RNA polymerase II acts as an RNA-dependent RNA polymerase to extend and destabilize a non-coding RNA

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RNA polymerase II (Pol II) is a well-characterized DNA-dependent RNA polymerase, which has also been reported to have RNA-dependent RNA polymerase (RdRP) activity. Natural cellular RNA substrates of mammalian Pol II, however, have not been identified and the cellular function of the Pol II RdRP activity is unknown. We found that Pol II can use a non-coding RNA, B2 RNA, as both a substrate and a template for its RdRP activity. Pol II extends B2 RNA by 18 nt on its 3'-end in an internally templated reaction. The RNA product resulting from extension of B2 RNA by the Pol II RdRP can be removed from Pol II by a factor present in nuclear extracts. Treatment of cells with α -amanitin or actinomycin D revealed that extension of B2 RNA by Pol II destabilizes the RNA. Our studies provide compelling evidence that mammalian Pol II acts as an RdRP to control the stability of a cellular RNA by extending its 3'-end.

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Introduction

Polymerase II (Pol II) transcribes mRNAs as well as several classes of non-coding RNAs (ncRNAs), making it the central enzyme responsible for transcribing much of mammalian genomes. A breadth of literature describes both the mechanism and regulation of Pol II as a DNA-dependent RNA polymerase. Several reports have shown that yeast Pol II can also function as an RNA-dependent RNA polymerase (RdRP) (Dezelee *et al.*, 1974; Johnson and Chamberlin,

1994; Lehmann *et al.*, 2007). Most strikingly, Cramer and colleagues showed that yeast Pol II extends the 3'-ends of RNAs in a template-specific manner (Lehmann *et al.*, 2007). They created RdRP 'scaffolds', which contained RNA–RNA duplexes with 5' single-stranded regions; the 3'-end of a duplex could be extended sequence specifically by yeast Pol II using the 5' single-stranded region as an RNA template. These scaffolds were created using sequences from both a synthetic aptamer known to bind Pol II, as well as a region of the hepatitis δ virus (HDV) antigenome. HDV, as well as plant viroids, do not encode a RdRP and yet they undergo RNA replication once inside host cells; α -amanitin sensitivity has suggested that Pol II is involved in replicating the HDV genome (Taylor, 2003; Lai, 2005), as well as plant viroid genomes (Rackwitz *et al.*, 1981). *In vitro*, a segment of HDV antigenomic RNA can serve as a template for α -amanitin-sensitive RdRP activity in nuclear extracts (Filipovska and Konarska, 2000; Chang *et al.*, 2008).

B2 RNA is an ~ 180 nt ncRNA transcribed by RNA Pol III in mouse cells. It is encoded by B2 SINEs (short interspersed elements), which are repeat sequences present in high copy number throughout the mouse genome (Kramarov and Vassetzky, 2005). In response to heat shock, as well as other cellular stresses, levels of B2 RNA increase (Liu *et al.*, 1995; Li *et al.*, 1999). We have shown that B2 RNA mediates transcriptional repression of mRNA genes in response to heat shock (Allen *et al.*, 2004). It does so by binding directly to RNA Pol II and assembling into complexes at the promoters of repressed genes where it renders the polymerase inactive (Espinoza *et al.*, 2004). A second ncRNA, B1 RNA, is also transcribed by RNA Pol III from mouse SINEs after heat shock (Liu *et al.*, 1995; Li *et al.*, 1999). Like B2 RNA, B1 RNA binds directly to Pol II; however, it is not able to repress transcription (Mariner *et al.*, 2008; Wagner *et al.*, 2010).

Biochemical experiments have revealed the molecular mechanism by which B2 RNA inhibits transcription. It forms a tight and kinetically stable complex with core Pol II, and via this interaction, prevents Pol II from properly engaging the DNA during the assembly of complexes on the promoter (Espinoza *et al.*, 2004; Yakovchuk *et al.*, 2009). This results in promoter-bound complexes that contain Pol II, B2 RNA, and general transcription factors; however, the complexes are transcriptionally inert because the proper polymerase–DNA contacts are not established. Nearly all Pol II–promoter contacts are disrupted by B2 RNA, suggesting that B2 RNA interacts with Pol II in a region that overlaps with the DNA-binding cleft (Yakovchuk *et al.*, 2009). Indeed, recent electron microscopy studies localized B2 RNA to the active site cleft of Pol II (Kassube *et al.*, 2012).

Here, we tested nuclear extracts for the presence of an activity that can dissociate B2 RNA from highly purified core Pol II. We found that activity-facilitated dissociation depended on all four NTPs, leading us to hypothesize that Pol II could use B2 RNA as an RdRP substrate. This hypoth-

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esis was tested and confirmed using a series of biochemical experiments with highly purified Pol II. Subsequent experiments were performed to determine the function of the Pol II RdRP activity in controlling B2 RNA levels in cells. These studies provide the first evidence that human Pol II can use a natural, mammalian ncRNA as a substrate for RNA-dependent RNA polymerization, thereby controlling its cellular metabolism.

Results

Facilitated dissociation of the B2 RNA/Pol II complex requires all four NTPs and is inhibited by α -amanitin

The complex between B2 RNA and purified Pol II is kinetically stable *in vitro*, with a half time for dissociation of over 1 h (Espinoza *et al*, 2004). It seems likely that dissociation of this interaction is controlled by other factors in cells. To test this possibility, we formed the complex between 32 P-labelled B2 RNA and purified human Pol II and incubated it with a small amount of nuclear extract prepared from human cells.

NTPs were included in some reactions to test whether they might be required for facilitated dissociation, for example by a helicase in the extract. B2 RNA/Pol II complexes were resolved by electrophoretic mobility shift assays (EMSAs). As shown in Figure 1A, the extract disrupted the B2 RNA/Pol II complex in the presence of NTPs. Neither extract nor NTPs alone caused loss of the B2 RNA/Pol II complex.

We partially purified the activity that dissociated B2 RNA from Pol II using conventional chromatography. Using the fraction containing the activity we asked which NTP(s) was required for dissociation of B2 RNA from Pol II. As shown in Figure 1B, the dissociation activity required all four NTPs; when each of the NTPs was individually omitted, facilitated dissociation of B2 RNA from Pol II did not occur. Given that all four NTPs were required, we hypothesized that Pol II catalytic activity was involved in the facilitated dissociation of B2 RNA from Pol II, perhaps functioning as an RdRP using B2 RNA as the substrate. To test this we added α -amanitin to the assays, which inhibits Pol II DdRP and RdRP activity (Lehmann *et al*, 2007). As shown in Figure 1C, α -amanitin

Figure 1 An activity in nuclear extracts removes B2 RNA from Pol II in an NTP-dependent reaction. (A) NTPs and a nuclear extract facilitate dissociation of the Pol II/B2 RNA complex. Complexes containing 32 P-labelled B2 RNA and Pol II were formed then incubated in the presence of nuclear extract and/or NTPs. Complexes and free RNA were resolved by electrophoresis through a native polyacrylamide gel. (B) Facilitated dissociation of B2 RNA from Pol II requires all four NTPs. Complexes containing 32 P-labelled B2 RNA and Pol II were formed then incubated in the presence of a partially purified factor and NTPs. NTPs were individually omitted from reactions where indicated. (C) α -Amanitin inhibits facilitated dissociation of B2 RNA from Pol II. Reactions were assembled as in (B); the reaction in lane 2 also contained α -amanitin (100 μ M). (D) Facilitated dissociation occurs when ATP is replaced by AMP-PNP. NTPs in the combinations indicated were added to reactions. Source data for this figure is available on the online supplementary information page.

blocked facilitated dissociation of the B2 RNA/Pol II complex by the partially purified activity. These data are most consistent with the dissociation activity requiring the RdRP catalytic activity of Pol II. Facilitated dissociation also occurred when ATP was replaced by AMP-PNP, an ATP analogue that can be used for RNA synthesis by Pol II but lacks a hydrolysable β - γ -phosphoanhydride bond (Figure 1D). Together, the experiments in Figure 1 show that nuclear extracts contain an activity that can dissociate B2 RNA from Pol II in a manner that depends on Pol II catalytic activity, most likely acting as an RdRP using B2 RNA as a substrate.

B2 RNA is a substrate for RNA-dependent RNA polymerization by Pol II

To directly test whether Pol II can use B2 RNA as an RdRP substrate, we formed a complex between 32 P-labelled B2 RNA and highly purified Pol II, and subsequently added NTPs. As shown in Figure 2A, under these conditions B2 RNA was shifted to a slower migrating band (B2 RNA*) that ran at a distinct position in a denaturing gel and was ~20 nt longer than the input B2 RNA (compare lane 2 with lane 1). Similarly, when a complex between unlabelled B2 RNA and Pol II was incubated with NTPs containing [α - 32 P]-CTP, B2 RNA was radioactively labelled (lane 3) and it migrated at a position similar to that observed in lane 2. B1 RNA/Pol II complexes were also incubated with NTPs; however, 32 P-labelled B1 RNA did not increase in size, nor did unlabelled B1 RNA become labelled (Figure 2A, lanes 4–6). The increased size of B2 RNA after incubation with Pol II and NTPs, and the absence of detecting smaller RNAs in the gel, is most consistent with Pol II extending the 3'-end of RNA as opposed to *de novo* synthesis of a new RNA.

To ensure that the increased size of B2 RNA was due to the polymerization activity of Pol II, we titrated α -amanitin into reactions containing unlabelled B2 RNA and 32 P-labelled NTPs (Figure 2B). α -Amanitin completely blocked labelling of B2 RNA (lanes 6–8 compared with lanes 9 and 10). Inhibition of the DdRP activity of Pol II by α -amanitin is shown as a control (lanes 1–5). We conclude that the observed extension and labelling of B2 RNA are due to Pol II acting as an RdRP.

We next considered how RdRP activity affected the binding of B2 RNA to Pol II. We knew that addition of NTPs to B2 RNA/Pol II complexes did not cause dissociation of the complex in EMSAs (Figure 1). If Pol II remained bound to B2 RNA after using it as an RdRP substrate, then the RdRP reaction should exhibit properties of a single-round reaction. To test this we titrated either Pol II or 32 P-labelled B2 RNA into reactions containing NTPs. When Pol II was titrated (Figure 3A), we found that low amounts of polymerase were not able to extend all of the B2 RNA in the reaction, indicating that when Pol II was subsaturating (i.e., at a concentration below the K_D for binding B2 RNA (Espinoza *et al*, 2004)) the reaction did not undergo multiple-rounds of synthesis and release. As the amount of Pol II was increased all of the B2 RNA was extended. The reciprocal titration also supports a single-round model; as concentrations of B2 RNA were increased, unextended B2 RNA accumulated (Figure 3B). Together, these data show that extended B2 RNA remains committed to Pol II.

Figure 2 Pol II uses B2 RNA as a substrate for RNA-dependent RNA polymerization. (A) Pol II can extend B2 RNA. RNAs detected by phosphorimager are indicated. B2 RNA* designates B2 RNA that has been extended via RdRP activity. (B) α -Amanitin inhibits extension of B2 RNA. Increasing concentrations of α -amanitin (0.1, 1, and 10 μ M) were added to DdRP and RdRP reactions. Unextended 32 P-labelled B2 RNA in lane 11 was used as a size standard. RNAs detected by phosphorimager are indicated. Source data for this figure is available on the online supplementary information page.

Figure 3 Extension of B2 RNA by Pol II is a single turnover reaction. (A) At a low concentration, Pol II cannot extend all the B2 RNA in a reaction. Reactions contained 0.05 nM B2 RNA and 1, 2, 4, or 8 nM Pol II. (B) The amount of extended B2 RNA remains constant as the amount of B2 RNA added to reactions increases. Reactions contained 3.5 nM Pol II and 0.2, 0.4, 0.8, 1.6, or 3.2 nM B2 RNA. Source data for this figure is available on the online supplementary information page.

Extension of B2 RNA by Pol II occurs from the 3'-end and is internally templated

Extension and labelling of B2 RNA should require its 3' hydroxyl; to test this we treated B2 RNA with periodate prior to performing RdRP assays. Periodate treatment oxidizes the 2' and 3' hydroxyl groups on the 3' ribose into aldehydes (Figure 4A) such that the 3'-end of B2 RNA can no

Figure 4 The 3'-OH is needed for Pol II to extend B2 RNA. (A) The schematic illustrates the product of periodate oxidation, which contains 2' and 3' aldehydes in place of hydroxyls. (B) Oxidation of B2 RNA inhibits the RdRP reaction. B2 RNA was treated with periodate and subsequently used in RdRP assays. RNAs were detected by phosphorimager after incubation with Pol II and 32 P-labelled NTPs. Source data for this figure is available on the online supplementary information page.

longer serve as a substrate for polymerization. When unlabelled, oxidized B2 RNA was added to reactions containing Pol II and NTPs, the RNA did not become labelled; however, both mock treated and untreated B2 RNA were labelled and extended (Figure 4B). These data show that the native 3'-end of B2 RNA is required for its extension.

The extension of B2 RNA could result from either templated or non-templated polymerization. Templated polymerization of ~20 nt would require all four NTPs, since within B2 RNA a 20-nt region composed of fewer than four different nucleotides does not exist. By contrast, non-templated polymerization would not be expected to require all four NTPs to obtain the fully extended product (Johnson and Chamberlin, 1994). The results in Figure 1B suggested that extension of B2 RNA occurred in a templated reaction. To directly test this we formed unlabelled B2 RNA/Pol II complexes and added different sets of NTPs containing [α - 32 P]-CTP. When either ATP, UTP, or GTP was omitted from reactions, little or no labelling occurred (Figure 5A). The weakly labelled bands observed in the absence of ATP or UTP likely resulted from the incorrect addition (mis-incorporation) of [α - 32 P]-CTP into a small portion of partially extended B2 RNA. The requirement for all four NTPs to observe full extension and labelling is indicative of template-specific RNA-dependent RNA polymerization.

To determine the region of B2 RNA that serves as a template as well as the sequence added to the 3'-end, we performed a series of nucleotide omission reactions with 32 P-labelled B2 RNA (Figure 5B). In the presence of all four NTPs, B2 RNA was extended by 18 nt. Individually omitting CTP or GTP from reactions resulted in 14 and 7 nt being added to the 3'-end of B2 RNA, respectively. From these data we can define two important parameters of the template sequence: The first 14 nt in the template lack G, and the first 7 nt in the template are entirely A and U. There is only one region of B2 RNA that fits these parameters; it is near the 3'-end of the RNA. Figure 5C shows this region, with the sequence added to the 3'-end of B2 RNA underlined. Individually omitting ATP or UTP from reactions resulted 3 and 2 nt being added to the 3'-end of B2 RNA (Figure 5B), respectively, which does not perfectly fit the first three nucleotides of the predicted extended sequence (Figure 5C). We believe that in the presence of limited nucleotides, single A and U residues can be

Figure 5 A specific region of B2 RNA serves as the template for 3'-end extension by the Pol II RdRP. (A) Extension of the 3'-end of B2 RNA by Pol II requires all four NTPs. Individual nucleotides were omitted from reactions as indicated, and all reactions contained [α - 32 P]-CTP. (B) Identification of the sequence added to B2 RNA by the Pol II RdRP. Individual nucleotides were omitted from RdRP reactions containing 32 P-labelled B2 RNA. (C) Sequence of the template and extended (underlined) regions of B2 RNA. (D) Mutation of C155 to G confirms the region of B2 RNA used as a template by the Pol II RdRP. Individual nucleotides were omitted from RdRP reactions containing 32 P-labelled mutant (mt) or wild type (wt) B2 RNA, as indicated. Source data for this figure is available on the online supplementary information page.

mis-incorporated during the addition of the first three nucleotides to the extended B2 RNA. To directly test whether the region of B2 RNA identified serves as a template for extension, we replaced the C at position 155 with a G. This would change the location at which CTP is first required from position 15 to position 8, as well as change the location at which GTP is first required from position 8 to position 12. As shown in Figure 5D, when the mutant 32 P-labelled B2 RNA was used in RdRP assays, the mutation had the predicted effect on the positions at which GTP and CTP are first required.

Extended B2 RNA can repress transcription, but with decreased potency

We examined whether extended B2 RNA maintained the ability to repress DNA-dependent transcription by Pol II. In the past, we primarily studied the mechanism of transcriptional repression by B2 RNA using a highly purified *in vitro* transcription system and a reporter containing a G-less cassette (Espinoza *et al*, 2004, 2007). B2 RNA is a potent transcriptional repressor in this system, but would not be fully extended or labelled by RdRP activity due to the lack of GTP in reactions. To determine whether B2 RNA was capable of repressing transcription in a reconstituted system under conditions where it could be fully extended, we assembled reactions containing TBP, TFIIB, TFIIF, Pol II, and a promoter contained on negatively supercoiled DNA in the absence and presence of B2 RNA. All four NTPs were added and the RNA produced was monitored by reverse transcription coupled to real-time PCR. As shown in Figure 6A, B2 RNA strongly repressed transcription in these reactions. As a further test we also used a linear heteroduplex template and monitored runoff transcription with all four NTPs, including [α - 32 P]-CTP. Under these conditions, B2 RNA repressed transcription and was labelled during the course of the reaction, indicating that B2 RNA was extended (Figure 6B).

Figure 6 The RdRP extended B2 RNA still functions as a transcriptional repressor. (A) B2 RNA represses promoter DNA-dependent transcription in the presence of all four NTPs. Products from *in vitro* transcription reactions were detected by reverse transcription followed by real-time PCR. The bars are the average of three reactions, and the errors are 1 s.d. (B) B2 RNA represses transcription by Pol II from a heteroduplex DNA template. Shown are the transcripts produced from the DNA template (runoff transcript) as well as B2 RNA*, which was labelled during the course of the reaction. (C) Extended B2 RNA represses transcription *in vitro*, but with decreased potency. The 18 nt addition to the 3'-end of B2 RNA had the sequence shown in Figure 5C. B2 RNA*, B2 RNA, and B2 RNA(3-73), as a negative control, were titrated into transcription reactions. In all, 390 nt G-less RNA product was quantitated, and normalized to product made in the absence of ncRNAs, and plotted versus ncRNA concentration. (D) Extended B2 RNA blocks crosslinking of Pol II to promoter DNA but not to the same extent as B2 RNA. Experiments were performed with promoter DNA that contained a photoactivatable crosslinker between the +3 and +4 positions. ncRNAs (4 nM) were added to reactions before the assembly of closed complexes. Products were resolved by 5% SDS-PAGE and visualized by phosphorimager. (E) Extended B2 RNA represses transcription in cells, but not to the same extent as B2 RNA. Cos cells were cotransfected with a firefly luciferase reporter and either an expression construct for B2 RNA, B2 RNA*, or the parental pUC plasmid as a control. Each bar is the average of at least eight transfections and each error bar is the s.e.m. Source data for this figure is available on the online supplementary information page.

Together, the data in Figures 6A and B are consistent with a model in which B2 RNA retains the ability to repress DNA-dependent transcription by Pol II after being extended by the Pol II RdRP. As a further test of this model, we generated B2 RNA* (i.e., extended B2 RNA) using T7 RNA polymerase and titrated it into G-less transcription reactions. In this assay, the 5' half of B2 RNA (nucleotides 3-73) was used as a negative control (Espinoza *et al*, 2004). As shown in Figure 6C, B2 RNA* repressed transcription, but with a potency approximately three-fold lower than that of B2 RNA. We investigated whether this decreased potency would be reflected in experiments that probe the mechanism by which B2 RNA represses transcription. B2 RNA disrupts contacts between Pol II and the promoter DNA, as evidenced by site-specific crosslinking and DNase I footprinting studies (Yakovchuk *et al*, 2009).

We tested the extent to which B2 RNA and B2 RNA* inhibited crosslinking between the largest subunit of Pol II (Rpb1) and the promoter DNA at a position near the transcription start site. Each ncRNA was assembled into complexes containing TBP, TFIIB, TFIIF, and Pol II on ³²P-labelled promoter DNA with a photoactivatable crosslinker between positions +3/+4. As shown in Figure 6D, B2 RNA* inhibited Pol II-promoter crosslinking, but not to the same extent as B2 RNA. This suggests that the overall mechanism of repression was unchanged by the additional sequence on the 3'-end of B2 RNA. These crosslinking results correlate with the transcriptional inhibition data in Figure 6C.

Our *in vitro* experiments show that B2 RNA* represses transcription with a reduced potency compared with B2 RNA; moreover, the extension reaction makes B2 RNA a better

substrate for facilitated dissociation from Pol II (Figure 1). These data suggest that in cells B2 RNA* would inhibit transcription to a lesser extent than B2 RNA. To test this we performed transient transfection assays in Cos cells (which do not have endogenous B2 RNA) and measured the effect of ectopically expressed B2 RNA or B2 RNA* on expression of a luciferase reporter. As shown in Figure 6E, B2 RNA* did not repress the reporter to the same extent as B2 RNA. Therefore, both in cells and *in vitro*, the 3'-end extension on B2 RNA decreases its potency as a transcriptional repressor. In cells, it is unclear whether this decreased potency is due to facilitated dissociation of B2 RNA* from Pol II or from changes in the repressive properties of the ncRNA itself.

3'-End extension by Pol II destabilizes B2 RNA in cells

We next asked whether extended B2 RNA was present in mouse cells. To do so, we designed a primer for reverse transcription that would specifically anneal to extended B2 RNA and performed RT-PCR with RNA isolated from the nuclei of NIH 3T3 cells. As shown in Figure 7A, extended B2 RNA is present in mouse cells, and detection of the RNA required reverse transcriptase and the RT primer. Importantly, the mouse genome does not contain any B2 SINEs with the RdRP appended sequence; therefore, the extended B2 RNA detected by RT-PCR could not have been directly transcribed from the genome.

Since α -amanitin inhibits extension of B2 RNA by Pol II *in vitro* (Figure 2B) and B2 RNA is a Pol III transcript, we asked how treatment of cells with α -amanitin affected levels of B2 RNA and extended B2 RNA. The amount of α -amanitin used was sufficient to inhibit Pol II transcription, but not Pol III transcription; indeed, levels of Myc mRNA decreased 37-fold after α -amanitin treatment (data not shown). Unexpectedly, treatment of cells with α -amanitin caused a significant increase in total Pol III transcribed B2 RNA (17-fold), whereas extended B2 RNA increased only slightly, resulting in a 13-fold decrease in the ratio of extended B2 RNA to total B2 RNA (Figure 7B). As controls, levels of other Pol III transcripts were monitored: 7SK RNA, 7SL RNA, and a tRNA^{Leu}. None of these transcripts substantially increased upon α -amanitin treatment, showing the effect on B2 RNA was specific, and none substantially decreased, confirming the α -amanitin did not target Pol III.

These data show that α -amanitin treatment causes a dramatic increase in total B2 RNA and a corresponding decrease in the ratio of extended B2 RNA to total B2 RNA. It was possible that the increase in B2 RNA was due to increased Pol III transcription due to a stress response; B2 RNA is well known to increase in cells after heat shock and other cellular stresses (Liu *et al*, 1995; Li *et al*, 1999) and perhaps α -amanitin treatment stressed the cells. To assess this, we monitored the levels of B1 RNA before and after α -amanitin treatment. Like B2 RNA, B1 RNA is a stress-induced Pol III transcript (Liu *et al*, 1995; Li *et al*, 1999). As shown in Figure 7B, however, α -amanitin treatment did not increase the level of B1 RNA, rather its level was slightly decreased. Importantly, B1 RNA cannot serve as a substrate for RdRP-catalysed extension *in vitro* (Figure 2A). Therefore, we favour a model in which the increase in B2 RNA levels after α -amanitin treatment is due to inhibition of RdRP extension of its 3'-end by Pol II, and not due to induced transcription of B2 SINEs in response to cellular stress.

Figure 7 Extension of B2 RNA by Pol II decreases its stability in cells. (A) The 3' extended B2 RNA is present in mouse cells. Nuclear RNA was isolated from NIH 3T3 cells and subjected to RT-PCR with an RT primer specific for the sequence of the extended B2 RNA. Control reactions were performed in the absence of either reverse transcriptase or the oligo used to prime reverse transcription. (B) Treating cells with α -amanitin causes an increase in total B2 RNA and a decrease in the ratio of extended B2 RNA to total B2 RNA. NIH 3T3 cells were treated with α -amanitin for 18 h, nuclear RNA was isolated and quantitated by either RT-PCR (extended B2 RNA, 7SL RNA, and tRNA^{Leu}) or primer extension (total B2 RNA, B1 RNA, and 7SK RNA). The columns are the averages of two replicates. Each error bar represents the range of the two replicates. (C) Actinomycin D does not inhibit extension of B2 RNA by Pol II *in vitro*. RdRP reactions were performed with unlabelled B2 RNA and Pol II in the absence or presence of actinomycin D (5 μ g/ml). Labelled B2 RNA was quantitated. The columns are the averages of three replicates, which were normalized by the amount of labelled RNA produced in the absence of actinomycin D. The error bars are 1 s.d. (D) Extended B2 RNA decays more rapidly than total B2 RNA. Actinomycin D (5 μ g/ml final concentration) was added to cultures of NIH 3T3 cells. Nuclear RNA was harvested at the time points shown. Extended B2 RNA and total B2 RNA were quantitated using RT-PCR and primer extension, respectively. Source data for this figure is available on the online supplementary information page.

One explanation for how inhibition of RdRP-catalysed 3'-end extension causes increased levels of B2 RNA is that the extension reaction destabilizes B2 RNA. To measure the stability of B2 RNA in cells we used actinomycin D, which blocks DNA-dependent RNA synthesis by all three nuclear RNA polymerases (Bensaude, 2011). Although it was unlikely that actinomycin D would block the Pol II RdRP activity, we

tested this directly in a biochemical assay using purified B2 RNA and Pol II. As shown in Figure 7C, labelling of B2 RNA by Pol II was unaffected by actinomycin D.

To determine the stabilities of extended and total B2 RNA in cells we blocked Pol III transcription by treating cells with actinomycin D and monitored levels of total B2 RNA and extended B2 RNA over time. As shown in Figure 7D, levels of total (dashed line) and extended (solid line) B2 RNA both decreased to ~50% of their initial levels after 20 min of treatment. At this point, extended B2 RNA continued to decrease and was not detectable by 2 h. In contrast, total B2 RNA decreased only slightly after 20 min. These data show that extended B2 RNA is much less stable than total B2 RNA in cells. Moreover, it appears that B2 RNA exists in two pools, one that can be extended and is unstable and a second that cannot be extended and is stable. These data also provide an explanation for the increase in total B2 RNA that is observed upon treatment of cells with α -amanitin. The α -amanitin blocks Pol II RdRP extension of B2 RNA, thereby eliminating a means to destabilize B2 RNA, which results in an overall increase in the steady-state level of total B2 RNA.

Discussion

We discovered that cells contain an activity that can dissociate B2 RNA from Pol II, and that this facilitated dissociation requires all four NTPs and Pol II catalytic activity. This observation led us to test whether B2 RNA serves as a template and/or substrate for the RdRP activity of human Pol II. Our studies revealed that Pol II adds 18 nucleotides of defined sequence to the 3'-end of B2 RNA. The extension reaction is single-round *in vitro* and extended B2 RNA can still act as a transcriptional repressor *in vitro* and in cells, albeit with lower potency. Inhibition of Pol II extension of B2 RNA in cells caused a dramatic increase in the overall level of B2 RNA. Treatment of cells with actinomycin D showed that extension of B2 RNA decreases its half life. Together, these studies provide the first example of a mammalian cellular RNA that serves as a substrate for the Pol II RdRP and demonstrate that the Pol II RdRP activity can control the cellular stability of an ncRNA.

The observation that B2 RNA is extended by Pol II indicates that the 3'-end of B2 RNA sits in the active site of the polymerase. This is consistent with our previous studies showing that B2 RNA disrupts contacts between Pol II and the promoter DNA (Espinoza *et al*, 2007; Yakovchuk *et al*, 2009), as well as the observation that B2 RNA is localized in the DNA-binding cleft of Pol II (Kassube *et al*, 2012), which contains the active site (Cramer *et al*, 2000). In contrast to B2 RNA, B1 RNA is not extended, which is interesting given that cryo-EM studies have shown that B1 RNA also localizes to the DNA-binding cleft of Pol II (Kassube *et al*, 2012). Perhaps B1 RNA is oriented such that its 3'-end is not near the active site, or perhaps the secondary structure of B1 RNA does not allow 3'-end extension to occur.

We were surprised to observe that extension of B2 RNA stopped at a relatively well-defined position that was part way up a stem in the secondary structural model for B2 RNA that we previously defined experimentally (Espinoza *et al*, 2007). We do not yet understand why this occurs. It is likely that extension changes the secondary structure in the 3' region of B2 RNA, which could provide a barrier to

further polymerization. The number of nucleotides added during RdRP extension could also be limited by a combination of the length of the new duplex formed and/or the conformation of the B2 RNA/Pol II complex. Previous biochemical studies of the DNA-dependent transcriptional activity of Pol II could provide insight into this matter. *In vitro*, when Pol II was assembled on template DNA annealed to a 9-nt RNA primer, the catalytic activity of the complex sharply decreased after the RNA was extended to 20 nt (Kireeva *et al*, 2000). Other studies found that 18 nt is the maximum size the melted DNA bubble obtains prior to its upstream collapse during early transcription (Pal *et al*, 2005). It is possible that structural transitions that occur during RdRP extension of B2 RNA are similar to those that occur during early DNA-dependent transcription by Pol II, in which case the former could provide an *in vitro* tool to investigate Pol II structure/function relationships.

It was unexpected to find that total B2 RNA levels dramatically increased when cells were treated with α -amanitin to block Pol II activity, whereas levels of extended B2 RNA did not significantly change. This phenomenon was unique to B2 RNA because other Pol III transcripts did not substantially change upon treatment with α -amanitin, including B1 RNA. Because the stress-inducible B1 RNA did not increase with α -amanitin treatment, the increase in B2 RNA is unlikely to be attributable to new transcription in response to cellular stress. Rather, our data support a model in which inhibition of the Pol II RdRP activity by α -amanitin increases B2 RNA levels. Extension of B2 RNA by the Pol II RdRP decreases its half life in cells; hence, blocking extension causes levels of total B2 RNA to increase. It remains possible that a portion of the increase in B2 RNA observed after α -amanitin treatment results from increased Pol III transcription of specific B2 SINES. This would then couple with a substantial decrease in the rate of decay of B2 RNA due to inhibition of the Pol II RdRP activity to give rise to the large increase in B2 RNA levels after α -amanitin treatment.

Using actinomycin D to measure the stability of extended and total B2 RNA revealed that the population of B2 RNA that can be extended is less stable than total B2 RNA. Upon actinomycin D treatment, total B2 RNA decreased rapidly at first (to ~50%), then the remaining B2 RNA decayed far more slowly. These data suggest that approximately half of the B2 RNA is in a form that is already extended or can be extended, and that this pool of B2 RNA is significantly less stable due to extension of the 3'-end by Pol II. The remainder of the B2 RNA is in a form that cannot be extended, and as a result, decays much slower. The cause of two pools of nuclear B2 RNA is unknown. It is possible that the extendable and unextendable B2 RNA exist in two distinct protein-nucleic acid complexes. Alternatively, it has previously been shown that polyadenylation of exogenously expressed B2 RNA results in increased stability in cells (Borodulina and Kramerov, 2008). Perhaps half of the B2 RNA is polyadenylated, and this pool of B2 RNA would not likely serve as a substrate for the Pol II RdRP.

We have previously shown that B2 RNA serves as a repressor of Pol II transcription during the cellular response to heat shock (Allen *et al*, 2004). The research presented here generates many new ideas regarding the interplay between RdRP extension of B2 RNA and transcriptional repression by B2 RNA during the heat shock response. For example, B2 RNA levels increase significantly upon heat shock due to

increased transcription of B2 SINEs (Fornace and Mitchell, 1986); it is possible that decreased extension of B2 RNA under conditions of cellular stress contributes to the observed increase in B2 RNA levels after heat shock. To repress transcription during heat shock, B2 RNA binds Pol II and localizes to the promoters genes (e.g., the actin and hexokinase II genes) (Allen *et al*, 2004; Mariner *et al*, 2008). It remains to be determined whether the extended B2 RNA is stably bound to Pol II in cells and/or if it is present at the promoters of genes repressed upon heat shock. *In vitro* studies showed that once Pol II has engaged promoter DNA, B2 RNA is no longer able to bind the polymerase and repress transcription (Espinoza *et al*, 2004; Yakovchuk *et al*, 2009). This model predicts that cellular Pol II that is not already engaged in transcription is responsible for extending B2 RNA. Studies in HeLa cells found that ~40% of the total Pol II molecules are not chromatin-bound (Kimura *et al*, 1999), and it is this population that we imagine extends B2 RNA.

Extended B2 RNA can function as a transcriptional repressor *in vitro*, however, with a reduced potency compared with B2 RNA. This was also true in transient transfection assays. In addition, we found that an activity in nuclear extracts can dissociate the B2 RNA/Pol II complex in a process that depends on RdRP-mediated extension of B2 RNA (Figure 1). Therefore in the transfection assays, it is possible that such an activity dissociated B2 RNA* from Pol II, leading to the reduced transcriptional repression. Determining this will require identifying the factor(s) responsible for the dissociating activity and characterizing its cellular function, which are topics for future study.

B2 RNA-binding Pol II and repressing transcription is loosely analogous to bacterial 6S RNA-binding bacterial RNA polymerase and repressing transcription (Wassarman and Storz, 2000; Wassarman, 2007; Goodrich and Kugel, 2010). Bacterial RNA polymerase uses its RdRP activity to release 6S RNA by using it as a template to carry out *de novo* synthesis of short RNAs (Wassarman and Saecker, 2006). Pol II RdRP activity is involved in dissociating the B2 RNA/Pol II complex; however, an additional factor is required to remove extended B2 RNA from Pol II. In cells, such an activity could control transcriptional repression by B2 RNA.

Beyond controlling B2 RNA stability, it is possible that Pol II's RdRP activity plays a broader role in cellular processes. It seems likely that Pol II will extend other cellular RNAs. Determining the breadth of Pol II's RdRP activity will require identifying other RNA substrates for Pol II and deciphering their functions in cells.

Materials and methods

Plasmid construction and RNA preparation

Construction of pUC-T7-B2, pUC-T7-B1, and pUC-T7-B2(3–73) and their restriction digestion to generate templates used for T7 RNA polymerase reactions are described elsewhere (Allen *et al*, 2004). For the experiment shown in Figure 5B, a DNA template encoding B2 RNA was generated from pUC-T7-B2 using PCR with a reverse primer containing 2'-methoxyl A residues at the 5' terminal two positions (Kao *et al*, 2001). For the experiment shown in Figure 5D, a DNA template encoding B2 RNA(C155G) was generated from pUC-T7-B2 using PCR. For the experiment shown in Figure 6C, a DNA template encoding B2 RNA* was generated from pUC-T7-B2 using PCR.

Unlabelled and body-labelled ncRNAs were synthesized *in vitro* by T7 RNA polymerase and gel purified as previously described (Allen *et al*, 2004). For 5'-end labelling, purified unlabelled ncRNAs

were treated with 15 units of Antarctic phosphatase (NEB) in the supplied buffer for 1.5 h at 37°C. The enzyme was heat inactivated at 65°C for 15 min and RNA was ethanol precipitated. For Figure 4, B2 RNA was oxidized by incubation in 0.1 M sodium acetate pH 5.0 and 10 mM Na-m-periodate (Fluka) for 1 h at room temperature in the dark (Langland *et al*, 1995).

Partial purification of dissociation activity

HeLa cells were grown in 21 flasks in JMEM with 10% newborn calf serum (NCS) plus penicillin/streptomycin (250 µg/ml each) in 5% CO₂ at 37°C. Nuclear extract was prepared as described previously (Dignam *et al*, 1983). All chromatography was performed in buffer D (40 mM HEPES, 20% glycerol, 1.5 mM MgCl₂, 1 mM DTT, 0.2 mM PMSF, 0.2 mM EDTA) with variable concentrations of KCl. HeLa nuclear extract was flowed onto a DEAE-cellulose column in buffer D containing 50 mM KCl. The factor was eluted in buffer D containing 300 mM KCl. Pooled fractions were dialyzed in buffer D containing 100 mM KCl and then loaded on a phosphocellulose (P11) column. The factor was eluted in buffer D containing 300 mM KCl. Pooled fractions were dialyzed in buffer D containing 100 mM KCl and subsequently loaded on Source 15Q HPLC column. A linear salt gradient from 100–500 mM KCl was applied to the column. The dissociation activity fractionated as a single peak at ~300 mM KCl. Fractions containing activity were pooled, dialyzed into Buffer D containing 100 mM KCl, and subsequently loaded on a Affi-Gel heparin HPLC column. A linear salt gradient from 100 to 500 mM KCl was applied to the column. The dissociation activity fractionated as a single peak at ~250 mM KCl. Fractions containing activity were pooled and concentrated prior to loading on a Superose-6 size exclusion column, which was run in buffer D containing 100 mM KCl. The dissociation activity eluted in a single peak with an approximate molecular weight of 200–400 kDa when compared with protein molecular weight standards.

EMSA assays

Native human Pol II was prepared as described previously (Lu *et al*, 1991). ³²P-labelled B2 RNA (0.2 nM) was added to Pol II (2 nM) in buffer A containing 15 units of RNA Guard (GE Biosciences) in a volume of 20 µl and incubated at 30°C for 10 min. TFIIF (1 nM) was included in reactions testing the partially purified factor. HeLa nuclear extract or the partially purified factor and NTPs (1 mM each, where indicated) were added after 10 min. After 25 min, calf thymus DNA (1.2 µg) was added to each reaction, prior to loading onto a 4% polyacrylamide gel containing 0.5 × Tris-borate-EDTA buffer, 5% glycerol, and 5 mM magnesium acetate and subjected to electrophoresis as previously described (Espinoza *et al*, 2004).

RdRP assays

Reactions (20 µl) were assembled in buffer B containing 10% glycerol, 10 mM Tris (pH 7.9), 10 mM HEPES (pH 7.9), 50 mM KCl, 8 mM MgCl₂, 1 mM DTT, 25 µg/ml BSA. Unless indicated otherwise, Pol II (4 nM) and ncRNA (0.05–0.1 nM) were incubated at 30°C for 10 min, followed by the addition of NTPs for 45 min (either 625 µM ATP, 625 µM UTP, 625 µM GTP, and 25 µM [α -³²P]-CTP (5 µCi per reaction), or 1 mM each NTP). Reactions were stopped and RNA was resolved by denaturing PAGE as previously described for *in vitro* transcription reactions (Espinoza *et al*, 2007).

DNA-dependent *in vitro* transcription and crosslinking assays

Transcription reactions (20 µl) were assembled in buffer A and contained factors and template DNA at the following final concentrations: 3.5 nM TBP, 10 nM TFIIB, 2 nM TFIIF, 2 nM Pol II, and 1 nM DNA template. Transcription factors were prepared as previously described (Kugel and Goodrich, 1998; Weaver *et al*, 2005). The DNA template consisted of either negatively supercoiled plasmid pBSMLP (Figure 6A) (Goodrich and Tjian, 1994), an 88-bp linear DNA containing the Adenovirus major late promoter (AdMLP; –53 to +10) with a mismatched region from –10 to –5 (Figure 6B), an 88-bp linear DNA containing the AdMLP (–53 to +10) with a mismatched region from –9 to –3 (Figure 2B), or a negatively supercoiled plasmid DNA containing the AdMLP fused to a 380-bp G-less cassette (Figure 6C) (Goodrich and Tjian, 1994). For all experiments except that shown in Figure 2B, DNA templates were preincubated with TBP at 30°C for 4 min. TFIIB, TFIIF, Pol II, and ncRNA (5 nM, unless otherwise indicated) were incubated together in a separate tube at 30°C for 4 min. For the experiment shown in

Figure 2B, DNA template or B2 RNA were incubated with Pol II and TFIIF at 30°C for 20 min. NTPs were then added and the incubation was continued for 20 min. In Figure 2B (lanes 1–6), 6B, and 6C NTP concentrations and detection of transcripts by denaturing PAGE were as previously described (Espinoza *et al*, 2004; Yakovchuk *et al*, 2009). For the experiment in Figure 6A, 1 mM of each of the four NTPs was used, transcripts were ethanol precipitated; transcription reactions performed without NTPs were used to establish background. After DNase treatment, reverse transcription was performed using MultiScribe Reverse Transcriptase (Applied Biosystems), which was followed by real-time PCR with SYBR Green (Applied Biosystems) detection. The primer sequences used were (5′–3′): reverse transcription and PCR reverse, GCGAATTG-GAGCTCCACCGCG; PCR forward, GCTGTCTGCGAGGGCCAG. For the experiment in Figure 6D, site-specific protein–DNA photo-cross-linking was performed with a linear DNA template containing the AdMLP and a photoactivatable crosslinker between positions +3/+4 as previously described (Yakovchuk *et al*, 2009).

RNA isolation from cells, detection of specific transcripts, and transfection assays

NIH 3T3 cells were maintained in 5% CO₂ at 37°C in DMEM containing 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamate. In the experiment shown in Figure 7B, NIH 3T3 cells were treated with 50 µg/ml α-amanitin for 18 h. In the experiment shown in Figure 7D, NIH 3T3 cells were treated with either 5 µg/ml actinomycin D or 0.1% DMSO. Nuclei were isolated by resuspending cells in 80 µl of NP buffer (2 mM MgCl₂, 10 mM Tris–HCl (pH 7.4), 10 mM NaCl, and 0.5% (v/v) NP-40) per 1 million cells and incubating for 5 min on ice. Nuclei were harvested by centrifugation and washed once in an equal volume of NP buffer. Nuclear RNA was extracted with Trizol Reagent (Invitrogen). Total B2 RNA, B1 RNA, and 7SK RNA were detected by primer extension as previously described (Allen *et al*, 2004). For RT-PCR, RNA samples were treated with DNase I (14 units) at 37°C for 10 min and then heat inactivated. The RNA was added to 50 µl reactions containing 0.5 µM of the appropriate primer (5′-AGTGTA CTTACATATAATAAAG for extended B2 RNA or random decamers

for Myc mRNA, 7SL RNA, and tRNA^{Leu}) and 125 units of MultiScribe Reverse Transcriptase (Applied Biosystems). Reactions were incubated at 25°C for 10 min, 37°C for 2 h, then 85°C for 5 min. Where indicated, parallel reactions were performed in the absence of either reverse transcriptase or the primer. Myc cDNA was quantitated using real-time PCR and TaqMan probes. 7SL and tRNA^{Leu} cDNA were quantitated by ethidium staining PCR products (7SL forward 5′-GGTGTCCGCACTAATTCGGCATCAATA TGG; 7SL reverse 5′-TATTCACAGGCGCGATCCCACTACTGATC; tRNA^{Leu} forward 5′-GTCAGGATGGCCGAGTGGTCTAAG; tRNA^{Leu} reverse 5′-CCACGCTCCATACGGAGACCAGAATCCCC). For detection of cDNA made from extended B2 RNA, a nested PCR approach was used. First, 25 rounds of PCR were performed with primers (0.5 µM) that amplified the region of the extended B2 cDNA from 3 to 196 (forward primer, 5′-GCTGGTGAGATGGCTCAG; reverse primer, 5′-AGTGTACTTACATATAATAAAG). Second, primers (5 µM) were added, which amplified the 3–81 region of B2 cDNA (forward primer, 5′-GCTGGTGAGATGGCTCAG; reverse primer, 5′-CATGTG GTTGCTGGGATTG). PCR was continued for 10 rounds. Products were resolved on ethidium-stained agarose gels and quantitated. Transfection and luciferase reporter assays were performed as previously described (Mariner *et al*, 2008).

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Conflict of interest

The authors declare that they have no conflict of interest.

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