October 2, 2011

Fred Russell Kramer

Personal

Birth	July 7, 1942 – New York City
Family	Married forty years, widowed, two children, four grandchildren

Education

1956 - 1959	The Bronx High School of Science
1959 - 1964	University of Michigan – B.S. with Honors in Zoology
1964 - 1969	The Rockefeller University – Ph.D. (with Vincent Allfrey)
1969 - 1972	Columbia University – Postdoctoral training (with Sol Spiegelman)

Appointments

1962 - 1964
Laboratory Technician, Cytogenetics Laboratory
Carnegie Institution of Washington, Ann Arbor, Michigan

1969 - 1986
Department of Genetics and Development
and Institute of Cancer Research
College of Physicians and Surgeons
Columbia University

1969 - 1971	Fellow of the American Cancer Society
1971 - 1972	Research Associate
1972 - 1973	Instructor
1973 - 1980	Assistant Professor
1980 - 1983	Senior Research Associate
1983 - 1986	Research Scientist

1986 - present
The Public Health Research Institute

1986 - present	Member, Department of Molecular Genetics
2000 - 2006	Director, Office of Technology Transfer
2006 - 2010	Associate Director for Technology Transfer

1987 - present
Department of Microbiology, New York University School of Medicine

1987 - 2003	Research Professor
2003 - present	Adjunct Professor

2003 - present
Professor of Microbiology and Molecular Genetics
New Jersey Medical School
University of Medicine and Dentistry of New Jersey

Awards

2005 Jacob Heskel Gabbay Award in Biotechnology and Medicine

Professional groups

American Association of University Professors
American Society for Biochemistry and Molecular Biology
American Society for Microbiology
Association for Molecular Pathology
New York Academy of Sciences
Society of the Sigma Xi
The RNA Society

Fred Russell Kramer

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RESEARCH SYNOPSIS

For the past forty-two years, our laboratory has been exploring nucleic acid structure to understand the role that it plays in macromolecular interactions that control biological processes. The work has led to the design of novel nucleic acid molecules and the development of experimental techniques that enable the construction of extremely sensitive and specific molecular diagnostic assays. More than one hundred people have worked in the laboratory or participated as close collaborators. The following paragraphs sketch the significant research themes that the laboratory has pursued.

Mechanism of RNA replication

The laboratory studied the mechanism of RNA-directed RNA synthesis catalyzed by the bacteriophage polymerase, Qβ replicase. No one knew the mechanism by which the viral replicase selectively copies Qβ genomic RNA, while ignoring the vast number of other RNA molecules that are present in the bacterial host. Qβ RNA was too large to be studied with the techniques that were then available. However, we discovered a much smaller RNA (MDV-1 RNA) in Qβ-infected *Escherichia coli* that is replicated in the same manner as Qβ RNA (Kacian *et al*., 1972). Using classical enzymatic and electrophoretic techniques, we determined the complete nucleotide sequence of both complementary strands of MDV-1 RNA (Mills *et al*., 1973). This was the longest nucleic acid that had ever been sequenced. Knowledge of the sequence enabled experiments to be carried out that provided insights into the mechanism of RNA replication. We discovered that each complementary strand of MDV-1 RNA possessed extensive secondary structures (Klotz *et al*., 1980). We demonstrated that the rate of RNA synthesis was determined by pauses in polymerization that occur where secondary structures form in the nascent strand (Mills *et al*., 1978), and we showed that structural reorganizations occur during product strand elongation (Kramer & Mills, 1981). We also developed an electrophoretic technique for separating the complementary strands that enabled the elucidation of the overall mechanism of RNA-directed RNA synthesis (Dobkin *et al*., 1979), and we utilized chemical and enzymatic nucleic acid modification methods to identify the sequences and structures that are required for the selective recognition of the RNA by the replicase, and for the initiation of product strand synthesis (Mills *et al*., 1980; Bausch *et al*., 1983; Nishihara *et al*., 1983).

Novel nucleic acid sequencing techniques

Rapid nucleic acid sequence analysis was essential to further studies of replication. We developed a chain-termination method for RNA sequence analysis (Kramer & Mills, 1978) at the same time that Fred Sanger developed a chain-termination method for DNA sequence analysis. Knowledge of the extensive secondary structure of MDV-1 RNA led us to realize that both of these sequencing techniques were compromised by the persistence of strong secondary structures during electrophoretic separation of the partially synthesized strands. We introduced a widely adopted solution to this problem, which was based on the use of modified nucleosides, such as inosine, that form weaker secondary structures (Mills & Kramer, 1979). Years later, we conceived novel techniques that enable entire genomes to be sequenced in a concerted manner by hybridization to oligonucleotide arrays (Chetverin & Kramer, 1993; 1994). These techniques were licensed exclusively to the Affymetrix Corporation (U.S. Patents 6,103,463 and 6,322,971).

In vitro evolution of replicating RNA populations

The *in vitro* replication of RNA by Qβ replicase provides a model system for studying precellular evolution. When MDV-1 RNA is replicated *in vitro*, the number of RNA molecules doubles every 20 seconds, resulting in an exponential increase in the number of RNA strands. Occasionally, errors occur during replication, producing RNA molecules with a mutated nucleotide sequence. When replication is carried out in the presence of an inhibitor of replication, mutant molecules that resist the inhibitor have a selective advantage, and if allowed to replicate for hundreds of generations, these mutants become predominant in the RNA population. Since phenotype and genotype reside in the same molecule, sequence analysis of the selected RNAs provided insights into the mechanism of Darwinian evolution.

Our laboratory carried out extensive studies on the *in vitro* evolution of replicating populations of MDV-1 RNA. Utilizing serial transfer techniques, hundreds of replicative generations could be completed in a day. By imposing different selective pressures, different variants emerged. Sequence analysis of the replicating RNA populations at different times during their evolution elucidated how the nucleotide changes that occurred conferred resistance to the particular inhibitor that was used (Kramer *et al*., 1974). Parallel molecular evolution experiments carried out in the presence of the chain elongation inhibitor, ethidium bromide, confirmed that many different genotypic pathways lead to the same phenotypic result, just as in the evolution of organisms. These experiments laid the foundation for modern *in vitro* selection techniques that are used to isolate nucleic acid molecules possessing predetermined catalytic activities.

The results of the *in vitro* evolution experiments also provided useful insights into the structural constraints that are required for an RNA to be replicatable. Though mutations occur everywhere in an RNA, the only mutations selected during the evolution of MDV-1 RNA occurred in single-stranded regions of the molecule, indicating that double-stranded structures are essential to the replicative process. When ribonuclease T1 was used as a selective agent, the mutants that arose were significantly resistant to the nuclease. The macromolecular dimensions of both the nuclease and the RNA limited cleavage to only a few sites on the exterior of the RNA molecule. The selected RNAs possessed non-cleavable nucleotide substitutions at just those exposed sites. These experiments elucidated the tertiary structure of MDV-1 RNA, enabling us to design exponentially amplifiable recombinant RNAs.

Recombinant RNAs

Many investigators wished to use the exponential amplification of RNA by Qβ replicase to synthesize large amounts of any mRNA or any genomic RNA. However, Qβ replicase is highly specific for Qβ phage RNA. We devised a scheme that enabled the replication of any heterologous RNA. Novel RNA templates were constructed by covalently inserting heterologous RNA sequences within the MDV-1 sequence at a single-stranded site that occurs on the exterior of the MDV-1 RNA molecule (Miele *et al*., 1983). The resulting recombinant RNAs possessed all of the secondary and tertiary structures that are required for replication, and the presence of the inserted sequence on the exterior of the molecule did not interfere with access to the structures required for replication. Consequently, Qβ replicase was able to catalyze the exponential synthesis of the entire recombinant RNA. Moreover, the recombinant RNAs were bifunctional, in that they retained the biological activity of the inserted sequence, as well as the replicatability of the MDV-1 RNA.

We constructed recombinant RNAs that contained the entire mRNA sequence encoding chloramphenicol acetyltransferase. These recombinant molecules were amplified exponentially *in vitro* by incubation with Qβ replicase, and the replicated RNA served as template for the cell-free synthesis of enzymatically active chloramphenicol acetyltransferase (Wu *et al*., 1992). We demonstrated that these recombinant mRNAs could be continuously synthesized and that large quantities of biologically active protein could be produced in a coupled replication-translation system that contained both Qβ replicase and bacterial ribosomes (Ryabova *et al*., 1994). We also constructed amplifiable recombinant RNAs that contained entire viroid genomes (U.S. Patent 5,871,976), and the recombinant, by itself, was infectious when placed on the leaves of tomato plants.

Extremely sensitive gene detection assays

With the advent of the AIDS crisis, it became imperative that very sensitive assays be developed for the detection of pathogenic retroviruses. We realized that an attractive strategy for detecting rare targets is to link a nucleic acid probe to a replicatable reporter that can be amplified exponentially after hybridization to reveal the presence of the target (Chu *et al*., 1986). We therefore covalently linked MDV-1 RNA to an oligonucleotide probe that was complementary to a predetermined genetic target. The resulting molecules were used in assays in which the probes bind specifically to target sequences, unbound probes are washed away, and the probe-target hybrids are incubated with Qβ replicase to generate a large number of easily detected reporter molecules. Since as little as a single molecule of MDV-1 RNA can serve as template for the exponential synthesis of millions of RNA copies by Qβ replicase, these assays were extremely sensitive.

We also realized that it was simpler to perform these assays with recombinant MDV-1 RNA molecules in which a probe sequence is embedded within the MDV-1 RNA, rather than being attached to the RNA by a linker. We constructed recombinant-RNA probes and demonstrated that they were bifunctional, in that they bound specifically to their targets, and after they were bound they served as templates for their own exponential amplification (Lizardi *et al*., 1988). We demonstrated that recombinant-RNA hybridization probes could be used in sensitive gene detection assays (Lomeli *et al*., 1989; Kramer *et al*., 1992). The inclusion of intercalating fluorescent dyes, such as ethidium bromide, in the reaction mixtures to detect the reporter RNA enabled the assays to be carried out in real-time under homogeneous conditions in sealed tubes (Kramer & Lizardi, 1989; Lomeli *et al*., 1989). We also demonstrated that the time required to synthesize a given quantity of reporter RNA is inversely proportional to the logarithm of the number of target molecules originally present in a sample, thus enabling quantitative determinations over an extremely wide range of target concentrations (U.S. Patent 5,503,979). This quantitative analytical technique has found wide application in real-time clinical assays that utilize polymerase chain reactions.

The sensitivity of Qβ replicase assays employing recombinant RNAs was limited by the inability to wash away every unbound probe. Persistent nonhybridized probes were amplified along with hybridized probes, generating a background signal that obscured the presence of rare targets. We investigated a number of different ways to eliminate this background (Kramer & Lizardi, 1989; Blok *et al*., 1997; U.S. Patents 5,118,801 and 5,312,728). Rather than trying to improve existing washing techniques (which were already quite efficient), we altered the design of the probes so that they could not be replicated unless they were hybridized to their target. We divided the recombinant-RNA probes into two separate molecules, neither of which could be amplified by itself, because neither contained all of the elements of sequence and structure that are required for replication by Qβ replicase. The division site was located in the middle of the embedded probe sequence. When these "binary probes" were hybridized to adjacent positions on their target sequence, they could be joined to each other by incubation with an appropriate ligase, generating a replicatable reporter RNA, which was then exponentially amplified by incubation with Qβ replicase. Nonhybridized probes, on the other hand, because they were not aligned on a target, could not be ligated, and signal generation was strictly dependent on the presence of target molecules. Because there were no background signals, the resulting assays were extraordinarily sensitive. As little as a single HIV-1 infected cell could be detected in samples containing 100,000 uninfected lymphocytes (Tyagi *et al*., 1996). This technique was licensed to Abbott Laboratories (U.S. Patents 5,759,773 and 5,807,674) and has been used in automated assays that detect the genes of many different infectious agents in human clinical samples.

Molecular beacons

We invented novel hybridization probes called "molecular beacons," which enable the direct detection of specific nucleic acids in living cells and in diagnostic assays (Tyagi & Kramer, 1996). These probes are hairpin-shaped oligonucleotides with a fluorophore at one end and a nonfluorescent quencher at the other end. When they are not bound to a target nucleic acid, the fluorophore is in contact with the quencher and the probes are dark. When these probes bind to their targets, they undergo a conformational reorganization that separates the fluorophore from the quencher, resulting in a bright fluorescent signal that indicates the presence of the target. Because these probes

only fluoresce when they are bound to target sequences, there is no need to isolate the probe-target hybrids to determine the amount of target present in a sample.

We showed that the mechanism of fluorescence quenching involves the transient formation of a nonfluorescent fluorophore-quencher complex, thus any desired fluorophore can be used as a label (Tyagi *et al*., 1998; Marras *et al*., 2002). When a set of molecular beacons are prepared, each specific for a different target sequence, and each labeled with a differently colored fluorophore, different nucleic acid targets can be detected simultaneously in the same assay tube or in the same cell. Moreover, by taking their thermodynamic behavior into consideration (Bonnet *et al*., 1999), molecular beacons can be designed so that they are significantly more specific than corresponding conventional linear hybridization probes. Molecular beacons can be designed in such a manner that the presence of even a single nucleotide substitution in a target sequence prevents the formation of a probe-target hybrid (Tyagi *et al*., 1998; Marras *et al*., 1999).

Our laboratory demonstrated the advantages of using molecular beacons as amplicon detector probes in quantitative, real-time, exponential amplification assays. We designed extremely sensitive, multiplex, clinical PCR assays that simultaneously detect four different pathogenic retroviruses in blood (Vet *et al*., 1999); and we designed "wavelength-shifting" molecular beacons (Tyagi *et al*., 2000) that enable many different genetic targets to be detected simultaneously in the same sample, utilizing simple instruments that possess a monochromatic light source. We also pioneered the use of molecular beacons for high-throughput "spectral genotyping" (Kostrikis *et al*., 1998); and we demonstrated the ease with which molecular beacons can distinguish single-nucleotide polymorphisms in PCR assays (Marras *et al*., 1999). We showed that molecular beacons work well in NASBA assays (Van Beuningen *et al*., 2001), as well as in PCR assays; and we demonstrated how molecular beacons can be used to monitor *in vitro* transcription in real time (Marras *et al*., 2004).

Our laboratory also designed a panel of assays that identify mutations in potential parents that cause Tay-Sachs disease and cystic fibrosis in the children of Ashkenazi Jews; and we developed a single-tube, multiplex assay that utilizes molecular beacons for the detection of bacteria that can be used as agents of bioterror: *Yersinia pestis*, *Bacillus anthracis*, *Burkholderia mallei*, and *Francisella tularensis*. We have also developed a single-tube version of a PCR assay that rapidly identifies multidrug-resistant *Mycobacterium tuberculosis* in sputum samples (El-Hajj *et al*., 2001). This assay has undergone clinical trials (Varma-Basil *et al*., 2004) and is being developed for commercial distribution. And finally, we have worked on the development of assays that detect hospital-acquired infections caused by pathogenic fungi and by methicillin-resistant and vancomycin-resistant *Staphylococcus aureus*.

Highly multiplex screening assays

Our laboratory has developed multiplex screening assays that utilize color-coded molecular beacons in single-tube gene amplification reactions that identify which infectious agent, if any, is present in a clinical sample (U.S. Patent Application 10/426,556). The first assay of this type is able to identify the 15 most prevalent bacterial species that are found in blood samples taken from febrile patients (Marras *et al*., 2007). Unlike classical blood cultures, which take many days to yield results, these “molecular blood cultures” require only two hours to complete. Each of the 15 species-specific molecular beacons is labeled with a unique combination of two differently colored fluorophores selected from a palette of six differently colored fluorophores. The two-color fluorescence signal that arises during the course of a PCR assay that amplifies a segment of the bacterial 16S ribosomal RNA gene uniquely identifies the species that is present. Future assays will utilize three differently colored fluorophores (selected from a palette of seven colors) to uniquely label each of 35 species-specific molecular beacons. This will enable simultaneous screening for the presence of both common species and rarely seen species, such as agents of bioterror. Widespread use of these assays will enable the rapid identification of common infectious agents, while at the same time providing an early warning system that will help contain the spread of major epidemics.

We have also highly multiplex screening assays based on a different principle. In these assays, only four differently colored molecular beacons are present during the amplification of a segment of the bacterial 16S ribosomal RNA gene. Unlike the assays described above, these molecular beacons

contain relatively long probe sequences, enabling them to bind to amplified 16S ribosomal RNA gene segments generated from many different bacterial species. The stability of each of the four resulting probe-target hybrids depends upon how well each of the molecular beacons matches the amplified target sequence. After amplification, the mixture of fluorescent probe-target hybrids is melted apart by raising the temperature and simultaneously determining, for each of the four differently colored probes, the temperature at which each hybrid falls apart (seen as a loss of fluorescence). The resulting set of four melting temperatures serves as a unique spectral signature that identifies which species is present (U.S. Patent Application 10/110,907). We demonstrated that 27 different species of *mycobacteria* can be uniquely identified with the aid of only four of these “sloppy” molecular beacons (El-Hajj *et al*., 2009); and we demonstrated that sloppy molecular beacon probes can identify 94 different species of bacteria (across 64 genera) in rapid, PCR assays designed to detect and identify bacterial species that can cause sepsis if present in the blood stream (Chakravorty *et al*., 2010).

Detection of rare cancer cells in otherwise wild-type tissue samples

Our laboratory is also developing PCR assays that use allele-discriminating gene amplification primers to determine the proportion of cells in a biopsy or blood sample that contain rare mutations indicative of cancer. We have utilized the knowledge gained from molecular beacons to design hairpin-shaped primers that are extraordinarily specific, enabling amplification products to be synthesized only from nucleic acids possessing single-nucleotide substitutions indicative of cancer. By comparing the number of rounds of amplification needed to synthesize detectable amplicons with a mutant-specific primer to the number of rounds of amplification needed to synthesize detectable amplicons with a wild-type specific primer, the proportion of cells that are cancerous can be measured. As little as one cancer cell in the presence of 100,000 wild-type cells can be seen (U.S. Patents 6,277,607 and 6,365,729). This technique will be useful for monitoring the effectiveness of cancer therapies.

Self-reporting oligonucleotide arrays

We have demonstrated that molecular beacons are useful for the determination of gene expression profiles (Manganelli *et al*., 1999; Dracheva *et al*., 2001). We are exploring the use of arrays of molecular beacons for the simultaneous quantitation of hundreds of different mRNAs in a sample. Each molecular beacon is immobilized at a different location on the surface of a glass chip. Instead of enzymatically adding a fluorophore to the target mRNAs and hybridizing those targets to an array of linear probes, when an array consists of immobilized molecular beacons, the mRNAs need not be labeled and the molecular beacons become fluorescent when the targets bind to them. Hairpin-shaped probes are significantly more specific than linear probes, and the intensity of the fluorescence generated by the molecular beacons is directly proportional to the number of mRNAs that are bound.

We are also investigating distributed array formats, in which many different molecular beacon probes are used at the same time. Each type of molecular beacon probe is immobilized on a different microbead, and tens of thousands of beads are used in an assay. After hybridization to a mixture of mRNAs, the fluorescence of each bead is rapidly read by a spectral analyzer that determines the number of target mRNAs bound to each bead from the fluorescence of the molecular beacons on its surface. In order to facilitate this approach, we have developed a rapid method for telling which bead contains which probe (U.S. Patent Application 20040248163). In this technique, additional hairpin-shaped nucleic acids possessing quenchers and differently colored fluorophores at each end are also immobilized on the surface of each bead. These additional hairpins do not serve as probes; instead the presence or absence of each hairpin serves as a binary element in a “serial number” that identifies the bead to which they are attached. For example, three different-length hairpins can be used, each labeled with one of five differently colored fluorophores, for a total of 15 distinctive elements that can be present or absent on the surface of the bead. The serial number of each bead in a collection of perhaps 100,000 beads is then simultaneously read by raising the temperature and noting, for each bead, which fluorescent colors appear on the surface of the bead as the temperature is raised, causing the three different-length hairpins to denature. The availability of practical gene expression profiling arrays should enable the identification of gene ensembles that control development, the discovery of new metabolic pathways, the exploration of cellular responses to viral and bacterial infection, and the development of high-throughput assays that identify new therapeutic agents.

Detection of mRNAs in living cells

One of the most exciting programs in the laboratory is the direct detection of mRNAs in living cells. Conventional *in situ* hybridization techniques require the "fixing" of cells to enable the unbound probes to be washed away. Fixing denatures and crosslinks the proteins, resulting in cell death. Thus, *in situ* hybridization provides a static view of mRNA distribution and is not effective for the investigation of dynamic processes. Because molecular beacons only become fluorescent when they bind to their target, there is no need to fix and wash the cells, and the synthesis, movement, localization, and disappearance of mRNAs can be viewed as a function of time. We have shown that molecular beacons are excellent probes for visualizing mRNAs in living cells, and we have used them in experiments with many different cell types. We found that molecular beacons can be synthesized from modified nucleotides that do not occur naturally, such as the 2'-*O*-methylribonucleotides, in order to prevent digestion of the molecular beacons by cellular nucleases and to prevent cleavage of the target mRNAs by cellular ribonuclease H. We also found that the interfering effects of autofluorescence from cellular components can be overcome by using wavelength-shifting molecular beacons, which have large Stokes shifts that enable them to fluoresce at longer wavelengths (Tyagi *et al*., 2000). Furthermore, molecular beacons are not toxic to cells, and different mRNAs in the same cell can be visualized simultaneously with differently colored molecular beacons. And finally, we have linked molecular beacons to tRNA sequences in order to ensure that the probes are retained within the cytoplasm (Mhlanga *et al*., 2005).

The injection of molecular beacons into living cells allows the expression of particular genes to be monitored as a function of genetically programmed development, or as a response to external stimulation. With the aid of deconvolving and confocal fluorescence microscopy, we used molecular beacons to visualize the formation, transport, and localization of *oskar* mRNA in living *Drosophila* embryos (Bratu *et al*., 2003). We also used molecular beacons to follow the movement of β-actin mRNA into growing lamellipodia as lymphocytes move across surfaces. Currently, we are using molecular beacons to track the movement and localization of CaMKII, Map-2, β-actin, and Arc mRNA in primary cultures of rat hippocampal neurons, in order to understand how the stimulation of presynaptic dendrites leads to mRNA localization and to the long-term potentiation of postsynaptic dendrites, which is an attractive model system for studying cellular mechanisms of memory formation (Batish *et al*., 2011). In addition, we are following the transport of specific mRNAs from the neuronal nucleus to postsynaptic dendritic sites, to determine the kinetics of mRNA movement and to elucidate the mechanism by which mRNAs are localized in stimulated dendrites.

Tracking Individual mRNA molecules

Although the fluorescence from a single molecular beacon bound to an mRNA is not sufficiently bright to be seen above the background fluorescence in a living cell, we devised a method that enables 96 molecular beacons to bind to a single mRNA molecule, which allows specific mRNAs to be seen and followed as they are synthesized, processed, and move within the nucleus and through the nuclear pores to the cytoplasm (Vargas *et al*., 2005). The technique that we developed involves the cloning of a synthetic sequence into the region of a target gene that encodes the 3'-untranslated region of the particular mRNA molecules that we wish to see and follow. The synthetic sequence contains 96 tandemly repeated molecular beacon binding sites. The presence of 96 probes on the 3' end of each mRNA does not prevent the binding of nuclear proteins. The motion of these individual mRNA-protein complexes were recorded by time-lapse photography. Analysis of their tracks demonstrates that they move freely by Brownian diffusion within the extranucleolar, interchromatin space. Experimental manipulation of the cellular environment by lowering the temperature and altering the availability of ATP, enabled us to conclude that occasionally these particles become trapped on the surface of the chromatin, and that the expenditure of metabolic energy is required for the particles to resume their motion. We are now introducing tandemly repeated molecular beacon target sites into different genes in order to study the mechanism of transport and localization of particular mRNAs in different cell types. This method will also aid in the identification of cellular sites where other processes central to gene expression occur. Examples of such processes are mRNA splicing, maturation, export, and decay. The ability to simultaneously track different mRNAs tagged with different multimeric target sequences, using differently colored molecular beacons in the same cell, will be especially useful in this regard.

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