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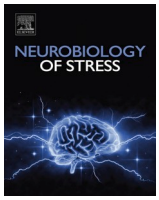

B2 SINE RNA as a novel regulator of glucocorticoid receptor transcriptional activity

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ABSTRACT

Glucocorticoids are a key component to the cellular response to stress. Glucocorticoids act via glucocorticoid receptors found ubiquitously in the brain and body. Glucocorticoid receptors can bind to response elements throughout the genome to elicit changes in transcription, an adaptation observed at the cellular level. Yet, the transcriptional changes as a consequence of glucocorticoid receptor activation are variable across brain regions, stress conditions and recurrent bouts of glucocorticoid exposure. Here we describe a non-coding RNA, B2 SINE, which is regulated by glucocorticoids and can in turn regulate glucocorticoid receptor transcriptional activity. We show that activated glucocorticoid receptors interact directly with B2 SINE RNA via a decoy response element contained within the transcript sequence and alter receptor binding to response elements in the genome and, subsequently, changes in loci expression.

1. Introduction

Glucocorticoids are an indispensable component of the physiological stress response. In the brain, glucocorticoids are one of the primary cellular mediators regulating the organismal response and adaptation to stress. Glucocorticoids act on receptors found ubiquitously in the brain and body (Sánchez et al., 2000). Canonically, the glucocorticoid receptor (GR) functions as a ligand-dependent transcription factor, upon activation translocating to the nucleus and binding to response elements found in the genome to regulate transcription (Gray et al., 2017). Glucocorticoid response elements (GREs) comprise a large fraction of glucocorticoid receptor binding sites and are characterized by palindromic sequence not atypical of steroid receptor binding motifs (Polman et al., 2012, 2013). Binding to GREs is a primary way by which the GR and in turn glucocorticoids and stress regulates transcription in stress sensitive regions of the brain. Several mechanisms have been proposed to explain regional and context specific differences in transcription elicited by stress and glucocorticoids including local changes in chromatin, GR-cofactor recruitment and varying levels of receptor abundance (Bartlett et al., 2019). It has long been noted that many steroid receptor binding sites derive from transposon insertions, particularly short interspersed nuclear elements (SINE), and the role of transposons in both genome and epigenome evolution is a significant one (Hunter, 2020; Lapp and Hunter, 2016). Further, Our own work has shown that stress and glucocorticoids regulate the expression of at least some of these elements (Hunter et al., 2012; Bartlett et al., 2021a).

More recently, a novel mechanism for GR-induced transcriptional regulation has been discovered at the RNA level. Kino et al. first described the functions of the non-coding RNA GAS5, a transcript produced under growth arrest and starvation conditions at the cellular level (Kino et al., 2010). The GAS5 RNA contains a so called “pseudo-GRE” that is a motif resembling the GRE sequence found across the genome. Upon activation, GR is able to interact directly with GAS5 RNA at the pseudo-GRE site contained within the transcript. The consequences of this interaction are the sequestration of the receptor, activated GR binds preferentially to the GAS5 transcript and not to GREs within the genome attenuating GR-induced transcription of the locus (Kino et al., 2010), thus acting as an antagonist for GRs transcriptional activity. Using C6 glioblastoma cells, which express high levels of GR and have been used previously in our lab to model transcriptional interactions between transposable elements and GR (Beaumont, 1985) (Bartlett et al., 2021a), we sought to examine the interactions between B2 SINE RNA and the GR. We have found that B2 SINE RNA contains a similar pseudo-GRE motif of which activated GR interacts with directly and herein describe the consequences of B2 SINE RNA on GR-induced transcription, which display a higher degree of complexity than the simple antagonism

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observed with GAS5.

2. Materials and methods

2.1. Cell culture and nucleic acid transfections

C6 cells (ATCC) were cultured in DMEM supplemented with 10% fetal bovine serum (ThermoScientific). Cells were otherwise cultured and passaged according to standard protocols (ATCC) (Bartlett et al., 2021a) (Benda et al., 1968). Cells were maintained in log-phase growth and switched to serum deprived DMEM media 24 h prior to experiments. On the day of an experiment, cells were transfected using lipofectamine3000 (ThermoScientific) with 0.5 pmol of RNA for 3 h. Then dexamethasone to a final concentration of 10 nM or DMSO was added to media. After 1.5 h, a subset of cells were fixed by addition of formalin to a final concentration of 1% for 10 min. Cross-linking of nucleic acids to associated proteins was then quenched by the addition of 1 mL of 2M glycine for 5 min. Fixed cells were then pelleted and frozen for either RNA IP or chromatin IP. After 3 h, the remaining cells were washed with PBS twice and RNA was extracted using Trizol. All measurements performed contained at least 3 replicates.

2.2. RNA in vitro transcription

For RNA in vitro transcription we used the AmpliScribe T7 High Yield Transcription kit (Lucigen) applying a 6hr incubation at 42degC and using a template to the following B2 RNA sequence: 5′- TAATACGACTCACTATAGGGGCTGGAGAGATGGCTCAGCGGTTAAGAGCACCCGACTGCTCTTCCAGAGGTCATGAGTTCAATTCCCAGCAACCACATGGTGGCTCACAACCATCTGTAAAGAGATCTGATGCCCTCTTCTGGTGTATCTGAAGACAGCTACAGTGTACTTATATATAATAAATAAATAAATCTTTAAAAAAAAAA-3′, $\Delta_{30\text{-}45del}$B2 RNA sequence: 5′-TAATACGACTCACTATAGGGGCTGGAGAGATGGCTCAGCGGTTAAGTCCAGAGGTCATGAGTTCAATTCCCAGCAACCACATGGTGGCTCACAACCATCTGTAAAGAGATCTGATGCCCTCTTCTGGTGTATCTGAAGACAGCTACAGTGTACTTATATATAATAAATAAATAAATCTTTAAAAAAAAAA-3′, scrB2 RNA sequence: 5′-TAATACGACTCACTATACCTAACAGGGATCGTGAGGCTTGCGTGTTAAGACCAGCACACCTGTAAGTTAGGTGTACTTTACCGTAACTGAATAATGTCCCTATTATGAAAACCATTGTTCCACCTGGGCCTGGAAAAATGCTGGATCGATCCAATAAATAACGAAAGTCATACCTTATTCATCAAAGGAAGGCACGAGTACTAGAA-3’. DNA template for the transcription was a PCR amplified product derived from a gBlock (IDT) using as a forward primer the T7: 5′-TAATACGACTCACTATA-3′ and a reverse primer for B2 RNA and $\Delta_{35\text{-}45del}$B2 RNA: 5′-TTTTTTTTTTAAAGATTTATTTATTTATTATATAAGTACACA-3′ and a reverse primer for scrB2 RNA: 5′- TTCTAGTACTCGTGCCTTCCTTTG-3’. RNA transcripts were purified using the Qiagen miRNA kit and concentration determined using a Nanodrop2000 (Thermofisher).

2.3. RNA immunoprecipitations (RIP)

Fixed cells were lysed and subjected to immunoprecipitation according the manufacturer’s protocol (Magna RIP kit, EMD Millipore). Inputs were refrigerated and lysates were precipitated with 10ug of G5-GR antibody (SantaCruz) at 4degC with rotation overnight. After washes, both input and IP samples were digested with proteinase K for 2 h at 65degC and then incubated for 10 min at 95degC to inactivate proteinase K (ThermoScientific). RNA was purified using Qiagen miRNA extraction columns (Qiagen). RNA was eluted in RNAse free water prior to cDNA synthesis (Quantitect Reverse Transcription kit, Qiagen). Genomic (gDNA) DNA was digested prior to cDNA synthesis to remove the possibility of genomic contamination. RNA binding to GR protein was assessed following qPCR using the percent input method. For statistical analysis, statistical significance was determined using a two-tailed student’s t-test. Primer sequences can be found in Supplemental Table 1.

2.4. Chromatin immunoprecipitation

Fixed cells were lysed and subjected to immunoprecipitation as previously described (Bartlett et al., 2021a; Bartlett and Hunter, 2019). In short, lysates were sheared with the Covaris M220 using the Covaris truChIP kit (Covaris) to produce achieve a chromatin smear between 200 and 600bp. Inputs were refrigerated and lysates precipitated with 10ug of G5-GR antibody (SantaCruz) at 4degC with rotation overnight. After washes, both input and IP samples were digested with proteinase K overnight at 65degC. The next day samples were incubated at 95degC to inactivate proteinase K. Samples were then purified using phenol-chloroform extraction followed by ethanol precipitation. DNA binding was assessed following qPCR using the percent input method. Primer sequences can be found in Supplemental Table 1.

2.5. RNA expression

Residual gDNA was removed via DNase treatment from extracted RNA prior to cDNA synthesis. cDNA was synthesized using random hexamers according the manufacturer’s protocol (Quantitect Reverse Transcription kit, Qiagen). Using a AppliedBiosystems StepOnePlus system, relative quantification of RT-qPCR data was done using the deltaCT method using rat 7SK for housekeeping gene expression. 7SK was chosen based on previous work in the lab to determine which housekeeping gene is an appropriate control for RNA polIII driven transcripts like B2, which itself interacts directly with RNA polII, confounding most standard housekeeping primers (Bartlett and Hunter, unpublished data). Previous work has also shown that commonly used housekeeping genes such as b-actin and GAPDH show substantial variation between biological sexes (Bartlett, Lapp and Hunter in preparation). Sybr green master mix (ThermoScientific) was used as per manufacturers instructions. Primers were either those used in previous publications (Hunter et al., 2012; Bartlett et al., 2021a), or designed using IDT-DNAs software (Integrated DNA Technologies). Primers were also synthesized by IDT. Primer sequences can be found in Supplemental Table 1.

2.6. Quantification and statistical analysis

For RIP-qPCR statistical analysis, statistical significance was determined by $p < 0.05$ using a two-tailed student’s t-test, or. For statistical analysis of ChIP-qPCR and RT-qPCR, data is judged to be statistically significant when $p < 0.05$ by Tukey’s test following significant main effect(s) or interaction term in a two-way ANOVA.

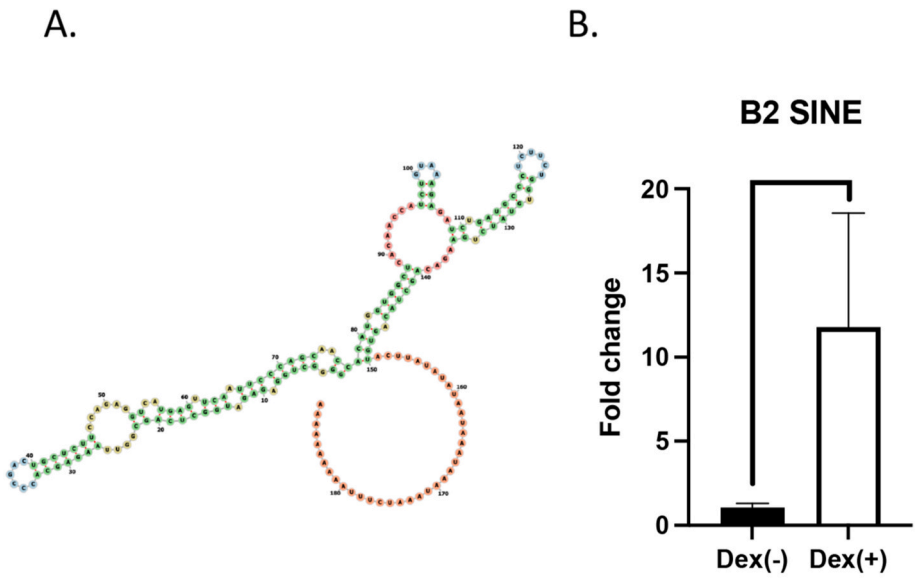
3. Results

3.1. B2 SINE RNA is induced by GR-activation and contains a pseudo-GRE

The secondary structure of B2 SINE RNA as predicted by RNAfold is shown (Fig. 1A) (Lorenz et al., 2011). In alignment with our previous findings, we observed that B2 SINE expression is induced by activation of the glucocorticoid receptor in C6 cells ((Bartlett et al., 2021a), Fig. 1B). Using FIMO (Find Individual Motif Occurrences), a motif detection algorithm, we predicted that the consensus sequence for B2 SINE RNA contains a putative pseudo-GRE at the 30-45bp region (Fig. 1C) (Grant et al., 2011; Bailey et al., 2009).

3.2. Activated GR binds to B2 SINE RNA

The overview of our RNA-immunoprecipitation experiment is briefly



C.

Motif ID	Alt ID	Sequence Name	Strand	Start	End	p-value	q-value	Matched Sequence
1	NAGAACAGNCTGTTCT	B2_SINE	+	30	45	0.000864	0.15	AGCACCCGACUGCUCU
1	NAGAACAGNCTGTTCT	B2_SINE	-	31	46	0.000864	0.15	AAGAGCAGTCGGGTGC

Fig. 1. The secondary structure of B2 SINE RNA generated with RNAfold is shown (Fig. 1A). B2 SINE RNA expression is increased in C6 cells following 1hr of dexamethasone treatment demonstrating B2 is hughly regulated by glucocorticoids(*$p < 0.05$)(Fig. 1B). B2 SINE RNA contains a putative GRE identified with motif analysis using FIMO from the MEME suite (Fig. 1C).

described (Fig. 2A). In short, we generated full length B2 SINE transcripts and B2 SINE transcripts lacking the predicted pseudo-GRE, that is a B2 SINE with a deletion of the 30-45bp region, using an in vitro transcription kit. These transcripts were introduced to C6 cells transiently and cells were stimulated with dexamethasone or vehicle and

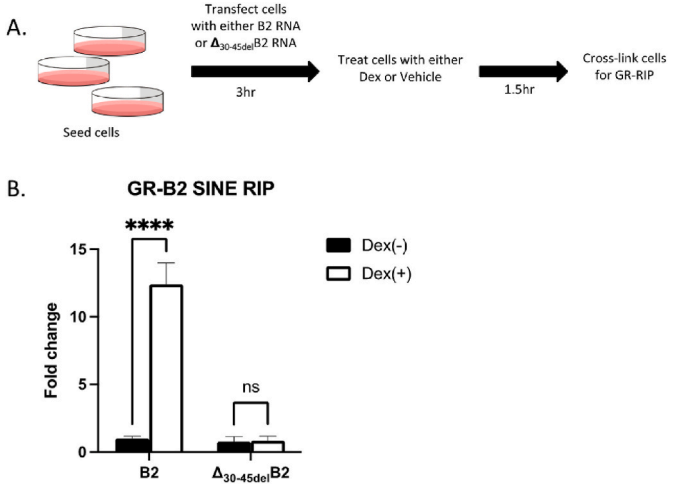


Fig. 2. Experimental overview for B2 SINE RNA-glucocorticoid receptor immunoprecipitation is shown (Fig. 2A). Briefly, cells were transfected with either B2 RNA or B2 RNA (B2) with the putative GRE deleted (Δ30-45del). Three hours after transfection they were treated with either vehicle or dexamethasone (DEX), then, 1.5 h after DEX treatment all four groups were treated to cross-link RNA and associated proteins and chromatin harvested for GR RNA-immunoprecipitation to demonstrate B2 binding to the GR is specific and dependent on the presence of the putative GRE. Following DEX administration, glucocorticoid receptor association with the full length B2 SINE transcript is observed, but no significant association with the Δ30-45del RNA was observed (****$p < 0.001$; Fig. 2B).

nucleic acids subsequently crossed-linked to associated proteins with formaldehyde for RNA-IP. We observed that upon activation GR binds to the full-length B2 SINE transcript but does not bind to the B2 SINE transcript featuring the deletion of the predicted pseudo-GRE (Fig. 2B).

3.3. *B2 SINE RNA alters GR-binding to GREs and GR-induced transcription*

We developed an experiment to test the idea that B2 SINE RNA interactions with the GR has functional consequence for GR activity (Fig. 3A). We transiently transfected cells with either full length B2 SINE RNA or RNA generated from the scrambled B2 SINE sequence. Following transfection, we stimulated cells with either dexamethasone or vehicle and either cross-linked for chromatin-immunoprecipitation or harvested for RNA expression. We then examined RNA expression and GR binding at 4 genes (Per1, Mt2a, Sgk1 and Gilz) known to be reliable GR targets across a variety of tissues, which is an important consideration given than most GREs are not conserved across tissues and cell types (Polman et al., 2013; Bartlett et al., 2019, 2021a). We found that unlike GAS5, B2 SINE elicits a wide variety of changes in GR-binding and GR-induced expression across the loci we evaluated (Fig. 3B–I). For Per1, we found that completely blocked Per1 expression (Fig. 3B, $F(1,8) = 20.06$, $n = 3$, $p < 0.05$; main effect of B2, $p < 0.0005$; and Dex $p < 0.002$, interaction $p < 0.02$) while B2 SINE RNA enhanced GR-binding to the Per1 GRE (Fig. 3F, $F(1,8) = 5.42$, $n = 3$, $p < 0.05$). For Mt2a, we found that B2 SINE RNA did not alter B2 SINE RNA alter Mt2a expression (Fig. 3C) nor did it affect GR-binding to the Mt2a GRE (Fig. 3G). For Sgk1, we found that B2 did increase Sgk1 expression (Fig. 3D, $F(1,8) = 82.24$, $n = 3$, $p < 0.0001$) but did not enhance GR-binding to the Sgk1 GRE (Fig. 3H, $n = 3$). For Gilz, we found that B2 SINE RNA increased Gilz expression (Fig. 3D, $n = 3$,main effect of Dex $F(1,8) = 26.3$, $p < 0.001$; B2 $F91,8) = 6.79$, $p < 0.03$; interaction $F(1,8) = 10.1$, $p < 0.02$) and enhanced GR-binding to the Gilz GRE (Fig. 3I, $n = 3$, main effect of Dex $F(1,8) = 20.46$, $p < 0.002$; B2 $F(1,8) = 33.26$, $p < 0.0004$; interaction $F(1,8) = 20.06$, $p < 0.0025$).

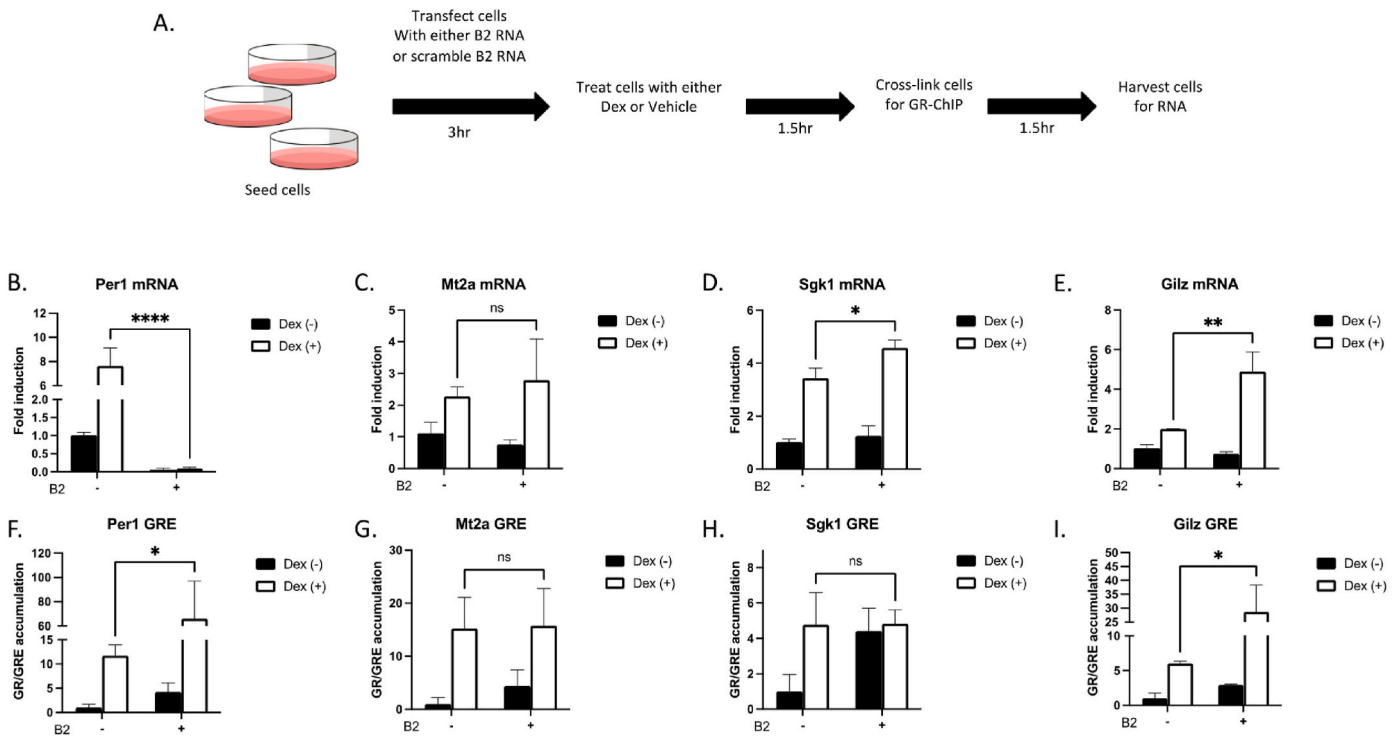


Fig. 3. Experimental overview for elucidation B2 SINE RNA effects on glucocorticoid receptor binding and induced transcription is shown (Fig. 3A). In brief, the cells were treated with either B2 RNA or a scrambled version of it, followed with DEX or vehicle treatment for 1.5 h. After 1.5 h, half the cells were harvested for GR-ChIP. After 1.5 h more (total of 3 h) the remaining cells were harvested and processed for RT-PCR to demonstrate the transcriptional effects of B2 on GR at the selected target genes. These genes (Per-1, Mt2a, Sgk1 and Gliz) were chosen as they contain strong GREs known to be active in C6 cells and a variety of tissues. Glucocorticoid receptor induced transcription for several responsive genes following transfection and dexamethasone stimulation is shown (Fig. 3B–E). Glucocorticoid receptor binding to several glucocorticoid receptor response elements following transfection and dexamethasone stimulation is shown (Fig. 3F–I). (ns: non-significant, *$p < 0.05$, **$p < 0.01$, ****$p < 0.001$).

4. Conclusions

We have found that B2 SINE RNA is a novel regulator of GR transcriptional activity. To our knowledge, this is only the second transcript that has been identified to act in a binding site "decoy" fashion for the GR. Unlike GAS5, which has been described as a blanket ribo-repressor sequestering activated GR, B2 SINE RNA does not appear to function in such a linear fashion. We have observed that B2 SINE RNA has widely divergent effects on GR-binding and GR-induced transcription. These include having a strong repressive effect on Per1 expression, while simultaneously increasing GR binding to the Per1 GRE, *increasing* expression of Sgk1 and Gilz RNA, and enhancing dexamethasone induced binding of GR to the latter promoter, while failing to do so at the latter. Clearly, B2 is in a more complex class of transcriptional regulator than that of a simple repressor like GAS5.

We have repeatedly observed that B2 SINE expression itself is sensitive to glucocorticoids. We have also found evidence that B2 SINE expression is tightly regulated at the chromatin level in both cultures and in the rodent hippocampus, a target of stress and glucocorticoids. These findings are seemingly in line with other groups reporting that B2 SINE transcripts are elevated following acute stress (Hunter et al., 2012; Bartlett et al., 2021a). For instance, B2 SINE expression is increased in the dentate gyrus is cells that recently fired following exposure to a novel environment (Lacar et al., 2016). The same group has confirmed these findings and added that in this context the time scale for B2 SINE expression seems to mirror that of immediate early genes and that B2 SINE expression is "bona fide": that is to say it is driven by internal RNA pol III elements not a spurious artifact of read through transcription (Linker et al., 2020).

B2 SINE RNA has been widely described in the context of heat shock as a master transcriptional regulator. B2 SINE RNA is highly expressed during heat shock while so called "housekeeping genes" are heavily down-regulated (Espinoza et al., 2004). B2 SINE facilitates this rapid transcriptional response by interacting directly with RNA pol II at housekeeping gene transcription start sites leading to polymerase pausing (Allen et al., 2004). In sum, the variable effects of B2 SINE RNA on GR mediated transcription may be due to interactions with transcriptional machinery beyond their interaction directly with GRs (see Fig. 4 for a schema of B2 RNA interactions with GR). Others have shown that B2 SINE itself functions as a self-cleaving ribozyme suggesting that the transcript itself has an intrinsic built-in negative feedback loop (Zovoilis et al., 2016; Hernandez et al., 2020). This may indicate that glucocorticoid-induced expression of B2 SINE represents an additional epigenetic transcriptional feedback loop contributing dynamically and transiently to subsequent glucocorticoid or stress exposure.

We and others have long argued that repetitive elements like B2 SINE represent far more than genomic fossils (Bartlett and Hunter, 2018; Bartlett et al., 2021b; Fedoroff, 2012; Chuong et al., 1038; Frank and Feschotte, 2017). The evolution of transcription factor binding sites, like the glucocorticoid receptor, is directly tied to that of repetitive transposable elements (Feschotte, 2008; Feschotte et al., 2002; Lynch et al., 2015). While the focus of these analyses has been to link genomic elements as vectors to transcription factor binding site proliferation, the mere fact these elements contain binding site motifs suggests the capacity for nascent transcripts from these elements to interact with transcriptional machinery. In an endocrinological context, Alu SINE RNA, which are the primate analog of B2 SINEs, contains a pseudo-steroid receptor motif and are necessary for progesterone receptor binding to progesterone receptor half-sites found within the genome (Jacobsen et al., 2009). This is all to say that the function of transposon-derived RNAs in interacting with transcriptional regulators has yet to be fully realized, and are likely to emerge as significant

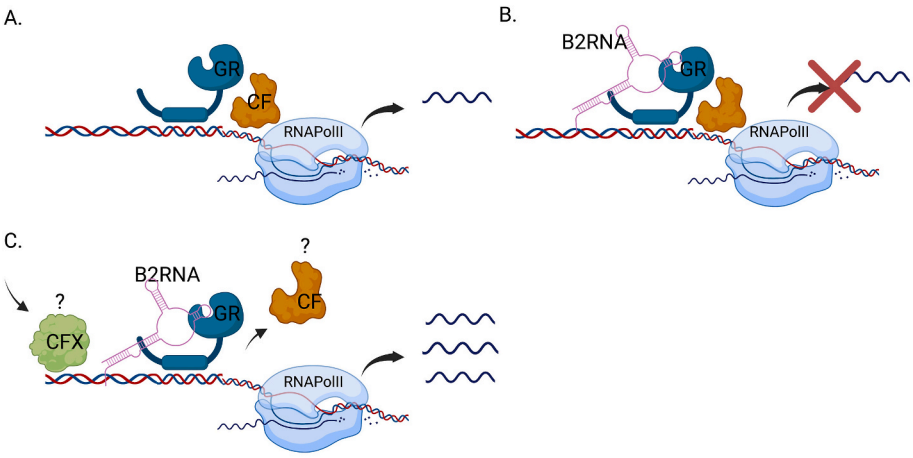


Fig. 4. Potential schema of B2 RNA interactions with GR. Panel A. represents the activity of GR in the absence of interactions with B2 RNA. GR with its co-factors (CF) binds to GREs on target genes and induces RNA polymerase II (RNAPolII) dependent transcription of RNA. At some GREs (4B.), such as Per1, B2 RNA interaction with GR allows GR to bind to its target GRE, but transcription is blocked. At other targets (4C.) B2 appears enhance transcription (e.g. Gilz and Sgk1) potentially via recruitment of other cofactors (CFX), conformational change in the GR itself, or dissociation from normally co-recruited cofactors (CF). Figure created with BioRender.com.

regulators of steroid dependant gene transcription in a number of contexts.

CRediT authorship contribution statement

Andrew A. Bartlett: designed and performed experiments, Formal analysis, and wrote this MS. **Guia Guffanti:** designed experiments and edited this MS, Writing – review & editing. **Richard G. Hunter:** designed experiments, wrote and revised this MS, Writing – review & editing.

Declaration of competing interest

No authors have any conflict of interest to disclose, and there has been no financial support that might influence this work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ynstr.2023.100522.

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