DNA Tumor Viruses and Their Contributions to Molecular Biology

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ABSTRACT This summer marks the 51st anniversary of the DNA tumor virus meetings. Scientists from around the world will gather in Trieste, Italy, to report their latest results and to agree or disagree on the current concepts that define our understanding of this diverse class of viruses. This article offers a brief history of the impact the study of these viruses has had on molecular and cancer biology and discusses obstacles and opportunities for future progress.

KEYWORDS cancer, molecular biology, tumor viruses

On a hot muggy day in 1969, 81 scientists gathered at the Cold Spring Harbor Laboratory (CSHL) to discuss tumor viruses. This initial meeting, held from 7 to 9 August, was organized by Joe Sambrook of the CSHL and Lionel Crawford of the Imperial Cancer Research Fund. The meeting was coincident with a tumor virus workshop held from 31 July to 15 August that year. The 14 students attending the workshop were treated to lectures by 29 top scientists in virology and molecular biology from around the world. The hope was that the intense study of this class of viruses would lead to an understanding of fundamental life processes. How do eukaryotes replicate their DNA? What is the structure of a eukaryotic gene, and how is gene expression regulated? How do cells orchestrate these processes during development and adult homeostasis to maintain the viability of the organism, and how do perturbations of this regulation lead to disease, including cancer? This year, as we mark the 51st anniversary of that meeting, which is still an annual event, it is appropriate to reflect on the contributions the field has made to biology, to define the challenges to our next level of understanding, and to chart a path forward.

BACTERIOPHAGE SET THE STAGE

The role of bacterial systems, and bacteriophage in particular, in the initial stages of molecular biology has been well documented. Those studies demonstrated the power of combining genetics with biochemistry to define the nature of genes and the mechanisms that control their expression, as well as the multiple components required for DNA replication. A critical factor that allowed rapid advances in bacteriophage research was the ability to grow the host of these viruses *in vitro*. This made the detailed study of bacterial viruses tractable, allowing genetic studies of both host and virus, thus leading to deep insights into the interplay of these systems. Perhaps less appreciated is the role that bacteriophage played in inspiring the development of animal virology, especially tumor virology.

A key moment in virology occurred in 1945, when Max Delbrück, Salvador Luria, and Alfred Hershey formed what became known as The Phage Group. Each year, they gathered at the Cold Spring Harbor Laboratory, and this eventually morphed into the bacteriophage meetings. These investigators and their colleagues realized that the focused study of a few bacteriophage could lead to rapid advances in the fundamental questions of molecular biology. Their insights were made possible because both the

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host, especially *Escherichia coli*, and the viruses could be cultured. This allowed the development of the plaque assay and quantitation of virus input and yield, which in turn led to the single-step growth curve experiment. One of the stunning revelations of these experiments was the eclipse phase of bacteriophage infection, as viruses do not divide by binary fission but rather disassemble and later generate progeny using the released DNA as a template. It is telling that the 1969 bacteriophage meeting at CSHL had 293 participants, far more than the attendance at the initial tumor virus meeting that same year.

EMERGENCE OF TUMOR VIRUSES AS MODEL SYSTEMS

Curiously, animal and human viruses were discovered and effective viral vaccines to rabies virus and yellow fever virus were developed and put into broad use long before the molecular nature of viruses was uncovered. The discovery of the first tumor virus, Rous sarcoma virus, was published in 1911 (1), while Shope papillomavirus, the first described DNA tumor virus, was detected in 1933 as a filterable agent capable of inducing papillomas in cottontail rabbits (2). However, tumor virology could not really develop as a mature discipline until methods for culturing the host cells became available. The first tissue culture systems were described in the late 1800s, but they required the establishment of mouse L cells and the demonstration of their clonal growth to realize the full power of viruses as a tractable system (3). The first human cell line (HeLa) was established in 1953 (4), and soon, a number of people developed and optimized cell growth media, allowing the culture of different cell types from a variety of animals (5–8).

Improved cell culture methodologies and the resulting expansion of available human and animal cell lines were major events in the development of tumor virology, enabling the discovery of murine polyomavirus (MPyV) and human adenovirus in 1953, and of simian virus 40 (SV40) in 1960 (9–12), which in turn led to the development of the plaque assay for animal viruses (5). The ability to synchronously infect cultured cells allowed for time course experiments, which defined the sequence of events in productive infection and thereby the delineation of the stages of viral infection.

The properties of cultured cells, such as doubling time, saturation density, serum and growth factor requirements, growth arrest at confluence, and anchorage-dependent or -independent growth, could then be assessed. These assays allowed the comparison of normal cultured cells with those that were rendered tumorigenic by viruses. These studies also led to the realization that viral transformation in culture generally occurred in cell types that were nonpermissive for productive infection. Now, the full power of the genetic techniques developed for the study of bacteriophage could be applied to animal viruses. The first temperature-sensitive mutants of MPyV were reported in 1965. This landmark study also demonstrated that continuous expression of a viral gene is required to maintain the transformed phenotype, a phenomenon now termed oncogene addiction (13). Shortly after, temperature-sensitive mutants of SV40 and adenovirus were generated (14–18).

EXPLOSIVE IMPACT OF DNA TUMOR VIRUSES ON BIOLOGY

The 15 years following the initial tumor virus meeting at the CSHL saw a series of technical and conceptual advances that heavily influenced research in the biological sciences (see a special issue of *Virology* [19]). The first advance was powered by the discovery of restriction enzymes and their use in generating physical maps of viral genomes (20, 21). The first detailed map of the restriction enzyme recognition sites of a tumor virus, SV40, is shown in Fig. 1. Restriction enzyme mapping was also key to defining the first viral origin of DNA replication and to generating the first viral transcription map (22, 23).

Mutants have always been a key tool for studying the biology of bacteriophage and animal viruses, but now, rather than treating viruses with mutagens and selecting for a mutant phenotype, viral DNA could be manipulated with restriction enzymes to generate and map mutations (24–28). This culminated in the development of site-

FIG 1 Restriction endonuclease map of the SV40 genome. Restriction endonuclease recognition sites are depicted as arrows. These data were generated prior to the sequencing of the SV40 genome, so the sites are represented as map units relative to the single EcoRI recognition site. This figure appeared as the back cover of the abstracts for the 1975 Tumor Virus Meeting held on 13 to 17 August 1975 at the Cold Spring Harbor Laboratory; courtesy of Ludmila Pollock, Cold Spring Harbor Laboratory, reprinted with permission of the publisher.

directed mutagenesis, the ability to target specific mutations to a preselected region of the genome (29). These developments had broad influences on all of the biological sciences by changing the way genetics was done, which in turn greatly impacted the study of DNA tumor viruses.

Like all of biology, tumor virology advanced with the development of DNA sequencing (30, 31), which resulted in the first complete genomic sequence of an animal virus, that of SV40 (32, 33). In addition to providing definition to genetic landmarks, such as transcriptional promoters, introns, coding regions, and the predicted amino acid sequence of viral proteins, sequencing allowed precise knowledge of the effects of mutations on these elements. DNA sequencing also greatly accelerated work on viral systems that were previously intractable because of a lack of cell culture systems that

supported infection. Thus, although significant work with important human pathogens such as human papillomavirus (HPV) and the polyomaviruses JC virus (JCV) and BK virus (BKV) had to await the advent of cloning, sequencing of these genomes allowed the first view of their genetic organization.

A number of new technologies emerged in the 1970s that greatly accelerated research in molecular biology and had huge impacts on tumor virology. These included the development of Southern blot analysis (34), which enabled visualization of integrated viral genomes; advances in DNA and RNA enzymology, which led to the development of nuclease protection assays and facilitated mRNA analysis; and the development of Northern blot analysis to analyze RNA (35). Among the most surprising results stemming from these assays, coupled with electron microscopy, was the discovery of mRNA splicing (36, 37), quickly followed by the discovery of transcription factors, transcriptional enhancers, and polyadenylation signals (38–41). Finally, the development of systems supporting cell-free replication of SV40 and adenovirus DNA opened the door to the discovery of factors required for eukaryotic genome replication (42, 43). The key role that tumor viruses played in these advances has been extensively reviewed (19, 44).

Another major advance occurred during this period, the first cloning of an animal virus genome, SV40, into a bacterial plasmid and the demonstration that infectious virus could be produced from that clone (45). Furthermore, it was shown that cloned SV40 DNA amplified in bacteria efficiently induced cellular transformation. This enabled detailed genetic studies of DNA tumor viruses, which in turn impacted all of virology. For instance, cloning of viral genomes allowed the propagation and subsequent study of growth-defective viral mutants so that mutations could be introduced into cloned viral DNA and analyzed in mammalian cells. Because cloned viral DNA was propagated in bacteria, and thus carried bacterial modification marks, it was readily distinguishable from viral DNA that replicated in mammalian cells and did not carry these marks. This led to the development of the DpnI assay, which was used to identify and explore *cis*- and *trans*-acting elements required for viral DNA replication (45).

Coapplication of genetics and biochemical studies of proteins encoded by DNA tumor viruses contributed to dramatic changes in understanding protein structure and function. The work of Anfinsen and colleagues on the denaturation and refolding of RNase first suggested that proteins were globular entities whose structure was dependent on amino acid interactions governed solely by their amino acid sequence (46). Therefore, disruption of any of the critical interactions by mutation would result in the loss of the protein's activity. This view was challenged by the work of Wakil and colleagues, who demonstrated that fatty acid synthetase consisted of multiple domains, each carrying a specific enzymatic function and each retaining the ability to fold independently of each other (47). However, the broad significance of these studies was initially not widely appreciated.

Early experiments on the large tumor antigens (LTs) encoded by SV40 and MPyV demonstrated that these proteins have a remarkable number of activities. Not only were they required for viral DNA replication and transcriptional control, but they also stimulated cellular DNA synthesis, altered patterns of cellular gene expression, and were required for viral tumorigenesis. A key early question thus posed was, how does a single protein elicit so many biological effects? One view was that LTs acted as pleiotropic effectors, targeting a key cellular regulatory switch that would lead to multiple biological effects (48). Alternatively, LTs could be multifunctional proteins possessing independent biochemical activities, each contributing alone or cooperatively to different biological functions. Subsequent work demonstrated that the LT proteins were, in fact, a combination of both. Examples of independent activities include the LXCXE motif, which governs the interaction with the Rb family of tumor suppressors, altering the expression of multiple cellular genes and contributing to S-phase entry; the carboxy-terminal host range domain (or adenovirus helper function), which renders monkey epithelial cells permissive for the growth of human adenovirus (49–51); and the nuclear localization signal (52, 53). In contrast, some LT structural

domains, such as the J domain, origin-binding domain, and ATPase domain, act in coordination to effect viral DNA replication (54–57).

Finally, studies on DNA tumor viruses have made important contributions to viral immunology. For instance, the first demonstration that viruses downregulate the major histocompatibility system was shown in adenoviruses (58), and a combination of computational, genetic, and molecular biology studies led to the discovery of the SV40-encoded microRNAs and linked them to both viral gene regulation and recognition by cytotoxic T cells (59).

VIRAL DISEASE AND CANCER

Shortly after their discovery in 1953, adenoviruses were associated with human diseases. Similarly, human polyomaviruses JCV and BKV were linked to progressive multifocal leukoencephalopathy and nephropathy, respectively, coincident with their discoveries in 1971 (60–62). Although the first DNA virus to be associated with human cancer was Epstein-Barr virus, with its contribution to Burkitt's lymphoma (63), the clearest example of a human DNA virus being associated with a human cancer stemmed from the seminal studies of Harald zur Hausen (64), who discovered novel HPV types that were later shown to be the cause of cervical cancer. However, MPyV was the first DNA tumor virus shown to induce cancer in its natural host. More recently, a human polyomavirus (Merkel polyomavirus) has been linked to some forms of human cancers (65).

The early observations demonstrating that viruses can induce cancer in animals and transform cells in culture opened the door for exploring fundamental questions of neoplastic diseases. What exactly defines a tumor cell? How do cancer cells differ from their normal counterparts? What is the molecular basis for these differences? What properties do virus-induced cancers share with cancers with a nonviral etiology, and what are the distinct features of each of these diseases? Tumor viruses, coupled with the development of robust techniques for culturing cells, have provided a path toward answering these fundamental questions. It is more than a little disconcerting that many aspects of these fundamental questions remain unanswered, and that in other cases, the answers accepted by the field are too broad and descriptive to generate predictive hypotheses. Nevertheless, advances that can be directly traced to work on tumor viruses have had an enormous impact on basic molecular biology and on biomedical research. A prime example is the current rapidly developing field of personalized cancer therapies, which is the direct outgrowth of the increase in DNA (and RNA) sequencing capacities and the identification of the cellular targets altered in individual cancers.

Studies using temperature-sensitive mutants of SV40 and murine polyomavirus established that DNA virus-transformed cells expressed specific virus-encoded proteins and that continuous expression of these proteins was required to maintain the transformed phenotypes. But how was expression of these proteins maintained in all the transformed cells? Several studies suggested that viral DNA was integrated in the chromosomes of transformed cell lines. If true, what was the mechanism of integration? It was already known that bacteriophage lambda DNA integrated at specific locations in both the phage and bacterial genomes in a lysogenic infection, and that integration required the action of specific phage genes. Whether tumor virus integrations occurred by a similar mechanism was definitively answered by Southern blotting experiments. Transformed cells harbored integrated viral DNA, and the integration occurred at random sites on both the viral and cellular DNA (66, 67). Furthermore, integration did not require viral gene activity but rather was the consequence of cellular functions resulting in the “willy-nilly” joining of DNA ends (68). This study, led by John Wilson, was the first description of what is now known as nonhomologous end joining (NHEJ).

The realization that somatic mammalian cells efficiently integrated exogenously added DNA led to the isolation of 293 cells, which are permissive cells transformed by human adenovirus DNA (69), and the development of COS cell lines, which express SV40 large T antigen (70). Those and companion studies provided the first demonstrations that viral transformation is incompatible with productive infection. Permissive

cells expressing SV40 T antigen could only be isolated if viral replication functions were inactivated, either by mutating the origin of replication or by eliminating the replication activity of the T antigen. The development of COS and 293 cells and derivative lines had a major impact on both basic molecular biology and the emergence of the biotechnology industry, where 293 cells have been used to produce retroviruses, papillomaviruses, and defective adenoviruses for use in gene therapy and vaccines.

The discovery of tumor suppressors is among the most impactful events that emerged from the study of DNA tumor viruses. Both p53 and pRb, and their roles in viral tumorigenesis, were uncovered because of their direct physical interaction with viral transforming proteins (71–74). Another important finding was the realization that some viral oncoproteins induce tumorigenesis by stimulating the targeted degradation of their binding partners (75, 76). The contributions that these and subsequent studies have made to cancer biology have been extensively reviewed (19, 44). Less appreciated is the fact that the multiple experimental strategies used in these studies, such as the biochemical isolation and identification of multiprotein complexes and the mutational analyses that directly linked the complexes to specific biological consequences, marked the beginnings of the field now called proteomics. Another important tool derived from the study of DNA tumor viruses was the development of transgenic mice that express viral oncoproteins in specific tissues. The first of these systems focused on the SV40 large T antigen (77, 78). These experiments moved the field beyond observations of clonal cell lines to the more complex multicellular environment of animal tissues and provided the first view of how oncogene and tumor-suppressing activities drive specific steps of tumorigenesis (79–82).

THE NEXT 50 YEARS: OVERCOMING BARRIERS TO PROGRESS

There has never been a better time to be a scientist. How do we move forward? A large part of advancing our understanding of nature depends on identifying and overcoming technical and conceptual barriers that limit progress. Rapid technical advances are allowing us to view many aspects of nature that were previously obscured or totally hidden.

Among these technical advances are developments in high-resolution structural biology coupled with incredible increases in computer power, which will continue to enhance our understanding of macromolecular structures and dynamics. In fact, advances in computer hardware and software have enabled an array of new biological research strategies, including single-cell science. The ability to explore viral infections at the single-cell level now allows the detailed study of many complex systems (such as infections of tissues consisting of multiple cell types) that were previously unapproachable. At another level, advances in computational biology have enabled the study of large-scale questions, including metagenomics, microbiomes, and the study of the interplay between viral and human genetics. The study of metagenomes and microbiomes is in the early stages of development and suffers from the growing pains associated with any new scientific field. However, the impact is likely to be enormous. The role that host factors play in virology is also an issue of tremendous importance, given that host genetics and epigenetics are undoubtedly important determinants of infection outcome.

One factor inhibiting our understanding of viruses is the inability to generate infectious stocks for the vast majority of viral species known to exist. Metagenomic sequencing is a powerful tool for studying unculturable agents, and efforts in this arena need to be increased. However, a much deeper understanding of viral infection requires systems that enable *in vitro* studies that do not currently exist for most viruses. To overcome this block, new cell culture and organoid systems need to be developed that (i) support viral productive infection so that infectious virions can be produced, and (ii) mimic the conditions viruses encounter in host organisms so that the complexity of their interactions with tissues composed of many cell types can be studied. To accomplish this will require a much deeper understanding of abortive infections and thus the factors that limit viral infection in nonpermissive

FIG 2 The years and locations of the first 50 tumor virus or DNA tumor virus meetings are shown. The initial meeting, at Cold Spring Harbor Laboratory (CSHL), was organized by Lionel Crawford and Joe Sambrook. The organizers of subsequent meetings at the CSHL (in alphabetical order) included Tom Benjamin, Michael Botchan, Terri Grodzicker, Warren Levinson, David Livingston, Malcom Martin, Carol Prives, Phil Sharp, Howard Temin, and James Watson. The meetings at Cambridge University were held at Churchill College and were organized by Mike Fried. The meetings in Madison were held at the University of Wisconsin and were organized by Paul Lambert. The meetings in Trieste were sponsored by the International Centre for Genetic Engineering and Biotechnology and were organized by Lawrence Banks and Miranda Thomas. The meeting in San Diego was held at the Salk Institute for Biological Studies and was organized by Matthew Weitzman. The meeting in Oxford was held at St. Catherine's College and organized by Alan Storey and Mike Fried. The meetings in Montreal were held at the McGill Cancer Research Centre and organized by Jose Teodoro, Phil Branton, Jacques Archambault, and Paola Blanchette. The meetings in Birmingham were held at the University of Birmingham and were organized by Joanne Parish and Sally Roberts.

cell types. Finally, it is important to realize that the vast majority of viral genes, even for some of the better-studied viruses, have unknown functions. Often, these genes are labeled as “nonessential” because they are not required for productive infection in specific cell culture systems. Yet, the fact that they have been evolutionarily conserved in the highly selective environment of the virosphere speaks to their importance.

The lack of studies of these important areas is partly due to cultural and conceptual barriers that have emerged in virology. In fact, the influence of these barriers extends beyond virology and impacts all of biology. First among these is the tendency to emphasize only topics of obvious clinical relevance. Ironically, this tendency has done as much to slow progress in the development of new therapies for human conditions as any other factor. In virology, it has led to an incredible focus on agents that cause human disease while ignoring the larger biological questions as to why some viruses induce disease while others do not. This has led the field to deemphasize or ignore studies of mature systems. For example, the tumor-virology field has largely moved away from studies of murine polyomavirus. This is one of the few systems that allows the study of productive infection and tumorigenicity in a natural host. There are many other similar examples. Second, there is a current overemphasis on reductionist approaches to science. Hypothesis-driven research is certainly critical to advancing our understanding of nature. However, it is imperative not to forget that the hallmark of our field, the scientific method, starts with observation. Only then do experiments that distinguish competing hypotheses to explain observations come into play. An array of new tools are now available that allow observations of phenomena to which we were previously blind. The use of these tools needs to be greatly expanded.

The next 50 years of tumor virology are ripe with opportunities and promise. If we have learned anything from the previous 50 years, it is that good things happen when smart and motivated individuals are set free to intensely focus on questions and goals that they themselves define. However, it is important to appreciate that scientific progress is incremental. The concept of “breakthroughs” and “transfor-

mative discoveries" are for the most part artifacts of hindsight through which time and ego filter the many observations that in aggregate dramatically change thinking, and thus, the direction of inquiry. What matters is the frequency of the increments. The field needs to continue to display vision and courage if the complex and seemingly unapproachable scientific questions that confront us are to be dissected and understood.

CONCLUDING REMARKS

This article attempts to briefly summarize the contributions DNA tumor viruses have made to molecular biology and biomedical research. For a more comprehensive review of this topic, see the excellent review by DiMaio (83). The 50 previous tumor virus (and later, DNA tumor virus) meetings have served as a powerful catalyst for progress. Indeed, many of the discoveries summarized in this Gem were first reported at these meetings. It is important to remember that these conferences were made possible by the support of the institutions that hosted them, and more importantly, by the scientists who sacrificed their time and energy to ensure their success. Figure 2 depicts a history of where and when each of these meetings occurred. The gathering of scientists in Trieste, Italy, for the 51st DNA tumor virus meeting presents an opportunity to reflect on the contributions this field has made to the biological sciences and medicine. More importantly, the talks, posters, and informal discussions (and sometimes outright arguments) will chart a path forward.

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