

EXPRESSION ANALYSIS OF GENOME

Structure

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16.1 INTRODUCTION

You learned about the analysis and modification of the genome in Unit 15. In the present Unit, you will learn about the principles of genomic expression analysis. The simplest way to define gene expression analysis is the study of how genes are transcribed to produce functional gene products, such as functional RNA species or proteins. Understanding gene control helps distinguish between abnormal or unhealthy cellular processes and normal ones such as differentiation.

Objectives

After studying this Unit, you would be able to:

- ❖ describe the concept and function of reporter genes,
- ❖ comprehend the basics of temporal and site-specific gene expression,
- ❖ explain the concept of gene silencing,
- ❖ explain the basics and mechanism of RNA interference,
- ❖ comprehend the biological functions of RNA interference, and
- ❖ discuss the applications of RNA interference.

16.2 REPORTER GENES

A reporter gene (commonly called as reporter) in molecular biology is a gene that is attached to a regulatory sequence of another gene of interest in plants, animals, cell cultures, or bacteria that researchers pay attention to. These genes are referred to as reporters because the expressed traits in an organism can be easily detected and quantified, or they are the selectable markers. Reporter genes are frequently used to study whether a specific gene has been taken up or expressed in a targeted cell or population. Figure 16.1 shows the mechanism of the expression of a reporter gene.

The gene of interest and the reporter gene are cloned in a DNA construct before being transferred into the cell or organism. This construct typically takes the shape of a plasmid, a circular DNA molecule that is present in prokaryotic or bacterial cells in a culture. The expression of the reporter gene is considered as a signal for successful uptake of the gene of interest by the cell or organism and hence its study is essential.

Fluorescent and luminous proteins are frequently utilized as the reporter genes that produce visually recognizable properties. Examples include the enzyme luciferase, which catalyzes a reaction with luciferin producing luminescence, green fluorescent protein (GFP) gene of the jellyfish expresses a protein that exhibits green fluorescence when exposed to light in the blue to ultraviolet range, and the red fluorescent protein from the gene dsRed [fr]. Research related to the genetic transformation of plants has traditionally used the GUS gene, although luciferase and GFP are also increasingly popular.

One of the most commonly utilised reporter genes in bacteria is the lacZ gene of *E. coli*, which codes for the protein beta-galactosidase. The enzyme expressed by this gene gives bacteria a blue colour when they are grown on medium containing the substrate analogue X-gal. Another example of a bacterium-selectable marker that also serves as a reporter is the chloramphenicol acetyltransferase (CAT) gene, which confers resistance to the antibiotic chloramphenicol.

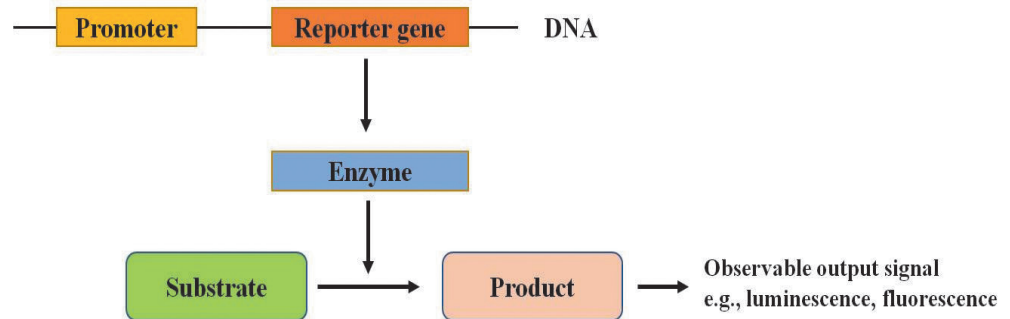


Fig. 16.1: The mechanism of the expression of a reporter gene

16.2.1 Transformation and Transfection Assays

Only a small percentage of the population responds well to several transformation and transfection techniques, which are used to express a modified or foreign gene in an organism. Therefore, a technique for locating those rare instances of effective gene uptake is required. For instance, the galactosidase system can be injected with IPTG to produce constitutive or inducible reporter gene expression. When utilized in this manner, reporter genes generally express themselves under the regulation of their own promoters, irrespective of the inserted gene of interest's promoter (DNA region that triggers gene transcription). As a result, the expression of the reporter gene is unrelated to the expression of the gene of interest, which is advantageous when the gene of interest is expressed only under specific conditions circumstances or in difficult-to-access tissues.

16.2.2 Gene Expression Assays

Reporter genes can result in the production of a protein that has minimal direct effects on the organism or cell culture. Reporter genes can be activated, that is, they can be expressed constitutively by cloning the gene of interest together with the reporter gene where both the genes are regulated by the same promoter. This results in the transcription of a messenger RNA that code for two protein-coding sequences (fusion protein). Despite being fused, it is crucial that both proteins are able to correctly fold into their active conformations and engage in interactions with their substrates. In order to ensure that the reporter and gene product do not restrict each others function, a piece of DNA encoding a flexible polypeptide linker region is typically inserted while creating the DNA construct. Additionally, reporter genes may be induced to express throughout growth. In these situations, the reporter gene is expressed by the use of trans-acting elements, such as transcription factors.

Researchers need to find pathways, small molecule inhibitors, and activators of protein targets to develop new drugs. Also, reporter gene assays are increasingly being used in high throughput screening (HTS). As the reporter enzymes, such as firefly luciferase, can be direct targets of minute molecules and obstruct the interpretation of high-throughput sequencing data, novel coincidence reporter designs featuring artefact suppression have been developed.

16.2.3 Promoter Assays

Reporter genes can be employed to test for a specific promoter's activity in an organism or cell. There isn't a distinct "gene of interest" in this instance; the reporter gene is simply put under the target promoter's control, and the reporter gene product's activity is measured. Results are typically expressed in terms of activity under "consensus" promoters, which are known to strongly drive gene expression.

SAQ 1

Fill in the blanks:

- These genes are referred to as reporters because the expressed traits in an organism can be easily detected and quantified and hence can be used as
- The lacZ gene of *E. coli* codes for the protein
- A bacterium selectable marker that also serves as a reporter is the gene, which confers resistance to the antibiotic chloramphenicol.

16.3 TEMPORAL AND SITE-SPECIFIC GENE EXPRESSION

The action that converts a gene's informational content into the creation of a functional product, often a protein, is referred to as the gene expression process. Although certain genes, such as those that code for transfer RNAs, ribosomal RNAs, and a few other short RNAs, have RNA as their functional output but majority of genes in cells code for proteins. The mechanism of gene expression through the processes of genetic transcription and translation is represented in Figure 16.2.

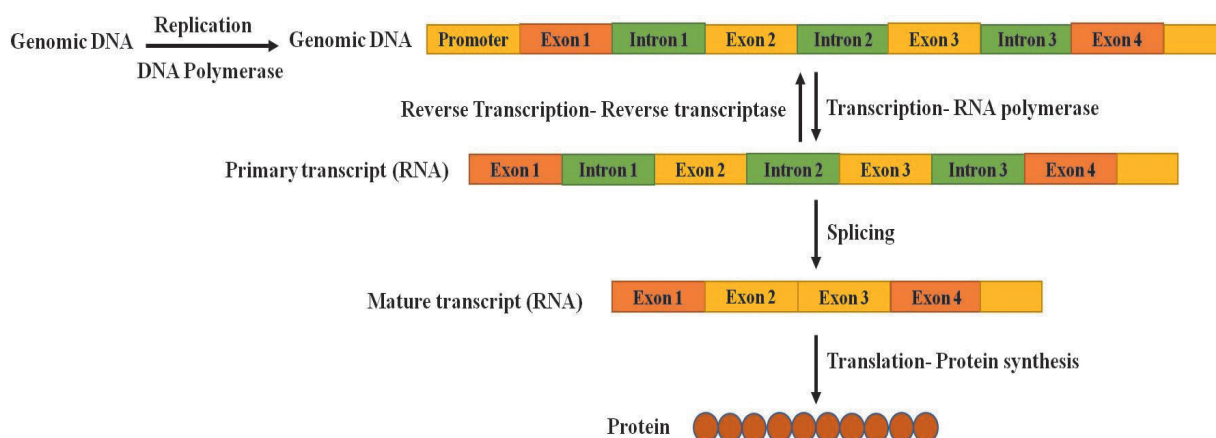


Fig. 16.2: Gene expression: A gene's or genes' phenotypic expression through the processes of genetic transcription and translation

16.3.1 Temporal Analysis

The triggering of genes inside an organism's particular tissues at particular developmental junctures is known as spatiotemporal gene expression. The intricacy of gene activation patterns varies greatly. Some are simple and unchanging, like the pattern of tubulin, which is always expressed in living cells. The expression of some, on the other hand, varies drastically within seconds or from cell to cell, making them extremely complicated and challenging to anticipate and model. Since a cell's identity is determined by the collection of genes it actively expresses, geographical and temporal variation is essential for the diversity of cell types present in mature organisms. There could only be one kind of cell if gene expression was constant over time and space.

For example, the wingless gene, a member of the *wnt* gene family, is expressed in alternating stripes separated by three cells in the fruit fly *Drosophila melanogaster* during the early stages of embryonic development. This pattern disappears by the time the embryo becomes a larva, but the wingless gene is still present in certain tissues, such as the imaginal discs of the wings, which are patches of tissue that eventually develop into the adult wings. The spatiotemporal pattern of wingless gene expression is determined by a network of regulatory interactions, which includes the effects of multiple unique genes such as even-skipped and Krüppel.

What causes variations in a gene's expression throughout time and space? A retrogressive challenge exists in determining what resulted in the initial changes in gene expression since present expression patterns only depend on past expression patterns. Symmetry breakdown is the process resulting in temporal and geographical variation in uniform gene expression. For instance, the messenger RNA (mRNA) for the genes *nanos* and *bicoid* in *Drosophila* embryonic development is deposited in the poles of the egg by maternal cells prior to implantation, resulting in asymmetric expression of these genes in the oocyte.

16.3.2 Site-specific Analyses

Analyses of gene expression at specific sites are crucial for comprehending tissue functions. Although DNA-related technologies have advanced quickly, it is still difficult to analyze a tissue's full genome for expression at a given space. Information on cell types and site-specific gene expression is necessary for a complete knowledge of tissue functions. Imaging techniques like *in situ* hybridization have proved effective for analyzing the site-specific gene expression of tissue. However, with these techniques, only a few marker genes may be evaluated concurrently. It is necessary to employ a next-generation DNA sequencer together with an adequate site-specific sampling technique in order to analyze many genes at once. Furthermore, site-specific multi-omics (such as genomes, transcriptomics, and proteomics) studies will be required to fully comprehend tissue functions. The tiny sample sizes in these procedures necessitate the employment of a sensitive analyzer. However, a number of extremely sensitive analytical methods have been created recently. For instance, utilizing next-generation DNA sequencers,

specific gene expression, or genome analysis approaches for single cells have been described. More information on the corresponding functions is now available than was previously possible, due to the combination of tissue microdissections and high throughput examination of single cells. To fully use analytical technologies, a technique for site-specific cell or microdissection collection from a tissue should be established.

SAQ 2

Fill in the blanks:

- a) The action that converts a gene's informational content into the creation of a functional product, often a protein, is referred to as the process.
- b) The triggering of genes inside an organism's particular tissues at particular developmental junctures is known as gene expression.
- c) Imaging techniques like have proved effective for analyzing the site-specific gene expression of tissue.

16.4 GENE SILENCING

A unique gene regulation mechanism called RNA silencing controls the number of transcripts either by inhibiting transcription *via* transcriptional gene silencing (TGS), or by causing sequence-specific RNA to degrade *via* posttranscriptional gene silencing (PTGS), or by RNA interference (RNAi). Silencing of particular genes has been linked to regulatory processes such as antiviral defence mechanisms, chromosomal remodeling, transposon silencing, and gene regulation. It has also been linked to two primitive processes, quelling in fungi and co-suppression in plants.

The first PTGS/RNAi studies were made in plants, but afterward, nearly all eukaryotic species, including parasites, insects, protozoa, nematodes, flies, mouse, and human cell lines, were shown to be experiencing RNAi-related events. Quelling in fungi, co-suppression or PTGS in plants, and RNAi in the animal kingdom are the phenotypically diverse but mechanistically related forms of RNA interference. Recently, it has been discovered that other aspects of eukaryotic cells' naturally occurring RNAi processes, such as microRNA production and heterochromatinization, also exist.

Gene silencing caused by RNAi is a two-step mechanism, as per the extensive genomic and pharmacological study. In the first phase, an enzyme that mimics RNase III breaks down dsRNA into small interfering RNAs (siRNAs), which are 21 to 23 nucleotides long. The siRNAs join RISC (RNA-induced silencing complex), an RNase complex that acts on the corresponding mRNA and destroys it, in the second phase. In various organisms, helicases, dsRNA endonucleases, Dicer, and RNA-dependent RNA polymerase have all been found to play significant roles in RNAi. A few of these elements also

manage the maturation of multiple species by processing a significant number of non-coding RNAs, or microRNAs. MicroRNA biosynthesis and function share common characteristics with RNAi processes. Because of its exceptional effectiveness and specificity, RNAi is being viewed as a crucial tool for gene-specific therapeutic activities that affect the mRNAs of disease-related genes, as well as for functional genomics.

16.4.1 Unravelling RNA Silencing

It would be wise to give a general summary of the homology-dependent RNA silencing mechanism and highlight its salient characteristics in order to comprehend it.

Post-Transcriptional Gene Silencing in Plants

RNA silencing in plants came to light accidentally while looking for transgenic petunia blooms that were supposed to be more purple. The goal of R. Jorgensen's lab in 1990 was to increase the activity of the chalcone synthase (*chsA*) gene, an enzyme essential for the synthesis of anthocyanin. Unexpectedly, few transgenic petunia plants carrying the *chsA* coding area controlled by a 35S promoter lost both transgene and endogenous chalcone synthase activity, leading to the development of white or variegated sectors in many of the flowers. Run-on transcription assays in isolated nuclei showed that the decrease in cytosolic *chsA* mRNA was not associated with decreased transcription. The term "**co-suppression**" was created by Jorgensen to characterize the loss of mRNAs from both the transgene and the endogene. Both sense and antisense transgenes have the potential to cause PTGS, and biochemical data show that comparable processes may be at play in both situations. It is important to note that the co-suppression phenomena have been proven in metazoans and mammals in addition to plants.

RNA Interference and Quelling

With increasing reports of PTGS in plants, independent findings of homology-dependent gene silencing in fungal systems were also made. They were referred to as quelling. Quelling was discovered while attempting to alleviate the output of an orange pigment produced by the *Neurospora crassa* gene *al1*. A plasmid containing a 1,500-bp segment of the *al1* gene's coding sequence was used to transform a strain of *N. crassa* with a wild-type *al1+* gene (orange phenotype). A few transformants had their phenotypes stabilized to albinism. While the native *al1* mRNA was significantly decreased in the *al1*-quelled strain, the quantity of unspliced *al1* mRNA was comparable to that of the wild-type strain, demonstrating that quelling and not the transcription rate changed the level of mature mRNA in a homology-dependent manner.

Owing to the discovery of Fire et al., who firmly showed the biological nature of inducers in gene silencing by administering pure dsRNA directly into the body of *Caenorhabditis* worms, the phenomena of RNAi initially gained attention.

16.4.2 Important Features of RNA Silencing

Investigations on numerous organisms with labels such as quelling in fungi, PTGS in plants, RNAi in mammals, and virus-induced gene silencing (VIGS)

have independently led to the discovery of a universal paradigm for gene control. The dsRNA serves as the inducer and the target RNA is destroyed in a homology-dependent manner. Additionally, the degradative machinery needs a set of proteins that are common to most species in terms of both structure and function. siRNA production and systemic transmission of silencing from its site of initiation are two characteristics that are present in the majority of these activities.

16.4.3 Components of Gene Silencing

Understanding the silencing mechanism has been attempted using genetic and molecular methods. In order to look for mutants deficient in RNA interference, quelling, or PTGS, genetic screens were performed on the algae *Chlamydomonas reinhardtii*, the nematode *Caenorhabditis elegans*, the fungus *Neurospora crassa*, and the plant *Arabidopsis thaliana*.

Dicer

Members of the RNase III family are one of the few nucleases which specifically target dsRNAs and cleave them with 3'-hydroxyl and 5'-phosphate termini and 3' overhangs of 2 to 3 nucleotides. This enzyme was given the name Dicer because it can convert dsRNA into evenly sized short RNAs (siRNA). The nucleases in question have been preserved throughout evolution in flies, worms, fungi, animals, and plants. Dicer has four diverse domains, including an amino-terminal helicase, a dsRNA binding domain, two RNase III motifs, and a PAZ domain (a 110-amino-acid domain found in proteins like Argo, Piwi, and Pinhead/Zwille). It also shares this domain with the QDE2/RDE1/Argonaute family of proteins, which has been genetically linked to RNAi by separate studies. Dicer's tandem RNase III domains are suggested to catalyze cleavage.

RNase III enzyme fully degrades dsRNA to produce fragments of 12 to 15 bp, which are about half as large as siRNAs. The RNase III enzyme functions as a dimer, whereas the Dicer enzyme contains two catalytic domains in each monomer, one of which differs from the consensus catalytic sequences. The Dicer enzyme breaks down dsRNA with the help of two compound catalytic centers. The model for the production of 23- to 28-mer diced siRNA products were developed as a result of the recent discovery of the crystal structure of the RNase III catalytic domain. According to this model, the two internal domains with selective sequence identity forfeit their functional relevance as the dimeric Dicer folds on the dsRNA substrate to yield four compound catalyst sites, while the two terminal sites with the best levels of homology to the consensus RNase III catalytic sequence continue to function. As a result, the diced products, which are twice as large as the typical 12- to 15-mer pieces, appear as digests of the RNase III enzymes. A similar model suggests that specific adjustments to the Dicer structure may alter the distance joining the two active terminal sites, leading to the production of siRNAs with different sizes and species-specific imprints. Clearly, this model has to be verified using the Dicer crystal structure.

RNA-Induced Silencing (RISC) Complex and the Guide RNAs

The inability of cellular isolates exposed to a Ca^{2+} dependent nuclease (micrococcal nuclease, that can digest both RNA and DNA) to degrade the homologous mRNAs and the lack of this impact with DNase I treatment served as evidence that RNA was a critical element of the nuclease activity. The RNA-induced silencing complex (RISC) was named after the sequence-specific nuclease activity that was seen in the cellular extracts and was responsible for abasing target mRNAs.

SAQ 3

Fill in the blanks:

- Quelling in fungi, co-suppression or PTGS in plants, and RNAi in the animal kingdom are the phenotypically diverse but mechanistically related forms of
- The biosynthesis and function share common characteristics with RNAi processes.
- The term was created by Jorgensen to characterize the loss of mRNAs from both the transgene and the endogene.
- Independent findings of homology-dependent gene silencing in fungal systems were referred to as
- The enzyme convert dsRNA into evenly sized short RNAs (siRNA).
- The was named after the sequence-specific nuclease activity that was seen in the cellular extracts and was responsible for abasing target mRNAs.

16.5 RNA INTERFERENCE

RNA interference (RNAi) is the procedure by which the targeted mRNA molecules are neutralized by RNA molecules to block the targeted gene expression. RNAi involves the targeting of RNA molecules for the sequence-specific inhibition of gene expression by double-stranded RNA *via* transcriptional or translational repression. Andrew Fire and Craig C. Mello split the 2006 Nobel Prize in Physiology or Medicine for their 1998 publication of RNAi in the nematode *Caenorhabditis elegans*. Since, RNAi and its regulatory capabilities were discovered; it has become clear that RNAi has enormous prospects in suppressing desired genes. It is currently acknowledged that RNAi is precise, stable, and effective. Small interfering RNA (siRNA) and microRNA (miRNA) are two classes of tiny ribonucleic acid (RNA) molecules that are essential to the RNAi pathway. Post-transcriptional silencing happens as a result of mRNA degradation because protein translation is halted. The pre-transcriptional silencing process of RNAi can limit transcription by catalyzing DNA methylation at genomic sites corresponding to complex miRNA or siRNA. RNAi plays a crucial part in protecting cells against parasitic nucleotide sequences (such as transposons and viruses) and also affects the organisms development.

Numerous eukaryotic and animal cells naturally include the RNAi pathway. It is started by the enzyme Dicer, which breaks lengthy dsRNA molecules into short double-stranded pieces called small interfering RNAs (siRNAs), which have 21 to 23 nucleotides. The sense, that is, passenger strand and the antisense, that is, guide strand of each siRNA are unwound to form two single-stranded RNAs (ssRNAs), respectively. The protein Argonaute 2 then cleaves the passenger strand (Ago2). The guide strand is integrated into the RISC while the passenger strand is destroyed. The target mRNA is then bound and degraded by the RISC assembly. The guide strand interacts with a complementary strand in an mRNA molecule, which activates Ago2, a catalytic subunit of the RISC, to initiate cleavage.

16.5.1 Mechanism of RNA Interference

It is a method for gene regulation that restricts the amount of transcript in two ways:

- Reducing transcription (**transcriptional gene silencing**);
- Decreasing the quality of the RNA generated (**post-transcriptional gene silencing**)

The following steps can be used to explain RNA interference's mechanism:

- With the help of an enzyme known as **Dicer**, long, double-stranded RNA is cut into manageable pieces. Small interfering RNA (siRNA), is the name given to these tiny bits.
- The **siRNAs** are transported through the complex of RNA-induced silencing. The RNA is triggered as the duplex unwinds.
- One of the strand of the double-stranded RNA is deleted when the siRNA binds to the **Argonaute** protein, which promotes RNA breakdown and prevents translation. The mRNA target sequences are bound by the remaining strand.
- To control the target sequence, the Argonaute protein either cleaves the mRNA or enlists the help of other agents.

16.5.2 Cellular Mechanism

Short double-stranded RNA molecules engage with the catalytic RISC component argonaute in the cytoplasm of a cell to begin the RNA-dependent gene silencing process known as RNA interference. RNAi is regulated by RISC and is started by these interactions (Fig. 16.3). If the dsRNA is exogenous (either from laboratory manipulations or infection with an RNA-based virus), the RNA is transported straight into the cytoplasm and cuts into brief fragments by Dicer. Similar to pre-microRNAs generated from RNA-coding genes inside the genome, endogenous dsRNA can likewise originate within the cell. The main transcripts from these genes are first processed in the nucleus to create pre-distinctive miRNA's stem-loop structure before being released to the cytoplasm. Thus, the foreign and endogenous dsRNA routes meet at the RISC.

By making the ribonuclease Dicer more active, which binds to and cleaves human short hairpin RNAs (shRNAs) or exogenous dsRNAs into double-stranded fragments with 20–25 base pairs and a 2-nucleotide overhang at the 3' end, exogenous dsRNA triggers RNAi. The RISC-Loading Complex subsequently divides these siRNAs into single strands and incorporates them into an active RISC (RLC). Dicer-2 and R2D2 are parts of RLC, which is necessary to connect RISC and Ago2. TATA-binding protein-associated factor 11 (TAF11) induces Dcr-2-R2D2 tetramerization, which increases the binding affinity to siRNA and facilitates RLC formation tenfold. The R2-D2-Initiator (RDI) complex would change into the RLC by association with TAF11. R2D2 possesses tandem double-stranded RNA-binding domains that enable it to recognise the thermodynamically stable end of siRNA duplexes, while Dicer-2 recognises the opposite, less stable extremity. Asymmetric loading is brought about by Ago2's MID domain, which locates the thermodynamically stable end of the siRNA. The "passenger" (sense) strand, whose 5' end is abandoned by MID, is emitted, and the "guide" (antisense) strand is preserved and collaborates with AGO to build the RISC. After joining the RISC, siRNAs base-pair to their target mRNA and cleave it to prevent it from being used as a translation template. In contrast to siRNA, a miRNA-loaded RISC looks for possible complementarity among cytoplasmic mRNAs. miRNAs bind to mRNAs in the 3' untranslated region (UTR) where they normally show weak complementarity, obstructing ribosome access and preventing translation. Exogenous dsRNA is recognised and bound by an effector protein called R2D2 in *Drosophila* and RDE-4 in *C. elegans*, which increases dicer activity. It is uncertain what mechanism results in this length selectivity in this protein, which only binds to long dsRNAs.

siRNA

A class of double-stranded RNA (20–24 bp) known as small interfering RNA (siRNA) functions in the RNA interference pathway by preventing the production of genes that have complementary nucleotide sequences to siRNA, hence causing mRNA degradation. A short RNA species (siRNA) with both sense and antisense polarity was found as the by-product of RNA degradation by PTGS, providing a critical insight into the process. The siRNA molecules with specific chemical structures are produced and assembled as dsRNA molecules. In contrast to non-silenced control plants, siRNAs were initially identified in plants that were either undergoing virus-induced gene silencing or co-suppression. Later on, siRNAs were discovered in both *Drosophila* embryo extracts which were showing RNAi *in vitro*, and *Drosophila* embryos which had received dsRNA injections. These siRNAs were found in *Drosophila* tissue culture cells where RNAi was triggered by adding >500-nucleotide-long exogenous dsRNA. The production of siRNA was shown to be the distinguishing characteristic of any homology-dependent RNA-silencing method.

miRNA

MicroRNAs are short noncoding RNAs with a length of about 22 nucleotides that are involved in posttranscriptional gene silencing, regulation of gene expression and have important biological functions, particularly during development. The phenomena of RNAi comprises of both the silencing

mechanisms caused by the external dsRNA and the endogenously produced gene silencing effects of miRNAs. Although mature miRNAs share structural similarities with siRNAs made from external dsRNA, miRNAs must first go through a significant amount of post-transcriptional modification. A miRNA is produced in the cell nucleus from a much longer RNA-coding gene as a **primary transcript called a pri-miRNA**, which is then processed by the microprocessor complex into a 70-nucleotide stem-loop structure called a pre-miRNA. This complex is made up of the dsRNA-binding protein DGCR8 and the **RNase III enzyme Drosha**. Since Dicer binds to and cleaves the dsRNA part of this pre-miRNA to create the mature miRNA molecule which can be incorporated into the RISC, siRNA and miRNA, all have similar downstream biological mechanisms. Epstein-Barr virus (EBV) was the first human virus shown to express miRNAs. Since then, several microRNAs in viruses have been identified.

Since miRNAs, particularly those found in animals, frequently have imperfect base pairing to a target and impede the translation of several other mRNAs with identical sequences, siRNAs produced from lengthy dsRNA precursors vary from miRNAs in this regard. Comparatively, siRNAs often base-pair flawlessly and cause mRNA cleavage in only one particular target. Different argonaute proteins and dicer enzymes are used in *C. elegans* and *Drosophila* to handle siRNA and miRNA.

Three prime untranslated regions and microRNAs

Regulating sequences in the 3' untranslated regions (3'UTRs) of mRNAs are often involved in RNAi post-transcriptionally. Such 3'-UTRs frequently have binding sites for regulatory proteins and miRNAs. miRNAs can reduce the number of certain mRNAs that are expressed by binding to particular locations in the 3'-UTR and either preventing translation or result in the degeneration of the transcript. Additionally, the 3'-UTR may contain silencer sequences that bind repressor proteins that prevent mRNA production. Frequently, microRNA response elements (MREs) are found in the 3'-UTR. The miRNAs attach to the MRE sequences. These 3'-UTRs frequently include these motifs. MREs account for nearly half of all regulatory motifs found in the 3'-UTRs.

RISC activation and catalysis

During RISC activation, the other anti-guide strand, also known as the passenger strand, is weakened. Although strand selection is unaltered by the direction in which dicer cleaves the dsRNA prior to RISC integration, the guide strand is often the one whose 5' end is less firmly linked to its counterpart. Instead, the more stable 5' end of the passenger strand may be bound by the R2D2 protein, which would then operate as the distinguishing factor.

It is unclear how the active RISC finds mRNAs that are compatible in a cell. Translation of the mRNA target is not necessary for RNAi-mediated degradation, despite the fact that it has been suggested that the cleavage process is tied to translation. P-bodies, also known as GW bodies or cytoplasmic bodies, are areas of the cytoplasm where argonaute proteins are localized and miRNA activity is likewise concentrated. P-bodies have high rates of mRNA degradation. P-bodies are thought to be a crucial location in the RNAi process since their disruption reduces RNAi's effectiveness.

16.5.3 Transcriptional Silencing

Many eukaryotes employ RNAi pathway components to maintain the structure and organization of their genomes. Pre-transcriptional downregulation of genes is achieved by modification of histones and the ensuing induction of heterochromatin formation; this procedure is known as **RNA-induced transcriptional silencing (RITS)**, and it is carried out by a protein complex known as the RITS complex. It is unclear how the RITS complex influence the development and organization of heterochromatin. To sustain the existing heterochromatin regions, RITS assembles a complex of siRNAs that are homologous to the localized genes and firmly attach to the methylated histones. This complex also acts co-transcriptionally to damage any nascent pre-mRNA transcripts that are started by RNA polymerase.

Dicer is necessary to produce the first complement of siRNAs that target future transcripts; therefore, it makes sense that while its maintenance is not dicer-dependent, the development of such a heterochromatin area is. It has been proposed that heterochromatin maintenance works as a self-reinforcing feedback loop, with new siRNAs being produced from sporadic nascent transcripts by RdRP and incorporated into regional RITS complexes.

16.5.4 Variation Among Organisms

The capacity of various organisms to absorb foreign dsRNA and utilize it in the RNAi pathway varies. RNAi effects can be systemic and heritable in *C. elegans* and plants in contrast to *Drosophila* or humans. In plants, siRNAs are assumed to be transferred between cells by plasmodesmata to allow RNAi to spread. Heritability results from the methylation of promoters that RNAi has targeted; the altered pattern of methylation is replicated in each subsequent cell generation.

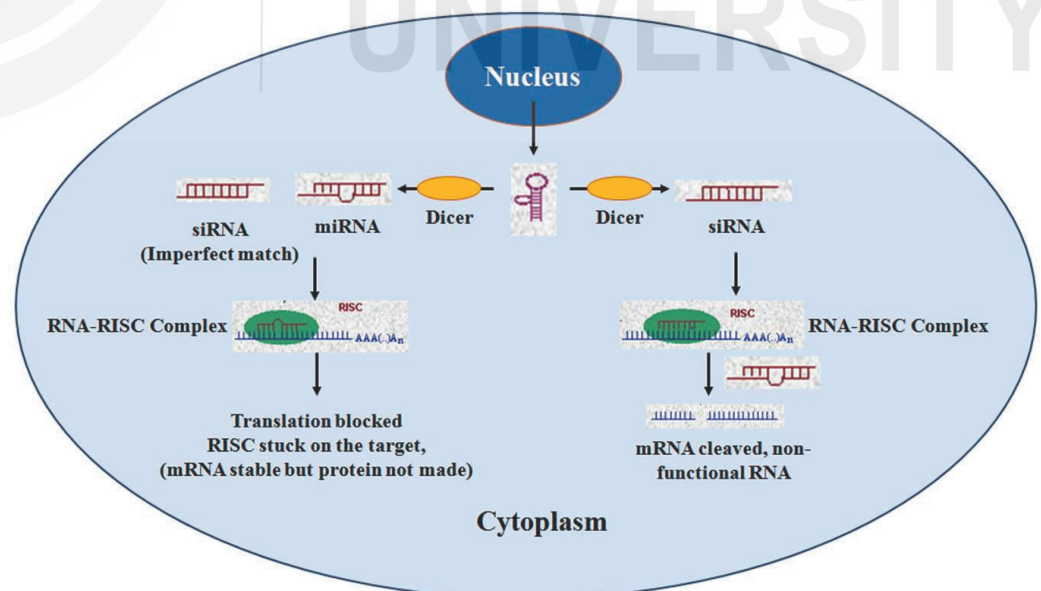


Fig. 16.3: The mechanism of RNA Interference

The targeting of endogenously produced miRNAs can be used to draw broad general distinctions between plants and animals. While in plants, miRNAs usually cause direct mRNA fragmentation by RISC because they are perfectly

or almost perfectly complementary with their target genes. In animals, miRNAs typically have more divergent sequences and suppress translation. It is possible to prevent translation initiation factors from interacting with the polyadenine tail of the mRNA in order to produce this translational effect.

Leishmania major and *Trypanosoma cruzi* are two examples of eukaryotic protozoa that completely lack the RNAi pathway. Several fungi, including the model organism, *Saccharomyces cerevisiae*, lack any or all of the components

16.5.5 Biological Functions of RNAi

Immunity

RNAi is a crucial component of the immune system's defence against viruses and other foreign genetic material, particularly in plants where it may additionally stop transposons from self-replicating. Many dicer homologs are expressed by plants like *Arabidopsis thaliana*, which are adapted to respond differently to various viruses. It became apparent that induced gene silencing in plants affected the whole plant and could be transferred by grafting from stock to scion plants before the RNAi pathway was well understood. Since then, it has been determined that this process is an attribute of the plant immune system that allows the entire plant to respond to a virus after the first localised contact. As a result, several plant viruses have evolved highly effective defences against the RNAi response. These include viral proteins that bind dicer-produced small dsRNA fragments with single-stranded overhang ends. In some plant genomes, endogenous siRNAs are also produced in response to bacterial infection. These effects might be a component of a broader response to pathogens that inhibits any host metabolic activity that promotes infection.

RNAi can cause an antiviral response in some animals, despite the fact that plants typically express more variations of the dicer enzyme than mammals. RNAi is crucial for antiviral innate immunity in juvenile and adult *Drosophila* and is effective against viruses like the *Drosophila* X virus. Worms that overexpress components of the RNAi process are immune to viral infection and produce higher levels of argonaute proteins in response to viruses.

There is a paucity of information on the function of RNAi in mammalian innate immunity. There may be evidence for an RNAi-dependent mammalian immune response in the presence of viruses that can disrupt the RNAi response in mammalian cells, regardless of the theory's criticism that it lacks sufficient proof. It has been demonstrated that mammalian cells have a functioning antiviral RNAi pathway.

Downregulation of genes

The two main functions of endogenously expressed miRNAs are morphogenesis timing and maintaining undifferentiated or partially differentiated cell types, such as stem cells, which include both intronic and intergenic miRNAs. This involves the regulation of development and translational repression. Since the bulk of the genes controlled by miRNAs in plants are transcription factors, their activity is extremely broad and controls the expression of important regulatory genes, such as transcription factors and

F-box proteins, to regulate whole gene networks during development. miRNAs are connected to the development of cancers and the disruption of the cell cycle in many species, including humans. Here, miRNAs can act as both tumor suppressors and oncogenes.

Evolution

Parsimony-based phylogenetic analysis suggests that an early RNAi pathway was likely already present in the most recent common ancestor of all eukaryotes; the absence of the process in some eukaryotes is regarded to be a derived trait. This primitive RNAi system contained at least one dicer-like protein, one argonaute, one PIWI protein, and one RNA-dependent RNA polymerase, possibly with other biological functions. These elements were most likely present in the eukaryotic crown group and may have closer functional connections with RNA degradation mechanisms like the exosome. This is supported by a large-scale comparative genomics investigation. Additionally, this research reveals that the RNA-binding argonaute protein family is homologous to and originally descended from components of the translation initiation machinery and is found in eukaryotes, the majority of archaea, and some bacteria (including *Aquifex aeolicus*).

16.5.6 Applications of RNAi

RNAi pathway for gene knockdown

Gene knockdown is a technique for reducing the expression of particular genes in an organism. This is accomplished by the naturally occurring RNAi mechanism. A double-stranded siRNA molecule that is generated with a complementary sequence to the gene of interest is used in the gene knockdown method. Once the Dicer enzyme begins to break down siRNA, the RNAi cascade commences. The procedure results in the degradation of the mRNA and the elimination of any instructions needed to produce specific proteins. Using this technique, researchers can partially, but not completely, suppress the expression of a particular gene.

Medications

The strategy of using RNAi treatments to shut down genes has proven to be effective, as demonstrated by randomised controlled clinical trials. The treatments in this class, which are expanding, work by reducing the expression of the proteins that particular genes are able to encode using siRNA. To date, regulatory agencies in the US and Europe have authorized four RNAi drugs: patisiran (2018), givosiran (2019), lumasiran (2020), and inclisiran (2020 in Europe with anticipated US approval in 2021).

While all of the RNAi therapeutics that have been currently approved by regulatory bodies targeting liver-related illnesses; other drugs that are still in the research phase focus on a variety of other conditions, such as cystic fibrosis, cardiovascular problems, carcinoma, bleeding issues, gout, alcohol use disorders, and eye problems.

Delivery mechanisms

For RNA interference to achieve its therapeutic potential, siRNA must be efficiently delivered to the cells of the target tissues. However, a number of

obstacles need to be removed before it may be applied therapeutically. For instance, "naked" siRNA is prone to a number of challenges that lower its therapeutic efficiency. Additionally, bare RNA can activate the innate immune system and be destroyed by serum nucleases once siRNA has reached the circulation. Unmodified siRNA molecules cannot easily cross the cell membrane because of their size and extremely polyanionic (carrying negative charges at several locations) nature. Therefore, siRNA needs to be synthetic or enclosed in nanoparticles. If therapeutic dosages are not adjusted, siRNA transport across the cell membrane may result in unexpected toxicities, and siRNAs may have off-target effects (e.g., unexpected suppression of genes with partial sequence complementarity). Since their effects are diminished with each cell division, frequent treatment is necessary even after they have entered the cells. Lipid nanoparticles and conjugates are two strategies that aid in siRNA distribution to target cells in response to these possible problems and hurdles.

Lipid nanoparticles

The core of lipid nanoparticles (LNPs) is modelled following liposomes, which are lipid shell-encased aqueous cores. Large unilamellar vesicles (LUVs), which can be 100 nm in size, are the resting place for a subset of liposomal structures utilized to carry medications to the target tissues. Plasmids, CRISPR, and mRNA are examples of LNP delivery systems that may be used to encase nucleic acids.

Conjugates

Targeted delivery for RNAi therapies using siRNA conjugates is an alternative to LNPs (e.g., aptamers, carbohydrates, GalNAc, peptides, antibodies). In addition to other cardiometabolic disorders including hypertension and non-alcoholic steatohepatitis, therapeutics utilizing siRNA conjugates that are developed for uncommon or inherited diseases such as hemophilia, acute hepatic porphyria (AHP), hereditary ATTR amyloidosis (NASH), and primary hyperoxaluria (PH).

Biotechnology

There have been numerous documented other applications for RNAi, such as the manufacturing of insecticides, crops, and food. The RNAi pathway has produced a wide range of products, including nutrient-fortified plants, arctic apples, decaffeinated coffee, nicotine-free tobacco, and hypoallergenic crops. A variety of new products could be made with the help of RNAi based technology.

Viral infection

The creation of two unique antiviral therapies was one of the first uses of RNA interference in medicine. The first type targets viral RNAs. Targeting viral RNAs has been shown in multiple studies to reduce the replication of multiple viruses, including adenovirus, hepatitis A, HIV, HPV, hepatitis B, SARS coronavirus respiratory syncytial virus (RSV), SARS-CoV, influenza virus, and measles virus. Targeting the host cell's genes is the second strategy used to stop early viral invasions. For example, blocking the chemokine receptors (CXCR4 and CCR5) can stop HIV entry.

Cancer

Conventional chemotherapy can kill cancer cells with effectiveness, but since it lacks the ability to differentiate between normal and malignant cells, it frequently results in serious side effects. Various studies have shown that RNAi can give a more targeted method of preventing tumor growth by targeting genes relevant to cancer (i.e., oncogene). Additionally, it has been proposed that RNA interference (RNAi) may increase the susceptibility of cancer cells to chemotherapeutic agents, providing a complementary therapeutic approach to chemotherapy. Inhibiting cell invasion and migration is yet another possible RNAi-based therapy. RNA interference therapies treat cancer by suppressing particular genes that promote malignancy. By complementing the cancer genes with RNA interference (RNAi), for example, by keeping the mRNA sequences consistent with the RNAi drug, this is achieved. RNA interference (RNAi) sequences should ideally be chemically altered to enhance their ability to bind to cancer cells. RNAi uptake is regulated and monitored by the kidneys.

Neurological diseases

Neurodegenerative illnesses may potentially be treated using RNAi techniques. The amount of A peptide, which is connected with the origin of Alzheimer's disease, may be considerably lowered by selectively targeting Amyloid beta-producing genes (such as BACE1 and APP) *via* RNA interference, according to studies conducted in cells and mice. Additionally, these silencing-based methods for treating Parkinson's illness and polyglutamine disorder show encouraging outcomes.

Transgenic plants

Transgenic crops express dsRNA that has been carefully chosen to silence important genes in insect targets. These dsRNAs are exclusively meant to affect insects that express specific gene sequences. As a proof-of-concept, a 2009 study showed that the dsRNAs could kill any one of four species of fruit flies while harming none of the others.

Insecticides

RNAi is being developed as a pesticide using a range of methods, including genetic modification and topical treatment. Certain insects' midgut cells absorb the dsRNA molecules as part of the environmental RNAi mechanism. Some insects experience systemic effects as a result of the signal reaching every part of their body (referred to as systemic RNAi).

There are no harmful effects in animals given RNAi at dosages millions of times higher than what is anticipated for human exposure. RNAi affects various Lepidoptera species (butterflies and moths) in different ways. To understand how RNAi functions in certain taxa of insects, models such as *Drosophila* spp., *Spodoptera* spp., *Locusta* spp., *Tribolium castaneum*, *Helicoverpa armigera*, *Nilaparvata lugens*, *Bombyx mori*, and *Apis mellifera* have been extensively used. While *Glossina morsitans* contains three Ago2 genes, *Musca domestica* only has two.

Food

RNAi has been used to genetically modify plants such that they produce less natural plant toxins. These methods make use of the RNAi phenotype that is persistent and heritable in plant populations. Cotton seeds are a good source of dietary protein; however, they should not be consumed by humans since they naturally contain the hazardous terpenoid gossypol. A critical enzyme in the production of gossypol, delta-cadinene synthase, has been reduced in cotton stocks using RNA interference (RNAi), without impacting the production of the enzyme in other parts of the plant, where gossypol is crucial for protecting plant against pest damage. The amounts of allergens in tomatoes have been successfully reduced through development efforts, and plants have been fortified with nutrient-rich antioxidants.

Stimulation of immune response

The innate immune system, which may be further separated into acute inflammatory responses and antiviral responses, is in charge of controlling siRNA. Small signaling molecules known as cytokines provide messages that trigger the inflammatory response. Tumor necrosis factor (TNF-), interleukin-6 (IL-6), interleukin-1 (IL-1), and interleukin-12 (IL-12) are a few of them. Inflammation and antiviral responses produced by the innate immune system result in the release of pattern recognition receptors (PRRs). These receptors aid in classifying infections as bacterial, fungal, or viral. More PRRs should be included in siRNA and the innate immune system in order to assist it in identifying various RNA structures. In the event of an infection, the siRNA is therefore more likely to trigger an immunostimulant response.

SAQ4

Fill in the blanks:

- The is the procedure by which the targeted mRNA molecules are neutralized by RNA molecules to block the targeted gene expression.
- The and are two classes of tiny ribonucleic acid (RNA) molecules that are essential to the RNAi pathway.
- A miRNA is produced in the cell nucleus from a much longer RNA-coding gene as a primary transcript called a
- The was the first human virus shown to express miRNAs.

16.7 SUMMARY

- A test gene with quantifiable expression is known as a reporter gene. It can be present on plasmids that have their T-DNA integrated into the genome of a cell. Examining how those genes are expressed after a cell has undergone a transformation is important.

- Reporter genes are those sequences that may be examined to ascertain how altered genes are expressed. It is possible to do a reporter gene test by calculating the total amount of protein synthesised. They often have luminous properties and provide visual clues for precise estimates. Examples include, Green fluorescent proteins, luciferase, octopine synthase, etc.
- The method through which a gene in a cell is activated to produce RNA and proteins is called gene expression. The RNA or the protein generated from the RNA, as well as the function of the protein in a cell can be used to quantify gene expression.
- RNA interference has been called post-transcriptional gene silencing, transgenic silencing, and quelling in the past.
- RNA interference, also known as "RNA-mediated interference," is a method for RNA-guided control of gene expression. It involves double-stranded RNA suppressing the gene expression with similar nucleotide sequences.
- The enzyme dicer, which breaks down double-stranded RNA (dsRNA) into 20–25 base pair-long short double-stranded fragments, starts the RNAi pathway. The RNA-induced silencing complex (RISC) is subsequently formed by incorporating one of each fragment's two strands, referred to as the guide strand, which base pairs with complementary sequences.
- Post-transcriptional gene silencing occurs when a messenger RNA (mRNA) molecule pairs with the guide strand base, causing the mRNA to be cut down by argonaute, which is the RISC's catalytic component.
- When created by RNA-coding genes in the cell's own genome, short RNA fragments are referred to as microRNAs (miRNA) and small interfering RNAs (siRNA) accordingly.
- Many model species, including *Drosophila melanogaster*, *Arabidopsis thaliana* and *Caenorhabditis elegans* have been used to study the RNAi process.
- Both in cell culture and in live animals, the selective and powerful impact of RNAi on gene expression makes it an invaluable research tool. Synthetic dsRNA put into cells can cause the suppression of the target genes of interest.

16.8 TERMINAL QUESTIONS

1. What are reporter genes? Describe their role in transformation and transfection assays.
2. Describe the role of reporter genes in gene expression and promoter assays.
3. Briefly explain about the type of RNAs that play role in RNA interference?

4. Explain the concept of temporal and site-specific gene expression and their analysis.
5. Briefly explain the mechanism of gene silencing.
6. Define RNA interference. Describe the mechanism and biological functions of RNAi.
7. Describe few applications of RNAi.

16.9 ANSWERS

Self-Assessment Questions

1. a) selectable markers, b) beta-galactosidase, c) chloramphenicol acetyltransferase (CAT)
2. a) gene expression, b) spatiotemporal, c) *in situ* hybridization
3. a) RNA interference, b) microRNA, c) co-suppression, d) quelling, e) Dicer, f) RNA-induced silencing complex (RISC)
4. a) RNA interference (RNAi), b) Small interfering RNA (siRNA); microRNA (miRNA), c) pri-miRNA, d) Epstein-Barr virus (EBV)

Terminal Questions

1. Refer to Section 16.2 and Subsection 16.2.1.
2. Refer Subsections 16.2.2 and 16.2.3.
3. Refer Subsection 16.5.2.
4. Refer to Section 16.3.
5. Refer to Section 16.4.
6. Refer to Section 16.5, Subsections 16.5.1 and 16.5.5.
7. Refer Subsection 16.5.6.