Published in final edited form as:

Methods. 2009 January ; 47(1): 73–77. doi:10.1016/j.ymeth.2008.10.009.

ROMA: an *in vitro* approach to defining target genes for transcription regulators

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Abstract

We describe an *in vitro* transcription-based method called ROMA (run-off transcription-microarray analysis) for the genome-wide analysis of transcription regulated by sigma factors and other transcriptional regulators. ROMA uses purified RNA polymerase with and without a regulatory protein to monitor products of transcription from a genomic DNA template. Transcribed RNA is converted to cDNA and hybridized to gene arrays allowing for the identification of genes that are specifically activated by the regulator. We discuss the use of ROMA to define sigma factor regulons in *Bacillus subtilis* and its broad application to defining regulons for other transcriptional regulators in various species.

Keywords

RNA polymerase; regulon; sigma factor; microarray

1. Introduction

The regulation of gene expression at the level of transcription initiation is essential for all organisms. In bacteria, transcription initiation requires σ factors that interact with core RNA polymerase (RNAP) and DNA [1,2] to direct transcription of a specific set of genes (a regulon) by activation of target promoters [3]. Much of this primary regulation is influenced by transcription factors that bind to DNA or RNAP holoenzyme and modulate transcriptional activity [4,5].

Defining the complete regulons of specialized or general transcriptional factors remains a challenge, but this task has been favorably influenced by the availability of gene microarray technology and increasingly sophisticated computational approaches to identify DNA regulatory motifs and co-regulated genes. Traditional microarray analyses involve quantifying *in vivo* variations in the levels of mRNA that result from genetic or environmental differences between test samples. Here, we describe a microarray-based technique called ROMA (run-off transcription- microarray analysis) [6] that uses genome-wide patterns of RNA expression from *in vitro* transcription reactions to help define the regulons of transcriptional regulators.

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ROMA uses purified RNAP holoenzyme to direct transcription from purified genomic DNA *in vitro*. Transcribed RNA species are then identified by the use of oligonucleotide or PCR product-based microarrays representing part or all of the genome. The regulon of both positive- or negative-acting transcription factors can be investigated by supplementing the *in vitro* transcription reaction with the factor under investigation and by quantifying changes in RNA expression. ROMA is a powerful technique in itself, but is particularly effective when combined with other global analytical techniques such as *in vivo*-based microarray analyses and bioinformatic methods.

2. Development and use of ROMA

The current concept of ROMA finds its genesis in early attempts to define the *Bacillus subtilis* σ^D regulon in the Chamberlin laboratory. These workers used purified σ^D holoenzyme and *B. subtilis* genomic DNA as template in *in vitro* transcription reactions. The resulting pool of ^{32}P -labelled RNA was used to probe a genomic λ library leading to the identification of several σ^D regulon members [7]. Subsequently, a similar strategy was used to identify members of the *B. subtilis* σ^E regulon [8].

ROMA combines this genome-wide transcription strategy with the transcript identification power of DNA macro- or microarrays. To date, most experiments have used arrays with one double-stranded PCR product or one single-stranded oligonucleotide per annotated gene but extending this technology to high-density tiling arrays should be straightforward. ROMA has proven useful in helping to define the regulons of specialized transcriptional regulators such as σ factors in *Bacillus subtilis* [6,9] and the cAMP-receptor protein (Crp) in *Escherichia coli* [10]. A related method has been used to study recruitment of the TATA-binding protein to gene promoters and activation of transcription in an archaeal system [11].

We have focused on using ROMA to help define regulons for extracytoplasmic function (ECF) σ factors [12] in *B. subtilis*. As part of a comprehensive strategy to define the regulons of σ^W , σ^X , and σ^Y for example, ROMA was used along with traditional *in vivo* macroarray and *in silico* promoter identification techniques [6,9,13]. In these cases, ^{32}P -labelled mRNA populations were hybridized to nylon membranes spotted by PCR products representing each annotated gene (DNA macroarrays). For σ^W , 30 specific operons (about 60 genes) were ultimately identified as belonging to the σ^W regulon [6,14]. In the case of σ^X , ROMA expanded the known regulon [15,16] by several genes and ultimately informed the current view that the σ^X regulon acts in part to mediate *B. subtilis* resistance to cationic antimicrobial peptides [9]. σ^Y activated only a small set of genes as detected by ROMA including its own operon and one other unlinked gene, *ybgB*, which was subsequently confirmed as a bona fide target for σ^Y [13].

Recently, we used ROMA together with oligonucleotide-based DNA microarrays as part of a comprehensive strategy to define the *B. subtilis* σ^M regulon. Many of the genes in the σ^M regulon play a role in responding to cell wall stresses mediated by various chemical and physical challenges [17]. In section 4, we use these σ^M -based experiments to illustrate relevant technical aspects of a typical ROMA experiment. ROMA has also been used to help define the regulon of a newly discovered σ factor in *B. subtilis*, YvrI, which activates a total of five genes. In this case, ROMA largely confirmed what had already been deduced using other genetic and bioinformatic methods but importantly, the comprehensive and global nature of ROMA suggested that other regulon members had not been overlooked [18].

In *E. coli*, the cAMP-receptor protein (Crp) regulates the expression from >100 promoters but the use of traditional *in vivo*-based microarray analyses to define the Crp regulon has been complicated by the complex intracellular regulatory networks that are activated after Crp induction [10]. ROMA was used to define primary Crp-dependent genome-wide transcription

events [10] leading not only to the identification of 152 previously unrecognized Crp-regulated operons but to a broadened understanding of the physiological role of Crp in the cell [10,19]. The use of genomic DNA as a template for *in vitro* transcription reactions has also been reported in an Archaeal system to investigate the ability of the Pt2 regulator to activate TATA-binding protein-dependent transcription [11]. This suggests that ROMA and related techniques may ultimately be extended to the global analysis of transcription in archaeal [20,21] and even eukaryotic systems.

3. Experimental methods - general considerations

ROMA uses purified RNAP holoenzyme to transcribe specific RNA messages from genomic DNA *in vitro* and microarray technology to identify these RNA species. Consequently, there are four major components to a ROMA experiment: i) preparation of genomic DNA template, ii) purification of RNAP and the required transcriptional regulator, iii) design of the *in vitro* transcription reaction and iv) use and interpretation of microarray data. We consider these aspects below.

3.1. Preparation of DNA

Genomic DNA should be of high quality prepared either by an extraction kit or by traditional means followed by at least two phenol:chloroform extractions. For ease of analysis, it may be desirable to reduce the size of the *in vitro* transcription products and this can be accomplished by mechanical shearing or by enzymatic digestion of the template DNA. Mechanical shearing can be accomplished by vigorous pipetting, by sonication, or by using a nebulizer (Invitrogen cat. no. K7025-05) and a pressurized gas source [22]. In all cases, shearing should be monitored by periodically examining the DNA by electrophoresis to visualize the extent of shearing and to determine the size of the predominant shear product. Restriction enzymes are an alternative means of cutting the DNA and benefit from the fact that cutting is positionally constant and predictable. The frequency of cut sites for restriction enzymes varies according to the genome and enzymes should be picked that cut on average every ~4 or more kb. This will typically allow transcription to proceed far enough to overlap the probe(s) for the first one or two genes in each target operon, but will reduce the extent of signal from downstream genes that may or may not be physiologically relevant. Restriction digestion suffers from the fact that a target promoter might carry internal or proximal cut sites that prevent promoter recognition by RNAP or truncate the transcription unit prior to the first relevant probe. This was not a significant problem with our early experiments which used long PCR-based probes and DNA macroarrays. In this case, we used DNA in separate experiments that was digested with either of two enzymes with different specificities (*EcoRI* and *HindIII*). In all cases, genomic DNA should be subjected to phenol:chloroform extraction, precipitated, and re-suspended in RNase-free water to a concentration of at least 1 mg ml⁻¹.

In experiments using arrays with single oligonucleotide probes for each gene we have used undigested DNA that was sheared to an average size of several kb. This enables transcription to reach the first gene (or more) in most target operons. However, transcription termination is comparatively inefficient *in vitro* and this can lead to signals that extend into downstream transcription units that are not dependent on the test transcription factor in the cell. With ssDNA oligonucleotide arrays, these signals only detect co-directional genes. With PCR-based probes, we frequently observed apparent activation of convergently transcribed genes, likely due to antisense transcription. In practice, and with knowledge of gene organization, likely false positives stemming from read-through transcription can usually be discerned [17].

3.2. Preparation of RNAP and transcription factors

ROMA requires purified RNAP and purification of the σ factor or transcription factor under investigation. Details regarding the purification of transcription factors are beyond the scope of this review but are increasingly routine. The purification of RNAP from most bacteria is rather straightforward using a combination of polyethyleneimine precipitation, heparin sepharose or anion exchange chromatography, and size exclusion chromatography [23,24]. Tagged variants of RNAP where a subunit (most commonly the beta' subunit) carries a polyhistidine tag can also facilitate purification of the holoenzyme [25]. A variety of ion exchange chromatography steps can be used to obtain fairly pure core polymerase free from substantial sigma factor contamination [26–28]. The use of purified core polymerase tends to simplify the spectrum of transcription products eventually obtained but is not strictly required. For some species, purified holoenzyme or core polymerase is commercially available. Naturally, every effort should be made to purify required proteins free from endogenous nucleases. Generally, protein concentrations of 1 mg ml^{-1} stored in buffered 50% glycerol at -20°C should be suitable for use in ROMA.

3.3. In vitro transcription

In general, the reaction conditions required for ROMA are the same as those for conventional *in vitro* transcription reactions (excepting the use of radiolabeled nucleotide) and the best conditions for particular holoenzyme or promoter types must be empirically determined. The concentration of salt (usually NaCl or KCl) in the reaction buffer can sometimes have a dramatic effect on transcription. To enable transcription of the widest possible set of target promoters, we recommend using a potassium glutamate-based buffer system at least initially since the salt concentration-dependence of transcription is much broader in such buffers [29]. If at least one target promoter is known, it may be worthwhile to optimize transcription conditions using this DNA and radiolabeled nucleotide. The genes for ECF σ factors are frequently preceded by an autoregulated promoter thus providing a suitable test promoter even in the absence of additional regulon information [12]. It is worth noting however, that transcription from a test promoter will only act as a general guide in deducing permissive transcription conditions and that the relative efficiency of transcription from other promoters in a regulon may vary.

As a starting strategy for ROMA we generally supplement reactions with $1 \mu\text{g}$ sheared genomic DNA, 100 nM (approximately $2 \mu\text{g}$) RNAP, and $2 \mu\text{M}$ (a ~ 20 -fold excess over RNAP) of σ factor in a $50 \mu\text{l}$ reaction volume. An excess of σ factor maximizes holoenzyme formation and acts to out-compete binding by other σ factors that may contaminate even purified core preparations. It is advisable to pre-mix RNAP and σ factor and incubate on ice or at RT for 15 minutes to allow holoenzyme reconstitution before addition to the reaction volume. This is followed by the addition of DNA and a 15 minute incubation at the transcription reaction temperature (usually $\sim 37^\circ\text{C}$) to allow holoenzyme-promoter binding. The reaction is started by the addition of NTPs and multiple rounds of RNA synthesis proceed over a 15–20 minute incubation.

3.4. Transcript detection and data interpretation

Original versions of ROMA relied on the application of radioactively labeled mRNA pools to DNA macroarrays but the broader availability of high quality microarrays and the use of fluorescently labeled cDNAs make this the better choice when possible. Traditional microarray data is usually reported in terms of fold-induction but in ROMA, identification of gene transcription relies on the comparison of relative transcript abundance between original transcription samples either lacking or containing the regulator under investigation. Therefore, we have typically calculated the difference in signal intensity (rather than the ratio) when analyzing the resulting datasets (c.f. [17]). mRNAs from both samples (e.g. transcribed with

and without added σ factor) are independently reverse transcribed to cDNAs and differentially labeled with either Alexa Fluor 555 or Alexa Fluor 647 before hybridization to a microarray slide. Those genes corresponding to a large increase in signal intensity in the presence of the added regulatory protein are likely targets of transcriptional activation.

We have exclusively used ROMA to probe the regulatory roles of ECF σ factors in *B. subtilis*. Most if not all purified RNAP preparations will be variably contaminated with one or more σ factors. For this reason, the control ROMA reaction (the reaction lacking the σ factor under investigation) will often show positive hits representing transcription events catalyzed by contaminating σ factor(s). In the test reaction (the reaction containing the σ factor under investigation) many of these positive hits will disappear since the test σ factor is added in excess and will out-compete less abundant σ factors for limiting core RNAP. The interpretation of ROMA data in these cases are therefore focused on those new signals that appear in the presence of the test σ factor. An analysis of loss of signal in the presence of the test σ factor is a rather more complex situation that may be difficult to definitively interpret. In the case of non- σ factor transcriptional regulators, it may be easier to detect the effect of negative regulation. In their ROMA-based analysis of the Crp regulon in *E. coli*, Zheng et al. [10] identified 16 operons that were negatively regulated by this factor.

Naturally, the signal intensity observed for legitimate transcription events will vary amongst regulatory targets. Signal strength in these instances is not correlated with transcription efficiency *in vivo* and of course does not yield any information regarding the stability of the message or the efficiency with which it is translated *in vivo*. Therefore, inferences regarding gene expression *in vivo* should not be made based on signal intensities observed during ROMA experiments.

Our previous σ^W regulon analysis [6] illustrates three important principles when using ROMA as part of a multi-faceted approach to regulon identification. First, a comprehensive strategy involving the use of *in vivo* macro/microarray analysis, computational promoter prediction, and ROMA led to cross-corroboration of hits amongst the methods and this reduced the frequency of false positive data. Second, no one experimental or computational method was able to identify more than ~80% of what was ultimately judged to be the entire regulon [6]. The inclusion of traditional *in vivo* array and computational methods with ROMA therefore reduces the frequency of false negative data. Figure 1 is a graphical depiction [6,30] of the intersections between the σ^W regulon members identified by *in vivo* microarray, computational promoter prediction, and ROMA and conceptionally illustrates the utility of using a comprehensive approach to regulon prediction. Third, as with all methods, positive hits (especially those not strongly corroborated by all three strategies) should be confirmed by other means such as analyzing putative promoter-*lacZ* fusions *in vivo* and mapping of deduced transcriptional start sites using primer extension, 5'-RACE or related techniques.

4. Using ROMA to define the σ^M regulon in *B. subtilis*

σ^M is a *B. subtilis* ECF sigma factor that plays a role in mediating responses to chemical and physical cell wall stressors [17,31–35]. To define the σ^M regulon, we used ROMA together with promoter consensus search procedures and *in vivo* microarray-based comparisons of the antibiotic (vancomycin) stimulon in wild-type and σ^M mutant strains [17].

To identify σ^M -activated genes, each 50 μ l ROMA reaction contained 18 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 10 mM NaCl, 100 mM KCl, 1 mM DTT, 10 μ g ml^{-1} acetylated BSA, 5% glycerol, and 40 units RNasin (Invitrogen). Two parallel reactions contained 100 nM RNAP with and without 2 μ M σ^M , respectively. After incubation on ice to allow holoenzyme formation, each reaction was supplemented with 1 μ g of *B. subtilis* genomic DNA (sheared using vortexing and vigorous pipetting) and incubated for a further 10 minutes at 37°C.

Reactions were started by the addition of an NTP mixture containing 800 μM each of CTP, GTP, ATP and UTP. Transcription was allowed to proceed for 20 minutes at 37°C and reactions were stopped by the addition of 200 μl stop solution (2.5 M sodium acetate, 10 mM EDTA, 15 $\mu\text{g ml}^{-1}$ linear acrylamide). Reactions were subjected to phenol:chloroform extraction, precipitated, resuspended in water and treated with DNase to remove genomic DNA. After another round of extraction and precipitation, the mRNA was dissolved in 20 μl RNase-free water. Fifteen μl of this was reverse transcribed into cDNA using the SuperScript Plus Indirect cDNA Labeling Kit (Invitrogen) according to manufacturer's instructions. cDNA from the sample lacking σ^M was labeled with Alexa fluor 555 and cDNA from the sample supplemented with σ^M was labeled with Alexa fluor 647. The labeled samples were quantified using a Nanodrop spectrophotometer, mixed, and hybridized to microarray slides carrying 65-mer oligomers representing each annotated open reading frame in the *B. subtilis* W168 genome in duplicate. The slides were scanned with a GenePix 4000B array scanner and subsequently analyzed using the GenePix Pro 4.0 software package. Figure 2 shows representative results. Note the appearance of several strong signals that reflect specific transcription of σ^M -dependent genes.

ROMA played an important part in helping define the σ^M regulon. Of the 19 newly identified σ^M -dependent promoters identified in this study, 14 (74%) were obtained in the ROMA analysis [17]. Five additional hits obtained using ROMA were not corroborated by computational prediction or by the *in vivo* microarray analysis and these remain to be validated as legitimate σ^M targets. σ^M -dependent genes are upregulated in response to cell wall-acting antibiotics such as vancomycin and to several other chemical and physical challenges. A comprehensive cataloging of the σ^M regulon afforded by ROMA and other techniques, combined with knowledge about the conditions that induce σ^M expression, informs the current view that this σ factor mediates a cellular response to agents that damage cell wall structure or perturb cell wall metabolism in *B. subtilis* [17].

5. Discussion

5.1. Advantages and limitations of ROMA

ROMA provides several benefits over traditional *in vivo*-based transcriptional profiling methods. First, it can include, but does not demand, genetic manipulation of the host bacterium genome. This is of particular importance in the post-genomic era where knowledge of genome biology has outstripped the species-specific development of genetic tools and methods. Second, ROMA detects primary transcription events and, unlike other microarray-based methods, does not generate downstream effects that can arise from prolonged chemical or physical induction treatments of cells or from secondary gene expression in the cell. Third, ROMA involves the application of purified proteins to genomic DNA and is therefore free from the influence of negative regulators. Fourth, ROMA can be used to test the influence of positively-acting regulators that may not be amenable to natural or artificial induction *in vivo*. Theoretically, one could also test the influence of regulator (e.g. σ factor) competition for RNAP by modulating protein concentrations in the ROMA reactions. Finally, as an *in vitro* transcription-based technique ROMA may betray transcription events that may be masked *in vivo* by the general complexity of the system. For example, ROMA, particularly when used with high-density tiled arrays or arrays with dsDNA probes, can detect antisense transcripts, regulatory RNAs, and other short-lived RNA species that are often difficult to detect *in vivo*.

ROMA does require purified RNAP and purified regulator although only in rare instances should this provide an insurmountable challenge. Transcription events that require unknown chemical or protein activators can also provide a limitation when using ROMA. Some promoters are significantly dependent on DNA supercoiling for their activity and such promoters may ultimately be under-represented in the final analysis. Finally, ROMA can

generate false positive hits due to inefficient transcription termination *in vitro*. In our experience, transcription can proceed upwards of 10 kb downstream of a legitimately activated promoter [18]. While downstream, co-directional genes may also be co-transcribed *in vivo*, this situation may require further analysis using, for example, Northern blotting. However, read-through transcription into convergent genes appears to occur with significantly higher frequency *in vitro* than in the cell, as expected given the lower efficiency of transcription termination observed *in vitro*. Efficient shearing or the judicious use of restriction enzymes to cut the genomic DNA can reduce this problem. As with all methods used to identify specifically activated promoters, results should be confirmed using alternative means such as reporter fusions *in vivo* or promoter-specific *in vitro* transcription reactions.

5.2 Conclusions

Since ROMA obviates the need for genetic manipulation of the host organism it will be particularly useful for the many known or emerging organisms for which genetic techniques have not been developed. ROMA also provides an indication of primary transcription events and does not suffer from complicating secondary reactions that can arise during *in vivo* experiments from environmental effects on cellular metabolism or hierarchical gene expression in the cell. In many cases, although transcriptional regulators can be recognized in the genome, conditions leading to their induction in the cell may not be known. ROMA therefore provides a strategy for determining the transcriptional role of these regulators even in the absence of additional physiological information. Indeed, the identification of regulon members using ROMA may lead to testable predictions concerning the environmental conditions that ultimately induce expression of a regulator. ROMA is most effective when included with complementary methods of regulon identification such as traditional *in vivo*-based microarray analyses and genome-wide computational promoter prediction methods. Alternatively, it can also be viewed as a good first step towards presumptive identification of co-regulated promoters in the genome when a paucity of basic information precludes the concurrent use of computational promoter prediction techniques.

Acknowledgements

This work was supported by a grant from the National Institutes of Health (GM-047446).

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Figure 1.

Graphical depiction of the intersections amongst three strategies to determine the *B. subtilis* σ^W regulon. Twenty-eight of thirty σ^W -dependent gene promoters (filled circles) deduced using *in vivo* microarray analyses, computational promoter prediction, and ROMA are shown. Re-drawn from [6].

Figure 2.

False-color image of a microarray slide for the analysis of σ^M -activated transcription by ROMA. The mRNA sample corresponding to the σ^M -supplemented reaction is visualized as Red while the control sample is Green. Oligonucleotide probes corresponding to annotated *B. subtilis* genes were spotted in duplicate as described in [17]. The pixel intensity in the Green channel was subtracted from that in the Red channel to generate a graphical depiction of the magnitude of the transcriptional stimulation as shown in Fig. 2 in ref. [17].