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# Review

# Antisense transcription: A critical look in both directions

**T. Beiter[a,] *, E. Reich[a], R. W. Williams[b] and P. Simon[a]**

[a] Molecular Biology Lab, Medical Clinic, Department of Sports Medicine, University of Tuebingen, Wilhelmstr. 31, 72074 Tuebingen (Germany), Fax: +49-7071-295162,
e-mail: thomas.beiter@med.uni-tuebingen.de
[b] Department of Anatomy and Neurobiology, University of Tennessee Health Science Center, Memphis, TN (USA)

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**Abstract.** The mammalian genome contains a large layer of hidden biological information. High-throughput methods have provided new insights into the regulatory networks that orchestrate the "when, where and how" of gene expression, revealing a complex interplay between proteins, regulatory RNAs, and chemical and structural alterations of the genome itself. Naturally occurring antisense transcription has been considered as an important feature in creating transcriptional and hence cellular and organismal complexity. Here, we review the current understanding of the extent, functions and significance of antisense transcription. We critically discuss results from genome-wide studies and documented examples of individual antisense transcripts. So far, the regulatory potential of gene overlaps has been demonstrated only in a few selected cases of experimentally characterized antisense transcripts. Facing the large-scale antisense transcription observed in eukaryotic genomes, it still remains an open challenge to distinguish transcriptional noise from biological function of gene overlapping patterns.

**Keywords.** Antisense transcription, natural antisense transcripts, sense-antisense pairs, overlapping genes, transcriptome, gene regulation.

## Introduction

Since the first analysis of the draft human genome sequence was published in 2001 [1, 2], the number of protein-coding genes has been constantly revised downwards from about 35 000 to some 21 000, a number much lower than initially expected ([3, 4], www.ensembl.org). Large-scale studies of diverse eukaryotic genomes have revealed that the number of protein-coding genes alone is not sufficient to account for differences in molecular and cellular complexity. So what makes humans different from the tiny roundworm *Caenorhabditis elegans* (~19 000 protein-coding genes) [5], the inconspicuous plant *Arabidopsis thaliana* (~27 000 protein-coding genes) [6] or the fruit fly *Drosophila melanogaster* (~13 500 protein-coding genes) [7].

This enigma can only partly be explained by higher levels of alternative pre-mRNA splicing and post-translational modifications of proteins. Quite obviously, the transcriptional output of a genome is orchestrated by a complex network of functional, structural and regulatory elements. Many scientists now believe that the key to complexity lies not exclusively in the protein-coding genes and their mRNAs transcripts, but also in the tremendous reservoir of non-coding RNAs (ncRNAs) that are

* Corresponding author.

estimated to represent >90 % of the transcriptional output of the human genome [8–11]. It has become evident that there are numerous classes of ncRNAs with diverse known and putative functions, and the ncRNA world has become the new playground of molecular scientists. In recent years, attention has been drawn to the fact that a significant fraction of the transcriptome comprises RNAs containing sequences that are complementary to other endogenous RNAs. These natural antisense transcripts (NATs) can have protein-coding properties but mainly represent ncRNAs. NATs have been implicated with diverse regulatory functions at various levels, including imprinting, X-inactivation, RNA processing, RNA export, and transcriptional regulation. The number of genome-wide screening approaches to assess the proportion and diversity of NATs in eukaryotic transcriptomes has increased rapidly, but this increase is unfortunately not yet matched by experimental confirmation regarding the function and physiological relevance of these transcripts.

In this review, one of our main goals is to provide an overview of what is actually known about the extent and function of antisense transcription and sort out some of the key observations, hypotheses, and speculations. First, we summarize current estimates of the amount of antisense transcription and discuss potential pitfalls in interpreting results from high-throughput screening and expression analyses. We also survey recent data on the regulation and evolution of NAT transcription and its potential contribution to organismal complexity. Finally, we discuss putative antisense regulatory mechanisms and emphasize the problem of generating good evidence of causes and effects in sense-antisense (SAS) relationships.

## Screening for antisense transcription: How to separate the wheat from the chaff

NATs can be divided into *cis*-NATs, which are transcribed from the opposite strand of the same genomic locus as the sense RNA, and *trans*-NATs, which are transcribed from separate loci and display short or imperfect complementarity with their corresponding sense transcripts [12]. Most studies on antisense transcription have focused primarily on *cis*-NATs because relationships are easier to identify. In several cases, the *cis-trans* nomenclature might be rather misleading, as some *cis*-NATs may not be functionally linked to their ascribed sense counterpart or exert effects both in *cis* and in *trans*, as we discuss later. Our review is restricted to *cis*-NATs, although we are well aware of the ambiguity of the term. By common definition, the term SAS pair refers to a pair of transcripts produced from the same locus on the chromosome, but from the DNA strands opposite to each other. SAS pairs can be arranged in diverse overlapping patterns, and NATs can range in size from a hundred to several thousand base pairs, and can represent coding or ncRNA. They can undergo splicing or can derive from intronless genes.

During the past 6 years, numerous publications have addressed the extent of antisense transcription in the human and mouse genomes using different detection strategies. Basic studies using mRNA and expressed sequence tag (EST) libraries combined with information like exon–intron splicing structures and poly(A) signals estimated the extent of the NAT phenomenon in mammals to be in the range between 15 % and 25 % of all transcriptional units [12–15]. However, because there is only partial overlap among published data sets, and because a large fraction of non-polyadenylated and unspliced NATs is generally excluded from these studies for the sake of higher stringency, it is highly likely that numbers of NATs are considerably higher. Many complementary sources of expression data including cap analysis of gene expression (CAGE), serial analysis of gene expression (SAGE), massively parallel sequencing of cDNA pools, and tiling arrays, are now being used to expand the catalog of NATs in the mammalian transcriptome [16–21]. It has been estimated that at least 40 % of all transcriptional units may have concurrent overlapping antisense partners [22]. However, experimental validation is poor, and it is appropriate to ask if these figures make sense.

Given that 10–20 % of EST sequences in UniGene are annotated in the wrong direction [23], reliable *in silico* screenings have to be based on a stringent set of parameters to identify the correct orientation of transcripts and therefore tend to underestimate the real amount of antisense transcription. In contrast, microarrays and tiling arrays are prone to produce false positive artifacts due to genomic contamination of RNA samples, cross hybridization, or unintended double-stranded labeling of RNA [24, 25]. In a recent study, Perocchi et al. [26] showed that about half of all antisense signals observed in a conventional high-density tiling array were experimental artifacts, most probably due to spurious synthesis of second-strand cDNAs that occurred during reverse transcription (RT). Although it is a standard method in molecular biology, RT reactions are still poorly understood. Several studies have exploited strand-specific RT-PCR from randomly selected SAS candidate pairs to evaluate their screening strategy [20–22, 27]. However, with conventional strand-specific RT-PCR absolute strand-specificity cannot be achieved due to priming artifacts generated by unspecific binding of

the RT-primer or by small degraded RNA or DNA fragments, which can act as primers during the RT step [28, 29]. Given that the majority of NATs are supposed to be expressed at lower levels than their sense counterparts [21, 30], RT-PCR signals may be deceptive, especially when using the same amplicon for both transcripts of an SAS pair. Endogenous or false priming artifacts might also feign positive coregulation of sense and putative antisense transcripts, which several authors consider to be the prevalent expression pattern of SAS pairs [17, 31–33]. More sophisticated protocols have been established for the detection of viral antisense transcripts [34, 35], and should be considered in future studies of antisense transcription.

We of course do not question the widespread occurrence and the particular extent of antisense transcription. However, as only few SAS pairs have been experimentally validated (an overview of the few well-investigated human NATs is given in Table 1), it would be unwise to disregard the contribution (real or potential) of experimental and biological artifacts. Large-scale transcription profiling studies provide us with a directory of addresses of NAT candidates. The main challenge now is to confirm these candidates and to obtain much more information about their partnerships, relatives, ancestors, and potential functions.

## Antisense transcription: The key to complexity?

The widespread occurrence of antisense transcription in the mammalian genome raises several important questions: (a) Are NATs key factors of a complex control architecture required for the coordination and modulation of gene expression and consequently directly linked to organismal complexity? (b) Is antisense transcription basically a spatial phenomenon resulting from combined usage of regulatory elements and/or from a transcriptionally favorable local chromatin structure? (c) Is there a direct or indirect *cis*-regulatory interaction between the transcripts of an SAS pair?

As we demonstrate here, at present none of these questions can be answered satisfactorily.

## NATs: A conglomeration of multiple transcripts

As mentioned above, antisense transcription is a deliberately vague term that describes a basic arrangement of two transcriptional units localized on opposite strands in a genomic locus. Therefore, any attempt to draw universally valid conclusions regarding function, evolution and interactions of NATs will probably fail. We first have a closer look at the ingredients of the antisense melting pot and the contribution of the different groups to the "multi-ethnic" transcriptome society.

So far, we have used the term NAT without defining which gene in an SAS pair we consider the sense transcript and which the antisense. Conventionally, the term sense transcript refers to the protein-coding version in cases where only one partner of the SAS pair is an ncRNA. When both are either non-coding or coding, the distinction is arbitrary. Generally, the sense gene is presumed to be the more abundant and more widely expressed partner that usually has a better characterized or more direct function [78]. Often, the sense strand is considered the one that undergoes splicing or has longer intronic sequences [79].

Not surprisingly, estimates on the contribution of ncRNA to antisense transcription are wide ranging [12, 13, 33] due to different screening procedures and difficulties in discriminating coding and non-coding transcripts. It is agreed that the dominant fraction of NATs is made up of ncRNA. Moreover, several studies have shown that a significant amount of NATs are not polyadenylated and have a restricted nuclear localization [20, 31, 33]. This is hardly surprising given that the diversity and complexity of the nuclear transcriptome is significantly greater than that of cytosolic RNA [80].

NATs can be divided into different categories based on the orientation of SAS pairs: head-to-head or divergent (overlapping 5'ends), tail-to-tail or convergent (overlapping 3' ends), or embedded (one gene included within the region of the other) [81]. While earlier works reported convergent SAS pairs to be more prevalent in the mammalian genome [13–15, 82, 83], more recent studies argue in favor of the divergent orientation [12, 23, 31, 84]. A comprehensive census of types of NATs is limited by the fact that overlapping patterns are still difficult to define because most genes give rise to transcript variants that are not well represented in sequence databases. A significant fraction of SAS partnerships result from 3' UTR polyadenylation variants [25] or first exon splice variants that can be located several kilobases away from the annotated 5' end [85, 86]. These may escape detection depending on the screening strategy. In summary, we conclude that there is no clearly predominant overlapping pattern, and there are many distinct subsets of SAS pairs that may play different biological roles in mammalian genomes.

## SAS pairs: True relationship or marriage of convenience

A tissue- or stimuli-specific expression pattern is generally regarded as a prima facie indicator of the functional relevance of transcripts. Several studies

**Table 1.** Results of a PubMed search to retrieve individual human natural antisense transcripts (NATs) that have been experimentally characterized in more detail.[a]

| NAT | Sense transcript | SAS pattern | Coding capacity of NAT | Putative NAT regulatory function | Experimental evidence for putative NAT regulatory mechanism | References |
|---|---|---|---|---|---|---|
| p15AS | p15 (CDKN2B, cyclin-dependent kinase inhibitor 2B; NM_078487) | embedded | non-coding | transcriptional silencing of sense transcript *via* heterochromatin formation | conclusive | [36] |
| Zeb2 NAT | Zeb2 (zinc finger E-box binding homeobox protein 2; NM_014795) | divergent | non-coding | intron retention in sense transcript | conclusive | [37] |
| KCNQ1OT1 (LIT1) (KCNQ1 overlapping transcript 1; NR_002728, AK123073) | KCNQ1 (potassium voltage-gated channel, KQT-like subfamily, member 1; NM_000218) | embedded | non-coding | imprinting control | conclusive | [38–40] |
| sONE/NOS3AS (AY515311/ NM_173681) | NOS3 (eNOS) (nitric oxide synthase 3; NM_000603) | convergent | putative protein-coding | post-transcriptional down-regulation of sense transcript | conclusive | [41, 42] |
| naPINK1 | svPINK1 (short variant of PTEN induced putative kinase 1; NM_032409) | embedded | non-coding | stabilizing or promotion of the expression of the sense transcript | conclusive | [43] |
| ATXN8OS (SCA8; KLHL1AS) (AF126749) | KLHL1 (kelch-like 1; NM_020866) | divergent | non-coding | regulation of sense transcript expression | suggestive | [44, 45] |
| rTSα (ENOSF1, enolase superfamily member 1; NM_017512) | TYMS (thymidylate synthetase; NM_001071) | convergent | protein-coding | down-regulation of sense transcript by site-specific cleavage | suggestive | [46] |
| Msx1-AS | Msx1 (msh homeobox 1; NM_002448) | embedded | non-coding | negative regulation of sense transcript | suggestive | [47] |
| HASNT (HA synthase 2 antisense; BI829151, AI761403) | HAS2 (hyaluronan synthase 2; NM_005328) | divergent | putative protein-coding | negative regulation of sense transcript | suggestive | [48] |
| E2F4-AS | E2F4 (E2F transcription factor 4; NM_001950) | embedded | putative non-coding | negative regulation of sense transcript | suggestive | [49] |
| SAS-ZFAT (AB167742, NR_002438) | TR-ZFAT (truncated form of zinc finger gene in AITD susceptibility region (ZFAT); NM_001029939) | embedded | non-coding | negative regulation of sense transcript | suggestive | [50] |
| aHIF (U85044) | Hif1α (hypoxia-inducible factor 1, alpha subunit basic helix-loop-helix transcription factor; NM_001530) | convergent | non-coding | negative regulation of sense transcript | suggestive | [51, 52] |
| NR1D1 (RevErbAα; NM_021724) | TRα2 (thyroid hormone receptor, alpha isoform 2; NM_003250) | convergent | protein-coding | inhibition of alternative splicing of sense transcript | suggestive | [53] |
| UBE3A-AS | UBE3A (ubiquitin protein ligase E3A; NM_000462) | convergent | non-coding | imprinting control | suggestive | [54, 55] |
| GNAS1-AS (AJ251759) | GNAS1 (GNAS complex locus; NM_016592) | divergent | non-coding | imprinting control | suggestive | [56] |

**Table 1** *(Continued)*

| NAT | Sense transcript | SAS pattern | Coding capacity of NAT | Putative NAT regulatory function | Experimental evidence for putative NAT regulatory mechanism | References |
|---|---|---|---|---|---|---|
| DMPK-AS (AK309505?) | DMPK (dystrophia myotonica-protein kinase (DMPK); NM_001081563) | convergent | non-coding | chromatin control | suggestive | [57] |
| HAR1Ra/b (DQ860410/ DQ860411) | HAR1F (RNA gene involved in cortical development; DQ860409) | divergent | non-coding | negative regulation of sense transcript | poor | [58] |
| SFRS2 (splicing factor, arginine/serine-rich 2; NM_003016) | MFSD11 (major facilitator superfamily domain containing 11; NM_024311) | divergent | protein-coding | regulation of sense transcript splicing | poor | [59–61] |
| SLC22A18-AS (BC030237, NM_007105) | SLC22A18 (solute carrier family 22 (organic cation transporter), member 18; NM_002555, NM_183233) | divergent | putative protein-coding | imprinting control | poor | [62, 63] |
| anti-BDNF (BDNFOS, multiple transcript variants; NR_002832) | BDNF (brain-derived neurotrophic factor; NM_170731, NM_001709, NM_170733, NM_170735) | convergent | non-coding | regulation of sense transcript expression | poor | [64] |
| HFE-AS | HFE (hemochromatosis protein isoform 1 precursor; NM_000410) | divergent | putative non-coding | regulation of sense transcript expression | poor | [65] |
| ABOAs (CK821046, BI792887, BI793155) | ABO blood group (transferase A, alpha 1-3-N-acetylgalactosaminyltransferase; transferase B, alpha 1-3-galactosyltransferase; NM_020469) | divergent | putative protein-coding | regulation of sense transcript expression | poor | [66] |
| FGF-AS (NUDT6; NM_007083) | FGF-2 (fibroblast growth factor 2 (basic); NM_002006) | convergent | protein-coding | regulation of sense transcript expression | poor | [67, 68] |
| DAMS (AF071111) | SMAD5 (NM_001001419) | divergent | putative protein-coding | regulation of sense transcript expression | poor | [69] |
| MKRN2 (makorin, ring finger protein, 2; NM_014160) | RAF1 (v-raf-1 murine leukemia viral oncogene homolog 1; NM_002880) | convergent | putative protein-coding | regulation of sense transcript expression | poor | [70] |
| EMX2OS (NR_002791, AY117413) | EMX2 (empty spiracles homeobox 2; NM_004098) | divergent | non-coding | regulation of sense transcript expression | poor | [71] |
| MYCNOS (BC002892) | N-MYC (v-myc myelocytomatosis viral related oncogene, neuroblastoma derived, NM_005378) | divergent | non-coding | regulation of sense transcript expression | poor | [72] |
| TSIX (X (inactive)-specific transcript, antisense on chromosome X; NR_003255) | XIST (X (inactive)-specific transcript on chromosome X; NR_001564) | embedded | non-coding | unknown (X-inactivation control in mice) | | [73–75] |
| anti-BDMA | BCMA (tumor necrosis factor receptor superfamily, member 17, TNFRSF17; NM_001192) | embedded | putative protein-coding | unknown | | [76] |

**Table 1** *(Continued)*

| NAT | Sense transcript | SAS pattern | Coding capacity of NAT | Putative NAT regulatory function | Experimental evidence for putative NAT regulatory mechanism | References |
|---|---|---|---|---|---|---|
| MFSD11 (major facilitator superfamily domain containing 11; NM_024311) | SFRS2 (splicing factor, arginine/serine-rich 2; NM_003016) | divergent | protein-coding | unknown | | [61] |
| IGF2-AS (NM_016412) | IGF2 (insulin-like growth factor 2; NM_001007139) | embedded | protein-coding | unknown | | [77] |

[a] The search was restricted to human NATs using the keywords: antisense transcript, antisense transcription, NAT, antisense RNA, sense-antisense. Thirty one described human NATs could be retrieved. Available papers and cross-references were thoroughly analyzed and categorized according to experimental confirmation of the predicted NAT function. Note that categorization is done based on a subjective assessment of the authors. A certain categorization does by no means refer to the quality of the cited paper. The terms "conclusive", "suggestive" and "poor" describe if an assumed mechanism has been experimentally verified, concluded from experimental observations, or proposed. NCBI RefSeq or GenBank accession numbers of representative sequences are given if available.

conclude that NATs tend to be expressed in a cell/tissue-specific manner and/or display a tendency to be linked to the expression pattern of their sense counterparts [13, 17, 30–33, 87, 88]. However, quite obviously, there is no consistent scheme in terms of a uniform expression relationship that can be applied to SAS pairs in mammals. Mammalian RNAs that form SAS pairs have been reported to frequently exhibit reciprocal expression patterns [27, 88], but other studies conclude that NATs display a prevalent tendency to be positively correlated with the expression of their corresponding sense counterparts [30–32]. We already considered the potential noisiness of experimental expression data that would likely give an overestimate of the fraction of coexpressed and coregulated SAS pairs. In high-throughput screenings it is also often difficult to prove that both genes are actually transcribed in the same cell, and the fraction of non-coding NATs is generally underrepresented in most studies. Regarding putative SAS regulatory mechanisms, one should be aware that correlations—positive or negative—are not a sufficient predictor of a regulatory SAS interaction. There is increasing evidence that gene order in eukaryotic genomes is not random [89–91], and genes that have similar and/or coordinated expression are often clustered along the genome. Coexpression can be mediated on the small scale by usage of shared regulatory elements, like common enhancers or bidirectional promoters [92–96], or on a larger scale *via* chromatin-mediated processes [97–100]. The biological relevance of coexpression is evident only in a few cases, and Hurst and his colleagues suggested that many incidences of low-to-moderate level coexpression of linked genes might well be coincidental or spurious rather than an indication of functional coordination [101].

Coregulation of divergent SAS pairs has been implicated with bidirectional promoter activity extending to the 5' end of the first intron of the sense gene [84]. CpG-island-associated bidirectional promoters are generally associated with a broad range of alternative transcription start sites (TSSs) that can be spread over ~100 bp [95]. It is questionable whether an observed overlap of transcripts initiated from these promoters provides an additional layer of transcriptional control. Considering non-coding NATs, one can also not exclude a certain level of transcriptional noise that arises from bidirectional promoter activity. Most promoters are probably not intrinsically directional [102] and inappropriately orientated transcription may be an occasional byproduct of chromatin remodeling associated with transcriptional activity. On a larger scale, regional chromatin remodeling and modification processes that are associated with general transcriptional activity may also promote an extended level of transcriptional background activity arising from internal cryptic promoters. This may account for some observed non-coding NATs. However, the contribution of nonfunctional, neutral, or 'noisy' transcripts to the total transcriptional output in eukaryotic genomes is still controversial [85, 103–108].

## Considerations on NAT evolution

Antisense transcription has been reported in viruses [34, 109], and a wide range of organisms from bacteria [110, 111] to protozoa [112], fungi [113, 114], plants [115–117], invertebrates [118], and mammals. If NATs provide an additional layer of transcriptional regulatory complexity, one would expect conservation of evolutionarily advantageous SAS patterns, and perhaps even a concordant increase in abundance of

NATs as a function of organismal complexity. Efforts to compare the amount of antisense transcription in different metazoan genomes based on comprehensive *in silico* approaches offered a considerable variability in the proportion of SAS transcripts but no evidence for a link between antisense transcription and organismal complexity [23, 119]. The variation of estimated numbers may be partly technical and partly due to species-specific differences in transcriptome annotation and transcript sequence integrity. Perhaps for the same reasons, the numbers of apparent conserved SAS patterns between human and mouse are surprisingly low, ranging from a few hundred [21, 120–122] to somewhat more than a thousand conserved SAS pairs [22, 25].

Several mechanisms have been proposed to explain the evolution of SAS orientation patterns, including translocation and transposition events, gene extensions by adoption of transcription initiation or termination signals from the opposite strand, and the formation of new genes and splice variants [121, 123–125]. A recent comparative analysis of seven vertebrate genomes provided evidence that most gene overlaps evolve stochastically and without any positive pressure for overlap [121]. The authors reasoned that *cis*-regulatory SAS interactions are rather exceptional and evolve merely by chance as a consequence of new gene arrangements. This seems conclusive for overlapping protein-coding transcripts; however, it is debatable if, and to what extent, this conclusion is applicable to non-coding NATs. As a matter of fact, there are still numerous human non-coding, structured and conserved RNA genes that remain to be discovered and fully annotated [126], and, compared to protein-coding genes, there might be a considerable higher fraction of non-coding transcripts that might have acquired lineage-specific function due to specific SAS overlapping patterns. The underlying evolutionary constraints might not be evident at the primary sequence level, as cross species conservation of the transcribed region of ncRNA genes generally is remarkable weak [127, 128]. Apart from acting as passive transmitters of protein sequence information, single-stranded RNAs have some unique properties that make them suitable for regulatory roles that might contribute basic layers of regulatory complexity. The quick and easy production, as well as the rapid degradation of RNA, the conformational flexibility of RNA molecules, and their ability to interact with nucleic acid and protein holds the potential for rapid and sophisticated coupling, processing, and storage of endogenous and environmental information [129, 130]. The evolutionary constraints that act on regulatory RNA genes might be fundamentally different from protein-coding genes [9, 131], and ncRNA genes are supposed to evolve significantly faster with conserved features being limited to transcriptional regulatory motifs, short variable elements or RNA secondary and tertiary structure properties [128, 132–135].

## Antisense transcripts: Copilots or stowaways of the transcriptional machinery?

Despite the widespread use of artificial antisense oligonucleotides to block expression or alter pre-mRNA splicing of targeted genes [136, 137], surprisingly little is known about the regulatory action and the underlying mechanisms of endogenous NATs. NATs have been implicated in numerous regulatory mechanisms, affecting, directly or indirectly, virtually all levels of transcriptional control. Yet, most experimental approaches are not up to the task of distinguishing cause and effect in SAS relationships (see Table 1). To date, even the fundamental question of whether or not endogenous SAS sequence pairs actually hybridize in eukaryotic cells is largely unresolved, even for the more extensively investigated NATs [138]. In the following, we discuss the different regulatory mechanisms that have been associated with antisense transcription. So far, conclusions about the regulatory potential of NATs have been drawn only from a small set of overlapping genes in different species.

### RNA masking

In principle, duplex formation between partners of an SAS pair may lead to post-transcriptional masking of key regulatory features within either transcript and thereby inhibiting interaction with trans-acting factors required for splicing, export, or transcript stability. Many SAS overlapping patterns involve at least one exon-intron border and, among SAS pairs involving splice variants, a remarkably high percentage have their pairing regions affected or completely eliminated by alternative splicing [12, 25]. So, is there a relationship between alternative splicing and antisense-directed regulation (Fig. 1A)? So far, only two mammalian examples have been published that provide such as link, namely the RevErbAα transcript that is believed to inhibit alternative splicing of the thyroid hormone receptor TRα2 mRNA [53], and the transcriptional factor *Zeb2* NAT that has been implicated in intron retention in the *Zeb2* mRNA [37].

### RNA editing

Hypothetically, long and perfect intermolecular double-stranded RNA (dsRNA) duplexes might also serve as substrates for RNA-editing adenosine deaminases (ADARs) that act on dsRNA to hydrolytically

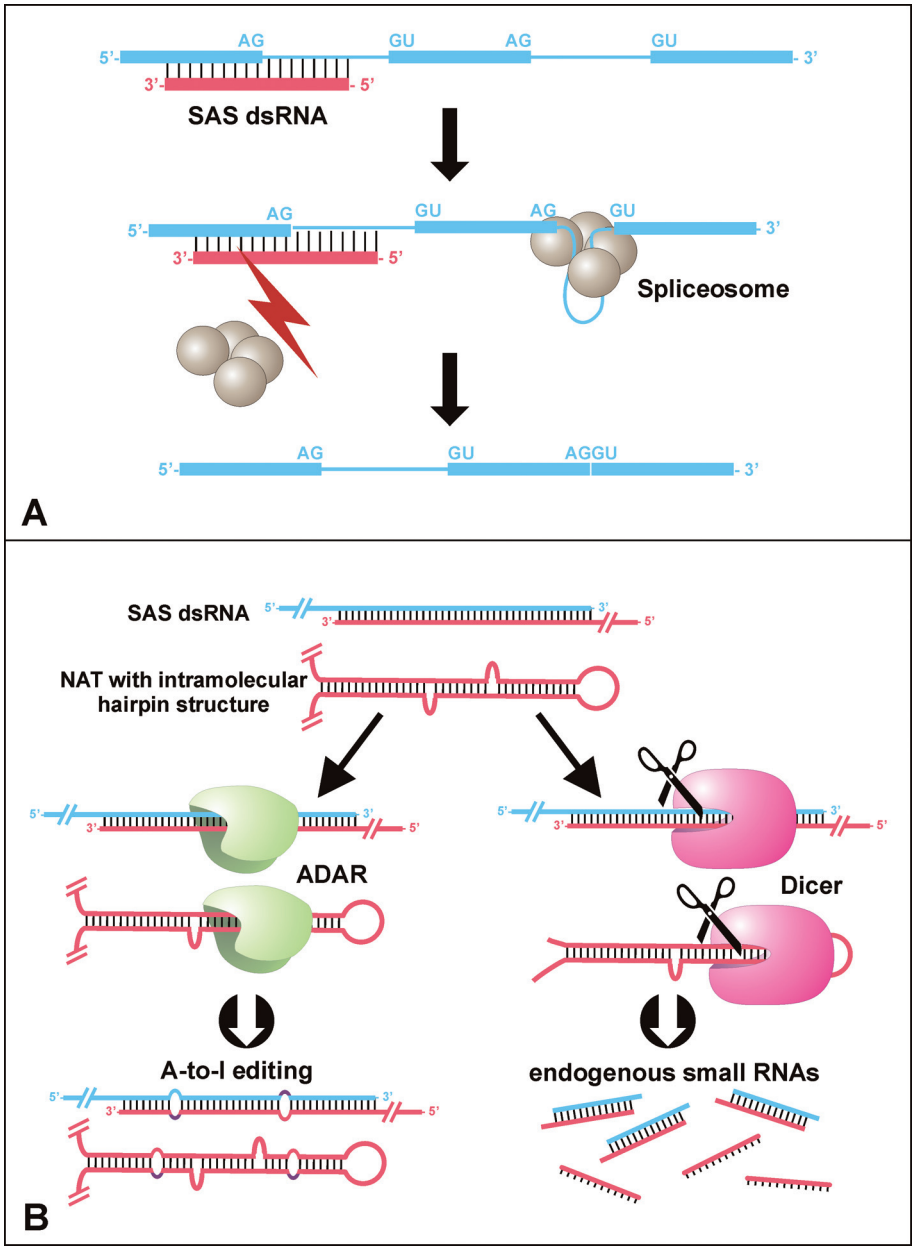


**Figure 1.** (A) RNA masking: A *cis*-regulatory element in the sense transcript (blue) is masked by hybridization with the natural antisense transcript (NAT) (red). In this example, sense-antisense (SAS) duplex formation prevents binding of the spliceosome, and consequently, the intron is retained. (B) A-to-I editing and RNA interference: Like intramolecular hairpin structures, intermolecular SAS RNA duplexes might constitute potential substrates for RNA-processing enzymes like ADAR and DICER.

deaminate adenosine to inosine (A-to-I editing) (Fig. 1B). As any RNA that is at least partially double-stranded is thought to represent a potential substrate for A-to-I editing, it has been suggested that naturally occurring antisense dsRNA duplexes should be heavily edited *in vivo*, raising a possible general role for antisense transcripts in the regulation of nuclear retention, stabilization or degradation of the sense transcript [139, 140]. However, several independent studies of the human transcriptome have confirmed that virtually all of the editing sites reside within inverted elements (mainly Alu repeats) that form intramolecular fold-back hairpin structures [141–143], and NATs, apart from inverted repeats within them, show no editing sites that indicate a connection between antisense transcription and RNA editing in mammals [144, 145]. Although some edited sequences that are rapidly degraded or retained in the nucleus might not be represented in expressed sequence data sets [144], it seems unlikely that natural SAS RNA hybrids represent prominent substrates of the RNA editing machinery.

## RNA interference

It is tempting to speculate that SAS RNA hybrids, as well as NATs that form intramolecular hairpin structures, might serve as precursors of small regulatory RNAs, like short interfering RNAs (siRNAs),

Piwi-interacting RNAs (piRNAs) or microRNAs (miRNAs), that can induce targeted down-regulation of homologous genes at the transcriptional and post-transcriptional level through inhibition of translation, induction of mRNA degradation, or silencing of promoters *via* heterochromatin formation [146–149]. Studies in plants and flies have revealed that SAS pairs and NATs have the potential to become substrates for the ribonuclease III-like enzyme Dicer to produce siRNAs and miRNAs [150–152], indicating that mammalian SAS pairs might also play a role in small RNA-based gene silencing pathways (Fig. 1B). In fact, recent studies in mice provided first evidence for mammalian endogenous siRNAs derived from intermolecular dsRNAs [153, 154]. These reports show that endogenous siRNAs in mammals can be products of the interaction of SAS pairs from the same or different loci, the latter obviously arising from hybridization between an mRNA and its transcribed pseudogene [155]. The extent and significance of endogenous siRNAs in the mammalian genome remain elusive, and one should be aware that the occurrence and regulation of these siRNAs might be largely species specific even in cases of cross-species conserved mammalian SAS partnerships [156].

**Transcriptional interference**

Duplex formation between sense and antisense transcript is not necessarily a prerequisite for putative antisense regulation, because the transcriptional process itself may be sufficient to exert biological activity. There are a number of conceivable modes of action but the boundary between speculation and fact is still broad and fuzzy. Several putative mechanisms have been grouped under the term "transcriptional interference" (TI), describing a direct suppressive influence of one transcriptional process on another in *cis* [157]. TI can occur at the initiation phase of transcription by competition between two interfering promoters for occupation of regulatory elements and binding of RNA polymerase II (RNA Pol II), and at the elongation stage when "oncoming traffic" emanating from one strand hinders the passage or initiation of the RNA Pol complex on the opposite strand (Fig. 2A). Atomic force microscopy made it possible to demonstrate that transcription from convergently oriented promoters can cause collision of RNA Pol complexes, thus halting the transcriptional process [158]. Examples of transcriptional collision in regulation of SAS pairs have been described in yeast [159, 160], and TI has been proposed a key feature of mammalian gene regulation [161]. Considering the broad range of interweaving regulatory possibilities, proof of principle is difficult to obtain, and it is impossible to draw generalized conclusions from an observed overlapping pattern on a certain mode of action. For example, transcription over a promoter or other regulatory regions (activators, silencers, insulators) in some cases might perturb interaction with binding proteins [106], while in other cases binding might be promoted by improving chromatin accessibility [162].

**Targeting of chromatin complexes**

Some NATs may not feed back directly to the overlapping gene but may act indirectly by recruiting factors that promote or inhibit transcription in *cis* or *trans*. These NATs may function as some form of guide RNA by targeting an effector-complex to a specific nucleic-acid sequence. Recently, Yu et al. [36] reported the existence of a p15-NAT that was inversely expressed with the tumor suppressor gene p15 (*CDKN2B*) in human leukemia cells. Using different reporter constructs, they showed that the p15-NAT is capable of down-regulating p15 in a Dicer-independent manner by triggering transcriptional silencing through heterochromatin formation.

Regulation of gene expression through chromatin structure can affect individual genes, chromosomal domains, and entire chromosomes. The dynamic of chromatin state is coordinated by a combination of processes including nucleosome positioning, chromatin remodeling, incorporation of specific chromatin components (alternate histone variants), reversible modifications of histone tails, and binding of chromatin-associated proteins [163, 164]. Actively transcribed genes are commonly devoid of nucleosomes at their transcriptional initiation site, and their core promoters and proximal downstream coding regions are highly enriched in acetylated histones. Moreover, several histone methylation patterns have been found to be associated with varying effects depending on the modified residues (reviewed in [165–168]). Methylation of histone 3 lysine 9 (H3K9) and lysine 27 (H3K27), and methylation of histone 4 lysine 20 (H4K20) has been implicated in heterochromatin formation and gene silencing [169]. Dimethylated H3K4 (H3K4me2) has been correlated with promoter CpG islands, and may have protective effect against DNA methylation [85, 170]. H3K4me3 localizes at transcriptional start sites and is associated with the initiated form of RNA Pol II, while H3K79me2 and H3K36me3 accumulate at intragenic regions and 3' ends of actively transcribed genes, and therefore are considered as hallmarks of ongoing transcription elongation [85, 165, 169, 171–173]. There seem to be complex interactions among DNA-binding proteins, RNA Pol II, chromatin, and chromatin-associated proteins to orchestrate open-

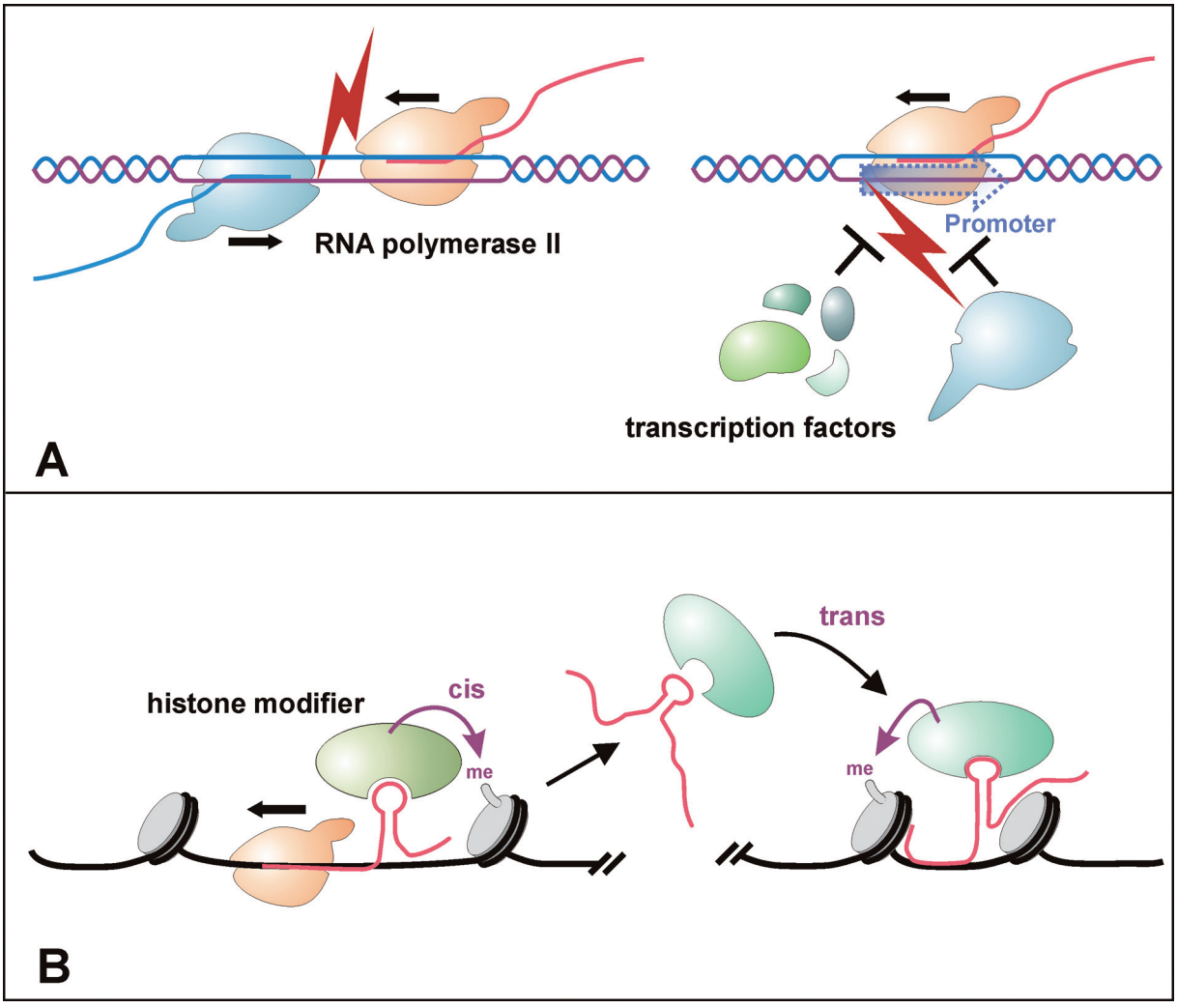


**Figure 2.** (A) Transcriptional interference: Elongating polymerase from one gene directly interferes with transcription elongation (left) or initiation (right) of an overlapping transcriptional unit. (B) Epigenetic modification: The NAT forms a structure that is recognized by distinct histone-modifying complexes. Recruited complexes can act in *cis* or may be guided by the ncRNA to distant genomic loci.

ing of chromatin for transcriptional initiation [174–176], disruption of chromatin structure for transcriptional elongation [85, 165, 169, 171–173], as well as restoration of chromatin structure following the passage of RNA Pol II [177–179]. Considering the complex network of overlapping sense and antisense transcripts, it is apparent that effects of local alterations in chromatin structure are not exclusively confined to insulated transcriptional units, but may also influence, deliberately or incidentally, the expression of adjacent or intersecting transcribed regions on both strands of a genomic locus.

Histone modifications have been implicated in regulation of higher order chromatin structure and attraction of effector complexes to specific chromatin domains. The significance and hierarchic position of distinct histone modification patterns in the complex traffic guidance system that coordinates transcription are still unclear [180]. Whatever their exact contribution, histone modifiers, as well as enzymes that trigger DNA methylation, must be recruited to their target genes. It has been postulated that specific ncRNAs and NATs may function to guide DNA- and histone-modifying enzymes to discrete genomic loci [181, 182] (Fig. 2B). Thus, NATs could play a central role in gene regulation through altering the dynamic properties of chromatin and the establishment of epigenetic chromatin marks.

## Regulation of monoallelic expression and developmental control

Several studies have shown that antisense transcription appears to be pivotal to the mechanisms governing dosage compensation and genomic imprinting in the mammalian genome (reviewed in [106, 183]).

While these studies emphasize the regulatory potential of NATs they also illustrate the spectrum of obstacles and caveats one encounters when dealing with antisense transcription.

**X chromosome inactivation.** Probably the most prominent mammalian ncRNA is the X-inactive specific transcript (*Xist*) that is expressed exclusively from the inactivated X chromosome (Xi) and plays a central role in the initial phase of sex-chromosomal dosage compensation in female mammals. When X inactivation is initiated, *Xist* RNA spreads from its site of transcription to coat the X chromosome, and this spread correlates with the transcriptional silencing of the Xi [184]. The exact mechanisms by which *Xist* accomplishes silencing of the Xi are still unknown [185, 186]. It is believed that *Xist* functions by recruiting histone-modifying complexes, thereby helping to establish multiple repressive layers of histone modifications, histone variants, and DNA methylation on the Xi [187–189]. In mouse, the *Tsix* gene encodes a non-coding NAT that overlaps the entire *Xist* locus, and has been shown to regulate *Xist* expression [190]. *Tsix* is initially expressed on both X chromosomes in females and is down-regulated at the onset of cell differentiation on the Xi. In contrast, its expression persists transiently on the future active X chromosome [191]. Loss of *Tsix* expression on the future Xi is a prerequisite for the up-regulation of the *Xist* transcript, whereas initially persistent expression of *Tsix* enables the second X chromosome to remain active. Knockout studies and premature truncation of the *Tsix* transcript in mutant mouse embryonic stem (ES) cells demonstrated a *cis*-regulatory function of *Tsix* on the *Xist* locus [192–196]. Several studies indicate that *Tsix* function acts *via* complex chromatin remodeling activities within the *Xist/Tsix* locus [191, 197–199]. However, the underlying mechanisms are not well understood. Recently, Ogawa et al. [200] reported that *Xist* and *Tsix* can form duplexes that are processed to small RNAs (25–42 nucleotides) in a Dicer-dependent manner. The authors propose a model whereby on the active X these small RNAs locally repress *Xist* in *cis* at the onset of X inactivation by a yet unknown mechanism.

The situation in human X inactivation is, if anything, even more unclear. Although the presence of both *Xist* and *Tsix* analogues has been verified, only the first seems to have retained a crucial function in humans. Surprisingly, the human equivalent of *Tsix*, i.e. *TSIX*, is coexpressed with *XIST* from the human Xi and has no regulatory influence on *XIST* transcription and X inactivation [74]. It has to be noted that, although random X inactivation seems to be a conserved feature among placental mammals, species-specific differences exist in some aspects of the mechanism. In rodents the extraembryonic cell lineages differ from somatic tissues in that X inactivation is imprinted, preferentially silencing the paternal X chromosome, whereas in humans, X inactivation occurs randomly among cells in both placenta and somatic tissues [201]. *TSIX* may have become functionless in the course of primate evolution in association with this key developmental change.

**Genomic imprinting.** Several studies suggest an intimate relationship between antisense transcription and the regulation of genomic imprinting, a process whereby certain genes are expressed monoallelically from either the maternal or paternal chromosome [13, 23, 31, 202]. Obviously, the fundamentals of X chromosome inactivation are mechanistically closely related to the processes that govern genomic imprinting. It has been shown that, in both human and mouse, SAS pairs are significantly less prevalent on the X chromosome than on any autosome [13, 23, 31, 202]. According to Reik and Lewis [203], autosomal imprinting and X-chromosome inactivation co-evolved with placentation during the divergence between ancestral egg-laying and placental mammals. On the X chromosome, inactivation then spread to involve the whole chromosome while on the autosomes imprinted clusters evolved, marked by an abundance of associated NATs.

In mammals, only a small number of genes are imprinted, and most imprinted genes are clustered in chromosomal domains that are broadly conserved between humans and mice [204]. There is growing evidence that relatively short DNA sequences, called imprinting control regions (ICRs), govern monoallelic expression across the entire imprinted domain. ICRs are characterized by differentially methylated regions (DMRs) that are acquired during male or female gametogenesis and maintained only on one parental allele after fertilization [106].

Several ICRs have been shown to contain promoters for long ncRNAs (mostly NATs) whose expression correlates with the repression of some or all imprinted protein-coding genes. Generally, antisense transcription seems to play a pivotal role at sites of genomic imprinting, and several NATs have been implicated in the silencing not only of overlapping genes but also of reciprocally imprinted non-overlapping genes. Here we briefly discuss some well-characterized NATs in imprinted gene clusters. More detail is provided by Yang and Kurado [183] and Pauler et al. [106].

The insulin-like growth factor receptor type 2 gene (*Igf2r*) encodes for a transmembrane receptor in mammals that binds both mannose-6-phosphate-tagged proteins and IGF2 through independent binding

sites. Two DMRs at the murine *Igf2r* locus are associated with the paternally imprinted (maternally expressed) *Igf2r.* DMR1 includes the *Igf2r* promoter, while DMR2 is located within the second intron of the *Igf2r* gene including the promoter for a non-coding paternally expressed NAT, termed *Air* that has been implicated in imprinting control of the *Igf2r* locus [205, 206]. In peripheral tissues, targeted deletion of the *Air* promoter and premature truncation of the *Air* transcript results not only in loss of silencing of the paternal *Igf2r* allele but also in biallelic expression of *Slc22a2* and *Slc22a3*, two paternally imprinted genes that do not overlap with *Air* [207–209]. Paradoxically, in the mouse brain *Igf2r* is biallelically expressed despite the presence of the paternally expressed *Air* transcript, and in humans, *IGF2R* is biallelically expressed in all tissues despite the presence of a differentially methylated CpG island in intron 2 [210, 211].

Another well-investigated NAT that acts as a bidirectional silencer of multiple genes is located in the mouse *Kcnq1* imprinted domain. Almost all of the imprinted genes in the *Kcnq1* domain are maternally expressed, except the paternally expressed NAT *Kcnq1o1* (*Lit1*) [38, 212]. The promoter of *Kcnq1o1* has been mapped to an ICR (KvDMR1) that is located in intron 10 of the *Kcnq1* gene. KvDMR1 is methylated on the maternal chromosome and unmethylated on the paternal chromosome and acts as a long-range repressor for a cluster of 8 paternally imprinted genes [213]. Similar to the *Igf2r* locus, the maternally methylated KvDMR1 is associated with the promoter of the paternally expressed NAT *Kcnq1o1.* Evidence for a role of *Kcnq1ot1* in gene silencing was obtained by targeted deletion of the antisense promoter and experimental truncation of the *Kcnq1ot1* RNA, resulting in loss of imprinted expression of the genes known to be under the control of KvDMR1 [40, 214–216]. In a recent study, the bidirectional silencing property of *Kcnq1ot1* has been mapped to a highly conserved repeat motif within the 5' end of the *Kcnq1ot1* RNA, which directs transcriptional silencing by RNA/chromatin interactions [217]. In this model, the NAT coats the chromatin of flanking sequences in *cis*, followed by the recruitment of the heterochromatin machinery, which in turn targets the linked chromosomal domains to distinct nuclear compartments to maintain the repressive chromatin throughout successive cell divisions.

It is generally agreed that expression of *Kcnq1ot1* paves the way for imprinted silencing at the KvDMR1 cluster. However, very recently, a thorough analysis of allelic expression in *Kcnq1ot1* truncation mice revealed that antisense transcription is not the sole regulatory mechanism that is operational at the KvDMR1 domain, suggesting a more complex picture in the imprinted regulation of different subsets of genes in different cell lineages or at different stages of development [39]. In addition to the antisense promoter, two binding sites for the insulator protein CTCF have been reported within KvDMR1, which are occupied only on the unmethylated paternal allele [218]. This raises the possibility that KvDMR1 may also function as a chromatin insulator, in a manner similar to the *H19* DMR reported in imprinting of the *Igf2* locus. The mechanism that governs the expression of the reciprocally imprinted genes *Igf2* and *H19* is commonly referred to as the enhancer competition model. *Igf2* is exclusively expressed from the paternal allele, whereas the non-coding *H19* is maternally expressed [219]. Monoallelic expression of the *Igf2* gene is regulated by a DMR upstream of the *H19* promoter and by an *H19* downstream enhancer [220]. On the maternal chromosome the hypomethylated *H19* DMR acts as an insulator sequence that binds CTCF, thereby preventing enhancer/promoter interaction at the *Igf2* locus and allowing transcription at the *H19* promoter. On the paternal allele, methylation of the DMR prevents CTCF binding, leading to *Igf2* expression and *H19* silencing. This mechanism is based on a network of intra- and interchromosomal interactions at the DMR [221–223]. Unlike *Kcnq1ot1*, the *H19* transcript itself, although being conserved and highly expressed among mammals, seems to play no role in the imprinted regulation of *Igf2* [224].

**Regulation of Hox gene expression.** Probably the most striking example of a seemingly intricate network of overlapping coding and non-coding transcripts can be found in the regulation of the homeobox (*Hox*) genes that encode key regulators in embryonic development. Mammals possess four similarly organized *Hox* clusters (A–D) on four different chromosomes that consist of 9–11 *Hox* genes. The expression of *Hox* genes is precisely regulated and synchronized during development to specify positional values that control the anteroposterior patterning [225]. Detailed transcriptional analyses of the human *HOX* clusters revealed the presence of a remarkable number of ncRNAs, mainly transcribed from genic and intergenic regions in the direction opposite to the *HOX* genes [226–228].

One heretical question to ask in this context is whether antisense transcription is actually a central theme in developmental control or whether the prevalence of reported SAS pairs has been catalyzed by the intensive study of the *Hox* cluster. In any case, expression of *Hox* genes not only requires a sophisticated spatial and temporal regulation but also needs to be maintained for the duration of their patterning functions.

At least part of this transcriptional cellular memory appears to rely upon chromatin-remodeling proteins that play an integral role in epigenetic regulation and maintenance of the *Hox* expression pattern [163, 229]. Two opposing groups of histone-modifying complexes, the trithorax group (TrxG) and the Polycomb group (PcG) proteins, have been implicated in maintaining this cellular memory as regions of open and closed chromatin domains in the Hox loci [230]. In this context, the large number of ncRNAs in the Hox clusters may be instrumental by altering the accessibility of DNA sequences important for TrxG and PcG binding, or by recruiting these complexes to discrete chromatin loci. However, it cannot be excluded that expression of some ncRNAs merely reflects transcriptional 'background noise' of these domains. One of these transcripts has been functionally characterized in more detail by Rinn et al. [226]. This highly conserved 2 kb ncRNA, termed *HOTAIR*, is transcribed from an intergenic region in an antisense manner with respect to the canonical HoxC genes. Surprisingly, knockdown of *HOTAIR* showed no changes in the *HoxC* cluster, but instead showed a loss of transcriptional repression from a 40-kb region of the *HoxD* cluster, accompanied by a global loss of H3K27me3 over this locus. It could be demonstrated that *HOTAIR* interacts with the polycomb repressive complex 2 (PRC2) that mediates tri-methylation of H3K27. Although it is transcribed from an intergenic region, HOTAIR highlights the potential of antisense transcripts to mediate epigenetic silencing in *trans* by guiding a repressive mark to a distant chromosomal domain.

## Conclusions

It remains an open challenge to ascribe pattern-associated mechanisms to individual SAS pairs and to find suitable experimental approaches to distinguish between controller, actuator, and by-product in SAS relationships. Experimental perceptions may be shaped unconsciously by theory preference, and in view of the extensive network of overlapping regulatory pathways, determination of biological functionality of a given NAT is quite difficult. Unfortunately, disproving functional significance can be even harder, as some NAT regulatory mechanisms seem to be highly species-specific and spatially and temporally restricted, irrespective of the observed expression pattern.

If we are looking for the hidden pathways that lead from genotype to phenotype, we have to abandon the heavily traveled roads reserved for protein-coding transcripts. Unfortunately, evolution is robust but messy and there is a risk that many of these paths might have been abandoned long ago, leading us into blind alleys and long diversions, or are only temporarily accessible.


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