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Noncoding DNA is not 'Junk" but a necessity for origin and evolution of biological complexity

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Abstract

All eukaryotic genomes contain, besides the coding infor-mation for amino acids in different proteins, a significant amount of noncoding sequences, which may or may not be transcribed. In general, the more evolved or biologically complex the organisms are, greater is the proportion of the noncoding component in their genomes. The popularity and success of "central dogma of molecular biology" during the last quarter of the 20th century relegated the noncoding DNA sequences to a mortifying status of "junk" or "selfish", even though during the pre- "molecular biology" days there were good indications that such regions of the genome may function in as yet unknown ways. A resurgence of studies on the noncoding sequences in various genomes during the past several years makes it clear that the complex biological organization demands much more than a rich proteome. Although the more popularly known noncoding RNAs are the small microRNAs and other similar species, other types of larger noncoding RNAs with critical functions in regulating gene activity at various levels are being increasingly identified and characterized. Many noncoding RNAs are involved in epigenetic modifications, including imprinting of genes. A comprehensive understanding of the significance of noncoding DNA sequences in eukaryotic genomes is essential for understanding the origin and sustenance of complex biological organization of multicellular organisms.

Keywords : noncoding RNA, microRNA, Xist, nuclear speckles, hsr-omega.

Introduction

For the most part of their life cycle, the unicellular organisms need to regulate their genetic programmes only in temporal axis but the advent of multicellularity, and consequent division of labour between cells of an organism, necessitated an integration of the temporal regulation with the spatially different genetic programmes of the various cell types present in the organism. The increasing biological complexity depends not only on evolution of novel "functions" or proteins but also on more complex regulatory networks. A greater importance of more complex regulatory networks over creation of "new" genes in generation of biological complexity was emphasized by evolutionary biologists and geneticists even in the "pre-molecular biology" era (e.g., see Mayr[1]). Paradoxically, however, the advent of "central dogma of molecular biology" and the consequent excitement resulting from understanding of functions and regulations of individual protein-coding genes during the last quarter of 20th century undermined studies on the regulatory networks. A strong faith in the "central dogma" resulted in the common perception that sequences of DNA in the genome that do not have a protein-coding function or are not involved in production of proteins are likely to be irrelevant. Consequently, a majority of molecular biological studies during the past few decades were generally driven by the concept that the noncoding DNA is "junk" or "selfish" or "parasitic"[2-4]. The sequencing of genomes of large number of species, ranging from bacteria to human, reestablished the earlier inferences of classical geneticists and cytologists that much of the DNA in genomes of higher organisms does not carry typical "genes" or protein-coding genetic information. Interestingly, while to some workers this confirmation appeared to strengthen the concept of "junk" DNA, it also fuelled a greater curiosity for possible functions of the noncoding DNA because the proportion of noncoding DNA in the genome has, in general, increased with increasing biological complexity. Thus while noncoding DNA is almost non-existent in bacteria, it can make up as much as 90% or more of the genome in higher organisms[5-7].

Results of recent comparative genomic studies and better appreciation of cellular networks reconfirm that the genetic differences between any two related species are more due to changes in the 'noncoding" DNA rather than in the protein-coding genes. Thus while human genome has ~25 fold more DNA compared to the fruit fly, the number of protein-coding genes appears to be even less than 2-fold greater. Enhancing the complexity of regulatory circuits allows a greater variety of combinations of similar numbers of proteins and thus more complex networks, which are required to fashion more diverse structures and organizations.

The primary way in which the genetic information affects cell function is by the process of transcription resulting in the formation of RNA. Classical studies employing metabolic labeling of newly synthesized RNA and/or reassociation kinetics indicated that a large proportion of nuclear DNA, substantially greater in diversity than the estimated number of protein-coding genes, was actually transcribed and surprisingly, bulk of this heterogeneous nuclear RNA (hnRNA) species was found to never reach the cytoplasm and thus not translated into proteins[8-12]. Recent genomic and RNomic studies have reconfirmed these studies[5, 13-15].

The discovery of post-transcriptional gene silencing and subsequent understanding of the phenomenon of RNAi[16-19] sparked a remarkable appreciation of the functional significance of noncoding DNA sequences, especially the noncoding RNAs. In keeping with this excitement, numerous reviews on significance of noncoding DNA are now available[5, 20-32]. In the following, the major pathways through which the noncoding sequences in genomes may affect cell functions are briefly discussed. Specific roles of noncoding RNAs in imprinting are discussed by P. K. Gupta in another article in this issue (page 51).

Some noncoding DNA sequences regulate transcription of genes and are essential for biological complexity

The sizes of cis- and trans-regulating DNA sequences (promoters, enhancers, silencers, boundary elements etc) which regulate the expression of protein-coding (or other) genes is variable and in many cases, these are actually longer than the transcribed parts to provide for modular regulation. As stated earlier, much of the evolutionary differences in related species depend on modulating the regulation of a given gene rather than the structure or function of the protein encoded by it. Since transcription factor binding sites are short sequences, rather rapid alterations in regulation of genes can occur by small changes in the base sequences. Thus new target sites for binding of the transcription factors may be created or the existing ones eliminated by a few base changes or by insertion/mobilization of transposable elements (see Wray[33] for a recent discussion). Thus one of the very important functions of the noncoding DNA sequences is to provide for networked regulation of transcriptional activity of genes. Additionally, the physical organization of chromatin and its higher order packaging in the 3-dimensional space of the nucleus are critical for cell type specific gene expression patterns[34-36]. However, the extent and nature of genomic DNA that is required for providing this kind of "information" is as yet little understood.

Roles of noncoding but transcribed sequences in protein coding genes

Less than 2% of human genomic DNA actually accounts for amino acid-coding regions. However, the genes coding for proteins also often contain substantially greater lengths of transcribed but untranslated regions like introns, 5'- and 3'-untranslated regions (UTRs) etc. These noncoding but transcribed introns and the UTRs, have very significant roles in generation of protein diversity (through alternative splicing of introns), in regulating the half-lives of mRNA and in their location/targeting in cells[37, 38]. A particularly remarkable example of the great potential of introns in generating protein diversity is the *Dscam* gene of *Drosophila* which has the potential to generate, through alternative splicing, as many as ~38000 varieties of protein isoforms; these numerous isoforms appear to be involved in guiding the different axons as they grow to reach their target sites[39, 40].

A large variety of noncoding RNAs with vital functions are produced in cells

One group of noncoding RNAs, which has a long history of acceptance as being essential for RNA processing and translation, includes *transfer RNAs* (tRNA), *ribosomal RNAs* (rRNA*), small nuclear RNAs* (snRNA), *small nucleolar* RNAs (snoRNA), small *Cajal body-specific RNAs* (scaRNA) etc[41-43].

The noncoding DNA sequences, which are independently transcribed but are neither translated nor directly involved with the process of translation, are intriguing. As mentioned earlier, the existence of such transcripts has been known since 1960s[20, 21] but these were generally ignored because of the bias against "selfish" or "junk" DNA. However, the recently renewed interest continues to reveal an increasing number of noncoding RNA species, which are essential for a variety of basic and vital functions in cells.

Small noncoding RNAs

The small noncoding RNAs include several classes with overlapping or distinct functions. *MicroRNAs* (miRNA) and *short-interfering RNAs* (siRNA) are the smallest functional RNAs ranging in size from 19 to 25 nucleotides and have received considerable attention during the last ten years since they regulate gene activity through RNA interference at multiple levels like chromatin organization, transcription, post-transcriptional processing, stability and translation of mRNA etc. In view of their multiple roles, these short RNAs integrate the regulatory networks and thus play significant role in

the origin and evolution of biological complexity[44-52]. *Efference RNA* (eRNA) and *mirtrons* refer to noncoding RNAs derived from intronic sequences of protein coding or other noncoding genes. They also function like miRNAs to regulate networks of gene activity by interfering with transcription or translation of the target[7, 53, 54].

Piwi-interacting RNAs (piRNAs) or *repeat-associated short interfering RNAs* (rasiRNAs) are germ cell-specific 26-31 nucleotide long RNA molecules involved in silencing of mobile genetic elements and repetitive sequences[55].

Promoter RNAs (pRNA) are associated with the promoter regions of genes and are required for RNA-directed epigenetic remodeling and transcriptional silencing of RNA-targeted promoters by direct binding of the antisense strand of siRNAs either to DNA or to a sense-stranded RNA corresponding to the promoter[56].

tmRNAs (tRNA-like and mRNA-like RNA), also known as 10Sa RNA or ssrA RNA, are complex, dual-functional, small, stable RNA species present in bacteria that mimic both a tRNA and mRNA. They recognize and recycle the stalled ribosomes[57, 58].

Guide RNAs (gRNA) are involved in RNA editing seen in certain organisms/genes and are part of the editosome; they are partially complementary to the pre-mRNAs to be edited[59, 60].

Signal recognition particle RNA or the 4.5S and SRP RNA in bacteria and eukaryotes, respectively, is a major structural component of the signal recognition particle (SRP) RNA-protein complex in the cytoplasm of cells that binds to the mRNA of proteins destined for secretion from the cell[61, 62].

Large noncoding RNAs

Besides the above families of small noncoding RNAs, a variety of larger transcripts with diverse functions are known and their number is steadily increasing (see http://biobases.ibch.poznan.pl/ncRNA[62]). Some large noncoding RNAs, whose functional properties are better understood, are discussed below.

Some noncoding RNAs regulate chromatin organization and transcriptional status of entire chromosomes or chromosome regions

Organisms with XX/XY chromosome sex-determination system, like *Drosophila* and mammals, need dosage compensation to compensate for difference in the dosages of sex-chromosome-linked genes between the homo- and hetero-gametic sexes[63]. In mammals, dosage compensation is achieved by inactivation of one of the two X-chromosomes in somatic cells of females[64], while in *Drosophila*, single X-chromosome in male cells becomes hyperactive to attain the transcriptional level of the two X-chromosomes in female cells[65, 66]. In spite of these opposing operative mechanisms, it is interesting that in both cases, noncoding RNA species are primarily responsible for the specific chromatin organization that determines the activity levels of the X-chromosomes. The large nucleus-limited noncoding RNA of the human *Xist* gene (or its homologue in other mammals) is produced only by the inactive X-chromosome and the Xist transcripts spread in cis to paint the entire length of the X-chromosome. The Xist transcripts provide a platform for binding of chromatin remodeling complexes to keep that X-chromosome in an inactive state. It is interesting that the decision to allow *Xist* transcription from only one of the two X-chromosomes is also based on production of another noncoding RNA, the Tsix RNA from the complementary strand of the *Xist* gene (for recent reviews, see Yang and Kuroda[67] and Wutz and Gribnau[68]). In the case of *Drosophila*, the single X-chromosome in male cells becomes hyperactive because the roX1 and roX2 noncoding RNA species "paint" the entire X-chromosome and thereby allow the assembly of protein complexes (the Msl complex) which set the X-chromosome transcription in male cells at a higher level[69-71]. Thus both in mammals and *Drosophila*, noncoding RNAs provide a mechanism for organized recruitment of the required proteins, which establish the desired chromatin organization, condensed for inactivity of female mammalian X chromosome or loose for hyperactivity of male *Drosophila* X-chromosome.

In an analogous manner, the monoallelic expression of many imprinted genes in mammalian cells requiring locally altered chromatin organization is regulated by noncoding transcripts[67] (also see P. K. Gupta in this issue).

Some noncoding RNA species bind with different families of proteins and regulate their activity

The elaborate RNA processing events in nucleus and subsequent transport of the mature mRNA to cytoplasm involve a large variety of proteins, many of which are RNA-binding. The high dynamicity of gene expression, and consequently of the RNA processing events, requires the different RNA processing and transporting proteins to dynamically shuttle between different protein complexes and sometimes to also remain in an inactive state. Since most of the RNA-processing proteins have long half-lives and are cyclically recruited to different sites in the nucleus, these proteins need to be sequestered when inactive. It is likely that their sequestration is catalyzed by some RNA molecules specifically

produced for such purposes. Some insights into these events have been obtained through studies on stressed cells since it is known that RNA processing and transport are dramatically inhibited in cells exposed to heat shock or similar other cellular stresses[72, 73].

A complex and dynamic organization of the processing factors within the nucleus is reflected in the presence of a variety of nucleoplasmic speckles and other bodies, besides the more obvious nucleolus and chromatin[74, 75]. A large number of studies have shown that conditions which inhibit nuclear transcriptional and RNA processing activities, cause the different classes of RNA processing proteins to get sequestered in distinct nuclear compartments or speckles. Some examples are: i) aggregation of the various speckled domains like interchromatin granules or splicing speckles[76-78], omega speckles[21, 79], Lamin A/C speckles[77, 80, 81], coiled or Cajal bodies[82, 83], paraspeckles[84] or other heterogeneous nuclear RNA binding protein (hnRNP) speckles[85] etc, ii) release of snRNPs from the splicing speckles[76], iii) formation of novel nuclear stress bodies in human cells[86], iv) formation of cytoplasmic stress granules in plant and several animal cell types[87]. Studies on some of these structures have helped identify a few noncoding RNA species which regulate the dynamic redistribution of RNA processing proteins in relation to cellular activity.

Studies in our laboratory with *Drosophila* cells have shown that the heat shock RNA omega-nuclear or hsrω-n noncoding RNA is essential for organizing a special nuclear compartment, the omega speckles, for storage/sequestration of the members of hnRNP family and other related proteins in normal as well as stressed cells[21, 79, 88]. During development, this noncoding RNA is expressed, in a regulated manner, in almost all cells types of *Drosophila*[89, 90]. Both over and under-expression of this noncoding RNA has severe consequences for the organism[91] (Mallik and Lakhotia, unpublished).

A somewhat similar function seems to be carried out by the noncoding satellite III (sat III) transcripts in human cells following heat shock[92]. Heat shock induces transcription of the noncoding sat III sequences, located on centromeric heterochromatin of human chromosomes 9 and 11[86]. A variety of RNA processing proteins, RNA polymerase II and heat shock transcription factor etc get sequestered with these transcripts as stress granules in heat shocked human cells[92].

In both the above cases, the non-coding hsrω-n or the sat III transcripts appear to serve an important function through sequestering specific classes of proteins when they are not required to be active. Paraspeckles are also known to contain at least one noncoding RNA species[84]. Such noncoding RNA species provide a paradigm for regulation of the RNA processing machinery in cells[92]. It is highly likely that analogs of the hsrω-n, sat III or the paraspeckle RNA species are involved in regulating activities of the other classes of cellular proteins involved in RNA metabolism. These remain to be discovered.

A different kind of trans-regulation is seen in the case of the non-coding *Xlsirts* RNA, which along with the coding VegT RNA is involved in organization of the cytokeratin network within the vegetal cortex of *Xenopus* oocytes, indicating that some ncRNAs help maintain the structural integrity of eukaryotic cytoskeleton[93].

Another paradigm for the role of noncoding RNAs in regulating protein function is the recent discovery of the noncoding HSR1 transcripts in mammalian, *Xenopus*, *Drosophila* as well as *Caenorhabditis* cells whose binding with the heat shock factor is essential for activation of the heat shock genes under conditions of cellular stress[94, 95].

ncRNA and epigenetic changes

Many recent studies show that a relatively large proportion of ncRNAs are essential for several complex molecular and structural epigenetic modifications that take place in eukaryotic cells. The modulation of chromatin organization leading to establishment and maintenance of silencing or hyperactivation of the entire X-chromosome in mammals or *Drosophila*, respectively, noted above, is one example of the epigenetic changes regulated by ncRNAs. “Opposite strand transcription” also plays significant roles in epigenetic modifications of the Hox gene clusters and thus regulation of their developmental expression[96, 97]. Comparable “opposite strand transcripts” are involved in regulation of the imprinted genes[67]. pRNA mediated gene regulation at the chromatin level through histone methylation/acetylation is another instance of epigenetic regulation by ncRNAs[56]. The double stranded *NRSE* smRNA (Neuron Restrictive Silencing Element small modulatory RNA) functions as an endogenous inducer of neuronal differentiation by directing multipotent neural stem cells towards a neuronal lineage thereby acting as a key regulator of cell fate choice[98]. Some ncRNAs also have been implicated in DNA methylation and heterochromatin formation in centromeric and other regions[99, 100].

Noncoding RNAs as hubs for coordination of different cellular networks

It is known that most of the RNA-binding proteins recognize their target RNAs through small

sequence and/or structural motifs[101]. Thus a noncoding RNA may provide binding sites for a variety of proteins and thereby act as a hub to regulate the functional competence of a variety of proteins belonging to different networks in the cell[91]. It is significant to note that many of the large noncoding RNAs are characterized by rapid sequence divergence even between related species, a feature that was initially taken as evidence for their being "junk" or "selfish". However, when viewed from the viewpoint of evolution of new regulatory circuits, this property becomes significant. Since these RNAs affect cellular activities by binding with different proteins, which need only short target sites, bulk of the sequence of such noncoding genes is less constrained and, therefore, may accumulate random mutations without compromising functions of their RNA products. Further, as in the case of evolution of new promoter activities, some of these mutations in noncoding transcripts may create new protein binding sites resulting in "novel" functions. Thus the large noncoding RNA species, like the small miRNA etc, act as "hubs" which regulate and integrate a variety of cellular networks.

Conclusions

Understanding gene function in terms of protein synthesizing activity has been a major achievement of modern biology. It is also obvious that the effective proteome in any species can be much larger than that originally encoded by the genome due to the versatile processes like alternative splicing (including trans-splicing), RNA-editing, post-translational modifications of proteins etc. Nevertheless, the enormous diversity in the structure and organization of multicellular organisms cannot be explained only in terms of the proteome. The complexity of biological organization calls for much greater complexity of regulation. A correlation between the proportion of noncoding DNA and biological complexity by itself indicates that such DNA sequences, either through their cis-regulatory actions or through the production of noncoding RNAs, have very significant roles in origin and sustenance of biological complexity. The elucidation of functions of several noncoding RNA species in recent years has encouraged more in depth studies on the noncoding DNA component in genomes. In this context, a remark of great evolutionary biologist, Ernst Mayr[1], made prior to the "molecular biology revolution", is worth noting: "The rate of evolutionary change in the macromolecules of important structural genes is presumably largely controlled by the system of regulatory genes. The number of new questions this opens up is legion". Appreciation of significance of the noncoding DNA has indeed opened up this "legion". Mayr[1] further observed "The day will come when much of population genetics will have to be rewritten in terms of interaction between regulator and structural genes. This will be one more nail in the coffin of beanbag genetics. It will lead to a strong reinforcement of the concept that the genotype of the individual is a whole and that the genes of a gene pool form a unit". While during the past three decades the success stories of discovery of new proteins and their genes by the followers of "central dogma" did not generally encourage integrative studies on genome's functions, the time is now ripe to fulfill Mayr's hope of understanding the genomes as integrated entities rather than "beanbags" of protein coding genes. In the absence of such holistic and integrative understanding, biotechnological gene manipulation programmes will remain ineffective.

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