# How to create transgenic animals

## Introduction

Reverse genetics relies on engineering the genome through adding, subtracting, and replacing genes. There are three major approaches to reach these goals: the generation of transgenic animals (adding genes), generation of knockouts (deleting genes), and genome editing (replacing or deleting genes).

In this lesson, we will focus on how modified versions of a gene or a foreign gene – what we call a transgene - can be introduced into animal cells, a process called transgenesis.

## Genome engineering - an overview

There are several ways how a gene of interest can be altered. First, the gene of interest can simply be deleted from the genome (although for diploid organisms this means that both copies of the gene have to be deleted). Such gene knockouts are especially useful if the gene is not essential. The other way is to replace the gene of interest by one that is expressed in the wrong tissue or at the wrong time during development.

Further, genes can also be engineered so that they are expressed normally in most cells and tissues, but deleted in certain tissues. This is especially useful when a gene has different roles in different tissues. We will discuss all these ways in the following lessons (lessons 3 and 4).

It is also possible to create new types of proteins in an animal, which allows following where, when, and how a gene of interest is expressed in an organism. For this purpose, reporter constructs are created and introduced into the genome. A reporter gene (often just called reporter) consists of a gene that is attached to a regulatory sequence of another gene of interest. Certain genes are chosen as reporters, because the characteristics they confer on organisms expressing them are easily identified and measured, or because they are selectable markers. To introduce a reporter gene into an organism, scientists place the reporter gene and the gene of interest in the same DNA construct and insert it an organism. It is important to use a reporter gene that is not natively expressed in the cell or organism under study, since the expression of the reporter is being used as a marker for successful uptake of the gene of interest.

Commonly used reporter genes that induce visually identifiable characteristics usually involve fluorescent or luminescent proteins. Examples include the gene that encodes jellyfish green fluorescent protein (GFP), which causes cells that express it to glow green under blue light. In such a reporter construct, the gene of interest is fused to the coding region of GFP (see figure 2-1). When this reporter is introduced into the genome, the GFP-tagged protein of interest can be tracked inside cells by monitoring its fluorescence. We call this a translational reporter, because here, the translated fusion protein is the readout.

On the contrary, transcriptional reporters consist of a promoter fragment from a gene of interest that drives the expression of another gene, for example, the gene *lacZ*, which encodes the bacterial protein beta-galactosidase. When this enzyme is expressed, it produces blue color by converting the substrate analog X-gal (see figure 2-2). Thus, the blue color indicates where the gene of interest is expressed within a tissue or animal. Alternatively, transcriptional reporter constructs can also be created that drive the expression of GFP. Typically, promoter fragments of a few kilobases immediately upstream of the start codon contain a significant portion of the cis-regulatory information necessary to provide a tentative expression pattern of the endogenous gene under study.

A crucial aspect of gene expression studies is choosing an adequate type of reporter for the purposes of the experiment. Each reporter differs in the amount of information it provides about the expression of a gene. Transcriptional reporters can be used to visualize the expression pattern for a gene of

interest. Fusing 5′-upstream sequences to GFP can be done in a number of ways and usually presents no technical challenge. Compared to translational reporters, however, promoter fusions may not give a complete representation of the real expression pattern of a gene, both spatially and temporally. Since not the original gene but a different one (e.g., *lacZ* or GFP) is expressed, modifications of the expression pattern of the gene by external influences or by developmental clues cannot be studied if these modifications affect the protein level (for example, protein degradation or protein modifications, such as phosphorylations or acetylations).

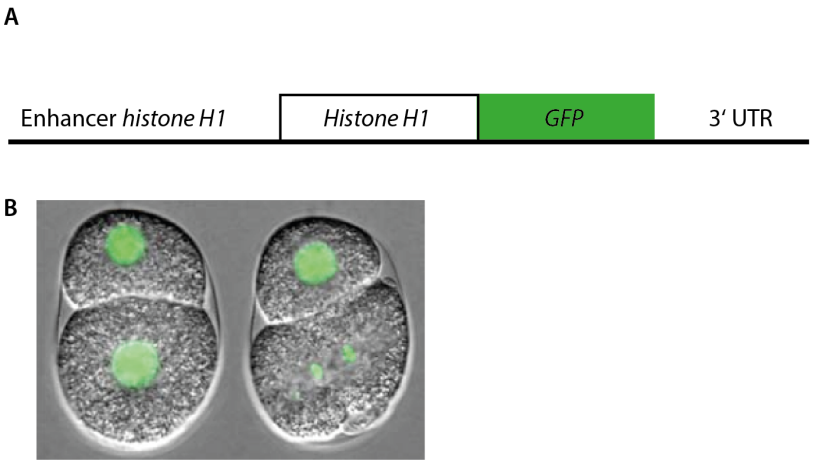


**Figure 2-1 Example for a GFP translational reporter. (A)** Translational reporters are in-frame gene fusions between the coding sequence of GFP and a gene of interest (here, Histone H1). GFP can be inserted at any point in the open reading frame, preferably at a site that does not disrupt protein function or topology. **(B)** Expression of the GFP reporter in dividing mouse oocytes, where expression of the fusion protein is seen in the nucleus.

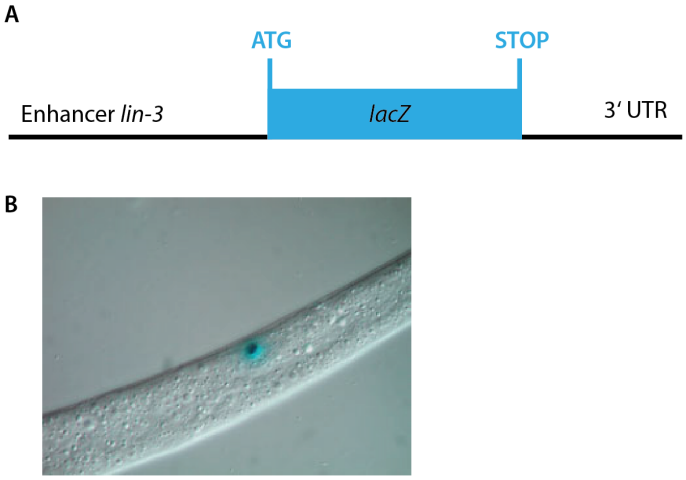


**Figure 2-2 Example for a transcriptional reporter using *lacZ*. (A)** Promoter fragments of a few kilobases immediately upstream of the start codon contain a significant portion of the *cis*-regulatory information (enhancer) necessary to provide expression of the gene of interest. Here, the enhancer of the *C. elegans* homolog of the epidermal growth factor (lin-3) is used. The coding sequence of *lin-3* is replaced by the coding sequence of *lacZ*. **(B)** The expression of the reporter gene (detected using X-Gal) is seen in cells where the *lin-3* enhancer is active (the anchor cell).

Translational reporters can provide information about a gene's expression pattern, because additional regulatory information that may be present in introns or 3′-UTRs is included in such reporter constructs. In addition, translational gene fusions can also provide information about subcellular localization and the temporal aspects of gene regulation. However, in some cases, the fusion of a gene to GFP can sometimes disrupt protein function or even lead to toxicity of the chimeric product. Furthermore, the fusion protein may fold differently than the "normal" one, which may affect its binding to interaction partners, mask potential phosphorylation sites or affect protein stability, which will lead to a false interpretation of the expression pattern.

## Transgenesis: How transgenic animals are generated

A transgene is a foreign or modified gene that has been added to the genome to create a transgenic organism. Transgenes can be introduced into cells in a variety of ways (see figure 2-3). Electroporation is one method used to introduce DNA into bacteria or cells in culture. Here, a brief electric shock renders the cell membrane temporarily permeable, allowing foreign DNA to enter the cytoplasm. In plant cells, genes are frequently introduced by a technique called particle bombardment: DNA particles are shot through the cell wall into the cell with a special modified gun. Microinjection is used to introduce DNA into mammalian cells, fly embryos or worms. Transgenes can also be brought into cells by viral transfer, a technique frequently used in plant and mammalian cells, which we will not discuss here.

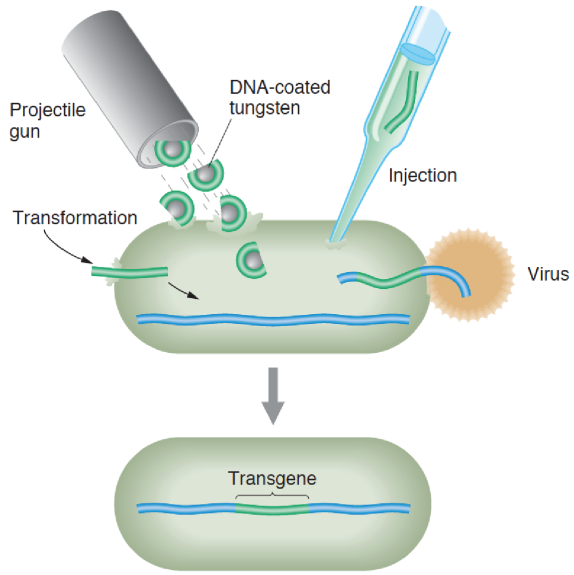


**Figure 2-3** Some of the ways to introduce foreign DNA into a cell.

To be most useful for the following experiments, the altered gene, once it is introduced into a cell, must recombine with the cell's genome to be stably maintained after cell division. Furthermore, the altered gene must be integrated into the germ line so that it can be inherited to the next generation.

Here, we will briefly discuss the various methods used to produce transgenic organisms and will discuss in more detail transgenesis in *Drosophila* and the mouse.

# Delivering DNA into host cells

## Electroporation efficiently transforms single cells

In electroporation, cells are mixed with DNA and an electric field is applied to cells in order to increase the permeability of the cellular membrane, allowing the DNA to be introduced into the cell. Electroporation is often used to transform bacteria or yeast, but it is also highly efficient for the introduction of transgenes into tissue culture cells, especially mammalian cells.

Electroporation allows cellular introduction of large highly charged molecules such as DNA which would never passively diffuse across the hydrophobic bilayer core. During electroporation, the lipid molecules are not chemically altered but simply shift position, opening up a pore which acts as the conductive pathway through the bilayer as it is filled with water (see figure 2-4).

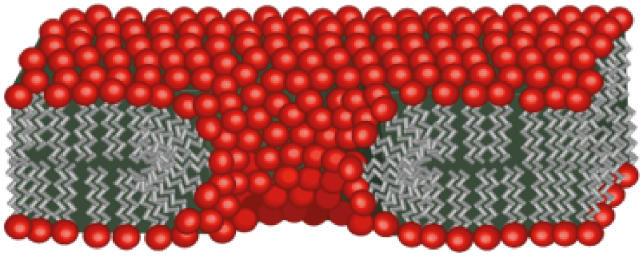

**Figure 2-4** Schematic showing the theoretical arrangement of lipids in a hydrophilic pore induced by a short strong electrical pulse.

When the short electrical pulse is applied, the membrane charges like a capacitor through the migration of ions from the surrounding solution, which leads to a localized rearrangement in lipid morphology. The resulting structure is believed to be a "pre-pore" since it is not electrically conductive but leads rapidly to the creation of a conductive pore. DNA can enter the cell though these pores. If the pore heals and the bilayer reseals, the cell recovers and survives and now contains the transgenic DNA (see figure 2-5).

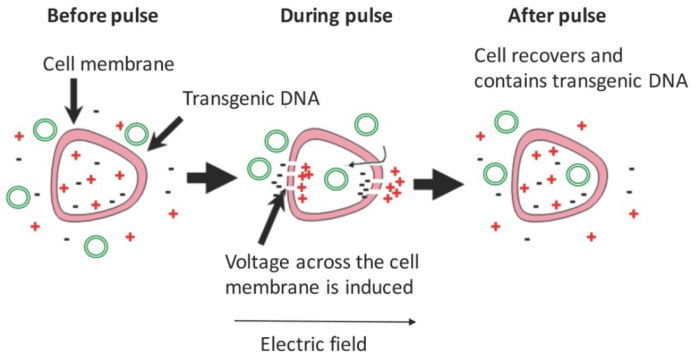


**Figure 2-5** Principle of electroporation to deliver DNA into cells.

## Gene gun: the method of choice to generate transgenic plants

The gene gun (also called gunbiolistic particle-delivery system) is a mechanical method to deliver DNA into the cells using a gene gun. It was originally designed for the transformation of plants, but

is able to transform almost any type of cell, and is not limited to transformation of the nucleus; it can also transform organelles, including plastids. Small heavy metal particles (gold or tungsten) are coated with DNA and shot into the target cells under high pressure (see figure 2-6).

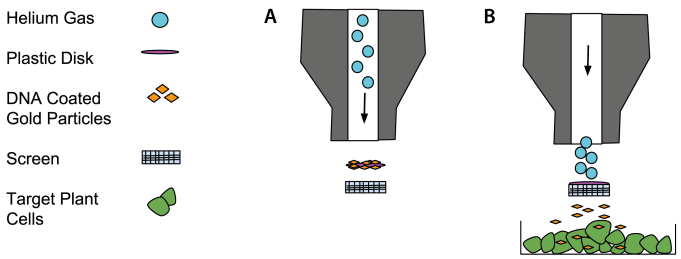


**Figure 2-6 A gene gun is used for delivery of transgenic DNA to cells.** This method is known as 'biolistics'. Gene guns can be used effectively on most cells but are mainly used on plant cells. **(A)** The chamber is filled with Helium to build pressure on the rupture disk. **(B)** When the rupture disk breaks, the resulting burst of helium propels the DNA/gold-coated macrocarrier ('Plastic Disk') into the stopping screen. When the macrocarrier hits the stopping screen, the DNA-coated gold particles are propelled through the screen and into the target cells. (adapted from Wikipedia, Gene gun)

The gene gun has become a common tool for labeling subsets of cells in cultured tissue. In addition to being able to transfect cells with DNA plasmids coding for fluorescent proteins, the gene gun can be adapted to deliver a wide variety of vital dyes to cells. Gene gun bombardment has also been used to transform *C. elegans* as an alternative to microinjection.

## DNA microinjection: the method of choice for many animal models

### Microinjection in *C. elegans*

In *C. elegans*, the transgenic DNA is simply injected directly into the worm. The injection strategy is determined by the worm's reproductive biology: the gonads of the worm are syncytial, meaning that there are many nuclei sharing the same cytoplasm. In *C. elegans*, the gonads form two syncytial regions, one on one arm of the gonad and one on the other (see figure 2-7A). These nuclei do not form individual cells until meiosis when they differentiate into individual eggs or sperm. The DNA solution is injected into the syncytial region of one of the arms (no matter which one), thereby exposing more than 100 nuclei to the DNA. By chance, a few of these nuclei take up the DNA (because during cell division, the nuclear membrane breaks down). The transgenic DNA can now be present in the nucleus in two different ways: first, the DNA can form an array with multiple copies of the DNA that exists as independent units that are not part of the chromosomes (called extrachromosomal array, see figure 2-7B). Alternatively, the transgenic DNA can be integrated into a chromosome. Since this integration is random, the incorporation of transgenic DNA as a multi-copy array may disrupt genes present on the chromosome, which may lead to lethality if an egg or a sperm with such an integration take place in fertilization.

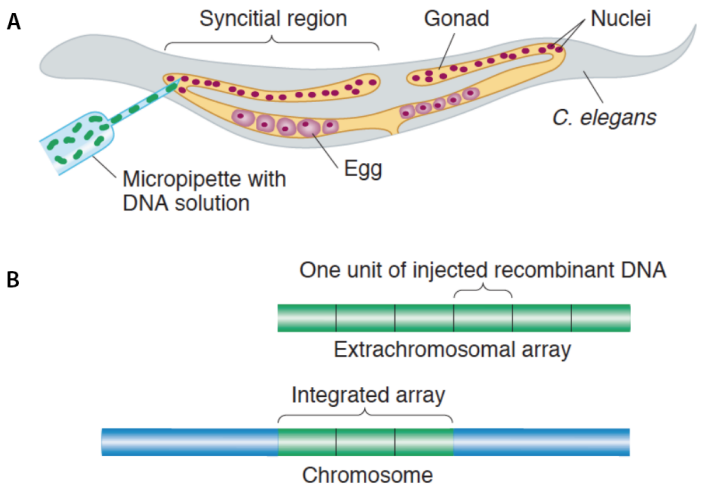


**Figure 2-7 Microinjection in *C. elegans*.** Transgenic animals are created by injecting transgenic DNA directly into a gonad. **(A)** Method of injection. **(B)** The two main types of transgenic results: extrachromosomal arrays and arrays integrated into a chromosome.

### Microinjection in *Drosophila*

A similar injection scenario is used for the generation of transgenic flies. Remember that during the syncytial stage of *Drosophila* development, the fertilized egg cell contains many nuclei that are not separated by cell membranes. Thus, *Drosophila* embryos are injected while they are still in the syncytial stage, which is simply visible under the microscope as one multinucleate cell without any cell membranes present between the cells. At this stage, cells that will become the future germ cells are located at the posterior position of the egg. Injecting the transgenic DNA at this end of the embryo will result in a few nuclei that incorporate the DNA (see figure 2-8). If these cells are destined to form the future germ cells, the fly that develops from this embryo will have the gene inserted into their germ line. Crossing this fly with wild-type flies will result in progeny that now carries the transgene as a transgenic fly.

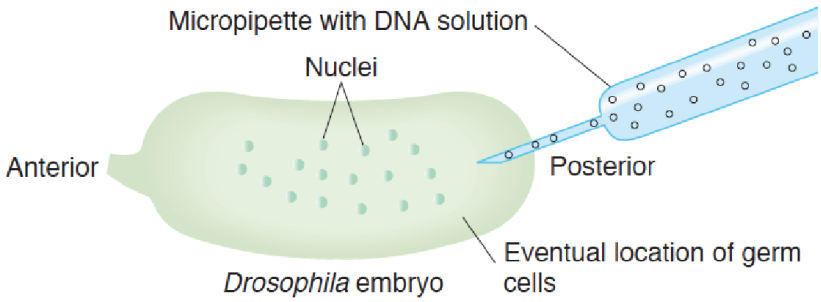


**Figure 2-8 Microinjection in *Drosophila*.** The transgenic DNA is injected into embryos at the syncytial blastoderm stage at the posterior end of the embryo where the future germ cells will form.

But, once we have injected our transgenic DNA, how can we know which flies contain the transgene? The trick is that the transgenic construct contains a marker gene (e.g., one that confers an eye color different from the one of the injected flies). If the transgene has integrated into the DNA in germ cells, flies that hatch from the injected embryo will not show the marker, because their somatic cells

will be wild type and only their germ cells (eggs in females and sperm in males) will contain the transgene. However, if we mate these flies with flies that do not contain a marker, some of the flies in the resulting progeny will show the marker (see figure 2-9). Often, the marker used for transgenesis is a gene conferring red eyes, because transgenic flies can easily be detected by simply looking for red eyes in a microscope. However, this implies that the embryos used for injection derive from flies that have white eyes (the genotype of these flies is *white*$^-$). In the lab, *white*$^-$ strains are commonly used as a reference for many experiments, because it allows to detect the presence of transgenes or other genome insertions that are often marked with the *white*$^+$ gene.

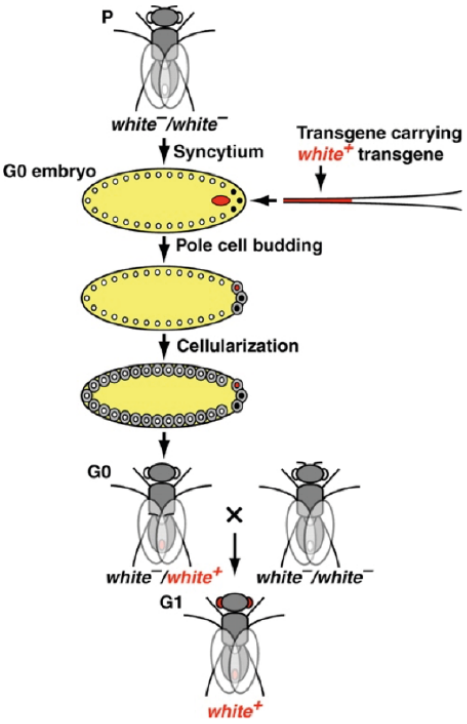


**Figure 2-9 *Drosophila* transgenesis.** Transgenic DNA containing the *white*$^+$ transgene (red) is is injected into Drosophila embryos (G0), which are less than one hour old (syncytial stage) and have been obtained from a parental (P) generation. Before cellularization, pole cells (black) bud off at the posterior end. For germ line transmission to occur, the transgenic DNA must be taken up into the pole cells that will become germ cells. Transgenic DNA integrated into a pole cell (red pole cell) can be transmitted from one generation (G0) to the next (G1). The integration events are identified using a marker, such as as *white*$^+$ that confers red eyes. When used in a mutant *white*$^-$ strain, this marks transgenic flies by giving them a red eye color.

In this approach, the insertion of the transgene into the DNA is random, thus, the transgene can be inserted into any of the four chromosomes. Thus, for further analysis of these flies, it is important to determine on which of the chromosomes the transgene has inserted.

## Determining the insertion site of transgenes

In the example mentioned above, the flies hatching from the injected embryos would be crossed with flies that are *white*$^-$, but also carry a balancer for one of the chromosomes. Usually, one starts with one balancer (e.g., for the second chromosome) already when crossing the G0 generation to save time.

As an example, let's look at a cross using a balancer for the second chromosome that you have already encountered before. The balancer carries the mutation Cy, which confers bent up wings, and the X-chromosomal *cis*-homozygous mutation $w^-$, which confers white eyes. For the flies in G1, we can't yet tell the genotype. From the phenotype we know that they contain the balancer and the transgene (TG); however, these flies could have different genotypes:

- If the TG is on the second chromosome, these flies would have the genotype TG / Cy
- If the TG is NOT on the second chromosome, these flies would have the genotype + / Cy; TG (the TG is now on another chromosome, with the ; separating the different chromosomes)

Therefore, we need another cross to determine where the TG is inserted. We therefore cross the red-eyed Cy flies with balancer flies (white eyes, Cy). For the progeny of this cross, there are two possibilities: Three phenotypes occur if the TG inserted on the 2nd chromosome (left in figure 2-10); four phenotypes occur if the TG inserted on the 3rd chromosome (right in figure 2-10). Thus, if white-eyed, non-curly flies occur in the progeny, we know that the TG is not on the 2nd chromosome, because in all other flies, either the TG or the balancer are present. If the Cy marker and the transgene segregate independently, they are on different chromosomes (see figure 2-10).

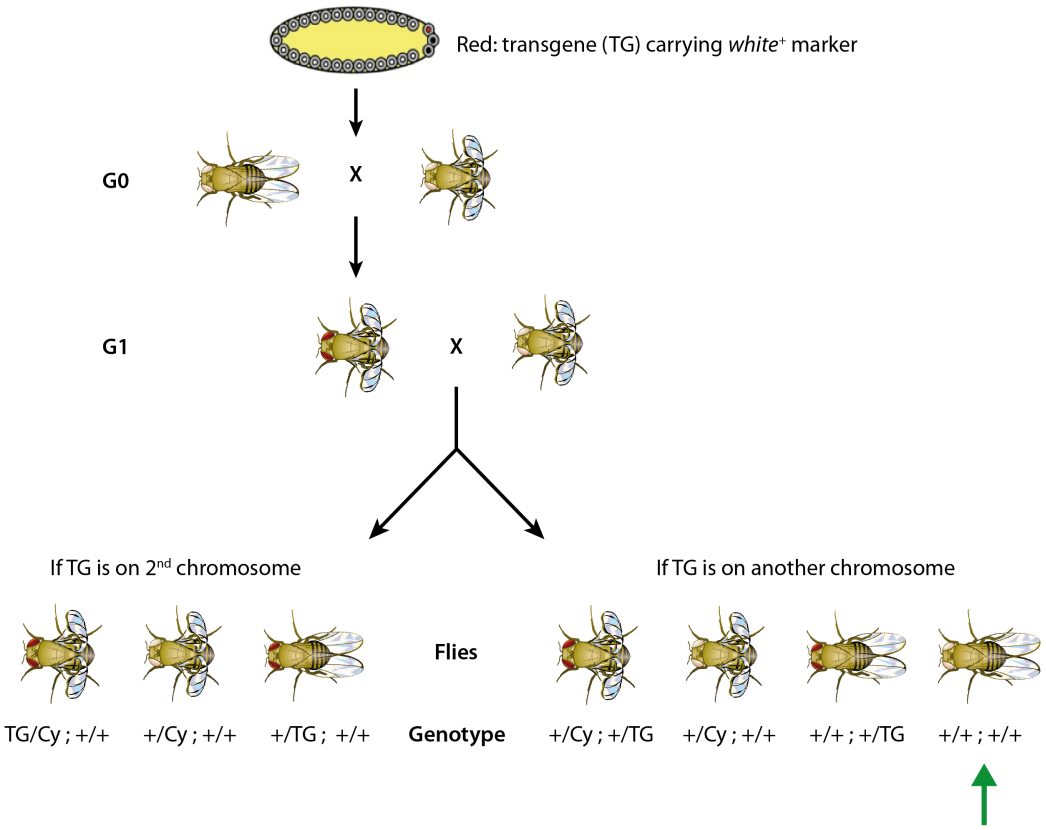


**Figure 2-10 Crossing scheme to determine the insertion site of transgenes in *Drosophila*.** After injecting the transgene into embryos, these embryos develop into adult flies (G0). G0 flies are crossed with flies containing a balancer chromosome, here, one for the 2nd chromosome, carrying the dominant marker *Cy* that confers bend up wings. In the G1 generation, flies with red eyes will appear, indicative of transgenic animals. To determine whether the transgene is on the 2nd chromosome, G1 flies are crossed again with flies containing the balancer. There are two different possibilities for the phenotypes of flies in the G2 generation (left or right), with only one phenotype that is different between both cases (green arrow). This is the one determining the location of the transgene. In both cases, both chromosomes (second and third) are indicated.

## Summary

In this lesson, we have discussed how DNA is delivered into cells to produce transgenic organisms. In the next lessons, we will discuss different strategies that were developed to insert transgenes into the genome in different organisms, and how these strategies are used to develop important experimental tools.