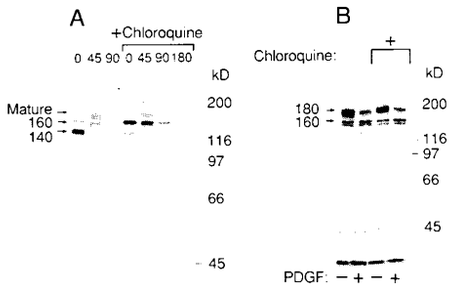

Fig. 4. Effect of chloroquine treatment on PDGF receptor metabolism in NRK and v-*sis*–transformed NRK cells. Cell monolayers were treated with Dulbecco's minimum essential medium (DMEM) with (+) or without 25 μM chloroquine for 1 hour at 37°C. v-*sis*–transformed NRK cells (**A**) were biosynthetically labeled with [^{35}S]methionine as described in Fig. 1 in the absence or presence of chloroquine. NRK cells (**B**) were labeled with [^{35}S]methionine with or without chloroquine at 37°C for 2 hours in the absence (−) or presence (+) of a saturating concentration of PDGF, which causes receptor internalization and degradation. Cell extracts were immunoprecipitated with antiserum to PDGF receptor as described in Fig. 1.

siently expressed at the cell surface and then degraded or if they are shunted directly to degradation pathways. Additionally, we cannot exclude the possibility that a small percentage of receptors is expressed at the plasma membrane before activation and then immediately degraded, thus evading detection by our methodology. In v-*sis*–transformed cells, however, the majority of PDGF receptor activation clearly occurred in intracellular compartments, before receptor maturation was complete and before receptors could have reached the cell surface. Our data are consistent with reports showing that chronic exogenous PDGF treatment, by itself, will not transform cells (*14*). These findings also help explain why attempts to reverse v-*sis*–mediated transformation with antisera to PDGF have had limited success (*15*). This novel, intracellular mechanism of activation of a growth factor receptor is likely to be responsible for v-*sis*–induced cell transformation. Attempts to reverse transformation by v-*sis*, therefore, should be directed to intracellular receptors.

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12. Previous ^{125}I-labeled PDGF binding studies (*2*) have shown that suramin "up-regulates" cell surface PDGF receptors in v-*sis*–transformed cells. Although suramin treatment did not inhibit intracellular receptor activation in v-*sis*–transformed cells, in separate experiments (*8*) we found that suramin prolonged the half-life of the mature form of the receptor in these cells. In the presence of suramin, mature receptor proteins were detectable at the cell surface as shown by their sensitivity to trypsin (data not shown). As noted previously, suramin inhibits binding of PDGF to cell surface receptors (*2*). However, suramin also has access to intracellular compartments (*13*). It is not clear, therefore, if an intracellular or an extracellular mechanism accounts for suramin's capacity to up-regulate cell surface PDGF receptors in v-*sis*–transformed cells.
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Juvenile Hormone Action Mediated in Male Accessory Glands of *Drosophila* by Calcium and Kinase C

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In an in vitro system for the *Drosophila melanogaster* male accessory gland, it was found that $10^{-9}M$ juvenile hormone III could accurately mimic the copulation-induced response of increased protein synthesis in glands from virgin flies. Stimulation by this hormone required calcium in the medium. Experiments with tumor-promoting phorbol esters indicated that activation of protein kinase C can also cause the glands to increase protein synthesis. Stimulation of protein synthesis by juvenile hormone did not occur in mutants deficient in kinase C activity. These results suggest a membrane-protein–mediated effect of juvenile hormone that involves calcium and kinase C.

THE MALE ACCESSORY GLANDS OF *Drosophila melanogaster* produce and secrete proteins that are found in the seminal fluid and have important effects on female reproductive behavior (*1*). In our in vitro system, the incorporation of [^{35}S]-methionine into trichloroacetic acid (TCA)–precipitable proteins increases throughout the 3 hours studied and is linear within the first 2 hours (Fig. 1A). Copulation induces an increase in protein synthesis (Fig. 1B) and RNA synthesis (Fig. 1C). Protein synthesis is increased in glands dissected immediately after copulation and is maximal in the flies 2 hours after copulation (Fig. 1B). Our in vitro measurements of the timing and magnitude of the stimulation of overall protein and RNA synthesis following copulation are in agreement with previous in vivo studies of the copulation-induced stimulation of protein and RNA synthesis in these glands (*2, 3*).

Since it is known that juvenile hormone (JH) effects the correct development, maturation, and functioning of the male accessory glands in other insects (*1, 4, 5*), and is important in the regulation of *D. melanogaster* female reproductive physiology (*6–9*), we examined the effects of JH on the male accessory glands in vitro. At concentrations of $10^{-9}M$ juvenile hormone III (JH III), the amount of incorporation of [^{35}S]methionine into TCA-precipitable proteins is maximally increased to nearly 300% of that of control values after a 1-hour incubation (Fig. 2A). There is a partial effect even at concentrations of $10^{-11}M$. The hormone 20-hydroxyecdysone at $10^{-7}M$ causes little or no increase in protein synthesis (Table 1). Each reagent listed in Table 1 was tested at

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more than one concentration; only results for the concentration that was maximally effective are shown. Combined treatment with ecdysone and JH III stimulates protein synthesis no more than treatment with JH III alone. Similarly, the activity of esterase 6 in ejaculatory ducts of *D. melanogaster* is elevated by treatment of isolated male abdomens with JH III or with 20-hydroxyecdysone, and ecdysone and JH do not act additively (*6, 10*).

These experiments can be directly compared to those with other insects in which removal of the corpus allatum or abdominal ligature, each of which eliminates the natural source of JH, influenced the proper maturation and function of the male accessory glands (*1, 10–12*). Correct glandular function including increased protein synthesis can be gained by topical application of JH. In our in vitro system, the natural source of JH is obviously removed but the addition of JH can cause the glands to increase their protein synthesis levels.

The *D. melanogaster* mutant, *apterous*^{4} (*ap*^{4}), is short-lived and known to be deficient in the production of JH III (*7, 13, 14*). The male accessory glands of 1-day-old *ap*^{4}/*ap*^{4} flies are substantially smaller than those from wild-type flies of a similar age. In vitro incubation of accessory glands from 1-day-old flies shows that glands from *ap*^{4}/*ap*^{4} males are only 30% as active in protein synthesis as wild type. The glands from *ap*^{4} flies show a 30 to 70% increase in protein synthesis when treated with 10^{-9} *M* JH III. We also treated intact flies topically with the metabolically more stable JH III analog, hydroprene, for 1 hour prior to dissection and labeling of the glands. These experiments gave the same results as the in vitro experiments and were more reproducible (Table 1).

JH III requires calcium in the medium to mimic the copulation-induced stimulation of protein synthesis (Fig. 2A). When male accessory glands were exposed to 10^{-9} *M* JH III in the presence of 0.1 m*M* calcium in the medium, a threefold increase over untreated glands was observed. Calcium alone, at concentrations of 1 or 5 m*M* in the medium, increased protein synthesis, but these concentrations did not enhance the level of JH III stimulation of protein synthesis over that seen at 0.1 m*M* calcium. Exposure of the glands to JH III without added calcium resulted in a lower stimulatory effect of JH III on protein synthesis. Preincubation of the glands in 1 m*M* EGTA (a calcium chelator) also decreased protein synthesis and eliminated any stimulation of protein synthesis by JH III. These results suggest that calcium bound to the glands

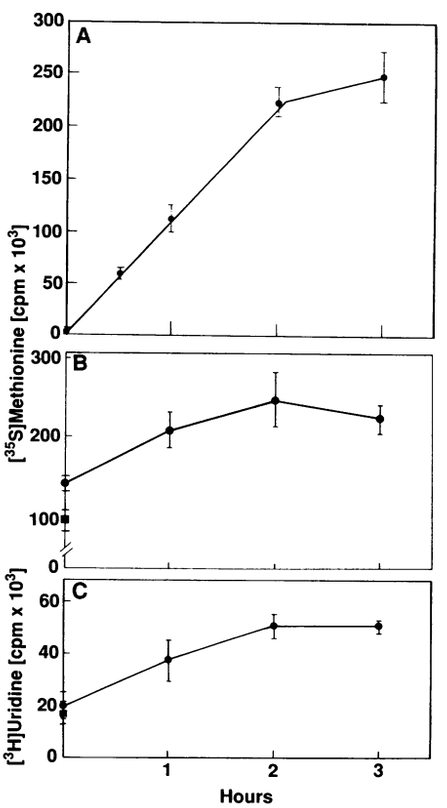

Fig. 1. (**A**) Incorporation of [^{35}S]methionine into accessory gland proteins in vitro. The accessory glands from virgin males were incubated in MOPS buffer containing 0.1 m*M* $CaCl_2$ and 2 μCi of [^{35}S]methionine. The mean values and standard deviations shown represent four to eight determinations with six glands per incubation. (**B** and **C**) Stimulation of in vitro protein and RNA synthesis after copulation. Six glands from copulated males were dissected at the times indicated and subsequently incubated in MOPS buffer containing 0.1 m*M* $CaCl_2$ for 1 hour. Closed circles: rates of incorporation of [^{35}S]methionine (2 μCi) into proteins (B); rates of incorporation of [^{3}H]uridine (10 μCi) into RNAs (C); squares represent values from virgin flies. Mean values and standard deviations were taken from at least four experiments. Wild-type *D. melanogaster* (Oregon-R) were grown at 25°C on a standard cornmeal-Karo-yeast agar medium supplemented with live yeast. After eclosion, flies were separated according to sex. In general, the experimental males were 10 to 11 days old, although flies from 7 to 12 days old were used on occasion. Accessory glands from six virgin males were dissected free of testicular and gut tissue and incubated at room temperature for 1 hour with gentle shaking in 60 μl of MOPS buffer (10 m*M* MOPS, *p*H 7.0, 80 m*M* NaCl, 10 m*M* KCl, 0.2 m*M* $MgCl_2$, and 0.1 m*M* $CaCl_2$) (*22*), and 2 μCi of [^{35}S]methionine (1125 Ci/mmol), or 10 μCi of [^{3}H]uridine (26.5 Ci/mmol). Copulated flies were virgin males whose glands were removed at varying times after copulation. Males that did not copulate within 1 hour were discarded. Experiments were performed in which ribonuclease treatment was included prior to TCA precipitation to assess the contribution of aminoacylated transfer RNAs in the protein assay; the results were the same as those obtained without ribonuclease treatment.

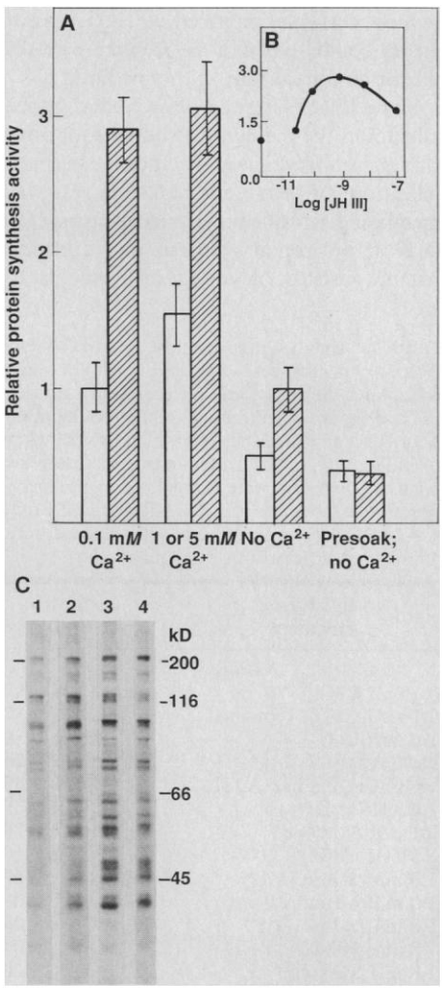

Fig. 2. (**A**) In vitro stimulation of accessory gland protein synthesis by JH III required calcium. Shaded bars represent incubations in the presence of 10^{-9} *M* JH III, open bars in the absence of JH III. Mean values and standard deviations from at least four determinations are shown. (**B**) The dose dependence of in vitro stimulation of protein synthesis by JH III is shown in the inset graph. Accessory glands from virgin males were incubated in siliconized vessels for 1 hour with MOPS buffer containing 2 μCi of [^{35}S]methionine and the indicated concentration of JH III and $CaCl_2$. In some experiments ("presoak"), external calcium was chelated by first placing the glands in MOPS buffer containing 1 m*M* EGTA and no calcium for 1 hour. They were then transferred to tubes containing MOPS buffer and [^{35}S]methionine for an additional hour. Values are means and standard deviations from six to eight determinations that were normalized to incorporation rates for glands incubated in 0.1 m*M* $CaCl_2$ MOPS buffer (without JH III). (**C**) In vitro incorporation of [^{35}S]methionine into accessory gland proteins following stimulation. One-dimensional gel electrophoresis was performed with 0.5-mm SDS slab gels of 10% polyacrylamide (*23*). Labeled proteins were extracted from glands of virgin flies incubated in MOPS with 0.1 m*M* $CaCl_2$ (lane 1); 1.0 m*M* $CaCl_2$ (lane 2); 0.1 m*M* $CaCl_2$ and 10^{-9} *M* JH III (lane 3); and, from glands of copulated flies (removed 1 hour after copulation) with 0.1 m*M* $CaCl_2$ (lane 4). In each case five glands per sample were incubated in a total volume of 40 μl containing 3 μCi of [^{35}S]methionine.

(which would be removed by EGTA treatment) could, when it is present, partially substitute for calcium in the medium.

Since JH III stimulation required added calcium in the medium to show its maximal effects, we investigated whether the permeabilization of both internal and external membranes to calcium could also cause an increase in protein synthesis in the glands. In the absence of added calcium, glands treated with the ionophore A23187 showed increased protein synthesis (Table 1). Additionally, when a tenfold excess of cobalt (a calcium antagonist) was added to the incubation medium with calcium, protein synthesis decreased to near the level of no added calcium in the medium (Table 1).

Table 1. Protein synthesis in the in vitro system. Accessory glands were incubated in MOPS buffer containing the indicated concentrations of calcium and other reagents and [^{35}S]methionine for 1 hour. Except where indicated, glands were taken from virgin flies. The protein synthesis results are relative values that were normalized to the incorporation of label into glands incubated in 0.1 m*M* $CaCl_2$. They represent means and standard deviations from at least four determinations.

Incubation medium	Protein synthesis
Wild type	
0.1 m*M* $CaCl_2$	1.00 ± 0.02
0.1 m*M* $CaCl_2$, copulated	2.50 ± 0.37
1.0 m*M* $CaCl_2$	1.53 ± 0.30
No $CaCl_2$	0.50 ± 0.20
No $CaCl_2$, 0.5 μ*M* A23187	2.64 ± 0.45
1.0 n*M* JH III*	2.88 ± 0.35
0.1 μ*M* ecdysone*	1.28 ± 0.20
1.0 m*M* $CoCl_2$*	0.65 ± 0.10
1.0 m*M* 8-Br-cAMP*	1.04 ± 0.07
1.0 m*M* DB-cAMP*	1.04 ± 0.08
1.0 m*M* 8-Br-cGMP*	0.81 ± 0.05
1.0 m*M* IBMX*	0.88 ± 0.03
1.0 μ*M* forskolin*	1.07 ± 0.20
20 μ*M* octopamine*	1.02 ± 0.07
2.0 m*M* serotonin*	1.11 ± 0.10
2.0 m*M* dopamine*	0.95 ± 0.10
5.7 n*M* insulin*	1.07 ± 0.06
0.1 m*M* $CaCl_2$, pretreated with 1.6 μg of hydroprene† ap^4/ap^4‡	2.02 ± 0.17
0.1 m*M* $CaCl_2$	0.32 ± 0.10
0.1 m*M* $CaCl_2$, pretreated with 1.6 μg of hydroprene *tur*	0.55 ± 0.12
0.1 m*M* $CaCl_2$	0.96 ± 0.20
0.1 m*M* $CaCl_2$, pretreated with 1.6 μg hydroprene§	1.13 ± 0.15

*Incubations were performed in 0.1 m*M* $CaCl_2$. The reagents were JH III (a racemic mixture, from Calbiochem, Boehring, San Diego, California); A23187; $CoCl_2$; 20-hydroxyecdysone; the cyclic nucleotide analogs 8-bromo adenosine 3′,5′-monophosphate (8-Br-cAMP), 8-bromo guanosine 3′,5′-monophosphate (8-Br-cGMP), and dibutyryl cyclic AMP (DB-cAMP); 3-isobutyl-1-methyl-xanthine (IBMX, an inhibitor of cAMP phosphodiesterase); forskolin (a simulator of adenylate cyclase); the insect neurotransmitters DL-*p*-hydroxyphenylamine (octopamine), 5-hydroxytryptamine (serotonin), and 3,4-dihydroxyphenethylamine (dopamine); and insulin (bovine). †Wild-type males were pretreated with hydroprene (a gift of Zoecon), an active analog of JH, which was applied topically to the abdomens of flies at a concentration of 1.6 μg in 0.3 μl of acetone. Accessory glands were dissected 1 hour after this treatment. Control flies were treated with the same amount of acetone only. ‡ap^4 males used were 1-day-old and are compared to 1-day-old wild-type males. This was necessary because the mutant flies die within a day or two after eclosion (*7*). The pretreated ap^4 males were pretreated with hydroprene as the wild type. §*tur* virgin males 11 days old were pretreated with hydroprene as the wild type.

As seen in the autoradiograms of one-dimensional gels of total protein from glands labeled in vitro (Fig. 2B), the banding patterns of proteins synthesized in glands from copulated flies, and from virgin flies treated with 10^{-9}*M* JH III or high levels of calcium, remained the same, although the amounts of protein synthesized clearly changed. This is the same pattern of protein synthesis found to occur in vivo (*2, 3*). The patterns of proteins synthesized in *Drosophila* accessory glands and testes were found to be identical in vitro as in vivo as determined by two-dimensional gel analysis (*15*).

A number of intracellular signaling pathways in which calcium plays a role have been described. One of these involves the breakdown of membrane phospholipids whose products cause the activation of protein kinase C and the release of free calcium stores. Tumor-promoting phorbol esters, such as phorbol-12,13-dibutyrate (PDBU), are known to elicit a diacylglycerol-like activation of protein kinase C (*16–18*). Figure 3 shows the stimulatory effects of PDBU on protein synthesis in the accessory glands in the absence and presence of added calcium (0.1 m*M*). However, at higher calcium levels (1.0 m*M*), the stimulatory effect of PDBU was not seen. Long exposures to phorbol esters can result in desensitization of protein kinase C (*19*). Glands exposed to PDBU for greater than 15 minutes showed no increase in protein synthesis; after a 1-hour exposure, protein synthesis was depressed. Incubation of the glands with a second tumor-promoting phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), gave identical results. Like stimulation by JH III, the stimulation by PDBU required calcium in the medium (Fig. 3). The non-tumor-promoting compound, phorbol, which does not activate kinase C, had no effect on protein synthesis in the glands. Experiments with turnip (*tur*) mutant flies known to be defective in kinase C activity (*20, 21*) showed that topical application of hydroprene to fly abdomens caused no significant increase in protein synthesis in the glands, whereas application to wild-type flies showed a stimulation (Table 1). Similar results were obtained in in vitro incubation experiments.

Other intracellular signaling pathways involve changes in the levels of cyclic nucleotides; however, none of the reagents best known to cause such changes have any substantial effect on protein synthesis in accessory glands (Table 1). Similarly, reagents such as the insect neurotransmitters dopamine, serotonin, and octopamine and the hormone insulin, showed no stimulation of protein synthesis (Table 1).

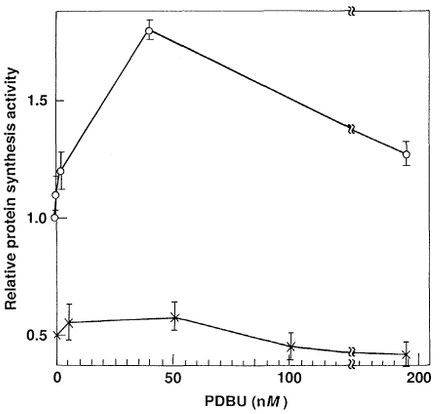

Fig. 3. In vitro stimulation of protein synthesis by PDBU required calcium. Accessory glands were preincubated in the indicated concentrations of PDBU in MOPS buffer for 10 minutes before being transferred to tubes containing MOPS buffer and label for an additional 1 hour. Mean values and standard deviations from at least four determinations are given and normalized to the value at 0.1 m*M* $CaCl_2$; (○) incubations in 0.1 m*M* calcium; (×) represent incubations in the absence of added calcium.

Recent results of Ilenchuk and Davey show that JH effects membrane Na^+,K^+-adenosinetriphosphatase in *Rhodnius* (*22*). Our results also suggest a membrane-protein-mediated activity for JH that involves calcium and kinase C.

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Technical Comments

Clathrin: A Matter of Life or Death?

In their Research Article, "Clathrin requirement for normal growth of yeast" (*1*), Sandra K. Lemmon and Elizabeth W. Jones describe the characterization of a yeast strain engineered to contain a deletion in the gene that encodes the heavy chain of clathrin (*CHC*, wild type; *chc*-Δ, deletion). Among the haploid progeny of a single diploid strain, they found a locus, referred to hypothetically as a "suppressor gene," that influences the ability of yeast cells to survive deletion of the *CHC* gene. This locus, genetically unlinked to *CHC*, exists in two forms: one that allows *chc*-Δ cells to live and grow slowly, and another that results in *chc*-Δ cell death. The original diploid contains one copy of each form. Inasmuch as clathrin is held to play important roles in membrane traffic within eukaryotic cells, it is tempting to suggest that the "normal" state of this gene results in the death of *chc*-Δ cells. Hence, "normal" yeast cells, and by extension all eukaryotic cells, would require clathrin to live.

Two years ago we reported results on clathrin deletion (*2*) that are largely confirmed by Lemmon and Jones with one important exception. Our strain of yeast survived deletion of the *CHC* gene, with no evidence of an unlinked genetic character influencing cell viability. We showed that clathrin-deficient cells are sickly, but that at least one aspect of membrane traffic, glycoprotein secretion, is preserved. From these data we argued that clathrin, although important for a normal rate of growth, is dispensable and possibly replaced by other structurally distinct molecules.

Now the question is, Which strain are we to accept as "normal"?

This question would be most difficult to answer with only the two strains that have been reported. For this reason, we have conducted a survey of more than ten strains from our collection and from three other laboratories (*3*). Many of these strains share a common parentage with the original strain used by us and by Lemmon and Jones. Each of the strains we examined survived deletion of the *CHC* gene. Although not mentioned in their article, we would ask if our colleagues also have examined other strains? While it is possible that all strains tested, save the one reported by our colleagues, contain genetic alterations that mitigate the lethal effect of clathrin deletion, we suggest rather that the unusual strain contains a cryptic mutation that is lethal only when clathrin is deleted. This could represent a function that replaces clathrin, or it could affect cell viability indirectly.

Given the large number of strains we have examined, the more representative phenotype appears to be survival of *CHC* deletion mutant cells. For this reason we believe it is appropriate to investigate clathrin function by examining membrane traffic in viable clathrin-deficient cells. To paraphrase Samuel Johnson, "It matters not how a *yeast cell* dies, but how *it* lives."

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Response: We are in the fortunate position of discussing important issues where the two sets of data in question (*1, 2*) are both correct and irrefutable. There seems to be no question that cells can survive clathrin deficiency in some genetic backgrounds and cannot do so in other genetic backgrounds.

Randy Schekman and Gregory Payne raise two issues of substance about the function of clathrin in yeast cells. The first issue is whether the *scd1* (suppressor of clathrin deficiency) allele suppresses the lethality associated with deletion of the clathrin heavy chain gene or whether the *SCD1* allele kills cells bearing the deletion. This is a semantic difference that cannot be resolved by segregational analysis. Only the determination of the molecular basis of *scd1* suppression will resolve this ambiguity.

The second issue raised is whether *SCD1* or *scd1* is the normal allele. Most of the yeast strains commonly used for genetic analysis are closely related to one another (*3*). To sample these strains will merely identify the more common allele in this highly inbred population. In this population, for example, the *ho* and *gal2* alleles (mutant forms of the genes that encode the mating type switching endonuclease and the galactose permease, respectively) are the more common alleles. In one sense it is not even very important which (*scd1* or *SCD1*) is the more common allele in the population at large. The important fact is that cells can survive without clathrin if they carry the *scd1* allele and must have clathrin if they carry the *SCD1* allele. The challenge is to discover the molecular basis that distinguishes these two states.

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