when annealed under certain conditions (Fedoroff et al., 1977). During the same period, there were numerous reports of small, translation-suppressing RNAs associated with inactive messenger ribonucleoproteins, called translation control RNAs (tcRNAs). In some experiments, these small tcRNAs behaved as if hydrogen bonded to the inactive mRNA (Heywood and Kennedy, 1976). The tcRNA work never really caught on at the time, but of course now seems provocative in retrospect.

Pre-miRNAs in the Genome

The endogenous pathway of mRNA silencing involves small RNAs, called microRNAs (abbreviated miRNAs) that are encoded in the organism’s genome and expressed in the appropriate developmental schedule (Moss, 2002; Pasquinelli, 2002); miRNAs are derived from larger precursor molecules in the nucleus, the most proximal of which are ~70-nt molecules consisting of imperfectly paired hairpins (Lee et al., 2002, 2003; Seitz et al., 2003) Very little is known about these “pre-miRNAs” are themselves produced but the available data are compatible with the derivation of the ~70-nt pre-miRNAs from considerably longer nuclear transcripts that lack translation open reading frames. It is interesting to recall in this respect that early studies on mammalian nuclear RNA revealed that a substantial portion of large, poly(A)-terminated molecules do not contain any sequences homologous to mRNA (Herman et al., 1976; Salditt-Georgieff et al., 1981). The functional significance of this puzzling population of nuclear RNA has remained elusive for a quarter of a century. Plausibly, it might harbor precursors of the pre-miRNAs, i.e., the “pre-pre-miRNAs.”
The initial studies of the intramolecular RNA duplex fraction of heterogeneous nuclear RNA (hnRNA) in HeLa cells operatively defined these regions by their resistance to RNases A and T1 (Jelinek and Darnell, 1972; Kronenberg and Humphreys, 1972; Ryskov et al., 1972; Calvet and Pederson, 1977; 1978) or RNases A and T2 (Roberston et al., 1977; Jelinek, 1977). One of these investigations scrutinized these double-stranded regions in particular detail, based on their relative resistance to single-strand–specific versus double-strand–specific conditions of RNase attack, and identified two distinct classes of dsRNA within hnRNA, one more perfectly base paired than the other (Calvet and Pederson, 1977). The pre-micro RNAs identified so far are ~70-nt RNAs with some base-pairing mismatches (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001; Mouro-latos et al., 2002). It is not possible at present to relate these identified nuclear pre-miRNAs to the previously characterized dsRNA regions of hnRNA, but this is a high priority for understanding the biogenesis of the miRNAs. In addition, there are other sequence families in mammalian genomes (Alu sequences in human) that produce, mostly via transcription by RNA polymerase III, small RNAs that contain stem-loop domains very similar to ones in the presently defined pre-miRNAs. Unfortunately, nothing else is presently known about the primary transcripts that give rise to pre-miRNAs, including the particular RNA polymerase that makes them (presumably pol II). A recent study demonstrated that a human pre-miRNA contains a 5'-phosphate, a 3'-OH, and a 1-4 nt 3' overhang, consistent with an RNase III-mediated derivation (Basyuk et al., 2003).

**Is RNA Interference Restricted to mRNA Targets?**

What is the target “reach” of RNA interference and mRNA silencing? Until recently, all the demonstrated RNA interference targets were messenger RNAs. For example, a recent study has demonstrated that certain endogenous small interfering RNA (siRNA)-like RNAs in the protozoan *Trypanosoma brucei* are complexed with polyribosomes in a manner that is dependent on active translation (Dijkeng et al., 2003). The extant reports using siRNAs to knock down RNA viral replication, a situation in which there are RNA species other than translating mRNA as potential targets, are almost certainly due to action on viral mRNAs (Dector et al., 2002; Jacque et al., 2002; Ge et al., 2003; Mccaffrey et al., 2003; Novina et al., 2003). Reports that translationally repressed, maternal mRNAs become susceptible to RNA interference only upon their developmental translational activation (Svodoba et al., 2000; Kennerdell et al., 2002) have fueled the idea of a direct connection between RNAi or mRNA silencing and the target’s active translational status. So, is there any basis for considering RNA interference or mRNA silencing more broadly than only targeting translating mRNAs? At first thought, the most plausible initial interpretation of the discovery of RNA interference was the one deriving from an evolutionary perspective, namely, that of an ancient defense against RNA genome-based infectious or parasitic organisms, a defense retained in extant organisms, but one that has also been tinkered with and toiled over ~2.5 billion years into a developmental gene regulation pathway in the metazoan Eukarya (Zamore, 2002). Such a mechanism would have likely first operated on an mRNA encoded (or brought in as genomic RNA) by a RNA virus. This idea sounds very plausible on evolutionary grounds. As for mRNA silencing by small RNAs, there is ample precedent in prokaryotes so perhaps this was an early feature of gene regulation that evolved before, or at least independently of, double-stranded RNA-triggered mechanisms.

**RNAi Hits non-mRNA**

The idea that the action of RNAi is restricted to translating mRNAs was perhaps not ever assumed by the field’s leaders, but it is certainly the prevailing operative view of most cell biologists using siRNAs to knockdown target mRNAs. By way of an example, in a recent collaborative study of mine in this journal (Wang et al., 2003), the use of siRNA to knock down a relevant mRNA in a mamalian cell line worked beautifully, as is very typical, but the more unorthodox possibility of using siRNA to knock down the small, RNA polymerase III transcripts that were of even greater interest in the study was not pursued.

The notion that siRNA targets must be mRNAs has recently been dramatically overturned (Liang et al., 2003). Ironically, one of the (three) organisms in which this study was done was the very same one in which the aforementioned link of RNAi action to translating mRNA was reported, namely, *T. brucei*, one of the first organisms in which RNA interference was described (Ngo et al., 1998). These investigators decided to target a group of small nuclear RNAs (snRNAs) that play a role in the biosynthesis of rRNA in the nucleolus (Bachellerie et al., 2002). They expressed siRNAs complementary to these snoRNAs and observed their destruction (Liang et al., 2003). This study is the first to demonstrate that siRNA can attack a nonpolysomal, non-mRNA target and in a cellular compartment other than the cytoplasm, the nucleus. Moreover, the target RNAs in this study have 5’ termini different than mRNA and also lack 3’ poly(A) tails, ruling out these ends of RNA as being required for siRNA action.

Can knocking down (or out) non-mRNA “housekeeping” RNAs, such as snoRNAs, be anything beyond just a good learning curve for understanding the molecular biology of cells? Consider a protein called Ro. It is a human autoantigen that is complexed with a group of small, cytoplasmic non-mRNAs in higher animals (Tan, 1982). When the gene for the Ro protein was knocked out of mice (by transgenic, heritable means, not transiently by RNA interference), the animals displayed phenotypes (Xue et al., 2003) that have the potential to contribute, with further work, to advances in our understanding of two of the most challenging and important current problems in all of medicine: innate immunity and immunological tolerance. Knockdown of the mRNA for Ro protein, or of the Ro RNAs themselves, by siRNA in mammalian cells or animals might offer additional experimental dimensions, now empowered by the transgenic knockout results. There are many other non-mRNAs of unknown function in eukaryotic cells, and it is possible that they too can be approached by RNA interference. Some of these may themselves turn out to be elements of RNA-mediated gene regulation. For example, can siRNAs knock down miRNAs?

The scale of RNA interference-mediated analysis of gene expression has now reached breathtaking levels in ideal organisms. In the first two large-scale applications of this approach, the mRNAs from ~5000 genes in *Caenorhabditis elegans* were systematically deleted, and more recently several studies of nearly comparable scale have been carried out in *Drosophila* cells (Pollard, 2003). Microarray-based approaches to the discovery of novel microRNAs are also now coming onto the scene (Krichevsky et al., 2005), so our understanding of the endogenous pathways of mRNA silencing and related miRNA functions, i.e., establishment of heterochromatin or genome reorganization, can be anticipated to rapidly expand during 2004.
Envisioning the Patient

Major questions remain about the utility of these discoveries for medicine, including the pharmacokinetics of administered siRNAs, the challenges of gene therapy-based approaches to implanting an intracellular supply of a miRNA in patients, and, most sobering of all, the implausibility of miRNA knockdown approaches for conditions based on loss of function mutations. Also to be borne in mind is the possibility that initially seductive mRNA targets revealed by microarray analysis may turn out not to be at the true center of the pathogenic process in the case of noninfectious disease (as opposed to the more immediately promising viral disease opportunities for siRNAs.) There is also the question of whether knockdown of a desired target, perhaps to the good, might bring with it an undesired shutdown of certain other, essential mRNAs in response, due to the operation of feedback controls that sense the targeted mRNA's presence or activity. These questions surrounding the utility of these discoveries for medicine (or agriculture) do not, of course, diminish the deserved excitement about the discoveries of RNA interference and RNA silencing as science. This essay has endeavored to add at least a small degree of perspective to this explosively emerging field. It seems likely that there is much more biology to be uncovered, and it is possible that major applications in medicine and agriculture may ensue, driven by the reliable engine of basic research.

ACKNOWLEDGMENTS

I thank my colleague Phillip Zamore for offering enabling comments on an early draft. My work is supported by grants GM-21395 and GM-60551 from the National Institutes of Health and a grant from the Human Frontier Science Program Organization. By the rules of the National Institutes of Health, I am required to affirm that the views expressed in this article do not represent the official position of the U.S. government. (If they did, it is amusing to ponder what America might be up to.) This article is dedicated to the memory of Charles A. Janeway, whose intellectual passion and outgoing generosity deeply touched me on several occasions over the years.

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