Viruses are capable of causing a wide variety of human diseases, ranging from rabies to smallpox to the common cold. The great majority of these infectious agents do harm through their ability to multiply inside infected host cells, to kill these cells, and to release progeny virus particles that proceed to infect other hosts nearby. The \textit{cytopathic} (cell-killing) effects of viruses, together with their ability to spread rapidly throughout a tissue, enable these agents to leave a wide swath of destruction in their wake.

But the peculiarities of certain viral replication cycles may on occasion yield quite another outcome. Rather than killing infected cells, some viruses may, quite paradoxically, force their hosts to thrive, indeed, to proliferate uncontrollably. In so doing, such viruses—often called tumor viruses—can create cancer.

At one time, in the 1970s, tumor viruses were studied intensively because they were suspected to be the cause of many common human cancers. This notion was not borne out by the evidence subsequently gathered during that decade, which indicated that virus-induced cancers represent only a minority of the cancer types affecting humans. Nonetheless, this line of research proved to be invaluable for cancer biologists: study of various tumor viruses provided the key for opening many of the long-hidden secrets of human cancers, including the great majority of cancers that have no connection with tumor virus infections.

Chapter 3

\textbf{Tumor Viruses}

A tumor of the chicken ... has been propagated in this laboratory since October, 1909. The behavior of this new growth has been throughout that of a true neoplasm, for which reason the fact of its transmission by means of a cell-free filtrate assumes exceptional importance.

Peyton Rous, 1911
As we will see, tumor virus research had a highly variable history over the course of the last century. These infectious agents were discovered in the first decade of the twentieth century and then retreated from the center stage of science. Half a century later, interest in these agents revived, culminating in the frenetic pace of tumor virus research during the 1970s.

The cancer-causing powers of tumor viruses drove many researchers to ask precisely how they succeed in creating disease. Most of these viruses possess relatively simple genomes containing small numbers of viral genes, yet some were found able to overwhelm an infected cell and its vastly more complex genome and to redirect cell growth in new directions. Such behavior indicated that tumor viruses have developed extremely potent genes to perturb the complex regulatory circuitry of the host cells that they infect.

By studying tumor viruses and their mechanisms of action, researchers changed the entire mindset of cancer research. Cancer became a disease of genes and thus a condition that was susceptible to analysis by the tools of molecular biology and genetics. When this story began, no one anticipated how obscure tumor viruses would one day revolutionize the study of human cancer pathogenesis.

3.1 Peyton Rous discovers a chicken sarcoma virus

In the last two decades of the nineteenth century, the research of Louis Pasteur and Robert Koch uncovered the infectious agents that were responsible for dysentery, cholera, rabies, and a number of other diseases. By the end of the century, these agents had been placed into two distinct categories, depending on their behavior upon filtration. Solutions of infectious agents that were trapped in the pores of filters were considered to contain bacteria. The other agents, which were small enough to pass through the filters, were classified as viruses. On the basis of this criterion, the agents for rabies, foot-and-mouth disease, and smallpox were categorized as viruses.

Cancer, too, was considered a candidate infectious disease. As early as 1876, a researcher in Russia reported the transmission of a tumor from one dog to another: chunks of tumor tissue from the first dog were implanted into the second, whereupon a tumor appeared several weeks later. This success was followed by many others using rat and mouse tumors.

The significance of these early experiments remained controversial. Some researchers interpreted these outcomes as proof that cancer was a transmissible disease. Yet others dismissed these transplantation experiments, since in their eyes, such work showed only that tumors, like normal tissues, could be excised from one animal and forced to grow as a graft in the body of a second animal.

In 1908, two researchers in Copenhagen reported their success in extracting a filterable agent from chicken leukemia cells and transmitting this agent to other birds, which then contracted the disease. The two Danes did not follow up on their initial discovery, and it remained for Peyton Rous, working at the Rockefeller Institute in New York, to found the discipline of tumor virology (Figure 3.1).

In 1909, Rous began his study of a sarcoma that had appeared in the breast muscle of a hen. In initial experiments, Rous succeeded in transmitting the tumor by preparing small fragments of tumor and implanting these into other birds of the same breed. Later, as a variation of this experimental plan, he ground up a fragment of a sarcoma in sand and filtered the resulting homogenate (Figure 3.2). When he injected the resulting filtrate into young birds, they too developed tumors, sometimes within several weeks. He subsequently found that these
induced tumors could also be homogenized to yield, once again, an infectious agent that could be transmitted to yet other birds, which also developed sarcomas at the sites of injection.

These serial passages of the sarcoma-inducing agent from one animal to another yielded a number of conclusions that are obvious to us now but at the time were nothing less than revolutionary. The carcinogenic agent, whatever its nature, was clearly very small, since it could pass through a filter. Hence, it was a virus (see Sidebar 3.1). This virus could cause the appearance of a sarcoma in an injected chicken, doing so on a predictable timetable. Such an infectious agent offered researchers the unique opportunity to induce cancers at will rather than relying on the spontaneous and unpredictable appearance of tumors in animals or humans. In addition to its ability to induce cancer, this agent, which came to be called Rous sarcoma virus (RSV), was capable of multiplying within the tissues of the chicken; far more virus could be recovered from an infected tumor tissue than was originally injected.

In 1911, when Rous finally published his work, yet another report appeared on a transmissible virus of rabbit tumors, called myxomas. Soon thereafter, Rous

**Figure 3.1 The young and the old Peyton Rous**

Peyton Rous began his work in 1910 that led to the discovery of Rous sarcoma virus (RSV). More than 50 years later (1966), he received the Nobel Prize in Medicine and Physiology for this seminal work—a tribute to his persistence and longevity. (A, from National Library of Medicine; B, from American Philosophical Society.)

**Figure 3.2 Rous’s protocol for inducing sarcomas in chickens**

Rous removed a sarcoma from the breast muscle of a chicken, ground it with sand, and passed the resulting homogenate through a fine-pore filter. He then injected the filtrate (the liquid that passed through the filter) into the wing web of a young chicken and observed the development of a sarcoma many weeks later. He then ground up this new sarcoma and repeated the cycle of homogenization, filtration, and injection, once again observing a tumor in another young chicken. These cycles could be repeated indefinitely; after repeated serial passaging, the virus was able to produce sarcomas far more rapidly than the original viral isolate.
and his collaborators found two other chicken viruses, and yet another chicken sarcoma virus was reported by others in Japan. Then, there was only silence for another two decades until other novel tumor viruses were discovered. The molecular nature of viruses and the means by which they multiplied would remain mysteries for more than half a century after Rous’s initial discovery.

Still, his finding of a sarcoma virus reinforced the convictions of those who believed that virtually all human diseases were provoked by infectious agents. In their eyes, cancer could be added to the lengthening list of diseases, such as cholera, tuberculosis, rabies, and sepsis, whose causes could be associated with a specific microbial agent. By 1913, the Dane Johannes Grib Fibiger reported that stomach cancers in rats could be traced to spirochete worms that

Sidebar 3.1 Viruses have simple life cycles The term “virus” refers to a diverse array of infectious particles that infect and multiply within a wide variety of cells, ranging from bacteria to the cells of plants and metazoa. Relative to the cells that they infect, individual virus particles, often termed virions, are tiny. Virions are generally simple in structure, with a nucleic acid (DNA or RNA) genome wrapped in a protein coat (a capsid) and, in some cases, a lipid membrane surrounding the capsid. In isolation, viruses are metabolically inert. They can multiply only by infecting and parasitizing a suitable host cell. The viral genome, once introduced into the cell, provides instructions for the synthesis of progeny virus particles. The host cell, for its part, provides the low–molecular-weight precursors needed for the synthesis of viral proteins and nucleic acids, the protein-synthetic machinery, and, in many cases, the polymerases required for replicating and transcribing the viral genome.

The endpoint of the resulting infectious cycle is the production of hundreds, even thousands of progeny virus particles that can then leave the infected cell and proceed to infect other susceptible cells. The interaction of the virus with the host cell can either be a virulent one, in which the host cell is destroyed during the infectious cycle, or a temperate one, in which the host cell survives for extended periods, all the while harboring the viral genome and releasing progeny virus particles.

Many viruses carrying double-stranded DNA (dsDNA) replicate in a fashion that closely parallels the macromolecular metabolism of the host cell (Figure 3.3). This allows them to use host-cell DNA polymerases to replicate their DNA, host-cell RNA polymerases to transcribe the viral mRNAs from double-stranded viral DNA templates, and host ribosomes to translate the viral mRNAs. Once synthesized, viral proteins are used to coat (encapsidate) the newly synthesized viral genomes, resulting in the assembly of complete progeny virions, which then are released from the infected cell.

Since cells do not express enzymes that can replicate RNA molecules, many RNA-containing virus particles encode their own RNA-dependent RNA polymerases to replicate their genomes. Poliovirus, as an example, makes such an enzyme, as does rabies virus. RNA tumor viruses like Rous sarcoma virus, as we will learn later in this chapter, follow a much more circuitous route for replicating their viral RNA.
they harbored. His work, for which he received the 1926 Nobel Prize, represented direct and strong validation of the idea, first indicated by Rous's work, that cancer was yet another example of an infectious disease.

Within months of Fibiger's 1926 Nobel award, he passed away and his scientific opus began to disintegrate. The stomach tumors that he had described were not tumors at all. Instead, they were found to be metaplastic stomach epithelia. As it turned out, these lesions were present in the rats' stomachs because of the profound vitamin deficiencies that these animals suffered; they lived in sugar refineries and ate sugar cane almost exclusively. Fibiger's Nobel Prize became an embarrassment to the still-small community of cancer researchers. They threw the proverbial baby out with the bathwater, discrediting both his work and the notion that cancer could ever be caused by infectious agents.

Interest in the origins of cancer shifted almost totally to chemically induced cancers. Chemicals had been discovered in the early twentieth century that were clearly carcinogenic (see Section 2.8). Study of Rous sarcoma virus and the other tumor viruses languished and entered into a deep sleep for several decades.

3.2 Rous sarcoma virus is discovered to transform infected cells in culture

The rebirth of Rous sarcoma virus research began largely at the California Institute of Technology in Pasadena, in the laboratory of Renato Dulbecco. Dulbecco's postdoctoral fellow Harry Rubin found that when stocks of RSV were introduced into Petri dishes carrying cultures of chicken embryo fibroblasts (CEFfs), the RSV-infected cells survived, apparently indefinitely. It seemed that RSV parasitized these cells, forcing them to produce a steady stream of progeny virus particles for many days, weeks, even months (Figure 3.4). Most other viruses, in contrast, were known to enter into host cells, multiply, and quickly kill their hosts; the multitude of progeny virus particles released from dying cells could then proceed to infect yet other susceptible cells in the vicinity, repeating the cycle of infection, multiplication, and cell destruction.

Most important, the RSV-infected cells in these cultures displayed many of the traits that had been previously associated with cancer cells. Thus, after RSV particles were applied to chicken fibroblasts in a culture dish, foci (clusters) of infected cells appeared. Under the microscope, these cells strongly resembled the cells isolated from chicken sarcomas (Figure 3.5). Thus, such cells showed a rounded morphology (shape, form) and had a metabolism reminiscent of that seen in cells isolated from tumors. This resemblance led Rubin, Howard Temin (a student in Dulbecco's laboratory; Figure 3.6), and others to conclude that the process of cell transformation—conversion of a normal cell into a tumor cell—could be accomplished within the confines of a Petri dish, not just in the complex and difficult-to-study environment of a living tissue.

These simple observations radically changed the course of twentieth-century cancer research, because they clearly demonstrated that cancer formation could be studied at the level of individual cells whose behavior could be tracked closely under the microscope. This insight suggested the further possibility that the entire complex biology of tumors could one day be understood by studying the transformed cells forming tumor masses. So, an increasing number of biologists began to view cancer as a disease of malfunctioning cells rather than abnormally developing tissues.

Temin and Rubin, soon followed by many others, used this experimental model to learn some basic principles about cell transformation. They were interested in the fate of a cell that was initially infected by RSV. How did such a cell proliferate
when compared with uninfected neighboring cells? After exposure of cells to a solution of virus particles (often called a virus stock), the two researchers would place a layer of agar above the cell layer growing at the bottom of the Petri dish, thereby preventing virus particles from spreading from initially infected cells to uninfected cells in other parts of the dish. Hence, any changes in cell behavior were the direct result of the initial infection by virus particles of chicken embryo fibroblasts.

The foci that Temin and Rubin studied revealed the dramatic differences in the behavior of normal versus transformed cells. When first introduced into a Petri dish, normal cells formed islands scattered across the bottom of the dish. They then proliferated and eventually filled up all the space in the bottom of the dish, thereby creating confluent cultures. Once they reached confluence, however, these normal cells stopped proliferating, resulting in a one-cell-thick (or slightly thicker) layer of cells, often called a cell monolayer (Figure 3.7).
The cessation of growth of these normal cells after forming confluent monolayers was the result of a process that came to be called contact inhibition, density inhibition, or topoinhibition. Somehow, high cell density or contact with neighbors caused these cells to stop dividing. This behavior of normal cells contrasted starkly with that of the transformants within the RSV-induced foci. The latter clearly had lost contact (density) inhibition and consequently continued to proliferate, piling up on top of one another and creating multilayered clumps of cells so thick that they could often be seen with the naked eye.

Under certain experimental conditions, it could be shown that all the cells within a given focus were the descendants of a single progenitor cell that had been infected and presumably transformed by an RSV particle. Today, we would term such a flock of descendant cells a cell clone and the focus as a whole, a clonal outgrowth, implying in both cases the descent of these cells from a common progenitor.

The behavior of these foci gave support to one speculation about the possible similarities between the cell transformation triggered by RSV in the Petri dish and the processes that led to the appearance of tumors in living animals, including humans: maybe all the cells within a spontaneously arising human tumor mass also constitute a clonal outgrowth and therefore are the descendants of a single common progenitor cell that somehow underwent transformation and then launched a program of replication that led eventually to the millions, even billions, of descendant cells that together formed the mass. As discussed earlier (see Section 2.5), detailed genetic analyses of human tumor cells were required, in the end, to test this notion in a truly definitive way.

3.3 The continued presence of RSV is needed to maintain transformation

The behavior of the cells within an RSV-induced focus indicated that the transformation phenotype was transmitted from an initially infected, transformed chicken cell to its direct descendants. This transmission provoked another set of questions: Did an RSV particle infect and transform the progenitor cell of the focus and, later on, continue to influence the behavior of all of its direct descendants, ensuring that they also remained transformed? Or, as an alternative, did RSV act in a “hit-and-run” fashion by striking the initially infected progenitor cell,
altering its behavior, and then leaving the scene of the crime? According to this second scenario, the progenitor cell could somehow transmit the phenotype of cancerous growth to its descendants without the continued presence of RSV.

Temin and Rubin’s work made it clear that the descendants of an RSV-infected cell continued to harbor copies of the RSV genome, but that evidence, on its own, settled little. The real question was, Did the transformed state of the descendant cells actually depend on some continuing influence exerted by the RSV genomes that they carried?

An experiment performed in 1970 at the University of California, Berkeley, settled this issue unambiguously. A mutant of RSV was developed that was capable of transforming chicken cells when these cells were cultured at 37ºC but not at 41ºC (the latter being the normal temperature at which chicken cells grow). Temperature-sensitive (ts) mutants like this one were known to encode partially defective proteins, which retain their normal structure and function at one temperature and lose their function at another temperature, presumably through thermal denaturation of the structure of the mutant protein.

After the chicken embryo fibroblasts were infected with the ts mutant of RSV, these cells became transformed if they were subsequently cultured at the lower (permissive) temperature of 37ºC, as anticipated. Indeed, these cells could be propagated for many cell generations at this lower temperature and continued to grow and divide just like cancer cells, showing their characteristic transformed morphology (see Figure 3.7B). But weeks later, if the temperature of these infected cultures was raised to 41ºC (the nonpermissive temperature), these cells lost their transformed shape and quickly reverted to the shape and growth pattern of cells that had never experienced an RSV infection (Figure 3.8).

The Berkeley experiments led to simple and yet profoundly important conclusions. Since the cells that descended from a ts RSV–infected cell continued to show the temperature-sensitive growth trait, it was obvious that copies of the genome of the infecting virus persisted in these cells for weeks after the initial infection. These copies of the RSV genome in the descendant cells continued to make some temperature-sensitive protein (whose precise identity was not known). Most important, the continuing actions of this protein were required in order to maintain the transformed growth phenotype of the RSV-infected cells.
This work showed that cell transformation, at least that induced by RSV, was not a hit-and-run affair. In the language of the tumor virologists, the viral transforming gene was required to both initiate and maintain the transformed phenotype of virus-infected cells.

### 3.4 Viruses containing DNA molecules are also able to induce cancer

RSV was only one of a disparate group of viruses that were found able to induce tumors in infected animals. By 1960, four other classes of tumor viruses had become equally attractive agents for study by cancer biologists. A new type of tumor virus was uncovered almost a quarter century after Rous’s pioneering work. This virus, discovered by Richard Shope in rabbits, caused papillomas (warts) on the skin. These were really benign lesions, which on rare occasions progressed to true tumors—squamous cell carcinomas of the skin.

By the late 1950s, it became clear that Shope’s virus was constructed very differently from RSV. The papillomavirus particles carried DNA genomes, whereas RSV particles were known to carry RNA molecules. Also, the Shope virus particles were sheathed in a protein coat, whereas RSV clearly had, in addition, a lipid membrane coating on the outside. In the decades that followed, more than 100 distinct human papillomavirus (HPV) types, all related to the Shope virus, would be discovered (Figure 3.9).

Research begun in the 1930s in Bar Harbor, Maine, yielded, three decades later, the agent (mouse mammary tumor virus, MMTV) that was responsible for the mother-to-offspring, milk-borne transmission of breast cancer susceptibility in certain strains of mice. The MMTV genome, like that of RSV, was found to be composed of RNA molecules and was therefore quite different from the papillomavirus genome.

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**Figure 3.8 Temperature-sensitive mutant and the maintenance of transformation by RSV** When chicken embryo fibroblasts were infected at the permissive temperature (37°C), at which the viral transforming function can be expressed, the cells became transformed. When the cultures containing these infected cells were shifted to the nonpermissive temperature (41°C), the viral transforming function was inactivated and the cells reverted to a normal, nontransformed morphology. Later, when the temperature of the culture was shifted back to the permissive temperature, the viral transforming function was regained and the cells once again exhibited a transformed morphology. The loss of the transformed phenotype upon temperature shift-up demonstrated that the continuous action of some temperature-sensitive viral protein was required in order to maintain this phenotype. The reacquisition of the transformed phenotype after temperature shift-down indicated that the viral genome continued to be present in these cells at the high temperature in spite of their normal appearance.

**Figure 3.9 Shope papillomavirus** (A) Electronic micrograph of Shope papillomavirus. (B) This cryo-electron micrograph, together with image enhancement, reveals the structure of a human papillomavirus particle. (A, courtesy of D. DiMaio; B, from B.L. Trus et al., Nat. Struct. Biol. 4:413–420, 1997.)
In the 1950s and 1960s, various other DNA tumor viruses were discovered (Table 3.1). Polyomavirus, named for its ability to induce a variety of distinct tumor types in mice, was discovered in 1953. Closely related to polyomavirus in its size and chemical makeup was SV40 virus (the 40th simian virus in a series of isolates). This monkey virus had originally been discovered as a contaminant of the poliovirus vaccine stocks prepared in the mid- and late 1950s (Figure 3.10A). Clever virological sleuthing revealed that SV40 particles often hid out in cultures of the rhesus and cynomolgus monkey kidney cells used to propagate poliovirus during the preparation of vaccine. In fact, the presence of SV40 was not initially apparent in these cell cultures. However, when poliovirus stocks that had been propagated in these monkey cells were later used to infect African green monkey kidney (AGMK) cells, SV40 revealed itself by inducing a very distinctive cytopathic effect—numerous large vacuoles (fluid-filled bubble-like structures) in the cytoplasm of infected cells (Figure 3.10B). Within a day after the vacuoles formed in an SV40-infected cell, this cell would lyse, releasing tens of thousands

| Table 3.1 Tumor virus genomes |
|-------------------------------|-----------------|
| **Virus family**              | **Approximate size of genome (kb)** |
| **DNA viruses**               |                               |
| Hepatitis B virus (HBV)       | hepadna 3                  |
| SV40/polyoma                  | papova 5                   |
| Human papilloma 16 (HPV)      | papova 8                   |
| Human adenovirus 5            | adenovirus 35              |
| Human herpesvirus 8 (HSV-8; KSHV) | herpesviruses 165 |
| Shope fibroma virus           | poxviruses 160             |
| **RNA viruses**               |                               |
| Rous sarcoma virus (RSV)      | retrovirus 9               |
| Human T-cell leukemia virus (HTLV-I) | retrovirus 9 |


Figure 3.10 SV40 virus  (A) As determined by X-ray diffraction, the protein capsid of the SV40 DNA tumor virus consists of three virus-encoded proteins that are assembled into pentamers and hexamers with icosahedral symmetry. The dsDNA genome of SV40 is carried within this capsid.  (B) SV40 launches a lytic cycle in permissive host cells, such as the kidney cells of several monkey species. The resulting cytopathic effect seen here, involves the formation of large cytoplasmic vacuoles prior to the death of the cell and the release of tens of thousands of progeny virus particles. (A, courtesy of Robert Grant, Stephen Crainic, and James M. Hogle; B, from A. Gordon-Shaag at al. J. Virol. 77:4273–4282, 2003.)
Viruses containing DNA molecules are also able to induce cancer

of progeny virus particles. (Because of SV40 contamination, during the course of poliovirus vaccine production, some poliovirus-infected cell cultures yielded far more SV40 virus particles than poliovirus particles!)

This lytic cycle of SV40 contrasted starkly with its behavior in cells prepared from mouse, rat, or hamster embryos. SV40 was unable to replicate in these cells, which were therefore considered to be nonpermissive hosts. But on occasion, in one cell out of thousands in an infected population, a transformant grew out that shared many characteristics with RSV-transformed cells, i.e., a cell that had undergone changes in morphology and loss of contact inhibition, and had acquired the ability to seed tumors in vivo. On the basis of this, SV40 was classified as a tumor virus.

By some estimates, between one-third and two-thirds of the polio vaccines—the oral, live Sabin vaccine and the inactivated, injected Salk vaccine—administered between 1955 and 1963 contained SV40 virus as a contaminant, and between 10 and 30 million people were exposed to this virus through vaccination. In 1960, the fear was first voiced that the SV40 contaminant might trigger cancer in many of those who were vaccinated; reassuringly, epidemiologic analyses conducted over the succeeding four decades indicated little, if any, increased risk of cancer among those exposed to these two vaccines (Sidebar 3.2).

Shope's papillomavirus, the mouse polyomavirus, and SV40 were grouped together as the papovavirus class of DNA tumor viruses, the term signifying papilloma, polyoma, and the vacuoles induced by SV40 during its lytic infection. By the mid-1960s, it was apparent that the genomes of the papovaviruses were all formed from circular double-stranded DNA molecules (Figure 3.11). This represented a great convenience for experimenters, since there were several techniques in use at the time that made it possible to separate these relatively

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**Sidebar 3.2 Is SV40 responsible for the mesothelioma plague?** The mesothelium is the membranous outer lining of many internal organs and derives directly from the embryonic mesoderm. Mesotheliomas—tumors of various mesothelial surfaces—were virtually unknown in the United States in the first half of the twentieth century. Beginning in about 1960, however, their incidence began to climb steeply. By the end of the century, the annual incidence had approached 2500 in the United States. Much of this increased incidence has been attributed to exposure to asbestos, specifically a subtype termed crocidolite. Thus, the disease is seen frequently among those who have worked with asbestos, which was used as a heat-resisting insulating material until its use was finally banned in the last two decades of the century. However, 20% of mesothelioma patients have no documented exposure to asbestos, a fact that has provoked a search for other etiologic (causative) agents.

SV40 is a plausible etiologic agent of mesothelioma. By 2003, forty-one laboratories across the world had reported SV40 DNA, RNA, or protein in mesothelioma cells. Traces of the virus are otherwise rarely found in human tumors, with the exception of certain types of brain tumors. Cultured human mesothelioma cells are readily infected by SV40, and this infection leads rapidly to their immortalization, that is, to the ability of these cells, which usually have limited proliferative potential in culture, to multiply indefinitely.

The presence of SV40 contamination of poliovirus vaccine stocks has raised concerns that mesothelioma may have been induced in many individuals as an unintended side effect of poliovirus vaccination. In fact, there are many instances of mesotheliomas in individuals who are highly unlikely to have been exposed to these vaccines. Moreover, the viral T-antigen protein, whose presence is invariably observed in SV40-transformed cells (see Section 3.6), is rarely detected in mesothelioma tumors. And attempts at demonstrating viral DNA have usually yielded DNA segments that are indicative of laboratory artifacts, for example, contamination of mesothelioma tumor samples by laboratory stocks of SV40 or by recombinant DNA plasmids that had previously been engineered to include segments of the viral genome. The discoveries of such contaminations provide increasing ammunition for those who are skeptical of SV40’s role in mesothelioma pathogenesis.

As many as 85% of humans are known to be infected with two viruses that are closely related to SV40—JC and BK—and antiseras that recognize the capsids of these viruses cross-react with the capsid of SV40, explaining many of the claims that SV40 is often present in human tissues. In immunosuppressed individuals, notably AIDS patients, JC virus can cause a fatal brain degeneration—progressive multifocal leukoencephalopathy (PML)—but there is no evidence that it is also carcinogenic.

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small, circular DNA molecules [about 5–8 kilobases (kb) in length] from the far larger, linear DNA molecules present in the chromosomes of infected host cells.

The group of DNA tumor viruses grew further with the discovery that human adenovirus, known to be responsible for upper respiratory infections in humans, was able to induce tumors in infected hamsters. Here was a striking parallel with the behavior of SV40. The two viruses could multiply freely in their natural host cells, which were therefore considered to be permissive. During the resulting lytic cycles of the virus, permissive host cells were rapidly killed in concert with the release of progeny virus particles. But when introduced into non-permissive cells, both adenovirus and SV40 failed to replicate and instead left behind, albeit at very low frequency, clones of transformants.

Other entrants into the class of DNA tumor viruses were members of the herpesvirus group. While human herpesvirus types 1 and 2 were apparently not tumorigenic (capable of inducing tumors), a distantly related herpesvirus of Saimiri monkeys provoked rapid and fatal lymphomas when injected into monkeys from several other species. Another distantly related member of the herpesvirus family—Epstein–Barr virus (EBV)—was discovered to play a causal role in provoking Burkitt’s lymphomas in young children in Equatorial Africa and New Guinea as well as nasopharyngeal carcinomas in Southeast Asia. Finally, at least two members of the poxvirus class, which includes smallpox virus, were found to be tumorigenic: Shope fibroma virus and Yaba monkey virus cause benign skin lesions in rabbits and rhesus monkeys, respectively. The tumorigenic powers of these viruses, which have very large genomes (135–160 kb), remain poorly understood to this day.

Researchers found that adenovirus and herpesvirus particles contain long, linear, double-stranded DNA (dsDNA) molecules, which, like the genomes of RSV, carry the information required for both viral replication and virus-induced cell transformation. Compared with the papovaviruses, the herpesviruses had genomes of enormous size (see Table 3.1), suggesting that they carried a proportionately larger number of genes. In the end, it was the relatively small sizes of papovavirus genomes that made them attractive objects of study by those interested in the molecular origins of cancer.

Most of the small group of genes in a papovavirus genome were apparently required to program viral replication; included among these were several genes specifying the proteins that form the capsid coat of the virus particle. This dic-
tated that papovaviruses could devote only a small number of their genes to the
process of cell transformation.

This realization offered the prospect of greatly simplifying the cancer problem
by reducing the array of responsible genes and causal mechanisms down to a
very small number. Without such simplification, cancer biologists were forced
to study the cancer-causing genes that were thought to be present in the
genomes of cells transformed by nonviral mechanisms. Cellular genomes
clearly harbored large arrays of genes, possibly more than a hundred thousand.
At the time, the ability to analyze genomes of such vast complexity and to iso-
late individual genes from these genomes was a distant prospect.

3.5 Tumor viruses induce multiple changes in cell
phenotype including acquisition of tumorigenicity

Like RSV-transformed cells, the cells transformed by SV40 showed profoundly
altered shape and piled up on one another. This loss of contact inhibition was
only one of a number of changes exhibited by virus-transformed cells (Table
3.2). As discussed later in this book, normal cells in culture will not proliferate
unless they are provided with serum and serum-associated growth-stimulating
factors in their culture medium. Cells transformed by a variety of tumor viruses
were often found to have substantially reduced requirements for these factors in
their culture medium.

Yet another hallmark of the transformed state is an ability to proliferate in cul-
ture for longer time periods than normal cells. Researchers discovered that nor-
mal cells have a limited proliferative potential in culture and ultimately stop
multiplying after a certain, apparently predetermined number of cell divisions.
Cancer cells seemed to be able to proliferate indefinitely in culture, and hence
were described as being immortalized (as discussed in Chapter 10).

When transformed cells were suspended in an agar gel, these cells were able to
proliferate into spherical colonies containing dozens, even hundreds of cells
(Figure 3.12). This ability to multiply without attachment to the solid substrate
provided by the bottom of the Petri dish was termed the trait of anchorage inde-
pendence. Normal cells, in contrast, demonstrated an absolute requirement for
tethering to a solid substrate before they would grow and were therefore consid-
ered to be anchorage-dependent. This ability of cells to grow in an anchorage-
dependent fashion in vitro usually served as a good predictor of their ability to
form tumors in vivo following injection into appropriate host animals.

This tumor-forming ability—the phenotype of tumorigenicity—represented the
acid test of whether cells were fully transformed, that is, had acquired the full

<table>
<thead>
<tr>
<th>Table 3.2 Properties of transformed cells</th>
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<tr>
<td>Altered morphology (rounded shape, refractile in phase-contrast microscope)</td>
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<tr>
<td>Loss of contact inhibition (ability to grow over one another)</td>
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<tr>
<td>Ability to grow without attachment to solid substrate (anchorage independence)</td>
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<td>Ability to proliferate indefinitely (immortalization)</td>
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<td>Reduced requirement for mitogenic growth factors</td>
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<td>High saturation density (ability to accumulate large numbers of cells in culture dish)</td>
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<td>Inability to halt proliferation in response to deprivation of growth factors</td>
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<td>Increased transport of glucose</td>
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<td>Tumorigenicity</td>
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</table>

repertoire of neoplastic traits. Tests for tumorigenicity could be performed when the cells used in an *in vitro* transformation experiment were prepared from a strain of mice and later injected into host mice of the same strain (Sidebar 3.3). Since the host and injected cells came from the same genetic strain, the immune systems of such syngeneic host mice would not recognize the transformed cells as being foreign bodies and therefore would not attempt to eliminate them—the process of tumor rejection (a process to which we will return in Chapter 15). This allowed injected cells to survive in their animal hosts, enabling them to multiply into large tumors if, indeed, they had acquired the tumorigenic phenotype.

Often, it was impossible to test the tumorigenicity of tumor virus-infected cells in a syngeneic host animal, simply because the cells being studied came from a species in which inbred syngeneic hosts were not available. This forced the use of immunocompromised hosts whose immune systems were tolerant of a wide variety of foreign cell types, including those from other species (see Sidebar 3.3). Mice of the Nude strain soon became the most commonly used hosts to test the tumorigenicity of a wide variety of cells, including those of human origin. Quite
frequently, candidate tumor cells are injected subcutaneously, i.e., directly under the skin of these animals. Since they also lack the ability to grow hair, nude mice provide the additional advantage of allowing the experimenter to closely monitor the progress of implanted tumor cells (Figure 3.13).

As mentioned, the small size of the genomes of RNA tumor viruses (e.g., RSV) and papovaviruses dictated that each of these use only a small number of genes (perhaps as few as one) to elicit multiple changes in the cells that they infected and transformed. Recall that the ability of a gene to concomitantly induce a number of distinct alterations in a cell is termed pleiotropy. Accordingly, though little direct evidence about gene number was yet in hand, it seemed highly likely that the genes used by tumor viruses to induce cell transformation were acting pleiotropically on a variety of molecular targets within cells.

3.6 Tumor virus genomes persist in virus-transformed cells by becoming part of host cell DNA

The Berkeley experiments (see Section 3.3) provided strong evidence that the continued actions of the RSV genome were required to maintain the transformed state of cells, including those that were many cell generations removed from an initially infected progenitor cell. This meant that some or all of the viral genetic information needed to be perpetuated in some form, being passed from a transformed mother cell to its two daughters and, further on, to descendant cells through many cycles of cell growth and division. Conversely, a failure to transmit these viral genes to descendant cells would result in their reversion to cells showing normal growth behavior.

Paralleling the behavior of RSV, cell transformation achieved by two intensively studied DNA tumor viruses—SV40 and polyomavirus—also seemed to depend on the continued presence of viral genomes in the descendants of an initially transformed cell. The evidence proving this came in a roundabout way, largely from the discovery of tumor-associated proteins (T antigens) that were found in cancers induced by these two viruses. For example, sera prepared from mice carrying an SV40-induced tumor showed strong reactivity with a nuclear protein that was present characteristically in tumors triggered by SV40 and absent in tumors induced by polyomavirus or by other carcinogenic agents. The implication was that the viral genome residing in tumor cells encoded a protein (e.g., the SV40 T antigen) that induced a strong immunological response in the tumor-bearing mouse or rat host (Figure 3.14).
The display of the virus-induced T antigen correlated directly with the transformed state of these cells. Therefore, cells that lost the T antigen would also lose the transformation phenotype induced by the virus. This correlation suggested, but hardly proved, that the viral sequences responsible for transformation were associated with or closely linked to viral sequences encoding the T antigen.

The cell-to-cell transmission of viral genomes over many cell generations represented a major conceptual problem. Cellular genes were clearly transmitted with almost total fidelity from mother cells to daughter cells through the carefully programmed processes of chromosomal DNA replication and mitosis that occur during each cellular growth-and-division cycle. How could viral genomes succeed in being replicated and transmitted efficiently through an unlimited number of cell generations? This was especially puzzling, since viral genomes seemed to lack the genetic elements that were thought to be required for proper allocation of chromosomes to daughter cells during mitosis.

Adding to this problem was the fact that the DNA metabolism of papovaviruses, such as SV40 and polyomavirus, was very different from that of the host cells that they preyed upon. When SV40 and polyomavirus infected permissive host cells, the viral DNAs were replicated as autonomous, extrachromosomal molecules. Both viruses could form many tens of thousands of circular, double-stranded DNA genomes of about 5 kb in size from a single viral DNA genome initially introduced by infection (see Figure 3.11). While the viral DNA replication exploited a number of host-cell DNA replication enzymes, it proceeded independently of the infected cells’ chromosomal DNA replication. This nonchromosomal replication occurring during the lytic cycles of SV40 and polyomavirus shed no light on how these viral genomes were perpetuated in populations of virus-transformed cells. The latter were, after all, nonpermissive and therefore prevented these viruses from replicating their DNA.

A solution to this puzzle came in 1968, when it was discovered that the viral DNA in SV40-transformed cells was tightly associated with their chromosomal DNA. Using centrifugation techniques to gauge the molecular weights of DNA molecules, it became clear that the SV40 DNA in these cells no longer sedimented like a small (~5 kb) viral DNA genome. Instead, the SV40 DNA sequences in virus-transformed cells co-sedimented with the high-molecular-weight chromosomal DNA of the host cells (Figure 3.15). In fact, the viral DNA in these

**Figure 3.15 Integration of SV40 DNA**

DNA molecules from SV40-transformed cells were isolated and sedimented by centrifugation through an alkaline solution stabilized by a sucrose gradient (used to prevent mixing of different fluid strata within the centrifuge tube). Under these conditions, the high–molecular-weight cellular DNA (blue) sedimented a substantial distance down the sucrose gradient (left side of graph). In contrast, the SV40 DNA isolated from virus particles (green) sedimented more slowly, indicative of its lower molecular weight. Forms I and II viral DNA refer to the closed-circular and nicked-circular DNAs of SV40, respectively (Figure 3.11). Use of nucleic acid hybridization revealed that the SV40 DNA sequences in SV40 virus-transformed cells co-sedimented with the high–molecular-weight chromosomal DNA of the virus-transformed cells (red). (Adapted from J. Sambrook et al., *Proc. Natl. Acad. Sci. USA* 60:1288–1295, 1968.)
transformed cells could not be separated from the cells’ chromosomal DNA by even the most stringent methods of dissociation, including the harsh treatment of exposure to alkaline pH.

These results indicated that SV40 DNA in virus-transformed cells had become covalently linked to the chromosomal DNA. Such integration of viral genome solved an important problem in viral transformation: transmission of viral DNA sequences from a mother cell to its offspring could be guaranteed, since the viral DNA would be co-replicated with the cell’s chromosomal DNA during the S (DNA synthesis) phase of each cell cycle. In effect, by integrating into the chromosome, the viral DNA sequences became as much a part of a cell’s genome as the cell’s own native genes.

Some years later, this ability of papovavirus genomes to integrate into host-cell genomes became highly relevant to the pathogenesis of one common form of human cancer—cervical carcinoma. Almost all (>99.7%) of these tumors were found to carry fragments of human papillomavirus (HPV) genomes integrated into their chromosomal DNA. Provocatively, intact viral genomes were rarely discovered to be present in integrated form in cancer cell genomes. Instead, only the portion of the viral genome that contains oncogenic (cancer-causing) information was found in the chromosomal DNA of these cancer cells, while the portion that enables these viruses to replicate and construct progeny virus particles was almost always absent or present in only fragmentary form.

3.7 Retroviral genomes become integrated into the chromosomes of infected cells

The ability of SV40 and polyomavirus to integrate copies of their genomes into host-cell chromosomal DNA solved one problem but created another that seemed much less solvable: how did RSV succeed in transmitting its genome through many generations within a cell lineage? The genome of RSV is made of single-stranded RNA (Figure 3.16), which clearly could not be integrated directly into the chromosomal DNA of an infected cell. Still, RSV succeeded in transmitting its genetic information through many successive cycles of cell growth and division (Sidebar 3.4).

![Figure 3.16 Genome structure of RNA tumor viruses](image)

Viruses like RSV carry single-stranded RNA (ssRNA) genomes. Uniquely, the genomes of this class of viruses were discovered to be diploid, i.e., to carry two identical copies of the viral genetic sequence. The organization of such genomes was revealed by, among other techniques, electron microscopy. (A) This electron micrograph shows the genome of a distant relative of RSV termed “baboon endogenous virus.” (B) As is indicated in the schematization, circular SV40 dsDNA molecules (red, top, bottom), to which oligo dT (oligodeoxythymidyline) tails were attached enzymatically, have been used to visually label the 3’ termini of the two single-stranded viral RNA molecules (blue). The viral RNA molecules (like cellular mRNA molecules) contains polyadenylate at their 3’ termini, and therefore anneals to the oligo dT tails attached to the circular SV40 molecules. As is also indicated, the two ssRNAs are associated at their 5’ ends. (Adapted from W. Bender and N. Davidson, Cell 7:595–607, 1976.)
This puzzle consumed Temin in the mid- and late-1960s and caused him to propose a solution so unorthodox that it was ridiculed by many, landing him in the scientific wilderness. Temin argued that after RSV particles (and those of related viruses) infected a cell, they made double-stranded DNA (dsDNA) copies of their RNA genomes. It was these dsDNA versions of the viral genome, he said, that became established in the chromosomal DNA of the host cell. Once established in this way, the DNA version of the viral genome—which he called a provirus—then assumed the molecular configuration of a cellular gene and would be replicated each time the cell replicated its chromosomal DNA. In addition, the proviral DNA, once established in the genome, could serve as a template for transcription by cellular RNA polymerase, thereby yielding RNA molecules that could be incorporated into progeny virus particles or, alternatively, could function as messenger RNA (mRNA) that was used for the synthesis of viral proteins (Figure 3.17).

The process of reverse transcription that Temin proposed—making DNA copies of RNA—was without precedent in the molecular biology of the time, which recognized information flow only in a single direction, specifically, DNA → RNA → proteins. But the idea prevailed, receiving strong support from Temin’s and David Baltimore’s simultaneous discoveries in 1970 that RSV and related virus particles carry the enzyme reverse transcriptase. As both research groups discovered, this enzyme has the capacity to execute the key copying step that Temin had predicted—the step required in order for RSV to transmit its genome through many cycles of cell growth and division.
It soon became apparent that RSV was only one of a large group of similarly con-
structed viruses, which together came to be called retroviruses to reflect the fact 
that their cycle of replication depends on information flowing “backward” from 
RNA to DNA. Within a year of the discovery of reverse transcriptase, the pre-

cence of proviral DNA was detected in the chromosomal DNA of RSV-infected 
cells. Hence, like SV40 and polyomavirus, retroviruses rely on integration of 
their genomes into the chromosome to ensure the stable retention and trans-
mission of their genomes.

There is, however, an important distinction between the integration mech-
nisms used by retroviruses like RSV and the DNA tumor viruses such as SV40 
and polyomavirus. Integration is a normal, essential part of the replication cycle 
of retroviruses. But in the case of DNA tumor viruses, chromosomal integration 
of their genomes is a very rare accident (<<1 per 1000 infections) that enables 
the perpetuation of viral genomes in the descendants of an initially infected cell; 
the rare SV40 genomes that do succeed in becoming established in chromoso-
mal DNA are found integrated in a haphazard fashion that often includes only 
fragments of the wild-type genome (Figure 3.18).

3.8 A version of the src gene carried by RSV is also present in uninfected cells

Because the genomes of retroviruses, like those of papovaviruses, were quite 
small (<10 kb), it seemed likely that the coding capacity of the retroviral 
genomes was limited to a small number of genes, probably far fewer than ten. 
Using this small repertoire of genes, retroviruses nevertheless succeeded in 
specifying some viral proteins needed for viral genome replication, others 
required for the construction and assembly of progeny virus particles, and yet 
other proteins used to transform infected cells.

In the case of RSV, the use of mutant viruses revealed that the functions of viral 
replication (including reverse transcription and the construction of progeny 
virions) required one set of genes, while the function of viral transformation 
required another. Thus, some mutant versions of RSV could replicate perfectly 
well in infected cells, producing large numbers of progeny virus particles, yet 
such mutants lacked transforming function. Conversely, other mutant deriva-
tives of RSV could transform cells but had lost the ability to replicate and make 
progeny virions in these transformed cells.

At least three retroviral genes were implicated in viral replication. Two of these 
encode structural proteins that are required for assembly of virus particles; a 
third specifies the reverse transcriptase (RT) enzyme, which copies viral RNA 
into DNA shortly after retrovirus particles enter into host cells. A comparison of 
the RNA genome of RSV with the genomes of related retroviruses lacking trans-
forming ability suggested that there was rather little information in the RSV 
genome devoted to encoding the remaining known viral function—transforma-
tion. Consequently, geneticists working with RSV speculated that all the viral 
transforming functions resided in a single gene, which they termed src (pronounced “sark”), to indicate its role in triggering the formation of sarcomas in infected chickens (Figure 3.19).

In 1974, the laboratory run jointly by J. Michael Bishop and Harold Varmus at the 
University of California, San Francisco, undertook to make a DNA probe that 
specifically recognized the transformation-associated (i.e., src) sequences of the 
RSV genome in order to understand its origins and functions (see Sidebar 3.5). 
This src-specific probe was then used to follow the fate of the src gene after cells 
were infected with RSV. The notion here was that uninfected chicken cells would 
carry no src-related DNA sequences in their genomes. However, following RSV
infection, src sequences would become readily detectable in cells, having been introduced by the infecting viral genome.

The actual outcome of this experiment was, however, totally different from expectation. In 1975, this research group, using their src-specific probe, found that src sequences were clearly present among the DNA sequences of uninfected chicken cells. These src sequences were present as single-copy cellular genes; that is, two copies of the src-related DNA sequences were present per diploid chicken cell genome—precisely the representation of the great majority of genes in the cellular genome.

The presence of src sequences in the chicken cell genome could not be dismissed as some artifact of the hybridization procedure used to detect them. Moreover, careful characterization of these src sequences made it unlikely that they had been inserted into the chicken genome by some retrovirus. For example, src-related DNA sequences were readily detectable in the genomes of several related bird species, and, more distantly on the evolutionary tree, in the

Sidebar 3.5 The making of a src-specific DNA probe In order to make a src-specific DNA probe, a researcher working in Bishop and Varmus’s laboratory exploited two types of RSV strains: a wild-type RSV genome that carried all of the sequences needed for viral replication and transformation, and a mutant RSV genome that was able to replicate but had lost, because of a major deletion of genetic sequences, the src sequences required for transformation (Figure 3.20). Using reverse transcriptase, he made a DNA copy of the wild-type sequences, yielding single-stranded DNA molecules complementary to the viral RNA. He then fragmented this DNA, and hybridized it to the RNA genome of the RSV deletion mutant that lacked src sequences, creating DNA–RNA hybrid molecules. He then retrieved the ssDNA molecules that failed to form DNA–RNA hybrids (discarding those that did form the hybrids). The result was ssDNA fragments that specifically recognized sequences contained within the deleted portion of the mutant RSV genome, that is, those lying within the src gene.

Because the initial reverse transcription of the wild-type RSV RNA was carried out in the presence of radiolabeled deoxyribonucleoside triphosphates, the src-specific DNA fragments (which constituted the src “probe”) were also labeled with radioisotope. This made it possible to discover whether DNAs of interest (such as the DNAs prepared from virus-infected or uninfected cells) also carried src sequences by determining whether the src probe (with its associated radioactivity) was able to hybridize to these cellular DNAs.

Figure 3.20 The construction of a src-specific DNA probe Wild-type (wt) RSV RNA (blue) was reverse-transcribed under conditions where only a single-stranded (ss) complementary DNA molecule (cDNA; red) was synthesized. The wt single-stranded viral DNA was then annealed (hybridized) to viral RNA (green) of the transformation-defective (td) mutant of RSV, which had lost its transforming function and apparently deleted its src gene. The resulting ds RNA:DNA hybrids were discarded, leaving behind the ssDNA fragment of the wtDNA that failed to hybridize to the RNA of the td mutant. This surviving DNA fragment, if radiolabeled, could then be used as a src-specific probe in order to detect src-related sequences in various cellular DNAs (orange).
DNAs of several mammals (Figure 3.21). The more distant the evolutionary relatedness of a species was to chickens, the weaker was the reactivity of the src probe with its DNA. This was precisely the behavior expected of a cellular gene that had been present in the genome of a common ancestral species and had acquired increasingly divergent DNA sequences as descendant species evolved progressively away from one another over the course of millions of years.

The evidence converged on the idea that the src sequences present in the genome of an uninfected chicken cell possessed all the properties of a normal cellular gene, being present in a single copy per haploid genome, evolving slowly over tens of millions of years, and being present in vertebrate species that were ancestral to both birds and mammals. This realization created a revolution in thinking about the origins of cancer.

3.9 RSV exploits a kidnapped cellular gene to transform cells

The presence of a highly conserved src gene in the genome of a normal organism implied that this cellular version of src, sometimes termed c-src (i.e., cell src), played some role in the life of this organism (the chicken) and its cells. How could this role be reconciled with the presence of a transforming src gene carried in the genome of RSV? This viral transforming gene (v-src) was closely related to the c-src gene of the chicken, yet the two genes had drastically different effects and apparent functions. When ensonced in the cellular genome, the actions of c-src were apparently compatible with normal cellular behavior and normal organismic development. In contrast, the very similar v-src gene borne by the RSV genome acted as a potent oncogene—a gene capable of transforming a normal chicken cell into a tumor cell.

One solution to this puzzle came from considering the possibility that perhaps the src gene of RSV was not naturally present in the genome of the retrovirus ancestral to RSV. This hypothetical viral ancestor, while lacking src sequences, was perfectly capable of replicating in chicken cells. In fact, such a src-negative retrovirus—avian leukosis virus (ALV)—was common in chickens and was capable of infectious spread from one chicken to another (see Sidebar 3.6). This suggested that during the course of infecting a chicken cell, an ancestral virus, similar to this common chicken virus, had acquired sequences from the host-cell genome (Figure 3.22), doing so through some genetic trick. The acquired cellular sequences (e.g., the src sequences) were then incorporated into the viral genome, thereby adding a fourth gene to the existing three genes that this retrovirus (like similar retroviruses) used for its replication in infected cells (see Figure 3.19).
Once present in the genome of RSV, the kidnapped src gene could then be altered and exploited by this virus to transform subsequently infected cells.

This scheme attributed great cleverness to retroviruses by implying that these viruses had the ability to pick up and exploit preexisting cellular genes for their own purposes. Such behavior is most unusual for a virus, since virtually all other types of viruses carry genes that have little if any relatedness to DNA sequences native to the cells that they infect (see Sidebar 3.7).

But there was an even more important lesson to be learned here, this one concerning the c-src gene. This cellular gene, one among tens of thousands in the chicken cell genome, could be converted into a potent viral oncogene following some slight remodeling by a retrovirus such as RSV. Because it was a precursor to an active oncogene, c-src was called a proto-oncogene. The very concept of a proto-oncogene was revolutionary: it implied that the genomes of normal vertebrate cells carry a gene that has the potential, under certain circumstances, to induce cell transformation and thus cancer.
The structures of the c-src proto-oncogene and the v-src oncogene were worked out rapidly in the years that followed these discoveries in 1975–1976. Just as the viral geneticists had speculated, all of the viral transforming sequences resided in a single viral oncogene. Within the RSV RNA genome, the v-src gene was found at the 3’ end of the genome, added to the three preexisting retroviral genes that were involved in viral replication (see Figure 3.19).

This scenario of acquisition and activation of c-src by a retrovirus led to three further ideas. First, if retroviruses could activate this proto-oncogene into a potent oncogene, perhaps other types of mutational mechanisms might operate to yield a similar outcome through their ability to reshape the normal c-src gene. Maybe these other mutational mechanisms caused the activation of a cellular proto-oncogene into an oncogene without removing the normal gene from its normal roosting site on the cellular chromosome. Maybe the information for inducing cancer was already present in the normal cell genome, waiting to be unmasked.

Second, it became clear that all of the transforming powers of RSV derived from the presence of a single gene—v-src—in its genome. This was of great importance, because it implied that a single oncogene could, as long suspected, elicit a large number of changes in the shape, metabolism, and growth behavior of a cell. More generally, this suggested that other cancer-causing genes could also act pleiotropically. Accordingly, if a transformed, tumorigenic cell differed from a normal cell in 20 or 30 distinct traits, perhaps these multiple changes were not dependent on the alteration of 20 or 30 different genes; instead, maybe a small number of genes would suffice to transform it to a tumorigenic cell.

Third, RSV and its v-src oncogene might represent a model for the behavior of other types of retroviruses that were similarly capable of transforming infected cells.

Sidebar 3.7 Where do viruses and their genes originate? The discovery by Varmus and Bishop’s laboratory of the cellular origins of the RSV src gene provoked the question of where most viral genes come from and how viruses originate. Viral genome replication—in both DNA and RNA virus replication cycles—is executed with far less fidelity than is the replication of cellular DNA. The consequence is that viral genome sequences evolve far more rapidly than the genomic sequences of metazoan cells. Hence, any traces of precursor gene sequences that may have been present in other viruses or in cells were erased by this rapid evolution hundreds of millions of years ago.

We will probably never know where and how most viral genomes originated. Presumably, viruses have been around since the cells that they parasitize first appeared. Some types of viruses may have begun as renegade cellular genes that broke away from cellular genomes and struck out on their own. RNA viruses may bear vestiges of early cellular life forms that used RNA molecules as genomes and, in the case of retroviruses, of a later stage of cellular evolution when a reverse transcriptase-like cellular enzyme enabled the transition from RNA to DNA genomes.

Whatever their origins, the great majority of viral genes have no obvious relatedness with the genes of their hosts. This highlights the uniqueness of the Varmus and Bishop finding that retroviruses can apparently acquire and transduce (carry in their genomes) cellular genes, seemingly promiscuously. This flexibility of their genomes suggested a novel application of retroviruses, which began to flourish in the mid-1980s: a variety of interesting genes were introduced into retroviral genomes using recombinant DNA techniques. The resulting viral genomes were then used as vectors to transduce these genes into cultured cells in vitro and into living tissues in vivo (the latter application often being referred to as “gene therapy”).
cells in vitro (in the culture dish) and inducing tumors in vivo (in living tissue). Perhaps these other transforming retroviruses had acquired other cellular genes unrelated to src. While c-src was certainly the first cellular proto-oncogene to be discovered, maybe other cellular proto-oncogenes were hiding in the vertebrate cellular genome, waiting to be picked up and activated by some passing retrovirus.

3.10 The vertebrate genome carries a large group of proto-oncogenes

An accident of history—an encounter in 1909 between a Long Island chicken farmer and Peyton Rous—made RSV the first tumorigenic retrovirus to be isolated and characterized in detail. Consequently, RSV was favored initially with the most detailed molecular and genetic analysis. However, in the 1950s and 1960s, a group of other chicken and rodent tumor viruses were found that were subsequently realized to be members, like RSV, of the retrovirus class.

The diversity of these other transforming retroviruses and the diseases that they caused suggested that they, like RSV, might be carrying kidnapped cellular proto-oncogenes and might be using these acquired genes to transform infected cells. So, within a year of the discovery of v-src and c-src, the race began to find yet other viruses that had traveled down a similar genetic path and picked up other, potentially interesting proto-oncogenes.

Another chicken retrovirus—the MC29 myelocytomatosis virus—which was known to be capable of inducing a bone marrow malignancy in chickens, was one of this class. MC29 was also found to carry an acquired cellular gene in its genome, termed the v-myc oncogene, which this virus exploited to induce rapidly growing tumors in infected chickens. As was the case with v-src, the origin of the v-myc gene could be traced to a corresponding proto-oncogene residing in the normal chicken genome. Like src, myc underwent some remodeling after being incorporated into the retroviral genome. This remodeling imparted potent oncogenic powers to a gene that previously had played a benign and possibly essential role in the life of normal chicken cells.

Of additional interest was the discovery that MC29, like RSV, descended from avian leukosis virus (ALV). This reinforced the notion that retroviruses like ALV were adept at acquiring random pieces of a cell’s genome. The biological powers of the resulting hybrid viruses would presumably depend on which particular cellular genes had been picked up. In the case of the large majority of acquired cellular genes, hybrid viruses carrying such genes would show no obvious phenotypes such as tumor-inducing potential. Only when a growth-promoting cellular gene, that is, a proto-oncogene, was acquired might the hybrid virus exhibit a cancer-inducing phenotype that could lead to its discovery and eventual isolation by a virologist.

Mammals were also found to harbor retroviruses that are distantly related to ALV and, like ALV, are capable of acquiring cellular proto-oncogenes and converting them into potent oncogenes. Among these is the feline leukemia virus, which acquired the fes oncogene in its genome, yielding feline sarcoma virus, and a hybrid rat–mouse leukemia virus, which on separate occasions acquired two distinct proto-oncogenes: the resulting transforming retroviruses, Harvey and Kirsten sarcoma viruses, carry the H-ras and K-ras oncogenes, respectively, in their genomes. Within a decade, the repertoire of retrovirus-associated oncogenes had increased to more than two dozen, many named after the viruses in which they were originally discovered (Table 3.3). By now, more than thirty distinct vertebrate proto-oncogenes have been discovered through this route.
The vertebrate genome carries many of proto-oncogenes

Table 3.3 Acutely transforming retroviruses and the oncogenes that they have acquired

<table>
<thead>
<tr>
<th>Name of virus</th>
<th>Viral oncogene</th>
<th>Species</th>
<th>Major disease</th>
<th>Nature of oncoprotein</th>
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<tr>
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<td>mpl</td>
<td>mouse</td>
<td>myeloproliferation</td>
<td>TPO receptor</td>
</tr>
<tr>
<td>Regional Poultry Lab v. 30</td>
<td>eyk</td>
<td>mouse</td>
<td>sarcoma</td>
<td>RTK; unknown ligand</td>
</tr>
<tr>
<td>Avian sarcoma virus CT10</td>
<td>crk</td>
<td>chicken</td>
<td>sarcoma</td>
<td>SH2/SH3 adaptor</td>
</tr>
<tr>
<td>Avian sarcoma virus 17</td>
<td>jun</td>
<td>chicken</td>
<td>sarcoma</td>
<td>transcription factor</td>
</tr>
<tr>
<td>Avian sarcoma virus 31</td>
<td>qin</td>
<td>chicken</td>
<td>sarcoma</td>
<td>transcription factor</td>
</tr>
<tr>
<td>AS42 sarcoma virus</td>
<td>maf</td>
<td>chicken</td>
<td>sarcoma</td>
<td>SH2-dependent ubiquitylation factor</td>
</tr>
<tr>
<td>Cas NS-1 virus</td>
<td>cbl</td>
<td>mouse</td>
<td>lymphoma</td>
<td>SH2-dependent ubiquitylation factor</td>
</tr>
</tbody>
</table>

Abbreviations: CSF, colony-stimulating factor; EGF, epidermal growth factor; G, GTP-binding; PDGF, platelet-derived growth factor; RTK, receptor tyrosine kinase; ser/th, serine/threonine; SH, src-homology segment; TK, tyrosine kinase; TPO, thrombopoietin.

Notes:

a) Not all viruses that have yielded these oncogenes are indicated here.

b) Ortholog of the mammalian fps oncogene.

c) Also causes carcinomas and endotheniomas.

d) Ortholog of the mammalian ras oncogene.

e) Ortholog of the avian c-myc oncogene.

f) Ortholog of the avian fps oncogene.

Abbreviations: CSF, colony-stimulating factor; EGF, epidermal growth factor; G, GTP-binding; PDGF, platelet-derived growth factor; RTK, receptor tyrosine kinase; ser/th, serine/threonine; SH, src-homology segment; TK, tyrosine kinase; TPO, thrombopoietin.

In each case, a proto-oncogene found in the DNA of a mammalian or avian species was readily detectable in the genomes of all other vertebrates. There were, for example, chicken, mouse, and human versions of c-myc, and these genes seemed to function identically in their respective hosts. The same could be said of all the other proto-oncogenes that were uncovered. Soon it became clear that this large repertoire of proto-oncogenes must have been present in the genome of the vertebrate that was the common ancestor of all mammals and birds, and that this group of genes, like most others in the vertebrate genome, was inherited by all of the modern descendant species.
Now, a quarter of a century later, we realize that these transforming retroviruses provided cancer researchers with a convenient window through which to view the cellular genome and its cohort of proto-oncogenes. Without these retroviruses, the discovery of proto-oncogenes would have been exceedingly difficult. By fishing these genes out of the cellular genome and revealing their latent powers, these viruses catapulted cancer research forward by decades.

### 3.11 Slowly transforming retroviruses activate proto-oncogenes by inserting their genomes adjacent to these cellular genes

As described above, each of the various tumorigenic retroviruses arose when a nontransforming retrovirus, such as avian leukosis virus (ALV) or murine leukemia virus (MLV), acquired a proto-oncogene from the genome of an infected host cell. In fact, the “nontransforming” precursor viruses could also induce cancers, but they were able to do so only on a much more extended timetable; often months passed before these viruses succeeded in producing cancers. The oncogene-bearing retroviruses, in contrast, often induced tumors within days or weeks after they were injected into host animals.

When the rapidly transforming retroviruses were used to infect cells in culture, the cells usually responded by undergoing the changes in morphology and growth behavior that typify the behavior of cancer cells (see Table 3.2). In contrast, when the slowly tumorigenic viruses, such as ALV and MLV, infected cells, these cells released progeny virus particles but did not show any apparent changes in shape or growth behavior. This lack of change in cell phenotypes was consistent with the fact that these viruses lacked oncogenes in their genomes.

These facts, when taken together, represented a major puzzle. How could viruses like MLV or ALV induce a malignancy if they carried no oncogenes? In 1981 this puzzle was solved.

The solution came from study of the leukemias that ALV induced in chickens, more specifically, from detailed analysis of the genomic DNAs of the leukemic cells. These cells invariably carried copies of the ALV provirus integrated into their genomes. Some researchers undertook to map the sites in the chromosomal DNA where the ALV proviruses had integrated. By the time these experiments began, a decade after Temin and Baltimore’s discovery, it had become clear that the integration of proviruses occurs at random sites throughout the chromosomal DNA of infected host cells. Given the size of the chicken genome, there might be many millions of distinct chromosomal sites used by ALV to integrate its provirus.

But molecular analysis of a series of ALV-induced leukemias, each arising independently in a separate chicken, turned up a major surprise. In a substantial portion (>80%) of these leukemia cell genomes, the ALV provirus was found to be integrated into the chromosomal DNA immediately adjacent to the c-\textit{myc} proto-oncogene (Figure 3.23)! This observation, on its own, was difficult to reconcile with the notion that provirus integration occurs randomly at millions of sites throughout the genomes of infected cells.

It soon became clear that the close physical association of the integrated viral genomes and the cellular \textit{myc} gene led to a functional link between these two genetic elements. The viral transcriptional promoter, nested within the ALV provirus, disrupted the control mechanisms that normally govern expression of the c-\textit{myc} gene (see Figure 3.23). Now, instead of being regulated by its own native gene promoter, the cellular \textit{myc} gene was placed directly under viral transcriptional control. As a consequence, rather than being regulated up and down
by the finely tuned control circuitry of the cell, c-myc expression was taken over by a foreign usurper that drove its expression unceasingly and at a high rate. In essence, this hybrid viral–cellular gene arising in the chromosomes of leukemic cells now functioned much like the v-myc oncogene carried by avian myelocytomatosis virus.

Suddenly, all the clues needed to solve the puzzle of leukemogenesis (leukemia formation) by ALV fell into place. The solution went like this. During the course of infecting a chicken, ALV spread to thousands, then millions of cells in the hematopoietic system of this bird. Soon, the infection was so successful that the bird would become viremic, that is, its bloodstream carried high concentrations of virus particles.

Each of these tens of millions of infections resulted in the insertion of an ALV provirus at some random location in the genome of an infected cell. Almost always, this provirus integration had no effect on the infected host cell, aside from forcing the host to produce large numbers of progeny virus particles. But on rare occasions, perhaps in 1 out of 10 million infections, a provirus became integrated by chance next to the c-myc gene. This jackpot event led to an explosive outcome—conversion of the c-myc gene into a potent oncogene. The cell

Figure 3.23 Insertional mutagenesis
The oncogenic actions of viruses, such as avian leukemia virus (ALV), that lack acquired oncogenes could be explained by the integration of their proviral DNA adjacent to a cellular proto-oncogene. (A) Analysis of numerous B-cell lymphomas that were induced by ALV revealed that a large proportion of the ALV proviruses were integrated into the chromosomal DNA carrying the c-myc proto-oncogene; the majority were integrated between the first noncoding exon of c-myc and the second exon, in which the myc reading frame begins. The integration sites are shown here by the filled triangles. As indicated, most but not all of the proviruses were integrated in the same transcriptional orientation as that of the c-myc gene. (B) This behavior could be rationalized as follows. In the course of ALV infection of chicken lymphocytes, ALV proviruses (green) become integrated randomly at millions of different sites in the chromosomal DNA of the lymphocytes. On rare occasions an ALV provirus becomes integrated (by chance) within the c-myc proto-oncogene (red). This may then cause transcription of the c-myc gene to be driven by the strong, constitutively acting ALV promoter. Because high levels of the Myc protein are potent in driving cell proliferation, the cell carrying this particular integrated provirus and activated c-myc gene will now multiply uncontrollably, eventually spawning a large host of descendants that will constitute a lymphoma.

carrying this deregulated \textit{myc} gene then began uncontrolled proliferation, and within weeks, some of the progeny cells evolved further into more aggressive cancer cells that constituted a leukemia.

This scenario explains the slow kinetics with which these leukemias arise after initial viral infection of a bird. Since activation of the \textit{c-myc} gene through provirus integration is a low-probability event, many weeks and many millions of infectious events are required before these malignancies are triggered. This particular mechanism of proto-oncogene activation came to be called \textbf{insertional mutagenesis}; it explains, as well, the leukemogenic powers of other slowly acting retroviruses, such as MLV. By now, study of avian and murine retrovirus-induced infections has demonstrated integration events next to more than 25 distinct cellular proto-oncogenes. Indeed, insertional mutagenesis can be used as a powerful strategy to find new proto-oncogenes (Sidebar 3.8).

Unvoiced by those who uncovered insertional mutagenesis was another provocative idea: Maybe it was possible that nonviral carcinogens could achieve the same end result as ALV did. Perhaps these other carcinogens, including X-rays and mutagenic chemicals, could alter cellular proto-oncogenes while these genes resided in their normal sites in cellular chromosomes. The result might be a disruption of cellular growth control that was just as destabilizing as the events that led to ALV-induced leukemias.

\section*{3.12 Some retroviruses naturally carry oncogenes}

The descriptions of retroviruses provided in this chapter indicate that there are essentially two classes of these viruses. Some, such as ALV and MLV, carry no oncogenes but can induce tumors that erupt only after a long latent period (i.e., many weeks) following the initial infection of a host animal. Other viruses, such as RSV, can induce cancer rapidly (i.e., in days or several weeks), having acquired an oncogene from a cellular proto-oncogene precursor.

In reality, there is a third class of retroviruses that conforms to neither of these patterns. Human T-cell leukemia virus (HTLV-I) infects about 1\% of the inhabitants of Kyushu, the south island of Japan. An endemic infection is also present, albeit at a lower rate, in some islands of the Caribbean. Lifelong HTLV-I infection carries a 3–4\% risk of developing adult T-cell leukemia, and the virus seems to be maintained in the population via milk-borne, mother-to-infant transmission.

There are no indications, in spite of extensive molecular surveys, that HTLV-I provirus integration sites are clustered in certain chromosomal regions. Accordingly, it appears highly unlikely that HTLV-I uses insertional mutagenesis to incite leukemias. Instead, its leukemogenic powers seem to be traceable to one or more viral proteins that are naturally encoded by the viral genome. The best understood of these is the viral \textit{tax} gene, whose product is responsible for activating transcription of proviral DNA sequences, thereby enabling production of progeny RNA genomes. At the same time, the \textit{tax} gene product appears to activate transcription of two cellular genes that specify important growth-stimulating proteins—IL-2 (interleukin-2) and GM-CSF (granulocyte macrophage colony-stimulating factor). These “growth factors,” to which we will return in Chapter 5, are released by virus-infected cells and proceed to stimulate the proliferation of several types of hematopoietic cells. While such induced proliferation, on its own, does not directly create a leukemia, it seems that populations of these HTLV-I–stimulated cells may progress at a low but predictable frequency to spawn variants that are indeed neoplastic. In this instance, the expression of certain viral oncogenes, notably \textit{tax}, appears to be an intrinsic and essential component of a retroviral replication cycle within host animals, rather than the consequence of rare genetic accidents that yield unusual hybrid genomes, such as the genome of RSV.
Sidebar 3.8 Insertional mutagenesis uncovers novel proto-oncogenes

As described earlier (see Section 3.10), the analysis of the genomes of rapidly transforming retroviruses enabled investigators to identify a large cohort of proto-oncogenes. The phenomenon of insertional mutagenesis, first discovered through the insertion of an ALV genome adjacent to the c-myc proto-oncogene, offered an alternative strategy for discovering these cellular genes. Thus, a researcher could study a series of independently arising tumors, all of which had been induced by a retrovirus, such as ALV or MuLV, that was known to lack its own oncogene. More specifically, this researcher could analyze the locations of the host-cell sequences that lay immediately adjacent to the integrated proviruses in the chromosomal DNA of tumor cells. The hope was that the proviruses might be found to be integrated repeatedly next to a (possibly still-unknown) cellular gene whose activation was triggered by the transcriptional promoter of the provirus. The adjacent gene could be readily cloned, since it was effectively tagged by the closely linked proviral DNA.

The initial fruits of this strategy came from studying the breast cancers induced by mouse mammary tumor virus (MMTV), another retrovirus. Researchers mapped the integration sites of MMTV proviruses in the genomes of mouse breast cancers that had been induced by this virus. Most of the proviruses were found to be integrated in one of three alternative genomic locations, clustering next to cellular genes that were then called int-1, int-2, and int-3 (Table 3.4). Each of these genes was later discovered to encode a protein involved in stimulating cell proliferation in one way or another. The deregulated expression of each of these genes, resulting from nearby MMTV provirus integration, seemed to be responsible for triggering the cell proliferation that led to the appearance of mammary tumors.

The int-1 gene, which was found to be homologous to the wingless gene of Drosophila, was renamed Wnt-1, and was the forerunner of a whole series of Wnt genes that have proven to be important vertebrate mitogens and morphogens, i.e., factors important for controlling morphogenesis. More recently, this search strategy has been used to uncover a large group of other cellular genes, each of which, when activated by insertional mutagenesis mediated by MLV, triggers leukemias in mice (Table 3.4).

Table 3.4 Examples of cellular genes found to be activated by insertional mutagenesis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Insertional mutagen</th>
<th>Tumor type</th>
<th>Species</th>
<th>Type of oncprotein</th>
</tr>
</thead>
<tbody>
<tr>
<td>myc</td>
<td>ALV</td>
<td>B-cell lymphoma</td>
<td>chicken</td>
<td>transcription factor</td>
</tr>
<tr>
<td>myc</td>
<td>ALV, FeLV</td>
<td>T-cell lymphoma</td>
<td>chicken, cat</td>
<td>transcription factor</td>
</tr>
<tr>
<td>nov</td>
<td>ALV</td>
<td>nephroblastoma</td>
<td>chicken</td>
<td>growth factor</td>
</tr>
<tr>
<td>erbB</td>
<td>ALV</td>
<td>erythroleukemia</td>
<td>chicken</td>
<td>receptor TK</td>
</tr>
<tr>
<td>mos</td>
<td>IAP</td>
<td>plasmacytoma</td>
<td>mouse</td>
<td>ser/thr kinase</td>
</tr>
<tr>
<td>int-1a</td>
<td>MMTV</td>
<td>mammary carcinoma</td>
<td>mouse</td>
<td>growth factor</td>
</tr>
<tr>
<td>int-2b</td>
<td>MMTV</td>
<td>mammary carcinoma</td>
<td>mouse</td>
<td>growth factor</td>
</tr>
<tr>
<td>int-3</td>
<td>MMTV</td>
<td>mammary carcinoma</td>
<td>mouse</td>
<td>receptor&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>int-Hint-5</td>
<td>MMTV</td>
<td>mammary carcinoma</td>
<td>mouse</td>
<td>enzyme&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>pim-1</td>
<td>Mo-MLV</td>
<td>T-cell lymphoma</td>
<td>mouse</td>
<td>ser/thr kinase</td>
</tr>
<tr>
<td>pim-2</td>
<td>Mo-MLV</td>
<td>B-cell lymphoma</td>
<td>mouse</td>
<td>ser/thr kinase</td>
</tr>
<tr>
<td>bmi-1</td>
<td>Mo-MLV</td>
<td>T-cell lymphoma</td>
<td>mouse</td>
<td>transcription repressor</td>
</tr>
<tr>
<td>tpl-2</td>
<td>Mo-MLV</td>
<td>T-cell lymphoma</td>
<td>mouse</td>
<td>non-receptor TK</td>
</tr>
<tr>
<td>lck</td>
<td>Mo-MLV</td>
<td>T-cell lymphoma</td>
<td>mouse</td>
<td>non-receptor TK</td>
</tr>
<tr>
<td>p53</td>
<td>Mo-MLV</td>
<td>T-cell lymphoma</td>
<td>mouse</td>
<td>transcription factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>IAP</td>
<td>myelomonocytic leukemia</td>
<td>mouse</td>
<td>growth factor</td>
</tr>
<tr>
<td>IL2</td>
<td>GaLV</td>
<td>T-cell lymphoma</td>
<td>gibbon ape</td>
<td>cytokine&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL3</td>
<td>IAP</td>
<td>T-cell lymphoma</td>
<td>mouse</td>
<td>cytokine</td>
</tr>
<tr>
<td>K-ras</td>
<td>F-MLV</td>
<td>T-cell lymphoma</td>
<td>mouse</td>
<td>Small G protein</td>
</tr>
<tr>
<td>CycD1</td>
<td>F-MLV</td>
<td>T-cell lymphoma</td>
<td>mouse</td>
<td>G1 cyclin</td>
</tr>
<tr>
<td>CycD2</td>
<td>Mo-MLV</td>
<td>T-cell lymphoma</td>
<td>mouse</td>
<td>G1 cyclin</td>
</tr>
</tbody>
</table>

<sup>a</sup>Subsequently renamed Wnt-1.
<sup>b</sup>Subsequently identified as a gene encoding a fibroblast growth factor (FGF).
<sup>c</sup>Related to notch receptors.
<sup>d</sup>Enzyme that converts androgens to estrogens.
<sup>e</sup>Cytokines are GFs that largely regulate various types of hematopoietic cells.

Abbreviations: ALV, avian leukosis virus; FeLV, feline leukemia virus; F-MLV, Friend murine leukemia virus; GaLV, gibbon ape leukemia virus; GF, growth factor; IAP, intracisternal A particle (a retrovirus-like genome that is endogenous to cells); Mo-MLV, Moloney murine leukemia virus; MMTV, mouse mammary tumor virus; ser/thr, serine/threonine; TK, tyrosine kinase.

3.13 Synopsis and prospects

By studying tumors in laboratory and domesticated animals, cancer biologists discovered a wide array of cancer-causing viruses during the twentieth century. Many of these viruses, having either DNA or RNA genomes, were found able to infect cultured cells and transform them into tumorigenic cells. These transforming powers pointed to the presence of powerful oncogenes in the genomes of the viruses, indeed, oncogenes that were potent enough to induce many of the phenotypes associated with cancer cells (see Table 3.2). Moreover, the ability of these viruses to create transformed cells in the culture dish shed light on the mechanisms by which these viruses could induce cancers in the tissues of infected host animals.

A major conceptual revolution came from the detailed study of RNA tumor viruses, specifically, Rous sarcoma virus (RSV). Its oncogene, termed v-src, was found to have originated in a normal cellular gene, c-src. This discovery revealed the ability of nontransforming, slowly tumorigenic retroviruses, such as ALV (avian leukosis virus), to acquire normal cellular genes and convert these captured genes into potently transforming oncogenes. The hybrid viruses that arose following these genetic acquisitions were now able to rapidly induce tumors in infected hosts.

Even more important were the implications of finding the c-src gene. Its presence in a normal cellular genome demonstrated that the cellular genome carries a proto-oncogene that can be converted into an oncogene following alterations to the sequences of the normal gene. (The details of these alterations will be described in the next several chapters.) Soon a number of retroviruses of both avian and mammalian origin were discovered to carry other oncogenes that had been acquired in similar fashion from the genomes of infected cells. While each of these proto-oncogenes was found initially in the genome of one or another vertebrate species, we now know that all of these genes are represented in the genomes of all vertebrates. Consequently, the generic vertebrate genome carries dozens of such normal genes, each of which has the potential to become converted into an active oncogene.

Yet other proto-oncogenes were discovered by studying the integration sites of proviruses in the genomes of tumors that had been induced by nontransforming retroviruses, such as murine leukemia virus (MLV) and ALV. The random integration of these proviruses into chromosomal DNA occasionally yielded, through the process of insertional mutagenesis, the conversion of a proto-oncogene into an activated oncogene that could readily be isolated because of its close linkage to the provirus. On many occasions, insertional mutagenesis led to rediscovery of a proto-oncogene that was already known because of its presence in an acutely transforming retrovirus; myc and avian myelocytomatosis virus (AMV) exemplify this situation. On other occasions, truly novel proto-oncogenes were discovered through study of provirus integration sites; the int-1 gene activated by MMTV provides a striking example of this route of discovery. In fact, the process of insertional mutagenesis remained little more than a laboratory curiosity, of interest to only a small cadre of cancer biologists, until it was reported to lead to tumors in patients being treated by gene therapy (Sidebar 3.9).

These discoveries of proto-oncogenes and oncogenes, as profound as they were, provoked as many questions as they answered. It remained unclear how the retrovirus-encoded oncogene proteins (called oncoproteins) differed functionally from the proteins encoded by corresponding proto-oncogenes. The biochemical mechanisms used by these oncoproteins to transform cells were also obscure.

The molecular mechanisms used by DNA tumor viruses to transform infected cells were even more elusive, since these viruses seemed to specify oncoproteins that were very different from the proteins made by their host cells. Such
differences suggested that these viral oncoproteins could not insinuate themselves into the cellular growth-regulating machinery in any easy, obvious way. Only in the mid-1980s, ten years after this research began, did their transforming mechanisms become apparent, as we will see in Chapters 8 and 9. For many cancer researchers, and for the public that supported this research, there was a single overriding issue that had motivated much of this work in the first place: did any of these viruses and the proto-oncogenes that they activated play key roles in causing human cancers? As we will learn, about one-fifth of the human cancer burden worldwide is associated with infectious agents. Hepatitis B and C viruses (HBV, HCV), as well as human papillomaviruses (HPVs), play key roles in triggering commonly occurring cancers. Indeed, even infrequently occurring human tumors that seem to be familial have been traced in recent years to viral infections (Sidebar 3.10). So the recognized role of viruses in cancer pathogenesis is substantial and growing.

Still, even if RNA and DNA tumor viruses were not responsible for inciting a single case of human cancer, the research into their transforming mechanisms would have been justified. This research opened the curtain on the genes in our genome that play central roles in all types of human cancer. It accelerated by decades our understanding of cancer pathogenesis at the level of genes and molecules. It catapulted cancer research from a descriptive science into one where complex phenomena could finally be understood and explained in precise, mechanistic terms.

Sidebar 3.9 Gene therapy can occasionally have tragic consequences Gene therapy has been found to be most applicable to diseases of the hematopoietic system. Thus, children who are born with a severe immunodeficiency due to a germ-line–specified defect in one or another critical component of the immune system can, in principle, be cured if the missing gene is transduced into their bone marrow stem cells using retrovirus vectors (see Sidebar 3.7). After infection in vitro by a gene-transducing retroviral vector, stem cells are introduced into the afflicted children, in whose bone marrow these cells become stably engrafted. The differentiated progeny of these engrafted, genetically altered stem cells are then able to supply missing immune functions, thereby reversing the congenital immunodeficiency.

Just such a therapeutic approach was launched in France, in which bone marrow stem cells from ten children suffering from a congenital immunodeficiency—X-linked severe combined immunodeficiency—were infected with a Moloney murine leukemia virus (MLV)–derived retroviral vector that transduced a gene specifying the gene product that they lacked—the γc protein. Nine of these children responded by showing a dramatic reconstitution of their immunological function. However, as reported in 2003, the two youngest children in the trial developed T-cell leukemia 30 and 34 months after the initiation of the gene therapy trial.

In both cases, analyses of the DNA of leukemic cells revealed a provirus derived from the viral vector that had become integrated within several kilobase pairs of the first exon of the LMO2 gene, a proto-oncogene that was previously known to be activated in human T-cell leukemias. Given the known role of the LMO2 oncogene in leukemogenesis, these inserted proviruses were almost certainly responsible for triggering the two leukemias. Hence, insertional mutagenesis leading to oncogenesis, which had long been feared as a possible but remote risk incurred by this gene therapy strategy, became a grim reality and may ultimately limit the options available to those interested in correcting inborn defects through gene therapy.
Sidebar 3.10 Classic Kaposi’s sarcoma appears to be a familial disease

Some virus-induced malignancies are limited largely to small subpopulations and thus resemble familial cancer syndromes. We will encounter many of these syndromes in Chapters 7, 8, 9, and 12. There, we will learn that inheritance of mutant alleles of tumor suppressor genes or DNA repair genes can create a strong, inborn cancer predisposition.

Prior to the onset of the AIDS epidemic, the disease of Kaposi’s sarcoma (KS)—apparently a malignancy of cells related to those forming the endothelial lining of lymph ducts—was confined largely to small subpopulations, notably men of Mediterranean and Jewish descent. This resembled a familial cancer, in which cancer-predisposing alleles were present only in the gene pools of certain ethnic subpopulations. After the onset of the AIDS epidemic, however, KS became 1000-fold more common, and at least this form of KS could be associated with an infectious agent—human herpesvirus-8 (HHV-8), also known as KSHV (KS herpesvirus). This virus, along with a number of other infectious agents, is an opportunistic pathogen that thrives in the bodies of those lacking a functional immune system. Because of the AIDS epidemic in Africa, KS has now become the fourth most common infection-induced cancer worldwide.

The virology of HHV-8 failed to explain how the “classic,” pre-AIDS KS is transmitted in immunocompetent populations. Indeed, these tumors could also be associated with HHV-8 infections. Examination of the various sub-strains of HHV-8, as defined by sequence polymorphisms in viral DNAs, has recently revealed that different sub-strains of the virus are present in different subpopulations of Jews; one sub-strain predominates among Ashkenazic Jews (of recent European descent), while a second is common among Sephardic Jews (of North African and Middle Eastern descent). Both populations have infection rates that are as much as 10 to 20 times higher than in non-AIDS Western populations. Provocatively, within these sub-groups, the transmission of specific HHV-8 sub-strains is correlated with inheritance of certain mitochondrial DNA polymorphisms far more strongly than with inheritance of Y-chromosome polymorphic markers. Mitochondrial DNA is transmitted maternally, indicating that maternal transmission of virus (occurring possibly via saliva) has played a major role in creating pockets of disease in family lineages that are likely to extend back to founder populations that existed more than 2000 years ago. [Another maternal transmission route may explain the high incidence of adult T-cell leukemia (caused by the HTLV-I retrovirus) in southern Japan (see Section 3.12).]

Hence, certain geographically and ethnically localized malignancies, such as classic KS and adult T-cell leukemia, are actually due to viruses that spread poorly “horizontally” (i.e., from one adult to another) but can be transmitted “vertically” (between parent and offspring) through long-term, intimate contact. This echoes the behavior of certain strains of mice that have high rates of breast cancer. As first shown in Bar Harbor, Maine, in 1933, transmission of disease from parent to offspring occurred when females of high-incidence strains were mated to males of low-incidence strains, but not following the reverse matings. Also, when female pups of high-incidence strains were transferred to low-incidence foster nursing mothers within 24 hours of birth, only 8% eventually developed breast cancer, compared with a 92% incidence exhibited by mice that had been nursed by mothers from a high-incidence strain. This led to the conclusion that this breast cancer susceptibility was transmitted from one generation to the next by a milk-borne infectious agent, which was later identified as mouse mammary tumor virus (MMTV).

Key concepts

- The notion that cancer might be an infectious disease gained favor with Peyton Rous's 1910 discovery that a virus—the Rous sarcoma virus (RSV)—could induce tumors in chickens, but the idea lost credibility in 1926 when the stomach lesions of Fibiger's rats were found to result from vitamin deficiency and not spirochete infection.

- Decades later, Howard Temin and Harry Rubin's discovery that cultured cells infected with RSV were transformed into tumor cells resurrected tumor virus research and led to the realization that cancer could be studied at the level of the cell.

- Transformed cells in culture show several characteristics: (1) unlike normal cells, transformed cells lack contact inhibition and consequently manifest as a multilayered clump known as a focus; and (2) the focus is a clonal outgrowth, with all its cells descended from a single, common progenitor. (3) The cells in a focus can often grow in an anchorage-independent fashion.

- Tumorigenicity in a host animal is the acid test for full cellular transformation.

- The transformation phenotype induced by RSV infection was found to be transmitted to progeny cells, and experiments using a temperature-sensitive (ts) mutant of RSV showed that retention of the transformed state depends on the continued activity of an RSV gene product.
In addition to RNA viruses like RSV, several classes of DNA viruses—including papovavirus, human adenovirus, herpesvirus, and poxvirus—were found to induce cancers.

While the genomes of RNA viruses consist of single-stranded RNA, the genomes of DNA viruses consist of double-stranded DNA (dsDNA). The papovaviruses—which include Shope papillomavirus, mouse polyomavirus, and SV40—have circular dsDNA genomes, while adenoviruses and herpesviruses have long, linear dsDNA. Like SV40, human adenovirus induces cell lysis after replicating in its natural, permissive host, but cannot replicate in nonpermissive host cells and instead may transform these cells.

Since replication of viral DNA genomes occurs independently of the host cells’ DNA and since viral genomes lack the elements to properly segregate during mitosis, the transmission of DNA tumor virus genomes from one cell generation to the next posed a conceptual problem, until it was discovered that DNA tumor virus genomes integrate into host-cell chromosomal DNA.

Since the genomes of RNA viruses consist of single-stranded RNA that cannot be incorporated into host DNA and re-infection does not explain the persistence of the transformed state in descendant cells, Temin postulated that RNA viruses make double-stranded DNA copies of their genomes—the heretofore unheard of process of reverse transcription—and that these DNA copies are integrated into the host’s chromosomal DNA as a part of a normal viral replication cycle. This is a major distinction from DNA tumor viruses, for which integration is a very rare and haphazard event that is not an integral part of viral replication.

Because their replication cycle depends on information flowing backward (i.e., from RNA to DNA), RNA viruses came to be called retroviruses and the DNA version of their viral genomes was called a provirus.

Working with RSV, researchers found that viral replication and cell transformation were specified by separate genes, with the transforming function residing in a single gene called src.

Using a DNA probe that specifically recognized the transformation-associated (i.e., src) sequences of the RSV genome, researchers made the unexpected discovery that src-related sequences were present in the DNA of chicken cells not infected by RSV. Further research indicated that the src gene was a normal, highly conserved gene of all vertebrate species (as later proved true of many other such genes).

The difference between the actions of the cellular version of src (c-src), which supports normal cell function, and the viral version (v-src), which acts as an oncogene, can be explained if v-src were altered after it was plucked from a cellular genome by an ancestor of RSV.

Because it can serve as a precursor to an oncogene, c-src was called a proto-oncogene, a term that carried the startling implication that normal vertebrate cells contain genes that have the intrinsic potential to induce cancer.

The acquisition and activation of src by a retrovirus indicated that a single oncogene could act pleiotropically to evoke a multiplicity of changes in cellular traits, as well as the possibility that other mutation mechanisms might activate proto-oncogenes that continued to reside in their normal sites in cellular chromosomes.

Some retroviruses can induce cancer although they do not carry oncogenes in their genomes; these viruses work much more slowly to induce cancer than those bearing oncogenes. Such nontransforming, slowly tumorigenic retroviruses activate proto-oncogenes by inserting their genomes adjacent to these cellular genes in cellular chromosomes, a process called insertional mutagenesis. This chance occurrence places the proto-oncogene under the control of the viral transcriptional promoter, which deregulates the gene’s expression.
and leads to uncontrolled cell proliferation. Insertional mutagenesis can be exploited to find new proto-oncogenes.

- In addition to the nontransforming retroviruses (which work via insertional mutagenesis) and the acutely transforming ones (which work via acquired oncogenes), retroviruses exist whose carcinogenic powers are traceable to their own normal gene products. A case in point is human T-cell leukemia virus (HTLV-I), whose tax gene encodes a protein that activates transcription of proviral DNA and, as a side effect, also stimulates expression of cellular growth factors that induce cell proliferation.

**Thought questions**

1. What observations favor or argue against the notion that cancer is an infectious disease?
2. How can one prove that tumor virus genomes must be present in order to maintain the transformed state of a virus-induced tumor? What genetic mechanisms, do you imagine, might enable this process to become “hit-and-run”, in which the continued presence of a tumor virus is not required to maintain the tumorigenic phenotype after a certain time?
3. Why are oncogene-bearing viruses like Rous sarcoma virus so rarely encountered in wild populations of chickens?
4. What evidence suggests that the phenotypes of cells transformed by tumor viruses in vitro reflect comparable phenotypes of tumor cells in vivo?
5. What logic suggests that the chromosomal integration of tumor virus genomes is an intrinsic, obligatory part of the replication cycle of RNA tumors viruses but an inadvertent side-product of DNA tumor virus replication?
6. What evidence suggests that a proto-oncogene like src is actually a normal cellular gene rather than a gene that has been inserted into the germ line by an infecting retrovirus?
7. How do you imagine that DNA tumor viruses and retroviruses like avian leukemia virus arose in the distant evolutionary past?
8. Why do retroviruses like avian leukemia virus take so long to induce cancer?

**Additional reading**


