MOLECULAR CHARACTERIZATION OF AN UNKNOWN P-ELEMENT INSERTION IN *DROSOPHILA MELANOGASTER*

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ABSTRACT

In the Molecular Characterization of an Unknown P-element Insertion in *Drosophila melanogaster*, a plasmid rescue was performed in order to identify the location of the target gene in the fly genome. Based on phenotypic evidence, this gene causes sterility in homozygous female flies. The purpose of this team project was to clone the P-element and the gene in which it was contained. After DNA isolation and cleavage, the DNA fragments were ligated to form circular plasmids and transformed into *E. coli* bacteria to be cloned. After incubation for 24 hours on ampicilin-containing agar plates, no bacterial colony growth was observed.

INTRODUCTION

Drosophila melanogaster

Model organisms are widely available species used for various research applications. Experiments with model organisms are particularly helpful because their results can be applied to humans. Because all modern organisms evolved from a common ancestor, there is a great deal of similarities throughout genomes that have been conserved over time. Typical model organisms include *E. coli*, nematodes, Wisconsin Fast Plants, and *Drosophila melanogaster* [1].

Drosophila melanogaster was the organism of choice for this particular experiment. The name of the species is derived from the Greek phrase meaning "black-bellied dew-lover" [2]. These flies are often referred to as simply *Drosophila*, despite the fact that the genus actually contains over 1500 species [3]. These flies are approximately three millimeters long and are found around rotten fruit. *Drosophila* mature relatively quickly, reaching adulthood in about a week (Fig. 1) [4]. Female flies reach sexual maturity within 12 hours. The flies are surprisingly easy to grow in a laboratory setting, and their periods of development are variable with temperature. There are also multiple similarities between the fruit fly genome and the human genome, making research on *Drosophila* especially significant [5].

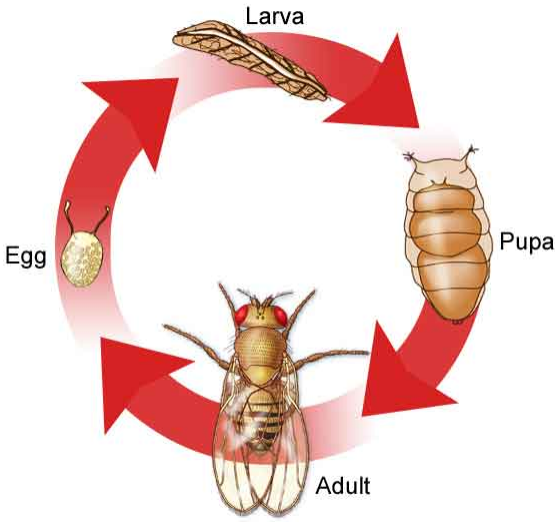

Fig. 1 Life cycle of *Drosophila.*

Fruit flies have been extensively studied, and the sequencing and mapping of the *Drosophila* genome, containing approximately 13,600 genes, was completed in 2000. However, the scientific community has described the functions of only a small percentage of these genes [1]. Accordingly, many *Drosophila* researchers are eager to continue exploring the seemingly infinite characteristics of this organism. The importance of *Drosophila* research to humanity was acknowledged in 1995 when the Nobel Prize in Medicine and Physiology was awarded for work with fruit flies [5].

Mutagenesis

Mutagenesis, the formation of mutations in an organism's genome, is a process used by scientists to determine the function of a particular gene. In general, there are two forms of mutagenesis: spontaneous and induced. Spontaneous mutagenesis is a random process that involves changes in certain base sequences [6]. Induced mutagenesis is a process that can have several causes including chemicals, radiation, and transposons [6]. Transposon mutagenesis involves the insertion of a transposon into an organism's genome. A transposon is a sequence of deoxyribonucleic acid that can move around to different parts of the cell genome. The insertion of a transposon disrupts and often inactivates the gene into which the transposon is inserted. This can change the expression of the gene in the cell [6].

For this study, flies with a transposon inserted into their genomes were used. This caused sterility in the female flies homozygous for this transposon. We hypothesized that the transposon was inserted into and had inactivated a gene involved in oogenesis or embryogenesis [1].

The transposon used in this experiment is a modified transposon. Natural transposons contain two parts: a gene encoding for transposase, an enzyme that catalyzes the mobilization of the transposons, and the recognition sequences for transposase action. The unmodified P-element contains the gene for transposase, the wild type *white* gene, the pBR322 *E. coli* plasmid, and six different restriction enzyme sites. The modified P-element lacks the transposase gene and is unable to move on its own, preventing any unpredictable movement [1].

Transposons and *P{lacW}*

DNA transposons are cut and inserted by the transposase enzyme to other regions of the genome. The transposase enzyme cleaves the sequence and produces two sticky ends which are then attached to a new portion of the genome by DNA polymerase and DNA ligase [1]. The transposon is cut by transposase at the site of an inverted terminal sequence. This produces the sticky ends, which are unevenly cut strands of DNA that help it to bind to other areas.

The transposon *P{lacW}* was injected into *Drosophila melanogaster* zygotes (Fig. 2) [7]. The *white* gene causes the flies to develop red eyes, thus functioning as a marker to identify the flies that possess the P-element. The ampicillin resistant plasmid pBR322 is used to clone sequences of genomic DNA adjacent to the *P{lacW}* [1].

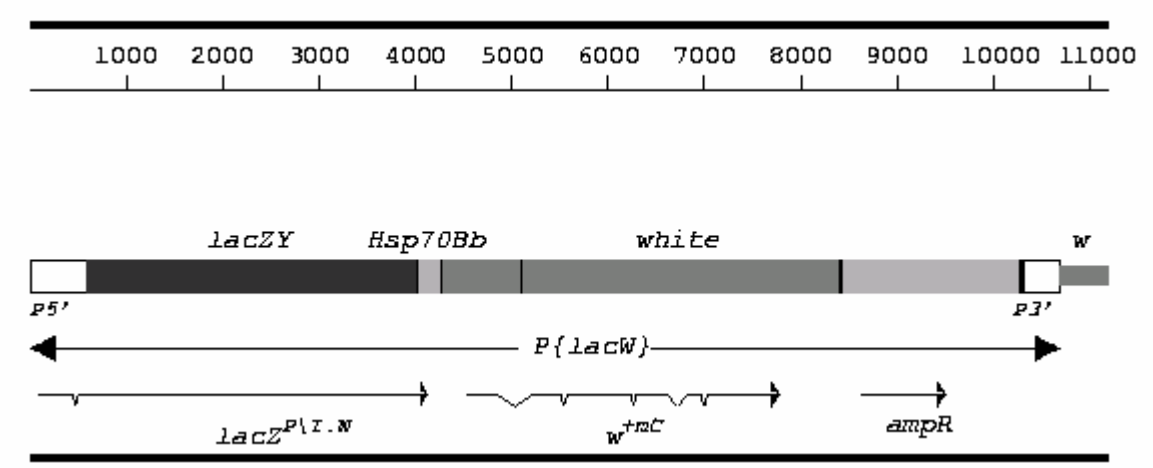

Fig. 2 The transposon *P{lacW}*with amp^R and *white* gene.

The reasons for using *P{lacW}* in *Drosophila melanogaster* include its enzyme recognition sites for six different restriction enzymes, origin of replication (the place at which DNA replication is initiated), and ampicilin resistance [1]. The six restriction enzymes enable isolation of the segment of pBR322 [7]. Two of the enzyme recognition sites, EcoRI and SacII, cut upstream of pBR322 (3' end), while the remaining four, Xba1, BGlII, PstI, and BamHI, cut downstream of pBR322, removing pBR322 and the portion

of the genome until the next recognition site [8]. These restriction enzymes cut out the P-element and some of the genome adjacent to it [1].

Plasmid Rescue

The purpose of recovering a plasmid, in the case of our project, is to obtain genomic DNA sequences adjacent to the *P{lacW}* transposon. Eventually, these cloned sequences will be used to determine what gene is disrupted by *P{lacW}* and hopefully responsible for female sterility in fruit flies [1]. The basic steps for plasmid rescue are: 1) isolation of genomic DNA, 2) cleavage of DNA into linear fragments, 3) ligation of these fragments into circular plasmids, and 4) transformation of the plasmids into bacteria (Fig. 3). In the last step, transformation, these new plasmids are cloned in bacteria [1].

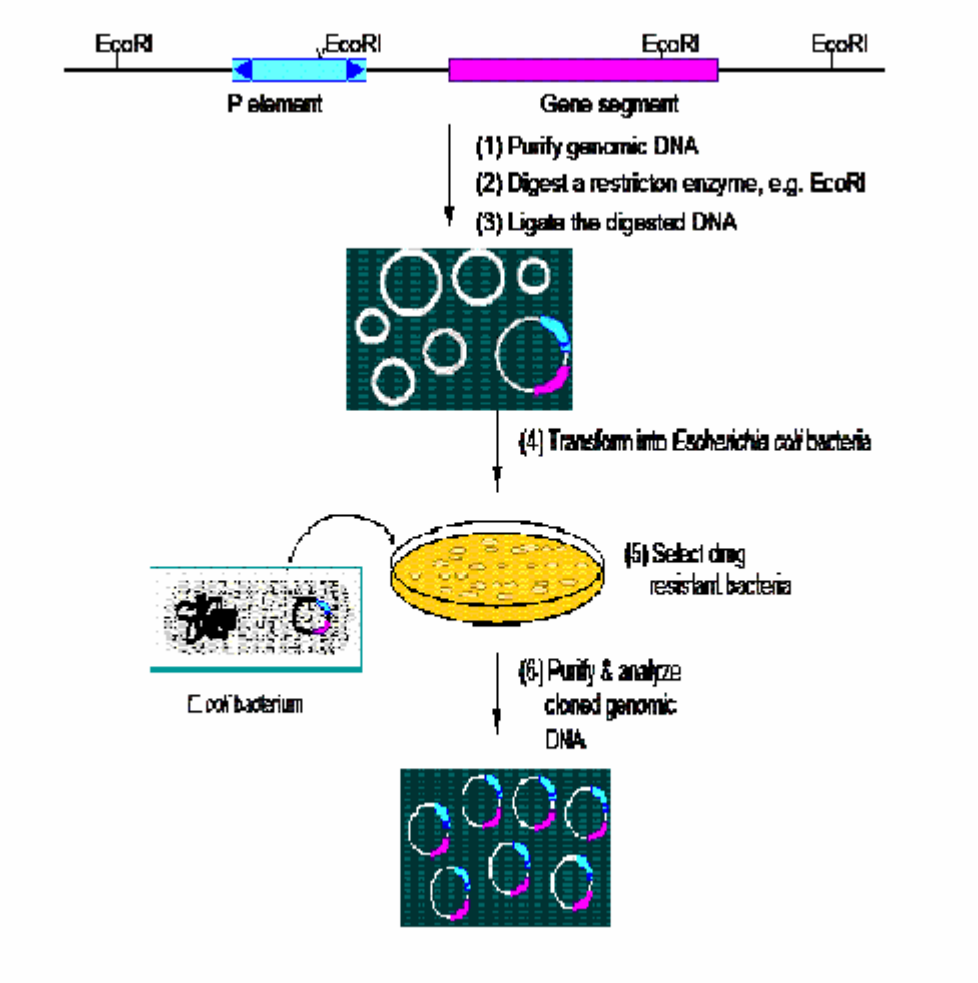

Fig. 3 Steps of plasmid rescue and transformation.

Transformation

Transformation is the process by which cells take up DNA from the environment. Naked DNA is DNA without associated proteins or histones and is outside of a cell body [9]. If a bacteria cell naturally takes up naked DNA from the environment, it is considered to be competent. Some cells, however, are not capable of ingesting foreign DNA, and they must first be treated to induce competence. Once the plasmids are taken up by the bacteria cells, the bacteria will replicate them along with the original genome, giving scientists a greater amount of plasmids with which to work. In order to have a successful cloning, there are three important characteristics the plasmids must show: enzyme recognition sites, origins of replication, and ampicillin resistance. The enzyme recognition sites allow for the restriction enzymes to cut the original genomic DNA into manageable linear fragments. The origin of replication will allow for the plasmid to replicate inside the bacteria. As the bacteria cells grow and reproduce, they will also replicate the plasmid, but only if the origin is present since it contains the sequence necessary to initiate DNA replication. The bacteria cells are placed into agar cultures with ampicillin and can only survive to produce colonies if the plasmids with the ampicillin resistance were successfully transformed. Therefore, the cells with the gene of study will be able to produce a bacterial colony which can be extracted and run through electrophoresis. The results of an electrophoresis would show whether the cloning of the target plasmid was successful [1].

MATERIALS AND METHODS

The plasmid rescue involves four basic steps: 1) isolation of genomic DNA, 2) cleavage of the DNA using the restriction enzymes, 3) ligation reactions to form circular plasmids from fragmented DNA, and 4) transformation of plasmids into E. coli bacteria.

Isolation of DNA

To isolate genomic DNA, approximately 25 homozygous *p{lacW}* flies per sample were ground in 100 µl of grinding buffer (5% sucrose; 80 mM NaCl; 100 mM Tris, pH 8.5; 0.5% SDS; 50 mM EDTA). The sample was incubated at 70ºC for 30 minutes. Thirty five µl of 8M KOAc (potassium acetate) were added, and the sample was incubated on ice for 30 minutes. Large debris was removed by centrifuge at 14,000 rpm at 4ºC for ten minutes. 150 µl of isopropanol were added to the supernatant, incubated for five minutes at room temperature, centrifuged for ten minutes. This was followed by the removal of the supernatant. 500 µl of 70% ethanol were added to wash away excess salt. The supernatant was recovered and treated with ribonuclease H (RNase H) in Tris-EDTA (TE) buffer for one hour at 37ºC. The sample was then treated with 2.5 µl (0.5 mg/ml) of proteinase K to degrade proteins. The sample was incubated at 65ºC for one hour. The volume was brought up to 500 µl with TE buffer. An equal volume (500 µl) of a 1:1 mix of phenol:chloroform was added. The sample was extracted twice with phenol:chloroform followed by two extractions with chloroform. The sample was centrifuged for 30 seconds at room temperature. Then, the aqueous (upper) layer was transferred to a sterile tube. The remaining volume was doubled by adding an equal

volume of chloroform. The sample was then spun at 14k for 30 seconds at room temperature. The upper layer was once again transferred to a clean tube. This step was repeated twice [1].

The volume of the DNA sample was estimated. One tenth of the volume was added as 3M sodium acetate (pH 5.2). Two volumes of ice cold 100% ethanol were added. DNA was precipitated by incubating the sample on ice for ten minutes. The sample was then spun for 10 minutes at 4ºC. The liquid was discarded, and 1 volume of 70% ethanol was added. The sample was spun for five minutes, and the supernatant was removed once again. The pellet was air dried for 15 minutes, and the DNA pellet was resuspended in 25 µl TE buffer [1].

The purity as well as the quantity of DNA in the samples was determined by measuring the UV absorbance of the DNA sample at both 260 nm and 280 nm using a UV spectrophotometer. Both nucleic acids and proteins absorb different wavelengths of UV light. The ratio of different absorptions indicates the purity of the DNA. The DNA sample was diluted with sterile TE buffer so that the final volume of the dilution was 500 µl. One ml of TE buffer was added to one cuvette. The cuvette was placed so that the optical surfaces were perpendicular to the path of light. The DNA sample was transferred to a second cuvette, and the absorbances were measured [1].

Three µg of DNA were digested in 40 µl total volume with 20 units of enzyme for one to two hours at 37ºC. The reagents were added in the following order: 10x buffer (4 µl), Bovine Serum Albunim (BSA, 1 µl), sterile distilled H_2O (28 µl), DNA (5 µl), and enzyme (2 µl). Each of the six groups used a different enzyme and its corresponding buffer. The six restriction enzymes included BamHI (with New England Biolabs 2, NEB2), Bg1II (NEB3), EcoRI (NEB4), PstI (NEB3), SacII (NEB4), and XbaI (NEB2) [1].

Ligation Reactions

Sixty µl of 10x ligase buffer, 500 µl of sterile distilled water, and 1 µl of T4 DNA ligase (New England Biolabs) were added to 40 µl of the digestion sample to join together ends of DNA. To promote intramolecular circularization/ligation of linear DNA fragments, we performed the ligation reactions under dilute conditions (in a total volume of 601 µl). Four control ligations were also set up: no DNA added, linear plasmid without the ligase, linear plasmid with ligase, and uncut plasmid DNA. The sample was concentrated by alcohol precipitation and resuspended in 20 µl TE buffer [1].

Transformation

Ethanol precipitation was performed in order to concentrate the sample. Half of each ligation reaction was transferred into two 1.5 µl tubes. To each tube, 1/10 volume 3M sodium acetate (pH 5.2) was added. Additionally, 2 volumes of ice cold 100% ethanol were added to each tube. Both tubes were then placed on ice for 20 minutes to precipitate the DNA. The sample was spun for 10 minutes at 4ºC. The liquid was

discarded, and an additional 500 µl of 70% ethanol were added. The sample was once again spun for five minutes at room temperature, and the supernatant was removed. The pellets were then combined and resuspended in 20 µl of TE buffer. Transformation competent *E. coli* bacterial cells were purchased from Invitrogen. Ten µl of DNA plasmids were added to 100 µl of bacteria and incubated on ice for 30 minutes. The samples were then heat shocked at 42°C for 45 seconds to induce the uptake of the DNA into the cells. The sample was diluted immediately in 500 µl of LB medium and incubated for 30 minutes at 37ºC. Cells were plated on a Luria-Bertani medium containing 100 µg/ml ampicillin [1].

RESULTS

Plasmid rescue was unsuccessful. The plasmid rescue transformation showed no growth of colonies on any plate except for the 400 µl agar plate of XbaI. The remaining eleven experimental plates exhibited no growth. None of the 4 µl plates displayed any bacteria colony growth. The 400 µl XbaI showed satellite growth (Fig. 3). The experimental plate results are summarized in Table 1.

Fig. 3 Satellite growth on the 400 µl XbaI plate.

Table 1: Summary of Bacteria Growth in Experimental Plates

Enzyme	**Site of Restriction Enzyme Cleavage**	**Observed (4 µl)**	**Observed (400 µl)**	**Expected**
BamHI	Left Side (5’ end)	No growth	No growth	Colony growth
BglII	Left Side (5’ end)	No growth	No growth	Colony growth
EcoRI	Right Side (3’end)	No growth	No growth	Colony growth
PstI	Left Side (5’ end)	No growth	No growth	Colony growth
SacII	Right Side (3’end)	No growth	No growth	Colony growth
XbaI	Left Side (5’ end)	No growth	Satellite growth	Colony growth

The four control groups were 1) no DNA added, 2) linear plasmid without ligase, 3) linear plasmid with ligase, and 4) uncut plasmid DNA. The control plate results are summarized in Table 2.

Table 2: Summary of Bacteria Growth in Controls

Control Type	**Site of Restriction Enzyme Cleavage**	**Observed (4 µl)**	**Observed (400 µl)**	**Expected**
No DNA added	None	No growth	No growth	No growth
Linear plasmid without ligase	EcoRI – Right Side (3'end)	No growth	No growth	No growth
Linear plasmid with ligase	EcoRI – Right Side (3'end)	No growth	No growth	Colony growth
Uncut plasmid DNA	None	No growth	No growth	Colony growth

Therefore, there was no significant growth of bacteria colonies. The plasmid transformation was unsuccessful in creating ampicillin resistant *Escherichia coli*.

DISCUSSION

UV spectrophotometric analysis indicated that DNA was present in the samples. For our sample of DNA, the ratio of A_{260}:A_{280} was 2.063. Based on the spectrophotometry results, we can infer that although our sample contained traces of RNA, it was composed mostly of DNA. The correct ratio of a pure sample of DNA is usually between 1.8 and 1.9. Deviations in this ratio can result from protein or RNA contamination. Higher ratios indicate that the sample has been contaminated with RNA while lower ratios indicate that the sample contains protein. The spectrophotometry reading indicates that DNA was most likely present for the transformation of the bacteria.

Even though the DNA was present, the bacterial colonies failed to grow on any of the plates including the controls. Four controls were used in this experiment to determine if various steps of the procedure worked. The first control contained the plasmid with ampicilin resistance gene but no ligase. The plasmid was cut with EcoRI, a ligation reaction was performed, and the DNA was precipitated using ethanol. The bacteria transformed with this sample did not grow into colonies. This was the expected result because DNA remained in linear segments due to the absence of ligase, and bacteria are unable to take in linear DNA. In the second control, there was no DNA, and, after following the same procedure as the first control, colonies did not grow. This was the expected result because the ampicillin resistance gene was not transformed into the *E. coli* bacteria. As a result the bacteria were unable to grow in a medium containing ampicillin, a bacteriostatic antibiotic. In the third control, the bacteria were transformed with pBR322 but did not undergo the digestion and ligation reactions. This was used to test if the pBR322 plasmid was effective in providing the bacteria with ampicillin resistance. In theory, the pBR322 should have been transformed into the bacteria and, as

a result, allowed the bacteria to grow on the medium with ampicillin. However, in our lab, the colonies did not form. The fourth control included pBR322, EcoRI, and ligase. This sample, when transformed, should have allowed the bacteria to grow colonies on the ampicillin medium. As in the third control, the colonies of *E. coli* did not form.

There are several errors that might have prevented the formation of the colonies in the third and fourth controls. The bacteria might have been destroyed while we were plating. However, it is unlikely that all six lab groups killed their bacteria while plating. Also, the plasmid might have been degraded. This too is improbable because the plasmid taken from the same source was used successfully previously, and the plasmids were kept at appropriate temperatures during the procedure. Errors in the production of the agar plates as well as errors in performing the ethanol precipitation are also possible but unlikely. Ethanol precipitations were successfully performed in the beginning steps of the lab. Most likely, the cells were not competent, and they were unable to take in the plasmid. This is the most probable source of error because no plate, not even the controls, exhibited bacterial growth.

CONCLUSION

Although our experiment was unsuccessful, we still gained valuable experience from using experimental biological methods and lab techniques. We learned how to isolate DNA from the *Drosophila melanogaster*, and how to purify the DNA using phenol/chloroform extraction. Through UV spectrophotometry we were able to quantify and assess the purity of the DNA sample. We discovered how to use restriction enzymes to cleave the desired sections of DNA from the fly genome and how to use DNA ligase to form circular plasmids from the linear DNA fragments. We learned how to make competent *E. coli* cells that were able to be transformed by taking in plasmids. Since the plasmid that we studied had a gene that coded for ampicillin resistance, we found that by plating the transformed bacteria in an ampicillin medium we should have been able to grow bacteria with the desired plasmid and clone the DNA segment that we wished to study. Although no bacterial colonies grew on our plates, we analyzed our results by comparing them to the controls.

If the experiment had been successful and if we had more time for future studies, we would have extracted the plasmids from the *E. coli* and sequenced the plasmid DNA. Using this information, we would have been able to determine the location of the transposon in the fly genome. With this information we would have been able to identify specifically the gene that, when mutated, causes female sterility. Since there are many similarities between the *Drosophila* and human genomes, this knowledge could have been extended to increase the understanding of human genes and their functions.

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