

Coupled pre-mRNA and mRNA dynamics unveil operational strategies underlying transcriptional responses to stimuli

Amit Zeisel, Wolfgang Köstler, Natali Molotski, Jonathan Tsai, Rita Krauthgamer, Jasmine Jacob-Hirsch, Gideon Rechavi, Yoav Soen, Steffen Jung, Yosef Yarden, Eytan Domany

Corresponding author: Eytan Domany, Weizmann Institute of Science

Review timeline:

Submission date:	22 March 2011
Editorial Decision:	05 May 2011
Revision received:	29 June 2011
Accepted:	17 July 2011

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision

05 May 2011

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the three referees whom we asked to evaluate your manuscript. As you will see from the reports below, the referees find the topic of your study of potential interest and they appreciate your approach. However, they raise several concerns on your work, which should be convincingly addressed in a revision of the present work. The recommendations provided by the reviewers are very clear in this regard.

In particular, there is a need for a more rigorous and detailed presentation of the computational analysis and to justify or even relax some of the key assumptions used in the analysis (eg as constant degradation rates or consistent elongation rates). Finally, reviewer #3 makes the relevant point that a deeper functional analysis of the results would strengthen the biological insights gained from this study.

*** PLEASE NOTE *** As part of the EMBO Publications transparent editorial process initiative (see our Editorial at <http://www.nature.com/msb/journal/v6/n1/full/msb201072.html>), Molecular Systems Biology will publish online a Review Process File to accompany accepted manuscripts. When preparing your letter of response, please be aware that in the event of acceptance, your cover letter/point-by-point document will be included as part of this File, which will be available to the scientific community. More information about this initiative is available in our Instructions to Authors. If you have any questions about this initiative, please contact the editorial office msb@embo.org.

If you feel you can satisfactorily deal with these points and those listed by the referees, you may wish to submit a revised version of your manuscript. Please attach a covering letter giving details of the way in which you have handled each of the points raised by the referees. A revised manuscript

will be once again subject to review and you probably understand that we can give you no guarantee at this stage that the eventual outcome will be favourable.

Yours sincerely,

Editor
Molecular Systems Biology

Referee reports:

Reviewer #1 (Remarks to the Author):

To say this in the beginning: this is a very good paper. The authors address the problem of how mRNAs can be rapidly regulated even if they are long-lived. They start with mRNA time series profiles measured by microarrays, and use the fact that on new Exon arrays, probe sets cover also introns. They then use the introns as a measure of pre-mRNA, which turns out to be a proxy for transcription rate - since pre-mRNA is rapidly processed into mRNA. They show that peaks of pre-mRNA typically precede that of mRNA, and often pre-mRNA shows pronounced overshoots. They interpret this overshooting as a mechanism for rapid upregulation of mRNAs. They then investigate a few genes more closely, by rt-PCR and fit mathematical models to these.

While I find the experimental approach and data very convincing, I am less satisfied by the mathematical analysis and data fitting. Here are my points concerning this:

- The mathematical analysis should be at least briefly described in the main text (this is journal frequently read by theoreticians, thus no need to hide it)
- The rationale behind the choice of the fitted function (shown in the supplement) is unclear to me, and not described at all. E.g. why is there a $3 \ln(2)$? This entire section of the supplement (or then maybe the materials and methods of the main text) should be rewritten, so that it becomes clear what was actually done and why
- It remains completely unclear how the models were fitted - are the equations of the appendix used? Are the equations that use the derivative of the measurements used? How are the derivatives estimated?
- The models heavily rely on pre-existing measurements of mRNA-stability (Friedel et al). This may lead to strange results, such as for example in 3C, where the decay rate is rapidly changing, which may be an artefact. Also, the half-life at 0 is always a multiple of 30. I hardly believe that Friedel et al rounded these numbers. Can this dependence on initial decay rates somehow be eliminated, or the decay rates be measured exactly for these cells at these conditions? What is the influence of wrong measurements of the decay rate at $t=0$?
- The authors stress everywhere in their manuscript that they are able to measure decay rates without labelling - this is untrue, as the estimates rely on a measured decay rate for $t=0$. These statements should be removed since they are wrong.
- It should be possible to estimate a confidence interval for the time-resolved parameters, e.g. by a bootstrapping approach. How much of the changed decay rates are actually significant?

Further comments:

- The authors should discuss which mechanisms may lead to such pulse-like induction. Are these, as some authors discuss, transcriptional feedbacks, post-translational feedbacks, or feed-forward loops? There are a few papers around that address these possibilities, and they should be discussed.
- The authors show how mRNAs can be rapidly upregulated. But mRNAs should also show a peaked response, if protein should be rapidly upregulated. This should be discussed.
- The color code for the heatmap is unclear - is it somehow representing FC? Why is FC at $t=0$? Is it normalised? This should be stated.
- The impact of the quantiles (50%,90%) chosen should be discussed more in depth, and examples (maybe heatmaps?) for different quantiles should be shown in the supplement.

Reviewer #2 (Remarks to the Author):

The authors present a rather interesting idea for measuring mRNA production and decay rates: Instead of using special treatments (transcriptional shut down) or labeling for measuring mRNA production and decay rates, they suggest using intron spots on microarrays to determine the production levels of new mRNAs (which they refer to as pre-mRNA). The idea is that introns are

only present in newly synthesized mRNAs and so can be used to distinguish these from mRNAs that have been produced before. Unlike prior methods this method is less disruptive and, perhaps more importantly, can be applied to any experiment that uses the microarrays the author use (whereas transcriptional shut down, for example, would likely require additional experiments to identify regulatory cascades etc.).

Using the new method the authors identify several genes for which pre-mRNA production increases or decreases prior to mRNA level increase (decrease) and note that many of these genes display 'overshooting' effects, that is pre-mRNA production fold change (FC) that is much higher than the overall mRNA fold change.

I found the overall idea to be innovative and the results were validated by a number of independent follow up experiments. Still there are several issues associated with the new methodology and analysis the authors need to clarify.

1. The method only applies to species where most genes are spliced which excludes many species from using the analysis. Even for mice cells, which the authors use, the method can only be applied to a subset of the genes for which enough introns exist. Of course, for genes that do not have any introns the method is not applicable. However, even for those with introns, it might be that they are degraded very rapidly which prevents the identification of pre-mRNAs. This is especially true for genes with small copy numbers. I thus think it is important to quantify the size of this group of genes. Specifically, can you provide a breakdown for the variance in pre-mRNA levels (or FC) based on: 1. The number of introns a gene has (from least to most) and 2. The levels of mRNAs (not the FC but the actual array reads for each gene).
2. The fact that the analysis relies on FC and not on actual expression level is a major shortcoming of the method. It has been demonstrated, over and over again when analyzing expression data that FC is extremely noisy, especially for genes with low copy number. Is there any way to quantify the confidence in these FC values? Any way to compute p-values?
3. The authors discuss the possible problems with previous methods for measuring mRNA decay. However, in Results they use transcription shut down to validate their finding. Can they elaborate on the disagreements between the results of the two methods? If they agree, as seems to be the case, could you really say that transcription shutdown is problematic or that such a disruptive technology leads to errors as you mention in the Introduction?
4. Can you compute the correlation between the pre-mRNA and mRNAs (Figure 2)? They seem pretty similar to me and I am not sure about the claim (page 9) of 'markedly different profiles'. What is the average cc for upregulated genes?
5. The main conclusion from this method, which the authors highlight, is that several genes 'overshoot' their response in terms of pre-mRNA production. While this may not have been demonstrated before experimentally, it seems almost unavoidable. Consider a gene with a long half life, a baseline production rate of 10 copies for some time unit and 10% degradation rate which will lead to a steady state of roughly 100 copies. To increase this mRNA by 2 FC in a short time would require the production of 50-100 copies in a short period of time which clearly would lead, for this method that measures FC to a 5-10 FC for the pre-mRNA. Why is it surprising to see such a result? What is a possible alternative model that these results contradict? Any previous citations that have used such contradicting assumption or that claims that overshoot does not or cannot occur?
6. A possible alternative direction is to use the method to determine degradation rates (this is what the others methods the authors discuss attempt to do). It seems like the authors assume a fixed degradation rate for each mRNA. Is there anyway to relax this assumption using this method? While it is clear that production rates are changing upon treatment, it is not clear how exactly degradation rates are regulated. It is likely that these rates may be regulated, at least for some genes and identifying these genes may aid in network reconstruction algorithms that currently assume steady rates.

To conclude, I found the method innovative and the analysis convincing though the new method has its limitations. I was somewhat disappointed by the results. While it may be that no previous method demonstrated production overshoot, it seems to me (maybe in hindsight?) almost obvious that this has to be the process. So the question is, what else can we gain from this method? Can these results be used for modeling or for determining post transcriptional regulation? If not, what should it be used for in the future?

Reviewer #3 (Remarks to the Author):

This study attempts to address an important set of issues in gene expression that are both experimentally and computationally challenging, that of understanding the underlying processes that determine effective messenger RNA levels in mammalian cells. The authors' focus on the progressive responses of immediate-early versus delayed early genes following cell stimulation. Many previous studies have examined these questions on a genome-wide level and determined that waves of functionally related classes of genes ("RNA regulons"?) are expressed (initially transcriptional regulators) followed by functionally responsive genes that differ with the types of cells being examined (e.g. inducers of differentiation or macrophage activation, etc.), followed by a wave of mRNAs encoding repair/remodeling functions to resolve the stimulated state. In many cases in the literature, simple mathematical relationships have been used and tested to investigate these time-dependent changes of transcripts, and these authors use a similar approach. For example, they attempt to measure temporal changes in transcript levels by quantifying pre-messenger RNAs, exons, and mature mRNAs. However, I have several questions and issues with this manuscript that concern me deeply.

1. The study is based upon four assumptions, the first two of which I am not prepared to accept outright. A) The first assumption involves zero-order production kinetics of transcripts and the consistency of transcript elongation events. Recent studies using mammalian cells have reported that the kinetics of transcriptional burst size are entirely due to gene-specific on and off switches per the "random telegraph model", that reflect wide differences in the *cis* DNA sequences; and that elongation rates of transcripts vary over a wide range (e.g. 3X during cell cycle in yeast). Therefore, the authors need to consider and to cite the findings of two papers that appeared in the April 22, 2011 issue of *Science* by DM Suter and by DR Larson. B) under the second assumption of the authors', I am not convinced that mRNA compartmentalization or export are not relevant factors in these calculations. Since the authors do not give us their rationale for this assumption, it is difficult to counter their argument in this critique. But it has been documented in literature that there are different export rates associated with different mRNA classes; and RNA capping and polyadenylation rates are variable across mRNAs as well. Therefore, while it may be fair for the authors to state an assumption for the sake of building a model, it is not fair for them to state these "do not need to be taken into account..." without justifying the basis for the assumption.

2. I am also concerned that the authors criticize the use of actinomycin D and 4-SU labeling to quantify mRNA decay rates, but then use actinomycin in their own experiments. There is excellent support in the recent literature that using reasonable levels of 4-SU does not cause undesirable changes in cellular functions of yeast or mammalian cells. On the contrary, actinomycin D is exceedingly problematic. Thus, it seems unfair to trash both methods and then to use the one with the poorest track record in the paper. I do feel that experimental data of the type shown in Figure 5 are necessary to validate the conclusions of this study. It is good that the actinomycin D data are consistent with the other conclusions in the paper but it seems disingenuous to "contrast" those methods to their new, improved method but to then use actinomycin D anyway. I would prefer to see 4-SU used in this study as it performs very well. But, I would compromise this position by suggesting the authors keep the actinomycin D data and just soften the criticism of both procedures.

3. One of the strongest benefits for understanding these approaches is to discern novel functionally related genes (classes) that are coordinated either transcriptionally or posttranscriptionally during cell activation/perturbation. The Hao and Baltimore paper achieves that goal as explained in the News & Views to that paper and in the followup *Nature Reviews Immunology* articles by P. Anderson. Indeed, this manuscript touches upon this point in a couple of places, including page 8 and on 10 where the authors discuss the overshooting genes being enriched in mRNAs encoding cell adhesion and motility proteins. This kind of finding could be further developed here and would greatly strengthen the paper by giving it a more functional orientation.

We thank all three reviewers for their careful reading of the paper and their comments, which were most helpful. We appreciate particularly their overall positive reaction to the paper. We have accepted nearly all the suggestions that were made and have revised the paper accordingly.

This letter of response is organized as follows. We list each reviewer's numbered comments, explain our point of view (in red type) and provide a precise pointer to the location in the paper and the Supplementary Information where the revision made in response to the comment can be found. Page numbers preceded by S (e.g. page S27) refer to the Supplementary Information. Otherwise (e.g. page 7) to the main text of the paper.

Reviewer 1

General comments:

"To say this in the beginning: this is a very good paper."

We are very happy that the Reviewer liked the paper; thanks for telling us!

"While I find the experimental approach and data very convincing, I am less satisfied by the mathematical analysis and data fitting. Here are my points concerning this:"

1. *"The mathematical analysis should be at least briefly described in the main text (this is journal frequently read by theoreticians, thus no need to hide it)"*

We fully accepted this criticism and have now added a subsection titled "Mathematical modeling of the transcription process" in the Results section of the manuscript (page 6). We describe in this subsection briefly the dynamic equations for pre-mRNA and mRNA concentrations and the assumptions on which our simple model is based. The equations are then re-written for fold-change variables (which we actually measure) and an outline of the inversion procedure (used to derive production and degradation profiles from the data) is provided, with a detailed description given in a subsection titled "Inference of transcript production and degradation profiles" in the *Materials and Methods* section (page 21).

2. *"The rationale behind the choice of the fitted function (shown in the supplement) is unclear to me, and not described at all. E.g. why is there a $3 \ln(2)$? This entire section of the supplement (or then maybe the materials and methods of the main text) should be rewritten, so that it becomes clear what was actually done and why"*

Again, we fully accept the criticism. Our revision and response appear at several places in the new version: The manner in which the fit was carried out ("*what was actually done and why*" is described in *Materials and Methods* (page 21-22, see point 1 above), while the functions used for the fit are described in the Supplementary Information, subsection "Functions used to fit the qPCR time course profiles", pages S27-S29, where we also provide our rationale for selecting these specific functional forms. Our main reason for choosing the constant $3 \ln(2)$ about which

the Reviewer was wondering was one of convenience – to reduce the number of parameters used for the fit. Now we have introduced a parameter (a_4) whose value is also found in the course of fitting the pre-mRNA fold change data. Actually it makes a lot of sense to use this parameter, since this allows fitting the initial slope of the function $\hat{P}(t)$ independently of its asymptotic long-time value.

3. *“It remains completely unclear how the models were fitted - are the equations of the appendix used? Are the equations that use the derivative of the measurements used? How are the derivatives estimated?”*

The criticism is well-taken. Our presentation could easily be misinterpreted. The way we invert the data to infer the production and degradation profiles is now explained in the *Materials and Methods* section (pages 21-22), as mentioned above. To answer briefly the specific question asked by the Reviewer: we first find the parameters of a function $\hat{P}^{(fit)}(t)$ that gives the best fit to the measured pre-mRNA fold change. Then, in order to calculate the production profile, we indeed take (analytically) the derivative of $\hat{P}^{(fit)}(t)$. The procedure used to derive the degradation profile $\hat{\alpha}_2(t)$ is more complicated, but we do not take derivatives of $\hat{M}(t)$ to accomplish this. We hope that the detailed description given in *Materials and Methods* is understandable now.

4. *“The models heavily rely on pre-existing measurements of mRNA-stability (Friedel et al). This may lead to strange results, such as for example in 3C, where the decay rate is rapidly changing, which may be an artefact. Also, the half-life at 0 is always a multiple of 30. I hardly believe that Friedel et al rounded these numbers. Can this dependence on initial decay rates somehow be eliminated, or the decay rates be measured exactly for these cells at these conditions? What is the influence of wrong measurements of the decay rate at $t=0$?”*

The Reviewer has found a mistake in the submitted version of the paper, and we thank him for this. The statement made in the caption of Figure 3 was wrong: the Friedel et al data were *not* used in order to set the parameters $\alpha_2(0)$ (or the pre-stimulus mRNA half life) in the calculations that produced these Figures. In the revised version we determine for each gene, presented in Fig 3, the value of its $t_{1/2}(0) = \ln 2 / \alpha_2(0)$ as part of the fitting procedure, as mentioned whenever these values are used: first in the last sentence of the “Mathematical modeling...” subsection in *Results* (page 8) and then in the subsections “Inferring transcript production and degradation dynamics from pre-mRNA and mRNA profiles” of *Results* (first paragraph, page 14) and “Production overshoot accelerates the induction of mRNAs” (pages 16-17) and in the legend of Figure 3 (page 25). The manner in which $\alpha_2(0)$ was determined by our fitting procedure is described in the *Materials and Methods* section, in the subsection “Inference of transcript production and degradation profiles” (page 22). The values determined by the fitting procedure are now given in each panel of Figure 3, and they have been rounded to integers. In fact, our model and inference of production and degradation do not “heavily rely on

pre-existing measurements of mRNA-stability” – as elaborated below (in response to the next point). The effect of using different estimates of pre-stimulus degradation times is addressed briefly in the last sentence of page 16 and at the end of the legend to Supplementary Figure S7.

5. “The authors stress everywhere in their manuscript that they are able to measure decay rates without labelling - this is untrue, as the estimates rely on a measured decay rate for $t=0$. These statements should be removed since they are wrong.”

See our response to the previous point. In the revised version we estimated the mRNA decay rates $\alpha_2(0)$ in the course of the fitting process. The labeling results of Friedel et al were now used only for Supplementary Figure S7 (which appeared as Figure 6 of our originally submitted manuscript), to assess the effect of grouping genes according to their decay times as estimated by us versus by labeling. Hence we do not believe that our statements are wrong, as our main results were derived without using methods that involve either transcription arrest or labeling (except for the conversion coefficient, see page 14, first paragraph).

6. “It should be possible to estimate a confidence interval for the time-resolved parameters, e.g. by a bootstrapping approach. How much of the changed decay rates are actually significant?”

We thank the reviewer for the useful suggestion, but think that including such information on the main figure will overload it and dilute the main message. We did perform the analysis he suggested, mention the fact in *Results* (the last sentence of the first paragraph on page 14), and in *Materials and Methods* (on page 22, bottom). Detailed description of the manner in which these errors are estimated is given in the Supplementary Information, page S29, in the last paragraph of the section “Functions used to fit the qPCR time course profiles”. Supplementary Figure S11 presents the resulting estimated error bars for one gene, *NR4A1* which exhibits non-trivial dynamics of both production and degradation profiles. The results indicate that the inferred changes in decay rates are highly likely to be statistically significant.

7. “The authors should discuss which mechanisms may lead to such pulse-like induction. Are these, as some authors discuss, transcriptional feedbacks, post-translational feedbacks, or feed-forward loops? There are a few papers around that address these possibilities, and they should be discussed.”

We are well aware of these papers and cite them in the Introduction. Our study does not focus on the structure of the network but rather on establishing the correct dynamics of the production and degradation processes. For example, papers from Uri Alon’s group (e.g. Rosenfeld et al JMB 2002) show how a high initial level of production, attenuated by negative auto-regulation, may accelerate response time in bacteria. The resulting production profiles call for a large discontinuous initial step, which we do not observe. Hence we believe that our observed production profiles originate from some more complex network structure. The very short time scale of the observed induction and repression of production suggests that it is likely to be governed by signaling alone (i.e. without feedback). We express such an opinion

in the Discussion (page 19), but additional experimental and theoretical studies are needed in order to determine reliably whether there is feedback or not, and what kind of network structure is involved.

8. *“The authors show how mRNAs can be rapidly upregulated. But mRNAs should also show a peaked response, if protein should be rapidly upregulated. This should be discussed.”*

Protein production dynamics is beyond the scope of the present work. The Reviewer’s conjecture, that an accelerated mRNA production has the functional purpose of accelerating protein production, is most probably correct. Interestingly, recent genome-scale measurements of synthesis rates of mRNAs (using 4sU labeling followed by sequencing) and the encoded proteins (by means of SILAC and mass spectrometry) have attributed changes in the cellular abundance of proteins predominantly to translational control (Schwanhaussner et al, 2011). Clearly, more work is needed to establish that peaked mRNA induction induces rapid protein-level response.

9. *“The color code for the heatmap is unclear - is it somehow representing FC? Why is FC at $t=0$? Is it normalised? This should be stated.”*

We agree that this is a confusing point. The fact that there is a FC at $t=0$ is due to the normalization used. We hope that the explanation provided in the legend of Figure 2B (page 24) is clear. Briefly stated, the reason is that since the heatmaps present the temporal behavior of a large set of genes, we had to center and normalize the entries in each row separately. Otherwise some very large FC values (e.g., the overshoot in pre-mRNA dynamics) would have completely suppressed the colors elsewhere. The price we pay for displaying all profiles in our heatmaps is the loss of amplitude information.

10. *“The impact of the quantiles (50%, 90%) chosen should be discussed more in depth, and examples (maybe heatmaps?) for different quantiles should be shown in the supplement.”*

In fact these points *are* discussed in the Supplementary Information, in subsection “Determining gene level exon/intron fold change from the gene’s PS”, page S25, and the requested heatmaps are shown in Figure S10

Reviewer 2

General comments

"The authors present a rather interesting idea for measuring mRNA production and decay rates.... I found the overall idea to be innovative and the results were validated by a number of independent follow up experiments."

We appreciate the Reviewer's positive comments.

"Still there are several issues associated with the new methodology and analysis the authors need to clarify."

1.0 "The method only applies to species where most genes are spliced which excludes many species from using the analysis. Even for mice cells, which the authors use, the method can only be applied to a subset of the genes for which enough introns exist. Of course, for genes that do not have any introns the method is not applicable."

We thank the Reviewer for raising this point. In response, we performed "intron statistics" on the genomes of six eukaryotic organisms, ranging from yeast to human. We knew that our method is applicable to the human genome and assumed that it is not applicable for yeast. So we tried to resolve the question raised by the Reviewer – where does the borderline pass? We generated the distributions of the number (per gene) of exons, the total number of intronic nucleotides and the lengths of mRNAs and pre-mRNAs (Supplementary Figure S2). We found that in terms of the number of genes with introns and the size of those introns, *C. elegans* is already much closer to human than to yeast, implying that the method is probably applicable to most multicellular organisms. This result came as a surprise to us, and hence we included a paragraph mentioning it in the main manuscript in the subsection titled "Measuring pre-mRNA and mRNA expression using intronic and exonic probe sets" of the Results section (page 10, last paragraph)

1.1 "However, even for those with introns, it might be that they are degraded very rapidly which prevents the identification of pre-mRNAs. This is especially true for genes with small copy numbers. I thus think it is important to quantify the size of this group of genes. Specifically, can you provide a breakdown for the variance in pre-mRNA levels (or FC) based on: 1. The number of introns a gene has (from least to most) and 2. The levels of mRNAs (not the FC but the actual array reads for each gene)."

The limitations on using the method for the human transcriptome, raised by the Reviewer, are now discussed in a paragraph at the end of the subsection titled "Measuring pre-mRNA and mRNA expression using intronic and exonic probe sets" of the Results section (page 10, last paragraph). The main bottleneck is the number of intronic probesets placed by the manufacturer on the existing platform. We could reliably detect gene-level exonic FC for about 8000 genes and, for nearly half of them, also their intronic signal. A more detailed discussion is

given in the Supplementary Information, in subsection “Microarray preprocessing”, page S24 and in subsection “Determining gene-level exon/intron fold change from the gene’s PS”, see page S26. The requested dependences of our ability to detect intronic and exonic signals are shown in a new figure (Supplementary Figure S3).

2. *“The fact that the analysis relies on FC and not on actual expression level is a major shortcoming of the method. It has been demonstrated, over and over again when analyzing expression data that FC is extremely noisy, especially for genes with low copy number. Is there any way to quantify the confidence in these FC values? Any way to compute p-values?”*

We explain in detail, in the subsection titled “Mathematical modeling of the transcription process” in the Results section (page 7, bottom) that all hybridization-based measurement techniques suffer from this “major shortcoming”, precluding really quantitative analysis. Different genomic sequences have different amplification and hybridization efficiencies and hence provide only relative values. Thus, all our measured profiles used attempt to define a profile involved the use of Fold Changes of the same transcript with respect to a reference condition (measurements at $t=0$). In general, the FC form of the model makes it practical for a larger community. Nevertheless, our model is written in a general way such that if the user can perform absolute measurements he/she can use the non-normalized form of the model.

In order to overcome noise associated with fold-change measurements we applied rather strict filters on “detected” PS, as explained in the Supplementary Information, end of subsection “Determining gene-level exon/intron fold change from the gene’s PS”, see page S26. In the same subsection we explain the manner in which we responded to the Reviewers request for noise estimation of the measured FC. The estimated intensity –dependent standard deviation of the FC is presented in Supplementary Figure S3D. We show that a FC of two is 4 standard deviations from the “no change” null hypothesis. Note that we also performed microarray experiments from biological triplicates. The ‘noise’ level for individual PS from a particular gene is indicated by error bars in Figure 2D, denoting standard deviation of biological triplicates. We are convinced that defining the intronic and exonic FC for each gene by taking the signals of multiple intronic/exonic PS into account gives fairly reliable estimates.

3. *“The authors discuss the possible problems with previous methods for measuring mRNA decay. However, in Results they use transcription shut down to validate their finding. Can they elaborate on the disagreements between the results of the two methods? If they agree, as seems to be the case, could you really say that transcription shutdown is problematic or that such a disruptive technology leads to errors as you mention in the Introduction?”*

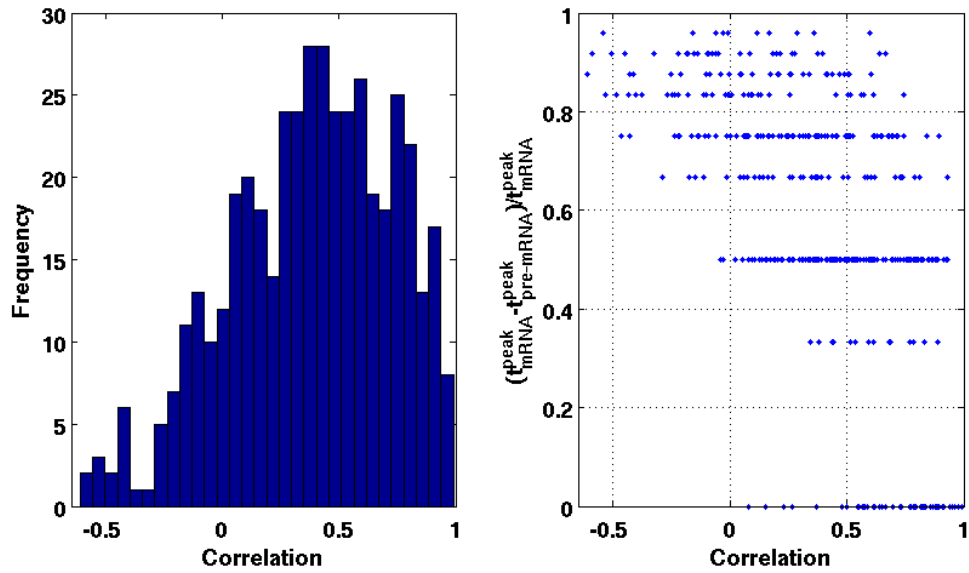
As described in *Results*, subsection titled “Inferring transcript production and degradation dynamics from pre-mRNA and mRNA profiles” (page 14, first paragraph), of all the parameters used for inference of production and degradation profiles, only the conversion coefficients α_1 were determined using transcription arrest. Since the conversion times are very short, we do not worry about transcription arrest affecting them in a significant way. In any case, α_1 enters

only into inference of the production profile and as long as it is on the scale of a few minutes, changing it will affect only the time interval by which $\hat{\beta}(t)$ is shifted with respect to the fitted $\hat{P}^{(fit)}(t)$ (see Supplementary Figure S1A, and the last paragraph of the Mathematical modeling subsection, page 8).

Results of a study that used labeling were used by us for grouping the genes by their mRNA decay times, for Supplementary Figure S7 (formerly Figure 6). We included Figure S7 for the sake of comparison with the new Figure 6, where mRNA degradation coefficients were estimated from fitting our microarray data. Finally, as noted by the reviewer, we used transcription arrest to estimate the mRNA decay times of a few genes (shown on Supplementary Figure S6B), in order to validate our findings. These points are mentioned at the end of the subsection titled “Inferring transcript production and degradation dynamics from pre-mRNA and mRNA profiles” (page 14, last paragraph). The requested comparison of our results with those obtained by transcription arrest is presented in the same paragraph. In brief, we find that measurements by transcription arrest confirm our inference of the change in degradation rates. Where we find no change – no change is found also using ACTD (e.g., compare Figure 3 and Supplementary Figure S6B), and our inference of mRNA stabilization at long times (for *AREG* and *HBEGF*) is also confirmed. The absolute values of the degradation times are, however, markedly different. Consistently with previous knowledge, methods that use transcription arrest may significantly overestimate mRNA half-lives.

4. *“Can you compute the correlation between the pre-mRNA and mRNAs (Figure 2)? They seem pretty similar to me and I am not sure about the claim (page 9) of 'markedly different profiles'. What is the average cc for upregulated genes?”*

We thank the Reviewer for this suggestion. We calculated the correlations as requested and show them now in Figure 2B in the rightmost bar. Within each group of transcripts that have the same mRNA peak time and same pre-mRNA peak time, we reordered the genes according to their correlations (as mentioned in the subsection “Genome-wide time dependent pre-mRNA and mRNA transcriptional responses to EGF stimulation” (page 11), and the Supplementary Information, subsection “Estimating correlations between exon and intron profiles in Figure 2B” (page S27). As expected, when the two peak-times are the same, the correlation is close to 1, but as the difference between the peak-times increases, the correlation decreases significantly (to zero and even to negative values). Hence most profiles are indeed “markedly different”. In particular, the variation of the correlation demonstrates beautifully one of our central results, that one cannot infer the timing of transcript production from the timing of the peak in mRNA abundance. The distribution of correlations for up-regulated genes and their dependence on the gap between peak-times is shown in the Figure below.



5. “The main conclusion from this method, which the authors highlight, is that several genes ‘overshoot’ their response in terms of pre-mRNA production. While this may not have been demonstrated before experimentally, it seems almost unavoidable. Consider a gene with a long half life, a baseline production rate of 10 copies for some time unit and 10% degradation rate which will lead to a steady state of roughly 100 copies. To increase this mRNA by 2 FC in a short time would require the production of 50-100 copies in a short period of time which clearly would lead, for this method that measures FC to a 5-10 FC for the pre-mRNA. Why is it surprising to see such a result? What is a possible alternative model that these results contradict? Any previous citations that have used such contradicting assumption or that claims that overshoot does not or cannot occur?”

We appreciate the reviewer’s intuition and take the liberty to build a more detailed example:

Consider a gene with medium mRNA half –life of 120 minutes $\Rightarrow \alpha_2 = \frac{\log(2)}{120} = 0.0058 \frac{1}{\text{min}}$,

and a typical pre-mRNA to mRNA conversion time of 3 minutes, $\Rightarrow \alpha_1 = \frac{\log(2)}{3} = 0.23 \frac{1}{\text{min}}$.

These parameters define the ratio of pre-mRNA to mRNA at steady state

$\Rightarrow \frac{P}{M} = \frac{\alpha_2}{\alpha_1} = \frac{3}{120} = \frac{1}{40}$, meaning that for each copy of pre-mRNA there are 40 copies of

mRNA. For a steady state production rate $\beta = 10 \frac{\text{copies}}{\text{min}}$ the corresponding number of mRNA

copies at steady state will be $M^{ss} = \frac{\beta}{\alpha_2} = \frac{10}{0.0058} \approx 1724 \text{ copies}$ and the number of pre-mRNA

copies will be $P^{ss} = \frac{\beta}{\alpha_1} = \frac{10}{0.23} \approx 43 \text{ copies}$. A simple way to increase the number of mRNA copies by 2 fold (to ~3400 copies) - without overshooting - is to increase production by 2-fold to $\beta = 20 \frac{\text{copies}}{\text{min}}$. This will result in a very fast response of the pre-mRNA level and after a short time (approximately 15 minutes which are 5 times the typical conversion time) the pre-mRNA level will approach its new steady state $P^{new} = \frac{\beta}{\alpha_1} = \frac{20}{0.23} \approx 87 \text{ copies}$. From this moment and on, the number of pre-mRNA copies will remain almost constant, but the number of mRNA copies will continue rising with a source term of $\alpha_1 P^{new} \approx 87 \times 0.023 = 20 \frac{\text{copies}}{\text{min}}$ until it balances the degradation term, $\alpha_2 M$, and reaches the new level of $M^{new} = \frac{20}{0.0058} \approx 3448 \text{ copies}$. This process involves producing much more than the new 1724 copies (as degradation of mRNA will occur during the rise), and will take approximately 9-10 hours (see Figure 1), and will not involve production overshoot. The overshoot we describe in this paper has the meaning of increasing production for a short time to more than 20 copies/minutes and then down to the level corresponding to maintaining the new steady-state. In terms of this example, using $\beta = 100 \frac{\text{copies}}{\text{min}}$ for a short time will quickly provide a source term of $\alpha_1 P^{new} \approx 430 \times 0.023 \approx 100 \frac{\text{copies}}{\text{min}}$, which will cause a rapid increase in mRNA. Then, when mRNA is close to the desired 2 fold level, the production level is reduced to 20 copies per minute, needed to maintain the 2 fold increase in mRNA abundance.

In summary, we agree with the reviewer that production overshoot is a very intuitively appealing way to achieve a rapid response. However, previous models hardly considered such a scenario, probably because the high dynamic range of production rates we report here was not considered.

6. "A possible alternative direction is to use the method to determine degradation rates (this is what the others methods the authors discuss attempt to do). It seems like the authors assume a fixed degradation rate for each mRNA. Is there anyway to relax this assumption using this method? While it is clear that production rates are changing upon treatment, it is not clear how exactly degradation rates are regulated. It is likely that these rates may be regulated, at least for some genes and identifying these genes may aid in network reconstruction algorithms that currently assume steady rates."

Actually, we did not assume fixed mRNA degradation rates. On the contrary, one of the outputs of our method is the inference (for each mRNA) of interesting time dependent degradation profiles $\hat{\alpha}_2(t)$, as shown in gold curves in Figures 3, 4 and 7. In agreement with previous reports

(e.g. Rabani et al 2011), we consider these non-monotonic degradation rates as important determinants in shaping the profiles of transcriptionally induced genes. Please note that in the revised version we inferred degradation rates (both at the initial steady state and throughout the time courses after stimulus) from fitting our data.

7. *“To conclude, I found the method innovative and the analysis convincing though the new method has its limitations. I was somewhat disappointed by the results. While it may be that no previous method demonstrated production overshoot, it seems to me (maybe in hindsight?) almost obvious that this has to be the process. So the question is, what else can we gain from this method? Can these results be used for modeling or for determining post transcriptional regulation? If not, what should it be used for in the future?”*

Our method allows direct inference of production and degradation profiles for mammalian cells. The procedure is simple, non-perturbing and direct – no need for labeling or for transcriptional arrest. As far as we know, no previous work demonstrated the wide-spread use of production overshoot to accelerate production of transcripts with long degradation times.

Another new finding on which we elaborate more in the revised version of our manuscript is that pre-mRNA levels of most up-regulated mRNAs had already increased by 20 minutes after EGF stimulus (see heatmaps in Figure 2B). This finding suggests that the initial regulation of these genes occurs via the primary transcriptional response, whereas the amplitude and duration of induction of these genes may be differentially shaped by newly synthesized transcriptional and posttranscriptional regulators. This argues against a frequently assumed linear cascade of induced transcription factors, which ultimately induce secondary response genes. Rather, the latter transcription factors may serve to sustain/amplify the initial induction of genes in the course of the primary response. These findings are in agreement with a recent report, which used global run-on and sequencing (Gro-seq) in MCF7 cells after estrogen stimulation (Hah et al, 2011). That study also found that a large fraction of the transcriptome was regulated, upon estrogen treatment, after a 10 minute interval. Our finding, of a wide range of delays between pre-mRNA and mRNA profiles, is also new. Hence, analysis of different experimental systems by our method (and metabolic labeling techniques) will likely reveal different modes of regulation underlying transcriptional induction.

Reliable quantitative measurements of production and degradation profiles, such as provided by our method, constitute an essential pre-requisite of any investigation aimed at proposing some model for the molecular mechanisms that may produce such profiles. We expect that the method will be widely used to study detailed transcription dynamics in a large class of organisms, under widely varying conditions and stimuli. These points are presented in the Discussion.

Reviewer 3

General comments:

"This study attempts to address an important set of issues in gene expression that are both experimentally and computationally challenging, that of understanding the underlying processes that determine effective messenger RNA levels in mammalian cells. The authors' focus on the progressive responses of immediate-early versus delayed early genes following cell stimulation. Many previous studies have examined these questions on a genome-wide level and determined that waves of functionally related classes of genes ("RNA regulons"?) are expressed (initially transcriptional regulators) followed by functionally responsive genes that differ with the types of cells being examined (e.g. inducers of differentiation or macrophage activation, etc.), followed by a wave of mRNAs encoding repair/remodeling functions to resolve the stimulated state. In many cases in the literature, simple mathematical relationships have been used and tested to investigate these time-dependent changes of transcripts, and these authors use a similar approach. For example, they attempt to measure temporal changes in transcript levels by quantifying pre-messenger RNAs, exons, and mature mRNAs."

In fact, previous studies that identified these "transcriptional waves" were based on the assumption that mRNAs peaking at the same time were also produced at the same time. No previous study has established that this is not the case, and that one cannot infer the timing of production from the timing of the peak in mRNA abundance. We did not find any previous study that used the two coupled simple differential equations for pre-mRNA and for mRNA, on which our work is based.

1.A *"The study is based upon four assumptions, the first two of which I am not prepared to accept outright. A) The first assumption involves zero-order production kinetics of transcripts and the consistency of transcript elongation events. Recent studies using mammalian cells have reported that the kinetics of transcriptional burst size are entirely due to gene-specific on and off switches per the "random telegraph model", that reflect wide differences in the cis DNA sequences; and that elongation rates of transcripts vary over a wide range (e.g. 3X during cell cycle in yeast). Therefore, the authors need to consider and to cite the findings of two papers that appeared in the April 22, 2011 issue of Science by DM Suter and by DR Larson."*

We thank the Reviewer for pointing out our unnecessary usage of a terminology that does not reflect our message. We explain all our assumptions carefully, in the subsection titled "Mathematical modeling of the transcription process" of the Results section (pages 6-7). The expression "zero-order production kinetics" has been removed from the paper. We do not allow explicit dependence of the production rate of a particular transcript on the concentration of the same or another transcript. We do allow, however, a general time-dependent form of the production rate, which effectively incorporates all the important dependencies listed by the Reviewer. We hope that the present version is not misleading and that our explanation is clear.

The two papers mentioned by the reviewer, which were published while our manuscript was under review, are now discussed and cited (page 7, first paragraph).

1.B *"under the second assumption of the authors', I am not convinced that mRNA compartmentalization or export are not relevant factors in these calculations. Since the authors do not give us their rationale for this assumption, it is difficult to counter their argument in this critique. But it has been documented in literature that there are different export rates associated with different mRNA classes; and RNA capping and polyadenylation rates are variable across mRNAs as well. Therefore, while it may be fair for the authors to state an assumption for the sake of building a model, it is not fair for them to state these "do not need to be taken into account..." without justifying the basis for the assumption."*

All the biological processes that involve conversion of pre-mRNA into mRNA are absorbed in a single conversion coefficient α_1 . These processes include splicing, RNA capping and polyadenylation. Since we do not assume that all transcripts have the same value of α_1 , in fact our model *does* allow different splicing, RNA capping and polyadenylation rates for different mRNAs. We do assume that these rates do not depend on time and are not affected by the stimulus, which, arguably, represents an oversimplification. As to the effect of export, the criticism is well-taken and we removed the statement cited by the reviewer. Indeed, not having measured or inferred directly the export rates, we cannot make categorical statements about it. We state our assumptions clearly in the subsection titled "Mathematical modeling of the transcription process" of the Results section (page 7), where we also describe the main implications of our assumptions and present our justification for making them. We also softened the statement on influences that need to be considered, accepting the Reviewers criticism.

2. *"I am also concerned that the authors criticize the use of actinomycin D and 4-SU labeling to quantify mRNA decay rates, but then use actinomycin in their own experiments. There is excellent support in the recent literature that using reasonable levels of 4-SU does not cause undesirable changes in cellular functions of yeast or mammalian cells. On the contrary, actinomycin D is exceedingly problematic. Thus, it seems unfair to trash both methods and then to use the one with the poorest track record in the paper. I do feel that experimental data of the type shown in Figure 5 are necessary to validate the conclusions of this study. It is good that the actinomycin D data are consistent with the other conclusions in the paper but it seems disingenuous to "contrast" those methods to their new, improved method but to then use actinomycin D anyway. I would prefer to see 4-SU used in this study as it performs very well. But, I would compromise this position by suggestin the authors keep the actinomycin D data and just soften the criticism of both procedures."*

The comment is well-taken. On pages 5 - 6 in the subsection "Inference of production *and* degradation profiles requires measurement of pre-mRNA *and* mRNA abundances" we have now discussed the two methods separately and are not "*trashing*" usage of labeling by 4-SU. As to using ACTD in our own measurements, this point has been raised also by the other Reviewers.

We repeat here our response to point 3 of Reviewer 2 (in case these responses are not sent to Reviewer 3).

As described in *Results*, subsection titled “Inferring transcript production and degradation dynamics from pre-mRNA *and* mRNA profiles” (page 14, first paragraph), of all the parameters used for inference of production and degradation profiles, only the conversion coefficients, α_1 were determined using transcription arrest. Since conversion times are very short, we do not worry about transcription arrest affecting them in a significant way. In any case, α_1 enters only into inference of the production profile and as long as it is on the scale of a few minutes, changing it will affect only the time interval by which $\hat{\beta}(t)$ is shifted with respect to the fitted $\hat{P}^{(fit)}(t)$ (see Supplementary Figure S1A, and the last paragraph of the Mathematical modeling subsection, page 8). We used transcription arrest also in order to estimate the mRNA decay times of a few genes (shown on Supplementary Figure S6B), in order to validate our findings (of EGF induced time-dependent variation of mRNA degradation).

As to labeling, indeed we used results of a study based on labeling, but only for grouping the genes by their mRNA decay times, for Supplementary Figure S7 (formerly Figure 6). Figure S7 was included for the sake of comparison with the new Figure 6, where mRNA degradation coefficients were estimated from fitting our own microarray data (see bottom paragraph on page 16).

All these points are mentioned at the end of the subsection titled “Inferring transcript production and degradation dynamics from pre-mRNA *and* mRNA profiles” (page 14, last paragraph). The requested comparison of our results with those obtained by transcription arrest is presented in the same paragraph.

3. “One of the strongest benefits for understanding these approaches is to discern novel functionally related genes (classes) that are coordinated either transcriptionally or posttranscriptionally during cell activation/perturbation. The Hao and Baltimore paper achieves that goal as explained in the News & Views to that paper and in the followup Nature Reviews Immunology articles by P. Anderson. Indeed, this manuscript touches upon this point in a couple of places, including page 8 and on 10 where the authors discuss the overshooting genes being enriched in mRNAs encoding cell adhesion and motility proteins. This kind of finding could be further developed here and would greatly strengthen the paper by giving it a more functional orientation.”

Our finding, to which the Reviewer refers, of enrichment of overshooting transcripts by genes coding for cell adhesion and motility related proteins, is a first step towards a functional understanding of the particular system studied (MCF10A cells responding to EGF). Such functional interpretation, which was not the focal point of our study, necessitates detailed extensive experimental probing of that particular system.

Hao and Baltimore focused on a particular very interesting system: the inflammatory response. Their findings suggest that inherent mRNA stability is a key factor in shaping this transcriptional response. P. Anderson refers in his News and Views to this coordinated response as “composing the inflammatory symphony”. Our work focuses on obtaining the actual time dependent transcriptional response to any general stimulus and we try to understand the underlying operational strategy.

Our method allows direct inference of production and degradation profiles for mammalian cells. The procedure is simple, non-perturbing and direct – no need for labeling or for transcriptional arrest.

From a biological regulation point of view we present new findings, on which we elaborate more in the revised version of our manuscript. We show that pre-mRNA levels of most up-regulated transcripts increase already 20 minutes after EGF stimulus (see heatmaps in Figure 2B). This finding suggests that the initial regulation of these genes occurs via the primary transcriptional response, whereas the amplitude and duration of induction of these genes may be differentially shaped by newly synthesized transcriptional and posttranscriptional regulators. To demonstrate the significance of this finding in the context of the Hao and Baltimore paper, note that possibly genes that they identified as “slow risers” in the inflammatory response, are actually produced at early times. In fact, we demonstrate such behavior of immune system genes in our study of the response of murine dendritic cells to stimulus by LPS (see Figures 7E and 7F and Supplementary Figure S9). In agreement with Hao and Baltimore, we also find that the stability of mRNA indeed shapes its profile. It is impossible, however, to infer *two* profiles – of both production and degradation - from *one* measured time-course (say mRNA abundance). Our observation that for about 80% of the genes up-regulated by EGF production starts during the first hour, argues against a frequently assumed linear cascade of induced transcription factors, which ultimately up-regulate secondary response genes. Rather, the early-induced transcription factors may serve to sustain/amplify the initial induction of the genes of the primary response. These findings are in agreement with a recent report, which used global run-on and sequencing (Gro-seq) in MCF7 cells after estrogen stimulation (Hah et al, 2011). That study also found that a large fraction of the transcriptome was regulated, upon estrogen treatment, after a 10 minute interval. Our finding, of a wide range of delays between pre-mRNA and mRNA profiles, is also new. Hence, analysis of different experimental systems by our method (and by metabolic labeling techniques) will likely reveal different modes of regulation underlying transcriptional induction.

Reliable quantitative measurements of production and degradation profiles, such as provided by our method, constitute an essential pre-requisite of any investigation aimed at proposing some model for the molecular mechanisms that may produce such profiles. We expect that the method will be widely used to study detailed transcription dynamics in a large class of organisms, under widely varying conditions and stimuli. These points are presented in the Discussion.

References

Hah N, Danko CG, Core L, Waterfall JJ, Siepel A, Lis JT, Kraus WL (2011) A rapid, extensive, and transient transcriptional response to estrogen signaling in breast cancer cells. *Cell* **145**: 622-634

Rabani M, Levin JZ, Fan L, Adiconis X, Raychowdhury R, Garber M, Gnirke A, Nusbaum C, Hacohen N, Friedman N, Amit I, Regev A (2011) Metabolic labeling of RNA uncovers principles of RNA production and degradation dynamics in mammalian cells. *Nat Biotechnol* **29**: 436-442

Schwanhausser B, Busse D, Li N, Dittmar G, Schuchhardt J, Wolf J, Chen W, Selbach M (2011) Global quantification of mammalian gene expression control. *Nature* **473**: 337-342

Acceptance letter

17 July 2011

Thank you again for sending us your revised manuscript. We are now satisfied with the modifications made and I am pleased to inform you that your paper has been accepted for publication.

Thank you very much for submitting your work to Molecular Systems Biology.

Sincerely,

Editor
Molecular Systems Biology

Reviewer #1 (Remarks to the Author):

The authors have addressed my main concerns sufficiently.