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Cellular Oncogenes and Multistep Carcinogenesis

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Summary. Two dozen cellular proto-oncogenes have been discovered to date through the study of retroviruses and the use of gene transfer. They form a structurally and functionally heterogeneous group. At least five distinct mechanisms are responsible for their conversion to active oncogenes. Recent work provides experimental strategies by which many of these oncogenes, as well as oncogenes of DNA tumor viruses, may be placed into functional categories. These procedures may lead to definition of a small number of common pathways through which the various oncogenes act to transform cells.

Two independent lines of work, each pursuing cellular oncogenes, have converged over the last several years. Initially, the two research areas confronted problems that were ostensibly unconnected. The first focused on the mechanisms by which a variety of animal retroviruses are able to transform infected cells and induce tumors in their own host species. The other, using procedures of gene transfer, investigated the molecular mechanisms responsible for tumors of nonviral origin, such as those human tumors traceable to chemical causes. We now realize that common molecular determinants may be responsible for tumors of both classes. These determinants, the cellular oncogenes, constitute a functionally heterogeneous group of genes, members of which may cooperate with one another in order to achieve the transformation of cells.

Retrovirus-Associated Oncogenes

An initial insight into cellular oncogenes came from study of Rous sarcoma virus (RSV). Retroviruses such as RSV have been studied intensively for the past decade, in part because of their unusual molecular biology involving reverse transcription and the high-efficiency integration of their genomes into the cellular chromosome. Another of their traits, still poorly understood, opened up study of cellular oncogenes: retroviruses are able to pick up and transduce cellular genetic information.

Upon dissecting the genome of RSV, Stehelin, Varmus, Bishop, and Vogt found two distinct portions (*1*). The first portion includes the genes responsible for viral replication, which involves the complex processes of reverse transcription, integration, and progeny virus particle formation. The other portion contains the *src* gene, which enables the virus to induce sarcomas in vivo and to transform chicken fibroblasts in monolayer culture. This *src* gene is now known to encode the structure of the tyrosine kinase termed pp60*src* (*2*). As these workers showed (*1*), the *src* oncogene is not a bona fide viral gene at all, but rather stems from a closely related gene residing in the genome of the chicken. This antecedent gene, sometimes termed a proto-oncogene, is a normal cellular gene and an integral part of the chicken genome (*3*).

This work proved that the cellular genome contains a gene that can exhibit strong transforming properties when properly activated. RSV served as a paradigm for more than 30 other animal retroviruses, each of which was also shown to have acquired a cellular oncogene during its brief evolution. Retroviruses thus represent useful devices to scan the cellular genome for the presence of proto-oncogenes. It seems that these hybrid transforming retroviruses usually exist ephemerally, picking up and activating a host proto-oncogene, inducing a tumor, and dying together with the afflicted host. Timely isolation of the virus from a tumor-bearing host

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can save the virus and its associated oncogene from oblivion.

A current listing of the various retrovirus-associated cellular oncogenes is shown in Table 1. As new transforming retroviruses are isolated and characterized, the list grows, but only in small increments. Characterization of many of the recent viral isolates has led to the rediscovery of proto-oncogenes already known from the study of other viruses. For example, the *myc* proto-oncogene is known to us from its association with no fewer than four distinct avian retrovirus strains (*5*). A recently characterized feline sarcoma was found to harbor the *sis* oncogene, known from earlier work with simian sarcoma virus, while another feline sarcoma virus carries the *abl* gene, originally described as part of the genome of Abelson murine leukemia virus (*6*). Therefore, we may be exhausting the repertoire of proto-oncogenes that can be retrieved from the cellular genome by retroviruses.

Transfected Tumor Oncogenes

A more recent body of work, begun 5 years ago, revealed a second group of cell-associated oncogenes. The initial experiments in this area were designed to demonstrate the molecular determinants that are responsible for transformation of cells exposed to chemical carcinogens. The design of these experiments was simple. Samples of DNA were extracted from chemically transformed cells and introduced into appropriate, untransformed recipients. The monolayer cultures of these transfected recipient cells were then scanned for foci of transformants. Induction of such foci would indicate the presence of dominantly acting, transforming information in the donor cell DNA.

The gene transfer experiments soon showed that certain types of chemically transformed cells carry oncogenic sequences in their DNA (*7*). The existence of such transforming sequences has subsequently been demonstrated in the DNA of a large number of different human tumor cell lines and tumor biopsies (*8, 9*). For example, the DNA of a human bladder carcinoma cell line could be used to induce a number of foci on mouse fibroblast monolayers (*10, 11*). Cells of the foci grew out into fibrosarcomas

Table 1. Cellular oncogenes. The retrovirus-associated oncogenes are grouped into three gene families (*src* to *raf*; Ha- and Ki-*ras*; *myc* and *myb*) and a group of genes having no known homology to one another or to any other oncogene. The *mam* (*30*) and *neu* (*29*) genes have not yet been isolated by molecular cloning. Each gene is presumably found in one or more copies in the genomes of all vertebrates, although this is not yet documented for many genes in this table. A group of other genes that may function as oncogenes has been discovered but is still incompletely characterized: three genes, *erb*A, *ets*, and *mil* are found together with *erb*B, *myb*, and *myc* oncogenes in the genomes of avian erythroblastosis, E26, and MH2 virus, respectively; *int 1* and *int 2* are altered by mouse mammary tumor virus provirus insertion; *MLVI 1* and *MLVI 2* are altered by murine leukemia virus provirus insertion. These last four genes may therefore be activated in a fashion similar to the avian leukosis virus-mediated activation of *myc* (*33–35*). Two other sequences, human Ha-*ras* 2 and Ki-*ras* 1, are closely related to two genes listed in this table; it remains unclear whether they are complete genes or pseudogenes.

Acronym	Origin	Species of isolation	Chromosomal location		Subcellular localization (*69, 73*) of virally encoded protein	Activity of virally encoded protein (*74*)
			Human (*24, 40, 71*)	Mouse (*72*)		
src	Rous sarcoma virus	Chicken	20		Plasma membrane	Tyrosine kinase
yes	Y73 sarcoma virus	Chicken				Tyrosine kinase
fps(=*fes*)	Fujinami (ST feline) sarcoma virus	Chicken (cat)	15	7	Cytoplasm	Tyrosine kinase
abl	Abelson murine leukemia virus	Mouse	9	2	Plasma membrane	Tyrosine kinase
ros	UR II avian sarcoma virus	Chicken			Cytoplasmic membranes	Tyrosine kinase
fgr	Gardner-Rasheed feline sarcoma virus	Cat				Tyrosine kinase
*erb*B	Avian erythroblastosis virus	Chicken				
fms	McDonough feline sarcoma virus	Cat	5		Cytoplasm	
mos	Moloney murine sarcoma virus	Mouse	8	4	Cytoplasm	
raf	3611 murine sarcoma virus	Mouse	3	6		
Ha-*ras*1	Harvey murine sarcoma virus	Rat	11	7	Plasma membrane	Guanosine diphosphate or guanosine triphosphate binding
Ki-*ras*2	Kirsten murine sarcoma virus	Rat	12		Plasma membrane	
myc	Avian MC29 myelocytomatosis virus	Chicken	8	15	Nuclear matrix	
myb	Avian myeloblastosis virus	Chicken	6		Nuclear matrix	
fos	FBJ osteosarcoma virus	Mouse	2		Nucleus	
ski	Avian SKV770 virus	Chicken	1			
rel	Reticuloendotheliosis virus	Turkey				
sis	Simian sarcoma virus	Woolly monkey	22	15	Cytoplasm	
	Related oncogenes known from sequence hybridization or transfection					
N-*myc*	Neuroblastomas	Human	2			
N-*ras*	Neuroblastoma, leukemias, sarcomas	Human	1			
	Unrelated oncogenes known only from transfection					
B*lym*	Bursal lymphomas	Chicken	1			
mam	Mammary carcinomas	Mice, human				
neu	Neuro-, glioblastomas	Rat				

when inoculated into young mice. Such foci were not observed when DNA of nontumor origin was tested in the transfection-focus assay.

The calcium phosphate transfection technique of Graham and van der Eb (*12*) has been used in these experiments. The recipient cells have generally been cells of the NIH 3T3 mouse fibroblast line. These cells were originally chosen because they were found to be particularly efficient at taking up and fixing exogenous, transfected DNA (*13*). As we discuss below, other properties of these cells have assumed increasing importance.

Many types of tumor cells develop transforming sequences in their DNA during their progression from the normal to the cancerous state. A list of these tumors includes carcinomas of the bowel, lung, bladder, pancreas, skin, and breast; fibro- and rhabdomyosarcomas; glioblastomas; a neuroblastoma; and a variety of hematopoietic neoplasms (*7–11; 14–18*). The oncogenes associated with these tumors are presumed to be important in inducing the transformed phenotypes of the tumor cells, but that role remains unproved. The existing experiments only demonstrate that these oncogenes can transform foreign cells into which they have been introduced by gene transfer.

The donor tumor cell yielding the transforming DNA may differ substantially from the cell used as recipient in a transfection. For example, an oncogene derived from a human bladder carcinoma can transform an NIH 3T3 mouse fibroblast (*10, 11*). This suggests that this particular oncogene can transform cells from a variety of tissues, but it also points out a weakness in the existing experiments: another oncogene, able to transform only bladder epithelial cells, would never have been detected in these experiments. This may help to explain the fact that only 20 percent or so of the tumor cell lines tested have yielded active oncogenes in NIH 3T3 transfection assays. Perhaps the remaining 80 percent of the tumors harbor oncogenes that require specialized recipient cells in order to register in a transfection focus assay. In work published to date, only rodent fibroblasts have been used, and these may not be responsive to such specialized oncogenes. Other explanations could be entertained for the negative results. For example, certain oncogenes may act as recessive or weakly transforming alleles and totally escape detection in the currently used transfection-focus assay.

Several of the active tumor oncogenes have been isolated by molecular cloning. These include the oncogene of the T24/EJ human bladder carcinoma cell line (*19–21*), the B*lym* oncogenes of a chicken lymphoma and a Burkitt's lymphoma (*22*), an oncogene of a human lung carcinoma (*23*), and one from a human neuroblastoma (*23*) that has been found as well in leukemias and sarcomas (*23–25*). In each case, a simple and fundamental truth has emerged. Each oncogene is closely related to a counterpart DNA sequence present in the normal cellular genome. Once again, one speaks of oncogenes and antecedent proto-oncogenes, although in this case the mechanism of activation does not involve intervention by a retrovirus.

Relationships Between the Two Groups of Proto-Oncogenes

The study of retroviruses and the use of transfection has allowed delineation of two groups of cellular proto-oncogenes. The two groups are, however, not separate and distinct. Instead, we now realize that they have some members in common. As first shown last year, the Ki-*ras* oncogene carried by Kirsten murine sarcoma virus is homologous to oncogenes detected by transfection in the DNA of human lung and colon carcinomas (*26*). The Ha-*ras* oncogene of Harvey murine sarcoma virus is the homolog of the well-studied oncogene of the human EJ/T24 bladder carcinoma cell line (*26, 27*). These relationships made it clear that certain cellular proto-oncogenes can become activated in two alternative ways. They may become associated with retroviruses, or they may become altered via mutational events that depend on nonviral mechanisms.

Examination of Table 1 reveals at least 18 different cellular genes that have been activated into oncogenes by various retroviruses. Curiously, only two of these, the Ha- and Ki-*ras* genes, have also been detected by transfection of tumor DNA's. This might suggest that the remaining 16 cellular genes are not readily activated by the mutational mechanisms that occur during nonviral carcinogenesis; or this might indicate a weakness in the existing transfection-focus assay, which may not register the presence of various oncogenes in tumor DNA.

Fortunately, other techniques are available for detecting the presence of active oncogenes in tumor DNA. Thus, examination of gene structure by the Southern technique has revealed altered (and probably activated) versions of the cellular *myc, myb*, and *abl* genes in several types of human and mouse tumors of nonviral etiology. These oncogenes, originally known from their association with the avian myelocytomatosis and myeloblastosis viruses and Abelson murine leukemia virus, are not readily detected in a transfection test involving NIH 3T3 cells. Other procedures will undoubtedly reveal more genes of this retrovirus-associated group that are active as well in spontaneous or chemically induced tumors.

As shown in Table 1, other oncogenes have been detected by transfection, but these genes have no counterparts among the oncogenes carried by known transforming retroviruses. These include oncogenes from chicken and Burkitt's lymphomas (*22, 28*), rat neuro- and glioblastomas (*11, 29*), and a group of mammary carcinomas (*30*). Perhaps these cellular genes have a structure or physiology that is incompatible with their mobilization by retroviruses.

Mechanisms of Activation of Proto-Oncogenes

The proto-oncogenes and the proteins that they specify form a structurally and functionally heterogeneous group. It is therefore not surprising that various molecular mechanisms are involved in activation of these genes. In fact, five separate mechanisms of proto-oncogene activation have been found to date.

The first mechanism to be documented involves over-expression of a proto-oncogene following acquisition of a novel transcriptional promoter. As Vande Woude and his colleagues showed, the *mos* proto-oncogene of mice, which is biologically inactive after molecular cloning, can be converted experimentally into a potent oncogene by addition of a strong transcriptional promoter (*31*). Another example of this mechanism comes from analogous activation of the Ha-*ras* proto-oncogene of rats (*32*). These oncogenes, created by ligation of cloned DNA segments, acquire activity because their transcripts are produced at much higher levels than those afforded by the promoters associated with the related normal proto-oncogenes.

This theme is repeated in oncogenes created by more natural processes. Thus, the *myc* and *erb*B proto-oncogenes present in several avian hematopoietic neoplasias have become activated after adjacent integration of an avian leukosis proviral DNA segment. This viral segment provides strong transcriptional promoter which, once again, replaces the indigenous promoters of these

genes (*33–35*). Many retroviruses may activate acquired cellular genes by forcing overexpression via the viral transcriptional promoter.

A second mechanism of activation involves overexpression due to amplification of the proto-oncogene or oncogene. The *myc* proto-oncogene is amplified 30 to 50 times in the human promyelocytic leukemia cell line HL-60 (*36, 37*), and is present in comparable amounts in a neuroendocrinal tumor of the colon (*38*). A Ki-*ras* gene is amplified three to five times in a human colon carcinoma cell line (*15*) and as much as 60-fold in an adrenocortical tumor of mice (*39*). Recently, 30 to 100 copies of a newly discovered relative of the *myc* gene, termed N-*myc*, were found in a number of human neuroblastomas (*40*) and a human chronic myelogenous leukemia cell line has been found to carry extra copies of the cellular *abl* gene (*41*). In these cases, the increased gene copy number is presumed to cause corresponding increases in transcript and gene product.

A third mechanism influences levels of transcription and, in turn, the amounts of gene product. This mechanism depends on the poorly understood mechanism of action of "enhancer" sequences, which can increase utilization of transcriptional promoters to which they become linked. The linked promoter may be as far as several kilobases away, and the enhancer may be positioned upstream or downstream of the promoter (*42, 43*). One example of this is the presence of retrovirus genome fragments downstream from the *myc* gene in avian lymphomas (*34*). Here the retrovirus elements appear to act by contributing not a promoter but an enhancer sequence.

Yet another mechanism involves the *myc* oncogene: recent work on mouse plasmacytomas and human Burkitt's lymphomas has demonstrated the juxtaposition of *myc* and immunoglobulin domains following chromosomal translocation. This appears to result in deregulation of the *myc* gene, which loses regulatory sequences of its own and acquires instead normally unlinked sequences involved in immunoglobulin production. This mechanism is explored by Leder in this issue (*44*). Rearranged *myb* sequences have been found in certain mouse plasmacytomas (*45*), but their detailed structure and mechanism of activation remains to be elucidated.

The fifth mechanism depends on alteration in the structure of the oncogene protein. This mechanism is most well documented in the case of the oncogene proteins encoded by the *ras* genes. In the case of the human bladder carcinoma oncogene of the T24/EJ cell line, it is clear that a simple point mutation converted the Ha-*ras* proto-oncogene into a potent oncogene. This G to T transversion caused the glycine, normally present as the 12th residue of the encoded 21,000-dalton protein, to be replaced by a valine (*46*). Another activated version of this gene encodes an aspartate residue at this position (*47*). Recent work on related oncogenes of the Ki-*ras* group also shows that alterations of the 12th residue of the encoded p21 protein lead to oncogenic activation (*48*). A slightly different result stems from study of a human lung carcinoma Ha-*ras* oncogene which carries a mutation affecting amino acid 61 of the p21 protein (*49*). It appears that these changes do not affect the levels of expression of these genes, only the structure of the encoded proteins.

These published results, along with as yet unpublished work of others, suggest that the codons specifying residues 12 and 61 represent critical sites which, when mutated, will often create oncogenic alleles. It seems that point mutations elsewhere in the proto-oncogenes may only serve to inactivate these genes instead of converting them into potent oncogenes.

Although the structures of these various activated oncogenes have been explored in great detail, the precise mechanisms responsible for their creation in spontaneously arising tumors remain obscure. It is widely assumed that these oncogenes are formed by somatic mutation. However, we have little direct proof of this. For example, there is no published comparison of an activated *ras* tumor oncogene with the homologous sequences prepared from the DNA of adjacent normal tissue.

Tissue Specificity of Oncogene Activation

One explanation for the existence of many different oncogenes might be related to the variety of tumors that arise in the body. Each oncogene might become activated only in certain tissue compartments and be specialized in transforming cells of that tissue. The existing results on the *ras* genes and the *myc* gene are not compatible with such a scheme. For example, the N-*ras* oncogene has been detected via transfection in DNA of sarcomas, lymphomas, leukemias of the myeloid lineage, a neuroblastoma, and a colon carcinoma (*14, 16, 23–25*). And the cellular *myc* oncogene has been implicated in the transformation of various hematopoietic cells as well as a neuroendocrinal tumor of the colon (*33, 34, 36–38*). This means that the N-*ras* and *myc* proto-oncogenes are susceptible to activation in a variety of tissues. Moreover, each of the resulting activated oncogenes seems able to affect the behavior of a variety of cell types. This suggests that by studying the effects of *myc* and *ras* on one type of cell (for example, fibroblasts), one may obtain data that are applicable to the transformation of a variety of cell types.

The lack of tissue specificity of these *myc* and *ras* oncogenes does not set the pattern for all oncogenes. Other, less well-characterized oncogenes have been found to date only in association with specific types of tumors. These include an oncogene of chicken lymphomas (*28*), one of rat neuro- and glioblastomas (*29*), one associated with various mammary carcinomas (*30*), and a group of oncogenes, each member of which is associated with tumors representing a specific stage of lymphoid differentiation (*50*).

Limited Powers of a Single Oncogene

The study of cellular oncogenes has generated a long list of these important agents of cellular transformation. But this list reveals little about the complex processes of tumorigenesis in vivo. The creation of a tumor cell within a tissue would seem to require far more than the activation of one of these oncogenes within the cell. Spontaneous or chemically induced tumorigenesis is known to be a multistep process, while the activation of an oncogene such as Ha-*ras* seems to occur as a single, discrete event.

This discrepancy has led to a suspicion that activation of an oncogene such as Ha-*ras* may represent only one component of a multistep process. Furthermore, questions can be raised about the NIH 3T3 cells which are forced into the tumor state after acquisition of only one oncogene (*7–10*). Investigators in several laboratories have pursued this issue by monitoring the activities of an Ha-*ras* oncogene in cells other than NIH 3T3. Their work provides direct demonstration of the limited powers of a single oncogene, acting alone, and of the necessity for cooperative interaction between different oncogenes (*51, 52, 53*).

In our own laboratory we used secondary rat embryo fibroblasts (REF's) as recipients of transfected oncogenes (*51*). Such cells are only several cell generations removed from those present within the rat embryo, and probably deviate only minimally from fully "normal" cells. When the Ha-*ras* oncogene was

applied to REF's in monolayer culture, no foci of transformed cells grew out in the following weeks. Cultures of established cells of the rat-1 or NIH 3T3 lines responded to transfection of the oncogene by producing hundreds of foci under comparable conditions. These results were not due to an inability of the transfected gene to establish itself within the REF's. Rather, it was clear that the REF's could not respond to the acquired gene and encoded gene product by yielding detectable foci of transformants.

The *ras* oncogene was not totally silent in these REF's. If the transfected REF cultures were dispersed and suspended in soft agar, colonies of transformants grew out. This indicated that one transformation phenotype, that of anchorage independence, could indeed be produced by the *ras* oncogene. The presence of the *ras* oncogene could also be revealed in another way. The *ras* oncogene was transfected together with the Ecogpt gene that confers resistance to the cytostatic effects of mycophenolic acid (*54*). The co-transfection protocol ensures that the small number of cells that acquired the Ecogpt gene also took up the *ras* oncogene (*55*). A small number of mycophenolic acid–resistant colonies grew out, and as many as 80 percent of these colonies contained morphologically transformed cells. Therefore, if the growth of the surrounding, untransfected cells was suppressed, then the change in cellular morphology due to the *ras* oncogene could be observed.

Transformants could be isolated from the colonies growing in soft agar and foci growing in the mycophenolic acid–treated monolayers. However, attempts to expand these various transfected cells into larger cell populations failed almost without exception. The *ras*-carrying REF's usually grew for several more cell-doublings and entered a crisis leading to death of all the cells. Attempts at seeding tumors with these cells invariably failed.

The powers of this Ha-*ras* oncogene were thus very limited when the gene was expressed within REF's. However, if recipient cells used for transfection had been previously established and immortalized in culture, as was the case with the rat-1 (or NIH 3T3) cells, a subsequently introduced oncogene was able to force the cells into a fully transformed, tumorigenic state in a single step. Stated differently, it appeared that one consequence of establishing or immortalizing cells in culture was the activation of cellular functions that could cooperate with the *ras* gene to create the full transformation phenotype. The established cells thus appeared to possess all of the traits required for tumorigenicity save those few that the acquired oncogene would specify. A similar conclusion has been reached independently by others (*52*).

These results bore on the issue of multistep carcinogenesis. They showed that a single genetic alteration, such as one leading to creation of a *ras* oncogene, was insufficient to achieve tumorigenic conversion of a normal fibroblast. By implication, other cooperating alterations of the cell were required, the precise nature of which was unclear.

Cooperation Between *ras* and Viral Oncogenes

Work on several DNA tumor viruses, notably polyoma and adenovirus, had demonstrated viral genes that could induce a cell to grow continuously in culture (*56, 57*). Thus, the poorly understood changes that are achieved when a cell line becomes established in culture can be mimicked by specific, well-defined viral genes. These oncogenes, unlike those associated with retroviruses, are truly viral, having evolved in association with the viral genomes over extended periods of time.

In the case of polyoma virus, three separate proteins, the small, middle, and large T antigens, are encoded by the "early" replicative region of the genome that is also active in virus-transformed cells. Dissection of these genes from one another had been hindered by the fact that they overlap within the viral genome. Kamen and his colleagues circumvented this problem by constructing clones that were, in effect, reverse transcripts of each of the three early viral messenger RNA's (mRNA's) (*58*). When the biological activity of these clones was tested by Rassoulzadegan, Cuzin, and their colleagues, distinct biological properties could be assigned to the middle T and large T antigen clones (*56*).

The middle T antigen was found to induce morphological alteration and anchorage independence, while the large T antigen altered serum dependence and life-span in culture. These and other results (*57*) were of great importance, since they showed that some of the critical traits associated with transformation could be assigned to distinct, separable viral genes. Perhaps the phenotypes of establishment and immortalization which rendered cells reactive to the *ras* oncogene could be elicited as well by one or another of these viral oncogenes.

When the middle T and *ras* oncogene clones were co-transfected into REF's, no new phenotypes were observed beyond those induced by *ras* alone. But the large T antigen clone and *ras* together achieved dramatic results. Rapidly expanding foci of transformed cells were induced in the co-transfected cultures. These foci, containing morphologically altered cells, were easily developed into mass cultures and seeded rapidly growing tumors upon inoculation into nude mice (*51*). While the *ras* gene alone behaved like an incomplete oncogene, it was clear that the two oncogenes together achieved complete conversion to tumorigenicity.

While this work was under way, analogous experiments were performed by H. E. Ruley of Cold Spring Harbor Laboratory, in which he examined the cooperation of the *ras* oncogene with the Ela early gene of adenovirus (*53*). Further experiments in our laboratory soon confirmed Ruley's finding that this adenovirus gene could replace the polyoma large antigen gene in a co-transfection with the *ras* oncogene. In both cases, the conversion of a normal cell into a tumor cell could be achieved by the cooperation of two distinct genes, one cellular and one viral.

Cooperation Between *ras* and a Second Cellular Oncogene

These experiments proved that *ras* could induce tumorigenicity when aided by a viral oncogene, and suggested mechanisms whereby DNA tumor viruses might contribute to tumorigenesis by providing one or more of the oncogenes required for this process. However, these data shed little light on those types of carcinogenesis that have no apparent viral involvement. Were there cellular genes which, like large T or Ela, could cooperate with *ras* in creating the tumorigenic state?

An obvious candidate was suggested by earlier work on the cellular *myc* oncogene. Cooper and Neiman had found that chicken lymphomas carried an oncogene capable of fibroblast transformation (termed B*lym*) in addition to the leukosis virus-activated *myc* oncogene (*59*). In our own laboratory, an active *ras* oncogene [called N-*ras* (*23*)] had been found to coexist with altered versions of the *myc* in both a promyelocytic leukemia and in an American Burkitt's lymphoma (*25*). In all these instances, an apparently activated *myc* gene was found together with an oncogene that was capable, like Ha-*ras*, of transforming NIH 3T3 fibroblasts. Perhaps the coexistence of these

active oncogenes within each tumor reflected essential roles that they played together during the tumorigenic process.

This suggested that we try to aid the *ras* gene by introducing it together with an active *myc* oncogene into the REF's. A molecular clone of the provirus of avian MC29 myelocytomatosis virus (*60*), provided by J. M. Bishop, was used as a source of an activated *myc* oncogene. When this *myc* clone was applied to rat-1 cells or REF's, no apparent effect on cellular phenotype was observed. However, when the Ha-*ras* and *myc* oncogene clones were applied together to the REF cultures, dense foci of morphologically transformed cells were found. Acting together, *myc* and *ras* were able to do what neither could do on its own. These co-transfected cells expanded into vigorously growing cultures and seeded rapidly growing tumors in nude mice. This provided some of the first experimental evidence for explaining why multiple cellular oncogenes were found activated in certain tumors—each must perform a distinct function which is required for successful tumorigenesis. Moreover, such experiments provide some explanation at the molecular level of the multistep process of carcinogenesis: each step may reflect a requirement for the activation of a distinct cellular gene, such as an oncogene.

Further Implications of the *ras-myc* Synergism

These results showed that cellular oncogenes, like their counterparts in the genomes of DNA tumor viruses, are functionally heterogeneous. Different oncogenes appear to exert qualitatively distinct effects on the cell. This requires a rethinking of the term oncogene, which cannot simply imply a gene that evokes morphological alteration and focus-formation. Instead, as is obvious from the earlier work with the DNA tumor viruses (*56, 57*) and the presently described experiments (*51, 53*), oncogenes may contribute in a variety of ways to the conversion of a normal cell into a tumor cell.

This raises the question of how many different oncogene functions must cooperate in order to convert a normal cell into one that is tumorigenic. The present results might be taken to indicate that two cellular genes, *ras* and *myc*, are able in concert to achieve this end point. But we are reluctant to conclude this after detailed examination of the tumors induced by *ras* plus *myc*. Initial observations showed that these tumors grew to a substantial size (2 centimeters in diameter) and then stopped growing; in contrast, the *ras* plus large T antigen tumors grew until they killed the host animal. Perhaps the large T antigen contributes multiple functions that are required for full transformation, only one of which corresponds to a function provided by *myc*.

A tumor cell may thus require additional functions beyond those several provided by the *ras* and *myc* genes. The search for a third type of oncogene function may require new biological assays. Most encouraging is the prospect that the number of separate cellular genes involved in the entire process is limited to as few as three. Activation of each of these genes may define an essential step in the carcinogenic process.

Categorization of Viral and Cellular Oncogenes

As mentioned earlier, the number of distinct cellular proto-oncogenes and associated oncogenes now exceeds 20, scattered throughout the cellular genome (Table 1). At least ten different oncogenes have been reported as parts of the genomes of various DNA tumor viruses (*61*). Does this imply the existence of 30 separate physiological functions, or can the number of distinct oncogene functions be very small?

One measure of simplification comes from comparison of structures of the various genes and their encoded proteins. Structural homology often implies functional analogy. In the case of three *ras* genes, Ha-*ras*, Ki-*ras*, and N-*ras*, this principle seems to be on firm footing: although the three genes are widely diverged in overall sequence, the encoded proteins are almost 90 percent identical in amino acid sequence (*23, 62*).

A second group of cellular genes includes those that have demonstrable tyrosine kinase activity and several structurally related genes whose products have not yet been associated with an enzymatic activity. This is the gene group that includes *src, yes, fes/fps, abl, ros, fgr, erbB, fms, mos*, and *raf*. While these genes may exhibit structural homologies, conclusions concerning functional analogy are problematical: the homologies are only vestigial (*63*); the encoded gene products are associated with different cellular sites (Table 1) (*64*); and these oncogenes are all distantly related to the gene encoding the catalytic subunit of the cyclic AMP (adenosine 3′,5′-monophosphate)–dependent protein kinase (*65*), whose functioning appears to be quite unrelated to cellular transformation. Evolution may have conferred distinct functions on the diverse members of this group, and attempts at associating all these genes with one type of transforming function are unjustified at present.

Further complexity is encountered when attempting to categorize the oncogenes of the DNA tumor viruses, and to relate these genes and encoded functions to those oncogenes of cellular origin. These viral oncogenes have not been acquired from a cellular genome within the past decades, as is the case with the retrovirus-associated genes. Instead, they are truly viral, having been evolved independently by these viruses, probably over many millions of years. Within a family of such viruses (for example, adenoviruses), clear and obvious homologies and analogies can be discerned. But between the families of DNA tumor viruses (that is, adeno-, herpes-, and papovaviruses) and the cellular genome no obvious homologies have been defined. One important and intriguing exception to this has been recently reported—a vestigial homology between the Ela antigen of adenovirus and the cellular *myc* and *myb* genes (*66*).

One resolution of these complexities may come from *functional* assays of these disparate genes. An example of such an assay is provided by the co-transfection test described above in which genes can be defined by their ability to help *ras* or *myc* to transform REF's. Using such criteria, we have placed Ha-*ras*, N-*ras*, and polyoma middle T in one functional group, each member of which is able to cooperate with *myc* in transformation. Conversely, we have assigned *myc*, large T, Ela, and the ill-defined cellular "establishment/immortalization genes" to a second group, since each member of this group helps *ras* to transform REF's (*51, 53*). This categorization is being extended by way of co-transfection tests with a variety of cloned viral or cellular oncogenes.

Other types of functional tests could be envisioned as well. For example, one assay might depend on the ability of cloned viral or cellular oncogenes to complement mutant viral genes required for a full cycle of viral replication. One test of this type has already been performed. An immediate early gene of pseudorabies virus is able to provide functions lacking in an Ela mutant of adenovirus (*67*). Perhaps this gene will eventually be placed in the functional classes including Ela, *myc*, and large T.

A third strategy has also yielded important insights into the functional relations among oncogenes (*68*). Revertants

of virally transformed cells have been isolated which resist attempted retransformation by viruses carrying Ha-*ras*, Ki-*ras*, *fes*, or *src* oncogenes. However, these cells can be retransformed by viruses carrying the *sis*, *mos*, or *fms* oncogenes. This suggests that the activities of the first four oncogenes converge on a common target that is bypassed by the last three. Taken in concert, the results of these tests may allow one to allocate the large number of oncogenes to a small number of groups, each group containing the genes whose functions impinge on a common regulatory pathway.

One aspect of the already established groupings is most intriguing. This concerns the cellular localization of the gene products encoded by the oncogenes that have been categorized by the co-transfection tests. The proteins made by one group—*myc*, large T, and Ela—are all associated with nuclear structures, perhaps the nuclear matrix (*69*). In contrast, the *ras* proteins and the middle T antigen are attached to the inner surface of the plasma membrane (*70*). This is compatible with the presence of one vital cellular target of oncogene action in the nucleus and another near the plasma membrane. Perhaps both targets must be acted on by oncogene proteins in order to achieve full transformation of the cell.

Prospects

The procedures of gene transfer and molecular cloning have made it possible to dissect out some of the centrally important determinants of the cancer process. These determinants—the oncogenes—act pleiotropically, since their gene products clearly affect complex regulatory cascades within the cell. Many of these cascades will be understood over the next decade, and with this will come insight into the molecular bases of some of the well-known idiosyncracies of the cancer cell, including its altered shape, substrate interaction, growth factor dependence, and energy metabolism.

It also appears that other peculiarities of the carcinogenic process may be explained in terms of the sequential activation of certain oncogenes. The experimental induction of cancer involving initiators and promoters may reflect requirements for activation of specific genes. For example, recent work in Great Britain demonstrated that tumor cells could be created in a two-step process involving initial immortalization by a chemical carcinogen followed by introduction of a cloned oncogene (*52*). The progression of tumors from precancerous growths, such as papillomas and adenomas, into autonomously growing cancers may also have an underlying molecular basis involving oncogenes.

This is not to say that all aspects of the cancer process will be readily understood in terms of the oncogenes with which we are now familiar. Cancer cells can modulate their antigenicity to evade the immune defenses. They can also acquire an ability to break off from a primary tumor and seed secondary growths at distant sites. Such cancer phenotypes do not represent initial derangements in growth control, but rather secondary adaptations that favor survival and clonal expansion. The precedent of the oncogenes leads us to the belief that even these complex biological phenomena will also be traced back to alteration of specific genes.

The eventual development of novel therapeutics against cancer cells will require discovery of agents that recognize targets that are present only in the cancer cell and are at the same time essential for the continued growth of this cell. Oncogenes and their proteins represent good candidates for targets of this sort. These deviant forms of the proto-oncogenes may be specific to cancer cells. And unlike a variety of other cancer cell traits, such as certain surface antigens, oncogenes may be indispensable for the ongoing growth of the tumor cell. By learning how the oncogene-encoded proteins work, we may learn how to antagonize their functioning and one day know how to reverse the engines that drive cancer cells forward.

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Yeast RNA Polymerase II Genes: Isolation with Antibody Probes

Richard A. Young and Ronald W. Davis

Summary. Genes encoding yeast RNA polymerase II subunits were cloned. Efficient isolation of these genes was accomplished by probing a phage λgt11 recombinant DNA expression library with polyvalent antibodies directed against purified yeast RNA polymerase II. The identity of genes that specify the largest RNA polymerase II subunits, the 220,000- and 150,000-dalton polypeptides, was confirmed by competitive radioimmune assay. Both of these genes exist in single copy in the yeast *Saccharomyces cerevisiae*.

Three distinct classes of RNA polymerase are responsible for the transcription of DNA into RNA in eukaryotes (*1, 2*). RNA polymerase I synthesizes ribosomal RNA; RNA polymerase II is responsible for the transcription of messenger RNA (mRNA); and RNA polymerase III synthesizes small RNA's such as transfer RNA and 5*S* ribosomal RNA. The RNA polymerase within each class is composed of 8 to 12 subunits, some of which belong only to that class and some of which are shared by polymerase from the other classes (*3*). Attempts to confirm and extend these observations have been hampered by the structural and functional complexities of these enzymes, by the limitations of current in vitro biochemical assays, and by the paucity of RNA polymerase mutants and difficulties in their isolation.

A thorough understanding of the processes controlling transcription and thus gene expression requires a detailed understanding of the components of the transcription apparatus. RNA polymerase subunits have been defined empirically as the smallest number of protein components that copurify and retain DNA-dependent RNA synthesis activity in vitro. However, this approach does not distinguish between proteins that are required for activity and those that simply copurify. Moreover, the assays used to define RNA polymerase subunits have not been useful in identifying proteins required for transcription initiation and termination activities, nor do they reveal other possible functions in which RNA polymerases might participate (for example, RNA processing).

Several factors make a systematic investigation of RNA polymerase subunit structure and function compelling in yeast. Yeast RNA polymerases have undergone careful biochemical scrutiny at the subunit level (*4, 5*). In addition, yeast RNA polymerases appear structurally and functionally very similar to those of higher eukaryotes; by immunological criteria, the two large RNA polymerase II subunits (220,000 and 150,000 daltons in yeast) are particularly well conserved (*6*). Finally, yeasts are amenable to study with a combination of biochemical and genetic tools. Thus, the isolation of genes encoding yeast RNA polymerase subunits should facilitate a genetic and biochemical definition of the enzyme's structure and function in eukaryotes.

As a means of cloning gene sequences efficiently when antibodies are used as probes of their polypeptide products, a method has been developed that permits rapid screening of large libraries of recombinant DNA in the phage expression vector λgt11 (*7*). This method was used with two modifications to isolate RNA polymerase II subunit genes (Fig. 1). Antigen produced in λ phage plaques rather than in λ lysogen colonies was immobilized on nitrocellulose filters. Host cells carrying multiple copies of the lac repressor gene, *lac*I, were used to conditionally regulate the potentially deleterious expression of the foreign genes controlled by the *lac*Z promoter. Only after taking this latter precaution was it possible to isolate some of the genes of interest.

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