Biochemistry

 Volume 28, Number 21 October 17, 1989

Perspectives in Biochemistry

Positive and Negative Controls on Cell Growth†

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Received May 15, 1989; Revised Manuscript Received June 12, 1989

The oncogenes that have attracted great attention over the past decade would seem to provide us with much of the explanation at the molecular level of the origins of cancer. We now count as many as 50 distinct cellular oncogenes, many of which are able to induce at least some of the cell phenotypes associated with the neoplastic state. Of these, about a dozen are directly implicated in the etiology of human cancer. The remainder have been found associated with a variety of mammalian and avian retroviruses, having been abstracted from the cellular genome by these transducing viruses. Yet others in this large group have been uncovered by virtue of nucleic acid homologies with previously known oncogenes (Bishop, 1983; Varmus, 1984). Taken together, these genes leave an impression of a richly complex cellular growth regulatory circuitry that can be perturbed at many points by the actions of oncogenes and their encoded proteins.

In spite of this already well-documented complexity, there are reasons to believe that oncogenes can provide at best only part of the explanation of the molecular and genetic mechanisms of cancer. These reasons stem from two attributes that are shared by all these genes. First, oncogenes act in one way or another as positive effectors of cell growth. As such, the oncogene paradigm overlooks the possible existence of an equally complex and important cellular growth regulatory network that is dedicated to suppressing or constraining cell growth. Such negative regulatory genes, to the extent that they exist, could become involved in cancer when they are lost or inactivated; such loss would remove a normally existing brake or constraint on the cell's growth, thereby triggering the runaway growth of neoplasia.

Second, oncogenes invariably arise as consequences of somatic alterations of the target cell genome. Thus, in human tumors, the cellular oncogenes studied have been found to arise as a consequence of well-defined somatic mutations of normal growth regulating genes often termed protooncogenes. A minority of human tumors acquire viral oncogenes following infectious events occurring in one or another target organ; such infections also represent somatic changes in the cell genome. Yet it is clear that inborn genetic determinants can also strongly affect the probability of tumor formation during an organism's lifetime. The existence of inherited cancer genes that are passed through the germline is also not addressed by the oncogene paradigm.

A class of genes is described here that promises to address both deficiencies. Altered forms of one of these genes are indeed transmitted through the germline and can strongly affect tumor incidence. Moreover, these genes behave as if they were negative regulators of growth, thus acting in a fashion directly opposite to that of the well-studied oncogenes. Genes in this new class have been termed "tumor-suppressor" genes, "recessive oncogenes", "antioncogenes", "emerogenes", and "growth-suppressor" genes (Klein, 1987). None of these terms is ideal; indeed, because we do not understand the normal functions of these genes, use of any specific term is a bit presumptuous. Most acceptable is the term growth-suppressor gene, which will be used here.

Evidence of Genetic Loss during Tumor Formation

Two lines of work have converged on the idea that loss of genetic functions underlies at least some of the steps of carcinogenesis. The first of these derived from the use of somatic cell hybridization, which was achieved by fusion of cells in monolayer culture. The resulting genetic hybrids provided the first indication that many tumor cells lack critical genetic elements that are present and functional in their normal counterparts. Upon fusion of tumor cells with normal cells, the great majority of resulting hybrids are found to be nontumorigenic. Such loss of tumorigenicity has been observed for a wide range of inter- and intraspecific cell hybrids (Harris, 1988), including hybrids of tumor cells fused with normal cells from the same tissue. These results imply that the normal parent used in the hybridization process is contributing genes

†Some of the work described here was supported by Outstanding Investigator Grant 5-R35-CA39826 of the National Cancer Institute and by American Cancer Society Grant CD355 to R.A.W., who is an American Cancer Research Professor.

0006-2960/89/0428-8263$01.50/0

to the tumorigenic partner that impose normal growth control on the latter. One presumes that these normal genes replace similar or identical genes that were previously lost from the tumor cell during its progression from normalcy to malignancy.

Results of this sort are difficult to interpret with precision, as they involve the comingling of two entire cell genomes. More focused conclusions come from work showing that only a small number of genes derived from the normal parent are required for the reimposition of normal growth control on the tumorigenic partner. Such observations are made possible by the karyotypic instability of certain cell hybrids, which tend to lose preferentially the chromosomes of one or another parent. In hybrids in which the normal parent's chromosomes are preferentially lost, one may often observe the reemergence of tumorigenic cell clones. This reacquisition of a tumorigenic phenotype can be correlated with the loss of one or another specific chromosome originating with the normal parent. This suggests the presence of a gene or small number of genes on this chromosome which act(s) to impose normal growth control on the tumor cell (Stanbridge et al., 1981).

Even more persuasive than these correlative experiments are those involving microcell fusions that enable an investigator to introduce selectively a specific normal chromosome into the tumorigenic cell. In the case of human HeLa cells, loss of tumorigenicity is observed upon introduction of a normal chromosome 11. Indeed, a single copy of this normal chromosome suffices to restore more normal growth control (Saxon et al., 1986). The nature of the chromosome 11 associated growth controlling gene(s) remains obscure.

The second line of work underscoring the importance of genetic loss during tumor pathogenesis has involved study of a small set of relatively rare tumors—retinoblastoma and Wilms' tumor. Both are tumors of children, and their biology suggests that the tumor cells represent embryonic cell types that have not succeeded in their normal progression to a differentiated state.

Here the indications of genetic loss are even more direct: karyological analysis of certain tumors of each type has revealed the presence of interstitial deletions that repeatedly affect specific chromosomal bands. In the case of retinoblastoma, deletion of 13q14 is occasionally observed (Yunis & Ramsay, 1978); Wilms' tumors often show deletion of chromosomal material around band 11q13 (Riccardi et al., 1978). These occasionally observed karyotypic abnormalities must underrepresent the true frequency of genetic loss. After all, millions of DNA base pairs need be lost before the microscopic morphology of a chromosome is affected. Many other deletions involving loss of smaller DNA segments may occur much more frequently but escape detection by the karyologist. In the case of retinoblastoma, these various chromosome 13q14 alterations have been presumed to involve a specific locus termed Rb.

The Case of Retinoblastoma

Retinoblastoma tumors are relatively rare, occurring in only one of 20000 children, and are almost always seen from birth up to the age of 5. The disease occurs as two distinct clinical entities. The "familial" form of retinoblastoma is seen in children having a similarly afflicted parent. These children usually show multiple independent foci of tumor formation affecting both eyes. In "sporadic" retinoblastoma, only a single focus of tumor formation is observed, and then in a child having no family history of this disease.

Alfred Knudson provided a unifying genetic explanation of these two forms of disease by proposing that both depend upon a common genetic mechanism involving two critical mutations (Knudson, 1971). He argued that the appearance of sporadic retinoblastomas depends upon two somatic mutations sustained by a single retinal stem cell, the descendants of which then proliferate to form the tumorigenic cell clone. In the case of familial retinoblastoma, he proposed that one of the required mutations is already present in the conceptus, having been acquired from sperm or egg. This mutation, by necessity, is implanted in all cells of the developing retina. Knudson suggested that the second of the required mutations is then sustained in one or another retinal cell, creating the doubly mutated cell whose genotype now favors neoplastic growth. The occurrence of doubly mutated retinal cells is obviously strongly favored in those children who already carry one mutant allele in each of their retinal cells. In this sense, one can say that the presence of such an allele from the moment of conception strongly predisposes to tumor onset.

We now realize that the two target genes postulated by Knudson are in fact the two copies of the Rb gene on chromosome 13 (Sparkes et al., 1983; Godbout et al., 1983; Cavenee et al., 1983; Dryja et al., 1984). The mutations leading to retinoblastoma involve molecular changes that cause loss of Rb gene function. This was already apparent from the aforementioned karyological studies that showed frequent deletion of the chromosomal region carrying the Rb locus. Because loss of both Rb gene copies favors malignant cell transformation, this would suggest, as mentioned earlier, that the intact Rb gene functions normally to suppress the runaway growth of retinal cells. Indeed, a single intact copy would appear to suffice to orchestrate normal cell growth, since the bulk of the retina in a child afflicted with familial retinoblastoma appears to be perfectly normal.

The Rb gene has been isolated by molecular cloning (Friend et al., 1986; Lee et al., 1987; Fung et al., 1987; Bookstein et al., 1988). It is a large gene of 190 kilobases that is affected by gross structural alterations in as many as 30% of tumors. These estimates of the frequency of Rb gene alterations in retinoblastomas have been obtained by Southern blotting analysis of genomic DNAs, a procedure that provides a relatively insensitive assay of mutation. Recent studies of Rb alleles found in tumor cells and having a grossly normal structure have revealed subtle changes in gene sequences including point mutations that affect splicing of Rb mRNA precursors and result in the deletion of exons from the Rb mRNA (Horowitz et al., 1989). The most sensitive assays of gene inactivation are those designed to analyze the Rb protein through use of immunoprecipitation. One recent study has revealed the absence of the Rb protein from 18 out of 18 retinoblastoma cell lysates although this protein is readily demonstrable in normal retinoblasts, the apparent precursors of the tumor cells (J. Horowitz and R. A. Weinberg, unpublished results). Taken together, these studies suggest that inactivation of both Rb alleles occurs during the genesis of virtually all retinoblastomas. A functional proof that the cloned DNA indeed represents the Rb gene has been produced recently through the introduction of the cloned gene into retinoblastoma and osteosarcoma cells (Huang et al., 1988). These cells lose their tumorigenicity following reacquisition of the gene; this in turn strongly supports the conclusion that display of the tumor phenotype depends upon loss of this gene from these cells.

Puzzles and Paradoxes Surrounding the Rb Gene

The Rb gene is involved in far more than the genesis of retinoblastomas. This was already apparent from long-term followups of children cured of the familial type of retinoblastoma early in life. These children suffer various types

of sarcoma later in life at rates greatly above normal (Draper et al., 1986; Toguchida et al., 1988). Accordingly, Rb hemizygosity predisposes to tumors in at least two developmental lineages. Moreover, use of the cloned Rb gene as probe has also shown RB inactivation occurring frequently in osteosarcomas and soft tissue sarcomas that appear to arise purely from somatic mutational accidents (Friend et al., 1987; Weichselbaum et al., 1988).

This picture is further complicated by the more recent findings that Rb inactivation is also found in a very high proportion of small cell lung carcinomas, in about one-third of bladder carcinomas, and in a smaller proportion of mammary carcinomas (Harbour et al., 1988; Lee et al., 1988; T'Ang et al., 1988). These gene losses all appear due to somatic mutation. This creates an interesting paradox since these particular tumor types are not known to occur with increased rates in children born with defective Rb alleles. Thus, we have no idea why the loss of the surviving Rb allele in these children, which must occur with substantial frequency throughout their bodies, does not cause them to contract these other types of tumors at unusually high rates.

The Rb gene is expressed in a wide range of tissues throughout the body (Bernards et al., 1989). Such expression would suggest an important role of Rb in regulating the proliferation of a wide variety of cell types. Once again a puzzle arises, in that Rb inactivation triggers tumor formation in only a relatively narrow range of tissue types. This might suggest that intactness of the Rb gene is not critical to the growth regulation of many of the cell types in which it is expressed.

Yet a third puzzle surrounding the Rb gene concerns the species distribution of the retinoblastomas: it has been seen only in human beings even though there have been extensive opportunities to observe the disease in many other mammals. Not unexpectedly, the Rb gene is represented in the genomes of all other mammals and even more distantly related chordates. The mouse Rb homologue encodes a protein that is 90% identical in its amino acid sequence to its human counterpart (Bernards et al., 1989). Clearly Rb is an important regulator of growth in other mammals which must also sustain occasional homozygous losses of the Rb gene. Perhaps some idiosyncrasy of human embryogenesis makes our retinas particularly susceptible to tumor formation; alternatively, the molecular biology of our retinal cells may cause them to be especially dependent on Rb function for their growth regulation.

The Rb-Encoded Protein

The gene product of Rb is a 105-kilodalton nuclear phosphoprotein that has DNA binding properties (Lee et al., 1987; Whyte et al., 1988). These facts focus one's thinking on mechanisms of action involving regulation of transcription. Although plausible, such mechanistic speculations are still premature, since no specific transcriptional factor or target gene is known to be affected by this protein, which is termed p105-Rb.

One year ago, insight into the functioning of this protein came in a dramatic fashion and from a totally unexpected quarter. The work providing this insight originated in laboratories studying the molecular mechanisms of cell transformation by DNA tumor viruses, specifically human adenovirus type 5. This virus induces upper respiratory tract infections in humans, but in certain rodents it can act as a potently tumorigenic agent. Its ability to induce tumors is traceable to two oncogenes carried in the viral genome, termed E1A and E1B.

The E1A oncogene is the most intensively studied of these by virtue of its multiple effects on cells. When introduced in rodent embryo cells, it has an "immortalizing" power that enables these cells to grow indefinitely in culture, in this way avoiding the senescence that is the fate of normal embryo cells. The E1A oncogene can also collaborate with a *ras* oncogene in converting a fully normal embryo cell into a tumorigenic one (Ruley, 1983). Finally, E1A-encoded proteins can act as transcriptional regulators, inducing the expression of some viral and cellular genes and repressing yet others (Berk, 1986).

These multiple powers of the E1A oncogene required a molecular explanation. Two laboratories studying E1A function found an important clue in the observation that the E1A-encoded proteins are able to form stable complexes with as many as six distinct cellular proteins (Yee & Branton, 1985; Harlow et al., 1986). Thus, antibodies specifically reactive with the viral oncoproteins precipitate both these proteins and cellular proteins from adenovirus-transformed cells. The presence of these six cellular proteins in the immunoprecipitates is not due to some adventitious cross-reactivity of the precipitating antibodies, which recognizes none of these cellular proteins in lysates of cells not transformed by adenovirus.

Such observations are not without precedent in the field of DNA tumor virology. The SV40 large T antigen oncoprotein is known to complex with the host cell protein, p53 (Lane & Crawford, 1979; McCormick & Harlow, 1980). Similarly, the middle T oncoprotein of polyomavirus complexes with and activates the cellular $pp60^{src}$ protein (Courtneidge & Smith, 1983; Bolen et al., 1984). Clearly, tumor viruses have evolved an ability to alter host cell metabolism by developing proteins that are able to complex with specific target proteins of host cell origin. One assumes that the functioning of these host cell targets is perturbed following complex formation with the viral oncoproteins.

In the case of the adenovirus E1A protein, its wide range of activities may be attributable to its ability to complex with six or more cellular proteins, each of which may serve as a controller of one or another important cellular regulatory pathway. The results of last summer revealed that one of these host cell targets is p105-Rb, the same protein that is lost from retinoblastomas and osteosarcomas through genetic alteration of the Rb gene (Whyte et al., 1988).

This confluence of two apparently unrelated areas of research revealed a fully unexpected confrontation between the gene product of an oncogene and a gene product ostensibly involved in contraining normal cellular growth. The first of these acts as a growth agonist while the second would seem to act as a growth antagonist. One simple model would state that, by complexing with p105-Rb, the E1A oncoprotein is able to neutralize its growth-suppressing effects and, in so doing, liberate the cell from previously operative constraints on growth. All this encourages those who would call Rb an "antioncogene", but this term still falls short, since it would not seem that the normal function of the Rb gene is to counteract oncogene action.

Coming close on the heels of the discovery of the E1A·p105-Rb complex, findings from two other groups extended this paradigm by demonstrating that this p105-Rb also forms complexes with two other, distinct viral oncoproteins. The SV40 large T oncoprotein (SV40 LT) forms a complex with p105-Rb in addition to its previously mentioned ability to complex with the cellular p53 protein (De Caprio et al., 1988). In addition, the E7 oncoprotein of human papillomavirus type 16 also has binding affinity for p105-Rb (Dyson et al., 1989). This latter virus is implicated as an etiologic agent in a sub-

stantial proportion of cervical carcinomas.

These more recent findings attach even greater significance to p105-Rb. The three oncogene-encoded proteins are structurally very distinct and are specified by viruses that otherwise have little in common. This ability of their respective oncoproteins to complex with p105-Rb would seem to have arisen through some process of convergent evolution governed by the central position of p105-Rb in regulating cell growth and the importance of its deregulation to various viral growth cycles. Thus, by altering p105-Rb function, these viral oncoproteins may allow a quiescent cell to progress to a more active growth state that results in a more hospitable intracellular milieu for virus replication.

Biological tests had previously shown that these three viral oncogenes function analogously in cell transformation. Like E1A, the other viral oncogenes act to immortalize embryo cells. In addition, like E1A, the SV40 LT and HPV E7 oncogenes can each collaborate with a *ras* oncogene to effect the conversion of embryo fibroblasts to a tumorigenic state (Land et al., 1983; Phelps et al., 1988). Hence, the shared physiological functions of these three oncogenes can now be traced to a common biochemical substrate.

One final set of data underscores the relevance and importance of these complexes to the transforming powers of the oncogene proteins. Mutations of the E1A and SV40 LT oncogenes that knock out their transforming abilities simultaneously ablate their ability to complex with p105-Rb (De Caprio et al., 1988; Whyte et al., 1989). This connection between complex formation and transforming ability is highlighted most dramatically by a simple point mutation in SV40 LT that wipes out both traits with one blow (De Caprio et al., 1988). Accordingly, these complexes cannot be dismissed as adventitious aggregation artifacts; instead, they clearly reflect important functional interactions.

Models of p105-Rb Function

These various transforming mechanisms involving either alterations of the Rb gene or perturbation of its encoded protein force one to confront the role of gene and protein in the normal cell. How indeed do these act to constrain normal cell proliferation? And what are the precise consequences of removing p105-Rb from the cell or altering its function through complex formation?

Two quite different models come to mind. The first holds that, in nongrowing cells, p105-Rb acts as a suppressor of growth, perhaps by repressing the expression of genes essential for proliferation. Upon receiving mitogenic stimuli, signals are conveyed to p105-Rb that neutralize its function and thus allow the derepression of functions essential for growth. These signals impinging upon p105-Rb might include cellular proteins that act analogously to the viral oncoproteins described earlier. For example, increased levels of the cellular *myc* protein are induced by mitogens, and the *myc* protein is said to have certain structural and functional similarities with the viral oncoproteins described earlier (Phelps et al., 1988; Moran, 1988; Ralston & Bishop, 1983; Figge et al., 1988; Vousden & Jat, 1989). Thus, a *myc*-like protein could interact with p105-Rb and act to depress growth functions.

Following an alternative model, p105-Rb sits in an inactive state in growing cells. When these cells encounter growth-inhibitory signals in their environment, such signals may be transduced to the nucleus where they activate p105-Rb molecules that then proceed to down-regulate cellular functions including the transcription of genes essential for continued growth. Currently available evidence does not allow us to choose one of these models over the other.

Two other biochemical/molecular phenomena merit mention. First, no evidence obtained to date directly implicates p105-Rb as a transcription regulator. The E1A protein that interacts with p105-Rb is undeniably a regulator of transcription, but site-directed mutagenesis indicates that this activity is assignable to domains of the E1A protein that are distinct from the regions involved in transformation and p105-Rb binding (Lillie et al., 1987). Second, p105-Rb represents a collection of distinct molecular species that differ in their state of phosphorylation. Since SV40 LT oncoprotein binds only to the unphosphorylated form of p105-Rb (Ludlow et al., 1989), this might suggest that this subfraction of the p105-Rb pool represents its biologically active form that mediates growth regulation.

Growth Suppressor Genes Masquerading as Oncogenes

The Rb gene would appear to be only one example of a large class of cellular genes that act analogously to down-regulate cell growth. Most of these genes are known only from indirect types of genetic analysis. However, it now appears that, unbeknownst to us, two of these antigrowth genes have been in the hands of molecular biologists for many years, each masquerading as a growth-stimulating oncogene.

The first of these is *erb*A, an oncogene known originally from its presence in the genome of avian erythroblastosis virus. The *erb*A oncogene resides in this viral genome with a second viral oncogene, *erb*B, which like *erb*A is a transduced cellular gene that has acquired oncogenic potential following its mobilization by the retrovirus. Each of these viral oncogenes stems from a well-known cellular progenitor: the normal cellular version (i.e., the protooncogene) of *erb*A encodes the thyroid hormone receptor while the cellular progenitor of *erb*B specifies the epidermal growth factor (EGF) receptor (Sap et al., 1986; Weinberger et al., 1986; Downward et al., 1984).

Altered versions of the two receptor proteins conspire with one another to induce malignant conversion of avian erythroid precursors. The *erb*B oncoprotein acts as a mitogen to drive the proliferation of undifferentiated precursors of the avian erythrocytes. However, these erythroblasts tend to differentiate with high frequency into erythrocytes, which lack proliferative potential. Because of this, erythroid precursors transformed by *erb*B alone are poorly tumorigenic. *erb*A acts in a distinct and synergistic way on these erythroid cells by blocking their ability to differentiate. As such, it traps these cells in a pool of undifferentiated red cell precursors whose size is continuously expanded by the growth-stimulatory effects of the *erb*B oncoprotein (Graf & Beug, 1983).

Recent biochemical studies have generated a provocative model of how the *erb*A oncoprotein acts to block differentiation: it actively blocks the functioning of its normal counterpart, the thyroid hormone (triiodothyronine, T3) receptor (K. Damm, C. C. Thompson, and R. M. Evans, submitted for publication). It would appear that, during normal avian erythropoiesis, ambient levels of the T3 hormone cause the receptor to activate expression of a bank of differentiation-specific genes. In this way, the normal T3 receptor acts as a growth suppressor since the resulting differentiated cells, in this instance red cells, irreversibly lose proliferative potential. In the presence of the aberrantly functioning *erb*A oncoprotein, normal T3 receptor present in the same cell is unable to interact productively with its bank of responder genes, and thus the path to differentiation is blocked. In genetic terms, the oncogenic (i.e., *erb*A) allele of the T3 receptor gene would appear to act as a dominant negative, blocking the functioning of its wild-type counterpart.

The *p53* gene, mentioned previously in this discussion, represents a second growth-suppressing gene that until recently was viewed as a growth agonist. The *p53* gene is named after its encoded protein. This protein is known largely from its association with the SV40 LT oncoprotein in virus-transformed rodent cells. SV40 LT avidly binds this host cell protein (Lane & Crawford, 1979; McCormick & Harlow, 1980). In so doing, it increases the half-life of p53 from ca. 15 min to more than 24 h. As a consequence, the steady-state level of p53 is increased by as much as 2 orders of magnitude (Oren et al., 1981).

This large increase in p53 protein levels has parallels to the deregulation of expression levels seen upon activation of oncogenes like *myc* and *fos* (Adams et al., 1985; Coppola & Cole, 1986; Baumbach et al., 1986; Ruther et al., 1987; Verma & Sassone-Corsi, 1987). These oncogenic activations are achieved through deregulation of transcript levels of the respective genes. SV40 LT achieves a similar end result, but through the expedient of stabilizing the gene product. The increased level of gene product was presumed in turn to have a growth-promoting effect on the cell, resulting ultimately in cell transformation. Moreover, *p53* mRNA levels increase in the cell in response to mitogens (Reich, 1984), further strengthening the analogy with the *myc* and *fos* genes, which are also turned on by serum factors.

An apparently conclusive proof of *p53*'s growth-agonistic function was provided several years ago when three groups showed that an oncogene can be created by fusing *p53* cDNA with a strong constitutive transcriptional promoter. The resulting chimeric construct behaved like clones of the *myc*, E1A, SV40 LT, and HPV E7 oncogenes: it could immortalize cells and could collaborate with a *ras* oncogene in malignant transformation (Eliyahu et al., 1984; Parada et al., 1984; Jenkins et al., 1984).

A discordant note soon came from reports that the *p53* gene is deleted or grossly damaged in certain tumor cell genomes (Mowat et al., 1985). This is hardly consistent with the proposed role of *p53* as a growth-stimulating gene. More to the point were detailed characterizations of the cDNAs used in the earlier experiments, which were found unexpectedly to carry point mutations (Finlay et al., 1988; Eliyahu et al., 1988, 1989; Hinds et al., 1989). Moreover, when p53 cDNAs carrying the bona fide wild-type sequence were used in attempts to construct oncogenes, these were found to be actively inhibitory for growth (Eliyahu et al., 1989; Finlay et al., 1989).

These data suggest that the normal *p53* gene acts as a growth-suppressing element in the cell and that mutant forms of its encoded protein act in a quite opposite way to stimulate growth. In strong analogy with *erb*A, discussed above, it now appears that mutant alleles of *p53* act as dominant negatives to inhibit functioning of wild-type *p53* alleles. How this is achieved on a molecular level is not yet totally clear. One clue may be provided by the observation that p53 molecules form stable oligomers (Kraiss et al., 1988). In summary, two genetic mechanisms can involve *p53* in tumorigenesis: reading frame mutations that create dominant negative alleles that neutralize function of the surviving wild-type allele, and mutations that inactivate both copies of this gene. In either event, the consequence of this is the physiological inactivation of p53 function, resulting in turn in a release from its growth-constraining effects.

Genetic Mechanisms That Involve Growth-Suppressor Genes in the Cancer Process

We have described two distinct genetic mechanisms involving growth-suppressor genes that favor the malignant outgrowth of tumor cells: the inactivation of both gene copies and the creation of dominant negative alleles that succeed in neutralizing the activity of the surviving wild-type allele. In the case of the Rb gene, the inactivation of gene copies creates recessively acting null alleles. When both gene copies are reduced to this state, then runaway cell growth is unleashed. To date, dominant negative alleles of Rb have not been reported although they may well exist. In the case of *p53*, the neutralization of gene function can be achieved both through gene inactivation and through the creation of dominant negative alleles. A dominant negative allele of *erb*A has been documented; null alleles have not yet been seen in tumor cell genomes and presumably await discovery. The end result of all these various genetic alterations is the same—removal of a previously operative barrier to cell proliferation.

As suggested earlier, these three genes appear as representatives of a much larger class of cellular genes that normally function as growth suppressors. The indications for the existence of these genes rest largely on indirect genetic arguments that are worth describing in outline. Many of these genes appear to behave like the Rb in tumorigenesis: conversion of both of their copies to null alleles triggers cancer in one or another organ.

Clues pointing to the existence of these genes derive from the specific genetic mechanisms believed responsible for the elimination of the two functional copies of these genes. To repeat the pattern described for sporadic retinoblastoma, we note the following: an initial random somatic mutation inactivates one Rb copy; subsequently, a second somatic event removes the surviving wild-type allele. Critical to our discussion is the mechanism responsible for loss of the *second* allele. It could in principle depend upon a mutational event that creates a novel null allele unrelated to the inactive allele generated earlier on the other chromosome. The probability of each of these two mutational events would be equal and very low (e.g., 10^{-6} per cell generation), and the probability of nullizygosity of the cell would be the product of these two small probabilities.

A far easier route to losing the second gene copy is provided by mechanisms involving chromosomal loss or mitotic recombination. Here the chromosomal region carrying the surviving wild-type allele is lost wholesale and replaced by a duplicated copy of the corresponding region on the homologous, already mutated chromosome. Such events may occur as frequently as 10^{-3} per cell generation and now result in two identical copies of the initially mutated, defective allele. Importantly, any previous heterozygosity that existed in the chromosomal region surrounding the growth-suppressing gene is lost, since all chromosomal markers in this region are now present in two identical copies. Thus, this chromosomal region has suffered a "reduction to homozygosity".

All this leads to a powerful and simple genetic analysis that involves searching tumor cell genomes for chromosomal regions that repeatedly suffer reductions to homozygosity (Hansen & Cavenee, 1987). Repeated observation of chromosome-specific loss of heterozygosity in a series of tumors represents prima facie evidence for the presence of a growth-suppressor gene on this chromosome, recessive alleles of which are able to affect the neoplastic phenotype when the dominant wild-type alleles are lost. The chromosomal markers most readily used to detect loss of heterozygosity are restriction fragment length polymorphisms (RFLPs), detected through Southern blotting and use of auspiciously chosen probes. In this fashion, growth-suppressor genes involved in acoustic neuromas have been assigned to chromosome 22 and genes involved in colon car-

Table I: Chromosomal Locations of Putative Growth Suppressor Loci Based on Tumor-Specific Reduction to Homozygosity

neuroblastoma	1 p
small cell lung carcinoma	3 p
colon carcinoma	5 q
Wilms' kidney tumor	11 p
bladder carcinoma	11 p
retinoblastoma, osteosarcoma	13 q
ductal breast carcinoma	13 q
astrocytoma, colon carcinoma	17 p
meningioma, acoustic neuroma	22 q
colon carcinoma	18

cinoma assigned to chromosomes 17 and 18 (see Table I for a larger list). These molecular analyses are powerful in being able to scan the whole genome for chromosomal regions carrying possible suppressor genes. Nonetheless, these results remain at best indirect proofs of the existence of such genes. Only molecular cloning of these genes, which will require enormous experimental effort, will provide thoroughly convincing proof that all these genes exist and function as hypothesized.

Precis and Prospect

Various lines of evidence have converged in the last years on the idea that our genome carries a group of genes that is normally involved in negatively regulating cellular proliferation. Each of these genes would seem to act as a critical guardian of normal growth in only a subset of the body's many cell types. We suspect this because loss of both gene copies is associated in each case with a particular, idiosyncratic subset of tumor types.

The true size of this class of genes is unknown and at present unknowable, since we have only imperfect means for discovering them. Stated simply, we know about most of these genes only when the cell is deprived of their functions. Experimentally, the bulk of these genes has become apparent through somatic cell hybridization, karyologic studies, and RFLP analysis. In addition, two well-studied oncogenes, *p53* and *erb*A, are now realized to be aberrant versions of growth-suppressing genes. Taken together, this class still composes only a dozen or so genes. Many others may escape detection by the admittedly limited types of analysis currently available to us.

Granting our imperfect understanding of growth-suppressor genes, it must be said that these genes may ultimately be seen as more important in tumorigenesis than the intensively studied oncogenes. After all, it is far easier to inactivate gene function than it is to create the hyperactive forms of genes that we study as oncogenes. Creating oncogenes often involves precise and subtle tinkering with specific nucleotide sequences. Contrast the limited number of sites at which point mutations (and these mutations alone) can create a *ras* oncogene (Barbacid, 1987) with the almost unlimited number of genetic changes that can act to cripple the 190-kb-long Rb gene.

Most interesting are the biochemical and cell physiological mechanisms that enable these genes and their encoded proteins to constrain cell growth. Perhaps it is more than mere coincidence that the three suppressor genes isolated to date, *Rb*, *erb*A, and *p53*, all encode nuclear proteins that appear as candidates for transcriptional regulators (*Rb*, *p53*) or are known to function in this manner (*erb*A).

One might argue that cell growth is governed by separate, parallel growth-agonistic and growth-antagonistic signaling pathways that converge upon the ultimate decision determining cell growth or quiescence. But the recent work demonstrating direct physical interactions between oncoproteins and growth-suppressing proteins suggests a different scenario—that these two types of proteins are interwoven in a common signaling pathway that is responsible for making the big decisions about proliferation and differentiation, the decisions that lie at the heart of morphogenesis and the dysmorphogenesis that we know as cancer.

Acknowledgments

I thank Ehry Anderson for excellent help in the preparation of the manuscript.

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