

RESEARCH HIGHLIGHT

Transcription, one allele at a time

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Abstract

A recent study presents a technique allowing one to image transcription from a single gene copy in live cells, and highlights the dynamic nature of transcriptional regulation.

Transcription of a gene into its messenger RNA occurs through a series of defined steps, from transcription factor binding at the promoter to pre-initiation complex formation, entry into elongation and finally termination [1]. Most of the players in these processes, such as the general transcription factors associated with the RNA polymerase, elongation factors, and termination factors, are now well known. However, transcription is inherently a highly dynamic process [2], which sets obvious experimental challenges to most biochemical methods. In this context, imaging techniques have emerged as important tools to study the rapid succession of events that constitute transcription in real time [3]. Watching single cells over time also discloses details that are obscured in a typical test-tube experiment in which the outputs of millions of cells are averaged. Such details can indeed be quite dramatic, such as the high-amplitude oscillations observed in the case of the signaling pathway that activates the transcription factor NFκB [2]. Negative-feedback loops within the NFκB pathway generate cyclic subcellular accumulation of signaling proteins, which results in oscillations in the transcriptional activity of their target genes.

In a recent study published in *Nature Methods*, Yaron Shav-Tal and colleagues (Yunger *et al.* [4]) push the limits of transcription imaging further. They take advantage of the MS2 system developed a decade ago [5], in which a DNA sequence from the bacteriophage MS2 - the MS2 binding site (MBS) - is inserted into a gene of interest. When transcribed, this sequence folds into a stem-loop structure that can be bound with high affinity by the

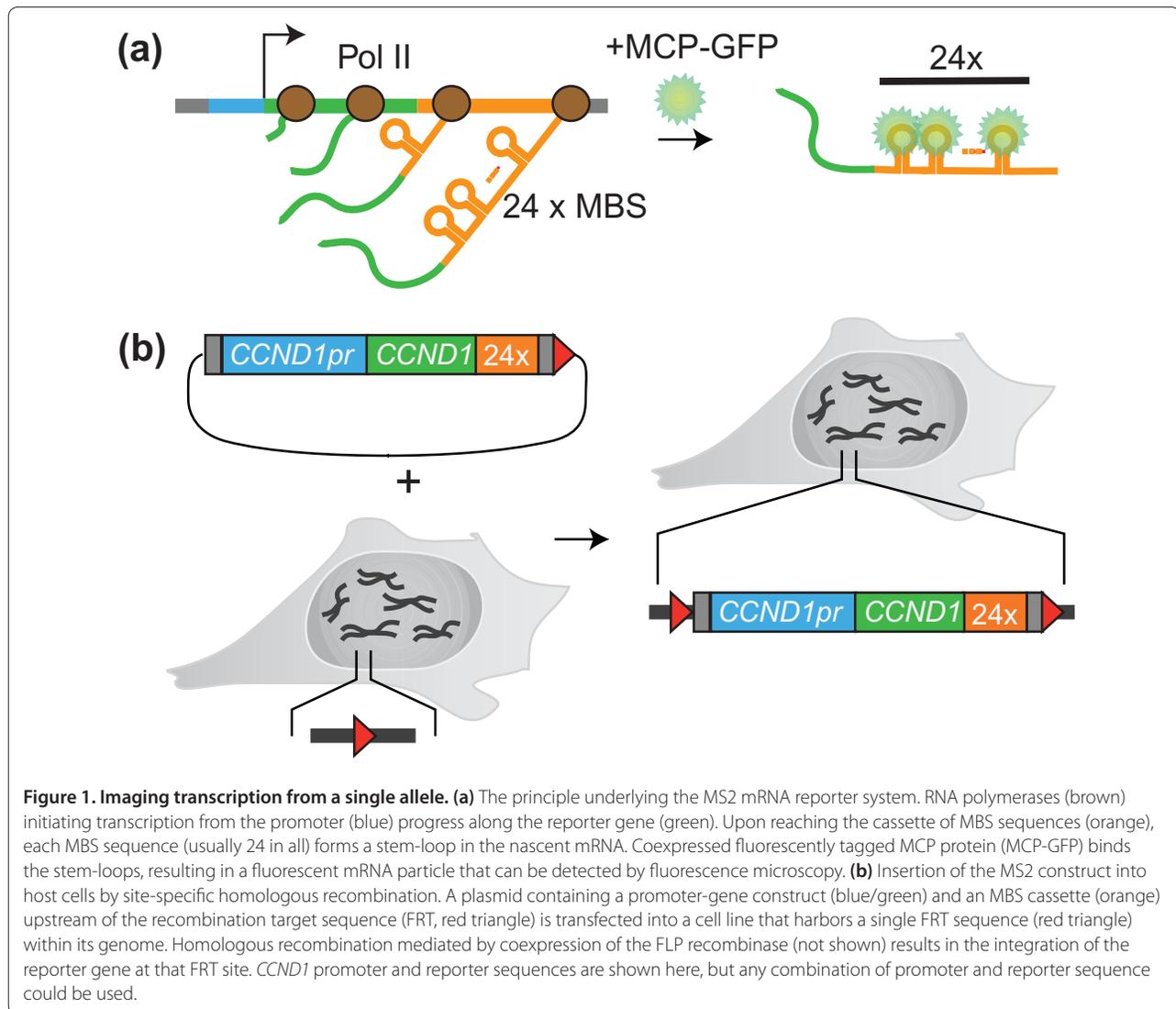
bacteriophage capsid protein (MCP). Coexpression of the MBS-gene construct with one in which MCP is fused to a fluorescent protein gives you an endogenous reporter system that allows the detection of single molecules of mRNA (Figure 1a).

The MS2 RNA reporter system combined with site-specific recombination

Using the MS2 system, details of mRNA transcription, diffusion and nuclear export have been revealed in many different organisms, from bacteria to fruit flies to mammalian cells [3]. Up to now, however, genomic integration of MS2-tagged gene constructs using traditional techniques (such as plasmid integration or viral infection) has resulted in the integration at a random genomic location of an array containing multiple copies of the gene of interest [6]. Although these arrays confer high amplification of fluorescent signal, they come with drawbacks. First, the real-time signal from one fluorescent mRNA gets blurred by signals from the hundreds of unsynchronized fluorescent mRNA molecules present at the gene array. Second, features specific to repeated sequences, or to the locus of integration (which is random), might interfere with transcriptional regulation of the gene of interest. Advances in light microscopy and fluorescent probe development have now brought the detection of single mRNA molecules within reach, and as a result, the limitations of the gene arrays are beginning to outweigh their advantages. However, all studies at the single-gene level in mammalian cells are hindered by the complexity of the genetic techniques required (for example, the need to make transgenic animals).

To overcome the problem of repeated gene sequences and random insertion, Yunger *et al.* [4] combined the MS2 system with a site-specific recombination system. They start from a host human cell line the genome of which harbors a single specific recognition site (FRT) for the yeast FLP recombinase. Co-transfection of the cell line with plasmids that contain the recombinase and the MBS-tagged gene flanked by the FRT sequence results in a single insertion of the construct at the genomic FRT site. A host cell line with the desired characteristics and with a stable site for MS2-tagged gene insertion can readily be prepared by integrating the recombinase-recognition site sequence FRT into a cell line of choice

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(the FRT site itself inserts at random, however, and this initial step cannot be controlled).

In this way, they were able to insert just a single copy of a gene of interest along with its MBS repeats at a specified location in the genome of a host human embryonic kidney cell line (Figure 1b). The other player of the system, the fluorescent MCP, was expressed through transient transfection. This approach considerably simplifies the generation of standardized mammalian cell lines that express reporter mRNAs at a consistent genomic locus. As proof of efficacy, the authors used their new technique to compare the expression of the same reporter gene (human cyclin D1) transcribed under the control of two different promoters: the cytomegalovirus (CMV) promoter (*CMVpr*), which is broadly used to achieve high expression levels of exogenous proteins; and the cyclin D1 promoter (*CCND1pr*), which is apparently active at a constant level throughout the cell

cycle [7]. The genetic construction guarantees that the genomic context is identical in the two cell lines, and that the observed differences originate only from promoter-dependent regulation. The authors then demonstrate the versatility of their system using a battery of fluorescence imaging techniques. The *CCND1* and *CMV* promoters were chosen to drive expression of the cyclin D1 gene for this particular experiment, but in principle any gene-promoter combination could be used in this system.

In addition to providing proof of principle of the new technique, the experiments presented by Yunger *et al.* [4] describe how two promoters can differ in their control of transcription. The average number of reporter mRNA molecules per cell in the *CMVpr* cell line is more than double that in the *CCND1pr* cell line (114 compared with 41). Consistent with the total numbers of mRNA molecules, more nascent mRNA chains were observed on average at the reporter gene locus in the *CMVpr* cell

line compared with the *CCND1pr* cell line (average of 14 versus 7 nascent chains over a cell population).

Burst-like activity of the cyclin D1 promoter

The authors went on to measure the intensity of the fluorescence emitted by the nascent chains at the reporter gene locus in real time, which correlates with the number of RNA polymerases present on the gene downstream of the MBS cassette. In the *CMVpr* cell line, the intensity fluctuated slightly over time around its mean value, reflecting small variations in the number of elongating polymerases present. The *CCND1pr* cell line displayed strikingly different behavior. Periods of intense transcriptional activity alternated with periods where no fluorescence was detected. These bursts of activity occurred over periods ranging from minutes to hours - the average duration of the ON state was 200 minutes, while the duration of the OFF state averaged 22 minutes. This is a direct observation showing that promoters not only tune the global output of transcription (the average level) but also its kinetics: the gene associated with the *CMV* promoter remains on at all times, with a high number of engaged polymerases. The *CCND1* promoter, in contrast, alternates between ON and OFF states, with an overall lower number of engaged polymerases. These bursts are not necessarily incompatible with previous observations of constant cyclin D1 transcription levels over the cell cycle [7]: as the pulses occur at time scales shorter than that of the cell-cycle phases, fluctuations might average out over time to generate a constant output.

Kinetic modeling suggests that the differences observed in the number of nascent RNA chains at the gene results from variation in the initiation frequency (one transcript initiated every 22 seconds for the *CMVpr* compared with one every 52 seconds for the *CCND1pr*), rather than to differences in elongation rate. These results confirm the common view that the main lever for transcription regulation is the efficiency of the promoter-dependent recruitment of elongating complexes (PolII combined with its necessary cofactors).

Taken together, these quantitative measurements draw a detailed picture of the regulation of mRNA production from two different promoters. The initiation rate t_i combined with the mean number of molecules of a given mRNA per cell (m) can be used to estimate the RNA's half life t_d ($t_d = mt_i$), which is 42 minutes for RNA transcribed from the *CMV* promoter and 32 minutes for the same RNA transcribed from the *CCND1* promoter, values close to the 30 minutes estimated for endogenous *CCND1* mRNA [8]. Interestingly, the lifetime of these mRNA molecules is longer than the OFF state of the *CCND1* promoter: in this case, transcription does not shut down long enough to significantly deplete the cell of its mRNAs. The outcome for the *CCND1* promoter is a

wide but single-peaked distribution of the number of mRNAs per cell, and shows that OFF states are not a stable feature leading to a phenotype of low mRNA copy number, but instead contribute dynamically to regulate the number of mRNA molecules within the cell.

Pulses of transcription have already been observed in reporter systems in bacteria and in endogenous genes in the slime mold *Dictyostelium discoideum* and in cultured mammalian cells, whereas data on constitutively expressed genes in yeast suggest that these are transcribed via a constitutive, single-step initiation process [9]. Even if not the rule, bursts of transcription seem to be a possible mode of transcription regulation in higher eukaryotes. One possible advantage is that generating bursts of transcripts provides the cell with more regulatory options: increasing the average level of expression can be achieved by increasing the burst frequency, the burst duration or the number of molecules per burst. Each of these options can be governed by different molecular interactions involving different cofactors.

What could be the mechanism behind such transcription pulses? The fact that this phenomenon was only observed at one of the two promoters studied rules out bursting as an intrinsic feature of eukaryotic gene expression - for example, to effect a large-scale transition to 'open' transcribable chromatin. Low-frequency binding of transcription factors could be one explanation. Detailed statistics of the times spent in the OFF/ON states in the case of the *CCND1* promoter could provide further information on the number of hidden biochemical steps leading to gene activation or shutdown, and therefore help to build a better model describing the kinetics of transcription and its contribution to mRNA copy-number variability [10].

Transcription and DNA replication

Another important question is what happens during DNA replication when the replication fork has to go through an actively transcribing gene. Do RNA polymerases get displaced? In contrast to prokaryotes, little is known about how replication and transcription interact at the molecular level in mammalian cells. While Yunger *et al.* [4] were observing cells in S-phase (DNA replication) and the following G2 phase, they would sometimes observe a site of transcription separate into two less intensely fluorescent sites, suggesting that these sites were the replicated copies of the gene on the sister chromatids. Analysis of the fluorescence intensity at the gene up to 3 hours before such site duplications revealed no transcriptional shut down, indicating that the passage of the replication fork might not fully displace engaged polymerases from their template. Interestingly, measuring fluorescence recovery after photobleaching (FRAP) at the duplicated sites revealed slower kinetics of recovery

than at unduplicated sites. All these observations suggest how the MS2 system could be put to use to study the molecular details of collisions between elongating RNA polymerases and replisomes, for example, by multiplexing the imaging of nascent mRNA with that of replication-fork progression.

The technique presented by Yunger *et al.* [4] provides an important tool for single-gene imaging studies in cell lines: a convenient system that provides a standardized genomic context, in which all inserted constructs experience the same local environment (such as chromatin state or the influence of enhancers). Any promoter-gene combination can potentially be inserted (along with its MBS cassette) into the host cell line, opening up approaches to live-cell transcription studies. By combining the MS2 system with a site-specific recombination system, Yunger *et al.* have considerably simplified and standardized its use as a quantitative, single-molecule mRNA fluorescent reporter. Given the richness of detail provided by their experiments, we anticipate that their system will be a valuable tool for those studying transcription. It also constitutes an important step towards the ultimate goal of studying the expression of a fully endogenous gene in single cells over time.

Acknowledgements

TL is supported by an HFSP long-term Fellowship. RHS acknowledges the support of NIH grants GM86217 and GM57071.

Published: 27 August 2010

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doi:10.1186/gb-2010-11-8-129

Cite this article as: Lionnet T, Singer RH: **Transcription, one allele at a time**. *Genome Biology* 2010, **11**:129.