Cell. Mol. Life Sci. 64 (2007) 1793 – 1800
1420-682X/07/141793-8
DOI 10.1007/s00018-007-7084-0

Cellular and Molecular Life Sciences

Visions & Reflections (Minireview)

Useful 'junk': *Alu* RNAs in the human transcriptome

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Received 16 February 2007; received after revision 28 March 2007; accepted 25 April 2007
Online First 18 May 2007

Abstract. *Alu* elements are the most abundant repetitive elements in the human genome; they have amplified by retrotransposition to reach the present number of more than one million copies. *Alu* elements can be transcribed in two different ways, by two independent polymerases. 'Free *Alu* RNAs' are transcribed by Pol III from their own promoter, while 'embedded *Alu* RNAs' are transcribed by Pol II as part of protein- and non-protein-coding RNAs. Recent studies have demonstrated that both free and embedded *Alu* RNAs play a major role in post-transcriptional regulation of gene expression, for example by affecting protein translation, alternative splicing and mRNA stability. These discoveries illustrate how a part of the 'junk DNA' content of the human genome has been recruited to important functions in regulation of gene expression.

Keywords. *Alu* elements, *Alu* RNA, *Alu* RNP, alternative splicing, A-to-I editing, translation regulation, miRNAs, UTR, gene expression.

Introduction

In 2001, the completion of the human genome sequencing revealed that less than 2% of its nucleotide sequence code for proteins, while 55% of its mass are exclusively composed of repetitive elements [1]. With more than 1 million copies, *Alu* elements are the most abundant repetitive elements in the human genome; altogether, they represent about 10% of the genome mass and belong to the SINE (short interspersed elements) family of repetitive elements.

Modern *Alu* elements are approximately 300 bp in length and have a dimeric structure. They are composed of two similar but distinct monomers (left and right arms) joined by an A-rich linker (Fig. 1a). *Alu* elements are specific to primates; they emerged more than 55 million years ago from the 7SL RNA gene, which encodes the RNA moiety of the signal recognition particle (SRP) [2, 3]. *Alu* elements amplified via RNA intermediates by a mechanism of retrotransposition that is currently under investigation. As they do not encode any protein, their amplification has probably been dependent on the transposition machinery of other retrotransposing elements, and it has recently been shown that they could use LINE-1 elements for this purpose [4]. Very few *Alu* elements are able to retrotranspose; they emerged from a limited number of *loci* that are considered as 'master genes' of *Alu* amplification. These master genes were active at different time periods during primate evolution, and *Alu* elements can therefore be classified into subfamilies of different ages, based on their primary sequence similarities (for a review see [5]).

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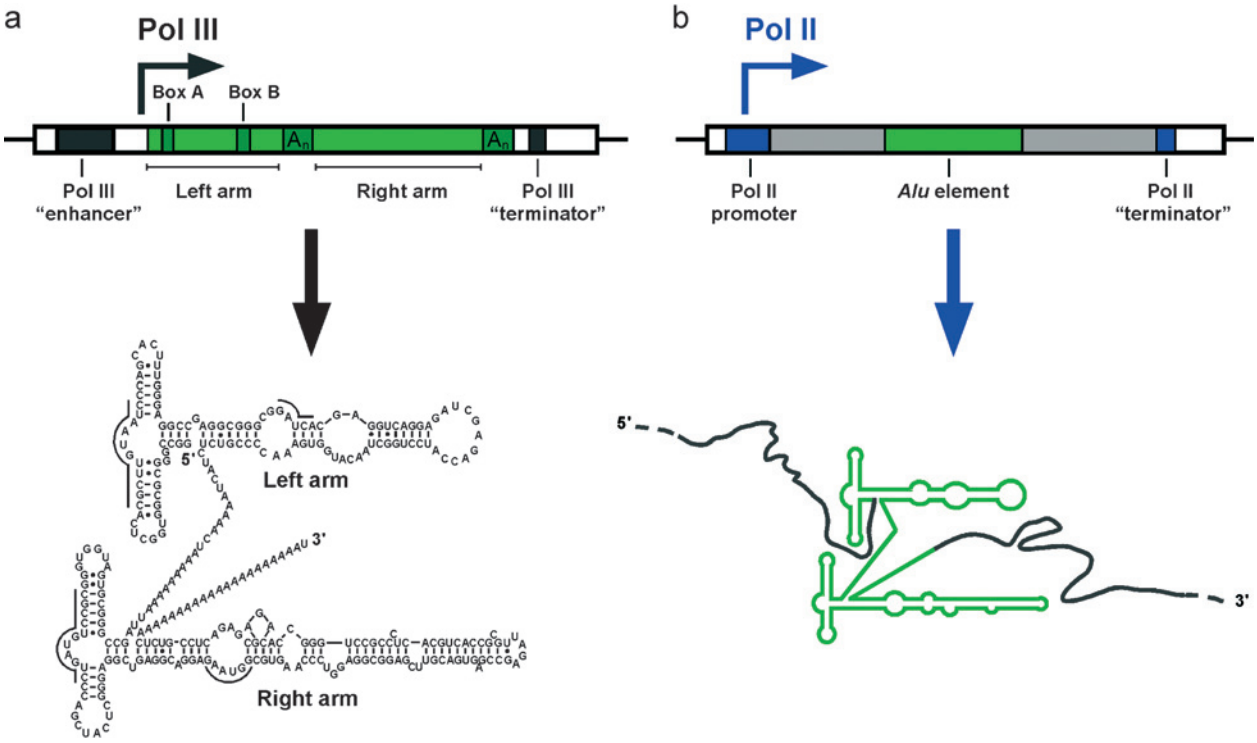

Figure 1. Transcription of *Alu* elements by Pol II and Pol III. (*a*): Free *Alu* RNA. This hypothetical *Alu* element, shown in green, is transcribed by its internal Pol III promoter elements (Box A and Box B), which are helped by an upstream Pol III enhancer (or upstream promoter elements) for efficient transcription. A Pol III terminator (TTTT-3′) is depicted downstream of the *Alu* element. Both Pol III enhancer and terminator are provided by the locus of integration of the *Alu* element. The secondary structure of the resulting *Alu* RNA was drawn based on a previously determined secondary structure [78] and adapted to the sequence of the *Alu* element of intron 4 of the α-Fetoprotein gene [79], which was shown to bind the SRP9/14 proteins [11,14]. Underlined letters indicate the binding sites of SRP9/14 by analogy to SRP RNA [80]. (*b*) Embedded *Alu* RNA. This hypothetical *Alu* element, shown in green, is located inside of a protein-coding gene. It might be inserted into an intron, a UTR, and exceptionally into a coding region. This *Alu* element is then transcribed by Pol II, embedded inside a larger transcription unit. Depending on the sequence environment, as well as on its own sequence, the embedded *Alu* RNA might adopt a typical *Alu* fold, as depicted here.

Alu elements have had a major impact on the architecture of the human genome, not only because they amplified by retrotransposition, but also because, due to their high sequence conservation, they provide an abundant source for homologous non-allelic recombination events, resulting in duplications and deletions of large chromosomal regions (for further discussion see [6] and references therein). This review focuses on the role of *Alu* sequences in influencing protein diversity and gene expression at the posttranscriptional level.

Alu RNA transcription by Pol II and Pol III

It has been known for a long time that *Alu* RNAs, transcribed from *Alu* elements, are present in the cytosol of primate cells. *Alu* elements indeed inherited the internal A and B boxes of the RNA polymerase III (Pol III) promoter from the 7SL RNA gene. However, this internal promoter is not sufficient to drive transcription *in vivo*, and *Alu* elements are dependent for expression on sequences flanking their site of insertion [7, 8]. At normal cell growth, *Alu* RNAs accumulate at very low levels (10^3–10^4 molecules per cell), but their abundance increases up to 20-fold under various stress conditions such as adenovirus infection, heat shock and cycloheximide exposure [8–10]. These types of Pol III-transcribed *Alu* RNAs will thereafter be referred to as 'free *Alu* RNAs' (Fig. 1a). *Alu* elements integrated into various locations in the human genome and are therefore also transcribed as part of larger transcription units of protein- and non-protein-coding RNAs. For example, messenger RNAs (mRNAs) contain *Alu* sequences. These *Alu* elements are therefore transcribed by RNA polymerase II (Pol II) and will thereafter be referred to as 'embedded *Alu* RNAs' (Fig. 1b).

Over the last years, both free and embedded *Alu* RNAs have been shown to play an important role in post-transcriptional regulation of gene expression. The best-described examples are discussed below.

Free *Alu* RNAs and protein translation

The typical *Alu* RNA is a dimer of related but non-equivalent arms that are joined by an A-rich linker and followed by a short poly(A) tail (Fig. 1a). Each arm is related to the *Alu* portion of SRP RNA in terms of sequence and secondary structure and can bind the cognate SRP protein SRP9/14 *in vitro* [11] and *in vivo* [12, 13]. Recent results from our group revealed that synthetic *Alu* RNPs (ribonucleoproteins), composed of *Alu* RNA in complex with SRP9/14, and naked *Alu* RNA have opposite effects on protein synthesis in cell-free translation systems. While *Alu* RNA stimulates the translation of reporter mRNAs, *Alu* RNP acts as a general inhibitor of protein translation [14]. Further investigations of the mechanism by which they influence protein translation showed that both of them act at the level of translation initiation [14]. A large fraction of the protein SRP9/14 is not assembled into SRP in primate cells [12]. The 'free' SRP9/14 may bind *Alu* RNA and the occurrence of *Alu* RNP *in vivo* seems therefore very likely. In addition, *Alu* RNA migrates as a high molecular weight complex in density gradients of HeLa cell extracts [12,15], and *Alu* RNAs expressed in response to adenovirus infection are assembled in SRP9/14-containing RNPs [13].

Expression of free *Alu* RNA *in vivo* was shown to stimulate translation of co-transfected reporter genes [16, 17]. These data were actually the first to suggest that transcribed *Alu* elements may serve specific functions in translation regulation. The region of *Alu* RNA required for stimulating reporter gene expression is located in the right arm [17], far away from the SRP9/14 binding site. Inferred from the results obtained *in vitro*, these data might suggest that the expressed *Alu* RNA was not bound to SRP9/14.

The results discussed above underscore a potentially critical role of SRP9/14 in regulating the activity of free *Alu* RNAs *in vivo*. *Alu* RNA and SRP9/14 should therefore not be considered independently, but rather be regarded as an ensemble. One piece of missing data is then the regulation of SRP9/14 expression. The excess of SRP9/14 over SRP was only observed in immortalized cell lines [12], and the expression levels of SRP9/14, relative to SRP, will have to be investigated in different tissues and under different physiological conditions to better characterize the potential regulation of *Alu* RNA activity. Moreover, as most of the previous studies concerning *Alu* RNA transcription were performed using cultured cancer cells [8, 9, 15, 18, 19], it would also be interesting to measure the level of *Alu* RNA in different tissues or in cultured primary cells. It was indeed recently shown that the level of *Alu* transcripts is much lower in normal than in cancerous hepatic cells [20]. Another piece of missing data is the potential of *Alu* RNAs to bind SRP9/14 *in vivo*. It not only depends on the availability of SRP9/14 but also on the capacity of *Alu* RNAs to fold into the right structure to be recognized by the proteins. Certain *Alu* RNAs may harbor mutations that interfere with folding and protein binding, or may hybridize to other RNAs, which may interfere with SRP9/14 binding. It will therefore be essential to study binding of SRP9/14 to *Alu* RNA comprehensively *in vivo*.

The exact mechanisms by which *Alu* RNP and *Alu* RNA influence translation initiation remain to be unraveled. The involvement of the dsRNA-dependent protein kinase, PKR, in this mechanism is unclear, because if *Alu* RNA indeed activates PKR at low concentrations, it can inhibit it at higher concentrations [21]. Moreover, it was shown that *Alu* RNA stimulates translation of reporter genes in PKR knockout cells [17]. More recently, it was proposed that the effect of *Alu* RNA could also be mediated by poly(A)-binding proteins (PABP) sequestering [22]. Global control of translation, as observed for *Alu* RNP, is usually exerted by regulating phosphorylation or availability of initiation factors [23]. Based on current data, stimulation of translation by *Alu* RNA [14, 17] might be restricted to certain mRNAs. In this case, target mRNAs, which are so far unidentified, might share some common features, at the level of sequence or secondary structure, which would be recognized by *Alu* RNA, possibly in conjunction with other regulatory proteins. In any case, further characterization of the roles of *Alu* RNP and *Alu* RNA, as well as better understanding of their biological function, will require identification of cellular factors interacting with *Alu* RNP and *Alu* RNA.

Intronic *Alu* RNAs and alternative splicing

Alternative splicing is a major source of protein diversity; it has been estimated that 30–60% of all human genes produce alternative exons [24]. The use of alternative splice sites in pre-mRNAs generates multiple variants of proteins from a single gene, and it was estimated that the *Drosophila* axon guidance receptor gene, Dscam, potentially generates up to 38000 isoforms by alternative splicing [25]. Among the different ways by which a gene acquires new alternative splice sites, the prevalent one is the mutation of pre-existing intronic sequences that results in the recruitment of intronic regions in the mature mRNA. This process is called exonization. By providing alternative splice sites in pre-mRNAs, *Alu* RNAs embedded into introns were shown to be

important mediators of alternative splicing (for detailed reviews see [26, 27]).

The *Alu* consensus sequence contains 9 potential 5′ splice sites and 14 potential 3' splice sites [28, 29], most of them being present on the minus strand. By genome-scale computational studies [29], Sorek and colleagues determined that 5.2 % of all identified alternative exons derive from *Alu* elements [29]. Sequence comparison of *Alu* exons revealed that not all potential splice sites were used at the same frequency [29]; the most favored sites were positions 275 and 279 used as 3' splice sites and position 158 used as 5' splice sites. The results of sequence analysis were confirmed *in vivo* by experiments using an ADAR2 minigene, which contains an alternative *Alu* exon [30]. Site-directed mutagenesis of the ADAR2 minigene helped to determine the nucleotide composition that governs the use of alternative splice sites in an antisense embedded *Alu* RNA for the 3' [31], and 5′ [32] positions. Other studies showed that the mechanism of *Alu* exonization might also be influenced by auxilliary splicing enhancer [33], and silencer [34] sequences, which are present on the *Alu* consensus sequence.

Alu RNAs embedded into introns might then be considered as a large reservoir of potential alternative exons, providing primates with an enhanced capacity to increase protein diversity. The other side of the coin is that single-base mutations may change an alternative into a constitutive splice site [31, 32]. Pathologies such as the Alport [35] and the Sly syndromes [36] are known to be caused by mutations that result in constitutive inclusion of an *Alu* exon. More recently, it was even discovered that alternative inclusion of an *Alu* exon might lead to a genetic disease. A mutation in intron 6 of the CTDP1 gene, creating an alternatively spliced *Alu* exon, results in CCFDN (congenital cataracts, facial dysmorphism and neuropathy) syndrome [37].

A-to-I editing of embedded *Alu* RNAs

Among the different RNA modifications that occur co- or post-transcriptionally, modification of adenosine (A) to inosine (I) is one of the best characterized. This base conversion reaction is catalyzed *in vivo* by members of the ADAR (Adenosine Deaminase Acting on RNA) family of enzymes. Despite the apparent mass of inosine found in the transcriptome [38], very few positions edited by ADAR were identified until recently, when three independent groups discovered that embedded *Alu* RNAs were responsible for most (more than 90%) of the A-to-I editing events in the human transcriptome (for detailed reviews see [26, 39, 40]). Using elaborated genome-scale computational approaches, Kim and colleagues identified 30085 substitutions in 2674 different transcripts [41], Levanon and colleagues identified 12'723 substitutions in 1637 different transcripts [42], and Athanasiadis and colleagues found 14500 substitutions in 1445 mRNAs [43]. Most of the A-to-I substitutions occurred within *Alu* RNAs embedded in non-coding regions of mRNAs [42]. To test the accuracy of these computational approaches, a certain number of previously unknown editing sites were experimentally confirmed [42].

ADAR enzymes preferentially edit adenosines located in double-stranded regions of RNA molecules and do not have a strict sequence requirement at the editing site (for reviews see [44, 45]). Interestingly, it was determined that adenosine editing inside an embedded *Alu* RNA is favored when another embedded *Alu* RNA, in the opposite orientation, is present in the same RNA molecule at a distance shorter than 2 kb [43]. This observation suggested that two neighboring embedded *Alu* RNAs base pair and become a substrate for ADAR. This hypothesis was further supported by studies on the editing patterns of cyclin M3 intron 2 and NFκB1 intron 16, which showed that the base pairing between two embedded *Alu* RNAs occurs intramolecularly, and not intermolecularly [46].

Interestingly, these genome-scale studies failed to detect any of the known editing sites in the coding region of glutamate [47] and serotonin receptor [48] mRNAs, strongly suggesting that not all editing sites of the human transcriptome have been uncovered. Similar studies with mouse and fly transcriptomes revealed that the high levels of editing are primate-specific [49]. The high incidence of editing events in primates could conveniently be explained by the presence of a large number of embedded *Alu* RNAs in the human transcriptome, which, with their high potential to base pair intramolecularly due to their low sequence divergence, provide ideal substrates to ADAR [50].

The precise role of RNA editing is still speculative, but it might affect gene expression at several steps, such as mRNA stability, splicing and protein translation. Knowing that ADAR knockouts are incompatible with normal life [51, 52], and that aberrant editing is found in several neurological disorders [53, 54], the embedded *Alu* RNA-mediated A-to-I editing of the human transcriptome, albeit incompletely understood in its precise function, may represent a fundamental process of post-transcriptional regulation of gene expression. Very recently, an interesting connection between *Alu* editing and *Alu* exonization was discovered. It was shown that an *Alu* exon in the human

Nuclear prelamin A Recognition Factor (NARF) is strictly dependent on a tissue-specific A-to-I editing for its exonization [55].

Alu RNAs embedded in 5′ and 3′ UTRs of human mRNAs

Untranslated regions (UTRs) are known to play crucial roles in post-transcriptional regulation of gene expression, including translation efficiency, subcellular localization and stability of mRNAs [56]. *Alu* RNAs embedded in untranslated regions of mRNAs might therefore be at the right position to modulate gene expression at different levels.

In an attempt to identify *Alu* RNAs embedded in UTRs, more than ten years ago, Yulug and colleagues identified only 87 complementary DNAs (cDNAs) by database searching [57], while in 2001 Landry and colleagues mentioned that they identified about 1700 UTRs harboring embedded *Alu* RNAs, by screening the non-redundant UTR database [58]. More recently, by screening the human transcriptome for the presence of *Alu* elements transcribed in 5′ and 3′ UTRs of human mRNAs, we identified 291 *Alu* RNAs embedded in 5′ UTRs of 244 different transcripts, and 2142 *Alu* RNAs embedded in 3′ UTRs of 1548 different mRNAs (http://www.unige.ch/sciences/biologie/bicel/Strub/research/Alu.html). Taking into account the length of UTRs, we calculated that there is one *Alu* element per 24000 bases in 5′ UTRs and one per 14000 bases in 3 UTRs. These numbers showed a certain preference for the accumulation of *Alu* elements in 3′′ UTRs as compared to 5′ UTRs. A gene ontology analysis of these datasets showed that there is no under- or overrepresentation of these elements in UTRs of mRNAs involved in specific molecular functions or biological processes (http://www.unige.ch/sciences/biologie/bicel/Strub/research/Alu.html). Their function, if any, might then be specific to each mRNA.

For *Alu* RNAs embedded in 5′ UTRs of specific mRNAs, a role in inhibiting translation has been recognized. A transcript isoform of the DNA repair protein BRCA1, which is specifically expressed in breast cancer tissue, contains an *Alu* RNA embedded in its 5′ UTR [59]. This *Alu* RNA has been shown to be responsible for the translational defect of this mRNA, because it forms stable secondary structures that partially block translation initiation. Similarly, an antisense *Alu* element in the 5 UTR of ZNF177 mRNA, a zinc finger protein of unknown function, has been shown to strongly decrease the translation efficiency of the mRNA [58]. It was also suggested that an *Alu* element in the 5′ UTR of human growth hormone receptor (hGRH) could regulate the translation of this mRNA [60], and an *Alu* RNA embedded in the 5′ UTR of a contactin mRNA isoform was recently shown to inhibit protein translation [61]. All these examples suggest that the presence of *Alu* RNAs embedded in 5′ UTRs inhibits protein translation; this is most likely due to stable secondary structures formed by the *Alu* RNA, which block translation initiation by preventing proper ribosomal assembly on the 5′ UTR.

A role for *Alu* RNAs embedded in 3′ UTRs has been suggested in regulating mRNA stability. It was proposed that antisense *Alu* elements inserted in 3′ UTRs could generate adenine- and uracil-rich elements, or AREs [62]. AREs are known to destabilize certain mRNAs, thereby affecting their expression levels. Although the exact mechanism of ARE-enhanced mRNA degradation is still unknown, it is thought to occur through the exosome pathway [63, 64]. Antisense *Alu* elements harbor a Poly(T) which is complementary to the terminal A stretch, and it was estimated in this study that half of the longest AREs derived from antisense *Alu* elements [62]. These results still need to be experimentally confirmed, but they suggest a new role for embedded *Alu* RNAs in regulating mRNA stability. This role was also suggested by an isolated study showing that an *Alu* RNA embedded in the 3'UTR of LDL receptor can bind the chemical PMA (phorbol-12-myristate-13-acetate) and increase the mRNA half-life [65]**.** Interestingly, recent results from our group suggest that SRP9/14 can bind to some *Alu* RNAs embedded in 3′ UTRs and, in this way modulate the stability of these transcripts [J. H., T. S., K. S., unpublished results].

Altogether, these results suggest that there is no general rule as to how *Alu* RNAs embedded in UTRs influence mRNA expression. From the few examples described so far, it appears that *Alu* RNAs in 5′ UTRs may repress translation, whereas *Alu* RNAs in 3′ UTRs may regulate mRNA stability. However, a much larger sample of mRNAs will have to be studied on a case-by-case basis to obtain a more conclusive view of these potential effects. Accumulating data in this research field might ultimately lead to drawing more general conclusions on regulation of gene expression by *Alu* RNAs embedded in UTRs.

Alu RNAs and miRNAs

MicroRNAs (miRNAs) are endogenous, short (about 22 nt) non-coding RNAs. They play a major role in regulation of gene expression by translational repression or mRNA cleavage (for review see [66]). miRNAs are transcribed as large precursors, which are processed

in small mature miRNAs. The mature miRNAs trigger translational repression or mRNA cleavage, depending on the level of complementarity with their target site [67]. Recently, two independent studies linked both free and embedded *Alu* RNAs to miRNA expression and function.

miRNA precursors are currently assumed to be transcribed by Pol II from their own promoter, or in intronic regions of host mRNAs [68]. By studying an miRNA cluster on chromosome 19 (C19MC) which encodes 54 miRNAs, Borchert and colleagues recently showed that *Alu* elements are responsible for transcribing a certain number of these miRNAs. C19MC is a primate-specific miRNA cluster [69], in which more than two-thirds of the 100-kb sequence is composed of *Alu* elements. As previously mentioned, *Alu* elements harbor an internal Pol III promoter; however, they lack the TTTT-3′ Pol III terminator. By a variety of experimental approaches, Borchert and colleagues provided evidence that, in some cases, transcription from the *Alu* Pol III promoter can pass through the *Alu* element and transcribe the downstream miRNA precursors before a Pol III terminator is encountered [70]. This study does not only show for the first time that miRNAs can be transcribed by Pol III, but also reveals that *Alu* transcription might be tightly linked to miRNA expression.

An *in silico* analysis looking for sequence complementarity between miRNAs and embedded *Alu* RNAs revealed that about 30 miRNAs exhibit a typical short-seed complementarity with a specific sequence contained within sense *Alu* RNAs embedded in 3′ UTRs of human mRNAs [71]. Using different algorithms, the authors determined that *Alu* RNAs embedded in 3′ UTRs are probable miRNA targets. This discovery needs to be experimentally validated, but it opens a new perspective in which embedded *Alu* RNAs might influence gene expression by being miRNA targets.

Free *Alu* RNAs: *trans* effectors of mRNA expression?

As previously mentioned, free *Alu* RNAs have been shown to modulate gene expression by influencing translation initiation [26]. Recently, another regulatory function of Pol III-transcribed *Alu* RNAs was proposed. The ncRNA 21A, which represents an *Alu*Jb, was found to downregulate the expression of CENP-F, a kinetochore protein that plays a central role in mitosis [72]. This regulation appears to occur through sequence complementarities of 21A RNA with intronic sequences of the CENP-F gene, suggesting that 21A RNA may interfere with CENP-F mRNA maturation. This discovery is, so far, the only reported example of a free *Alu* RNA modulating gene expression by sequence complementarity with the mRNA. But it might represent a new mechanism by which free *Alu* RNAs post-transcriptionally act on gene expression.

Concluding remarks

The above-mentioned examples highlight the impact of transcribed *Alu* elements on gene expression at the post-transcriptional level. However, *Alu* elements were also shown to influence gene expression at the transcriptional level, mainly by providing new enhancers, regulatory and polyadenylation signals to neighboring genes (for reviews see [73, 74]). This gain of regulatory function by retroelements was previously described as 'exaptation' [75].

Whereas the results discussed here focus on the beneficial effects of *Alu* element insertion, the same process could also be detrimental for the organism. By inserting into essential genes, *Alu* elements can indeed annihilate important physiological functions; moreover, because of their high level of sequence homology, they can also mediate important disease-causing genomic rearrangements [76]. The duality of the effect of *Alu* element insertion demonstrates that *Alu* amplification has not been a neutral process; on the contrary, that it has been actively participating in genome evolution. This strategy of genome evolution might be metaphorically compared to a sustainable development policy. While modern societies start recycling chemically-usable waste in order to preserve natural resources and to save energy, the genome has been successfully recycling a part of its 'junk DNA' content into useful regulatory functions, with the net result of gaining complexity at low cost by taking advantage of the fortuitous integration of repetitive elements into favorable locations.

This analysis has only focused on *Alu* repetitive elements; however, it might be extended to other retrotransposed elements. LINE-1 elements, for example, were also proposed to influence gene expression at various levels (for review see [77]), and further investigations might well show that their impact on gene expression is as important as that of *Alu* elements.

Acknowledgments. Work on *Alu* RNAs in the laboratory of K.S. is supported by grants from the Swiss National Science Foundation and from the Canton of Geneva. We are indebted to Drs. J. Brosius and E. A. C. Lucken for critical reading of the manuscript and helpful comments.

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