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Review Article

GENE TRAPPING: A POWERFUL TOOL OF FUNCTIONAL GENOMICS TO IDENTIFY NOVEL GENES

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Abstract- Gene trapping is a type of insertional mutagenesis that disrupts gene function by the integration of a vector in the intergeneric sequences. It provides an important and unique method for studying the relationship between gene expression and function and a powerful tool to characterize novel genes and analyze their importance in biological phenomena. It is performed with gene trap vectors that simultaneously mutate and report the expression of the endogenous gene at the site of insertion. Based on the component of gene expression cassette which they exploit, trap vectors are classified as: enhancer trap vectors, promoter trap vectors, gene trap vectors, poly A trap vectors and secretory trap vectors. Vectors are introduced in embryonic stem cells in mice and leaves or floral parts in case of plants by electroporation or virus-mediated transformation. The transformed cells are selected on the basis of selectable markers. Insertion events are detected and the trapped lines are established. This technique has been used to identify tissue specific and temporally regulated genes in plants and mice. It proves to be a powerful tool of functional genomics.

Keywords- Trap vectors, insertional mutagenesis, novel genes, tissue –specific, reporter genes.

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Introduction

Gene trapping is a unique method that helps to identify novel gene by producing random gene disruption by inserting a DNA element, which contains a reporter gene and a selectable marker, throughout the genome. It is a type of insertional mutagenesis that disrupts gene function by the integration of a vector in the intergeneric sequences. (Insertional Mutagenesis is a strategy that uses the insertion of DNA to mutagenize genes at the insertion site. The inserted sequence acts as a tag to clone the mutated gene). It is an insertion-based gene discovery method that utilizes random integration of reporter gene constructs into the genome and produces a dominant expression phenotype. Lee and co- workers [1] described it as a high-throughput approach of generating mutations in murine ES cells through vectors that simultaneously disrupt and report the expression of the endogenous gene at the point of insertion [2]. It provides an important and unique method for studying the relationship between gene expression and function and a powerful tool to characterize novel genes and analyze their importance in biological phenomena [3].

Gene trapping is accomplished by using gene trapping vectors which mutate endogenous gene at the site of insertion and identify the site of integration and mutated gene by reporting the expression of the mutated gene. The mutation is generated by inserting a trap vector construct into an intronic or coding region of genomic DNA. It contains selectable reporter tags which are used to identify cell lines in which the vector has successfully interrupted a gene. The random insertional mutations generated in the genome are immediately accessible to molecular characterization through the reporter tags, which can also be useful for further experimentation in cells for instance as a DNA tag for cloning of the disrupted gene. This technique was originally used for studies of insertional mutagenesis in the mouse. In addition to generating a bank of mutations in genes, gene trapping also helps in gene identification, generating insertions into novel and previously uncharacterized transcript, studying the expression patterns of the genes and used to identify and characterize genes regulated by exogenous stimuli or during development.

Methodology of Gene Trapping

Gene trapping is based on the principle of stable, random integration of a DNA construct in the cellular genome that is capable of highlighting integration and function of genes. To achieve this, special vectors composed of reporter genes and selectable markers but lacking a specific functional feature are used. The reporters in these are normally inactive. Full functionality is only acquired when they have properly integrated into the gene that provides the missing function. Thus, they are able to exploit the endogenous gene function and hence the term 'gene trapping'. The gene expression cassette in eukaryotes consists of an enhancer sequence and promoter sequence upstream of structural genes which are controlling elements of gene expression. The structural gene is made up of exons (coding regions) and introns (non-coding) regions whose boundaries are marked by splice acceptor and splice donor regions. A gene trap vector exploits any one of these components of gene expression cassette [4].

Gene trapping in mouse

For gene trapping in mouse, the suitable vector is selected and constructed. The embryonic stem (ES) cells are isolated from inner layer of blastocyst of the mouse embryo and cultured *in vitro* to produce ES cells lines. These are transformed with the gene trap vector using electroporation or retroviral infection. The transformed cells are selected using selectable marker due to their differential growth ability. The transformed cells are subjected to two types of screen- molecular techniques for identification of the interrupted gene and the regeneration of these cells to produce transgenic off springs either by production of aggregation chimeras or

introduction of cells in morula. The embryos can then be used for expression analysis of reporter and identification of the location of the trapped gene using the reporter gene. The transgenic off springs are used to maintain the trapped cells. They are also used for phenotypic analysis of the trapped gene [Fig-1].

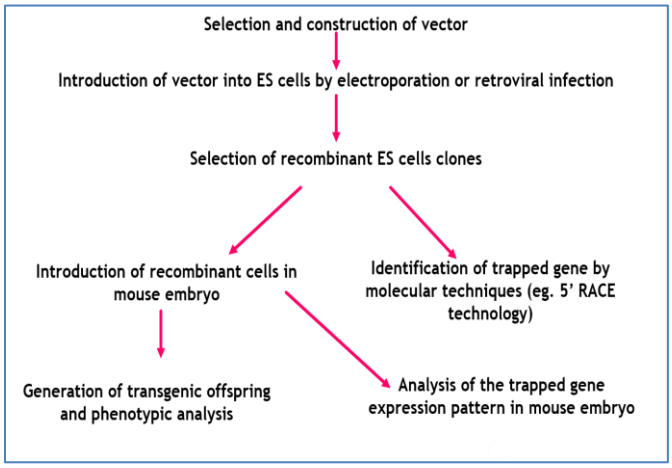


Fig-1 Procedure of gene trapping in mouse

Gene trapping in plants

For gene trapping in plants, the vectors used are mostly T-DNA based. These are introduced in plants using electroporation or T-DNA mediated transformation. The transformed cells are selected and grown to produce T mutants. These are subjected to reporter assay to identify the location of trapped gene (T1 mutants). The transformants are also subjected to PCR and gel blot analysis for identification of trapped genes. The T1 mutants are screened and trapped lines are established [Fig-2].

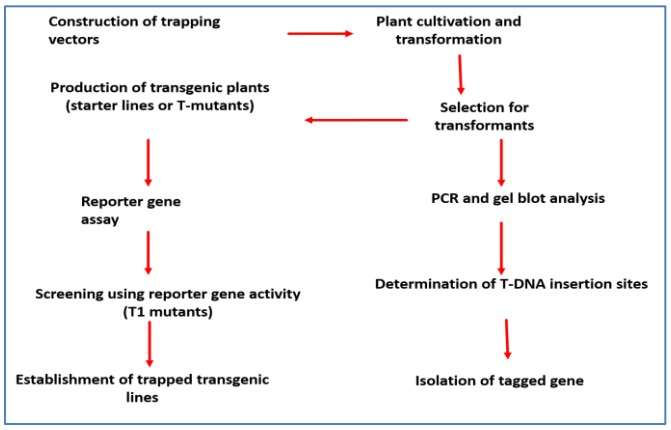


Fig-2 Procedure of gene trapping in plants

Trap Vectors

The purpose of gene trapping is to tag and detect cis-regulatory sequences by locating the reporter gene within an endogenous gene, which is achieved by 'trap vectors'. A variety of trap vectors or entrapment vectors have been developed for this purpose, but all essentially work by insertion of a reporter gene into an endogenous gene. Any gene trap vector must disrupt function of the trapped gene, should provide a reporter as a tag to easily detect endogenous gene expression and should allow rapid identification of the trapped gene by serving as a molecular tag. Due to their small size, gene trap vectors only marginally affect the expression of genome. Also, we can identify genes that show no or only subtle phenotypes when mutated, by monitoring the reporter gene expression. The term 'gene trap vector' is a misnomer since any vector which affects the gene expression cassette is termed as gene trap vector. Based on the component of gene expression cassette which they exploit, trap vectors are classified as: enhancer trap vectors, promoter trap vectors, gene trap vectors, poly A trap vectors and secretory trap vectors. Based on the carrier construct, they are classified as: plasmid based vector, retroviral based vectors, transposon based vectors and T-DNA based vectors in plants

Enhancer trap vectors

Gene expression is tightly regulated by a set of short sequence elements located in the proximity of promoter, which are termed as ,'enhancers'. Enhancer trap vectors help to investigate how and when enhancer DNA sequences can affect gene regulation, and also aid in the determination of their possible location. These vectors contain a reporter gene with a weak or minimal promoter. The minimal promoter is not itself sufficient to drive expression of reporter gene but requires the vector to insert near a cis-acting enhancer element to produce expression of reporter gene. In addition to a reporter gene, these vectors also contain selectable markers with a constitutive promoter and polyadenylation sequence (poly A) [Fig-3]. When the vector inserts in the proximity of a chromosomal gene, the result is expression of the reporter gene under control of the neighboring chromosomal enhancers. The expression of the reporter gene is thus activated by chromosomal genes. Thus, it results in transcriptional fusion.

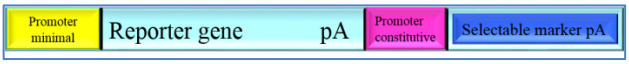


Fig-3 Schematic representation of an enhancer trap vector

Examples of enhancer trap vectors

p3LSN enhancer-trap vector has LacZ as reporter gene. Truncated heat-shock inducible minimum (hsp68) promoter immediately upstream of lacZ is the minimal promoter. Neomycin phosphotransferase gene acts as selectable marker with constitutive promoter Herpes simplex virus thymidine kinase (HSV-tk). This vector usually generates hypomorphic rather than null mutations. [5]. *Enhancer trap in Drosophila* uses transposon *P* element. It carries reporter gene β-galactosidase with minimal reporter. This vector can hop into genome. *Plant enhancer trap vectors* are mostly T-DNA based and contain reporter gene and selectable marker between left and right border of disarmed T-DNA. Bacterial β -D-glucuronidase (uid A) is mostly used as reporter gene because of the absence of endogenous β-glucuronidase (GUS) activity in most plants and the opportunity to visualize the presence of the enzyme by sensitive histochemical techniques [6]. Selectable markers are generally NEO, Hygro or βgeo (A fusion of lacZ gene and the neo gene). Jellyfish green fluorescent protein gene (GFP) and firefly luciferase gene also used as selectable marker. The best known example of such vector is enhancer trap element DsE which is based on Ac/Ds transposon of maize and GUS fused to a minimal promoter derived from the 35S promoter of CaMV is used as reporter gene. NPTII gene, which confers resistance to kanamycin serves as a selectable marker. It was used for the identification and characterization of genes that are expressed in different developmental processes in *A. thaliana* [7]. Another such example is cET-1 which is a T-DNA vector derived from an enhancer trap Ds element. GUS reporter adjacent to the left border and is preceded by a minimal 35S promoter containing a ribosome entry site. It has been used to tag genes in Poplar trees [8].

Expression of the reporter gene inserted by an enhancer trap vector is under the control of enhancer of the endogenous gene. Thus, this method can effectively trap enhancer sequences. But by using this vector, it may not be easy to identify and locate the enhancer causing the specific expression pattern, since the enhancer element could be in either side of the vector and could also be at a far off distance. The insertion of the vector occurs at random sites which results in disruption of endogenous genes and hence affect gene expression and phenotype. For the gene trapping by this method, the insertion should necessarily take place near the 5' end of the gene thus, shows a 5' bias. This technique is not very mutagenic. Also, it requires screening of a very large population and is able to trap only active genes. Transcription ally silent genes in the target cells are thus missed by this strategy.

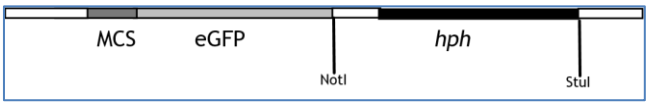
Promoter trap vector

Promoter is a combination of short sequence elements upstream of structural gene, to which RNA polymerase binds in order to initiate transcription of a gene. A

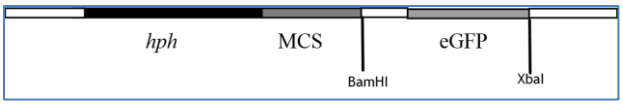
promoter trap is able to exploit the promoter and enhnacer of endogenous gene.The promoter trap in its minimum configuration contains a promoterless reporter gene and a poly A sequence.The promoter trap must bind in exonic region of the endogenous active gene in correct orientation, for transcription of reporter gene. The expression of reporter gene is thus under the control of the promoter and enhancer of the active gene which it interrupts. It also contains selectable marker with constitutive promoter [Fig-4]. These vectors are highly efficient in studying differential expression of the genes. Following insertion into exon of gene, chimeric RNA consisting of upstream endogenous exonic sequence and the reporter gene, is produced which results in production of fusion protein. When the insertion occurs in intronic region, entire trap sequence is spliced out of the mature mRNA. Thus, it results in transcriptional as well as translational fusion.
Examples of promoter trap vectors

Fig-4 Schematic representation of a promoter trap vector

The promoter trap vectors for bacteria and animals are mostly based on plasmids and virus respectively. For instance *Pβgal promoter trap vector* is a plasmid based vector and has a promoter less LacZ gene as reporter gene. Neomycin resistance gene with Phosphoglycerate 1(PGK1) constitutive promoter serves as selectable marker. It needs to be inserted into the coding sequence of gene to activate transcription of lacZ. *U3neo promoter trap provirus* contains the ampicillin resistance gene (amp) and a plasmid origin of replication flanked by the neo gene in each LTR. The selection is based on neomycin gene.*U3βgeo*, another vector based on U3, has reporter gene β geo (βgeo encodes a fused protein having β-galactosidase and neomycin phosphotransferase activities), polyadenylation signal (pA), U3 region of enhancerless Moloney murine leukemia and *E. coli* supF tRNA gene (Sup5). *HIV based lentiviral and MLV based retroviral promoter trap vectors* have reporter gene PuroR- GFP gene between a strong cellular splice acceptor (SA) and a polyadenylation (polyA). These elements are present in reverse orientation into SIN MLV10 and HIV14 vectors upstream of modified 3' LTR. Reverse orientation prevents reporter expression from the 5' LTR of the transduced vector.
The T-DNA vector used for promoter trapping in plants uses selectable markers NEO, Hygro or βgeo gene under control of a constitutive promoter. Promoterless reporter gene is placed at the right border for visualizing the trapped promoter. It has been used for gene detection and cloning in tobacco and *Arabidopsis.* The promoter-trapping vector pEGFPHPH [Fig-5a] and pCBGFP [Fig-5b] have reporter gene in it is eGFP ene**.** BamHI-XbaI eGFP fragment is cut from pEGFP and fused with selectable marker hygromycin B (hph) which itself is NotI-StuI hph fragment amplified from pCB1003. MCS: Multiple cloning sites, including BamHI, KpnI, SacI,SmaI, AgeI restriction sites are present. Vectors can be linearized by these restriction enzymes. The two vectors differ in orientation of the reporter and selectable markers. They have been used for promoter trapping in *Magnaporthe grisea* [9].



a) **pEGFPHPH**



b) pCBGFP

Fig-5 Promoter trap vector in plants

Promoter trap vectors can be effectively used to study tissue-specific, developmental stage-specific and stress-induced promoters. This method has high mutagen city rate as compared to enhancer trap. This approach has also led to the identification and isolation of several cryptic promoter elements [10-11]. This method helps in detection of insertions in UTR's and thus enable detection of hidden mutations. They can also be used for gene targeting ('targeted trapping') [12]. But reporter expression requires both transcriptional fusion and in-frame, translational fusion with a coding region of the interrupted gene, so frequency with which promoter-trap vectors insert into exons is exceedingly low. It cannot be used to trap transcription ally active genes. Insertion in intronic regions results in complete degradation of reporter mRNA.

Gene trap vectors

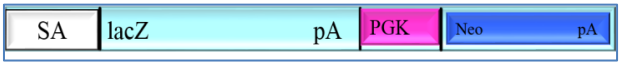
Gene traps are plasmid or retrovirus-based vectors containing a reporter gene that is only expressed upon integration in a functional gene. The gene trap cassette contains a promoter less reporter construct preceded by a splice acceptor site at the 5' end and is followed by a selectable marker with polyadenylation signal at the 3' end. Gene trap vectors contain a polyadenylation signal at the 3' end that causes the mRNA to be truncated and non-functional. They can be expressed even on integration in intronic regions since they can take advantage of the splicing apparatus by having a splice acceptor site. Both transcription and translation of reporter gene is under the control of endogenous promoter and regulatory sequences. Integration of the vector in exonic or intronic region results in production of fusion protein having a part of endogenous gene at N-terminal and reporter gene at C-terminal. Some vectors have an internal ribosome entry site (IRES) which help in translation of reporter gene even without fusion with endogenous gene. Second generation gene trap vector contain selectable marker with constitutive promoter which help them to trap transcription ally inactive gene. A gene trap vector can successfully trap promoter, splicing apparatus, exons and introns. Integration of the vector in exonic or intronic region results in production of fusion protein having a part of endogenous gene at N-terminal and reporter gene at C-terminal. Conventional gene trap vectors use a splice acceptor to take advantage of endogenous transcription and truncate the mRNA, leaving the gene 5' of the insertion site intact, followed by the vector sequence containing the selection/reporter construct. The splice acceptor interrupts normal splicing and causes the downstream vector sequence to be transcribed. A polyA signal is placed at the 3' end of standard vectors, causing translation to end and producing a truncated fusion protein. Conventional gene trap vectors will produce a null fusion protein that is regulated in the same manner as the trapped gene. In addition, the insertion site of the gene trap vector will affect the protein as well, with some truncated proteins retaining partial functionality depending on the intragenic location of functional domains [13].

Examples of gene trap vectors

βgal gene trap [Fig-6a] is one of the first vector used for gene trapping which contains a splice acceptor (SA) site immediately upstream of a promoter less lacZ gene followed by a neomycin resistance gene as selectable marker with phosphoglycerate kinase constitutive promoter. *PGT4.5 gene-trap* vector [Fig-6b] also contains a splice acceptor (SA) site immediately upstream of a promoter less lacZ gene and Neomycin resistance gene as selectable marker but with human βactin constitutive promoter. It has been used to study mouse as a model of human genes. *ROSA–GFNR gene trap* [Fig-6c] is a retroviral trap vector derived from ROSA βgal. It has GFNR gene as reporter which is a fusion between enhanced green fluorescent protein (EGFP) and *Escherichia coli* Nitroreductase gene (NTR). It has been widely used for surveys of transcription ally regulated genes in mouse [14]. The gene trap vectors based on βgeo are *IRESβgeo*, *SAβgeo gene trap* and *pU-21T*. While IRESβgeo was constructed by introducing IRES from the EMC (encephalo myocarditis) virus between the splice acceptor and the βGeo sequences. SAβgeo gene trap [Fig-6d] has a splice acceptor (SA) from the intron1/exon2 of mouse En-2 gene and βgeo reporter. Bacteria initiation codon in βgeo is replaced by the protein translation initiation sequence from the Moloney murine leukemia virus (MoMuLV) End gene. SAβgeo gene trap cassette is efficient for both electroporation and retrovirus based gene transfer methods. It has been used to display the expression of the trapped endogenous gene. Trap vector pU-21T [Fig-6e] was constructed from pU-17 vector. It contains βgeo gene as reporter which has polyadenylation (pA) sequence from the SV40 T-antigen

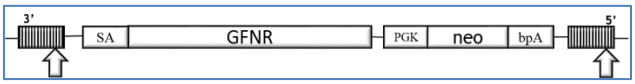
and SA site of the mouse En2 gene. Sequence of the En2 exon was changed to give it three stop codons—one in each frame. *lox*71 site was moved further 30, to 300 bp upstream of the SA site. FRT site was placed before each of the *lox*P and *lox*2272 sites. Vector was linearized at the single *Spi*el site before electroporation. It was used for studying gene trapping in Japanese wild mouse [15].

In plants, gene trap element DsG [Fig-6f] was based on Ac/Ds transposon of maize and T-DNA. It contains an intact promoter less GUS reporter gene (GUS) and NPTII gene (KAN) as a selectable marker. It also contains triple splice acceptor (A) and *Arabidopsis* intron (I). It acts an exon and intron trap. It was used for the identification and characterization of genes that are expressed in different developmental processes in *A. thaliana* [7]. Another vector, yy322 based on T-DNA vector was used in plants. Luciferase genes in it act as reporter and kanamycin as selectable marker. It contains triplet splice acceptor site. It was used for studying gene expression in *Arabidopsis* [17]. It also works as both exon and intron trap. cGT-1 [Fig-6g] vector derived from an enhancer trap Ds element has GUS reporter gene adjacent to the right border and is preceded by splice acceptor sites and a short plant intron. Kanamycin resistance gene in it acts as selectable marker. It was used to tag genes in Poplar trees [8]. Similar to other two vectors, it also works as both exon and intron trap.

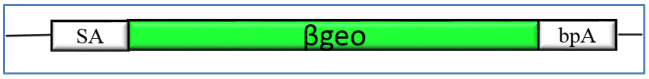


a) βgal gene trap

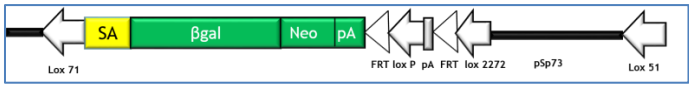
b) pGT4.5 gene-trap



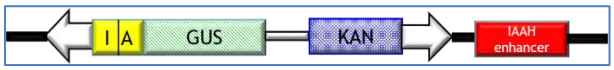
c) ROSA–GFNR



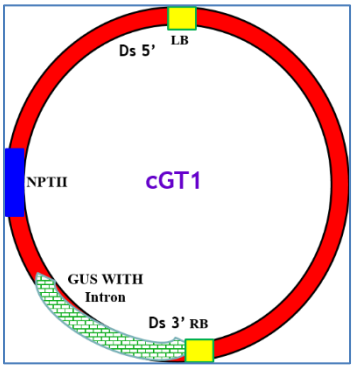
d) SAβgeo gene trap



e) Trap vector pU-21T



f) Gene trap element DsG



g) cGT1

Fig- 6 Gene trap vectors in plants and animals

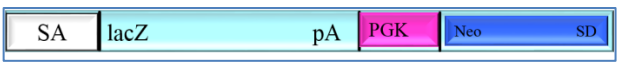
Gene trapping through gene trap vectors leads to simultaneous identification and mutations of genes. This technique has high mutagen city and trapping efficiency. It can detect insertion events in both exons and introns. The presence of IRES in certain vectors helps in translation of the reporter without in frame fusion with the gene, but the reporter gene can disrupt the gene activity (*i.e.* produce inactive proteins). Insertions could be lethal to the cell/organism. Insertion in an intron can lead to alternative splicing, leading to lower levels of wild-type transcripts and often resulting in hypomorphic alleles. Since insertion near 3'region leads to production of fully functional endogenous protein leading to null mutation, it can cause mutation only when inserted in correct orientation. It cannot trap transcription ally inactive genes.

Poly A trap vectors

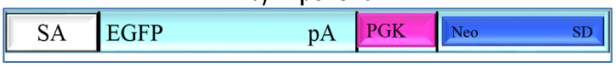
A PolyA trap vector can capture polyadenylation/terminator sequence of endogenous gene. Polyadenylation involves addition of typically 200 Adenine residues to the 3' end of a mRNA. The poly(A) tail is important for stabilizing mRNA. A poly A trap vector consists of reporter and selectable marker under the control of constitutive promoter. Instead of pA signal, it has a splice donor (SD) sequence. A spliced PolyA signal from an endogenous gene is therefore required to generate stable mRNA. Integration of the vector in exonic or intronic region results in production of fusion protein having a part of endogenous gene at N-terminal and reporter gene at C-terminal and also a fusion transcript between marker and 3' exons. Several termination codons following the selectable marker prevent the translation of the 3' trapped exons. It can trap genes that are not normally expressed, or are expressed at very low levels under experimental conditions. It can also trap the 3' ends of genes. Poly A trap vector system represents transcriptional and translational fusion.

Examples of Poly A trap vectors

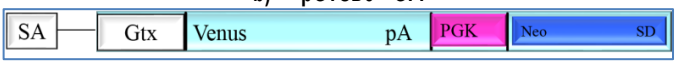
Most of the PolyA trap vectors are based on βgal polyA trap. This vector contains a adenoviral splice acceptor (SA) site immediately upstream of a promoter less lacZ gene and polyadenylation signals (pA) from SV40. Neomycin resistance gene acts as a selectable marker with phosphoglycerate kinase1 constitutive promoter. HPRT splice donor (SD) helps to trap polyA sequence. pGTSD5 is the best example [Fig-7a] . pGTSD5—GFP [Fig-7b] is derived from pGTSD5 by replacing lacZ with EGFP-pA which contains EGFP cassette with a Kozak consensus sequence at the translational initiation codon and two stop codons between the SA site and the initiation codon. Expression occurs only on integration at 5' end. Another two vectors pGT1V1 [Fig-7c] and pGT1V2 [Fig-7d] are also derived from pGTSD5 by replacing lacZ with bicistronic reporter Gtx-Venus (Gtx-9nt sequence in 5'UTR of home box gene Gtx, Venus-a variant of EYFP). SA is linked to reporter through synthetic intervening sequences which helps to increase reporter activity by minimizing conformational interference. pGT1V1 has polyA from SV40 (pA) while pGT1V2 has pA from bovine growth hormone (bpA). PolyA trap vectors IRESβgalNeo –pA [Fig-7e] and +pA [Fig-7f] were constructed by Salminen and co-workers [16] and are useful in the search for genes responding to specific regulatory factors. In plants, yy323 and yy327 polyA trap vectors are T-DNA based vector. They contain triplet splice acceptor site, Luciferase gene as reporter and kanamycin as selectable marker. Terminator sequence of NPTII is deleted. They were used for studying gene expression in *Arabidopsis* [17].



a) pGTSD5

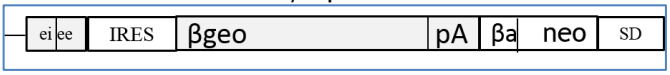


b) pGTSD5—GFP

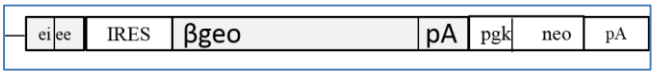


c) pGT1V1

d) pGT1V2



a) IRESβgalNeo –pA



b) IRESβgalNeo +pA

ei-eng2 intron; e: eng2 exon(splice acceptor), β geo: lacZ-neo fusion; pA: polyadenylation signal; IRES: internal ribosomal entry site; βa: human L-actin promoter; SD: mouse Pax2 splice-donor site; pgk: phosphoglycerate kinase promoter

Fig-7 Different PolyA trap vectors

PolyA trap vectors can trap genes that are not expressed in undifferentiated cells and help to lose background intergenic insertions since only insertions in genes generates resistant clones. Any gene can potentially be identified at almost equal probability by using this method regardless of the relative abundance of its transcripts in target cells. These vectors can successfully trap 3' region of the gene. But they show a 3' bias. This bias tends to limit the mutagen city of the insertions. 3' bias is a result of nonsense-mediated decay (NMD) [18]. Nonsense-mediated decay targets transcripts with premature termination codons for destruction by the cellular machinery and is presumably a cellular defense mechanism. For instance because the polyA trap insertions generates fusion transcripts of host gene exons downstream of the neo gene, the stop codon in neo will lead to NMD of transcripts other than those close to the 3' end. NMD can be suppressed by introducing a floxed internal ribosome entry site (IRES) sequence upstream of initiation codons in all the reading frames inserted between the neo gene and the splice donor sequence of UPATrap polyA trap vector.

Secretory trap vector

Secretory trap vectors are specially designed to detect integrations in genes that encode cell surface proteins and uses protein sorting to detect integration events. These special vectors contain a transmembrane portion fused to reporter genes which help its incorporation in the membrane following insertion in genes that encode cell surface proteins. The best example is pGT1.8TM vector developed by Skarnes [19]. It contains a transmembrane of the CD4 type I protein (TM) domain immediately downstream of a splice acceptor (SA) site, followed by the β-geo reporter with its own polyA site. Integration produces fusion protein. When a fusion protein contains a secretory signal (SS) sequence, it is translocated into the cytosol where β-gal activity can be assayed. Integration of the vectors in genes that lack a signal sequence produces a fusion protein with an internal TM domain. Insertions of this kind expose βgeo to the lumen of the ER, where β-gal activity is lost. This technique has been used to capture netrin 1 and neuropilin 2 genes. A disadvantage of this technique is that it cannot differentiate between no insertion and insertion in non-secretory gene. So, improved strategy of detecting insertion in such genes was designed for central nervous system cells. The modified secretory-trap vector incorporates a transmembrane (TM) domain, an internal ribosome entry site (IRES) and an alkaline phosphatase (PLAP) gene.A bicistronic transcript is produced, encoding first a fusion between the endogenous protein and β-geo that localizes to the neuronal cell body and second the PLAP protein,which localizes to the entire cell surface, including the axon. It can distinquish between no insertion and insertion in non secretory gene.

Special vector

Apart from vectors described in proceeding section, there are certain vectors which have been designed to perform special functions.

Flip Rosaβgeo

It is a conditional gene trap vector with two directional site-specific recombination systems. Saβgeo reporter gene resides between pairs of inversely oriented heterotypic frt (frt/F3) and lox (loxP/lox5171) recombinase target sites (RTs). RTs enable two directional inversions of the SaβgeopA cassette when exposed to FLP and Cre recombinases in succession. The conditional gene trapping approach has been implemented for high throughput mutagenesis by the International Knockout Mouse Consortium (IKMC). Equipped with two directional site-specific recombination systems, the vectors enable temporally and spatially restricted mutagenesis in somatic cells [2].

Plasmid rescue vector

These are the most important vectors which are used to trap single-exon genes and identify the trapped gene. Eg. U3NeoSV1 promoter-trap provirus for mouse. It contains ampicillin resistance (amp) gene and a plasmid origin of replication (Ori) flanked by the neomycin resistance (neo) gene in each long terminal repeat (LTR). Selection of mutant cells is by neo gene. Genomic DNA is isolated from mutagenized clones, digested with *EcoRI*, and then ligated and used to transform bacteria. Plasmid isolated from transformed bacteria (ampicillin resistant) and sequenced to identify the gene mutated by the insertion of the promoter-trap vector. The plasmid rescue vectors in plants are based on T-DNA. They contain the reporter gene, selectable marker for both plant and bacteria and an origin of replication which helps it to replicate in bacteria. Genomic DNA from mutant plant is digested with an appropriate enzyme and religated. Ligation products are introduced into *E. coli* and clones are selected on appropriate antibiotic selection. Plasmid isolated from such bacteria thus contains the linked plant DNA.

Removable exon trap vector (pRET)

The RET vector uses an improved poly A-trap strategy for the efficient identification of functional genes regardless of their expression status in target cells. A combination of a potentially very strong splice acceptor and an effective polyadenylation signal assures the complete disruption of the function of trapped genes. Inclusion of a promoter less GFP cDNA in the RET vector allows the expression pattern of the trapped gene to be easily monitored in living cells. Finally, because of loxP-containing LTRs at both ends, the integrated proviruses can be removed from the genome of infected cells by Cre- mediated homologous recombination. Hence, it is possible to attribute the mutant phenotype of gene-trapped cells directly to RET integration by inducing phenotypic reversion after provirus excision. RET virus carries the following three elements in the reverse orientation relative to virus transcription: (i) a gene terminator cassette consisting of a strong splice acceptor (SA), multiple stop codons, an IRES sequence, a GFP cDNA and an efficient poly A signal; (ii) a complete and constitutive HSV to cassette for virus titration and negative selection; and (iii) a unique NEO cassette for the enhanced poly A trap, containing a splice donor (SD) and an mRNA instability signal, but acking a poly A signal. (C) Structure of residual provirus elements following Cre-mediated excision.

Creation of Gene Trapped Lines

For producing gene trapped lines the suitable vectors are transformed in the target cells and transformed cells are screened for mutations to identify insertion events. The selected strains are then subjected to expression and genotypic screen for identification and isolation of trapped genes. These cells are further regenerated to establish 'trapped lines'.

Vector delivery

Vectors are introduced in embryonic stem cells in mice by electroporation or retroviral mediated transfer. In plants they are introduced by electroporation or T-DNA mediated transformation. While electroporation is used for plasmid based vectors, retroviral mediated transfer is used for retroviral trap vectors. The electroporation carries out random insertion of the construct, is better for generating allelic series and results in multiple insertions. The gene trapping by electroporation method does not induce gene silencing or ectopic expression of the reporter gene, but electroporated plasmid DNA is often digested by exonuclease making cloning of insertion site problematic. On the other hand retroviral based method shows a biased insertion into the 5'portion of a gene, including the 5' untranslated region and first intron, produces a higher percentage of null mutations and ensures the integration of a single copy of the entire vector rather than multiple copy. Since the proviral DNA always retains the LONG TERMINAL-REPEAT (LTR) sequence, cloning can be achieved efficiently. But, transfer by this method leads to retroviral-mediated gene silencing and ectopic reporter gene expression.

Identification of insertion events

The transformed cells are selected on the basis of selectable marker. Selectable

markers are genes that confer differential growth ability to the transformed cells and allow them to grow under conditions which are otherwise lethal for the cells. The commonly used selectable markers in gene trapping are neomycin phosphotransferase II(NPTII or neo), kanamycin resistance gene(KAN), hygromycin phophotransferase (hptI) and puromycin resistance (PURO) genes. All of them are antibiotic resistance genes.

Genetic screen

The transformed cells are subjected to genetic screens for identification of the trapped gene and its expression. These screens are broadly divided into two types: expression screen and genotypic screens.

Expression screen

A genetic screen of mutants based solely on the expression pattern of the mutated gene is termed as expression screen. It is done by reporter gene assay, aggregation chimaeras, expression-trapping screen, induction trapping, FACS etc. The transformed cells are subjected to reporter assay to identify the location of the trapped gene. These are grown under controlled conditions to study the expression pattern of the trapped gene and ascertain its function in the organism. Thus, it helps in identification of the gene. Some of the commonly used reporter are βgal/LacZ (*E.Coli* LacZ gene), GUS (*E.Coli* gusA gene or uidA gene), LUC (Firefly *Photinus pyralis* luc gene) and GFP (Gfp gene of jellyfish *Aequorea Victoria*).

There are other methods of expression screening. For instance *Fluorescent activated cell sorting* is also used in gene trapping to separate the cells with different localization of the trapped gene. This technique has been used for cell fractionation and sorting of cell on the basis of their fluorescent activity. *Aggregation chimaeras* are simple and inexpensive technique to generate mouse strains derived from embryonic stem (ES) cells, in which clumps of about eight ES cells are fused with eight-cell-stage embryos, developing into morphologically normal embryos that contain cells derived from both the ES cells and the donor embryo. *Expression-trapping screen* is a gene-trap screen that identifies clones with trapped events in genes expressed in specific lineages. Eg. Secretory trap. *Induction trapping* identifies clones with trapped events in specific signaling pathways.

Genotypic screen

A genetic screen of mutants based solely on the sequence of the mutated gene is the genotypic screen. It is done by using various variants of polymerase chain reaction. Most common is *reverse transcriptase PCR* which involves isolation of mRNA from the cells and using it to produce cDNA through PCR using a trap specific primer and an oligodT primer complementary to the polyA tail of mRNA. RT-PCR generates a cDNA efficiently but the ends of the mRNA may be missed. To avoid this, RACE is carried out. In *5'RACE (Rapid amplification of cDNA ends)* a trap specific primer which anneals to internal sequence is used to produce ssc DNA. This DNA is tailed with an anchored oligoA sequence using terminal transferase enzyme. The RNA is degraded and dscDNA is obtained using a primer that anneals to the anchor sequence. Further amplification is carried out using a primer that anneal to the trap sequence and anchor sequence. The 3' end of these DNA corresponds to the 5' end of the mRNA. The *3'RACE* technique is similar to 5' RACE expects for the primers. Amplification is achieved by a trap specific primer and a primer that anneals to the anchor of the oligodT primer used for synthesis of first strand. *INVERSE PCR* is also used for genotypic screening. In this type of PCR the interrupted gene and the vector is excised using restriction digestion. It is religated to produce circular DNA. The PCR is carried out using primers which are directed in opposite directions and are trap specific. In *ADAPTOR PCR*, the interrupted gene is excised and the adaptors are ligated ate the cut ends. Amplification is achieved by nested primers which are trap specific. Other methods are *TAIL PCR (Thermal Assymetric Interlaced PCR)* and *SPLINKERETTE PCR*.

Establishment of trapped lines

After screening of the progeny for stable reporter gene assay, the trapped lines are established as hypomorphic, loss of function and gain of function allele. Various collaborative works throughout the world are being carried out for trapping every possible gene in an organism. For mouse, trapped embryonic stem cells can be obtained from International Gene Trap Consortium website. The International Gene Trap Consortium website gives a list of all publicly available gene trap cell lines. These lines are available for non-collaborative distribution on nominal handling fees.

Gene Trapping in Use

Gene-trapping methods have been used for the classification of genes based on expression analysis [20-21] development of molecular markers for specific cell types or developmental stages [22-23]; promoter hunting [24]; preparation of knockout mutants for functional analysis [25-26] and for generation of insertional mutants. Some of the genes isolated by gene trapping in mice are TEF-1, Fug 1, Netrin, α-E-Ctenin, Jmj, Bcl-w, Apaf1, HSP90β, Neurophilin 2 and others and in plants are A1 EM, HVT 1, Pyk20, tcup, Lj Cbp 1, eIF-4A 1, EXORDIUM (EXO) and Bidirectional promoter controlling ovule development [13].

Gene trapping has been invariably used in plants to identify novel genes and promoters which can be of immense importance in crop improvement. This method has been used to identify tissue specific and temporally regulated genes and promoters in *Arabidopsis thaliana* [7, 27, 17]. Xiao-hong and co-workers [9] used two promoter-trapping vectors pCBGFP and pEGFPHPH, to transform protoplasts of *Magnaporthe grisea* and established that the promoter trapping technique can be used as a tool for functional genomics analysis. Groover and co-workers [8] reported the use of gene and enhancer trap vectors (cGT-1 and cET-1 respectively) carrying the β-glucuronidase (GUS) reporter gene into the poplar genome via *Agro bacterium tumefaciens* transformation to identify a new class of vascular-expressed gene tagged by enhancer trap line cET-1-pop1-145. They also identified and characterized gene and enhancer trap lines defining genes expressed during primary and secondary vascular development. Their results indicate that many genes expressed during secondary growth and wood formation are also expressed in vascular tissues in other parts of the plant, suggesting that differential or subtractive screens that exclude genes expressed in leaves or other organs discard genes that could play a fundamental role in wood formation. Furthermore poplar gene and enhancer traps provide a new resource allowing direct reference to the poplar genome sequence and help in identifying novel genes of interest in forest biology. Similarly, Filichkin and co-workers [28] isolated a novel gene ET304 (representing a large family of putative transcription factors containing a conserved AT-hook motif and DNA binding domain) from *Populus* (*Populus*: aspens and cottonwoods) using an enhancer trap approach. A gene-trapping vector carrying a GUS/Luciferase dual reporter gene was developed by [29]. Using this system in *Arabidopsis*, 3,737 trapped lines from 26,900 individual T-DNA insertion lines were identified. Sequence determination of the T-DNA insertion loci in the genome of 78 trapped lines identified GUS/Luc fusions with 27 annotated *Arabidopsis* genes which included a subset of transcription factors, protein kinases, regulatory proteins and metabolic enzymes. Their results established the utility of the GUS/Luc dual reporter system as a gene trap reporter for studying plant genome function and also as a convenient dual reporter system for study of gene expression.

Jiang and Ramachandran [30] utilized maize transposon Dissociation (Ds) insertion lines for functional genomics of rice pollen and seed development and as alternative germplasm resources for rice breeding. They established a two-element Activator/Dissociation (Ac/Ds) gene trap tagging system and generated around 20,000 Ds insertion utilization of the maize transposon Ac/Ds gene trap system used to obtain trapped lines with enhanced yield in rice. Using this system certain transcription factors and genes of unknown functions were trapped. Their results suggested that rice could be improved not only by introducing foreign genes but also by knocking out its endogenous genes. Their results also suggested that gene trapping can be used as a technique for improving rice varieties. Similarly, Sharma and co-workers [31] used T-DNA promoter trap lines in *A. thaliana* to identify ovule specific gene. For this, GUS-650 line with uidA reporter gene was inserted in head-to-head orientation in the At3g17140 gene. T-DNA insertion did not affect this gene and the 668-bp

intergenic region functioning as a bi-directional promoter was identified. This experiment helped to establish that 672-bp fragment separating the uidA and At3g17140 genes is a bi-directional promoter specific to ovules. Such a strong tissue-specific promoter can be utilized for many important biotechnological applications like it could be used to drive a pair of antibody fragments to ensure that the two types of molecules are produced in equimolar quantity in the same cell/tissue. Being specific to ovules and developing seeds, this promoter could be employed in improving nutritional quality of seeds through engineering of metabolic pathways. Further, the ability of this promoter to drive gene expression in the embryo sac during early stages of differentiation could be deployed to manipulate reproductive pathways in plants.

Limitations

The genes which are not expressed in ES cells cannot be trapped by gene trapping method in mice. Gene trapping does not always generate null alleles. Trapping is not entirely random but shows preference for large transcription units and genes and all the genes cannot be targeted. Phenotypes of the mutants are unpredictable. One major shortcoming of gene trap approach is that it may not always produce a loss-of-function mutation as alternative splicing could happen, resulting in low levels of wild-type transcripts and often a hypomorphic allele. Due to the time and work necessary for the production of chimeras, one can only study the expression pattern of a very limited number gene of embryonic stages and not of adults. It cannot be used for genes which are permanently switched off and multiple copies of the trap vector can be integrated. The particular gene of interest may not be mutated by this method. Insertion of the trap vector near 5' end results in production of non- functional fusion protein while insertion near the 3' end results in fusion protein which can retain its functional ability. Another concern is inactivating multiple genes. This can happen if the vector insertion is in a region where the coding regions of multiple genes overlap, or is upstream of an RNA gene such as a microRNA that would normally be transcribed along with the gene that has been identified as inactivated. Genes coding on the opposite strand can also be inactivated by vector insertion. One more problem is associated specifically with retroviral based vectors. The retroviral based vectors are subject to gene silencing. If the reporter gene gets integrated in a gene that is part of alternative splicing, resulting in low levels of wild-type transcripts and often a hypomorphic allele. One way to minimize such risk is to work with two or more different ES cell lines with insertions in different parts of the gene at the same time in order to increase the chance of obtaining a complete loss-of-function allele.

Conclusion

Originally developed as a method of random insertional mutagenesis, gene trapping has been extended as a method to monitor gene expression profiles. The ability to efficiently trap, sequence and detect the expression of genes, regardless of their transcriptional activity, has made gene trapping an exceptional tool for gene discovery. The gene trap methodology is a powerful tool to characterize novel genes and analyze their importance in biological phenomena. A rapid and cost-efficient method that is ideally suited for large-scale mutagenesis and full-genome exploration. It is gene-oriented and the mutated gene is known from the beginning due to its reverse genetics approach. Trapping generates a single-cell reporter of transcriptional activity, rather than assessing messenger RNA (mRNA) abundance in a cell population. Functional studies may easily be accomplished directly on the trapped cells or on organisms derived from them. The expression pattern of the trapped gene can be studied by observing the reporter gene activity. The reporter gene can be used to study spatial and temporal regulation of the trapped genes. Reporter expression has also been used to study environmental and hormonal responsive genes. The vector sequences provide a molecular tag for characterization of the disrupted gene. Gene trap can easily establish an allelic series consisting of hypomorphic, loss-of-function allele and even gain-of-function allele with the different gene trap lines for the same gene. Gene trap mutagenesis represents a powerful tool for reproductive genetics and for reproductive disease modelling. It is especially powerful for mutating genes essential for embryonic development and the reproductive process. Genes expressed at very low levels can also be identified. Insertional mutagenesis with trap vectors yields experimental data that are otherwise obtained in two separate approaches, namely mutagenesis and reporter gene expression. Due to their small size gene trap vectors change the genomic context of the trapped gene only minimally and a faithful expression pattern is obtained. Gene trap insertions create null alleles in most cases. Gene traps have some intrinsic properties that make them complementary to RNA-based approaches such as DNA microarrays. It is possible to monitor the reporter gene expression already in the heterozygous state and to identify genes that show no or only subtle phenotypes when mutated. Gene trap vectors used for this technique are very efficient in creating mutations as well as studying expression of the trapped genes. Compared to other mutagenesis technique, this technique is efficient in creating widespread mutations but often lead to unpredictable phenotypes.

Application of review: The review describes the significance of gene trapping in identifying novel genes. This article emphasizes the ability of gene trapping to efficiently trap, sequence and detect the expression of genes, regardless of their transcriptional activity and establish it as an exceptional tool for gene discovery.

Review Category: Regulation of gene expression

Abbreviations: Ac/Ds-Associaton/Dissociation; CaMV-Cauliflower mosaic virus; DNA- Deoxoribose nucleic acid; ES- embryonic stem; ET- Enhancer trap vector; FACS- Fluorescent activated cell sorting; GFP- green fluorescent protein gene; GT- Gene trap vector; GUS- β-glucuronidase; IRES- Internal ribosome entry site; lacZ- β-lactose galactosidase gene; LTR- Long terminal repeat; MCS-Multiple cloning site; NMD-Nonsense mediated decay; NPTII-Neomycin phosphotransferase II gene; pA- polyadenylation site; PCR- Polymerase chain reaction; PGK- Phosphoglycerate; RACE- Rapid amplification of cDNA ends; RNA- Ribosenucleic acid; SA-Splice acceptor site; SD-Splice donor site; T-DNA-Transferred DNA; uid A- β -D-glucuronidase gene.

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Ethical Approval

We hereby declare that this article does not contain any studies with human participants or animals performed by any of the authors.

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