

Different Types of Transgene Silencing in Animals: A Natural Foundation for RNAi Technology

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Abstract

The RNAi technology is a revolutionized and powerful mechanism for in built alternate cell defence of an organism. The foreign DNA intruders form double stranded RNA intermediate during their propagation, which further cleaved in small antisense RNA by the action of dicing enzyme Dicer and further coupled with the complementary RNA for nucleation. Now a days lot more transgene were developed that involved frequently different silencing tricks, in extreme cases threshold induced silencing, DNA elimination etc. In few organisms the hall mark of silencing are involved in unpaired DNA silencing in somatic cells. The simple co-suppression events and double stranded mediated RNA degradation creates a new technology RNAi for a readymade knock down methods, However beyond limiting unfold silencing strategies of different transgenes in transcriptional silencing and RNAi technology has imported connection that are subject to answer in the review. We imagine new more powerful technology may generate for readymade activation or silencing based on the recently invented different types of transgene silencing.

Keywords: Co-suppression; Threshold induced silencing; Unpaired DNA silencing; RNAi; *Drosophila melanogaster*

Introduction

Gene silencing led to a lot of hustle and bustle in the life science community since its first accidental observance in the *Petunia* flowers in the early 1990s when an attempt to increase the intensity of the purple pigment by addition of extra copies of the anthocyanin gene exogenously resulted into a wide range of colours from strong purple to even white. Thus, when one or more copies of a transgene introduced into an organism culminate into reduced or no expression of the transgene, along with the homologous endogenous gene, from where the transgene is derived, the phenomenon of transgene induced silencing is defined. A simple cross talk between multiple transgenes inserted in different locations in the genome or precursor endogenous genes undergoes silencing, which is referred to as 'transgene silencing' or 'co-suppression'. The complete mechanism of transgene silencing is not fully understood however recent work has thrown light on its multiple facets and added to our understanding of it. The sequence homology is the major criteria for co-suppression. We have slowly started gaining knowledge about the mechanisms involved and the complexity of their interactions with one another. Silencing has been observed in a wide spectrum of organisms ranging from plants, fungi to higher animals. In plants, after discovery of silencing in *Petunia*, the experiments began with the introduction of T-DNA vector encoding a tobacco virus sequence into the tobacco plant and a later infection by the virus did not affect the plant while infection by another virus resulted into disease condition. Also molecular analyses showed that the viral sequences underwent transcription but the mRNA did not accumulate leading to the speculation that the phenomenon occurred at the posttranscriptional level [1]. Using potato virus Y and GUS reporter gene it was shown that in plants, double stranded RNA (dsRNA) formation was important for initiation of silencing [2-4]. Later small interfering RNAs (siRNAs) were found to be the intermediates in silencing in plants [5]. Thus the silencing pathway is known in detail for plants which starts with the introduction of a transgene resulting into formation of dsRNA which is further processed into siRNAs responsible for the sequence specific degradation of the targeted RNA molecules during silencing. Discovery of silencing in *Neurospora crassa*, known as 'quelling' also was made almost simultaneous to that in plants. However it took quite a long time to make the journey from silencing in plants to silencing in animals. This was mainly due to the fact that silencing

was thought to have evolved as a cellular defence against different factors that may tamper with the organism's original genetic information like a viral attack and as higher organisms possess a highly sophisticated and fully functional immune system it was believed that this alternate cellular defence mechanisms was not required by them. Also the detection of RNAi technology in mammals was prolonged by the numerous ds RNA (more than 30 nucleotides) induced mechanisms that lead to non-specific gene suppression. In fact, any introduction of more than 30nt long nucleic acid was recognized by alpha interferon in animal immune system and destroyed as parasitic elements. It was only in 1997 that co-suppression was discovered in *D. melanogaster* [6] as well as in *C. elegans* in 1998 sharing the idea of co-suppression in fly [7] and since then *C. elegans* and *D. melanogaster* has served as great model for dissecting the silencing mechanism in animals. An extensive work in this direction has opened up new dimensions to understand the numerous pathways and interplays through which this silencing is achieved by the cells and given us important tools to further unravel the hitherto unknown secrets from nature's treasure box. A few pages of this article can never do justice to a mechanism as vast and diverse as silencing, however, here we try just to provide an overview of the mechanism for a simpler understanding.

Transgene silencing in *C. elegans*

The copies of a same transgene are introduced into *C. elegans* that caused both transgene and endogenous gene to have a reduced expression. Both transgene and endogenous gene have a sequential identity on which the effect of these expressions depends. Important initiator and a target in these phases is RNA, as they are probably mediated by those molecules [7]. In wild type *C. elegans* co-suppression

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of transgene leading to feminization of the germ line genomes shown by high repetitive transgene containing multiple copies of *fem-1* gene, photocopying loss of functional mutation of *fem-1* gene. This depends on the promoter region of *fem-1*. No feminization occurs when this is absent [8]. Some genes are identified in the mutants of *C. elegans* where defective condition in transposon silencing and RNA interference such as *mut-2*, *mut-7*, *mut-8* and *mut-9* which are resistant to co-suppression. This may be a line to prove that co-suppression in *C. elegans* and RNA interference may be guided by same molecular pathways. Transposon silencing mutants (*mut*) have an RNAi resistance, the phenotype is called as RDE and some *rde* mutants have a mutator phenotype called as MUT. It may be noted that all *rde* mutants are not *Mut* same way as all *mut* mutants are not *Rde* [9-11]. RRF-1 a worm dependant RNA dependent polymerase (RdRP) is involved in some virus induced gene or transgene in nematode worm *C. elegans* to produce single stranded siRNAs in a *dicer* independent manner. Virus induced gene silencing in *C. elegans* is inheritable and can generate profound epigenetic consequences in future generations [12].

In *Drosophila melanogaster*

In *Drosophila* it has been found that suppression is proportional to transgene dosage, i.e., gene regulation depends on the number of transgenes inserted. Here, they used *white* regulatory sequences (as a promoter) and the structural part of *Alcohol dehydrogenase (Adh)* to make hybrid construct (transgene) and introduced it into different locations in *Drosophila melanogaster* genome in an *Adh* null mutant background. When number of the transgene *w-Adh* increased from one to six copies then level of *Adh* message gradually decreased in quantity which was concluded from high copy numbers of the transgenes assayed by northern blots and RNase protection assays. Flies containing six copies had only about 15% of *Adh* message level as compared to flies with one copy [6]. Furthermore, with increasing number of *w-Adh* transgene it was found that it lowers its own expression as well as also lowers that of the endogenous *Adh* gene held at one normal copy [6]. Mechanistically co-suppression is Polycomb complex dependent, which is account for generalized transcriptional repressor. In addition [13] it was also found *Drosophila* that two non-homologous reciprocal fusion genes, *w-Adh* and *Adh-w*, exposed co-suppression using the endogenous *Adh* sequence as an intermediary that is mutually inclusive to partial homology of both transgenes. Deletion of the endogenous *Adh* gene eliminated the interaction, while reintroduction of *Adh* fragment restored the silencing. Using truncated *Adh* constructs, a non-transcribed segment in the *Adh* regulatory region, was found to be one of the sequences required for homology recognition. The silenced transgenes are associated with the Polycomb group complex of chromatin proteins. Further experiments [14] exposed that *Alcohol dehydrogenase (Adh)* transcription unit has two types of transgene silencing. Transcriptional gene silencing (TGS) is polycomb dependent and happens when *Adh* is driven by the *white* eye colour gene promoter. While, Full length *Adh* transgene are silenced post-transcriptionally at high copy number or by pulsed increase over threshold. The piwi protein controls both TGS and PTGS mechanisms. Similarly Argonaute proteins function in gene silencing induced by double-stranded RNA (dsRNA) in various organisms [14]. They showed that the presence of multiple copies of EGFP triggers PTGS and probably TGS of the EGFP transgenes in cultured S2 cells. In this study, they also found that AGO1 and AGO2 depletion caused the accumulation of multi-copied enhanced green fluorescence protein (EGFP) transgene transcripts in *Drosophila* S2 cells. Depletion of AGO1, the essential factor for miRNA biogenesis, led to an increased transcriptional rate of the transgenes. In contrast, depletion of AGO2, the essential factor

for siRNA-directed RNAi, resulted in EGFP mRNA stabilization with concomitant shortening of the EGFP mRNA poly (A) tail and EGFP protein levels were increased as EGFP mRNA. Thus, new outcome raise the possibility that in addition to their role in RNAi, AGO2 and Dicer2 also function in PTGS of multi-copied transgenes and in the protection of the poly (A) tails from being shortened. The possibility that AGO2 and Dicer2 involve, at least in part, poly(A) length maintenance of transgene mRNA suggests a potentially important link between transgene silencing and poly(A) tails [14]. Linked reporter gene (*mini-white*) in transgenic *Drosophila* usually silenced by regulatory DNA from the *Drosophila* gene *engrailed* [15]. This silencing is strengthened in flies homozygous for the transgene and has been called "pairing-sensitive silencing." The pairing-sensitive silencing activities of a large fragment (2.6 kb) and a small sub-fragment (181 bp) were explored. Since pairing-sensitive silencing is often associated with Polycomb group response elements (PREs), here they tested the activities of each of these *engrailed* fragments in a construct designed to detect PRE activity in embryos. Both fragments were found to behave as PREs in a *bx-d-Ubx-lacZ* reporter construct, while the larger fragment showed additional silencing capabilities. Using the *mini-white* reporter gene, a 139-bp minimal pairing-sensitive element (PSE) was defined. Hence, a lot still remains to be discovered with respect to transgene induced silencing in *Drosophila melanogaster* in upcoming future.

In mammals

While considerable information is available about transgene silencing in plants and lower eukaryotes, efforts are being made to explore the same in higher animals, especially mammals. As discussed above, dsRNA has a crucial role in silencing. However, when a dsRNA longer than 30 nucleotides is introduced into a mammalian cell a parasitic response is triggered. The longer nucleic acid sequences are recognized as foreign genetic elements by the interferon and destroyed. A dsRNA induces Interferon synthesis and activates PKR and 2',5'-oligoadenylate synthetases both of which produce the activators of RNaseL that stops the total protein synthesis non-specifically but dsRNAs shorter than 30 nt or the siRNAs do not elicit this response making them useful in inducing RNAi in mammals [16-18]. Transcription of shRNAs by RNA polymerase III has been shown as another approach to solve the limitation presented by dsRNAs as these shRNAs form siRNAs that trigger silencing. The number of transgenes introduced into the cells also plays an important role in deciding the effect of this phenomenon. When many copies of homologous transgenes are present in a concatameric array, a decrease in the gene expression occurs in the mammals as these repeated copies are detected by cells, the repression being mediated by the PcG complex or PTGS involving dsRNA [19,20]. In a study, it appeared that transgene silencing was dependent on transcription and epigenetic mechanisms mediated it [21]. It is very essential to maintain the suppression for a prolonged period to achieve its successful biological application. Green mice and rats were used to probe into the possibility of finding a system to maintain the suppression effect for a longer time using a Green to Red (pGtoR) transgenic construct and comparing it with the effect of synthesised siRNA. The result highlighted that the silencing effect lasted longer with the transgenic approach and that a decline in endogenous gene expression can be achieved by this transgenic RNAi system in every organ at all developmental stages [22]. Retroviral and lentiviral vector based delivery approaches were tried for transgene as well as siRNA and shRNA delivery as transfection can sometimes be unpredictable. However, retroviral vectors can sometimes themselves get silenced resulting in low expression of the transgenes and although lentiviral vectors do not get silenced during development [23,24] they

too have some other limitations. Despite the limitations, these vectors have been used successfully for delivery of transgenes into mammalian system in numerous cases. Hence a lot still remains to be discovered with regards to transgene induced co-suppression in mammals.

Modes of Co-suppression

Co-suppression is a widely observed phenomenon now and it is also an equally diverse phenomenon with respect to the mechanisms through which it is achieved in different organisms. Thus, an endogenous gene or a transgene can be used as an alternate element for variation of different silencing events. Co-suppression is a type of repression in expression by another transgene in multiple ways and several scientific studies have battered our understanding of them. Some important pathways through which the silencing proceeds have been discussed below.

Non-homologous co-suppression

Co-suppression is a phenomenon in which the endogenous gene gets silenced along with the exogenously introduced homologous transgene. However, co-suppression of non-homologous genes has also been found. Presence of an endogenous sequence that has resemblance to parts of both the non-homologous transgenes is necessary for their co-suppression [13]. The researchers went ahead to show that not the decrease in this endogenous gene RNA but its deletion disrupts the said non-homologous co-suppression. In a separate study, it was shown that the expression of functional I retro-transposons or I factors was repressed by transgenes containing an internal fragment of the I factor when both were introduced into *Drosophila* lacking such elements; displaying a trait of homology dependent gene silencing (or co-suppression). The extension of this study gave another instance of non-homologous co-suppression by showing that the same transgene induced the silencing of a non-homologous reporter gene containing a 100bp I promoter that steers the expression of the reporter gene. In agreement with the study by PAL-BHADRA et al. (1999) mentioned above, four sequences were identified that might serve the function of mediators in this co-suppression mechanism [25]. Thus, the endogenous intermediary sequence has a very important role in carrying out the non-homologous co-suppression successfully.

Threshold induced silencing

The existence of threshold is indicated by a study that showed doses of dsRNA below a certain level did not lead to silencing in *Drosophila*

embryo extract, while a ten-fold increase in the doses induced silencing [26]. Very few experiments exploring the connection between transgene doses and silencing have been carried out till date. In adult flies as well as the embryos, when two copies of the *Adh* gene along with *hsp70-Adh* gene were introduced and expressed with the help of heat treatment, the RNA level increased as predicted. However, when the stock containing four copies of the same gene with an *hsp70-Adh* gene was heat treated there was a fast decrease in *Adh* mRNA. This showed that the heat treatment increased the mRNA expression from a level below the threshold to a level above it which led to silencing [27] (Figure 1). Double stranded RNAs, when given in smaller doses, lead to only a partial change in phenotype in zebrafish embryos, while higher doses led to partial (>50%) and complete changes in phenotype (about 35%). This provided another evidence of threshold induced silencing [28,29].

Meiotic silencing and unpaired DNA silencing

The process of meiotic silencing by unpaired DNA examines the entire genome and supervises the pairing between homologous DNA sequences during early meiosis. A study showed that in *Neurospora*, the presence of unpaired copies during the prophase of meiosis obstructed the as co-spore maturation as these unpaired sequences led to the silencing of both paired and unpaired DNA copies homologous to them [30]. The genes SAD-1 (an RNA-directed RNA polymerase) and SAD-2 are involved in meiotic silencing by unpaired DNA and work interdependently. A mutation in any one of these genes suppresses this silencing effect [31]. Similar surveillance mechanism in mammals leads to meiotic sex chromosome inactivation (MSCI); by inactivation of the transcription of certain genes in the heterologous XY chromosomes (in males) that do not synapse during meiosis. It has been shown that ATR and its complicated interaction with other silencing effectors have a very important role in this meiotic silencing occurring in mammals and that a decrease in its level does not disturb the silencing maintenance. The study further adds that the silencing consists of two stages among which one is dynamic and reversible while the other is stable and irreversible [32]. A strategy has been described for silencing the expression of a particular gene of interest in the germline using MSCI approach [32]. Recently we found that unpaired DNA silencing in somatic *Drosophila* cells is common. The mechanism is required for a device of cell surveillance against foreign parasitic DNA. We compare the expression of multiple *Adh*-promoter-*white* reporter (*Adh-w*) inserts in paired and unpaired configurations in *Drosophila* somatic cells (Figure 2). The unpaired copies exhibit a clear repression at the transcriptional level relative to paired gene dosage effect, which is dependent upon long noncoding RNA, *Polycomb* and *piwi*. Long noncoding RNA found from the *Adh* promoter is abundant in the unpaired condition. It serves as an attachment environment for at least two proteins POLYCOMB and Piwi. The functional attachment RNA-Piwi might create a silencing chromatin configuration by accumulating histone modifying enzymes and its precursor at the *Adh-w* promoter. The distinct transcriptional silencing characteristic for unpaired DNA represents a novel mechanism to repress new transposon and foreign DNA insertions for protection of genome integrity (Utpal Bhadra submitted data).

DNA elimination

DNA elimination has been widely studied in Tetrahymena, a ciliated protozoan, at the sexual reproduction stage when it produces new macronucleus from the germline micronucleus. Extensive remodelling of the somatic genome that involves fragmenting of the chromosomes and removal of external eliminated sequences (IESs) is seen during the

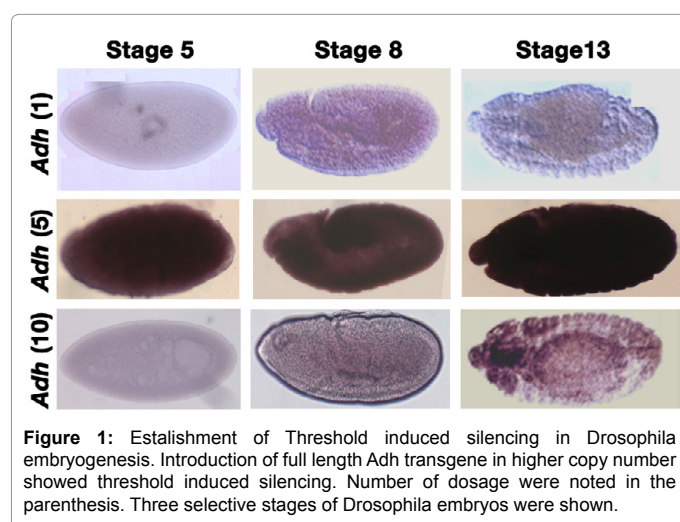


Figure 1: Establishment of Threshold induced silencing in *Drosophila* embryogenesis. Introduction of full length *Adh* transgene in higher copy number showed threshold induced silencing. Number of dosage were noted in the parenthesis. Three selective stages of *Drosophila* embryos were shown.

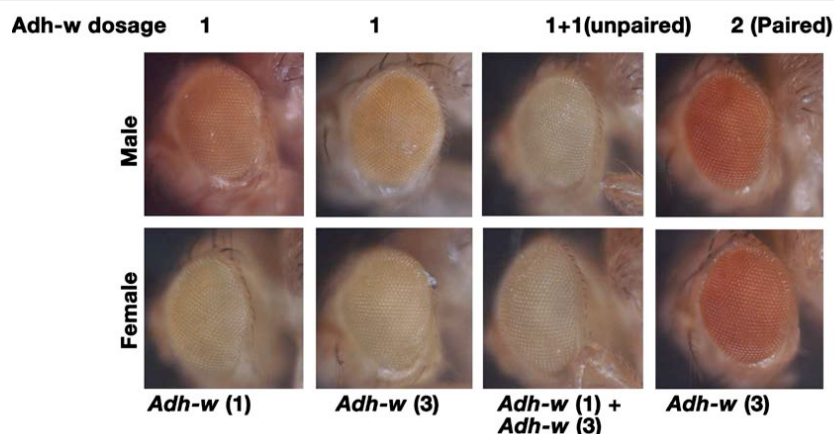


Figure 2: Unpaired DNA silencing in *Drosophila* somatic cells. The adult eye colour of *Adh-w* transgene showed unpaired gene silencing in different copy number variation. Number of parenthesis were noted in the chromosomal location. *Adh-w* dosages were on the top of the panel. In *Adh-w* one copies males showed the dosage compensation than that of one copy female.

nuclear development stage. Transposase proteins have been found to be used by cells for the elimination of unwanted DNA from their somatic macronucleus; the process being driven by homologous recognition of these sequences by small RNAs produced during meiosis from the germline nucleus [33]. As the internal eliminated sequences are widely distributed in the promoter regions and also in the introns, they impart great importance to the genome restructuring [34]. The mediators of DNA elimination, scnRNAs, arise specifically from the micronucleus, from a range of chromosomal locations and this production of scnRNAs is IES-biased due to IES-biased transcription. This biased production of scnRNAs and their degradation in the parental macronucleus is responsible for determining the entire framework of DNA elimination mechanism [35]. More studies directed towards elucidating similar mechanism in mammals and higher organisms are needed.

Box 1:

Co-suppression: A simple silencing cross talk between multiple homologous transgenes inserted in different genomic location, even that also silence endogenous homologous gene from where transgene are derived. In general terms, co-suppression is the suppression of any two or more things simultaneously. Similarly, in the context of genetics, co-suppression refers to the simultaneous repression of at least two copies of same genes i.e. the transgene and the homologous endogens.

Non-homologous co-suppression: Non homologous co-suppression showed that direct homology between the transgene sequences is not indispensable criteria for co-suppression to occur. The suppression of the two transgenes occurs that do not have any direct sequence homology with each other (i.e. two non-homologous transgenes) is called non-homologous co-suppression. Moreover one of the endogene which is partially homologous to each transgene works as a mediator in the silencing event.

Threshold induced silencing: Silencing initials by the excess expression product of transgenes. Once expression super side a threshold limit for live cells and produce a toxic effect. The threshold limit for each gene is different. Introduction of less copy number of transgenes does not induce silencing, however when this transgene number passes a certain threshold, silencing is triggered. This phenomenon is known as Threshold induced silencing (Figure 1).

Unpaired DNA silencing: Major parasitic threats of the host genome leads to integration of genome to host chromosome by

unpaired means. Later a self-replication can take place which produced paired copy. The frequent initial integration of Parasitic DNA in the host genome was reacted by a silencing effect. Therefore unpaired copy undergoes silencing with paired copies. One single unpaired copy is sufficient to reduce the expression of paired copies (Figure 2).

Pairing sensitive silencing: The phenomenon is identically opposite to unpaired DNA silencing. Paired copies in the somatic cells undergo silencing whereas unpaired or single transgene copy is normally expressed. Two genes in the same allelic combination producing a silencing effect.

DNA elimination: In some animals, external sequences are eliminated from the somatic macronucleus leading to remodelling of the somatic genome. This mechanism is called DNA elimination.

RNA interference: A readymade knock down technology. Truly, it is Double stranded RNA dependent silencing. The dsRNA of a gene of interest reduce the expression of the same gene. It is a tool that can be used for genetic manipulation and has great biological applications. It shows great resemblance to PTGS mechanism.

Polycomb group gene and pre containing construct

Polycomb group genes are highest conserved regulatory factors that are responsible for the maintenance of silent states of homeotic genes [36,37]. The development of molecular probes in the early 1980s led to the discovery that PcG genes are not needed to establish but rather to maintain repression of HOX genes outside their normal expression domains [38]. When *w-Adh* transgene was introduced into an *Adh* null mutant background of *Drosophila melanogaster* genome, it was found that the *w-Adh* transgenes were repressed suggesting an involvement of Pc-G genes. The Pc and Pcl mutations were tested as heterozygote, and each was found to reduce the degree of co-suppression by approximately half. This result suggests that the silencing is dependent on the Pc-G gene products. Further analysis on the involvement of Pc-G was done by testing the Pc-G protein association by antibody probing of polytene chromosomes. Under repressing conditions, but not with the single constructs, *w-Adh* sites show labeling with anti-Pc and anti-ph antibodies [6] so, the mutational and binding studies provide evidence for an involvement of the Pc-G complex in co-suppression of this type [6]. The association of the Pc-G on the transgene may act as a mechanism for maintenance of a set level of histone acetylation [39-42]. In addition, the same scientist found after two years that the

repressive effect of *w-Adh* transgenes on the *Adh-w* transgenes also produces accumulation of Pc on the polytene chromosomes at the site of *Adh-w* insertion [6]. PcG proteins form multimeric complex that exert their respective function by modifying chromatin structure [36]. Till now three distinct *Drosophila* PcG protein complexes have been biochemically purified and characterized; that is polycomb repressor complex 1 and 2 (PRC1 and PRC2) and, most recently, Pho repressive complex (PhoRc) [43]. All three complexes contain multiple subunits encoded by PcG genes that are crucial for *Hox* gene silencing [43]. The core of *Drosophila* PRC2 contains the three PcG proteins E(z) (enhancer of zeste), Su(z)12 (suppressor of zeste 12) and Esc (Extra sex combs), and this complex function as a histone methyltransferase (HMTase) that specifically methylates lysine 27 of histone H3 (H3K27) in nucleosomes *in vitro* [44]. The core of *Drosophila* PRC1 contains the PcG proteins Ph (Polyhomeotic), Psc (posterior sex combs), Sce (sex combs extra) and Pc, and it inhibits nucleosome remodeling and transcription during *in vitro* assays [45].

From the dissection of cis regulatory sequences of *Hox* genes in reporter gene assays [46,47], and the use of chromatin immunoprecipitation (ChIP) assays [48] converged to reveal that PcG proteins associate with specific cis regulatory sequences that are needed for PcG repression; hence, these sequences were called Polycomb response elements (PREs). In *Drosophila* *Hox* genes the first PREs identified. Recent studies suggests that PREs are largely devoid of nucleosomes and that pre DNA serves as an assembly platform for many different PcG protein complexes through DNA-protein and protein-protein interactions. The coming out picture suggests that the binding and modification of chromatin by PcG proteins is needed for interaction of PRE tethered PcG protein complexes with nucleosomes in the flanking chromatin in order to maintain a Polycomb repressed chromatin state at promoters and coding regions of target genes [43]. From many years ago it found that Fab-7 is a well-characterized, PRE-containing element that is involved in the regulation of the homeotic gene Abdominal-B [49]. After inserted into the fly genome, Fab-7 containing transgenes can ectopically recruit PcG proteins, leading to PcG-dependent silencing of reporter genes [50]. When present in multiple copies in the genome, Fab-7 can induce long distance gene contacts that enhance PcG dependent silencing. It demonstrated that components of the RNA interference (RNAi) machinery are involved in PcG mediated silencing at Fab-7 and in the production of small RNAs at transgenic Fab-7 copies. They are specifically required for the maintenance of long range contacts between Fab-7 copies. This concludes the novel role of the RNAi machinery in regulating the nuclear organization of PcG chromatin targets [51].

Co-suppression and pairing sensitive silencing

It is suggested by some experiments that many PREs are composite elements of sites important for silencing and important for “pairing” or bringing together distant DNA elements. Both activities may be necessary for PRE function. In a related condition, fragments of DNA included within P-element vectors can cause those transposons to insert themselves in the genome near the parent gene of the included DNA (transposon homing). It is expressed as a hypothesis that DNA fragments that cause transposon homing or pairing-sensitive silencing are bound by protein complexes that can interact to bring together distant DNA fragments.

Studies suggest that Regulatory DNA from *Drosophila* gene engrailed causes silencing of a linked reporter gene in transgenic *Drosophila*. The silencing is more in flies homozygous for the transgene and thus has been called pairing sensitive silencing, as pairing is

important for the enhancement of silencing in most cases [15]. Pairing sensitive silencing is often associated with Polycomb group response elements. Both fragments were found to behave to behave as PREs in a bxd-Ubx-lacZ reporter construct and layer fragment showed more capabilities.

However it was found that some PREs do not cause pairing sensitive silencing but some fragments of DNA may cause pairing sensitive silencing and those do not act as PREs [52]. Studies found that *Drosophila* PcG protein Pleiohomeotic binds to specific sites in a silencer of the homeotic gene Ultrabithorax. It was found that point mutations in these Pleiohomeotic binding sites results in disappearance of PcG repression *in vivo* in an Ultrabithorax reporter gene. Thus it is found that insertion of other non-binding PcG proteins to homeotic gene silencers contains a function of DNA bound Pleiohomeotic protein [53]. It is found that altered gene expression due to allelic pairing is observed due to transvection of different foreign elements. The *white* locus of the genome exhibit it in two ways firstly gain of function mutation *zeste-1* reduce when pairing of two copies are present in the genome and secondly certain alleles of white exhibit a partial expression when pairing is possible [6]. The *w-Adh* gene also exhibit pairing sensitive silencing in this respect. Total expression of collective homologous transgene declines due to increase in copy number in the nucleus. Thus *w-Adh* transgene when in single copy expressed more than when homozygous at any location in genome. Studies suggest that it may involve recognition for homology at some point and followed by change of chromatin state [54].

Link Between Co-Suppression and RNAi

The natural mechanism of RNA interference has two parts: Dicer, the RNase III family nuclease, identifies and cleaves the dsRNA into 21-23 nucleotide double stranded fragments, siRNAs and these siRNAs then become a part of the RNA induced silencing complex (RISC) that cleaves the target mRNA into small fragments. Cosuppression involves introduction of transgene that leads to silencing of the endogenous gene due to homology. This co-suppression proceeds via two mechanisms: Transcriptional and Post-transcriptional. In plants, transcriptional silencing occurs when there is a promoter homology between the transgene and the endogene, while the posttranscriptional silencing occurs when homologous regions are protein encoding ones. Although both TGS and PTGS are triggered by dsRNA, the posttranscriptional gene silencing greatly resembles the RNAi mechanism and proceeds through small 21-24 nucleotide RNAs similar to the siRNAs [5,55-63]. In *D. melanogaster*, a relation between TGS and PTGS has been shown [27]. There have been some new additions to these silencing types: Threshold induced silencing and meiotic and unpaired DNA silencing. Threshold induced silencing is greatly dependent on the amount of transgene that is introduced while the meiotic and unpaired DNA silencing works through improper pairing of genes in the chromosomes. Insertion of dsRNA for silencing of genes is a co-suppression mechanism that greatly mirrors RNA interference. A study on *C. elegans* showed that mutants incapable of transposon silencing and RNA interference were also resistant to co-suppression. This suggested that RNA interference and co-suppression might involve same molecular machinery as their mediators but at the same time the results also showed that co-suppression and RNAi are two distinct phenomena [9]. Another experiment established that increased germ cell death takes place due to silencing resulting from co-suppression or RNAi and that both transgenes and dsRNAs cannot enhance apoptosis in a silencing-blocked genetic background [64]. It has been shown that transgene suppression works in a similar fashion in both plants as well as *C. elegans* and this working requires transcriptional

activity of the transgene which further indicates that co-suppression advances through an RNA intermediate [9]. Involvement of dsRNA in co-suppression has been supported by the observation that inverted repeat transgenes are more susceptible to co-suppression [65]. RNAi and co-suppression both rely on the same genes for their performance indicating that transgenes and dsRNAs belong together [66]. Therefore RNAi is a true technological device while co-suppression is the endogenous application of the RNAi technology. They will follow the same mechanistic hall marks.

Transgenic Effect on Transposable Elements

The transposable element (TE or transposon) has a DNA sequence that changes its position within the genome, sometimes by creating or reversing mutations and altering the cell's genome size. Transposition often results in duplication of the TE. Barbara

McClintock's discovery of these 'jumping genes' of maize in 1983.

A major influence on the structure of genome during evolution is transposable elements. Studies suggest that they can cause mutations and led to the concept of selfish DNA which is useful to manipulate gene transfer vectors. There are mainly 2 types 'copy and paste' retrotransposons that are mobilized by transcribing an RNA copy and then reverse transcribing and integrating somewhere else in the genome (Figure 3). The other one, in contrast, is 'cut and paste' transposable elements that transpose by the direct excision from DNA and insert elsewhere in genome (Figure 4) [67]. An internal promoter and coding sequence for the integration and reverse transcription of the specific protein is required by retrotransposable elements. There are different effects on the regulation of transposable elements like in *Caenorhabditis elegans* it is silenced through post transcriptional modification whereas in *Arabidopsis* the silencing is done by chromatin remodelling factors

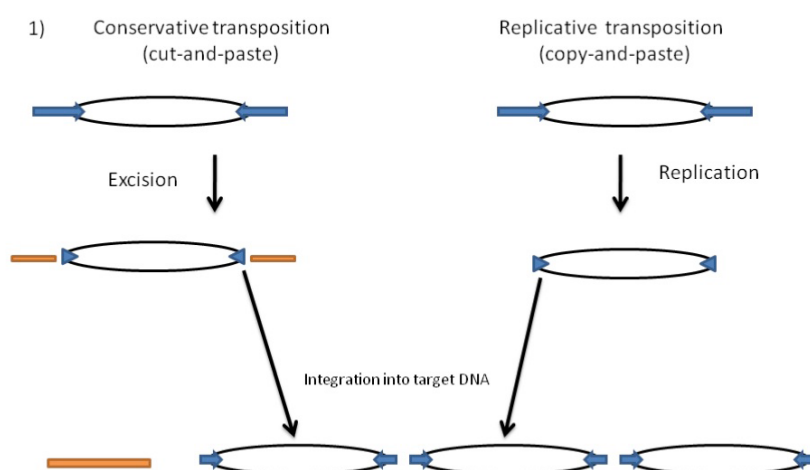


Figure 3: Mechanism of transposition of transposable elements. schematic representation of the two major mechanisms of transposition. Conservative transposition the TE is excised from the donor DNA, and integrates into a new target DNA. Ligation of the broken ends of the DNA reconstitutes the donor locus. Replicative Transposition involves amplification of the TE by copying through transcription followed by reverse transcription. The newly made copy gets inserted elsewhere in the genome, but the donor element does not move.

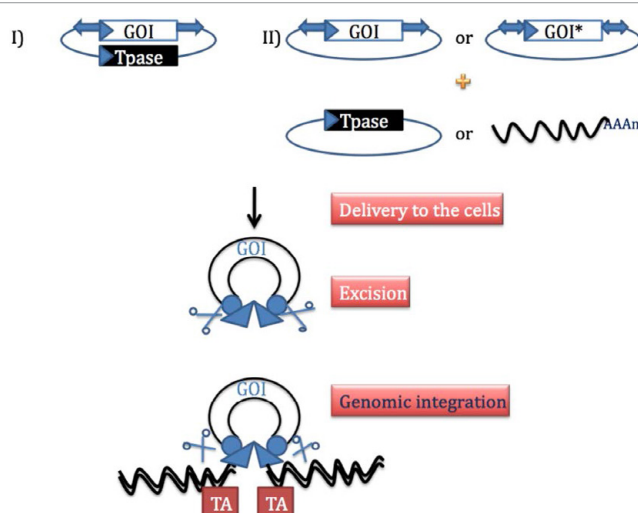


Figure 4: The diagram of a SB plasmid based transposon system combines naked DNA and viral vectors to its purpose; due to stable genomic insertion of expression cassettes it causes long term and efficient transgene expression. It has two functional elements a specific DNA that causes a gene to be inserted into cell's genome and a transposase that moves the transposase, that bind the terminal inverted repeats flanking the gene of interest (GOI) and causes excision and integration of the transposon.

of which many are studied [68]. Transposable elements can be inserted into virgin genomes within a few generations and after that only limited transposition activity is retained. Studies found that after the introduction of I element, a retro-transposon which is similar to mammalian LINE elements in *Drosophila melanogaster* genomes, without such elements, initially caused high frequency transposition from the incoming TE, high mutation rate, chromosome non-disjunctions and female sterility which is referred as hybrid dysgenesis. Study suggests that High frequency transposition is transient as the number of I elements reaches a finite value and transposition ceases after 10 generations. It is proposed by some experiments that I elements encode a factor that negatively regulate their own transcription, but evidence supporting such claims is still lacking [69]. It was found that the activity of the I element can be repressed by prior introduction of transgenes which expresses a small internal region of I element. This mechanism will act as a characteristic feature of homology dependent gene silencing. This indicates that Transposable Elements can be tamed by homology dependent gene silencing, a process which developed itself as a specific defence mechanism against these elements [70].

Recent results confirm that long term expression of therapeutic transgenes can be achieved using a transposon based system in stem cells and *in vivo*. A natural DNA transfer vehicle capable of efficient gene insertion is produced. The latest generation calls it Sleeping Beauty transposon based hyperactive vector (SB100X), after the Grimm brothers' famous fairy tale is capable to address the problem of non-viral approaches in low efficiency of stable gene transfer. The combination of Transposon based non-viral gene transfer coupled with latest delivery system can give us access to long term therapeutic effect without compromising biosafety [71,72].

Conclusion and Perspective

A steady work is constantly going on to resolve the mysteries surrounding this mechanism and in near future we will get a complete and orchestrated picture of the entire endogenous profile of the process. Our group's experience of working with different transgenic effect on *Drosophila melanogaster* for more than a decade led us to understand how important the field is in the prospect of research in genetics and finding new information that would help in the future of RNAi research. We found that the core components of RNAi factors work as an interlink between Transcriptional Co-suppression and RNAi mechanism which led us to believe that transgene co-suppression might be the natural foundation for RNAi mediated mechanism. The purpose of the review was to give an overview of the relative events that transgene co-suppression played in the discovery and understanding the real hall mark of RNAi and more powerful related mechanism. Different transgenic stocks and their distinct behaviours and their crosses revealed two different types of silencing namely threshold included silencing and silencing of unpaired DNA in animals. It was found out that the integration of foreign DNA in host genome by an unpaired copy and was increased by subsequent replication to a paired copy was a constant threat for foreign parasites but it was eliminated by the host cellular defence mechanism. Second each gene was synchronously expressed in live cells according to their immediate requirements. Any excess expression of the protein product was detected by the cell sophisticated surveillance system and reduces its toxic vigour immediately. The limitation of excess protein product was reduced by a threshold dependent mechanism, when threshold induced silencing was initiated. However the threshold differs for each specific gene and the range of threshold was very wide spread. Therefore one or two copies insertion of the same transgene does not instigate threshold induced gene silencing. The threshold limit only

instigates when cell surveillance measures the toxic level for instance close to five copies of endogenous Adh gene. These mechanisms give us advanced ways for future researchers to develop protective measures in animals against ample production of parasitic protein products and hybrid constructs etc. The review discusses different ways to consolidate different silencing procedure. It also asks the question whether co-suppression is the founder of RNAi technology. The main motive of the future researchers being to develop new technology that will be able to shut down or activate the gene of interest according to their will for the treatment of different gene expression modulated diseases and will develop new devices for cellular defence in the host.

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