Visualizing the structure and motion of the long noncoding RNA HOTAIR

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ABSTRACT

Long noncoding RNA molecules (lncRNAs) are estimated to account for the majority of eukaryotic genomic transcripts, and have been associated with multiple diseases in humans. However, our understanding of their structure–function relationships is scarce, with structural evidence coming mostly from indirect biochemical approaches or computational predictions. Here we describe direct visualization of the lncRNA HOTAIR (HOX Transcript Antisense RNA) using atomic force microscopy (AFM) in nucleus-like conditions at 37°. Our observations reveal that HOTAIR has a discernible, although flexible, shape. Fast AFM scanning enabled the quantification of the motion of HOTAIR, and provided visual evidence of physical interactions with genomic DNA segments. Our report provides a biologically plausible description of the anatomy and intrinsic properties of HOTAIR, and presents a framework for studying the structural biology of lncRNAs.

Keywords: atomic force microscopy; HOTAIR; long noncoding RNA

INTRODUCTION

Long noncoding RNA (lncRNA) molecules are defined as RNA transcripts longer than 200 nt, which lack an evident ORF and are transcribed by RNA polymerase II. These large transcripts are often also polyadenylated and spliced. Thousands of lncRNAs are annotated to date, but only a few have been studied and defined as functional. Functional lncRNAs are involved in almost every stage of gene expression (Amaral and Mattick 2008; Dinger et al. 2008; Hung and Chang 2010; Fatica and Bozzoni 2014) and have been implicated in a variety of diseases such as cancer and neurodegenerative disorders.

Despite their abundance and emerging importance, our knowledge concerning lncRNA structure is poor. Existing information on the structure of large RNA molecules in general is scarce, with <7% of all RNA structures in the Protein Data Bank being in the size range between 200 and 5000 nt, most of these being ribosomal RNA subunits (Ban et al. 2000) studied by X-ray crystallography. The structure of MALAT1 has been resolved by crystallography (Brown et al. 2014), but available information on other lncRNA structures derives mostly from indirect methods. For example, the structure of SRA (Novikova et al. 2012) and HOTAIR (Somarowthu et al. 2015) were depicted through biochemical methods. Another direction taken to further study lncRNAs such as XIST (Wutz et al. 2002) and GAS5 (Kino et al. 2010) was domain analysis. On the other hand, it has recently been suggested, using a statistical approach, that lncRNAs may not have a structure at all (Rivas et al. 2017). This discrepancy may result from a lack of solid structural information, and its resolution could shed light on the biology of this important class of molecules.

In this work we aimed to obtain such information using one of the most studied lncRNAs, HOTAIR, as a test case. HOTAIR has been shown to bind PRC2 and LSD1 (Tsai et al. 2010) to drive chromatin modification at specific genomic sites (Chu et al. 2011), thus playing a key role in genome silencing. HOTAIR DNA binding sites are focal, specific and numerous, implying HOTAIR as a silencing selector element pinpointing genomic locations to modification (Chu et al. 2011). HOTAIR was shown to be...
required for the epithelial-to-mesenchymal transition (Pádua Alves et al. 2013; Battistelli et al. 2016), thereby defined as an oncogene and a negative prognostic marker in various cancers (Liu et al. 2013; Wan 2013; Wu et al. 2014).

Our central tool in this study was atomic force microscopy (AFM). AFM has been used to study nucleic acid structures in both fluid and air (Lyubchenko et al. 2011), including the genomic RNA of human immunodeficiency virus (HIV)-1 (Andersen et al. 2004). While AFM is limited in resolution compared with X-ray crystallography or NMR, it enables direct visualization by physically probing native, large molecules under biological conditions. AFM also allows statistical analysis of structurally diverse molecules, as previously suggested to be the case for lncRNAs (Novikova et al. 2013).

RESULTS

HOTAIR has a distinct anatomy

We first generated HOTAIR molecules by in vitro transcription (IVT), using multiple templates and multiple IVT systems in order to avoid method-biased observations. The resulting transcripts were analyzed by gel electrophoresis and RNA-seq and found to be intact and to fully map to the HOTAIR gene sequence in a human reference genome (Supplemental Notes 1, 2). HOTAIR molecules were then scanned by AFM in fluid, under conditions that mimic the chemical environment of the nucleus as reliably as possible (Alberts et al. 2002; Cowan 2002; J Cowan, pers. comm.) and at 37°C (Supplemental Note 3).

AFM scanning demonstrated that under these conditions, HOTAIR molecules assume a distinct anatomy (Fig. 1A,B), based on a four-limbed body which ends in a branched U-shaped motif, which we termed the U-module. Visualization of HOTAIR by Cryo-EM showed the same anatomy and flexibility observed in AFM (Fig. 1C; Supplemental Note 4). This anatomy was reproduced in seven independent synthesis and scanning repeats. In contrast, a random RNA transcript formed by scrambling HOTAIR sequence formed indistinct shapes and aggregates, with some even not folding (Supplemental Note 5). The archetypal HOTAIR anatomy could be reliably assigned to ∼66% of the observed objects that were intact based on size, with excluded ones being either clear but eccentric or too vague to be reliably assigned. The presence of eccentric forms could reflect that not all HOTAIR products fold properly in the nucleus and are subsequently dysfunctional; however, the folding of HOTAIR in the nucleus could be facilitated by yet unidentified cellular factors. In addition to the whole molecule, the functional HOTAIR modules suggested in previous studies (Tsai et al. 2010; Chu et al. 2011; Wu et al. 2013; Kalwa et al. 2016) were also scanned, showing distinct structures (Supplemental Note 6, Movie S1–S3).

Our HOTAIR model divides the molecule into nine structural segments, which we name as follows: neck (N), torso (T), pelvis (P), leg long (LL), leg short (LS), hand long (HL), hand short (HS), U-module long (UL), U-module short (US) (Fig. 1D). Total length of all segments across multiple samples was constrained to 252 ± 9.4 nm, but the observed variance within segments was 5% to 50% of the mean segment length (shorter segments exhibited higher variance) (Fig. 1E). The high degree of flexibility in our proposed model of HOTAIR may seem contradictory to the conventional meaning of a molecule having a structure; however, this notion is contained within a set of objects that are well-defined anatomically but are globally flexible. A useful analogy is shown here by human dancers frozen in various configurations (Fig. 1F), who also combine these two properties.
Dimensions of HOTAIR

The molecular dimensions of HOTAIR were measured by an algorithm that combined the absolute size calculated by the AFM with a dsDNA molecule as an internal size reference. We chose to use a dsDNA molecule termed HOTAIR-binding DNA 1 (HBD1) (Liu et al. 2013), which we synthesized for this study (Supplemental Note 7). HBD1 is a 433 bp molecule existing preferentially at the B-DNA geometry (helical rise of 3.5 Å per base with 10.5 bp/turn, yielding a longitudinal density of 3 bp/nm) under the study conditions, thus mapping to 144 nm in length. In contrast, HOTAIR, a 2158 nt RNA molecule which is mostly dsRNA, preferentially exists at the A-DNA geometry (helical rise of 2.6 Å per base with 11 bp/turn, yielding a longitudinal density of 4.23 bp/nm).

Our measurements (Supplemental Note 8) showed a mean length of 142 nm for HBD1, and 252 nm for HOTAIR, the latter translating to 1066 bp of dsRNA, which theoretically unfold to 2132 nt of ssRNA. Taken together, this calculation represents a \(~1.5\%\) error in molecular measurements by AFM under the study conditions.

Interaction between HOTAIR and DNA

It is critical to note that our aim in this study was to report intrinsic properties of HOTAIR, particularly its ability to interact with genomic DNA, dissociated from the suggested role of auxiliary proteins in this function, which is still unclear. For example, a recent study reported that genome targeting by HOTAIR is independent of at least one specific protein it interacts with, EZH2, although it did not rule out other proteins such as those that are part of the complex LSD1 (Chu et al. 2011). With that in mind, our aim here was first to describe the structural “baseline” of HOTAIR, on top of which future investigations of the functional complexes it forms inside the cell could be carried out.

To this end, two dsDNA sequences from a previous study (Chu et al. 2011) were used, one that was found to highly associate with HOTAIR and one that was found not to, termed HOTAIR-binding DNA 1 (HBD1) and HBD4, respectively (Supplemental Note 7). HBD1 and HBD4 were allowed to interact with HOTAIR for short times, up to 30 min at various HOTAIR:HBD ratios, and fast AFM scanning was used to count temporally and positionally defined interactions (remaining bound at the same position along several scan frames). AFM scans demonstrated clear physical interaction between HOTAIR and DNA (Fig. 2A–C; Supplemental Movies S4–S6). Several interesting features of these interactions were observed. First, assignments based on plausible configurations combined with length measurements revealed that HOTAIR:DNA interactions appear to be mediated by the U-module.
bonds HOTAIR (Supplemental Fig. S6). In some cases, a second interaction is seen mediated by an H segment (Fig. 2A,C). Second, the 1200 nt domain of HOTAIR, previously shown to associate with DNA (Kalwa et al. 2016), exhibited this ability under the study conditions, suggesting that the U-module maps to this specific domain (Fig. 2F; Supplemental Movie S7). Third, the occupancy of HBD1 by HOTAIR was ratio-dependent (Fig. 2G), suggesting a real biological phenomenon. Finally, the occupancy of HBD1 by HOTAIR was 3.2-fold higher than that of HBD4 (46 occupied out of 60 total HBD1 molecules counted vs. 16 occupied out of 66 total HBD4 counted) (Fig. 2H). Interestingly, HBD1 occupancy by the 1200 nt domain was 18 out of 32 total HBD1 counted, suggesting that an additional domain, such as an H segment as observed here, contributes to the higher binding of the whole molecule. The HOTAIR:DNA interactions observed by AFM were corroborated by flow cytometry, showing that HBD1, but not HBD4, binds HOTAIR (Supplemental Fig. S6).

Evidence for a triplex helix mediating HOTAIR:DNA interaction

HOTAIR:DNA interactions have been proposed to be mediated through a triplex helix structure (Chu et al. 2011). A more recent study (Kalwa et al. 2016) showed by electrophoretic mobility shift assay that HOTAIR segments may form RNA–DNA–DNA triplexes, however in a nonbiological system (e.g., boiling to 60°C and cooling). Hoping to shed light on this mechanism, we initially used the Triplexator (Buske et al. 2012) package to perform sequence-based predictions of potential sites for triplex formation between HOTAIR and HBD1. Triplexator retrieved three potential triplex scenarios, all within the HBD1-binding, 1200 nt domain of HOTAIR. Out of these, two were biologically probable, that is, sequences with sufficient guanine residues to support Hoogsteen and reverse-Hoogsteen base-pairing, and antiparallel configuration. Examination of the highest-scoring combination of oligonucleotides by circular dichroism (CD) spectroscopy showed evidence of a formed triplex involving ssRNA and dsDNA, namely a negative peak at 210 nm and a shifted positive peak at 280 nm (Fig. 2I; Supplemental Note 9).

Motion of HOTAIR using fast-scan AFM

Our observations of HOTAIR revealed a highly flexible molecule, which exhibited a striking diversity of configurations, albeit converging to the same anatomy. A central question is thus whether the known mechanics of RNA allows for such flexibility. Previous works have reported that the flexibility of dsRNA is lower than that of dsDNA, and measurements by orthogonal techniques have yielded persistence length values around 62 nm (Hagerman 1997; Abels et al. 2005), which is longer than the observed discrete segments of HOTAIR. However, these estimations may not properly reflect the behavior of biological RNA molecules. Computational predictions, as well as recent experimental works that include biochemical methods and direct imaging (Andersen et al. 2004; Somarowthu et al. 2015), show that these molecules are rich in unpaired loops of varying sizes, which can be thought of as mechanical joints that allow for the observed flexibility. In order to quantify this freedom of motion within our experimental system, we measured the range of movement of a single RNA joint in the AFM. We scanned 12-nt RNA joints connected at the edges of DNA origami rectangles used as AFM imaging guides. Segments connected by these joints were able to pivot up to approximately ±100° in the study conditions (Supplementary Note 10). Given the fact that, based on biochemical analysis as well as computational predictions applied locally (Supplementary Note 11), HOTAIR is rich in such joints, its actual flexibility is likely significantly larger than that predicted for an idealized dsRNA shaft.

We utilized the fast scanning capability of our AFM system in order to quantitate the dynamics of minimally constrained HOTAIR molecules under biological conditions. The mica surface was covered with poly-L-ornithine in order to pin down, as quickly as possible, the DNA–RNA complexes that formed during the short incubation time. Once the complex is captured by the mica, DNA is no longer moving, whereas RNA appears as softer, smaller, shows a complex 3D structure with multiple nonbinding points allowing it to move more freely and adopt a range of possible configurations. Our initial observations revealed a very diverse range of HOTAIR morphologies (Fig. 3A), and further investigation into the dynamics of these molecules demonstrated that this diversity most likely derives from movement, and not degradation or misfolding (Supplementary Movies S8–S10). All limbs of HOTAIR are capable of pivoting around joints, extending, or retracting (Fig. 3B; Supplementary Movie S8), including the U-module, which exhibited pincer-like motion and at least one clear joint in segment UL (Fig. 3C). These movements occupied a radius of up to 20 nm from body (Fig. 3D,E).

Visualizing RNA:DNA structures by AFM

Our AFM scans enabled direct observations of structural configurations of DNA and RNA under the study conditions, as well as the identification of differences between their material properties using phase imaging. In this imaging mode, the phase difference between the cantilever and the drive signal is measured, a measurement sensitive to the stiffness or softness of the sample, providing an additional layer of information in samples where height may be equal throughout, but that are made of various materials. Phase imaging of our samples was able to discriminate between DNA and RNA based on the fact that the DNA is...
more rigid than RNA (Fig. 4A,B). For this reason, we frequently used phase imaging in complex samples to be able to reliably determine molecular identity of sample objects.

Multiple scans have repeatedly shown the right-handed, double helical structure of DNA under the study conditions in exquisite detail (Fig. 4C–E). Our measurements yielded a dsDNA helical twist angle of 31.89° relative to helix plane, which is within the observed range (Dickerson and Klug 1983) of 27.7°–42.0° (Fig. 4F). Measured mean helical pitch was 3.76 nm, within 3 Å of the accepted value of 3.4 nm for B-DNA (Fig. 4G,H). Measurements of dsRNA (Fig. 4I,J) yielded a helical twist angle of 18.86° relative to helix plane, within the observed range of 16.1°–44.1°, and a helical pitch of 2.89 nm, with the accepted value being 2.82 nm for A-DNA (Fig. 4K,L). Interestingly, dsRNA exhibited a lower profile than dsDNA due to its relative softness.

**DISCUSSION**

LncRNA molecules are emerging as abundant and important players at multiple levels of regulation over gene expression. Our ability to study structure-function relationships in this new group of molecules would be critical to our understanding of their biology, their roles in health and disease, and the potential ways to correct their malfunction.

Our conclusions regarding structure-function relationship of HOTAIR need to be taken carefully for several reasons. First, our model does not take into account HOTAIR-protein interactions, which may be indispensable to its cellular state and functionality. Such interactions have been previously described, for example, with the proteins PRC2 and LSD1 (Tsai et al. 2010), which are involved in specific chromatin modifications. While our observations indicate that HOTAIR has an inherent structure, this structure could be very well modulated in the cell by such interactions. Future studies could build on the approach we describe here, and incorporate these proteins in the study systems.

Second, we chose to visualize HOTAIR in conditions that mimic, to the best of our knowledge and ability, the chemical conditions inside the nucleus. Particularly, divalent and monovalent ions are expected to play important roles for lncRNA molecules, that is, stabilizing long-range interactions. Our study conditions included, for example, a low magnesium concentration; a higher magnesium concentration could theoretically produce different anatomies and configurations than the ones observed here.

Third, because of the observed flexibility of HOTAIR, the molecule orients freely and displays variable anatomy. This phenotype highlighted the need to rely on multiple parameters, such as measured lengths and position of modules relative to each other, while eliminating shapes that cannot be assigned beyond a reasonable likelihood. Despite that, image-based assignment of molecular identities is an inherent limitation of molecular imaging in AFM, and our conclusions in this report should be taken with this limitation in mind. Better assignment could be achieved by adding information to the system, for example, by chemical modification of the molecule under observation. A simple way to do that is by...
hybridizing a nanoparticle-labeled DNA or RNA probe to a specific part of the molecule; the nanoparticle could be relatively easily identified in the AFM, indicating with high probability the identity of the part it binds to. However, such methods constantly face the risk of the modification perturbing the native conformation of the molecule, in a molecular analog of Heisenberg’s uncertainty principle. In this study we chose to do careful assignment as natively as possible. Further studies using additional methods could further improve our understanding of the complex structure and behavior of molecules such as HOTAIR.

With that said, our observations of HOTAIR produce a biologically plausible model of its anatomy, quantitate its motility, and confirm it can intrinsically target genomic DNA. Moreover, our findings demonstrate that structural study of lncRNAs can be done using AFM as a tool of choice, owing to its ability to enable direct, high-resolution, and dynamic visualization of nucleic acids in a liquid, cell-like environment, and at physiological temperature. Although it was introduced more than three decades ago (Binnig et al. 1986), AFM is still not a mainstream technique in molecular, cellular, and structural biology. Our findings make a convincing case in favor of adding this versatile tool to X-ray crystallography, NMR, and cryo-EM, in order to enable new forms of understanding of the behaviors of biological molecules.

MATERIALS AND METHODS

In vitro transcription (IVT) and RNA-seq

L2RS-HOTAIR and pCDNA3-HOTAIR were a kind gift from Prof. Howard Chang. pJ-HOTAIR was purchased from DNA2.0. IVT templates were either plasmids linearized with EcoRI restriction enzyme (NEB), or PCR amplicons. IVT was carried out using two separate kits (from New England Biolabs and Megascript). IVT was carried out for 3 h at 37°C and followed by DNA template digestion (using DNase included in the kits). Samples were analyzed by gel electrophoresis using formamide as denaturing agent to verify purity from prematurely terminated transcripts. Purity was confirmed by HPLC using an Agilent 1100 series instrument. RNA was purified using MegaClear kit. RNA-seq was performed at the Nancy and Stephen Grand Israel National Center for Personalized Medicine (G-InCPM) at the Weizmann Institute of Science. Library preparation was done using in-house protocols.

AFM and Cryo-EM

Samples were analyzed using a NanoWizard ULTRA Speed AFM (JPK Instruments) mounted on an inverted optical microscope (Nikon Eclipse TE2000-U or Zeiss AxioObserver.A1), or equipped with a JPK TopViewOptics. Samples were imaged in buffer at ambient temperature in amplitude-modulation or phase-modulation AC mode. Fast-scanning high-resonant ultra-short cantilevers (USC-F0.3-k0.3, NanoWorld) with a nominal resonance frequency of 300 kHz in air, spring constant of 0.3 N/m, reflective chromium/gold-coated silicon chip, and high-density carbon tips with a radius of curvature of 10 nm were used. Prior to deposition on substrate, RNA and HBDs molecules were incubated in filtered nuclear-like buffer (NLB; 5 mM Tris-HCl, 140 mM K+, 0.5 mM Mg2+, pH = 7.2) for 30 min at 37°C. For cryo-EM, HOTAIR-bearing grids were plunge-frozen in liquid ethane cooled by liquid nitrogen, using a Leica EM-GP plunger (4 sec blotting time, 80% humidity), and imaged at liquid nitrogen temperature on an FEI Tecnai TF20 electron microscope operated at 200 kV with a Gatan side entry 626 cryo-holder. Images were recorded on a K2 Summit direct detector (Gatan) mounted at the end of a GIF Quantum energy filter (Gatan). Images were collected in counting mode, at a calibrated magnification of 16,218 yielding a pixel size of 3.083 Å. Additional detailed methods can be found in the Supplemental Notes.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.
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Author contributions: R.S.S. spearheaded research. R.S.S., D.S., L.A., H.H., and J.G. designed and performed experiments and analyzed data. L.A. and H.J. performed computational modeling. I.B. wrote the manuscript.

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