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# Activated Proto-onc Genes: Sufficient or Necessary for Cancer?

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*Summary.* Proto-onc genes are normal cellular genes that are related to the transforming (onc) genes of retroviruses. Because of this relationship these genes are now widely believed to be potential cancer genes. In some tumors, proto-onc genes are mutated or expressed more than in normal cells. Under these conditions, proto-onc genes are hypothesized to be active cancer genes in one of two possible ways: The one gene–one cancer hypothesis suggests that one activated proto-onc gene is sufficient to cause cancer. The multigene–one cancer hypothesis suggests that an activated proto-onc gene is a necessary but not a sufficient cause of cancer. However, mutated or transcriptionally activated proto-onc genes are not consistently associated with the tumors in which they are occasionally found and do not transform primary cells. Further, no set of an activated proto-onc gene and a complementary cancer gene with transforming function has yet been isolated from a tumor. Thus, there is still no proof that activated proto-onc genes are sufficient or even necessary to cause cancer.

A primary objective of cancer investigators is the identification of cancer genes. Despite fierce efforts, this objective has not met with much success (*1, 2*). The only known cancer genes are the transforming (onc) genes of retroviruses. Typically, these viruses initiate and maintain cancers with autonomous onc genes that are dominant in susceptible cells (*3*). These onc genes are nonessential to the multiplication of the virus and hence are not maintained and are not allowed to evolve in retroviruses (*3*). The viral onc genes are the products of rare recombinational accidents between retroviruses and normal cellular genes, which have since been termed proto-onc genes. The term is used here exclusively for those cellular genes which share sequences with any of the 20 known viral onc genes. Other investigators have used the term also to describe potential cancer genes that are unrelated to retroviral onc genes. The normal function of proto-onc genes is poorly understood. On the basis of in vitro assays, one of them appears to be a growth factor, another a growth factor receptor, and two others resemble genes of the yeast cell cycle (*4, 5*).

The discovery of autonomous onc genes as dominant determinants of cancer in retroviruses, beginning in the early 1970's with the *src* gene of Rous sarcoma virus, has set a precedent that has pervaded cancer gene research (*3*). It has become the basis for the one gene–one cancer hypothesis although the relevance of single-gene models to natural cancers except those caused by retroviral onc genes is as yet unknown. For example, it is now widely believed that upon "activation" any of the 20 known cellular proto-onc genes may function like a viral onc gene. Activation is assumed to convert a normal proto-onc gene to a functional equivalent of a viral onc gene either by enhanced transcription or by mutation. Thus this hypothesis assumes that proto-onc genes are not only relevant to the very few natural tumors caused by retroviruses with onc genes, but also to virus-negative tumors. The discovery that molecularly defined or cloned DNA species from some tumors are capable of transforming the morphology of certain cell lines, typically the preneoplastic mouse NIH 3T3 cell line has also led to the hypothesis that such DNA's are autonomous cancer genes (*6, 7*). The coincidence that one 3T3-transforming DNA species found in certain tumors is a known proto-onc gene, related to the onc genes of Harvey and Kirsten sarcoma viruses, has lent further support to the one gene–one cancer hypotheses (see below).

However, circumstantial evidence suggests that most cancers are not caused by single genes but are the products of multiple events that probably involve multiple genes. These events and their presumed target genes, have been formally divided into initiation and promotion or maintenance genes (*1, 2*). Retroviruses without transforming genes, or chronic leukemia viruses, and DNA viruses have long been thought to function as initiation or maintenance genes in multigene carcinogenesis, because these viruses increase the cancer risk of infected animals. Recently, it has been hypothesized that activated proto-onc genes play a role either as initiation or maintenance genes in multigene carcinogenesis, rather than being autonomous cancer genes because, under the conditions tested, activated proto-onc genes did not transform primary cells (see below).

Despite the popularity of these hypotheses, it is pointed out here that there is as yet no convincing evidence that activated proto-onc genes even are necessary, much less sufficient, for carcinogenesis. The evidence against single-gene models is based on data indicating that activated proto-onc genes are not consistently associated with tumors, and cannot transform primary cells per se. The problem with multigene carcinogenesis by proto-onc genes is that activated proto-onc genes do not correlate with specific tumors and that complementary cancer genes have not been identified.

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## Retroviral onc Genes and Normal Proto-onc Genes

Retroviruses containing onc genes (as a part of the total genome) are the fastest acting carcinogens known to date. Such viruses are obligatory carcinogens in susceptible cells and have only been isolated from animals with neoplasms. By contrast all other retroviruses and all DNA viruses with oncogenic potential are regularly isolated from man or animals without neoplasms. This is consistent with single-gene carcinogenesis by retroviruses with onc genes and possible multigene carcinogenesis with all other viruses.

Indeed, retroviral onc genes are the only genes known that initiate and maintain cancers. That they are necessary for transformation has been proved genetically with temperature-sensitive (ts) mutants of Rous (RSV) (*8*), Kirsten (KiSV) (*9*), and Fujinami sarcoma viruses (*10, 11*), with avian erythroblastosis virus (*12*), and with deletion mutants of these and other retroviruses (*13–19*). The most convincing argument, that they are also sufficient to initiate and maintain neoplastic transformation, is that all susceptible cells infected by retroviruses with onc genes become transformed shortly after they are infected. This high transformation efficiency virtually excludes selection of preneoplastic cells initiated by another gene.

The structural characteristic of retroviral onc genes is a specific sequence that is unrelated to the three essential virion genes *gag, pol,* and *env*. This onc-specific sequence of retroviruses is related to one or several proto-onc genes. Typically the onc-specific sequence replaces essential virion genes and thus renders the virus replication-defective and helper virus–dependent. In the special case of RSV the *src* gene is added to the three essential virion genes (*3, 13, 14, 20*). Since onc genes are parasitic, they are readily eliminated by spontaneous deletion. Similarly, defective oncogenic viruses are dissociated from helper viruses and then lost without affecting the survival value of the essential retrovirus vector (*3, 20*). Therefore, retroviruses with onc genes are subject to extinction unless maintained in laboratories.

Almost all known viral onc genes are hybrids of coding regions from proto-onc genes linked to coding regions from essential retroviral genes (*20*). Only a few viral onc genes consist of coding regions from proto-onc genes linked to retroviral control elements. The identification of hybrid onc genes provided the first unambiguous clues that viral onc genes and corresponding cellular proto-onc genes are different in that proto-onc genes are neither related to, nor linked in the cell to, elements of essential retrovirus genes (*21, 22*). Sequence comparisons of cloned genes have since confirmed and extended that viral onc genes and corresponding proto-onc genes are not isogenic (*3, 25, 26;* see also *37–40*). All known viral onc genes are subsets of proto-onc genes linked to regulatory and coding elements of virion genes.

The first among viral onc genes to be diagnosed as a hybrid gene was the onc gene of avian carcinoma virus MC29 (*21, 23*) (Fig. 1). It is the only known gene encoded by MC29. About one-half of its information (1.5 kilobases) is derived from the *gag* gene of retroviruses; the other half (1.6 kb) termed *myc* is derived from the proto-*myc* gene. The gene is defined by a 110,000-dalton Δ*gag-myc* protein, termed p110 (*23, 24*). The proto-*myc* gene of the chicken has at least three exons. The boundaries of the first exon are at present undefined (*25–27*). The *myc* region of MC29 shares with proto-*myc* four codons, possibly from the 3′ end of the first exon, and all codons of the second and third proto-*myc* exons (Fig. 1). Three other avian carcinoma viruses MH2, OK10, and CMII also have onc genes with *myc* sequences (*24*). The *myc* sequence of CMII is part of a Δ*gag-myc* hybrid gene similar to that of MC29 (*24*). The *myc*-related gene of MH2 is derived from the second and third proto-*myc* exon and includes the splice acceptor of the first proto-*myc* intron (Fig. 1) (*25, 28, 29*). It also appears to be a hybrid consisting of six *gag* codons up to the splice donor of the *gag* gene (*30*). It is expressed via a subgenomic messenger RNA (mRNA) as a p57 *myc*-related protein product (*31–33*). In addition, MH2 contains a second potential transforming gene Δ*gag-mht*. The *mht* sequence is very closely related to the onc gene of murine sarcoma virus MSV3611 (*28, 29*). Whether both genes are necessary for transforming function is still unclear. The *myc* sequence of OK10 is part of two overlapping genes (Fig. 1) (*34*). One is expressed via a spliced mRNA as a p57 protein (*32–35*) and is isogenic with the *myc* gene of MH2. The other is part of a large hybrid onc gene, *gag-*Δ*pol-myc*, and includes as coding region the proto-*myc* derived intron and splice acceptor. This hybrid gene is defined by its expression of a 200,000-dalton protein termed p200 (*24*). Again, it remains to be determined whether both of these two onc gene

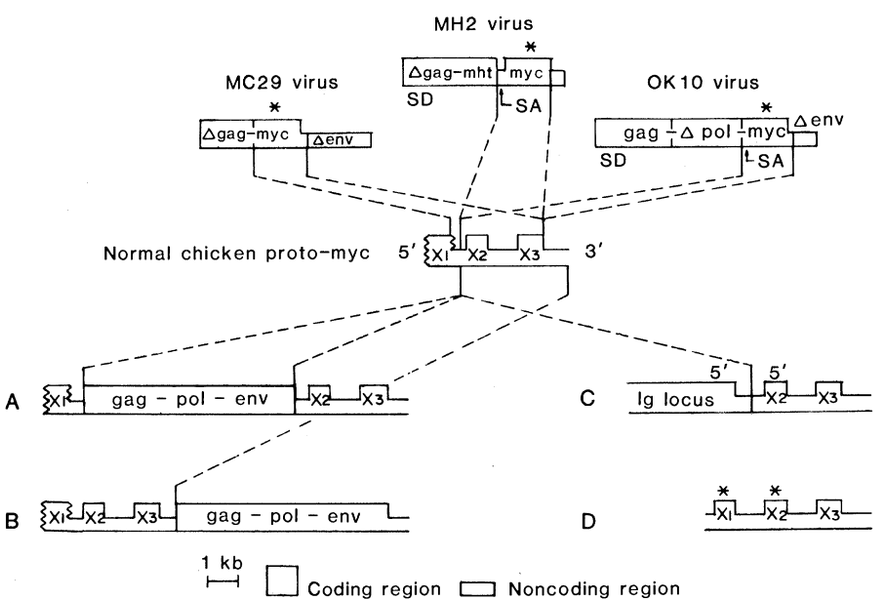


Fig. 1. *Myc*-related genes in avian carcinoma viruses and in normal and lymphoma cells. The common and specific *myc* domains of avian carcinoma viruses MC29 (*22, 25, 26*), MH2 (*28, 29*), and OK10 (24, 34), of normal chicken proto-*myc* (*25, 26*), and of the proto-*myc* genes of avian leukosis (*65, 75*) and human Burkitt's lymphoma (*71, 73, 81*) are graphically compared. Proto-*myc* has three exons (X1, X2, X3), the first of which is thought to be noncoding (*25, 71, 89*). The proto-*myc* genes of chicken and man are related but not identical: Their first exons are different; there are major differences between their second exons and minor differences between the third exons (*25*). The wavy vertical lines indicate that the borders of chicken proto-*myc* X1 are as yet undefined. *Gag, pol, env* are the three essential virion genes of retroviruses and Δ marks incomplete complements of these genes. SD is a splice donor and SA is a splice acceptor. (A) and (B) designate proto-*myc* altered by retroviruses in chicken leukosis. (C) and (D) designate human proto-*myc* rearranged with the Ig locus (C) or mutated (*) in X1 or X2 in Burkitt's lympohoma (D). The asterisk indicates point mutations compared to proto-*myc*.

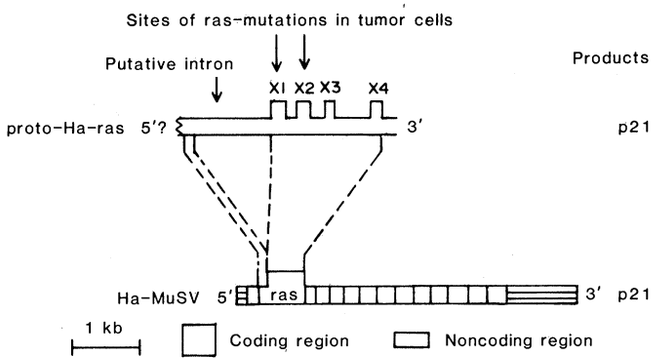


Fig. 2. Comparison of the genetic structures of the human proto-Ha-*ras* gene (*104, 110*) and the 5.5-kb RNA genome of Harvey sarcoma virus (Ha-MuSV) (*139*). The proto-*ras* gene of rats contains four exons which are colinear with that of the human counterpart, differing only in silent mutations (*109, 128, 138*). It is not known whether proto-*ras* of rats shares a 5′ region of homology with Ha-MuSV. Ha-MuSV is a genetic hybrid of the proto-*ras* gene of rats, a 30*S* defective retrovirus RNA from rat cells and of Moloney leukemia virus (*109, 142*). Wavy vertical line indicates that the 5′ border of proto-*ras* is as yet unknown. *X*, exon; □□□, Ha-MuSV sequences derived from 30*S* defective rat–retrovirus RNA; ▤, Ha-MuSV sequences derived from Moloney murine leukemia virus. p21 is the 21,000-dalton protein produced by Ha-MuSV and by proto-*ras*.

products are necessary for transforming function. It would appear that MH2 and OK10 are the first known examples of oncogenic retroviruses, with two genes possibly being essential for transforming function. [Avian erythroblastosis contains a gene that is not essential for transforming function of its primary onc gene but enhances oncogenicity (*3*).]

Thus, all *myc*-related viral onc genes are subsets of proto-*myc* linked to large or small retroviral coding regions and regulatory elements. Sequence comparisons indicate that the viral *myc* sequences of MC29, MH2, and OK10 each differ from proto-*myc* in private point mutations, but not in a common, virus-specific mutation (*34*). Therefore, the gross structural differences between the viral and cellular genes, rather than mutations, appear to be relevant for activation of the viral *myc* genes.

As yet, no virus with a *myc*-related onc gene has been isolated from a mammalian species. However, *myc*-containing feline proviruses with unknown biological activity have been detected by hybridization of lymphoma DNA from feline leukemia virus–infected cats (*36*). All other examples of hybrid onc genes that have been studied also fit the definition that viral onc genes and proto-onc genes are not isogenic (*3, 16, 24*).

The coding regions of a few viral onc genes, presumably the *src* gene of RSV and probably the onc genes of the Harvey and Kirsten sarcoma viruses (Ha-*ras* and Ki-*ras*) are derived entirely from proto-onc sequences (Fig. 2). Nevertheless, even these onc genes differ from proto-onc genes in extensive deletions and point mutations. Moreover recent work indicates that the *src* gene of RSV is a tripartite hybrid of genetic elements derived from two proto-*src* genes and from a nontransforming retrovirus, which contributed five 3′ terminal codons (*3, 40*).

Two arguments indicate that these structural differences between onc and proto-onc genes are essential for transforming function of the viral genes. (i) There is the overwhelming positive evidence that many proto-onc genes are regularly expressed in normal cells without altering the normal phenotype (*3, 41*). (ii) There is more indirect negative evidence that retroviral or plasmid vectors carrying more or less those proto-onc gene regions that are related to viral onc genes do not transform normal diploid cells. For example, phage or plasmid vectors carrying an incomplete copy of the largest proto-*src* gene do not transform cells although a *src*-related protein is expressed (*42–45*). The clones tested included the 3′ half of an approximately 4-kb proto-*src* gene (*3*). This part of the gene shares the 519 amino terminal codons with *src* but lacks the 12 codons that make up the carboxyl terminus of *src* (*42–45*). The same major proto-*src* region also fails to transform in a RSV vector (*46*) or in a reticuloendotheliosis virus vector (*47*). Further, molecularly cloned proto-*fos*, the precursor of the transforming gene of FBJ murine osteosarcoma virus (*48*), or proto-*fps*/*fes*, the precursors of avian Fujinami and feline sarcoma viruses (*49, 50*), or proto-*myc* the precursor of avian MC29 virus (*22, 51*) do not transform cells in culture. Proto-Ha-*ras*, the precursor of Harvey murine sarcoma virus (Ha-MuSV) also fails to transform in a reticuloendotheliosis virus vector in which the viral onc gene has transforming function (*47*).

Apparent exceptions are proto-*mos* and proto-*ras* which, after ligation to retroviral promoters, transform the preneoplastic NIH 3T3 cell line (*52, 53*). The proto-*mos* and proto-*ras* regions used in these constructions are essentially the same as those found in Moloney and Harvey sarcoma viruses but are not complete proto-onc genes (Fig. 2). Conceivably, the proto-onc regions that were not included in these constructions and are not in the viruses might in the cell suppress transforming potential of the complete proto-onc genes. Moreover, as discussed later, transforming function in 3T3 cells is not a reliable measure of transforming function in diploid embryo cells or in the animal. Neither the proto-*ras* nor the proto-*mos* construction were found to transform diploid embryo cells (*54–56*).

Thus, normal proto-onc genes and viral onc genes are related, but are structurally and functionally different. Clearly, viral onc genes are more than the sum of their retroviral and cellular parts. The question is now whether there are conditions under which proto-onc genes can cause cancer like viral onc genes.

## Search for Activation of Proto-onc Genes to Cancer Genes

The only clear, although indirect, proof for activation of proto-onc genes to cancer genes is based on the rare cases in which proto-onc genes functioned as accidental parents of retroviral onc genes. On the basis of structural analyses of retroviral genes and proto-onc genes, it has been deduced that viral onc genes were generated by transduction of specific domains from proto-onc genes (*3, 20*). Because no significant sequence homology exists between nontransforming retroviruses and proto-onc genes, such transductions must proceed via two rare, nonhomologous recombinations (*3, 25*). In addition, it appears that only a few cellular genes are proto-onc genes or can function as progenitors of viral onc genes since the same proto-onc sequences have been found in different viral isolates (*29*). It is probably for these reasons that proto-onc gene transductions or "activations" are extremely rare, even though all cells contain proto-onc genes and many animal species contain retroviruses without onc genes. Only 50 to 100 sporadic cancers from which retroviruses with onc genes were isolated have been reported, and no experimentally reproducible system of transduction has ever been described (*3, 57, 58*). The known retroviruses with onc genes, which were isolated from these sporadic cancers, are fossil records of such rare recombinational accidents.

Transduction of cellular genes or domains thereof is not a prerogative of retroviruses. Transduction of complete cellular genes was first observed in bacteriophage lambda. Transductions of cellular sequences have been frequently observed in animal viruses such as SV40 and polyoma (*59*). It is conceivable that some SV40 and polyoma virus transductions are as oncogenic as retroviral onc genes. However, such transductions will not be oncogenic viruses in nature, because the transduced DNA's substitute essential virion genes and render the viruses dependent on nondefective helper viruses which are cytocidal. Thus, the noncytocidal nature of retroviruses is an essential criterion of viral oncogenicity.

The roles of proto-onc genes as accidental progenitors of retroviral onc genes has made them the focus of the search for cellular cancer genes. The postulated role of cellular proto-onc genes as cancer genes was initially tested by many investigators in view of a "one gene–one cancer" hypothesis and more recently in view of a "multigene–one cancer" hypothesis. The one gene–one cancer hypothesis postulates that activation of inactive cellular oncogenes is sufficient to cause cancer. It is based on the oncogene hypothesis of Huebner and Todaro (*60*). Some investigators have postulated that activation is the result of increased dosage of a given proto-onc gene product. This view, termed the quantitative model (*3, 24*), received support from early experiments which suggested that the *src* gene of RSV or the *myc* gene of MC29 and the corresponding proto-onc genes and their products were equivalents (*61–65*). In the meantime, significant structural and functional differences between these genes have been found (*3, 40, 42–46*; see above). Others have suggested that proto-onc genes are activated by mutations or rearrangements in the primary DNA sequence (*66, 67*). This view is termed the qualitative model (*3, 24*).

The multigene–one cancer hypothesis postulates that an activated proto-onc gene is necessary but, unlike the corresponding viral gene, not sufficient to cause cancer. A quantitatively or qualitatively activated proto-onc gene is postulated to function either as initiation or as maintenance gene together with another gene, in a multistep process (*54, 55, 68–74*). This hypothesis fits the view of how virus-negative tumors are thought to arise in general and provides identifiable candidates to test the hypothesis. However, since retroviral onc genes have yet to be dissociated into initiation and maintenance functions, this hypothesis is without functional precedent.

Two kinds of assays have been performed to test these hypotheses. One assay correlates transcriptional activation and mutation of proto-onc genes with cancer; the other directly measures transforming function of proto-onc genes upon transfection into certain recipient cells, typically the preneoplastic mouse NIH 3T3 cell line (*6, 7, 54, 55*). Such experiments have most frequently linked cancers with alterations of proto-*myc* and proto-*ras*.

*Role of proto-*myc *activation in B-cell lymphomas*. Transcription of proto-*myc* is frequently enhanced in retroviral lymphomas of chicken. On the basis of this observation it has been postulated that transcriptional activation of proto-*myc* is the cause of B-cell lymphoma (*65, 75*). The chicken B-cell lymphoma is a clonal cancer that appears in a small percentage of animals infected by one of the avian leukosis viruses (which have no onc genes) after latent periods of more than 6 months (*58*). The hypothesis, termed downstream promotion, postulates that the gene is activated by the promoter of a retrovirus integrated upstream (Fig. 1) and that activated proto-*myc* behaves functionally like the transforming gene of MC29 (*65*). In accord with the hypothesis, hybrid RNA's consisting of 5′ viral and cellular proto-*myc* sequences are found in some tumors (*65*). Subsequently, samples were found in which the retrovirus was integrated 3′ of proto-*myc* or 5′ in the opposite transcriptional direction. In these cases, no hybrid RNA's are found, and the virus is thought to function like an enhancer of proto-*myc* (Fig. 1) (*75*).

However, proto-*myc* differs structurally from the 3-kb Δ*gag-myc* gene of MC29 (Fig. 1) (*25, 26*). It has been argued (*3*) that the hypothesis does not explain (i) the origin of about 20 percent of viral lymphomas in which proto-*myc* is not activated (*65*); (ii) the discrepancies between the phenotype of the disease and the cancers caused by MC29; (iii) the clonality of the tumors as defined by a single integration site of the retrovirus with regard to proto-*myc*; and (iv) the long latent period of the disease. Given about $10^6$ kb of chicken DNA and activation of proto-*myc* by retrovirus integration within about 5 kb of proto-*myc* (*27, 75*), one in $2 \times 10^5$ infections should generate the first tumor cell. Since the chicken probably has more than $10^7$ uncommitted B cells and many more virus particles, the critical carcinogenic integration event should occur after a short latent period. The tumor should also not be clonal, since integration by retroviruses is not site-specific, and there could be numerous infections during the latent period of about 6 months. Further, the model has not been confirmed in murine (*76, 77*), feline (*36*), and bovine (*78*) leukemia. Instead, the high percentage of virus-negative feline (*36*) and bovine (*79*) lymphomas indicates that a retrovirus may not even be necessary for the disease.

It has also been suggested that point mutations, rather than a virus, may activate avian proto-*myc* because mutations have been observed in viral lymphoma (*80*). However, the proto-*myc* mutations have not been shown to be specific or functionally relevant to viral lymphoma. The absence of specific point mutations compared to proto-*myc* indicates that oncogenic function of *myc*-related viral genes is probably not due to point mutations (*34*).

Activation of proto-*myc* has also been postulated to cause the retrovirus-negative human Burkitt's lymphomas and mouse plasmacytomas. In these cases, chromosome translocation has been proposed as a mechanism of activating proto-*myc* function (*71, 72, 81, 82*). The human proto-*myc* is distinct from that of the chicken from which carcinoma viruses have been derived (Fig. 1). The two genes have entirely different first exons, similar second exons except for a few regions, and colinear third exons (*25*). In humans, proto-*myc* is located on chromosome 8, and an element of this chromosome is reciprocally translocated in many Burkitt's lymphoma lines to immunoglobulin (Ig) loci of chromosome 14 and less frequently to Ig loci of chromosome 2 or 22. Since the crossover points of chromosome 8 are near proto-*myc*, translocation was initially suspected to activate proto-*myc* transcriptionally by rearranging proto-*myc* (Fig. 1) or by altering its immediate environment and thus bringing it under the influence of new promoters or enhancers (*81*). In many lymphomas however, rearranged proto-*myc* is not linked to a new promoter; instead the first presumably noncoding exon is replaced by the Ig locus, linked to it 5′-5′ in the opposite transcriptional orientation (*81*) (Fig. 1). This model does not explain how proto-*myc* would be activated when the complete proto-*myc* gene, including its known promoters and flanking regions, is translocated (*71, 73, 81*); neither can the model explain certain Burkitt's lymphomas in which proto-*myc* remains in its original chromosomal location while a region 3′ of proto-*myc* is translocated (*83–87*). Thus there is no consistent alteration of

proto-*myc* in Burkitt's lymphoma that could explain its activation to a cellular oncogene.

There appears to be no consensus as to whether proto-*myc* expression is enhanced in Burkitt's lymphoma cells, as compared to normal control cells. Some investigators report elevated expression compared to normal B lymphoblasts or lines (*88*), while others report essentially normal levels of proto-*myc* mRNA (*71, 86, 87, 89, 90–93*). Enhanced proto-*myc* transcription is not specific for B-cell lymphomas, since high levels of proto-*myc* expression are seen in non-Burkitt's lymphomas (*92*), in other tumors (*74*), and in chemically transformed fibroblast cell lines in which proto-*myc* is not translocated or rearranged (*41*). The view that enhanced expression of proto-*myc* may be sufficient to cause Burkitt's lymphoma can also be challenged by the observations that proto-*myc* transcription either reaches cell cycle–dependent peak levels in certain cell lines (*41, 94*) or maintains constitutively high levels in embryo cells similar to those in tumor cells (*95*).

The possibility that mutations of proto-*myc* may be correlated with Burkitt's lymphoma has also been investigated. In some Burkitt's cell lines, mutations have been observed in translocated, but not rearranged, proto-*myc* (Fig. 1) (*93, 96*). Initially it was proposed that these mutations may activate proto-*myc* by altering the gene product (*96*), but in at least one Burkitt's lymphoma line the coding sequence corresponding to proto-*myc* exons 2 and 3 was identical to that of the normal gene (Fig. 1) (*89*). It has since been proposed that mutations in the noncoding exon may activate the gene (*93, 97*). However, there is no functional evidence for this view, and an activating mutation that is characteristic of Burkitt's lymphomas has not been identified. A sequence comparison between translocated proto-*myc* of a mouse plasmacytoma with the germline proto-*myc* indicated that the two genes were nearly identical—there was a difference of one nucleotide in the first exon. It was concluded that proto-*myc* mutations are not required for oncogenesis (*98*). It is also an open question at this time whether the first human proto-*myc* exon is indeed noncoding (*89*) or has possibly an open reading frame capable of encoding a major protein (*25, 99*).

Thus, there appears to be no translocation, rearrangement, elevated expression, or characteristic mutation of proto-*myc* that is common to all Burkitt's lymphomas investigated. This casts doubt on the concept that any of the known proto-*myc* alterations are a sufficient cause (or even necessary) for Burkitt's lymphoma.

The question of whether proto-*myc* has transforming function has been tested directly by means of the 3T3 cell transformation assay with DNA from chicken or human B-cell lymphomas. However, no *myc*-related DNA was detected even though its presumed functional equivalent, the Δ*gag-myc* gene of MC29, is capable of transforming 3T3 cells (*100, 101*) and other rodent cell lines (*102*). Instead, another DNA sequence termed *Blym* was identified by the assay (*68, 103*). On the basis of these results, the role of proto-*myc* in lymphomas has been interpreted in terms of a two-gene hypothesis. It has been suggested that activated proto-*myc* is necessary but not sufficient to cause the lymphoma (*69, 74*). It is postulated to have a transient early function that generates a lymphoma maintenance gene, *Blym*, which appears to be the DNA that transforms 3T3 cells and is thought to maintain the B-cell tumor. There is no proof for this postulated role of proto-*myc* as a lymphoma initiation gene, because the 3T3 cell transformation assay does not measure proto-*myc* initiation function, and because there is no evidence that the two genes jointly (or alone) transform B cells. Furthermore, the hypothesis does not address the question of why proto-*myc* should have any transforming function at all, if it is not like MC29. (MC29 does not require a second gene to transform a susceptible cell.) It is also not known whether *Blym* is altered in primary Burkitt's lymphomas, since all of the transfection experiments were done with DNA from cell lines.

Chromosome translocation involving the proto-*myc* chromosome 8 may conceivably be a specific but not a necessary consequence, rather than the cause of the lymphoma (*104*). Human B-cell lymphomas with translocations that do not involve chromosome 8 have been described (*105, 106*). In the case of clonal myeloid leukemias with consistent translocations (as in the "Philadelphia" chromosome), it has been convincingly argued that translocation is preceded by clonal proliferation of certain stem cells with the same isoenzyme markers as leukemic cells but without chromosomal abnormalities (*107*). Thus further analyses of primary Burkitt's lymphomas are required before the question of whether proto-*myc* alteration contributes to Burkitt's lymphoma can be answered.

*Proto-*ras *mutations and the cause of human and rodent carcinomas*. Use of the 3T3 cell assay to measure transforming function of DNA from a human bladder carcinoma cell line has identified DNA homologous to the *ras* gene of Harvey rat sarcoma virus (Ha-MuSV) (Fig. 2) (*67, 108*). On the basis of the viral model, the proto-Ha-*ras* gene is thought to be a potential cancer gene because it encodes a 21,000-dalton protein, p21, which is colinear with an onc gene product p21 of Ha-MuSV (Fig. 2) (*109*). The proto-Ha-*ras* gene from the bladder carcinoma cell line differs from normal proto-Ha-*ras* in a point mutation which alters the 12th p21 codon in exon 1 from normal Gly to Val (*67, 110*). This mutation does not cause overproduction of the *ras* gene product (p21) in the 3T3 cell line (*67*) and does not change known biochemical properties of p21 (*111*). The single base change is thought to activate the gene to a functional equivalent of Ha-MuSV and to be the cause of the carcinoma because it is the apparent cause for 3T3 cell-transforming function (*67, 112*). However, this mutation has not been found in a survey of more than 60 primary human carcinomas, including 10 bladder, 9 colon, and 10 lung carcinomas (*113*), in 8 other lung carcinomas (*114*), and 14 additional bladder and 9 kidney carcinomas (*115*). Further, the mutated human proto-Ha-*ras*, which transforms 3T3 cells, does not transform primary rat embryo cells (*54, 70*) and, more significantly, does not transform human embryo cells (*116*). Transformation of primary cells would be expected from a gene that causes tumors in animals. Thus the mutated proto-*ras* gene does not correspond to the viral model which transforms primary mouse, rat (*117, 118*), and human cells (*119–123*). In addition, Val expressed by the 12th codon of 3T3 cell–transforming proto-ras is different from the Arg of the viral counterpart (*110*).

Other mutations have since been found to confer 3T3 cell-transforming function to proto-Ha-*ras* DNA. Proto-Ha-*ras* with a mutation in codon 61 was isolated from a human tumor cell line (*124*). Proto-Ha-*ras* DNA's that transform 3T3 cells were also isolated from 2 out of 23 primary urinary tract tumors analyzed. One of these contained a mutation in codon 61, the other was not identified (*125*). The mutations were not found in the normal tissue of the respective patients. Nevertheless, this does not prove that 3T3 cell–transforming function of proto-*ras* was necessary for tumor formation since each was associated with only 1 out of 23 histologically indistinguishable tumors.

A 3T3 cell–transforming proto-Ha-*ras* DNA was also found in some (not all) chemically induced benign papillomas

and malignant carcinomas of mice (*126*). Since only a small (5 to 7 percent) portion of the benign tumors progressed to carcinomas, it would appear that 3T3 cell–transforming proto-*ras* was not sufficient to cause the carcinomas, and since not all carcinomas contained the mutation, it would appear that it was not necessary either. A high proportion, that is, 14 out of 17 methylnitrosourea-induced mammary carcinomas of rats, were found to contain 3T3 cell–transforming proto-Ha-*ras* DNA (*127*). This suggests that the mutation is not necessary for the tumor, although it may be important for tumor progression. The original study reported nine out of nine positives (*128*). However, the hormone-dependence and high tissue specificity of the carcinogen in this study suggests that other genes must be involved, because mutated proto-*ras* has been found in association with other tumors and transforms 3T3 cells without hormones. It is plausible that other genes, which may be involved in tumorigenesis but which do not register in the 3T3 assay, were also altered by the carcinogen. No activated *ras* genes were found in 20 chemically induced liver carcinomas of rats (*129*).

As the result of an effort to explain why mutated proto-Ha-*ras* transforms preneoplastic 3T3 cells, but not rat or human embryo cells, the proposal was made that mutated proto-Ha-*ras* is only one of at least two activated genes that are necessary to induce cancer (*54, 55, 70*). This two-gene hypothesis was tested when primary rat cells were transfected with a mixture of the mutated human proto-Ha-*ras* and either MC29 provirus or activated proto-*myc* from mouse plasmacytoma (*54*), or with the EIA gene of adenovirus (*70*) as helper genes. None of these genes were able to transform rat embryo cells by themselves, but some cells were transformed by the artificially mixed genes. The study in which the adenovirus virus helper gene was used showed that proto-*ras* expression varied from high to normal levels in transformed cells and that normal proto-*ras* was inactive in the assay (*70*). The study with *myc*-related helper genes did not show that the transformants contained and expressed the added DNA's; not tested in this study was the question of whether unaltered forms of proto-*ras* or proto-*myc*, together with an altered helper gene, are sufficient to register in this assay. This question appears to be particularly relevant since a proto-*myc* clone from a mouse plasmacytoma with an SV40 enhancer at its 3′ end but without its natural promoter (*72*) was reported to be active (*54*) although such a construction is not expected to activate proto-*myc*.

The *myc*-related genes were proposed to convert rat embryo cells to cells that are capable of dividing indefinitely, like 3T3 cells, a function termed immortalization (*54, 55*). However, the immortalization function of MC29 or of activated proto-*myc* was not demonstrated independently. The proposal did not explain why an immortalization gene was necessary. Obviously, immortalization is necessary to maintain cells in culture. However, immortalization is not necessary for focus formation and probably not for tumor formation since embryo cells are capable of sufficient rounds of mitoses (up to 50) in cell culture and in the animal to develop tumors (*130*). In the avian system, MC29 transforms primary cells and causes tumors in chicken independently without the benefit of secondary oncogenes, and most MC29 tumor cells are not immortal if tested in cell culture. The failure of maintaining cells from many human tumors in cell culture, under conditions where cells from similar tumors survive, also suggests that immortality may not be an essential criterion of a tumor cell (*131*). There is also no precedent for a function of proto-*ras* in a multistep transformation mechanism, because the transforming genes of Harvey or Kirsten sarcoma viruses transform rat and mouse embryo cells (*117, 118*) or human embryo cells (*119–123*) with single-hit kinetics and without helper genes. Likewise, there is no precedent for the artificial mixtures of the two activated proto-onc genes in any natural tumors.

Other 3T3 cell–transforming proto-*ras* genes, namely, proto-Ki-*ras* which is more closely related to the *ras* gene of Kirsten sarcoma virus than to Harvey virus, and N-*ras*, which is related to both viruses, have also been found in tumors or cell lines (*132*). Proto-Ki-*ras* encodes a p21 protein that is related to the p21 protein encoded by proto-Ha-*ras* (*110, 132, 133*). One group has found 3T3 cell–transforming proto-Ki-*ras* DNA in three primary human tumors and five tumor cell lines out of 96 samples tested (*114, 134*). The same group also found 3T3 cell–transforming proto-Ki-*ras* DNA in one out of eight lung carcinomas tested (*114*). The DNA from this tumor, but not that from normal tissue of the same patient, had a mutation in the 12th codon. The low percentage of 3T3 cell–positives among these tumors raises the question of whether the mutations were necessary for tumorigenesis.

In a study of human melanomas, only one of five different metastases from the same human melanoma patient was found to contain 3T3 cell–transforming proto-Ki-*ras* DNA (*135*). A 3T3 cell–transforming Ki-*ras* DNA was also detected in a metastatic variant but not in a primary methylcholanthrene-induced T-cell lymphoma of mice (*136*). An example of a spontaneous proto-*ras* mutation appearing in tumor cells cultured in vitro has now been described (*137*). This suggests that these proto-*ras* mutations were consequences rather than the causes of these tumors. The view that *ras* mutation is a consequence of tumorigenesis is also consistent with the results that only one *ras* allele is mutated in some primary tumors (*113, 125, 133*), whereas both alleles are mutated in typical tumor cell lines (*113, 114*).

Since 3T3 cell–transforming or mutated proto-*ras* genes are only rarely associated with human and murine tumors and since mutated proto-Ha-*ras* does not transform human or rat embryo cells (*54, 70, 116*) (proto-Ki-*ras* was not tested), there is as yet no proof that mutated proto-*ras* is sufficient or even necessary for any of the above tumors.

The failure of the mutated proto-Ha-*ras* or proto-Ki-*ras* to behave like the viral model indicates that the mutated proto-*ras* genes from tumor cells and Harvey or Kirsten sarcoma viruses are not equivalent carcinogens. The probable basis for this functional difference is that the cellular and viral genes are not isostructural and also differ in virus and cell-specific point mutations: (i) Normal proto-*ras* genes of rats and humans each have four colinear exons which differ only in silent point mutations (*109, 110, 128, 138*). The 5′ ends of both genes are not yet defined. The better known human proto-*ras* contains possibly another upstream exon that includes a virus-related region of 120 nucleotides (*110*) (Fig. 2). The proto-*ras* genes are transcribed into 1.2- and 5-kb mRNA's compared to the genomic viral mRNA of 5.5 kb (*58, 139–141*) (Fig. 2). Moreover, *ras* is only about 10 percent of the genomes of Harvey and Kirsten sarcoma viruses. Each viral RNA contains about 3 kb of genetic information, derived from a rat 30*S* defective retrovirus RNA (*142*), which is presumed to be noncoding but which may nevertheless contribute to the oncogenicity of these viruses (Fig. 2). (ii) The point mutations that confer 3T3 cell–transforming function to proto-Ha-*ras* genes from tumors are all different from those that set apart viral *ras* genes from proto-*ras* (*143*) (Fig. 2). The *ras* gene of Harvey virus (*144*) differs

from proto-*ras* of rats (*128, 138*) and humans in the 12th (Arg/Gly) and the 59th (Thr/Ala) codon. The *ras* genes from tumors carry single mutations either in the 12th or in other codons, which are different from those found in viruses (*143*). As yet no proto-*ras* mutation was found in codon 59 (*143*).

Experiments are in progress which indicate that the differences between Harvey virus and the proto-*ras* genes from tumor cell lines are functionally significant. For example, a Harvey virus in which the 12th *ras* codon has been changed to that of normal proto-*ras* still transforms 3T3 cells and rat primary cells (*145*). Thus either the virus-specific 59th *ras* codon or other virus-specific elements or both activate the *ras* gene of this Harvey virus. The 3T3 cell transforming *ras* gene from the human bladder carcinoma cell line which is unable to transform rat primary cells per se (*54*) was recently shown to transform primary cells when linked to a viral enhancer (*146*), or to cause sarcomas in mice after it was integrated into a retrovirus vector (*147*). It follows that the particular mutation that activates 3T3 cell–transforming function of the proto-*ras* gene (that is, *Val* in codon 12) is not sufficient to convert the gene to a carcinogen equivalent of Harvey virus. Possibly the deletion of cellular elements flanking *ras* or the addition of virus specific elements are necessary to convert the gene to an autonomous carcinogen.

There are other discrepancies between mutated cellular *ras* genes and viral *ras* genes. It has been argued that mutated proto-*ras* is a recessive transforming gene, because both *ras* alleles are mutated in typical tumor cell lines although only one allele is mutated in some primary tumors (*114, 132, 143*). By contrast, the viral onc gene is dominant. A definitive answer to the question whether *ras* mutations are dominant or recessive 3T3 cell–transforming genes could be obtained by simultaneous transformation with mutated and normal *ras* genes. Finally, Ha- and Ki-MuSV are not obvious models for proto-*ras* genes with hypothetical carcinoma function, since these viruses cause predominantly sarcomas.

## Conclusions

The preponderance of 3T3 cell–transformation negatives among the above-described tumors suggests that either no genes have caused the negative tumors or that the assay failed to detect them. That only *ras*-related proto-onc genes have been detected in human tumors indicates another limitation of the 3T3 assay. Since the proto-*ras* mutations detected by the 3T3 assay do not transform primary cells, it is possible that they are not relevant for tumor formation. Available data suggest that these are coincidental or consequential rather than causative mutations occurring in tumor cells, because the mutations are not consistently correlated with specific tumors and because in some cases they precede tumor formation and in others they evolve during tumor progression. Despite its effectiveness to transform 3T3 cells mutated proto-*ras* is not an autonomous cancer gene, similar to a viral onc gene. Thus the 3T3 test is inadequate for determining whether proto-*ras* genes cause tumors in animals. The efficiency of the assay to identify cancer genes unrelated to proto-onc genes (*6, 7*) remains to be determined.

The proto-onc genes are sometimes mutationally or transcriptionally altered in tumor cells. Because there is no functional evidence that such proto-onc genes transform embryo cells or cause tumors and no consistent correlation between altered proto-onc genes and a specific tumor, the one-gene hypothesis (that altered proto-onc genes are sufficient to cause tumors) lacks support. As yet, viral onc genes are the only "activated" proto-onc genes that are sufficient to cause tumors and that act as autonomous, dominant cancer genes in susceptible cells.

The observations that altered proto-onc genes do not behave like viral onc genes and that in some tumors multiple proto-onc genes are altered (*74*) have been interpreted in terms of a multigene hypothesis. Altered proto-*myc* has been proposed to cooperate with the *Blym* gene to cause chicken and human B-cell lymphoma (*69*). Along with other genes altered proto-*ras* has been proposed to cause carcinomas and reported to cooperate in an artificial system with altered proto-*myc* to transform rat embryo cells in culture (*54, 55*). The spliced *myc* genes of MH2 (*28, 29*) and OK10 viruses (*34*), but not that of MC29 (Fig. 1), may indeed be models for onc genes that are not sufficient for transforming function. However, several reservations about a role of altered proto-*myc* or proto-*ras* in multigene carcinogenesis are possible: (i) There is no functional evidence that a combination of altered *myc* and *Blym* forms lymphomas or that altered *ras*, together with another gene from carcinomas, transform appropriate normal test cells. An artificial combination of altered *ras* in combination with a *myc*-related or an adenovirus gene was reported to transform primary rat cells. However, it was not reported whether both genes are present and functional in all transformants, and there is no evidence that these artificial *ras*-helper genes are models for the hypothetical helper genes in tumors with altered *ras*. (ii) The observations that proto-*myc* alterations are not consistently associated with B-cell lymphomas and that proto-*ras* mutations are only rarely associated with specific carcinomas support the proposition that these proto-onc gene alterations may not be necessary for these tumors. (iii) The proposals that altered proto-onc genes are necessary but not sufficient for tumor formation are a significant departure from the original view that they were equivalents of viral onc genes. They do not address the question why these genes are assumed to have oncogenic functions different from those of the viral models. Ironically, these proposals suggest that activated proto-onc genes are functional subsets of viral onc genes, whereas viral onc genes are structural subsets of proto-onc genes. Functional proof for multiple, synergistic transforming genes and consistent correlations between altered proto-onc genes and specific tumors are needed to support the view that proto-onc genes are necessary for multigene carcinogenesis.

It may be argued that the proto-onc gene alterations associated with some cancers play a nonspecific, but causative, role in carcinogenesis that could be substituted for by another gene. To support this view, it would be necessary to know which other genes could substitute for the role that altered proto-onc genes are thought to play in the origin of cancer. Further, one would have to know whether proto-onc gene alterations are more typical of cancer cells than alterations of other genes and which other genes characteristically undergo alterations in tumor cells. Unknown events, additional to the known alterations of resident proto-onc genes, may be required for the development of cancer (*3, 148*).

The fact that proto-onc genes share common domains with viral onc genes remains a persuasive argument that proto-onc genes may, under certain conditions, be changed into cancer genes. The evidence that most normal proto-onc genes are expressed in normal cells suggests that cell-specific domains of proto-onc genes may suppress potential oncogenic function. Thus, mutation or

removal of suppressors could activate a proto-onc gene, as has been predicted for Burkitt's lymphoma. Clearly, the identification of such suppressors would depend on a complete genetic definition of proto-onc genes. To date, we do not know both termini of any proto-onc gene [except for human proto-*myc* (*89*), which is not a prototype of a known oncogenic virus]. In addition, virus-specific onc gene elements may also be essential to activate a proto-onc gene. In this case, a retrovirus without an onc gene (leukemia virus) could activate a proto-onc gene by a single illegitimate recombination which would form a hybrid onc gene. Such an event would be more probable than the generation of a retrovirus with an onc gene for which at least two nonhomologous recombinations are necessary.

DNA technology has made it possible to convert nontransforming DNA from viral or cellular sources to synthetic genes that transform cell lines or normal cells. Examples are the proto-*mos* and proto-*ras* retroviral long terminal repeat (LTR) recombinants that transform 3T3 cells (*52, 53*), the proto-*ras, myc*, and adenovirus DNA combinations that transform rat embryo cells (*54, 70*), the LTR-mutant proto-*ras*-SV40 construction that transforms rat embryo cells (*146*), or a synthetic mutant *ras* virus that causes sarcomas in mice (*147*). Another example is a synthetic gene that consists of a mouse proto-*myc* gene in which all or part of the first exon is replaced by the LTR of mouse mammary tumor virus. Upon introduction into the germline, this gene was expressed in 11 transgenic mice. Only two of these developed mammary tumors after two pregnancies, and not in all mammary glands. It was suggested that the gene may be necessary but not sufficient for the development of these tumors (*149*). Clearly, the integrity of proto-onc genes was altered in these constructions, since only subsets of proto-onc genes were included. It is as yet unclear how the products of these truncated and conjugated proto-onc genes differ from normal counterparts. There is also no genetic evidence yet that the synthetic genes encode transforming proteins or transform indirectly like chemical carcinogens. In order to assess the relevance of such iatrogenic transformations to cancer, it would be helpful to determine whether the number of DNA species that can be converted to transforming variants is large or small, and it would be necessary to determine whether any such DNA's ever occur in natural tumors. The most important challenge now is to develop functional assays for cellular cancer genes.

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**RESEARCH ARTICLE**

# DNA Elements Are Asymmetrically Joined During the Site-specific Recombination of Kappa Immunoglobulin Genes

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During B-cell differentiation, a series of site-specific recombinations assemble the variable (V) region exons of immunoglobulin genes (*1–3*). Recombinational assortment of gene segments expands the coding capacity of a locus, enabling a large repertoire of gene products to be generated from a relatively small number of components. This appears to be a common feature of the immune system loci that encode antigen-binding proteins; the variable region exons of T-cell–specific antigen receptor genes also assemble recombinationally (*4, 5*) as do those in another locus that is also thought to be involved in immune recognition (*6*).

The gene segments targeted for rearrangement at these loci are flanked by characteristic DNA sequences. The putative "joining signal" consists of a heptamer, a spacer region, and a nonamer (*1–5*). The heptamer and nonamer elements are evolutionarily conserved, being similar in different vertebrate classes (*7*), as well as in genes that rearrange in different cell lineages (*1–5*). Joining signals always have one of two forms; the heptamer and nonamer sequences are separated by an approximately 12-base spacer, or by an approximately 23-base spacer. Gene segments linked to joining signals with 12-base spacers appear to recombine only with those linked to joining signals containing 23-base spacers and vice versa (*1–5*). The presence of joining signals and the adherence to the 12–23 spacer rule have been taken to indicate that similar, perhaps identical, enzymes catalyze recombination at all six loci that are known to rearrange during immunodifferentiation (*4*).

The kappa immunoglobulin locus provides a simple and well-characterized system in which to study the details of the recombination process. Only two component parts, $V_\kappa$ and $J_\kappa$, recombine in forming a complete kappa variable region exon. Rearrangement of the kappa locus generates two distinct classes of recombinant junctions (*8–13*). One product of rearrangement is a "coding joint," which is the junction between $V_\kappa$ and $J_\kappa$ coding sequences in an assembled variable region exon. The other product we refer to as a "reciprocal joint": it consists of the two joining signals derived from $V_\kappa$ and $J_\kappa$ fused to one another at their (formerly) coding-proximal borders (*8*) (Table 1). The existence of reciprocal joints at the kappa locus indicates that gene rearrangement may be a reciprocal process, yet reciprocal joints apparently

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