A History of Cancer Research: Retroviral Oncogenes

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The discovery and characterization of retroviral oncogenes were important milestones in cancer research. The viruses turned out not to be key causes of cancer in humans, but the oncogenes they carried provided key clues to the role cellular genes, the proto-oncogene counterparts of these sequences, played in tumorigenesis. In this excerpt from his forthcoming book, Joe Lipsick looks back at early work on retroviruses, such as the experiments that distinguished their ability to infect and transform cells, the groundbreaking work on Src, and some of the controversy surrounding the Nobel Prize awarded for these discoveries.

A TRINITY OF ROUS SARCOMA VIRUSES

The discovery that acutely transforming retroviruses contain oncogenes of cellular origin revolutionized the field of cancer research. Instead of being the long-sought cause of human cancer, retroviruses instead became powerful tools for identifying and studying normal genes that are mutated by other mechanisms to cause human cancer. This scientific revolution resulted from an unexpected convergence of animal virology, genetics, molecular biology, and molecular evolution. Although this discovery would eventually have been made by studying other acutely transforming retroviruses, some unusual features of Rous sarcoma virus (RSV) were particularly favorable for the identification of retroviral oncogenes and of their normal cellular progenitors (proto-oncogenes).

In 1958, Howard Temin and Harry Rubin used their focus assay to deduce that infection with a single viral particle was sufficient to transform a normal cell into a cancer cell (see Lipsick 2021). However, the isolation of biological clones of viruses and of infected cells from individual foci provided some surprises. In 1963, Temin reported that viral replication was not required for transformation. He had isolated "non-producer" cells infected by RSV at a low multiplicity of infection that were morphologically transformed but did not produce infectious virus (Fig. 1).

In 1962, Peter Vogt in the Rubin laboratory discovered that the Bryan high-titer strain of RSV (BH-RSV) contained an avian leukosis virus (ALV) similar to that described by Bang and Ellerman (see Lipsick 2021). Infection by this Rous-associated virus (RAV) could be assayed in cell culture because RAV-infected cells became resistant to transformation by the BH strain, a phenomenon known as "interference." Harry Rubin had previously described a replica-

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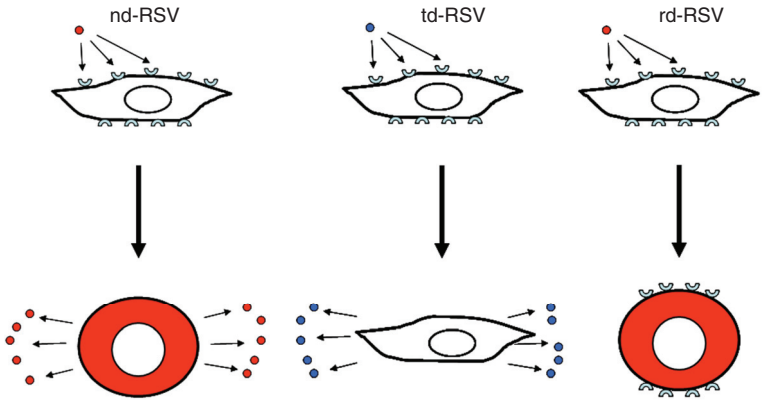

Figure 1. The biology of nondefective (nd), transformation-defective (td), and replication-defective (rd) forms of Rous sarcoma virus (RSV). Note that avian leukosis virus (ALV), Rous-associated virus (RAV), and replication-inhibiting factor (RIF) all behave like the td-RSV mutant shown here. Round, red cells are morphologically transformed. Cell surface receptors available for viral infection are indicated by light blue semicircles. These receptors are no longer available following infection with nd-RSV or td-RSV (interference). The Bryan strain of RSV contains a mixture of td-RSV and rd-RSV genomes within virions containing envelope proteins encoded by td-RSV. (Modified from Martin GS. 2004. *Oncogene* **23:** 7910–7917, with permission, from Springer Nature.)

tion-inhibiting factor (RIF) present in some but not all chicken embryos that prevented transformation by the BH strain. RIF turned out to be a congenitally transmitted form of ALV.

The following year, Hidesaburo and Teruko Hanafusa in the Rubin laboratory used limiting dilution assays to further dissect BH-RSV. The Rous transforming virus (BH-RSV) was itself incapable of replication, as Temin had reported. However, infection of BH-RSV "non-producer" cells with RAV resulted in the production of a viral stock capable of both transformation and replication. RAV was therefore a "helper" virus required for the replication of BH-RSV. These results led to the conclusion that the transforming component of RSV was defective for replication.

This conclusion was questioned when Robert Dougherty and Henry DiStefano reported in 1965 that "non-producer" cells infected by BH-RSV produced viral particles when examined by electron microscopy. Peter Vogt and Robin Weiss both showed that such cells did indeed produce infectious transforming virus that could be assayed using cells from quail or from particular inbred strains of chicken. This production of infectious virus was due to an endogenous retrovirus that was dubbed RAV(0).

The Hanafusa laboratory then discovered a "chicken helper factor" present in cells of some chickens that did not produce virus themselves but did permit the replication of BH-RSV. As Hanafusa and Weiss later showed, this factor was an envelope protein encoded by a retrovirus-related endogenous gene. Cells from chickens lacking this envelope protein could still be transformed by the BH strain of RSV. However, the resulting "non-producer" cells were truly incapable of producing infectious virus. These results showed that BH-RSV was indeed replication-defective (rd). The elegance of Hidesabora and Teruko Hanafusa's work in this field was summarized by Peter Vogt in a memorial tribute:

> I am not going to describe this part of Saburo's research except to say that it elicited awe for its ingenious experimental design and the precision with which it was carried out. A commentary in the News and Views section of *Nature* around 1970 already referred to Saburo as "legendary." The story goes that Saburo's papers on helper factors were understood by only 3 other people: his wife and scientific collaborator Teruko, and the 2 avian tumor virologists Steve Martin and Robin Weiss. I think there is a grain of truth in this.
>
> … Saburo and I were very good friends. It was one of those friendships that grew out of the

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type of intense competition that generates deep respect and then turns into genuine, mutual affection. (Vogt PK. 2010. *Genes & Cancer* **1:** 6–11)

Ray Bryan had isolated his high-titer strain of RSV by repeated passage in chickens. Because this strain produced many foci in the Temin and Rubin assay, it became the "gold standard" for American researchers. However, Rous had also distributed his original virus to other laboratories around the world. Unlike the BH strain, the Carr–Zilber, Prague, and Schmidt–Ruppin (SR) strains maintained in Europe were all capable of causing tumors in rodents. The independently isolated but closely related Bratislava avian sarcoma virus (B77) behaved similarly. In 1963, Jan Svoboda extended these observations by isolating a cell line from a rat tumor (XC) caused by the Prague strain of RSV. This cell line did not produce infectious virus, but a replication-competent transforming virus could be isolated by co-cultivation with uninfected chicken cells. These results were consistent with the hypothesis that viral replication was not required for oncogenic transformation. However, these results also suggested that the transformed rat cells might harbor a nondefective RSV (nd-RSV).

In 1966, Alice Goldé showed that the SR and the Prague strains of RSV were indeed nondefective (Fig. 1). Unlike the two components of the BH strain, biologically cloned stocks of these viruses were capable of both replication and transformation of chicken cells in culture. The Carr–Zilber and B77 strains were also then shown to be nondefective. Therefore, defectiveness for viral replication was not a requirement for transformation after all. Importantly, these nondefective viruses provided a much simpler system for studies of retroviral genetics and molecular biology than the BH strain, which contained both a replication-defective transforming virus (BH-RSV) and a replication-competent nontransforming helper virus (RAV).

THE FRUITS OF RETROVIRAL GENETICS

In the 1940s and 1950s, the phage group led by Max Delbrück, Alfred Hershey, and Salvador Luria used the viruses of bacteria and the quantitative logic of physics to discover fundamental principles of bacterial genetics and molecular biology. The isolation and characterization of mutants of host cells (bacteria) and their viruses (bacteriophages) provided very powerful tools for these studies. The same strategies were then applied to study the viruses of animals (see Lipsick 2021).

Peter Vogt and Hidesaburo Hanafusa each established their own laboratory after training with Harry Rubin. In the 1960s and 1970s they were instrumental in the development of retroviral genetics, with an emphasis on experimental rigor and careful reasoning. A major question concerned the mechanism by which RSV infected chicken cells. Vogt found that the BH strain of RSV contained two different replication-competent transformation-defective (td) helper viruses, RAV-1 and RAV-2. Remarkably, these viruses did not interfere with each other. Cells infected by RAV-1 were resistant to transformation by BH-RSV/RAV-1 but not by BH-RSV/RAV-2. The converse was true for RAV-2. Neutralizing antisera defined serologic subgroups that correlated with these differences in biological activity. These results led to a model in which genetic variants of the viral envelope protein mediated binding to different receptor proteins on the surface of the host cell. Previously infected cells produced sufficient envelope protein to occupy its specific receptor, thereby causing interference (Fig. 1).

Some inbred strains of chickens were found to be resistant to RAV-1 (subgroup A), RAV-2 (subgroup B), or both. Careful study revealed two different mechanisms of intrinsic resistance. First, the host cells could be deficient for expression of the specific receptor. This could be due to the absence of a dominant susceptibility allele of the receptor gene that permitted infection. It could also be due to cell-type specificity of receptor expression (e.g., macrophages were resistant to infection by subgroup A viruses even in animals that were genetically susceptible to infection). Second, the host cells could be resistant to infection owing to expression of endogenous retroviral genes that produced intracellular envelope proteins that occupied their cellular receptors.

Further studies eventually revealed the existence of at least ten different subgroups of avian retroviruses, some of which are commercially important in agriculture. Only a few specific subgroups permitted the infection of rodent cells by these avian viruses. This work provided the paradigm for studies that later identified CD4, CXCR4, and CCR5 as receptors for HIV, the retrovirus that caused the human AIDS epidemic. Remarkably, individuals homozygous for a recessive allele of *CCR5* are resistant to infection with HIV, just as some recessive alleles of receptor genes in chickens conferred resistance to infection by specific subgroups of avian retroviruses.

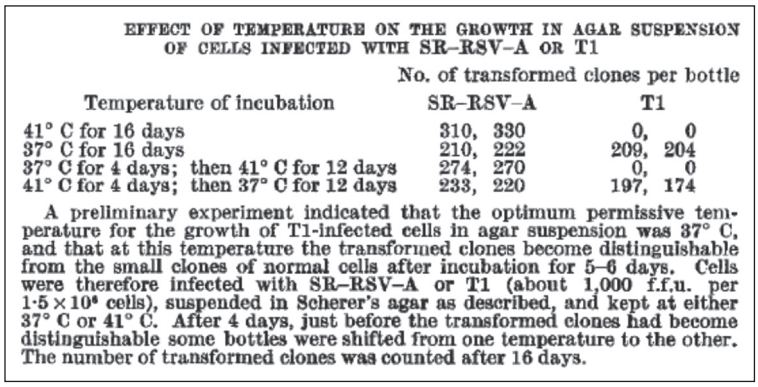
The Vogt and Hanafusa laboratories used the nd-RSV from Europe to demonstrate genetic recombination with transformation-defective-RAV helper viruses. Recombination between co-infecting viruses had been described in bacteriophages and permitted a form of genetic mapping akin to that used in diploid plants and animals. The relatively high frequency of recombination in retroviruses was due in part to their diploid genome. The size of the RSV genome had been controversial until 1968 when Peter Duesberg used biochemistry and electron microscopy to show that two major noncovalently linked single-stranded RNA molecules of similar size (3×10^6 MW) were present in RSV. Following the discovery of reverse transcriptase (RT) in 1970, it seemed likely that template switching by this viral enzyme was the cause of retroviral recombination.

The next logical step in retroviral genetics was the isolation of mutants to define functional genes and create a genetic map. A question of particular interest was to identify the gene(s) required for transformation. Temin had used natural variation to isolate viral mutants that affected the morphology of cells transformed by the BH strain of RSV (see Lipsick 2021). The identification of nd-RSV provided a much simpler system for mutant analysis. In 1966, Alice Goldé used irradiation to create replication-defective mutants of nd-RSV that behaved like the transforming component of BH-RSV. The Goldé and Vogt groups both then showed that irradiation could also create transformation-defective mutants that behaved like RAV and ALV. Together these results confirmed that while viral replication was compatible with transformation, it was not required for transformation.

Studies in a variety of genetic model organisms had shown the power of conditional mutants. Among the most useful of these were temperature-sensitive (ts) mutants. The most common forms of such mutants are heat-sensitive, because the protein encoded by the mutant allele functions properly at a lower (permissive) temperature, but not at a higher (nonpermissive) temperature. In the early 1960s, Alan Campbell and Bob Edgar had isolated conditional ts-mutants of bacteriophages. In 1965, Mike Fried reported the first ts-mutant of a tumor virus, the murine polyoma virus. However, both viral replication and transformation were affected.

In 1969, Peter Vogt's laboratory reported the isolation of the first ts-mutants of RSV. After treating cells transformed by nd-RSV with a chemical mutagen, the focus assay was used to identify mutant viruses. From 152 isolates, Kumao Toyoshima and Vogt identified two ts-mutants of RSV. These mutants were ts for both transformation and viral replication. Meanwhile, Steve Martin performed a very similar experiment, but with a bit more luck. From 256 isolates, he identified six that were ts for oncogenic transformation. One of these was recloned, studied in detail, and in 1970 reported to have a very interesting phenotype. The mutant called T1 replicated similarly to its isogenic nondefective parent, SR-RSV-A, at both 37°C and 41°C. However, unlike the parental virus, the T1 mutant transformed cells at 37°C but not at 41°C (Fig. 2).

Remarkably, morphologic transformation by T1 was reversible upon temperature shift. Cells transformed at 37°C reverted to a normal phenotype at 41°C. Conversely, cells infected at 41°C that did not exhibit a transformed phenotype did so when shifted to 37°C. These results provided further evidence that viral replication could be dissociated from transformation. Importantly, they also showed that a viral gene function not required for replication was required for both the initiation and maintenance of transformation. In



EFFECT OF TEMPERATURE ON THE GROWTH IN AGAR SUSPENSION OF CELLS INFECTED WITH SR–RSV–A OR T1

Temperature of incubation	No. of transformed clones per bottle SR–RSV–A	T1
41° C for 16 days	310, 330	0, 0
37° C for 16 days	210, 222	209, 204
37° C for 4 days; then 41° C for 12 days	274, 270	0, 0
41° C for 4 days; then 37° C for 12 days	233, 220	197, 174

A preliminary experiment indicated that the optimum permissive temperature for the growth of T1-infected cells in agar suspension was 37° C, and that at this temperature the transformed clones become distinguishable from the small clones of normal cells after incubation for 5–6 days. Cells were therefore infected with SR–RSV–A or T1 (about 1,000 f.f.u. per $1{\cdot}5 \times 10^6$ cells), suspended in Scherer's agar as described, and kept at either 37° C or 41° C. After 4 days, just before the transformed clones had become distinguishable some bottles were shifted from one temperature to the other. The number of transformed clones was counted after 16 days.

Figure 2. A temperature-sensitive mutant of Rous sarcoma virus revealed that a gene function not required for viral replication (not shown here) was required for both initiation and maintenance of transformation. (Reprinted from Martin GS. 1970. *Nature* **227:** 1021–1023, with permission, from Springer Nature.)

1972, John Bader described a mutant of the replication-defective BH-RSV that was ts for transformation. Transformation by this mutant was also reversible upon temperature shift. The following year, John Wyke described a method for the selection of ts-mutants of RSV by using bromodeoxyuridine plus light to kill transformed cells that continued to divide at the nonpermissive temperature. This permitted him to isolate new ts-mutants with far less effort than required for "brute force" screening in the absence of any selection.

Additional evidence for viral genes required for transformation but not replication came from comparative studies of the transforming properties of different retroviruses. Following the isolation of RSV by Peyton Rous, several other acutely (rapidly) transforming retroviruses were isolated from chickens and later from other animals, including mice, rats, cats, and monkeys. Like RSV, some caused fatal fibrosarcomas. Others, like the avian myeloblastosis virus (AMV), caused rapidly fatal leukemia (cancer of the blood cells) but not fibrosarcoma. Eventually, investigators developed cell culture assays for the leukemic transformation of normal blood cells. In 1961, Marcel Baluda reported that AMV could transform macrophages (a type of blood cell), but not fibroblasts. Conversely, RSV was shown to transform fibroblasts, but not macrophages. Nevertheless, both viruses could replicate in both types of cells. These results suggested that these two retroviruses likely contained transforming genes with specificities for different cell types.

The critical tools for identifying and discovering the source of the transforming genes of retroviruses were discovered fortuitously. In 1971, Vogt reported that during the passage of biologically cloned stocks of nd-RSV, spontaneous td-RSV mutants arose at high frequency in the absence of any mutagenic treatment. Similar results were reported by Martin and Duesberg in 1972. Intriguingly, the nd-RSV stocks contained a large RNA subunit called "a" that was no longer present in the td-RSV mutants that arose either spontaneously or following irradiation, whereas a smaller RNA subunit called "b" was still present. By contrast, one of Martin's chemically induced mutants that was ts for transformation but not for replication had retained its larger "a" subunit at both the permissive and nonpermissive temperatures.

ONCOGENIC ALGEBRA: a = b + *x*

The spontaneously arising transformation-defective mutants of nd-RSV strains provided isogenic pairs of viruses that appeared to differ only in their ability to morphologically transform normal chicken embryo fibroblasts. The Duesberg and Vogt laboratories together showed that biologically cloned nd-RSV of the Prague C strain contained only the larger "a" subunit, whereas the corresponding td-RSV mutant contained only

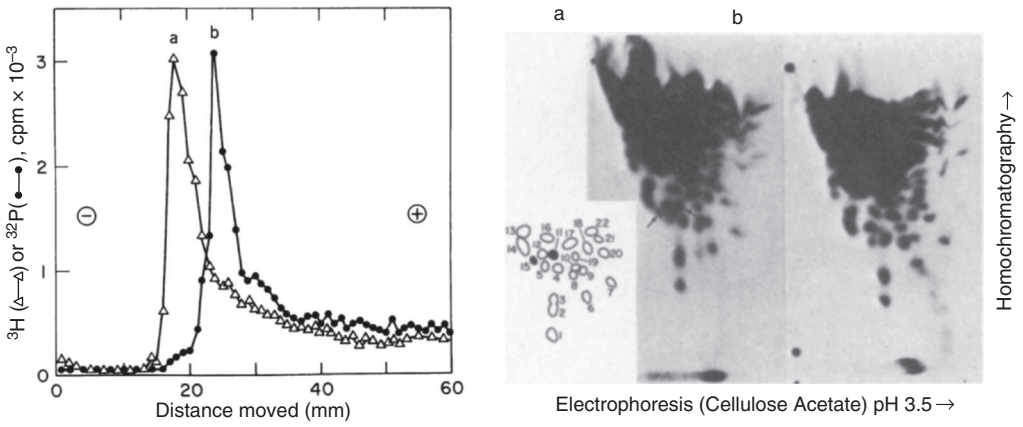


Figure 3. Comparative analyses of genomic RNA from an isogenic pair of Prague C Rous sarcoma viruses (RSVs), the parental nondefective (nd) strain (a) and a mutant transformation-defective (td) strain (b). (*Left*) Co-electrophoresis of nd-RSV labeled with [^{3}H]-uridine and td-RSV labeled with [^{32}P]-phosphate. (*Right*) Two-dimensional "fingerprinting" of [^{32}P]-phosphate-labeled RNA fragments created by digestion with T1 RNase. The shaded dots and the arrows indicate oligonucleotide spots unique to the nd virus. (Reprinted from Lai MC, et al. 1973. *Proc Natl Acad Sci* **70:** 2266–2270, with permission.)

the smaller "b" subunit when assayed by gel electrophoresis (Fig. 3, left). Similar results were obtained with isogenic pairs of nd-RSV and td-RSV derived from the SR A and the B77 strains. These results showed that, in all three cases, the td-RSV mutants were likely to have arisen by deletions of ~10% of their RNA genome.

The nature of these deletions was explored further by two additional methods. Two-dimensional "fingerprinting" of radioactive RNA fragments invented in the laboratory of Fred Sanger several years earlier was used by Michael Lai in the Duesberg laboratory to compare enzymatically digested fragments of isogenic pairs of nd-RSV and td-RSV (Fig. 3, right). The majority of resolvable T1 RNase-resistant oligonucleotide spots were present in both viruses, whereas a small number of additional spots were present only in nd-RSV. Once again, similar results were obtained with isogenic pairs of viruses from three different strains of RSV.

Because only a relatively small number of large oligonucleotides were well resolved by RNA fingerprinting, nucleic acid hybridization was used to assess the overall homology of nd-RSV and td-RSV. cDNA fragments of td-B77 genomic RNA were synthesized in vitro using the viral RT. These fragments were then hybridized to either nd-B77 or td-B77 genomic RNA that had been radioactively labeled. Hybridization was then assayed by the resistance of double-stranded RNA–DNA duplexes to pancreatic RNase. The results showed that at least 60% of the genomes were shared between nd-B77 and td-B77, whereas ~10% of the nd-B77 genome was not present in td-B77. The latter result was consistent with the gel electrophoresis experiments.

Taken together, these results led to two conclusions. First, the td-RSV mutants arose by deletion of the nd-RSV genome. Second, this deletion contained specific sequences required for oncogenic transformation. These conclusions were summarized algebraically as "$a = b + x$", where x represents the additional genetic material required for transformation by nd-RSV. This work provided the first physical evidence for an oncogene, a term first used in 1969 by Robert Huebner and George Todaro to describe the transforming genes of retroviruses.

A variation on the technique of RNA fingerprinting together with retroviral genetics was used to map the oncogene of RSV. The discovery of polyadenylation of vaccinia virus RNA by Joseph Kates in 1970 soon led to the realization that most eukaryotic mRNAs were polyadenylated at

their 3′ end. Furthermore, oligo-dT cellulose could be used to greatly enrich poly(A)-containing mRNAs from pools of total cellular RNA. The poly(A) tail of retroviral RNA permitted Lui-Hai Wang in the Duesberg laboratory to map the location of oligonucleotides within the RSV genome by determining the shortest poly(A)-containing RNA fragment that contained each oligonucleotide. The oligonucleotides present in nd-RSV but not in td-RSV were located near the 3′ end of the genome. A similar analysis of strains with mutations that affected other functions of the virus revealed the gene order of nd-RSV to be 5′-*GAG-POL-ENV-ONC*-3′ (Fig. 4). A few years later, Alan Bernstein and Steve Martin used retroviral recombination to map the chemically induced ts-mutants of RSV to the same ONC region that was deleted in td-RSV.

The success of these landmark studies depended on two unusual quirks of RSV. First, RSV is the only naturally isolated retrovirus that is nondefective for both viral replication and oncogenic transformation in cell culture. Many other acutely transforming retroviruses were subsequently discovered, almost all of which contained novel sequences specific for oncogenic transformation and were replication-defective (Fig. 4). An interesting exception was the spleen focus-forming virus (SFFV) discovered by Charlotte Friend that caused erythroleukemia in mice as a result of a mutant envelope protein that interacted with the erythropoietin receptor.

Second, by far the most common naturally arising td-RSV variants turned out to have sustained complete deletions of the viral oncogene. This is likely due to repeated sequences that were later shown to flank the oncogene of RSV. Had commonly occurring td-RSV variants resulted from point mutations, small insertions, or small deletions, no consistent differences would likely have been detected by gel electrophoresis, RNA fingerprinting, or nucleic acid hybridization.

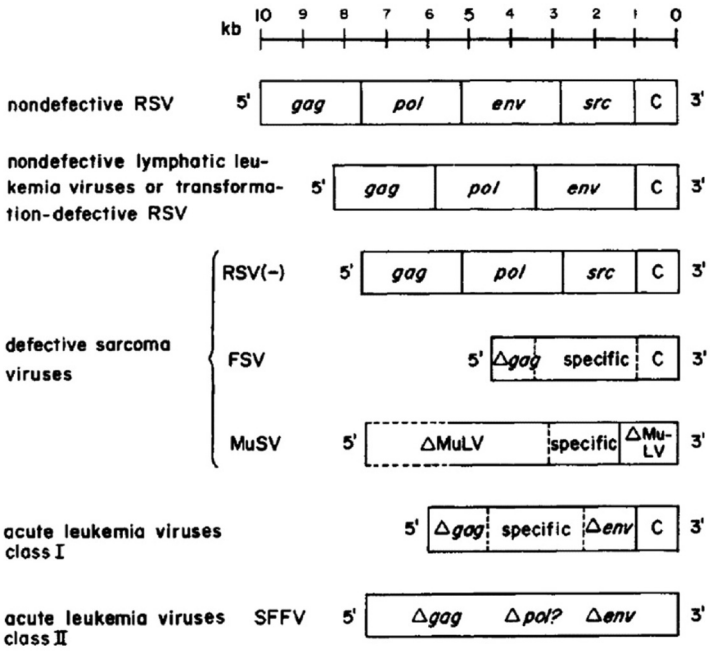


Figure 4. Transformation-specific sequences of representative retroviruses identified by RNA footprinting and nucleic acid hybridization. Only nondefective Rous sarcoma virus (nd-RSV) has all three genes required for replication in addition to its oncogene (*src*). RSV(–) indicates a replication-defective form of RSV present in the Bryan strain. The Friend spleen focus-forming virus (SFFV) did not contain novel transformation-specific sequences. (Reprinted, with permission, from Duesberg PH. 1980. *Cold Spring Harb Symp Quant Biol* **44:** 13–29, © Cold Spring Harbor Laboratory Press.)

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THE VIROGENE–ONCOGENE HYPOTHESIS

In the early 1960s, the cancer research establishment in the United States shifted its attention to the discovery and prevention of putative human leukemia viruses. Several developments prompted this redirection of efforts and resources. In the world of laboratory science, Ludwig Gross published the first description of a murine leukemia virus (MuLV) in 1953. Although leukemia viruses had been discovered by Ellerman and Bang in 1908 (see Lipsick 2021), these viruses occurred in chickens and similar viruses had not previously been found in mammals. Gross succeeded where others had failed by using the inbred AK strain of mice created by Jacob Furth that had a 90% incidence of spontaneous leukemia. In addition, Gross injected newborn rather than adult mice with cell-free filtrates of leukemia cell extracts. Soon, others were able to reproduce his results, leading to the isolation of several new MuLVs that were eventually named for their discoverers—Friend, Schwartz, Graffi, Moloney, Rauscher, and Abelson.

Meanwhile, the success of the polio virus campaign by the National Foundation for Infantile Paralysis (later rebranded as the March of Dimes) brought societal pressure to apply similar "directed research" efforts to bear on other diseases, particularly those that affected children. The apparent clustering of childhood leukemia cases together with the recent discovery of MuLVs led to the hypothesis that childhood leukemia was a transmissible viral disease, and therefore might be prevented by vaccination. In 1961, the National Cancer Institute established the Laboratory of Viral Oncology, a new intramural research program directed by Ray Bryan, who had developed the high-titer strain of RSV. A large amount of funding was also devoted to a new Virology Research Resources Branch. This industrial-style operation was intended to produce a vaccine against a yet unidentified human leukemia virus. Reports of the identification and isolation of such viruses initially generated a lot of excitement, but these turned out to be laboratory artifacts or contamination by viruses from other animal species. Although two bona fide human leukemia viruses (HTLV-1 and -2) were eventually isolated in the 1980s by the laboratories of Robert Gallo and Yorio Hinuma, these retroviruses are rare causes of human disease.

The failure to find horizontally transmitted human leukemia viruses led to a new theory about the origin of human cancer. Several lines of evidence were consistent with the vertical transmission of leukemia viruses in mice. Inbred strains of mice with a high incidence of leukemia (AKR and C58) were viremic even prior to birth. By contrast, inbred strains of mice with a low incidence of leukemia (C3H and C57) did not express detectable virus until late in life. Progeny of crosses between high-incidence (100%) and low-incidence (<1%) strains had an intermediate incidence (50%) of leukemia, suggesting a Mendelian pattern of inheritance. Remarkably, mice treated with irradiation or chemical carcinogens developed high titers of leukemia viruses. In 1969, the Todaro laboratory reported that serial passage of virus-negative mouse embryo cells in culture resulted in spontaneous transformation that was sometimes accompanied by the release of MuLV. Taken together, this evidence led Heubner and Todaro to propose the virogene–oncogene hypothesis:

> It is postulated that the viral information (the virogene), including that portion responsible for transforming a normal cell into a tumor cell (the oncogene), is most commonly transmitted from animal to progeny animal and from cell to progeny cell in a covert form. Carcinogens, irradiation, and the normal aging process all favor the partial or complete activation of these genes. (Huebner RJ, Todaro GJ. 1969. *Proc Natl Acad Sci* **64:** 1087–1094)

Rather than human leukemia and other cancers being caused by transmission of a virus from person to person, cancer was now thought to be caused by the activation of latent viruses already present in normal cells. The discovery of RT by the Temin and Baltimore laboratories in 1970 provided vindication for Temin's controversial provirus hypothesis (see Lipsick 2021). This proposal that the RNA genome of RSV was copied into a DNA provirus that became part of the host cell genome offered an attractive

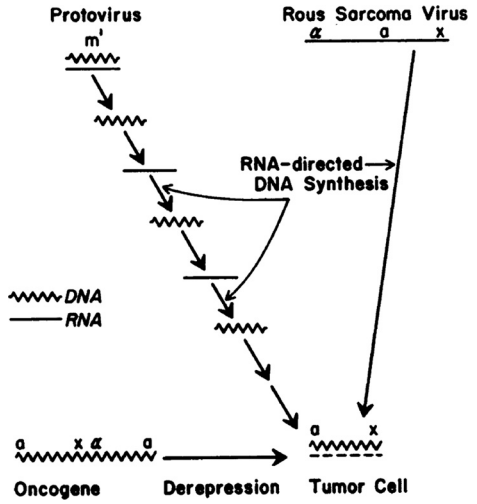


Figure 5. Models for neoplastic transformation. The DNA sequence from a to x expresses an RNA molecule that causes transformation. Infection with Rous sarcoma virus inserts this DNA sequence into infected cells. In the virogene–oncogene hypothesis, this DNA sequence (the oncogene) exists in a repressed state within a virogene present in all normal cells. In the protovirus hypothesis, the oncogenic information is created de novo by repeated rounds of transcription and reverse transcription of an initially nononcogenic protovirus. (Reprinted from Temin H. 1972. *Proc Natl Acad Sci* **69:** 1016–1020.)

explanation for the "covert" form of Huebner and Todaro's putative virogene. Temin himself favored a different model that he referred to as the protovirus hypothesis (Fig. 5). Rather than a preexisting oncogene-containing virogene within cells being activated by carcinogens or during aging, Temin thought that repeated rounds of transcription and reverse transcription of a noncarcinogenic protovirus might generate the sequences required for oncogenesis.

These competing theories were hotly debated. Jan Svoboda, a virologist who had worked under difficult conditions behind the Iron Curtain in what is now the Czech Republic, wrote of a scientific meeting that he attended in Amsterdam in 1972, "I remember Bob Huebner shouting at Howard Temin 'You do not understand virology!' and the reply was 'You do not understand oncology!'" (Svoboda J. 2008. *Adv Cancer Res* **99:** 1–32).

AN ANCIENT ENEMY WITHIN

A critical prediction of the virogene–oncogene hypothesis was the presence of oncogene-containing retrovirus-related sequences in uninfected cells. In 1972, both Marcel Baluda and Harold Varmus used nucleic acid hybridization to show that uninfected chicken cells contained multiple copies of sequences related to those of avian retroviruses. Some of these sequences could be transmitted in the germline and were located on specific chromosomes. However, by using the entire RSV genome as a radioactive probe, one could not distinguish between the predictions of the protovirus hypothesis and the virogene–oncogene hypothesis. One needed to know whether any of these endogenous virus-like sequences contained the oncogenic region of nd-RSV that Duesberg and Vogt had shown was missing in td-RSV.

The key to answering this burning question was the preparation of a nucleic acid probe specific for the oncogenic region of nd-RSV. Following in the footsteps of Ramareddy Guntaka, another postdoctoral fellow in the joint Bishop–Varmus laboratory, Dominique Stehelin succeeded in preparing a nucleic acid probe specific for the region of nd-RSV that was missing in td-RSV. The viral genome within an RSV virion consists of two single-stranded RNA molecules synthesized by the RNA polymerase II of the host cell that produced the virus. Following infection of a host cell, these plus-stranded viral mRNAs are used as templates for RT to produce a double-stranded RNA–DNA hybrid. A viral enzyme then degrades the RNA, leaving a single-stranded minus-strand DNA template. RT then uses this minus-strand DNA as a template to synthesis plus-strand DNA. The result is a double-stranded proviral DNA that can integrate into the genome of the infected host cell.

In 1970, the Bishop laboratory had shown that actinomycin D could inhibit the DNA-dependent polymerase activity of RT, but not its RNA-dependent polymerase activity. Therefore, in the presence of actinomycin D, the RT present in detergent-disrupted virions could be used to prepare a radioactively labeled minus-strand DNA probe that was complementary to the

plus-strand RNA genome of the virus. A radioactive minus-strand DNA probe specific for all the sequences shared by nd-RSV and the shorter td-RSV was generated by this method from td-RSV. To make a probe specific for the unique region of nd-RSV, radioactive minus-strand DNA fragments prepared from nd-RSV virions were hybridized to nonradioactive plus-strand RNA from td-RSV. Hydroxyapatite chromatography was then used to separate the shared sequences present in double-stranded RNA–DNA hybrids from the unhybridized single-stranded DNA sequences unique to nd-RSV, which were referred to as *sarc* (Fig. 6).

Stehelin found that both of these probes hybridized to genomic DNA from uninfected chickens (Fig. 7A). These results appeared to be consistent with the virogene–oncogene hypothesis. However, when the same probes were hybridized to genomic DNA from other species of birds, the results were quite different (Fig. 7B). The *sarc* probe identified similar sequences in DNA from quail, turkey, duck, and emu (obtained from the Sacramento Zoo), whereas the td-RSV probe did not. Furthermore, the relative strength of hybridization correlated with the inferred evolutionary distances among these species of birds (Fig. 7C).

The implications of this experiment were profound and unexpected. The *sarc* sequences that had been deleted in td-RSV were evolutionarily conserved. However, this was not the case for the sequences that were shared by both nd-RSV and td-RSV. The simplest explanation was that RSV had arisen by the capture (transduction) of cellular DNA sequences by an ALV-like replication-competent chicken retrovirus. Although the initial publication of these results did not provide evidence for the presence of similar sequences in humans and other mammals, such evidence was soon forthcoming. Within a few years, the Bishop–Varmus, Geoffrey Cooper, Hidesaburo Hanafusa, and Anna Marie Skalka laboratories showed that the transformation-specific sequences within nd-RSV contained an intronless copy of a normal chicken gene (Fig. 8). The evolutionarily conserved cellular gene (c-*src*) from which the viral oncogene (v-*src*) had arisen became known as a proto-oncogene.

The careful study of animal tumor viruses had turned the world of experimental oncology upside down. It has been said that serendipity is when one looks for a needle in the haystack, and instead finds the farmer's daughter (or son). Rather than cancer being caused by external infectious agents, it now seemed likely that human cancer was caused by the mutation of normal proto-oncogenes. In a 1981 review article, Mike Bishop dubbed these proto-oncogenes "enemies within," a sinister phrase that recalled the United States' fear of Communists and the alien-invader movies of the 1950s (Bishop JM. 1981. *Cell* **23:** 5–6).

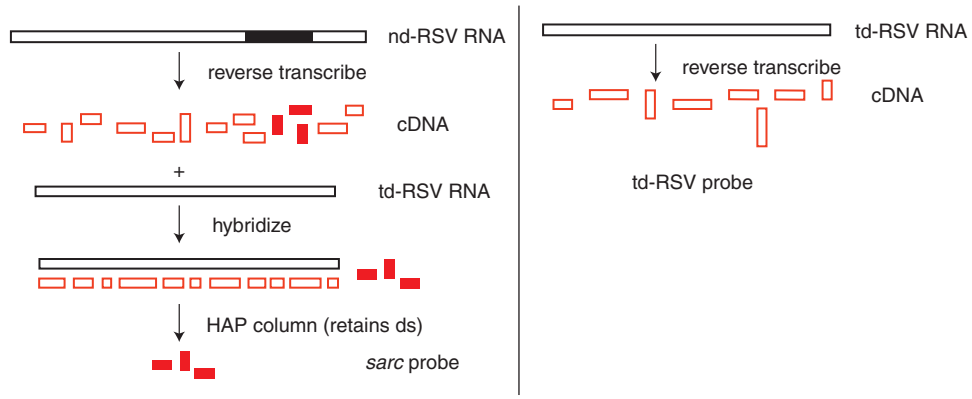
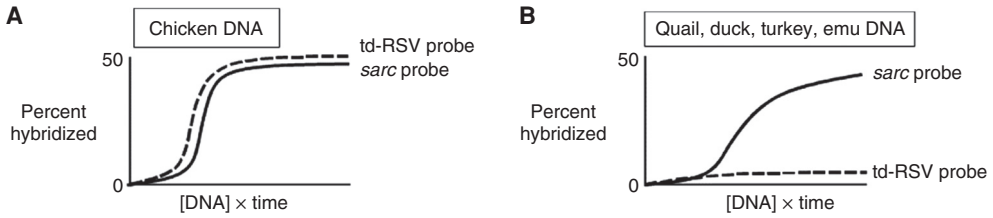


Figure 6. Preparation of *sarc* probe by subtractive hybridization (*left*). Preparation of transformation-defective Rous sarcoma virus (td-RSV) probe without subtraction (*right*). The shaded boxes indicate sequences that had been deleted from nondefective RSV (nd-RSV) during the formation of td-RSV. RNA is shown in black. Radioactive DNA is shown in red.



C

Table 2 Thermal stabilities of duplexes between $cDNA_{sarc}$ and normal DNAs

DNA	$\%cDNA_{sarc}$ in duplexes	T_m	ΔT_m	Phylogenetic distance from chicken (Myr)
Provirus (XC)	56	81	0	
Chicken	52	77	− 4	0
Quail	37	74	− 7	35–40
Turkey	30	72	− 9	40
Duck	16	71	−10	80
Emu	15	70	−11	100

Figure 7. Hybridization of genomic DNA from different species of birds with the *sarc* and transformation-defective Rous sarcoma virus (td-RSV) probes. (*A,B*) Schematic representations of nucleic acid hybridization experiments. (*C*) Correlation of strength of hybridization of the *sarc* probe with evolutionary distances inferred from the fossil record and antigenic relationships among proteins (provided by Allan Wilson). The XC control was Jan Svoboda's rat cell line containing a single-copy nondefective RSV (nd-RSV) provirus. (Table reprinted from Stehelin D, et al. 1976a. *Nature* **260:** 170–173, with permission, from Springer Nature.)

Interestingly, Peter Vogt had written in 1972, "There is thus no reason to assume that the oncogenic information of class a subunits [nd-RSV] is necessarily viral in origin; rather, this information could have arisen from the cell" (Vogt PK. 1972. *J Natl Cancer Inst* **48:** 3–9). There was ample precedent for the transduction of cellular genes by bacterial viruses, a phenomenon discovered in 1952 by Norton Zinder and Joshua Lederberg that became a powerful tool for studying the genetics of bacteria. However, when the cellular origin of the oncogene of RSV was discovered in 1975, Vogt was quite surprised (P Vogt, pers. comm.).

Peyton Rous himself had been strongly opposed to the idea that alterations of normal cellular genes were the cause of cancer. During his Nobel Prize acceptance speech in 1966 he had said, "What can be the nature of the generality of neoplastic changes… . A favorite explanation has been that oncogens [carcinogens] cause alterations in the genes of the cells of the body, somatic mutations as these are termed. But numerous facts, when taken together, decisively exclude this supposition." Ironically, the virus that Rous had isolated from the tumor of a sick chicken in 1911 ultimately paved the way for the discovery of the very genes that, when mutated, cause cancer.

During the 1970s, Hidesaburo Hanafusa and his colleagues at Rockefeller University showed that infection of chickens with td-RSV could lead to the recovery of transformation-competent nd-RSV. These experiments also argued strongly in favor of a cellular origin for retroviral oncogenes. However, these experiments did not distinguish between recombination with oncogenic proviral sequences versus the transduction of a normal cellular gene.

Prior to the discovery of c-*src*, Edward Scolnick's laboratory at the NCI had used nucleic acid hybridization to characterize the genomes of acutely transforming rat sarcoma viruses (RaSVs) that had arisen by passage of non–acutely transforming MuLVs in rats. Scolnick's group was able to show that two different RaSVs contained nucleic acids related to those of rats rather than mice. However, they could not determine whether these transduced sequences were derived solely from endogenous rat retroviruses or contained additional rat genes. In the absence of appropriate mutants, the relationship of these rat-derived sequences to oncogenic

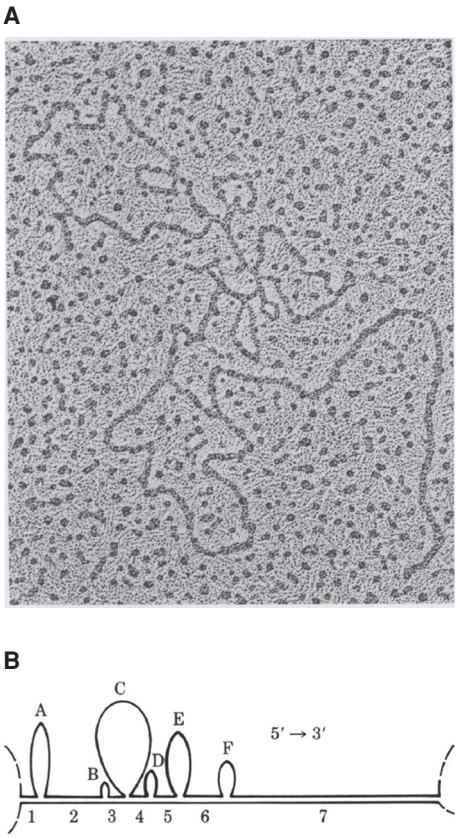


Figure 8. (*A*) Electron micrograph and (*B*) schematic diagram of a heteroduplex between molecular clones of c-*src* and v-*src*. The loops represent introns present in c-*src* but not v-*src*. (Reprinted from Parker RC, et al. 1981. *Proc Natl Acad Sci* **78:** 5842–5846, with permission.)

transformation also remained unclear. They later showed that the two different strains of RaSV contained repetitive DNA sequences of rats (VL30) along with similar but not identical oncogenes of cellular origin that become known as H-*ras* and K-*ras* (for Harvey and Kirsten RaSVs).

Paul Neiman's laboratory had also used nucleic acid hybridization to investigate the relationships among nd-RSV, td-RSV, and cellular DNA. In 1974, they reported that td-RSV was missing ~13% of the sequences present in nd-RSV. However, they concluded that these missing sequences were not present in normal chicken DNA. This failure to identify the c-*src* proto-oncogene may have been due to their experimental strategy, in which radioactive viral RNAs were hybridized to excess cellular DNA. The presence of multiple copies of endogenous retroviruses versus only one copy of c-*src* per genome perhaps obscured the existence of the latter, which was soon discovered by the *sarc*-specific subtractive hybridization approach of the Bishop–Varmus laboratory described above.

Studies of other acutely transforming retroviruses of chickens, mice, cats, and monkeys eventually identified approximately 30 different retroviral oncogenes and the cellular proto-oncogenes from which they had been transduced (Fig. 9). Some proto-oncogenes had been transduced more than once, sometimes in more than one species of animal. These results implied that the total number of proto-oncogenes might be relatively small. In all cases, homologs of these proto-oncogenes were present within the human genome.

NOBEL, NON BELLE?

It became increasingly likely that the discovery of viral oncogenes and then of cellular proto-oncogenes would result in big scientific prizes (Fig. 10). In 1982, the Albert Lasker Awards (named for the advertising man who had spread addiction to cigarettes far and wide) were given to Michael Bishop and Harold Varmus, along with three other retrovirologists, Raymond Erickson, Robert Gallo, and Hidesobura Hanafusa. A Lasker Award is sometimes a precursor to a Nobel Prize (named for the man who invented dynamite and smokeless gunpowder). Scientists waited expectantly for the other shoe to drop. In 1989, the Nobel Prize in Physiology or Medicine was awarded to Michael Bishop and Harold Varmus.

The most coveted coin in the realm of science is publication. Currently the most desirable positions in the order of authorship of biology papers are the first (generally the person who did most of the work) and the last (often the person in charge of the laboratory in which the work was performed). The order of authorship on the paper that described the Nobel Prize–winning discovery of cellular proto-oncogenes was Dominique Stehelin, Harold Varmus,

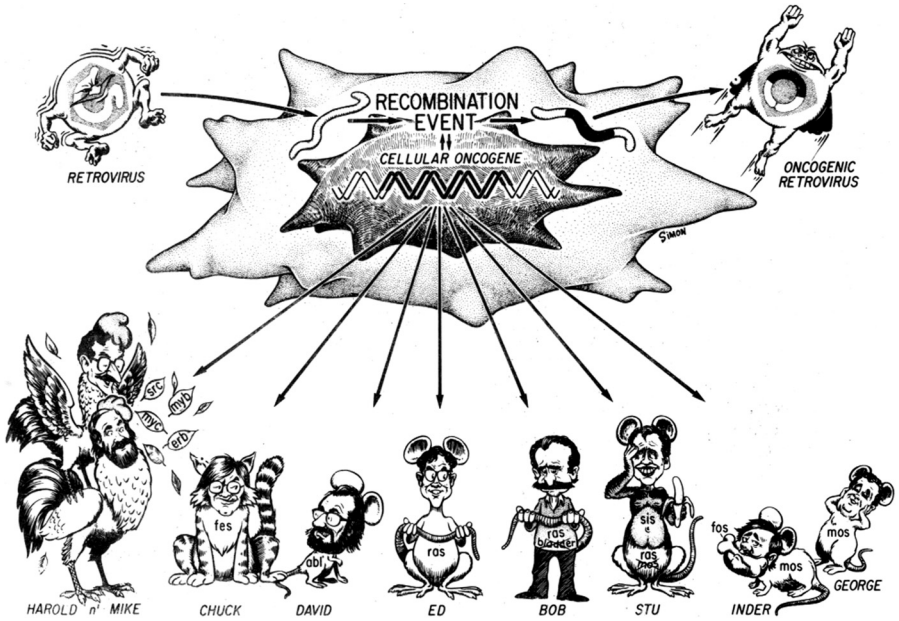


Figure 9. Cover of the abstract book for the meeting on RNA Tumor Viruses at Cold Spring Harbor Laboratory on May 25–29, 1983. Some (but not all) of the discoverers of various retroviral oncogenes are depicted as the animals from which the viruses were isolated. (Illustration © Jamie Simon.)

Michael Bishop, and Peter Vogt. However, as was the custom at that time, the authors were grouped by home institution, the first three from UC San Francisco and the last from the University of Washington.

Alfred Nobel's bequest stated that a prize was to be given each year for a discovery occurring in the previous year and to no more than three people. The Nobel committee has often ignored the former but never (at least so far) the latter stipulation. In this case, only the middle two authors were judged deserving. Peter Vogt, a virologist's virologist who had generated and characterized the critical mutants of RSV, seemed happy to see the importance of the work acknowledged. Dominique Stehelin, the postdoctoral fellow in the Bishop–Varmus laboratory who had performed the key molecular biology experiments, felt differently, saying:

> I am very disappointed. I find all that very unfair and rotten. I did the work all by myself, from A to Z. I spent three years in their San Francisco lab, from 1972 to 1975, at a time when nobody other than me was working on the subject and I am not even associated in this distinction. This work belongs to me and I don't know why the scientific community refuses to attribute the discovery to me. Undoubtedly because working in Lille is judged less prestigious than being a researcher in San Francisco. (*Los Angeles Times*, October 9, 1989)

Stehelin launched a campaign to right this perceived wrong. Some prominent members of the French scientific community publicly supported his claims. In an open letter to the Nobel Committee, Stehelin wrote of the joy of discovery that drives scientists to continue working in the face of repeated failure—"I suspect that few have the privilege of enjoying such a moment when one is intensely and profoundly aware that a major step forward in Science has been made, and that one has contributed to it." Sadly, that enjoyment was not enough. Joy due to the awarding of a prize (or lack thereof) had been replaced by anger and indignation. For better or worse, there is no court of appeals for Nobel Prizes. In response to Stehelin's letter, a member of the Nobel Committee said that Stehelin had not done any important work since leaving the Bishop–Varmus laboratory. Stehelin responded that he had dis-

Figure 10. Cover of the abstract book for The Third Annual Meeting on Oncogenes at Hood College in Frederick, Maryland, on July 7–11, 1987. The smell of gold was in the air. (Illustration © Jamie Simon; lyrics by Tony Hunter.)

covered the cellular homologs of several other retroviral oncogenes (*myc*, *myb*, *erbA*, *erbB*, *ets*) after establishing his own independent laboratory in Lille, France. He had also won several other important scientific awards, including the Louis-Jeantet Prize, a European equivalent of the Lasker Award.

This unfortunate episode raises some difficult questions about the winner-take-all nature of science as currently practiced. What are the benefits and detriments of scientific prizes? What motivations for doing science are likely to best serve individual scientists? What rewards for scientists are likely to best serve society as a whole? As is always the case, each discovery is built upon the discoveries of others.

When asked how he would celebrate his Nobel Prize, Harold Varmus said, "I'm hoping to watch a victorious Giants [baseball] game." Initially things went well. The San Francisco Giants won a playoff series with the hapless Chicago Cubs. The Giants then faced their cross-bay rivals, the Oakland As, in the 1989 "World" Series, the championship of American professional baseball. In recognition of their Nobel Prize, Varmus and Bishop were invited to throw ceremonial pitches prior to game four of the best-of-seven series that was dubbed "The Battle of the Bay." However, the devastating Loma Prieta earthquake occurred minutes before the start of game three. Ten days later, game three was played and the ceremonial pre-game pitches were thrown by policemen and

firefighters. Willie Mays, the hall-of-fame New York and then San Francisco Giants' outfielder, had been bumped from game three to game four. Varmus and Bishop were bumped to game five. Mike Bishop later wrote that he had pleaded with the commissioner of baseball to share the game four honors with Willie Mays. The commissioner declined, putting the value of a Nobel Prize in proper perspective by replying, "Doc, get real." And there was no game five. The Giants lost the series in four.

SUGGESTED READING

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A History of Cancer Research: Retroviral Oncogenes

Joseph Lipsick

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