Viral Oncology

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Introduction

Few fields in modern biology and certainly no other field in cancer research can be traced back to the work of one man in the same way that the foundations in the field of viral oncology can be traced back to the work of Peyton Rous in 1911. The fact that he was awarded the Nobel Prize for his work in 1968, as the first nonagenarian to receive this honor, reflects the span of time that was needed to appreciate the full impact of Rous' discovery. For several decades, Rous' work was kept on the shelf of curiosa; did it really matter that some avian tumors could be transmitted with cell-free filtrates? And yet, Rous' original work showed more than that. Rous isolated not just one virus (the sole modern survivor, virus nr. 1, now designated as RSV) but at least half a dozen, derived from different primary tumors that were brought to him directly by the chicken farmers. Rous pointed out that the agents were not only competent to induce tumors, but also imprinted the phenotypic properties of the original tumor on the recipient transformed cell. Was this the first observation that reflected the action of *sarc* and other, analogous pieces of the cellular genome that can be picked up and transduced by the transforming RNA tumor viruses?

At no point did Rous believe that viruses caused all tumors or that a virus necessarily pushed the transformed cell all the way from the normal to the fully autonomous tumor cell. He coined the term "tumor progression," whereby "tumors go from bad to worse." Later, progression was defined by Foulds "as an irreversible qualitative change in one or more of the characteristics of the neoplastic cells." Foulds' conclusion that ". . . the properties and behavior of tumors are determined by numerous unit characters, which, within wide limits, are independently variable, capable of combination in a great variety of ways, and liable to independent progression" clearly reflects a Darwinian process in a nutshell, based on variation and selection of the fittest cell, if disobedience to host control can be regarded as fitness.

The *directly transforming RNA tumor viruses,* equipped with different kinds of inserts or additions derived from the cellular genome, are represented in this book by the chapters on the avian and murine sarcoma viruses and by a special chapter on the surprising Abelson virus. In addition to their significance for neoplastic transformation, work on these viruses proved that some kind of genetic transduction can take place between the somatic cells of higher organisms. They were also the first known to violate the "central dogma of molecular biology" that stated, i.a., that DNA can make RNA, but that the reverse is impossible. After the ingenious prediction of Temin, disbelieved and even ridiculed for a decade, the spectacular discovery of reverse transcriptase, independently made by Temin and Baltimore in 1970, led to the immediate and total collapse of this part of the dogma and to another Nobel Prize.

The somewhat poorer cousins of the family, *the nontransforming* but nevertheless "oncogenic" *leukemia and mammary tumor viruses* are equally competent to transcribe their RNA into DNA and to integrate it with the genome of the recipient cell, but they lack the extra sequences that appear to be required for a direct transforming effect. Clearly, these viruses contribute to tumor development by more indirect mechanisms, as indicated also by the long latency periods and the important influence of modifying hormonal, genetic, and immunological host factors. In the course of the long latency period, latent preneoplastic cells accumu-

late and viral genomes tend to be amplified in the target tissue. When the tumor finally emerges, it often shows nonrandom chromosomal changes, frequently trisomies. Could it be that the viruses initiate and accelerate a process where the ultimate and decisive event takes place at the cytogenetic level?

It is interesting to note that the first major discovery in this area, the identification of the mammary tumor virus by Bittner, did not lead to any major enthusiasm or breakthrough in the field in 1935. This was probably because the major importance of conditioning host factors had been emphasized from the beginning. It was proved that the virus was neither necessary nor sufficient to cause mammary tumors by itself. In contrast, the next major milestone, the discovery of murine leukemia virus by Gross in 1951 *did* change the entire climate of opinion. The apparently direct transmission of murine leukemia with cell-free filtrates discovered in a mammalian species was as dramatic as the discovery of avian leukosis virus by Ellerman and Bang, made 40 years earlier in a submammalian species. It can be said, in retrospect, that the difference in the impact of Bittner's and Gross' discovery was more the result of the design, interpretation, and presentation of the experiments than to any fundamental difference between the murine leukemia virus and mammary tumor virus systems. In both cases, the oncogenic retrovirus is carried by genetically susceptible strains. Although the mode of transmission can be (but does not have to be) different, the transmission of integrated proviruses and/or large infectious virus loads to newborn animals is a common feature of both systems. Host factors contribute to the likelihood of tumor development in several ways in both cases. More or less well defined genetic factors are known to influence viral replication, target cell susceptibility, and the immune response of the host to the neoplastic or the preneoplastic cells.

Agents of this category are discussed in the chapters on the avian, murine, feline, bovine, and simian C-type viruses, and in the section on the mammary tumor agent. The complex interactions between the virus, the target cell, and the host are considered in the chapter on the pathogenesis of murine leukemia and in the section on the mammary tumor agent.

The second part of the book is devoted to the *oncogenic DNA viruses.* The earliest precursor of this field was the discovery of the cottontail rabbit papilloma virus by Shope in 1933. This work, performed half a century ago, uncovered many important principles, although the full significance of some was not understood at the time and others were soon forgotten. It is important to recall some lessons learned from the Shope system because they are, *mutatis mutandis,* clearly relevant to modern tumor virology. They include the persistence of the viral DNA in the proliferating, basal cell layer, in the absence of any sign of virus maturation; the dependence of the viral cycle on the differentiation process of keratinization; the rare focal progression of a virus induced benign papilloma to an apparently virus-independent malignant carcinoma; and the cell-mediated rejection of the immune rabbit, directed against the proliferating, virus-DNA-carrying but nonproducer papilloma cells.

The relationship of the oncogenic DNA viruses to their host cell and their transforming effect differs from oncorna-virus induced transformation in some fundamental ways. Part of the difference is due to the iron-clad rule that all known oncogenic DNA viruses afflict irreversible damage on their host cell from the moment they enter the viral cycle. In contrast, all known RNA tumor viruses can multiply in their host cells without impairing the host's ability to proliferate. It follows that the oncogenic effect of the DNA viruses must be entirely dependent on the prolonged latency of the viral genome and the suppression of most viral functions. This relationship is often referred to as "nonpermissive" and is attributed to the inherent inability of the target cell to support the viral cycle, or the defectiveness of the viral genome, or both. This may not be the entire story, however, nor is nonpermissiveness

necessarily the right word. It is true that DNA-viral transformation can be achieved in entirely nonpermissive and usually artificial virus-host systems (e.g., polyoma/hamster cell), and it is also true that defective viral genomes can sometimes transform permissive cells of the natural host (e.g., SV40/African green monkey cells). Both situations are the products of the laboratory, however. Like all other DNA, the genome of the DNA tumor viruses has been selected for survival in their natural habitat, i.e., the potentially permissive host that carries the complete viral genome (e.g., polyoma/mouse, EBV/man). One or several viral genomes per cell are preserved in certain host cells in a completely latent form over long periods of time and multiply only in coordination with the host cell genome. The regulation of this latency is still an almost completely unexplored area, important to understand, and full of future promise. Studies on some of the larger oncogenic DNA viruses (Epstein-Barr virus in particular) suggest a subtle interplay between viral and cellular control mechanisms. We may look forward to the eventual unraveling of finely poised interactions; they will no doubt turn out to be equally or more complex than the mechanisms evolved by the better known symbiotic virtuosi, the lysogenic phage/prokaryote systems, although they will probably be quite different in detail.

It can be easily seen why the maintenance of viral genomes in a state of prolonged latency, inaccessible to host antibodies, would provide a survival advantage to the viral DNA. A latent viral infection may also provide certain advantages to the host; for example, it may provide a constant source of antigenic stimulation and protection from reinfection. But what happens to the cell that harbors the viral genome *in vivo?* Is it phenotypically changed ("transformed")? Is it oncogenic or potentially oncogenic? If the question is phrased in the strict sense, i.e., exclusively in relation to the latent virus carrying cell in the living animal, we can scarcely find any answer at all at this time. We must content ourselves with extrapolations from *in vitro* systems. Interesting as they may be in their own right, such extrapolations must be taken with reservations because they may not be representative of the *in vivo* situation.

In vitro transformation usually implies immortalization, i.e., the emancipation of the cell from its previous dependence on exogenous mitogenic stimuli for growth and survival. In addition, many types of transformed cells are relatively frozen in their differentiation. In spite of what is often tacitly assumed, they are not, however, necessarily tumorigenic *in vivo.* To grow as an autonomous tumor, the transformed cell must be not only immortal but also resistant to negative growth regulating feedback signals in vivo. Although the control mechanisms of the host are largely unknown (except in special cases such as some of the hormonally controlled tissues), it is clear that all cells must obey a variety of stop signals *in vivo.*

The three major categories of the DNA tumor viruses (papova-, adeno-, and herpesviruses) are discussed in the corresponding chapters. Although these viruses are very different in size and viral strategy, their transforming action may not be so different as it would seem at first sight. In all three systems, the main candidate transforming function is intimately related to readily detectable nuclear antigens, the only viral function regularly present in all transformed cells. Designated as T-antigens in the papova- and adenovirus-transformed cells and as EBNA, HUPNA, and HATNA in the various lymphotropic herpesvirus systems, these DNA-binding, highly antigenic, nonhistone proteins have been identified in all DNA virus transformed cell systems in which they have been sought. They have not been found in any RNA virus transformed cells. In the most thoroughly studied papovavirus systems, several subtypes of the T-antigen were shown to exist. The nature of these antigens and their biological significance form a continuously recurring theme in all chapters on the biology of the oncogenic DNA viruses.

Directly or indirectly, they may also play an immunological role. It is clear that the prolonged

coexistence of certain animal species with ubiquitous, potentially highly oncogenic DNA viruses has led to the establishment of the most watertight immune surveillance mechanisms against potentially neoplastic cells that are presently known. Polyoma in mice, EBV in man, and *Herpesvirus saimiri* in the squirrel monkey are some of the most interesting examples, discussed in the corresponding chapters. An understanding of these surveillance mechanisms is equally important for the tumor immunologist and the virologist. How does nature do it when it does it well? What mechanisms were fixed by selection to achieve these almost perfectly harmonious, symbiotic systems where the "immunological orchestra" of all normal individuals of an entire species is geared to recognize relatively minor changes in the surface membrane of virally transformed cells with the utmost efficiency and where the otherwise often antagonistic forces of the various immune effectors are coordinated to achieve faultless protection, allowing tumor development only in relation to rare biological accidents such as immunosuppression or other forms of immunopathology? How is the generation of suppressor cells avoided? What cells perform the killer function? Viral oncology can only raise these questions; the solutions will have to come from tumor immunology, a discipline that is all too often preoccupied with seeking evidence for immune rejection in other (i.e., nonviral) tumor systems where they are much less prevalent and, when they exist, are often haphazard or inefficient.

A joint consideration of all virally induced tumors leaves the clear impression that viral transformation is very often only the first step in a complex series of events that leads to tumor development. Host controls participate at many different levels during the subsequent process; they include both immunological and nonimmunological mechanisms. The unraveling of these mechanisms requires many disciplines other than virology. It is also clear, however, that full understanding of virally induced transformations would be very important, not only because they often represent the first step in the chain of events that leads to a tumor, but perhaps more importantly because they lend themselves for a more precise analysis than the nonviral systems. Transforming viruses carry neat packages of genetic information that act in a similar way in each transformation event. The molecular analysis of such events has already advanced much more quickly than the study of chemical carcinogenesis where the crucial changes are still hidden in the blackbox of the cellular genome. In this context, the significance of viral oncology cannot be overemphasized, no matter whether and to what extent viruses contribute to the etiology of human tumors, a question that appears to hang over the entire field like Damocles' sword. To take only one concrete example, the cellular insert of the transforming RNA viruses may be useful in identifying critical areas of the cell genome involved in growth control and, in nonviral tumors, in transformation itself. Can directly transforming viruses that carry cellular genes induce cytogenetically unaltered, diploid tumors? When corresponding tumors are induced by nonviral agents or by viruses that do not directly transform, do they show corresponding changes in the dosage of homologous cellular genes brought about by chromosome duplications or translocations? Or, to mention a specific case, does the large cellular insert of Abelson virus genome, known to induce diploid leukemias after short latency periods, carry the same segment of chromosome 15 that is known to become regularly duplicated (trisomic) in murine lymphomas induced by a wide variety of agents? These and similar questions that are now open to experimental approach may bring the field of viral oncology to bear on other fields of carcinogenesis that appear to be quite remote at first sight. The distance is more spurious than real. The biological problem is common to chemical carcinogenesis, viral transformation, and spontaneous transformation of normal to malignant cells. Viral oncology may be the Trojan horse that can open the way to a detailed comprehension of transformation in general, at the most basic (DNA) level.

Although the field has progressed considerably since 1911, and many of the advances were made in the last few years, some of the basic questions remain the same. The Editor hopes that this book that turns particularly to the trained biological scientist who wishes to enter the field of viral oncology will give some partial answers, and more importantly, convey the present rapid growth of the field, the excitement of continuous new discoveries, and will help to formulate the questions that still face us as major challenges.

I would like to thank all contributors for their painstaking work.

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Contents

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Avian Oncoviruses: Genetics

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In this chapter a broad overview of the genetics of the avian oncoviruses will be presented. This group of RNA tumor viruses is included in the subfamily Oncovirinae within the family Retroviridae (100,101,357) as viruses with RNA genomes that replicate via DNA intermediates. The relatively new term *oncovirus* and the more commonly used term *RNA tumor virus* will be used interchangeably throughout this chapter.

In comparison with some of the bacterial viruses, the avian oncoviruses remain genetically relatively undefined. Nevertheless, the research of the past decade has produced a significant increase in our understanding of the genetics and genetic interactions of this group of animal viruses, and from both the molecular biology and genetic points of view the oncoviruses may be regarded as a unique and fascinating group.

The avian RNA tumor viruses are the natural causes of neoplastic disease in fowl. The variety of tumors induced by these viruses will be discussed in another chapter, but for simplicity the avian oncoviruses can be divided pathologically into those that induce solid tumors of connective tissue (avian sarcoma viruses, ASV) and those that induce neoplastic transformation of hematopoietic cells (leukemia viruses). The ASVs can malignantly transform fibroblasts in tissue culture, and thus they provide an excellent system for studying neoplastic conversion of cells. Some of the leukemia viruses (acute leukemia viruses) can also transform cells in culture, and these have been the subjects of much research in recent years.

Avian oncoviruses are enveloped RNA-containing viruses with unique life cycles and genetic properties. Virions of this group contain two subunits of RNA, each of which contains a complete set of the viral genetic information required for productive transforming infection. Thus virus is genetically diploid. Retrovirus particles carry a unique enzyme, reverse transcriptase (an RNA-dependent DNA polymerase), that is essential for replication of the virus. Shortly after the virus infects a permissive cell, the single-stranded RNA genome is transcribed into a double-stranded DNA copy that migrates from its site of synthesis in the cytoplasm to the nucleus, where it becomes covalently associated with the chromosomal DNA. A host cell enzyme, RNA polymerase II, is responsible for the synthesis of new genomic RNA and viral messenger RNA.

All avian oncoviruses that are capable of replicating independently have at least three genes: *gag*, coding for the internal structural proteins of the virion that bear group-specific antigenic determinants common to all members of an avian oncovirus group; *pol*, coding for the RNA-dependent DNA polymerase; *env*, coding for the envelope glycoproteins of the virus. In addition, the nondefective ASVs carry a fourth gene, *src*, coding for a nonstructural sarcoma-inducing protein that is also responsible for the morphological transformation of cells in culture. In each of these genes

a number of conditional mutants and nonconditional markers exist. These have provided insight into the virus-coded functions involved in virus replication and cell transformation and have facilitated genetic mapping experiments.

Avian oncoviruses are capable of two types of stable genetic interaction. The first is that interaction between the virus and the host cell chromosomal DNA such that the genomic information becomes stably associated with the cell for the remainder of its life. The second is recombination between viruses, which is observed at high frequency after coinfection of cells with genetically different avian oncoviruses. Recombination occurs between genes and within genes via some form of intergenomic crossing-over. The underlying mechanisms involved in the two types of stable genetic interaction are not understood at the present time.

In this chapter we will first discuss the nature of the viral genome as it is within the virion, then review the physiology of the viral genes together with conditional and nonconditional markers within those genes, and finally consider the genetic map of the avian oncoviruses and the genetic interactions observed within this group of viruses. Several excellent reviews have recently been published in these areas, and the reader is encouraged to consult them for a wider discussion of some of the topics covered (29,48,142,355,361,367).

GENOMES OF AVIAN ONCOVIRUSES

The genomic RNA of the avian oncoviruses can be extracted from virions as a high-molecular-weight component with a sedimentation coefficient of 60S–70S. On heating or treatment with DMSO, this 60S–70S complex dissociates to yield smaller components that sediment at 30S–40S (79,96,241,276). The 60S–70S RNA thus appears to be a multimeric structure held together by hydrogen bonding. Various methods, including particle weight determination (21), electron microscopy (74, 202,222), and a combination of sedimentation analysis and electrophoretic mobility analysis in polyacrylamide gel (198), have been used to determine the molecular weights of these RNAs. Although the values obtained depend on the virus being studied and the method used, it is generally accepted that the molecular weight (MW) of the 60S–70S complex is between 4.5 and 7.5×10^6. The MWs of the 30S–40S subunits range from 2.2 to 3.5×10^6, the lowest value being that for oncoviruses in which one or more genes have been deleted, and the highest being that for the nondefective ASVs that carry the additional gene *(src)* responsible for cell transformation (80,85,198).

The 30S–40S subunit RNAs resemble eukaryotic messenger RNA in that the 3′ end of each molecule consists of poly(A), approximately 200 nucleotides long (206,269). The 5′ terminus is a cap structure in which a guanosine (methylated in the 7 position) is linked in a 5′-5′ manner via a triphosphate to a nucleotide methylated in the 2′-0 position (114,197). Furthermore, subunit-length RNAs can be translated both *in vivo* and *in vitro* (115,364). The genomic RNAs of these viruses must therefore be regarded as having positive polarity.

In a discussion of the nature of the avian RNA tumor virus genome, Vogt (356) first raised the possibility that this group of viruses might possess a polyploid genome in which each 30S–40S RNA subunit would contain a complete set of viral genes, a situation quite different from that of the haploid segmented genomes of other virus groups such as the orthomyxoviruses. Because the genetic complexity of genomic RNA can be determined experimentally, evidence has rapidly accumulated to support the concept of polyploidy. If the 60S–70S RNA represents a polyploid genome, then its genetic complexity will be equal to that of one 30S–40S subunit, whereas if the genome is haploid, its complexity will correspond to that of the 60S–70S RNA. Evidence from three independent techniques (RNA:DNA reassociation kinetics, transfection, and RNase T_1 oligonucleotide analysis) provides compelling support for polyploidy.

For the oligonucleotide analysis, virion RNA was uniformly labeled with ^{32}P and completely digested with ribonuclease T_1. The large unique oligonucleotides generated were separated by two-dimensional electrophoresis or by a combination of electrophoresis and homochromatography, and from their specific activities the total amount of unique genetic information in the RSV genome was calculated according to the following formula:

$$\frac{CPM^{\text{genome RNA}}}{CPM^{\text{unique oligonucleotide}}} = \frac{MW^{\text{genome RNA}}}{MW^{\text{unique oligonucleotide}}}$$

The data from several laboratories yielded a value of 3-4 $\times$ 10^6 as the MW of RNA required to contain all of the unique genetic information in the genome (28,87,268).

A quite different approach employed the technique of infecting cells with naked DNA (transfection). Infectious viral DNA can be isolated from RNA-tumor-virus-infected cells, thus indicating that a complete copy of the viral genome is associated with the cellular DNA, and in experiments in which this infectious DNA was sheared, the smallest size capable of infecting cells with single-hit kinetics was equivalent to one 30S–40S RNA subunit (65,163,214).

Calculations of genetic complexity based on the reassociation kinetic data obtained when viral RNA is hybridized to a complementary DNA copy have yielded more variable results. However, the most widely accepted value from this approach is 3 $\times$ 10^6 d (14).

Comparison of the MW of a 30S–40S subunit with that of 60S–70S RNA suggests that there are only two subunits in each 60S–70S complex. Therefore the oncoviruses are referred to as diploid rather than polyploid. MW determinations are in agreement with electron microscopic observations (23,202,203), particularly for the mammalian RNA tumor virus genomes, where the two subunits remain stably associated even after they have been "spread" to remove much of the secondary structure. By use of an elegant tagging technique (24) it has been possible to demonstrate that the 5′ termini of the two subunits are linked, whereas the poly(A)-containing 3′ ends are free. In mammalian viruses the 5′-5′ linkage can be observed as a Y-shaped configuration termed the "dimer linkage structure" (203). No such structures have been observed with the avian oncoviruses (23), and it would appear that the linkage in these viruses is unstable under the conditions used for spreading the molecules for electron microscopy. Nevertheless, it is assumed that the avian viruses have a similar arrangement of subunits in the 60S–70S RNA.

In the avian RNA tumor viruses, DNA synthesis is primed by a 4S RNA molecule with the structural and functional properties of transfer RNA (tRNA). This essential component of the genomic RNA is identical with the cellular tRNA for tryptophan ($tRNA^{tryp}$) (71,97,154). It binds strongly to homologous 30S–40S RNA, and the melting temperature of this complex is higher than that for other 4S RNA species that are bound to the viral genome (70,99). The binding site is close to the 5′ terminus of the 30S–40S RNA and extends from nucleotides 103 to 118 (67,156, 301,327). Deoxyribonucleotides are added to the 3′ terminus of this primer by the virion-associated RNA-dependent DNA polymerase (326), and since after addition of a little over 100 bases the enzyme encounters the 5′ terminus of the 30S–40S RNA, a mechanism must exist to transfer the growing chain to the 3′ end of the viral genome. This is facilitated by the presence of identical nucleic acid sequences at the 5′ and 3′ ends of the virion RNA (51,104,156,290,323). This terminal redundancy involves up to 20 nucleotides, the exact sequences of which may differ among various avian oncovirus strains (318,371). The role of the terminally redundant sequences and the possible mechanisms involved in transcription of the single-stranded RNA genome into a double-stranded DNA copy will be discussed in more detail in another chapter. However, it will be relevant to discussions