

# B2 RNA binds directly to RNA polymerase II to repress transcript synthesis

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**B2 RNA is a small noncoding RNA polymerase III transcript that represses mRNA transcription in response to heat shock in mouse cells. Here we define the mechanism by which B2 RNA inhibits RNA polymerase II (Pol II) transcription. Using a purified Pol II transcription system, we found that B2 RNA potently inhibits transcription by binding to core Pol II with high affinity and specificity. Through this interaction, B2 RNA assembles into preinitiation complexes at the promoter and blocks RNA synthesis. Once B2 RNA is removed from preinitiation complexes, transcriptional activity is restored. Our studies describe a previously unobserved mechanism of transcriptional repression by a small RNA and suggest that B2 RNA associates with Pol II at promoters in heat shocked cells to actively inhibit transcription.**

Transcription of protein-encoding genes into mRNA by Pol II is a complex process that must be tightly regulated to maintain normal pathways of cellular growth and differentiation. Promoter-specific transcription requires numerous auxiliary factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH)<sup>1</sup> and is regulated by the combined actions of positive and negative regulatory factors, such as site-specific activators and repressors, coactivators, corepressors and chromatin-associated proteins<sup>2,3</sup>. The Pol II transcription reaction consists of multiple steps, the first of which is formation of the preinitiation complex. During this step, the general transcription factors and Pol II assemble at the promoter and the DNA around the start site is melted. In the presence of nucleoside triphosphates, preinitiation complexes initiate RNA synthesis and the polymerase proceeds through a series of steps that minimally include escape commitment, promoter escape and transcript elongation<sup>4-6</sup>. Each step in the transcription reaction has the potential to be regulated by transacting activators and repressors.

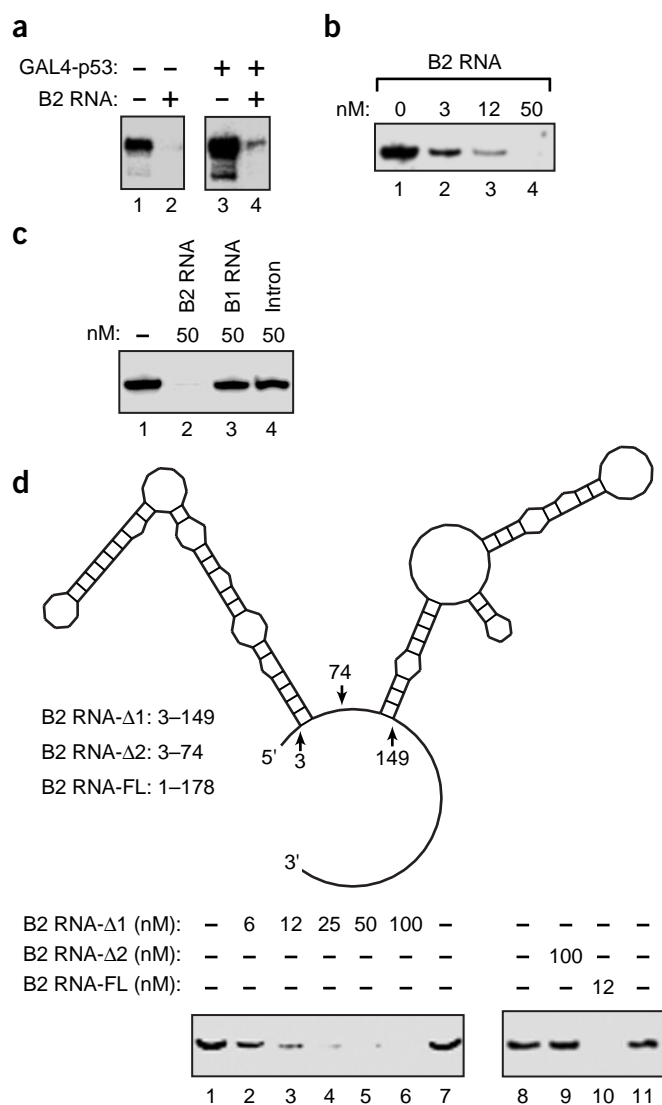
The vast majority of transcriptional regulators are proteins, and the mechanisms by which protein factors regulate transcription have been extensively studied. In a paradigm shift, RNA regulators of the transcription reaction are now being identified<sup>5,7-11</sup>. 7SK RNA targets transcript elongation by regulating the activity of the positive transcription elongation factor (P-TEFb)<sup>9,10</sup>. Along with the HEXIM1 protein, 7SK RNA binds to phosphorylated P-TEFb and inhibits its kinase activity, thereby repressing transcription<sup>12,13</sup>. U1 small nuclear RNA associates with TFIIH and stimulates transcription initiation *in vitro*<sup>8</sup>. We previously identified a synthetic polyguanosine RNA oligonucleotide that binds to Pol II and specifically inhibits the escape commitment step of the transcription reaction<sup>5</sup>. In *Escherichia coli*, the 6S RNA binds directly to *E. coli* RNA polymerase and represses transcription from  $\sigma^{70}$  promoters during the stationary phase of growth<sup>11</sup>. From this limited set of examples, it is already apparent that

RNA regulators have diverse mechanisms for controlling the transcription reaction.

We recently found that a small mouse RNA, B2 RNA, can act *in trans* to repress mRNA transcription after heat shock in mouse cells<sup>14</sup>. In response to heat shock, general Pol II transcription is repressed, while a subset of heat shock genes are strongly activated<sup>15-24</sup>. For example, actin, histone H1 and hexokinase II transcript levels decrease sharply after heat shock, whereas levels of hsp70 and other hsp transcripts increase<sup>21-24</sup>. The level of B2 RNA, an RNA polymerase III transcript encoded by short interspersed elements (SINES), also increases after heat shock<sup>25-29</sup>. We found that treating mouse cells with an antisense oligonucleotide against B2 RNA attenuated the decrease in actin and hexokinase II transcription normally observed in response to heat shock<sup>14</sup>. *In vitro*, recombinant B2 RNA specifically inhibited Pol II transcription in mouse nuclear extracts, confirming that B2 RNA acts as a transcriptional repressor. In addition, B2 RNA coimmunoprecipitated with Pol II specifically after heat shock. These results showed that B2 RNA inhibits Pol II transcription in response to heat shock in mouse cells; however, many questions remained regarding the mechanism by which this occurs.

Here we used a highly purified *in vitro* transcription system to characterize the mechanism by which B2 RNA inhibits the mRNA transcription reaction. Specifically, we asked which transcription factor(s) B2 RNA targets, what step in the transcription reaction it inhibits, and whether repression by B2 RNA is reversible. We found that B2 RNA binds to core Pol II with high affinity, specificity and kinetic stability, thereby resulting in potent transcriptional inhibition. Through this interaction B2 RNA can incorporate into preinitiation complexes at promoters and render them inactive. Removal of B2 RNA from preinitiation complexes restores transcriptional activity. Our data suggest a model whereby after heat shock in mouse cells B2 RNA binds Pol II

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**Figure 1** B2 RNA represses mRNA transcription with specificity *in vitro*. **(a)** Mouse B2 RNA represses basal and activated Pol II transcription when added to a nuclear extract from human Jurkat cells. B2 RNA was added at a final concentration of 400 nM. **(b)** B2 RNA represses Pol II transcription in a minimal reconstituted transcription system. Reactions contained highly purified human core Pol II, TBP, TFIIB and TFIIF. 390-nt G-less RNA transcript is shown. **(c)** B1 RNA and a group I intron RNA do not repress Pol II transcription. B2 RNA, B1 RNA or the P4–P6 region of the group I intron was added to reactions before formation of preinitiation complexes. 390-nt G-less RNA is shown. **(d)** B2 RNA-Δ1 (nucleotides 3–149) inhibits transcription whereas B2 RNA-Δ2 (nucleotides 74–149) does not. Full-length B2 RNA (B2 RNA-FL) and two deletion mutants were added to transcription reactions before formation of preinitiation complexes. 390-nt G-less RNA is shown.

promoter and the recombinant B2 RNA was PAGE-purified. B2 RNA inhibited both basal transcription (Fig. 1a, compare lanes 2 and 1) and GAL4-p53 activated transcription (compare lanes 4 and 3) when added to nuclear extracts prepared from human Jurkat cells, as assayed by primer extension. Notably, this level of inhibition was similar to that observed when B2 RNA was added to mouse nuclear extracts<sup>14</sup>.

We next asked whether B2 RNA could inhibit transcription in a reconstituted human system consisting of the minimum set of factors required to support promoter specific basal transcription: TBP, TFIIB, TFIIF and core Pol II<sup>4,5,30</sup>. Low nanomolar concentrations of B2 RNA strongly inhibited Pol II transcription in the minimal system (Fig. 1b). To test the specificity of this inhibition we added two other small structured RNAs to *in vitro* transcription reactions: the 136-nt mouse B1 RNA and the 158-nt P4–P6 region of the *Dunaliella* group I intron<sup>34</sup>. B1 RNA, like B2 RNA, is transcribed from SINEs and is upregulated upon heat shock<sup>25,28</sup>, but unlike B2 RNA, B1 RNA does not inhibit transcription when added to mouse nuclear extracts<sup>14</sup>. B1 RNA and group I intron RNA had little effect on the levels of transcript RNA produced in this system (Fig. 1c). To further test the specificity of inhibition by B2 RNA we produced two deletion mutants. The deletions were based on the predicted secondary structure of B2 RNA generated using mfold version 3.1 (Fig. 1d)<sup>35,36</sup>. B2 RNA-Δ1 (nucleotides 3–149) lacks a predicted single stranded 3' tail and two 5' nucleotides. B2 RNA-Δ2 contains nucleotides 3–74, and this region of the RNA is predicted to fold into the same secondary structure as seen in the full-length RNA. B2 RNA-Δ1 inhibited transcription nearly as well as full-length B2 RNA (Fig. 1d, lanes 2–6), whereas B2 RNA-Δ2 did not inhibit transcription when added at concentrations up to 100 nM (lane 9). The results in Figure 1 show that B2 RNA is both a potent and specific inhibitor of Pol II transcription. Moreover, B2 RNA inhibits transcription by targeting one or more of the four factors in the minimal transcription system.

### B2 RNA binds directly to core Pol II

We previously found that polyguanosine RNA oligonucleotides inhibit the escape commitment step of the transcription reaction by binding directly to core Pol II<sup>5</sup>. We therefore hypothesized that Pol II was the target of B2 RNA during transcriptional inhibition. To test this we carried out *in vitro* transcription reactions under three conditions: (i) our standard conditions (ii) with excess TFIIB and TFIIF and (iii) with excess Pol II. Under each of the three conditions, B2 RNA was titrated into transcription reactions and the IC<sub>50</sub> for inhibition was determined (Fig. 2). Similar amounts of B2 RNA were required to inhibit transcription under our standard conditions and with excess TFIIB and TFIIF; the IC<sub>50</sub> values were 1.3 nM and 2.1 nM, respectively. In the latter case TFIIB and TFIIF were present at 20 nM, well above the IC<sub>50</sub>

and assembles into transcriptionally inactive preinitiation complexes at specific promoters, leading to repression of transcription until the B2 RNA is removed.

## RESULTS

### B2 RNA inhibits transcription in a reconstituted system

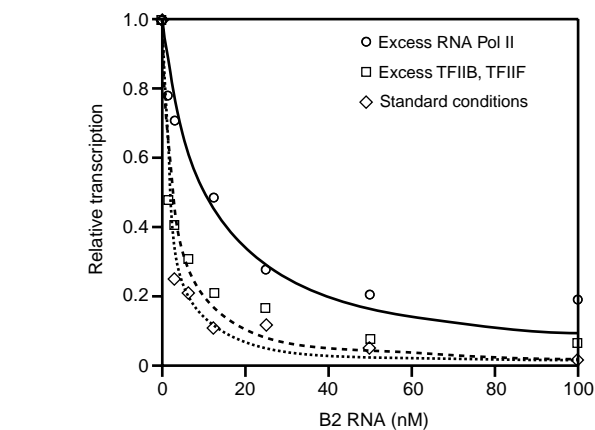
In our initial studies of mouse B2 RNA we found that it repressed basal and activated Pol II transcription when added to mouse nuclear extracts, and that it coimmunoprecipitated with Pol II exclusively after heat shock<sup>14</sup>; however, the mechanism of transcriptional inhibition remained to be determined. Defining this mechanism and identifying the direct target(s) of B2 RNA required the use of a reconstituted Pol II transcription system that responded to B2 RNA. In other studies, we have extensively used a Pol II transcription system reconstituted from highly purified human components to investigate the mechanism of transcription and its regulation by protein factors<sup>4,5,30–33</sup>. Before testing B2 RNA in our reconstituted transcription system it was important to ask whether the potency of B2 RNA transcriptional repression in human nuclear extracts was similar to that previously observed in mouse nuclear extracts. B2 RNA (178 nucleotides (nt)) was produced from a mouse B2 gene cloned downstream of a bacteriophage T7

for inhibition. By contrast, when the amount of Pol II was increased five-fold (to 15 nM, circles with solid line), the  $IC_{50}$  for inhibition by B2 RNA increased to 10.2 nM. These results show that B2 RNA targets Pol II to inhibit *in vitro* transcription.

To directly test whether B2 RNA binds Pol II we used electrophoretic mobility shift assays (EMSA). Pol II shifted B2 RNA (Fig. 3a, lane 5), but TBP, TFIIB and TFIIF did not (Fig. 3a, lanes 2–4). By varying the concentration of Pol II we found that it bound to B2 RNA with high affinity (Fig. 3b,  $K_{d,app} \leq 2$  nM). This estimate of the affinity is similar to the  $IC_{50}$  for B2 RNA transcriptional inhibition; this is consistent with Pol II being the primary target of B2 RNA. We next determined the effect of increasing KCl concentration on formation of the Pol II–B2 RNA complex. Increasing KCl minimally affected formation of complex; only at 1 M KCl was there an appreciable decrease in the amount of complex observed (Fig. 3c). We also measured the kinetic stability of the Pol II–B2 RNA complex by forming complexes containing  $^{32}P$ -labeled B2 RNA, challenging with a 1,000-fold excess of unlabeled B2 RNA, and measuring the rate of dissociation. After 1 h, 50% of the complexes remained (Fig. 3d,  $k_{off} = 1.3 \pm 0.1 \times 10^{-4} s^{-1}$ ). In testing the specificity with which the Pol II–B2 RNA complex forms, we found that a 50-fold excess of group I intron RNA reduced the amount of Pol II–B2 RNA complex by only 50% (data not shown). Taken together, our results confirm that B2 RNA binds core Pol II with high affinity, specificity and kinetic stability.

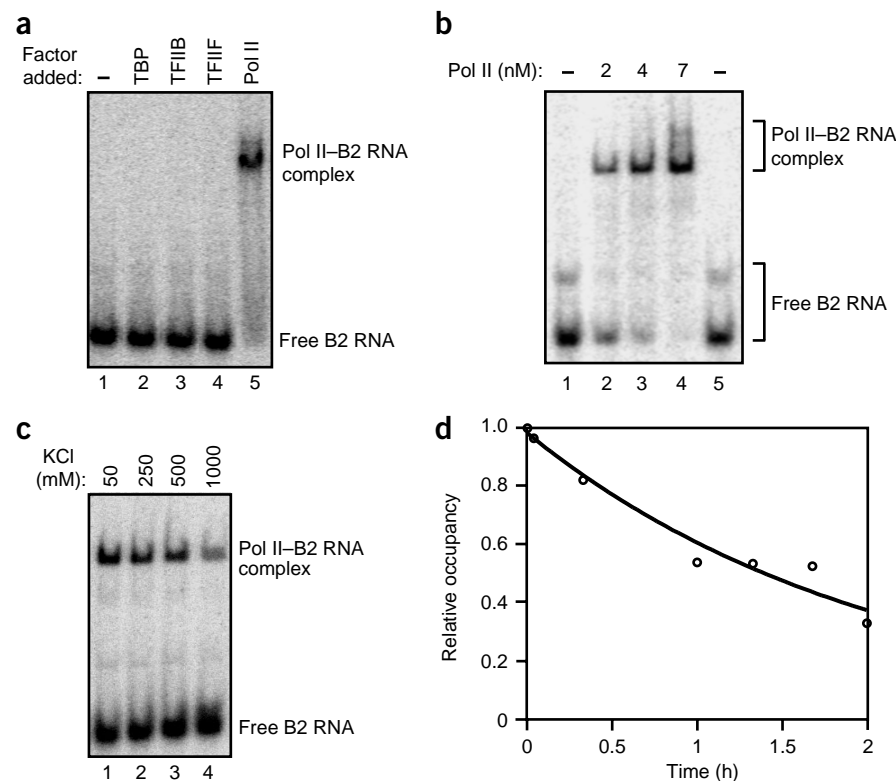
### B2 RNA binds an RNA docking site on Pol II

We previously found an RNA docking site on Pol II that tightly binds polyguanosine RNA oligonucleotides, resulting in inhibition of transcription<sup>5</sup>. This docking site is distinct from the DNA-binding channel and RNA exit groove. Although polyguanosine oligonucleotides bind this site with high affinity, other homopolymeric oligonucleotides show little or no binding to the polymerase<sup>5</sup>. To ask whether B2 RNA binds to this RNA docking site, we titrated a fluorescently labeled RNA



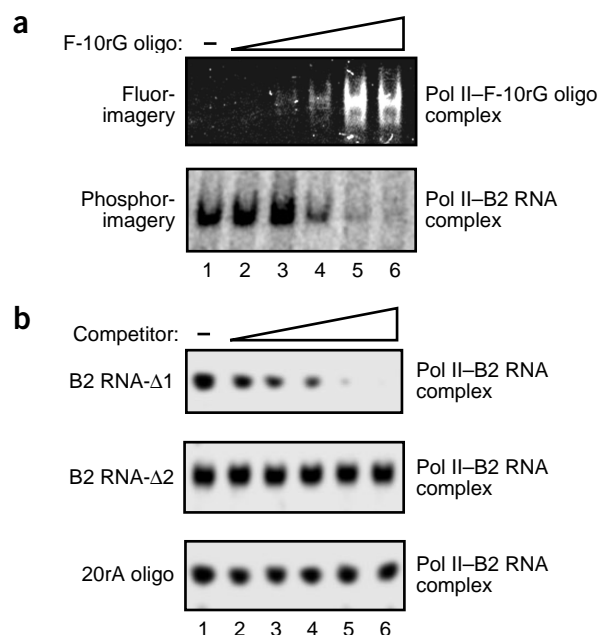
**Figure 2** B2 RNA targets Pol II to inhibit transcription. B2 RNA was titrated into transcription reactions containing either the standard amounts of transcription factors, excess TFIIB and TFIIF, or excess Pol II. The amount of 390-nt G-less product was quantified by phosphorimetry and plotted.

oligonucleotide consisting of ten guanines (F-10rG) into reactions containing a constant amount of  $^{32}P$ -labeled B2 RNA and subsequently added Pol II. Complexes were resolved on a native gel and visualized by both fluorimetry and phosphorimetry (Fig. 4a). The F-10rG oligonucleotide efficiently competed with B2 RNA for association with Pol II as seen by a decrease in the Pol II–B2 RNA complex (phosphorimager scan) and an increase in the Pol II–F-10rG complex (fluorimetry scan). These data indicate that B2 RNA binds the previously identified RNA docking site on Pol II. As controls we also tested whether unlabeled B2 RNA- $\Delta 1$ , B2 RNA- $\Delta 2$ , and an RNA oligonucleotide consisting of 20 adenines (20rA) would compete with B2 RNA for binding Pol II. Of these RNAs, B2 RNA- $\Delta 1$  competed, as seen by a decrease in the in the Pol II–B2 RNA complex, whereas B2 RNA- $\Delta 2$  and the 20rA oligonucleotide did not (Fig. 4b). These data



**Figure 3** B2 RNA binds Pol II with high affinity, specificity and kinetic stability. (a) Core Pol II binds B2 RNA, whereas TBP, TFIIB and TFIIF do not. Recombinant  $^{32}P$ -labeled B2 RNA was incubated separately with TBP, TFIIB, TFIIF and core Pol II. Free B2 RNA was resolved from protein–RNA complexes using native gel electrophoresis. (b) B2 RNA binds Pol II with high affinity. EMSAs were carried out with  $^{32}P$ -labeled B2 RNA (0.5 nM) and purified human Pol II. In this gel, free B2 RNA resolved as two distinct bands that probably resulted from two conformations, both of which are bound by the polymerase. The relative abundance of these two conformations observed in gels is variable. (c) Complexes between B2 RNA and Pol II can form in high salt.  $^{32}P$ -labeled B2 RNA (3 nM) was incubated with purified Pol II (2 nM) in reactions containing KCl at the indicated concentrations and EMSAs were carried out. (d) The complex between Pol II and B2 RNA is kinetically stable. Pol II–B2 RNA complexes were formed, challenged with a 1,000-fold excess of unlabeled B2 RNA for variable times, and analyzed by native gel electrophoresis. The rate constant for dissociation is  $1.3 \pm 0.1 \times 10^{-4} s^{-1}$ .





**Figure 4** B2 RNA binds a previously identified RNA docking site on Pol II. (a) A fluorescently labeled polyguanosine RNA oligonucleotide competes with  $^{32}\text{P}$ -labeled B2 RNA for binding Pol II. Pol II (3.5 nM) was incubated with  $^{32}\text{P}$ -labeled B2 RNA (5 nM) in the absence (lane 1) or presence of increasing concentrations of F-10rG-oligo labeled with Alexa Fluor 555 (lanes 2–6: 0.5, 2.5, 5, 25 and 50 nM F-10rG-oligo, respectively). The same gel was analyzed by both fluorimetry (top) and phosphorimetry (bottom) and the region of the gel containing the Pol II–B2 RNA complex and the Pol II–F-10rG-oligo complex is shown for both scans. (b) Unlabeled B2 RNA- $\Delta$ 1 competed with  $^{32}\text{P}$ -labeled B2 RNA for binding Pol II, whereas B2 RNA- $\Delta$ 2 and the 20rA oligonucleotide did not. Concentrations of Pol II and RNAs were the same as in a. The region of the native gel containing the Pol II–B2 RNA complex is shown.

show that the competition with the F-10rG oligonucleotide and B2 RNA- $\Delta$ 1 is specific and support the conclusion that B2 RNA binds the previously identified RNA docking site on the polymerase.

### B2 RNA blocks an early step in transcription

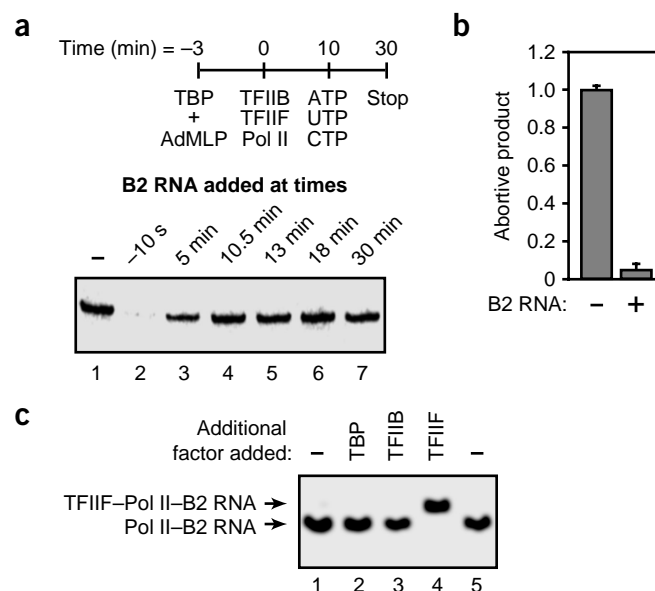
Because B2 RNA binds the polymerase, it has the potential to inhibit any step in the transcription reaction from formation of preinitiation complexes through transcript elongation. To begin to identify the step(s) that B2 RNA inhibits, we carried out the experiment shown in Figure 5a. B2 RNA was added to *in vitro* transcription reactions either before preinitiation complex formation (lane 2), before nucleotide addition (lane 3) or after the addition of nucleotides (lanes 4–7). The addition of TFIIB, TFIIF and Pol II to reactions started the assembly of preinitiation complexes (designated as time zero). When B2 RNA was added to reactions before formation of preinitiation complexes, transcription was completely inhibited (compare lanes 2 and 1). By contrast, when B2 RNA was added at any point after preinitiation complexes had formed, little or no inhibition was observed (lanes 3–7). Several conclusions can be made from these results. First, B2 RNA cannot associate with existing preinitiation complexes to inhibit transcription (lane 3). Second, B2 RNA cannot actively disrupt ternary transcribing complexes after they have formed (lanes 4–7). Third, B2 RNA acts rapidly to inhibit transcription. Under these conditions TFIIB, TFIIF and Pol II form preinitiation complexes with promoter-bound TBP within 10 s (ref. 30). Therefore, B2 RNA must

act within 10 s to block transcription. These data show that B2 RNA, when present during assembly of preinitiation complexes, blocks transcription by preventing the formation of functional initiation complexes.

To determine whether B2 RNA blocks the earliest steps of transcript synthesis we carried out abortive initiation assays in which preinitiation complexes are provided with limited nucleotides such that only 3-nt RNA products can be made<sup>37</sup>. Over the course of the reaction, 3-nt RNAs are continuously synthesized and released. When B2 RNA was present during preinitiation complex formation, the production of abortive 3-nt RNA was inhibited ~20-fold (Fig. 5b). We conclude that inhibition by B2 RNA occurs prior to or coincident with initiation. Therefore, B2 RNA either inhibits the assembly of preinitiation complexes or it enters preinitiation complexes and inhibits initiation.

If B2 RNA inhibited preinitiation complex assembly, it could do so by blocking the association of Pol II with one or more of the general transcription factors. To begin to test this, we asked whether TBP, TFIIB or TFIIF could supershift the Pol II–B2 RNA complex in native gels. TFIIF supershifted the B2 RNA–Pol II complex, but TBP and TFIIB did not (Fig. 5c). This suggests that the mechanism of B2 RNA transcriptional inhibition does not involve blocking the interaction of TFIIF with Pol II. The lack of supershifts with TBP and TFIIB does not preclude the possibility that these factors associate with the TFIIF–Pol II–B2 RNA complex in solution.

**Figure 5** B2 RNA prevents Pol II from forming functional initiation complexes. (a) B2 RNA acts before the formation of initiation complexes. Reactions were assembled as diagrammed in the schematic and B2 RNA was added at the time points indicated above the gel. 390-nt G-less transcript is shown. (b) Abortive initiation was inhibited when B2 RNA was added before formation of preinitiation complexes. The 3-nt abortive product was quantified by phosphorimetry, normalized, and the averages of three reactions ( $\pm$  1 s.d.) were plotted. (c) TFIIF can associate with the Pol II–B2 RNA complex. Core Pol II and the indicated general transcription factor were incubated with  $^{32}\text{P}$ -labeled B2 RNA. Complexes were resolved by native gel electrophoresis. The region of the gel containing the Pol II–B2 RNA and the TFIIF–Pol II–B2 RNA complexes is shown.



**Figure 6** B2 RNA associates with preinitiation complexes. **(a)** B2 RNA causes preinitiation complexes to migrate faster in native gels. Preinitiation complexes containing TBP, TFIIB, TFIIF and core Pol II were formed on fluorescently labeled AdMLP DNA in the absence and presence of B2 RNA. Complexes were resolved by EMSA and analyzed by fluorimetry. The positions of the preinitiation complex (PIC), the faster-migrating band (PIC-B2 RNA), and the free DNA are indicated. **(b)** Formation of the faster-migrating complex requires TBP, TFIIB, TFIIF and Pol II, in addition to B2 RNA. B2 RNA and fluorescently labeled AdMLP DNA were incubated with TBP, TFIIB, TFIIF and core Pol II (lane 3) or in the absence of one of the factors (lanes 4–7). Complexes were resolved by EMSA and analyzed by fluorimetry. Lanes 1 and 2 show free DNA and the preinitiation complex in the absence of B2 RNA, respectively. **(c)** B2 RNA migrates at the same position as the AdMLP DNA in the faster-migrating complex. Preinitiation complexes were formed on fluorescently labeled AdMLP DNA in the absence or presence of  $^{32}$ P-labeled B2 RNA (100 nM final concentration). Complexes were resolved by EMSA and analyzed by fluorimetry (left) and phosphorimetry (right). **(d)** B2 RNA- $\Delta 2$  does not associate with preinitiation complexes. Full-length B2 RNA and two deletion mutants were added during formation of preinitiation complexes, complexes were resolved by EMSA and analyzed by fluorimetry. Preinitiation complexes formed in the absence of B2 RNA are shown in lanes 1 and 5.

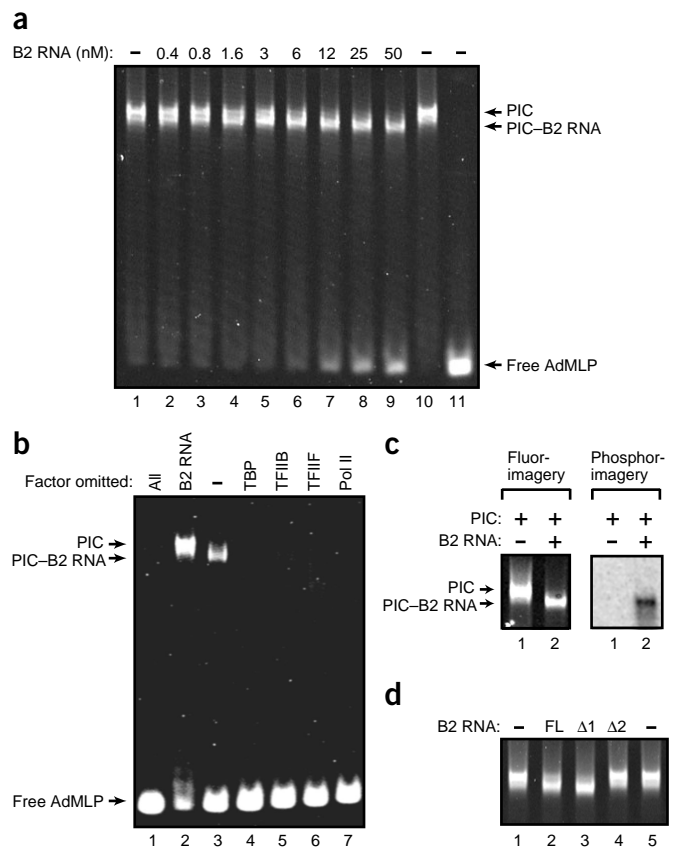
### B2 RNA associates with preinitiation complexes

To directly test whether B2 RNA inhibits the formation of preinitiation complexes, we used EMSAs with fluorescently labeled AdMLP DNA (Fig. 6a). Increasing amounts of B2 RNA were incubated with Pol II, TFIIF and TFIIB, and then added to a preformed TBP-AdMLP complex. Preinitiation complexes that were formed in the absence of B2 RNA are shown in lanes 1 and 10 of the fluorimetry scan (Fig. 6a). Formation of these complexes strictly depended on TBP, TFIIB, TFIIF and Pol II (data not shown). As B2 RNA was titrated into reactions (lanes 2–9), a new band emerged that migrated faster than the preinitiation complex.

We hypothesized that the faster-migrating complex was a preinitiation complex containing B2 RNA. If so, its formation would depend on all of the general transcription factors and Pol II. To test this we individually omitted TBP, TFIIB, TFIIF and Pol II from reactions containing B2 RNA and the AdMLP. The faster-migrating complex did not form when any one of the four transcription factors was omitted (Fig. 6b).

We next determined whether B2 RNA was present in the faster-migrating complex by asking whether  $^{32}$ P-labeled B2 RNA and fluorescently labeled AdMLP DNA comigrate in native gels. Alignment of a fluorimetry scan and a phosphorimetry scan of a single native gel (Fig. 6c, left and right panels, respectively) shows that  $^{32}$ P-labeled B2 RNA migrated at the same position as the fluorescent DNA in the faster-migrating complex (lane 2 in both panels). For comparison, lane 1 of the fluorimetry scan shows preinitiation complexes without B2 RNA. We conclude that B2 RNA incorporates into preinitiation complexes containing the general transcription machinery and promoter DNA to form inhibited complexes that migrate more quickly than functional preinitiation complexes in native gels.

To test the specificity with which the faster-migrating complex forms, as well as to determine whether it correlates with the inhibition of transcription, we tested the two deletion mutants of B2 RNA. We showed above that B2 RNA- $\Delta 1$  inhibited transcription whereas B2 RNA- $\Delta 2$  did not (Fig. 1d). Incubating B2 RNA- $\Delta 1$  with the transcription factors during assembly of preinitiation complexes resulted in the faster-migrating complex as analyzed by fluorimetry (Fig. 6d, lane 3), whereas B2 RNA- $\Delta 2$  had no effect (lane 4). These data demonstrate that B2 RNA associates with preinitiation complexes with specificity and correlate the formation of the faster-migrating complex with transcriptional inhibition.



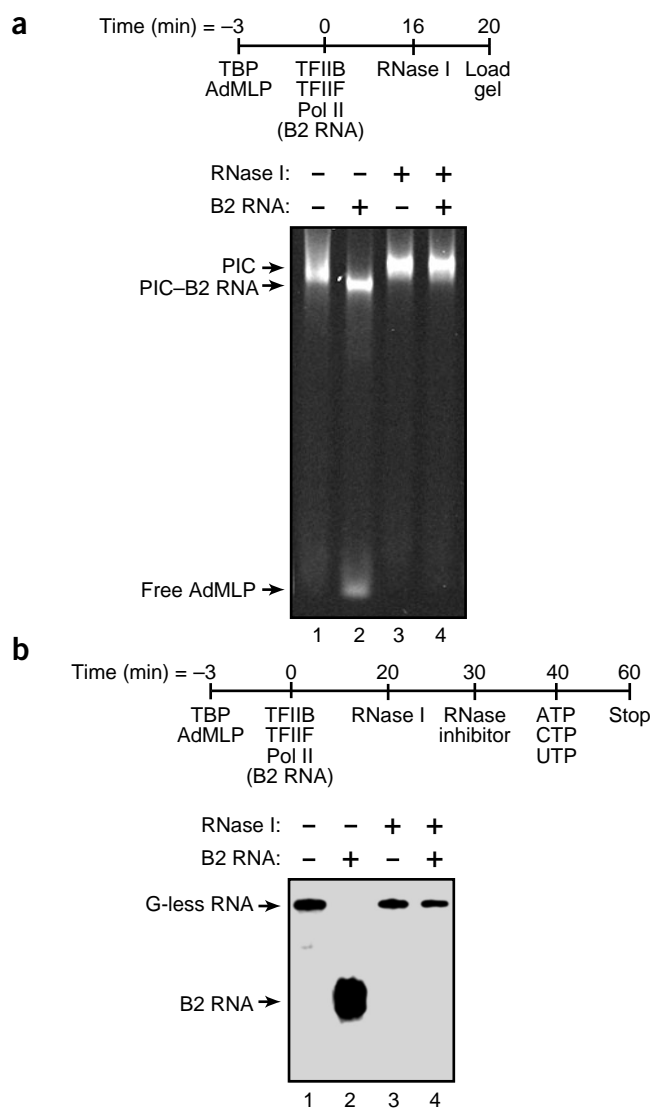
### The effects of B2 RNA are reversible

As a biological repressor of Pol II transcription, the inhibitory effect of B2 RNA on preinitiation complexes must be reversible as cells recover from heat shock and return to homeostasis. We asked whether the effect of B2 RNA on migration of preinitiation complexes and *in vitro* transcription could be reversed using an RNase to degrade B2 RNA. Preinitiation complexes formed on fluorescently labeled AdMLP in the absence and presence of B2 RNA were treated with RNase I and resolved by EMSA (Fig. 7a). Degrading B2 RNA shifted the faster-migrating band up to the position of functional preinitiation complexes. Phosphorimetry scans showed that the RNase I treatment completely degraded the B2 RNA (data not shown). RNase I treatment did not affect the position at which preinitiation complexes migrated in the absence of B2 RNA (lane 3). These data show that the continued presence of B2 RNA is required to observe the faster-migrating complex, consistent with its presence in the complex.

To determine whether degrading B2 RNA in preinitiation complexes results in recovery of transcriptional activity, we formed preinitiation complexes with and without B2 RNA, treated with RNase I and monitored transcription (Fig. 7b). Notably, an RNase inhibitor was added to reactions after treatment with RNase I to protect G-less transcripts synthesized during the reaction from degradation. When B2 RNA was removed by RNase I treatment, Pol II transcriptional activity was restored (lane 2 versus lane 4). Together, the results in Figure 7 show that the effects of B2 RNA on both the migration of preinitiation complexes and their transcriptional activity are reversible.

### DISCUSSION

The studies presented here define the mechanism by which B2 RNA represses transcription. B2 RNA binds directly to Pol II and assembles



**Figure 7** The effects of B2 RNA on migration of preinitiation complexes and transcriptional activity can be reversed with RNase treatment. **(a)** RNase treatment restored the migration of preinitiation complexes. Preinitiation complexes formed in the absence or presence of B2 RNA (100 nM final concentration) were treated with RNase I (1 Unit) and subsequently resolved by EMSA and analyzed by fluorimetry. Lanes 1 and 2, control reactions that were not treated with RNase I. **(b)** The transcriptional inhibition caused by B2 RNA can be reversed by treatment with RNase. Preinitiation complexes formed in the presence or absence of  $^{32}\text{P}$ -labeled B2 RNA (100 nM final concentration) were treated sequentially with RNase I (1 Unit) and the RNase inhibitor Supersasin (40 Units) before the addition of NTPs. The positions of the 390-nt G-less RNA and B2 RNA are indicated.

gels compared with complexes not containing B2 RNA. It is possible that negative charge contributed by the presence of bound B2 RNA causes preinitiation complexes to migrate faster. Alternatively, B2 RNA could induce a conformational change in the preinitiation complex, resulting in a change in migration. It is also possible that B2 RNA triggers release of a subunit within the preinitiation complex, thereby causing the complex to migrate faster. The cause of the faster migration is reversible because removing B2 RNA by ribonuclease digestion restores the migration of the inhibited complex to that of the preinitiation complex.

B2 RNA inhibits the transcription reaction at a point after stable complexes form and prior to RNA synthesis. B2 RNA could directly inhibit initiation. It is also possible that B2 RNA prevents the promoter DNA from melting, thereby blocking formation of open complexes. Alternatively, B2 RNA could block the binding of incoming nucleotides into the active site of the polymerase. The abortive initiation data showed that B2 RNA blocks multiple-turnover synthesis of short 3-nt RNAs; however, it is possible that a 3-nt RNA is made once in the presence of B2 RNA but the resulting complex is unable to both abortively release the short RNA and further extend it. It remains to be determined which of these steps B2 RNA inhibits.

Our *in vitro* data provide a model for how B2 RNA represses the transcription of specific genes in mouse cells after heat shock. During heat shock, Pol II transcription is transiently repressed at genes such as actin and hexokinase II<sup>14,21–24</sup>. We propose that upon heat shock in mouse cells, B2 RNA binds Pol II and forms inactive complexes at the promoters of repressed genes. How B2 RNA-mediated repression is relieved as cells recover from heat shock remains unclear. We found that the transcriptional repression caused by B2 RNA is reversible *in vitro* (Fig. 7). In cells, we propose that a factor exists to remove B2 RNA from Pol II, thereby relieving transcriptional repression. This factor could be an RNase, a helicase, a second regulatory RNA, or a protein that binds to B2 RNA displacing it from the polymerase. The relief of B2 RNA-mediated repression in cells will be the topic of future studies.

Upon heat shock, a mechanism must exist to allow genes such as *hsp70* to be transcribed despite the presence of B2 RNA. Such a mechanism might involve the hypothesized factor discussed above or a factor specific to genes activated during heat shock. Upregulation of genes encoding hsp proteins involves the transcriptional activator HSF (heat shock factor), which binds to heat shock-responsive promoters to stimulate transcription<sup>38</sup>. It is possible that HSF directly prevents the inhibitory effect of B2 RNA; however, previous attempts to observe an interaction between HSF and B2 RNA were unsuccessful<sup>29</sup>. Alternatively, HSF could recruit a factor that removes B2 RNA from polymerases that are bound at the promoters of genes activated during heat shock. The *hsp70* gene has also been found to harbor Pol II paused during early transcription<sup>39</sup>. It is possible that the transcrip-

into stable preinitiation complexes on promoter DNA. These complexes are transcriptionally inactive; they cannot produce both long RNAs and short abortive transcripts. The continued presence of B2 RNA is required to maintain the inhibited state as removal of B2 RNA restores transcriptional activity. These findings describe a previously unobserved mechanism whereby a small RNA regulates transcription. In addition, our results suggest a model for how B2 RNA can directly inhibit the activity of Pol II at promoters in heat shocked cells.

Our results show that B2 RNA incorporates into preinitiation complexes and renders them nonfunctional, thereby describing a unique mechanism of transcriptional repression by a regulatory RNA. The use of both fluorescent and  $^{32}\text{P}$  labels in EMSA experiments allowed us to determine that promoter DNA and B2 RNA comigrated; this strongly indicates that they are present in a single complex that migrates faster than preinitiation complexes. Our other data are consistent with this model. Treatment with RNase I showed that the continued presence of B2 RNA was required for the faster-migrating complex, indicative of its existence in the complex. In addition, the formation of the faster-migrating complex depended on the presence of all three of the general transcription factors and Pol II. It remains unclear why inhibited preinitiation complexes migrate faster in native

tional pause is involved in the mechanism by which these genes overcome B2 RNA repression.

We have now identified two dissimilar RNAs (one natural and one synthetic<sup>5</sup>) that bind tightly to an RNA docking site on Pol II and inhibit transcription. The observation that a polyguanosine RNA oligonucleotide can block B2 RNA binding to the polymerase (Fig. 4), suggests that B2 RNA and the oligonucleotide bind the same or overlapping sites. We previously found the RNA docking site on Pol II to be removed from the DNA-binding channel and the RNA exit groove<sup>5</sup>, but a biological function was not ascribed to this site. Because B2 RNA is a natural RNA regulator of transcription, our current studies provide a biological rationale for why Pol II has an RNA docking site. Notably, B2 RNA and polyguanosine RNA oligonucleotides inhibit transcription via distinct mechanisms: the former blocks all detectable RNA synthesis and the latter blocks synthesis of the fourth phosphodiester bond<sup>5</sup>. Our studies raise the possibility that cells harbor a class of small RNAs that bind a common docking site on core Pol II to regulate transcription in diverse ways (such as activation, repression and/or derepression) and by a variety of mechanisms.

## METHODS

**Construction of plasmids and preparation of RNA and fluorescently labeled DNA.** Construction of pUC-T7-B2 and pUC-T7-B1, encoding full-length B2 RNA and B1 RNA, respectively, is described elsewhere<sup>14</sup>. This method was also used to construct pUC-T7-B2-Δ1 and pUC-T7-B2-Δ2. Isolation of fragments for *in vitro* transcription and production of RNAs using T7 RNA polymerase is also described elsewhere<sup>14</sup>.

Fluorescently labeled AdMLP DNA was prepared from two complementary oligonucleotides (Integrated DNA Technologies) containing AdMLP sequence from -40 to +20. The template strand oligonucleotide was purchased with a six-carbon linker and a primary amine on its 5' end. This modified oligonucleotide was phenol-chloroform extracted, ethanol precipitated, and dissolved in water at a concentration of 4 mM. Oligonucleotide solution (4 μl) was added to 41 μl of 0.1 M sodium borate, pH 8.5. Alexa Fluor 647 (purchased as a succinimidyl ester from Molecular Probes) (100 μg) was dissolved in 7 μl of DMSO. The oligonucleotide and dye were mixed and agitated overnight in the dark. After ethanol precipitation, the fluorescently labeled oligonucleotide was purified away from free dye and unlabeled oligonucleotide by chromatography on a C18 column (Supelco, 25 cm × 4.6 mm) using 0.1 M triethylammonium acetate and an acetonitrile gradient (5–20% (v/v)). The purified, labeled template strand was annealed to the nontemplate strand oligonucleotide. The F-10rG oligonucleotide was prepared in a similar manner except Alexa Fluor 555 (Molecular Probes) was used.

***In vitro* transcription reactions in nuclear extracts.** Transcription reactions were carried out as described<sup>31</sup> using p(GAL4)<sub>5</sub>-E1b-CAT (100 ng), which contains five GAL4-binding sites and the adenovirus E1b TATA box upstream of the gene encoding CAT<sup>40</sup>. Nuclear extracts (10 μg total protein) from Jurkat cells were added to reactions. Nuclear extracts<sup>41</sup> and GAL4-p53 (ref. 42) were prepared as described. When included, B2 RNA and control RNAs were added to reactions before the addition of nuclear extract.

***In vitro* transcription and abortive initiation reactions in a purified transcription system.** Human TBP, TFIIB, and TFIIF and core Pol II were prepared as described<sup>30</sup>. The DNA template was negatively supercoiled plasmid DNA containing the AdMLP core promoter (-53 to +10) fused to a 380-bp G-less cassette<sup>37</sup>. Transcription reactions were done as described<sup>30</sup> with the following modifications. TFIIB, TFIIF, Pol II and B2 RNA (when present) were combined in one tube. AdMLP template DNA and TBP were combined in a second tube. The two tubes were then transferred to 30 °C and incubated for 3 min, after which the contents of the two tubes were mixed and incubated an additional 20 min at 30 °C before nucleotide triphosphates were added and transcription was allowed to occur for 20 min. Abortive initiation reactions were assembled as described above and products were treated as described<sup>5</sup>.

**Electrophoretic mobility shift assays.** EMSAs were carried out in 20-μl reactions under conditions similar to those of the *in vitro* transcription assays. Binding occurred at 30 °C for 10–20 min. Ficoll (2 μl of 20% (w/v)) was added to each reaction and complexes were resolved on native polyacrylamide gels (4% (w/v), 35.5:1, 5% (v/v) glycerol, 5 mM magnesium acetate and 0.5× TBE) at 150 V for 3 h using borosilicate gel plates (CBS Scientific).

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## COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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