Genome-Wide Profiling of Yeast DNA:RNA Hybrid Prone Sites with DRIP-Chip

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Abstract

DNA:RNA hybrid formation is emerging as a significant cause of genome instability in biological systems ranging from bacteria to mammals. Here we describe the genome-wide distribution of DNA:RNA hybrid prone loci in *Saccharomyces cerevisiae* by DNA:RNA immunoprecipitation (DRIP) followed by hybridization on tiling microarray. These profiles show that DNA:RNA hybrids preferentially accumulated at rDNA, Ty1 and Ty2 transposons, telomeric repeat regions and a subset of open reading frames (ORFs). The latter are generally highly transcribed and have high GC content. Interestingly, significant DNA:RNA hybrid enrichment was also detected at genes associated with antisense transcripts. The expression of antisense-associated genes was also significantly altered upon overexpression of RNase H, which degrades the RNA in hybrids. Finally, we uncover mutant-specific differences in the DRIP profiles of a Sen1 helicase mutant, RNase H deletion mutant and Hpr1 THO complex mutant compared to wild type, suggesting different roles for these proteins in DNA:RNA hybrid biology. Our profiles of DNA:RNA hybrid prone loci provide a resource for understanding the properties of hybrid-forming regions in vivo, extend our knowledge of hybrid-mitigating enzymes, and contribute to models of antisense-mediated gene regulation. A summary of this paper was presented at the 26th International Conference on Yeast Genetics and Molecular Biology, August 2013.

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Introduction

Elevated DNA:RNA hybrid formation due to defects in RNA processing pathways leads to genome instability and replication stress across species [1–7]. R loops threaten genome stability and often form under abnormal conditions where nascent mRNA is improperly processed or RNA half-life is increased, resulting in RNA that can hybridize with template DNA, displacing the non-transcribed DNA strand [8]. A recent study also found that hybrid formation can occur in *trans* via Rad51-mediated DNA-RNA strand exchange [9]. Persistent R loops pose a major threat to genome stability through two mechanisms. First, the exposed non-transcribed strand is susceptible to endogenous DNA damage due to the increased exposure of chemically reactive groups. The second, more widespread mechanism, identified in *Escherichia coli*, *Saccharomyces cerevisiae*, *Caenorhabditis elegans* and human cells, involves the R loops and associated stalled transcription complexes, which block DNA replication fork progression [3,4,8,10,11]. R loop-mediated instability is an area of great interest primarily because genome instability is considered an enabling characteristic of tumor formation [12]. Moreover, mutations in RNA splicing/processing factors are frequently found in human cancer, heritable diseases like Aicardi-Goutieres syndrome, and a degenerative ataxia associated with Senataxin mutations [13–17].

To avoid the deleterious effects of R loops, cells express enzymes for the removal of abnormally formed DNA:RNA hybrids. In *S. cerevisiae*, *RNH1* and *RNH201*, each encoding RNase H are responsible for one of the best characterized mechanisms for reducing R loop formation by enzymatically degrading the RNA in DNA:RNA hybrids [8]. Another extensively studied anti-hybrid factor is the THO/TREX complex which functions to suppress hybrid formation at the level of transcription termination and mRNA packaging [4,11,18,19]. In addition, the Senataxin helicase, yeast Sen1, plays an important role in facilitating replication fork progress through transcribed regions and unwinding RNA in hybrids to mitigate R loop formation and RNA polymerase II transcription-associated genome instability [3,20]. Several additional anti-hybrid mechanisms have also been
DNA:RNA hybrid prone loci in *S. cerevisiae*

The genomic distribution of DNA:RNA hybrids

These genome-wide analyses enhance our understanding of DNA:RNA hybrid-forming regions *in vivo*, highlight the role of cellular RNA processing activities in suppressing hybrid formation, and implicate DNA:RNA hybrids in control of a subset of antisense regulated loci.

Results

The genomic distribution of DNA:RNA hybrids

DNA:RNA hybrids have been previously immunoprecipitated at specific genomic sites such as rDNA, selected endogenous loci, and reporter constructs [2,5]. Subsequently, DRIP coupled with deep sequencing in human cells has demonstrated the prevalence of R loops at CpG island promoters with high GC skew [26]. To investigate the global profile of DNA:RNA hybrid prone loci in a tractable model, we performed genome-wide DRIP-chip analysis of wild type *S. cerevisiae* (ArrayExpress E-MTAB-2388) using the S9.6 monoclonal antibody which specifically binds DNA:RNA hybrids, as characterized previously [43,44]. DRIP-chip profiles were generated in duplicate (Spearman’s r = 0.78 when comparing each of over 2 million probes after normalization and data smoothing, Supplementary Figure S1) and normalized to a no antibody control.

Overall, our DRIP-chip profiles identified several previously reported DNA:RNA hybrid prone sites including the rDNA locus and telomeric repeat regions [Figure 1, Supplementary Tables S1, S2] [2,29–31]. DNA:RNA hybrids were also observed at 1217 open reading frames (ORFs) (containing greater than 50% of probes above the threshold of 1.5 and found in both wild type replicates) (Supplementary Table S3). These were generally shorter in length than average (p = 4.29e-50), highly transcribed (Wilcoxon rank sum test p = 2.21e-06), and had higher GC content (p = 2.52e-50) [Figure 2A, 2B and 2C, Supplementary Figure S2]. Importantly, despite the correlation between DNA:RNA hybrid association and transcriptional frequency, the wild type DRIP-chip profiles compared to the localization profile of the RNA polymerase II subunit RpH3 revealed very low correlation (p = 0.0097; [45]). This suggests that the DRIP-chip method was not unduly biased towards the short DNA:RNA hybrids that could theoretically have been captured within active transcription bubbles. Importantly, because genes with high GC content also have high transcriptional frequencies (Supplementary Figure S3), it is not clear from our findings whether GC content or transcriptional frequency contributed more to DNA:RNA hybrid forming potential. Furthermore, we observe that DNA:RNA hybrid prone loci do not encode for mRNA transcripts with particularly long half-lives (Supplementary Figure S2D), suggesting that the act of transcription is vital to DNA:RNA hybrid formation and supporting the notion of co-transcriptional hybrid formation as the major source of endogenous DNA:RNA hybrids.

Our data also revealed DNA:RNA hybrids highly associated with Ty1 and Ty2 subclasses of retrotransposons (Figure 2E, Supplementary Table S4). Consistent with our findings at ORFs, the levels of DNA:RNA hybrids correspond well with the known levels of expression of these elements. In general, Ty1 which constitutes one of the most abundant transcripts in the cell has the highest levels of DNA:RNA hybrids. Ty3 and Ty4 that are only slightly expressed have much lower levels of hybrids, and the lone Ty3 retrotransposon which is transcriptionally silent is not enriched for DNA:RNA hybrids [Figure 2E] ([46–48]). In contrast to the trends observed with ORFs, GC content in retrotransposons is not highly correlated with the levels of expression, suggesting that expression is the main contributor to

Author Summary

RNA processing factors are mutated in human cancers, inherited developmental disorders and neurodegenerative syndromes. Defects in RNA processing have been associated with increased levels of mutations and DNA damage in part via the formation of DNA:RNA hybrids. Although it is likely that specific regions of the genome are more prone to DNA:RNA hybrid formation, a map of hybrid-prone regions is not available. In this study, we describe the genome-wide distribution of DNA:RNA hybrids in both normal and mutant *Saccharomyces cerevisiae* cells. The resulting profiles contribute to both our understanding of the general properties of hybrid-forming loci and to our knowledge of hybrid-mitigating enzymes. Interestingly, significant DNA:RNA hybrid enrichment was detected at genes associated with antisense transcription. We show that overexpression of RNAse H, which degrades the RNA in hybrids, significantly affects the expression of genes associated with antisense transcripts. These findings support a role for DNA:RNA hybrids in regulation of gene expression by antisense transcripts.

identified including topoisomerases and other RNA processing factors [2,6,7,9,21–23].

To add to the complexity of DNA:RNA hybrid management in the cell, hybrids also occur naturally and have important biological functions [24]. In human cells, R loop formation facilitates immunoglobulin class switching, protects against DNA methylation at CpG island promoters and plays a key role in pause site-dependent transcription termination [25–28]. Transcription of telomeres by RNA polymerase II also produces telomeric repeat-containing RNAs (TERRA), which associate with telomeres and inhibit telomere elongation in a DNA:RNA hybrid-dependent fashion [29–31]. Noncoding (nc)RNA such as antisense transcripts, perform a regulatory role in the expression of sense transcripts that may involve R loops [32]. The proposed mechanisms of antisense transcription regulation are not clearly understood and involve different modes of action specific to each locus. Current models include chromatin modification resulting from antisense-associated transcription, antisense transcription modulation of transcription regulators, collision of sense and antisense transcription machineries and antisense transcripts expressed in trans interacting with the promoter for sense transcription [32–40]. More recently, studies in *Arabidopsis thaliana* found an antisense transcript that forms R loops, which can be differentially stabilized to modulate gene regulation [41]. Similarly, in mouse cells the stabilization of an R loop was shown to inhibit antisense transcription [42].

Here we describe, for the first time, a genome-wide profile of DNA:RNA hybrid prone loci in *S. cerevisiae* by DNA:RNA immunoprecipitation followed by hybridization on tiling microarrays (DRIP-chip). We found that DNA:RNA hybrids occurred at highly transcribed regions in wild type cells, including some identified in previous studies. Remarkably, we observed that DNA:RNA hybrids were significantly associated with genes that have corresponding antisense transcripts, suggesting a role for hybrid formation at these loci in gene regulation. Consistently, we found that genes whose expression was altered by overexpression of RNAse H were also significantly associated with antisense transcripts. A small-scale cytological screen found that diverse RNA processing mutants had increased hybrid formation and additional DRIP-chip studies revealed specific hybrid-site biases in the RNAse H, Sen1 and THO complex subunit Hpr1 mutants.

Figure 2A, 2B and 2C, Supplementary Figure S2D, suggesting that the act of transcription is vital to DNA:RNA hybrid formation and supporting the notion of co-transcriptional hybrid formation as the major source of endogenous DNA:RNA hybrids.

Genome-Wide Profiling of Yeast DNA:RNA Hybrids
DNA:RNA hybrid formation. Specifically, Ty3 retrotransposons have the highest GC content but have only modest levels of expression and DNA:RNA hybrids.

DNA:RNA hybrids are significantly correlated with genes associated with antisense transcripts

Certain DNA:RNA hybrid enriched regions identified by our DRIP-chip analysis such as rDNA and retrotransposons are associated with antisense transcripts [49,50]. Therefore, we checked if this was a common feature of DNA:RNA prone sites by comparing our list of DNA:RNA prone loci to a list of antisense-associated genes [51]. Because the expression of antisense-associated transcripts may be highly dependent on environmental conditions, we based our analysis on a list of transcripts identified in S288c yeast grown to mid-log phase in rich media which most closely mirrors the growth conditions of our cultures analyzed by DRIP-chip ([51]). DNA:RNA hybrid enriched genes significantly overlapped with antisense-associated genes, suggesting that DNA:RNA hybrids may play a role in antisense transcript-mediated regulation of gene expression (Fisher's exact test $p = 1.03\times10^{-12}$) (Figure 3A, 3B and 3C, Supplementary Table S5).

RNase H overexpression reduces detectable levels of DNA:RNA hybrids in cytological screens and suppresses genomic instability associated with R loop formation presumably through the degradation of DNA:RNA hybrids [7,52,53]. To test for a potential role of DNA:RNA hybrids in antisense-mediated gene regulation, we performed gene expression microarray analysis of...
Figure 2. DNA:RNA hybrids are enriched at protein-encoding genes and retrotransposons of higher transcriptional frequency. (A) Average gene profile of DNA:RNA hybrids at ORFs enriched for DNA:RNA hybrids under wild type conditions. (B–D) CHROMATRA plots of DNA:RNA hybrid distribution along genes sorted by their length (B), grouped into five transcriptional frequency categories as per [69]) (C) or grouped into four GC content categories (D). Genes were aligned by their TSSs. (E) The average DNA:RNA hybrid score at Ty1, Ty2, Ty3, Ty4 and Ty5 retrotransposons in the left panel shows higher enrichment at Ty1 and Ty2 retrotransposons. The average profile of DNA:RNA hybrids at all retrotransposons under wild type conditions is shown in the right panel.
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Figure 3. Genes associated with DNA:RNA hybrids were significantly associated with antisense transcripts. (A) Antisense association of DNA:RNA hybrid-enriched genes in wild type. The p-value indicates significant enrichment (Fisher’s exact test) of antisense-associated genes among all genes enriched for DNA:RNA hybrids in WT.
DNA:RNA hybrid-enriched genes compared to the Yassour et al. 2010 antisense-annotated dataset ([51]). (B) CHROMATRA plots of DNA:RNA hybrid distribution along genes sorted by their length and separated by whether they are antisense associated or not. Genes were aligned by their TSSs. (C) Average gene profile of DNA:RNA hybrids at genes associated with antisense transcripts. (D) Genes with increased mRNA levels upon RNase H overexpression were significantly associated with antisense transcripts compared to all transcripts represented by the microarray. (E) Antisense-associated DNA:RNA hybrid-enriched genes in wild type have lower transcription frequency compared to non-antisense-associated DNA:RNA hybrid-enriched genes. Genes up-regulated at the transcript level by RNase H overexpression have lower transcription frequency compared to all genes on the expression microarray. Intervals indicate range of the 95% of genes closest to the average in each sample. Averages stated above each bar. P values indicate significant decrease in transcriptional frequency (Wilcoxon rank sum test). (F) Overlap between DNA:RNA hybrid-enriched genes and RNase H-modulated transcripts sorted by antisense association according to the Yassour et al. 2010 database. For genes that are both hybrid-enriched and modulated at the transcript level by RNase H overexpression, the antisense association (100%) is significantly higher (Fisher’s exact test p = 2.2e-16) than those of the parent datasets (37.4% for DNA:RNA hybrid-enriched genes, 43.9% for RNase H-modulated genes).

dnaRNA hybrids using the S9.6 antibody. Importantly, previous work in our lab has shown that all of the mutants screened exhibit chromosome instability (GIN), which would be consistent with increased hybrid formation [53]. Significantly elevated hybrid levels were found in 22 of the 40 mutants tested compared to wild type, including a STB2 mutant which has been previously linked to R loop formation (Figure 5, [4]). We also assayed some of the well-characterized R-loop forming mutants, RNase H, Sen1 and Hpr1, as positive controls for elevated DNA:RNA hybrid levels (Figure 5).

In our screen, we detected hybrids in mutants affecting several pathways linked to DNA:RNA hybrid formation such as transcription, nuclear export and the exosome (Figure 5, Table 1). Consistent with findings in metazoan cells, we also observed hybrid formation in some splicing mutants (Figure 5, Table 1; [56]). Several rRNA processing mutants were enriched for DNA:RNA hybrids (7 out of the 22 positive hits), likely due to DNA:RNA hybrid accumulation at rDNA genes, a sensitized hybrid formation site (Figure 1; [2]). It is possible that, as seen in mRNA cleavage and polyadenylation mutants, DNA:RNA hybrid formation may contribute to their CIN phenotypes [6]. Currently, there are 52 yeast genes whose disruptions have been found to lead to DNA:RNA hybrid accumulation, 21 of which were newly identified by our screen (Table 1). The success of this small-scale screen suggests that most RNA processing pathways suppress hybrid formation to some degree and that many DNA:RNA hybrid forming mutants remain undiscovered.

**Figure 3D, Supplementary Table S5** and tended to have high GC content, similar to DNA:RNA hybrid enriched genes in wild type (Supplementary Figure S4). However, the genes with increased mRNA levels under RNase H overexpression and the antisense-associated genes enriched for DNA:RNA hybrids in our DRIP experiment both tended towards lower transcriptional frequencies (Figure 3E). These findings suggest that antisense-associated DNA:RNA hybrids moderate the levels of gene expression. Indeed, genes that were both modulated by RNase H overexpression and enriched for DNA:RNA hybrids were all found to be antisense-associated (Figure 3F).

The mechanism underlying altered gene expression in cells overexpressing RNase H remains unclear. While the association with antisense transcription is compelling, alternative models exist. One possibility is that the stress of RNase H overexpression triggers gene expression programs that coincidentally are antisense regulated. We analyzed gene ontology (GO) terms enriched among genes whose expression was changed by RNase H overexpression. Consistent with previous work, genes for iron uptake and incorporation were strongly activated by RNase H overexpression (p = 2.21e-15) (Figure 4A, Supplementary Table S6) and several of these iron transport genes (i.e. FRE4, FRE2, FFE3, FET3, FET4) are antisense-associated ([51,54]) suggesting that overexpression of RNase H activates transcription of these genes by perturbing antisense-mediated regulation. Alternatively, changes in RNase H levels may increase the cellular iron requirements since sensitivity to low iron concentration is associated with DNA damage and repair [55]. To test this alternative hypothesis, we tested the RNase H deletion and *sen1-1* mutants for sensitivity to low iron conditions compared to a *fet3A* positive control (Figure 4B). The *sen1-1* mutant, RNase H deletion or overexpression did not induce sensitivity to low iron ruling out the possibility that the transcriptional response in cells overexpressing RNase H was a result of cellular iron requirement. Collectively, our DRIP-chip and microarray analysis suggest that DNA:RNA hybrids may be an important player in antisense-mediated gene regulation.

**Cytological profiling of RNA processing mutants for R loop formation**

Transcription-coupled DNA:RNA hybrids have been shown to accumulate in a diverse set of transcription and RNA processing mutants involved in a wide range of transcription related processes (Table 1). To gain a broader understanding of factors involved in R loop formation, we performed a cytological screen of RNA processing, transcription and chromatin modification mutants for DNA:RNA hybrids using the S9.6 antibody. Importantly, previous work in our lab has shown that all of the mutants screened exhibit chromosome instability (CIN), which would be consistent with increased hybrid formation [53]. Significantly elevated hybrid levels were found in 22 of the 40 mutants tested compared to wild type, including a STB2 mutant which has been previously linked to R loop formation (Figure 5, [4]). We also assayed some of the well-characterized R-loop forming mutants, RNase H, Sen1 and Hpr1, as positive controls for elevated DNA:RNA hybrid levels (Figure 5).
mutating SEN1 resulted in higher levels of DNA:RNA hybrids at shorter genes (Figure 7A and 7B), which is consistent with Sen1’s role in transcription termination particularly for short protein-coding genes ([5,60,61]). The rnh1Δrmh201Δ mutant revealed higher levels of DNA:RNA hybrids at highly transcribed and longer genes (Figure 7A and 7B) which is supported by a wealth of evidence of RNase H’s role in suppressing R loops in long genes to prevent collisions between transcription and replication machineries ([8,62]).

Further inspection of our profiles also revealed that rnh1Δrmh201Δ and senl-1 mutants but not the hpr1Δ mutant had increased DNA:RNA hybrids at tRNA genes (two tailed unpaired Wilcoxon test p = 1.56e^{-19} in the rnh1Δrmh201Δ mutant and 1.68e^{-15} in the senl-1 mutant) (Figure 8A, 8B and 8C, Supplementary Table S7) and this was confirmed by DRIP-quantitative PCR (qPCR) of two tRNA genes in wild type and rnh1Δrmh201Δ (Supplementary Figure S5). Because tRNAs are transcribed by RNA polymerase III, this observation indicates that Hpr1 is primarily involved in the regulation of RNA polymerase II specific DNA:RNA hybrids while RNase H and Sen1 have roles in a wider range of transcripts. Mutation of SEN1 also led to increased levels DNA:RNA hybrids at snoRNA (two tailed unpaired Wilcoxon test p = 1.81e^{-19} (Figure 8D, 8E and 8F, Supplementary Table S8) consistent with its role in 3’ end processing of snoRNAs ([63]).

Discussion

The genomic profile of DNA:RNA hybrids

Identifying the landscape of genomic loci predisposed to DNA:RNA hybrids is of fundamental importance to delineating mechanisms of hybrid formation and the contributions of various cellular pathways. Although our profiles depend on the specificity of the anti-DNA:RNA hybrid S9.6 monoclonal antibody, this aspect has been well characterized [44] and several of our observations are consistent with what has been reported in the literature. Locus specific tests showed that DNA:RNA hybrids occur more frequently at genes with high transcriptional frequency and GC content [4,5,18]. Moreover, in rmh201Δ cells, there is an inverse relationship between GC content and gene expression
levels, suggesting that DNA:RNA hybrids accumulate at regions of high GC content and block transcription in the absence of RNase H [64]. Our work extends the knowledge of DNA:RNA hybrids from a few locus-specific observations to show that, in wild type, there are potentially hundreds of hybrid prone genes that tend to be shorter in length, frequently transcribed and high in GC content [2,4,56]. The latter is consistent with recent studies in human cells that demonstrated that genomic regions with high GC skew are prone to R loop formation, which plays a regulatory role in DNA methylation [26,27]. However, while we determined the relationship between GC content and DNA:RNA hybrid formation, we were unable to do the same analysis for GC skew, likely due to the low level of GC skew and lack of DNA methylation in Saccharomyces. This is unsurprising since the best characterized functional element associated with GC skew, CpG island promoters [26,27], are not found in yeast. Importantly, our findings at retrotransposons support the notion that expression levels and not GC content contribute more to DNA:RNA hybrid forming potential. Additionally, DRIP-chip analysis of wild type cells identified hybrid enrichment at rDNA, retrotransposons, and

| Yeast gene linked to DNA:RNA hybrid formation | Reference |
|---------------------------------------------|-----------|
| Exosome and RNA degradation: DIS3, RPB6, XRN1 | This study, [7,22] |
| Helicase: SEN1, SRS2 | [5,9] |
| mRNA cleavage and polyadenylation: CLP1, CFT2, FIP1, PCF11, RNA14, RNA15, TRF4 | [6,22,75] |
| mRNA export: MEX67, MTR2, NAR2, NUP133, RNA1, SAC3, SRR1, SUB2, SUS1, TRF1, YRA1 | This study, [6,19,22,76,77] |
| Other processes: ESC2, KAE1, PSH1, STS1 | This study |
| RNA Polymerase II transcription and chromatin modification: LEO1, MED12, MED13, MDT1, NPL3, RTT103, SPS3, SNR3, SPT2, SPS5 | This study, [7,9,23,78] |
| RNase H: RHH201, RH1 | This study, [6,7] |
| rRNA processing factors: DPB6, DPB7, IMP4, RPF1, SNU13, SNU66 | This study |
| Splicing: MUD2, SNU141, PRP31, YH1C, SNU13, SNU66 | This study |
| THO transcription elongation: THO2, HPK1, MFT1, THP2 | [6,18,58] |
| Topoisomerase: TOP1 | [2] |

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Figure 5. DNA:RNA hybrid cytological screen revealed high DNA:RNA hybrid levels in RNA processing and chromatin modification mutants. Asterisks indicate mutants with significantly increased levels of DNA:RNA hybrids compared to wild type (p<0.000024). Error bars indicate standard error of the mean. Representative chromosome spreads are shown: blue stain is DNA (DAPI) and the red foci are DNA:RNA hybrids.
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telomeric regions. Along with previous studies, our DRIP-chip analysis confirms that rDNA is a hybrid prone genomic site and suggests that many factors of rRNA processing and ribosome assembly suppress potentially damaging rDNA:rRNA hybrid formation [2,7]. The presence of TERRA-DNA hybrids at telomeres is supported by our observation of significant hybrid signal at telomeric repeat regions across all DRIP-chip experiments.

Antisense association of DNA:RNA hybrids

The DRIP-chip dataset is a resource for future studies seeking to elucidate the localization of DNA:RNA hybrids across antisense-associated regions and the impact of DNA:RNA hybrid removal on genome-wide transcription. We observed that genes associated with antisense transcripts were significantly enriched for DNA:RNA hybrids and modulated at the transcript level by RNase H overexpression. Antisense regulation has been reported at mammalian rDNA and yeast Ty1 retrotransposons, loci that were also enriched for DNA:RNA hybrids in our DRIP-chip [49,50]. The role of DNA:RNA hybrids and RNase H in antisense regulation is currently unclear. However, there are several non-exclusive models of antisense gene regulation. One model proposes that the physical presence of the antisense transcripts is crucial to antisense gene regulation. For instance, \textit{trans}-acting antisense transcripts have been shown to control Ty1 retrotransposition, reverse transcription and retrotransposition [65]. Another study has further shown that \textit{trans}-acting antisense transcripts that only overlap with the sense strand promoter can block sense transcription, potentially by hybridizing with the non-template DNA strand [33]. These suggest that antisense

Figure 6. Genome-wide profiles of DNA:RNA hybrids in revealed similar enrichment of rDNA, retrotransposons and telomeres in wild type and mutants. DRIP-chip chromosome plot of DNA:RNA hybrids in wild type, \textit{mnh1}\textsuperscript{D}, \textit{hpr1}\textsuperscript{D} and \textit{sen1-1} at chromosome XII. The average of two replicates per strain is shown. Bars indicate ORFs (grey), rDNA (purple), retrotransposons (green) or genes associated with an antisense transcript (red) [51,54]). Grey boxes delineate telomeric repeat regions. Y-axis indicates relative occupancy of DNA:RNA hybrids. X-axis indicates chromosomal coordinates. P indicates probability of observing a number of enriched features below what was observed (P>0.99997).
doi:10.1371/journal.pgen.1004288.g006
Figure 7. Mutant specific trends in protein-coding genes prone to DNA:RNA hybrid formation. (A–C) CHROMATRA plots of DNA:RNA hybrid distribution along genes sorted by their length (A) grouped into five transcriptional frequency categories as per [69] (B) or grouped into four GC content categories (C). Genes were aligned by their TSSs.

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transcription in cis is not necessary as long as the antisense transcript is present. It is possible that DNA:RNA hybrids may be formed by the antisense or the sense transcript with genomic DNA. Moreover, DNA:RNA hybrids may play a functional role in antisense transcription regulation as shown by antisense-associated genes both enriched for DNA:RNA hybrids and affected transcriptionally by RNase H overexpression. Experiments comparing the ratio of antisense versus sense transcripts and
determining the amount of DNA:RNA hybrid formation by either transcript under conditions known to regulate the particular gene will further elucidate the role of RNase H and DNA:RNA hybrids in antisense regulation.

**DRIP-chip analysis of hybrid-resolving mutants**

Our investigation of mutant-specific DNA:RNA hybrid formation sites was consistent with the existing literature on Hpr1, Sen1 and RNase H. Significantly, the hpr1Δ and rnh1Δnhkl201A mutants exhibited increased DNA:RNA hybrid levels along the length of long genes, while the sen1-1 mutant exhibited increased DNA:RNA hybrid levels along the length of short genes (Figure 7A). This coheres with Hpr1’s function in transcription elongation and mRNA export, and RNase H’s role in preventing transcription apparatus and replication fork collisions, which carry greater consequence for long genes ([4,57–59,62]). In contrast, Sen1 is particularly important for transcription termination at short genes ([61]).

In addition, the RNase H deletion and sen1-1 mutants had increased hybrids at tRNA genes, suggesting that they are both required to prevent tRNA:DNA hybrid accumulation. Interestingly, a recent study found that the mRNA levels of genes encoding RNA polymerase III and proteins that modify tRNA are increased in an rnh1Δnhkl201A mutant [64], which may be in response to a lack of properly processed tRNA transcripts. The finding that both tRNA and snoRNA genes were enriched for hybrids in sen1-1 highlights the role of Sen1 in RNA polymerase I, II and III transcription termination and transcript maturation [60,63,66]. More broadly, our data and the literature support the notion that transcripts from RNA polymerases I, II and III can be subject to DNA:RNA hybrid formation especially in RNA processing mutant backgrounds.

**Perspective**

Factors regulating ectopic, genome destabilizing DNA:RNA hybrids are best characterized in yeast, although less is known about the functions of native R loop structures. The genome-wide maps of DNA:RNA hybrids presented here recapitulate the known sites of hybrid formation but also add important new insights to potential functions of R loops. Most importantly, we demonstrate the usefulness of DRIP profiling for detecting biologically meaningful differences in mutant strains. Therefore, DRIP profiling of yeast genomes in various mutant backgrounds will be key to understanding the causes and consequences of inappropriate R loop formation and how these are modulated by other cellular pathways.

**Methods**

**Strains and plasmids**

All strains are listed in Supplementary Table S9. For RNase H overexpression experiments, recombinant human RNase H1 was expressed from plasmid p425-GPD-RNase H1 (2μ, LEU2, GPDpr-RNase H1) and compared to an empty control plasmid p425-GPD (2μ, LEU2, GPDpr) [7].

**DRIP-chip and qPCR**

Briefly, cells were grown overnight, diluted to 0.15 OD600 and grown to 0.7 OD600. Crosslinking was done with 1% formaldehyde for 20 minutes. Chromatin was purified as described previously [67] and sonicated to yield approximately 500 bp fragments. 40 μg of the anti-DNA:RNA hybrid monoclonal mouse antibody S9.6 (gift from Stephen Leppla) was coupled to 60 μL of protein A magnetic beads (Invitrogen). For ChIP-qPCR, crosslinking reversal and DNA purification were followed by qPCR analysis of the immunoprecipitated and input DNA. DNA was analyzed using a Rotor-Gene 600 (Corbett Research) and PerleCTa SYBR green FastMix (Quanta Biosciences). Samples were analyzed in triplicate on three independent DRIP samples for wild type and rnh1Δnhkl201A. Primers are listed in Supplementary Table S11.

For DRIP-chip, precipitated DNA was amplified via two rounds of T7 RNA polymerase amplification ([68]), biotin labeled and hybridized to Affymetrix 1.0R S. cerevisiae microarrays. Samples were normalized to a no antibody control sample (mock) using the rMAT software and relative occupancy scores were calculated for all probes using a 300 bp sliding window. All profiles were generated in duplicate and replicates were quantile normalized and averaged. Spearman correlation scores between replicates are listed in Supplementary Table S10. Coordinates of enriched regions are available in Dataset S1/S2/S3/S4/S5/S6/S7/S8. DRIP-chip data is available at ArrayExpress E-MTAB-2388.

**DRIP-chip analysis**

Enriched features had at least 50% of the probes contained in the feature above the threshold of 1.5. Only features enriched in both replicates were reported. Transcriptional frequency [69], GC content ([70]) and gene length were compared using the Wilcoxon rank sum test. Antisense association was analyzed by the Fisher’s exact test using R. Statistical analysis of genomic feature enrichment was performed using a Monte Carlo simulation, which randomly generates start positions for the particular set of features and calculates the proportion of that feature that would be enriched in a given DRIP-chip profile if the feature were distributed at random [67]. 500 simulations were run per feature for each DRIP-chip replicate to obtain mean and standard deviation values. These values were used to calculate the cumulative probability (P) on a normal distribution of seeing a score lower than the observed value by chance.

**DRIP-chip visualization**

CHROMATRA plots were generated as described previously ([71]). Relative occupancy scores for each transcript were binned into segments of 150 bp. Transcripts were sorted by their length, transcriptional frequency or GC content and aligned by their Transcription Start Sites (TSS). For transcriptional frequency transcripts were grouped into five classes according to their transcriptional frequency described by Holstege et al 1998. For GC content transcripts were grouped into four classes according to their GC content obtained from BioMart ([70]). Average gene, tRNA or snoRNA profiles were generated by averaging all the probes that were encompassed by the features of interest. For averaging ORFs, corresponding probes were split into 40 bins while 1500 bp of UTRs and their probes were split into 20 bins. For smaller features like tRNAs and snoRNAs corresponding probes were split into only 3 bins. Average enrichment scores were calculated using in house scripts that average the score of all the probes encompassed by the feature.

**Gene expression microarray**

Gene expression microarray data is available at GEO GSE46652. Strains harboring the RNase H1 over-expression plasmid or empty vector were grown in SC-Leucine at 30°C. All profiles were generated in duplicate. Total RNA was isolated from 1 OD600 of yeast cells using a RiboPure Yeast kit (A&B Applied Biosystems), amplified, labeled, fragmented using a Message-Amp III RNA Amplification Kit (A&B Applied Biosystems) and hybridized to a GeneChIP Yeast Genome 2.0 microarray using
the GeneChip Hybridization, Wash, and Stain Kit (Affymetrix). Arrays were scanned by the Gene Chip Scanner 3000 7G and expression data was extracted using Expression Console Software (Affymetrix) with the MAS5.0 statistical algorithm. All arrays were scaled to a median target intensity of 500. A minimum cut off of p-value of 0.05 and signal strength of 100 across all samples were implemented and only transcripts that had over a 2-fold change in the RNase H over-expression strain compared to wild type were considered significant. The correlation between duplicate biological samples was: control \((r = 0.9955)\), RNase H over-expression \((r = 0.9719)\). For statistical analysis, GC content, transcription frequencies and antisense association were analyzed as for DRIP-chip analysis.

**Yeast chromosome spreads**

Cells were grown to mid-log phase in YEPD rich media at 30°C and washed in spheroplasting solution (1.2 M sorbitol, 0.1 M potassium phosphate, 0.5 M MgCl₂, pH 7) and digested in spheroplasting solution with 10 mM DTT and 150 µg/mL Zymolase 20T at 37°C for 20 minutes similar to previously described \([72]\). The digestion was halted by addition of ice-cold stop solution (0.1 M MES, 1 M sorbitol, 1 mM EDTA, 0.5 mM MgCl₂, pH 6.4) and spheroplasts were lysed with 1% vol/vol Liposol and fixed on slides using 4% wt/vol paraformaldehyde/3.4% wt/vol sucrose \([73]\). Chromosome spread slides were incubated with the mouse monoclonal antibody S9.6 (1 µg/mL in blocking buffer of 5% BSA, 0.2% milk and 1X PBS). The slides were further incubated with a secondary Cy3-conjugated goat anti-mouse antibody (Jackson Laboratories, #115-165-003, diluted 1:1000 in blocking buffer). For each replicate, at least 100 nuclei were visualized and manually counted to obtain the fraction with detectable DNA:RNA hybrids. Each mutant was assayed in triplicate. Mutants were compared to wild type by the Fisher’s exact test. To correct for multiple hypothesis testing, we implemented a cut off of \(p<0.01\) divided by the total number of mutants compared to wild type, meaning mutants with \(p<0.00024\) were considered significantly different from wild type.

**BPS sensitivity assay**

10-fold serial dilutions of each strain was spotted on 90 µM BPS plates with FeSO₄ concentrations of 0, 2.5, 20 or 100 µM and grown at 30°C for 3 days \([55]\).

A summary of this paper was presented at the 26th International Conference on Yeast Genetics and Molecular Biology, August 2013 \([74]\).

**Supporting Information**

**Dataset S1** Wild type replicate 1 enriched region coordinates. (XLSX)

**Dataset S2** Wild type replicate 2 enriched region coordinates. (XLSX)

**Dataset S3** \(sen1-1\) replicate 1 enriched region coordinates. (XLSX)

**Dataset S4** \(sen1-1\) replicate 2 enriched region coordinates. (XLSX)

**Dataset S5** \(hpr1A\) replicate 1 enriched region coordinates. (XLSX)

**Dataset S6** \(hpr1A\) replicate 2 enriched region coordinates. (XLSX)

**Dataset S7** \(mh1A\) \(mh201A\) replicate 1 enriched region coordinates. (XLSX)

**Dataset S8** \(mh1A\) \(mh201A\) replicate 2 enriched region coordinates. (XLSX)

**Figure S1** Spearman correlation scatter plot of wild type replicates. (EPS)

**Figure S2** Box plots comparing the distribution of (A) gene length, (B) transcription frequency, (C) GC content, and (D) mRNA transcript half-life of ORFs enriched for DNA:RNA hybrids versus ORFs not enriched for DNA:RNA hybrids. The p values calculated by the Wilcoxon rank sum test are shown. (EPS)

**Figure S3** (A) Distribution of % GC content of all ORFs sorted by transcriptional frequency. (B) Distribution of % GC content of ORFs enriched for DNA:RNA hybrids in WT sorted by transcriptional frequency. Intervals indicate 95% confidence intervals. (EPS)

**Figure S4** Distribution of % GC content of all genes represented on the expression microarray \((n = 5657)\), and transcripts up- \((n = 212)\) or down-regulated \((n = 88)\) by RNase H overexpression. Intervals indicate 95% confidence intervals. Averages are stated above each sample. The p-value indicates a significant increase in GC content of upregulated genes compared to all microarray transcripts (Wilcoxon rank sum test). (EPS)

**Figure S5** Relative quantities of (A) \(SUF2\) tRNA gene and (B) \(tV/UCD\) tRNA gene detected in WT or \(rnh1A\) \(rnh201A\) as detected by DRIP-quantitative PCR (qPCR). Error bars indicate standard deviation. (EPS)

**Table S1** List of tRNA enriched for RNA:DNA hybrids in wild type, \(rnh1A\) \(rnh201A\), \(hpr1A\) and \(sen1-1\). (XLSX)

**Table S2** List of telomeric repeat regions enriched for RNA:DNA hybrids in wild type, \(rnh1A\) \(rnh201A\), \(hpr1A\) and \(sen1-1\). (XLSX)

**Table S3** List of ORFs enriched for RNA:DNA hybrids in wild type, \(rnh1A\) \(rnh201A\), \(hpr1A\) and \(sen1-1\). (XLSX)

**Table S4** List of retrotransposons enriched for RNA:DNA hybrids in wild type, \(rnh1A\) \(rnh201A\), \(hpr1A\) and \(sen1-1\). (XLSX)

**Table S5** Lists of open reading frames (ORFs) and antisense-associated ORFs enriched for RNA:DNA hybrids in wild type or modulated at the transcript level by RNase H overexpression. (XLSX)

**Table S6** GO function sorting of genes modulated at the transcript level by RNase H overexpression. (XLSX)

**Table S7** List of tRNA genes enriched for RNA:DNA hybrids in wild type, \(rnh1A\) \(rnh201A\), \(hpr1A\) and \(sen1-1\). (XLSX)
Table S8  List of snoRNA genes enriched for RNA:DNA hybrids in wild type, rnh1ΔΔrnh201A, hpr1Δ and smo1-1.

Table S9  Strains used in this study.

Table S10  Spearman correlation scores.

Table S11  DRIP-qPCR primers.

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Author Contributions
Conceived and designed the experiments: YAC MJA AH PCS. Performed the experiments: YAC MJA ZL AH. Analyzed the data: YAC MJA PCS. Contributed reagents/materials/analysis tools: MJA PYTL ZL MSK PH. Wrote the paper: YAC MJA PCS PH.

Genome-Wide Profiling of Yeast DNA:RNA Hybrids

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