

# Review

## Sometimes the Result Is Not the Answer: The Truths and the Lies That Come From Using the Complementation Test

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### ABSTRACT

It is standard genetic practice to determine whether or not two independently obtained mutants define the same or different genes by performing the complementation test. While the complementation test is highly effective and accurate in most cases, there are a number of instances in which the complementation test provides misleading answers, either as a result of the failure of two mutations that are located in different genes to complement each other or by exhibiting complementation between two mutations that lie within the same gene. We are primarily concerned here with those cases in which two mutations lie in different genes, but nonetheless fail to complement each other. This phenomenon is often referred to as second-site noncomplementation (SSNC). The discovery of SSNC led to a large number of screens designed to search for genes that encode interacting proteins. However, screens for dominant enhancer mutations of semidominant alleles of a given gene have proved far more effective at identifying interacting genes whose products interact physically or functionally with the initial gene of interest than have SSNC-based screens.

### A BRIEF HISTORY OF THE COMPLEMENTATION TEST

The early fly geneticists were certainly aware of a small number of genes that appeared to be defined by multiple alleles, in the sense that these mutations were recombinationally inseparable and produced the same or similar phenotypes (MULLER 1932; STONE 1935; STERN and SCHAEFFER 1943). Moreover, these geneticists did indeed carefully analyze the phenotypes of various double heterozygotes in an attempt to understand gene function. Among other things these studies led to the discovery both of intragenic recombination (GREEN and GREEN 1949, 1956; LEWIS 1951; GREEN 1990) and of complex interactions between various allelic combinations (*cf.* STERN and SCHAEFFER 1943; GREEN 1961). But given the small number of genes defined by multiple alleles, it is not clear that these workers saw the construction of double heterozygotes as a tool for addressing the single question that underlies the complementation test: namely, among a set of independently arising mutations causing the same or similar phenotypes, which of those mutations define the same gene(s)?

The first experimental approach designed to address this question was referred to as the *cis-trans* test (PONTECORVO 1958). According to Pontecorvo, the

*cis-trans* test was first proposed by Ed Lewis (LEWIS 1951) to describe those cases where heterozygotes for two tightly linked mutations exhibit a phenotype when the mutations are arranged as a *trans*-heterozygote but not as a *cis*-heterozygote. To quote PONTECORVO (1958, p. 37), “if a heterozygote having two different allelic recessives (arisen by independent mutations), one on one chromosome and the other on the homologous chromosome (*trans* or repulsion arrangement), has a recessive phenotype, or a more nearly recessive phenotype than the corresponding double heterozygote with both recessives on one chromosome and a normal homolog,” then the two mutations define the same gene. LEWIS (1951) refers to this as “position pseudoallelism,” while Pontecorvo refers to it simply as the “Lewis effect.” Despite Pontecorvo’s attribution of the idea to Lewis, Lewis himself attributes the origin of this idea to Seymour Benzer, who both coined the term “*cis-trans* test” in the course of his studies of the T4 *rIIa* and *rIIb* genes and popularized its usage (BENZER 1955, 1962; LEWIS 1967). It is unclear, at least to us, which of these two scientists truly deserves credit for devising the *cis-trans* test, and so we suggest that credit be shared equally<sup>2</sup>.

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<sup>2</sup>However, we note that the online encyclopedia Wikipedia has decided the issue in favor of Ed Lewis (<http://en.wikipedia.org/wiki/Complementation>). That must surely settle the issue.

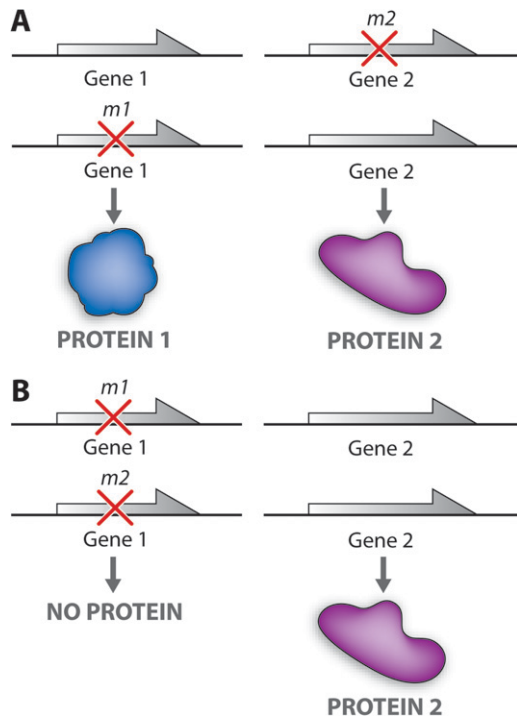


FIGURE 1.—The *trans*-component of the complementation test in its simplest form.

The power of the classical *cis-trans* test is that performing the *cis* component of the test ( $m1\ m2/+ +$ ) as well as the *trans* component ( $m1\ +/+ m2$ ) effectively rules out a potentially serious error that might occur in the case of two tightly linked genes with related functions (such as the *rIIa* and *rIIb* genes in phage T4). One could imagine a case where heterozygosity at both loci created combined haplo-insufficiency, misleading the investigator into believing that the mutations were allelic. But if this were the case, then the same mutant phenotype would be observed in *cis*- as well as *trans*-heterozygotes. Unfortunately, the *cis-trans* test is rarely done in higher eukaryotes both because it is critical only in the case of very tightly linked and functionally related loci and because when two mutations are tightly linked it is often difficult, if not impossible, to obtain the necessary doubly mutant chromosomes by recombination<sup>3</sup>.

The modern version of the complementation test was first described in detail by GILES (1958) and FINCHAM (1958) in articles published in the Proceedings of the 10th International Congress of Genetics. The test rapidly became a mainstay of genetic analysis in fungi (*cf.* CASE and GILES 1960) and in higher organisms, such as *Drosophila* (*cf.* GREEN 1961). The basic form of this test is described in Figure 1. Consider two independen-

<sup>3</sup>However, it might well still be worth the effort of doing the *cis* component of the test in at least some cases—see the discussion below of cases of combined haplo-insufficiency involving tightly linked genes in the mouse.

tly isolated mutations,  $m1$  and  $m2$ . Both of these mutations are fully recessive, and  $m1/m1$  and  $m2/m2$  homozygotes manifest similar mutant phenotypes (for example, a change in wing structure in flies or auxotrophy for histidine in yeast). As shown in Figure 1A, if  $m1$  and  $m2$  are not in the same gene then the wild-type (+) alleles of both genes are still present in the double heterozygote and fully functional forms of both proteins are produced. In this instance, the two mutations are said to “complement each other” and to define different genes. However, Figure 1B displays the case where both mutations  $m1$  and  $m2$  lie within the same gene. In this case the double heterozygotes ( $m1/m2$ ) possess only mutant copies of this gene and will thus manifest only the mutant phenotype<sup>4</sup>. In this case, we say that  $m1$  and  $m2$  to “fail to complement each other” and that they define the same gene.

The value of this test cannot be overrated. For example, it allows the mutants obtained in a given screen to be quickly sorted into complementation groups. To consider a small-scale screen, our laboratory used a germline clone assay to screen 50,000 EMS-treated chromosomes for a meiotic defect. Among the 12 mutants recovered, complementation testing revealed that fully a third were alleles of a single gene (*ald*), two more were alleles of a previously identified meiotic gene [*c(2)M*], while the remaining six appear to define novel genes. For the most dramatic example of the utility of this test, consider the case of the classic screen by NUSSLEIN-VOLHARD *et al.* (1984), where the use of the complementation test allowed the authors to sort >250 new zygotic lethal mutants into a tractable number of complementation groups. Similarly, the classic screen by HARTWELL *et al.* (1973) for mutants that control the progression of the cell through the cell cycle yielded 149 mutants that were sorted into a much smaller number of genes (32), or complementation groups, solely by the complementation test. It is this test that makes the analysis of the outcome of mutant screens, both large and small, a tractable process.

That said, there are two ways in which the complementation test can “lie to you.” First, as detailed below, it is possible for mutations at two different loci to interact in such a way as to produce a mutant phenotype in  $m1\ +/+ m2$  heterozygotes, even though  $m1$  and  $m2$  define separate genes. This phenomenon is discussed in detail below. Second, it is also possible for two mutants in different domains of the same gene to fully complement

<sup>4</sup>There are several well-described instances in which the phenotype of a double heterozygote for two mutations within a given gene is considerably more extreme than that of either of the two homozygotes. This phenomenon is referred to as “negative complementation” (FINCHAM 1966). Negative complementation presumably reflects the ability of the two abnormal protein products to form a dimer or multimer that is not only nonfunctional (as are homodimers of the two mutant proteins) but poisonous as well. For a modern example of this phenomenon in *Drosophila* see BICKEL *et al.* (1997).

each other, provided that they define separate functional elements of either the gene or its protein product. This phenomenon is referred to as intragenic complementation<sup>5</sup>.

However, despite these possible errors, we would be at a loss without the complementation test. Moreover, a variant of the complementation test, transformation rescue, is considered to be the gold standard for proving that one has indeed identified, at the DNA sequence level, a gene previously defined only by genetic mutations. This type of complementation test asks the question: Can a wild-type copy of gene *X* rescue the function of the mutant allele that is believed to define gene *X*? If you can show that a homozygote for the mutant of interest can be “rescued” by the addition of a wild-type copy of your candidate gene, and you have identified one or more mutant alleles at the level of DNA sequence alterations, then you have cloned the gene defined by that mutant.

#### CAVEATS FOR THE USE OF THE COMPLEMENTATION TEST

The complementation test is straightforward, but there are three simple caveats for its use. First, as originally conceived the complementation test should be done *only* when both loss-of-function mutations are fully recessive. Although the test has also been used in cases in which one of the mutations exhibits a dominant or semidominant phenotype due *solely* to haplo-insufficiency, there is a serious concern that a failure to see complementation with a second mutation might not reflect allelism, but rather simply the ability of a mutation in a second gene to enhance the phenotypic

effects of the first mutation (see below). In those cases where one wishes to determine whether or not two truly anti- or neomorphic mutations are allelic or whether one such mutation is allelic to loss-of-function mutations that map in the same interval, the only alternative is to “revert” the dominant in hopes of creating a testable loss-of-function, and thus recessive, mutation (*cf.* RASOOLY *et al.* 1991).

Second, the complementation test does not require that the two mutants have the same phenotype. As we have noted previously, different mutations in the same gene can produce rather different phenotypes. Sometimes a mutation that alters, but does not destroy, function will have a weaker effect on the organism’s phenotype than does a null or “knockout” mutation (for example, one might have alleles of a gene that result in lethality when homozygous and weaker alleles that cause a visible phenotype). In these cases the double heterozygote usually exhibits the phenotype associated with the weaker of the two alleles.

Third, it is important to note that the complementation test is only a test of gene function and provides no information regarding the nature or position of the mutations. Two mutations that alter the same base pair in the same gene will fail to complement, just as will two loss-of-function mutations at different sites in the same gene. Moreover, any nearby mutation or aberration that acts to inactivate a given gene will fail to complement other alleles of that gene even if the mutation or the breakpoint is some distance away.

A particularly illustrative example of this phenomenon arose in our lab. As noted above, a recent mutagenesis in our lab recovered four mutants that fell into a single complementation group. All four mutations were found to map to a small interval that included the *ald* gene, which encodes the fly *mgs1* homolog (GILLILAND *et al.* 2005). Mutations in the *ald* gene cause defects in both meiosis and mitosis, and all four new *ald* alleles failed to complement the original *ald* allele in terms of the meiotic defect. All four mutants were also rescued by a single copy of our *ald*<sup>+</sup> transgene construct. However, there were clear differences among the four mutants. Two mutants displayed both the meiotic and mitotic phenotypes and had DNA sequence changes in the *ald* gene (in one case an early stop codon and in the other a nine-codon deletion). However, although the other two mutants exhibited obvious meiotic defects, they had no mitotic defects, nor could we find DNA sequence changes associated with these two mutations either in the *ald* gene itself or in the region delimited by the rescue construct.

The answer to this paradox came when we discovered that both of these unusual mutants contained a new insertion of a transposable element known as a *Doc* in the next gene downstream of *ald*, a gene called *CG18212*. These insertions were 1638 or 2042 nucleotides downstream from the end of the *ald* transcript and

<sup>5</sup>The phenomenon of intragenic complementation is usually used to describe a case in which the protein of interest has two or more different and separable functional domains, such that a gene with a loss-of-function missense mutation in domain 1 might complement a missense mutation in domain 2. This can occur if the protein of interest has two or more separable functional domains. A loss-of-function missense mutation affecting domain 1 may fully complement a missense mutation affecting domain 2 since both domains are present in fully functional form in the doubly heterozygous diploid. A second form of intragenic complementation may occur even in proteins with single functional domains, so long as the protein contains two or more identical subunits. In the double heterozygote, the two differently mutant subunits are thought to mutually ameliorate their respective defects by entering into an oligomeric complex whose structure is more nearly normal than that found in either homozygote. Numerous examples of this phenomenon in lower eukaryotes have been described by SCHLESINGER and LEVINTHAL (1965) and by ZABIN and VILLAREJO (1975), but for a more recent consideration see HEHIR *et al.* (2000). Examples of this phenomenon in eukaryotes have been described by HAWLEY and WALKER (2003). However, a very different type of intragenic complementation is exhibited in cases in which one of the two mutations lies in an upstream regulatory element and the other lies within the coding region. These effects appear to reflect the ability of various enhancer-like elements to function in *trans* in an organism like *Drosophila*, which has ubiquitous somatic pairing. In *Drosophila* this type of intragenic complementation can be disrupted by heterozygosity for chromosomal rearrangements, a phenomenon referred to as transvection (for review see MORRIS *et al.* 1999 and DUNCAN 2002). Similar effects have been noted in a variety of organisms (TARTOF and HENIKOFF 1991; MATZKE 2001).

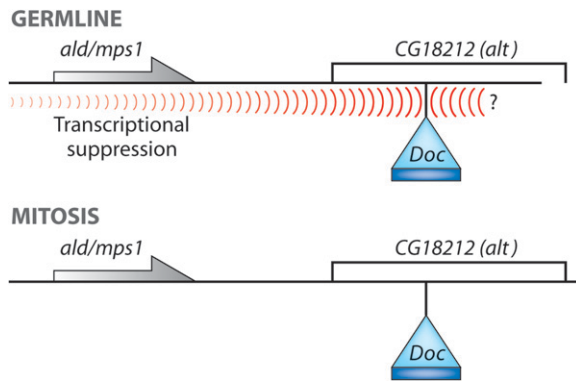


FIGURE 2.—Germline-specific inactivation of the wild-type copy of the *ald*<sup>+</sup> gene causes it to fail to complement a mutant allele of *ald* carried by the homologous chromosome, but only in the germline.

were thus well outside of the region included in our fully functional *ald*<sup>+</sup> transgene. We had considered *CG18212* as a candidate gene when we were cloning the *ald*<sup>l</sup> mutation and had ruled it out by creating a *P*-element excision that deleted virtually all of the gene, demonstrating that this mutation fully complemented the original *ald* mutation. As shown in Figure 2, it turns out that the answer to this riddle lay in our discovery that these *Doc* elements can suppress the function of at least one nearby gene (*ald*) in *cis* in the germline, but not in the soma<sup>6,7</sup>. As a result of this *cis*-inactivation in the germline, the *Doc* insertions in *CG18212* fail to complement canonical *ald* mutations in terms of the meiotic, but not the mitotic defect. As novel as this might seem, it is really no different from the inactivation of genes by moving them close to a block of heterochromatin, a phenomenon known as position-effect variegation (PEV). By moving the gene near the heterochromatin one can inactivate its function without damaging the gene—and that strongly variegating (inactivated) gene will fail to complement a loss-of-function allele of the same gene borne by its homolog, despite the fact that the gene itself is structurally intact.

There is a similar cautionary tale regarding interpreting transformation rescue experiments. For example, repair-deficient mutations in the *Drosophila* *mus309* gene were mapped to a small genetic region that con-

tained the fly homolog of the *Ku* gene, which encodes a well-characterized protein involved in DNA repair. Moreover, these mutants were rescued by a single-copy insertion of a gene encoding the DNA repair protein *Ku* (BEALL and RIO 1996). It thus seemed at the time a reasonable inference that the *mus309* gene encoded the fly *Ku* protein. The only missing piece of data was the sequencing of *mus309* alleles to identify the expected sequence lesion. Unfortunately, as shown in Figure 3, it turns out that *mus309* mutations are not alleles of the *Ku* gene. Rather, they were shown to be alleles of the adjacent *Dmblm* locus (KUSANO *et al.* 2001). *Dmblm* is a homolog of the human Bloom syndrome gene, which encodes a helicase of the RECQ family. Both genes function in the repair of double-strand breaks and increasing the dose of *Ku*, even by 50%, can suppress the effects of homozygosity for loss-of-function mutations at the *Dmblm* gene.

If there is a lesson to be learned from either story, it is the need to identify the structural lesions responsible for the individual mutations that define any gene or complementation group. To do otherwise might both allow one to be seriously misled and, more importantly, preclude the discovery of a valuable novelty.

## SECOND-SITE NONCOMPLEMENTATION

In rare cases, two mutations in unlinked genes, which by themselves are fully recessive, can create a mutant phenotype in double heterozygotes. In other words, although individuals heterozygous for either *m1* or *m2* alone (*m1*/+ and *m2*/+) are wild type, doubly heterozygous individuals (*m1*/+; *m2*/+) exhibit a mutant phenotype. This phenomenon is referred to as non-allelic noncomplementation or second-site noncomplementation (SSNC). Since the term SSNC seems to have the wider usage, we use it here. HAWLEY and WALKER (2003) have divided SSNC into three separate categories (types 1–3) that differ in terms of allele specificity at the two loci.

In type 1 SSNC, the interaction is restricted to specific missense alleles at *both* loci, and neither allele can be a null. The double-mutant combination may be thought of as a “synthetic antimorph” because only the combination of the two specific mutant proteins produces a poisonous gene product (see Figure 4). As intriguing as this phenomenon can be, to the best of our knowledge it has been demonstrated in only two cases (see below).

In type 2 SSNC, allele specificity is required at only one locus (gene *A*), but the mutation at the other gene (*B*) can be any loss-of-function allele, including a deletion. This phenomenon is thought to identify physical interactors by multiple mechanisms. Two of these cases, which are documented below, include examples in which the mutant form of protein *A* sequesters some of a limited quantity of protein *B* into inactive complexes or in which normal levels of protein *A* are

<sup>6</sup>This interpretation was confirmed once antibodies against Ald/Mps1 protein were available. Western blotting demonstrated that ovaries from mutant homozygous females have far lower amounts of *ald* protein than wild type, yet larval brains have normal amounts of protein (W. GILLILAND and R. S. HAWLEY, unpublished data).

<sup>7</sup>As a postscript to this story, we decided to assign a name to *CG18212*. As this gene is uninvolved in anything of any importance to female meiosis, but two deceitful interlopers (the two *Doc* elements) attempted to mislead us into thinking that it was critically important, we decided to name this gene *alt*, which stands for *aluminum tubes*. Because when someone presents you with pictures of aluminum tubes and tells you that they are intended for nuclear uses, you must be very careful about the inferences you make.



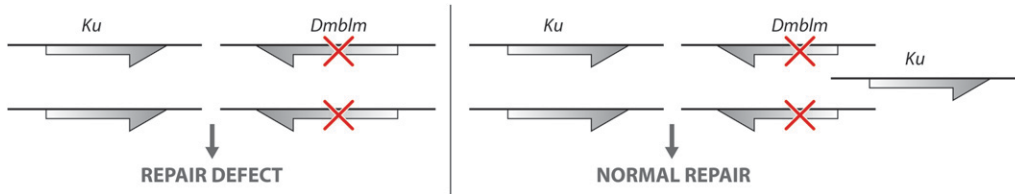


FIGURE 3.—An extra copy of the nearby *Ku* gene rescues the phenotypic defects caused by homozygosity for mutation at the *Dmblm* gene.

required to allow a mutant form of protein B to form the homotetramer that is required for function. In addition to identifying interactions between protein-encoding genes, there is no reason to preclude examples of this type of SSNC in which one mutation occurs in a gene encoding an RNA product (although no such examples of this phenomenon have yet been found).

In type 3 SSNC, also known as “combined haploinsufficiency,” the interaction is allele independent at both loci. The phenomenon can be, and often is, observed when both mutations are nulls. This type of SSNC neither requires nor implies the physical interaction of the two proteins. (Moreover, as in the case for type 2 SSNC, there is no reason that these types of interactions need be limited to genes that encode protein rather than RNA products.) Rather, it suggests only that reducing the dosage of the product of gene A does not create a phenotype, unless the cell or organism is further crippled by a reduction in the dosage of gene B. Although this type of SSNC is probably the most common, and sometimes the least interesting, it has uncovered important examples in which the proteins encoded by the noncomplementing genes do indeed act in functionally related processes.

**Type 1 SSNC (poisonous interactions)—the interaction is allele specific at both loci:** Type 1 SSNC is both the rarest and the most interesting of these phenomena. By definition it requires allele specificity at both genes, and neither allele can be a null mutation. Although this type of interaction can be explained by asserting that the two mutant proteins physically interact to produce a poisonous protein dimer or complex (see Figure 4), as shown below it need not always imply such an interaction. For illustration, we describe two cases in which type I SSNC did indeed reflect physical interaction and a third case in which it most certainly did not.

The first case of SSNC that did confirm a physical interaction was the discovery of dual allele-specific SSNC by mutations in the yeast  $\alpha$ - and  $\beta$ -tubulin genes. In 1988 Tim Stearns and David Botstein (STEARNS and BOTSTEIN 1988) screened for mutants that displayed cold-sensitive SSNC in the presence of a recessive cold-sensitive allele of  $\beta$ -tubulin. Over 20,000 mutagenized cells were screened, and only one second-site noncomplementer was found. This new mutant indeed turned out to carry a mutation in the  $\alpha$ -tubulin gene that fails to complement one of the  $\beta$ -tubulin mutants in an allele-specific fashion. The failure of complementation presumably

reflects the combination of the two mutant proteins to form a defective and poisonous tubulin subunit whose incorporation into a growing microtubule leads to severe defects (see Figure 4). The STEARNS and BOTSTEIN (1988) article is widely viewed as the hallmark on SSNC research, primarily because the screen did identify a mutation in a gene whose product,  $\alpha$ -tubulin, physically interacted with  $\beta$ -tubulin<sup>8</sup>.

A second case in which dual allele-specific noncomplementation appears to reflect the physical interaction of two proteins is provided by the work of Berg and his collaborators on the genes encoding the yeast proteins RFA (the large subunit of the heterotrimeric single-stranded DNA-binding protein RPA) and the DSB repair protein Rad52 (FIRMENICH *et al.* 1995). These authors identified a missense allele of the *RFA* gene (*rfa1-44*) that displayed a number of unusual interactions with the *RAD52* gene, including the failure to complement a specific missense allele of *RAD52* (*rad52-34*). HAYS *et al.* (1998) went on to demonstrate that the RFA and RPA proteins did indeed physically interact, at least as assayed by the yeast two-hybrid system. Moreover, the *rad52-34* mutation lies in the region of *RAD52* required to bind RFA, and this mutation blocks the binding of mutant Rad52 protein to wild-type RFA. (Unfortunately, the coexpression of both mutant proteins was toxic to yeast, and thus it was not possible to ascertain whether or not the protein encoded by the *rfa1-44* mutation does indeed bind to the mutant Rad52 protein to create a poisonous complex, but this seems a likely possibility.)

Unfortunately, one can also observe dual allele-specific SSNC even in cases where the two proteins do not physically interact. The classic example of this phenomenon stems from the efforts of David Drubin and his collaborators, who set out to identify actin-interacting proteins by screening for second-site mutations that failed to complement either of two temperature-sensitive

<sup>8</sup>We feel compelled to point out that despite the heuristic value of this article in terms of its genetic elegance, the finding that  $\alpha$ - and  $\beta$ -tubulin physically interact did not exactly come as a huge surprise to our colleagues in cell biology. Nonetheless, the finding of the  $\alpha$ -tubulin mutant turned out to be of real value to yeast geneticists because, prior to this study, no viable mutant alleles of the  $\alpha$ -tubulin gene were available. Moreover, Stearns and Botstein continued their efforts by screening for second-site noncomplementers of the  $\alpha$ -tubulin mutation. This screen also yielded both a new cold-sensitive allele of the original  $\beta$ -tubulin gene, and, more critically, the first recovered point mutation in the second  $\alpha$ -tubulin gene (*TUB3*). The only previous existing alleles of *TUB3* were null mutations generated by gene disruption.

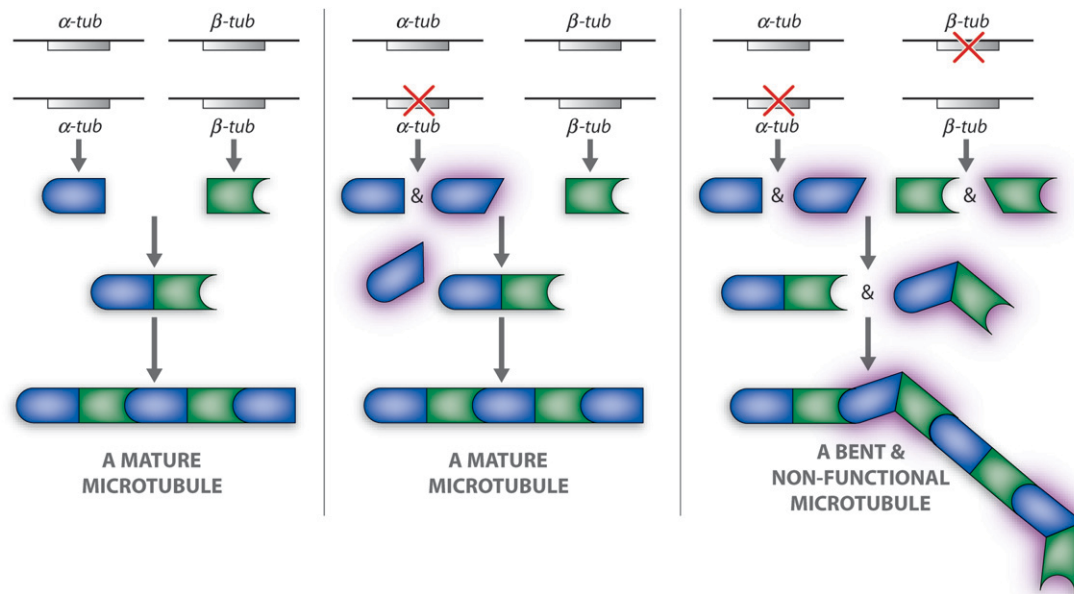


FIGURE 4.—Two missense alleles of the  $\alpha$ - and  $\beta$ -tubulin genes encode proteins that combine to create a poisonous tubulin monomer.

alleles of the yeast actin gene (VINH *et al.* 1993; WELCH *et al.* 1993). The screening of >55,000 mutagenized colonies yielded a total of 14 extragenic noncomplementing mutants. Although one of these mutations (*anc1*) caused several properties that made its product likely to be an actin interactor (such as defects in actin filament formation on its own), the Anc1 protein turns out *not* to be a physical partner of actin. Rather, the Anc1 protein is a yeast-specific subunit of the transcription factor TAF14, a component of the mediator complex (CAIRNS *et al.* 1996). Deletion of ANCI/TFG3 results in diminished transcription. Thus *anc1* mutants appear to interact with actin mutants not by physical interaction of mutant gene products, but instead by global effects on the transcription process.

Thus we are aware of but two true examples in which dual-allelic-specific second-site noncomplementers did indeed identify proteins that physically interact. To our knowledge, no other *bona fide* examples of this phenomenon exist in the literature and screens. For this reason, and because of the type of interactions observed by Drubin and his collaborators, screening for dual-allelic-specific SSNC as a means for identifying physically interacting proteins is rarely done and indeed has largely, and appropriately, been replaced by far more powerful proteomics-based methodologies.

**Type 2 SSNC—the interaction is allele specific at one locus:** In this second type of second-site noncomplementation, we observe allele specificity only for one of the two genes. The mutation at the other gene needs only to be a loss-of-function allele; indeed, a deficiency of the second gene usually will work just fine. There are a variety of interactions that can produce this result.

Although we discuss but two examples, there may be many more possibilities. In the first case, which involves the interaction of mutations in the  $\alpha$ - and  $\beta$ -tubulin genes in *Drosophila*, the failed complementation resulted from the ability of the mutant  $\alpha$ -tubulin protein to sequester  $\beta$ -tubulin subunits into inactive and non-functional complexes. This case is considered in detail below. In a second case, digenic retinitis pigmentosa in humans and mice, a mutant protein (RDS1) can form only functional homotetramers in the presence of normal levels of a second protein called ROM. In the presence of reduced levels of ROM the mutant RDS1 protein fails to form the requisite heterotetramers, and retinal degeneration is observed (KAJIWARA *et al.* 1994; GOLDBERG and MOLDAJ 1996; KEDZIERSKI *et al.* 2001). In both of these cases, type 2 SSNC does indeed imply physical or functional interaction.

The classic example of SSNC by sequestration of functional protein A into inactive complexes by mutant protein B is provided by Fuller and her collaborators, who recovered a mutation in a *Drosophila*  $\alpha$ -tubulin gene that failed to complement mutations in a spermatogenesis-specific  $\beta$ -tubulin gene in flies (HAYS *et al.* 1989). Flies heterozygous for the mutant  $\alpha$ -tubulin gene and for various recessive alleles of the  $\beta$ -tubulin gene, including a known null, are sterile (HAYS *et al.* 1989). In other words, although heterozygotes for mutations at either gene are fertile, doubly heterozygous flies are sterile. The interaction of this specific  $\alpha$ -tubulin mutation with  $\beta$ -tubulin mutations is not mimicked by a deficiency for the  $\alpha$ -tubulin gene, but rather requires a specific allele of the  $\alpha$ -tubulin gene. Fuller and her colleagues advanced the hypothesis that the  $\alpha$ -tubulin

protein acts to sequester the normal  $\beta$ -tubulin subunits into inactive dimers. As a result, the total number of functional tubulin dimers is reduced below some critical threshold. Approximately half of normal levels, as would be observed in the presence of heterozygosity for the  $\beta$ -tubulin null mutant alone, is apparently good enough. But reducing that concentration further, to one-quarter of normal levels, impairs spermatogenesis.

The observed interaction between  $\alpha$ - and  $\beta$ -tubulin mutations provided an obvious basis for a screen for mutations in other genes whose protein products might interact with either  $\alpha$ - or  $\beta$ -tubulin. Aware that many such interactions might be spurious, these workers wisely focused their attention on those interactions that met two further quite stringent criteria. First, the interaction had to be allele specific for at least one of the two loci, and second, the noncomplementing (*nc*) mutation had to cause a defect in male fertility on its own (in the absence of the tubulin mutation). Although mutations meeting these criteria were found (*cf.* REGAN and FULLER 1988; GREEN *et al.* 1990), as detailed below it is not clear that any of the proteins encoded by these genes has a functional, much less a physical, relationship to  $\beta$ -tubulin.

We first consider the noncomplementing mutation that defined the *whirligig* gene (GREEN *et al.* 1990). Although this allele of *whirligig* (denoted *wrl<sup>nc4</sup>*) was initially identified on the basis of its failure to complement a deletion that included the  $\alpha$ -tubulin gene, it was subsequently shown to fail to complement  $\beta$ -tubulin mutations as well. (In fact, it also fails to complement one of the other tubulin noncomplementing mutations, *haywire*, which is described below). The interaction of *whirligig* mutations with  $\beta$ -tubulin null mutations is allele specific in the sense that the *wrl<sup>nc4</sup>* mutation fails to complement some  $\beta$ -tubulin mutations and not others. Moreover, *whirligig* mutants display multiple anomalies in microtubule organization, including defects in spermatid differentiation, disordered spermatid components, disrupted flagellar axonemes, and axonemes lacking one or both central pair microtubules (GREEN 1990). Strengthening the case for a functional interaction between the Whirligig protein and tubulin, although a deletion for *whirligig* is a dominant male sterile, that sterility is rescued by heterozygosity for the  $\beta$ -tubulin null mutation. Thus, sterility can result from any combination of mutations that alters the relative concentrations of these two proteins, and fertility can be restored by making the concentration of these proteins more equal. Unfortunately, attempts to clone *whirligig* have been complicated by the observation that the recessive male sterility and the  $\beta$ -tubulin noncomplementing phenotypes are recombinationally separable, suggesting that the two phenotypes may be caused by two distinct mutations. (J. A. BRILL, personal communication). Thus, it is not clear that the gene defined by the *B2t* noncomplementing mutation plays a direct role

in spermatogenesis, nor is there yet any compelling evidence that its product directly interacts with tubulin.

A similarly frustrating story evolved from the study of one of the best characterized of these second-site noncomplementing genes, the *haywire* gene. Again, the interaction of *haywire* mutations with  $\beta$ -tubulin null mutations is allele specific (REGAN and FULLER 1988), and males homozygous for these *haywire* mutations have defects in several major microtubule-based processes. Nonetheless, when the *haywire* gene was cloned, it was shown to encode a general RNA polymerase II transcription factor that is also essential for nucleotide excision repair (MOUNKES *et al.* 1992; MOUNKES and FULLER 1999). Presumably, as was the case for the actin noncomplementer described in the previous section, the aberrant *haywire* gene product results in the impaired expression of some gene or set of genes in a fashion that cripples meiosis and spermatogenesis in a cell with reduced levels of  $\beta$ -tubulin.

The reason that this type of screen for interactors pulls up proteins involved in gene expression is fairly obvious. Once the normal level of a tubulin subunit has been reduced by a mutation in that gene, any second mutation that further diminishes the expression of tubulin or tubulin-interacting genes is likely to create a phenotype. For example, many, if not most, yeast screens for mitotic mutants have identified splicing genes. This observation was explained by BIGGINS *et al.* (2001), who noted that the yeast  $\alpha$ -tubulin gene contains an intron; it is thus one of the few yeast genes that must be spliced. Therefore mutants with defects in the splicing machinery might well be expected to have mitotic defects. Indeed, when BIGGINS *et al.* (2001) made an intronless TUB1 gene, the splicing mutants no longer had mitotic defects.

Taken together, these observations suggest that sequestration-based examples of SSNC are relatively rare. They also provide a warning that screens for second-site noncomplementers of null alleles are unlikely to identify genes encoding proteins whose products interact functionally, much less physically, but rather are far more likely to identify genes whose products are required for the expression of the gene defined by the original null allele.

**Type 3 SSNC (combined haplo-insufficiency)—the interaction is not allele specific at either locus:** This type of SSNC neither requires nor implies the physical interaction of the two proteins. Rather, it suggests only that reducing the dosage of the product of gene A does not create a phenotype unless the cell or organism is further crippled by a reduction in the dosage of gene B. Combined haplo-insufficiency does not require allele specificity at either gene, and it is sometimes created by using null alleles or deficiencies at one or both genes. This type of SSNC is probably the most common and sometimes the least interesting. One could imagine, for example, a case where the mutation in gene B simply

depressed the rate of transcription of gene A, thus decreasing the level of A protein production below some threshold of function.

However, there are also important examples in which the observation of combined haplo-insufficiency does indicate that two genes do act in functionally related processes. Consider, for example, a case involving *Hoxb-5* and *Hoxb-6* genes that has been reported by RANCOURT *et al.* (1995). *Hoxb-5* and *Hoxb-6* are adjacent genes in the mouse *HOXB* locus and are members of the homeotic transcription factor complex that governs establishment of the mammalian body plan. Although loss-of-function mutations at the *Hoxb-5* and *Hoxb-6* genes are fully recessive, *hoxb-5*, *hoxb-6* trans-heterozygotes (*Hoxb-5*<sup>-</sup>, *Hoxb-6*<sup>+</sup>/*Hoxb-5*<sup>+</sup>, *Hoxb-6*<sup>-</sup>) display a mutant phenotype, presumably as a result of combined haplo-insufficiency. There are in fact many other examples of SSNC in *HOX* clusters besides these two genes (R. KRUMLAUF, personal communication). For examples in both mice and humans of severe phenotypic abnormalities in limb development that result from combined haplo-insufficiency involving mutations at the *Hoxd-13* and *Hoxa-13* genes see DEBEER *et al.* (2002), FROMENTAL-RAMAIN *et al.* (1996), GOODMAN and SCAMBLER (2001), and DAVIS and CAPECCHI (1996).

A second such example can be found in the analysis of genes required for spindle pole body function in yeast, namely *MPS1* and *SPC42*. The *SPC42* gene encodes a core component of the spindle pole body whose function is directly regulated via phosphorylation by the Cdc28 kinase. However, in addition to this direct form of regulation by Cdc28, Cdc28 also regulates Spc42 function indirectly by a pathway that involves phosphorylation of Mps1 (JASPERSEN *et al.* 2001). Neither of these two independent pathways is essential on its own for cell viability, but disruption of both pathways can lead to defects in spindle pole body duplication. While mutations in the *MPS1* or *SPC42* gene that block Cdc28-dependent phosphorylation are viable in either haploids or diploids, diploids that are doubly heterozygous for both mutations are not viable (JASPERSEN *et al.* 2001). Apparently, as a consequence of the changes in spindle pole body size that accompany the shift from a haploid to a diploid cell, the combined haplo-insufficiency for Cdc-28 phosphorylatable forms of these two proteins becomes a lethal event (S. JASPERSEN, personal communication).

Such examples aside, anyone devising a screen for functionally related genes on the basis of searching for mutants that exhibit combined haplo-insufficiency is likely to be frustrated by the rather large number of nonspecific modifiers described in the first paragraph of this section. Only when such a screen is coupled with a powerful secondary screen, such as demanding that the new mutations cause relevant phenotypes as homozygotes in the absence of a mutation at the original locus, is there a good likelihood of identifying interesting new

loci. However, the recent development of techniques designed by Boeke and his collaborators (*cf.* YE *et al.* 2005) may allow the development in yeast of high-throughput means to identify examples of SSNC that can be sorted through by exactly such secondary screens.

#### DOMINANT ENHANCEMENT: THE TECHNIQUE THAT ACTUALLY WORKS!

Although screens for second-site noncomplementers that demand that both mutations be fully recessive have not proven terribly successful in identifying proteins that physically interact or even in identifying proteins that function in the same or parallel pathways, real successes have been obtained from screens for dominant enhancer mutations of semidominant mutations. These screens are by definition not screens for noncomplementation at all, because they begin with a semidominant or weakly poisonous antimorphic mutation and then screen for dominant enhancers of that mutation. (Recall that a true complementation test requires that both mutations be *fully* recessive!) In addition, one could expand the types of “bait” mutants employed in such screens to include the sorts of overexpression constructs created in *Drosophila* by Rorth and her colleagues (RORTH *et al.* 1998) or conversely to screen a collection of overexpressing lines for their ability to enhance a given “threshold” mutation.

For example, in *Caenorhabditis elegans* YOOK *et al.* (2001) observed strong interactions between heterozygous mutations in the genes encoding the physically interacting synaptic proteins UNC-13 and syntaxin/UNC-64, but only when at least one mutation encoded a partially functional, but weakly poisonous, gene product. In this instance, dominant enhancement was observed only if the altered gene products impair the protein complexes with which they are normally associated (YOOK *et al.* 2001). These authors then tested mutant alleles of other genes, whose protein products were also known to interact with syntaxin/UNC-64, for their ability to interact with a poisonous allele of this gene. Although the strongest effects were observed between loci encoding gene products that bind to one another, interactions were also observed between proteins that do not directly interact but are members of the same complex. YOOK *et al.* (2001) also observed strong dominant enhancement between genes that function at distant points in the same pathway, implying that physical interactions are not required for this process. However, mutations in genes that function in different processes such as neurotransmitter synthesis or synaptic development did complement one another. Thus, this genetic interaction was specific for genes acting in the process of synaptic vesicle trafficking.

A similar approach was taken by Dan Kiehart and his colleagues who used dominant enhancement to identify a group of genes whose protein products interact



with nonmuscle myosin in *Drosophila* (HALSELL and KIEHART 1998; HALSELL *et al.* 2000). In *Drosophila*, the *zipper* (*zip*) gene encodes the nonmuscle myosin protein. Mutations in this gene produce malformed legs in the adult. Indeed, a missense allele (*zip[Ebr]*) proved useful as bait in a screen for dosage-sensitive enhancers or suppressors. Kiehart and colleagues scanned through 70% of the *Drosophila* genome by testing a large collection of deficiencies for their ability to modify the expression of a semidominant *zip[Ebr]* and identified three chromosomal deficiencies that strongly enhanced the phenotype associated with the *zip[Ebr]* mutation. More importantly, the interaction of those deletions with *zipper* can be explained as the result of reducing the dose of specific genes located within the regions defined by these deficiencies. These include genes encoding cytoplasmic myosin, collagen IV, and the signal transduction protein RhoA.

Similarly, Fehon and his collaborators identified a number of interactors for the *Drosophila* homolog of the human neurofibromatosis type 2 protein (NF2), a gene known as *Merlin*, by screening for dominant enhancers of semidominant effects on head, eye, wing, and leg morphology, of a dominant-negative allele of this gene (LA JEUNESSE *et al.* 2001). From a screen of 100,000 mutagenized chromosomes they recovered 29 dominant enhancers of *Mer<sup>3</sup>*, 23 of which fell into five complementation groups. In many cases these mutations also modified the expression of a hypomorphic *Mer<sup>3</sup>* mutation as well. Finally, mutations in two of these complementation groups were shown to modify the cellular localization of wild-type Merlin protein and thus clearly do define genes whose products interact with Merlin.

But perhaps the most impressive use of this method has been the work by Rubin and his collaborators to dissect the role of the Ras signaling pathway in eye development in *Drosophila*. For example, SIMON *et al.* (1991) performed an elegant screen for mutations in genes whose products interacted with the protein encoded by the *sevenless* gene in *Drosophila*. The product of the *sevenless* gene specifies a membrane receptor tyrosine kinase whose function is required for the proper differentiation of one type of photoreceptor cell (R7) that is present in each of the 800 repeating units that compose the fly's compound eye. SIMON *et al.* (1991) used site-directed mutagenesis to screen for dominant enhancers of a temperature-sensitive *sevenless* allele. Twenty such enhancers, defining seven lethal complementation groups, were recovered from >30,000 treated chromosomes. The genes defined by these mutations encode critical components of a Ras-dependent signaling process that acts downstream of the Sevenless protein to mediate R7 differentiation.

Of course, not all such interactions will define interacting functions. Consider, for example, the case of *Minute* mutations in *Drosophila*, which define the genes

that encode ribosomal proteins. There are numerous examples of mutations that would be recessive in a non-*Minute* background but cause a phenotype, even as a heterozygote, in the presence of the *Minute* mutation. Obviously, screens based on dominant enhancement, just like those based on canonical SSNC, must include rigorous secondary screens to filter out those genes that just alter the expression of the bait gene. Moreover, as we noted in the preceding section, the development of high-throughput screens, such as the RNAi-based screen for dominant enhancers recently carried out in *C. elegans* by LEHNER *et al.* (2006), may provide a rapid method for identifying such mutations and facilitate their rapid characterization.

## SUMMARY AND CONCLUSIONS

To quote LEWIS (1967, p. 20), "In many situations the *cis-trans* test works well." Indeed, we would go a bit further and argue that in the vast majority of cases the assignment of new mutations to individual genes by use of the complementation test works well. Nonetheless, misleading results can be obtained quite easily, as a result of the failure of mutations in different genes to complement. The existence of several fascinating examples of SSNC has led various investigators to perform a number of screens designed to identify functionally related genes on the basis of second-site noncomplementation. Unfortunately, such screens have not been terribly successful or at least not as successful as screens for dominant enhancers of weakly dominant alleles of the gene of interest.

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