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The molecular analysis of tko, a behavioral mutation in Drosophila melanogaster

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THE MOLECULAR ANALYSIS OF *tko*, A BEHAVIORAL MUTATION IN *DROSOPHILA MELANOGASTER*

by

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DISSERTATION

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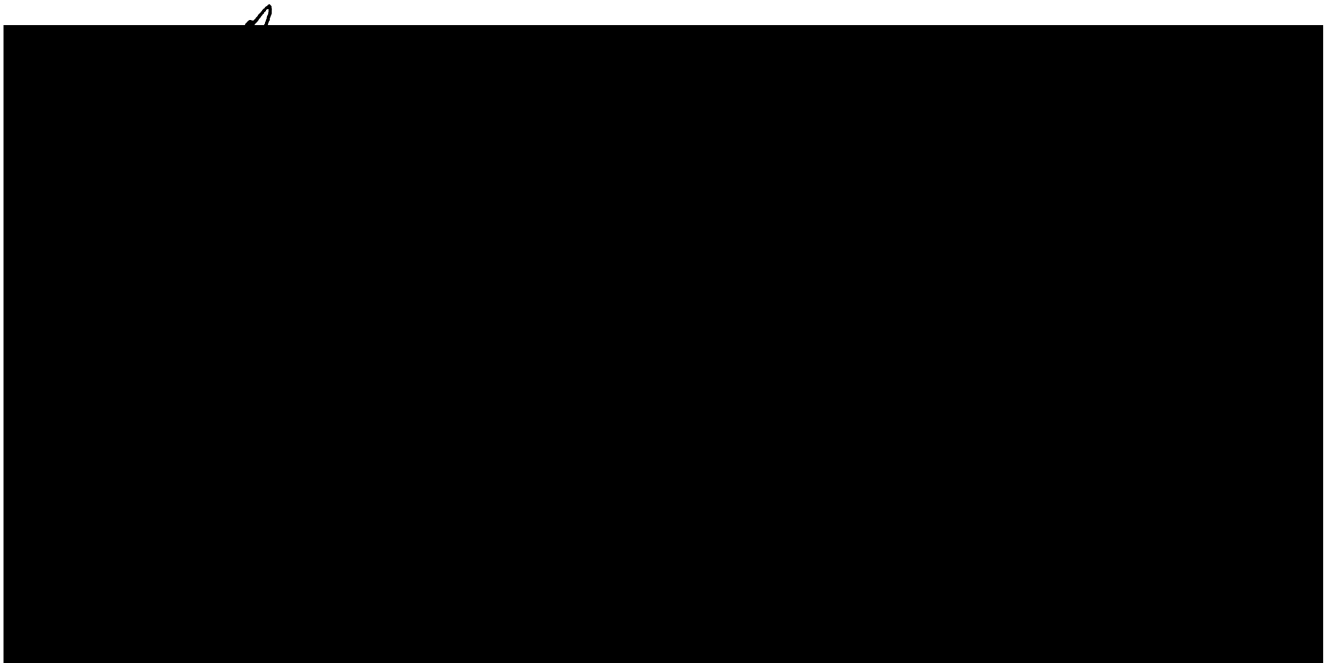

Dedicated to my husband, Robert Colgrove.

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ABSTRACT

The technical knockout mutation, *tko,* is one of a family of behavioral mutations called "bang-sensitive" mutations. Flies carrying these mutations are paralyzed temporarily by physical shocks, such as a jolt of the culture vial. Indirect arguments based on electrophysiology and studies with double mutants suggest that *tko* might affect neuronal excitability. I began to study the gene coding for *tko* to gain some insight into the defects which cause bang sensitivity.

The *tko* gene has been mapped genetically to band 3A2 of the X chromosome just distal to the well known *zeste* gene. I used P-element transformation to show that a 3.1 kb fragment of genomic DNA from this region complements two alleles of *tko*: $l(1)^{tko}zw64k11$, a lethal allele, and tko^{25t}, which causes the behavioral defect. Northern blot analysis indicated that this 3.1 kb fragment contains only one complete transcript, 0.68 kb in length. I isolated cDNA clones corresponding to this transcript and sequenced these clones, as well as the corresponding genomic DNA.

The cDNA sequence contains an open reading frame coding for a protein with remarkable homology to ribosomal protein S12 from *Escherichia coli* and *Euglena gracilis* chloroplast. This homology raises the possibility that the *tko* gene product may be a mitochondrial ribosomal protein. A defect in mitochondrial function could affect ion gradients and energetics in neurons and muscles, resulting in behavioral abnormalities. This can explain several of the defects seen in *tko* flies. Alternatively, the *tko* gene product could have evolved from S12 into a non-ribosomal pro-

tein of similar function.

Cytochrome oxidase assays showed that *tko* flies have much less activity than wild type flies. This is consistent with the hypothesis that *tko* codes for a mitochondrial ribosomal protein. Antibodies were generated against a fusion protein and a synthetic peptide containing parts of the *tko* sequence. These antibodies bind to a protein with apparent molecular weight of 17 kD, which may be the *tko* gene product. The implications of the hypothesis and further experiments to test it are discussed.

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CHAPTER 1

INTRODUCTION

1.1 Study of Behavioral Mutations

The normal operation of the nervous system depends on the complex interplay of many molecules and cells. The advent of new techniques in molecular biology has made it possible to study the roles that individual molecules play in the function and development of the nervous system. The molecules studied using these techniques include ion channels, neurotransmitters and their receptors, trophic factors and adhesion molecules. The use of genetics combined with molecular analysis has proven to be a powerful approach for identifying and studying molecules important for nervous system function. The fruitfly, *Drosophila melanogaster*, has several advantages for this approach. It has been extensively studied genetically. Many mutations and chromosomal rearrangements have been analyzed and mapped on its chromosomes. The phenotypes of these mutations are useful in two ways. First, they provide a wide variety of genetic markers for use in genetic manipulation and for mapping genes of interest. Second, the phenotypes of interesting genes can provide insight into the function of these genes. Over the last decade, many powerful molecular techniques have been established for *Drosophila*. These include the creation of genomic libraries for chromosomal walking, cDNA libraries for the analysis of transcripts, and the establishment of P-element transformation. The repertoire of genetic and molecular techniques available makes it possible to clone and study

almost any gene in the fly. Also, unlike other organisms that are used for their genetic and molecular advantages, such as yeast and *E. coli*, the fly has a complex nervous system and exhibits complex behaviors and learning. Thus the fruitfly has become a favorite organism for studying the nervous system at the molecular level.

To study genes involved in normal nervous system function, one must first identify these genes. In *Drosophila*, researchers have looked for mutations that affect the behavior of the fly, because abnormal behavior may reflect a primary defect in the proper development or operation of the nervous system. The type of behavioral abnormality determines what type of gene function one expects to find. Studies of the *per* locus, learning mutants and the *Shaker* locus illustrate how the selection of a behavioral mutation coupled with molecular analysis has increased our knowledge and understanding of nervous system operation.

To study the genes involved in biological clocks, Konopka and Benzer (1971) looked for mutations affecting the periodicity of the fly's circadian rhythms. They identified three mutations that affected the periodicity of the flies' activity cycles. One increased the length of the cycle, one decreased the length of the cycle and the third destroyed all cyclic behavior. Because these all mapped to a single site, the *per* (period) locus, they reasoned that this gene was crucial for the functioning of the biological clock. Recent cloning and sequencing of this gene has revealed that it codes for a protein similar to a proteoglycan (Jackson et. al., 1986; Reddy et. al., 1986) and could be involved in gap-junction formation (Bargiello et. al., 1987). If this hypothesis proves true then it points to the importance of gap junctions in the circuits that regulate the biological rhythms.

To study the mechanisms of learning and memory, Quinn and his colleagues developed a screen that would identify flies with an inability to learn or remember (Aceves-Pina et. al., 1983). Using this screen they identified several mutations, *dunce, rutabaga, turnip* and *cabbage*, that affected learning or memory in the fly. The cloning of the *dunce* gene led to its identification as a phosphodiesterase (Byers et. al., 1981; Kauvar, 1982; Chen et. al., 1986). Biochemical studies of another mutant, *rutabaga*, showed that flies carrying this mutation had decreased adenylate cyclase activity (Livingstone et. al., 1984). These discoveries demonstrated the importance of cyclic-AMP phosphorylation for learning mechanisms and complemented the work of Kandel in *Aplysia* (Kandel et. al., 1983).

Another example of the power of genetic and molecular techniques in *Drosophila* for studying the nervous system came from the laboratory of Lily and Yuh Nung Jan. Using electrophysiological techniques they determined that the behavioral mutant, *Sh* (*Shaker*), has a defect in a potassium channel at the larval neuromuscular junction (Jan et. al., 1977). Further experiments by Salkoff and Wyman (1981) showed that the *Shaker* flies lacked a particular potassium current, the A-current. The different types of mutations at the *Shaker* locus, affecting either the amount or kinetics of the A-current, suggested that *Shaker* was a structural gene coding for a component of the potassium channel. The cloning and expression of the *Shaker* gene has confirmed this hypothesis (Papazian et. al., 1987; Tempel et. al., 1987; Timpe et. al., 1988). Because the potassium channel is present in the nervous system in small amounts and because there is no known toxin with high affinity for this channel, it was not accessible to purification and analysis using standard

biochemical techniques. Therefore the cloning of the *Shaker* gene represents a significant advance since it allows detailed study of the structure and regulation of a potassium channel, an important component of the nervous system.

All of the above examples point to the utility of using genetics combined with molecular biology for studying nervous system function. I have chosen to take this approach to study a particular behavioral mutation, *tko* (technical knockout). This mutation is one of a family of mutations know as "bang-sensitive" mutations. Flies carrying these mutations are sensitive to physical jolts, such as a sharp rap on the culture vial or the standard assay of vortexing for ten seconds. Normal flies are unaffected by these manipulations but bang-sensitive flies will become paralyzed for thirty seconds to five minutes. This paralysis is intriguing in itself and suggests some defect in the nervous system or musculature of the flies. Several other pieces of evidence, described in the next section, suggest that the bang-sensitive mutations affect the excitability of the nervous system. By cloning and analyzing one of these genes I hoped to gain insight into its function and to understand how a defect in the gene could cause this interesting behavioral abnormality.

CHAPTER 2

BANG-SENSITIVE MUTATIONS

There are five known bang-sensitive loci on the X-chromosome: *bas* (bang sensitive), *bss* (bang senseless), *eas* (easily shocked), *kdn* (knockdown), and *tko* (technical knockout). Each locus has one or two alleles which cause the bang-sensitive behavior. It is probable that there are more bang-sensitive loci scattered throughout the genome, but they have not yet been detected because the autosomes have not been screened for bang-sensitive mutations. In addition to their bang-sensitive phenotype, these mutants have several other phenotypes that give clues to the functions of these genes. The following sections describe what is known of the phenotype of the bang-sensitive mutants.

2.1 Bang Sensitivity

All bang sensitive mutants share a common trait in their sensitivity to physical jolts. When vortexed at high speed for ten seconds they become immobile for a period of time. The different mutants differ in the length of time they remain immobile and in the length of the refractory period following the "banging" during which they will be insensitive to a second jolt. For example, half of *bss* flies can be paralyzed again only 7 minutes after the first paralysis (Ganetzky and Wu, 1982), while *tko* flies are refractory for up to an hour (Judd et. al., 1972). These differences indicate a difference in the severity of the defects caused by the mutations and could arise from a difference in the mechanisms by which the bang sensitivity

occurs.

In general the sensitivity within a population of flies is quite variable. In fact the sensitivity of a single fly will vary when tested at different times. This variability probably results from several factors. The most obvious factor is the refractory period mentioned above. If a fly has recently experienced a physical jolt in the culture vial, such as falling off the side of the bottle, then it may still be refractory and thus less sensitive to physical jolts when tested. Because *tko* flies have such a long refractory period they are more likely than *bss* flies to be refractory during a test. It is difficult to control for this, since the flies are awake and active before each test, and therefore there is always the possibility that they could fall and knock themselves out.

In studying *tko*, I have noticed that their activity level seems to affect their sensitivity to shock. During high activity periods they tend to wake up much sooner than during low activity periods (unpublished observation). This could mean either that a high level of activity directly antagonizes the sensitivity to shock or, more likely, it could be related to the refractory period. During times of low activity the flies are less likely to knock themselves out and therefore are less likely to be in a refractory state when tested.

One final observation about bang sensitivity is that the flies often go through a period of uncontrolled hyper-activity as they are recovering from the paralysis. This activity includes waving their legs about furiously or vibrating their wings in such a way as to propel them into the side of the culture vial. This hyper-activity may result from a hyper-excitability of the nervous system discussed in section 2.5.

2.2 Temperature Sensitivity

All of the bang sensitive mutants exhibit some temperature sensitivity, although not all in the same way (Burg and Wu, 1987). The mutants *bas* and *tko* are both sensitive to high temperature, becoming paralyzed at 37 °C. On the other hand *eas* and *bss* are normal at high temperature but are more sensitive to low temperatures. These flies are paralyzed at 10.5 °C and 13 °C respectively, while wild type flies are not immobilized at this temperature. Although it is not clear exactly what this temperature sensitivity means, it suggests that the different mutations fall into two distinct groups. These groups may have different defects responsible for their bang sensitivity.

2.3 Sensitivity to Anesthetics

While working with tko^{25t} flies I noticed an apparent insensitivity to ether anesthesia as compared to wild type. That is, given equal exposure to a given volume of ether, the *tko* flies appeared to wake up faster than wild type flies. I tested this by exposing flies for 2 minutes to 0.2ml of ether, which had been allowed to evaporate in a closed flask for 2 minutes, and then timing how long it took for the flies to wake up. The sensitivity of flies to ether depended on many variables, including temperature, age and sex of the flies, but when all these factors were controlled the *tko* flies consistently woke up faster than the Oregon R (OR) wild type flies. This was true whether one looked at the $t_{1/2}$ (the time for 1/2 of the anesthetized flies to wake) or at the mean time for waking. The pooled data for 68 *tko* flies and 60 Oregon R flies give a mean waking time of 303±110 (seconds±standard deviation) for the *tko* flies and 546±211 seconds for Oregon R. This difference is highly

significant statistically ($p<0.001$, Student's t test).

Most of the other bang-sensitive flies did not show as big an effect as the *tko* flies, with the exception of *bss* which woke significantly slower than Oregon R. The data for *bss* show a mean waking time of 910±199 seconds. The results for all the ether sensitivity experiments are graphed in figure 1. The slight differences between Oregon R and *eas*, *bas* and *kdn* are not statistically significant. It is odd that *bss* is actually more sensitive to ether than wild type while *tko* appears less sensitive. This again points out the possible difference in these two mutations as seen in the temperature sensitivity results.

In contrast to the ether sensitivity results, observations indicate that all of the bang-sensitive mutants appear to be more sensitive to anesthesia by CO_2 than wild type. They all take much longer to recover from this anesthesia than OR flies. This has not been quantified, but the effect is large enough to be noticeable from direct observation of flies following CO_2 anesthesia. Although it is not obvious what these various sensitivities indicate about the defects caused by the bang sensitive mutations, these observations could help in interpreting data obtained from cloning the genes. They also may lead to some insights on the mechanism of ether anesthesia in the fly.

2.4 General Viability

Not surprisingly, bang sensitive flies tend to be less viable than wild type flies. The adult *tko* flies are clearly weaker than normal and appear to have difficulty doing normal activities such as climbing the sides of the culture bottle, frequently falling off the side. They also fly very poorly, usually maintaining a diagonal

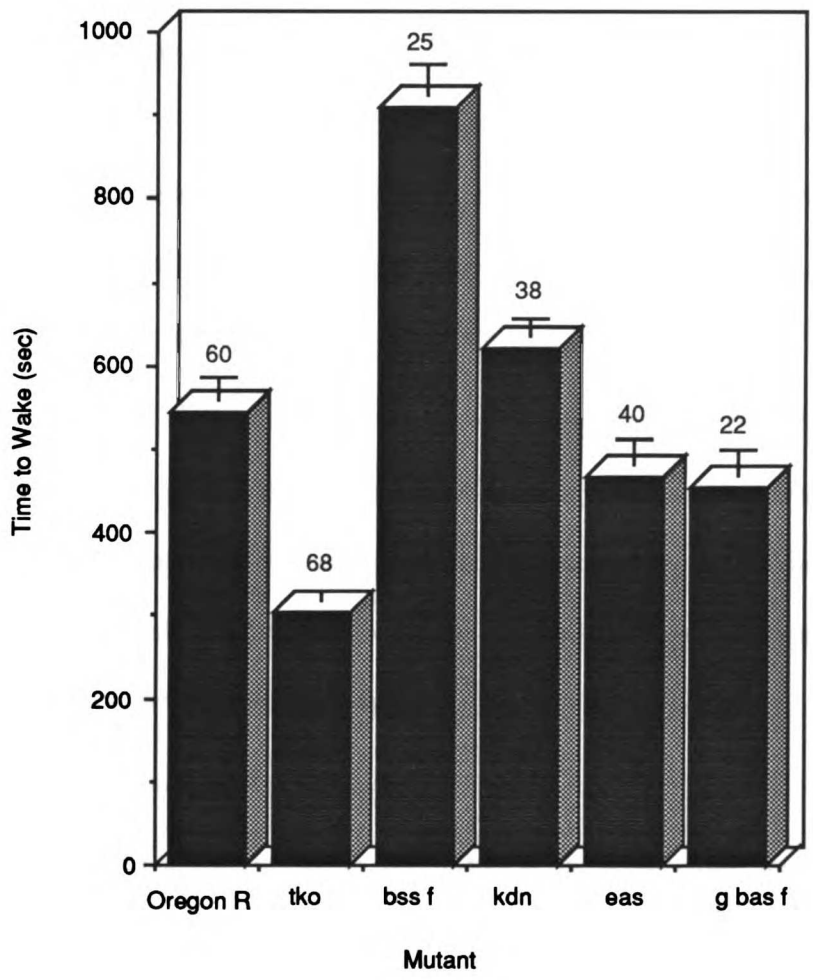


Figure 1. Time to wake from etherization for bang-sensitive mutants.
This graph shows the mean time to wake from ether anesthesia for bang sensitive mutants. The number above each column indicates the number of flies tested for each genotype. Error bars indicate the standard error of the mean.

trajectory downward until they reach the ground. Furthermore, they appear to tire easily. For example, if one repeatedly scares a *tko* fly by hitting the table next to it, its escape attempts will get progressively shorter until, after five or six escapes, it will cease to react (unpublished observation). Normal flies will continue to jump and fly away indefinitely. This general weakness certainly contributes to the *tko* mutants' decreased life span as these flies are much more prone to accidents such as falling off the side of the culture vial and getting stuck in the food.

The fertility of *tko* flies appears to be normal, but the larvae grow slowly. At 25 ° C, tko^{25t} flies take 14 days to grow from embryos to adults; wild type flies take only 10 days. This could in part be due to the general weakness described above, as it could simply take the larvae longer to eat enough food to grow large enough to reach each new developmental stage.

2.5 Electrophysiology

In order to study synaptic mechanisms in *Drosophila*, Jan and Jan (1978) developed a method of recording from the larval neuromuscular junction. Using this method they found an interesting defect in *bss* larvae. The method consists of dissecting a larva to expose the body wall muscle and the attached nerves. They then impale a muscle fiber on the body wall with a recording electrode while stimulating the nerve with a suction electrode. This approach can be done easily, since the muscle fibers on the *Drosophila* body wall are quite large. These experiments are done in a low Ca^{++} saline solution to avoid muscle twitches.

Using this method, Jan and Jan (1978) demonstrated two types of facilitation at the larval neuromuscular junction. The first, which they named short-term

facilitation, occurred when the motor nerve was stimulated twice in rapid succession. In this case, the second ejp (excitatory junctional potential) would be larger than the first. When they stimulated the nerve at high frequency, e.g. 10 Hz, for a long time, e.g. 20 seconds, they saw a second increase in the ejp which they called long-term facilitation. The two types of facilitation differed in several respects. The short-term facilitation required external Ca^{++} while the long-term facilitation arose even in the absence of external Ca^{++}. The short-term facilitation did not require external Na^{+}, occurring even in the presence of tetrodotoxin or when Na^{+} was replaced by sucrose. However long-term facilitation failed to develop under each of these conditions. Long-term facilitation seemed to result from an increased duration of Ca^{++} sensitivity in the nerve terminal following an action potential. Furthermore, the long term facilitation was affected by manipulations which affect the activity of the Na^{+}/K^{+} ATPase. Manipulations which decreased pump activity, such as the addition of the toxin Oubain, an increase in external Na^{+} or a decrease in external K^{+}, tended to increase the ease in which the facilitation developed. In other words, lower frequencies and shorter stimulation times produced the long term facilitation. Conversely, manipulations which increased pump activity, such as increasing external K^{+}, eliminated the long-term facilitation. These results suggested that the long-term facilitation resulted from the build up of Na^{+} in the nerve terminal during the high frequency stimulation.

When they recorded from the *bss* larvae (referred to in the publication as bas^{MW1}), Jan and Jan found some interesting anomalies. First, the frequency of

mejps (miniature excitatory junctional potentials) is lower in the mutant than in the wild type. Second, they could elicit long-term facilitation much more easily in these mutants than in wild type. While wild type larvae required stimulation at 10 Hz for tens of seconds for long-term facilitation to appear, the mutants showed facilitation after stimulation at 4 Hz for only three to four seconds. Wild type larvae never showed facilitation when stimulated at 4 Hz. The mutants appeared to have completely normal short-term facilitation. The long-term facilitation in the mutants was affected the same way as wild type by addition of Oubain, with long-term facilitation developing even more rapidly. Similarly, addition of TTX to the bathing solution eliminated the long-term facilitation in the mutant as it did in the wild type. Ganetzky and Wu (1982) also saw this abnormal facilitation, and in addition they showed that the facilitation in *bss* was associated with the generation of multiple action potentials in the motor nerve.

The results of these experiments are consistent with the hypothesis that *bss* causes some defect in the Na^+/K^+ ATPase. However this is not the only possible explanation. For example, this mutation could increase the Na^+ sensitivity of the machinery responsible for long-term facilitation. Alternatively, *bss* could affect some other cellular function necessary for the proper operation of the Na^+/K^+ ATPase. It is important to note that the facilitation defect may not be related to the bang-sensitivity of the fly. Both effects could be secondary results of some central defect in the fly. However these results show two important points with respect to the bang-sensitive flies. First, they show that one of the bang-sensitive mutants does have a defect in the nervous system. Second, they point to a type of hyper-

excitability in the mutant in the form of enhanced facilitation. It is possible that bang-sensitivity could result from this hyper-excitability. This is suggested by the fact that high frequency stimulation, which might be similar to the nerve stimulation caused by a physical jolt, caused an increased muscle response. Further experiments are necessary to confirm or negate this hypothesis.

Although no abnormalities have been detected in the larval neuromuscular junctions of the other bang-sensitive mutants, they all show some evidence of hyper-excitability, both in their behavior and in the interaction of these mutations with other mutations (see sections 2.1 and 2.6). It's possible that these mutations all have similar defects to *bss* but only in the central nervous system where it is difficult to study.

2.6 Phenotype of Double Mutants

One can often learn a great deal about mutations by seeing how they interact with other mutations. In the case of the bang-sensitive mutations, something can be learned by the suppression or enhancement of the bang sensitivity by other mutations.

2.6.1 Suppression by nap^{ts}

Ganetzky and Wu (1982) obtained a very interesting result when they examined the interaction of the bang-sensitive mutations with a second mutation, nap^{ts} (no action potential, temperature sensitive). The nap^{ts} mutation causes a temperature dependent paralysis in the flies. At 37 °C these flies become completely immobile due to a block in the Na^+ conductance of neurons. Ganetzky and Wu con-

structed several double mutant strains, each with the nap^{ts} mutation as well as one of the bang-sensitive mutations or the *Shaker* mutation. They showed that the *nap* mutation indirectly suppresses the phenotype of *Shaker* and all of the bang-sensitive mutations even at a temperature permissive for the no-action-potential phenotype. This was true not only at the behavioral level, but for *Shaker* and *bss* the nap^{ts} mutation suppressed the abnormal electrophysiological phenotype seen at the larval neuromuscular junction.

Ganetzky and Wu suggested that this suppression was due to a counter-balancing of membrane excitability defects, with *Shaker* and the bang-sensitives increasing membrane excitability of the neurons and nap^{ts} decreasing it. They could show this with *Shaker*, which is known to have a defective potassium channel (Salkoff and Wyman, 1981). This abnormality normally leads to a prolonged ejp in the muscle, accompanied by multiple action potentials in the nerve, after a single stimulus to the motor nerve. When combined with nap^{ts}, both the nerve response and muscle response look normal. Similarly, the abnormal facilitation in the *bss* larval neuromuscular junction is completely eliminated in the *bss;*nap^{ts} double mutant.

These results suggest that the bang-sensitivity in the bang-sensitive mutants is indeed caused by a hyper-excitability in the nervous system. If the hyper-excitability were merely a secondary defect unrelated to the bang-sensitive behavior then decreasing the neuronal excitability with the nap^{ts} mutation should not have eliminated the behavioral phenotype. The results also underscore the similarity between all the bang sensitive mutants. They are all suppressed by nap^{ts}, and this

suggests they all have an underlying hyper-excitability causing their bang-sensitivity.

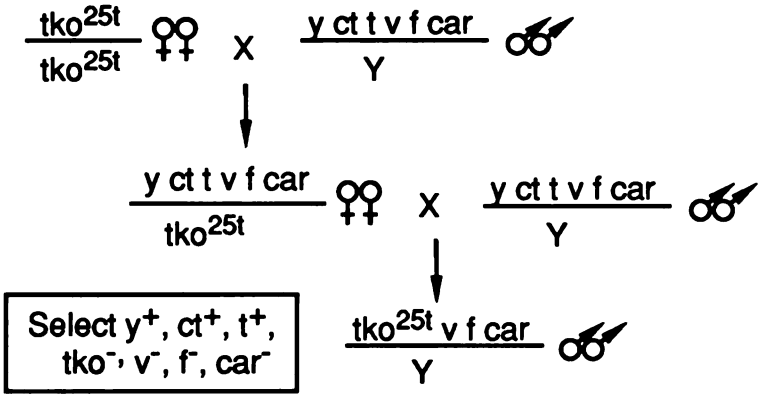
2.6.2 Interactions Between Bang-sensitive Mutations

Constructing double mutant strains can help to determine whether two genes interact with one another. In order to determine whether the bang-sensitive loci interact with one another, I constructed double mutants between tko^{25t} and the other bang sensitive mutations. The crossing scheme I used to generate the double mutant ***tko,eas*** is shown in figure 2. Since *eas, bss, bas, kdn* and *Shaker* all lie between *vermillion* (*v*) and *forked* (*f*) I used the same scheme for each construction. I was able to construct double mutant lines for the combinations of tko^{25t} with eas^{2}, ***kdn***, bss^{B910}, and Sh^{102}. I did not retrieve any double mutants of the genotype ***tko,bas***. This implies that these double mutants are either so sick that survival to adulthood is extremely rare, or they are completely inviable.

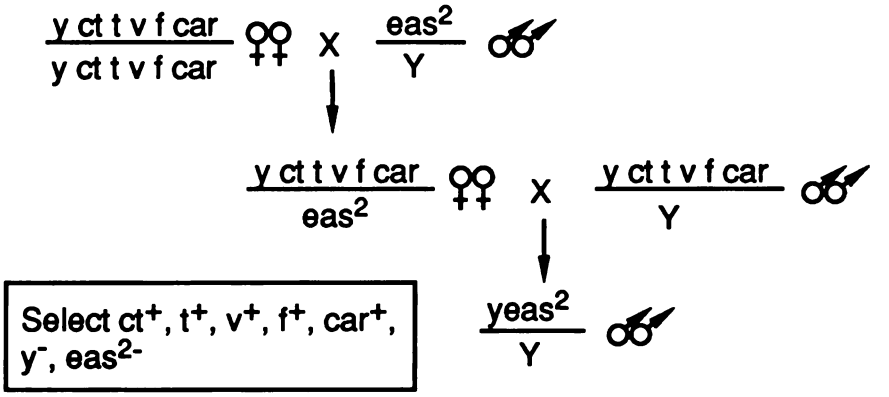
Qualitatively, these double mutant strains are extremely weak and extremely bang-sensitive. Very few double mutant flies survive to adulthood, and those that do tend to have very short life spans. They have difficulty walking and cannot fly. They have extreme difficulty emerging from the pupal case, and they often do not succeed. They often fall if one inverts the vial they are in and the force of this fall will often cause a paralysis lasting several minutes.

Burg and Wu (1987) did a quantitative study of several of these strains. They also found that the ***tko,bas*** combination was inviable. In addition they were unable to recover any ***tko,eas*** flies, supporting the idea that these combinations cause such extreme disabilities for the flies so that they are nearly inviable. When they tested

A. Cross to generate tko^{25t} v f car



B. Cross to generate y eas^2



C. Cross to generate tko^{25t} eas^2

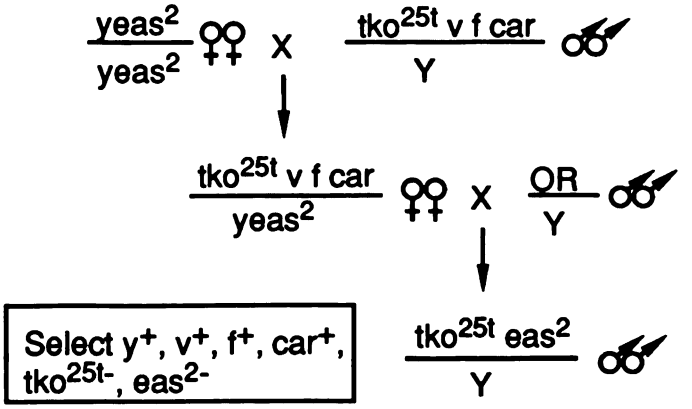


Figure 2. Crosses to Generate Double Mutant Flies.

tko,bss and *bss,bas* and *bas,eas* flies for bang sensitivity, they found a synergistic effect between each combination of mutations. The double mutant flies remained paralyzed after banging for much longer than either of the single mutants alone or than the sum of the single mutant paralysis times. For example, for the *tko,bss* combination, the double mutant remained paralyzed for an average of 6 minutes, while *tko* only remained paralyzed for 1.25 minutes and *bss* only for 2.5 minutes. Interestingly, the double mutant seemed to receive temperature sensitivity from both mutations, so that it was temperature sensitive to both high (from *tko*) and low (*bss*) temperatures.

This analysis fits well with my qualitative observations, and suggests that all the bang-sensitive mutations cause the behavioral abnormality through a similar defect. This defect may be hyper-excitability of the nervous system, which could in turn be caused by a variety of gene defects. It is interesting that the *tko,Sh* combination was also very weak and extremely bang sensitive, which suggests that adding any extra excitability to *tko* no matter what the mechanism will enhance the bang sensitivity of the flies. So although the synergism between the bang sensitive mutations suggests a similar mechanism for bang sensitivity, the mechanism may be a secondary effect, such as membrane hyper-excitability, caused by different primary defects.

2.7 Summary

The bang-sensitive mutations are a family of mutations which cause flies to be sensitive to a physical jolt. The phenotypes of these mutations indicate both similarities and differences among the different genes. They all share a distinctive

behavioral phenotype, bang-sensitivity, and this phenotype is synergistically enhanced in double mutants carrying two bang-sensitive mutations. However there are differences in temperature sensitivity, sensitivity to anesthetics, and electrophysiological results which suggest that the affected genes may have different functions. Some of the results suggest two classes of bang-sensitive mutations; one class consisting of *tko* and *bas* and the other consisting of *bss* and *eas*. Many of the results, particularly the results from the nap^{ts} double mutants, suggest that all the bang-sensitive phenotypes may result from a hyper-excitability of the nervous system. It is possible that all the bang-sensitive mutations share the hyper-excitability, but differ in the exact defect which leads to the hyper-excitability. This could account for the prevalence of both similarities and differences among the bang-sensitive mutations. Further experiments are needed to determine exactly how similar these genes are and how mutations in them lead to bang-sensitivity. Cloning and molecular analysis of these genes should lead to some insights into their functions and their similarities to one another.

CHAPTER 3

GENETIC ANALYSIS OF *tko*

3.1 Alleles of *tko*

There are three known alleles of the *tko* gene. Only one of these alleles, tko^{25t}, produces a viable adult fly with the bang-sensitive phenotype described in the previous chapter. The other two alleles, $l(1)^{tkok11}$ and tko^{15p}, are lethal (Judd et. al., 1972). All of the alleles, including 25t, are lethal in combination with the deficiencies *Df(1)*w^{rJ1}, *Df(1)X12, Df(1)65j26,* and *Df(1)62g18* (Judd et. al., 1972). In addition, tko^{25t} is lethal in combination with $l(1)^{tkok11}$. Because tko^{25t} is completely recessive, my working hypothesis is that tko^{25t} is a hypomorph, underproducing the product or producing a product which is only partially active. Also, because $l(1)^{tkok11}$ behaves like a deficiency for *tko*, i.e. $tko^{25t}/l(1)^{tkok11}$ has the same phenotype as $tko^{25t}/Df(1)w^{rJ1}$, $l(1)^{tkok11}$ is probably a null mutation, producing none or extremely little of the active tko gene product.

In contrast to *tko*, Ganetzky and Wu (1982) suggest that the mutation bss^{MW1} is a gain of function mutation. This hypothesis is based on the partial dominance of this mutation. This distinction between the two mutations could account for many of the phenotypic differences discussed in chapter 2.

The allele $l(1)^{tkok11}$ causes lethality in the larval stage (Shannon et. al., 1972). These mutants grow slowly until they reach second instar. Their molt to the second

instar stage is delayed about 24 hours (Shannon et. al., 1972). After reaching second instar they grow to reach full size for this stage, then stop growing and eventually die. A few reach third instar, but they do not grow following this molt and also eventually die. None pupate.

3.2 Genetic Mapping of *tko*

Based on recombination mapping, the *tko* locus lies on the tip of the X-chromosome, 0.006 map units distal to the zeste locus and 0.037 map units proximal to the giant locus (Judd et. al., 1972). This region has been extremely well studied, and a large number of deletions and duplications have been constructed throughout the region. Some particularly useful deletions include $Df(1)w^{rJ1}$, which uncovers *tko* but not *gt* (giant), *Df(1)64c4* and $Df(1)w^{g58\text{-}11}$, both of which uncover zeste, but not the *tko* locus (see figure 3). These deletions will prove extremely important in locating the position of the *tko* locus on a physical map of the genomic DNA as discussed in Chapter 4. The cytological position of *tko* was placed at band 3A2 by Judd et. al. (1972) based on the breakpoint of the deletion $Df(1)w^{rJ1}$, and this was later confirmed by the molecular data of Mariani et. al. (1985).

3.3 Mosaic Analysis of *tko*

Shannon et. al. (1972) constructed gynandromorphs of $l(1)^{tkok11}$ through loss of a ring X chromosome. They found no mutant tissue on the adult mosaic flies except for patches on the abdominal tergites which lacked bristles and appeared etched, like the *bobbed* phenotype (Shannon et. al., 1972). This suggests that for most tissues the k11 allele of *tko* is probably an autonomous cell lethal. It also indicates that the *tko* gene product is probably expressed throughout the adult and is

necessary in almost all tissues.

3.4 Summary

The *tko* locus lies at the tip of the X-chromosome just distal to the zeste locus. The availability of deficiencies in this region makes it an attractive area for molecular analysis. The null phenotype of *tko* is lethal, probably similar to the k11 phenotype. The viable 25t allele is probably a hypomorph. Mosaic studies suggest that the *tko* product is expressed throughout the adult and lack of the product causes cell lethality for almost all tissues in the adult except the abdominal tergites.

CHAPTER 4

MOLECULAR ANALYSIS OF *tko*[1]

To learn more about the causes of bang-sensitivity, I set out to clone the *tko* gene. The genomic region around *tko* has been recently cloned in a genomic walk designed to extract the zeste locus, which neighbors *tko*. (Mariani et. al., 1985; Gunaratne et. al., 1986). V. Pirrotta provided me with lambda clones containing DNA from this region. I used this DNA to further define the location of the *tko* gene through the mapping of deficiency breakpoints and P-element transformation as described below. Once the gene was localized, I was able to analyze the transcripts in the region and isolate a cDNA corresponding to the most probable *tko* transcript. Sequencing of this cDNA and comparison of the protein translation product implied by this sequence with other known proteins led to an intriguing hypothesis concerning the possible function of this tko gene product.

4.1 Breakpoint Mapping

Figure 3 shows a map of the genomic region near *tko*. To localize the *tko* gene on this physical map I mapped breakpoints that fall close to or within the *tko* gene. Using Southern blots, I confirmed that the position of the Df(1)$yw^{258\text{-}11}$ breakpoint lies between two Bam HI sites at positions -3.5 and -1.0 (figure 3), as previously reported (Mariani et. al., 1985). I also delimited the position of the Df(1)w^{rJ1} break-

[1]Most of the material in this chapter has been previously published (Royden et. al., 1987).

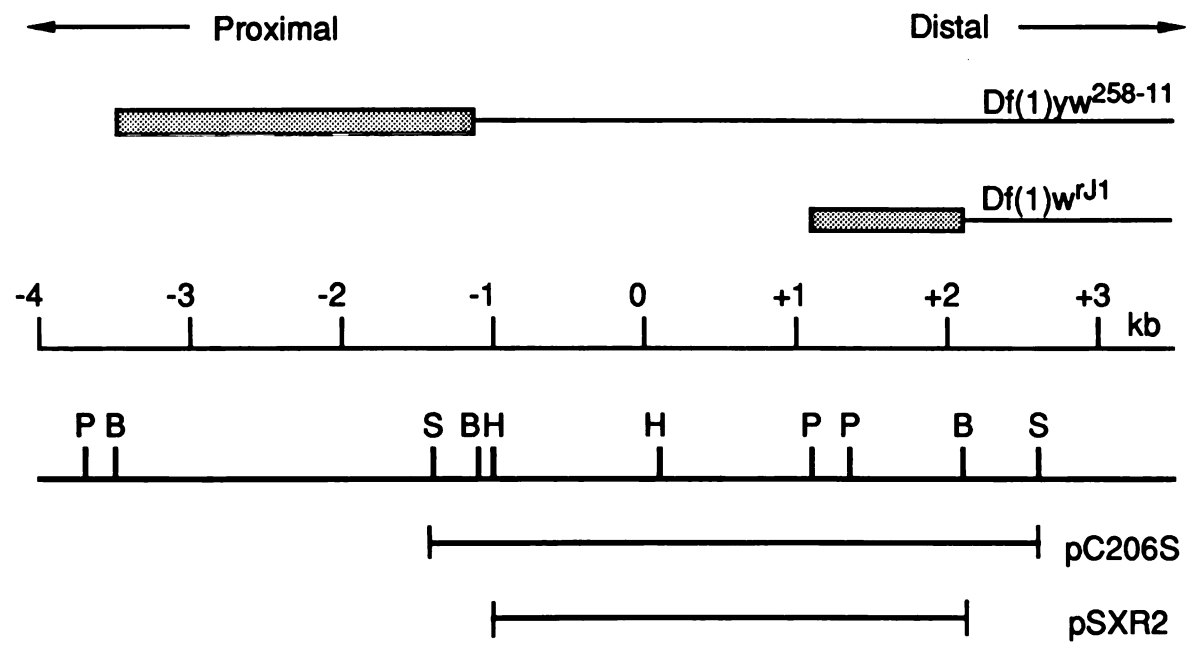


Figure 3. Map of the *tko* region showing fragments used for transformation.
Restriction sites for the enzymes BamHI (B), SmaI (S), HindIII (H), and PstI (P) are shown on the lower horizontal line. The scale is given in kilobases with the origin chosen as the start of the longest cDNA. The top two lines indicate the breakpoints for the deficiencies Df(1)$yw^{258\text{-}11}$ and Df(1)w^{rJ1}. The regions within which the breaks occur are indicated by the shaded bars. The solid line represents the DNA still present in these deficiencies. The lower two bracketed lines show the extent of the fragments used in the transformation experiments.

point to within 1kb, from the Pst I site at position +1.1 to the Bam HI site at position +2.1 (data not shown). Since Df(1)$yw^{258\text{-}11}$ is tko^{+} and Df(1)w^{rJ1} is tko^{-} (Judd et. al., 1972), these two breakpoints define the proximal end of the *tko* gene to within a 5.6 kb region, between the Bam H I sites at positions -3.5 and +2.1 (figure 3). To further define the extent of the *tko* locus, I undertook P-element mediated transformation.

4.2 P-element transformation

Using the Carnegie 20 vector (Rubin and Spradling, 1983) which contains the entire *ry* (rosy) gene, I transformed flies with one of two genomic fragments. The first was a 3.1 kb fragment extending from the Xho I site at position -1 to the Bam HI site at position +2.1. The second was a 4.0 kb Sma I fragment extending from -1.5 to +2.5 (Figure 3). I isolated 5 independent lines transformed with the 4.0 kb Sma I fragment and 7 independent lines transformed with the 3.1 kb fragment. All of the flies transformed on the 2nd or 3rd chromosome were tested for the ability to complement the lethality of $l(1)^{tkok11}$ (see experimental procedures). Table 1 shows that in all the strains tested this DNA did complement $l(1)^{tkok11}$, as demonstrated by the survival of adult males carrying this mutation on their X chromosome. These rescued flies were phenotypically normal. They showed no signs of bang sensitivity, appeared healthy, and were fertile.

The transformants were also tested for the ability to complement the behavioral defect of tko^{25t}. Flies of the genotype tko^{25t}/Y; P(ry^{+},tko^{+})/+ were tested for bang sensitivity. In preliminary tests all of the transformed strains complemented tko^{25t}, but the number of flies tested was small. I chose to amplify three

COMPLEMENTATION OF *l(1)^{tkok11}* BY TRANSFORMATION					
Plasmid	Strain	Chromosome	FM7c males	l(1)^{tko} males	Complementation
	P[(ry, tko)]22	II	39	38	+
	P[(ry, tko)]43	III	42	27	+
pC206S	P[(ry, tko)]44	X	55	0	N.D.
	P[(ry, tko)]101.1	X	29	0	N.D.
	P[(ry, tko)]101.3	N.D.	18	10	+
	P[(ry, tko)]201.1	III	32	19	+
	P[(ry, tko)]203.1	II	41	29	+
	P[(ry, tko)]236.1	X	33	1*	N.D.
pSXR2	P[(ry, tko)]236.2	II	22	43	+
	P[(ry, tko)]250.1	II	15	14	+
	P[(ry, tko)]250.2	X	50	0	N.D.
	P[(ry, tko)]281	X	25	1*	N.D.

Table 1. Complementation of *l(1)^{tkok11}*

The first column indicates the plasmid used for transformation. The second column shows the strain tested and the third indicates the chromosome into which the P-element inserted for each strain. Columns four and five show the number of male offspring of each genotype which arose from crossing *l(1)^{tkok11}*/FM7c ; +/+ ; +/+ females with +/Y ; P(*ry*$^{+}$,*tko*$^{+}$)/+ ; ry^{506}/ry^{506} males. Similar crosses were done for flies transformed on the X or 3rd chromosome. The asterisk indicates flies that I think are of an X/0 genotype caused by non-dysjunction of the X chromosome in their mothers. These flies were sterile as would be expected of X/0 males. N. D. indicates that complementation was not determined for that strain.

COMPLEMENTATION OF *tko^{25t}* BY TRANSFORMATION				
Plasmid	Strain	# tested	Avg time Immobile(sec)	Complementation
	OR	25	0.6	N.A.
	tko^{25t}	131	102	N.A.
pC206S	P[(ry, tko)]22.1	116	0	+
pSXR2	P[(ry, tko)]236.2	106	0.28	+
pSXR2	P[(ry, tko)]250.1	85	0	+

Table 2. Complementation of the behavioral mutant *tko^{25t}*.

For the transformants listed the flies tested were of the genotype *tko*^{25t}/Y ; P(*ry*$^{+}$,*tko*$^{+}$)/+. The mean times for the flies to right themselves following vortexing are given in column three. N. A. means not applicable.

strains for more extensive complementation tests. Table 2 shows the results of these tests. All flies carrying the P-element showed wild type behavior. In fact, of all 307 flies tested, only one was immobilized at all, staying immobile for 30 seconds. In contrast, 95% of the tko^{25t} flies were immobilized for periods longer than 30 seconds. All of the tko^{25t} flies carrying the P-element appeared normal in all other respects. Hence both DNA fragments complement the behavioral defect of tko^{25t} as well as the lethality of $l(1)^{tkok11}$. Therefore it is likely that the 3.1kb XhoI-BamHI genomic fragment provides all the function of the *tko* gene.

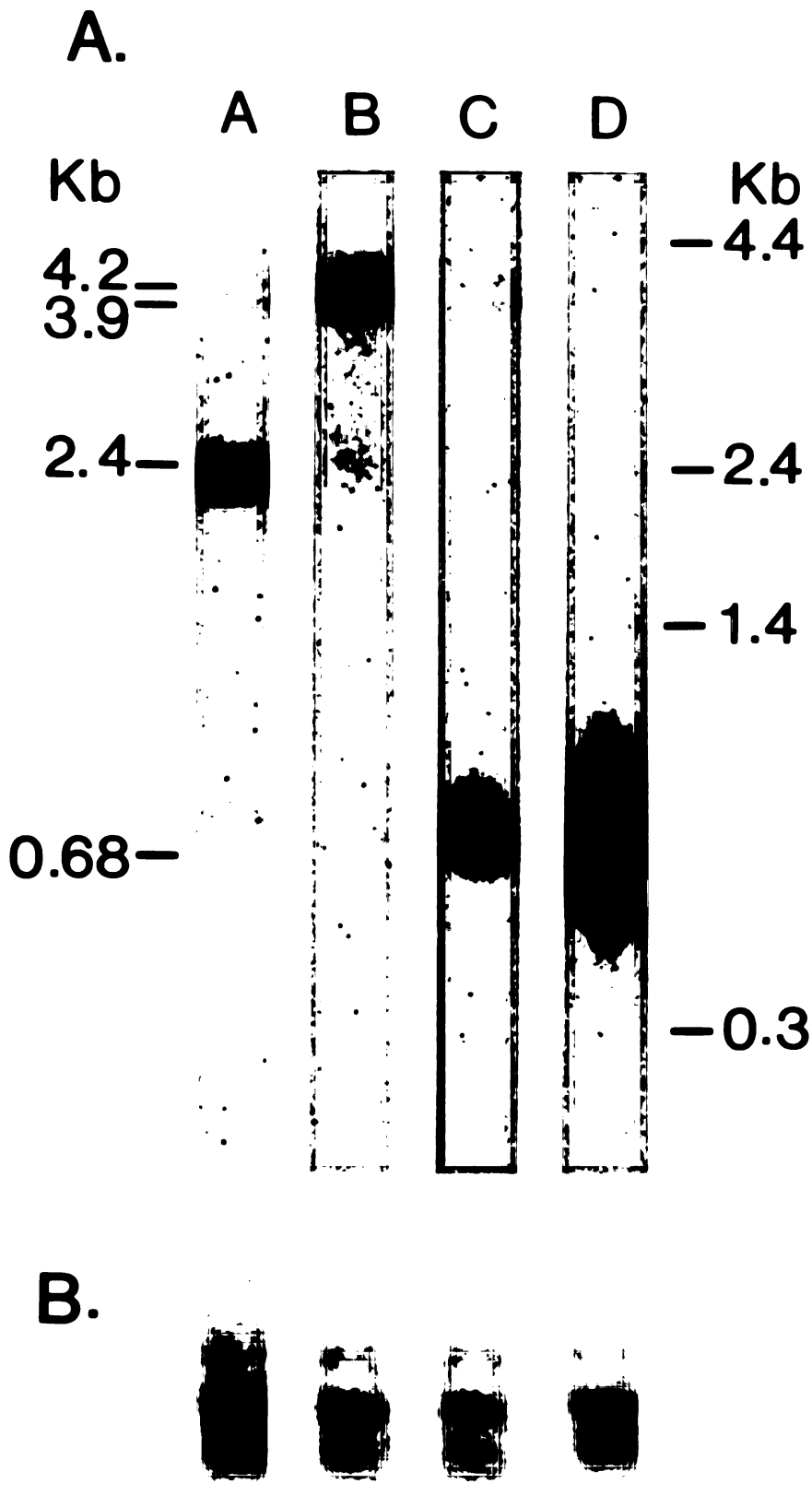
4.3 Transcript Analysis

To determine which transcripts are likely candidates to be products of the *tko* gene I used Northern blot analysis to map the transcripts in the *tko-zeste* region. Figure 4A shows the results of Northern blots hybridized with probes from this region and these results are summarized schematically in figure 5. The 3.2 kb Bam HI fragment extending from -1.1 to +2.1 hybridizes to three transcripts 0.68, 3.9, and 4.2 kb in length. The 3.9 and the 4.2 kb transcripts have been reported previously (Mariani et. al., 1985; Gunaratne et. al., 1986). The 0.68 kb transcript has previously been referred to as 0.9 kb (Mariani et. al., 1985; Gunaratne et. al., 1986), however I think 0.68 kb is a more accurate estimate of the size (see experimental procedures). The 0.68 kb and the 3.9 kb transcripts are expressed throughout development and the 4.2 kb transcript appears in the third instar and pupal stages (Gunaratne et. al., 1986). The two larger transcripts appear to be coextensive. Their 5-prime ends appear to lie within the 4.0 kb Bam HI fragment (figure 4A, lane A) and thus are at least 2.5 kb proximal to the 3.1 kb XhoI-BamHI fragment.

Figure 4. Transcripts in the *tko-zeste* region.

A. Northern blots of pupal poly-A^+ RNA. 10μg of poly-A selected pupal RNA was used per lane. Each lane was probed with the following fragments: Lane A - 4.0 kb Bam HI fragment (position -7.5 to -3.5). Lane B - 2.4 kb Bam HI fragment (position -3.5 to -1.1). Lane C - 3.2 kb Bam HI fragment (position -1.1 to +2.1). Lane D - This was probed with the longest of the larval cDNAs. The size estimate for each band is shown at the left of the figure. The positions of the RNA molecular weight markers (BRL) used for these Northern blots are shown on the right.

B. Actin control for RNA quantity. The same blots shown in part A were reprobed with a probe made from the *Drosophila* actin gene. This probe hybridized to three bands in all four blots. The relative intensities of these bands in the different lanes is an indication of the relative amounts of RNA in the lanes. The small variation in the intensity of the signals in these blots indicates that there is an approximately equal amount of RNA in each lane.


A.
A
B
C
D
Kb
4.2
3.9
2.4
0.68
Kb
4.4
2.4
1.4
0.3
B.

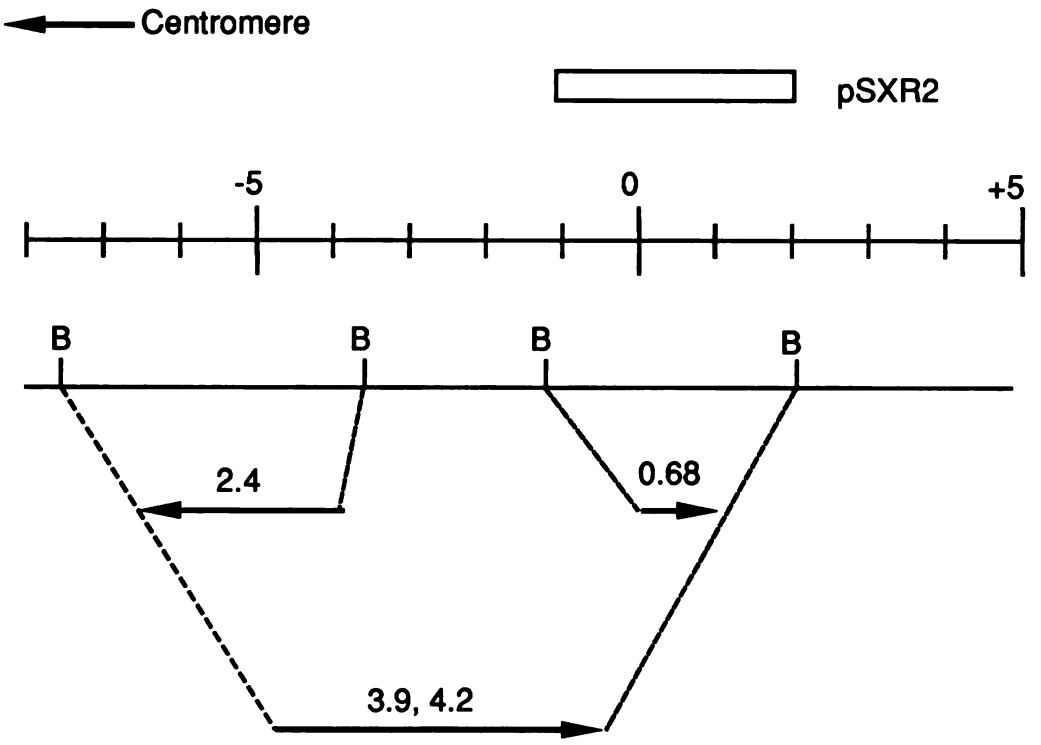


Figure 5. Summary of the transcripts in the *tko-zeste* region.
The restriction map shows sites for BamHI (B). The dotted lines indicate the extent of the genomic regions to which transcripts, indicated by arrows, were mapped in 4A. Arrow heads indicate the direction of transcription as determined by probing Northern blots with single-stranded probes. The open bar above the restriction map shows the extent of the 3.1 kb fragment that complements tko.

Moreover, the 2.4 kb Bam HI fragment hybridizes much more strongly to these large transcripts than do either the 4.0 kb Bam HI fragment or the 3.2 kb Bam HI fragment, indicating that the majority of these transcripts lie outside the 3.2 kb Bam HI fragment. Therefore the transcription start site for these larger transcripts would be deleted in the deficiency Df(1)$yw^{258\text{-}11}$ which is wild type for *tko*. Also, the 3.1 kb genomic fragment lacks the transcription start sites for these transcripts as well as the large portion of the transcripts which lies within the 2.4 kb Bam HI fragment, and yet it complements *tko* completely. I conclude from this that it is highly unlikely that the large 3.9 and 4.2 kb transcripts are involved in *tko* function. This leaves the 0.68 kb transcript as the only probable candidate to provide the *tko* function. My remaining studies have focused on this transcript.

4.4 Isolation and Characterization of cDNAs

To learn about the function of the *tko* product, I first determined its primary structure through the analysis of cDNAs. Using the 3.2 kb Bam fragment (position -1.1 to +2.1 in figure 3) as a probe, I screened a pupal cDNA library (Yedvobnick et. al. 1985) and a larval library (Poole et. al. 1985). I isolated three cDNAs, all 0.6 kb in length, from the pupal library and six, ranging in size from 0.6 kb to 0.65 kb, from the larval library. Restriction mapping revealed no differences between the three pupal cDNAs, so only one was studied further. This pupal cDNA was completely contained within the longest larval cDNA.

I sequenced the pupal cDNA, the longest two larval cDNAs and the corresponding regions of the genomic DNA. Figure 6A shows the structure of the transcript determined by this comparison between the cDNA sequence and the

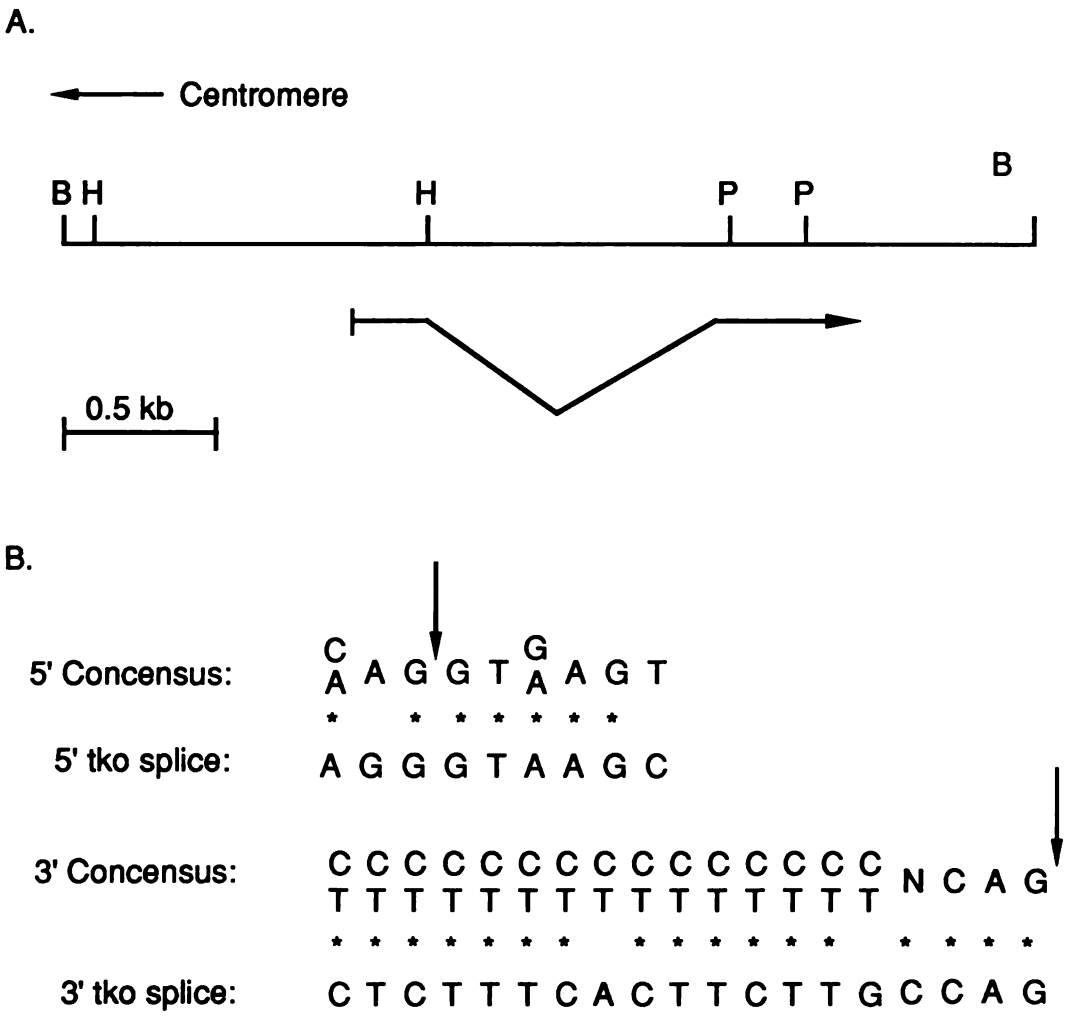


Figure 6. Structure of the *tko* transcript as determined from cDNA.
A. A restriction map of the 3.2 kb BamHI genomic fragment, which ranges from position -1.1 to +2.1 in Figure 3, shows restriction sites for BamH1 (B), HindIII (H), and PstI (P). The line below it shows the structure of the message as determined by comparison of cDNA and genomic sequences. The arrow indicates the direction of transcription as determined by probing Northern blots with single-stranded probes and by the position of the poly(A) tail seen on the larval cDNA. The 5' end shown in this diagram represents the end of the longest cDNA.
B. The *tko* splice sites determined from sequencing the genomic DNA are compared to the consensus splice sites (Mount, 1982). The vertical arrow indicates the position of the splice. Asterisks denote exact matches between the *tko* and the consensus splice sequences. N indicates that all four nucleotides are equally likely in that position.

genomic sequence. This comparison reveals one 1 kb intron which has typical consensus sequences at both splice junctions as shown in figure 6B. The total length of the message represented by the cDNAs is 643 bases. Probing Northern blots of embryonic, larval or pupal poly-A^+ RNA with a larval cDNA revealed only the 0.68 kb message (figure 4). The size of this message is so close to the length of the cDNAs, especially when one takes into account the extra length in the poly-A tail, that I feel the cDNAs must represent nearly the entire message. This cDNA does not hybridize to the other 3.9 kb and 4.2 kb transcripts in this region which indicates that there is little overlap between the cDNA and these two large messages.

4.5 Sequence and homology of cDNAs

The sequence of the cDNA along with the predicted amino acid sequence of the *tko* protein is shown in figure 7. I know the direction of transcription from hybridization of single-stranded probes to Northern blots and from the position of the poly-A tails on the two larval cDNAs. The frame chosen is the largest open reading frame which codes for 140 amino acids commencing with the first ATG in the cDNA. This ATG is preceded by STOP codons in all frames, and the sequence surrounding it (G A G C A T G -) is reasonably close to the consensus start of translation in *Drosophila* (C/A A A A/C A T G -) (Cavener, 1987). None of the downstream ATGs fit this consensus as well. Thus, since the first ATG in the sequence also has the best consensus start site, it is probably used as the start for translation. The second longest open reading frame codes for 99 amino acids, but contains no methionine codon and so is unlikely to be used.

```
  1   agc aac agc tga ttg tca cat tag aat tga att gga att aga tta tta aat att

 55   tta gcg ctt tta taa cac aaa agg ccg act aaa tag ctt ccg agc aag cgc cac

109   caa tcc gag cga cgg ctg gta cta caa act gaa cag aaa act act gaa caa aac

163   tcc gag agc ATG AAT TTT CTG CGG CAA TCC TTT GGC ATT ACG AAA CAG TTG GCT
                  Met Asn Phe Leu Arg Gln Ser Phe Gly Ile Thr Lys Gln Leu Ala

217   TCG CAG GCC ATC CAG TGC AGC TAT GAG ACC GCC GTC CGT GGA ATG GCA TCG CTG
      Ser Gln Ala Ile Gln Cys Ser Tyr Glu Thr Ala Val Arg Gly Met Ala Ser Leu

271   CAG CAG ATG CAC CGC AGC GGA CCG CAC ATA AAG ACG CGT CCG CCG CGC CAA CCG
      Gln Gln Met His Arg Ser Gly Pro His Ile Lys Thr Arg Pro Pro Arg Gln Pro

325   CTG GAT GGA AAG CCC TTC GCC AAG GGC GTT GTC CTG AAG ACG CTG ATA AAG AAG
      Leu Asp Gly Lys Pro Phe Ala Lys Gly Val Val Leu Lys Thr Leu Ile Lys Lys

379   CCC AAG AAG CCG AAC TCG GCC AAT CGA AAG TGC GTG CTG GTG CGC CTC TCC ACC
      Pro Lys Lys Pro Asn Ser Ala Asn Arg Lys Cys Val Leu Val Arg Leu Ser Thr

433   GGC AAG GAG ATG GTG GCC TAC ATC CCC GGC ATC GGG CAC AAC CTG CAA GAG CAC
      Gly Lys Glu Met Val Ala Tyr Ile Pro Gly Ile Gly His Asn Leu Gln Glu His

487   AAC ATT GTA CTG TGC CGC GTG GGG CGT CTG CAG GAC GTG CCC GGC GTT AAG CTG
      Asn Ile Val Leu Cys Arg Val Gly Arg Leu Gln Asp Val Pro Gly Val Lys Leu

541   AAG GCT GTG CGC GGA GTC TAC GAC TTG GCG CAC GTC GTC AAG AAG AGC CAA TAG
       Lys Ala Val Arg Gly Val Tyr Asp Leu Ala His Val Val Lys Lys Ser Gln AM

595   tta tta acc act tcc taa tca cgt tgt tca aat gca ttg ctt cac aat c
```

Figure 7. *tko* cDNA sequence and predicted amino acid sequence.

The portions of the cDNA sequence which are non-coding are given in lower case; The coding sequence is in upper case. The predicted amino acid sequence is directly below the DNA sequence.

Comparison of the predicted protein with other protein sequences in the Dayhoff data bank (NBRF release #11, December, 1986) using the program dfastp (Lipman and Pearson, 1985) revealed an interesting finding. The protein coded for by the *tko* locus has 41% amino acid identity with *Escherichia coli* ribosomal protein S12 and 46% amino acid identity with *Euglena gracilis chloroplast* ribosomal protein S12 (figure 8). The *E. coli* ribosomal protein S12 is the product of the *strA* locus which is mutated in streptomycin resistant bacteria (Post and Nomura, 1980). It is involved both in translational fidelity and in the initiation of translation (Ozaki et. al., 1969). It is notable that Lys(32) of the *E. coli* protein, one of the streptomycin binding sites (Funatsu and Wittmann, 1972), is conserved in the *tko* protein and the region around it is also highly conserved. The second streptomycin binding site at Lys(87) has been changed to a glutamine in the *tko* protein, but once again it is within a highly conserved region. One possibility raised by this homology is that the *tko* gene codes for a mitochondrial ribosomal protein with homologous function to S12 in *E. coli*. Consistent with this hypothesis is the finding that the thirty amino acids on the N-terminal of the *tko* protein have the characteristics of a signal sequence for mitochondrial matrix proteins (Hurt and van Loon, 1986). First, the region is rich in positively charged amino acids, lysine and arginine, and these are spaced at regular intervals. Second, it is rich in serine and threonine. Finally, it has no acidic amino acids throughout most of its length, although there is one glutamate residue toward the end of this sequence. The possibility of *tko* being a nuclear gene coding for a mitochondrial ribosomal protein is discussed below.

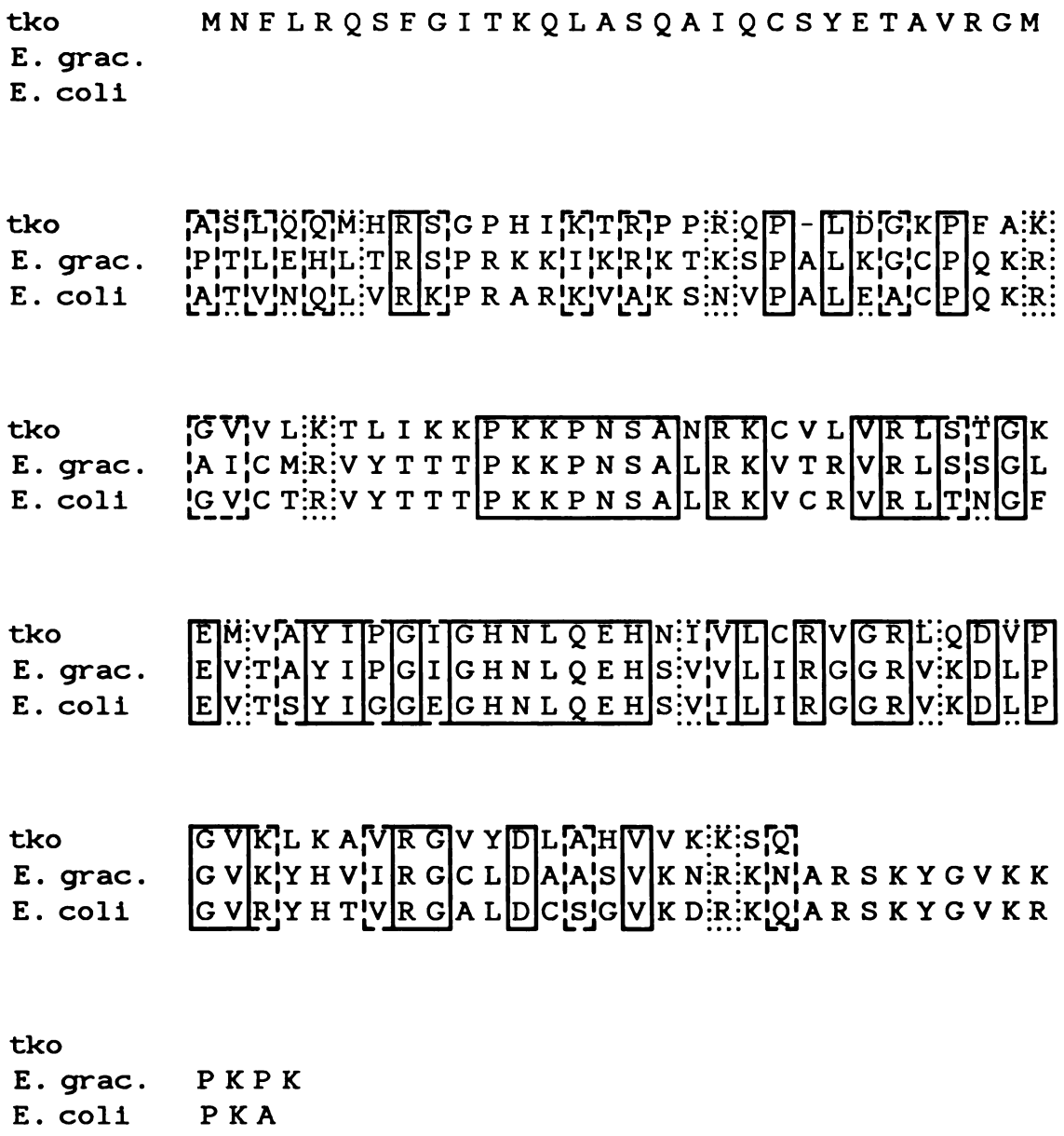


Figure 8. Homologies between the *tko* protein and ribosomal protein S12 from *E. coli* and *E. gracilis* chloroplasts.

Identical amino acids between the three proteins are boxed with solid lines. Dashed boxes indicate an identical amino acid in the *tko* protein and in one of the two S12 proteins. Dotted boxes indicate a conservative change between the *tko* protein and one or both of the S12 proteins. The dash indicates a gap inserted into the sequence by the alignment program to generate the best match between the two proteins.

4.6 Experimental Procedures

4.6.1 Construction of transformation vectors

To construct pSXR2, the 3.2 kb BamHI fragment (position -1.1 to +2.1 in figure 3) was subcloned into M13mp8. This construct was then digested with XhoI and SalI to generate a 3.1 kb fragment containing the 3.1 kb XhoI/BamHI fragment from the *tko* DNA and a small piece of the mp8 polylinker from the BamHI site to the SalI site. This XhoI/SalI fragment was then ligated into the SalI site of the Carnegie 20 vector (Rubin and Spradling, 1983). To generate pC206S, the 4.0 kb SmaI fragment was ligated into the Hpa I site of Carnegie 20. Ligated DNA was transformed into the HB101 strain of *E. coli*. DNA was extracted from ampicillin resistant colonies by standard techniques (Maniatis et. al., 1982), and analyzed by restriction digests to determine the presence and orientation of the inserts. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs.

4.6.2 Generation of Transformants

The transformants were generated by microinjection of supercoiled cloned DNA into pre-blastoderm embryos (Rubin and Spradling, 1982; Spradling and Rubin, 1982). Each plasmid, pSXR2 and pC206S, was co-injected with the plasmid pπ25.7wc which provides transposase function, but is itself unable to integrate (Karess and Rubin, 1984). The vector DNA was at a concentration of 450 μg/ml for pC206S, 330 μg/ml for pSXR2 and the helper plasmid pπ25.7wc was at a concentration of 100 μg/ml. Microinjection was carried out using the methods of Rubin and Spradling (1982). With each DNA vector, I injected approximately 400 embryos homozygous for ry^{506}.

The injected embryos were grown to adulthood and the surviving adults were individually mated to ry^{506} flies. The offspring from these crosses were screened for eye color, and those with wild-type eye color were characterized and maintained as transformed stocks. These stocks were then used for the complementation tests.

4.6.3 Fly stocks and Crosses

The tko^{25t} stock was provided by B. Ganetzky. The $l(1)^{tkok11}$ stock and the deficiency stocks were provided by B. Judd. The chromosomal positions of the transformed P-elements were determined by standard segregation analysis using balancer chromosomes containing dominant mutations in stocks homozygous for *ry*. These stocks were provided by G. Rubin. For complementation tests for $l(1)^{tkok11}$, male flies carrying the P-element on the second or third chromosome were crossed to virgin females of genotype $l(1)^{tkok11}$/FM7c. The male offspring were screened for the number not carrying the FM7c chromosome, which must have received the $l(1)^{tkok11}$ chromosome instead. Flies which were transformed on the X chromosome were also tested as a control.

To test for complementation of tko^{25t}, male flies carrying the P-element over a balancer chromosome marked with a dominant mutation were crossed to virgin females homozygous for tko^{25t}. All male offspring that did not receive the balancer chromosome, and so must have received the P-element, were tested for bang-sensitivity.

4.6.4 Behavioral Testing

Males were anesthetized with CO_2 and collected into vials at 0 to 3 days after eclosion from their pupal cases. They were tested at 5 to 9 days after eclosion. To test for bang-sensitivity, flies were transferred to an empty vial and immediately vortexed for 10 seconds at the highest setting of the vortexer. The time for each fly to right itself after vortexing was recorded.

4.6.5 Northern Analysis

10 μg of poly-A selected pupal RNA was electrophoresed on a formaldehyde agarose gel. This was then blotted onto Genescreen (NEN Research Products) filters. Probes were prepared by the random primer method (Feinberg and Vogelstein, 1983) from specific fragments which had been purified from agarose gels. Each blot was pre-hybridized for 20 hours at 50 ° C in a solution containing 50% formamide, 5X SSC (Maniatis et. al., 1982), 5% SDS, 50mM $NaPO_4$ pH 6.5, 5X Denhardt's (Maniatis et. al., 1982), and 200 μg/ml salmon sperm DNA. They were then hybridized for 20 hours at 50 ° C in the same solution containing 2.2 x 10^6 Cherenkov cpm of probe. An equal number of counts of probe was used for each blot. Filters were washed 2 times for 15 minutes at room temperature in 1X SSC, 0.1% SDS, then once for one hour at 65 ° C in 0.5X SSC, 0.1% SDS, and finally for one hour at 65 ° C in 0.1X SSC, 0.1% SDS. All filters shown in figure 4 were exposed to the same film for the same length of time.

In order to verify that all lanes of the blot contained equal amounts of RNA, the blots were reprobed with the Drosophila actin gene (Figure 4B). The blots were boiled for 5 minutes in pre-hybridization solution, and then prehybridized for 20

hours at 50°C as described above. They were then hybridized for 20 hours in hybridization solution containing 2.5 x 10^6 Cherenkov cpm of the actin probe. The blots were washed as described above.

Sizes of the bands were determined using SP6 generated RNA markers 0.68 kb and 1.4 kb in length or RNA molecular weight standards purchased from BRL and ranging from 0.3 kb to 9.5 kb in length. Both sets of markers gave us the same size estimate for the 0.68 kb transcript. The discrepancy between the size I report for the *tko* transcript, 0.68 kb, and that reported previously, 0.9 kb, could be due to the fact that the sizes of the standards reported in the previous papers were all much larger than this transcript, at least 1.2 kb (Mariani et. al., 1985; Gunaratne et. al., 1986). So the larger size reported previously could have resulted from error due to extrapolation.

4.6.6 cDNA Library Screening

The larval cDNA library was provided by S. Artavanis-Tsakonas and the pupal cDNA library was provided by T. Kornberg. I used standard methods (Maniatis et. al., 1982) to screen 1.4 x 10^5 phage from the pupal library and 1.8 x 10^5 phage from the larval library. The probe was made from the 3.2 kb BamHI fragment which was isolated by agarose gel electrophoresis. This fragment was radioactively labeled using nick translation (Maniatis et. al., 1982) to screen the pupal library or the random primer method (Feinberg and Vogelstein, 1983) to screen the larval library. The nitrocellulose filters were prehybridized for 12 hours at 42°C in hybridization solution consisting of 50% Formamide, 5X SSC, 5X Denhardt's solution, 50mM $NaPO_4$ pH 6.5, 100 μg/ml Salmon sperm DNA, and 0.1% SDS. The

filters were then hybridized for 24 hours at 42 ° C in the same solution containing 1-2 x 10^5 cpm/ml cherenkov counts of the labeled 3.2 kb probe. After hybridization, the filters were washed once for 30 minutes at room temperature and three times for 30 minutes at 50 ° C in 0.1X SSC, 0.1% SDS.

Phage that gave a positive signal from the autoradiography were replated and repurified once by the same method as above. They were then grown and their DNA isolated by standard techniques (Maniatis et. al., 1982). The inserts were analyzed by restriction analysis and southern blotting. The largest inserts were subcloned into M13mp18 and M13mp19 for sequencing.

4.6.7 Sequencing

Sequencing was carried out by the dideoxy chain termination method (Sanger et. al., 1980) on fragments of the cDNAs and the genomic DNA which had been subcloned into M13. All of the genomic DNA corresponding to the cDNA was sequenced on both strands.

4.7 Summary

I have demonstrated that a 3.1 kb genomic fragment complements two separate alleles of *tko*. Only one complete transcript, 0.68 kb in length, is contained in this fragment. This transcript fulfills all the requirements to be the *tko* gene. The majority of it is deleted in the tko^- deficiency, Df(1)w^{rJ1}, and is completely present in the tko^+ deficiency, Df(1)$yw^{e58\text{-}11}$. It is also completely contained within the fragments that complement the *tko* phenotype by P-element mediated transformation. No other detectable transcript in the region fulfills all of these require-

ments. Therefore, this 0.68 kb transcript almost certainly provides all of *tko* function. Two other transcripts overlap this 0.68 kb transcript but they are unlikely to be involved in *tko* function. More precise mapping of this transcript and of the mutations causing the *tko* defects is required before I can rule out completely the involvement of these transcripts. The sequence of a cDNA corresponding to the *tko* transcript implies a 140 amino acid protein with considerable similarity to the ribosomal protein S12 from *E. coli* and chloroplasts from *E. gracilis*. This sequence homology led me to the hypothesis that the *tko* gene codes for a mitochondrial ribosomal protein.

CHAPTER 5

ANALYSIS OF THE *tko* PROTEIN

The preceding analysis led to the tantalizing hypothesis that *tko* codes for a mitochondrial ribosomal protein. In this chapter, I discuss some experiments that examine some properties of this protein. Using an assay for cytochrome oxidase activity I test a prediction made from my hypothesis concerning the function of the *tko* protein. I also use antibodies to detect a protein band that is probably the *tko* gene product. I suggest experiments that make use of these antibodies to further test the hypothesis.

5.1 Cytochrome Oxidase Assay

If *tko* codes for a mitochondrial ribosomal protein, then one would expect the primary defect in tko^{25t} mutants would be mitochondrial. Specifically, one would expect to find that the enzymes coded on the mitochondrial DNA are either defective or of low abundance in these mutants. The sequence of mitochondrial DNA from *Drosophila yakuba* contains coding sequences for cytochrome b, three subunits of cytochrome c oxidase, and two subunits for an ATPase. In addition, this DNA contains seven reading frames for unidentified proteins (Clary and Wolstenholme, 1985). This is similar to other animals, including humans, whose mitochondrial DNA also codes for the same subunits of cytochrome c oxidase, cytochrome b and ATPase (Attardi et. al., 1986). It is reasonable to assume that *D. melanogaster* mitochondrial DNA also codes for these of proteins. A defect in the mitochondrial

ribosomes could lead to defects in these proteins or to decreased abundance in the mitochondria. To address this possibility I tested whether tko^{25t} flies have a defective cytochrome oxidase by assaying cytochrome oxidase activity from adult flies. I compared the activity in *tko* mutants to that from wild type Oregon R adults.

To test cytochrome oxidase activity I collected Oregon R and tko^{25t} female adult flies from 0 to 2 days old. Five groups of five flies each were assayed for each genotype. I homogenized these using a teflon pestle to break open the cells while leaving the mitochondria intact. I then centrifuged this solution to remove nuclei and unbroken cells. An aliquot of the supernatant was solubilized in 0.1% sodium cholate. I measured the activity of cytochrome oxidase by measuring the optical density at 550 nanometers (OD_{550}) of a cytochrome c solution following the addition of this solubilized aliquot. I measured the cytochrome oxidase activity with respect to the total protein in the cytoplasmic/mitochondrial supernatant. I found that tko^{25t} adults contained only 46% of the cytochrome oxidase activity of Oregon R adults as shown in figure 9A. I have repeated this experiment several times, and although the absolute magnitude of the cytochrome oxidase activity varied from day to day, probably due to large fluctuations in room temperature and differences in the age of the flies, the *tko* activity consistently remained at around 50% of the wild type activity.

This experiment indicates that tko^{25t} adults have significantly less cytochrome oxidase activity than wild type. The decrease in activity could be due to a decrease in the total number of mitochondria per μg protein or due to a decrease in cytochrome oxidase activity per mitochondrion or both. Ideally, it would be very

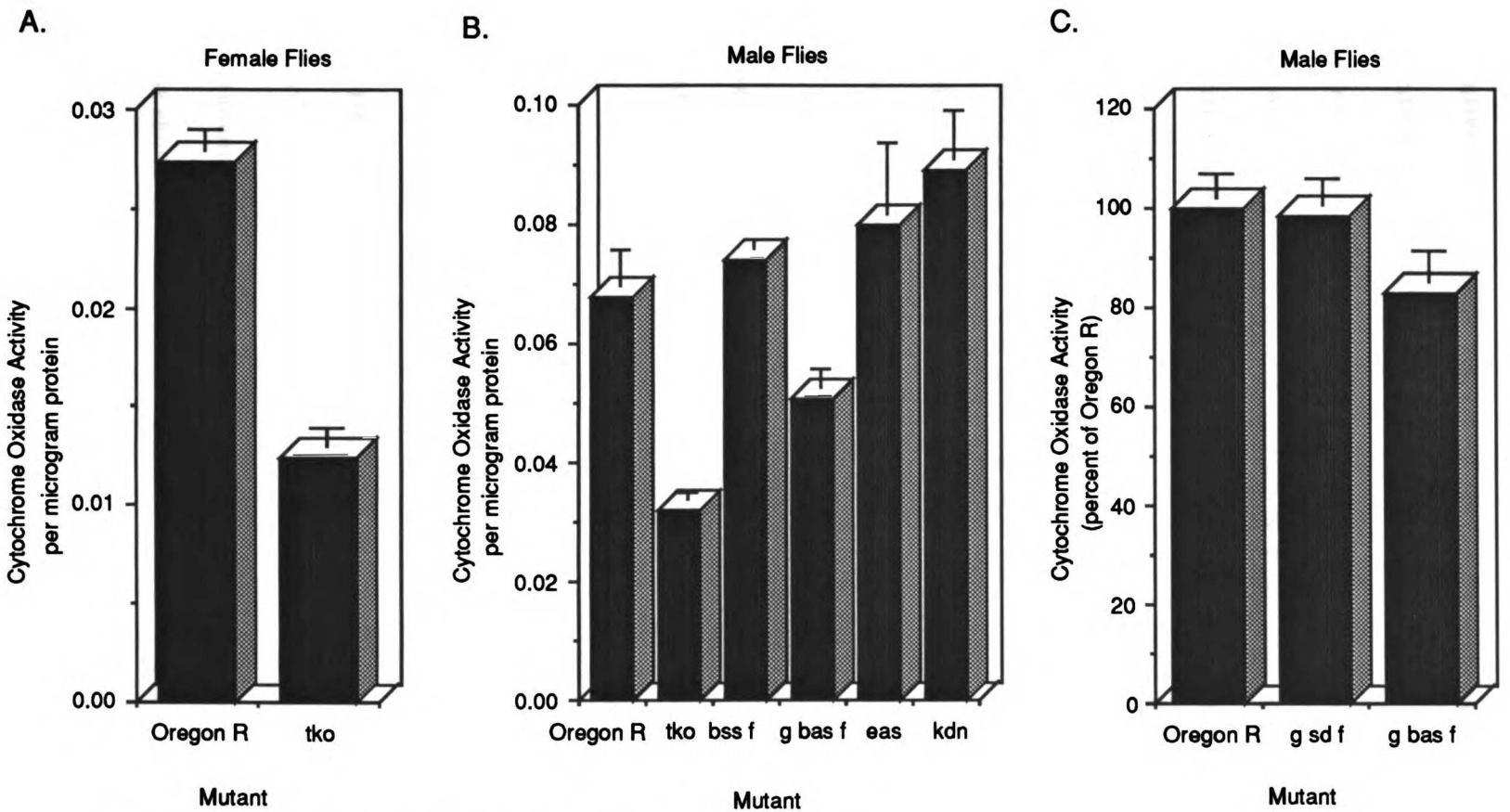


Figure 9. Cytochrome oxidase activity in bang-sensitive mutants.
A. Cytochrome oxidase activity in Oregon R and *tko*. Eight groups of five flies each were tested. Activity per microgram protein is shown. B. Cytochrome oxidase activity in bang sensitive mutants. Three groups of five flies each were tested. Activity per microgram protein is shown. C. Cytochrome oxidase activity in *bas* flies. Eight groups of five flies each were tested for *g bas f* and Oregon R flies and four groups of five flies each were tested for *g sd f* flies. Activity is shown as percentage of Oregon R activity. Error bars indicate the standard error of the mean.

informative to have some marker of the actual number of mitochondria in each preparation so that one could measure the cytochrome oxidase activity per mitochondrion. For example, one could use an antibody against a nuclear encoded mitochondrial protein or count the number of mitochondria in a given aliquot of the preparation under a microscope. Such an assay would distinguish the above possibilities.

I repeated the cytochrome oxidase assay for all the other bang sensitive mutations, using male adult flies from 4-6 days old. Three groups of 5 flies each were assayed for each genotype. The results of this experiment are shown in figure 9B. The *tko* males show about the same decrease in activity with respect to wild type (47%) as the females. The results for the other genotypes are less dramatic. Although there is some fluctuation around the wild type value, none of the changes are statistically significant. Because *g bas f* flies showed some decrease in cytochrome oxidase activity I repeated the assay for eight more groups of five flies each of Oregon R and *g bas f*. To control for the possibility that either of the markers *garnet* (*g*) or *forked* (*f*) were responsible for the decrease in activity, I also tested flies with *g sd f* (*sd* is *scalloped*) genotype. When this data is normalized so that Oregon R has 100% activity and combined with the previous data, the *g bas f* flies have only 83% of the Oregon R activity. This decrease is statistically significant ($p < 0.05$, Student's t-test). The flies carrying *g sd f* showed no decrease in activity. This result is shown in figure 9C. The decrease in cytochrome oxidase activity caused by *bas* could mean that *bas* also directly affects mitochondrial function. However, because the magnitude of the decrease is so small compared to *tko*, the argument for

a direct mitochondrial effect is much weaker. It is possible that the effect on mitochondrial function is indirectly caused by a defect in some other cellular function.

Of all the bang-sensitive mutants tested, only *tko* and *bas* show a decrease in cytochrome oxidase activity. All the other mutants tested, *bss*, *eas*, and *kdn*, clearly differ from *tko* in that they show either little change in activity or a slight increase. Further experiments with a larger sample size could reveal whether any of these increases are statistically significant. It is not clear exactly what an increase in cytochrome oxidase activity would signify, but it certainly indicates that these mutants differ from *tko* with respect to this particular mitochondrial function. This result does not rule out other mitochondrial defects for the mutants *bss*, *eas* and *kdn* but it suggests that they are probably not defects in the ribosomal machinery of mitochondria.

5.2 Antibody Studies

The most direct approach to study the *tko* gene product is to generate antibodies against this protein and use these antibodies to localize the protein in the cell. I have used two separate techniques to produce antibodies against the *tko* protein. First, I constructed a fusion of β-galactosidase and part of the *tko* protein by inserting part of the *tko* cDNA into the vector pUR291 (Ruther and Muller-Hill, 1983). The constructed plasmid, pUR291.24F, is diagrammed in figure 10. Figure 11 shows the *tko* protein sequence and indicates the region used in this construct. The fusion protein was produced in transformed *E. coli* induced with IPTG and subsequently isolated from the lysed cells by polyacrylamide gel electrophoresis. The gel purified protein was injected into rabbits and serum collected.

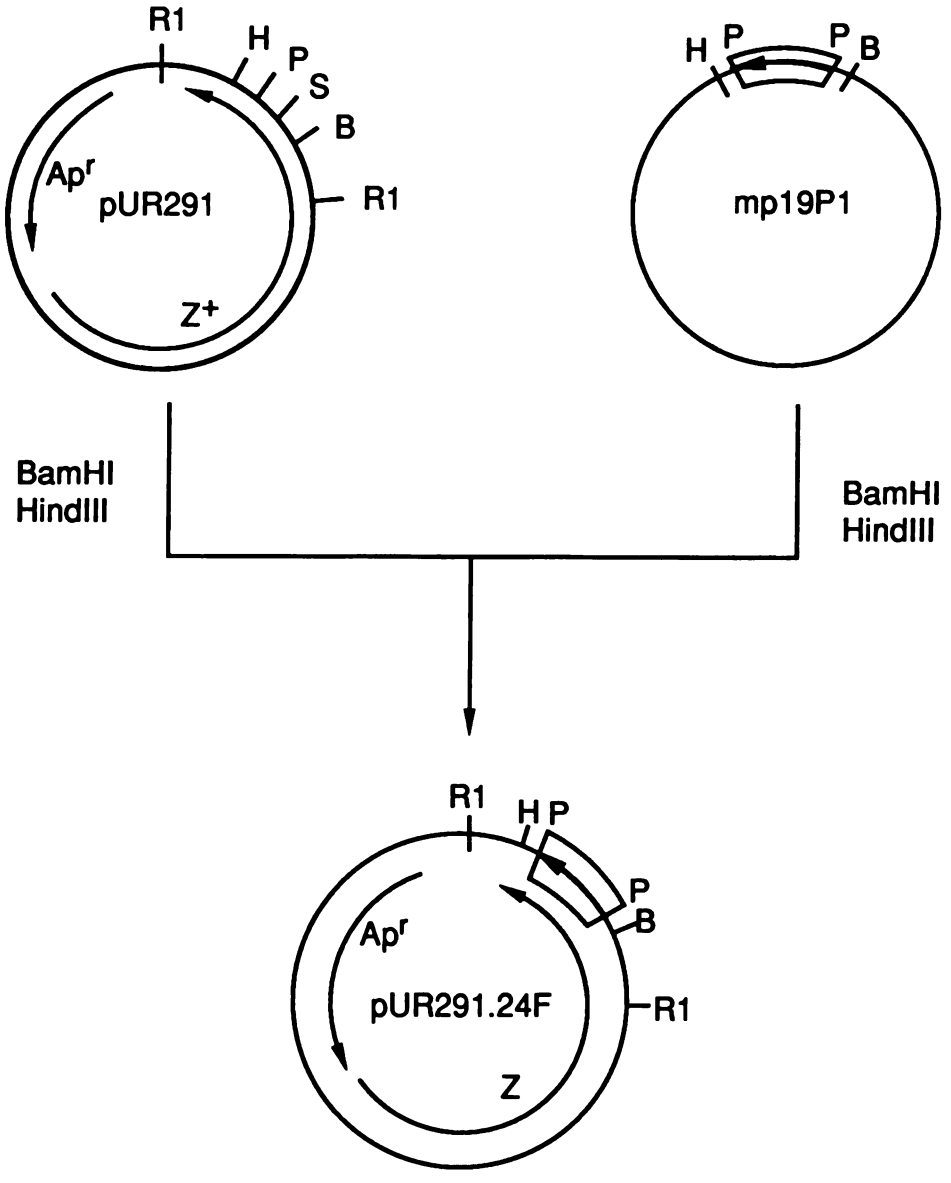


Figure 10. Construction of pUR291.24F

Ampicillin resistance gene (Ap^r) and beta-galactosidase gene (Z^+) are indicated by arrows within the plasmid pUR291. The open box indicates to fragment of Drosophila DNA. The arrow shows the direction of the *tko* transcription. mp19P1 is an M13 vector with the 0.24 kb Pst fragment inserted. The orientation of the fragment was determined by sequencing. B stands for Bam HI, H for Hind III, P for Pst I, R1 for Eco R1 and S for Sal I.

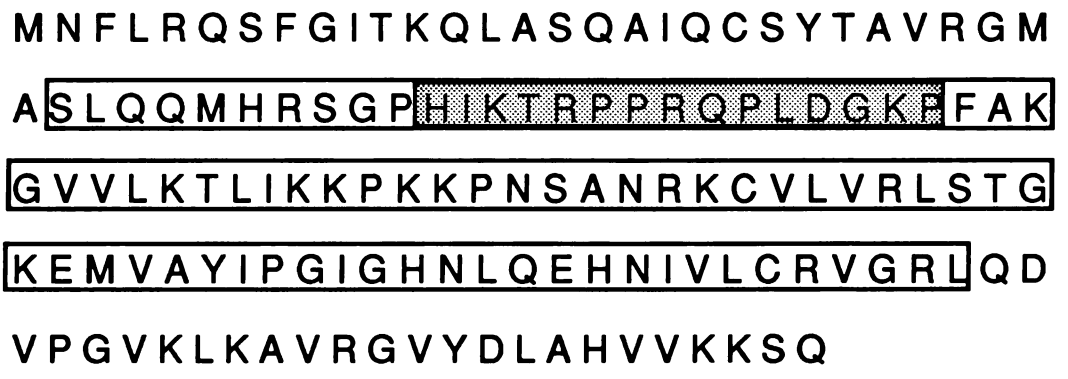


Figure 11. Fusion protein and synthetic peptide sequences.
The entire predicted protein sequence for *tko* is shown. The sequence included in the fusion protein is within the box. The synthetic peptide sequence is shaded.

The second approach to obtain antibodies used a synthetic peptide. The 15 amino acid stretch used for this is shown in figure 11. It was chosen because it was near to the N-terminus of the protein, but not part of the putative signal sequence. For the purposes of choosing a good region for the generation of antibodies I defined the signal sequence as the N-terminal region of the *tko* protein not represented in the *E. coli* ribosomal protein S12. This peptide was synthesized and, following conjugation to Keyhole lympet hemocyanin (KLH), injected into rabbits for the production of antibodies.

The crude antisera collected from the immunized rabbits were analyzed on western blots of the fusion protein. Antibodies against the fusion protein detected as little as 5 ng (50 fmole) of the fusion protein and antibodies against the synthetic peptide detected 10-20 ng (100-200 fmole) as shown in figure 12. These antisera were analyzed on western blots of embryonic proteins separated on 15% polyacrylamide gels. All antisera recognized a large number of bands on these westerns, however several criteria point to one particular band, of apparent molecular weight of 17kD, as the *tko* gene product. First, this band is one of a few bands in the correct size range for the *tko* protein. The apparent molecular weight of this band is somewhat higher than predicted from the amino acid sequence of this protein. Based on the predicted sequence the molecular weight should be about 15.5kD including the putative signal sequence and 12.3kD without it. However, it is known that some highly positively charged proteins, such as histones, migrate more slowly on SDS-polyacrylamide gels than would be predicted from their known molecular weights (Weber and Osborn, 1975). This affect of charge is greater for small molec-

Figure 12. Western blots showing titration of anti-*tko* antibodies.

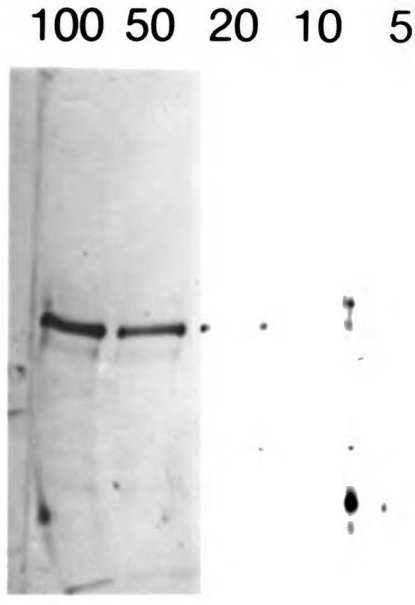
Each blot shows the titration of one antiserum against different amounts of the *tko*-β-galactosidase fusion protein. In each blot the first lane contains 100 ng of fusion protein; the second contains 50 ng; the third contains 20 ng; the fourth contains 10 ng; and the fifth contains 5ng. Each antiserum was diluted 1:100 for binding to the Western blots.

A. Western blot bound with anti-peptide antiserum, bleed 2.

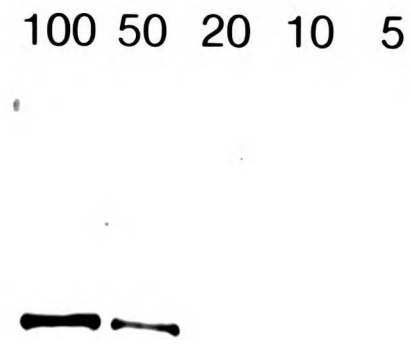
B. Western blot bound with anti-peptide antiserum, bleed 1.

C. Western blot bound with anti-fusion protein antiserum.

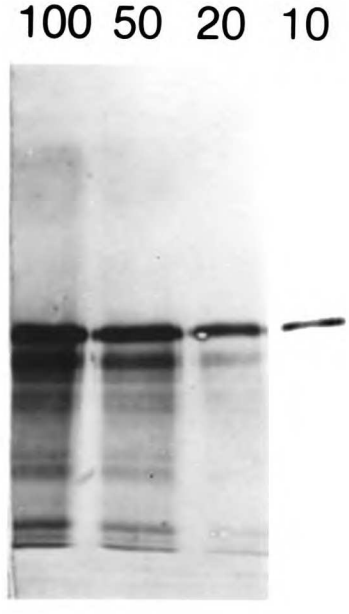
A.



B.



C.



ular weight proteins, with molecular weights less than 15 kD (Weber and Osborn, 1975). Therefore it is not surprising that the apparent molecular weight of the *tko* protein, which is small and highly positively charged with many arginine and lysine residues, is somewhat higher than the predicted molecular weight. Second it is the only band recognized by antisera to both the peptide and the fusion protein, and it is not recognized by the preimmune serum of either. Third, staining by the antisera against the synthetic peptide can be eliminated by preabsorption with the synthetic peptide. In adults a band of the same molecular weight is detected by antisera to both the fusion protein and the synthetic peptide. This protein seems to be a smaller fraction of the total protein in adults than in embryos, but this is not surprising since embryos are growing rapidly and probably have a larger number of mitochondria per cell than adults. I have not detected this protein band in larvae, but this could be due to effects of dilution. If the protein is a smaller proportion of the total protein in larvae than it is in embryos or adults then it might not show up on western blots.

Further analysis using western blots revealed is no detectable change in size or amount of this 17kD protein in tko^{25t} mutants. Furthermore, none of the other bands on these westerns differ between Oregon R and tko^{25t}. This is not surprising, since this allele is probably the result of a point mutation. If the result was merely the change of one amino acid in the protein then no change in size or abundance would be expected. Attempts to localize this protein by cellular fractionation have been inconclusive. Two different methods for fractionation by differential centrifugation were tried and depending on the method the majority of this protein pelleted

either with the nuclear pellet or the mitochondrial pellet. Due to difficulties inherent in a crude centrifugal fractionation this result does not disprove or support the hypothesis that the protein is mitochondrial. In fact, cytochrome oxidase assays of the fractions from the method taken from Alziari et. al. (1981) revealed that only a third to a half of the total cytochrome oxidase activity was concentrated in the mitochondrial pellet, with the rest equally distributed between the nuclear pellet and the supernatant fraction. The fact that the 17kD protein always appears in a pellet rather than in the supernatant fraction suggests that it is associated with some cellular organelle or the nucleus, as opposed to being a soluble enzyme. This is consistent with the mitochondrial hypothesis but it does not rule out many other possibilities.

The most direct experiment to address the hypothesis that *tko* is a mitochondrial ribosomal protein is to isolate mitochondrial ribosomes and use the antibodies against the *tko* protein to test whether it is present in the mitochondrial ribosomes. This experiment relies on the purification of the antibodies to eliminate the large amounts of background now seen on the western blots. These purified antibodies could be used to confirm the hypothesis that the 17kD protein is the *tko* gene product and to determine whether the *tko* protein is a constituent of mitochondrial ribosomes.

5.3 Materials and Methods

5.3.1 Cytochrome Oxidase Assay

Groups of five adult flies were collected by CO_2 anesthetization. After recovery from the anesthesia, they were cooled for about one minute and

immediately added to the homogenization buffer. They were homogenized using a teflon pestle in 250μl mitochondrial extraction buffer containing 0.25M Sucrose, 10mM Tris pH 7.4 and 1mM EDTA as in the method of Alziari et. al. (1981). The homogenate was transferred to a new tube and the homogenizer rinsed with 250μl extraction buffer, which was added to the homogenate. The homogenates were spun at 300 g for 5 minutes to remove unbroken cells and nuclei and the supernatant collected for cytochrome oxidase assay. The pellet was resuspended in extraction buffer to assay residual cytochrome oxidase activity. The total activity in the preparation was calculated both from an aliquot of the total homogenate taken before fractionation and from the total activity in the supernatant and pellet.

The cytochrome oxidase assay was performed by the method of Cooperstein and Lazarow (1951). A 1.7 x 10^{-5} M solution of cytochrome c in 0.03 M phosphate buffer, pH 7.4 was completely reduced by the addition of 10 μl of a freshly made solution of sodium hydrosulfite (1.2 M) per 3 ml cytochrome c solution. This was shaken vigorously for two minutes and allowed to sit for at least 5 minutes before the assay. 1 μl of 0.5% Na cholate was added to 4 μl of sample to solubilize the mitochondria. An aliquot of 0.6ml of the cytochrome c solution was added to the solubilized sample 2 minutes after the addition of Na cholate. The OD_{550} was read every 30 seconds for 6 minutes. The sample was then completely oxidized by the addition of a few grains of potassium ferricyanide, and the OD_{550} redetermined. The cytochrome oxidase activity was determined by finding the rate constant for the reaction from the equation:

$$k=\frac{\Delta\log[ferrocytochrome\ c]}{\Delta t}=\frac{log(D_{t_1}-D_{ox.})-log(D_{t_2}-D_{ox.})}{t_2-t_1}$$

The total cytochrome oxidase activity was then divided by the total protein in the sample, determined by the Amido Schwarz method (Schaffner and Weissman, 1973), to find the activity per μg protein.

5.3.2 Construction and Purification of Fusion Protein

The fusion protein was constructed by ligating the 0.24 kb Pst I fragment from the *tko* cDNA (see figure 10) into the vector pUR291 (Ruther and Muller-Hill, 1983), which contains a polylinker near the C-terminal of the gene for β-galactosidase. To insure that the fragment would insert in the correct orientation to produce the *tko* protein sequence, the 0.24 kb Pst I fragment was first subcloned into the phage M13 mp19, and then reisolated by cutting with the enzymes Bam HI and Hind III, which cut within mp19 on either side of the Pst I site. This fragment was ligated into the pUR291 vector which had been cut with Bam HI and Hind III. The *E. coli* strain DG101 was transformed with the constructed plasmid and three colonies that contained the insert were isolated. These clones were grown in L-Broth overnight, and 1.5ml centrifuged for 5 minutes to pellet the cells. This pellet was resuspended in Sample buffer (62.5 mM Tris-Cl pH 6.8, 10% glycerol, 2% Sodium dodecyl sulphate, 5% β-mercaptoethanol, .0015% bromphenol blue) and boiled for 5 minutes. The DNA was spun down and 20 μl of the supernatant loaded onto a 5% polyacrylamide gel. Staining with coomassie blue indicated that the constructed plasmid produced a protein slightly bigger than β-galactosidase as expected.

To isolate the protein, 100 ml of L Broth was inoculated with 1 ml of an over-

night culture of DG101 transformed with the pUR291.24F plasmid. This was grown 2 hours at 37 °C (OD_{550} = 0.44). The bacteria were then induced to produce the fusion protein by addition of 0.5 ml of IPTG (Isopropylthio-β-galactoside, purchased from Bethesda Research Laboratories) and the culture was grown for another 2 hours at 37 °C. The cells were pelleted by spinning at 6.5 K rpm for 5 minutes at 4 °C and resuspended in 2.6 ml of Solution 1 (25% Sucrose, 2 mM EDTA, 40 mM Tris pH 7.6, and 1 mM DTT). After the addition of 0.5 ml of lysozyme (2 mg/ml in solution 1) the sample was rocked for 1 hour at 4 °C. 40 μl of a 10% solution of Triton X 100 was added and the solution rocked for another hour. The solution was then spun for 20 minutes at 18 K rpm in a Sorvall SS-34 rotor. The supernatant was discarded and the pellet resuspended in 1.5 ml 2X sample buffer, and placed in a boiling water bath for 20 minutes. After spinning out the debris, the supernatant was loaded on a preparatory 5% acrylamide gel. The location of the fusion protein band was determined by staining the edges of the gel with coomassie stain. The fusion protein band was then cut out of the remaining part of the gel and the fusion protein eluted by electrophoresis. This purified protein was used to inoculate rabbits for the production of antibodies.

5.3.3 Synthesis of Synthetic Peptide

The synthetic peptide was made by the Biomolecular Resource Center at UCSF. The extent of the peptide is indicated in figure 11. The peptide was then conjugated to the KLH protein using gluteraldehyde by Berkeley Antibody Company. This conjugated peptide was used to inject the rabbits.

5.3.4 Production of Antibodies

The antibodies were generated in rabbits by the Berkeley Antibody Company. They injected rabbits with the proteins and boosted and collected sera at regular intervals. They also performed ELISA assays on the collected sera to determine the immunoreactivity to the original antigens.

5.3.5 Western Blots

Embryos were collected for 24 hour period and dechorionated for 90 seconds in 50% chlorox. They were homogenized in a dounce homogenizer in 2X sample buffer and boiled for 10 minutes. After spinning out the debris, 10 to 20 μl were loaded onto a 15% polyacrylamide gel and the proteins separated by electrophoresis. Adults were cooled on ice and immediately added to 2X sample buffer, after which they were treated the same as the embryos. Prestained molecular weight standards (Bethesda Research Laboratories) were used to measure the apparent molecular weights of protein bands on the western blots. When it was necessary to load equal amounts of protein per lane, the protein concentration was determined by the Amido Schwarz method (Schaffner and Weissmann, 1973). The proteins were then electrophoresed from the gel onto nitrocellulose. The blots were washed for 1 hour in a blocking solution containing 2% BSA (Bovine Serum Albumin) and 5% Goat serum in TBST (10mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween-20). The blocking solution was then replaced with primary antibody against either the fusion protein or the synthetic peptide diluted 1:100 in TBST. The blot was incubated at room temperature for 30 minutes in the primary antibody. The primary antibody solution was removed and the blots washed three times for 5 minutes in TBST. The

secondary antibody, goat anti-rabbit conjugated to alkaline phosphatase, was then applied and incubated for 30 minutes. Following the removal of the secondary antibody the blot was again washed three times in TBST. The filters were then incubated from 2 to 20 minutes in 10 ml AP buffer (100 mM Tris pH 9.5, 100 mM NaCl, 5 mM MgCl) containing 66 μl of NBT (Nitro-blue-tetrazolium, 50 mg/ml) and 33 μl of BCIP (5-bromo-4-chloro-3-indolyl phosphate, 50 mg/ml; Substrates NBT and BCIP from Protoblot kit from Promega Biotec). After the protein bands appeared, the reaction buffer was removed and the reaction stopped by washing the blots in a solution of 20 mM Tris pH 8.0, 5mM EDTA. The filters were then dried and stored.

5.6.6 Isolation of Mitochondria for Western Blots

Two fractionation techniques were used to isolate mitochondria. In the first (Bygrave et. al., 1975), embryos were collected and dechorionated in 50% chlorox bleach. They were then washed and transferred to mitochondrial extraction buffer (154 mM KCl, 2mM Tris pH 7.4, 1mM EDTA, and 0.3% BSA) and homogenized with a teflon pestle. The nuclei and unbroken cells were removed by spinning for 3 minutes in an HS4 rotor at 800 rpm (120g). The supernatant was then transferred to a new tube and spun for 5 minutes at 4700 rpm (4300g) in an HS4 rotor. The pellet was taken up in Wash Buffer (154 mM KCl, 2mM Tris pH 7.4, 0.3% BSA) and spun once more for 5 minutes at 4700 rpm. The pellet was then resuspended in wash buffer. 15 μl of mitochondrial sample was mixed with 15 μl 2X Sample buffer and boiled for 10 minutes. The debris was removed by centrifugation and 10 μl loaded onto a polyacrylamide gel for electrophoresis.

In the second technique (Alziari et. al., 1981), adults or dechorionated embryos were homogenized with a teflon pestle in buffer containing 0.25 M Sucrose, 10 mM Tris pH 7.4, and 1 mM EDTA. Homogenates were spun for 5 minutes in the HS4 rotor at 1250 rpm (300g) to remove nuclei and unbroken cells. The supernatant was collected and spun for 10 minutes at 4000 rpm (3000g) to pellet the mitochondria. Cytochrome oxidase assays of the fractions from this method revealed that the cytochrome oxidase activity per μg protein was enriched 4-5 fold in the mitochondrial pellet. However the mitochondrial pellet contained only 1/3 to 1/2 of the total cytochrome oxidase activity, with the remainder distributed equally between the nuclear pellet and the supernatant fractions. The fractions were mixed with sample buffer and subjected to electrophoresis as in the above method.

5.7 Summary

I have carried out some initial experiments to test whether the *tko* protein is a mitochondrial ribosomal protein. The results of these experiments are encouraging. Cytochrome oxidase assays suggest that the 25t allele of *tko* does cause a metabolic defect, the decreased function of the cytochrome c oxidase. This is consistent with the hypothesis that tko^{25t} causes a defect in a mitochondrial ribosomal protein. The only other bang-sensitive mutant with a decrease in cytochrome oxidase activity is *bas*, which shows a slight decrease. This result adds to the list of similarities between *tko* and *bas*. Antibodies to portions of the *tko* protein recognize a protein band of apparent molecular weight 17 kD which is present in both embryos and adults. Future experiments with purified antibodies should be able to determine whether the *tko* protein is actually a mitochondrial ribosomal protein.

CHAPTER 6

DISCUSSION

I set out to clone the *tko* gene in hopes of discovering a factor involved in the regulation of nervous system excitability. I succeeded in cloning the gene and demonstrating that a small 0.68 kb message is sufficient to complement the *tko* phenotypes. By sequencing this message and looking for homologies with other known proteins I discovered that the *tko* gene product bears a remarkable resemblance to the *E. coli* ribosomal protein S12, as well as to the ribosomal protein S12 of *E. gracilis* chloroplasts. While this result was quite surprising it led me to the hypothesis that the *tko* gene product is a mitochondrial ribosomal protein. In this chapter I discuss in detail the basis for this hypothesis and the its implications for our understanding of bang-sensitivity, mitochondrial protein synthesis and evolution.

6.1 Basis for Mitochondrial Ribosome Hypothesis

The similarity between the *tko* gene product and the ribosomal protein S12 from *E. coli* and chloroplasts implies that the *tko* gene product is probably involved in protein synthesis. The large number of amino acid identities between these proteins indicate that they are almost certainly homologues arising from the same ancestral protein. However, it is not obvious from a superficial consideration whether the *tko* gene product is more likely to be a part of the eukaryotic, cytoplasmic ribosome or a part of the mitochondrial ribosome. Three arguments

strongly suggest that the *tko* gene product is in fact a mitochondrial ribosomal protein. The first is based on the functional similarities between prokaryotic and mitochondrial ribosomes. The second is based on the evolutionary theory that mitochondria arose from eubacteria living in symbiosis with the original eukaryotic organisms. Finally, the presence of a possible mitochondrial signal sequence at the N-terminus of the *tko* protein supports the hypothesis that it is a mitochondrial protein.

6.1.1 Functional Considerations

6.1.1.1 Ribosomal Protein S12

To understand what role the *tko* gene product might play in *Drosophila*, one must first examine the function of the *E. coli* ribosomal protein S12 in prokaryotic protein synthesis. Ribosomal protein S12 (referred to hence as S12) is a product of the *strA* locus in *E. coli* (Ozaki et. al., 1969). Mutations in either of two amino acids in this protein leads to resistance to the antibiotic streptomycin (Funatsu and Wittman, 1972), which inhibits protein synthesis in bacteria by inhibiting the initiation of translation and decreasing the fidelity of translation (Ozaki et. al., 1969). This suggests that the S12 protein is somehow involved in initiation of translation and in maintaining the fidelity of translation.

Ozaki et. al. (1969) isolated the protein that contains the the binding site for Streptomycin and that is the affected protein in streptomycin resistance. They showed that this protein, S12, plays an important role in initiation of protein synthesis and in maintenance of fidelity. By reconstituting the small (30S) ribosomal subunit from purified proteins, they showed that 30S subunits lacking the S12

protein (referred to in their paper as P10) have considerably decreased ability to initiate translation. They demonstrated further that this defect was due to a decreased ability to bind the formylated methionyl-tRNA (f-met-tRNA^{Met}) used by prokaryotes for initiating translation. This importance in the binding of f-met-tRNA^{Met} provides a clue as to the probable function of the *tko* gene product as discussed below.

Ozaki et. al. (1969) also examined the involvement of S12 in the fidelity of translation. They looked at the frequency of mistakes in the translation of poly-U message. They found that ribosomes containing protein S12 from a streptomycin resistant strain had a lower frequency of errors than those containing S12 from a streptomycin sensitive strain. They further discovered that when they reconstituted 30S subunits lacking the S12 protein, the ribosomes also had a lower frequency of errors in translation. This, coupled with the streptomycin data, underscores the importance of ribosomal protein S12 in translational fidelity.

6.1.1.2 Protein Synthesis

The protein synthesis machinery in prokaryotes, eukaryotes, chloroplasts and mitochondria is very similar. All of these use ribosomes to translate messenger RNA into polypeptide chains. All of the ribosomes consist of two subunits made up of ribosomal RNA and a large number of ribosomal proteins. The ribosomes all guide tRNA molecules charged with amino acids to specific sites so that the amino acids can be linked together to form peptide chains. However if one looks at the mechanisms of translation from these different ribosomes in more detail, one discovers that prokaryotic ribosomes appear more similar to mitochondrial ribosomes

than to eukaryotic cytoplasmic ribosomes. This similarity suggests that the *tko* protein is more likely to be mitochondrial than cytoplasmic.

6.1.1.3 Ribosomal structure

The gross structure of all ribosomes is fairly uniform. They all consist of two subunits, usually of different sizes which are made up of ribosomal RNA and proteins. However, prokaryotic, eukaryotic and mitochondrial ribosomes all differ from one another with respect to the total size of the subunits, the amount of RNA and number of proteins as well as the ratio of RNA to protein. Chloroplast ribosomes are so similar to prokaryotic ribosomes that I will not discuss them separately.

The ribosomes from a typical prokaryote, e.g. *E. coli*, consist of a large (50S) subunit and a small (30S) subunit. The large subunit contains a 23S and a 5S rRNA (ribosomal RNA) and about 34 proteins. The small subunit contains a 16S rRNA and about 21 proteins (Buetow and Wood, 1978). In contrast, the eukaryotic cytoplasmic ribosomes tend to be larger than the prokaryotic ones, with the large subunit having a sedimentation value of 60S and the small subunit 40S. The large subunit consists of three rRNAs, 28S, 5.8S and 5S, and about 45 proteins. The small consists of an 18S rRNA and about 33 proteins (Alberts et. al., 1983).

It is much more difficult to generalize about mitochondrial ribosomal structure because they vary greatly even between closely related species. The S values of the large and small subunits, respectively, range from 39S and 29S in rat liver mitochondria to 50S and 37S in yeast (*Saccharomyces cerevisiae*) mitochondria to 60S and 44S in Maize (Curgy, 1985). The large and small rRNAs range from 16S and 12S in mammalian mitochondria to 21S and 15S in yeast to 26S and 18S in higher

plants such as Maize (Curgy, 1985). In general, mitochondrial ribosomes tend to be smaller than their cytoplasmic counterparts and they tend to have a higher ratio of protein to RNA than the cytoplasmic ribosomes (Curgy, 1985). One feature common to all mitochondrial ribosomes except those from higher plants is that they all lack the 5S and 5.8S rRNA (Curgy, 1985). However, it is possible that the large mitochondrial rRNA can carry out the function of the 5S rRNA. This is suggested by structural similarity between one part of the yeast large mitochondrial rRNA and *E. coli* 5S rRNA. Further, the organization of the rRNA genes in the genome of human mitochondria, tRNA-12S mrRNA-tRNA-16S mrRNA-tRNA, is similar to that in *E. coli*, 16S rRNA-tRNA-23S rRNA-5S rRNA-tRNA (Curgy, 1985) if one assumes that the 5S has fused to the large RNA in the human mitochondrial ribosome.

Prokaryotic, eukaryotic and mitochondrial ribosomes all differ from one another structurally to some extent. Mitochondrial ribosomes vary widely across different species, and structurally differ in some important features, such as lack of a 5S rRNA, from both cytoplasmic and prokaryotic ribosomes. So, if one were to rely only on the structure of ribosomes, one might conclude that the eukaryotic and prokaryotic were most similar to one another and that the mitochondrial ribosomes have diverged a great distance from either of the other types. However, as described in the next sections, when one examines the mechanisms of translation in the various ribosomes then the mitochondrial ribosomes appear more closely related to prokaryotic ribosomes than either mitochondrial or prokaryotic ribosomes are to cytoplasmic ribosomes.

6.1.1.4 Initiation of Translation

In order to initiate translation, prokaryotic ribosomes form an initiation complex consisting of the small ribosomal subunit, the mRNA, the formylated methionyl tRNA, and several initiation factors (IF2 and IF3) and GTP (Haselkorn and Rothman-Denes, 1973). This complex then binds the large subunit to form the complete ribosome and translation continues following the release of the initiation factors. Mitochondrial initiation of translation is very similar to this, also using the formylated met-tRNA^{Met} for initiation. Also, the binding of f-met-tRNA^{Met} by the *Neurospora* mitochondrial ribosomal subunits will occur even with initiation factors supplied from *E. coli* (Buetow and Wood, 1978). In contrast, eukaryotic cytoplasmic ribosomes differ somewhat in initiation. They do not use a formylated met-tRNA^{Met} and initiation factors cannot be exchanged between cytoplasmic and mitochondrial ribosomes (Buetow and Wood, 1978). Because S12 in prokaryotes is involved in the binding of the fmet-tRNA^{Met} to the initiation complex and because this process is so similar in prokaryotes and mitochondria, it seems likely that mitochondrial ribosomes would have an analogue to S12. The initiation process differs with respect to the binding of the initiator tRNA in the eukaryotic cytoplasmic ribosomes, so these are less likely to have a highly conserved analogue to S12.

6.1.1.5 Elongation

Elongation consists of three steps: binding of the aminoacyl tRNA, formation of the peptide bond and translocation. Although the S12 protein probably does not have much function for these steps except in the maintenance of fidelity, there is some resemblance between mitochondrial and prokaryotic ribosomes in this process,

that emphasizes the similarity between these two translation systems. The first likeness is the use of elongation factors. Mitochondrial ribosomes can generally function using the supernatant fractions containing elongation factors from *E. coli*, and the reverse is also sometimes true (Buetow and Wood, 1978). However, cytoplasmic ribosomes generally cannot function when supplied with the supernatant fractions of either *E. coli* or mitochondrial ribosomes. This again points out the fact that mitochondrial ribosomes appear to be more closely related functionally to prokaryotic ribosomes than either is to eukaryotic ribosomes.

6.1.1.6 Sensitivity to Antibiotics

Another similarity in translation between prokaryotic and mitochondrial ribosomes is their sensitivity to antibiotics. Several antibiotics which inhibit protein synthesis in prokaryotes also inhibit protein synthesis in mitochondria. For example, chloramphenicol inhibits the peptidyl transferase activity in mitochondrial ribosomes from *Neurospora*, yeast and rat liver as well as in *E. coli* ribosomes (Buetow and Wood, 1978). Mitochondrial protein synthesis is also inhibited by other antibiotics, such as tetracyclines, neomycin, erythromycin and streptomycin (Curgy, 1985). Cytoplasmic ribosomes are not sensitive to these antibiotics, but are instead inhibited by cyclohexamide which does not affect prokaryotic or mitochondrial ribosomes. The mitochondrial sensitivity to streptomycin is especially interesting in the present case, since S12 is the binding site for streptomycin. The sensitivity of mitochondrial ribosomes to this antibiotic strongly suggests that there is a protein very like S12 in structure in the mitochondrial ribosomes. The *tko* gene product is a likely candidate to be this protein.

6.1.2 Evolutionary Considerations

The second argument that the *tko* gene product is likely to be a mitochondrial ribosomal protein is based on evolutionary considerations. The endosymbiotic theory that mitochondria originated from prokaryotes living in symbiosis with eukaryotes has been widely accepted for several reasons (Curgy, 1985). The first comes from functional similarities between mitochondrial and prokaryotic ribosomes as discussed above, particularly their common sensitivity to antibiotics that inhibit protein synthesis. These functional analogies suggest that mitochondria may have arisen from prokaryotes.

A second argument for the endosymbiotic hypothesis comes from the similarity of various proteins, such as ATPases (Ovchinnikov et. al., 1984), cytochromes (Mathews, 1985) and pyruvate dehydrogenase (Keha et. al., 1982) in both mitochondria and prokaryotes. A particularly relevant example of protein conservation is revealed from the recent cloning of a mitochondrial ribosomal protein in yeast, which shows considerable homology to the *E. coli* ribosomal protein S14 (Myers et. al., 1987). This protein has 24% amino acid identity with the *E. coli* S14. This is somewhat less than the amount of amino acid identity between the chloroplast S14 and its *E. coli* counterpart (43% amino acid identity). This finding in yeast parallels the data for the *tko* protein, which has less similarity to the *E. coli* S12 (41% amino acid identity) than does the chloroplast S12 (68% amino acid identity, Montandon and Stutz, 1984). The yeast result shows that mitochondrial ribosomes do in fact contain proteins that are homologous to the prokaryotic ribosomal proteins. That they show less homology than their chloroplast counterparts is not surprising,

since chloroplast ribosomes have not diverged as far from their prokaryotic ancestors as have the mitochondrial ribosomes (Alberts et. al., 1983; Gray et. al., 1984). The differences between the mitochondrial proteins and their prokaryotic homologues could be due to the fact that the structure of the mitochondrial ribosomes differs in some respects from that of prokaryotic ribosomes. For example, mitochondrial ribosomes have higher protein content than the prokaryotic ribosomes (Curgy, 1985). The S14 and S12 proteins in mitochondrial ribosomes must differ from their bacterial homologues to interact with these different structural features. This result from yeast adds strong support to my hypothesis that the *tko* gene product is a mitochondrial ribosomal protein.

The final argument for the endosymbiotic theory, and perhaps the most complete study, comes from the comparison of ribosomal RNA from various genetic systems: eubacteria, archaebacteria, chloroplasts, mitochondria and eukaryotic cytoplasmic ribosomes. Kochel and Kuntzel (1981) compared the sequence for the small rRNA in *A. Nidulans* mitochondria with that for the *E. coli* 16S rRNA. They found that the two sequences had 59% homology in the primary sequence and considerable similarity in the potential secondary structure. The mitochondrial rRNA was more closely related to the bacterial rRNA than to the nuclear encoded small rRNA. They also found a similar result for the large ribosomal RNA from *A. Nidulans* and *E. coli* (Kochel and Kuntzel, 1982). Relying on similarities in secondary structure among large ribosomal RNAs from various species, they constructed a phylogenetic tree based on the homologies within one domain of the large rRNA that is conserved throughout all species. This tree shows mitochondrial rRNA from six species, (rat,

human, mouse, Paramecium, yeast and Aspergillus) are all more closely related to *E. coli* than to yeast nuclear ribosomal rRNA.

Gray et. al. (1984) took advantage of the fact that all small ribosomal RNA molecules have a highly conserved core in their secondary structure. This core consists of eight domains which are always present in this rRNA. They used this core to compare rRNAs from a wide range of genetic systems, including eubacteria, archaebacteria, chloroplasts, mitochondria, and cytoplasmic ribosomes from eukaryotes. This method eliminated the problems of aligning RNAs of greatly different sizes. From their results they concluded that mitochondria from mammals and fungi are related to each other and arose from eubacteria, while mitochondria from higher plants arose separately from eubacteria. While their results are quantitatively different from those of Kochel and Kuntzel, the conclusion that mitochondria arose from eubacteria is the same. Therefore, the structure of these phylogenetic trees constructed from this analysis is apparently quite robust, with the same patterns obtained even when different data sets and different methods of comparison are used.

Thus the evolutionary evidence suggests that mitochondrial ribosomes are more closely related to eubacterial ribosomes than to eukaryotic cytoplasmic ribosomes. This adds support to the hypothesis that *tko* is a mitochondrial ribosomal protein.

6.1.3 Mitochondrial Signal Sequences

One final piece of data that is consistent with the hypothesis that the *tko* gene product is a mitochondrial ribosomal protein is the existence of a possible

mitochondrial signal sequence at the N-terminal of the putative *tko* amino acid sequence. Most mitochondrial matrix proteins that are coded in the cell nucleus and translated in the cytoplasm have a signal sequence that directs them into the matrix of the mitochondria. In most cases this sequence is cleaved at the end of the transport process (Alberts et. al., 1983). Although the sequences that have been studied vary greatly, they share some common features which are also present in the *tko* N-terminal sequence. They are rich in the positively charged amino acids, arginine and lysine; they are rich in serine and threonine, with these amino acids regularly spaced throughout the sequence; and they are devoid of acidic residues (Hurt and van Loon, 1986). Except for one glutamate residue near its C-terminal end, the putative *tko* signal sequence fulfills all of these conditions. The existence of this glutamate residue does not negate the possibility that this is a signal sequence, since it may not be a part of the signal sequence. Signal sequences can be as short as 9 residues (Hurt and van Loon, 1986) and so the 23 amino acids preceding this glutamate could serve as the entire signal. The presence of this possible signal sequence adds weight to the hypothesis, since if the *tko* protein is a mitochondrial ribosomal protein then it should be made as a precursor form with a signal sequence in the cytoplasm. If such a sequence were not present it would be more difficult to accept the possibility that the *tko* protein is a mitochondrial protein.

6.2 Alternative Functions for *tko*

Based on the above functional and evolutionary considerations it seems quite likely that mitochondria arose from prokaryotes and that the *tko* gene product is the mitochondrial homologue to the bacterial S12 protein. The evidence from cyto-

chrome oxidase assays suggests that there is a mitochondrial defect in tko^{25t} flies. I cannot rule out the possibility that the *tko* protein has some other function in the fly, such as a function in the cytoplasmic ribosomes or some other RNA binding function. The similarity of patches carrying the $l(1)^{tko k11}$ mutation to tissue in *bobbed* mutants suggests a defect in cytoplasmic protein synthesis, since *bobbed* mutants lack some cytoplasmic ribosomal RNAs (Ribossa et. al., 1966). This may be misleading because mitochondrial biogenesis and protein synthesis depend on cytoplasmic protein synthesis. The similarities between the two mutations could both be due to defects in mitochondrial biogenesis. A definitive assignment of the *tko* protein to a particular function awaits further experiments with the antibodies.

6.3 Implications of Hypothesis

6.3.1 Explanation of *tko* Phenotype

The hypothesis that the *tko* gene product is a mitochondrial ribosomal protein can explain several of the defects seen in flies carrying the tko^{25t} mutation. Since several proteins essential for oxidative phosphorylation, including subunits of cytochrome oxidase, are encoded on mitochondrial DNA (Clary and Wolstenholme, 1985), a defect in the protein translation machinery in the mitochondria should lead to defects in the metabolism of the fly. Such a defect could result in the weakness seen in the mutant flies. This weakness might in fact be due to a lack of energy in the muscles themselves and not necessarily be related to a nervous system defect. A metabolic defect could similarly explain the sensitivity to CO_2 seen in the tko^{25t} mutants. If the mutant flies use O_2 less efficiently than wild type, then they might

be more severely affected and take longer to recover when there is little oxygen available as in CO_2 anesthesia. This same metabolic defect could easily explain the general slow growth and inviability of the flies.

One interesting phenotype of the lethal allele of *tko* is the fact that it lives until second instar before dying. However, the mosaic studies suggest that this mutation is cell lethal for almost all cells in the fly. This apparent dichotomy can be explained by the fact that the embryo inherits many mitochondria and much mRNA from its mother. All of the maternal mitochondria will have functional ribosomes; perhaps enough to allow each mitochondrion to reproduce several times. If, in addition the embryo has a large amount of maternal mRNA which could code for functional *tko* gene product, then this combination could conceivably be sufficient to keep the mitochondria dividing normally for a significant length of time. Once the maternal mRNA had been used up or degraded, the mitochondria might still divide for a while using their good ribosomes. It would only be after these ribosomes have been diluted out by mitochondrial divisions that the mitochondria would cease to be able to grow properly. All of this could take a rather long time and it is conceivable that the larvae survive as long as they do with the maternal mitochondria and mRNA.

I do not know specifically how a mitochondrial defect would cause the bang-sensitive phenotype. The nervous system uses a large amount of energy, so it is possible that under the stress of "banging" it simply uses up its available energy stores. This argument, however, does not explain the hyper-excitability of *tko* flies mentioned in chapter 1. As *tko* flies are recovering from paralysis, they often go through

a period of uncontrolled wing vibration, as though their flight muscles were being over stimulated by the motor neurons. This would seem opposite what one would expect from a depletion of energy stores. One explanation for this hyper-excitability would be that in *tko*^{25t} a defect in the mitochondrial ribosomal protein might lead to the disruption of calcium sequestration by mitochondria or of other ATP-dependent calcium uptake mechanisms in the nerve terminals. It is known that mitochondria have active calcium uptake systems in both vertebrates and insects (Dawson et. al., 1971; Wohlrab, 1974; Bygrave et. al., 1975; Akerman and Nicholls, 1983), and may act to buffer the overall concentration of calcium in nerve terminals (Nicholls and Crompton, 1980; Akerman and Nicholls, 1983). There are also several other ATP-dependent mechanisms for the removal of calcium from the cytoplasm (Kendrick et. al., 1977; Walz, 1982a and 1982b) which could be adversely affected by this mutation. It is possible that the *tko* defect exerts effects similar to those of poisoned mitochondria. Poisoning mitochondria with inhibitors of oxidative phosphorylation at motor nerve terminals causes an increase in the frequency of miniature end-plate potentials as well as an increase in the amount of transmitter released with each nerve impulse (Alnaes and Rahamimoff, 1975). This is presumably due to an increased level of intracellular free calcium. These conditions can give rise to the seemingly opposite effects of paralysis and hyper-excitability. For example, prolonged stimulation, as in "banging" could cause a large increase in intracellular calcium in the terminal, which could lead to depletion of transmitter at susceptible synapses in the brain (Zucker, 1985) which could result in paralysis. The hyperactivity that appears as the flies recover from "banging" could then occur if the

transmitter pool was restored before the levels of calcium in the nerve terminals returned to a more normal level. Alternatively, the paralysis could result from hyper-activity of inhibitory interneurons that synapse on excitatory interneurons or motor neurons. In this case, if the activity of the inhibitory interneurons decayed more rapidly than that of the excitatory interneurons or the motorneurons then this could lead to the hyper-activity seen as the flies recover. Further physiological and biochemical studies are necessary to determine whether abnormalities associated with mitochondrial function are present in *tko* mutants. For example, one can test both metabolic activity and calcium uptake in mitochondria from tko^{25t} to see whether these are indeed defective.

6.3.2 Relation to Other Bang Sensitive Mutations

Although the hypothesis that the *tko* product is a mitochondrial ribosomal protein is compelling, there are many ways which this kind of defect could lead to bang-sensitivity, as discussed above. Therefore, it is unclear whether all or any of the other bang-sensitive mutations cause defects in mitochondrial proteins. On one hand, there are more than eighty mitochondrial ribosomal proteins. If mutations in some of them lead to defects in mitochondrial ribosomes which then cause bang-sensitivity, then this would explain the prevalence of bang-sensitive genes in the fly. On the other hand, if the proximal cause of bang-sensitivity is not a metabolic defect but rather some neural defect such as general hyper-excitability, as suggested by many of the results described in chapter 1, then it is conceivable that none of the other bang-sensitive mutants affect the mitochondria, but instead affect the excitability of the neurons by some other mechanism.

Of the other bang-sensitive mutations, *bas* is most similar to *tko* and therefore is most likely to have a mitochondrial defect. Several similarities have been discussed. Both *tko* and *bas* are sensitive to high temperatures, whereas *bss* and *eas* are sensitive to low temperatures. Double mutants with *tko* and *bas* are apparently inviable whereas double mutants between *tko* and *eas*, *bss* or *kdn* are viable. Finally, *tko* and *bas* are the only bang-sensitive mutants that show a decrease in cytochrome oxidase activity compared to wild type flies. The correspondence in phenotype between *tko* and *bas* suggests that they may have defects in the same function, possibly a mitochondrial ribosomal function. Although *eas*, *bss* and *kdn* appear to differ from *tko* in several respects, these mutations may cause defects in mitochondrial functions other than cytochrome oxidase activity or protein synthesis. However it is equally likely that these mutants have defects in completely different cellular functions. The definite answer will await the cloning of the other bang-sensitive genes. Either result will be informative. If the other bang-sensitive mutants turn out to have mitochondrial defects, it will stress the importance of mitochondrial function for proper operation of the nervous system. If they turn out to have some other defects, those defects may tell us something about the regulation of excitability in neurons.

6.3.3 Mitochondrial Translation

The cloning of a mitochondrial ribosomal protein is important for the field of mitochondrial biogenesis because it will help us to understand the mechanisms of mitochondrial translation. It is difficult to clone mitochondrial ribosomal proteins, since almost all of them are coded for in the nucleus (Schieber and O'Brien, 1985).

Because null mutations are likely to be lethal, and it is difficult to know what lethal phenotype to look for in a mitochondrial ribosomal mutant it has been difficult to isolate the genes for these proteins. A great deal of progress has been made recently in yeast, since yeast can survive without the use of their mitochondria. By analyzing the *pet* genes in yeast, nuclear genes that are required for the morphogenesis of respiratory competent mitochondria, Tzagaloff and Myers (1986) have identified several genes that probably code for mitochondrial ribosomal proteins. Recently they cloned one yeast gene for a mitochondrial ribosomal protein that is homologous to *E. coli* S14. The analysis of these proteins in yeast will yield much information about mitochondrial protein synthesis. However yeast mitochondrial ribosomes differ both in size and protein content from animal mitochondrial ribosomes. In order to verify that the animal systems are similar to the yeast system, one must also clone mitochondrial ribosomal proteins in higher eukaryotes. The cloning of *tko* is very important since it is the first mitochondrial ribosomal protein to be cloned in higher eukaryotes. It emphasizes the similarity between mitochondrial ribosomes and prokaryotic ribosomes, and enhances the data from yeast which also suggests that mitochondrial ribosomes are closely related to prokaryotic ones.

The *tko* result and the yeast result suggest that more mitochondrial ribosomal proteins will be found that have homology to bacterial ribosomal proteins. This suggests that one could clone some of them based on homologies to bacterial proteins. This experiment must be performed with caution because, although the *tko* coding DNA contains 46% identical bases to the *E. coli* S12 coding region and 43% identical bases to the *E. gracilis* chloroplast S12 coding sequence, these matching

bases are distributed throughout the coding regions and not clustered together. The *tko* gene differs from its bacterial and chloroplast counterparts in many of the third positions of codons, which can often change without affecting the protein sequence. For example, out of 48 identical amino acids between *tko* and *E. coli* S12, 28 (58%) differ in the third position of their codons in the DNA sequence. The difference is even more dramatic between *tko* and the *E. gracilis* chloroplast S12 where 44 (85%) of the 52 identical amino acids differ in the third positions of their codons. These differences appear to be due to a bias in the *tko* gene to use G or C in the third position and a bias in the *E. gracilis* gene toward A and T in the third position. 83% of the codons in the *tko* coding region have G or C in the third position while 79% of the codons in E. gracilis chloroplast S12 coding region have A or T in this position. In the *E. coli* gene G and C are used with about the same frequency as A and T (49% versus 51% respectively) in these positions. Because of the high frequency of changes in the third position of codons coding for identical amino acids there are few long stretches of identical DNA sequence between *E. coli* and *tko*. The longest stretch of identical DNA sequence consists of 11 bases. Therefore, one would have to be careful designing probes and determining stringencies for hybridization to perform this type of experiment.

6.3.4 Evolution

I have used the endosymbiotic hypothesis as support for the idea that the *tko* gene product codes for a mitochondrial ribosomal protein. There is a great deal of support for this hypothesis from the analysis of ribosomal RNA. If further experiments show that the *tko* gene product is indeed a mitochondrial ribosomal protein,

then its homology with a bacterial ribosomal protein will add much weight to the endosymbiant hypothesis. The result with the yeast homologue to bacterial S14 already points in that direction, and I suspect that as more mitochondrial ribosomal proteins are cloned and sequenced we will continue to see many that resemble the prokaryotic ribosomal proteins. This data should aid in supporting this important theory of evolution.

6.4 Summary

I have cloned and analyzed the gene coding for the *tko* gene product. Its homology to *E. coli* ribosomal protein S12 suggests that it is a mitochondrial ribosomal protein. This hypothesis is supported by functional similarities between prokaryotic and mitochondrial ribosomes as well as by the endosymbiotic theory of evolution. If this hypothesis is true it can explain many of the phenotypes seen in the *tko* mutants, including their weakness, and it suggests mechanisms for the bang-sensitivity and hyper-excitability. Of the other bang-sensitive mutants, *bas* is the most similar to *tko*, suggesting that it may also cause a mitochondrial defect. Further experiments are necessary to determine whether the other bang-sensitive mutations also affect mitochondria or if they cause bang-sensitivity through some other defect. Finally, if the *tko* hypothesis holds up, it is another example of the similarity between prokaryotic and mitochondrial ribosomes. It will add to our understanding of mitochondrial protein synthesis and it will add support to the endosymbiotic theory of the origin of mitochondria.

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