Aberrant Gene Silencing in Tumor Progression: Implications for Control of Cancer

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Although it is clear that genetic alterations are critical for the initiation and maintenance of human cancer, it is also becoming evident that epigenetic changes may be essential for the development of these diseases as well. The best studied of these latter processes is heritable transcriptional repression of genes associated with aberrant DNA hypermethylation of their promoters. Herein we review how very early occurrence of these gene silencing events may contribute to loss of key gene functions which result in disruption of cell regulatory pathways that may contribute to abnormal cell population expansion. These altered regulatory events may then provide a setting where mutations in the same disrupted pathways may be readily selected and serve to lock tumor progression into place. This hypothesis has potential impact on means to prevent and control cancer and for the use of epigenetic markers for cancer risk assessment and early diagnosis.

It is apparent, from work outlined in multiple papers in this volume, that cancer is a disease driven not only by genetic, but also by epigenetic changes. All types of human cancers have broad shifts in chromatin patterns, as compared to the normal cells from which they derive, and these abnormalities potentially contribute to heritable increases and decreases in gene expression (Jones and Laird 1999; Jones and Baylin 2002; Herman and Baylin 2003). This paper focuses on the decreases that provide an alternative mechanism to gene mutations for deriving loss of tumor suppressor gene function (Jones and Laird 1999; Jones and Baylin 2002; Herman and Baylin 2003). Although the molecular determinants for this loss of gene expression are still being elucidated, the best-understood component, at present, is an abnormal increase in DNA methylation involving CpG islands in gene promoters and which is associated with the transcriptional repression of involved genes (Jones and Laird 1999; Jones and Baylin 2002; Herman and Baylin 2003). This brief review outlines how such epigenetically silenced genes are being discovered and how the consequences of the lost gene expression for tumor progression are being outlined. Understanding of the position of the lost gene function in tumor progression is a particular focus, as is definition of the abnormalities of cell control pathways that ensue. Finally, current understanding of the molecular events in chromatin regulation of cancer epigenetic gene silencing that may initiate and maintain the abnormal gene transcriptional repression is examined.

EPIGENETIC GENE SILENCING IN CANCER

The shifts in DNA methylation that occur in tumor cells and the promoter-localized increases that accompany abnormal decreases in gene transcription are outlined in this volume by J. Herman and T. Tlsty. Briefly, despite widespread losses of DNA methylation in regions where this DNA modification should normally be present, local gains of methylation are simultaneously found in CpG islands in the promoter regions of a growing list of genes in all cancer types (Jones and Laird 1999; Jones and Baylin 2002; Herman and Baylin 2003). The association of this local promoter change with loss of gene function important for tumor development has at least three historical phases. First, the concept gained attention as promoter DNA hypermethylation began to be associated with well-recognized tumor suppressor genes which, when mutated in the germ line of families, lead to inherited forms of cancer. Almost half of such genes, which can be mutated in tumors of somatic cell origin, have now been well characterized to also be inactivated in this setting by epigenetic gene silencing (Jones and Laird 1999; Jones and Baylin 2002; Herman and Baylin 2003).

Second, the recognition of epigenetic silencing of the above genes has led to a host of candidate tumor suppressor genes being associated with this type of loss of function in cancer. Such genes are generally recognized when searches for mutations fail to find such genetic changes and loss of expression of the genes in question is documented at the level of mRNA transcripts. At least 30 such genes, involving a widespread distribution over many chromosomes in cancer cells, have now been defined (Jones and Laird 1999; Jones and Baylin 2002; Herman and Baylin 2003). This group of genes, as discussed below, presents a challenge for discovering the true importance of the associated transcriptional silencing for tumor progression steps.

The third groups of genes are those being discovered through a growing list of technologies designed to randomly screen cancer genomes for all types of epigenetic abnormalities, including those associated with promoter hypermethylation and transcriptional silencing. These techniques include hybridization chip procedures for detection of CpG island methylation, methylation-sensitive restriction-enzyme-based production of DNA fragments representing alterations in patterns of DNA methylation, and expression microarray approaches to detect re-expression of abnormally silenced genes when the chro-

matin events mediating transcriptional repression are pharmacologically reversed (Akama et al. 1997; Huang et al. 1999; Toyota et al. 1999; Costello et al. 2000; Salem et al. 2000; Suzuki et al. 2002; Yamashita et al. 2002; Shi et al. 2003; Ushijima 2005; Yu et al. 2005). Again, the major challenge when such searches identify promoter hypermethylated genes is to elucidate the true importance of the loss of gene expression for tumor development.

Several important themes have emerged from all of the above gene discoveries that are beginning to alter our view of cancer as a disease strictly arising through genetic changes. First, more loss of tumor-suppressor-like gene function may occur in cancer via epigenetically mediated heritable transcription repression events than via frank gene mutations (Jones and Laird 1999; Jones and Baylin 2002; Herman and Baylin 2003). Thus, multiple genes are now being described in which mutations have not been observed but abnormal gene silencing is frequent in multiple cancer types (Jones and Laird 1999; Jones and Baylin 2002; Herman and Baylin 2003). Second, all major cellular control pathways which, when disrupted, contribute to tumorigenesis (Hanahan and Weinberg 2000), have now been shown to be involved with epigenetically mediated gene silencing (Jones and Laird 1999; Jones and Baylin 2002; Herman and Baylin 2003). Third, as expanded upon below, many abnormal gene silencing events may arise early in tumor progression, and specifically in preinvasive stages, and these may act to facilitate abnormal expansion of stem/progenitor-like cells in which subsequent genetic and epigenetic abnormalities arise and foster tumor progression (Kiyono et al. 1998; Romanov et al. 2001; Jones and Baylin 2002; Herman and Baylin 2003; Chen et al. 2004; Suzuki et al. 2004).

POSITION AND FUNCTION OF EPIGENETIC GENE SILENCING IN TUMOR PROGRESSION

In the best-characterized genes that are abnormally epigenetically silenced in cancer, there has been emphasis, through careful comparison of normal and abnormal tissues, on understanding the timing during tumor progression for onset of promoter hypermethylation and loss of gene function. From these studies, there is a growing list of such genes that appear to lose transcription very early during neoplastic development, often at precancerous stages of tumor progression (Belinsky et al. 1997, 1998; Kiyono et al. 1998; Nuovo et al. 1999; Jones and Baylin 2002; Herman and Baylin 2003; Holst et al. 2003; Chen et al. 2004; Suzuki et al. 2004). In addition, a possibly emerging theme is that such loss of gene function might be one mechanism through which neoplastic cells may become, somewhat in terms of a concept derived by Weinstein (2002), "addicted" to certain oncogenic driving pathways. A striking example of these concepts (Suzuki et al. 2004), outlined in Figure 1, is the appearance in very early lesions at risk for progression to colon cancer, atypical crypt foci (ACF), of abnormal promoter methylation in the *SFRP*s (secreted frizzled related proteins). These genes encode a family of proteins with ability to antagonize activation of the Wnt pathway at the level of cell membrane Wnt–Wnt ligand interaction (Finch et al. 1997; Melkonyan et al. 1997; Rattner et al. 1997). This hypermethylation, and associated gene silencing, were found to be present in one or more of the *SFRP*s, and usually multiple ones, in virtually all primary colon cancers examined (Suzuki et al. 2004). In a series of biology studies with cultured colon cancer cells, it

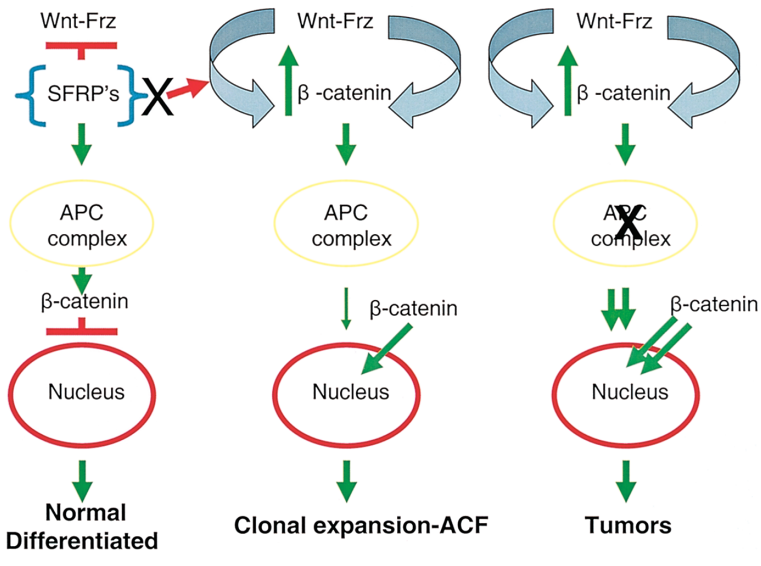

Figure 1. Model depicting how epigenetic gene silencing, very early in the preinvasive stages of colon tumor evolution, as discussed in the text, can "addict" cells to overactivity of the Wnt pathway, even in the absence of downstream mutations. In the far left panel, normal expression of the SFRPs, as colon epithelial cells differentiate, blunts interaction of Wnt with the receptor Frz and thus helps to down-regulate the pathway. This leads to low cytoplasmic levels of the key downstream transcriptional initiator of Wnt pathway activation, β-catenin. This control cooperates with a properly functioning APC tumor suppressor complex that facilitates phosphorylation of β-catenin, which renders it susceptible to cytoplasmic degradation and prevents high levels of this protein from reaching the nucleus. Abnormal epigenetic silencing of the SFRPs (*large X*, *left panel*) leads (*red arrow*) to (*middle panel*) Wnt–Frz interaction, activation of the pathway (*large blue arrows*), increased cytoplasmic levels of β-catenin (*green arrow* at *top* of *middle panel*), and partial overriding of the APC complex leading to some increase in levels of this protein in the nucleus and abnormal, preinvasive, expansion of colon epithelial precursor cells (atypical crypt foci or ACF). A mutation (*large X*, *right panel*) in APC, other members of the complex, or an activating mutation of β-catenin, leads to further constitutive Wnt pathway activity via crippling of function of the APC complex. Thus, further increases in nuclear β-catenin and resultant progression to invasive tumors are selected for.

emerged that replacement of SFRPs in cells with absent expression blocked Wnt pathway-driven transcriptional activity and caused apoptosis (Suzuki et al. 2004). This occurred even in cells harboring the key mutations in downstream Wnt pathway genes that activate the pathway intracellularly and are thought to be the very early, or "gatekeeper," steps in colon cancer evolution (Kinzler and Vogelstein 1997; Gregorieff and Clevers 2005).

The picture that emerges from these studies of the SFRPs is that loss of these proteins early in colon cancer development leads to abnormal constitutive activation at the ligand level of the key developmental Wnt pathway that has the ability to abnormally expand stem/progenitor cells in the colon (Kinzler and Vogelstein 1997; Gregorieff and Clevers 2005). These cells, which would be, in essence, addicted to overactivity of the Wnt pathway, would be primed for readily selecting mutations in downstream Wnt pathway genes, which most commonly occur in the APC tumor suppressor gene, which would drive the Wnt pathway harder and facilitate tumor progression (Fig. 1). The above-mentioned cell biology of the SFRPs in colon cancer cells shows how this selection could occur. APC mutations cripple the intracellular complex that normally leads, in the cytoplasm, to phosphorylation and degradation of β-catenin, which otherwise can go to the nucleus and mediate the transcriptional events central to the activated Wnt pathway (Kinzler and Vogelstein 1997; Gregorieff and Clevers 2005). Such complex crippling, however, may depend for function on the levels of β-catenin presented to the complex; active Wnt ligand at the membrane, facilitated by loss of SFRPs, leads to elevation of intracellular levels of this protein (Kinzler and Vogelstein 1997; Suzuki et al. 2004; Gregorieff and Clevers 2005). In early preinvasive colon lesions, such SFRP loss may lead to enough constitutive increases in β-catenin to partially overwhelm even an intact APC complex, leading to early progenitor cell expansion. This SFRP loss also leads to increased levels of β-catenin in those colon tumors harboring activating mutations in this protein. In summary (Fig. 1), epigenetically mediated loss of the SFRPs may drive colon cancer progression from the earliest to latest stages by selecting for, and facilitating, definitive mutations in the Wnt pathway. The question may even be raised, albeit a heretical one, whether the mutations in Wnt pathway genes would even result in clonal cell expansion without the "epigenetic gatekeeper" role of loss of function in genes such as the *SFRP*s (Fig. 2).

The role of epigenetic gene silencing in early cancer development has also become apparent in data emerging from comparing primary human tumor analyses to animal models of epigenetic gene silencing. Such animal models are going to be increasingly utilized to determine the true role of genes discovered to be silenced in tumors in association with promoter methylation, but for which no significant incidence of mutations is found. Mouse knockout studies of such genes can be used to test the consequences for the role of the genes in processes such as embryonic and mature tissue development plus the consequences of loss of function for tumorigenesis. We have taken such an approach for a gene, HIC1 (Hypermethylated-in-Cancer

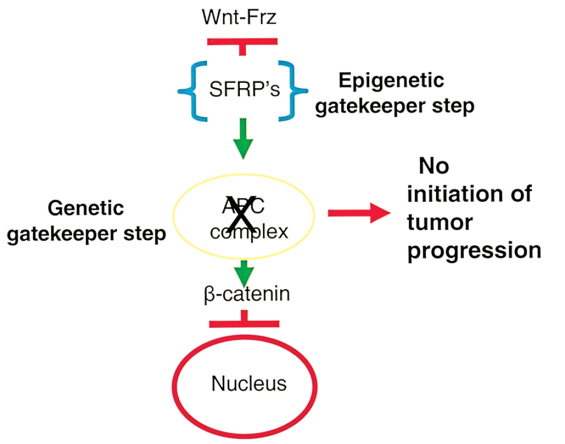

Figure 2. Model predicting, as described in the text, how a mutation in APC would not, without the epigenetic inactivation of the SFRPs or related proteins, begin to drive tumor progression.

1), which was discovered in a random search approach for hypermethylated genes possibly residing in a chromosome 17p region frequently deleted in multiple human cancer types (Wales et al. 1995). This region is distal to the tumor suppressor gene *p53* and is often deleted independent of deletion and/or mutations of *p53*. Interestingly, HIC1, which is hypermethylated early, in preinvasive stages of cancers such as breast (Fujii et al. 1998) and colon (Wales et al. 1995), is also a transcription activation target of p53 (Wales et al. 1995; Guerardel et al. 2000). The constitutive homozygous knockout of Hic-1 is lethal (Carter et al. 2000), but the heterozygotes get late-onset tumors in which the wild-type allele is universally retained and hypermethylated (Chen et al. 2003). Interestingly, there is a gender-associated tumor spectrum in which male mice get predominantly epithelial cancers and females get lymphomas and soft-tissue sarcomas (Chen et al. 2003).

The possible addiction of cells to pathways disrupted by the epigenetic silencing of HIC1 is apparent in the fact that this protein is involved in modulating activity of the powerful tumor suppressor, p53. This interaction may be viewed in two ways. First, crossing of Hic-1 heterozygous mice with p53 heterozygotes markedly modulates the time of appearance, virulence, and tumor spectrum as compared to either of the heterozygotes alone (Chen et al. 2004). Thus, both male and female double heterozygotes get new tumor types for the strain of mice studied, including osteosarcomas, breast tumors, and ovarian tumors (Chen et al. 2004). Again, the wild-type allele of *Hic-1* is almost always retained and hypermethylated, whereas the wild-type allele of *p53* is virtually always deleted (Chen et al. 2004). It appears that the epigenetic milieu, in this case epigenetic inactivation of Hic-1, determines the location, spectrum, and virulence of tumors induced even by genetic inactivation of the powerful tumor suppressor, p53 (Fig. 3).

Second, from a mechanistic standpoint in terms of one way in which HIC1 appears to function as a tumor suppressor, our recent data show that epigenetic loss of this

Figure 3. Model predicting, as described in the text, how mutations in the powerful tumor suppressor gene, *p53*, will drive tumorgenicity in directions determined by the epigenetic milieu.

protein leads to disruption of a complex network involving the key stress-sensing protein, SIRT1 (Frye 1999; Luo et al. 2001; Vaziri et al. 2001; Nemoto et al. 2004), and p53. SIRT1, a member of the class III family of sirtuins (Frye 1999; Vaziri et al. 2001), has p53 as one of its targets (Luo et al. 2001; Vaziri et al. 2001; Langley et al. 2002). Deactylation of p53 down-regulates the transcriptional function of this protein (Luo et al. 2001; Vaziri et al. 2001; Langley et al. 2002). We have recently found that HIC1 is a direct transcriptional repressor of SIRT1 and also complexes with SIRT1—both proteins are localized to the SIRT1 promoter (Chen et al. 2005). With epigenetic inactivation of HIC1, SIRT1 levels increase and acetylation of p53 is lost during DNA damage (Chen et al. 2005). This leads, in HIC1-deficient cells, to decreased p53 function and diminution of cell apoptotic response to DNA damage (Chen et al. 2005), providing a potential early oncogenic stimulus to cells. Again, this loss of HIC1 and diminished p53 function may drive cells down a pathway favorable for selection of genetic inactivation of p53, which then locks cells farther into tumor progression.

It appears evident from the examples provided above that the early role of epigenetic silencing, especially in the abnormal expansion of stem/progenitor cells which can precede and set the risk for cancer development, merits intensive continued investigation. The concept is that the likelihood of progression to frank malignancy or the virulence course of a particular tumor depends not only on given gene mutations, but also on the milieu of epigenetic alterations in which they occur. It must be remembered that these epigenetic changes may be ongoing not only in tumor cells or their precursors but also, as recently suggested (Hu et al. 2005), in stromal cells surrounding the neoplastic cells. Recent data have re-emphasized the importance of stromal cell abnormalities in contributing significantly to tumor progression (Tlsty 2001; Orimo et al. 2005). This concept of an epigenetic milieu of epigenetic abnormalities determining the course of tumor development has profound implications for prevention and therapeutic approaches to cancer, and for molecular strategies aimed at marker development for cancer risk assessment, early diagnosis, and prognosis prediction.

MOLECULAR STEPS IN ESTABLISHING AND MAINTAINING ABNORMAL HERITABLE TRANSCRIPTIONAL REPRESSION IN CANCER

There are no more important biological questions concerning epigenetically mediated loss of gene function in cancer than determining the molecular steps that establish and maintain the abnormal memory patterns during tumor development. We have come to understand much more about the latter than about the former, although both areas must undergo extensive clarification through ongoing research efforts.

With respect to causing initiation of the process of abnormal promoter methylation and associated silencing of tumor suppressor genes, we only have emerging clues. Certainly, there is building contribution to answering this question from the unraveling of molecular steps that cause gene promoters to be marked for silencing and maintained in a stably silent state. Critical to such silencing are studies of chromatin remodeling complexes, the histone modifications that separate active from inactive gene states, and the series of polycomb complexes under study from model organisms to man (Jenuwein and Allis 2001; Kouzarides 2002; Peters et al. 2003; Lund and van Lohuizen 2004). Particularly for the polycomb groups, we know that key components such as the protein Bmi1, complexes such as PRC4 which contains the histone methyltransferase, EZH2, and its partners, are increased in stem/progenitor cells and tumor cells that have properties of such cells (Varambally et al. 2002; Kirmizis et al. 2003; Kleer et al. 2003; Kuzmichev et al. 2005). Few specific targets for these silencing complexes, particularly in normal or neoplastic mammalian cells, are known. One key tumor suppressor gene that is frequently hypermethylated and silenced in cancer, *p16*, is a known indirect or direct target of Bmi1 (Lessard et al. 1999; Lund and van Lohuizen 2004), but the precise link as to how such targeting may be translated into the very stable silencing associated with promoter DNA methylation, or as to how and when such methylation appears during tumor development, is not known.

One emerging concept is that the course of epigenetic silencing of tumor suppressor genes may be a progressive one during tumor evolution in which degrees of silencing precede the imposition of abnormal DNA methylation on the promoter (Jones and Baylin 2002; Bachman et al. 2003). This concept encompasses key data, from model organisms to man, indicating that certain histone methylation marks, such as methylation of lysines 9 and 27 of histone H3 (methyl-K9-H3 and methyl-K27-H3), may be essential for recruiting DNA methylation (Selker et al. 1987; Bell et al. 1991; Tamaru and Selker 2001, 2003; Jackson et al. 2002; Johnson et al. 2002; Plath et al. 2003; Cao and Zhang 2004). Data from a study by Rauscher and colleagues (Ayyanathan et al. 2003), as we have reviewed (Fahrner and Baylin 2003), may be most relevant to the process under discussion. This study indicates that when transcriptional silencing of a promoter is transiently established, a very localized zone of histone transcriptional repression marks, and particularly methyl-K9-H3 and the recruitment of the protein HP1, is placed in and around the binding site for the transcriptional repression complex. If the complex is removed, most clones of cells revert to active transcription of the targeted gene and concomitant loss of the transcriptional repression marks. However, some clones retain the promoter repression marks, have absent or diminished transcription, and with

continued growth, cells from such clones evolve very stable transcriptional repression mediated by recruitment of promoter DNA methylation. It is possible that these dynamics derived in an experimental cell model system are highly pertinent to the dynamics ongoing in natural tumor progression which lead to abnormal heritable transcriptional silencing of genes.

With respect to maintenance of heritable silencing of tumor suppressor genes in tumor cells, we know much more than how the process is established (for review, see Jones and Baylin 2002; Herman and Baylin 2003). We, and other investigators, have established that the promoters of such silenced genes have localization of classic transcriptional silencing marks which include, in addition to CpG island methylation, presence of deactylated K9-H3 and K14- H3 and methyl-K9-H3. In turn, key activating marks, including methyl-K4-H3 and acetyl-K9 and K14-H3, are absent in such genes but are present to the virtual exclusion of the repressive marks when the same genes are expressed in a cancer cell (Fahrner et al. 2002; Ghoshal et al. 2002; Koizume et al. 2002; Nguyen et al. 2002; Bachman et al. 2003; Kondo et al. 2003). In this layering of transcriptionally repressive chromatin, the DNA methylation appears to be quite dominant; this is an important point with respect to selection during tumor progression of the most stably silenced state. The clearest example of this point is that some degree of DNA demethylation of the promoter must be achieved before transcription can be restored and key transcriptional activation marks, such as acetyl K9-H3 and K14-H3 and methyl-K9-H3, appear and repression marks, such as deacetylated K9-H3 and K-14-H3 and methyl-K9-H3, decrease (Fahrner et al. 2002; Nguyen et al. 2002). This fits with earlier observations that cancer cell treatment with class 1 histone deactylase (HDAC) inhibitors fails to transcriptionally activate densely promoter-DNA-methylated tumor suppressor genes but will synergize with DNA demethylating agents to do so (Cameron et al. 1999). Despite all of these observations, however, our knowledge of all the molecular events that mediate maintenance of the abnormally heritable silencing state of cancer cells, and their hierarchical relationships to one another, is almost assuredly quite incomplete. This critical aspect of the epigenetic abnormalities in tumor progression thus merits intense investigation. It also has profound implications for the translational quest to target reversal of epigenetic gene silencing as a means for cancer prevention and treatment.

CONCLUSIONS

This paper has stressed the point that a series of epigenetic, as well as genetic, alterations are ongoing throughout the initiation and maintenance phases of human tumor progression. In terms of the former, although a complex array of chromatin patterns are altered during tumor evolution, the best understood, at present, is an abnormal heritable transcriptional repression of proven or candidate tumor suppressor genes. This repression is mediated by a layering of chromatin changes at the level of histone modifications upon which DNA methylation is imposed as the step that tightly locks the silencing in. Many of the gene silencing events appear to occur at very early stages of neoplastic progression and even serve as "epigenetic gatekeeper steps," or an epigenetic milieu, without which key tumor suppressor mutations might not be able to play their full roles in fostering tumor evolution. These epigenetic steps may addict cells to oncogenic stimuli from abnormal signal transduction pathway activation or partial crippling of tumor suppressor genes lying downstream from abnormally silenced genes. Understanding the molecular events that initiate and maintain this heritable gene silencing may allow it to be targeted for reversal in strategies aimed at the prevention and treatment of cancer. The component of abnormal promoter DNA methylation is also providing a promising molecular marker strategy for cancer risk assessment, early detection, and gauging of prognosis.

ACKNOWLEDGMENTS

This study is supported by grants from the National Cancer Institute (CA043318) and National Institutes of Environmental Health Sciences (ES11858). We thank Kathy Bender for her help with the manuscript preparation.

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Cold Spring Harbor Symposia on Quantitative Biology

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Cold Spring Harb Symp Quant Biol 2005 70: 427-433
Access the most recent version at doi:10.1101/sqb.2005.70.010