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Review Article

The Potential of *U6* and Its Copies in the Regulation of the Human Genome

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ABSTRACT

Non-coding RNAs are conformed by a large repertoire of RNA molecules with unimaginable tridimensional structures and functions. Small nuclear RNAs are an essential part of the spliceosome machinery, which is crucial for proper mRNA maturation. It is important to add that *U6*, one of the four snRNAs forming the spliceosome has been extensively studied. Full-length *U6* (*U6*-1) loci are widely dispersed throughout the genome (200-900 copies), but a few *U6* full-length loci have been identified to date as potentially active genes. The importance of *U6* to carry out, together with other snRNAs, the catalytic activity and recognition of annealing target sequences, its evolution in the genome and the fact that the genome has many *U6* copies and pseudogenes, its association with retrotransposition, as well as their implication in diseases is discussed in this review.

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Introduction

The human genome sequencing demonstrated that nearly 98-99% of the genome is actively transcribed, but only 1.5% produces a protein product [1-3]. Our genome is constituted by a great variety of functional elements, almost unknown until a few years ago [1, 3]. Furthermore, large fractions of the human transcriptome that do not codify for proteins form an amazing large group of functional non-coding RNAs (ncRNAs) [4]. These versatile molecules have a large repertoire of types and functions that make them key players in the regulation of gene expression and genome stability (Table 1) [4-6].

snRNAs (small nuclear RNAs) are ncRNAs sized ~150 nt in length that have a key function in the spliceosome. In addition, they are crucial for proper mRNA maturation [7]. *U6*, one of the five snRNAs forming the spliceosome, has been widely studied due to their peculiar promoter region organization, recognized by the Pol III machinery [7, 8]. Besides, contrary to the other snRNAs, which either show gene copies in tandem or are organized in homogeneous repeats, the full-length *U6* locus (*U6*-1, located at 15q23) is widely dispersed throughout the genome [7, 8]. Earlier studies showed ~200 *U6* copies dispersed throughout the genome; however, more recent studies estimate ~900 *U6* genes copies [9]. Furthermore, it became apparent that the large majority of these copies are *U6* pseudogenes, containing nucleotide substitutions and truncations [10]. Only a few *U6* copies have been identified to date as potentially active genes; therefore, further studies are necessary to understand the function, if any, of each of these copies and pseudogenes [10]. This poses a challenge, not only due to the number of copies, but also because of the sequence similarities.

Given the large repertoire of types and functions that have been described for ncRNAs, as well as their tissue and development specificity, they have been recently postulated as much more specific markers for pathologies, such as cancer, with respect to mRNA and protein biomarkers [11, 12]. ncRNAs seem to be good molecular tools to improve the classification of types and subtypes of cancer and other pathologies, such as neurodegenerative diseases [11-16]. However, given the high potential of ncRNAs for understanding pathologies, their biogenesis, modes of action, and signaling pathways - both canonical and non-canonical - must be studied in detail.

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Table 1: Human ncRNAs types and functions.

Name	Function	Length
microRNAs(miRNAs)	mRNAs regulation, transcriptional regulators, lncRNAs regulators, Toll receptor ligands, stabilization of Ago proteins -RISC complex-	25-100 bp
tRF-derived RNA fragments	**Regulatory roles in several biological contexts** post-transcriptional gene regulation.	40-200 nt
piRNA	Mostly involved in the epigenetic and post-transcriptional silencing of transposable elements.	26-31 nt
Small Cajal body-specific RNAs (scaRNA)	RNA modification guides	
Small nucleolar RNA (snoRNA)	RNA modification guides	60 to 170 nt
Transference RNA (tRNA)	Translation-related RNAs	76 to 90 nt
YRNA	Regulatory RNAs Immunity	24 to 34nt
Vault RNA	Regulatory RNAs	88 and 140 nt
Small nuclear RNA (snRNA)	Spliceosomal RNA	150 nt
RNA component of mitochondrial RNA processing endoribonuclease (RMRP)	Catalytic RNAs	
Signal recognition particle RNA (7SL)	Translation-related RNAs	
7SK	Regulatory RNAs	
Ribonuclease P (RNAseP)	Catalytic RNAs	
Long non-coding RNAs (lncRNAs)	**Large repertoire of functions** Decoy, signal, guide, enhancer, scaffold, enzyme regulator, splicing modulation, small RNA precursor, extracellular secretion, targeting mRNAs, targeting proteins and miRNAs sponge.	More than ~IK200 nt
rRNA	Translation-related RNAs	~1800-5000 nt

snRNAs

In general, the function of ncRNAs is exerted by base pairing with other nucleic acid molecule. This action can be accompanied by associated proteins also known as ribonucleoprotein complexes [17]. One of the most abundant types of ncRNAs that carry out their function in ribonucleoprotein complexes are snRNAs (sn)RNPs. snRNAs are highly abundant non-coding transcripts, characterized by not being polyadenylated, which carry out their function in the nucleoplasm [17].

These ncRNAs have been categorized in two distinct classes based on their sequences similarity, protein used as cofactors and the polymerase transcribing them: Sm-Class-Pol II and Lsm-class; Pol II-III (Table 2) [7]. Since their discovery as central molecules of the spliceosome machinery, further works have been mainly focused on the study of snRNAs biology and their mode of action. These studies showed a high complexity in snRNAs assembly, trafficking, and mechanisms of action [7, 17].

Table 2: snRNAs classes: Sm-Class-Pol II and Lsm-class; Pol II-III.

snRNA class	snRNA	Maturation	Processed by	Structure
snRNA Sm	*U1, U2, U4, U4atac*, *U5*, *U7*, *U11* and *U12*	Subnuclear structures. They need to leave the nucleus and return to it.	Transcribed by Pol II. It requires the union of general transcript factors, such as TFIIA, TFIIB, TFIIE and TFIIF.	Constituted by a 5′-trimethylguanosine cap, a 3′stem–loop and a hetero-heptameric ring structure -Sm site- proteins binding site.
snRNA Lsm	*U6* and *U6atac*	Only in subnuclear structures-they never leave the nucleus-	Transcribed by Pol III	Constituted by a monomethylphosphate cap and a 3′stem- loop, terminating in a stretch of uridines - proteins binding site.

I The Spliceosome

The spliceosome is a ribonucleoprotein complex essential for the correct mRNA maturation, constituted by five different subunits of uridine-rich small snRNAs (*U1, U2, U4, U5* and *U6*), by the canonical splicing machinery or (*U11, U12, U6atac*) non-canonical splicing machinery, by more than 150 proteins, interacting with snRNAs denominated snRNPs, and by many others accessory proteins [7, 8]. All these make up a specialized macro-ribonucleoprotein complex mediating intronic cleavage. Both the spliceosome biogenesis and function are regulated by specific chemical modifications regulating base-pairing interactions among snRNAs [7, 8, 18, 19].

II *RNU6*

RNU6 or *U6* (*U6-1*) is a uridine-rich small non-coding RNA sized ~ 61 nt in length. Interestingly, it is an essential ncRNA for nuclear intron splicing (Figure 1), having structural and functional similarities to that of the domain V of autocatalytic group II introns [7, 8, 20, 21]. The similarity with the V domain is observed in the AGC triad, forming the *U2-U6* fold, which is present and conserved at both genomic elements, favouring the same tertiary folding interactions. Group II introns are present in bacteria, archaebacteria, mitochondria, and chloroplasts, but are notably excluded from nuclear genomes [20, 21]. The degree of conservation of the secondary and tertiary structures between group II introns and the *U2-U6* fold at the active site level strongly suggest that these introns and the spliceosome share a common evolutionary origin [22]. In addition, GU oscillations at the beginning of the *U2-U6* fold and during the formation of the catalytic center of group II introns have been observed [21, 22]. *U6* is the most conserved spliceosomal snRNA with more than a billion years of eukaryotic evolution [23].

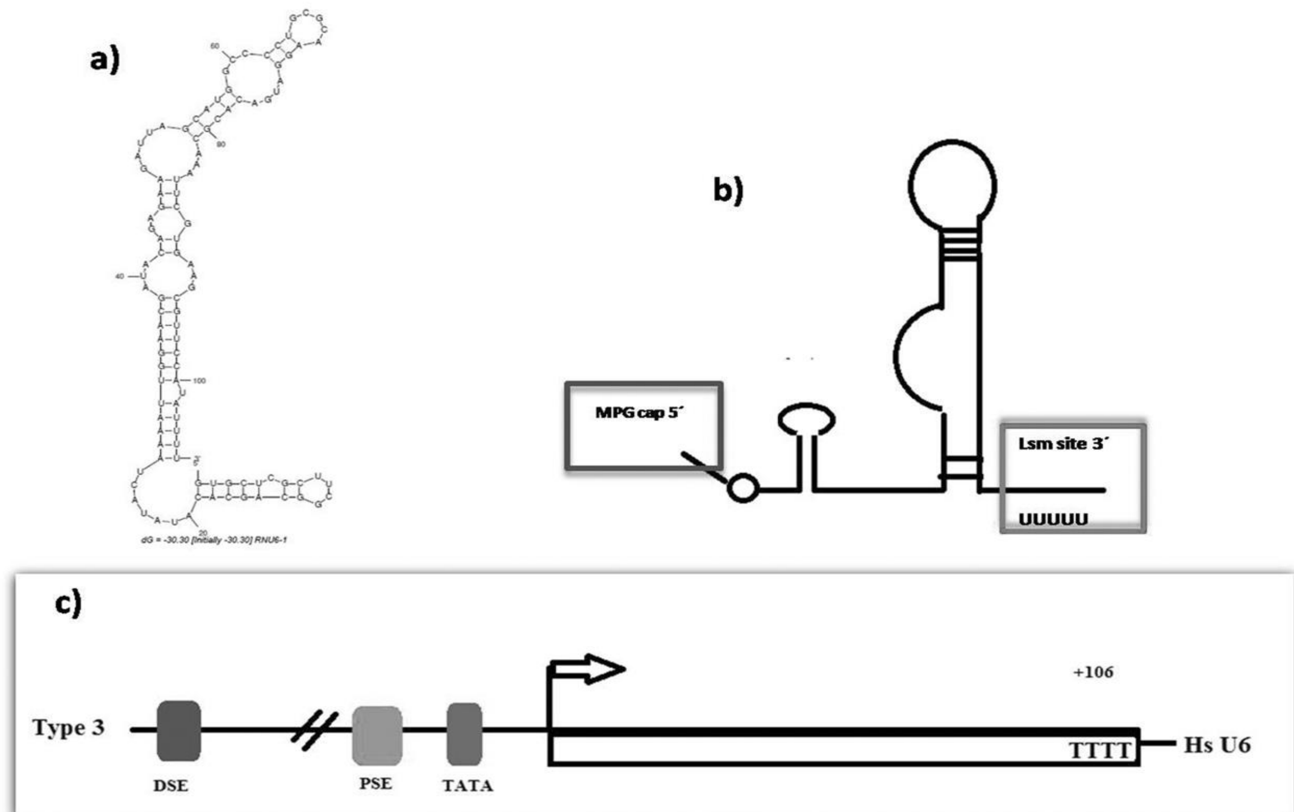

Figure 1: Putative secondary structure of human *U6* and the structure of the promoter gene. **a)** Secondary structure of human *U6* accordingly to The RNA Fold Web Server [59]. **b)** Secondary structure of human *U6* accordingly to Didychuk *et al.*, showing the 5′ γ-monomethyl cap and the 3′ U-tail present in Lsm class. **c)** Essential upstream elements of *U6* promoter including TATA box 30 base pairs upstream of the transcription start site, a proximal sequence element (PSE) 50 base pairs upstream, and a distal sequence element (DSE) 250 base pairs upstream [59].

RNU6 Interactome

RNU6 can interact with different molecules through the splicing cycle - three snRNAs; the pre-mRNA substrate; and more than 25 protein partners. Therefore, *RNU6* is a highly dynamic molecule [7, 8, 19, 21, 23]. These interactions occur throughout extensive structural *U6* rearrangements, including unwinding and reformation of stable internal secondary structure, which are attributed to *U6* post-transcriptional modifications during its biogenesis [8]. However, the precise effect of each modification has not been fully elucidated [8, 24]. Interestingly, *U6* is located in the core of the catalytic site, and it binds the magnesium ion needed for the spliceosomal machinery, regulating together with *U2* and *U5* the position of the substrate, which is fundamental for the reaction [7, 8, 19, 21, 23].

Genomic Organization

Regarding the genomic structure and organization, *U6* differs from other snRNAs not only in the type of promoter controlling its transcription, but also in the number of copies at the genome [25, 26]. For example, *U1* and *U2* are highly conserved genes, having 10-30 copies of true genes coding for snRNAs. Meanwhile, human *U6* has 200-900 copies coding for pseudogenes with many nucleotide substitutions and full-length truncations scattered throughout the genome and with unknown functions [9, 10].

U6 Promoter

Type III promoters – recognized by Pol III - were identified for the first time in the mammalian *U6* snRNA gene and in the human 7SK gene [27-29]. We have gained more knowledge about this kind of promoter since approximately 30 years ago. Few genes of our genome are recognized by Pol III, however, most of them are RNAs: catalytic, structural, and/or with unknown function, which are involved mainly in cell growth and cell cycle regulation [29, 30].

Based on the promoter region, specifically in the 5' terminal region, genes type 3 are divided in three distinct categories: i) promoter-gene-internal, ii) generally TATA-less, as those found in the VAI and tRNA genes and; iii) gene-external, containing a TATA box, as exemplified by the *U6* snRNA gene (Figure 1) [30]. Furthermore, the specificity of Pol III can change and alternate with Pol II through mechanisms, such as TATA box elimination or addition [9-26, 31]. It is believed that the recruitment of certain transcription factors used by Pol II and that are also necessary for PoI III influences chromatin remodeling. This, in turn allows the interaction of both polymerases [30]. Additionally, although the *U6* gene is highly conserved among organisms, its Pol III promoter structure is divergent. Studies performed in the human *U6* gene demonstrated the interaction between Pol II and Pol III and that this interaction at a site ~300 bp upstream is essential for its transcription [27-38]. Remarkably, the transcription of *U6atac* is also dependent on both Pol II and Pol III [31, 36]. Despite these, further studies are necessary to evaluate the transcriptional activity of *U6* and its involvement in different tissues and development stages and pathologies, given that specific tissue differences in the expression of *U6* have been reported [23, 25].

Mechanisms of *U6* Retrotransposition

Although the genome has 'allowed' and maintained many repeated sequences and copy number expansions, it is difficult to know the exact function they exert. However, the presence of these sequences along the human evolution has shaped the genome and new functional elements have been generated in most cases by a unique mechanism called retrotransposition [39]. Retrotransposition is the greatest remodeling force of the human genome, and it is currently represented by the Long Interspersed Nuclear Element 1 (LINE 1) [40-43]. These are 6-7kb elements that have all the machinery to be copied and mobilized into the genome. Generally, the 5'UTR has two promoters; the sense promoter directs the transcription of ORF1 and ORF2; and ASP directs the transcription of ORF0. The 3'UTR harbors both Alu and SVS elements, and the LINE 1 machinery to regulate its mobilization. The intrinsic nature of LINE 1 is to be copied and inserted itself into the genome, increasing genomic instability. In response to this, the cell has 'engineered' many mechanisms that control the levels of LINE1 retrotransposition; alterations in these regulatory systems can increase the mobility of LINE-1 and promote the formation of chimeric genes; however, in the case of *U6*, the conundrum is why a large spread of *U6* has been 'allowed' in the human genome [43].

In the 80s, the first studies of snRNA pseudogenes formation showed 4 different classes among which, three classes could be formed by using RNA as intermediate [43-47]. Recent studies have redefined the snRNAs pseudogene formation classification based on their structure organization (Table 3) Doucet *et al.* 2015 described in detail some processes by which LINE-1 elements can recruit RNA molecules, especially the *U6* sequence, promoting diversification and propagation of these sequences in the genome, not only in humans, but also in different species; however, the largest number of inserted and scattered copies are found in the human genome [42]. Interestingly, computational analysis demonstrated that the three LINE clades (L1, L2, and RTE) were able to form *U6* pseudogenes, even when L1 is actually the only active element in the genome, indicating this phenomenon has occurred throughout evolution. Remarkably, this has favoured the diversification of these sequences [42].

Table 3: Pseudogenes structure organization.

Group	Characteristics	Mechanism of amplification
Group I	Duplications of snRNA genes and their flanking sequence.	
Group II	Processed snRNA pseudogenes that generally end with an A-rich tail and are flanked by TSD.	Reverse transcription initiates directly on the snRNA by a template choice.
Group III	Processed pseudogenes that are heavily 30-truncated and flanked by TSD.	Reverse transcription initiates directly on the snRNA by a template choice.
Group IV	snRNA pseudogenes that form chimeras with a non-LTR retrotransposon.	Template switching.

Moldovan *et al.* reported the *U6*/L1 chimera can be generated more than once every fifteen retrotransposition events in HeLa cells, which according to the author it may suggest that the formation of *U6*/L1 chimera is a common event in the genome [47]. The *USB1* (*U6* snRNA phosphodiesterase) gene acts as exoribonuclease (RNase) responsible for trimming the (polyU) tract of the last nucleotides in the pre-*U6* snRNA molecule, leading to the formation of mature *U6* snRNA 3' end terminated with a 2′,3′-cyclic phosphate [48]. This terminal region is important because the authors demonstrated that the enzyme RTCB (RNA 2',3'-Cyclic Phosphate and 5'-OH Ligase) can facilitate the replacement of the 5' region of *U6*/L1 chimeras, mediated by the elimination of the 2′,3′-cyclic phosphate.

Interestingly, *U6*/L1 chimeric RNAs can arise independently of L1 retrotransposition and are formed through the 2′,3′-cyclic phosphate ligation on the 3′ end of the *U6* snRNA and 5′-OH on L1 RNA [48]. Chimeric *U6*/L1 RNAs are an important component of the transcriptome in multiple human cell lines [48]. As mentioned by Doucet *et al.*, the fact that *U6* has many copies and variants in the human genome has posed a challenge for the study of the possible role of each of these copies and variants. It is highly likely that these copies and variants have been omitted in many studies due to the lack of annotations in databases [48]. Many of these sequences have poor conservation among organisms and this is an important aspect to further understand their biological function [49]. Nevertheless, it has been observed in lncRNAs, which represent the less conserved and more recent sequences in the human genome with respect to other organisms, that although they do not keep their sequence identity, they maintain their function and are specifically transcribed in a variety of organisms [35, 49]. Importantly, recent findings regarding 'pseudogenes' have demonstrated the high regulatory capacity these sequences can exert in the genome [50]. So far, the genome has shown us that most of its transcripts are used to regulate gene expression in a very fine way. For this reason, the fact that the genome has been overly concerned with maintaining *U6* in mammals giving variability is challenging.

It has been reported that at least four *U6* human genes are transcriptionally active and the expression of a *U6* variant with nine substitutions and one deletion can be expressed under the control of an internal promoter [38, 39]. *U6atac*, a *U6* paralogue gene, proves that even when these sequences have a very low identity than other copies of *U6*, they can play a critical role in the cell. *U6atac* is a cofactor in the splicing of AT ± AC type introns and it is expressed at low levels in human cells [51, 52]. Based on all these, it can be postulated that many of the scattered copies of *U6* at the genome could also be having a biological function.

U6 Involvement in Diseases

U6 has been associated with some diseases, for example, mutations in *USB1* (*U6* SnRNA Biogenesis Phosphodiesterase 1), which is an exoribonuclease essential for the modification of spliceosomal small nuclear RNA (snRNA) *U6* by trimming its oligouridine tail and introducing a cyclic phosphate group, has been associated with accelerated *U6* decay and pre-mRNA splicing defects [53, 54]. It is likely that this deficiency induces *U6* 3′ end misprocessing. Mutations in this gene are associated with the genodermatosis Clericuzio-type poikiloderma with neutropenia (PN) [53, 54]. In a seemingly contradictory way, the *USB1* knockdown in HeLa cells showed no alteration in the expression levels of *U6* and an apparently correct pre-mRNA splicing; however, *U6* snRNA molecules became extended with a more heterogeneous length compared with controls. USB1 loss also modestly decreased *U6* snRNA stability [53, 55]. Accordingly with Moldovan *et al.*, the 3′ region is important because, as mentioned above, the enzyme RTCB can facilitate the replacement of this region of *U6*/L1 chimeras, mediated by the elimination of the 2′,3′-cyclic phosphate. The real implication of the modification exerted by *USB1* in this terminal domain remains to be elucidated.

Cancer

It has been reported in murine models of cancer an increased Pol III activity, which was later corroborated with transformed human cells, showing an increased transcript expression controlled by Pol III [56]. This seems to be strongly related to cellular transformation [56]. The latter is not surprising, since Pol III controls gene expression of transcripts involved mainly in cell growth and cell cycle regulation [27-30]. Importantly, Cabarcas *et al.* 2010, showed that *U6* transcription inhibition was mediated by PTEN (Phosphatase and Tensin Homolog) through the lipid-binding C2 domain of PTEN, and its interaction with BRF2 (BRF2 RNA Polymerase III Transcription Initiation Factor Subunit) [57]. Meanwhile, Puigdelloses *et al.*, showed the *U6* overexpression in the blood serum of adult patients with GBM (glioblastoma multiforme) and postulated this snRNA as potential biomarker to differentiate GBM from brain lesions that cannot be detected by imaging [58]. Due to cellular and cell growth regulation processes in which *RNU6* is involved, an increase in its expression seems to be essential for tumor growth [27-30, 56, 57]. Therefore, it is necessary to conduct further studies regarding the functions performed by *U6* to regulate cancer, as well as the functions performed by each *U6* copy and if there is a tissue-specific expression related to some pathology.

Conclusion

U6 is undoubtedly an snRNA with essential functions in the human genome, since during evolution it has considerably increased its number of copies and variants in the human genome. Although the study of *U6* represents a challenge for the scientific community, we firmly believe that it will elucidate many important functions of this RNA in health and in disease.

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Conflicts of Interest

None.

Competing Interests

None.

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