A reagentless biosensor for mRNA: a new tool to study transcription

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Key Words: Biosensor, Transcription, SSB, Myosin, RNA Polymerase, Gene Expression, mRNA

ABSTRACT

Gene expression, catalysed by RNA polymerases, is one of the most fundamental processes in living cells. Yet, the means to study their activity are currently limited. The majority of methods to quantify mRNA are based upon initial purification of the nucleic acid. This leads to experimental inaccuracies and loss of product. Here we describe the use of a reagentless mRNA fluorescent biosensor based upon the single stranded binding (SSB) protein. In this study, SSB showed similar binding properties to mRNA, to that of its native substrate, ssDNA. Furthermore, MDCC-SSB gave the same fluorescence response with both ssDNA and ssRNA, in a concentration dependent manner. When directly compared to RT-qPCR, we found the biosensor to be more reproducible with no product lost through purification. Therefore, the MDCC-SSB can be used as a comparative measurement of mRNA concentrations following *in vitro* transcription.

INTRODUCTION

The information to develop and sustain life is encoded within DNA. RNA polymerase II (RNAPII) facilitate the distribution of this information through the transcription of mRNA. The complex activity of RNAPII is reflected in its large size with a typical eukaryotic RNAPII comprising of 10-12 subunits, RPB1-12, these provide stability, regulation and the active site for the complex (1). Transcription is a multi-stage process; the formation of the preinitiation complex, the initiation of transcription, elongation of the mRNA transcript and finally termination. All of which require multiple accessory proteins such as the TFII family (2), CDK family (3-5) and the myosin motor proteins (6,7). These mechanisms have been very well characterised yet still protein-protein interactions occur that have undefined roles in this procedure.

In vitro transcription assays have allowed a biochemical characterisation of these complex multi-protein machines. Classically to measure the transcriptional activity of RNAPII *in vitro* studies have detected the presence and/or quantified the mRNA transcripts produced. This has revealed previously unknown information about the re-initiation (8) and termination steps (9), enhancement of transcription through molecular motors (6), and has provided a method to study transcription at a single molecule level (10).

In high yielding transcription assays such as the T7 polymerase, the presence of mRNA can be shown by gel electrophoresis. If the analysis requires quantification of RNA then spectroscopy is able to measure the nucleic acid concentration. However, the sample will need to be purified to remove both proteins and DNA from the solution, which leads to both a loss of total RNA yield and increased experimental error. Furthermore, both approaches require a high yield of mRNA and therefore are not suitable for eukaryotic transcription assays.

The use of radioactively tagged nucleotides incorporated into the transcript are common in eukaryotic *in vitro* transcription (11,12). Along with the requirement to purify the product there are additional safety factors and costs involved. Safer alternatives do include RNA specific dyes such as the Quant-iT RiboGreen reagent (13). Reverse Transcription quantitative PCR (RT-qPCR) is a very sensitive method to quantify transcripts (14). However, contaminating DNA from the *in vitro* transcription assay can lead to large errors in the quantification. This is also a multistep process which can lead to an increase in experimental error.

It is clear there is a need for a low cost, sensitive, easy to use reagent which can be added to directly compare in vitro transcription reactions without additional purification steps. Reagentless, fluorescence biosensors have been successfully employed in such roles in various biochemical assays (15-18).

A fluorescently labelled single stranded binding protein (SSB) from *Escherichia coli* has been successfully used as an ssDNA biosensor for monitoring helicase activity (19). *E. coli* SSB is a well characterised protein that is homo-tetrameric containing 4 OB-fold domains (20). The formation and nucleic acid binding properties of SSB vary depending on ionic conditions. It has two main binding modes, known as $(SSB)_{65}$ and $(SSB)_{35}$, which have been well characterised by Lohman *et al* (21). In high salt concentrations above 200mM NaCl SSB binds to ssDNA in the form of $(SSB)_{65}$. This case is also true when the nucleotide length is approximately 65 nucleotides long, causing the ssDNA to wrap completely around the protein. $(SSB)_{35}$ however is a different bonding mode where the ssDNA wraps only around half of the protein. This binding mode occurs in low salt concentrations less than 200mM NaCl and when the nucleotide length is around 35 bases (22). In the case of $(SSB)_{35}$ it is possible for two ssDNA molecules that are 35 nucleotides long to bind to one SSB tetramer (23). SSB has shown to have tight binding to ssDNA in both binding modes (24) and, whilst not as tightly as with ssDNA, it is also reported that SSB can bind to RNA in reference to its own mRNA (25).

Here we aim to extend the application of the SSB reagentless biosensor to show that along with ssDNA, it can also be used to measure mRNA. This will provide a low cost, rapid and sensitive alternative for directly measuring mRNA with minimal substrate isolation. To display the functionality and versatility of the biosensor we performed two example assays. Firstly we directly compare the biosensor to RT-qPCR methodology for determination of transcription products. Lastly, we use the biosensor to determine whether myosin motor activity is required during gene expression in complex with RNAPII.

MATERIALS AND METHODS

Reagents

Unless stated otherwise all reagents were purchased from Sigma Aldrich (Dorset, UK). Oligonucleotides are listed in the supplementary materials.

Protein expression and purification

SSB(G26C) was expressed from pET151 in *E.coli* BL21 DE3 cells (Invitrogen). The cells were grown to mid-log phase at 37 °C in LB media supplemented 100 µg. mL^{-1} ampicillin. IPTG was added to a final concentration of 1 mM and cells were grown overnight at 18 °C. The cells were harvested by centrifugation and re-suspended in 50 mM Tris·HCl (pH 7.5), 200 mM NaCl, 1 mM DTT, 20% Sucrose and 40 mM imidazole with 1 mM PMSF.

For purification, the cells were lyzed by sonication and purified from the soluble fraction by affinity chromatography (HisTrap FF, GE Healthcare). The pooled protein was further purified through a Superdex 200 16/600 column (GE Healthcare) equilibrated with 50 mM Tris·HCl (pH 7.5), 1 mM DTT and 150 mM NaCl. The purest fractions were concentrated by centrifugation and stored at -80 °C.

Labelling with MDCC

Labelling was adapted from Dillingham *et al* (19). 3mg of SSB was incubated with 1M DTT for 20 minutes at room temperature. DTT was removed using a PD10 column (GE Healthcare, Little Chalfont, UK) was equilibrated in labelling buffer (20mM Tris.HCl pH 7.5, 1mM EDTA, 500mM NaCl and 20% glycerol). After DTT incubation the solution was loaded onto the column and the column was eluted with the same buffer. 2-fold molar excess of MDCC was added and incubated for 4 hours at room temperature, with end over end mixing, while protected from light. Excess dye was removed using a PD10 column equilibrated in labelling buffer.

The concentration of SSB was taken using A_{280} with extinction coefficient of ε = 28,500 cm^{-1} M^{-1} per monomer and MDCC concentration was determined using A_{430} with extinction coefficient of ε = 44,800 cm^{-1} M^{-1}. Labelling efficiency was calculated using equation 1.

Equation (1) $$Labelling\ efficiency = \frac{A_x}{\varepsilon} \times \frac{MW\ of\ protein}{mg\ protein/mL} = \frac{moles\ of\ dye}{moles\ of\ protein}$$

Where A_x is the absorbance value of the dye at the absorption maximum wavelength. ε is the molar extinction coefficient of the dye at absorption maximum wavelength.

Electrophoretic Mobility Shift Assay (EMSA)

50nM SSB was incubated with 250nM $ssDNA_{70}$ or $ssRNA_{70}$ for 20 minutes at room temperature in 50mM Tris.HCl pH 7.5, 100mM NaCl, 3mM $MgCl_2$. Samples were loaded onto an acrylamide gel (12% acrylamide, Tris. Boric acid pH 7.5, 2.5mM Mg) (TBM) and ran in TBM buffer. SYBR®Gold (Invitrogen, Rochford, UK) stained the nucleic acids following the manufacturer's instructions.

Tryptophan fluorescence titration

$ssDNA_{70}$, or $ssRNA_{70}$, were titrated into 200nM SSB at 25˚C in 50mM Tris.HCl pH 7.5, 200mM NaCl and 3mM $MgCl_2$. Tryptophan fluorescence was measured using a Cary Eclipse Fluorescence Spectrophotometer (Agilent, Edinburgh, UK) at excitation 285nm and emission at 325nm. To calculate fluorescence quenched (%) used equation 2.

Equation (2)
$$Fluorescence\ quenched\ (\%) = \frac{F_i \times DF_i}{F_0} \times 100$$

Where F_0 is initial fluorescence intensity, F_i is the intensity after titration and DF_i is the dilution factor from the titration. The titration curves were fitted to a 1 site plus background equation:

Equation (3)
$$[Quenched\ \%] = \frac{Amplitude.[Nucleic\ Acid]}{K_d + [Nucleic\ Acid]} + Background$$

Titrations of Oligonucleotides to MDCC-SSB

All reactions were performed at 25 °C in a buffer containing 50mM Tris.HCl pH 7.5, 3mM $MgCl_2$ and 100mM, or 200mM NaCl, as defined with 50nM MDCC-SSB in a final volume of 100 μL. Measurements were performed using a ClarioStar Plate Reader (BMG Labtech). Fluorescence excitation was measured from 400.0 to 440.0nm, with a step-width of 1nm and emission at 770nm. Fluorescence emission was measured from 455-550nm, with a step width of 1nm and a fixed excitation of 430nm.The

fluorescence intensity was then taken at 471nm. Fluorescence change is presented as a ratio using equation 3.

Equation (4) $$Fluorescence\ change = \frac{F_i \times DF_i}{F_0}$$

Where F_0 is initial fluorescence intensity at 471nm and F_i is the intensity at 471nm after titration. DF_i is the dilution factor for that titration. The curves were fitted to Equation 3.

Stopped flow measurements

A HiTech SF61DX2 apparatus (TgK Scientific Ltd, Bradford-on-Avon, UK) with a mercury-xenon light source and HiTech Kinetic Studio 2 software was used. Excitation was at 435nm with emission through a 455nm cut-off filter (Schott Glass). In all experiments, the quoted concentrations are those in the mixing chamber, except when stated. All experiments were performed at 25°C in 50 mM Tris-HCl, 150 mM NaCl, 1 mM DTT, 3 mM $MgCl_2$, 10% glycerol and 5μM BSA. The dead time of the stopped-flow instrument was ~2 ms: during this initial time no change in fluorescence can be observed.

In vitro Transcription and RT-qPCR

T7 in vitro transcription was performed using the HiScribe™ T7 High Yield RNA Synthesis Kit (New England Biolabs, Hitchin, UK) with pET28-RecD2 as a template following manufacturer's instructions. The template was digested to yield the fragments as stated in the text. RNA polymerase II *in vitro* transcription was performed using the HeLaScribe kit (Promega). The DNA template was the pEGFP-C3 linearized plasmid containing the CMV promoter which would generate a 130 base run-off transcript. Reactions were performed according to the manufacturer's instructions. The reactions were performed for 60 min at 25°C. Reactions were also performed following pre-clearance of myosin VI by anti-myosin VI (Sigma HPA035483-100UL). Protein G Dynabeads (Invitrogen) were prepared according to manufacturer's instructions before being loaded with 4 μg antibody. Samples were incubated for 30 min on ice and beads were extracted immediately before performing the transcription

reaction. Where required, 25 μM Triiodophenol (TIP, Sigma) was added to the reaction mixture.

RNA was then purified using RNeasy® kit (Qiagen, Manchester, UK), or Gene Jet RNA purification kit (Thermo scientific), according to manufacturer's protocol. RT-qPCR was performed with one-step QuantiFast SYBR Green qPCR kit (Qiagen).

Cell Culture and Gene expression analysis

MCF7 cells were cultured at 37ºC and 5% CO_2, in Gibco MEM Alpha medium with GlutaMAX (no nucleosides), supplemented with 10% heat-inactivated Fetal Bovine Serum (Gibco), 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco). For MVI inhibition experiments, MCF7 monolayers were seeded to 30-50% confluency and then subjected to 25 μM TIP for 4 hours. Cells were then harvested for RT-qPCR analysis, as described above.

RESULTS

As previously mentioned, SSB has already been used as a biosensor to report on the unwinding of helicases. By carrying on the previous work done by Dillingham *et al* (19), this study will use the same point mutated SSB in which a glycine residue is replaced by a cysteine residue at the 26th position. This mutation does not affect the binding of SSB or the formation of its tetrameric shape (19). When SSB is referred to in this study, it is assumed the reference is to the G26C mutant.

SSB is an mRNA binding protein

Before developing the biosensor for use with mRNA, we first determined if SSB can bind to mRNA. SSB has been previously reported to bind mRNA (25) however, has not been discussed further. To this end, we performed qualitative electrophoretic mobility shift assay (EMSA) with $ssDNA_{70}$ and $ssRNA_{70}$. Here we show that SSB bound to $ssRNA_{70}$ in a manner indistinguishable from that of ssDNA (Figure 1A).

While the EMSA showed similar binding properties for both substrates, to confirm SSB does bind to mRNA and to define the kinetic parameters, tryptophan fluorescence quenching was used. Tryptophan residue 54 was found to be directly involved in binding to ssDNA (26) resulting in a fluorescence quenching. Titration of $ssDNA_{70}$ yielded 50% quenching and the K_d was limited by the concentration of SSB

(<200 nM). A breakpoint in the linear phase was observed once the stoichiometric complex of 1 tetramer to ssDNA was reached. The $ssRNA_{70}$ yielded a similar quenching response, albeit the binding affinity is weaker (K_d ~200 nM) (Figure 1B). The weaker binding may relate to the previous findings whereby SSB has preference for ssDNA over ssRNA, although binding is still possible. The consistent quenching response is indicative of a single binding site for ssDNA and ssRNA, as would be expected from the structure.

Overall we can conclude that ssRNA is a viable substrate for SSB and therefore SSB is a suitable scaffold for an mRNA biosensor.

MDCC-SSB, an mRNA biosensor.

To generate the biosensor, we first needed to select a fluorophore. We opted to use the commercially available environmentally sensitive fluorophore, MDCC. This fluorophore has similar properties to the previously published ssDNA biosensor with IDCC. Previously when IDCC was coupled to SSB through the G26C mutation a large fluorescence enhancement of 5.3 fold when SSB bound to dT_{20} occurred. G26C is positioned on a flexible loop in the apo-structure but the freedom is reduced in the DNA bound form. The slight change in conformation and the DNA wrapping around the surface are thought to lead to the fluorescence enhancement. Here the MDCC-SSB biosensor responded to the addition of $ssRNA_{70}$ and $ssDNA_{70}$ substrates when we recorded fluorescence spectra. A 1.9 fold increase is observed when 1μM of $ssDNA_{70}$ was added to 50nM MDCC-SSB tetramers and a 2.1 fold increase was observed with addition of 1μM $ssRNA_{70}$ to 50nM MDCC-SSB tetramers (Figure 2A). We observe a lower fluorescence change than previously published however the signal is still suitable for biochemical assays. Therefore we conclude that MDCC-SSB is a suitable biosensor for ssRNA detection.

Characterisation of the MDCC-SSB biosensor

All quoted concentrations for MDCC-SSB within this section refer to the concentration of MDCC-SSB tetramers, unless stated otherwise. To determine the suitability of MDCC-SSB as a biosensor to quantify mRNA, we first need to establish if there is a dependence between fluorescence intensity and ssRNA concentration. Here we use ssDNA as a control. The MDCC-SSB fluorescence increase was dependent on the concentration of $ssDNA_{70}$ (Figure 2B). Until a saturation point, there

was a linear increase in fluorescence that can be used to calibrate the biosensor. As seen with the tryptophan titrations, SSB is a tight binding protein, as a result, the interaction with ssDNA has a small K_d (within the low pM to low nM range) (27), therefore any free ssDNA should be bound by the biosensor. This tight binding theoretically means that a fluorescence signal should increase linearly up to the MDCC-SSB concentration used, at which point the signal will saturate. When titrating $ssDNA_{70}$ into MDCC-SSB there is a clear linear phase with a saturation point at 65 nM. This is consistent with a 1:1 complex between tetramer (50nM) and $ssDNA_{70}$. There is a mild salt dependence on the binding affinity whereby higher ionic strength displayed a slightly weaker binding. This is typical of DNA binding proteins.

A similar behaviour was also observed with $ssRNA_{70}$ (Figure 2C). A linear increase in fluorescence dependent upon RNA concentration. The saturation point was 101 nM, which is significantly higher than $ssDNA_{70}$. To determine if this saturation point is due to SSB dimers versus tetramers, we repeated the measurements at 200 mM NaCl. Here the saturation point was essentially identical at 109 nM. We conclude that SSB oligomerisation is not a factor in the ssDNA versus ssRNA difference. We suggest the 35 base binding mode may dominate with ssRNA or there is a slightly weaker interaction with ssRNA over ssDNA. This response is consistent with the difference in the tryptophan titrations. Overall the biosensor displays a large linear response which is independent to ionic strength between 100-200 mM NaCl.

Association kinetics of MDCC-SSB were measured to determine the suitability of the sensor to work in kinetic assays (Figure 2D). Measurements were performed under pseudo-first order conditions with a large excess of ssDNA, or ssRNA, over MDCC-SSB. For both substrates there was a linear relationship between rate constant and concentration. The association constants were ~10^8 M^{-1} s^{-1} suggesting rapid binding is independent of substrate. The association kinetics are lower than those previously published however glycerol was used in our reaction buffers to stabilise the protein.

Application of MDCC-SSB biosensor to measure in vitro transcription.

The biosensor has shown its ability to bind to mRNA and respond in a concentration dependent manner. To test the capability of the biosensor to detect concentrations of mRNA samples of different lengths other than the 70 nucleotide, an *in vitro* transcription assay was performed.

Transcription was driven from a T7 promotor which resulted in a 2225 nucleotide run-off transcript. Transcription assays were run for varying amounts of time resulting in different yields of mRNA product. The product was then purified and quantified by RT-qPCR. To establish if the biosensor can bind to the products and distinguish differences in concentration, 1μM of MDCC-SSB was added to each sample of purified mRNA. Fluorescence emission spectra were recorded and clearly demonstrate binding of the biosensor (Figure 3A). Moreover, there was a linear response in fluorescence intensity versus amount of mRNA showing that the biosensor can distinguish differences in transcription yield.

To test the suitability of the biosensor to work without the need to purify the mRNA and to judge the reproducibility, we setup several *in vitro* transcription reactions to generate different lengths of transcript. At the end of the reaction the sample was divided in two. Half was purified and then quantified with RT-qPCR, while MDCC-SSB was directly added to the remaining sample. The relative fluorescence intensity was recorded and this was converted to concentration of SSB binding sites (65 bases) based on a calibration of MDCC-SSB against known concentration of $ssRNA_{70}$ (Figure 3B). The RT-qPCR clearly displays larger variation in the quantification (Figure 3C). While there are uncertainties regarding the exact quantification, the large differences are likely resulting from loss of product during RNA purification and the setup of the qPCR reaction. Most of all, it can be clearly seen that the SSB biosensor can be used to qualitatively and quantitatively determine differences between *in vitro* transcription assays.

Myosin VI motor activity is critical for gene expression

Lastly we used the biosensor to investigate the impact of myosin VI perturbation upon RNAPII transcription. Myosin VI is critical for transcription (6), especially *in vitro,* yet its role remains elusive. Performing *in vitro* transcription, with a method for rapid analysis such as the SSB biosensor, can be used to determine the function of proteins such as myosin in this fundamental process.

We performed *in vitro* transcription assays using the HeLaScribe extracts. A 130 base run-off transcript was produced under the control of a CMV promotor. MDCC-SSB was added once the reactions were complete. Antibody-depletion of myosin VI has been used to perturb the activity of RNAPII (6). Our results are consistent with these findings whereby we observed a 60% decrease in MDCC-SSB

fluorescence within the depleted sample versus control (Figure 4A). To explore if the critical role of myosin VI will involve the motor domain, we performed measurements in the presence of a large excess of F-actin which would act to sequester the myosin motor domain. Indeed this led to a 50% decrease in transcript yield. Such a result indicates a potential role of motor activity in transcription. To further explore the specificity of this perturbation for myosin VI, we performed measurements in the presence of a small molecule inhibitor, TIP (28). This inhibitor has been shown to act specifically against myosin VI. Consistent with the depletion and actin measurements, we observed a 70% decrease in transcription. Thereby we concluded there is a dependence between transcription and the myosin VI motor activity.

To understand the significance of the *in vitro* measurements upon gene expression *in vivo*, we cultured MCF-7 cells in the presence of the myosin VI inhibitor. We the monitored the expression of several genes *PS2*, *GREB1*, *ESR1* and *ACTB*. All four genes showed a significant decrease in expression (Figure 4B). We therefore conclude that myosin VI motor activity is required for gene expression and the biosensor is able to report on this requirement.

DISCUSSION

During the production of this biosensor it was vital to compare the SSB binding to ssRNA with ssDNA. This study has reinforced the idea that SSB is able to bind to multiple single stranded nucleic acid substrates. RNA binding has already been previously shown by Shimamoto *et al* (25), within the context of SSB's ability to bind to its own mRNA. Whilst this has been reported it was important to reinforce this knowledge and compare the binding of both substrates that were designed to be of same lengths. As SSB binding occurs through its OB-folds, it is assumed that mRNA binding would occur, as this type of interaction does not distinguish between ssDNA and ssRNA substrates (29).

This study shows the addition of excess ssDNA, or ssRNA, substrates results in a large fluorescence enhancement of 1.9 and 2.1 fold, respectively. This is lower than previously described (19). This variation in fluorescence could be down to reduced labelling and/or the use of a lower salt concentration which could result in a difference in binding modes of SSB between the studies. The fluorescence changes indicate ssDNA and ssRNA are binding in a similar way, which is consistent with the

non-fluorescent protein measurements undertaken. Therefore, this result shows the biosensor is capable of reporting on the presence of mRNA.

The fluorescence increase occurs in a substrate concentration dependent manner for both the ssRNA and ssDNA substrates. There were some difference in the saturation point of the biosensor between the two substrates whereby ssRNA saturated at approximately 100 nM indicating the possibility of a 2:1 complex which could be interpreted as a 35 base binding mode. It may also indicate there is a weaker binding affinity for ssRNA, while this would be consistent with a preferential binding of ssDNA, it remains unclear as to whether affinity or binding mode are the cause of the difference. Nevertheless, the qualitative response of MDCC-SSB to ssDNA and ssRNA is similar and the defined binding mode is not a requirement for utilising a biosensor. Furthermore, due to this linear response the biosensor can be calibrated making it suitable for mRNA quantification.

The biosensor does not rely on transcripts that are of specific length. Whilst $ssRNA_{70}$ was used to characterise the biosensor, the post transcription result shows that multiple MDCC-SSB proteins are able to bind along the length of the *in vitro* transcripts and still produce a reliable signal.

The MDCC-SSB biosensor is suitable for two functions, as a qualitative comparative assay post *in vitro* transcription and as a quantitative assay following calibration. The former relies on the ability of the sensor to generate different intensity changes between different mRNA concentrations, samples can be normalised to a control and experiments can be matched accordingly.

Conversely, to accurately quantify the concentration of mRNA, the length of the transcript needs to be known. This knowledge is required to transfer the calibration titration of the fluorescence increase, performed with $ssRNA_{70}$, into a concentration of total transcripts. Whilst most transcripts will be full length there will be several smaller abortive or incomplete transcripts presence in any reaction. Therefore, total mRNA concentration can be determined in terms of SSB binding sites which are assumed to be 70 bases showing the MDCC-SSB can distinguish between the samples based upon absolute differences in the total number of SSB binding sites, along transcripts of various lengths.

Both RNA purification and RT-qPCR lead to a reduced transcription yield, large experimental errors and can be time consuming compared to solely using MDCC-SSB. The reagentless biosensor is stable within a varying salt concentrations and can be

used for high throughput analysis using a plate reader. This greatly improves the robust nature of analysing *in vitro* transcription experiments for the study of gene expression and allows for a simple addition and fluorescence emission intensity for accurate results. The benefit of this approach was exemplified by investigating the dependence of transcription on the myosin VI motor activity. We found that a small molecule inhibitor of myosin VI successfully decreased transcription to a similar level where myosin VI is depleted from the reaction. The significance of these findings on to the cell was represented by exposing mammalian cells to the inhibitor which led to a decreased expression of several genes tested here.

In summary, this study shows that MDCC-SSB is a suitable biosensor for measuring mRNA concentrations after transcription and eliminates the need for incorporation of radioactively labelled nucleotides and gel electrophoresis. It can be used to compare conditions without the need for quantification, or to quantify mRNA concentrations after calibration of the sensor; making it a rapid process capable of withstanding different salt buffers.

FUNDING

This work was supported by Medical Research Council [MR/M020606/1]; Royal Society [RG150801] and Leverhulme Trust [ECF-2014-688].

ACKNOWLEDGEMENTS

A.C thanks the University of Kent for funding his studentship. We thank Prof. M. Geeves for the F-actin sample.

AUTHOR CONTRIBUTIONS

A.C and C.P.T conceived the experiments. A.C, Y.H-G and C.P.T performed experiments and analysed data. A.C and C.P.T wrote the manuscript.

FIGURE LEGENDS

Figure 1: SSB can bind to single stranded RNA.

(A) EMSA Assay showing qualitative association of SSB with a 70 base ribonucleotide substrate. Bound species are depicted by *. (B) Tryptophan quenching monitored

while titrating ssDNA (red circles) or ssRNA (blue triangles). The curves were fitted as described in the methods.

Figure 2: MDCC-SSB is an mRNA biosensor.

(A) Fluorescence excitation and emission spectra for MDCC-SSB measured in the apo (Green), ssDNA bound (blue) and ssRNA bound (red) states. (B) MDCC-SSB fluorescence monitored while titration ssDNA into the biosensor in 100 mM NaCl (red) and 200 mM NaCl (blue). (C) Titration performed as in B but with ssRNA. (D) Stopped-flow pre-steady-state kinetics for MDCC-SSB binding ssDNA (red) and ssRNA (blue). Stopped-flow traces were fitted to single exponentials to yield the rate constants plotted in D. Association and dissociation rate constants were calculated from linear fits to the data. Data were averaged from three independent experiments.

Figure 3: Application of MDCC-SSB to measure *in vitro* transcription.

(A) Fluorescence emission spectra for MDCC-SSB and the products from 5 *in vitro* transcription assays. Experiments were performed for 20, 40, 60, 90 and 120 min then quantified using RT-qPCR using primers 1 and 2. (B) Comparison of RT-PCR (green) and MDCC-SSB (red) for determination of *in vitro* transcription yield. Measurements were performed as described in the Methods and text. RT-qPCR and MDCC-SSB was calibrated against known substrate concentrations. The concentration determined by MDCC-SSB refers to nano-molar binding sites. Error bars represent standard deviation from three independent experiments. (C) Comparison between the mean standard deviation arising from the measurements in B. This is displayed as a percent of the mean value determined by the respective method.

Figure 4: MDCC-SSB applied to study the impact of myosin VI motor activity upon RNA Polymerase II transcription.

(A) *In vitro* transcription by HelaScribe extracts. Reactions were performed following antibody depletion, with the addition of 5 μM F-actin, or in the presence of 25 μM TIP myosin VI inhibitor, as described in Methods. Samples were normalized to a non-depleted control reaction (error bars represent SEM). (B) Changes in gene expression following the addition of TIP to MCF-7 cells. Expression is plotted as a percentage of expression in mock cells (Error bars represent SEM).

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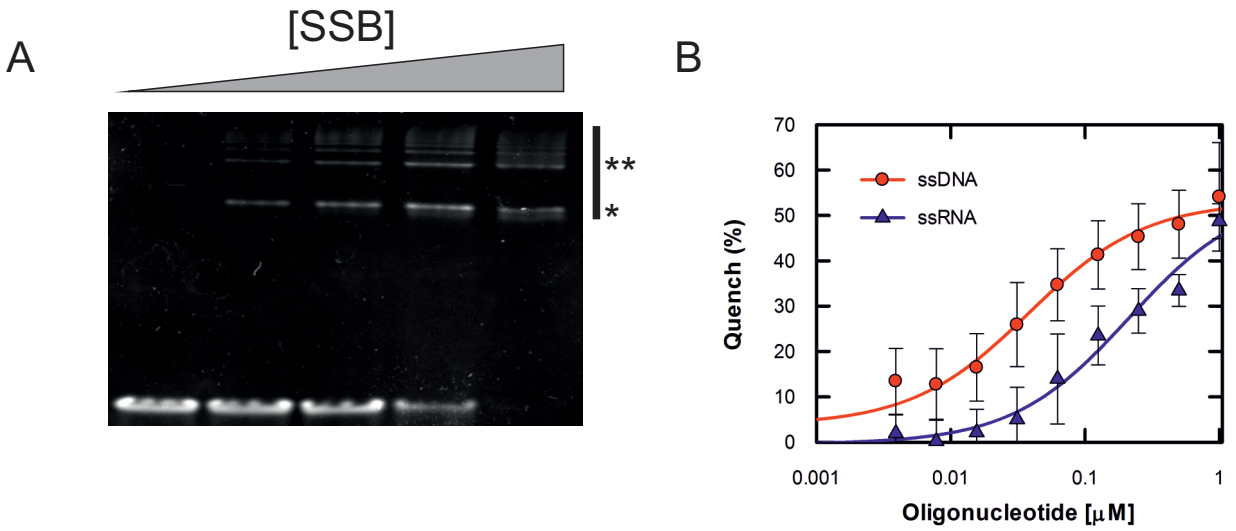


Figure 1

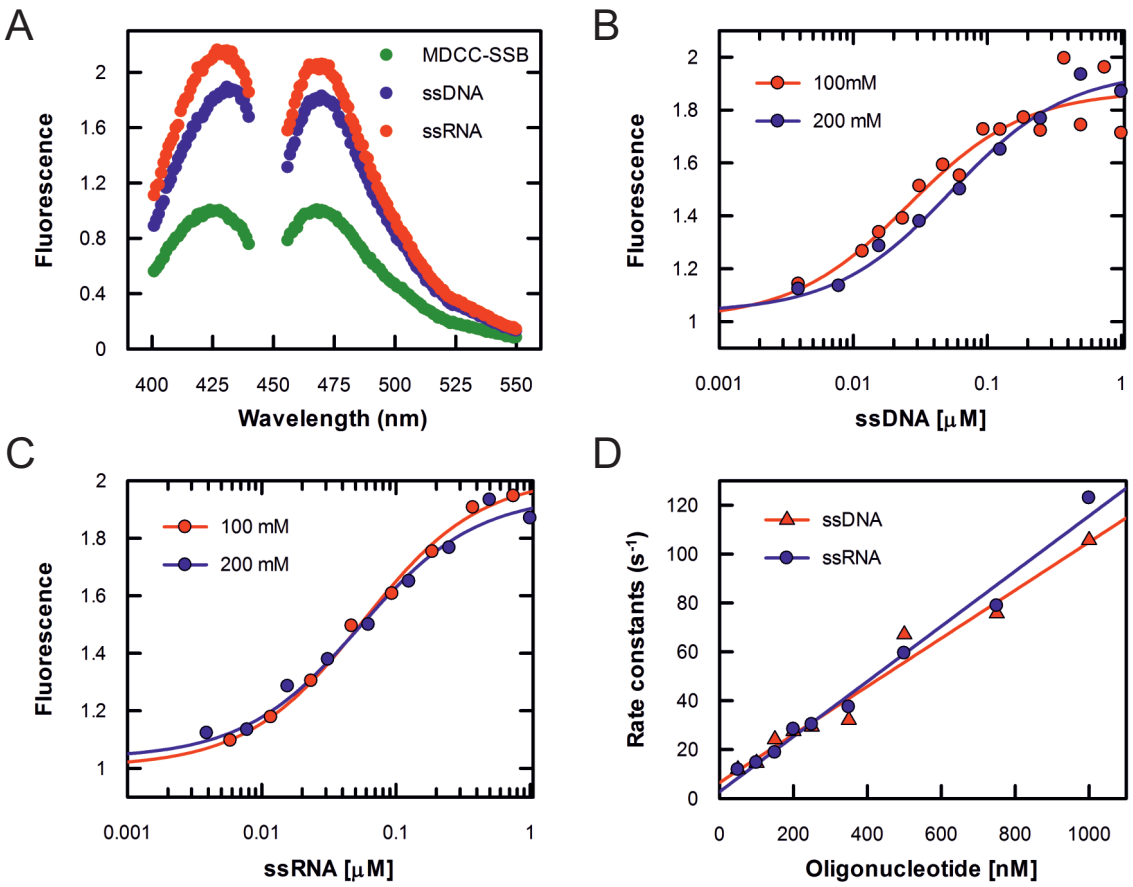
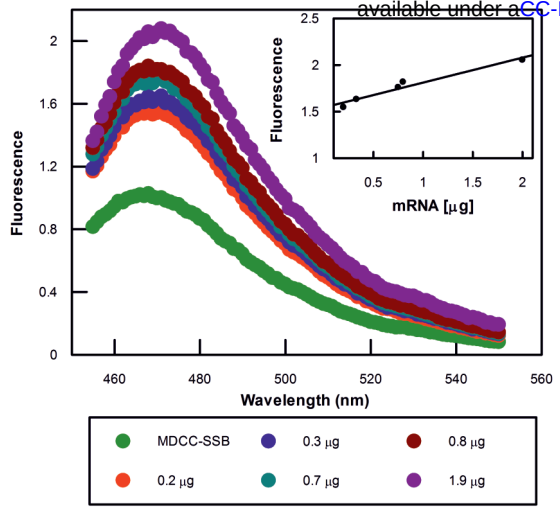


Figure 2

A



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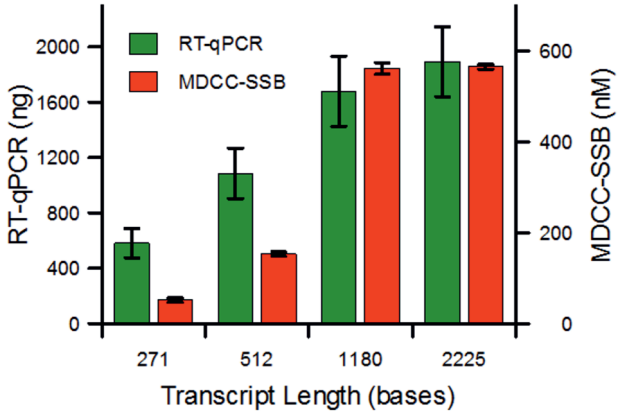

C

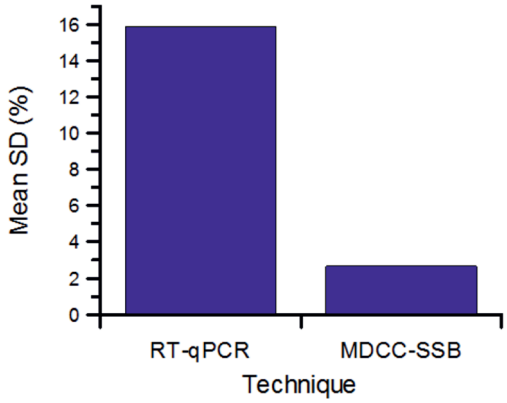

Figure 3

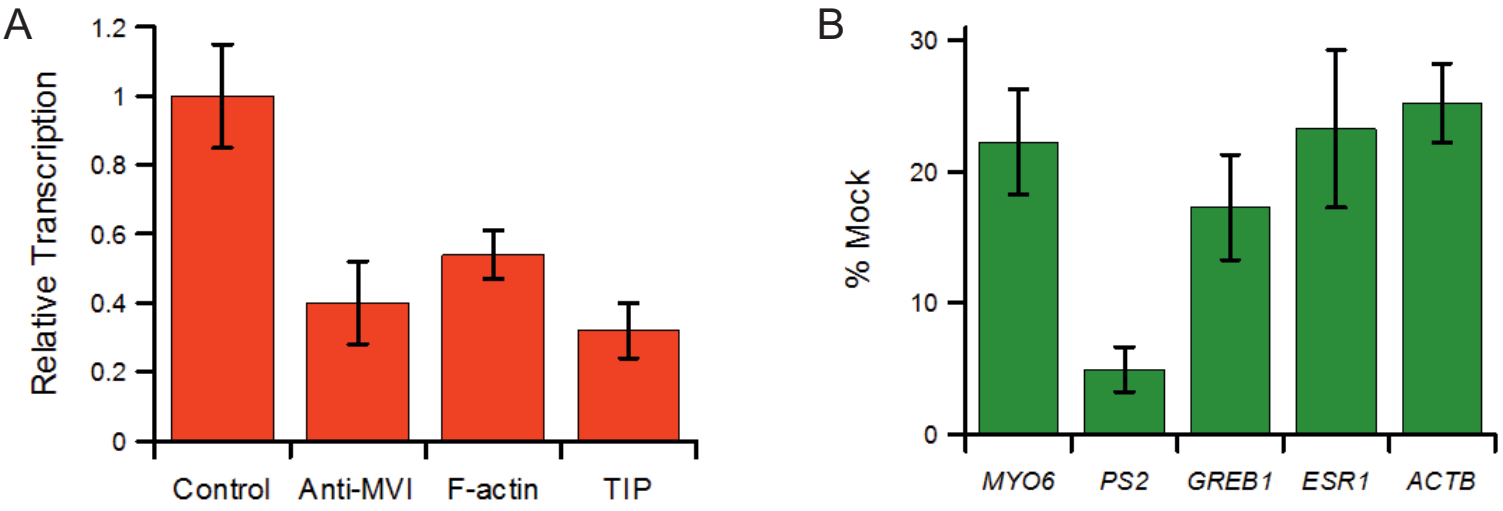

Figure 4