Haematology and Blood Transfusion Vol. 31
Modern Trends in Human Leukemia VII
Edited by Neth, Gallo, Greaves, and Kabisch
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Cancer Genes Generated by Rare Chromosomal Rearrangements Rather than Activation of Oncogenes *

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A. Introduction

In order to understand cancer, it is necessary to identify cancer genes. The search for such genes and for mechanisms that generate such genes must take into consideration that at the cellular level cancer is a very rare event. The kind of cellular transformation that leads to cancer in vivo occurs only in about one out of 2×10^{17} mitoses in humans and animals. The basis for this estimate is that most animal and human cancers are derived from single transformed cells and are hence monoclonal [1–5], that humans and corresponding animals represent about 10^{16} mitoses (assuming 10^{14} cells that go through an average 10^{2} mitoses), and that about one person in five dies from tumors [6].

The only proven cancer genes are the transforming (*onc*) genes of retroviruses. These are autonomous transforming genes that are sufficient for carcinogenesis [7, 8]. They transform susceptible cells in culture with the same kinetics as they infect tham, and they cause tumors in animals with single-hit kinetics [7, 8]. Therefore, these viruses are never associated with healthy animals and are by far the most direct and efficient natural carcinogens.

However tumors with retroviruses that contain *onc* genes are very rare in nature, as only less then 50 cases are recorded from which such viruses were isolated [5, 7–9]. Moreover these viruses have never been reported to cause epidemics of cancer. The probable reasons are that viral *onc* genes arise naturally only with great difficulty via two or more illegitimate recombinations, and that once arisen they are very unstable because they are not essential for virus replication [7, 8]. Nonessential genes are readily lost due to spontaneous deletion or mutation. Indeed, *onc* genes were originally discovered by analysis of spontaneous deletions of the *src* gene, the *onc* gene of Rous sarcoma virus (RSV) [10, 11]. Subsequently, about 20 other viral *onc* genes were identified in retroviruses [7–9, 12]. All these viral *onc* genes were originally defined by "transformation-specific" sequences that are different from the known sequences of essential virus genes [13].

Since *onc* genes are unstable, they must also be recent additions to retroviruses. Indeed, the cellular genes from which the transformation-specific sequences of oncogenic retroviruses were transduced have been identified in normal cells. This was initially done by liquid hybridization of transformation-specific viral sequences with cellular DNA [14–18], and later by comparing cloned viral *onc* and corresponding cellular genes [19]. Such cellular genes have since been termed proto-*onc* genes [7].

The cellular origin of the transformation-specific sequences of retroviral *onc* genes is frequently presented as a particular surprise [9, 12]. However, cells are the only known

* This manuscript is based on a previous publication [1] and was also presented at the Third International Symposium in Hematology and Oncology: Assessment and Management of Leukemia Cancer Risks in Stockholm, Sweden, May 1987.

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source of genetic material from which viruses could transduce genetic information, and viral transduction has been canonical knowledge since phage λ was first shown by the Lederbergs and Zinder to transduce β-galactosidase in the 1950s [110]. Indeed, viruses are themselves derivatives of cellular genes that have evolved away from their progenitor genes as they acquired their capacity of self-replication.

B. The Oncogene Concept

On the basis of the sequence homology between viral *onc* genes and proto-*onc* genes, viral *onc* genes have been postulated to be transduced cellular cancer genes, and proto-*onc* genes have been postulated to be latent cancer genes or oncogenes [20–29]. According to this view, termed the oncogene concept [29], proto-*onc* genes are not only converted to transforming genes from without by transducing viruses, but also from within the cell by increased dosage or increased function [20–29]. Activation of latent oncogenes from within the cell is postulated to follow one of five prominent pathways: (a) point mutation [30, 31]; (b) chromosomal translocation that brings the latent oncogene under the control of a heterologous enhancer or promoter [24, 32]; (c) gene amplification [28, 29]; (d) activation from a retroviral promoter integrated adjacent to the latent oncogene [9, 23–29]; or (e) inactivation of a constitutive suppressor [33]. Thus, this view predicts that latent cancer genes exist in normal cells. However, the existence of latent cancer genes is a paradox, because such genes would be the most undesirable genes for eukaryotic cells. The very essence of enkaryots is cellular cooperativity, rather than autonomy as is typical of cancer cells and prokaryotes.

The oncogene concept was a revision of Huebner's oncogene hypothesis, which postulated activation of latent oncogenic viruses instead of latent cellular oncogenes as the cause of cancer [34]. Nevertheless, Huebner's hypothesis remained unconfirmed because most human and animal tumors are virus-negative [9, 12]. Moreover, the retroviruses and DNA viruses that have been isolated from tumors are not directly oncogenic [5], except for the fewer than 50 isolates of animal retroviruses which contain *onc* genes [8, 9, 12].

The oncogene concept was highly attractive at first sight because it derived credibility from the proven oncogenic function of retroviral *onc* genes, the viral derivatives of proto-*onc* genes, and because it promised direct access to the long-sought cellular cancer genes in virus-free tumors with previously defined viral *onc* genes as hybridization probes. Predictably, the hypothesis has focused the search for cellular cancer genes from the 10^5–10^6 genes of eukaryotic cells to the 20 known proto-*onc* genes [8, 9, 23–29, 43].

The hypothesis makes four testable predictions, namely, (a) that viral *onc* genes and proto-*onc* genes are isogenic; (b) that expression of proto-*onc* genes would cause cancer; (c) that proto-*onc* genes from tumors would transform diploid cells as do proviral DNAs of viral *onc* genes; and above all (d) that diploid tumors exist that differ from normal cells only in activated proto-*onc* genes. Despite record efforts in the past 6 years, none of these predictions has been confirmed. On the contrary, in fact, the genetic and biochemical analyses that have defined essential retroviral genes, viral *onc* genes, and proto-*onc* genes during the past 16 years show in reference to (a) that viral *onc* genes and proto-*onc* genes are not isogenic [7, 8] (see below). As regards (b), it turned out that most proto-*onc* genes are frequently expressed in normal cells [8]. Contrary to the expectation in (c), none of the 20 known proto-*onc* genes isolated from tumors functions as a transforming gene when introduced into diploid cells. (The apparent exceptions of proto-*ras* and proto-*myc* are discussed below). By comparison, proviral DNAs of retroviral *onc* genes transform normal cells exactly as the corresponding viruses [9, 12]. And finally, no diploid tumors with activated proto-*onc* genes, as hypothesized in (d), have been found except for those caused by viruses with *onc* genes [35, 36]. Instead of activated oncogenes [8], clonal chromosome abnormalities are a consistent feature of virus-negative tumors [1–4, 37] and also of all those tumors that are infected by retroviruses without *onc* genes [5].

C. Claim that Proto-*ras* Genes Become Cancer Genes Due to Point Mutations

Harvey proto-*ras* is the cellular precursor of Harvey, Balb, and Rasheed murine sarcoma viruses, and Kirsten proto-*ras* is the cellular precursor of the murine Kirsten sarcoma virus [9, 12]. Both proto-*ras* and the viruses encode a colinear protein, termed p21, of 189 amino acids (Fig. 1) [38–44]. In 1982 it was discovered that Harvey proto-*ras* extracted from a human bladder carcinoma cell line, but not from normal cells, would transform the morphology of a few aneuploid murine cell lines, in particular the NIH 3T3 mouse cell line [30, 31]. Subsequently proto-*ras* DNAs from some other cell lines and from some primary tumors [8, 38–40] were also found to transform 3T3 cells. Since such proto-*ras* DNAs behave like dominant and autonomous cancer genes in this morphological assay, they were claimed to be cellular cancer genes [30, 31, 43]. The 3T3 cell transforming function of the Harvey proto-*ras* gene from the bladder carcinoma was reduced to a single point mutation that changed the 12th *ras* codon of p21 from the normal gly to val [30, 31]. In the meantime, more than 50 different point mutations in five different *ras* codons have been identified, all of which activate 3T3 cell transforming function [41, 42, 88]. Since the viral *ras* genes and proto-*ras* genes encode the same p21 proteins, whereas most other viral *onc* genes encode proteins that are different from those encoded by proto-*onc* genes (Fig. 1) [7, 8], this system has been considered a direct support for the hypothesis that viral *onc* genes and proto-*onc* genes are indeed isogenic and hence can become functionally equivalent by point mutations [26–31, 42–44].

However the following arguments cast doubt or the claims that point mutations are indeed necessary or sufficient to convert proto-*ras* to a dominant cancer gene:

1. Although most, but not all (see below), proto-*ras* genes with point mutations have been found in tumors or in certain cell lines, *ras* mutations are very rare in most spontaneous tumors [8, 38–40]. In fact, the gly to val mutation that was originally found in the human bladder carcinoma cell line [30, 31] has never been found in a primary tumor [43, 88]. Moreover, even in certain chemically induced or spontaneous tumors in which *ras* mutations are relatively frequent a consistent correlation between *ras* mutations and tumors has never been observed [8, 43–45].

Furthermore, it is not known whether in animals the origin of a *ras* mutation coincides with the origin of the tumor. For example, the *ras* mutation of the human bladder carcinoma [30, 31] was only found in a cell line 10 years after this line was derived from the original tumor [46].

On the basis of a numerical argument it is also unlikely that point mutations are sufficient to convert proto-*ras* genes to dominant cancer genes. The frequency of point mutations of eukaryotes is one in 10^8–10^{10} nucleotides per mitosis [47, 48]. Thus, about one in 10^7 mitoses is expected to generate mutant Harvey *ras* genes with dominant transforming function, since the diploid human cell contains about 6×10^9 nucleotides and since 50 different mutations can activate each of two sets of *ras* genes of diploid cells. By contrast, spontaneous transformation that leads to clonal tumors occurs in fewer than one out of about 2×10^{17} mitoses and only a small minority of these contain mutant *ras* genes.

It may be argued, however, that indeed one out of 10^7 mitoses generates a tumor cell with activated proto-*ras* and that the immune system eliminates these cells. However this is unlikely since a point mutation is not an easy target for immunity. Further, animals or humans who are tolerant to *ras* point mutations would be expected to develop tumors at a very early age, if point-mutated proto-*ras* genes were dominant cancer genes, as the 3T3 assay suggests. Instead, spontaneous human tumors with activated proto-*ras* are very rare and all were observed in adults [8, 38–40]. Moreover, the argument that cellular oncogenes exist that can be activated by point mutation and then controlled by immunity is hard to reconcile with the existence of athymic or nude mice which do not develop more spontaneous tumors than other laboratory mice [49]. Furthermore, this view is inconsistent with the evidence that immunosuppressive therapy or thymectomy does not increase the cancer rate of humans [50]. Finally, one would pre-

dict that in the absence of immunity, as in cell culture, one out of 10^7 normal cells should spontaneously transform due to point mutation of Harvey proto-*ras* alone and probably the same number due to mutation of Kirsten proto-*ras* [9]. Yet spontaneous transformation of diploid cells in culture is clearly a much less frequent event.

In an effort directly to test the hypothesis that *ras* genes are activated to dominant cancer genes by point mutation, we [41] analyzed whether the transforming function of *ras* genes does indeed depend on point mutations. Using site-directed mutagenesis we have found that point mutations are not necessary for the transforming function of viral *ras* genes and of proto-*ras* genes that had been truncated to be structurally equivalent to viral *ras* genes [41]. (See also Cichutek and Duesberg this volume.)

2. Contrary to expectation, the same proto-*ras* DNAs from human tumors that transform aneuploid 3T3 cells do not transform diploid human [51] or diploid rodent cells [52–54], the initial material of natural tumors. Thus transformation of 3T3 cells does not appear to be a reliable assay for transforming genes of diploid cells. Instead of initiating malignant transformation, mutated proto-*ras* genes merely alter the morphology and enhance tumorigenicity of aneuploid 3T3 cells. Apparently they activate one of the many morphogenic programs of eukaryotic cells. Observations that untreated 3T3 cells are tumorigenic in nude mice [55–57] are consistent with this view. Thus, proto-*ras* genes with point mutations are not sufficient to initiate malignant transformation. They only appear as dominant cancer genes in certain aneuploid cells, such as 3T3 cells, based on unknown biochemical effects that alter the morphology of these cells. Furthermore, morphological transformation of 3T3 cells is not *ras* gene specific. It occurs spontaneously [58] and also upon transfection with several DNA species derived from tumors or tumor cell lines that, like proto-*ras*, do not transform diploid cells [28, 43, 44]. Such DNAs are now widely considered as cellular cancer genes [28, 43, 44], although they are not related to viral *onc* genes and do not transform diploid cells.

3. Assuming that mutated proto-*ras* genes are cancer genes, like viral *onc* genes, one would expect diploid tumors that differ from normal cells only in *ras* point mutation. Contrary to expectation, chromosome abnormalities are consistently found in those tumors in which proto-*ras* mutations are occasionally found [2, 4]. The human bladder carcinoma cell line, in which the first proto-*ras* mutation was identified, is a convincing example. This cell line contains over 80 chromosomes (instead of 46) and includes rearranged marker chromosomes [46]. In view of such fundamental chromosome alterations, a point mutation seems to be a rather minor event. Indeed among diploid hamster cells transfected with mutated *ras* genes, only those that developed chromosomal abnormalities upon transfection were tumorigenic [59, 60].

Thus, proto-*ras* genes with point mutations are neither sufficient nor proven to be necessary for carcinogenesis and are not autonomous cancer genes as are viral *ras* genes. In addition, there is no kinetic evidence that the origin of the mutation coincides with the origin of the tumors in which it is found. It is consistent with this view that proto-*ras* mutations that register in the 3T3 cell transformation assay have been observed to occur in vivo in benign hyperplasias, as for example in benign murine hepatomas [61] or in benign, purely diploid mouse skin papillomas that differentiate into normal skin cells [62–66]. *Ras* mutations have also been observed to arise after carcinogenesis in aneuploid cancer cells [67–69], rather than to coincide with the origin of cancer. By contrast, viral *ras* genes are sufficient for transformation and thus initiate transformation of diploid cells in vitro and in vivo with single-hit kinetics and concurrent with infection [8, 70, 71].

This then raises the question as to why viral *ras* genes are inevitably carcinogenic under conditions under which proto-*ras* genes with point mutations are not. A sequence comparison between proto-*ras* genes and the known viral *ras* genes has recently revealed a proto-*ras*-specific exon that was not transduced by any of the known retroviruses with *ras* genes [41]. (See also Cichutek and Duesberg this volume.) It follows, that proto-*ras* and viral *ras* genes are not isogenic (Fig. 1). Since four different viral *ras* genes have been shown to lack the

same proto-*ras* exon and since point mutations are not necessary for transforming function, we have proposed that proto-*ras* genes derive transforming function for diploid cells by truncation of an upstream exon and recombination with a retroviral promoter ([41], see below).

D. Claim that the Proto-*myc* Gene Becomes a Cancer Gene Under the Influence of a Heterologous Cellular Enhancer

Proto-*myc* is the cellular precursor of four avian carcinoma viruses, termed MC29, MH2, CMII, and OK10, with directly oncogenic *myc* genes [8]. The transforming host range of viral *myc* genes appears to be limited to avian cells, as murine cells are not transformed by cloned proviral DNAs [52, 53, 72]. Nevertheless, it is thought that proto-*myc*, brought under the control of heterologous cellular enhancers or promoters by chromosome translocation, is the cause of human Burkitt's lymphoma or mouse plasmacytoma [32, 64, 73].

The following arguments cast doubt on whether such activated proto-*myc* genes are indeed necessary or sufficient for carcinogenesis:

1. The human proto-*myc* gene is located on chromosome 8. This chromosome is typically rearranged in B cell lines derived from Burkitt's lymphomas [8, 32, 64]. However, although chromosome 8 is subjected to translocations, proto-*myc* is frequently not translocated, and when translocated it is frequently not rearranged [8, 32, 64]. Moreover, no rearrangements of chromosome 8 were observed in about 50% of primary Burkitt's lymphomas; instead, other chromosome abnormalities were recorded [74]. Thus, proto-*myc* translocation is not necessary for lymphomagenesis.

2. Expression of proto-*myc* is not consistently enhanced in lymphomas [8].

3. As yet no proto-*myc* gene isolated from any tumor has been demonstrated to transform any cells [8]. In an effort to assay transforming function in vivo, a proto-*myc* gene that was artificially linked to heterologous enhancers was introduced into the germ line of mice [73]. Several of these transgenic mice developed lymphomas after 1–5 months, implying that activated proto-*myc* had transformed diploid cells. However, the lymphomas of the transgenic mice were all monoclonal [73]. Thus, if the activated proto-*myc* gene were indeed responsible for the lymphomas, it would be an extremely inefficient carcinogen, because only one of about 10^8 "control" B cells of the same mouse [75] with the same transgenic *myc* gene was transformed. Further, there is no deletion or mutation analysis to show that the activated proto-*myc* indeed played a direct role in the tumors of the transgenic mice [73]. In contrast, viral *myc* genes transform all susceptible cells directly and inevitably [8].

4. If translocated proto-*myc* were the cause of Burkitt's lymphomas, one would expect all tumors to be diploid and to carry only two abnormal chromosomes, namely, number 8 and the chromosome that was subject to reciprocal translocation with number 8. Instead, primary Burkitt's lymphomas exist with two normal chromosomes 8 that carry other chromosome abnormalities [74]. Thus, translocated proto-*myc* genes are not sufficient or proven to be necessary for carcinogenesis.

E. Probability of Spontaneous Transformation In Vivo Is at Least 10^9 Times Lower than Predicted from Proto-*onc* Gene Activation

It was estimated above that the probability of spontaneous transformation that leads to monoclonal tumors in humans is 2×10^{-17} per mitosis. One would expect activation of a preexisting, latent proto-*onc* gene to be a much more frequent event. For a given proto-*onc* gene, the probability of activation per mitosis would be the sum of the probabilities associated with each of the putative pathways [28, 29, 33] of proto-*onc* activation.

1. Since the probability of a point mutation per nucleotide per mitosis is about one in 10^9 [47, 48] per diploid cell, the probability that any one of the 20 known proto-*onc* genes is activated would be $2 \times 20 \times 10^{-9}$, assuming only one activating mutation per proto-*onc* gene. However, it would be 10^{-7} for Harvey-*ras* alone, since 50 different mu-

tations are thought to activate this gene to a dominant cancer gene (see above).

2. The probability of a given proto-*onc* gene to be activated by amplification is about one in 10^8, considering that about one in 10^3–10^5 mitoses leads to gene amplification in vitro and possibly in vivo and that about 10^3 out of the 10^6 kilobases (kb) of eukaryotic DNA are amplified [76, 77]. The probability that any one of the 20 known proto-*onc* genes would be activated by amplification would then be 2×10^{-7}.

3. The probability of oncogene activation by chromosome translocation depends largely on what distances between a proto-*onc* gene and a heterologous enhancer and which enhancers are considered sufficient for activation. Since distances >50 kb of DNA have been considered sufficient for activation of proto-*myc* [9, 64] and proto-*abl* [9, 78] (the proto-*onc* gene of murine Abelson leukemia virus [9]), and since an enhancer is likely to be found in every 50 kb of cell DNA, nearly every translocation within a 50-kb radius of a proto-*onc* gene should be activating. Thus the probability that a given proto-*onc* gene is activated per translocation would be 5×10^{-5} (50 kb out of 10^6 kb). The probability that one of the 20 known proto-*onc* genes is activated would then be 10^{-3} per translocation.

Translocation frequencies per mitosis are not readily available. In hamster cells, translocations are estimated to occur with a probability of 10^{-6} per mitosis [79, 80]. In cells directly derived from mice and humans, even higher frequencies (0.01–0.3) have been observed upon study in vitro [81–83]. The probability of a translocation per meiotic cell division in humans has been determined to be 10^{-3}–10^{-4}, based on chromosome abnormalities in live births [84]. Assuming one translocation in 10^4 mitoses, the probability that one out of the 20 known proto-*onc* genes is activated per mitosis by translocation would then be about 10^{-7}.

4. The probability that a proto-*onc* gene would be activated from without by the promoter or enhancer of a retrovirus integrated nearby is even higher than those associated with the intrinsic mechanisms. Since retrovirus integration within 1–10 kb of a putative latent cancer gene is considered sufficient for activation [9, 23–29], since retrovirus integration is not site-specific [10, 12], and since eukaryotes contain about 10^6 kb of DNA, a given proto-*onc* gene would be activated in at least one out of 10^6 infected cells [5, 8]. The probability that any one of the 20 known proto-*onc* genes would be activated would be 2×10^{-5} per infected cell.

The sum of these probabilities should reflect the spontaneous transformation frequency of cells per mitosis in vivo and in vitro. It would be between 10^{-5} and 10^{-7}. However, it should be at least 10^{-7} due to Harvey proto-*ras* mutations alone. Nevertheless, the actual number may be 10 times lower (or about 10^{-8}), depending on whether all or only some of these four putative mechanisms could activate a proto-*onc* gene and depending on whether a given cell is susceptible to transformation by a given *onc* gene or to a given retrovirus. Instead, spontaneous transformation per mitosis that leads to monoclonal tumors is only about 2×10^{-17} in vivo. Thus the expected probability of spontaneous transformation due to activation of preexisting oncogenes differs at least by a factor of 10^9 from that observed in diploid cells in vivo.

Again it may be argued that spontaneous malignant transformation does indeed occur at the above rates but that immunity eliminates nearly all transformants. However in this case athymic or nude mice should not exist and the cancer incidence should increase significantly upon immunosuppressive therapy or thymectomy; yet this is not the case [49, 50]. Moreover, diploid cells in culture have not been observed to transform at the above rates.

5. Certain cancers (e.g., retinoblastomas) are thought to be caused by activation of latent oncogenes that are normally suppressed by two allelic suppressor genes [33]. Cancers caused by such genes would be the product of inactivations of two allelic suppressors and thus very rare [33]. In individuals with genetic defects in one putative suppressor allele tumors such as retinoblastomas should occur due to inactivation of the second suppressor allele with the same frequencies as those estimated above for point mutation, translocation, and retrovirus insertion [33].

However, in over 80% of retinoblastomas that occur in individuals without prior genetic defect the putative suppressor genes

appear to be normal as judged by chromosome analysis [33], suggesting that other suppressors inhibit the putative retinoblastoma oncogene or that it does not exist. Instead, other chromosomal abnormalities that are always seen in such tumors [33] may be relevant to carcinogenesis (see below). Further, this activation hypothesis predicts that normal cellular DNA would cure retinoblastoma cells upon experimental transfection. Yet this has not been reported. Likewise, it would be expected that experimental, human-nonhuman heterokaryons that have lost chromosomes with suppressor genes would be transformed. It would also be expected that retinoblasts or other cells from individuals with a genetic defect in one suppressor allele would spontaneously transform with the probability of chromosome nondysjunction. Dysjunction has been observed to occur upon cultivation of biopsied murine [85] and human cells [86] with a probability of one in 10^{-3} (monosomies) to one in 10^{-4} (trisomies) per chromosome and mitosis. However, spontaneous transformations have not been described as occurring at this rate. Thus there is as yet no proof for suppressed cancer genes in normal cells.

F. Hypothesis that Activated Proto-*onc* Genes Require Unknown Complementary Genes for Carcinogenesis

Because of the consistent difficulties in demonstrating oncogenic function of proto-*onc* genes, a further revision of the oncogene concept has recently been favored. It proposes that "activated" proto-*onc* genes, like proto-*ras* or proto-*myc*, are not autonomous *onc* genes like their viral derivatives, but are at least necessary for the kind of carcinogenesis that requires multiple cooperating oncogenes [32, 52, 53, 64, 65, 87, 88]. Thus, activated proto-*onc* genes are proposed to be functionally different, yet structurally equivalent to viral *onc* genes. According to this theory, activated proto-*onc* genes would not be expected to register in transformation assays that detect single-hit carcinogens like viral *onc* genes [7, 8].

However, the hypothesis fails to provide even a speculative explanation as to why activated proto-*onc* genes are no longer to be considered functionally equivalent to viral *onc* genes [8]. Clearly, until the postulated complementary cancer genes are identified, this hypothesis remains unproven [8].

The hypothesis also fails to explain why among certain tumors, such as human carcinomas, individual carcinomas are only distinguishable from each other by the presence or absence of activated, putative oncogenes [8, 38–40, 42–44]. This implies either (a) that unknown oncogenes that do not register in the 3T3 cell assay would cause the same tumors as the putative oncogenes that do, or (b) that the putative oncogenes are not necessary for these tumors.

G. Viral *onc* Genes as Specific Recombinants Between Truncated Viral and Cellular Genes

Genetic and structural analyses of retroviral genes, viral *onc* genes, and proto-*onc* genes and direct comparisons between them have shown that viral *onc* genes and proto-*onc* genes differ both structurally and functionally. Therefore, we have proposed that viral *onc* genes are indeed new genes that do not preexist in normal cells, rather than being transduced cellular genes [7, 8, 13, 19] (Fig. 1). The original basis for this proposal was the definition of the transforming gene of avian carcinoma virus MC29 [89] as a genetic hybrid, rather than a transduced cellular oncogene [90]. It consists of a promoter and coding elements (*Δgag*) from an avian retrovirus linked to 3′ coding elements from cellular proto-*myc* (Fig. 1) [90]. Initially this became evident by comparing the structure and map order of MC29 with that of the three essential retrovirus genes, namely 5′*gag-pol-env* 3′ (Fig. 1) [91, 92].

Sequence comparison of the viral *Δgag-myc* gene with the chicken proto-*myc* gene provided direct proof that only a truncated proto-*myc* gene was present in MC29. Indeed a complete 5′ proto-*myc* exon was missing from the viral *Δgag-myc* gene [19]. This was apparently not an accident since the same 5′ proto-*myc* exon was also missing in the three other *myc*-containing avian carcinoma viruses MH2 [93, 94], CMII, and OK10 [8, 95]. Thus a viral and a cellular gene functioned as progenitors or proto-*onc*

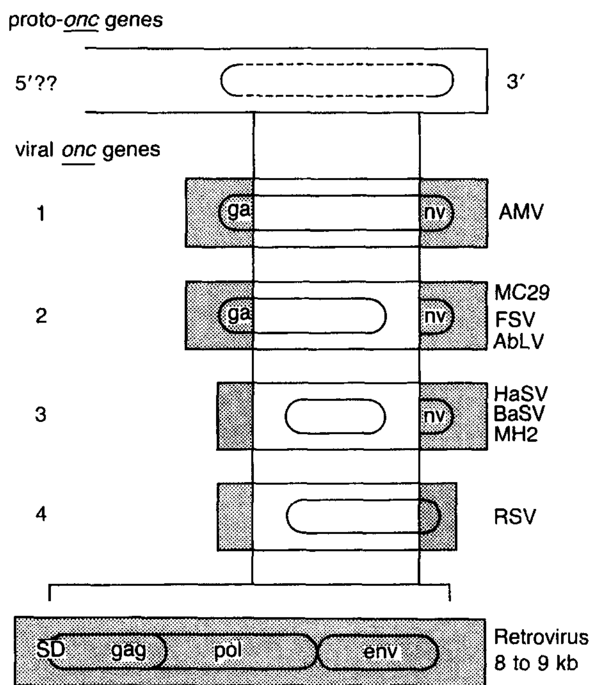

Fig. 1. The generic, recombinant structures of retroviral *onc* genes and their relationship to viral genes (*stippled*) and cellular proto-*onc* genes (*unshaded*). The genes are compared as transcriptional units, or mRNAs. All known viral *onc* genes are tripartite hybrids of a central sequence derived from a cellular proto-*onc* gene, which is flanked by 5′ and 3′ elements derived from retroviral "proto-*onc*" genes. Actual size differences, ranging from over 1–7 kb [9], are not recorded. The map order of the three essential retrovirus genes, *gag, pol,* and *env*, and of the splice donor (*SD*) are indicated. Four groups of viral *onc* genes are distinguished based on the origins of their coding sequence (⬭): *1*, The coding unit has a tripartite structure of a central proto-*onc*-derived sequence that is initiated and terminated by viral coding sequences; avian myeloblastosis virus (*AMV*) is an example [9, 96]. *2*, The coding unit is initiated by a viral and terminated by a proto-*onc* sequence; the *Δgag-myc* gene of avian carcinoma virus MC29 is an example [8, 9, 19, 90], as are hybrid *onc* genes of avian Fujinami sarcoma virus [97] and murine Abelson leukemia virus [9]. *3*, The coding unit of the viral *onc* gene is colinear with a reading frame of a cellular proto-*onc* gene; the *ras* gene of the murine Harvey and Balb sarcoma viruses [41] and possibly the *myc* gene of the avian carcinoma virus MH2 are examples [93, 94]. *4*, The coding unit is initiated by a proto-*onc* derived domain and terminated by a viral reading frame; the *src* gene of Rous sarcoma virus is an example [7, 9]. The transcriptional starts and 5′ untranscribed regulatory sequences (?) of all proto-*onc* genes are as yet not or not exactly known [8, 9]. There is also uncertainty about 5′ translational starts and open reading frames in some proto-*onc* genes (?) that are not transduced into viral *onc* genes of each of the viral recombinant *myc* genes (Fig. 1). More recently, the four known viral *ras* genes were each also shown to lack a 5′ proto-*ras* exon [41] (see above; Fig. 1).

Comparisons between the *onc* genes of other retroviruses and the corresponding proto-*onc* genes proved that, defined as transcriptional units, all viral *onc* genes are new genes. They are recombinants of proto-*onc* genes and retroviral genes (Fig. 1) [7–9]. Most but not all viral genes also encode new recombinant proteins. Based on the origin of their coding elements, the viral *onc* genes can be divided into the four groups illustrated in Fig. 1.

1. Those with amino and carboxy terminal domains from retroviruses and central domains from proto-*onc* genes. The *onc* gene avian myeloblastosis virus (AMV) is the prototype [9, 96].
2. Those with amino terminal domains from viral genes and carboxy terminal domains from proto-*onc* genes. The *Δgag-myc* gene of MC29 is the original example (see above). The *onc* genes of Fujinami sarcoma virus [97] and Abelson leukemia virus [9] also have the generic *Δgag*-X structure.
3. Those that are colinear with a reading frame of a proto-*onc* gene. The *ras* genes of Harvey and Balb murine sarcoma virus [41] and possibly the *myc* gene of avian carcinoma virus MH2 [93, 94] are examples.
4. Those with an amino terminal domain from a proto-*onc* gene and a carboxy terminal domain from the virus. The *src* gene of RSV is the prototype [7–9].

Since three of the four groups of recombinant viral *onc* genes also encode recombinant proteins, their specific transforming function can be directly related to their specific structure compared to that of proto-*onc* genes, as in proto-*myc* [98], proto-*src* [7], or proto-*ras* [41]. It is clear however that proto-*onc*-specific regulatory elements are always replaced by viral promoters and enhancers and that proto-*onc* coding sequences are frequently recombined with viral coding sequences. Thus, all viral *onc* genes are tripartite recombinant genes of truncated viral and proto-*onc* genes

gene products. The transforming function of the recombinant *onc* genes of group 3, which encode transforming proteins that are colinear with proteins encoded by proto-*onc* genes, cannot be explained in this fashion. However, all viral *onc* genes of this group each lack at least one proto-*onc*-specific 5′ exon like the avian carcinoma viruses with *myc* genes [8, 19, 93–95] or the murine sarcoma viruses with *ras* genes [41]. Conceivably elimination of transcribed or untranscribed suppressors or elimination of an upstream proto-*ras* cistron [41] or proto-*myc* cistron [98] and recombination with viral promoters are the mechanisms that generate transforming function (Fig. 1).

It follows that viral *onc* genes and the corresponding proto-*onc* genes are not isogenic. Viral *onc* genes are hybrid genes that consist of truncated proto-*onc* genes recombined with regulatory and frequently with coding elements from truncated retroviral genes. These consistent structural differences must be the reason why viral *onc* genes inevitably transform and why proto-*onc* genes are not transforming although they are present in all and are active in most normal cells [7, 8].

Clearly if cellular oncogenes preexist in normal cells, it would be much more likely to find retroviruses with intact cellular oncogenes than retroviruses with new *onc* genes put together from unrelated and truncated viral and cellular genes by illegitimate recombination. However, it may be argued that proto-*onc* gene truncations reflect packaging restrictions of transducing retroviruses rather than conditions to activate proto-*onc* genes. Such restrictions would have to be mostly sequence-specific, as most retroviruses with *onc* genes can accommodate more RNA – at least 10 kb, as in RSV [99] – than they actually contain, namely 3–8 kb [9]. But there is no evidence that retroviruses discriminate more against certain transduced or artificially introduced sequences [9] than against others, because retroviruses can accommodate very heterogenous sequences, such as the 20 different transformation-specific sequences [7–9, 13]. Yet all nonessential sequences of retroviruses are unstable [7, 8] unless selected for a given function.

Moreover, the fact that the same exons were selectively truncated from several proto-*onc* genes in independent viral transductions that have generated active *onc* genes indicates that specific truncations are necessary for transforming functions. Examples are proto-*myc*, the precursor of four avian carcinoma viruses [8, 19, 93], proto-*ras*, the precursor of three murine sarcoma viruses [41], proto-*myb*, the precursor of avian myeloblastosis and erythroblastosis viruses [9, 100], proto-*erb*, the precursor of three avian sarcoma and erythroblastosis viruses [9], proto-*fes*, the precursor of three feline sarcoma viruses [9], proto-*fps*, the precursor of three avian sarcoma viruses [9, 101], proto-*abl*, the precursor of Abelson murine leukemia and a feline sarcoma virus [9], proto-*mos*, the precursor of several Moloney sarcoma viruses [9, 102], and proto-*src*, the precursor of RSV and two other avian sarcoma viruses [103]. In some cases of independent transductions, the same proto-*onc* genes were even truncated at exactly the same breakpoints, as for example in two different avian sarcoma viruses derived from proto-*fps* [101].

The existence of at least seven retroviruses containing proto-*onc* sequences that had already been truncated by recombination with other cellular or viral genes prior to transduction lends further independent support to this view. Examples are the *onc* genes of avian carcinoma virus MH2 [8, 93, 94], of avian erythroblastosis and sarcoma virus AEV [9], of avian erythro- and myeloblastosis virus E26 [100], of the feline sarcoma virus GR-FeSV [9, 104], of RSV [7, 9] and of Harvey and Kirsten sarcoma viruses [9, 41]. Certainly the odds against transduction of rare, rearranged proto-*onc* genes instead of normal proto-*onc* genes are overwhelming. Yet seven out of the less than 50 known isolates of retroviruses with *onc* genes [9] contain previously rearranged proto-*onc* sequences, most likely because truncation is necessary for transforming function. Indeed, it may be argued that these viruses have transduced these rearranged proto-*onc* genes from a preexisting tumor that was generated by these rearrangements. Thus, the rearranged proto-*onc* genes of these seven oncogenic retroviruses may be "transduced cellular oncogenes" after all.

Therefore recombination of proto-*onc* genes with retroviral or cellular genes ap-

pears to be necessary to convert proto-*onc* genes to transforming genes. A definitive assessment of why viral *onc* genes transform and cellular proto-*onc* genes do not requires more than comparisons of primary structures and transforming tests with DNAs. It will be necessary to know what proto-*onc* genes do and whether they encode proteins that function alone or as complexes with other proteins.

I propose, then, that proto-*onc* genes that are transcriptionally activated or have undergone point mutations but retain a germline structure are not cellular cancer genes. I suggest that the hypothesis that proto-*onc* genes are latent cellular cancer genes that can be converted to active transforming genes by increased dosage or function is an exaggerated interpretation of sequence homology to structural and functional homology with viral *onc* genes.

This proposal readily resolves the paradoxes posed by the hypothesis that proto-*onc* genes are latent cellular cancer genes that can be activated by enhanced expression or point mutation. The proposal accounts for the frequent expression of proto-*onc* genes in normal cells [8]. The proposal is also entirely consistent with the lack of transforming function of "activated" proto-*onc* genes from tumors. The fact that mutated proto-*ras* changes the morphology and enhances tumorigenicity of aneuploid and tumorigenic 3T3 cells is an important observation, but not an exception to the experience that native proto-*onc* genes from tumors analyzed to date do not transform diploid cells. The proposal also provides a rationale for the chromosome abnormalities of tumor cells, as these appear to be microscopic evidence for cancer genes (see below) instead of the "activated" proto-*onc* genes identified to date.

H. Hybrid *onc* Genes of Retroviruses as Models of Cellular Cancer Genes

The proposal that proto-*onc* genes derive transforming function by truncation and recombination with retroviral or cellular genes predicts that recombinations among cellular genes could also generate transforming genes. The view that cellular cancer genes are rare recombinants of normal cellular genes is in accord with the fact that rearranged and abnormal chromosomes are the only consistent, transformation-specific markers of tumor cells [2–5, 37]. Further, the clonality of chromosome alterations, e.g., the marker chromosomes of tumors [2–5, 37], indicates that tumors are initiated with and possibly caused by such abnormalities as originally proposed by Boveri in 1914 [105].

A major difficulty with the view that specific recombination sites among rearranged chromosomes are markers of recombinant cancer genes is that neither the chromosome breakpoints nor the karyotypes of different tumors of the same cell lineage are the same. Although some tumors show typical nonrandom abnormalities, such as the Philadelphia chromosome of chronic myelogenous leukemia and the 8 to 14, 2 and 22 translocations of Burkitt's lymphomas, exceptions are always seen, and the chromosome breakpoints of two different tumors with the same karyotypes are not the same at the nucleotide level [43, 74, 106]. Such heterogeneity of breakpoints, and thus of mutation, among otherwise indistinguishable tumors argues either for different transforming genes in the same tumors or against chromosome breakpoints as markers of transforming genes. However, this argument does not take into consideration that together with the microscopic karyotype alterations other submicroscopic mutations may have occurred that could have produced cancer genes. It is consistent with this that tumor cells contain, in addition to microscopic chromosome abnormalities, submicroscopic deletions and restriction enzyme site alterations [107]. Thus, specific marker chromosomes may only be the tip of an iceberg of multiple chromosomal mutations that may have generated cancer genes as well as mutationally activated or inactivated growth control genes.

The generation of retroviral *onc* genes from viral genes and proto-*onc* genes appears to be a direct model for the process of how cancer genes may be generated by chromosomal rearrangements. Less than 50 isolates of retroviruses with *onc* genes have been recorded in history [8, 9, 12], although both potential parents of retroviral *onc* genes are available in many animal or hu-

man cells because retroviruses are widespread in all vertebrates [5, 9, 12]. This extremely low birth rate of retroviruses with *onc* genes must then reflect the low probability of generating de novo an oncogenic retrovirus from a proto-*onc* gene and a retrovirus by truncating and recombining viral and cellular genes via illegitimate recombinations [7, 8, 13]. Clearly, at least two illegitimate recombinations are required (Fig. 1): one to link a 3′ truncated retrovirus with a 5′ truncated proto-*onc* gene, the other to break and then splice the resulting hybrid *onc* gene to the 3′ part of the retroviral vector.

The first of these steps would already generate a "cellular" cancer gene that ought to be sufficient for carcinogenesis. The birth of such a gene would be more probable than that of an oncogenic retrovirus that requires two illegitimate recombinations, but it would be harder to detect than a complete replicating retrovirus with an *onc* gene. Nevertheless even this would be a rare event. Given that such a recombination would have to take place within the 8–9 kb of a retrovirus (Fig. 1) integrated into the 10^6-kb genome of a eukaryotic cell and also within an estimated 1–2 kb of a proto-*onc* gene (Fig. 1), and assuming that translocation or rearrangement occurs with a probability of 10^{-4} (see above), the probability of such a recombination per mitosis would be 8×10^{-6} $2 \times 10^{-6} \cdot 10^{-4}$, or 10^{-15}. That a second illegitimate recombination is required to generate a retrovirus with an *onc* gene would explain why the occurrence of these viruses is much less frequent than spontaneous transformation due to recombinant cancer genes. This probability may, nevertheless, be higher than the square of 10^{-15}, since the two events may be linked and since multiple integrated and unintegrated proviruses exist in most infected cells.

The probability that illegitimate recombination would generate cancer genes from normal cellular genes would also be very low, since most illegitimate recombination would inactivate genes. The above estimates for the probability of spontaneous transformation of 2×10^{-17} per mitosis and of translocation of 10^{-4}, which would be a minimal estimate for illegitimate recombination, suggest that 10^{13} translocations or rearrangements are needed to generate a transforming gene that causes a monoclonal tumor. This could be either a single autonomous transforming gene that is like a viral *onc* gene or a series of mutually dependent transforming genes [108, 109] that would each arise with a higher probability than an autonomous *onc* gene. The facts that multiple chromosome alterations are typically seen in tumors [2–4, 37, 74] and that as yet no DNAs have been isolated from tumors that transform diploid cells with single-hit kinetics suggest that most cellular cancer genes are indeed not autonomous carcinogens like viral *onc* genes. It is consistent with this view that most cellular genes are also not converted to autonomous cancer genes by retroviral transduction via illegitimate recombination and truncation. Only about 20 cellular genes, the proto-*onc* genes, have been converted to autonomous viral *onc* genes, although viral transduction via illegitimate recombination is a random event that does not benefit from sequence homology between retroviruses and cells [7, 8, 13].

Thus viral *onc* genes have not as yet fingered preexisting cellular cancer genes. No cellular gene is a structural or functional homolog of a viral *onc* gene, but the viral *onc* genes appear to be models for how cancer genes may arise from normal cellular genes by rare truncation and recombination.

Acknowledgments. I would like to thank S.A. Aaronson, S. Blam, M. Kraus, M. Pech, K. Robbins, S. Tronick and others from the Laboratory of Cellular and Molecular Biology, National Cancer Institute, Bethesda, Maryland, for critical and amusing discussions and generous support during a sabbatical leave, and B. Witkop, National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases, Bethesda, Maryland, for asking many of the basic questions that I try to answer in this manuscript. I also thank my colleagues H. Rubin for encouragement and K. Cichutek, R.-P. Zhou, D. Goodrich, S. Pfaff, and W. Phares, University of California, Berkeley, California, for inspiring comments and their work. This research has been supported by (OIG) National Cancer Institute Grant CA-39915A-01 and Council for Tobacco Research Grant 1547 and by a Scholarship-in-Residence of the Fogarty International Center, NIH, Bethesda, Maryland.

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